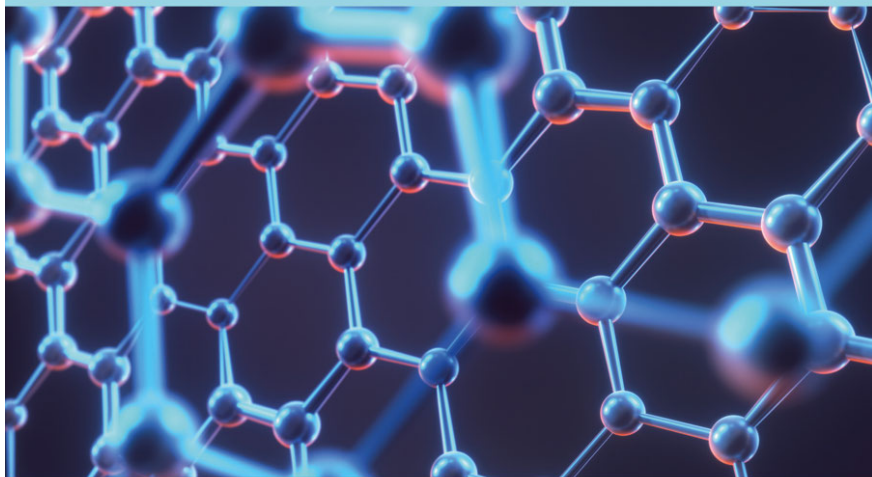


Developments in Applied Microbiology and Biotechnology



Emerging Technologies in Applied and Environmental Microbiology

Edited by
Maulin P. Shah and B.R.M. Vyas



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Editors

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Chapter 1: Experimental investigation of leaked potable water quality supplied by the leading water treatment plant in Bangladesh

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Abstract

Dhaka water supply and sewerage authority (DWASA) of Bangladesh is the primary water supply source to the Dhaka city inhabitants through its' distribution network after the water treatment process of Sitalakhaya river integrated with groundwater source while Sitalakhaya river water is treated by Sayedabad Water Treatment Plant (SWTP). The main objective of the current study is to analyze the physicochemical parameters of supplied water to Sayedabad Water Treatment Plant and drinking water to the consumers of the Jurain area, Dhaka city. Water quality parameters: pH, color, turbidity, alkalinity, hardness, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total dissolved solids and residual chlorine have been experimentally analyzed through the standard analytical method. The experimental investigation of the water quality assessment revealed that the physicochemical characteristics of water released from the water treatment plant were within quite an acceptable limit. Still, in the allocation unit, water quality was not in the acceptable range, and residual chlorine concentration was decreasing notably with an expansion in space from the water treatment plant. The study demonstrated that most of the vital water quality parameters except BOD_5 , chemical oxygen demand, and residual chlorine, were within the permissible limit, recommended by World Health Organization and BDS guidelines.

Keywords

Integrated water management; Residual chlorine; River water quality in Bangladesh; River water treatment

1. Introduction

Among all-natural resources cognizant on earth, water is one of the essential components. It is vital to all living creatures, most ecological systems, human health, food production, and economic progress. Human health is closely associated with the quality of drinking water and providing safe drinking water is one of the essential public health precedences. A comprehensive range of water problems faces nations and individuals around the world. The measurement revealed in 2000 by the World Health Organization (WHO), around the world, 1.1 billion people, lacked access to a better water supply. The consumption of contaminated water caused about 80% of all disorders and over one-third of deaths in developing countries. An approximate, as much as one-tenth of each person's adequate time is lost to water-affiliated disorder (Kundzewicz, 1997). The lack of clean water supply and sanitation is the main cause of infection. So, Freshwater is the major essential thing of human life. Drinking water quality is an essential concern for humankind since it is directly connected with public health. In developing countries, many water resources are risky since they bear harmful physical, chemical, and biological organic or inorganic organisms (Cheesbrough, 2006), especially in developing countries like Bangladesh. Dhaka is the capital of Bangladesh. In Dhaka city, DWASA supplies water to users' end through a network of underground pipelines. The main sources of this water are underground and treatment plants. Groundwater is extracted from the ground through deep tube wells, and the treatment plant supplies water to the city through pipes by purifying the water of the Sitalakhaya river. WHO discerns pure drinking water as that water having allowable characteristics in terms of its physical, chemical, and bacteriological parameters. The safe and potable water is, free from pathogenic microbes, and hazardous chemicals/substances, and aesthetically acceptable (i.e., pleasing to the sight, odorless, and good taste). It is also important that this type of water should be not only available but also be available in enough quantity all the time (Park, 2005). World Health Organization (WHO) has provided several rules to regulate the quality of pure water that must be followed by drinking water supply authorities and water users in each area (WHO, 2006).

Surface water is supplied to the treatment plant before sending it into the pipes. The water entering the pipe must be of good quality. In Dhaka city,

the pipe system is very outdated, and most of the pipes are in used condition. As the water quality at the users' end is of main concern, it is imperative to test water quality there. Water quality in the end should satisfy the Bangladesh Environment Conservation rules (1997) and be preferable to The WHO guidelines. So, we take attempt to determine the quality of water supplied by DWASA in the Jurain area in Dhaka city.

Physical, chemical, and biological parameters are the main basis for assessing water quality. Therefore, the physicochemical parameters (pH, turbidity, color, alkalinity, hardness, TDS, residual chlorine BOD₅, and COD) have been assessed in this study to determine the overall water quality (Egorov et al., 2002; Hossain & Mahmud, 2019; Momtaz, 2002; Saal et al., 2020; Sharma & Rather, 2015; Water Environmental Federation and American Public Health Association, 2005; Wu et al., 2020). Each of these parameters embeds a vital ingredient in the water quality assessment of this area. These results are compared to those of the BDS and WHO standards (Momtaz, 2002; WHO, 2006).

Jurain is a residential area in Dhaka city. Thousands of people are living here. They used to consume a bulk amount of water supplied by DWASA for drinking and daily uses. The water supplied to Jurain by DWASA comes mainly from the SWTP and underground water in the vicinity of Jurain, which is collected through a deep tube well and supplied with pipes. Due to the faulty distribution of the pipeline system, there may be contamination of water. So, it is vital to assess the water quality of this area for supplying pure water for drinking and other purposes to ensure the health and safety of this huge number of people. Thus, the objectives of the present study are as follows:

To assess water quality parameters of SWTP's water, groundwater, and also some residential house's water for drinking purposes.

- To analyze the water quality, and diversity from the source to consumer, which may be useful in discerning appropriate healing measures for warding off the drinking water from contamination and facilitating the safe drinking water supply to consumers.
- To make a comparative analysis between SWTP's water and water in the different residential houses of the Jurain area supplied by SWTP and groundwater.

1.1. Study area

Jurain is a large area in the southern neighborhood of Dhaka, Bangladesh. It is not too large with its area but quite a crowded neighborhood, with a number of residents close to 25,000 people. This area is located at $23^{\circ} 41' 17.7792''$ N, and $90^{\circ} 26' 35.3796''$ E. It is under the Dhaka Municipality area. Fig. 1.1 presented the location map of the studied area. The distance between SWTP and Jurain is 5.1km.

2. Materials and methods

2.1. Sampling

The collected water samples from different sources have been delineated in Table 1.1. In the present study, the procedure of sampling used was an assemblage of random and purposive sampling. The samples of water were collected from households located at different distances in the Jurain area from SWTP and after the sterilization of house taps. Samples were also collected from SWTP and the Underground water pumping station in Jurain. For sampling, high-density PVC bottles were used. All these bottles were closed carefully and kept airtight and transported to the laboratory. Water samples were collected from the outlet of the SWTP to measure the efficiency of this treatment plant and to assess the quality of water just before ingoing the transmission line. The samples are immediately transferred to the subsequent laboratory for testing and analysis.

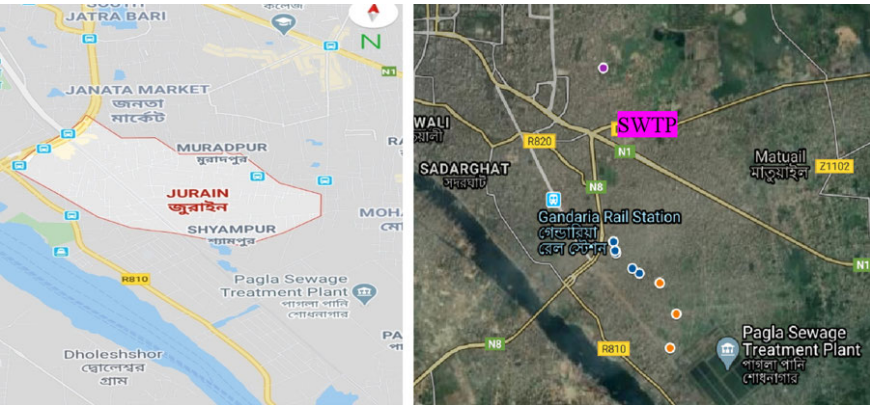


Figure 1.1 Location of the studied area and sampling point.

Table 1.1

Treatment plant and production pumps		Households	
Sample No	Location	Sample No	Location
Sayedabad Water Treatment Plant (SWTP)	5	124, Rajab Ali Sarder Road, Jurain.	1
East Juraine water	6	849, Fatema	2

pump		Manzil, Jurain	
Hazi Md Okaludin water pump	7	160- Bahadur Saheb Manzil, Jurain	3
Salaudddin water pump	8	51, Muradpur High School Road.	4
–	9	13- Rob Miah Manzil, Jurain	–
–	10	88- Md Romim vila, Jurain	–

2.2. Determination of physicochemical parameters

2.2.1. pH, turbidity and color

25mL water sample was taken in a clean tube for tests from the collected water sample from a different household, groundwater sources, and SWTP. The pH, turbidity, and color were measured by pH meter, turbidimeter, and spectrophotometer, respectively.

2.2.2. Alkalinity and hardness

150mL water was taken in a beaker for the test from the collected water sample. The titration procedure was used to determine the alkalinity of the water. Methyl orange concentration was used as an indicator of this titration. The hardness of water was determined by titrating with a standard solution of ethylene diamine tetra-acetic acid (EDTA) (Ali et al., 2019). 100mL water sample was taken in a beaker for this test. EDTA solution, standard CaCO_3 solution, Eriochromic Black-T indicator, and buffer solution were used as a reagent for this test.

2.2.3. TDS

The gravimetric method was used to determine the TDS of water. 50mL sample was taken in a standard glass-fiber filter and the filtrate was collected. The filtrate was evaporated to a constant weight condition in an oven maintained at a temperature of 180°C to remove mechanically occluded water. Hydrochloric acid was used as a reagent for this test. TDS was calculated by Eq. (1.1).

$$\text{TDS} = \frac{(\text{AD}) * 1000}{\text{S}} \quad (1.1)$$

Where, A=final 1800C weight of the dried residue+the tared dish, mg, D=tared dish weight, mg, and S=ml of sample volume.

2.2.4. Residual chlorine

The Starch-Iodine method was used to determine the residual chlorine of water. 200mL of the sample was taken in an Erlenmeyer flask for this test (APHA et al., 2017). The residual chlorine has been determined by Eqs. (1.2) and (1.3).

$$\text{Residual chlorine } \left(\frac{\text{mg}}{\text{L}} \right) = \text{mL of 0.025N sodium thiosulfate used} \times \text{M.F} \quad (1.2)$$

$$\text{M.F} = \frac{\text{Normality of Na}_2\text{S}_2\text{O}_3 * \text{equivalent wt of CL}_2 * 1000}{\text{mL of sample taken}} \quad (1.3)$$

Where, M.F- Microfiltration.

2.2.5. BOD₅ and COD

The BOD₅ was assayed by the 5-day test method, and the chemical oxygen demand (COD) was analyzed by the close reflux method. The sample sequence water was made by assessing 50mL of sample water into a BOD bottle bearing nutrient buffer. The bottle was sealed with sealing gel to ward off gaseous interferences and settled in an incubator at 20±°C. The primary DO quantity was filed. The DO quantity for every day thereafter was recorded for 5 successive days, using an automated DO probe. A COD reactor was pre-heated to 150°C, and a plastic shield settled frontward of the reactor. A 0.2mL reagent was pipetted into the sample, heated for 2h, and cooled in a water bath. The tube was then analyzed with a spectrophotometer. Volatile organic compounds (VOC) were entirely oxidized in this method, compared to the open system, because of the longer contact time with oxidants.

3. Result and discussions

The assessed values of the collected water samples in different physical and chemical water quality parameters are summarized in Table 1.2. The quality of water parameters is measured by comparing the test results with both Bangladesh Drinking Water Standard (BDS) and WHO guidelines for drinking water quality.

3.1. pH

pH significantly impacts biological activity. It additionally affects a few substances of the water body, the activity of creatures, and the hardness of toxic properties indicate in the aquatic environment. The determination of pH is anticipated to discern the corrosiveness of the water (WHO, 2006). WHO (2006) and BDS (1997) recommended values with a varying range of 6.2–6.8, the values of pH of all samples of various sampling locations were found in a permissible range of 6.5–8.5. A maximum pH of 6.80 was observed at SWTP and 124, Rajab Ali Sarder Road, Jurain. A minimum pH of 6.20 was recorded at Hazi Md Okaludin Water Pump. Among the 10 locations, the minimum pH value 6.2, 6.3, and 6.4 was found in a water sample collected from respectively, Hazi Md Okaluddin water Pump, East Juraine water pump, and 160- Bahadur Saheb manzil, below the permissible range of 6.5–8.5 according to WHO (2006) and BDS (1997), and also indicated the water could be little acidic (Momtaz, 2002; WHO, 2006). The mean pH values of water samples taken from different sampling locations are 6.51, indicating the water was fit for consumption.

3.2. Turbidity and color

The maximum turbidity of 9.70 NTU was found in 160- Bahadur Saheb Manzil, and a minimum of 1.86 NTU value was found in Salauddin Water Pump. The assessed results of turbidity of most water samples were within the allowable amount of 10 NTU according to BDS; however, many samples exceeded the permissible value of 5 NTU according to WHO Guideline value. Among the production Pumps, 66.67% of samples did not exceed the WHO permissible value of 5 NTU, and the remaining 33.33% of samples exceed the WHO permissible value but were within the range of BDS guideline value (10 NTU). In the case of household samples, 83.35% of samples were below 5 NTU, and the remaining 16.65% exceeded 5 NTU but remained within the range of 10 NTU WHO standards. The turbidity test result of

SWTP is 2.13, showing 100% below the permissible value of 5 NTU WHO Standard has been presented in Fig. 1.2. It is recommended that turbidity of more than 1 NTU will significantly impact the disinfection efficiency; the Naked eye can mark out turbidity of more than 5 NTU and is often alleged by the consumers. Maximum levels of Turbidity may provide protection for opportunistic microorganisms and pathogens (Aulicino & Pastoni, 2004). Turbidity shows an increase from SWTP and water pump toward end-users concerning distance from the treatment plant. This incident can be attributed to the accumulation of more suspended particles in the water toward end-users through faulty joints in pipelines. On the other hand, the color of water does not deed so many health problems, but it is aesthetically not to be taken. Color in water is mainly due to the appearance of colored organic matter, metals such as Fe, Mn, or highly industrial wastes (Hossain & Mahmud, 2019). The values of color difference within the range of 27Pt. Co to 49Pt. Co exceeding the limit of WHO (2006) and BECR'97 (Momtaz, 2002; WHO, 2006).

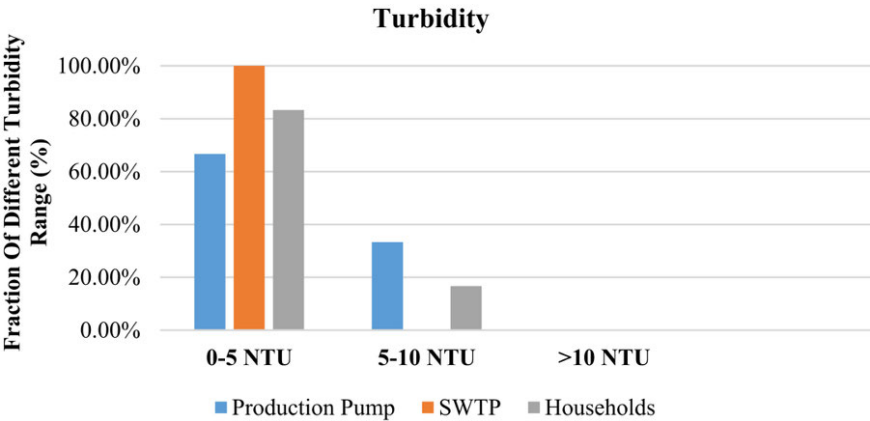


Figure 1.2 Turbidity of collected water samples.

Table 1.2

Water quality parameters:	pH	Turbidity	Color	Alkalinity	Hardness	TDS	Residual chlorine	BOD ₅	COD	
–	NTU	Pt. Co	mg/l	mg/L	ppm	mg/L	ppm	ppm	ppm	Units
6.5–8.5	10	15	500		1000	0.2	0.2	4		BDS

				200–500					(ECR 1997)
6.5–8.5	5	15		500	1000	0.6–1.0	–	–	WHO (2006)
6.8	2.13	27	101	132	153	0.7	0.2	3	Sample No
									1
6.3	2.70	42	118	206	341	0	0.18	2	2
6.2	8.30	38	116	192	312	0	0.22	4	3
6.6	1.86	35	100	248	480	0	0.14	3	4
6.8	2.84	29	112	303	514	0	0.25	3	5
6.52	3.15	44	118	202	446	0	0.70	1	6
6.4	9.70	37	144	160	222	0	1.50	5	7
6.51	1.98	49	161	184	228	0	1.30	16	8
6.54	2.45	41	122	208	403	0	1.00	12	9
6.5	3.12	31	155	310	416	0	1.40	15	10

3.3. Alkalinity

Alkalinity is the assessment of water's capacity to absorb Hydrogen ions (neutralize acids) without significant pH change. The acid titration method was used to determine the alkalinity of water samples. Among all samples, the maximum value of 161 was found in a water sample collected from a house connection at 51, Muradpur High School Road, Jurain, and the minimum alkalinity, of 100 were measured in water samples collected from Salauddin Water pump. All the collected samples had the measured alkalinity concentration within the range of 0–200 ppm, thoroughly favorable following WHO (2006) and BECR'97 water quality guideline values (Momtaz, 2002; WHO, 2006).

3.4. Total hardness

The range of the total hardness of water samples collected from various sampling locations was 132–310mg/L. The maximum value of the total hardness level was found at 88- Md Romim Vila (310mg/L), and the minimum standard was recorded at SWTP (132mg/L). Soft water was not found in the samples. The Water of SWTP was found in 100% moderately Soft. Hard and

very hard water was found at 66.67% and 33.33%, respectively, in household water samples where 100% of production pump water was hard. Among all the samples, 100% of water samples did not overcome the permissible value of 500mg/L following WHO (2006) and BDS (ECR'97) water quality guideline values. According to the hardness, the measured value of water may be classified as in Fig. 1.3 presented that dissolved calcium and magnesium salts from the surrounding ores are mainly responsible for the hardness of the water (Lin et al., 1996). The hardness impacts the taste of water, but the taste threshold varies from person to person. If the total hardness is too poor, the water can precipitate the pipe corrosion; the Boiler scale and water scale are shaped in the heating process when the total hardness is too high (higher than 200mg/L) (Yisa & Jimoh, 2010).

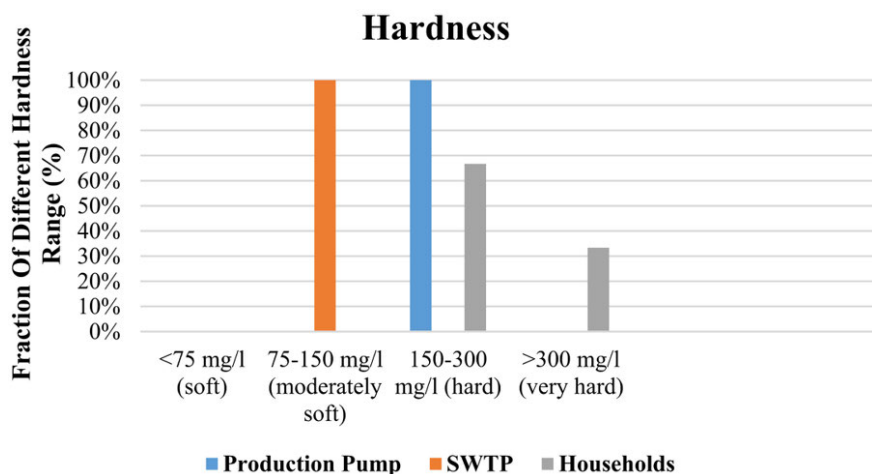


Figure 1.3 Hardness of collected water samples.

3.5. Total dissolved solids

Assessment of TDS of water samples collected from various locations fluctuated from 153 to 514ppm. According to the measured TDS values, water is classified as excellent, good, fair, poor, and unacceptable in Fig. 1.4. A maximum amount of 514ppm was found at 124, Rajab Ali Sarder Road, and a minimum value of 153ppm was recorded at SWTP. So, the measured value of TDS in SWTP is excellent. The measured value of the Production Pump was (100%) good. Most of the samples lie in good (300–600ppm) and also excellent (<300). 33.33% of samples of households were excellent, and 66.67% were good. All the samples are acceptable following the permissible

limits (1000ppm) of WHO (2006) and ECR (1997). TDS level was increased at a reasonable level at 124, Rajab Ali Sarder Road, which could be due to cross-contamination within the distribution system. High TDS can produce offensive odors, tastes, colors, and health problems that rely on the specific pollutants existing (Jensen et al., 2004). US-EPA (1978) suggests a treatment procedure when TDS concentrations exceed 500mg/L or 500ppm (Ali et al., 2019).

3.6. Residual chlorine

The concentration of residual chlorine in SWTP was found at 0.7mg/L, which demonstrates that treated water is acceptable following WHO and BDS (Momtaz, 2002). But there was no residual chlorine concentration in the production pump, and household water could have happened due to inadequate distribution systems of pipelines. The level of Concentration under 0.6mg/L is insufficient for disinfection, and this might cause pathogenic bacterial growth in the allocation system (Ali et al., 2019). So, the water of the households and also the production pump are not so qualified for disinfection. Cherepovets, there is a study in Cherepovets, Russia found that a decrease in residual chlorine concentration in the allocation system is caused by gastrointestinal illness (Egorov et al., 2002). Drinking water without chlorine was in serious danger of occurring cholera and diarrhea to the people of Colombia, as demonstrated by another study (Cárdenas Ayala et al., 1993).

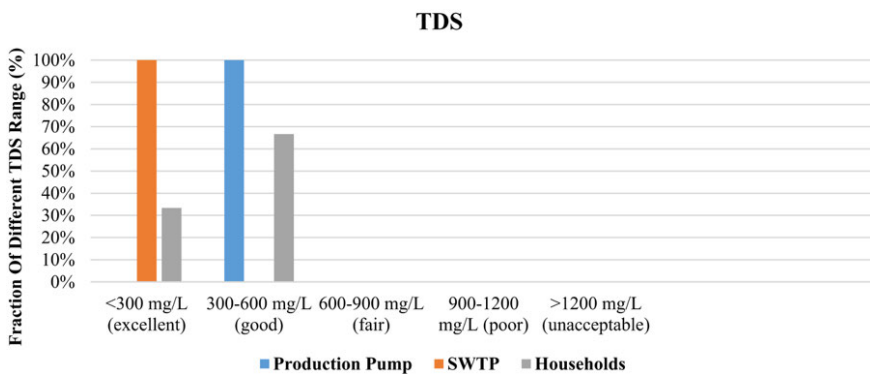


Figure 1.4 TDS of collected water samples.

3.7. BOD₅

Biochemical oxygen demand for 5 days (BOD₅) can be applied as a

criterion of the usefulness of wastewater treatment plants. The U.S. Clean water act list it as a conventional pollutant. All the collected water samples from several households in this area had a BOD_5 concentration more significant than the permissible value of 0.20ppm of BECR'97. The concentration of BOD_5 in SWTP was found at 0.2ppm, which is permitted according to BECR'97 (Momtaz, 2002). 66.67% of samples of the production pump were well within the permissible limit of 0.2ppm of BECR'97, and 33.33% of the sample exceeds this allowable limit. The maximum BOD_5 concentration was found at 1.50ppm in 160- Bahadur Saheb manzil, and the minimum was 0.14ppm in Salaudddin Water Pump. It could demonstrate that the maximum concentration of BOD_5 in SWTP and production pump were within the range of permissible value. Still, when the water was distributed from the pump and SWTP to the households, then it showed an increasing trend. Therefore, Due to cross-contamination through leaking pipes, disallowed connection, and inappropriate domestic storage facilities could be responsible for high BOD_5 concentration in the distribution system. The assessed value of BOD_5 level in water samples collected from several locations in Jurain is demonstrated in Fig. 1.5.

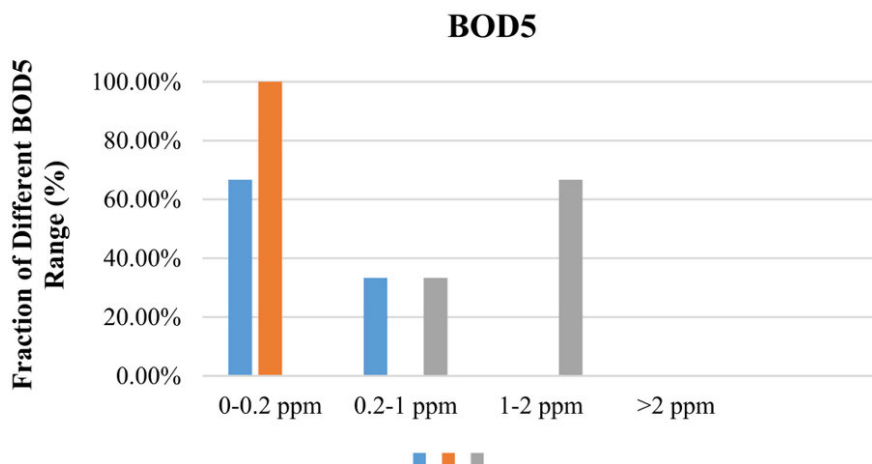


Figure 1.5 BOD_5 of collected water samples.

3.8. COD

The Chemical oxygen demand is an asses of oxygen equivalent to the necessity of oxidizing organic substance contents by a strong chemical agent. The COD test helps to identify toxic conditions and the appearance of

biologically resistant organic substances. The maximum value of COD concentration was found at 88- Md Romim Vila (15ppm), and the minimum level was recorded at 849, Fatema Manzil, Jurain (1ppm). The measured value of COD concentration at SWTP was found 3ppm, which is permissible for drinking water according to BDS (Momtaz, 2002). 100% of Samples of production pumps didn't exceed the permissible limit of 4ppm following BDS (Momtaz, 2002). In the case of household samples, 67% of samples were exceed 4ppm, and the remaining 33% below 4ppm. The measured COD concentration presented in Fig. 1.6 showed an increasing trend from SWTP and production pumps toward end-users. Cross-contamination within the distribution system could be responsible for it. The high level of contaminants present in water samples represents the high value of COD.

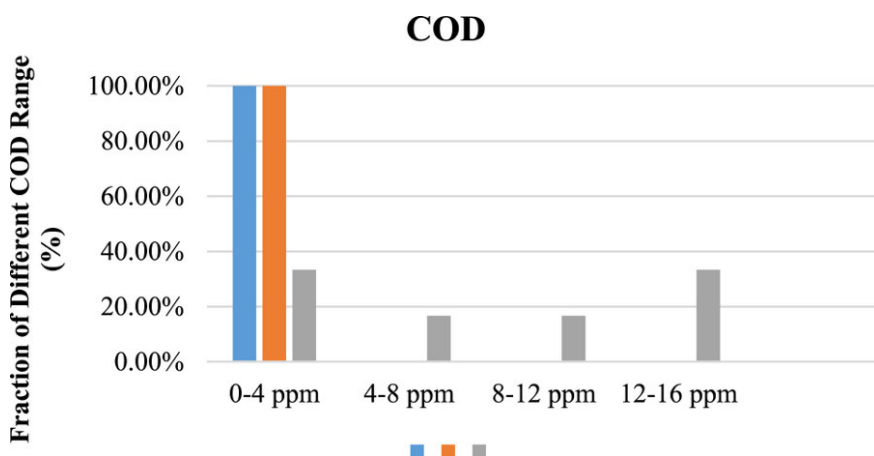


Figure 1.6 COD of collected water samples.

4. Conclusions

Dwellers of DWASA in Jurain area are not only troubled by insufficient water supply but also are asserted to significant threat due to the lack of safe water. The assessed value of water quality of random samples in DWASA's distribution system showed that the water quality of SWTP is satisfactory. Still, in some production tube well, it is not adequate and deteriorated during its flow through the distribution system. The assessed value of pH, turbidity, alkalinity, and hardness was mostly within the permissible range, but BOD₅ and COD value of almost all the selected household samples was significantly higher. No residual chlorine was not founded in the production Pump and household samples. That's indicated, that the water is not capable of disinfection and microbial contamination can easily happen here. So, it needs to be ensured that sufficient free chlorine residual is reaching the user end. Therefore, it can be concluded from the determination of this study that the DWASA should not exclusively depend on its treatment plant's sufficiency. DWASA's piped water supply systems are usually buried in complex reticulations, and they are challenging to operate and maintain. The authority should also lead regular monitoring programs to ward off possible contamination of water along with its distribution network by cross-connections, cross-contamination by leaking pipes, improper domestic storage, and others. Public response was quite interesting, as revealed by the questionnaire survey. People were very much anxious about the quality of drinking water they were consuming, as most of them gave suggestions about the stress of periodical monitoring by the government agencies. The assessed value of this study may help the government and related agencies to take proper remedies regarding appropriate chlorination practices in the area.

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Author's contributions

Joydeb Babu Pranta: Project Design, Conceptualization, Data Curation, Resources, Formal Analysis, Writing-Original Draft; **Protik Chakraborty:**

Methodology, Formal Analysis; **Nazia Hossain:** Supervision, Project Administration and Validation, Writing- Review and Editing.

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Chapter 2: Mass balance, energy balance, and economic analysis of groundwater supply for tannery industry: a case study

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Abstract

About 1000m³ groundwater treatment plant modeling was designed in this study. Merlimau of Malaysia was projected in this case study for groundwater supply to a tannery industry. This project dealt with simulations and calculations of every single detail of the process description, flow sheeting, suitability of tanks and their sizing, mass and energy balance, process control, hazard and operating studies, environmental management, and economic analysis. A specific pump was used to supply raw groundwater to tanks, and different types of water treatment tanks sizing were calculated based on 1000m³ water treatments. Chemical treatment was applied for water softening purposes to make it useable for industrial applications and inhibit microbial activity. The maximum energy, 54.9% of total energy investment was consumed by pumping of raw water from ground, 35.5% and 9.5% energy was for dose pumps, and mixer-flocculator-wash pumps, respectively. Besides, the comprehensive economic analysis of the groundwater plant establishment, yearly process handling related chemical, labor and maintenance cost has been demonstrated in this study. The simultaneous process of this whole 1000m³ groundwater treatments and supply was targeted as a realistic and cost-effective alternative from the hard-impure raw groundwater to soft-purified water for industrial applications.

Keywords

Economical analysis; Energy balance; Groundwater supply management; Integrated design project; Mass balance; Water plant design

1. Introduction

Groundwater is a significant potable and palatable water source that comes from beneath the ground. Two types of water sources: groundwater and surface water are existing in our world. Surface water includes all kinds of water sources on the surface, such as rivers, ponds, lakes, swamps, sea, ocean, and others. Surface water sources have been used for domestic purposes, irrigation, and industrial usages for centuries. Many regions within Asia and African even depend on surface water for water supply in city areas, while the countryside still depends on hand-pump or other types of pumped water from the ground (Hossain et al., 2020; Hossain & Mahmud, 2019; Prasad & Rao, 2018). In contrast, groundwater plays a significant role in water supplies, especially in winter within Europe and America. Many regions of Europe and America continents are solely dependent on groundwater upon divergent purposes such as community service, farming, and industrial uses (Al-Badaii et al., 2013; Hossain et al., 2018; Prasad & Rao, 2018).

For the scenario of commercial groundwater supply, the integrated design of water plant management is considered as the most significant sector. It should be noted that groundwater pumps and required water treatment methods varied for different purposes (Ramaswamy, 2008). Groundwater pumps and treatments were mainly performed for material-based industries such as tannery, glass factory, ceramics, plastic, and non-food-based manufacturing industries based on Malaysian groundwater quality in Malaysia. A Malaysian company involved in tanning process is requiring water supply system for their industrial activities and plant to treat the industrial waste effluent. The tannery is located at a very remote area to avoid public nuisance. However, water scarcity has become an issue to the company since no water supply is available near the area. Hence, an integrated water treatment is designed in order to supply the water into the tannery. Water will be supplied by using groundwater which is available at the area.

1.1. Aim and scope

This case study aimed to design a 1000m³ groundwater plant for water supply to the tannery industry in Merlimau, Malaysia. The comprehensive process elaboration, required tanks for water treatment and specifications, mass and energy balance, operation parameters, environmental and economic factors were simulated and analyzed in this study. Merlimau is located in an industry-based area, Malacca, in Malaysia. Leading chemical,

tannery, and other non-food-based industries were established in this region, and these industries demanded a large amount of water. Overuse of lake and river water caused severe water scarcity in the area. Therefore, groundwater has been highly recommended for industrial applications in the city. Currently, a plant-scale tannery industry has been planned to set up in Marlimau, remote from the urban area. Since the tannery industry demands adequate water supply, water supply management turned as the prerequisite step for this scenario. Hence, water plant design has been an initial measure to be taken. Since the tannery industry was projected to be set up in a plant scale, 1000m³ groundwater plant modeling has been designed initially for this case study.

2. Methodology

2.1. Plant location

The water treatment plant was designed to establish at barren area beside Merlimau Industrial Estate, Merlimau, Melaka state, Malaysia. Merlimau is a town in Malacca, Malaysia and it is about 22km southeast of Melaka City. The remote area of Melaka State, Malaysia, was preferred as a water plant incorporated with tannery industry because this area maintains suitable climate conditions, comparatively less populated and polluted as well as cheaper land cost. Fig. 2.1 presents the location map of Merlimau, and Fig. 2.2 presents the proposed area for the water treatment plant.

2.2. Data collection, process design, and flow sheeting

Process design and data collection is one of the most significant parameters of any case study (Hossain et al., 2019). The process plan of current case study was commanded by a Malaysian company involved in tanning process needing groundwater supply in Merlimau industrial state, Malaysia. The data related plant design, economic, and environmental analysis consisting ground water submerging pump, various types of tanks required for water treatment, mass and energy balance, required equipment and chemicals were assembled from diverse sources water treatment industries' reports and database, groundwater treatment experts, related journal articles, books and book chapters, technical datasheet, manufacturers and supplied, up-to-date websites for equipment and chemical price. All the simulations related tank design, mass and energy balance and economic analysis were analyzed by Microsoft excel.

The operations and technologies in current process modeling were adopted by model water plant design from the previous case study and technical set up in Malaysia as well as integration with the water treatment plant design of the introduction to the environmental engineering book (David, 1998). This study has conducted a comprehensive analysis of each water tank type and sizing from water collection to water supply. The whole process consisted of 5 types of tanks: rapid mixing basin (RM-1), reaction basin (RB-1), settling tank (ST-1), rapid sand filter (RF-1), and storage tank (S'T-1). The comprehensive mass and energy balance of this plant model incorporated the detailed mass and energy input-output through each tank of the process. Table 2.1 presents the parameters for the groundwater quality of the location. The integrated design in this study was designed based

on the treatments of this groundwater. Figs. 2.3 and 2.4 present the operational diagram and flowsheet of the groundwater supply for each tank, respectively. All the equipment to establish the plant and required power consumption for the plant has been simulated. Fig. 2.5 delineates energy distribution of the water plant. Apart from the flow-sheeting and tank design, this study also conducted an inclusive economic analysis for the plant set up and running conditions for the first year of the project, including water supply equipment, chemical costs, labor cost, and others. Overall process control, including piping and instrumentation (PID), hazard and operating studies (HAZOP), and environmental management for the plant, were also emphasized elaborately. Fig. 2.6 outlines the piping and instrumentation diagram of this integrated plant design.



Figure 2.1 Location map of Merlimau.



Figure 2.2 The *red* circle presented the proposed area for the groundwater treatment plant beside Merlimau industrial estate.

3. Results and discussions

3.1. Tanks description and sizing

In this case study, among 5 types of tanks, a rapid mixing basin (RM-1) is the most crucial physical operation affecting coagulant dose efficiency, and the chemical reaction in coagulation is completed in less than 0.1s. Usually, it distributes the coagulant chemicals throughout the water stream. Coagulant aids and enhanced coagulants typically are added to improve the density, and slow-settling flock, as well as help, maintain flock formation (David, 1998). Reaction basin (RB-1) is for the aeration into the water stream. In this groundwater supply management, forced-draft aeration or water flow to environment countercurrent wise has been implemented. To control microbes and eliminate intrinsic gases, air-ratio, and aeration period in the water tank was well-maintained (David, 1998).

Table 2.1

Sample parameter	Concentration (mg)
7.48	pH
28.66	Ca ²⁺
13.75	Mg ²⁺
26.62	Na ⁺
1.35	K ⁺
121.62	HCO ₃ ⁻
17.56	Cl ⁻
0.3	F ⁻
76.63	SO ₄ ²⁻
0.08	Fe ²⁺
0.05	Mn ²⁺
119.5	Total hardness

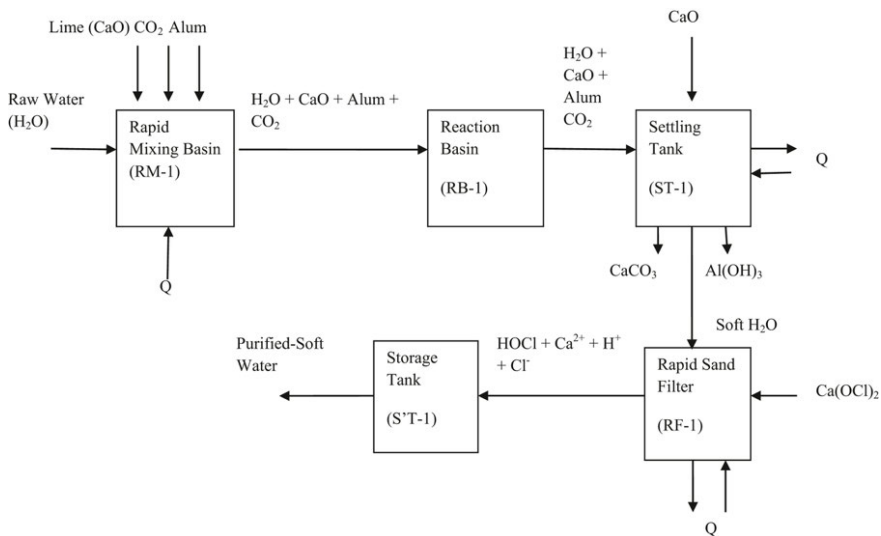


Figure 2.3 Operational diagram of ground water supply.

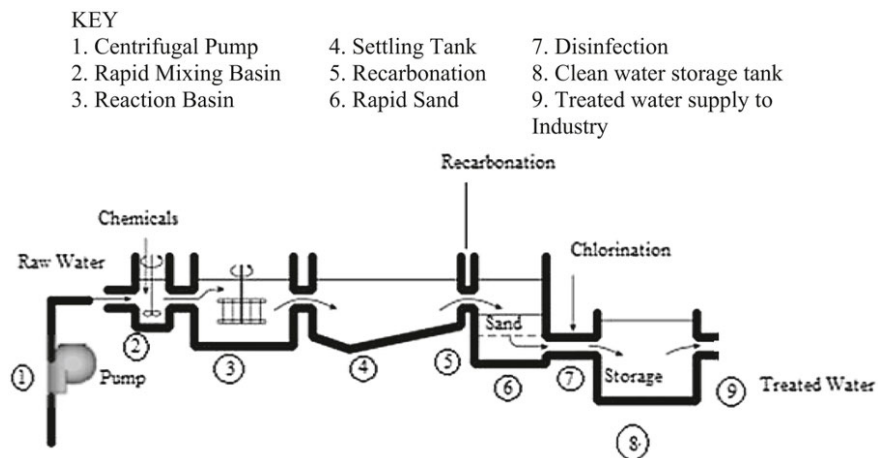


Figure 2.4 Flowsheet of ground water flow.

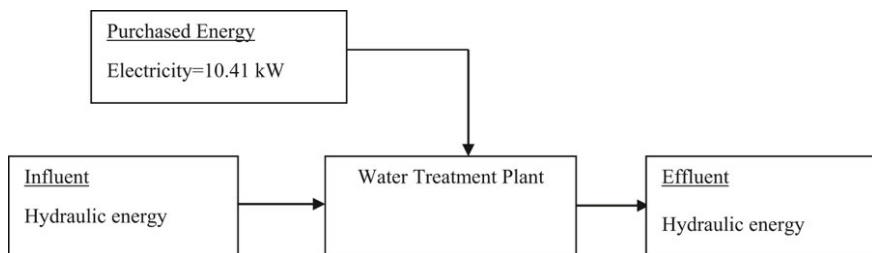


Figure 2.5 Energy distribution of the water plant.

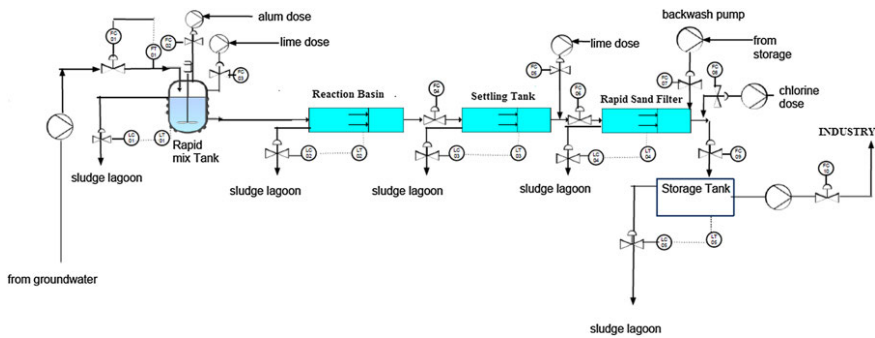


Figure 2.6 Piping and instrumentation diagram.

Settling tank (ST-1) was used to separate settleable and floating constituents. Recarbonation process was initiated immediately after settling step where carbonates were transformed into bicarbonates. This process usually introduces lime-soda ash softening by adding carbon dioxide to turn the solution stable against carbonate-precipitation (Chatzakis et al., 2006). In this project, Lime (CaO) and Alum (Aluminum Sulfate) were used as coagulants. Recarbonation played as a pH controlling role caused this pH change has influenced significant pH change in softened water and reactions in the water. Rapid Sand Filter (RF-1) consists of two types of layer media: sand layer and gravel layer to filter the water.

The upward water and air-flow rate through media enlarged the media bed and detached the solids from the media bed by causing a scrubbing activity. Disinfection was used in water treatment to reduce pathogens (diseases producing microorganisms) to an acceptable level. In this groundwater supply management process, calcium hypochlorite [$\text{Ca}(\text{OCl})_2$] was used as the disinfecting chemical because it is the most common, safe, and cheap disinfecting chemical.

Storage Tank (S'T-1) was used to store soft water for industrial usages. In this project, pipelines set up was one of the most complicated section designed to submerge water from the ground, handle softening processing through water tanks, store and supply water to the industries. Additionally, three different types of pumps were used in this process: centrifugal pump for capture water from underground, dose pumps to imply doses, and water-back pumps to wash tanks (David, 1998).

3.1.1. Sizing

In the groundwater treatment plant, the methods used were manual methods and involved a lot of calculations to determine dimensions. The critical

parameters for manual analysis were the flow rate of water and detention time. These parameters played a vital role in calculating the volume (Eqs. 2.1 and 2.2) needed for each tank (David, 1998). Tanks' types and sizing are mentioned in Table 2.2.

$$V=\frac{Q}{t}(2.1)$$

where Q is Flow rate, m³/s; t is Detention time, s; V is Volume, m³.

Table 2.2

Tanks	Sizing
Square tank Number of tank=2, diameter, D=1.42m, height, H=3m, freeboard=0.16m Impeller Rotational speed=30.45rpm, impeller diameter, Di=0.7m, ratio of Di and D=0.5 Rapid mixer A turbine, 6 flat blade, vanned disc	Rapid mix
Square tank Length=2.5m, width=2.5m, height=3m, freeboard=0.3m, Flocculator Diameter=0.5m, rational speed=39rpm, Baffle Height=2.2m, thickness=0.1m,	Reaction basin
Rectangular basin Number of tank=1, tank length, L=14.3m, tank width, W=7m, tank height, H=7.4m, freeboard=0.7m	Settling tank
Tank (2 units) Length=2.5m, width=2.6m, height=4m, freeboard=0.4m Media Sand depth=0.5m, gravel depth=0.3m	Rapid sand filter
Cylindrical tank	Storage

Number of tank=1, diameter, $D=24.3\text{m}$, height, $H=15\text{m}$, freeboard for 100m^3
--

Free board is the empty space inside the tanks.

$$\text{Dimension, } V = L \times W \times H \text{ (2.2)}$$

where L is Length, m; W is Width, m; H is Height, m.

3.2. Process description and flowsheet

To reduce total water hardness, three types of chemical dose (coagulants) by dose pump were used, and they are Lime dose (CaO), Alum (Aluminum Sulfate), and disinfectant Calcium Hypochlorite $\text{Ca}(\text{OCl})_2$. Alum may react in different ways to achieve coagulation. In this case, aluminum hydroxide $[\text{Al}(\text{OH})_3]$ precipitated forming a “sweep floc” that tended to apprehend total suspended solids as it settled out of suspension. Calcium hypochlorite $\text{Ca}(\text{OCl})_2$ was used as a disinfectant that exited out as CaCO_3 sludge, HOCl , Ca^{2+} , H^- , Cl^+ after refining the raw water. When calcium hypochlorite was added to water, a mixture of ions, and hypochlorous acid (HOCl) was formed (Rc.1) (David, 1998).



This reaction was pH-dependent and virtually complete within a very few milliseconds (David, 1998). The operational diagram and flowsheet showed here how raw groundwater was operated with a coagulant dose and moved from Rapid mix filter to storage treated water.

3.3. Mass and energy balance

3.3.1. Mass balance

Mass balance is also known as material or elemental balance. Mass balance implements the conservation total mass of a system into physical analysis by balancing between total material entering and leaving any system. Mass balance equalized the mass flow in this system as well as identified total mass accumulation, mass loss, and other factors. The total mass balance for each tank was calculated by Eq. (2.3) (David, 1998).

$$M_{in} = M_{out} \quad (2.3)$$

where M_{in} =Mass Input and M_{out} =Mass Output.

Mass Balance in Rapid Mix Tank

$$1000 \text{ m}^3 \text{ water} + 132.6 \text{ kg Lime(CaO)} + 3.3 \text{ kg Alum} = 132.6 \text{ kg Lime(CaO)} + 3.3 \text{ kg Alum} + 1000 \text{ m}^3 \text{ water}$$

Mass Balance in Reaction Tank

$$1000 \text{ m}^3 \text{ water} + 132.6 \text{ kg Lime(CaO)} + 3.3 \text{ kg Alum} = 132.6 \text{ kg Lime(CaO)} + 3.3 \text{ kg Alum} + 1000 \text{ m}^3 \text{ water}$$

Mass Balance in Settling Tank

About 18.1kg Lime was needed for Recarbonation to neutralize pH. As 5% Lime remains in water with dissolved form, so 11.47kg Lime addition with 5% remaining Lime is enough to neutralize pH. In this project, Total water volume=1000m³. So, 0.1% water=1m³ water. Mass balance was followed by Eq. (2.4) (David, 1998).

$$M_{in} = M_{acm} + M_{out} \quad (2.4)$$

where M_{in} =Mass Input, M_{acm} =Mass Accumulation and M_{out} =Mass Output.

$$\begin{aligned} (132.6 + 11.47) \text{ or } 144.07 \text{ kg Lime} + 3.3 \text{ kg Alum} + 1000 \text{ m}^3 \text{ water} &= (125.97 \text{ kg Lime Sludge} + 1 \text{ m}^3 \text{ water}) \\ &+ 3.3 \text{ kg Al(OH)}_3 \text{ sludge precipitated} \\ &+ (18.1 \text{ kg dissolved Lime} + 999 \text{ m}^3 \text{ water}). \end{aligned}$$

Mass Balance in Rapid Sand Filter and Storage

Eighty percent chlorine removal=1kg calcium hypochlorite sludge with dead microbes.

Mass Balance

$$\begin{aligned} 18.2 \text{ kg dissolved Lime} + 1.25 \text{ kg Calcium Hypochlorite} + 999 \text{ m}^3 \text{ water} \\ = (1 \text{ kg Calcium hypochlorite sludge} + 2 \text{ m}^3 \text{ water}) + (0.25 \text{ kg dissolved Calcium Hypochlorite} + 18.2 \text{ kg dissolved Lime} + 997 \text{ m}^3 \text{ water}) \end{aligned}$$

Water Loss or Addition

Ten percent water of total volume or (1000m³ × 0.1)=100m³ water for each tank might be evaporated on sunny days or might be added on rainy

days.

3.4. Energy balance

Groundwater utilities consumed a significant amount of power. Pumping consumed the most massive energy consumption for this water submerge and water treatment plant. Several pump stations have been conducted through a distribution water network for immersing the water from the ground, sending the raw water to the tank for treatment purposes and pumping softened water to the industrial sites. The water pump type, submerging capacity, location set up was designed based on the plant location, and water utility boundaries. Overall, energy consumption was influenced by the desired water treatment standard (David, 1998). For this groundwater treatment plant, 54.9% of energy consumption was for pumping of raw water from ground, 35.5% energy was for dose pumps, and 9.5% energy was for mixer-flocculator-wash pumps. Table 2.3 presents the equipment and power consumption.

3.5. Process control

The implementation of the control system in chemical engineering plays a major role in achieving the desired products with high quality and low cost. Process control acts as an optimization driving tool for any product generation in chemical engineering applications. Usually, “control” is conducted by automated devices like controllers, which are not human-driven manual intervention (Kamen, 1999). However, accurate control is strongly correlated with regular maintenance. For this project, sensors and transmitters were expected to be handled and calibrated time-to-time and their response period was maintained as per the requirement of the system. In this plant, the primary type of control system used is the feedback control system. Feedback control is the control of the output of the process and corrects any sensed deviation from there (Friedland, 1989). The primary drawback of feedback control was projected as the occurrence of disturbance by output variables before the initial response from the control system. So, feedforward control was needed to maintain the pH on set point value since feedforward control is performed as open-loop control that adjusted the process subsequently (e.g., amount of chemical dose) with the difference in any measured variable (e.g., the flow rate). In this water treatment, this control has performed as “pace-to-flow” control initially. The chemical dosing

pump has controlled the stroke length and dosing control frequency for maintaining an adequate dosing system. These functions can be controlled by applying both the control system, feedforward for keeping the plant flow rate and feedback to monitor the effect of chemical doses (Bequette, 2003).

Table 2.3

Equipment	Power consumption (kW)
0.19	Mixer and flocculator
5.72	Submersible pump (ground water)
1.2	Lime dose pump
1.5	Alum dose pump
0.8	Water back-wash pump
1.0	Chlorination pump
$\Sigma=10.41$	

3.6. Piping and instrumentation diagram (PID)

PIDs represented a specialized form of drawing the whole piping and instrumentation process into a documented approach. They were diagrammed based on “in-house rules” on the standard of Malaysia. P&IDs were drawn by chemical engineering drawing software. This process employed two specific symbol systems where one symbol represented processes and other components (e.g., tank, pump, valves) and additional denoted process signaling and overall network. In this process, PID, all PID components were numbered, and they were connected to the water plant maintenance set up (Bequette, 2003). In this water treatment plant, 3 types of controller ad 4 types of sensors were used. Fig. 2.3 presents the PID diagram.

Controllers: Feedback, Feedforward, and Feedback-Feedforward.

Sensors: FC=Flow Sensor, FT=Flow Transmitter, LC=Level Sensor, LT=Level Transmitter.

3.7. HAZOP

HAZOP are the formal systematic and critical examination of the process and engineering facets of a production facility for identifying causes and sequences. In many water treatments plants, chlorine gas is used for disinfection purposes and sometimes as alternative disinfectants; ultraviolet

radiation (UV) and ozone are also used to reduce the need for high amount of chlorine. But chlorine gas is the most hazardous substance for the community, and this has potential accidental release from delivery through the application, so this could be a security hazard as well as UV and ozone are dangerous as the same reason for chlorine gas (Davis, 2010). To avoid this unwanted hazard, in this project, Calcium Hypochlorite [$\text{Ca}(\text{OCl})_2$] was used as disinfectants though it is quite expensive than chlorine gas. For softening purposes, Lime dose (CaO) with 65% granular and Alum dose (Aluminum Sulfate) was used as it more natural an environment-friendly (Davis, 2010). Moreover, it was planned that the working staff would be an expert on maintenance and operational safety program. Preventive care, good housekeeping, and good record-keeping also will be maintained in this project.

3.8. Environmental management

Environmental management has been performed to maintain natural health around the area of this water plant. In this case study, the process maintained the ecological resources and emphasized minimizing any type of pollution like soil or water pollution instead of managing the environment itself (Davis, 2010). The main environmental objectives behind the proper site selection, establishment, and operation of water plants were:

- Sustain the environmental values of groundwater submerge in the ecological aspect, minimize the waste discharges such as inorganic solids, salts, and other chemicals to the environment containing as well as recycle the water purifying chemical components.
- Maintain the site of the groundwater process plant far away from the polluted area.

Minimization of effluent discharge to the ground will protect the plant area from soil degradation, erosion, oversalination, chemical-contamination, and water-logging (Davis, 2010).

3.9. Economic analysis

To carry out an economic analysis, all types of cost and investment must be mentioned. In this case study, water supply equipment capacity and capital cost relationship is shown in Table 2.4. The estimated cost for this project is done for the 1st year with fixed capital investment (FCI) as an

experimental period. The capital investment included the total acquisition of newly set physical properties such as equipment, machinery, goods inventories, plant building set up, and others. FCI included the investment for process-operation completion like installed process equipment, tanks, piping system, and others. It included direct costs and indirect costs (David, 2011). Total capital investment (TCI) was simulated by following Eq. (2.5).

$$\text{Total Capital Investment} = \text{Fixed Capital Investment} + \text{Total Annual Cost} \tag{2.5}$$

Fixed capital cost usually carries equipment capital cost, and the total annual cost is divided into 7 divisions such as total chemical cost, total yearly operational and maintenance cost, total engineering cost, other labor costs, managerial cost, total annual utility cost, and total annual tax. For this case study, the delivered equipment method is used to estimate capital investment. The fraction of the supplied equipment method used for this project is a fluid processing plant. Tables 2.5 and 2.6 represent the total chemical cost and total labor cost, respectively. The total annual cost is shown in Table 2.7. The details of each material, equipment, doses, and others are mentioned and calculated in the economic analysis datasheet. The prices of all chemicals and products are referenced from online shopping application www.Alibaba.com (Engineering and Construction Machinery, 2018).

Table 2.4

Equipment	Material of construction	Capacity measure	Price per item (US\$)	Number of items	Total cost (US\$)
SS	Power=5.72kW	3888.00	1	3888.00	Centrifugal pump (small including motor)
SS	Power=1.2kW	1620.00	2	3240.00	Lime dose pump (small)

SS	Power=1.5kW	1944.00	1	1944.0	Alum dose pump (small)
SS	Power=0.8kW	648.00	1	648.00	Water back-wash pump (small)
SS	Power=1.0kW	972.00	1	972.00	Chlorination pump
CS	Valve diameter (range: 5–12cm)	97.20	10	972.00	Valves
CS	Diameter=0.70m, impeller constant, $K_T=6.3$	129.60	1	129.60	Impeller
SS	Power=0.1W (3rd)	486.00	3	1458.00	Flocculator (including motor)
Brass or SS	Pressure=0–10bar,	113.4	9	1020.60	Flow rate controller
PVC (polypropylene coating)	Size: L×W=3×1mm	32.40	3	97.20	Level controller
SS (brass coating)	Opening size: 92×92×110mm, calibrations=2 points, measurement range=0–14 pH	388.80	1	388.80	pH controller
SS	Space=5cm, size: L×W=3×5m	38.88	1	38.88	Vertical flat bar
CS	Size: L×W=3×2m, opening size=0.5cm ²	145.80	1	145.80	Heavy wear mesh
Sand	Effective size=0.48mm, depth=0.5m,	1502.55	1	1502.55	Sand layer

	uniformity coefficient=1.7, Weight=6.625MT				
Gravel	Depth=0.3m, weight=3.98MT	902.67	1	902.67	Gravel layer
—	—	1200.00	2	2400.00	Computers
—	—	400.0	1	400.0	CCTV
		2000.0	-	2000.0	Software

CS, carbon steel; MT, metric ton; SS, stainless steel.

Table 2.5

Chemicals	Price per kg (US\$)	Total price to treat 1000m³ water (US\$)	Annual price (US\$)
0.30	(271/2) kg×0.30=40.65	3658.50	Dry lime (CaO)
2.40	(1.25/2) kg×2.40=1.50	547.50	Calcium hypochlorite (65% granular)
0.62	3.3kg×0.62=2.05	553.50	Aluminum sulfate (alum)

Table 2.6

Engineering cost		
Engineers	Hourly cost (US\$)	Annual cost (US\$)
55.00	93,600	System/software engineer
40.00	83,200	Application engineer
		Other labor cost
Hourly cost (US\$)	Annual cost (US\$)	Job levels
13.00	30,420	Production supervisor
10.00	23,400	Technician

8.00	18,720	Electrician
3.00	7020	Pipe fitter
3.00	7020	Security guard

Table 2.7

Purposes	Cost (US \$)
4759.50	Total chemical cost
25,212.00	Total annual operational and maintenance cost
176,800.00	Total engineering cost
56,190.42	Other labor cost
124,800.00	Managerial cost
3140.00	Total annual utility cost
400.00	Annual total taxes
391,301.92	Total annual cost

4. Conclusions

This chapter addressed all expected simulations to integrate groundwater pumping, water treatment, and water supply to industry. The estimation for this case study was considered from both the technical and economic point of view. The analyses were based on a rigorous process design that was carried out to assess tank-sizing, the mass-energy balances, and process control of all the tanks of the process. The minimum doses and energy were estimated to imply. The FCI and all operating costs were determined according to up-to-date information, and then economic analysis was carried out. It is expected that with these simulations, the process will be running for the 1st year. If the process gives proper water supply in the 1st year, it might be broadened with a higher volume of water further.

Declarations

Ethics approval and consent to participate

The facts and views in this chapter are solely mine, and I am totally responsible for authenticity, validity, and originality. I also declare that this manuscript is my original work, and I have not copied from anywhere else.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, Nazia Hossain, upon reasonable request.

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Chapter 3: Microbial communications in Gram-positive and Gram-negative bacteria

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Abstract

Bacterial gene expression is regulated by density dependent cellular communication known as quorum sensing (QS) for expression of various virulence factors and secondary metabolites during stress conditions. Both Gram-positive and Gram-negative bacterial species communicate with one another with the help of small signaling molecules. The regulatory machineries that are present in canonical signaling pathway have novel regulatory network designs. The Gram-positive cells mainly communicate with the help of small peptides known as autoinducer peptides or quorum sensing peptides. The Gram-negative groups of bacterial cells communicate by N-acyl homoserine lactone molecules. QS molecules are found not only to maintain interactions between the bacterial species but are associated with the cross talks existing within the host. Various types of peptides and chemical compounds are associated with the bacterial communications. Such communication accentuates competence, virulence, production of antibiotics, sporulation, motility, and formation of biofilm. Interference of QS by stalling signal generation is not very common, although effective quorum quenching can stop this communication.

Keywords

AHL; AIP; Gram-negative; Gram-positive; QIP; Quorum sensing

1. Introduction

Microbial cells possess the ability to live within myriad of stimuli from the environment. They often face harsh conditions owing to various physical changes in the environment like alteration in temperature, pH, depletion of media or chemical changes like presence of antibacterial agents, growth inhibitors, reactive oxygen species, etc (Bonilla, 2020). The bacterial species possess simple mechanism of adapting the environmental fluctuations that includes the use of two component phosphorelay schemes helping the microbial cells in responding to various types of environmental conditions with the activation of specific target genes (Stock et al., 2000). Even there could be other harmful, antagonistic or detrimental species around. To sustain in such a situation, bacteria have developed a survival mechanism by formation of biofilms (Johnson, 2008; Nag et al., 2021). Inside the biofilm bacterial communities are protected by the coating of extracellular matrix (ECM) consisting of three-dimensional network of macromolecules such as collagen, hydroxyapatite, glycoprotein, etc (Kokare et al., 2009). Formation of these biofilms, thereby optimal utilization of the surrounding to grow, multiply and disperse, are closely associated with the cell-to-cell communication system called quorum sensing (QS). The first QS mechanism was found in luminous bacterial strains—*Vibrio fischeri* and *Vibrio harveyi* by Nealson and Hastings (1979). Earlier this system was thought to be closely related to only aquatic organisms. But later, it was found out to be an inherent property of a wide range of organisms that results in synchronous control of gene expression depending on cellular density of the system.

Continuous secretion of stimulants, that is, certain low molecular weight signaling molecules, keeps on accumulating in the surrounding at a basal level. Therefore, high enough population density leads to threshold level of accumulation of signaling molecules that go through interaction with cascade of receptors or transcription factors causing coordinated induction/repression of target genes. These tiny, diffusible stimulants are termed as autoinducers (AIs) (Dickschat, 2010). With the help of these, AIs bacteria perceive the alterations in the environment and change the behavior of the colonies in biofilm community coordinating by controlling gene expression. By virtue of this mechanism, bacteria switch between two distinct states—that is, planktonic state (low cell density (LCD) stage promoting individual and asocial behavior) and sessile state (high cell density (HCD) stage

promoting social or group behavior) (Miller & Bassler, 2001; Oggioni et al., 2006). There are wide range of AIs found in both Gram-positive and Gram-negative bacteria, for example—N-acyl homoserine lactones (AHLs), furanosyl borate diester (AI₂), diketopiperazine (DKP), certain oligopeptides, etc (Dickschat, 2010). However, the inducers for gram positive and gram negative bacteria differ by nature. In Gram-negative bacteria AHLs are the most common inducers having different side chain acyl group based on the species. Most of these inducers are released from the cell and can diffuse through the membrane (Banerjee & Ray, 2017; Lee & Zhang, 2015). However, in case of Gram-positive bacteria, the inducers are peptides (AIPs), formed from precursor oligonucleotides, and hence needed to be secreted from the cell to accumulate outside. Signaling depends on two-component system (consisting of inducer and kinase receptor on the membrane) (Abee et al., 2011). A list of signaling molecules in different organisms is given in Table 3.1.

Understanding of the microbial communication system will help us developing modern therapeutic approach for treatment of infections caused by biofilms or even utilization of various beneficial roles of biofilms efficiently for our betterment. Hence, in this chapter would focus on the existing mechanism of cell-to-cell communication in Gram-positive and Gram-negative bacterial cells and the molecules responsible for such coordination.

Table 3.1

Inducer	Signaling molecule	Receptor system	Organism	References
N-acyl homoserine lactones	LuxI type and LuxR type activator system works in coordination	Gram-negative bacteria such as <i>Vibrio</i> sp., <i>Pseudomonas</i> sp., <i>Actinobacteria</i> , cyanobacteria etc.	Sharif et al. (2008) Polkade et al. (2016) Kirisits and Parsek (2006)	Autoinducer 1
Furanosyl borate	LuxS based	Found in wide	Wang	

diester/DPD	system	variety of Gram-positive and negative bacteria (such as <i>Streptococcus</i> sp).,	et al. (2018)	Autoinducer 2
Catecholamine-like molecules	Interacts with LEE like genes	Gram-negative bacteria such as <i>E.coli</i> , <i>Salmonella</i> , <i>Klebsiella</i> , <i>Vibrio cholarea</i> etc.	Hernandez and Sintim (2020)	Autoinducer 3
Small peptides with thiolactone ring	AgrC-AgrA based system	Found in Gram-positive bacteria <i>Staphylococcus</i> , <i>Enterococcus</i> , <i>Bacillus</i> etc.	Asad and Opal (2008)	Peptides

2. QS in Gram-negative bacteria

As stated already, AHLs are the major type of AIs associated with the mechanism of QS in Gram-negative bacteria. However, it is important to note that the AHL is a generalized term used here. In reality, different chemical forms of AHLs are found in various organisms that could be distinct in nature and hence promote intraspecies communication. Usually there are two types of genes that closely control this communication—I gene and R gene (Abisado et al., 2018). The bioluminescent bacterium *Vibrio fischeri* being the most extensively studied organism in this context, the role of these two genes and the associated ligand (i.e., the signaling molecule) is very well studied. In *Vibrio fischeri*, the genetic circuit of I/R system consists of *luxI* and *luxR* genes (Egland & Greenberg, 1999). *N*-3-oxohexanoylhomoserinelactone (OHHL) is the signaling molecule in this case which is produced by the LuxI protein, a product of the LuxCDABEG operon system (Meighen, 1994). These molecules can easily diffuse through the cell membrane and go outside of the cell. At low concentration of OHHL inside the cell, the translated LuxR proteins degrade rapidly (Visick et al., 2000). Depending on the cell density of the bacteria, at a certain point, the concentration of these released molecules (OHHL) becomes high enough to get diffused back to the cells. As a result, the rediffused OHHL interacts with the LuxR proteins localized in the cytoplasm forming a LuxR-OHHL protein complex which is responsible for bioluminescence as well as bacterial growth. On the other hand, the complex stimulates the production of LuxI, thereby inducing the quorum sensing circuit (Urbanowski et al., 2004). This mechanism gives an idea about the creation of positive feed forward loop of autoinduction when the surrounding environment of the microbial colonies is flooded with the QS signal.

However, the QS mechanism in different organisms could be a little different and quite complex than the above explained one. Molina et al. (2005) found the evidence of a putative AHL synthase gene (*eamI*) and a convergently transcribed *eamR* gene in *Erwinia amylovora* which were revealed to be homologous to *LuxI* and *LuxR* genes respectively (Molina et al., 2005). In *Pseudomonas aeruginosa* system, two different AHL-like molecules participate in QS mechanism. LasI produces *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) which interacts with the LasR receptor. It has been observed that RhII synthase produces *N*-butanoyl-L-homoserine lactone (BHL)

that interacts with RhlI protein. Along with these two, a third chemically distinct molecule 2-heptyl-3-hydroxy-4(1H)-quinolone (also known as *Pseudomonas* quinolone signal i.e., PQS) plays important role. LasR being activated by the signaling molecule successively activates the RhlR and PQS systems (Wilder et al., 2011) (Fig. 3.1).

3. QS in Gram-positive bacteria

Gram positive bacteria, unlike the gram-negative ones, use small autoinducer peptide (AIP) molecules to communicate with each other (Monnet & Gardan, 2015). As peptides cannot diffuse through the cell membrane, an ATP-binding cassette protein also known as ABC transporter plays important role in secretion of the produced peptides. The precursors of the AIPs are produced inside the cell are known as pro-AIPs which are very diverse in sequence and structure. But these pro-AIPs are processed by the transporter proteins to reduce the peptide length up to 5–17 amino acids which are then transported outside the membrane (Verbeke et al., 2017). When the extracellular AIP concentration reaches certain threshold, they are transported back to the cell with the help of another transporter protein where they interact with conserved histidine residues of the associated sensor kinase and autophosphorylate it. This is followed by cascade of phosphorylation and dephosphorylation to response regulators which control the gene expression of the proteins participating in this signaling pathway. This is known as a two-component QS system which auto-induces a feed forward loop. For example, a well-studied two component system is observed in *S. aureus* where agr operon controls the entire communication (Thoendel & Horswill, 2009). In this case, AgrD is the pro-AIP produced by the operon which is processed to AIP by the AgrB transporter and also transported out of the cell. When the AIP concentration in the environment becomes high enough, it is transported back to the cell by AgrC transporter. The response regulator AgrA interacts with the operon and induces production of regulatory RNA named as RNAIII which regulates production of virulence factors as well as formation of biofilms (Thoendel & Horswill, 2010) (Fig. 3.2).

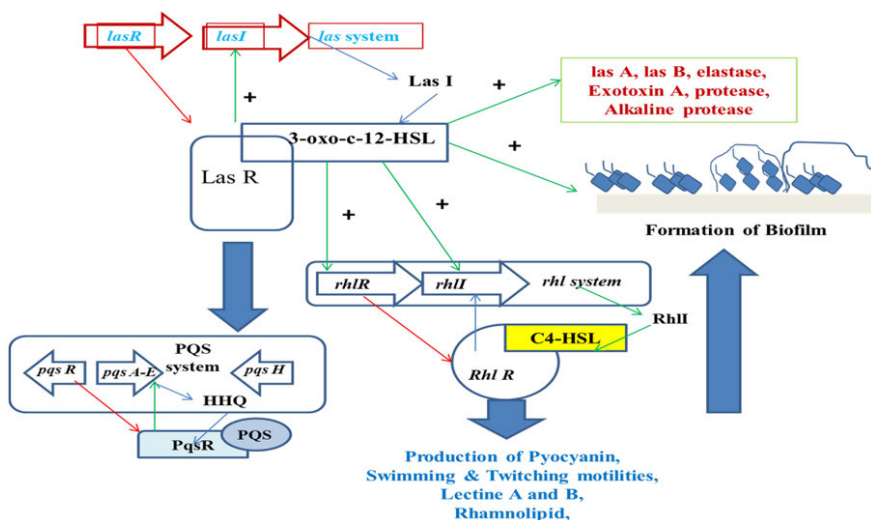


Figure 3.1 Mechanism of QS in Gram-negative bacterial cells.

Another system associated with the gram-positive quorum sensing is RNPP system which is an acronym of the consisting four major receptors—Rap, NprR, PlcR, and PrgX. Rap is a transcriptional activator protein, whereas NprR, PlcR, and PrgX are DNA binding transcription factors. Later with the discovery of Rgg-pheromone system, the acronym has changed to RRNPP. A detail of the various organisms and signaling molecules is noted in Table 3.2.

4. Interspecies communication

By species-specific QS mechanisms bacteria are able to detect self-signals. However, it has been found that most of the times bacteria survive in a polymicrobial community having mixed population. In such a population, bacteria also need to have an interspecies communication system for survival and efficient utilization of nutrients. Interspecies communication in bacteria was first noticed in *Vibrio harveyi*—one of the most studied organisms in the context of quorum sensing. Similar to gram-negative bacteria, *V. harveyi* produces and responds to AHL type Ais (Banerji et al., 2020). But it also possesses certain types of two component signaling system which is analogous to the gram-positive bacteria. AI₂ as mentioned in Table 3.1, can also be found in *V. harveyi*, which participates in interspecies communication. AI-2 (furanosyl borate diester) is produced by the enzyme LuxS. After being produced, the AI-2 signaling molecule reacts with LuxP and LuxQ proteins to relay the signal and control gene expression. S-adenosylmethionine (SAM) pathway requires the involvement of LuxS. When LuxS-type bacteria undergo SAM pathway, a signaling molecule DPD (4,5-dihydroxy-2,3-pentanedione) is generated (Kaur et al., 2018). In non-LuxS bacteria, S-adenosylhomocysteine (SAH) plays the role of producing DPD. DPD being an unstable molecule, the molecule is rearranged in different organisms to give rise to various derivatives of DPD. Distinct DPDs are produced in both *V. harveyi* and *S. typhimurium* suggesting the interspecies communication between these two organisms (Rader et al., 2011). Moreover, LuxS has been found in various organisms like *Bacillus*, *Actinobacillus*, *Clostridium*, *E. coli*, *Haemophilus*, *Helicobacter*, *Salmonella*, *Streptococcus*, *Vibrio*, *Klebsiella* sp. organisms (Kaur et al., 2018). LuxS system of all these organisms is capable of producing signals that can be detected by the AI-2 specific reporter system in *V. harveyi*.

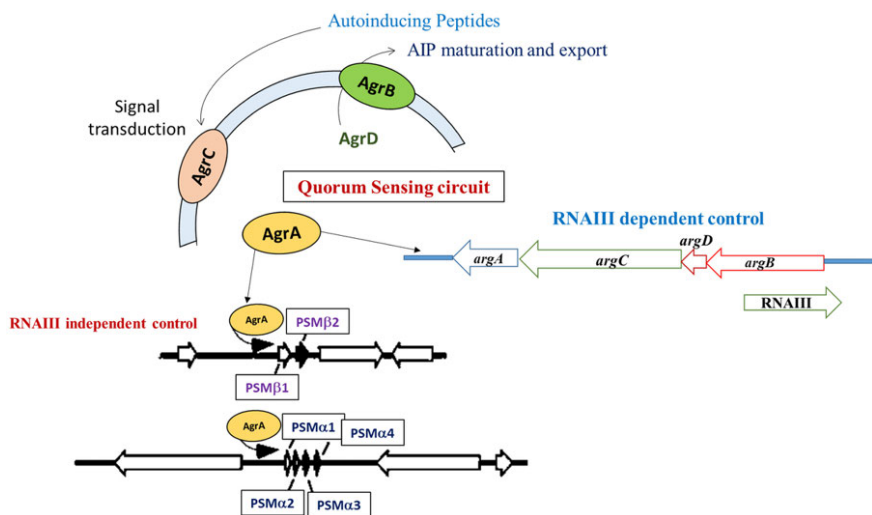


Figure 3.2 Schematic diagram of two-component quorum sensing in Gram-positive bacterial cells.

An interesting and notable thing in this context is the fact that organisms that do not possess LuxS, hence cannot produce AI-2, still can detect the neighboring AI-2 signals. A prominent example of this is *Pseudomonas aeruginosa* which does not have luxS gene but still can detect AI-2 signal. Boron-free AI-2 can be detected by PctA and TlpQ receptors via their dCACHE (double CALcium channels and CHEmotaxis) domain in *P. aeruginosa*. Histidine kinase of the dCACHE domain present in *B. subtilis* and diguanylate cyclase (DGC) present in *Rhodopseudomonas palustris* can also detect AI-2 signal (Zhang et al., 2020).

Table 3.2

System	Receptor proteins	Organism	Brief mechanism	References
AgrC-AgrA	<i>S. aureus</i>	AgrC transporter protein incorporates AIPs and AgrA is response regulator that promotes	Yarwood et al. (2001)	Two component system

		RNAIII which thereby controls the virulence and biofilm forming property of the cell	
<i>B. subtilis</i>	DegS-DegU interaction act as molecular switch. Phosphorylation of DegU promotes transcription of proteases (aprE, nprE) and inhibits SigD. Whereas, unphosphorylated DegU promotes competence development	Msadek et al. (1995)	DegS-DegU
<i>B. subtilis</i> , <i>Bacillus</i> <i>nematocida</i>	Activated by cell density signal. ComX is the AIP that interacts with the kinase receptor ComP activating the ComA transcription factor which thereby controls the expression of various genes	Deng et al. (2013)	ComP-ComA
<i>B. subtilis</i> ,	Pho-regulon	Martín	PhoP-PhoR

<i>Streptomyces lividans</i> , <i>Streptomyces coelicolor</i> , <i>M. tuberculosis</i>	genes are induced when PhoR kinase interacts with PhoP. Under phosphate starvation, this activity leads to regulation of <i>tuaA</i> promoters and synthesis of teichoic acid for cell wall	(2004)	
<i>Enterococcus faecalis</i>	A cyclic peptide GBAP acts as the AIP transported by FsrC. FsrA acts as a response regulator that controls expressions of multiple <i>fsr</i> genes and regulates virulence.	Ali et al. (2017)	Fsr system
<i>Xanthomonas campestris</i>	Signal DSF is detected by the RpfC leading to the activation of response regulator RpfG which regulates virulence by degrading cyclic-di-GMP and expressing the global regulator clp	He et al. (2009)	RpfC-RpfG

System	Receptor proteins	Organism	Brief mechanism	References
	<i>Xanthomonas campestris</i>	As a response to low oxygen tension, this system is activated. It works similar to the Rpf receptors and causes regulation of virulence	He et al. (2009)	RavS-RavR
	<i>Enterococcus faecalis</i>	Cytolysin acts as an AI which gets incorporated into the cell by transmembrane protein cylR1 interacting with cylR2 DNA binding domain, resulting in regulation of signaling cascade and gene regulation	Haas et al. (2002)	cylR1 and cylR2
Rap	<i>Bacillus</i> sp.	Rap receptors dephosphorylate SpooF-P to regulate sporulation. Some Rap receptors inhibit competence development by preventing the	Neiditch et al. (2017)	RRNPP system

		DNA binding activity of ComA protein. RapI interacts with ImmR, thereby regulating ICEBS ₁ excision		
<i>Streptococcus</i> , <i>Lactobacillus</i> etc	Rgg interacts with Shp2 and ShP3 followed by series of transcription activation and repression regulation of surface characteristics, virulence, and cyclic peptide production depending on the organism. It may also interact with ComR causing transcription activation sigX sigma factor leading to genetic competence.	Rgg		

Table Continued

System	Receptor proteins	Organism	Brief mechanism	References
	<i>Bacillus cereus</i> group	NprR binds to the NprX signal leading to		Npr

		regulation of some nutrient clearance genes and sporulation	
	<i>Bacillus</i> and <i>Lactobacillus</i> groups	In presence of PapR pheromones PclR undergoes confirmational changes to control transcriptional activation of virulence genes	PclR
	<i>Enterococcus</i> group	PrgX binds to both iCF ₁₀ and cCF ₁₀ . The PrgX-iCF ₁₀ complex causes blocking of RNA polymerase. Whereas, PrgX-cCF ₁₀ promotes transcription.	PrgX

5. Inter-kingdom communication

Bacteria not only interact with other bacteria, but also can communicate with organisms of another kingdom. There are various host-bacteria interactions that could be beneficial, that is, symbiotic or pathogenic in nature. It has been found that the QS molecules can significantly participate in these interkingdom interactions also, increasing the diversity of microbial communication. In our discussion, we focus on interaction with three major kingdoms—fungi, plant and mammals (humans):

5.1. Bacteria-fungi communication

One of the most closely studied bacteria-fungi interactions is between *P. aeruginosa* and *Candida albicans*. Both the organisms secrete QS signals and compete with each other (Padder et al., 2018). Transformation from mycelial stage to the hyphal form occurs in dimorphic fungi through the filamentation process. Cis-2-decenoic acid produced by *P. aeruginosa* inhibits the filamentation process of the fungi and causes biofilm dispersion. On the other hand, *C. albicans* can synthesize farnesol that inhibits the PQS system in *Pseudomonas* sp. and can also inhibit *S. aureus* biofilm formation (Hornby et al., 2001). Another such interaction is observed between fungus *Penicillium expansum* and bacteria *Acinetobacter calcoaceticus* and *Methylobacterium oryzae*. The fungus in this case produces patulin and both the bacteria produce *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C₁₂-HSL) an AI associated with LasI/R system. Afonso et al. (2020) has showed that patulin inhibits the growth of the bacteria, whereas HSL inhibits the growth of the fungus. *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C₁₂-HSL) is an AI associated with LasI/R system.

5.2. Bacteria-plant communication

Plants are constantly colonized by soil bacteria and therefore, the bacteria-plant communication is quite evident. Many of these interactions involve QS autoinducers that help bacteria infecting the plant but also can help the plant in various physiological processes like growth, nutrient accumulation, surviving oxidative stress, synthesis of various hormones and important metabolites etc. AIs 3-oxo-C₁₂-AHL and 3-oxo-C₁₆-AHL were when applied on to the roots of *M. truncatula* altered expression of more than 150 proteins dealing with oxidative stress, flavonoid and phytohormone metabolism (Zaytseva et al., 2019). Similar kind of effect was also observed on

Arabidopsis thaliana with 3-oxo-C8-AHL. The AI altered the protein accumulation level and increased the oxidative stress resistance ability of the plant. 3-oxo-C6-AHL was found to be involving in gene expressions of auxin and cytokinin pathway (Mathesius et al., 2003). 3-oxo-C14-AHL strengthens the cell wall of the plant cells by increasing the phenolic compound, cellulose and lignin content. With the help of DNA microchip technology, it was found out that when QS molecules are applied on the seedlings, it results into several hundred transcriptional level alteration of gene products (Zhao et al., 2016). Palmer et al. (2016) has showed rhizobia-legume interkingdom mutualism responsible for nodulation number and rate of nodulation.

5.3. Bacteria-higher eukaryote communication

AHL type autoinducers in mammalian cells involve in diverse functions in signaling pathways starting from calcium mobilization, activation of Rho GTPases, MAPK, to NF κ B. 3O-C₁₂-HSL produced by *P. aeruginosa* interact with p38 and p42/44 MAPK to disrupt cell junction associations leading to decrease in membrane integrity and increase in permeability (Vikstrom et al., 2006). AHLs cause various Rho family proteins—GTPases, Rac, Cdc42, etc. epithelial cell migration and reorganization of cytoskeleton. AHLs are also potent chemo-attractants for neutrophils and macrophages. *E. coli* can also detect and utilize small signaling molecules like epinephrine (Epi) or norepinephrine (NE). Enterohemorrhagic *E. coli* contains QseA regulating locus of enterocyte effacement abbreviated as LEE Pathogenicity Island. Along with QseBC this results in flagella activation and motility. LuxS mutant *E. coli* are unable to express *LEE* genes. But presence of Epi and NE at very high physiological concentration causes expression of *LEE* genes in the LuxS mutant bacteria (Hughes & Sperandio, 2008). Cells of *P. aeruginosa* can communicate with human cells using the small QS that will lead to a behavioral changes of human immune cells like phagocytic and major antigen processing cells that inturn help them to evade host immune response (Holm & Vikström, 2014).

6. Analytical techniques for detection of the QS molecules

6.1. Biosensor based techniques

Detection of various chemical species (biomarkers)—autoinducers, signaling peptides, regulatory proteins, etc. is crucial to study the quorum sensing mechanism in bacteria. Biosensors developed for this purpose can broadly be of two types—bacterial cell-mediated sensors and cell-free biosensors. The underlying mechanism of detection depends on UV-visible signal, fluorescence, and bioluminescence produced by the system (Steindler & Venturi, 2007). Fletcher et al. (2007) developed a *P. aeruginosa*-based assay for the detection of QS signal quinolones such as 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-heptyl-4-quinolone (HHQ). Upon interaction with the signaling quinolones, green pigment pyocyanin is produced giving rise to bioluminescence. As the biosensor bacteria are modified to not synthesize the AIs, they remain dark in color (Fletcher et al., 2007). Similar *P. aeruginosa* based biosensors are also being designed for detection of bacterial infections in urinary tract by using QS biomarker AHL. Functionalized ZnO-cysteine nanoparticles were used by Vasudevan et al. (2020) for real-time detection of photoluminescence with enhanced sensitivity and specificity. Recently, a synthetic biology method for cell-free protein expression was used to study cystic fibrosis lungs. The sputum samples contain the biomarker AI 3-oxo-C₁₂-HSL that was detected by a modular DNA-encoded biosensor. A specially designed plasmid pSB1A2:LasRV which continuously produces LasR protein that binds to the promoter LasRV. When this LasR interacts with the AHL in the environment, the promoter becomes free to synthesize a green fluorescence protein (GFP) which is easily detected by the fluorescence detectors (Wen et al., 2017).

6.2. Chromatography-based techniques

Sophisticated chromatography techniques can also be used for sensing QS molecules. Thin-layer chromatography (TLC) is a well-known process to use for the detection of AHLs. An *E. coli* biosensor was developed that utilizes the plasmid containing LuxR tagged with GFP for reporting. The GFP was overlaid on TLC silica sheets for detection of the fluorescence (Charlesworth et al., 2015). The biofilm formation and quorum sensing in activated sludge of waste water treatment can be detected by LC-MS/MS. Utilizing this method, three specific type of AHLs—C₄, C₆, and C₈-HSL

were studied by Sun et al. (2018) where they discovered the distinct distribution of AHLs in the treatment system. C₄-HSL was found to be predominantly present in the aqueous phase, whereas, C₆ and C₈-HSLs were preferentially distributed throughout the sludge phase (Sun et al., 2018). With the help of a hybrid gas chromatography and electron ionization mass spectrometry (GC/EI-MS), selective separation of the QS molecules as well as rapid detection of involved AHLs can be performed as shown by Cataldi et al. (2004) while working with *Burkholderia cepacia* strains. However, a modified LC-MS isotope dilution detection technique was introduced by May et al. where prior to analysis of the sample they introduced isotope-labelled internal standards which reduced the experimental requirements for constructing external calibration curve as well as improved the sensitivity of detection (Cataldi et al., 2004). Use of supercritical-fluid chromatography (SFC) coupled with high-resolution mass spectrometry (HRMS) can be used to simultaneous detection and quantitation of wide range of autoinducers. Hoang et al. (2020) detected 30 AHLs of *Paraburkholderia* sp. in a single run in few minutes along with picogram level of sensitivity.

6.3. Other techniques

Chromatography and the cell-based or cell-free biosensors are the two widely used methods for studying QS. However, there are few other methods that have been developed by scientists to detect the QS involved analytes. Among these other methodologies surface enhanced Raman scattering spectroscopy (SERS) is quite popular. This ultrasensitive analytical technology can be utilized for noninvasive and label-free detection of molecules. It can detect plasmonic nanostructures giving rise to characteristic curves. Bodelón et al. (2016) detected the heterocyclic pyocyanin—the interspecies communication analyte in *P. aeruginosa* using this method. Similarly, in-situ measurement of C₄ and C₆-AHL was successfully carried out by Pearman et al. (2007) by SERS (Pearman et al., 2007). On the other hand, a sensitive as well as cost effective method of detection would be to use ELISA to perform antibody-antigen interaction and link it to a chromogen that can be calorimetrically detected. However, raising monoclonal antibodies against the specific quorum sensing molecule or peptide is time consuming. Therefore, predeveloped monoclonal antibodies are required to perform the assay. Chen et al. (2010) used *Burkholderiacepacia* supernatant to detect 3-oxo-C₁₀-HSL by ELISA method. Another approach is to use

voltammetry for electrochemical detection of the signaling molecules. Simultaneous analysis of the QS analytes pyocyanin, PQS and HHQ was detected by Buzid et al. (2016) with Boron-doped diamond electrode. They performed differential pulse voltammetry scans for the detection of the AIs (Buzid et al., 2016).

7. Blocking of communication by forestalling QS

As a mechanism to resist the pathogenicity of bacteria, various other microbes, plants and animals have developed systems to block QS by interfering with the QS signaling pathway or generation of AIs. It has huge significance in medical field because these blocking agents or QS inhibitors (QSIs) have therapeutic effect and hence, can be used as a treatment strategy to fight against microbial infection, various diseases, and contamination. These compounds are also studied for development of modern drugs for the treatment of antibiotic resistance. Comprehensive lists of QS blocking agents or QSIs have already been reviewed by Asfour (2018), Hemmati et al. (2020) and Santhakumari and Ravi (2019). Few of these blocking agents (QSIs) are chemical compounds that target various proteins or transcription factors involved in QS signaling pathway. However, there are few enzymes that also work on reducing or inhibiting the production of QS signaling molecules thereby leading quenching of the QS signal. These are called quorum quenchers (QQs). Here, we try to provide a simple classification of the blocking agents based on their type for a brief overview on the various commonly found QS inhibition strategy (Table 3.3).

Table 3.3

Class	Inhibitor molecule	Inhibited organisms	Inhibition mechanism	References
Halogenated furanones	<i>P. aeruginosa</i>	Targets las QS circuit to repress lasB; reduces production of virulence factor; Mimiks AHL and competes with the AHL for binding site	Hentzer et al. (2002)	Furanone derivative
<i>Aeromonas hydrophila</i>	Ponnusamy et al. (2010)		2(5H)-furanone	
<i>P. aeruginosa</i>	Proctor et al. (2020)		(5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone	
RIPs (RNAIII inhibiting)	<i>S. aureus</i>	Interacts with TRAP in agr to	Gov et al. (2001)	RNAIII inhibitor

peptides); Rap binding peptides		inhibit RNAIII; reduces bacterial adherence to biotic and abiotic surfaces; inhibit Rap		
Yang et al. (2003)			Rap binding peptides	
Lactonase	<i>B. thuringiensis</i> , <i>P. aeruginosa</i> ; <i>Staphylococci</i>	Cleaves the lactone ring causing degradation of AHL	Anandan and Vittal (2019)	Autoinducer degrading enzymes
<i>Anabaena</i> sp.	Hydrolyses acyl chain to degrade AHL	Romero et al. (2008)	AHL acylase	
<i>P. aeruginosa</i>	Degrades 3-oxo-C12-HSL	Teiber et al. (2008)	Paraoxonase	
Microlides	<i>P. aeruginosa</i>	Suppresses lasB expression; reduces production of virulence factor	Hoffmann et al. (2007)	Antibiotic
<i>P. aeruginosa</i>	Targets AHL production; reduces virulence	Husain and Ahmad (2013)	Tetracycline	
Many plant based secondary metabolites like essential oils, alkaloids, flavonoids, saponin etc.	Works on diverse group of organisms	Interacts with various proteins associated with QS signaling pathway	Lahiri et al. (2019)	Phytocompound:

8. Conclusion

The process of chemical communication among the bacterial cells via the process of QS helps in the sustenance of the microbial species in adverse environmental conditions. The mechanism of QS helps in orchestrating the collective behavior of the microorganisms dwelling within the biofilm. The production of specific type of AIs is the major event in the mechanism of QS. This process also controls the formation of biofilm along with the development of virulence and multidrug resistance among the organisms. Being a unique feature, the process of QS helps in distinguishing one type of the bacterial cells from the other.

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Further reading

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Chapter 4: Microbial enzymes: a futuristic tools for a sustainable environment in 21st century

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Abstract

Since the most recent couple of many years, the wanton and uncontrolled utilization of oil and unified items prompted the aggregation of immense poison in the earthbound and marine climate. It raised the danger of ecological contamination and environmental change. The worry emerges more because of the wild and informal removal of toxin containing high subatomic weight, high harmfulness, and intricacy, which are hard to debase. Subsequently, the center is currently paid to look for proficient, eco-accommodating, ease squander the executive's innovation. Current microbial catalyst is just advanced apparatuses for maintainable climate in 21st century. An enormous number of enzymes from microbes, fungi, and algae have been accounted for to be engaged with the biodegradation of poisonous toxins. Bioremediation is a financially savvy and nature amicable biotechnology that is controlled by microbial catalysts. The examination movement around there would contribute toward creating progressed bioprocess innovation to decrease the poisonousness of the toxins and furthermore to acquire novel valuable substances. This book chapter part will talk about these issues, pinpointing explicit new advances in biocatalyst improvement, notwithstanding drawing the consideration of the biotechnology local area to the dynamic pursuit and find recently ecological biocatalyst, for bioremediation with novel green cycles.

Keywords

Bioremediation; Biotechnology; Green process; Microbial enzymes; Pollutant

1. Introduction

The biocatalytic capability of microorganisms has been utilized for quite a long time to deliver bread, wine, vinegar, and other regular items without understanding the biochemical premise of their fixings. Microbial enzymes have acquired interest for their far and wide uses in enterprises and medication inferable from their dependability, reactant action, and simplicity of creation and improvement than plant and creature chemicals. The utilization of compounds in different businesses (e.g., food, horticulture, synthetic substances, and drugs) is expanding quickly because of decreased handling time, low energy input, cost-viability, nontoxic and eco-accommodating qualities (Wu et al., 2021; Yang et al., 2021). Microbial enzymes are fit for debasing poisonous synthetic mixtures of modern and homegrown squanders (phenolic compounds, nitriles, amines, and so forth) either by means of corruption or transformation. Before, squanders were generally discarded by burrowing an opening and filling it with squander material. This method of garbage removal was hard to support inferable from the absence of another spot each an ideal opportunity to dump. New advancements for garbage removal that utilization high-temperature burning and compound decay (e.g., base-catalyzed dechlorination, UV oxidation) have developed. In spite of the fact that they can be powerful at lessening wide a scope of toxins yet at the same time have a few disadvantages. The related lacks in these strategies have centered endeavors toward tackling cutting edge bioremediation measure as a reasonable other option (Bhandari et al., 2021; Karigar & Rao, 2011) (Fig. 4.1).

Bioremediation is a microorganism-interceded change or debasement of pollutants into nonhazardous or less dangerous substances. The employability of different living beings like bacteria, fungi, algae, and plants for proficient bioremediation of contaminations has been accounted for (Bhandari et al., 2021). Bioremediation, a biotechnological interaction, is an organically interceded change in contaminations worried about reestablishing the recalculated climate and overseeing poisons by detoxification and mineralization (Karigar & Rao, 2011). The remediation of persistent organic pollutants (POPs) utilizing microbial chemicals is viewed as climate cordial, savvy, creative, and promising. Be that as it may, the bioremediation interaction has got a few constraints. It is a sluggish cycle, thus far, a couple of types of microorganisms fit for delivering explicit enzymes have shown

their strong capacity to corrupt contaminations (Chakraborty & Das, 2016; Gaur et al., 2018). Microorganisms are conveyed in the biosphere because of their metabolic capacity and are handily developed under a wide scope of natural conditions and produce chemicals. Different enzymes from high-impact microbes, for example, *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium* have regularly corrupted pesticides and hydrocarbons. Interestingly, compounds from anaerobic microscopic organisms have been utilized for bioremediation of polychlorinated biphenyls, dechlorination of trichloroethylene, and chloroform. As the increment of debased destinations represents a significant natural and human medical condition, it seems compulsory to sterilize the climate and to execute proficient purification techniques. The fundamental objectives of disinfecting ought to be the recuperation of soil wellbeing and richness, detoxification of ground water, reutilization of wastewater (mostly in nations with extreme water insufficiency), evacuation of adverse consequences on human and creature wellbeing, and creation of sound air. Thought of the current innovation for the expulsion of contaminations unmistakably shows that physical and synthetic techniques are regularly uneconomical. In any case, natural treatment innovation may offer an answer for this issue (Arslan et al., 2017).

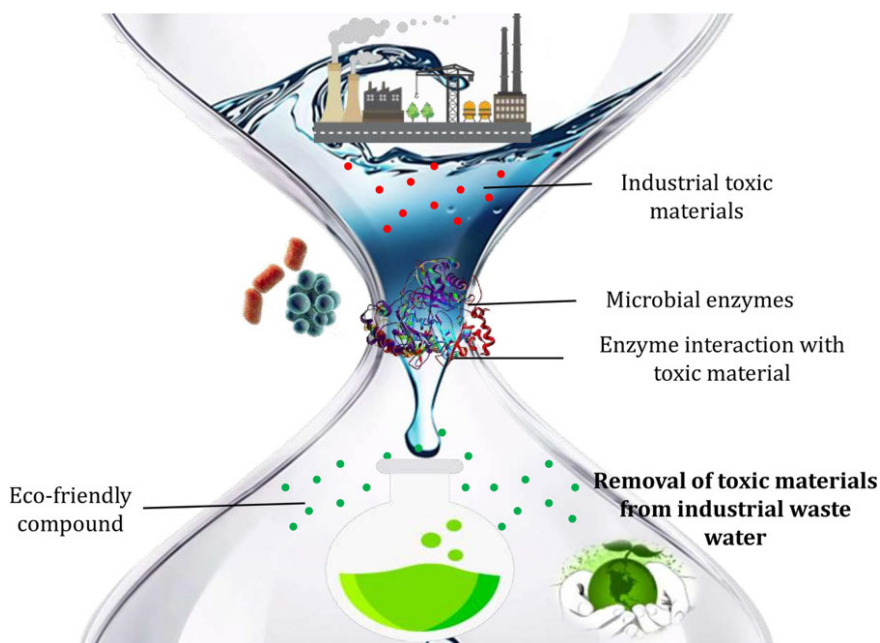


Figure 4.1 Bioremediation of industrial toxic material through

microbial enzymes.

The world relies upon oil. A tremendous sum is utilized, shipped, prepared and put away all throughout the planet. In 2003, the absolute world utilization of oil was over 13.1 billion liters each day. The United States Energy Information Administration projects world utilization of oil to increment to 98.3 million barrels each day ($15.63 \times 10^6 \text{ m}^3 \text{ day}^{-1}$) in 2015 and 118 million barrels day^{-1} ($18.8 \times 10^6 \text{ m}^3 \text{ day}^{-1}$) in 2030 (Varjani, 2017). With such intense usage, oil slicks are inescapable. Bioremediation is a micro-organism-inter-vened change or corruption of toxins into nonhazardous or less unsafe substances. The employability of different living beings like microorganisms, growths, green growth, and plants for proficient bioremediation of poisons has been accounted for many years. Borah and Yadav (Borah & Yadav, 2017) announced that bioremediation gives the most financially savvy and eco-accommodating estimations for the remediation of petrol defiled soil and water to bring back its local climate. Compound interceded measures are quickly acquiring interest due to decreased interaction time, admission of low energy input, financially savvy, nontoxic, and eco-accommodating attributes (ock Joo et al., 2015). Additionally, with the approach of recombinant DNA innovation and enzyme designing an organism can be controlled and refined in huge amounts to satisfy expanded need (Liu et al., 2013). Compounds are organic particles, enzymes in nature except for synergist RNA atoms (ribozymes), and go about as impetuses to help practically the entirety of the synthetic responses needed to support life. Catalysts are profoundly explicit; just speed up the pace of a specific response by bringing down the initiation energy without going through any perpetual change in them, and thus, are crucial biomolecules that help life (Singh et al., 2008; Stanzone et al., 2020).

2. Enzymes

2.1. Introduction to enzymes

Enzymes are organic impetuses that work with the transformation of substrates into items by giving good conditions that bring down the actuation energy of the response. A catalyst might be an enzyme or a glycoenzyme and comprises of at any rate one polypeptide moiety (Nelson & Cox, 2004).

2.2. Enzyme nomenclature

Enzyme names apply to a solitary synergist element, instead of to a progression of exclusively catalyzed responses. Names are identified with the capacity of the enzyme, specifically, to the sort of response catalyzed (Nelson & Cox, 2004).

2.3. Enzyme classification

A definitive recognizable proof of a specific chemical is conceivable through its catalyst bonus (E.C.) number. The task of E.C. numbers is depicted in rules set out by the International Union of Biochemistry. All realized enzymes can be categorized as one of these six classes. The six fundamental divisions are the oxidoreductases, the transferases, the hydrolases, the lyases, the isomerases, and the ligases (synthetases). Oxidoreductases catalyze the exchange of electrons and protons from a giver to an acceptor. Hydrolases work with the cleavage of C–C, C–O, C–N, and different bonds by water. Lyases catalyze the cleavage of these equivalent bonds by disposal, leaving twofold bonds (or, in the opposite mode, catalyze the expansion of gatherings across twofold bonds) (Nelson & Cox, 2004) (Fig. 4.2).

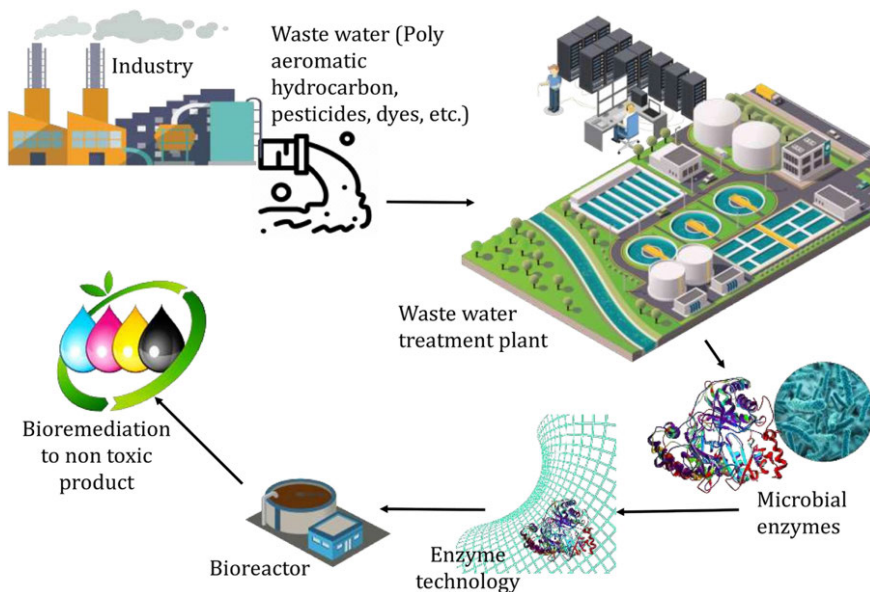


Figure 4.2 Schematic view of application of microbial enzymes as bioremediation.

3. Microbial enzymes in bioremediation

The detoxification of harmful natural mixtures by different microorganisms, growths, and higher plants through oxidative coupling is intervened with oxidoreductases. Microorganisms extricate energy through energy-yielding biochemical responses interceded by these enzymes to separate synthetic securities and to help the exchange of electrons from a diminished natural substrate (benefactor) to another substance compound (acceptor). The oxidoreductases take part in the humification of different phenolic substances that are delivered from the deterioration of lignin in a dirt climate. Similarly, oxidoreductases can likewise detoxify poisonous xenobiotics, for example, phenolic or aniline compounds, through polymerization, copolymerization with different substrates, or restricting to humic substances (Bhatt, 2019). Numerous microbes diminish the radioactive metals from an oxidized solvent structure to a decreased insoluble structure. During the cycle of energy creation, the bacterium takes up electrons from natural mixtures and utilizes radioactive metal as the last electron acceptor. Some the bacterial species lessen the radioactive metals in a roundabout way with the assistance of a transitional electron giver (Sharma et al., 2018).

Chlorinated phenolic compounds are among the most plentiful unmanageable squanders found in the effluents produced by the paper and mash industry. These mixtures are delivered upon the incomplete corruption of lignin during the mash blanching measure. Numerous parasitic species are viewed as reasonable for the expulsion of chlorinated phenolic compounds from debased conditions. The movement of organisms is fundamentally because of the activity of extracellular oxidoreductase catalysts, as laccase, manganese peroxidase, and lignin peroxidase, which are delivered from parasitic mycelium into their close by climate. Being filamentous, parasites can arrive at the dirt contaminations more successfully than microorganisms (Karigar & Rao, 2011; Vermelho et al., 2012) (Fig. 4.3).

3.1. Microbial oxygenases

Oxygenases have a place with the oxidoreductase gathering of enzymes. They take part in the oxidation of diminished substrates by moving oxygen from subatomic oxygen (O_2) using FAD/NADH/NADPH as a cosubstrate. Oxygenases are gathered into two classifications; monooxygenases and dioxygenases based on various oxygen atoms utilized for oxygenation. They assume a critical part in the digestion of natural mixtures by expanding their

reactivity or water dissolvability or achieving cleavage of the sweet-smelling ring. Microorganisms remove energy through energy-yielding biochemical responses intervened by these enzymes to sever synthetic securities and to help the exchange of electrons from a diminished natural substrate (contributor) to another substance compound (acceptor). During such oxidation-decrease responses, the pollutants are at last oxidized to innocuous mixtures (Sivaperumal et al., 2017; Skinder et al., 2020). The oxidoreductases take an interest in the humification of different phenolic substances that are delivered from the deterioration of lignin in a dirt climate. Similarly, oxidoreductases can likewise detoxify harmful xenobiotics, for example, phenolic compounds, through polymerization, copolymerization with different substrates, or restricting to humic substances (Park et al., 2006).

Numerous microorganisms diminish the radioactive metals from an oxidized dissolvable structure to a decreased insoluble structure. During the interaction of energy creation, the bacterium takes up electrons from natural mixtures and utilizes radioactive metal as the last electron acceptor. Some bacterial species diminish the radioactive metals by implication with the assistance of a halfway electron contributor. At long last, precipitant can be viewed as the consequence of redox responses inside the metal-diminishing microscopic organisms (Leung et al., 2010). Numerous parasitic species are viewed as reasonable for the expulsion of chlorinated phenolic compounds from tainted conditions. The movement of growths is basically because of the activity of extracellular oxidoreductase enzymes, as laccase, manganese peroxidase, and lignin peroxidase, which are delivered from contagious mycelium into their close by climate. Being filamentous, growths can arrive at the dirt poisons more adequately than microbes (Rubilar et al., 2008).

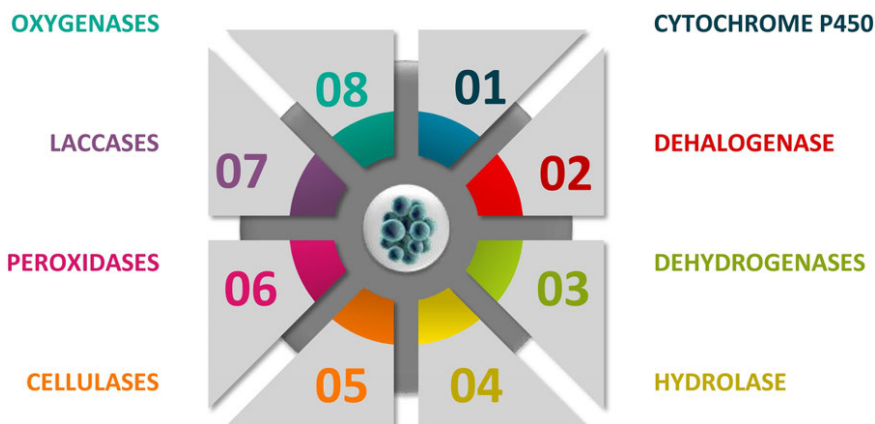


Figure 4.3 List of different microbial enzymes used for bioremediation.

3.1.1. Monooxygenases

Monooxygenases consolidate one atom of the oxygen atom into the substrate. Monooxygenases are grouped into two subclasses dependent on the presence of cofactor: flavin-subordinate Monooxygenases and P450 monooxygenases. Flavin-subordinate Monooxygenases contain flavin as a prosthetic gathering and require NADP or NADPH as a coenzyme. P450 Monooxygenases are heme-containing oxygenases that exist in both eukaryotic and prokaryotic creatures (Urlacher & Eiben, 2006). Most of monooxygenase concentrated beforehand is having a cofactor, yet there are sure Monooxygenases that work freely of a cofactor. These catalysts require just subatomic oxygen for their exercises and use the substrate as a lessening specialist. The desulfurization, dehalogenation, denitrification, ammonification, hydroxylation, biotransformation, and biodegradation of different fragrant and aliphatic mixtures are catalyzed by Monooxygenases. These properties have been investigated as of late for significant application in biodegradation and biotransformation of fragrant mixtures (Wei et al., 2018). Methane mono-oxygenase chemical is the best described one, among monooxygenases. This catalyst is engaged with the debasement of hydrocarbons like subbed methanes, alkanes, cycloalkanes, alkenes, haloalkenes, ethers, and sweet-smelling and heterocyclic hydrocarbons (Coleman et al., 2011; Fuentes et al., 2014). Under oxygen-rich conditions, mono-oxygenase catalyzes oxidative dehalogenation responses, while, under low oxygen levels, reductive dechlorination happens. Oxidation of substrate can prompt dehalogenation because of the development of labile

items that go through ensuing synthetic decay (Pimviriyakul et al., 2017).

3.1.2. Dioxygenases

Dioxygenases are multicomponent chemical frameworks that bring atomic oxygen into their substrate. Sweet-smelling hydrocarbon dioxygenases have a place with an enormous group of Rieske nonheme iron oxygenases. These dioxygenases catalyze enantio explicitly the oxygenation of a wide scope of substrates. Dioxygenases principally oxidize sweet-smelling compounds and, in this manner, have applications in ecological remediation. All individuals from this family have a couple of electron transport enzymes going before their oxygenase segments (Gibson & Parales, 2000; Guzik et al., 2013). The gem construction of naphthalene dioxygenase has affirmed the presence of a Rieske ($2\text{Fe}-2\text{S}$) bunch and mononuclear iron in every alpha subunit. The catechol dioxygenases fill in as a feature of nature's technique for debasing fragrant atoms in the Environment (Broderick, 1999). They are found in the dirt microscopic organisms and associated with the change of sweet-smelling antecedents into aliphatic items. The intradiol dividing chemicals use Fe(III), while the extradiol severing compounds use Fe(II) and Mn(II) in a couple of cases.

3.2. Microbial laccases

Laccases (p-diphenol: dioxygen oxidoreductase) comprise a group of multi-copper oxidases created by specific plants, organisms, bugs, and micro-organisms that catalyze the oxidation of a wide scope of diminished phenolic and sweet-smelling substrates with associative decrease of subatomic oxygen to water (Strong & Claus, 2011). Laccases are known to happen in numerous isoenzyme frames every one of which is encoded by a different quality, and, at times, the qualities have been communicated contrastingly relying on the idea of the inducer (Viswanath et al., 2014). Numerous microorganisms produce intra- and extracellular laccases equipped for catalyzing the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines just as some inorganic particles (Couto & Herrera, 2006; Dana et al., 2017). Laccases oxidize phenolic and methoxyphenyl acids, yet in addition decarboxylate them and assault their methoxy gatherings (demethylation). These catalysts are associated with the depolymerization of lignin, which brings about an assortment of phenols. Moreover, these mixtures are used as supplements for microorganisms or

depolymerized to humic materials by laccase (Kim et al., 2002). Among the organic specialists, laccases address an intriguing gathering of universal, oxidoreductase enzymes that show guarantee of offering extraordinary potential for biotechnological and bioremediation applications (Strong & Claus, 2011).

Laccases oxidize phenolic and methoxy phenolic acids as well as decarboxylate them and assault their methoxy gatherings (demethylation). These catalysts are engaged with the depolymerization of lignin, which brings about an assortment of phenols. Likewise, these mixtures are used as supplements for microorganisms or depolymerized to humic materials by laccase. The substrate explicitness and fondness of laccase can differ with changes in pH. Laccase can be hindered by different reagents like halides (barring iodide), azide, cyanide, and hydroxide (Mishra et al., 2019). Distinctive laccases seem to have varying resistance toward hindrance by halides, demonstrating differential halide availability. Laccase creation is touchy to the nitrogen focus in parasites (Brander et al., 2014).

Laccase can eliminate xenobiotic substances and produce polymeric items utilized for bioremediation measures. The major known toxin, PAHs, are circulated equitably in an indigenous habitat, comprising of a benzene ring organized straightly (Balcázar-López et al., 2016). Such contaminations, alongside their middle of the road, are of extraordinary ecological worry because of their poisonousness, ingenuity, mutagenicity, and cancer-causing nature in nature, created because of deficient ignition of nonrenewable energy sources and different mechanical squanders. Because of their low water dissolvability and helpless corruption rate, these fragrant hydrocarbons are xenobiotic (Mate & Alcalde, 2017). Microbial laccase has likewise been accounted for the corruption and decolorization of amino carbonyl complex containing refinery gushing, including the intricate combination of phenolic metals and detoxification of postmethanation refinery emanating and chloro lignin containing mash paper factory squander (Mehandia et al., 2020). An examination has shown that the recombinant CotA laccase from *E. coli* can decolorize recreated material effluents (STE). Both purged and rough recombinant CotA laccase effectively decolorized seven fundamentally various colors. At the point when STE was cradled at nonpartisan pH, the decolorization paces of filtered and rough CotA laccase were higher (Ma et al., 2017).

3.3. Microbial peroxidases

Peroxidases (contributor: hydrogen peroxide oxidoreductases) are omnipresent enzymes that catalyze the oxidation of lignin and other phenolic compounds to the detriment of hydrogen peroxide (H_2O_2) within the sight of a go between. These peroxidases can be haem and nonhaem enzymes. In well evolved creatures, they are associated with natural cycles like safe frameworks or chemical guideline. In plants, they are engaged with auxin digestion, lignin and suberin arrangement, cross-connecting of cell divider parts, safeguard against microbes, or cell lengthening (Koua et al., 2009). The heme peroxidases have been ordered into two particular gatherings as discovered distinctly in creatures and found in plants, organisms, and prokaryotes. The second gathering of peroxidases has been partitioned into three classes based on succession examination. Class I is intracellular chemicals including yeast cytochrome *c* peroxidase, ascorbate peroxidase (APX) from plants, and bacterial quality copied catalase peroxidases. Class II comprises of the secretory contagious peroxidases like lignin peroxidase (LiP) and manganese peroxidase (Mnp) from *Phanerochaete chrysosporium*, and *Coprinus cinereus* peroxidase or *Arthromyces ramosus* peroxidase (ARP). The principle job of class II peroxidases gives off an impression of being the debasement of lignin in wood. Class III contains the secretory plant peroxidases like those from horseradish (HRP), grain or soybean. These peroxidases appear to be biosynthetic catalysts engaged with cycles, for example, plant cell divider development and lignifications (Koua et al., 2009).

The heme peroxidases have been characterized into two particular gatherings as discovered uniquely in creatures and found in plants, growths, and prokaryotes. The second gathering of peroxidases has been partitioned into three classes based on grouping examination. Class I is intracellular catalysts including yeast cytochrome *c* peroxidase, ascorbate peroxidase (APX) from plants, and bacterial quality copied catalase peroxidases (Hofrichter et al., 2010). Class II comprises of the secretory contagious peroxidases like lignin peroxidase (LiP) and manganese peroxidase (Mnp) from *Phanerochaete chrysosporium*, and *Coprinus cinereus* peroxidase or *Arthromyces ramosus* peroxidase (ARP). Nonhaem peroxidases are not developmentally connected and structure five autonomous families. They are thiol peroxidase, alkyl hydro peroxidase, nonhaem haloperoxidase, manganese catalase and NADH peroxidase. Among every one of these thiol peroxidases is the

biggest and having two subfamilies as glutathione peroxidases and peroxy redoxins (Hofrichter et al., 2010).

3.4. Microbial cellulases

Cellulases presently guarantee the capability of changing over squander cellulosic material into food sources to meet expanding populace and have been the subject of extraordinary examination (Sharma et al., 2018; Urbanowicz et al., 2007). A few life forms produce cell bound, cell envelope related, and some extra cell cellulases. Extracellular cellulases, hemicellulases, and pectinases have been demonstrated to be constitutively communicated at exceptionally low levels by certain microbes and parasites (Jayasekara & Ratnayake, 2019). Cellulases are normally a combination of a few chemicals. At any rate: three significant gatherings of cellulases are associated with the hydrolysis interaction.

A. endoglucanase (EG, endo-1,4-D-glucanohydrolase) which assaults districts of low crystallinity in the cellulose fiber, making free chain closes;

B. exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase) which debase the cellulose atom further by eliminating cellobiose units from the free chain closes.

C. β glucosidase which hydrolyzes cellobiose to glucose units.

Alongside significant chemicals, some auxiliary enzymes are likewise present.

During the enzymatic hydrolysis, cellulose is corrupted by the cellulases to decreasing sugars that can be aged by yeasts or microbes to ethanol (Sun & Cheng, 2002). Cellulase chemicals are fit for debasing glasslike cellulose to glucose. Cellulases have been utilized in the assembling of cleansers since mid-1990s. Cellulases eliminate cellulose microfibrils, which are shaped during washing and the utilization of cotton-based materials. This is likewise seen as the shading lighting up and material relaxing in the material business. Basic cellulases are delivered by *Bacillus* strains and impartial and acidic cellulases by *Trichoderma* and *Humicola* organisms. In paper and mash industry, cellulases have been utilized for the expulsion of ink during reusing of paper (Karmakar & Ray, 2011). The cellulases are added during preparing to build the juice freedom from organic product mash and for the creation of ethanol from cellulosic biomass (Mewis et al., 2013).

3.5. Cytochrome P450

Cytochrome P450 (EC 1.14.14.1) is a superfamily of universal heme compounds found across the three organic spaces: Eukaryota, Bacteria, and Archaea, liable for a wide scope of capacities going from the union of complex common items and medication digestion in the living frameworks to bio-transformation of harmful synthetic substances in our biological system (Kumar, 2010). P450s have an inherent ability to debase xenobiotics through synthetic changes like aliphatic hydroxylations and epoxidations, dealkylations, dehalogenation, and different instrument based inactivations that are vital to bioremediation science. CYP101, CYP102, CYP1A1, CYP1A2, and CYP1B1 were known to use PAHs, of which CYP1A1 shows high action toward dibenzo-p-dioxin (DD) and mono-, di-, and trichloro-DDs while CYP1A1 freak, F240A, displays movement toward 2,3,7,8-tetrachloro (Kellner et al., 1997). Different enzyme designing and nonengineering concentrates on microbial P450s have been completed to bioremediate natural contaminations and hydrocarbons. Early surveys on designed P450s and the utilization of microbial P450s for biodegradation/hydroxylation of earth pertinent substrates were made (Kellner et al., 1997). Among the known microbial P450s, enzyme designing investigations on the model P450 from *Bacillus megaterium*, CYP102A1 (P450BM3), showed its capability to oxidize PAHs, for example, phenanthrene, fluoranthene, and pyrene to a combination of phenols and quinones (Jóźwik et al., 2016). Some microbial P450s showed potential for bioremediation of polyhalogenated aromatics. Makino et al. (Makino et al., 2014) examined hereditarily changed *Acinetobacter calcoaceticus* strain BD413 to communicate P450 xenobiotic-utilizing catalyst CYP105D1 from *Streptomyces griseus* ATCC 13,273 to include diligent contaminations, herbicides, and agrochemicals, which permitted micro-organisms to develop on these mixtures (Lamb et al., 2000).

P450s have the capacity, especially for the debasement of headstrong halogenated toxins, which are impervious to dioxygenases that the freaks F87W, F98W, Y96F, and V247L of heme monooxygenase CYP101A1 (P450-cam) from *Pseudomonas putida* showed movement with insoluble pentachlorobenzene, without the need of surfactants or natural solvents. In this way, the normal redesigning of wild-type CYP101A1 furnishes dynamic site freaks with an immeasurably improved capacity to oxidize polychlorinated benzenes into chlorophenol items, further debased by different

microorganisms. Subsequently, the CYP101A1 freaks could shape the premise of novel bioremediation frameworks for polychlorinated benzenes (Hrycay & Bandiera, 2015). Essentially, Chakraborty and Das (Chakraborty & Das, 2016) have announced a few microorganisms, for example, *Rhodococcus*, *Gordonia*, *Mycobacterium*, and *Pseudomonas* harbor catabolic qualities, plasmids, and genomes communicating P450s for the corruption and expulsion of POPs from the climate. Awad and Mohamed (Awad & Mohamed, 2019) found that P450 BM3 (CYP102A1) from *Bacillus megaterium*, designed from *E. coli* BL21, is valuable in the corruption of different natural gases poisons by immobilizing empty nanosphere particles of Pt/TiO₂-Cu under sun powered radiation where the debasement of isopropanol was discovered high (95%) at a pH of 7.0, encompassing temperature, and convergence of 20mg/L with the nonstop inventory of electrons through nanoparticles. P14 from raw petroleum polluted residue where the administrative articulation of CYP108J1 brought about PAHs' corruption, which can be utilized as the sole wellspring of carbon and energy. Further mutational investigation showed that NarL (nitrate-subordinate two-segment administrative factor) goes about as a novel repressor for the outflow of CYP108J1 during PAHs debasement (Peng et al., 2020).

3.6. Dehalogenase

Microbial dehalogenase (EC 3.8.1.5) has pulled in a lot of consideration as a result of its huge application in halogenated natural mixtures' bioremediation (Singh & Ward, 2013). A wide scope of halogenated compounds is debased utilizing dehalogenase chemical, which separates C-X securities through three systems, including hydrolytic, reductive, and oxygenolytic ones, which could perform dehalogenation by the substitution of the halogen molecule by hydroxyl bunch from water and a hydrogen iota from H₂, individually (van der Bolt et al., 1997). A pure strain of *Bacillus* sp. GZB, which has a remarkable capacity to simultaneously debrominate and mineralize the TBP, was isolated by Li et al. (2012) from the muck of a waste reuse site which has a great ability to all the while debrominate and mineralize the TBP. The debromination step has happened in two pathways, of which reductive bromination is the significant way and methyl bromination is the minor one. The reductive debromination continues with the debromination at both ortho and para positions to give 2,4-DBP and 2,6-DBP, individually. The mineralization item is CO₂, and the most noteworthy

mineralization proficiency of 29.3% was seen from 3mg/L TBP arrangement at 148h. The open understanding casing, specifically, ORFo5005, coding peptide ABC carrier substrate restricting enzyme, was anticipated to be engaged with the corruption pathway of TBP (Liang et al., 2017). The TBP dehalogenase quality arrangement from *Bacillus* sp. GZT was changed into equipped *E. coli* BL21 (DE3) to affirm TBP dehalogenase action. The refined compound has debased TBP inside 120min at 35°C at 200rpm, where 80% productivity was accomplished. H₂O₂, NADPH, Mn²⁺, and Mg²⁺ improved the dehalogenase exercises while EDTA, methyl viologen, Ni²⁺, Cu²⁺, Ca²⁺, and Fe²⁺ were unequivocally hindered. Also, the novel bacterial strain *Ochrobactrum* sp. when cloned with *tbbpaA* created recombinant strain *E. coli* BL21 (DE3) (Liang et al., 2017; Liang, Li, Mai, et al., 2019; Xiong et al., 2017). Furthermore, decontaminated dehalogenase compound from recombinant strain displayed high TBBPA debasing capacity (78%). +e quality of *Rhizobium* sp. encoding haloalkane dehydrogenase and haloalcohol dehydrogenase (HHDH) creating strain *Pseudomonas umsongensis* YCIT1612, both communicated in *E. coli*, additionally help in bioremediation (Liang, Li, Xiong, et al., 2019).

Also, Nelson et al. (Nelson et al., 2014) utilized the improvement culture of three *Dehalobacter* sp. strains (12DCB1, 13DCB1, and 14DCB1) to dehalogenate chlorobenzenes, dichlorotoluenes, and tetrachloroethene, the normal natural toxins, of which strain 12DCB1 specially dehalogenated separately flanked chlorines. Conversely, 13DCB1 dehalogenated the headstrong 1, 3, 5-trichlorobenzene to monochlorobenzene (MCB), and both these strains (unadulterated societies of *Dehalobacter* sp.) dehalogenated polychloroethene to cis-dichloroethene (Alfán-Guzmán et al., 2017). Strain 14DCB1 was the solitary strain to dehalogenate parasubstituted chlorines, and all strains dehalogenate 3, 4-DCT to mono chlorotoluene. These results showed that *Dehalobacter* sp. are adaptable dehalogenators. Zhang et al. (Zhang et al., 2013) detailed 2-haloacid dehalogenases (EC 3.8.1.2) from the marine bacterium *Pseudomonas stutzeri* DEH130; dehalogenase 1 and dehalogenase 2. The dehalogenase action test was completed for the two enzymes, and dehalogenase 2 was specially dynamic toward substrate L-2-CPA while they couldn't decontaminate dehalogenase appropriately. Dehalogenase 2 was more stereospecific toward halogenated substrate than dehalogenase.

3.7. Dehydrogenases

Dehydrogenase have a place with the group of oxidoreductase and are found in life forms going from microscopic organisms, yeast, plants to creatures, including people (Ye et al., 2019). The microbial liquor dehydrogenase (ADH; EC1.1.1.1) catalyze the reversible transformation of liquor to aldehyde or ketone and can be arranged as (a) NAD⁺ or NAD(P)⁺-subordinate dehydrogenases and (b) NAD⁺ or NAD(P)⁺-autonomous chemicals that utilization pyrroloquinoline quinone, heme, or cofactor F₄₂₀ as a cofactor. Likewise, aldehyde dehydrogenase (ALDHs, EC 1.2.1.3) catalyzes the NAD(P)⁺-subordinate oxidation of the aldehyde to a carboxylic corrosive (Akal et al., 2019; Chen et al., 2016).

Aldehyde dehydrogenase is discovered to be dynamic in the digestion of sweet-smelling compounds. The quality (orf9) from *Azoarcus evansii* liable for creating 3,4-dehydroadipyl-CoA semialdehyde dehydrogenase, which is utilized in oxidation 3,4-dehydroadipyl-CoA semialdehyde, has been communicated adequately (Fuchs et al., 2011). Ethylbenzene dehydrogenase (EBDH), an enantioselective chemical from the denitrifying bacterium *Azoarcus* sp. strain EbN₁ (to be renamed *Aromatoleum aromaticum*) catalyzes the oxygenindependent, stereospecific hydroxylation of ethylbenzene to 1-(S)-phenylethanol, the direct anaerobic oxidation of a nonactivated hydrocarbon, which helps in the biomineralization of ethylbenzene which is an item from raw petroleum (Fuchs et al., 2011; Weinert et al., 2011).

3.8. Hydrolase

Hydrolases break a synthetic bond using water and convert bigger particles to more modest ones to lessen contaminations' poisonousness. The work with the cleavage of C–C, C–O, C–N, S–S, S–N, S–P, C–P, and different bonds by water and catalyze a few related responses, including buildups and drunkards (Sharma, Sharma, et al., 2019). Hydrolytic enzymes have very assorted likely uses in various zones, for example, feed added substance, biomedical sciences, and synthetic enterprises. The breakdown of ester, amide, and peptide bonds by esterases, amidases, and protease may prompt items with next to zero harmfulness. Microbial hydrolase, for example, carbamate or parathion hydrolase from *Achromobacter*, *Pseudomonas*, *Flavobacterium*, *Nocardia*, and *Bacillus cereus* has been effectively utilized in the change of contaminations, for example, carbofuran, carbaryl or

parathion, diazinon, and coumaphos by the interaction of hydrolysis (Scott et al., 2008; Sharma, Sharma, et al., 2019; Sharma et al., 2018).

Cyanide-containing compounds are profoundly harmful and dangerous noxious; there are two diverse enzymatic pathways known for the debase-ment of nitrile compounds; one is a two-venture corruption including nitrile hydratase and amidase by means of an amide as a middle of the road, while the other is the immediate hydrolysis of nitriles to the relating acids and smelling salts, catalyzed by nitrilases (EC 3.5.5.1) (Sharma, Akhter, & Chatterjee, 2019). Such catalyst delivering microscopic organisms are *Nocardia* sp. furthermore, *Rhodococcus* sp. Phthalates are oftentimes utilized as a plasticizer and are omnipresent natural contaminations. Phthalate esters (PEs) and short-chain (C₁–C₄) alkyl esters of phthalates are a classification of poisonous natural mixtures that are generally utilized as added substances or plasticizers (conditioners) in the assembling of plastics. These have become incredible ecological concerns worldwide as a result of their suspected cancer-causing, estrogenic, and endocrine-upsetting properties (Huang et al., 2020; Jiao et al., 2013; Zhang et al., 2018).

Dang et al. (Dang et al., 2018) disengaged a thermophilic microbes *Bacillus* sp. BCBT21 from treating the soil horticultural remaining, which secretes hydrolases including chitinase, CMCase, protease, xylanase, and lipase, acts in high temperature, and assumes a critical part to corrupt biodegradable and oxo-biodegradable plastics from different assets.

3.8.1. Protease

Protease (E.C 3.4.21.12) has a place with the hydrolase group of catalysts that catalyze enzymes' peptide bonds. They can likewise be confined from microorganisms, for example, *Bacillus* sp., *Aspergillus* sp., and *Amycolatopsis* sp. Microbial proteases are vital as a result of their ease, high creation, and effective action. They are generally utilized in businesses like the cowhide business, the food business, and wastewater treatment (Okino-Delgado et al., 2019; Sivaperumal et al., 2017).

The protease compounds are additionally utilized in the bioremediation of marine shellfish squanders utilized in chitin extraction after deenzymeization. The antacid protease created by *Bacillus licheniformis* MP1 prompts 75% deenzymeization of shrimp squanders (Jellouli et al., 2011). The chitinase compound BsChi, created by *Bacillus subtilis* communicated in *E. coli*, debased glasslike chitin adequately to N-acetyl-Dglucosamine which

can be considered as a climate agreeable technique for the corruption of crab shell squanders (Jellouli et al., 2011; Wang, Li, et al., 2018). *Pseudomonas fluorescens* debase 3g/L of Impranil, water-dispersible polyurethane inside 4–5 days by protease action of enzyme displaying beta activity (Wang, Li, et al., 2018). Proteases diminish natural contamination by debasing and changing over the keratinous squanders and marine scavenger squanders into valuable items. Also, the utilization of harmful synthetic compounds has been supplanted by the catalyst's activity, diminishing its delivery into the climate. Hence, protease has a critical job in the bioremediation of a dirtied climate.

3.8.2. Lipases

Lipases (EC 3.1.1.3) have a place with the serine hydrolases and are a notable biocatalyst for the hydrolysis of the ester obligation of fatty substances into unsaturated fats and glycerol. Lipase debases lipids got from different microorganisms, creatures, and plants, along these lines lessening hydrocarbon from polluted soil. Lipolytic responses happen at the lipid-water interface, where lipolytic substrates ordinarily structure a harmony between monomeric, micellar, and emulsified states (Karigar & Rao, 2011; Sharma et al., 2018). Lipases can upgrade the bioremediation of oily effluents containing oils, fats, and enzyme released from different zones (Kumar et al., 2020). Lipase from *Acinetobacter* sp., *Mycobacterium* sp., and *Rhodococcus* sp. has been utilized to smother oil slicks (e.g., n-alkanes, fragrant hydrocarbons, and PAHs). Then again, the lipase from *Pseudomonas* sp. has been utilized in the bioremediation of soil sullied with mechanical waste oil, and the corruption of castor oil has been accounted for from the lipase created from *Pseudomonas aeruginosa* (Margesin et al., 2007; Sharma, 2018).

Lipases are additionally utilized in family clothing to diminish natural contamination and upgrade cleanser's capacity to eliminate extreme oil or oil smudges. The rough lipase from *Bacillus subtilis* strain is used in cleanser enterprises to limit phosphate-based synthetics in cleanser definitions (Saraswat et al., 2017). The corruption of manufactured polyester, polycaprolactone (PCL), by lipases got from *Lactobacillus brevis*, *Lactobacillus plantarum*, and their coculture uncovered that the *L. plantarum* lipase showed the most extreme PCL corruption productivity when contrasted with others. Lipase PL disconnected from *Alcaligenes* sp. catalyze the hydrolysis response from poly(L-lactide) (PLA) polymers to oligomers lastly to the

monomers (Mahmood et al., 2017; Wang, Liu, et al., 2018).

4. Possible strategies for overcoming problems associated with the use of enzymes

The utilization of a catalyst is conceivable if the result of the enzyme interceded response is less poisonous than the substrate. In addition, if the detoxification requires a multistep cycle, for example numerous enzymes acting consecutively, just explicit microorganisms are appropriate for accomplishing cleaning. Regardless of whether enzymes require cofactors, their utilization might be risky, except if an arrangement containing both the chemical and the individual cofactor is utilized. Another issue in the utilization of compounds to detoxify natural dirtied soil is given by the quick corruption of the free enzyme by proteases delivered by soil microorganisms (Chen et al., 1999). Enzymes may decrease or even lose their movement upon poison change or they may introduce a low steadiness and endurance under regularly cruel natural conditions. In the event that their rehashed use is required, chemicals may introduce low reusability, subsequently diminishing the proficiency of the entire treatment. Furthermore, whenever disconnected compounds are utilized, the expense of chemical confinement and purging incredibly hampers their useful application, mostly when their persistent taking care of is required (Rayu et al., 2012). For example, with especially obstinate contaminations, the chemical proficiency might be extraordinarily upgraded by the presence of extra mixtures going about as cosubstrates or arbiters. As announced above, for instance, this is the situation of laccase, whose potential might be improved by the expansion of a few middle people (Gianfreda et al., 1999). Torres-Duarte et al. (Torres-Duarte & Vazquez-Duhalt, 2010) examined the change of 12 halogenated pesticides (for example 2,4-DB, bromofenoxim, bromoxynil, dichlofenthion, dichlorophen, dinoterb, diuron, linuron, niclosamide, pentachlorophenol, picloram, and propanil) by a laccase from *Coriolopsis gallica* within the sight of nine distinct arbiters at various go between substrate rates.

The general outcomes show that the laccase–arbiter framework is by all accounts a decent option for the change of obstinate halogenated pesticides. For sure, dehalogenation of pesticides and development of pesticide–arbiter adducts happened, both adding to the decrease of pesticide ecological effect. The expansion of 1-hydroxybenzotriazol (HBT) to a laccase from *Trametes trogii* was the best among 11 middle people (for example

2,6-dimethoxyphenol: DMP), HBT, syringaldehyde, syringate, vanillin, vanillate, acetosyringone, m-, o- and p-coumarate) in the decolorization and detoxification of a material industry emanating (Khlifi et al., 2010).

Degradative enzymes with new or improved exercises and security under chosen conditions can be produced by hereditary designing techniques like sane site-coordinated mutagenesis and different DNA-rearranging strategies (e.g., the arbitrary fracture of a populace of freak qualities of a specific family followed by irregular reassembly). Compounds can be adjusted by coordinated advancement or by site explicit mutagenesis to improve existing biodegradation pathways or to create biocatalytic cycles for the creation of valuable items. For the debasement of hard-headed mixtures where no characteristic pathways are known, novel pathways can be created by joining pathway “tapes” from different hereditary sources (Kumar et al., 2020; Parales & Ditty, 2005). Nonetheless, restrictions are available for applying biomolecular designing procedures. For example, research is normally centered around changing compounds that can play out a response like the ideal one, however it very well may be hard to apply biomolecular designing to the bioremediation of novel poisons, which are not known to be biodegradable. By the by, it very well may be conceivable later on when our insight into the enzyme structure–work, collapsing, instrument and elements will be altogether improved (Singh et al., 2008; Stanzione et al., 2020).

5. Conclusions

Arising contaminations in nature are express and connected to the different ongoing inconveniences in biotic segments and accordingly weaken the environment as openness even in little focus and huge spaces of time. Explicit toxin substrate is perceived with explicit microbial chemicals at ideal condition (temperature/pH/contact time/fixation) so it effectively changes into various harmless items enzymatically by different enzymatic response systems including oxidation, decrease, end, and ring-opening. High-sub-atom- weight PAHs and polyhalogenated aromatics cooperate with cytochrome P450 chemical dynamic site and change into harmless items by oxidation. Low substrate explicitness and higher thermostability halotolerant limit of laccases changed different classes of contamination substrates like antiinfection agents, engineered colors, PAHs, and phenolic toxins by means of the oxidation cycle. The chemical dehydrogenase specially oxidized medium-chain auxiliary alcohols and manufactured polymers containing different hydroxyl bunches in their subatomic design into comparing aldehydes. The protease chemical debases the refractory keratinous squander biomass, marine shellfish squanders, colors, enzyme polymers, and oxo-biodegradable plastics utilizing the hydrolysis response instrument. Microbial hydrolases follow up on added substances or plasticizers, cyanides, and nitriles containing compounds and change the contamination into less hurtful items by buildups and alcoholysis responses. Catalysts present ecological benefits against synthetic compounds and microorganisms. They are: the biotransformation doesn't create harmful side items as is regularly the situation with synthetic and some microbiological measures; the chemicals are processed, in situ, by the native microorganisms after the treatment; the necessity to improve bio-accessibility by the presentation of natural cosolvents or surfactants is significantly more doable from an enzymatic perspective than utilizing entire cells; the creation of enzymes at a higher scale, with upgraded solidness or potentially action and at a lower cost is possible by utilizing recombinant-DNA innovation. Quick advances in different pathways, useable in microorganisms for the corruption of toxins, have illuminated their systems, and pathways have been described to create supportable bioremediation techniques for dirtying compounds. In any case, the designs of enzymes, administrative perspectives, and subatomic science actually require intensive arrangement.

Additionally, a lot of work is as yet needed to immovably set up all out comprehension of the subatomic reason for catabolic arrangements, to restrict inactivation of catalysts at high limit centralization of xenobiotics, to expand intensifies bioavailability in characteristic biological systems, and to improve the utilitarian capacity of the chose compound inside a thin scope of physico-substance conditions. The personal satisfaction on earth is connected inseparably to the general nature of the climate. Catalysts of microbial beginning have huge potential in squander the board, and subsequently in the advancement of green climate. The compounds are adequately used in numerous businesses for greater creations at sped up pace of response with harmless contamination and cost viability.

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Chapter 5: Characterization of wastewater and effluents remediation through nanotechnology for efficient reclamation and reuse

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Abstract

The exponential growth of the world population over the past few decades has led to a rampant increase in the demand of the water supply. This adverse situation of shortage of water has the potential to develop into a world water crisis, in the upcoming years. Therefore, reclamation and reuse of the wastewater have emerged as an urgent environmental requirement. To reuse the conventionally treated water, it should be of utilizable quality. The water that has been treated must meet the minimum safety standards and quality so that it can be used for specific reclamation purposes. Hence, the focus of the current research all over the world is on sustainable treatment approach for wastewater remediation. Research has shown that the complication of water treatment could be tackled with the use of an affordable, yet efficient technique known as nanotechnology. This chapter reviews the usage of applications of nanotechnology in the treatment of wastewater. Under the umbrella of nanotechnology, the procedures for the remediation of contaminants, the properties of the nanoparticles, and the recent advancements in the nano-technological treatment of wastewater have been discussed. The techniques for the remediation of the wastewater pollutants are adsorption, membrane filtration, photocatalysis, and the action of antimicrobial nanoparticles for the disinfection of the wastewater resources. The chapter comprehensively reviews the importance of nanotechnology in the remediation of the wastewater and how it can be effectual for the reclamation and reutilization of the wastewater.

Keywords

Nanotechnology; Reclamation and reuse; Remediation; Wastewater characterization and treatment

1. Introduction

The consumption of approximately 70% of the freshwater in industrial, agricultural, and domestic sectors has tremendously increased the demand of freshwater. In this process, high amounts of wastewater toxic effluents are produced, increasing the need of clean and fresh water. Therefore, upgradation in the conventional treatment techniques and wastewater recyclability is necessary for meeting the worldwide demand of pure water. There has been numerous research works focused on the development of physical, chemical, and biological conventional treatment methods which are used for the elimination of several contaminants from the wastewater such as ion-exchange processes, oxidation, precipitation, coagulation, flocculation, electrolytic methods, membrane filtration, adsorption, filtration, and reverse osmosis (Tchobanoglous et al., 2003). However, most of these methods are limited because of their high operational and capital cost, large amount of sludge generated and their tendency of failure in meeting the stringent environmental standards. In the past few years, molecular biology techniques such as 16S rRNA cloning, dot-blot hybridization, FISH (fluorescent in situ hybridization), polymerase chain reaction, etc. have shown significant results in the analysis of microbial diversity in wastewater (Dahiya et al., 2021). Recently, nanotechnology has emanated as an area of research that is being utilized for the removal of contaminants from the wastewater (Nassar, 2012).

Nanoparticles (NPs) exhibit unexpected and unique characteristics that vary from their bulk material counterparts. NPs can be functionalized with a variety of chemical groups to improve their affinity for a target contaminant particle (Xu et al., 2012). Consequently, these particles provide recyclable ligands and a high selectivity for the toxic contaminants in wastewater. They comprise of a shape/size dependent catalytic properties and large surface area which can help in absorption and catalysis for effective wastewater remediation. This chapter entails the wastewater remediation through nanotechnology for effective reclamation and reuse, synthesis of nanoparticles, and the recent advancements in the nanotechnological wastewater treatment. The nanotechnological applications include the synthesized nanoparticles as adsorbents for removing the toxicants and contaminants, as catalysts for the break-down and oxidation of noxious pollutants in wastewater, and as an integrated technology which is an amalgamation of the

conventional techniques and nanotechnology such as composite photocatalytic membrane technology which is a combination of photocatalytic activity of NPs and the membrane separation technique (Zhang et al., 2006). Nanotechnology can be enhanced by the incorporation of currently available techniques and thus can help in the production, conversion, and storage of energy, in the development of electronics, information, and communication technology, health and medical devices, agribusiness, materials and manufacturing, and minerals. Additionally, nanotechnology has the capability to solve many present-day issues by the virtue of lighter, stronger, smaller, faster, and better-performing components, materials, and systems. Therefore, the use of nanotechnology is increasing across numerous industry sectors worldwide and is expected to impact human health and everyday life.

The use of engineered nanomaterials is increasing throughout the world, for several applications. The advantages of nanomaterials or the ecological environment include the utilization of nanomaterials like titanium dioxide in wastewater treatment, selenium used in mercury adsorption, and zero-valent iron for the soil remediation. These nanomaterials enter the ecosystem through various pathways of water, air, and soil. Table 5.1 delineates the example of nanoparticles which are released in the environment through different pathways. These particles can be released by solid or liquid waste streams and through atmospheric emissions. The particles present in the wastewater effluents, solid wastes, direct discharges, or industrial spillages can move and become a part of marine ecosystems through rainwater runoff or wind.

Nanotechnology is a process of manipulating the matter at an atomic as well as molecular, levels to design a new product, system, and device with high-end optical, electronic, magnetic, mechanical, and conductive properties (Jain, 2017; Khan et al., 2019). This technology is being studied as a promising technology and has exhibited various advantages and accomplishments in numerous fields including wastewater remediation. Nanostructures help creating redox active media and more efficient catalysts for the purification of wastewater owing to their ease of functionalization. NPs have been efficient in the removal of several contaminants from the wastewater, such as color dyes, organic, and inorganic solvents, heavy metals, pathogens, and biological toxins that are responsible for various diseases (Kumar et al., 2014).

Table 5.1

Product used	Nanomaterials	Potential release in the environment
Cerium oxide	Exhaust emission	Fuel additives
TiO ₂ or ZnO	Used for skin application and the disposal of containers	Cosmetics
Hydrophobic nano-coatings and silver nanoparticles coatings	Absorption by the skin	Clothing
Carbon nanotubes	CNT emission	Utensils and toys
Ultrafine particles	Emission with the exhaust	Combustion processes
Byproducts in the nano range	Byproducts could be emitted locally	Nanomaterial production
Carbon nanotubes	E-waste will lead to emission of CNT	Electronics
Phosphorus and calcium and based nanoparticles	High local exposure and emission	Soil regeneration

2. Characterization of wastewater

Wastewater characterization is indispensable for wastewater treatment as it provides the information regarding concentration and type of contaminants present in the wastewater, which is crucial for the determination of the type of contamination. Wastewater characteristics can be classified into three categories, namely, physical, chemical, and biological which are briefly discussed below in Table 5.2.

2.1. Chemical characteristics of wastewater

2.1.1. Organic matter

Natural organic matter is composed of various sources of carbon compounds that are decomposable plant-based, engineered, and animal material.

2.1.1.1. Biological oxygen demand

Biological oxygen demand (BOD) is the amount of oxygen needed by the biological organisms present in wastewater, for the process of aerobic digestion. This process takes place in the presence of oxygen, degenerating the complex organic material and breaking it down into simpler compounds. There can be an increase in the BOD which is due to the biodegradation of the organic material exerting oxygen tension in water. The BOD increase can also be a result of animal as well as plant waste products, industrial effluents, household, and domestic sewage (Benit & Roslin, 2015).

Table 5.2

Characteristics	Description
<ul style="list-style-type: none">- The dark color of the wastewater is characterized by the amount of dissolved oxygen (DO) as well as by the precipitation of sulfurcompound present in the wastewater.- Septic conditions arise in low DO conditions, reflecting black color of the wastewater (Von Sperling, 2007).	Color
<ul style="list-style-type: none">- The presence of industrial effluents,	Odor

<p>operational by-products and anaerobic decomposition of the organic compounds are strong factors behind the foul odor of the wastewater.</p> <ul style="list-style-type: none"> - Strong offensive odors are marked by the presence of hydrogen sulfide and nitrogen-based compounds that have low solubility in the water. 	
<ul style="list-style-type: none"> - Increased amounts of total suspended solids (TSSs) decrease the dissolved oxygen (DO) levels, leading to a raise in the water temperatures. - Variations in the temperature of the wastewater can affect the microbial activity, liquid viscosity, and solubility of the gases. 	Temperature
<ul style="list-style-type: none"> - Increased wastewater turbidity is caused by suspended matter, organic-colored compounds, inorganic matter, planktons, and other microscopic organisms. - Turbidity can adversely affect the effectiveness of the treatment process as it can act as a barrier for the pathogens and the particles that cause turbidity which can in-turn result in harbor of bacteria and viruses. 	Turbidity

2.1.1.2. Chemical oxygen demand

Chemical oxygen demand (COD) is the total amount of oxygen consumed by strong oxidants present in the wastewater during the decomposition and chemical oxidation of organic/inorganic matter. COD can be used to identify the sensitivity of particulate matter toward the oxidation process as well as to determine the amount of inorganic chemical material present in the wastewater sample. The rise in COD levels of the wastewater is a result of

accumulation of solid waste, soluble organic compounds, antifreeze, residual food waste, emulsified oils and the dying bacterial cells that are responsible for the decomposition and release of DOC in the wastewater which in turn increases the COD.

2.1.1.3. Total organic carbon

Total organic carbon (TOC) is the magnitude of carbon present in an organic compound and is frequently used as a wide-ranging and nonspecific measure of water quality. The hike in the levels of TOC is because of escalating microorganism's growth, soil biota, root exudates as well as decomposition of plant and animal residues, leading to the depletion of oxygen supplies increasing the organic content in the water source.

2.1.2. Total solids

Total solids (TS) is a critical measure of the amount of dry waste material that is insoluble in the water sample and is crucial to be calculated to ideally monitor the operational productivity of wastewater treatment facilities. Total solids are composed of suspended, dissolved solids as well as settleable solids in water. The suspended solids include nonfilterable fine organic debris, clay, and slit particles, inorganic solids, algae, and other particulate matter. Dissolved solids are the organic and inorganic solids comprising of sulfur, iron, nitrate, chlorides, calcium, and other filterable solids. Furthermore, settleable solids are a part of organic/inorganic solids that settle at the bottom of the tank in the course of a normal detention period.

2.1.3. Total nitrogen

Total nitrogen (TN) is the combination of nitrite, nitrate as well as ammonia, and is an important nutrient for the growth of microorganisms in biological wastewater treatment process. TN can be used in the industrial and municipal wastewater treatment plants and can be regulated as an effluent parameter. In the first stage of the process, ammonia is produced during the decomposition of the organic nitrogen which is followed by the second stage where the oxidation of the ammonia takes place under appropriate biological conditions leading to a full conversion to nitrate in the final stage. At the point when ammonia nitrogen and nitrite are at highest concentration and the level of nitrate is at its peak then in that case the wastewater has been fully nitrified.

2.1.4. Total phosphorus

Wastewater is a rich resource of phosphorus compounds, and this compound is used for the growth of microorganisms. It exists in both organic and inorganic forms of Orthophosphates as well as Polyphosphates. The increase in the levels of phosphorus is an outcome of phosphates entering the waterbody in the form of various resources like detergents, agricultural fertilizers, industrial wastes, and domestic wastewater. The rise in the phosphorus content in the waterbody can lead to the algal growth causing oxygen depletion problems, which in turn can lead to the eradication of the marine organisms and can affect the taste and odor of the water and therefore phosphorus removal becomes an essential process in the wastewater treatment plants.

2.1.5. Chlorides

Chloride is one of the most common anions that is found in the wastewater and is a major constituent of dissolved solid waste. It infiltrates the water bodies from multiple sources like natural mineral deposits, brine salt, residential water softeners, run-offs of from agricultural or irrigation practices, industrial effluents, seawater intrusion and sewage discharges into the water. Elevated levels of chloride in the water stream can be toxic and deleterious in nature for the fauna and flora of the aquatic life. Moreover, higher concentrations of chloride can also increase the corrosivity in the water and this can adversely affect the drinking water quality and associated infrastructures.

2.1.6. Alkalinity

Alkalinity is the measure of ability of water to absorb the hydrogen ions and neutralize the acids. As the wastewater undergoes treatment, alkalinity plays an important role in maintaining the pH which further helps in determination of the point at which alkalinity of the wastewater comes to an end and free acidity begins. During the treatment of domestic and industrial wastewater, there are many elements that are responsible for the increase in the alkalinity. The major determinants that contribute to the alkalinity are suspended organic matter, dissolved inorganic as well as organic compounds and the bicarbonates present in the water. Higher alkalinity levels would affect the aquatic life by preventing the necessary pH changes that are harmful for the aquatic system and it influences the wastewater treatment

process by affecting anaerobic digestion.

2.1.7. pH

The “pH” is a measure of the activity of hydrogen ion in a solution and is an indicator of acidity as well as alkalinity of the wastewater. Being a chemical component of the wastewater, it directly impacts the wastewater treatability. The state in which the pH drops below 7 may indicate a septic condition of the wastewater which leads to the development of anaerobic bacterial environment mineralizing the waste in the water. Extreme pH values can damage the biological treatment units and therefore the pretreatment of the waste becomes essential.

2.2. Biological components of wastewater

2.2.1. Bacteria

Bacteria are microscopic, single-celled prokaryotes, with a simple internal structure and the ability to survive in diverse environments. Depending upon their oxygen requirements, bacteria can be further classified into facultative, aerobic, and anaerobic. During the wastewater treatment process, they are responsible for the reduction of pollutants and contaminants, for stabilization of organic compounds and for the purification of water by altering the chemical composition of the toxic waste material.

2.2.2. Fungi

Fungi are unicellular or complex multicellular eukaryotes that thrive on dead and decaying matter and form filamentous structures. They are valuable for the wastewater treatment process because of their potential to grow under low pH conditions. They have the capability to degrade the organic/inorganic matter which decreases the contaminant-associated risks and have the power to increase the bioavailability helpful in the remediation of heavy metals breaking them down to less toxic forms (Aung et al., 2018; Cai et al., 2013).

2.2.3. Viruses

A virus is a submicroscopic infective agent with a genetic code comprising of either a DNA or RNA. It uses the components of the host cell to replicate itself, damaging the host organism in the process. It plays an important role in wastewater remediation as it can impact the composition of the bacterial community by eliminating the bacteria, enabling transduction of genetic

material among the organisms, and altering the chromosomal composition of the bacteria as prophages (Petrovich et al., 2020).

2.2.4. Algae

Algae are nonvascular chlorophyll-bearing photosynthetic organisms that assimilate carbon mixotrophically and heterotrophically and derive solar energy from the atmosphere to form their biomass (Chandra et al., 2014; Subhash & Mohan, 2015). They can be used for the purification of wastewater as they have the capability of accumulation of pesticides, heavy metals, radioactive substances, plant nutrients, and organic/inorganic toxic materials (Jothinayagi & Anbazhagan, 2009).

2.2.5. Protozoa

Protozoa are microscopic eukaryotes with a unicellular organization. They have a complex internal structure, are either free-living or parasitic and feed on microorganisms, debris, organic tissues, and other organic matter. They help in the wastewater purification process by sustaining the bacterial population density with predation and maintaining an equilibrium among various groups, thus improving the water quality (Madoni, 2011).

3. Importance of nanotechnology in wastewater purification

Conventional wastewater treatment techniques such as reverse osmosis, filtration, and coagulation-flocculation are usually not effective enough in the elimination of target pollutants. Therefore, it is critical to design and implement advanced wastewater treatment techniques with high efficiency and low capital requirement. With the goal of improving the preexisting wastewater treatment techniques, the application of nanomaterial was investigated to formulate separating media of high value in terms of output and reactivity. Nanomaterials are utilized for the bioremediation of wastewater as well as disinfection of water (Mohan & Pittman, 2007). The nanotechnological treatments are more effective than their conventional counterparts because of their increased surface area to volume ratio, small size, surface and boundary effect, exceptional chemical properties, and their ability to treat the water in way that it could be further reused for general purposes (Baruah, 2019).

Nanomaterials exist ubiquitously in the surroundings because of anthropogenic sources as well as natural processes and are formed from the emerging nanotechnologies. Nanomaterials are the organic, inorganic, and organometallic components which display physical, chemical, and electrical properties that alter as function of organization of the material. It has been developed in numerous forms and shapes such as nanotubes, particles, films, colloids, nanowires, and quantum dots (Edelstein & Cammaratra, 1998). In the process of wastewater remediation, plethora of eco-friendly, effectual, and economic nanomaterials are being developed with distinctive functions for potential disinfection of ground water, surface water, and drinking water (Theron et al., 2008; Tyagi et al., 2017). Nanomaterials can be classified into four major categories based on their nature such as nano-adsorbents, nano-catalysts, nano-membranes, and green nanomaterials. Nano-adsorption includes the sorption of sorbate molecules on the sorbent surface with the help of molecular interactions along with diffusion of sorbate molecules (Bushra et al., 2016). In the past few years, countless valuable works have been published with the focus on the investigation of the elimination of the contaminants from the wastewater using nano-adsorbent materials (Kyzas & Matis, 2015; Tang et al., 2014; Zhang et al., 2014). The materials that can be utilized for the development of nano-adsorbents include clay, silica, modified compounds in composite form, carbon, and

metal oxides.

The second category of the nanomaterials is nano catalysts. These are used to increase the catalytic activity and are heterogenous catalysts that are broken into metal nanoparticles which are responsible for increasing the catalytic activity. Several types of nano-catalysts are used for the purification of the wastewater, such as Fenton based catalysts for amelioration of the chemical oxidation process of organic pollutants (Kurian & Nair, 2015), electrocatalysts (Dutta et al., 2014), and catalysts with antimicrobial properties (Chaturvedi et al., 2012).

The third category of the nanomaterials used for wastewater remediation processes include nano-membranes. This technique involves pressure-driven approach for the wastewater treatment, and this has been a proven technology to attain the desired water quality. There are many categories of membrane-filtration, among which the nano-filtration (NF) is widely used for the wastewater treatment in industries, due to reduced cost, high efficacy, small pore sizes, and high usage feasibility (Blanco et al., 2012; Ouyang et al., 2013). Nano-membranes can be created with the help of nanomaterials such as nano-carbon tubes and nano metal particles. In addition, nanofiltration has been introduced at various treatment plants to initiate the production of effluents that have low pollutant concentration (Van der Bruggen et al., 2008). It is also used for the biologically treated water generated from the pulp and paper production plants (Mänttari et al., 2006). Nanotechnology can be further used in treatment of the molasses-industry effluents with the help of nano-membranes and can be utilized for the remediation of organic dyes using ZnO particles (Satyawali & Balakrishnan, 2008).

The fourth class involves the incorporation of aforementioned nanotechnologies into biological methods. Production of green nanomaterials with the help of microbes and the extracts of other organisms has led toward the sustainable treatment of pollutants, for example Iron nanomaterial. Green nanoparticles can be utilized in the treatment process because of their redox potential while reacting with magnetic susceptibility, water, and nontoxic nature (Bolade et al., 2020). Fig. 5.1 illustrates the use of existing nano-techniques applied for the bio-treatment of industrial effluents.

4. Synthesis of nanoparticles

4.1. Liquid phase reduction

This is a frequently used methodology used for the generation of nano scale particles. In this process, the metal undergoes direct precipitation from its aqueous solution via chemical reduction of a metal cation. The reducing agents used for the process were solvated ABH_4 , gaseous H_2 , hydrazine hydrated ($\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$), hydrazine dihydrochloride ($\text{N}_2\text{H}_4 \cdot 2\text{HCl}$) and hydrazine hydrated ($\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$). The transition metal cation undergoes a reduction reaction can be expressed as:

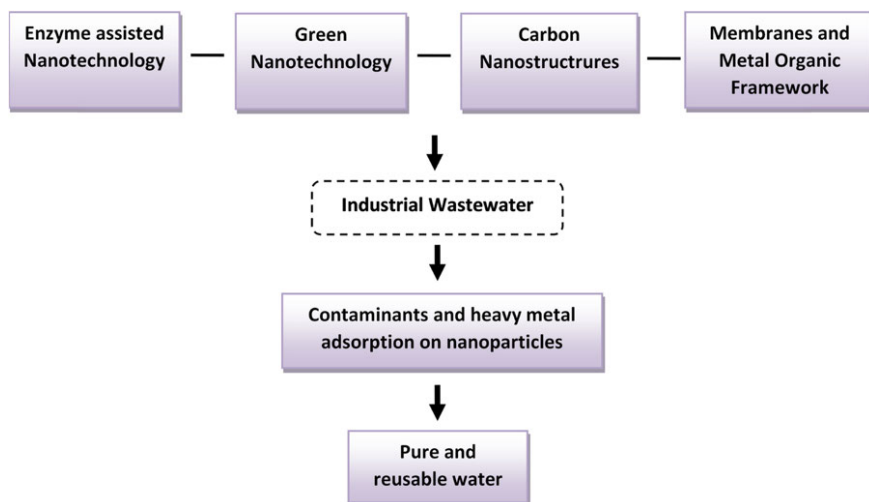
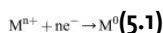


Figure 5.1 Schematic representation of nanotechnologies used for wastewater remediation.

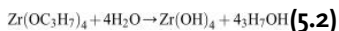
Other than this procedure, numerous studies explain similar process for the synthesis of nanoparticles. Studies have shown that cobalt nanoparticles (diameter: 400nm at 298K temperature) could be obtained using citric acid as capping agent and hydrazine as a reducing agent (Salman et al., 2014). However, nickel nanoparticles of (diameter: 3nm) can be produced using NaOH as reducing agent, hydrazine hydrate, and Poly vinyl pyrrolidone as a capping agent (Singh et al., 2011).

4.2. Sol-gel method

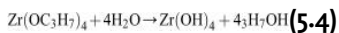
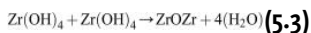
It is a wet chemical method that includes the sol formation based on partial condensation of metallic precursor and hydroxylation which is followed by the gel formation that forms 3-D metal oxide networks. This technique helps in the formation of a huge variety of metal oxides via processing of acetylacetonates, acetates, metallic alkoxide, and many inorganic salts as precursors. This procedure gives advantages such as good compositional homogeneity, high-phase purity, and high surface activity of the resulting powders.

Research shows that alumina nanoparticles can be synthesized effortlessly using sol gel method. In this technique, aluminum sulfate is dissolved in double distilled water and ammonia solution is added with continuous stirring resulting in a precipitate which is kept for 24h at 80°C in hot air oven. This ppt is then milled to procure gel powder, which is then calcined at 1100°C in a muffle furnace. This mass then undergoes the process of milling and sieving which then results in alumina nanoparticles of size in the range of 15–20nm (Sharma et al., 2008). This process can be used to synthesize nano-structured Zirconia powder and can be explained with a reaction below:

Hydrolysis:



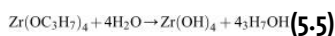
Condensation:



4.3. Solution combustion method

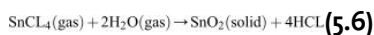
The method is used to produce nanoscale materials which are mostly used for the synthesis of ceramic powders for number of advanced applications. Nano oxide powder production is carried out by using metal nitrates in aqueous solution with fuel. The most utilized and suitable fuels are urea and

glycine because these have the capability to produce stable compounds with metal ions to increase the solubility and control selective precipitation of metal ions during the process of elimination of water. Solution combustion synthesis is an exothermic auto-propagated process which works on the principle that after the reaction is set off under heating, an exothermic reaction also starts and eventually forms a powder as a final product. This process turns out to be advantageous as it produces homogeneous and fine powders rapidly and is time as well as energy-saving technique. The parameters of the process including fuel-oxidant ratios and choice of fuel decide the characteristics of the powder which incorporates structure, size, purity, surface area and particle agglomeration. Previous research work has shown that the synthesis of alumina nanoparticles can be synthesized using glycine as a fuel and aluminum nitrate as precursor (Bhargavi et al., 2015). The overall reaction equation involved in the process of aluminum combustion synthesis is depicted below:



4.4. Chemical vapor synthesis (CVS)

It is one the most common methods which uses the gas-phase aerosol process for the synthesis of high-purity nanoparticles. This technique is similar to the chemical vapor deposition (CVD) process, where the parameters of the process are modified to synthesize solid thin film instead of nanoparticles. This process is carried out in cold wall and hot wall reactors at a sub-torr pressure, at 200–1600°C temperature and can be carried out with or without the carrier gas resulting in nucleation of particles within the vapor phase. For precursors, metal chlorides are utilized to produce oxides as they have low vaporization temperature. The equation for the above reaction is as follows:



The parameters that influence the performance and size of the nanomaterials are the precursor material, the total pressure, the precursor partial pressure, the energy source temperature, the precursor deliverance system,

the carrier gas, and the geometry of the reactor. The collection nanoparticles can be done by the means of electrostatic precipitators and filters. The nanoparticles that are synthesized by this method are usually generated in the form of aggregates because of their coagulation at the high temperature (Nakaso et al., 2003).

4.5. Hydrothermal treatment

In this heterogeneous reaction, the chemical precursor which is in aqueous phase is kept in the autoclave. With the use of hot water, this precursor undergoes pressure treatment that results in the precipitation of anhydrous and crystallized nanoparticles. This technique is advantageous as because of the generation of agglomerate free nanoparticles with an appropriate particle size and distribution. The hydrothermal fabrication of Fe_3O_4 water soluble nanoparticles requires a homogenous solution of sodium citrate in distilled water and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Polyacrylamide and ammonia are mixed with vigorous agitation and the resulting solution is sealed in an autoclave and is maintained at 200°C for 12h. After the completion of the reaction, a solid product is obtained. The particles procured this way are nanosized and are approximately the size of 20nm.

5. Wastewater effluents remediation through the applications of nanotechnology

Small size of nanomaterials makes them appropriate for wastewater remediation. There are various types of nanomaterials that can be helpful in wastewater remediation such as metal nanoparticles, polymeric nanoparticles, zeolite, self-assembled monolayer, oxides-based nanomaterials, carbon-based nanomaterials, and biopolymers (Gusain et al., 2020). These nanomaterials have specific physical, chemical, and biological properties that make them extremely useful for multiple wastewater treatment applications. There are certain nanotechnology-based techniques that can be used for wastewater treatment such as adsorption, nanofiltration, biosorption, photocatalysis, infection control, sensing, and monitoring (Palit & Hussain, 2020).

5.1. Adsorption and biosorption

Adsorption is a surface phenomenon and is an exothermic process which involves the phenomenon of phase transfer of either a molecule or an ion in gaseous or liquid state known as adsorbate, onto a solid, which is known as an adsorbent. This process forms a single molecular layer on the surface via chemical or physicochemical interactions under specific atmospheric conditions (Alawady et al., 2020; Gusain et al., 2020). Biosorption is a type of adsorption in which microorganisms such as bacteria, fungi and algae act as adsorbents because of their intrinsic ability to bind with a heavy metal. This process of biosorption involves ion exchange, microprecipitation, and cell-surface complexation (Fard et al., 2011).

5.1.1. Nano adsorbents

A wide use of nanoparticles as adsorbents has been observed in the past few years for the removal of deleterious pollutants from industrial wastewater. Nano-adsorbents can eliminate organic and inorganic pollutants and are classified mainly as carbon-based, metal-based, polymer-based nano adsorbents and zeolites as explained by Kumari et al. (2019).

5.2. Photocatalysis

Photocatalysis is a light-induced process which is accelerated and driven by a catalyst, and it includes a solid surface that absorbs the light photons to starts a chemical reaction (Bethi et al., 2016). This process is widely being studied for the wastewater treatment as photocatalysis can degrade an

extensive range of estrogens, organic materials, dyes, organic acids, pesticides, crude oil, microorganisms as well as few inorganic molecules and when this process is integrated with precipitation or filtration, it can also be used to eliminate metals (i.e., mercury) present in the wastewater. Some of the commonly used nanostructured semiconductor photocatalysts are Fe_2O_3 , TiO_2 , tungsten trioxide (WO_3), zirconium dioxide (ZrO_2), zinc sulfide (ZnS), ZnO , and cadmium sulfide (CdS) (Mahmoudian-Boroujerd et al., 2019).

The total amount of organic dyes such as RhB, MO, and acid orange (AO7) have started increasing in the wastewater from the last few years and therefore, it has become a matter of concern to treat and eliminate them. This can be dealt with the synthesis of a flower-like nanophotocatalyst for the deletion of AO7 from wastewater. In this case, Bismuth oxyhalides (BiOX) and Zinc ferrite (ZnFe_2O_4) can be used for the fabrication of P-BiOI/N- ZnFe_2O_4 which increases the fabrication efficiency for AO7 (Wang et al., 2020).

5.3. Nanofiltration

Nanofiltration is a liquid separation technology in which a pressure-driven membrane tends to reject the ionic and molecular species. Nanofiltration membranes such as organic membranes and ceramic membranes can be either porous or dense in nature. These membranes are a relatively recent innovation and have become the most preferred and used technique for the treatment of drinking water and wastewater remediation (Nagy, 2019). Nanofiltration membranes have a molecular weight cut-off (MWCO) for the uncharged particles falling under the nanometer range. These membranes have a pore size ranging from 0.5 to 2.0 nm and pressure ranging between 5 and 20 bars, with a decreased rejection of monovalent ions, higher valent ions, lower energy consumption compared to reverse osmosis, high flux value and higher rejection compared to ultra-filtration (Abdel-Fatah, 2018; Mulyanti & Susanto, 2018; Wu et al., 2017). These membranes are suitable for the filtration of organic and inorganic contaminants, including harmful microorganisms from the wastewater. There are two most used nanofiltration membranes including ceramic membranes and polymeric membranes. The polymeric membranes have an inferior chemical resistance and a high fouling rate whereas the ceramic membranes have higher chemical, mechanical, and thermal stabilities (Mostafavi et al., 2009). These

membranes are utilized for the nutrient-recovery from industrial effluents. NF₉₀ is a nanofiltration membrane that has the potential of rejecting 70% of phosphorus from the discharge of pulp and paper industry. In this case, the membrane fouling starts because of high amount of phosphorus content, therefore, the use of gold nanoparticles which is woven with a polymer blend of the nanofiltration membrane is suggested, in order to reach an increased recovery of phosphorous (Leo et al., 2011). This process can increase the rejection of trivalent phosphate up to 96% with high membrane hydrophobicity and fouling resistance (Shalaby et al., 2020).

5.4. Disinfection and microbial control

Disinfection is a process of elimination or reduction of the number of microbes present in the wastewater such as virus, bacteria, fungi, protozoa, prions, and rickettsia that pose a severe threat to human health (Yao et al., 2020; Zhang et al., 2018). This method is carried out onto the surface or a bulk material using physical or chemical methods (Zhao et al., 2020). The traditional disinfection techniques such as hypochlorite, chlorine, chloramines, chlorine dioxide, reverse osmosis, peracetic acid, and ozone are used for the wastewater treatment but these technologies are limited due to their enormous energy consumption, numerous DBPs, and requirement for expensive equipment which becomes a matter of concern (Qin et al., 2019; Zhang et al., 2020). This situation gives rise to a dire need of development of economical, sustainable, efficient, and time-consuming disinfection methods. Nanomaterials are equipped with functionality for the deletion or inactivation of the wastewater pathogens, such as specific reactivity and large surface areas which is not achievable using the conventional methods. The inactivation mechanism of these nanomaterials comprises of photochemical reactions and surface-based electrostatic interaction, which starts the disorganization of the cell wall, targeted disinfecting agent delivery and the production of reactive oxygen species (ROS) (Mauter et al., 2018; Wang et al., 2015). These nanomaterials have substantial surface properties and high reactivity which helps in easy pathogen inhibition in the water. Nanoparticles based on copper metal ions are one of the most potent and efficient antimicrobial agents. It has been observed in the previous studies that antibacterial copper nanoparticles can be synthesized using biowaste eggshell membrane with the help of biotemplated methods. The antimicrobial effect could be observed by the zone of inhibition for organisms like

S. aureus and *E. coli*. The fabricated CuO NPs give a high recyclability value (Amrane et al., 2020).

5.5. Water quality monitoring

The water has been contaminated with several toxic substances which are detrimental for the human health; therefore, it is necessary to monitor the water quality. However, it is a difficult task because of the low contaminant concentrations, variability, and the complexity of the wastewater matrices. Therefore, for these problems to be addressed, the scientific community has been developing nanomaterial-based sensors for monitoring the water quality. With the properties such as fast analysis and proficient trace contaminant recognition, nano-sensors can be explained as sensitive materials that react toward the changes in surrounding stimuli, such as mechanical stress, heat, chemical stress, volume change, gravitational and magnetic changes, concentration, and electrical force (Amrane et al., 2020; Scoville, 2013). These nano-sensors can convey chemical, physical, and biological information regarding the characteristics and behavior and of the nanoparticles from nanoscale to macroscopic level (Kurbanoglu & Ozkan, 2018). There are three major components of nanosensors, starting with a recognizing component (nanotubes, nanometals, nanowires, nanoparticles) which is connected to a transducer (amperometric, conductometric, voltametric, spectrophotometric) and a real-time monitoring display (Riquelme et al., 2017; Talari et al., 2021).

The content of organophosphorus (OP) compounds has increased in the wastewater from rivers, industrial discharge and in the agricultural runoff, as OPs are widely used as pesticides. This constant increase in the level of OPs is an alarming situation as it is neurotoxic and even a trace amount is harmful. Therefore, for the detection of OP compounds (diazinon pesticide) in agricultural and tap water, aptamer-based optical nanosensors are designed, that utilize reduced GO quantum dots (rGQDs) and MWCNTs.

6. Recent advancements in the nano-technological remediation of wastewater

6.1. Green nanotechnology

The chemically fabricated nanoparticles undergo self-agglomeration in aqueous solution and have other harmful disadvantages. Therefore, the use of fungal enzymes, bacterial enzymes, and plant extract can be a potential solution. Biofabrication of nanoparticles along with the use of microorganisms makes nanotechnology a useful, sustainable and environment friendly method for wastewater remediation. Green nanotechnology facilitates with tools that help in the transformation of biological systems to green nanotechnological approaches to nanomaterial synthesis, while eliminating the associated toxicity. The microorganisms act as biological systems that function like reductive agents for the metal complex salt and lead to the generation of metallic nanoparticles (NP). These NPs achieve superior solidity in a liquid environment because of the process of coprecipitation or by the addition of proteinaceous and bioactive elements onto the outer face of the nanoparticles.

Previous research works have put forward the biofabrication of iron oxide nanoparticles using *Aspergillus tubingensis* (STSP 25) which is acquired from the rhizosphere of *Avicennia officinalis* (Mahanty et al., 2020). The nanoparticles synthesized this way can eliminate more than 90% of the heavy metals [Ni (II), Pb (II), Cu (II), and Zn (II)] with a regeneration capacity of up to five cycles. The metal ions were chemically adsorbed on the nanoparticle surface in endothermic reactions. For example, the microorganisms can facilitate the reaction with catalytic enzymes which when combined with nanoparticles, help in wastewater effluent treatment. The use of nanotechnology and the assistance of microbes that help in synthesis of useful products are discussed below in Table 5.3.

6.2. Enzyme assisted nanotechnology

When enzyme molecules are incorporated into nanomaterials, they reduce their cellular interactions via steric hindrances and minimize the surface energy (Ding et al., 2015). Since enzymes provide a supplementary catalysis distinctiveness and are environment-friendly, making them more efficient and pliant in wastewater treatment and green energy production. The use of immobilized enzymes on nanomaterials makes them stable and sturdy because of their low vulnerability toward diffusional constraints, unfolding

resistance, being able to be used multiple times, and increased kinetic characteristics (Ding et al., 2015). The huge surface area of the nanomaterials increases the immobilization efficacy with the help of elevated enzyme loading. When the immobilizing matrix of magnetic nanomaterials is utilized, these immobilized enzymes are easy to be segregated from the reaction blend. Multimeric enzymes can also attain stability by the process of immobilization on nanomaterials. This immobilization of enzymes on solid substrates induces changes in the structures, leading to a decreasing α -helical structure and an increasing β -sheet structure. Studies have revealed that glutaraldehyde-modified iron oxide magnetic nanoparticles give temperature and pH stable immobilized enzymes. The immobilized peroxidase enzyme has the capability of removing red and green azo dyes (Darwesh et al., 2019).

Table 5.3

Nanotechnology applied	Associated microorganisms	Advantage	References
<i>Pseudomonas aeruginosa</i>	Biological dye removal; electrostatic interaction and chemisorption	Sarioglu et al. (2017)	Electrospunnaofibrous webs; Zircona nanoparticles
<i>Lysinbacillus</i> sp. <i>NOSK</i>	Bacterial bioremediation	San Keskin et al. (2018)	Electrospun cyclodextrin fibers
<i>P. ostreatus</i>	Oxidation by immobilized laccase	Ji et al. (2017)	Enzyme immobilized nanoparticles

6.3. Waste valorization

The waste conversion to useful products using technology has been studied frequently in the last few years. This method is being utilized for the reduction of waste and generation of useful products simultaneously. This approach is used for the generation of biogas, biohydrogen, clinker, adsorbents, and biomolecules (Gupta & Shukla, 2020). With the help of nanotechnology, an enhancement in the production rate has been observed for

effective conversion of waste into resources. Scientific works have shown that nanoparticles can be utilized to improve dark fermentation reactions for enhanced biohydrogen production (Kumar et al., 2019). Use of nanoparticles in place of fermentative bacteria has helped in the generation of biohydrogen from wastewater. Mixed culture bacteria can be utilized along with single, dual, and multiple nanoparticles for the generation of biohydrogen. In this case, the biohydrogen production is the maximum when several nanoparticles are utilized and the use of different nanoparticles increase the dehydrogenase and hydrogenase activity, which leads to an increased biohydrogen production (Elreedy et al., 2019). Therefore, nanotechnology can be used as a tool for the generation of green energy for an economical, environment-friendly production, and sustainable industrial growth.

7. Conclusion

Nanotechnology when combined with microorganisms has given a green remediation process and an eco-friendly approach toward the bio-treatment of industrial effluents. This chapter has discussed the synthesis of nanomaterials which provides superior results for sustainable and cost-effective wastewater remediation. Enzyme assisted nanotechnology provides highly active, stable, and long-lasting enzymes which offer several uses. The technique should be utilized at a commercial scale to its full potential. Future research work can be moved toward the production of bioelectricity and biohydrogen from the wastewater, which would be beneficial in improving industrial economy via green energy generation.

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Chapter 6: Enhanced Bioremediation of arsenic-contaminated groundwater using bacterial biosorption, sequestration, and phytoremediation techniques

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Abstract

Groundwater is a vital water resource for human consumption and for various activities in a global scale as it plays an important role in hydrological cycle. Contamination of groundwater is a worldwide concern. Arsenic contamination is stated as the “largest poisoning of a population in history.” It is estimated that every year, in India alone around 43,000 people die due to arsenic contamination. Because of various oxidation states (-3, 0, +3, and +5), it is difficult to remove arsenic from contaminated water. Bioremediation utilizes natural microbial mechanisms to transform a toxic heavy metal into a less harmful state. It relies on microbial activity to remove, mobilize, and contain arsenic through biosorption, sequestration, and phytoremediation techniques to efficiently treat arsenic contaminated groundwater. Green approach of microbial bioremediation has become a promising alternative for treatment of arsenic contaminated ground water. With new discoveries of hyper-tolerant arsenic *microbacterium*, there is a promising hope of next-generation solutions through bioremediation that the arsenic contamination would be addressed more efficiently by identifying and employing microbial capabilities.

Keywords

Arsenic; Bioremediation; Biosorption; Contaminated ground water; Green

approach; Toxic heavy metals

1. Introduction

1.1. Bacterial biosorption

Biosorption is a variant of sorption techniques in which the sorbent is a material of biological origin (Torres, 2020). This technique is an eco-friendly, cost-effective, and a passive process. Here the Arsenic (As) ions from the aqueous phase gets sequestered by the cell walls. The physio-chemical interaction between the functional groups present in the cell wall and the metal ions plays a significant role in determining the sorption mechanism on the cell wall surface (Dadwal & Mishra, 2017). The binding site for these metals is provided by the lipids, proteins, and polysaccharides present in the cell wall of the biosorbent (Prasad et al., 2013). In some cases, this process is exclusive and is independent of cell metabolism. It comes under metabolism independent biosorption while for others it comes under metabolism dependant biosorption. The other method is my metal accumulation. The advantages of biosorption over other technique includes, the reusability of biomaterial, removal of toxic metals in water present in low concentration with high efficiency, no postproduction of secondary compounds, low cost on operation, and a brief operation time (Bhargavi & Savitha, 2014). Different types of biomaterials such as plant biomass, agricultural waste, and microorganisms like bacteria, fungi, and algae are used to extract As ions from aqueous water.

Certain studies have suggested some arsenic contaminated sites might pose less of a threat to human health than others due to the microbial and geological factors that work combined to reduce the mobility of arsenic in waters (Reisinger et al., 2005). These improve the native sediments that are rich in Fe for the optimum sequestration of the arsenic contaminants from the water. Studies have also reported limited arsenic mobility by the anthropogenic introduction of nitrate. This acts as an oxidizing agent for reduced sediments and also results in the production of arsenic-sequestering ferric particles naturally (Senn, 2002). It is known that in various extreme environments, microbes play a role in reduction of nitrate for the production of ferrous solids (Weber et al., 2001). Anaerobic reaction vessels that contain native sediments from Upper Mystic Lake, Arlington, USA were created to experimentally determine the specific microbial community associated with arsenic sequestration during periods of nitrate-induced iron oxidation (Gibney & Nüsslein, 2007). In the study, during nitrate respiration, *Sulfurimonas*

denitrificans was found to be the most abundant 16S rDNA clone libraries. This is most possibly due to the oxidation of sulfide coupled with the reduction of nitrate implying that it has a little, if any, effect on arsenic mobility implying the bacteria possess high arsenic resistance. It is now considered that certain microorganisms under heavy metal stress were successfully able to develop resistance against them and have evolved various strategies to cope up with the extreme metal stress. Arsenic is detoxified in multiple ways such as by the uptake of As (V) in the form of phosphate by phosphate transporters, uptake of As (III) in the form of arsenite by aquaglyceroporins, reduction of As (V) to As (III) by arsenate reductase, oxidation and methylation of As (III) by arsenate oxidase and methyltransferase and finally extrusion or sequestration of As (III). It has been reported that many bacteria possess redox potential for arsenic detoxification (Satyapal & Rani, 2016).

2. Biosorption mechanisms

Biological materials are used as adsorbents in biosorption. It is likely that the microbial cell will acquire metal in a variety of ways due to the complex structure of microorganisms. It is not fully understood how biosorption occurs (Ahalya et al. 2003). Biosorption refers to the “ability of biomaterials to accumulate heavy metal and metalloid toxicants from aqueous state through metabolically mediated (i.e., by the use of ATP) or by spontaneous physico-chemical pathways of uptake (i.e., without the usage of ATP, metabolically independent), or as a property of certain types of inactive, nonliving microbial biomass which bind and accumulate heavy metals even from extremely dilute aqueous solutions.” It is a complex passive process that is dependent on numerous factors like cell physiology, physicochemical factors such as temperature, pH, contact time, ionic strength, metal concentration, chemistry of the metal ions, and cell wall composition of microorganisms. Studies are ongoing to better understand the biosorption of various kinds heavy metals, for example, cadmium, silver, lead, nickel, etc. by using microorganisms like fungi, algae, or bacteria by different research groups (Sulaymon et al., 2013). For the advancement of lignocellulosic biosorbents, lignocellulosic biosorption mechanisms are essential parameters. Biosorption of toxic pollutants is thought to be facilitated by the binding sites in cellulose and lignin, which are major constituents of lignocellulose biomass (Kumari et al., 2022).

Advantages of biosorption over conventional metal removal methods include cheaper production of biomass (bacteria or fungi), use of biomass for removal of heavy metals treatment of large volumes of wastewater, reduced volume of waste or toxic materials production and by being functional over wide range of conditions including temperature, pH, presence of other metal ions, etc (Gavrilescu, 2004). This technique has two main disadvantages, saturation of active sites of metal binding ligands and reversible sorption of metals on biomass.

2.1. Metabolism dependent biosorption

Metabolism-dependent biosorption is exhibited by living biological material. It involves various mechanisms like chelation; a specific way in which ions and molecules bind to metal ions and it involves the formation or presence of two or more separate coordinate bonds between a polydentate ligand and a single central atom, physical adsorption; adhesion of atoms, ions, or

molecules from a gas, liquid, or dissolved solid to a surface. This process creates a film of the adsorbate on the surface of the adsorbent. It is a surface phenomenon. Generally, the adsorption process is classified as physisorption, characteristic of weak van der Waals forces, or chemisorption, characteristic of covalent bonding. It may also occur due to electrostatic attraction, precipitation; it is the creation of a solid in a solution or inside another solid during a chemical reaction or by diffusion in a solid. When this reaction occurs in a liquid solution, the solid formed is called the “precipitate” and the chemical that causes the solid to form is called the “precipitant” or complexation. It consists of a central atom or ion, which is usually metallic and is called the coordination center, and a surrounding array of bound molecules or ions that are known as ligands or complexing agents (Shamim, 2018). Many metal-containing compounds, especially those of transition metals, are coordination complexes. There may involve a single process or combination of these processes. If the metal binding to cell wall is metabolism dependent then it involves energy from ATP. The ligands present on the cell wall of biological material such as phosphoryl, carboxyl, carbonyl, sulfhydryl, and hydroxyl groups immobilize the metal ion and then uptake occurs. Other factors that affect the metal uptake by living biomass include nature of heavy metals ions, conditions of the medium, cell wall composition, etc. The uptake process by living biomass involves adsorption to cell wall and entering into the cytoplasm (Gadd, 2009).

2.2. Metabolism independent biosorption

The metabolism independent process mostly occurs in biomass consisting of dead cells. The adsorption process is the main key point behind such physicochemical biosorption mechanism. The adsorption process can be ionic interactions or physiochemical adsorption. Presence of anionic ligands on bacterial cell wall (carboxyl, amine, hydroxyl, phosphate, and sulfhydryl groups) also plays an important role in metal biosorption. Living biological mass is preferred over dead mass, because living cells have ability for continuous metal uptake, and self-replenishment (Malik, 2004). Previously it is reported that adsorption is a rapid process while accumulation is slow and energy dependent. The fate of metal inside cell can be accumulation, detoxification and/or efflux depending on the nature of bacteria. In past few decades, many groups worked on heavy metal resistant bacteria that can be used for bioremediation. Many workers reported that

cells of bacteria of genera *Alcaligenes* and *Pseudomonas* can be used for bioremediation purpose (Sinha & Mukherjee, 2009).

2.3. Metal accumulation

In order to have the physiological effect on the growth of cells, heavy metals must enter the cell. Metal uptake system in bacteria is grouped in two types; one is fast and unspecific, constitutively expressed and does not require ATP. They are usually driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. The second type of uptake system is highly specific, slow, inducible and dependent on ATP, in addition to the chemiosmotic gradient. They are only induced in times of need, starvation or a special metabolic situation. As cell surface encounter metal ion, formation of a complex takes place, which is a prerequisite for uptake of metals by the organism. Once surface sorption takes place, the metal is transported into the periplasmic space of Gram-negative cells and transported further into the cytoplasm. When cell encounters high concentration of any heavy metal, the heavy metal ion is transported into the cytoplasm, accumulated inside the cell due to one type of metal uptake which is fast, unspecific, constitutively expressed and does not require ATP (Pabst et al., 2010).

3. Arsenic sequestration

Reduction of bacterial sulfate is a mechanism for sequestering metals in contaminated environments. It primarily takes place through precipitation of metal sulfides. When this sulfide is produced by bacteria, reduction in As (V) and precipitation of As (III) takes place in sulfide phases (Rochette et al., 2000). The other alternative process is when these sulfides react with As-bearing iron-oxide that results in oxidized S and dissolved Fe^{2+} as end-products. This released As at times gets readsorbed with remaining iron oxides or at times might result in the formation of arsenic-sulfide complexes to improve the solubility of arsenic. These interactions play a vital role as finally it determines the process by which sulfate reduction through sequestration affects the mobility of arsenic in an aqueous environment (Saalfield & Bostick, 2009).

3.1. Intracellular sequestration

Bacteria oxidizes As (III) into a lesser lethal form of arsenic, that is, As (V). The arsenite oxidation process is catalyzed by the arsenite oxidase enzyme. This oxidation takes place in the periplasm of the bacteria. AoxS is a sensor kinase that senses the presence of arsenite and consequently activates regulator protein, AoxR which controls the expression of the aox operon in association with the RpoN (an alternative σ_{54} of RNA polymerase). This RpoN is essential for the initiation of the arsenite oxidase transcription in *Agrobacterium tumefaciens*. In *Herminiimonas arsenoxidans* DnaJ, is a heat shock protein, is required for the proper folding of the AoxR regulators (Koechler et al., 2017). AoxAB complex is an arsenite oxidase that is synthesized normally after expression of the aox operon and then is exported to the periplasm through the Tat (Twin-Arginine Translocation) protein export pathway. Moreover in the periplasm, the arsenite oxidase plays a vital role in the oxidation of As (III) into the As (V) (Satyapal & Rani, 2016).

The arsenate reduction system in prokaryote is mainly of two types (a) - (a) -cytoplas- arsenate reduction and (b) periplasmin arsenate reduction. In the first type, Arsenate reductase enzyme, arsC is involved in the reduction of the As (V) to As (III), when As (V) is taken up by the pst and pit membrane transporters and afterward extrudates through the arsAB pump. Whereas in ArrA periplasmic respiratory system, the arsenate reduces the enzyme. Homologues of the arsC gene present in both; plasmids and the chromosomes. In cytoplasmic reduction ArsC utilizes glutaredoxins which

serves as source of reducing potential. In this reaction cascade, arsenate first binds to the anion site in the ArsC then it forms an arsenate thioester intermediate with the active site Cys12. It is then reduced in two steps by glutaredoxin and glutathione, producing the Cystic2-S-As(III) intermediate, which hydrolyzes to release arsenite (Kruger et al., 2013). The reduced As III is extruded from the cell or sequestered in the intracellular compartments, either as free arsenite or as conjugates with glutathione or other thiols.

3.2. Extracellular sequestration

Recent microbial studies on bacterial extracellular polymeric substances (EPS) have suggested them to possess a potential role in arsenic sequestration due to its metal binding properties. In the study both *Ochrobactrum ciceri* SW₁ and *Exiguobacterium profundum* PT₂, in the presence of arsenic, have shown to possess an increased an increased level of EPS production. This arsenic stress has increased the levels of carbohydrate and protein contents in the EPS. This is clearly indicated by the increase in the peaks of 1035–1218 and 1362–1613 wavenumbers as observed in the spectroscopy respectively to cope with the arsenic presence in the environment vicinity. Moreover, in the experiment, the shifting of peaks in the As (V) from the treated sample from 847 to 800 and 1211 to 1134 per cm has demonstrated the involvement of various macromolecules like, carbohydrates and proteins in the sequestration of arsenic due to these EPS. This was further confirmed by the electron scanning microscope of the EPS that revealed structural alteration. So, it is concluded that the increased production of bacterial EPS along with numerous polyanionic functional groups on the bacterial surface possess a tendency for arsenic sequestration. Furthermore, it is also proposed that these EPSs are suitable biosorbent material to sequester arsenic from contaminated water (Saba et al., 2019).

A similar study on dissimilatory iron reducing bacteria (DIRB) in a laboratory setting was done using strain *Klebsiella oxytoca* IR-ZA, *Shewanella putrefaciens* IAR-S₁ and *S. xiamenensis* IR-S₂. *Klebsiella oxytoca* IR-ZA released As into aqueous phase from As-bearing ferrihydrite, while strain *Shewanella putrefaciens* IAR-S₁ and *S. xiamenensis* IR-S₂ resequestrated As. Upon further analyzing, this was because of the formation of secondary minerals during ferrihydrite reduction. With the help of Fourier Transform Infrared Spectroscopy and high-performance liquid chromatography (HPLC), the characterization of these EPS was performed. Furthermore,

higher signal intensity at 1070–1120 per cm from a previous study also pointed out that IAR-S1 generates more polysaccharide with C–OH functional group than IR-ZA (Zhu et al., 2012). It was reported that the arsenic releasing strain IR-ZA generates more succinic acid than mannose compared to the arsenic immobilizing strains of IAR-S1 and IR-S2. Mannose was not detected with strain IR-ZA while strains of IAR-S1 and IAR-S2 generated 0.094 and 0.035mg of mannose in 24h respectively. This concluded that mannose and succinic acids are two important components in EPS that affects As mobilization in extracellular arsenic sequestration (Liu et al., 2020).

4. Arsenic phytoremediation

4.1. Soil–water–plant interaction

Inorganic arsenic (arsenate and arsenite) is highly toxic to plants because it detaches phosphorylation and inhibits phosphate uptake. At higher concentrations, arsenic interferes with plant metabolic processes and reduces their growth. Under certain conditions it may lead to plant death (Dermatas et al., 2004). The sensitivity of plants to arsenic appears to be determined by the absorption ability of the plants or nontranslocation of arsenic to shoots. Beans and other legumes are among the most sensitive plants to arsenic toxicity. The arsenic toxicity threshold limit for most plants is 40 and 200 mg/kg in sandy and clay soils, respectively (Tu et al., 2002). However, it is important to understand that possible toxicity of arsenic depends on its species, and it has been documented that 1 mg/L soluble arsenic causes damage to plants such as cow peas and barley. Plants can be classified into three basic groups based on the growth in contaminated soil: excluders, indicators, and accumulators (Baker, 1981), and they have shown four different conceptual strategies of trace element concentrations in plant crops in relation to the increasing of total metal/loids concentrations in the soil.

4.2. Translocation mechanisms from the root to the shoot

Translocation is the movement of materials from leaves to other tissues throughout the plant. Arsenic shows low mobility (unlike phosphorous) with respect to translocation from roots to shoots in plants, except for hyper accumulators (Zhao et al., 2003). The uptake of arsenic by plant roots varies for different plants or their parts (Stoltz & Greger, 2002). Arsenic ions are taken up, translocated from the roots to the shoot system via the xylem and then redistributed between tissues. The translocation of arsenic ions from root to shoot depends on root pressure and leaf transpiration (Raskin et al., 1997).

Passive uptake includes adsorption processes, ion exchange, and diffusion of the ions from the soil solution into the root endodermis (tissues) which can decrease the rate of transportation of some ions. Active uptake takes place against the concentration gradient, but requires metabolic energy and can therefore inhibit toxin production. Some ions have different and/or parallel patches of movement (symplastic, apoplastic, transcellular). Plants have the ability to actively select ions moving into cells and thereby excluding toxins. Arsenate taken up by roots is reduced to arsenite in

cytosol by glutathione (GSH) and translocated to the shoots (Caille et al., 2005; Sattelmacher, 2001; Thompson, 1993). Some authors reported that among vascular plants, dicotyledons transport more arsenic to their shoot system than monocots. The hyper accumulator fern *P. vittata* localizes arsenic in epidermal cells, mesophyll cells and xylem tissues (Bondada et al., 2007). The translocation factor (TF) or shoot: root quotient (SRQ) (Baker, 1981; Baker & Whiting, 2002) expresses arsenic concentration in the shoot in relation to arsenic concentration in the root as:

$$TF = [AS]_{shoot} / [AS]_{root}$$

Arsenic-tolerant plants translocate less than 2.8% arsenic to the parts above ground leading to low accumulation. The TF values for wetland plants from mine tailing impoundments were between 0.01 for *Salix* sp. up to 0.2 for *C. rostrata* (Stoltz & Greger, 2002). Other arsenic-tolerant plants had values between 0.17 and 0.15 (Fitz & Wenzel, 2002). A more comprehensive study carried out by including 46 plant species, reported a range from 0.01 to 0.9, with a median of 0.09, in arsenate-treated plants. Hyperaccumulators had values about two magnitudes higher, for example, 23.86 for *P. vittata*, and 90.91 for *Pityrogramma calomelanos* (Francesconi et al., 2002). TF values lower than 1 for plants indicates that arsenic transportation to the shoots is limited (Gonzaga et al., 2006). It reported lower arsenic concentration in the roots of basin wild rye (*Leymus cinereus*) at the evaluated phosphates level, with higher shoot concentration. Higher phosphate concentration stimulated higher root to shoot transfer and about 50% lower arsenic uptake in water fern (*Salvinia natans*) tissues has been observed in the presence of phosphates. However, there is little knowledge of the extent and mechanisms of As transport in the phloem. Particularly in rice (*Oryza sativa*), As concentrations decrease distinctly from roots to grain (roots>stems and leaves>husks>grain) (Abedin et al., 2002; Liu et al., 2006; Xu et al., 2008; Zhao et al., 2003) pointed out therefore that the contributions of xylem-versus phloem derived As to the grain need to be evaluated experimentally.

4.3. Vascular plants

Plant materials such as living plants (Hoffmann et al., 2004) their parts or their dried, seized, and chemically treated seeds (Kumari et al., 2006;

Pandey et al., 2009) and also the residue of vascular plants from industry or agriculture, such as rice polish, and orange wastes (Ghimire, 2003; Ranjan et al., 2009) were tested as biosorbent material for arsenic. Maximum sorption capacity for arsenic depends on the three factors; sorbate solution characteristic, biosorbent characteristic and process duration. Sorbate solution characteristics such as initial arsenic concentration, pH of the solid solution, occurrence of other co-ions, sorption time duration, and temperature, control the quantity of adsorption. Important biosorbent characteristic are: material porosity, particle size distribution, specific surface area, cation exchange capacity and surface functional groups. Arsenic sorption is limited by phosphate concentration (Ghimire, 2003; Kumari et al., 2006; Ranjan et al., 2009). Cadmium, selenium, and carbonate ions in solution above 200mg/L showed an improved arsenic biosorption. It is reported that optimum pH for *Moringa oleifera* seeds a biosorbent is different for arsenite and arsenate ion sorption, and was, respectively, 2.5 and 7.5mg/AsL (Kumari et al., 2006). Maximum biosorption capacity for living plants was observed to be a two-stage process, that is, rapid first phase, and a slow second phase. The first, rapid phase of reaching equilibrium was found for arsenate sorption by *Salvinia minima*, to be 6–12h (Hoffmann et al., 2004), and for arsenite sorption by *Salvinia natans* 48h, with different arsenic ions concentrations. The second phase was up to 96 and 12h. The adsorption of metal ions onto a plant surface was conducted in a relatively short time, while the uptake of metal ions took a long-time and was more complex reported chelation of As(III) with the –OH groups for fresh different parts of the biomass of *Momordica charantia*. Most of these experimental results were in good agreement with the Langmuir and Freundlich sorption models. However, the Dubinin–Radushkevich (D–R) sorption isotherm was applied to evaluate the nature of sorption and was used to explain the heterogeneity of surface energies (Ranjan et al., 2009).

4.4. Plant litter as biosorbent

The biosorbent biomass constitutes mainly lignin, cellulose and other elements stored in the cell walls and vacuoles. Fibers, lignins cellulose, and other substances bind to cell walls, such as phenols, cutin, suberins waxes, and others (Berg & McLaugherty, 2020) can be used as biosorbent. Certain cell wall components, especially lignin and pectin, are assumed to be connected with the sorption of metal ions (Bailey et al., 1999; Quek et al.,

1998; Volesky & Holan, 1995). Plant fibers are capacious for sorption of metal ions, and can be used in water cleaning (Bailey et al., 1999; Conrad, 2008). Mesocarp fibers from *Cocos nucifera* coir consists mainly of xylem and a fiber sheath. Cell wall lignin are distributed in cell walls of the fiber sheath, but in the xylem, they were smaller in the secondary walls than in the walls of the fiber sheath. Pectin was in detectable quantities only in the fiber sheath walls, middle lamella, cell corners, and extracellular matrix. In the xylem, pectin was regularly distributed in the wall, with a higher concentration in the middle lamella and cell corners. All cell walls facing the lacuna had a high content of pectin. The contaminant ions were mainly loaded on the xylem and tend to be loaded on the large fibers. Contaminants were distributed on and across the whole secondary wall (Conrad, 2008).

5. Arsenic hyper-tolerant *microbacterium*

One of the most abundant toxic metal in the environment is Arsenic. Even though, in general, it is toxic to living organisms, studies have reported that certain microorganisms have the ability to utilize arsenic compounds as electron donors or electron acceptors, and can also possess arsenic detoxification mechanisms (Anderson & Cook, 2004). Because of the ubiquitous nature of arsenic in the environment, it has led to the evolution of arsenic defense mechanism in microbes in arsenic rich environment. Therefore, a majority of studies dealing with arsenic resistant bacteria have been conducted in those environmental locations that have unusually high concentration of arsenic. To a certain extent common microorganisms such as *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* do show some tolerance to arsenic. A 2012 study has identified four *Microbacterium* Species that exhibit arsenic hyper-tolerance. They were isolated from the soil contaminated with textile effluent with arsenic rich areas of Sanganer, Jaipur, Rajasthan, India (Flora et al., 2012). With respect to the specific morphological characteristics of *Microbacterium*, they are yellow-pigmented Gram-Positive coryneform rods. They are found in diverse environmental sources including both soil and water samples. Furthermore, they rarely cause human infection apart from immunocompromised patients and catheter insertion sites (Chorost et al., 2018).

In another interesting study, four high arsenite tolerant bacteria were characterized by 16S rDNA gene sequencing which revealed their similarity to *Microbacterium paraoxydans* strain 3109, *Microbacterium paraoxydans* strain CF36, *Microbacterium* sp. CQ0110Y, *Microbacterium* sp. GE1017. In addition to being resistant to cobalt, nickel, zinc, chromium, selenium, and stannous, the four *Microbacterium* strains also displayed variable sensitivity to mercury, cadmium, lead, and antimony. *Microbacterium*, which has high tolerance to arsenite, is found in soil that is arsenically polluted and may play a role in soil bioremediation.

5.1. *Microbacterium paraoxydans* strain 3109

Actinobacteria are members of this phylum. Displays high tolerance to arsenite. Significant role in bioremediation of the soil.

5.2. *Microbacterium paraoxydans* strain CF36

It is a member of the phylum Actinobacteria. The G+C content of its DNA is

69.9mol%.

5.3. *Microbacterium* sp. CQ0110Y

This bacterium belongs to the phylum Actinobacteria and it also shows heavy resistance to Arsenic in soil.

5.4. *Microbacterium* sp. GE1017

It is a member of the phylum Actinobacteria ubiquitously distributed in various environments and includes plant-associated bacteria that are able to colonize tissue of agricultural crop plants.

The above four are the arsenite-resistant *Microbacterium* species that were detected to tolerate arsenite up to 80mM from the arsenic contaminated site. It is reasonable to say, these species could play a role in the biogeo-cycling of arsenic in the environments as the strains isolated from soil also exhibit tolerance to heavy metals like cobalt, nickel, zinc, chromium and selenium. Adaptation to polluted soils accounts for this polymetal resistance.

In the study it is hypothesized that these arsenite tolerant bacteria could possess transformation ability and could represent a good candidate for bioremediation of native polluted soil (Flora et al., 2012).

6. Contemporary trends and impending prospects

Eco-friendly, cost-effective, and passive process that produces no or negligible secondary pollutants are the current sustainable techniques to treat arsenic contaminated groundwater. The scope of research is wide open to further elucidate the exact underlying mechanism behind the metabolism-dependent and metabolism-independent biosorption techniques to pave way for the next generation efficient techniques. Extensive research is needed to find systematic methods for the optimal usage of Phytoremediation techniques including the efficient usage of specialized arsenic hyper-tolerant *Microbacterium* to treat arsenic contaminated groundwater in a well-planned manner.

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Chapter 7: Metal pollutants: an environmental hazard

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Abstract

A big environmental concern nowadays is metal waste in our environment. Metals such as arsenic, cadmium, mercury, and lead are some contaminants which are nontoxic at low concentrations and are commonly found in the biosphere. But as their concentration increases, they become harmful and also affect the food chain. Rapid urbanization and industrialization is majorly responsible for metal pollution. Anthropogenic activities add to their severity. Being non-biodegradable in nature, these metals damage the environment and human health. Bioremediation is an accepted technology for treating such pollutants as it is more effective, eco-friendly, and inexpensive compared to other techniques. Some other metal remediation methods include phytoextraction, phytostabilization, and rhizofiltration. This chapter emphasizes on the various types of metal contaminants, their mode of action in environment in addition to continuous monitoring and regulation of their hazardous effects.

Keywords

Environmental pollution; Global threat; Health effects; Metal pollutants; Remediation

1. Introduction

Environmental pollution is one of the most vital problems nowadays. Water pollution, soil pollution, air pollution, etc. account for the environmental pollution, but one of their major causative factors is the metal pollution. Environmental contamination is the existence of a contaminant in the environment like soil, water, and air which may be hazardous and bring about distress to living beings and the contaminate the surroundings (Aleksandra, 2011). Heavy metal pollution is covert, tenacious, and cannot be reversed (Li et al., 2014). Pollution like these not only destroy the well-being of the atmosphere, water system, and food crops, but also hampers the fitness as well as welfare of the living creatures by affecting the food chain (Li et al., 2014). Heavy metals are a crucial environmental pollutant that generates a serious threat to humans and animals by their constant presence in the environment (Laghlimi et al., 2015). Industrialization and technical expansions have created a fearsome freight on the nature by developing enormous quantity of perilous waste, heavy metals (cadmium, chromium, and lead), metalloids, and organic contaminants that have paved way to precarious risk in the ecosystem (Ayangbenro & Babalola, 2017). Heavy metal contamination is a man-made inevitable matter that mankind has scuffled to handle (Vareda et al., 2019). In this chapter, different types of heavy metals present in the environment, their sources, effects, and remediation measures are reviewed. Remediation policies can be undertaken to cleanse the polluted environments or for treating the effluents (Vareda et al., 2019). Heavy metal decomposition is a difficult process of concern due to metal's characteristics like solubility, oxidation-reduction properties, and complex formation. It is difficult to breakdown as it remains in either chemical or combined form. Properties like flexibility, accumulation, nonbiodegradability, and endurance makes metal pollution one of the paramount environmental load (Carolin et al., 2017).

2. Metal pollution

Metal pollution is a global area of concern, mainly in industrially developed countries, as it is normal to find overhead amount of metal ions there. Numerous environmental concerns due to heavy metal pollution have come up worldwide (Dhaliwal et al., 2020).

2.1. Metal pollutants

They are the metals that cause metal pollution. Because of their high atomic weight or density, they are referred to as heavy metals (Briffa et al., 2020). Subsequently, liberating from the lithosphere, metals continue to be in the nature for many years as they cannot degrade and exert destructive effects on all organisms (Wu et al., 2019). The rate of contamination of heavy metals in our atmosphere has presently become a major environmental issue of genuine concern (Rahman & Singh, 2019).

2.1.1. Characteristics of metal pollutants

Characteristic properties of some metal pollutants along with their effects on animals and plants, as well as their usage have been stated in Table 7.1 (Ayangbenro & Babalola, 2017; Briffa et al., 2020). The term “heavy metals” denotes an element with high density which is more than $4\text{--}5\text{g/cm}^3$ with an atomic number >20 (Laghlimi et al., 2015) and is dangerous to mankind even at minute concentrations (Dhaliwal et al., 2020).

Unlike the organic contaminants, most metals are nonbiodegradable and thus appear to pile up in living creatures. Lots of heavy metal ions, such as mercury, cadmium, lead, nickel, and chromium, are considered to be highly toxic and even cancer causing. The pernicious outcomes of heavy metals create anxiety about environmental pollution due to heavy metals among common people (Atieh et al., 2017). These metals are present in the earth's crust naturally as well as in soils, rocks, sediments, waters, and microbes with natural background concentrations. Man-made release of metal accounts for the highest concentration of pollutants in the environment. As heavy metals are difficult to be debased, they remain in the environment for long time (Aleksandra, 2011). Toxicological properties shown by heavy metals are listed in Fig. 7.1.

2.2. Causes of metal pollution

2.2.1. Anthropogenic activities

Human activities like mining, smelting operations, industrial and domestic

activities, agricultural usage of metals as well as metal holding mixtures are the main sources of heavy metals that bring about environmental contamination leading to human health issues (Jacob et al., 2018). Due to their soluble and mobile reactive forms, heavy metals formed by human activities usually have a rising bioavailability. Various anthropogenic sources include alloy manufacturing, deposition in the atmosphere, battery processing, biosolids, insulation, improper assembly of industrial waste matters, leather tanning, mining, pesticides, etc (Ayangbenro & Babalola, 2017).

2.2.2. Agricultural processes

Human influence on soil by means of agriculture is high. Plants desire micronutrients and macronutrients for their growth as well as to accomplish their life cycle. Metal ions which are a requisite for plant development and deficient in the soil have to be provided artificially either by foliar spray or by soil application. Various pesticides are used extensively in horticulture and agriculture implementations which contain substantial heavy metal concentration. For decades, lead arsenate was accustomed to sway off some parasitic insects in fruit orchards. Numerous formulations of As, Cr, and Cu are now used to protect timbers in many abandoned places. In future, such contamination will create problems if these areas are used for agriculture (Dhaliwal et al., 2020).

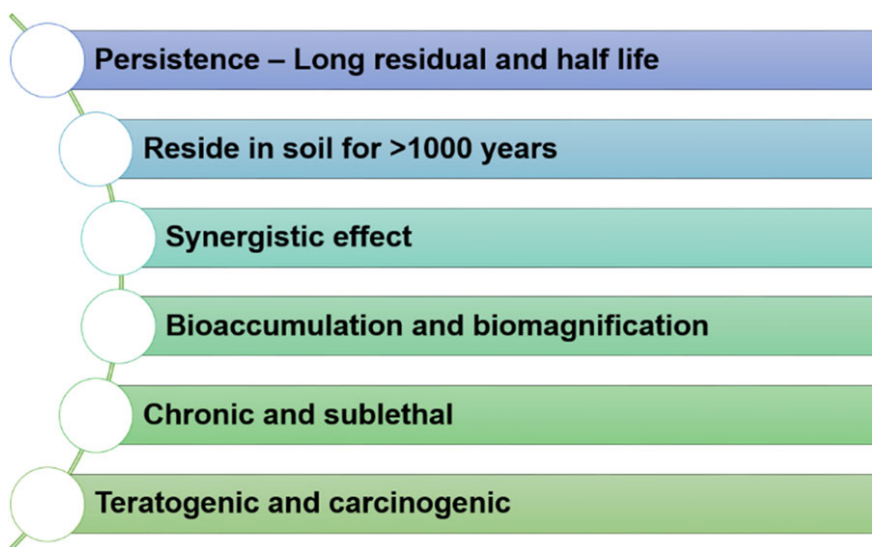


Figure 7.1 Toxicological properties of heavy metals.

Table 7.1

Metal	Properties	Effect on animals	Effect on plants	Uses
Iron-nickel sulfide: Pentlandite, Green hydrous nickel silicate: Garnierite, corrosion resistance at high temperatures silvery	Dermatitis, cardiovascular diseases, nasal cancer, kidney diseases, lung problems	Decline in enzyme activity, chlorophyll content, growth as well as uptake of nutrients	Making of coins and ornaments, stainless steel alloy, plated to prevent corrosion, and welding	Nickel
Siberian red lead was extracted as chromite ore. Solid, gleaming, and moderately active metal that reacts with all acids	Vertigo, gastrointestina ulceration, toxic nephritis, bronchitis, lung cancer pneumonia, etc.	Chlorosis, senescence, wilting, lesions, reduced growth and germination, oxidative stress	Alloy, leather tanning, electroplating, dye paint, synthetic rubies, color glass as green	Chromium
Extracted as a crude oil of organic complexes and iron ores from phosphate rocks, Ductile,	Impairment in nervous system	Inhibits enzyme activity	Steel shock and vibrant resistant springs, dietary supplements in the form of vanadyl sulfate and sodium metavanadate	Vanadium

reacts with hot acids like sulfuric acid				
Iron arsenic sulfide: Arsenopyrite Arsenic sulfide realgar also known as “Ruby of Arsenic” Bright silvery-gray color	Conjunctivitis, dermatitis, brain damage, skin cancer, cardiovascular and respiratory disorder	Membrane damage, loss of fertility, inhibits overall growth, interferes with metabolic processes and fruit production, oxidative stress.	Production of glass, wood insecticides formulation, doping agent in semiconductor, conversion of electric current into laser light.	Arsenic
Greenockite: Cadmium and sulfur, Present in combination with zinc	Bone disease, emphysema, kidney diseases, prostate cancer, microcytic hypochromic anemia, lymphocytosis, lung cancer, testicular atrophy	Chlorosis, reduce nutrient concentration, growth and seed germination	Fertilizer, nickel-cadmium batteries, pesticides, stabilizer in plastic processing, glassware coloration, corrosion-resistant plating	
Cinnabar: Mercury sulfide, Rare, silvery liquid metal	Attention deficit, ataxia, dementia, decrease in rate of fertility, blindness, deafness, gingivitis, dysphasia,	Affects antioxidative system, enhance lipid peroxidation, inhibit plant growth and nutrient uptake,	Barometers, manufacturing chlorine, thermometers, fluorescent lightbulbs, tooth fillings, gold recovery	Mercury

	gastrointestina irritation, pulmonary edema kidney problem	photosynthesis, induced genotoxic effect, homeostasis		
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Table Continued

Metal	Properties	Effect on animals	Effect on plants	Uses
Galena: Lead sulfide Combined with zinc, copper, and silver, Grey-silver alloy	Anorexia, neuron damage, chronic nephropathy, high blood pressure, hyperactivity, risk factor for Alzheimer's disease insomnia, learning deficits, low fertility, damage in renal system,	Chlorosis, inhibit photosynthesis, growth, enzyme activities, oxidative stress, and seed germination	Insecticides, beauty products, and lead glazes on pottery, cable sheeting, lead-acid batteries, computer screen-guard, lead crystal glass, ammunition, and projectiles	Lead

2.2.3. Industrial activities

Heavy metals have left an indelible mark in soil as a result of industrial activities such as coal and petroleum combustion (Dhaliwal et al., 2020). Since various heavy metals are used in specialist alloys and steel (V, Zn W, Mo, Co, Cu, Ni, Cr, Pb, Mn, Li, Ag, As), their processing, disposal, or even discarding in scrap metal may hasten environmental pollution (Hassaan et al., 2016). Mining has adversely affected the environment, leading to demolition and modification of the ecosystem, as well as loss of biodiversity and aggregation of contaminants in the environment. Ore processing and mining are major means of metal pollution in the soil (Ayangbenro & Babalola, 2017).

2.2.4. Other sources

Batteries (Zn, Sb, Pb), pigments and paints (Pb, Co, As, Sb, Cd, Mo, Si, Zn), catalysts (I, Co, I, Mo, Rh), polymer stabilizers (Zn Sn, Pb, Cd), and those used in printing and graphics (Se) are some other sources of metal pollutants in the ecosystem.

3. Effects of metal pollutants in environment

3.1. Global threat

In today's world, metal pollution is a major concern as the metal ions are nonbiodegradable and persist in the environment. The bioaccumulation of these heavy metals in the surrounding areas is lethal and becoming a major threat to the wellbeing of living organism. When compared to organic contaminants, heavy metals are unable to deteriorate by chemical or biological methods. Therefore, they can only converted into less toxic forms (Ayangbenro & Babalola, 2017). Among the heavy metals Pb and Cd are very dangerous for humans, plants, and crop cultivation. So, these metals should be reduced to nontoxic forms. Pb has a critical out turn in human health as it is a carcinogen and its potency can be easily identified in minute concentration (less than 2mg). This can lead to immune system damage and also change the properties of enzymes. For a wide range of organisms Cd is also a carcinogen, hence, its daily consumption should be reduced below 30µg. Other metals like Zn, Co, and Cu play a substantial role in living organisms but they can also turn harmful if consumed above a particular concentration. In case of enzymes, Cu and Zn are two essential cofactors wherein Zn plays an important role in protein synthesis and nucleic acid synthesis while Cu helps in neurotransmitter synthesis as well as production of energy. Co is the principle component of vitamin B₁₂. Daily consumption of Zn and Cu more than the range of 50–300 and 150–200mg, respectively, cause chronic and acute toxicity. Excessive use of Co and Cu can result in the formation of intense reactive oxygen species, which can oxidize membrane lipids, nucleic acids, and proteins (Bandiera et al., 2016). The human beings who are in contact with heavy metals are suffering from various ailments such as renal and gastrointestinal toxicity, melancholy, cardiovascular issues, tumors, tubular, osteoporosis, etc. Newborn babies, children, and young people are especially prone to heavy metal pollutants, bringing about developmental problems and low intuition balance. Many countries have formulated norms for permissible limit of heavy metals in the food to prevent its toxic consumption (Vardhan et al., 2019).

3.2. Mode of action in environment

The liberation of heavy metals into water bodies may bring about numerous biological, chemical, and physical reactions. They can be classified into two, which comprise the action of metals on the environment and the action of

environment on metals. The first grouping depends on the natural environment; where there could be an alteration in density, diversity structure of the community and species composition of population. The amount of heavy metal species in water and sediments has a significant impact on the degree and extent of the variations. As a result, physicochemical processes in effluents and water bodies have an important, though indirect, influence on biological reactions. The second category indicates that perhaps the conditions under which water bodies are obtained can cause a change in the speciation and immortality of heavy metals. Variable contributions of man-made and natural products, such as untreated wastewater, geochemical, and suspended solid particles, as well as chelator concentrations, are taken into account in such cases (Vardhan et al., 2019).

3.2.1. Mode of action in soil

Because of the accumulation of metalloids and heavy metals released from rapidly expanding industrial areas, soil becomes polluted. Swiftly enlarging industrial places, mine tailings, discarding of leaded gasoline, metal wastes, paints, animal manures, fertilizers, wastewater irrigation, sewage sludge, pesticides, and spillage of petrochemicals, coal combustion residues, and deposition in atmosphere are some of the causes. These heavy metals mostly become polluted in the environment because of various reasons such as follows: (i) in comparison with natural cycles, their rates of growth by anthropogenic cycles are faster, (ii) they are relocated from mines to diverse locations with high capacity for significant exposure, (iii) as compared to those in the receiving setting, the rate of metals in discarded items is relatively high, and (iv) chemical form of samples analyzed in the receiving environmental system can make a metal more bioavailable. Soil washing, immobilization, and phytoremediation are regularly listed as the best accessible methods for clearing up heavy metal polluted environment (Wuana & Okieimen, 2011). Remedial measures are necessary for shielding and reconstruction of soil ecosystem that is polluted by heavy metals (Abosede, 2017).

3.2.2. Mode of action in water

Two important offenders of pollution are urbanization and industrialization. Indistinguishable directives are seen in various countries, contamination of arsenic, for example, is still a global issue. The amount of arsenic in

groundwater is higher than that in surface water, where arsenic is mainly found in dissolved salts from weathered rocks and soils. Even if only trace amounts are carried to water sources, they can pose a serious threat to humans and perhaps other ecosystems. Heavy metals are abundant in raw water and do not degrade in the wastewater treatment process. These are eliminated either in the last effluent or in the resultant sludge formed. The characteristics and pollutant's level of the sewage that reaches the water depend on the sewage treatment efficiency. The nondegradable contaminants can then enter into the food web along with the aquatic sources like fish which can then be passed on to bigger fish, birds and mammals, including humans, which emigrate and transfer the pollutants to different habitats (Briffa et al., 2020).

3.2.3. Mode of action in air

Like water pollution, air pollution due to heavy metals is caused mainly due to urbanization and industrialization. Pollutants get into the atmosphere through different means. They can join as droplets, particles, or vapor, or in conjunction with particles or droplets. Human activities such as industrial actions, burning of fossil fuels, smelting, vehicle exhaust, etc. release a lot of metal pollutants into the environment. The coarse matters can create major health issues and also give rise to infrastructure degradation, acid rain formation, and corrosion, biodiversity loss due to particulate matters getting into the water sources when it rains, and even pave way to haze formation. Despite the fact that the troposphere and stratosphere are needed for contaminant movement, the atmosphere is divided into five major layers. Pollutants liberated next to the earth tend not to move far due to turbulence and restricted airflow. Because of the circulating air, contaminants emitted at a greater distance will travel farther. They can thus travel to a long distance when mixed with circulating air and can lead to global issues. Soluble particles will then interact with rain, falling into bodies of water and soil, contaminating the environment (Briffa et al., 2020).

3.3. Food chain depletion

The involvement of heavy metals in the food chain is a major source of concern due to the negative health effects they have on living creatures. Even in minute amounts, heavy metals are highly dangerous and can cause significant damage (Vardhan et al., 2019). Ingestion of tainted food, inhalation

from the environment, drinking polluted water, and skin absorption from agriculture, medical, fabrication, urban, and industrial zones are all the ways by which heavy metals can enter human life (Briffa et al., 2020). Metals are highly reactive even at minute amounts and can stack up in the food web, leading to serious public health problems (Ayangbenro & Babalola, 2017). Because of their noxious behavior and propensity to accumulate in the food chain, many heavy metal bands have become a source of concern. Fish, mollusks, and several other aquatic species at the bottom of the food chain can collect all of the metals and transfer them to humans via food, causing acute and chronic health problems (Hassaan et al., 2016).

4. Methods of remediation

Remediation is a technique used for lowering or eliminating the pollutants by attaching on the origin of pollutants to safeguard the nature and human beings from the hazardous actions of the pollutant (Parmar & Singh, 2015). Quantity of heavy metals in nature should be observed to prevent threats from reaching living beings. This can be achieved by the following means:

- by proper elimination of environmental contaminants from the industry,
- by analyzing the soil for metal contaminants before cultivation,
- by monitoring the unnecessary fumes from vehicles,
- by analyzing metal contaminants in food or water (Abosedo, 2017).

Various methods of remediation measures are in use for eliminating heavy metal pollutants from the surroundings, by means of both biological and nonbiological methods.

4.1. Nonbiological methods

4.1.1. Soil replacement

This technique includes the limited or absolute elimination of metal contaminants from the soil and is then replaced with contamination-free soil, popularly known as the “dig-and-haul” technique. The major disadvantages of these techniques are

- it can be done only for a limited area that is highly polluted,
- very costly,
- moves only the polluted medium to a different place, shifting the polluted region from a higher risk to one of lower risk (RoyChowdhury et al., 2018).

4.1.2. Chemical methods

4.1.2.1. Immobilization

Mostly in soil, heavy metals are separated into dissolved, transferrable, Fe/Al bound, Ca/Mg bound, organic material bound, and remnant types. Metals in soluble forms are easily treatable by organisms and are considered as the most significant pollutant remedial measure in the world.

Immobilization reduces solubility of metals and, as a result, their effectiveness in the soil. In soil, heavy metals are seen in different states such as soluble, organic matter bound, exchangeable, Ca/Mg bound, and residual forms. Among these, soluble forms of metals are easily available for biological organisms and these are figured as the foremost pollutant in the environment. This technique increases the soil potency by reducing the metal solubility.

4.1.2.2. Extraction

This approach entails adding chemical agents to carry metals into an aqueous state by enhancing metal solubility, allowing the metals to be removed from the entire system. This technique primarily employs a variety of inorganic eluents, chelating agents, and surfactants (RoyChowdhury et al., 2018).

4.1.3. Thermal process

The adsorption of contaminants takes place using a thermal method, which facilitates the disunion of gaseous pollutants present in the polluted environment. It is generally an ex situ mode of treatment, in which the polluted area is heated to an ultra-high temperature. During this, all gaseous pollutants, mainly organic matter, can be removed from the polluted plate. A major disadvantage of this technique is the release of air along with the gaseous pollutants. Anyhow, capturing and segregation methods aids in its reduction (Atieh et al., 2017).

4.1.4. Electrokinetics

Electrokinetics is a technique for clarifying the polluted environments, in which an electric current of lower intensity is passed in between the anode and cathode phase that are seen in the environment especially in the soil. Simultaneously, in between the electrodes small charged particles and ions are transferred. Hence, buffer solutions are used to maintain a constant pH. This technique is mostly conducted ex situ. From the electrode, the accumulated metals can be easily detached through precipitation or electroplating. This can be effectively and efficiently conducted in low permeable soils (Atieh et al., 2017).

4.2. Biological method (bioremediation)

Bioremediation is a technique which makes use of microorganisms for

removing heavy metals from environment. Adsorption and reduction or removal of pollutants from the environment with the help of biological resources is the main techniques undertaken (Jacob et al., 2018). A vast variety of pollutants which are both organic and inorganic can be remediated by microorganisms, it is important to appreciate from the start itself that microorganisms are not able to destroy metals. In any case, altering the physicochemical properties of the atmosphere is a good way to start in which microorganisms are capable of creating an impact on metals for its mobility (Eccles, 1999). Factors influencing bioremediating capability of microorganisms can be classified into biotic and abiotic components (Jacob et al., 2018), depicted clearly in Fig. 7.2.

4.2.1. Key factors in bioremediation

Bioremediation techniques are divided as, biomineralization, biotransformation, biosorption, and bio-accumulation which make use of microorganisms. During this process, the plants as well as microbes will attack the heavy metals enzymatically and nullify them into nonhazardous components.

4.3. Phytoremediation

It is a low cost and an eco-friendly process for making the polluted environment contamination-free. Conventional remediation methods like physical, thermal, chemical, and other techniques are extortionate, and in environment they may cause a huge contamination (Ahmed et al., 2018). Phytoremediation comprises of remediation methods that involve various treatment plans leading to pollution degradation, removal, and immobilization (Parmar & Singh, 2015). Fig. 7.3 depicts the schematic representation of bioremediation approaches. The water bodies and soils that have been polluted with heavy metals can be depolluted by phytoremediation as it is very cheap and more reliable. Regular remediation methods like physical chemical, thermal, and other methods are much expensive and may lead to environmental pollution when compared to phytoremediation (Ahmed et al., 2018).

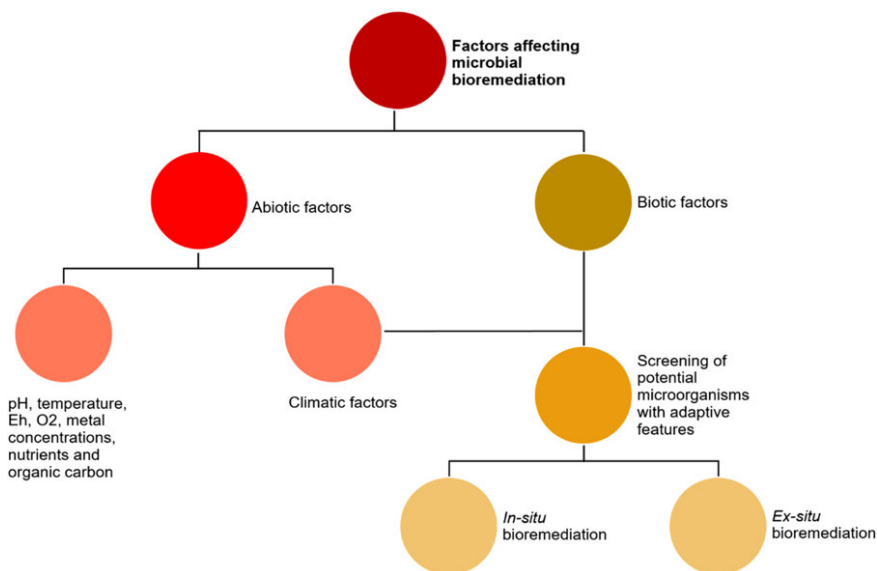


Figure 7.2 Factors affecting microbial bioremediation.

4.3.1. Phytoextraction

It is the process by which plants absorb contaminants from the atmosphere through their roots and aggregate those pollutants in shoots that are seen above the ground. It is also termed as phytoabsorption or phytoaccumulation. Phytoextractor plants have numerous attributes to withstand the effects of heavy metals and can also extract these from the polluted areas. By adapting phytoextraction, the problems faced by the plants and microbiome are reduced by acquiring the following factors (i) steady growth with elevated biomass, (ii) unchallenging cultivation, (iii) highly extended root system, (iv) adapting various environmental conditions, and (v) detestation to herbivores (Jacob et al., 2018).

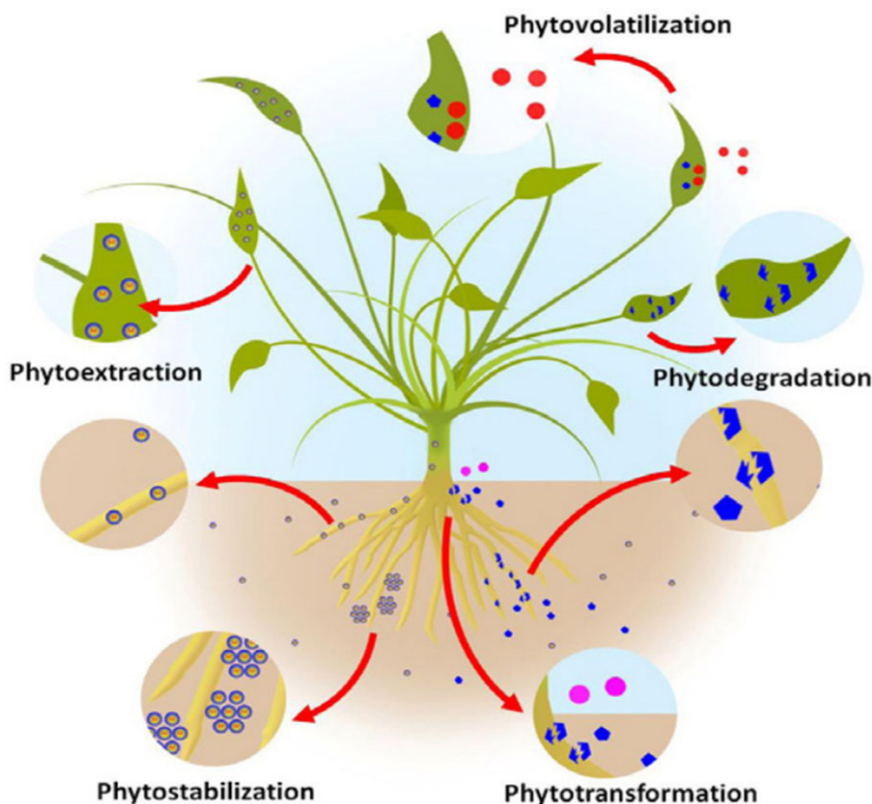


Figure 7.3 Schematic representation of bioremediation approaches (Parmar & Singh, 2015).

The plant selected for phytoextraction must have the following attributes:

- high levels of the metal adaptation;
- ability to assimilate elevated amount of metal in its harvestable parts;
- should possess fast growth;
- have an ability to form elevated biomass in the field;
- should possess an ample root system (Garbisu & Alkorta, 2001)

Phytoextraction can be classified into two groups, continuous phytoextraction and chelate-induced phytoextraction. Continuous phytoextraction necessitates the use of hyperaccumulating plants, whereas chelate induced phytoextraction necessitates the use of high biomass crop plants and chelating agents. Metal-accumulating plants are introduced or inserted into metal-polluted land and processed using traditional agricultural methods in case of continuous phytoextraction. Metal components are sucked up by the roots of developing plants and transferred to the aerial shoots, where they

will be assimilated. When metals are not available in their natural state in the ground for adequate plant absorption, chelates or highly acidic agents are added to the metals to facilitate their release into the soil solution, thus increasing the metal assimilation potential and intake speed of nonhyperaccumulating plants (Parmar & Singh, 2015).

4.3.2. Phytotransformation

Phytotransformation is also termed as phytodegradation, in which the neutralization of contaminants taken up by plants occurs via metabolic processes or the breakdown of contaminants outside the plant through the effect of compounds produced by the plants (Parmar & Singh, 2015). It accounts for the plant's intake of pollutants from water bodies and their transformation into organic components, which are of no or minute toxicity.

4.3.3. Phytostimulation

It is a method in which the plants expel out substances at their root phase (rhizosphere) to initiate degradation of fungal or microbial pollutants. This cascade is initiated when plants provide growth needs to microorganisms, which aids to support the reduction in pollutants. Consequently, it can be noticed that there is an initiation of a mutual or symbiotic relationship between plants and microorganisms toward pollutant degradation. The plants expel out natural components and oxygen at their rhizosphere which initiates growth of microbes, and thus microbes succeed in pollution degradation (Ahmed et al., 2018).

4.3.4. Phytostabilization

It is a technique for reducing heavy metal motility in the ecosystem by sorption and aggregation in the root system, precipitation within the root region, or a decline in metal valence in the rhizosphere, making them insignificant and thereby limiting their access to the food web and ground water (Wuana & Okieimen, 2011). During the cascade, pollutants, especially those seen in the soil system are immobilized by a variety of hyperaccumulating plants by means of root absorption and accumulation, along with precipitation at the root phase and the soil stabilization. This method is mainly used to treat polluted sediments, soils and sludge, and it widely relies on the capability of root nodules to stop the bioavailability and movement of the pollutants inside the nature.

Meanwhile, the major disadvantage of this method is that the pollutants

remain the same in soil. Because of the changes in soil conditions and dissemination of organic matter, there occurs an incomplete and slow discharge and leaching, affecting the supply of the metals to nearby places by means of soil erosion. Hence, stable and steady follow-up activities are required during the processes of phytostabilization to notice and detect the heavy metal bioavailability, movement, and ecological influence (Ahmed et al., 2018).

4.3.5. Phytovolatilization

The technique of deletion of gathered pollutants from the leaflets of plants to atmosphere by means of transpiration procedure is known as phytovolatilization (Jeevanantham et al., 2019). It makes use of plants which can evapotranspire pollutants, like Selenium (Se), Mercury (Hg), and gaseous hydrocarbons, from water bodies. Organic pollutants and heavy metals can be removed by phytovolatilization (Parmar & Singh, 2015). Plants uptake the pollutants from environment at the time of water intake and deliver them to their leaflets, where it is converted into a gaseous form into atmosphere at comparatively lesser quantity. This method is used for the remediation of areas polluted with mercury, which converts it into less hazardous mercuric ions (Atieh et al., 2017). Phytovolatilization is an advantageous method for eliminating Mercury (Hg) and Selenium (Se). During this process, the heavy metals are turned into its gaseous form and liberated into the atmosphere from leaves (Jacob et al., 2018). Moreover, one of its main disadvantage is that it does not degrade the pollutants entirely, it just changes its state from one form to another, that is, from soil into the atmosphere, where the contaminants can deposit again (Parmar & Singh, 2015).

4.3.6. Phytofiltration

Phytofiltration also known as rhizofiltration is a method in which pollutants are taken up by the root system from water sources with the aid of aquatic plants that are freely floating, submerged, or emergent (Jacob et al., 2018). In rhizofiltration, aquatic as well as terrestrial plants are used for adsorption, concentration, and precipitation of pollutants present in polluted water bodies with lesser amount of pollutants from the plant roots. The usage of terrestrial plants in rhizofiltration is widely accepted, compared to other plants, as they possess a larger surface area created by wrapping of their root follicles with an all-embracing fibrous root structure (Ahmed et al., 2018). The

main merit of this method is that it can be done either ex situ or in situ, and floral varieties which are nonhyperaccumulators can be utilized during this cascade. But the major drawback of this method is that the raised water level in water plants raises the trouble in drying, composting, and carbonization (Ahmed et al., 2018).

5. Conclusion

Metals are naturally found in nature in a certain concentration, but it turns toxic when its concentration exceeds the limit. Metal pollution is one of the major causes of environmental pollution. Metal pollutants present in the environment are a hazardous problem which may cause diseases and even death in humans and other life forms. These pollutants are formed due to various causes such as natural, industrial, and many more, but the main cause is believed to be the anthropogenic activities. Various remediation techniques have been developed ranging from biological to nonbiological methods. Biological measures of remediation are said to be more reliable and eco-friendly when compared to nonbiological methods.

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Chapter 8: Biotransformation of bisphenol A by laccase enzymes

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Abstract

Bisphenol A (2,2-bis(4-hydroxyphenyl) propane, BPA) belongs to the so-called emerging pollutants having endocrine-disrupting properties. In addition, it is suspected of having carcinogenic properties. BPA is used in the manufacture of several everyday products and, as a result, its discharge into the environment is expected. Thus, it is found in surface water, groundwater, and seawater at concentrations ranging from 5 to 200 ng/L, which are potentially harmful to humans and wildlife. Therefore, the removal of BPA from industrial wastewater to avoid BPA environmental pollution is a matter of serious worldwide concern.

The conventional wastewater treatment processes are costly and inefficient for the removal of BPA. This has driven the search for alternative methods such as those based on oxidative enzymes. In this context, laccases are attracting increasing interest in the last decades due to their broad substrate specificity and the fact that they only need molecular oxygen to exert their catalytic action producing water as the only by-product. This approach is believed to be a promising technology since it is cost-effective, environmentally friendly, and does not produce sludge. Detailed characterization of the intermediates and metabolites produced during enzymatic degradation of BPA as well as toxicity studies are of great importance to assess the effectiveness of the biodegradation process. This review reports the metabolites found by different researchers in the degradation of BPA by laccase enzymes.

Keywords

Biodegradation; Bisphenol A; Emerging pollutants; Laccase; Wastewater

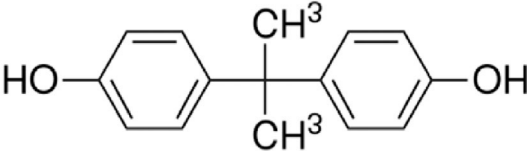
1. Introduction

Bisphenol A (BPA), chemically known as 2,2-bis(4-hydroxyphenyl)-propane (Table 8.1), is a monomer, manufactured from phenol and acetone in the presence of a catalyst, used as an intermediate to produce polycarbonate and epoxy resins. It is slightly soluble in water, 120–300mg/L at room temperature (U.S. Environmental Protection Agency, 2014) and bioaccumulates moderately in aquatic organisms (Cerkvenik-Flajs et al., 2018).

The superior properties of plastic made of BPA, such as heat and chemical resistance, durability, and strength at low temperature and easy processing, make this compound very suitable to manufacture a great variety of consumer goods (Global Bisphenol A, 2014). Thus, BPA is present in a huge amount of everyday life products such as plastic food and beverage containers (Abdel-Rahman et al., 2012), toys (Cao et al., 2010), thermal paper receipts (Konieczna et al., 2015) and electronic equipment (Global Bisphenol A, 2014). Thus, the annual worldwide production of BPA was estimated to be 8 million metric tons in 2016 and is expected to reach 10.6 million metric tons by 2022 (Bisphenol, 2016, p. 194). BPA enters the environment mainly due to the discharge of untreated and/or not properly treated effluents from the manufacturing plants of BPA and BPA-containing products (Cousins et al., 2002; Klecka et al., 2009; Staples et al., 1998). Other BPA environmental sources are the discharge of effluents and/or sludge from municipal wastewater treatment plants (WWTPs), leaching from landfills, combustion of domestic waste and plastic degradation in the environment (Flint et al., 2012; Fu & Kawamura, 2010; Teuten et al., 2009).

Table 8.1

Systematic chemical name	4,4'-isopropylidenediphenol
Bisphenol A	Synonyms
BPA	
Phenol, 4,4'-(1-methylethylidene)bis-4,4'-propane-2,2 diyldiphenol	
2,2-bis(4-hydroxyphenyl)propane	
80-05-7	CAS number
201-245-8	EC number

$C_{15}H_{16}O_2$	Molecular formula
	Structural formula
228g/mol	Molecular mass
120–300mg/L at 25°C	Solubility in water
38 (water), 75 (soil), 340 (sediment), and 0.2 (air) (Corrales et al., 2015)	Half-life (days)

The presence of BPA in the environment is of great concern since it has been identified as an endocrine disrupting chemical (EDC) by the US Environmental Protection Agency (EPA) and the World Wide Fund for Nature (WWF) and is suspected of having a carcinogenic activity (Barrios-Estrada et al., 2018; Shafei et al., 2018). Therefore, it is of utmost importance to remove BPA from effluents before discharging them into the environment. Consequently, in the last years, numerous methods to degrade BPA have been investigated. Advanced oxidation processes (AOPs), such as Fenton reaction (Mohapatra et al., 2011), ozonation (Wang et al., 2014), photocatalysis (Kuo et al., 2015; Tan et al., 2015), ultrasonics (Yu et al., 2014) and combined techniques (Nie et al., 2014), oxidize BPA efficiently due to the production of hydroxyl radicals. However, these processes present important drawbacks such as high cost, generation of toxic byproducts and are not environmentally friendly (Michałowicz, 2014). This has driven the search for novel and environmentally friendly technologies such as those based on enzyme catalysis (Cordova Villegas et al., 2016). In this sense, the use of ligninolytic enzymes, produced by a certain type of fungi, has been a topic of increased research in the last decades due to their broad substrate specificity that enables them to degrade a wide variety of recalcitrant pollutants (Mansur et al., 2003). Among such enzymes, laccases are particularly interesting because they only need oxygen from the environment to bring about their catalytic action producing water as the only by-product. In addition to this, their broad substrate specificity can be extended in the presence of low-molecular organic compounds known as redox mediators (Fabbrini et al., 2002). Hence, the use of laccases for BPA degradation seems very promising. To assess the effectiveness of the laccase-catalyzed BPA degradation a detailed characterization of the biotransformation products and

their toxicity is needed.

2. BPA occurrence and toxicity

The presence of the xenobiotic compound BPA in the environment has become ubiquitous due to its large production and its wide use in many manufactured products, supposing a wide and almost continuous exposure for humans (Chevalier et al., 2015; Legeay & Faure, 2017). The use of BPA in food and beverage contact materials leads to consumer exposure (Markis et al., 2013; Niu et al., 2012; Yoshida et al., 2001). Drinking water, air, dust inhalation and dermal contact are other ways of BPA contact by the population (Geens et al., 2012; He et al., 2009; Vandenberg et al., 2007). Thus, it was found that more than 95% of people have BPA in their bodies (Calafat et al., 2005). The European Commission (EU) has established a migration limit of 0.6mg/kg for BPA per kg of food (mg/kg) (Regulation (EU) No 10/2011, 2011) and has forbidden the use of BPA in the production of baby bottles (Regulation (EU) No 321/2011, 2011).

BPA is commonly found in the environment, including surface waters (Belfroid et al., 2002; Santhi et al., 2012), despite its relatively short half-life (Table 8.1). Some studies reported BPA concentrations in river waters in the ng/L range (Belfroid et al., 2002; Petrie et al., 2015; Santhi et al., 2012) whereas other authors found BPA concentrations higher than 1µg/L (Belfroid et al., 2002; Céspedes et al., 2005; Matsumoto, 1982; Staples et al., 2000; Vethaak et al., 2005). WWTPs are considered one of the major secondary sources of BPA pollution due to their incomplete removal or degradation by physicochemical and biological treatment (Tan et al., 2007). BPA can also be released into the environment from landfill leachate (Wintgens et al., 2003), paper mill effluents (Fürhacker et al., 2000) and the natural degradation of polycarbonate plastics (Dorn et al., 1987; Mohapatra et al., 2010). In Table 8.2 BPA concentrations reported in different matrices are gathered.

The estrogenic activity of BPA was discovered in 1936 (Dodds & Lawson, 1936) when scientists were looking for synthetic chemicals to replace the expensive natural estrogens in pharmacological applications. Therefore, its use as a drug was not developed but BPA found other applications such as the manufacture of polycarbonate plastics, becoming rapidly one of the most produced and used chemicals worldwide (Eladak et al., 2015).

It has been proven that BPA is not only an EDC, but it also causes damage to hepatocytes by oxidative stress (Huc et al., 2012). Furthermore,

exposure to BPA has been correlated with obesity, diabetes (Le Magueresse-Battistoni et al., 2018), cardiovascular diseases (Melzer et al., 2010), breast cancer (Brody & Rudel, 2003; Maffini et al., 2006) and prostate cancer (Tse et al., 2017). Moreover, BPA entry was updated in the Candidate List of Substances of Very High Concern (SVHCs) on January 15, 2018 to reflect its endocrine disrupting properties causing adverse effects on the environment.

3. Laccase enzymes

Laccase (EC 1.10.3.2, *p*-diphenol: oxygen oxidoreductase, lignin oxidase family 1, <http://www.cazy.org/Auxiliary-Activities.html>) is a ubiquitous enzyme in nature belonging to the multi-copper-containing oxidases. Laccase enzymes are found in plants, bacteria, fungi (Dwivedi et al., 2011) and a few insects (Xu, 1999). They catalyze the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions coupled to the reduction of molecular oxygen to water (Solomon et al., 1996; Yaropolov et al., 1994). Although known for a long time, laccases attracted considerable interest after the wood degradation studies by white-rot fungi and especially after the discovery that laccases were also able to oxidize nonphenolic structures in the presence of low-molecular-weight organic compounds (Bourbonnais & Paice, 1990; Call & Mücke, 1997), thus, expanding the range of substrates these enzymes can oxidize. Such low-molecular-weight organic compounds are called redox mediators and act as intermediate substrates for laccases. Thus, the first step is the oxidation of the mediator by the laccase enzyme. Then, the oxidized mediator oxidizes the nonlaccase substrate. Hence, the mediator acts as an electron shuttle between the substrate and the enzyme (Galli & Gentili, 2004; Widsten & Kandelbauer, 2008). Therefore, laccases are often used in combination with a redox mediator in the so-called laccase mediator system (LMS). More than 100 redox mediators have been reported so far, the most frequently applied being the synthetic compounds 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT). However, synthetic mediators are toxic and costly, which has driven the interest in finding natural mediators such as lignin-derived phenols (Cañas & Camarero, 2010). In Table 8.3 the chemical structures of some laccase redox mediators are depicted.

Table 8.2

Matrix	[BPA] range	Limit of detection (LOD)	Location	References
0.002–0.0297µg/L	0.5ng/L	France	Dupuis et al. (2012)	Drinking water treatment

				plant
<LOD-0.215µg/L 0.0035-0.0598µg/L	0.4ng/L	Malaysia	Santhi et al. (2012)	River water Tap water
0.01-44.65µg/L 0.37-491.54µg/kg dw	2.09ng/L 0.126ng/g dw	Taiwan	Lee et al. (2013)	River water River sediment
0.07-4.21µg/L	8.3ng/L	France	Colin et al. (2014)	PET bottled water
3400µg/L	–	Germany	Dsikowitzky et al. (2015)	Untreated paper effluents
0.00824-0.263µg/L	–	China	Zhang et al. (2014)	Songhua river
0.00039-0.281µg/L	–	Spain	Gorga et al. (2015)	River water
0.0151-0.190µg/L	–	Portugal	Rocha et al. (2015)	Tagus river
0.01357-3.3367µg/L	–	China	Wang et al. (2015)	Ningbo city rivers
0-12.729µg/L	–	Saudi Arabia	Al-Saleh et al. (2017)	Treated wastewater from WTPs
0.1-32.2µg/kg dm	–	Southern Baltic	Staniszewska et al. (2016)	Gulf of Dansk's marine sediment
0.0028-0.1177µg/L <DL-0.0931µg/L 0.0447-0.1713µg/L	0.61ng/L	Romagna area (Italy)	Pignotti et al. (2017)	Lamone river Fiumi Uniti rivers Marecchia river
0.03-0.55µg/kg 0.03-0.46µg/kg 0.01-0.3µg/kg 0.04-0.27µg/kg	0.03ng/g 0.01ng/g	Slovenian Coast	Cerkvenik-Flajs et al. (2018)	Farm mussels Open sea mussels

				Shells from farm mussels Shells from open sea mussels
8.50–16.84µg/kg dw	0.29ng/g dw	Malaysia	Omar et al. (2018)	Sediment
Maximum 0.117µg/L 3.6–38.7µg/g	–	United Kingdom	Petrie et al. (2019)	River water Digested sludge

dm, dry matter; *dw*, dry weight; *PET*, polyethylene terephthalate; *WTPs*, wastewater treatment plants.

Laccase catalysis is warranted by the presence of different copper centers in the laccase molecule (Fig. 8.1). Thus, laccases have four copper atoms in different sites: one Type 1 (T1) copper presenting an intense band at 600nm, which is responsible for the blue color of the enzyme, and one Type 2 (T2) copper with no bands in the absorption spectra and two Type 3 (T3) coppers, showing absorption bands at 330nm (Malmström, 1982; Thurston, 1994). The T2 and T3 coppers form a trinuclear cluster. In Fig. 8.2 the catalytic cycle of laccase is depicted. In typical interactions of laccase with a substrate, the catalytic site of laccase abstracts electrons from the substrate and releases an oxidized product. When a redox mediator is present, the mediator can be oxidized by laccase and further oxidizes another compound that is either a substrate or a nonsubstrate of laccase resulting in the formation of oxidized product(s) and the mediator regeneration (Banci et al., 1999).

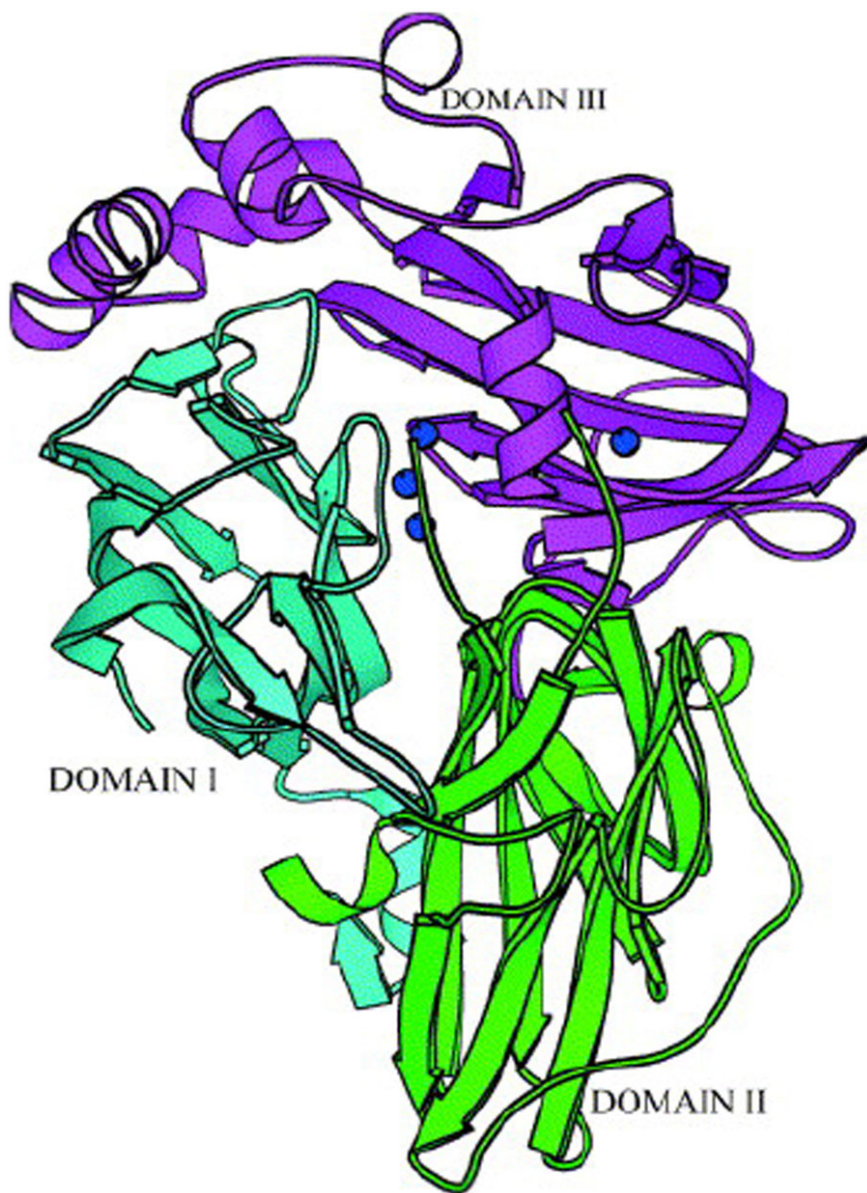


Figure 8.1 Ribbon representation of the overall structure of a laccase from the basidiomycete *Rigidoporus lignosus*. The copper atoms are depicted as *blue* spheres. The domains of *R. lignosus* laccase are indicated by roman numerals. Reprinted from Garavaglia, S., Cambria, M. T., Miglio, M., Ragusa, S., Lacobazzi, V., Palmieri, F., D'Ambrosio, C., Scaloni, A., & Rizzi, M. (2004). The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. *Journal of Molecular Biology*, 342, 1519–1531, Copyright (2004), with permission from Elsevier Ltd., UK.

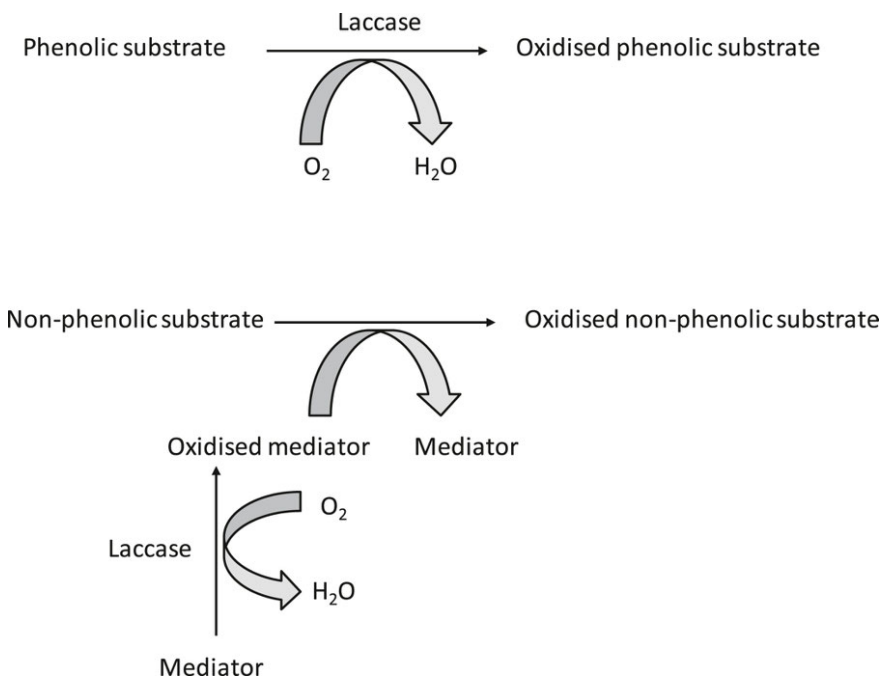


Figure 8.2 Mechanism of laccase action for both phenolic and nonphenolic substrates (Banci et al., 1999).

Table 8.3

Synthetic	
HBT (1-hydroxybenzotriazole)	ABTS (2,2'-azine-bis(3-ethylbenzothiazoline-6-sulfonic acid))
Natural	

<p><i>p</i>-coumaric acid (<i>trans</i>-4-hydroxycinnamic acid)</p>	<p>Acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone)</p>
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4. BPA degradation by laccases

Although there are several published papers devoted to the degradation of the xenobiotic pollutant BPA by laccase enzymes, only a few have investigated its biotransformation products. In Table 8.4 the resulting products of BPA biodegradation by laccase enzymes are presented.

Fukuda et al. (2001) studied the degradation of BPA by a purified laccase from a commercial preparation of *Trametes villosa* laccase expressed in *Aspergillus* sp. They found that the purified laccase (1.5 U/mL) degraded BPA (2.2mM) rapidly in 3h at pH 6 and 60°C with no need to add a redox mediator. They detected that BPA was transformed by laccase in high molecular weight (HMW) compounds, likely by oxidative condensation, and low molecular weight (LMW) compounds. One of the HML was identified as a BPA dimer (5,5'-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol) (Uchida et al., 2001) and one of the LMW as 4-isopropenylphenol (Fukuda et al., 2001; Uchida et al., 2001). They suggested that BPA laccase-catalyzed reaction may polymerize BPA to form oligomers followed by the addition of phenol moieties or the conversion of the oligomer in 4-isopropenylphenol. Later, Fukuda et al., (2004), proposed that the pathway of BPA biodegradation by laccase consisted of an oxidative condensation process to form different oligomers followed by a degradation process of the produced oligomers to release 4-isopropenylphenol and oligomer fragments with phenol molecules. Also, they observed that the BPA biodegradation products did not present estrogenic activity (luciferase reporter assay).

Table 8.4

Laccase source	Degradation products	Toxicity removal	References
HMW compounds and LMW compounds (4-isopropenylphenol, phenol)	nd	Fukuda et al. (2001)	<i>Trametes villosa</i> (DeniLite, Novozymes, Chiba, Japan)
HMW compounds (BPA dimer) and LMW compounds (4-isopropenylphenol)	nd	Uchida et al. (2001)	<i>T. villosa</i> (DeniLite, Novozymes, Chiba, Japan)
Oligomers, 4-isopropenylphenol	Yes	Fukuda et al.	<i>T. villosa</i> (DeniLite,

		(2004)	Novozymes, Chiba, Japan)
4-isopropylphenol and 4-isopropenylphenol	nd	Michizoe et al. (2005)	<i>Trametes</i> sp. (Daiwa Kasei, Shiga)
Dimers, trimers and tetramers	Yes	Cabana et al. (2007)	<i>Coriolopsis</i> <i>polizona</i> ; <i>C. polizona</i> +ABTS; <i>C. polizona</i> +HBT
2,3-bis(4-hydroxyphenyl)-2,3-dihydroxypropionaldehyde and p-1,2-dihydroxyisopropyl phenol	nd	Telke et al. (2009)	<i>Pseudomonas</i> sp.
Phenol, 4-isopropenylphenol	nd	Galliker et al. (2010)	<i>C. polizona</i>
Hydroquinone (traces), 4-isopropenylphenol and BPA-dimer	Yes	Torres-Duarte et al. (2012)	<i>Coriolopsis</i> <i>gallica</i>
4-isopropenylphenol Water insoluble product	nd	Chairin et al. (2013)	<i>Trametes</i> <i>polizona</i> <i>T. polizona</i> +HBT
4,4'-(2 hydroxy propane 1,2 diyl) diphenol, bis (4-hydroxyphenyl) butenal, 2-(1-(4-hydroxyphenyl) vinyl) pent-2-enal	nd	Chhaya and Gupte (2013)	<i>Fusarium</i> <i>incarnatum</i> UC-14
β -Hydroxybutyric acid Tartaric acid and pyroglutamic acid	nd	Daâssi et al. (2016)	<i>C. gallica</i> <i>C. gallica</i> +HBT
Methylpent-3-oic acid; ethyl-3-ethoxy propanoate; p-isopropenylphenol, 4-ethyl-2-methoxyphenol; hexadecane; ethyl-3-oxo-butanoate; benzaldehyde; nonadecane Methylpent-3-oic acid; ethyl-3-ethoxy propanoate; p-isopropenylphenol, 4-ethyl-2-methoxyphenol; hexadecane; 2,6-dimethyldecane; 2,6-dimethyldecane; p-hydroxybenzoic acid; 2-(2-hydroxypropyl)-benzene-1,4-diol; 2-(6-(tert-butyl)-1,1-dimethyl-2,3-dihydro-1 H-inden-4-yl) acetic acid	Yes No	de Freitas et al. (2017)	<i>Pleurotus</i> <i>ostreatus</i> <i>Pleurotus</i> <i>pulmonaris</i>
Oligomers	nd	Beck et al. (2018)	<i>Trametes</i> <i>versicolor</i> (Sigma-Aldrich)
Table Continued			

Laccase source	Degradation products	Toxicity removal	References
Benzoic acid; organic acids	Yes	Das et al.	<i>Bacillus</i> sp.

		(2018)	GZB (spore laccase and recombinant laccase)
Dimers and trimers	nd	Zdarta et al. (2018)	<i>T. versicolor</i> (Sigma-Aldrich)
Ethylbenzene, <i>p</i> -xylene, cyclohexanone, 1-methyl-4-isopropenyl-2-cyclohexenol	nd	Hongyan et al. (2019)	<i>T. versicolor</i>
Hydroquinone, 4-isopropenylphenol, 2,4-diterbutylphenol, 2-[2-(4-hydroxyphenyl)propan-2-yl]phenol	nd	Taghizadeh et al. (2020)	<i>T. versicolor</i> (Sigma-Aldrich)

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); HBT, 1-hydroxybenzotriazole; HMW, high molecular weight; LMW, low molecular weight; nd, not determined.

Michizoe et al. (2005) studied the degradation of BPA (200μM) by a commercial laccase (3μM) from *Trametes* sp. in isooctane (containing 4% v/v water) using reverse micelles. BPA was totally degraded in 1h leading to the products 4-isopropylphenol and 4-isopropenylphenol, which indicated the cleavage of the C–C bond.

Cabana et al. (2007) reported the total removal of BPA (0.022mM) at 50°C and pH 5 in 4h by crude laccase (10 U/L) from *Coriopsis polyzona*. The addition of the redox mediator ABTS (10μM) enabled the removal of 50% BPA in 1h. Laccase converted BPA into HMW compounds (dimers, trimers, and tetramers) through a radical polymerization mechanism. In addition, the estrogenic activity (YES assay) of BPA decreased by 35%, 90% and 100% after 1, 4 and 8h of laccase treatment, respectively.

Telke et al. (2009) reported the total BPA (160μM) degradation by a purified laccase (0.5 U) from the bacteria *Pseudomonas* sp. LBC1 in 5h. They suggested 2,3-bis(4-hydroxyphenyl)-2-hydroxy propionaldehyde, 2,3-bis(4-hydroxyphenyl)-2,3-dihydroxy-pionaldehyde and p-1,2-dihydroxyisopropyl phenol or isomers of these compounds as the degradation products of BPA.

Galliker et al. (2010) studied the BPA (1mM) degradation by *C. polyzona* laccase immobilized on silica nanoparticles. After 1h of laccase incubation, BPA was transformed into phenol and 4-isopropenylphenol together with other species that likely resulted from further reactions of BPA, phenol and

4-isopropenylphenol.

Torres-Duarte et al. (2012) reported that laccase from *Corioloopsis gallica* transformed BPA mainly into 4-isopropenylphenol and, in addition, its estrogenicity was lost (ER α and Zebrafish assays).

Chairin et al. (2013) investigated the degradation of BPA (0.01%) by a purified laccase (0.64 U/mL) from *Trametes polyzona* with 2mM HBT and with no redox mediator. They found that BPA was only removed by 12% in 1h in the absence of HBT whereas 66.8% and 100% were removed in 1 and 3h, respectively, in the presence of HBT (2mM). They determined that the major biodegradation product was 4-isopropenylphenol when no HBT was added. The addition of HBT led to the formation of 4-isopropenylphenol at the beginning of the reaction that completely disappeared at the end of the reaction (3h) while an insoluble product appeared as a result of 4-isopropenylphenol polymerization by the action of the HBT radical generated by laccase.

Chhaya and Gupte (2013) showed that a purified laccase from the mutant strain *Fusarium incarnatum* UC-14 entrapped in reverse micelles removed 91.4% of BPA (200mg/L) within 75min. The formation of 4,4'-(2 hydroxy propane 1,2 diyl) diphenol, bis (4-hydroxyphenyl) butenal and 2-(1-(4-hydroxyphenyl) vinyl) pent-2-enal indicated that BPA degradation occurred via two oxidation steps and one ring opening step.

Daassi et al. (2016) reported the biotransformation of BPA by *Corioloopsis gallica* laccase (1 U/mL), after 3h of incubation, into carboxylic acid derivatives such as tartaric acid and pyroglutamic acid in the presence of 1mM HBT and into β -hydroxybutyric acid in the absence of HBT.

de Freitas et al. (2017) studied the degradation of BPA by laccases from *Pleurotus ostreatus* and *Pleurotus pulmonaris*. They found that BPA at 100mg/L and 200mg/L were removed by 100% and 85%, respectively, in 1h, by both laccases (8 U/L). Thirteen aromatic and aliphatic compounds were identified as BPA degradation products including p-isopropenylphenol, methylpent-3-oic acid, ethyl-3-ethoxy propanoate and 4-ethyl-2-methoxyphenol. In addition, they observed that the treatment with *P. ostreatus* laccase decreased BPA toxicity (Microtox test) from 85% to less than 5%. However, *P. pulmonaris* laccase treatment did not reduce BPA toxicity, likely due to at least one of the BPA degradation products being as toxic as BPA.

Beck et al. (2018) found that the incubation of BPA (0.3mM) with a commercial laccase (0.175 U/mL) from *Trametes versicolor* for 24h at room temperature led to a precipitate product of BPA oligomers. They also pointed out that the reactivity of a compound toward laccase was more likely due to the redox potential difference between the compound and the laccase T1 copper than to their geometric and steric complementarity.

Das et al. (2018) studied the degradation of BPA (10mg/L) by the spore laccase (8.34mg/mL) from *Bacillus* sp. GZB as well as its recombinant laccase (367.74μg/L) expressed in *Echerichia coli*. BPA was totally degraded in 30h by the spore laccase and in 14h by the recombinant one. The quicker degradation obtained by the recombinant laccase may be due to the use of cell-free enzymes. In addition, BPA toxicity (luminescent bacteria) decreased. They hypothesized two possible pathways of BPA degradation by laccase. The first one would start with the oxidation of 4,4'-dihydroxy- α -methyl-stil-bene (an intermediate from BPA) to 2,4-dihydroxy-3-methy-lacetophe-none and benzaldehyde derivatives. The benzaldehyde derivative would be further oxidized to benzoic acid, the main intermediate of BPA formed by both spore and recombinant laccases. Finally, ring cleavage products of LMW organic acid derivatives would be produced. In the second one, 4-isopropenylphenol would be directly transformed to hydroquinone through a \bullet OH attack, which would be further oxidized to different organic acids.

Zdarta et al. (2018) studied the degradation of BPA (2mg/mL) by a commercial laccase from *T. versicolor* immobilized on *Hippospongia communis* spongin scaffolds. Under optimal conditions (pH 5, 30°C), almost 100% of BPA was removed in 24h, BPA dimers and trimers being the main biodegradation products.

Hongyan et al. (2019) reported that the transformation products of BPA degradation by *T. versicolor* laccase included ethylbenzene, *p*-xylene, cyclohexanone and 1-methyl-4-isopropenyl-2-cyclohexenol. They suggested that BPA degradation by *T. versicolor* laccase started from the C atoms between the two benzene rings that connect BPA. Also, they found a maximum BPA degradation of 97.7% after 1h of reaction under optimized reaction conditions (44.6°C, pH 5.20, 50 U/L laccase and 5mg/L BPA).

Taghizadeh et al. (2020) studied the biodegradation products of BPA by a commercial *T. versicolor* laccase immobilized on dealuminated zeolite.

They found that after 1h of treatment the highest BPA peak was broken and some minor peaks appeared, some of which were identified as hydroquinone, 4-isopropenylphenol and 2-[2-(4-hydroxyphenyl) propan-2-yl]phe-nol by GC-MS (Table 8.4).

According to the data presented in Table 8.4, most laccases led to 4-isopropenylphenol as a byproduct of BPA degradation. However, the biodegradation products obtained varied depending on the addition of redox mediators and the laccase source.

5. Conclusions

The environmentally ubiquitous compound bisphenol A (BPA) is known to act as an endocrine-disrupter, thus posing a serious threat to wide life and human health. Hence, this compound must be removed from effluents before discharging it into the environment. However, the traditional physical and chemical methods present some limitations such as their high cost and sludge generation. Therefore, greener, safer and cost-effective alternatives such as the use of enzymes are sought. Thus, laccase enzymes have proven to be very efficient in the degradation of BPA. However, more studies regarding the characterization of the intermediates produced during laccase biodegradation of BPA and their toxicity are needed to assess its true potential application in real scenarios.

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Chapter 9: A new emerging technology against foodborne pathogens: cold atmospheric plasma

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Abstract

Foodborne pathogens are one of the most troubling issues of safety in the food industry. Currently, many diseases caused by foodborne pathogens, such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp., have become a global problem for public health and the economy. In recent years, many nonthermal microbial inactivation methods have been developed as alternatives to traditional methods to ensure food safety. Cold atmospheric plasma (CAP) technology is one of these methods, which is being used effectively to control and inactivate bacteria, mold, and yeast. The microbial inactivation by the CAP system is due to the reactive groups formed during plasma production; these groups damage the microbial membranes, proteins, and DNA. In this chapter, the working principle of the CAP method, its role in microbial inactivation, and the studies and the possibilities of its use in the food industry have been summarized.

Keywords

Cold atmospheric plasma; Food safety; Foodborne pathogens; Foods; Microbial inactivation; Plasma systems

1. Introduction

It is estimated that the world population will reach 9.8 billion by 2050 (FAO, 2021; Katsigiannis et al., 2021). Due to the growing world population, sustainable access to food and food safety has become a global problem. Foodborne pathogens are one of the most important threats to food safety. Food spoilage by pathogens can cause huge losses to the economy and public health. Many diseases and deaths are being reported due to foodborne pathogens (CDC, 2021a). For example, *Salmonella*, one of the most frequently reported foodborne pathogens causes 93.8 million cases of salmonellosis each year, and 155,000 of these cases result in death (Kim et al., 2020). Hence, it is important to prevent food spoilage and ensure food safety (Ng et al., 2021).

Traditional technologies such as pasteurization, heating, and boiling have been used for many years in food processing. Additionally, chemicals such as iodine compounds, potassium sorbate, organic acids, and sodium hypochlorite are frequently used to control and inactivate foodborne pathogens (Barba et al., 2017; Loretz et al., 2011; Ng et al., 2021). However, these chemicals may cause changes in sensory properties such as color, odor, and texture of food. They may also decrease the nutritional value (Huang et al., 2020). On the other hand, nonthermal techniques are being developed that can improve the sensory, physicochemical, and nutritional properties of food, as well as, free them of microbes (Coutinho et al., 2021). Methods such as ionizing radiation, high hydrostatic pressure, pulsed electric field, supercritical carbon dioxide technology, and ultrasound are nonthermal food processing methods that have recently gained popularity (Kim et al., 2019; Misra et al., 2014; Osae et al., 2020). Although these methods are effective in inactivating foodborne pathogens, they have some disadvantages as well, such as high equipment costs, the need for trained personnel, and negative consumer perception (Heo et al., 2021; Misra et al., 2014). However, cold atmospheric plasma (CAP) technology so-called “Emerging Food Preservation Technologies” is promising because it is eco-friendly, inexpensive, efficient, and has low operating temperatures (Liao et al., 2017; Zhang et al., 2013; Ziuzina et al., 2015). Moreover, it allows scaling up to meet industrial needs (Katsigiannis et al., 2021).

Cold plasma processes were performed under vacuum until recently. The

technique was incompatible with food processing and was used in industrial processes such as bonding of plastics, electronic cleaning, and changing the surface properties of inorganic materials. With the development of cold plasma systems that can operate under atmospheric pressure, CAP has started to attract commercial and technical attention in the food industry (Friedrich, 2011; Zhang et al., 2013). CAP has been successfully applied for inactivating bacteria, mold, yeast, and viruses. However, CAP has great potential in inactivating the spores and biofilms of microorganisms, sterilizing surfaces and medical devices, and biomedical applications such as wound and cancer treatment (Costello et al., 2021; Ma et al., 2015; Patange et al., 2017).

Plasma is produced by exposing gas or gas mixtures to an electric field that accelerates charged particles. The role of the reactive species formed during the production of plasma in microbial inactivation has been studied in detail in the last three decades (Dasan et al., 2018; Misra et al., 2019). Studies have reported that foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Yersinia enterocolitica* can effectively inactivate CAP (Bourke et al., 2018; Gök et al., 2019; Katsigiannis et al., 2021; Ulbin-Figlewicz et al., 2015). Currently, CAP is being applied more frequently to control and inactivate pathogens in various foods such as milk and dairy products, meat (including poultry) and meat products, fresh vegetables and fruits, and cereals. The nonthermal nature of CAP and its application at atmospheric pressure is advantageous for sensitive and heat-sensitive foods (Costello et al., 2021).

This chapter summarizes the role and mechanism of CAP technology in microbial inactivation. Additionally, the fundamentals of CAP and its studies and possibilities of use in the food industry have been explained from a microbiological perspective.

2. Cold atmospheric plasma

2.1. Fundamentals of plasma technology

Plasma, the fourth state of matter, is formed as a partially or fully ionized gas produced by electrical discharges between electrodes (Heo et al., 2021; Katsigiannis et al., 2021; Wan et al., 2019). Plasmas can contain reactive species such as ultraviolet (UV) photons, electrons, free radicals, positive and negative ions, and excited or unexcited molecules and atoms (Min et al., 2017; Misra et al., 2018; Moutiq et al., 2020). Plasma can be produced by applying different energies such as electric or magnetic fields, microwave, radiofrequency, heat, and laser light (Coutinho et al., 2018). Applying an electric field is the most preferred method for conducting plasma processes under atmospheric conditions and at room temperature (Misra et al., 2019). The half-life of the reactive species formed in plasma is very short due to energy loss during a collision. Thus, to prevent the loss of the reactive species during plasma production, the plasma system must be continuously supplied with energy (Hertwig et al., 2018).

Plasmas can be classified into two large groups according to thermodynamics, namely thermal plasmas and cold plasmas (Hertwig et al., 2018; Misra et al., 2018; Yoo et al., 2021). In thermal plasmas (where the temperature can reach up to several thousand Kelvin such as 15,000K), the temperatures of the charged and neutral species are approximately similar. The situation varies as the number of neutral species is much higher than the number of charged species. In cold plasmas, the temperature of the different particles is unequal. Moreover, in cold plasmas, the temperature of electrons is around several thousand Kelvin, while the temperature of the ion and neutral species is close to the ambient temperature. Therefore, cold plasmas which are suitable for the treatment of heat sensitive materials are also called nonthermal plasmas (Hertwig et al., 2018; Mandal et al., 2018; Misra et al., 2019; Patange et al., 2018).

With the recent scientific and technological advancements in plasma, highly practical methods have been developed for cold plasma production under atmospheric conditions. Various electrode configurations such as geometry, number, and position have contributed to the development of many plasma systems. Atmospheric pressure plasma jets and dielectric barrier discharge (DBD) systems are currently the most widely used systems for CAP production (Heo et al., 2021; Hertwig et al., 2018; Jayasena et al.,

2015). DBD systems consist of a relatively simple structure compared to other systems and operate on efficient and economical power sources (Kim et al., 2013). Additionally, systems such as corona discharge, microwave discharge, and resistive barrier discharge are also used in CAP production (Gavahian et al., 2019; Katsigiannis et al., 2021; Misra et al., 2019). On the other hand, there are no standardized methods for CAP applications yet. CAP systems are generally designed for laboratory applications (Hertwig et al., 2018). Usually, gases such as air, argon, helium, and oxygen are used in food processing by the CAP method (Gan et al., 2021). Among the particles formed during CAP production, reactive nitrogen and reactive oxygen species (ROS) are highly effective in inactivating microbes in foods (Liao et al., 2017). Additionally, parameters such as the type of gas used in the CAP process, the processing time, the intensity of the electric current, the gas flow rate, the configuration of the plasma source, the surface structure, the humidity level, and the temperature of the system can affect microbial inactivation (Hertwig et al., 2018; Misra et al., 2019; Moritz et al., 2017). High gas flow rates and long processing times increase the collisions and effects of reactive species and promote microbial inactivation (Coutinho et al., 2021; Han et al., 2016). On the other hand, excessive increase in processing time may cause deterioration of protein structure of foods and oxidative degeneration of compounds such as ascorbic acid and phenolic acid (Xu et al., 2017). In CAP applications, it is important to optimize the process according to the sensitivity of the food (Ng et al., 2021).

One of the most commonly used gases in CAP applications is ambient air. Air plasma is a very good source of reactive nitrogen species (RNS) and ROS (Ziuzina et al., 2015). ROS found in humid air plasma may contain ozone, single oxygen, hydroxyl radical, hydrogen peroxide, superoxide anion, hydroperoxyl, alkoxyl, carbonate anion radical, and peroxy. Likewise, nitric oxide, peroxyxynitrite, nitrogen dioxide radical, alkyl peroxyxynitrite, and peroxyxynitrous acid are among those groups in RNS samples found in humid air plasma (Gilmore et al., 2018; Hertwig et al., 2018; Misra & Jo, 2017; Xu et al., 2020).

There are many reaction mechanisms responsible for the generation of active species during CAP production. These include processes such as ion-ion neutralization, electronic pulse processes, photoemission, photo absorption, ion-molecule reactions, and photoionization (Misra et al., 2019).

2.2. Plasma systems used in CAP production

The most commonly used methods for CAP production are DBD, plasma jets, corona discharge, and microwave discharge. CAP systems usually have an application cabinet, gas and pressure control systems, and an evacuation device. However, these systems differ in the shape, number, location, pressure conditions, and current type of electrodes (Coutinho et al., 2021).

The general principle of the DBD system is that plasma is formed between two electrodes separated by dielectric layers (Katsigiannis et al., 2021). The dielectric barrier between the electrodes limits the current flow and prevents the formation of sparks at the point of contact (Coutinho et al., 2018; Misra et al., 2019). Additionally, the DBD system is ideal for applying on large surfaces (Phan et al., 2017). It is possible to work directly with alternating current discharge in a DBD system. Moreover, gas pressures between 10^4 – 10^6 Pa, frequencies between 0.05 and 500kHz, and voltage of at least 10kV are the preferred parameters for operating the system (Coutinho et al., 2021). While producing CAP in the DBD system, air, inert gases, water vapor, and various gas combinations can be used (Coutinho et al., 2021). Some of the advantages of the DBD systems are that they are inexpensive, simple to build, flexible and scalable, and can be operated uniformly on large surfaces (Katsigiannis et al., 2021). Reports from several studies have shown that foodborne pathogens have successfully been inactivated by CAP produced using the DBD system (Los et al., 2017; Wan et al., 2019; Xu et al., 2017). The DBD system is schematically represented in Fig. 9.1. Studies have shown that DBDs are highly effective and are easy to design for making ROS and RNS (Table 9.1) (Misra & Jo, 2017).

Reports suggest that the demand for a flexible DBD system is rapidly increasing in the food industry. The food to be sterilized by CAP can be in a flexible bag. However, the electrode used for CAP production is rigid. Additionally, this system becomes expensive when used to produce disposable plasma generators. Therefore, there is a need to develop flexible DBD systems for the food industry that are cost-effective and flexible (can be applied anywhere) (Heo et al., 2021).

Plasma jets are one of the most widely used systems in CAP production. In this system, various configurations for gas discharge are possible, usually in an open electrode arrangement (Nishime et al., 2017). The plasma jet system is schematically represented in Fig. 9.2. While producing CAP in

plasma jets, the gas moves at an increasing speed between the two electrodes. The electrodes at the center are stimulated by a radio frequency (13.56MHz), while the electrodes at the sides are grounded. Gases such as argon and helium are generally used in plasma jets at increasing flow rates. The disadvantage of this system is that high gas flow rates are required to create a plasma flame, which is expensive (Coutinho et al., 2018; Souza et al., 2021). The advantages of plasma jets are that they allow direct application and fit into narrow spaces (Coutinho et al., 2021).

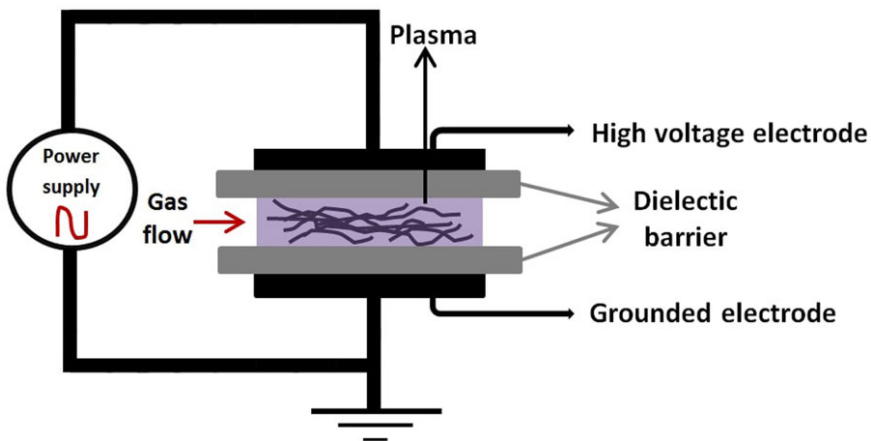


Figure 9.1 Dielectric barrier discharge system. Modified from Coutinho, N.M., Silveira, M.R., Rocha, R.S., Moraes, J., Ferreira, M.V.S., Pimentel, T.C., et al. (2018). Cold plasma processing of milk and dairy products. *Trends in Food Science and Technology*, 74, 56–68. <https://doi.org/10.1016/j.tifs.2018.02.008>.

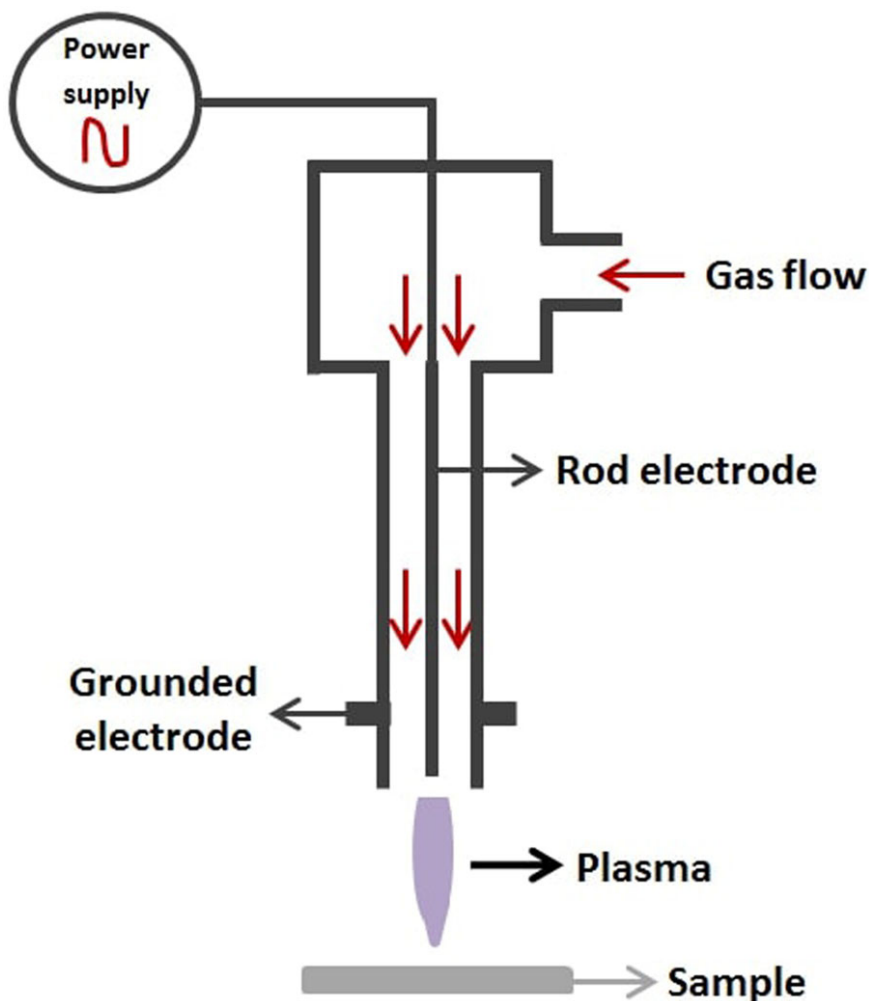


Figure 9.2 Plasma jet system. Modified from Coutinho, N.M., Silveira, M.R., Rocha, R.S., Moraes, J., Ferreira, M.V.S., Pimentel, T.C., et al. (2018). Cold plasma processing of milk and dairy products.

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<https://doi.org/10.1016/j.tifs.2018.02.008>.

Table 9.1

Food product	Type of plasma	Processing conditions	Treatment dose	Target foodborne pathogen	Maximum inactivation and result (log CFU/g)	References
Meat and meat product						
DBD	Voltage:	0.3min	<i>Listeria innocua</i>	1.6	Rød et al.	Beef, dry

	27kV Frequency 2.78kHz Power: 31W Feed gas: Ar/O ₂		(initial load 6.2 log)	log-reduction	(2012)	cured (Bresaola)
High Voltage Pulsed Discharge	Voltage: 25kV Frequency 42kHz Feed gas: Ar, O ₂ , Ar+O ₂ mixture gas	180–300s	<i>Staphylococcus aureus</i> <i>L. monocytogenes</i> (initial load 6 log)	0.85 and 0.83 log-reduction respectively	Gök et al. (2019)	Pastırma
Plasma jet	Frequency 1MHz, Voltage: 2–3kV Feed gas: Air, Ar	30, 60, 120, 180s	<i>Campylobacter jejuni</i> (initial load 6.00–7.00 log)	1.5–2.5 log-reduction	Rossow et al. (2018)	Chicken breast
Arc jet	Power: 50W Feed gas: N ₂	5min	<i>Escherichia coli</i> (initial load 6.00 log)	1.62 log-reduction	Yong et al. (2014)	Chicken breast
DBD	Frequency 15kHz Feed gas: Air	10min	<i>L. monocytogenes</i> (initial load 6.04 log)	2.36 log-reduction	Yong et al. (2017)	Beef jerky
			<i>E. coli</i> O157:H7 (initial load 5.93 log)	2.65 log-reduction		
High Voltage (CAP-Pen)	Voltage: 16kV Frequency 30kHz Feed gas: He, O ₂	8min	<i>L. innocua</i> (initial load 7.70 log)	3.30 log-reduction	Noriega et al. (2011)	Chicken muscle
DBD	Frequency 15kHz Power:	10min	<i>Salmonella</i> Typhimurium (initial load 5.51 log)	2.58 log-reduction	Jayasena et al. (2015)	Beef loin

	100W Feed gas: N ₂ /O ₂		log)			
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Food product	Type of plasma	Processing conditions	Treatment dose	Target foodborne pathogen	Maximum inactivation and result (log CFU/g)	References
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DBD	Power: 125W Feed gas: He, O ₂	60s	<i>E. coli</i> and <i>L. monocytogenes</i> (initial load 7.80 and 8.39 log, respectively)	5.12 log-reduction for <i>E. coli</i> , 6.69 log-reduction for <i>L. monocytogenes</i>	Kim et al. (2011)	Sliced bacon
		90s		4.80 log-reduction for <i>E. coli</i> , 5.79 log-reduction for <i>L. monocytogenes</i>		
DBD	Frequency RF Power: 200W Feed gas: Ar	10min	<i>S. aureus</i> (initial load 6.00–7.00 log)	~3.50 log-reduction	Kim et al. (2014)	Beef Jerky

Milk and dairy product

Corona discharge	Voltage: 9kV Feed gas: Air	20min	<i>E. coli</i> (initial load 7.78 log)	4.15 log-reduction	Gurol et al. (2012)	Milk, whole
DBD	Frequency 1.5kHz Power: 250W Feed gas: Air	10min	<i>S. Typhimurium</i> , <i>L. monocytogenes</i> (initial load 6.21 and 6.43 log, respectively)	2.46 log-reduction for <i>S. Typhimurium</i> and 2.40 log-reduction for <i>L. monocytogenes</i>	Kim et al. (2015)	Milk
DBD	Voltage:	5min	<i>L. innocua</i>	direct	Wan	Cheese

(High Voltage)	100kV Frequency 60Hz Feed gas: Dry air			treatment: 1.6 log-reduction indirect treatment: 0.8 log-reduction	et al. (2019)	(Queso fresco)
DBD	Voltage: 3.5kV Frequency 50kHz Feed gas: He, He/O ₂ mixture gas	1, 5, 10, 15min	<i>S. aureus</i>	0.05–0.45 and 0.08–0.91 log-reduction with He and He/O ₂ mixture, respectively	Lee et al. (2012)	Cheese
Fluidized reaction system	Voltage: 4.4kV Power: 480W Feed gas: N ₂	20–120s	<i>Cronobacter sakazakii</i>	1.17–3.27 log-reduction	Chen et al. (2019)	Nonfat dry milk powder

Table Continued

Food product	Type of plasma	Processing conditions	Treatment dose	Target foodborne pathogen	Maximum inactivation and result (log CFU/g)	References
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Fruits-vegetables and their juices

DBD	Voltage: 15 and 60kV Feed gas: Air	5, 10, 15, 30min	<i>E. coli</i>	6 log-reduction (for 60kV and 15min)	Prasad et al. (2017)	Tomato
DBD	Voltage: 42.6kV Feed gas: Air	10min	<i>E. coli</i> O157:H7 (initial load: ~5.4 log)	0.4–0.8 log-reduction for the 1-, 3-, and 5-layer configurations	Min et al. (2017)	Romaine lettuce
DBD	Frequency: 60Hz Voltage: 12kV Power: 40W Feed gas: Air	5min	<i>E. coli</i> O157:H7 (initial load 8.2 log)	4.6 log-reduction	Klockow and Keener (2009)	Spinach

Plasma jet	Voltage: High voltage (commercially available plasma system) Feed gas: N ₂	15min	<i>S. Typhimurium</i>	1.76 and 0.94 log-reductions on strawberry and potato, respectively	Fernandez et al. (2013)	Strawberry Potato
Corona discharge	Frequency: 0–2.4kHz Voltage: 35kV Feed gas: Air	3min	<i>Salmonella</i> sp. (initial load 6.93 log)	0.29 log-reduction	Hertrich et al. (2017)	Mixed salad
DBD	Frequency: 0–2.4kHz Voltage: 34.8kV Feed gas: Air (flexible)	5min	<i>E. coli</i> O157:H7, <i>Salmonella</i> sp., <i>L. monocytogenes</i> (initial load ~6 log)	0.9, 0.3 and 0.9 log-reduction for <i>E. coli</i> O157:H7, <i>Salmonella</i> sp., <i>L. monocytogenes</i> , respectively	Min et al. (2016)	Romaine Lettuce
DBD	Voltage: 26kV Frequency: 2500Hz Feed gas: Air	300s	<i>E. coli</i> O157:H7	Not detectable	Shah et al. (2019)	Baby kale leaves
Plasma jet	Power: 650W Feed gas: Air	120s	<i>E. coli</i> (initial load 6.16 log)	4.02 log-reduction	Dasan and Boyaci (2018)	Apple juice

Table Continued

Food product	Type of plasma	Processing conditions	Treatment dose	Target foodborne pathogen	Maximum inactivation and result (log CFU/g)	References
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Plasma jet	Voltage: 11kV Frequency: 1000Hz Feed gas: Ar, O ₂	6min	<i>Bacillus</i> sp.	7.2 log-reduction	Hou et al. (2019)	Blueberry juice
DBD (High Voltage)	Voltage: 90kV Feed gas: Air,	30, 60, 120s	<i>S. enterica</i> (initial load 5–8 log)	5 log-reduction	Xu et al. (2017)	Orange juice

	MA65 gas					
<i>Other food products</i>						
DBD	Power: 350W Frequency 15kHz Feed gas: Air, N ₂ , O ₂ , CO ₂ , CO ₂ /Ar (90/10%)	15min	<i>S. enteritidis</i> (initial load ~6.7 log)	Air: >5.0 log-reduction 4.8, 2.3, 2.0 and 3.0 log-reduction for O ₂ , CO ₂ , N ₂ and CO ₂ /Ar, respectively	Hertwig et al. (2017)	Almond
DBD	Frequency 15kHz Power: 250W Feed gas: Air	20min	<i>Bacillus cereus</i> , <i>B. subtilis</i> , <i>E. coli</i> O157:H7 (initial load 4.29, 4.23 and 5.26 log, respectively)	~1.30 log-reduction for <i>B. cereus</i> and <i>B. subtilis</i> ~2.30 log-reduction for <i>E. coli</i> O157:H7	Lee et al. (2016)	Brown rice
Corona discharge	Voltage: 17kV Feed gas: Ar	20min	<i>E. coli</i>	3.62 log-reduction	Santos Jr. et al. (2018)	Pumpkin puree
DBD	Frequency 50Hz Voltage: 80 Kv Feed gas: Air	5min	<i>E. coli</i> ATCC 25920 (initial load ~4.1 log)	3.67 log-reduction	Los et al. (2017)	Wheat, flour

CAP is produced by corona discharge when brightness and ionization occur close to the sharp electrode. The brightness occurs as a result of a weak light discharge at atmospheric pressure (Coutinho et al., 2018). Corona discharge systems can be operated in direct current or pulsed voltage mode. The advantages of this system are that the application and operating costs are low and it has a simple design. Additionally, in this system, the electric field is sufficient to accelerate the molecules or atoms in the gas

surrounding the randomly generated electrons up to the ionization energy level (Phan et al., 2017; Souza et al., 2021). On the other hand, the disadvantage of the system is that it has a limited processing capacity with a nonuniform configuration (Coutinho et al., 2021). The corona discharge system is schematically represented in Fig. 9.3.

The most distinctive feature of CAP produced by microwave discharge is that the discharge can occur without electrodes. Microwaves produced by a magnetron (operating at 2.45GHz) are used in this system (Coutinho et al., 2018). Microwaves combine with electrons, causing an increase in kinetic energy and enabling ionization. The microwave discharge system is easy to use. However, the disadvantage of the system is that it requires a series of discharges to directly decontaminate large areas (Coutinho et al., 2021). The microwave discharge system is schematically represented in Fig. 9.4.

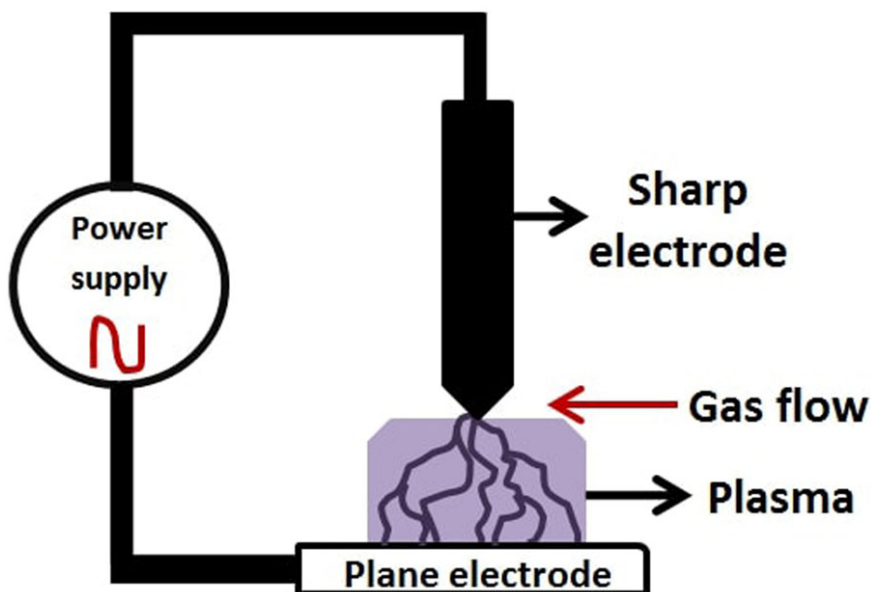


Figure 9.3 Corona discharge system. Modified from Coutinho, N.M., Silveira, M.R., Rocha, R.S., Moraes, J., Ferreira, M.V.S., Pimentel, T.C., et al. (2018). Cold plasma processing of milk and dairy products.

Trends in Food Science and Technology, 74, 56–68.

<https://doi.org/10.1016/j.tifs.2018.02.008>.

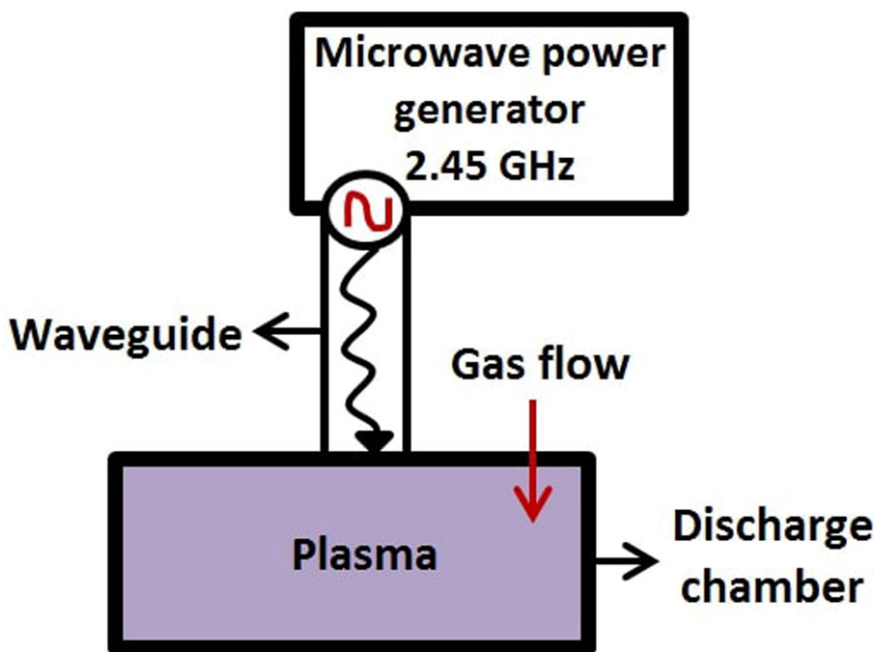


Figure 9.4 Microwave discharge system. Modified from Coutinho, N.M., Silveira, M.R., Rocha, R.S., Moraes, J., Ferreira, M.V.S., Pimentel, T.C., et al. (2018). Cold plasma processing of milk and dairy products.

Trends in Food Science and Technology, 74, 56–68.

<https://doi.org/10.1016/j.tifs.2018.02.008>.

2.3. The role and mechanism of cold atmospheric plasma in microbial inactivation

The use of CAP to inactivate microorganisms in foods has been extensively studied. In the CAP process, charged particles, high-energy UV photons, and reactive species (such as ROS, RNS, and hydrogen peroxide) are formed (Min et al., 2016; Misra et al., 2018; Patange et al., 2019). Charged particles and reactive species accumulated in the environment can damage the cell membrane and intracellular components such as protein and lipid of microorganisms through different mechanisms (Bourke et al., 2017). Additionally, these reactive species can also obstruct breathing (Dasan et al., 2018). Microbial inactivation of CAP is associated with enzyme inactivation, cell leakage (through electroporation), DNA fragmentation, and lipid peroxidation (Gan et al., 2021; Hertwig et al., 2018; Pankaj et al., 2017).

When reactive species and ions get excited, they collide with surfaces and other particles, or emit UV photons, causing them to lose their internal energy. CAP inactivates microorganisms by damaging DNA through the

emission of UV photons, degrading proteins and lipids via ROS/RNS, and also disrupting membranes through charged particles. Charged particles such as electrons and ions accumulate on the surface of bacterial cell membrane and induce its rupture due to electrostatic forces. Sometimes, DNA exposed to UV photons can change and consequently, result in unwanted cell replication (Dasan et al., 2018; Han et al., 2020).

Reactive oxygen and nitrogen species (RONS) formed by CAP are the main agents in plasma that control the microbes (Ng et al., 2021; Shaw et al., 2018). High oxygen concentrations in CAP have a strong inactivation effect on microorganisms because the oxygen-based reactive species, such as ozone and atomic oxygen, are very potent (Lu et al., 2016). The oxidative effect of RONS forms almost no harmful by-products during the CAP process. Thus, CAP technology is considered to be an eco-friendly microbial inactivation method (Xu et al., 2020).

It has been reported that ROS can break the peptidoglycan bonds of the bacterial cell wall, and thus, destroy it. Of all the macromolecules in the cell membrane, lipids are the most vulnerable to oxidation as they are found close to the surface. The hydroxyl radical can initiate peroxidation of the lipid layer in the cell membrane (Coutinho et al., 2021; Liao et al., 2017). Hydrogen peroxide is used as a bactericide for surface disinfection and oxidizes DNA, proteins, and lipids (Linley et al., 2012). Additionally, hydroxyl radicals can be formed by the photolysis of hydrogen peroxide. The formation of nitrogenous and nitric acids from RNS in plasma discharge is generally associated with the acidification of water (Ng et al., 2021; Niquet et al., 2018).

Intracellular accumulation of RONS occurs in two ways. In the first method, the RONS produced during the plasma discharge process enter the cell through active transport. At this stage, RONS are transported through temporary pores in the cell membranes of microorganisms (Ng et al., 2021). Pore formation by reactive species alters the transmembrane potential of the cell. Additionally, reactive species cause acidification by reducing the intracellular pH, which increases the permeability of the membrane (Coutinho et al., 2021; Hertwig et al., 2018). The other method is through the increase in the external oxidative stress, which affects the oxidative stress inside the cell. The proportion of RONS inside the cell increases with an increase in the external oxidative stress (Ng et al., 2021).

The efficiency of inactivation and models may differ due to variations in the CAP systems, working gas, and procedures (Xu et al., 2020). The gas selected for CAP production can change the reactive species and their amounts. If the selected working gas contains air or oxygen, the amount of ROS may increase, and hence, increase the inactivation rate (Han et al., 2016; Xu et al., 2020). It is difficult for microorganisms to simultaneously cope with all the stress factors originating from the plasma. Therefore, using gases in combination, such as He/O_2 , Ar/O_2 , N_2/O_2 , $\text{He}/\text{O}_2/\text{H}_2\text{O}$, $\text{N}_2/\text{N}_2\text{O}$, can be advantageous in microbial inactivation (Coutinho et al., 2021).

In the CAP process, changes in environmental conditions must be considered since the formation of several key RONS depends on humidity. Highly reactive hydroxyl groups can be produced in high humidity, thus increasing the inactivation of microorganisms (Katsigiannis et al., 2021; Misra et al., 2016). Tian et al. (2015) found that plasma-activated water (PAW) destroyed cell membrane integrity and membrane potential of *S. au-* *S. au-*. Additionally, it has been reported that PAW induces a significant increase in ROS, which causes oxidative stress in cells, and thus, increases inactivation (Tian et al., 2015).

Mechanisms of inactivation can vary among microorganisms due to differences in structural properties; for example, bacterial spores are more resistant than bacteria (Hertwig et al., 2018). It has been reported that CAP is more effective on Gram-negative bacteria than Gram-positive bacteria due to differences in the cell wall structures (Mai-Prochnow et al., 2016; Xu et al., 2020). The thick peptidoglycan layer of Gram-positive bacteria prevents reactive species from passing through the cell wall (Fig. 9.5) (Ng et al., 2021).

To summarize the microbial inactivation process of CAP, it has been shown that during CAP production, ROS reacts with lipids in the cell membrane and initiates lipid peroxidation. With the cell membrane destroyed by lipid oxidation, ROS flows into the cells. ROS accumulates in the cells and increases oxidative stress. This causes protein inactivation, mitochondrial dysfunction, and DNA fragmentation, which results in the release of DNA fragments and oxidized proteins through the destroyed cell membrane (Xu et al., 2020).

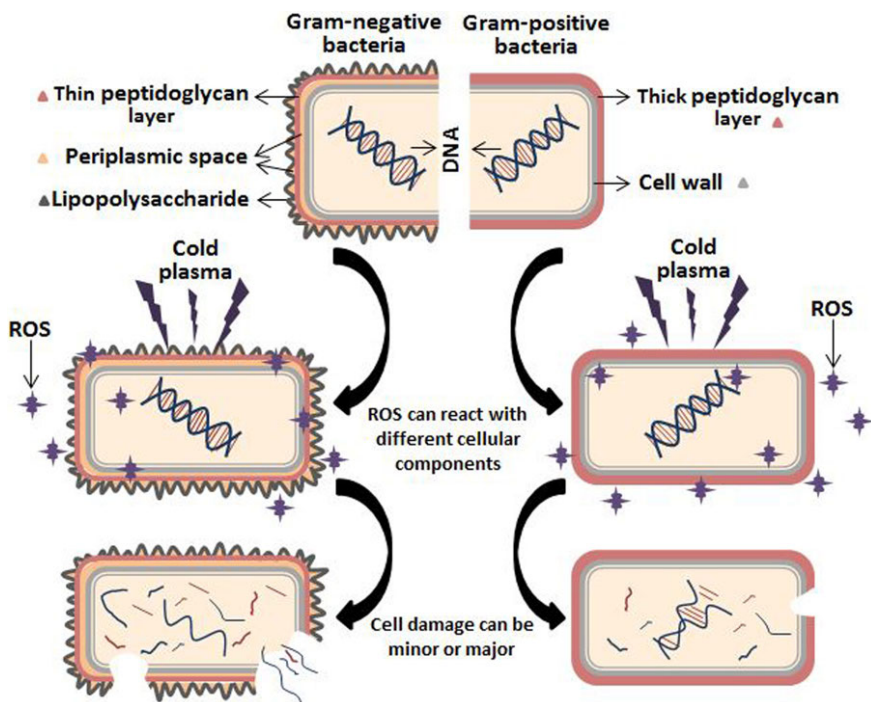


Figure 9.5 Mechanisms of action of atmospheric pressure cold plasma with Gram-positive and Gram-negative bacteria.

2.4. Effects of cold atmospheric plasma on microbial DNA

The effects of CAP on cellular DNA have been studied by many researchers. Changes in cell membrane integrity and pore formation by CAP disrupt the interaction between proteins. As a result, DNA is released from the cell and can be partially broken down by the electrical discharge. The effect of ROS on breaking single- and double-stranded DNA is an important factor in inactivating microbes (Gilmore et al., 2018). UV photons, emitted into the environment during CAP production, induce the dimerization of thymine bases in bacterial DNA strands and inhibit the growth of bacteria (Coutinho et al., 2021; Hertwig et al., 2018). Additionally, UV photons can also damage the DNA of bacterial spores (Coutinho et al., 2021). It has been reported that an increase in the DNA from spores is directly proportional to an increase in UV emission (Hertwig et al., 2015). UV photons can also cause intrinsic photodesorption as a result of breaking chemical bonds (Hertwig et al., 2018). However, single- and double-strand breaks in DNA can accumulate rapidly, which causes DNA to be rapidly fragmented into smaller oligonucleotides (Gilmore et al., 2018).

2.5. Applications of cold atmospheric plasma on foodborne pathogens

In recent years, many foodborne illnesses caused by contaminated foods have been reported. Reports from around the world have identified *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp. as target pathogens that cause serious human infections and deaths (CDC, 2021a; Kim et al., 2011; Ziuzina et al., 2014). Additionally, foodborne pathogens such as *Staphylococcus aureus*, *Clostridium botulinum*, *Bacillus cereus*, *Campylobacter* spp., *Clostridium perfringens*, *Shigella* spp., and *Yersinia enterocolitica* are reported to be responsible for foodborne infections and intoxications (CDC, 2021a; Yoo et al., 2021; Ziuzina & Misra, 2016).

Foodborne diseases are generally defined as diseases that occur as a result of consuming foods contaminated with pathogens or their toxins (Barba et al., 2017; Bintsis et al., 2017). For a foodborne pathogen to cause disease, the dose taken into the body must be above a certain level, which may vary with the characteristics of the pathogen. Furthermore, the clinical effects of the pathogen may differ between individuals. For elderly, pregnant, newborn, and immunocompromised individuals, the dose of infection may be lower than for other individuals. Foodborne diseases usually occur after 12–72h, and their effects can last between 4 and 7 days. Additionally, the symptoms of foodborne diseases are generally characterized by fever, abdominal pain, diarrhea, vomiting, and headache (Barba et al., 2017).

Foodborne pathogens can survive and grow during processing and storage. The increased tolerance of pathogens to antibiotics and disinfectants has reduced their impact on foods. Therefore, foodborne pathogens have become major targets in industrial food processing (Patange et al., 2019). Meat and meat products, milk and dairy products, fish and seafood, ready-to-eat foods, salads, poultry meat products, fruits, and vegetables can be counted among the foods at risk of contamination by foodborne pathogens (Kamble et al., 2021; Rød et al., 2012). Many outbreaks of foodborne diseases are reported around the world. According to CDC reports, contaminated foods such as mushrooms, peaches, ground beef, eggs, chicken, onions, papaya, melon, and chicken salad play a role in outbreaks of *Salmonella* spp. (CDC, 2021b). Similarly, outbreaks have resulted from the consumption of foods such as feta cheese, pork, deli ham, raw milk, packaged salad, and ice cream contaminated with *L. monocytogenes* (CDC, 2021c).

Additionally, many outbreaks have been reported due to the consumption of foods such as lettuce, alfalfa sprouts, salad, green leafy vegetables, ground beef, and butter contaminated with *E. coli* O157: H7 (CDC, 2021d). Therefore, many studies have focused on the inactivation of *Listeria* spp., *E. coli*, and *Salmonella* spp. (Table 9.1). The effect of CAP on foodborne pathogens in foods such as milk and dairy products, meat and meat products, fresh vegetables and fruits, fruit juices, and nuts has been investigated in recent studies. Table 9.1 compiles the studies on the inactivation of foodborne pathogens in food products by CAP treatment.

Large variations observed in the effects of CAP on microbes across studies may be due to factors such as gas type, equipment, food surface structure, processing time, gas flow rate, growth conditions of the microorganisms, and initial cell concentrations (Liao et al., 2017). This creates difficulty in comparing the efficacy of the methods based on the available data. Therefore, a standardized system for microbial inactivation with CAP is needed (Fernández et al., 2012).

Factors such as high osmotic pressure, low pH, and low temperature can trigger stress responses in microorganisms. In bacteria, that are exposed to such stressful conditions for a short time, the cellular physiology can change and make them resistant to inactivation methods applied in the future (Patange et al., 2019). Therefore, determining the factors that affect microbial resistance and developing appropriate strategies are important. It has been reported that the rate of microbial inactivation is generally inversely proportional to the initial cell concentration (Fernández et al., 2012). Additionally, it has been argued that the initial concentration of microorganisms may affect the inactivation efficiency of CAP (Xu et al., 2020; Ziuzina et al., 2014). A study by Kamgang-Youbi et al. (2008) reported that increasing the initial concentration of microorganisms decreases the efficiency of inactivation.

The surface structure of the food and the food matrix has a strong influence on the effect of CAP on microbes. Inactivating pathogens using CAP from structures like leaf stomata, or surfaces that are rough, cracked, and have clefts, is difficult (Bourke et al., 2018; Misra & Jo, 2017). Cells in a liquid carrier are more resistant to CAP inactivation than those on a solid surface. This is due to the need for reactive species to diffuse into the liquid (Smet et al., 2017). Thus, it is important to carefully select the

CAP-associated systems and applications to ensure that the CAP used for a certain food structure is efficient and safe (Bourke et al., 2018).

Relative humidity plays an important role in inactivating microbes using CAP by influencing the formation of reactive species (RONS). Increasing relative humidity causes an increase in peroxide and hydroxyl production (Coutinho et al., 2018; Liao et al., 2017) since a significant amount of water is required in the processing stages. Also, most foods have high moisture content and water activity (Ng et al., 2021). A study conducted on the inactivation of *Salmonella enteritidis* and *Salmonella typhimurium* in eggshells using CAP elucidated the importance of relative humidity (Ragni et al., 2010). It was found that a 90-minute plasma treatment (produced by DBD) at 35% and 65% relative humidity caused a maximum reduction of 2.5 and 4.5 log CFU/eggshell in the number of *S. enteritidis* and *S. typhimurium* in the eggshell, respectively (Ragni et al., 2010).

Higher input voltage and frequency values are associated with greater microbial inactivation by CAP (Dasan et al., 2016; Liao et al., 2017; Lu et al., 2014). Deng et al. (2007) found that increasing the voltage and frequency caused *E. coli* in samples to decrease at a higher rate. The same study reported that CAP produced at 16, 20, and 25kV in DBD systems reduced *E. coli* on unshelled almonds by 1.0, 2.43, and 4.12 logCFU/g, respectively (Deng et al., 2007). Additionally, the processing time also affects microbial inactivation. A study reported that the inactivation of *L. monocytogenes* was significantly dependent on the processing time of cold plasma (Lee et al., 2015). Similarly, it has been reported that CAP has a greater inactivation effect on planktonic cells when the applied voltage and treatment time are increased (Patange et al., 2017).

The effects of the gas or gas mixture used in CAP production are important for microbial inactivation and cost management (Misra & Jo, 2017). In a study where CAP was produced using a plasma jet, it was found that a 2-min treatment reduced *L. monocytogenes* on ham slices and chicken breast from 1.94 to 6.52 logCFU/g and 1.37 to 4.73 logCFU/g, respectively (Lee et al., 2011). In the same study, a combination of N₂ and O₂ was reported to be more effective than He, He mixed with O₂, and N₂ gas or gas mixtures (Lee et al., 2011). The presence of O₂ in the gas mixture has been reported to significantly increase microbial inactivation efficiency (Misra & Jo, 2017).

Many combined methods can be applied to inactivate foodborne

pathogens. Studies have used CAP in combination with natural antimicrobials (Costello et al., 2021; Cui et al., 2016; Yoo et al., 2021). The CAP-Nisin combination was investigated by Costello et al. (2021). Moreover, it has been reported that the combination of encapsulated CAP with clove oil has a synergistic effect on the inactivation of *E. coli* O157:H7 and *S. aureus* (Yoo et al., 2021). Similarly, Cui et al. (2016) studied the effect of thyme oil and cold nitrogen plasma combination on *Salmonella* in eggshells.

3. Conclusions

Currently, many technologies and methods are being used to inactivate foodborne pathogens. However, CAP technology has come to the fore in recent years due to its many positive features. Some of these features are its relatively simple design, eco-friendly nature, and its ability to provide microbial inactivation without much changes in the nutritional value, quality, and sensory properties of foods. Hence, CAP technology has attracted the attention of many researchers and has been successful in inactivating foodborne pathogens. It has been shown that many factors play a role in the inactivation of foodborne pathogens by CAP. The fact that CAP systems are not standardized and have many parameters creates difficulties in comparing the results of its effect on microbial inactivation across studies. Studies on the use of CAP (which has accelerated in the last decade) in microbial inactivation lay the groundwork for the widespread use of CAP technology in the food industry in the future. All the changes that occur in foodborne pathogens treated with CAP should be studied in detail. Particularly, determining the changes in the gene structure of the foodborne pathogen to which CAP is applied will immensely contribute to understanding the mechanism of the action of CAP. However, more studies are needed to achieve this goal.

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Chapter 10: Genetic engineering of algae

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Abstract

Nowadays, there is an increasing interest in the use of genetically engineered algae for industrial and biotechnological applications like wastewater treatment. Population overgrowth and industrial development has caused the release of organic pollutants and heavy metals into water bodies posing the ecosystem and human health at risk. Phycoremediation is seen as an effective way to reduce water pollution and producing algal biomass. Recently, algal biomass has been utilized as a sustainable and cost-effective feedstock for renewable biofuel production to decrease the negative environmental impact of fossil fuels. However, due to some limitations of algal native strains in cultivation, harvesting, and product extraction, which prevents reaching optimal biomass for phycoremediation and biofuel production at low costs, some investigation have been directed toward the genetic engineering of algae. Thus, some studies have shown the enhancement of phycoremediation systems using overexpression of enzymes involved in specific biochemical pathways to improve photosynthetic efficiency and growth in toxic concentrations of heavy metals. Also, in addition to using wastewater as a cheap and available nutrient supply for cultivation, genetic manipulation of algal biosynthetic pathways plays an important role in improving the growth and production rate of biofuel and reducing its cost. Therefore, finding and manipulating metabolic pathways of algae will elucidate some novel metabolically important genes which could enhance phycoremediation and its related byproducts. The present chapter is focused on genetic engineering of algae for improvement of phycoremediation and biofuel production. In addition, metabolic pathways and genetic engineering approaches are reviewed

and discussed.

Keywords

Algae; Biofuel; Biomass; Genetics; Heavy metals; Phycoremediation; Wastewater

1. Introduction

Since 1970s, modification of living cells and organisms using genetic engineering techniques has opened promising possibilities for agricultural and medicinal industries, bioremediation of industrial wastewater, energy production, and so on. Thus, many genetically engineered (GE) plants, animals, fungi and algae have been produced by stable integration of a specific piece of DNA derived from a different unrelated species into their genome (nuclear, mitochondrial or chloroplast genome). This foreign piece of DNA called “transgene” is inherited by their daughter cells and gives the organisms new properties including higher growth rate, increased productivity, enhanced biosynthetic pathways, and resistance to a particular disease or xenobiotic factors. There are currently global research efforts aimed at genetic modification of photosynthetic organisms like algae for different applications (Radakovits et al., 2010; Spicer & Molnar, 2018).

Microalgae (microscopic algae) have a great genetic potential for removing toxic pollutants from municipal and industrial wastewater as well as producing high added-value compounds like renewable biofuels (Bansal et al., 2018; Hlavova et al., 2015; Lü et al., 2011; Ng et al., 2017; Schiano di Visconte et al., 2019; Snow & Smith, 2012). Microalgae also lack cell differentiation and allelic genes in most of their vegetative stages (haploid stages). Therefore, genetic manipulation of microalgal strains is much simpler than that of higher plants and, thus, they are considered as an attractive system in genetic engineering researches (Nazari & Raheb, 2015). In this regard, a variety of molecular biology tools are utilized to develop genetically modified microalgae with improved properties (Gomaa et al., 2016). In this chapter, we discuss about the provided solutions to reduce some environmental problems through genetic engineering of microalgae.

2. Applied characteristics of algae

Algae are photosynthesizing organisms which vary from unicellular microalgae to multicellular plant-like macro algae. These organisms have potential to fix the atmospheric carbon dioxide using the sunlight energy and to efficiently utilize the produced organic substances to convert them into biomass. In addition, they can fix nitrogen oxides released from different sources (Bansal et al., 2018; Schiano di Visconte et al., 2019). For application purposes along with the essential requirements such as carbon, nitrogen, phosphorus and water, macronutrients and micronutrients are required for culturing algae. Another feature that makes algae worthy of attention is their ability to grow on lands and in water sources that are not suitable for the growth of other crops (Ahmad et al., 2011; Pittman et al., 2011). They can also grow in wastewater containing high amount of nitrogen and phosphorus and remove toxic pollutants as well as nitrogen and phosphorus. These characteristics make them great candidates for wastewater treatment and industrial CO₂ capture and utilization (Schiano di Visconte et al., 2019). Large-scale cultivation of algae for wastewater treatment leads to an increased biomass with an extra intrinsic value for other applications such as biochars and biofertilizers. In addition, high added-value products including various alternatives to fossil fuels such as bioethanol, biohydrogen and biodiesel can be produced from the generated biomass. This capacity is due to the cellular structure of algae, which have the ability to synthesize macromolecules like lipids and other active metabolites (Bansal et al., 2018). In addition, algae are not a world major food such as rice, wheat, jowar, bajra, small millets, cereals, grams, pulses, vegetables, and fruits, so using them as a feedstock for other practical purposes will not affect food prices. Thus, coupling a wastewater treatment plant with an algae cultivation system may turn the problems caused by environmental pollution into sustainable economic opportunities such as low-energy nutrient removal, valuable commodity production, and abatement of greenhouse gases (Schiano di Visconte et al., 2019).

3. The basis of algal genetic manipulation

Research about the culturing of algae as a high protein and inexpensive food dates back from 1950s. Since then, considerable knowledge has been obtained about the physiology and growth of algae as potential sources for wastewater treatment and production of various chemical and pharmaceutical compounds, biofuels, electricity, and bioindicators that help to minimize impurities in the environment (Bansal et al., 2018; Hlavova et al., 2015). However, up to recent years our knowledge has been mostly limited to algal native strains, which are traditionally used for different purposes. Among these wild-type strains, the fast-growing ones are used for biomass production while other ones are used to produce specific compounds such as astaxanthin, eicosapentaenoic acid (EPA), and oils. However, an economical production process requires the use of strains with high cell growth rate and maximum product formation simultaneously. Overall, it seems very difficult and uneconomical to achieve algal strains with both characteristics through simple strain selection or selective breeding technologies (Hlavova et al., 2015). To overcome this problem, one promising solution would be the rational genetic alteration of algal metabolic processes to obtain modified algal strains with optimized growth and production characteristics (Bansal et al., 2018; León-Bañares et al., 2004; Ng et al., 2017).

3.1. Remarkable aspects for the production of transgenic algae

Usually, different wild-type species of algae have the desired property. Therefore, a careful selection of the appropriate target species should be performed at the beginning of every algal genetic manipulation project. One of the most important aspects for choosing a particular alga species is the ease of its cultivation, especially under laboratory conditions. Therefore, small algae that grow rapidly within a short period of time in a synthetic medium under defined environmental conditions are preferred for genetic manipulation experiments. In addition, the applied target species should be able to grow in low-cost culture media to high densities, even under varying conditions. For transformation experiments, the repeat content of the ideal genome should be as low as possible, and in most cases haploid algae are favored. In addition, algae with mononuclear cells should be selected for genetic manipulation experiments. In addition to the previous molecular,

biochemical, physiological, and ecological knowledge of the target species, extensive information of its genome sequence is also very important for the production of transgenic algae. Although, no single alga species fulfills all of the above-mentioned requirements, several microalgae present some of them (Hallmann, 2007). Therefore, the following section is focused on the genetic modification of microalgae as proper target microorganisms for the above-mentioned purpose.

3.2. Genetic manipulation of microalgae

Microalgae including prokaryotic cyanobacteria and eukaryotic algae are a good source of pigments, vitamins, polysaccharides, proteins, pharmaceuticals and other biologically active compounds with high commercial value (Gomaa et al., 2016; Snow & Smith, 2012). Some of these microorganisms are able to tolerate variable environmental conditions including extreme environments. In addition, they can grow photoautotrophically, heterotrophically and mixotrophically depending on the type of available carbon source. These features make them very advantageous for different biotechnological processes such as wastewater treatment and cosmetic and pharmaceutical compound and biofuel production. Nonetheless, an effective and viable utilization of native microalgae for an industrial scale process requires the optimization of their growth conditions. For the cost-effective production of microalgal biomass, selected strains are usually grown under natural sunlight. However, variable light intensity and shading effect during a day can influence their productivity (Gomaa et al., 2016).

On the other hand, the cost and energy of the harvesting step of microalgal biomass production is considerably high. The harvested biomass should be dried to avoid decomposition, which can require specialized equipment. Therefore, biomass harvesting of microalgae along with the extraction of the desired product constitute the major part of the overall process cost. To minimize the cost and improve the yields of a given process at industrial scale, genetic engineering of metabolic/biosynthetic pathways of the selected microalgal strains is required (Gomaa et al., 2016). The results of some studies have shown that growth and cultivation of selected microalgal strains could be improved by increasing their potential for carbon fixation and light utilization through genetic manipulation of the photosynthesis pathways (Deng et al., 2014). Harvesting and extraction of the final desired product could also be improved in GE microalgal strains by

inducing flocculation, autolysis and continuous product secretion (Gomaa et al., 2016; Mendez et al., 2015). Therefore, the economic feasibility of phycoremediation as well as the production of its related byproducts, such as biofuels, can ultimately be improved by increasing microalgal growth and production rate through genetic modification. However, progress in genetic engineering of microalgae has been slow until recent years and, in addition to this, it has some drawbacks (Gomaa et al., 2016; León-Bañares et al., 2004). The main hurdles in developing transgenic microalgae are the low growth rate and the quantity of gene expression in these strains. Methods successfully applied for transformation of other organisms failed in microalgae due to considerable evolutionary distance between algae and those organisms. Therefore, all techniques including introduction of DNA into cells, selection of appropriate promoters, new discriminating marker genes and expression vectors had to be developed for microalgae exclusively (Nazari & Raheb, 2015). One solution for making microalgal production processes cost-effective is the simultaneous production of coproducts (e.g., fine chemicals) together with the main product (e.g., biofuel) (Otero et al., 2007).

Until now several genome-sequencing projects have been completed for different microalgal strains, resulting in assembled and annotated sequences which are publicly available in GenBank and other databases (see <https://www.ncbi.nlm.nih.gov/genome/browse/#!/overview/algae> and <https://www.ncbi.nlm.nih.gov/genome/?term=cyanobacteria>). Furthermore, sequencing of mitochondrial and chloroplast genomes was performed with more microalgal strains than genome sequencing projects due to their plastid genomes are much smaller (Hallmann, 2007). These organelle genome sequences are also available at NCBI organelle database (<http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/organelles.html>). Access to genome sequences which belong to microalgal strains with industrial applications greatly facilitates targeted DNA sequence manipulation for different purposes (Radakovits et al., 2010).

Many completed and ongoing genetics studies on microalgae have been directed toward the analysis and modification of photosynthetic and metabolic pathways. Successful results were achieved in this area and, thus, several examples of transgenic microalgae were constructed for biotechnological applications (León-Bañares et al., 2004). On the basis of some

research studies, the structural and physiological properties of the unicellular eukaryotic algae together with their potential for genetic manipulation have made them a perfect choice for recovery of heavy metals from industrial wastewater (Cai et al., 1999). The unicellular *Chlamydomonas reinhardtii* as a representative of eukaryotic microalgae has been extensively used to study diverse biological processes such as photosynthesis, light-induced gene expression, organelle biogenesis, light perception, cell–cell recognition and cell cycle control. *C. reinhardtii* can be an attractive system for the expression of target proteins due to the fact that its nuclear, its chloroplast and its mitochondrial genomes can be stably transformed (Mamedov & Yusibov, 2011). Some studies have shown that the trace-metal binding properties of *Chlamydomonas* can be enhanced through the expression of a foreign metallothionein or the expression of the moth bean pyrroline-5-carboxylate synthetase (P5CS) gene, which induces the accumulation of free proline in *Chlamydomonas* (Cai et al., 1999; León-Bañares et al., 2004; Siripornadulsil et al., 2002).

Diatoms are other unicellular eukaryotic microalgae which possess physiological potential to accumulate high amounts of lipids. Genetic manipulation of diatoms for improving lipid production was one of the first reported approaches for the production of biodiesel by transgenic microalgae (Dunahay et al., 1996). *Synechocystis* sp. PCC 6803 was also the first cyanobacteria (prokaryotic algae) to have its genome sequenced in 1996 (Kaneko et al., 1996). The availability of cyanobacterial genome sequences allowed developing these strains for the photosynthetic production of chemicals by rational genetic modifications. Furthermore, the lack of cell differentiation and the absence of allelic genes due to the haploid nature of prokaryotic cells make cyanobacteria a simple system for genetic manipulation (Pulz & Gross, 2004).

Pioneering research on the genetic manipulation of algal wild-type strains suggested potential applications of transgenic microalgae in phycoremediation and biofuel production (Randolph-Anderson et al., 1993). Up to now, further studies have been performed for those purposes and the following sections will be reviewed and discussed some of them in detail. In addition to naturally produced compounds, the use of microalgae as model eukaryotic hosts is also possible, especially in cases where specific genes cannot be expressed in yeasts (León-Bañares et al., 2004). In this regard,

microalgae can be used as a new type of bioreactor for high expression of recombinant proteins including vaccines, antibodies, enzymes, blood-clotting factors, immune regulators, growth factors, hormones, and other valuable products such as the anticancer agent Taxol (Yan et al., 2016). Since microalgae are not hosts for major pathogens, GE strains are specifically used for containment and controlled growth minimizing the level of contaminations and maintaining maximal productivity in bioreactor systems both under phototrophic and heterotrophic conditions (Beacham et al., 2017; Giardi et al., 2011; León-Bañares et al., 2004; Richmond, 2008; Zaslavskaja et al., 2001).

4. Ecological risks of genetically engineered microalgae

Potential applications of GE algae have given rise to new commercial opportunities and subsequently greater public scrutiny and government regulation. Therefore, transparent and formal risk assessments are urgently needed in decision-making processes for commercial use of GE microalgae. A main step in risk assessment is the identification of those sets of circumstances that may lead to an adverse effect(s) (Keese, 2008; Snow & Smith, 2012). GE microalgae, similar to their wild counterparts, are likely to disperse widely from open ponds through aerosolization (Genitsaris et al., 2011; Sharma & Singh, 2010), wildlife vectors (Kristiansen, 1996), turbulent weather that damages or destroys cultivation and processing facilities, accidents, human errors and other events and, on a smaller scale with less probability, from enclosed photobioreactors (Snow & Smith, 2012). Some biologists have predicted that most target traits of GE microalgae are unlikely to provide a selective advantage in nature, resulting in a rapid decline (Henley et al., 2013). Therefore, it is not expected that GE microalgae survive in the natural environment, outside of open ponds and enclosed bioreactors, so their ecological risk is minimal but not zero. However, if ecological and evolutionary assessments show some GE microalgae or their evolving progeny might have potential to thrive and persist in the natural environment after unintended release, it must be confirmed that their survival and growth are highly unlikely to cause environmental harm through transgenic DNA transfer to other organisms (Henley et al., 2013; Snow & Smith, 2012). Horizontal gene transfer (HGT) from GE microalgae to other organisms has been commonly cited as one potential risk, especially when cultivating GE cyanobacteria. HGT can occur between different cyanobacterial taxa, as well as between cyanobacteria and eukaryotic algae. Moreover, previous studies have shown the ability of some cyanobacterial strains for releasing and taking up “naked” DNA from their surroundings along with importing and exporting their DNA through viral vectors. However, it is important to notice that the transferred gene can provide a novel trait to the recipient organism which may result in negative, neutral or positive effects. Therefore, HGT is an event that may or may not harm (Henley et al., 2013; Keese, 2008; Snow & Smith, 2012). Over evolutionary time, gene transfers often facilitated the obtaining of functions in prokaryotes by eukaryotic organisms, allowing them colonizing new environments (Andersson, 2005).

However, HGT occurs less frequently between distantly-related organisms and the most infrequent HGT events were detected from eukaryotes to prokaryotes (Keese, 2008). Moreover, HGT can significantly occur between eukaryotic algae through viral transmission. Even HGT from algae to animals was observed (Ni et al., 2012; Snow & Smith, 2012). In 2008, Rumpho et al. (2008) reported that the algal nuclear gene of oxygenic photosynthesis, *psbO*, was transferred and integrated into the nuclear genome of the photosynthetic sea slug *Elysia chlorotica* by ingestion of its algal food source *Vaucheria litorea*. They came to such a conclusion by finding identical copies of *psbO* gene sequence in the sea slug and *V. litorea* nuclear genomes. Furthermore, the sequencing of *V. litorea* plastid genome demonstrated that the plastid metabolism in this organism depended on its nuclear encoded proteins. So, Rumpho et al. (2008) concluded that one of the reasons for the photosynthesizing activity of the ingested *V. litorea* plastids in the *E. chlorotica* digestive epithelium without access to the algal nucleoplasm was the existence and expression of *V. litorea psbO* gene in the above-mentioned mollusc's nuclear genome (Rumpho et al., 2008).

It seems that successful HGT events in cyanobacteria occur most frequently among genes involved in photosynthesis or respiration and specific genes of unknown function which have a critical role in environmental adaptations (Snow & Smith, 2012). Success of the above-mentioned HGT events is in agreement with the following hypothesis: HGT events often involve genes that directly affect the competition and adaptation of similar cyanobacterial species in neighboring niches (Yerrapragada et al., 2009). Thus, notable examples of GE microalgae which must be monitored for biosafety and environmental risks are those carrying transgenes encoding antibiotic resistance and enhanced growth under natural conditions (Spicer & Molnar, 2018). Fortunately, it is predicted that genetic and mechanical containment plus conditional matching of GE algal traits to unnatural cultivation conditions would further reduce the ecological risk. However, these hypothetical predictions must be confirmed through rigorous ongoing monitoring and mesocosm experiments by experts including ecologists to minimize risk and foster public and regulatory acceptance (Henley et al., 2013).

5. Methods for production of transgenic microalgae

Growing access to innovative and powerful genetic tools as well as availability of genomic information for microalgae have greatly facilitated the genetic engineering process for both prokaryotes and eukaryotes. Subsequently, fundamental study for genetic modification of microalgae metabolic/biosynthetic pathways has recently received much attention to open up new possibilities for industrialization of algae (Lü et al., 2011; Ng et al., 2017). The basic process for microalgae genetic manipulation includes host selection, gene targeting, plasmid construction, transformation tools, selection system and DNA editing tools. Among them, the transformation and selection methods are the most important steps in the genetic engineering of microalgae for the production of foreign proteins and modification of specific metabolic pathways (Ng et al., 2017; Schiano di Visconte et al., 2019). Despite high efficiency of transformation in bacteria, yeasts and even in higher plants, only few species of microalgae were able to be successfully transformed at the nuclear and chloroplast genome (León-Bañares et al., 2004). Therefore, efficient transformation systems are necessary to enhance the transformation potential of microalgae at both nuclear and chloroplast level. The main methods for transformation of microalgae have been adapted mainly from plant applications due to their similar cell wall (Schiano di Visconte et al., 2019). Each method has their advantages and disadvantages based on efficiency, integration and stability of the transgene (Ng et al., 2017). Generally, molecular tools for genetic engineering of microalgae were developed with focus on *C. reinhardtii*, for which stable genetic transformation at both nuclear (Debuchy et al., 1989) and chloroplast (Boynton et al., 1988) levels was firstly reported. Based on those initial findings, reliable and reproducible transformation protocols have been currently provided for prokaryotic and eukaryotic algal cells.

5.1. Transformation techniques

The oldest method reported for gene delivery to microalgae during the nuclear transformation process is the random insertion of transgene in the nuclear genome by vortexing algal cells in the presence of DNA and glass beads (Fig. 10.1). In this way, temporal permeability is created in the cell membrane which enables DNA molecules to enter the cell while preserving viability. Transformed cells can be selected by antibiotic resistance and phenotypic variation. Despite simplicity, high efficiency and incredibly low

cost, this method can only be used for transformation of cell-wall-deficient mutants and wild-type cells following enzymatic degradation of the cell wall. Therefore, it cannot be directly applied to many kinds of microalgae including *Chlorella*, *Nannochloropsis* and *Phaeodactylum* genera because their cell walls are rigid (León-Bañares et al., 2004; Ng et al., 2017). The other strategies including agitation with silicon carbide (SiC) whiskers, sonication in the presence of polyethylene glycol, electroporation and biologically mediated gene transfer by *Agrobacterium tumefaciens* are also suitable for nuclear transformation of microalgae (Ng et al., 2017). The SiC-mediated transformation technique is similar to the glass bead method and was used for the transformation of several higher plants and some microalgal species without the removal of the cell wall. Nevertheless, due to the high cost of SiC whiskers and their risk to human health, this method is not commonly used. Electroporation as a powerful transformation tool was widely used for importing foreign DNA fragments to many types of cells including wall-less and walled strains of *Chlamydomonas*. This method requires specific instrumentation for creating electrical impulses to deliver exogenous DNA into cells and optimization steps. However, the highest rate of nuclear transformation for microalgae was achieved using electroporation compared with the other transformation systems. Except for eukaryotic microalgae, prokaryotic microalgae such as cyanobacteria are also transformable by conjugation or electroporation in the lab. Cyanobacteria are a source of natural products such as proteins, vitamins, and chemical compounds such as 3-hydroxypropionic acid and succinic acid and can be engineered to synthesize substances of biotechnological interest (León-Bañares et al., 2004; Ng et al., 2017).

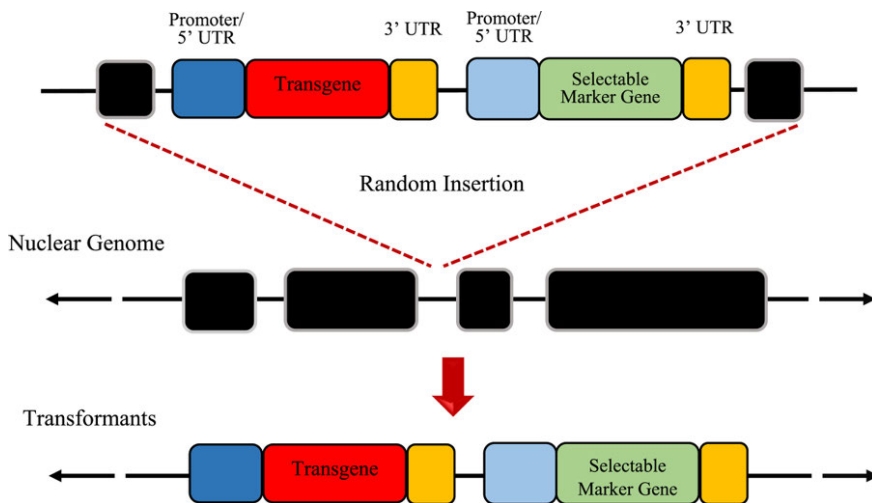


Figure 10.1 Transformation of microalgal nuclear genome by random insertion.

Another method applied for the transformation of microalgae is biolistics, which delivers nucleic acid to cells by high speed particle bombardment. This method is able to transform almost any type of cell including prokaryotic and eukaryotic cells and is not only limited to the transformation of the nucleus but it can also transform eukaryotic organelles, including chloroplasts and mitochondria. Since the chloroplast has multiple membranes, biolistics is the most efficient strategy for delivering foreign DNA fragments into its genome (León-Bañares et al., 2004; Ng et al., 2017). To use the above-mentioned method, firstly exogenous (transgenic) DNA is mixed with particles of a heavy metal, usually tungsten or gold. These fine particles stick to the negatively charged DNA and the DNA/metal particles are loaded onto one side of a plastic bullet (Fig. 10.2A) (Carter & Shieh, 2015). Then, a lawn of algal cells is spread onto an agar plate and placed into a vacuum chamber. This plate is bombarded with DNA-coated particles, which are shot into the target cells at high velocity by a gene gun or a biolistics particle delivery system (Fig. 10.2B). Some of these particles penetrate the cell wall, plasma membrane, and chloroplast membrane to deliver DNA fragments, which are then integrated into the plastid genome (Rasala et al., 2013). The first stable transformation of *C. reinhardtii* chloroplast genome was performed in 1988 using the biolistics method, and up to now has remained the most efficient and reliable method for chloroplast transformation (Boynton et al., 1988; Rasala et al., 2013). However, the number of transformants obtained was relatively low (i.e., 100 clones per 10^6 cells). With

particle bombardment, insertion of transgene into chloroplast DNA (cpDNA) is not random and carried out by homologous recombination. Therefore, the foreign DNA fragments would integrate into the cpDNA within left border (LB) and right border (RB) (Fig. 10.2C). In addition, biolistics has been successfully used for the transformation of diatoms. Unlike plants, *Agrobacterium*-mediated transformation is not widely used in microalgae and has only been reported for *C. reinhardtii*, *Haematococcus pluvialis*, *Chlorella vulgaris* and the oil-bearing marine algae *Parachlorella kessleri* (León-Bañares et al., 2004; Ng et al., 2017). Viruses known to infect unicellular *Chlorella*-like algae and brown seaweed can be proper candidates for the development of cloning and expression systems. The large size (150–330 kbp) of these double-stranded DNA viral genomes might permit carrying large sequences of foreign DNA and their insertion into microalgae. In 1994, virus-mediated transformation was successfully used in filamentous brown seaweeds (León-Bañares et al., 2004). However, up to now few studies have been done on this transformation method. Transposons, a group of mobile DNAs, have been also considered as powerful tools for genetic transformation of some microalgae. Artificial transposon systems—originally designed for in vitro mutagenesis—were developed by extracting essential elements from natural transposons. This system was extensively studied and the results obtained demonstrated its efficiency for improving the transformation of both prokaryotic and eukaryotic microalgae such as *Spirulina platensis* strain C₁ (*Arthrospira* sp. PCC9438) (2004), *Leptolyngbya* sp. strain BLO902 (2012) and *Nannochloropsis oceanica* (2019) by electroporation (León-Bañares et al., 2004; Lin & Qin, 2014; Nazari & Raheb, 2015; Ng et al., 2017; Osorio et al., 2019).

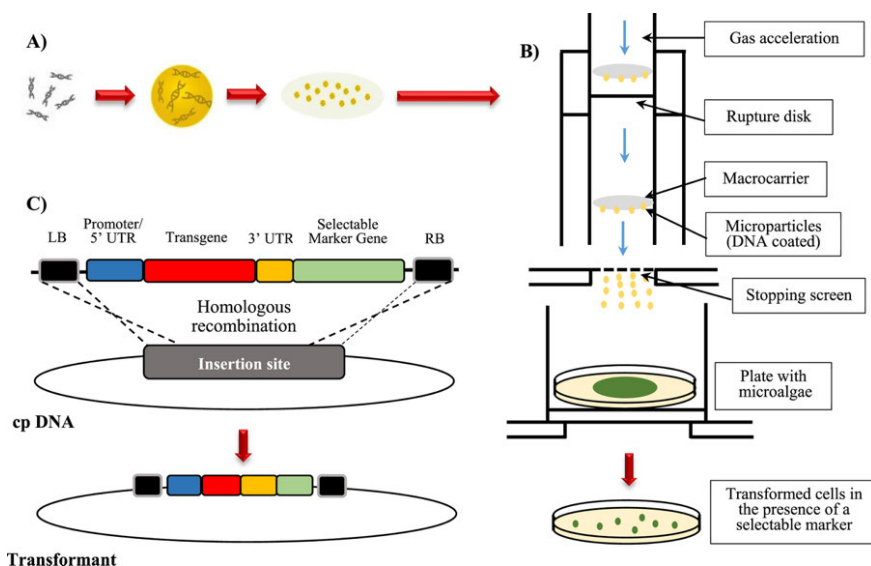


Figure 10.2 Chloroplast Transformation in Microalgae by the Biolistic Method. (A) DNA is mixed with particles of a heavy metal. DNA-coated particles are loaded onto a plastic bullet. (B) In a gene gun or biolistics particle delivery system, a pressurized gas, usually helium, provides the force for the gun. Gas pressure builds up until a rupture disk brakes, driving the plastic bullet down a shaft. The plastic bullet is abruptly stopped at the end of the shaft, but the DNA/metal particles emerge from the gun with great speed and force. If the gun is aimed at biological tissue, some of the metal particles will penetrate the cell membranes and deliver DNA constructs to cells. (C) Transformation of chloroplast genome by homologous recombination.

5.2. Integration and expression of transgenes

Once the heterologous gene entered the microalga cell and was integrated into its nuclear or chloroplastic chromosome, it has to be expressed (León-Bañares et al., 2004). Integration of a heterologous gene into the nuclear genome occurs primarily by random insertion through non-homologous end joining and at a low but measurable frequency by targeted gene insertion through homologous recombination (HR). After delivery of the foreign DNA to chloroplast through bombardment, homologous recombination occurs between the vector sequence and the corresponding genomic sequences (Boynton et al., 1988; Rasala et al., 2013). The expression of the integrated gene depends directly on its transcription to a messenger RNA (mRNA) and then the translation of the transcribed mRNA to the

related protein product. So, the transgene must be under control of a promoter and 5' and 3' untranslated regions (UTRs). The promoter region, where the regulatory elements bind to it, is responsible for initiating the transcription of DNA to mRNA from the transcription start site (+1) of the gene. While 5'UTR is the region of an mRNA that is directly upstream from the initiation codon and functions to regulate the ribosome association and the translation of a transcribed mRNA, the 3' UTR seems to influence mRNA stability and may interact with 5' UTR. Effective promoters for the expression of a heterologous gene in *C. reinhardtii* nuclear genome derive from endogenous or chimeric variants of endogenous promoters. Among them, the most effective promoters were $\beta 2TUB$ (the promoter region of the beta 2-tubulin gene), *NIT1*, (the *nit1* promoter region which is inducible by ammonium starvation), *LHCB1* (the promoter region of gene encoding a protein of the light harvesting complex of photosystem II), *HSP70A* (the promoter region of the gene encoding the heat-shock protein 70A), *RBCS2* (the promoter region of the gene encoding Rubisco small subunit), and *PSAD* (the promoter region of the gene encoding the subunit of photosystem I) (Rasala et al., 2013). Also, synthetic promoters constructed by combining the *HSP70A* gene from *Chlamydomonas* with other promoters have been used for transgene expression in microalgae (Nazari & Raheb, 2015). Furthermore, heterologous gene expression from the chloroplast genome seems to be primarily regulated at the translational level. The most successful regulatory elements which were identified for transgene expression in chloroplasts were endogenous promoters and 5' UTRs of *pbsA*, *atpA*, *atB*, *petD*, *rbcl*, *pbsD*, and *psaA* genes (Rasala et al., 2013). The similarity between the codon usage of the transcript and that of the host organism is another important aspect that should be considered and enhanced by codon-optimization to reflect the high frequency nuclear or chloroplast codon usage (León-Bañares et al., 2004; Rasala et al., 2013).

5.3. Selection of transformants

One of the most difficult steps in expressing heterologous genes in microalgae is the selection of the transformants. This step is primarily carried out through generating auxotrophic defective mutants and, then, transforming them with the wild-type authentic gene or by integrating a gene that induces resistance to an antibiotic or herbicide as a selectable marker (Ng et al., 2017). For several years, the only adequate selection method available for

microalgal transformation was the first one. However, this strategy is not applicable to wild-type or diploid microalgae (i.e., diatoms) due to the difficulties involved in generating mutants in which both alleles of a gene are defective (León-Bañares et al., 2004). Instead, antibiotic or herbicide screening for transformants is the most frequently used approach and a bacterial gene (*ble*) encoding zeomycin resistance is suitable for most microalgae. In this regard, different hosts and promoter strength of discriminatory marker genes would affect the selection efficiency. The type and concentration of a discriminatory marker or antibiotic for genetic transformation in various species of microalgae must be precisely determined. The commonly antibiotic used as a discriminatory marker for commercial vectors (i.e., pChlamy) is hygromycin (Ng et al., 2017). Zeocin, chloramphenicol, streptomycin, kanamycin, erythromycin, spectinomycin, and paromomycin were also successfully used for the selection of transformed *C. reinhardtii*, *Chlorella* sp., *Synechococcus* and *Nannochloropsis* sp. (Al-Haj et al., 2016; Díaz-Santos et al., 2016; Gan et al., 2018; Molina-Márquez et al., 2019; Newman et al., 1990; Ng et al., 2017) Furthermore, several different reporter genes such as β -glucuronidase (GUS) and adapted versions of the gene encoding green fluorescent protein (GFP) and luciferase have been fused to the genes encoding the desired protein. Then, those fusion proteins can be used for gene expression and protein localization studies in microalgae. Lack of requirement for an exogenous substrate as well as high expression of the GFP gene by codon optimization of its native gene have made this gene a favorite reporter for microalgae (Nazari & Raheb, 2015; Ng et al., 2017; Schiano di Visconte et al., 2019).

5.4. Stability of transgenes

Despite the successful and diversified genetic transformation of numerous algal species, the expression of a heterologous gene may be extremely low or nonexistent (León-Bañares et al., 2004; Ng et al., 2017). If all the required elements for the optimal transcription and translation are present, such disorder can be due to the protective behavior of most microalgae for recognizing a foreign gene, which leads to degrading or silencing it (Schiano di Visconte et al., 2019). The gene silencing has been attributed to a variety of epigenetic mechanisms that are heritable and reversible changes to control gene expression without alteration of its DNA sequence. These changes might occur at the transcription level through cytosine methylation

of the promoter regions or posttranscription level by an RNA-mediated process called RNA interference (RNAi). RNAi forms RNA-induced silencing complexes (RISCs) that promote mRNA degradation and silence gene expression (León-Bañares et al., 2004). Therefore, one of the great concerns in genetic manipulation of microalgae is the genetic stability of the transformants (Ng et al., 2017; Schiano di Visconte et al., 2019). Based on the results of previous research, *Chlamydomonas*, *Dunaliella*, *Chlorella*, and *Nannochloropsis* showed high genetic stability after transformation. On the contrary, other species of microalgae, including *Thalassiosira weissflogii*, *Ulva lactuca*, *Poryhyra miniata*, *Kappaphycus alvarezii* and *Gracilaria changii*, were not stable after nuclear transformation. Although integrated transgenes in the chloroplast genome of *Chlamydomonas*, *Chlorella*, *Porphyridium*, and *Euglena gracilis* are quite stable, more stable transformants have been reported in the nuclear transformation (Ng et al., 2017).

5.5. Microalgal genome editing

With the advent of the modern sequencing technology, the accessibility of microalgal genome sequences has simplified the metabolic engineering of their genetic and biosynthetic pathways. In the 2000s, control of microalgal metabolism was started by the development of genome editing and genome engineering tools (Ng et al., 2017; Schiano di Visconte et al., 2019). Up to now, several novel genetic manipulations including RNA interference, Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR-associated protein 9 (CRISPR-Cas9) systems have been developed for the precise genome editing. The CRISPR-Cas9 system, discovered in 2013, has attracted more attention than other genome editing tools due to its accuracy, simplicity and cost-effectiveness (Cai et al., 2019; Ng et al., 2017). This system provides an immunological weapon to bacteria and archaea against invading bacteriophages (viruses) and mobile genetic elements. The type-II CRISPR-Cas9 system from *Streptococcus pyogenes* has been extensively studied and well characterized (Cai et al., 2019; Ng et al., 2017). The Cas9 protein is an RNA-mediated endonuclease that is guided to the target sites by single guide RNA (sgRNA) which consists of a CRISPR targeting RNA (crRNA) and a transactivating crRNA (tracrRNA). Following the guide of sgRNA, Cas9 targets the genome specific sequence with three nucleotides protospacer adjacent motif (PAM) that is found immediately at

3' end of the desired locus in the genome and cleaves both strands of DNA. Breakdowns of double-stranded DNA must be quickly repaired to prevent cell death. DNA repair process is done through the nonhomologous end joining machinery (NHEJ) or homologous recombination (HR) of the DNA repair process. NHEJ repair process is normally caused by gene disruption by insertion or deletion mutation whereas HR repair approach enables the replacement or insertion of the desired sequences with the existence of donor DNA (Fig. 10.3A) (Cai et al., 2019).

Despite the high applicability of the CRISPR-Cas9 system, high genomic data to prevent off-target sgRNA design are required. So, more attention was paid to the modified form of this strategy (Jiang et al., 2014). The CRISPR interference (CRISPRi) system is a new approach that uses the same design of guide RNA with nuclease-deficient Cas9 which lacks the ability to cleave DNA and only functions as a DNA binding complex for gene interference instead of gene modification for gene regulation (Fig. 10.3B). Therefore, this system is similar to the traditional RNA interference (RNAi) such as silence RNA (siRNA) and micro-RNA (miRNA) and in the same way can repress or activate the gene expression (Ng et al., 2017).

6. Genetic manipulation of microalgae for biofuel production

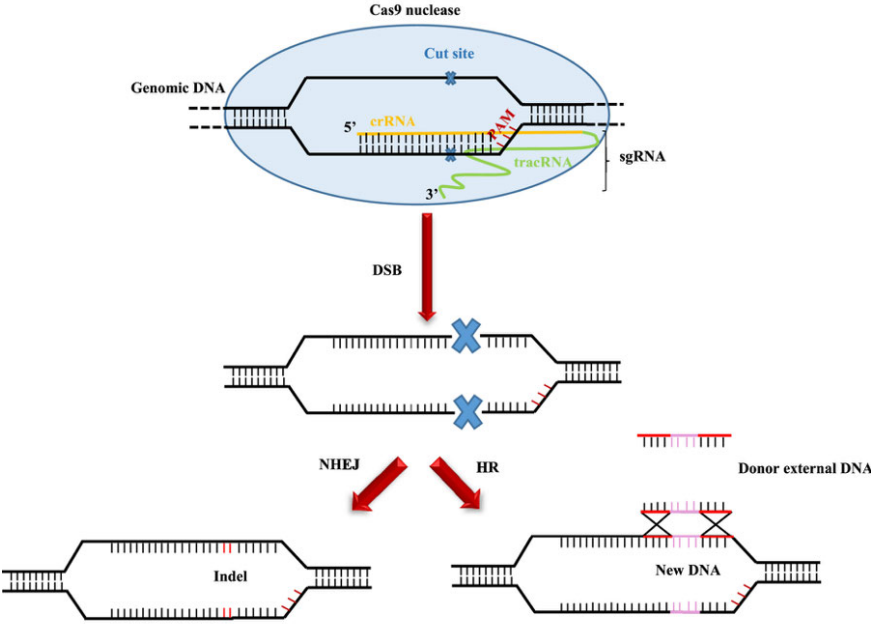
Recently there is a growing attention for renewable biofuel production mostly due to the global warming concern, the decreasing of fossil fuel resources, the increasing world-wide oil price and the risk of increasing atmosphere CO₂ levels as a result of fossil fuel consumption. Biomass produced from microalgae has appeared as a highly desirable source of renewable energy when compared with other clean energies such as solar and wind, since it can be easily conveyed and suitably stored. It was also reported that the use of biofuel produced from algae (micro and macro) decreased the release of CO₂ (78%) as well as the sulfur emissions (98%) (Banerjee et al., 2016). Biomass derived from microalgae can be managed in different pathways to generate biofuels such as biodiesel, bioethanol, and biomethane. As described below, here we focus on biodiesel and bioethanol obtained from microalgae.

7. Microalgae as a broad range of biofuel products

Microalgae have a great ability to use CO₂ as a carbon source to produce a broad range of biofuels (biodiesel, bioethanol, and biomethane) and can be grown on nonarable land using saltwater or even wastewater streams. Moreover, microalgae are able to produce a variety of added-value products such as antioxidants, polyunsaturated fatty acids and edible proteins to balance the cost of biofuel production. Like the higher plants, microalgae can capture solar energy and transform it into compounds with high chemical bound energy such as lipids, carbohydrates and proteins. Lipids (e.g., triglycerides) are one of the preliminary forms of energy storage in microalgal cells and can be employed to produce biodiesel via transesterification (Radakovits et al., 2010). In addition, carbohydrates (e.g., starch) can be used to produce bioethanol through fermentation processes. However, several challenges need to be conquered before using microalgae as a sustainable and cost-effective option for biodiesel and bioethanol production. The major obstacles include inconsistent production of biomass at a bulky scale of microalgal cultivation system, absence of profitable extraction techniques, identification of strains with appropriate biodiesel or bioethanol traits as well as inefficient light harvesting capability in microalgal growth conditions.

With regard to biodiesel derived from microalgal, many investigations have been directed to develop a microalga with high lipid content. Some of these studies were conducted by genetic manipulation of metabolic and biosynthetic algal pathways to overcome the above-mentioned challenges. Therefore, it is apparent to focus on improving lipid accumulation in microalgae to reach higher oil production. To achieve this purpose, a better understanding of the algal metabolic pathway for lipid production is vital. To increase lipid contents, microalgal lipid engineering can be achieved by different methods such as metabolic and genetic engineering.

A) CRISPR/Cas9



B) CRISPRi

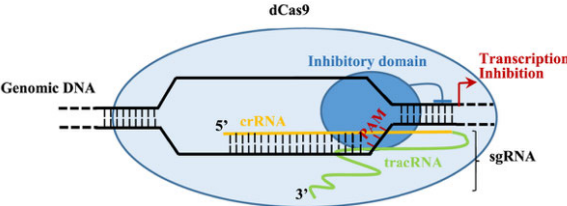


Figure 10.3 Comparing the Genome Editing with CRISPR/Cas9 and CRISPRi Systems. (A) In the CRISPR–Cas9-mediated genome editing system, the Cas9 and sgRNA form a complex in vivo and then bind on the target DNA sequence upstream of PAM sequence. The Cas9 nuclease domain then cleaves the target DNA sequence, forming a double-stranded break (DSB). DSB must be repaired via either NHEJ or HR immediately to avoid cell death (Indel: Insertion and Deletion). (B) In CRISPRi, a catalytically dead variant of Cas9 (dCas9) without endonucleolytic activity is coupled with designed small guide RNAs (sgRNAs) complementary to a desired DNA target. The dCas9-sgRNA complex acts to sterically hinder transcription of the targeted DNA, causing gene repression.

8. Lipid metabolism

8.1. Lipid biosynthesis

The lipid biosynthesis in microalgae starts in the chloroplast of their cells via the photosynthesis which uses atmospheric CO_2 , and then produces the building blocks of fatty acids (FAs) and triacylglycerols (TAGs). The FAs contain a carboxylic group along with an aliphatic chain which can be saturated or unsaturated (monounsaturated or polyunsaturated). Depending on the number of carbon atoms there are short, medium and long chain FAs. The initial step in FA biosynthesis contains acetyl-CoA carboxylase which catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate (Bilbao et al., 2017). Then, the malonyl group from malonyl-CoA is transferred to malonyl-ACP (ACP: acyl carrier protein) catalyzed by malonyl-CoA: ACP transacylase. Afterward, malonyl (acyl)-ACP in the presence of ketoacyl-ACP synthases and during four steps reactions (condensation, the first reduction, dehydration and the second reduction) forms Acyl-ACP which later is used as a substrate for the elongation of FA. After each condensation, the fatty acid synthase (FAS) complex catalyzes reduction, dehydration and second reduction steps. At the end of the elongation, FAs are transferred to the cytoplasm to be used as substrates of the acyl transferase to synthesize the neutral lipids named TAG. Because of having high energy acyl chains in TAG, they are considered as the main form of storing energy in microalgae (Bilbao et al., 2017).

8.2. Lipid catabolism

Lipid catabolism consists of two main phases that are lipolysis and degradation of FAs to acetyl-CoA. Lipolysis begins with the cleavage of FAs or the head groups from the glycerol backbone in structural and storage glycerolipids and is catalyzed by lipases on lipid droplets or at membranes. The complete degradation of released free FAs mostly occurs in peroxisomes through the β -oxidation pathway of algae. In the lipolysis, depending on the specific substrate, various lipases such as triacylglycerol (TAG) lipases, diacylglycerol (DAG) lipases, monoacylglycerol (MAG) lipases, phospholipases and galacto-lipases are involved. All these lipases belong to the carboxylic ester hydrolase category because they release free FAs while phospholipases C and phospholipases D are considered phosphoric diester hydrolases because they breakdown the phosphodiester bond in the phosphorylated head group. FA degradation which leads to the production of

acetyl-CoA, two-carbon acetyl units, occurs in peroxisomes of green microalgae and sometimes in mitochondria of some other algal species. The β -oxidation pathway consists of four repeating enzymatic steps including, oxidation, hydration, dehydrogenation and thiolitic cleavage of an acyl-CoA, which are catalyzed by three enzymes of acyl-CoA oxidase (ACX), multifunctional protein and 3-ketoacyl-CoA thiolase, then acetyl-CoA is formed as a final product (Kong et al., 2018).

8.3. Genetic engineering of algae for lipid improvement

Recently, the application of genetic engineering to increase lipid production and its accumulation in microalgae has received a great attention due to the higher is the lipid content in microalgae the higher is the biodiesel produced (Sharma et al., 2018).

The strategies to improve lipid synthesis in microalgae mostly rely on the manipulation of the enzymes-encoding genes involved in the lipid biosynthesis pathways with the aim of diverting the carbon and other reductive compounds to lipid biosynthesis. As FAs play a significant role in the regulation of the lipid biosynthesis, increasing the expression of the crucial enzymes associated with FA biosynthesis may lead to lipid improvement in microalgae (Sharma et al., 2018). The first attempt to increase the lipid yield was done in 1996 by Dunahay et al. (1996) through the overexpression of the acetyl-CoA carboxylase (ACCase) gene, which encodes a vital enzyme involved in converting acetyl-CoA to malonyl-CoA. Their results demonstrated no improvement in FA; however, an increase (up to threefold) was observed in the ACCase activity. So, they concluded that the sole overexpression and elevated activity of ACCase had no effect on lipid biosynthesis.

Later, Talebi et al. (2014) found that the concurrent overexpression of *accD* (gene encoding one subunit of ACCase) and ME genes (malic enzyme-gene responsible for the alteration of malate to pyruvate) increased total FA in microalgae. Furthermore, additional investigations found that manipulation in the expression of many enzymes-encoding genes resulted in the improvement of the lipid content in microalgae. For example, the overexpression of acetyl-CoA synthase (in *Schizochytrium*), glycerol kinase (in *Fistulifera solaris*) and NAD(H) kinase (in *Chlorella pyrenoidosa*) genes along with the suppression of phosphoenolpyruvate carboxylase (in *C. reinhardtii*) and pyruvate dehydrogenase kinase (PDK) (in *Phaeodactylum tricornutum*) genes were reported to improve the lipid content with no adverse effect on

cell growth (Deng et al., 2014; Fan et al., 2015; Ma et al., 2014; Muto et al., 2015; Sharma et al., 2018; Yan et al., 2013).

Two independent studies were performed in 2016 by Li et al. (2016) and in 2017 by Úbeda-Mínguez et al. (2017) on the diacylglycerol acyltransferase (DGAT) gene and their results confirmed that the overexpression of the above-mentioned gene resulted in increased levels of TAG. The former study was done in *Nannochloropsis oceanica* (Li et al., 2016) and the latter one in *Tetraselmis chui* (Úbeda-Mínguez et al., 2017). Later, an investigation by Zhu et al. (2018) was conducted to examine the expression effect of the exogenous phospholipid diacylglycerol acyltransferase (PDAT) fused with a chloroplast transit peptide in *C. reinhardtii* CC-137 on the production of FA and TAG. Their findings indicated a 22% and a 32% increase, respectively, in the content of FA and TAG of the recombinant *C. reinhardtii* in comparison to the wild-type strain. Afterward, an investigation by Takahashi et al. (2018), demonstrated that cellular lipid contents increased in the mutants of *Coccomyxa* sp. strain Obi which were isolated by the means of TALEN and were defective in the gene for the large subunit of ATP: α -D-glucose-1-phosphate adenylyl transferase (AGPL). Moreover, the findings of a study by Rengel et al. (2018) evidenced that the overexpression of the endogenous chloroplastic acetyl-CoA synthetase (ACS2) gene under the control of the strong constitutive RBCS2 promoter in *C. reinhardtii* led to increased levels of starch and acyl-CoA in nitrogen-replete cultures. The Cr-ACS2 transformants also showed higher levels of ACS2 transcript and accumulation of TAG than those of the untransformed controls. Therefore, they concluded that the manipulation of ACS2 gene was a suitable method to improve the accumulation of TAG (Rengel et al., 2018). Furthermore, the role of stramenopile-type lipid droplet protein (StLDP) on the improvement of neutral lipid accumulation in *Phaeodactylum tricornutum* was identified by other researchers (Yoneda et al., 2018). In Table 10.1 the gene manipulation for lipid improvement is summarized.

Table 10.1

Gene	Microalgae	Mode of action	References
<i>C. cryptica</i>	Overexpression	Dunahay et al. (1996)	ACCase

<i>D. salina</i>	Overexpression	Talebi et al. (2014)	accD and ME genes
<i>Schizochytrium</i> sp	Overexpression	Yan et al. (2013)	ACS
<i>C. reinhardtii</i>	Overexpression	Rengel et al. (2018)	ACS
<i>F. solaris</i>	Overexpression	Muto et al. (2015)	Glycerol kinase
<i>C. pyrenoidosa</i>	Overexpression	Fan et al. (2015)	NAD(H) kinase
<i>C. reinhardtii</i>	Suppression	Deng et al. (2014)	Phosphoenolpyruvate carboxylase
<i>P. tricornutum</i>	Suppression	Ma et al. (2014)	Pyruvate dehydrogenase
<i>Coccomyxa</i> sp	Suppression	Takahashi et al. (2018)	ADP-glucose pyrophosphorylase (sta6)
<i>P. tricornutum</i>	Overexpression	Yoneda et al. (2018)	Lipid droplet protein (StLDP)
<i>T. chui</i>	Overexpression	Úbeda-Mínguez et al. (2017)	PDAT
<i>C. reinhardtii</i>	Overexpression	Zhu et al. (2018)	PDAT
<i>N. oceanica</i>	Overexpression	Li et al. (2016)	DGAT

*ACC*ase, acetyl CoA carboxylase; *accD*, gene encoding one subunit of AC-Case; ACS, acetyl-CoA synthase; *DGAT*, diacylglycerol acyltransferase; *ME*, malic enzyme; *PDAT*, phospholipid diacylglycerol acyltransferase.

Among the studies associated with transcriptional regulation, the basic-region/leucine zipper (bZIP) transcriptional factor (TF) proved its significant role in regulating both signaling pathways and lipid metabolic pathways to increase the lipid yields (Dröge-Laser & Weiste, 2018). Li et al. (2019) discovered the novel bZIP₁ transcription factor, designated NobZIP₁, whose overexpression increased lipid production and oil secretion in the model microalga *Nannochloropsis oceanica*. They showed that the above-mentioned improvement was due to the role of the NobZIP₁ in up- and down-regulation of several key genes involved in lipogenesis and cell wall

polymer synthesis, respectively, which, in turn, induced lipid overproduction and oil secretion. According to their findings the NobZIP₁ up-regulated the expression of key genes, such as ACBP (acyl-coenzyme A (CoA)-binding protein), KAS (3-ketoacylacyl carrier protein synthase), LC-FACS (long-chain acyl-CoA synthetase) and LPAAT (lysophosphatidic acid acyltransferase) and led to an increase in lipid accumulation in NobZIP₁-overexpressing strains (Li et al., 2019).

On the other hand, the NobZIP₁ down-regulated the expression of CPS (putative capsular polysaccharide synthesis gene) and UGDH (UDP-glucose dehydrogenase: a key enzyme in the biosynthesis of cell wall polysaccharides) genes which are involved in the cell wall carbohydrate metabolism and, thereby, induced the lipid secretion due to impair cell wall polysaccharide formation. So, down-regulation of CPS and UGDH gene expression redirected the carbon flux toward both lipid biosynthesis and weakened the cell wall and facilitated concurrent lipid overproduction and secretion (Li et al., 2019).

Many studies stated that environmental stress as well as deficiency of nutrients decreased the microalgal proliferation to produce high energy products such as lipids and starch. In this regard, the overexpression of lipid synthesis genes under control of an inducible promoter would be of interest due to its activation when microalgal proliferation reaches a high mass. Two examples of these inducible promoters are a nitrate-responsive promoter in diatoms and a copper-responsive element in *C. reinhardtii* (Radakovits et al., 2010).

There are other targets such as improving the light use productivity in photosynthetic algae that indirectly affects lipid content via cell growth features (Sharma et al., 2018). In this sense, many efforts have been done to expand the photosynthetic productivity on microalgal growth. Among them, a study by Mussgnug et al. (2007) confirmed the role of RNAi to knock down genes encoding light-harvesting antenna complex (LHC) in *C. reinhardtii* which could be also used to many various microalgae much easier than a random mutagenesis technique. LHC in green algae is applied to maximize light capture under low-light conditions. But this LHC activity under intense sunlight can cause photodamage. So, under high-light conditions to protect such damage most of the absorbed photons by green algae are converted and wasted to fluorescence and heat which is a limitation in

the production process efficiency of mass culture. To solve this problem, Mussnug et al. (2007) down-regulated the entire LHC gene family of *C. reinhardtii* using RNAi technology and constructed a mutant Stm3LR3. Their result showed that the mutant Stm3LR3 had a significant reducing effect on transcription of LHCI and LHCII mRNAs as well as decreased the fluorescence levels while increasing the photosynthetic yield under high-light conditions (Mussnug et al., 2007). *Dunaliella bardawil*, a halotolerant wall-less microalga, is naturally able to grow in a wide range of NaCl concentrations (from 0.1 to 5M) through accumulation of intracellular glycerol in response to extracellular osmotic pressures. At high concentrations of salt (hyperosmotic stress), *Dunaliella* enhances glycerol photosynthesis; accumulation of intracellular glycerol leads to more assimilation of photosynthetically fixed carbon, faster removal of fixed carbon from the photosynthetic center and, thus, a further increase in fixation of CO₂. Fang et al. (2012) conducted an experiment using the *C. reinhardtii* *sedoheptulose 1,7-bisphosphatase* (*CrSBPase*) gene, which encodes a rate-limiting enzyme SBPase in the photosynthetic carbon reduction cycle (Calvin cycle). They introduced the *CrSBPase* gene to the glycerol-producing green alga *D. bardawil* through the *Agrobacterium tumefaciens*-mediated gene transfer system. They succeeded to enhance the photosynthesis and increased the glycerol production in *D. bardawil* via expression of the *CrSBPase* gene in the above-mentioned strain. They also demonstrated that the enhanced photosynthetic activity of the recombinant *D. bardawil* carrying the *CrSBPase* gene was due to the improvement of CO₂ assimilation following the osmoticum glycerol production (Fang et al., 2012). Furthermore, a significant role of the RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzyme in improving the photosynthesis as well as the carbon fixation in cyanobacterial *Synechocystis* cells PCC 6803 were confirmed by Liang and Lindblad (2017). They succeeded to improve RuBisCO levels in two recombinant strains of cyanobacterium *Synechocystis* PCC 6803 FL50 and FL52 by 2.1- and 1.4-fold, respectively. Their results demonstrated that the overexpression of the RuBisCO gene in *Synechocystis* PCC 6803 increased the level and activity of RuBisCO as well as photosynthesis and growth in recombinant strains of *Synechocystis* (Liang & Lindblad, 2017). Moreover, a study by Yang et al. (2017) proved the possibility of enhancing the photosynthetic productivity by the genetic manipulation of the Calvin cycle in the green microalga *C. vulgaris*.

Yang et al. (2017) introduced the cyanobacterial fructose 1,6-bisphosphate aldolase gene (involved in the Calvin cycle) into the *C. vulgaris* chloroplast with the aim of improving the photosynthetic potential and growth of this microalga (Yang et al., 2017). Their results indicated an increased photosynthetic capability (~1.2-fold) and cell growth in the recombinant *C. vul-* *C. vul-* compared to the non-recombinant strain. In addition, they concluded that the cyanobacterial aldolase overexpression may cause the regeneration of ribulose 1,5-bisphosphate in the Calvin cycle and energy transfer in photosystems (Yang et al., 2017).

Inhibition of lipid catabolism is another logical choice to enhance the lipid content regardless of the microalgal strain. A study by Trentacoste et al. (2013) using the diatom *Thalassiosira pseudonana* indicated that targeted knockdown of a gene encoding multifunctional lipase/phospholipase/acyl-transferase enzyme led to a lipid content increase with no effect on its growth. They demonstrated that knockdown strains resulted in more lipid yields compared to their wild-type counterparts under nutrient limitation conditions (silicon starvation) (Trentacoste et al., 2013).

Genes involved in the activation of both free FAs and TAGs as well as genes which were directly associated with β -oxidation of FAs could be inactivated, which resulted in an enhancement of the cellular lipid yield. The gene inactivation can be achieved through RNA silencing or by random mutagenesis, however, there were some problems with these strategies resulting in harmful effects on proliferation and cellular growth (Radakovits et al., 2010). Therefore, this approach can only be applied under controlled conditions in terms of external carbon source and constant light such as photobioreactors for improving lipid biosynthesis (Shahid et al., 2020).

9. Genetic engineering of microalgae for starch improvement

Starch is a primary energy storage compound for some algae and its production in plastids is catalyzed by specific enzymes such as ADP-glucose pyrophosphorylase. As it can be used for biofuel (bioethanol) production, many attempts have been done to enhance starch contents. One method to increase the starch storage in microalgae is introducing enzymes involved in starch-producing into the cytosol because it can provide more space for accumulation of starch granules. The only problem with cytosolic starch production is the far distance between AGPase from the pyrenoid, which is in the chloroplast, and could not be able to activate the 3-phosphoglyceric acid (3-PGA). However, genetic manipulation could help to overcome this problem by introducing AGPase, which has activity without the existence of PGA, such as Mos (1-198)/SH2 (Boehlein et al., 2009; Radakovits et al., 2010). The Mos (1-198)/SH2 is a maize/potato mosaic small subunit in which the first 198 amino acids are from the maize endosperm small subunit, while the carboxyl-terminal 277 amino acids come from the potato tuber AGPase small subunit. The most interesting characteristic of Mos (1-198)/SH2 is its enzymatic activity in the absence of 3-PGA which is considerably higher (up to 5-fold) than that of the wild type (Boehlein et al., 2009).

Reducing starch degradation (or blocking metabolic pathways) in microalgae is another logical option to improve the starch content. In this regard, two mechanisms have been suggested, hydrolation and phosphorylation, which have been examined on *Arabidopsis thaliana*. The hydrolytic mechanism requires α -amylase to hydrolase semicrystalline glucans at the surface of the insoluble starch particles. The α -amylase in *A. thaliana* contributes to starch degradation while this role is played by a homologous protein in *C. reinhardtii*. The second one needs glucan-water dikinases (GWD) and phosphoglucan water dikinases (PWD) to phosphorylate glucose residues on C-6 and C-3 positions at the amylopectin, respectively (Radakovits et al., 2010). The results of the studies performed by Yu et al. (2001) indicated that the disruption of the GWD in *A. thaliana* led to an increase in starch levels up to sixfold compared to those in its wild-type counterpart, whereas, Ritte et al. (2006) demonstrated disruptions in PWD resulted in a less extra amount of produced starch. These phosphorylation phases are excellent gene knockout targets for a starch accretion phenotype in microalgae (Radakovits et al., 2010; Ritte et al., 2006; Yu et al., 2001).

Therefore, genetic manipulation of starch biosynthetic pathways for improving microalgal biofuel (bioethanol) production seems logical.

10. Microalgal genome editing

Genome editing techniques such as CRISPR/Cas9, TALENs and ZFNs have become accurate tools for modification of targeted genes (gene replacement, gene knockout) in numerous organisms like microalgae (Ng et al., 2017).

A significant advantage of the CRISPR method in microalgae is its simplicity for controlling metabolic pathways. In regard to lipid engineering in microalgae, this method can enhance the lipid amount through concurrently inhibiting the metabolic pathways which compete to lipid synthesis such as lipid degradation and β -oxidation. The first successful application of (CRISPR)/Cas9 technique in microalgae was performed by Jiang et al. (2014). Their results confirmed that the transient expression of single guide RNA (sgRNA) and Cas9 genes in *C. reinhardtii* were transformed using the electroporation delivery system. Their experiments showed the accurate function of sgRNA and Cas9 genes in *C. reinhardtii* to bring about modifications in the targeted genes (an exogenous hygromycin resistance gene, an exogenous mutant GFP gene, an exogenous mutant Gaussia luciferase (Gluc) gene and an endogenous FKB12 gene) (Jiang et al., 2014). The first attempt to perform CRISPR technique in microalgae opened a door for further investigation associated with improving biofuel production as well as more effectiveness of phycoremediation.

Later, Baek et al. (2016) reported the transformation of *C. reinhardtii* by the DNA-free RNA-guided engineered nucleases (RGENs) preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) technique. Their RGEN RNP method led to consecutive CpFTSY and ZEP two-gene knockout and the creation of a strain constitutively producing zeaxanthin with better photosynthetic capacity. Moreover, the findings of Baek et al. (2016) confirmed the improvement of biomass production resulting of the genetic modification of microalgae. The results of another study by Shin et al. (2016) indicated the successful production of CRISPR/Cas9-induced mutations at the CpSRP43, MAA7 and ChlM loci by delivering Cas9 RNP containing the Cas9 protein and sgRNAs. They also specified that the productivity of those targeted mutations was 100-fold higher than that of the previously reported for CRISPR/Cas9-induced mutagenesis in *Chlamydomonas* (Shin et al., 2016). Moreover, Shin et al. (2016) proposed that their successful method can be used in other microalgal species for improvement of biofuel production.

Another genome-editing investigation was conducted by Wang et al. (2016) on the industrial oleaginous microalga *Nannochloropsis oceanica* applying nitrate reductase (NR). They established a CRISPR/Cas9-based targeted gene knockout method for the *N. oceanica* strain IMET₁ using gRNA directed Cas9 protein to sequence-specifically cleave targeted DNA sequences that matched the gRNA. Wang et al. (2016) demonstrated that the knockout of endogenous NR gene through this method resulted in growth failure in medium containing nitrate (NaNO₃) as sole nitrogen source, yet uncompromised growth under ammonium (NH₄Cl). Their findings indicated that NR-deletion mutants with the strict auxotrophic phenotype can possibly apply for both probing microalgal nitrogen metabolism and genetic frame as an eco-friendly advancement of transgenic feedstock (which can be assessed by controlling the nitrogen source) (Wang et al., 2016). In addition, applying multiple gRNAs in a single CRISPR array provides a high possibility of concurrent editing of several target sites on the genome. Alternatively, the specificity and effectiveness of the above-mentioned method can be enhanced by adjusting the vector design or tuning the gRNA parameters (Wang et al., 2016).

Nymark et al. (2016) developed an extremely effective CRISPR/Cas9 based system optimized for producing stable targeted gene knockouts in the marine diatom *Phaeodactylum tricornutum*. In that system, the diatom codon-optimized Cas9 was driven by a promoter for a fucoxanthin chlorophyll a/c-binding protein gene (LHCF2) and sgRNA was under control of the *P. tricornutum* U6 promoter (Nymark et al., 2016). They showed that using two BsaI cutting sites, which sited within the sgRNA cassette, a small adapter could be simply inserted into the sgRNA and made stable targeted gene knockouts in the *P. tricornutum*. Nymark et al. (2016) proposed that their method could be useful for other microalgal genome editing studies to modify lipid profile and consequently improve the biodiesel production.

Ajjawi et al. (2017) identified 20 transcription factors (TFs) with a negative regulatory effect on lipid production using the RNA-seq analysis of *Nannochloropsis gaditana* throughout nitrogen deficiency. One of those identified TFs was a transcriptional regulator of lipid accumulation, Zn(II)₂Cys₆ (ZnCys). Applying a CRISPR–Cas9 reverse-genetics pipeline allowed insertional mutagenesis of 18 of 20TFs (Ajjawi et al., 2017). Their results confirmed that knocking out a homolog of fungal ZnCys-encoding genes

enhanced partitioning of total carbon to lipids from 20% in wild type to 40%–55% for the mutant strains in nutrient-supplied situations, respectively (Ajjawi et al., 2017).

Baek et al. (2018) investigated the potential of the *C. reinhardtii* CC-4349 strain to improve its lutein and zeaxanthin production, which are dietary carotenoids with protective effect against age-related macular degeneration. They induced a knock-out mutation of the zeaxanthin epoxidase gene by preassembled DNA-free CRISPR-Cas9 ribonucleoproteins in the CC-4349 strain. Their results indicated a significantly elevated zeaxanthin content (56-fold) and productivity (47-fold) than those of the wild type. The finding of Baek et al. (2018) had no direct relation to the biofuel production, however considering the added-value products obtained by their study it can be beneficial to balance the production costs of biofuel. In addition, Yoshimitsu et al. (2018) succeeded to isolate several knockout lines of the FTSY gene of *Coccothyrax* sp. strain KJ, encoding a signal recognition particle-docking protein at a frequency of 0.01% by intracellular delivery of a ribonucleoprotein complex comprising recombinant Cas9 protein and guide RNA through an electroporator means. It was reported that under nitrogen depletion conditions *Coccothyrax* sp. strain KJ accumulated more TAGs than the 60% of its dry weight, therefore, it is a good candidate for biodiesel production (Yoshimitsu et al., 2018).

An investigation by Guzmán-Zapata et al. (2019) demonstrated that the transient expression of the *Streptococcus pyogenes* Cas9 gene and sgRNAs, targeted to the single-copy nuclear *apt9* gene encoding an adenine phosphoribosyl transferase (APT), resulted in the efficient disruption at the expected locus. Subsequently, introduction of *indels* (insertions and deletions) to the *apt9* locus led to cell insensitivity to the otherwise toxic compound 2-fluoroadenine (2-FA) (Guzmán-Zapata et al., 2019). Their successful results showed that transient expression of Cas9 and a sgRNA could be used as a highly efficient and economical approach for editing nuclear genomes (Guzmán-Zapata et al., 2019). An efficient method using both meganucleases and TALE nucleases (sequence-specific nucleases) was applied for targeted and stable editing of the marine diatom *Phaeodactylum tricornutum* genome by Daboussi et al. (2014) to produce an improved lipid-producing strain. Cotransformation of the above-mentioned strain with nuclease-encoding constructs and a selectable marker gene led to high rates of

genome modification with 56% for the colonies exhibiting targeted mutagenesis and 27% for targeted gene insertion (Daboussi et al., 2014). Applying the generated improved lipid producing strain showed 45-fold increase in TAG accumulation through the interruption of the UDP-glucose pyrophosphorylase gene compared to the wild type (Daboussi et al., 2014). This engineered strain would be useful for biofuel (biodiesel) production as well as helping researchers for a better understanding of synthetic biology in diatoms as photosynthetic microalgae.

On the other hand, an investigation by Sizova et al. (2013) was performed using the ZFN technique to target the *COP3* gene which encodes the light-activated ion channel channelrhodopsin-1 (Sizova et al., 2013). They designed ZFNs based on the accessible online tools and examined their capability to repair a nonfunctional aminoglycoside 3'-phosphotransferase VIII (*aphVIII*) gene conferring resistance to paromomycin (Pm-R) interspaced with a short *COP3* target sequence. Upon concurrent delivery of engineered ZFNs and *aphVIII* DNA template to *C. reinhardtii*, they could restore marker activity and select paromomycin resistant clones with expressing nuclease. Since, 1% of these Pm-R clones contained a modified *COP3*, the specific modification of *COP3* locus was possible by homologues recombination. The findings of that study provided evidence that the ZFN technique could modify any *Chlamydomonas* gene at any positions and engineered ZFNs could be useful for site-specific modification of a nuclear gene of this genus of green microalgae in which mutations are not selectable (Sizova et al., 2013). In Table 10.2 genome editing studies in microalgae are summarized.

11. Application of microalgae to phycoremediation

Phycoremediation indicates the process of pollutant removal from the aquatic environment by employing algae (macroalgae and microalgae). It is an eco-friendly procedure because the above-mentioned photoautotrophic microorganisms are able to use the phosphorus (P) and nitrogen (N) p- (N) p- in wastewater as nutritional sources. The amount of P and N, which mainly results from industry and household activities, can be decreased by microalgae to acceptable levels to reuse wastewater. This is due to the fact that microalgae need P, N and CO₂ to grow and synthesize carbohydrates, lipids and proteins that can be further transformed into biofuels (such as biodiesel and bioethanol) (Pacheco et al., 2015). The produced biomass, then, can be used to generate biofuels such as biodiesel (through transesterification of lipids), bioethanol (via fermentation of carbohydrates) and biogas (through anaerobic digestion) (Park et al., 2011).

Apart from the inorganic nutrients (P and N), there are other harmful compounds present in wastewater such as metals and pharmaceutical compounds. Microalgae have the ability to adsorb and metabolize trace metals, which make microalgae one of the most biologically sequestered trace metals in aquatic ecosystems. Several characteristics of microalgae including metal-binding groups on their cell wall, their large surface to volume ratio and efficient metal uptake as well as storage systems are related to their metal adsorption capability. Besides the vital trace metals needed for metabolism, microalgae can effectively sequester poisonous heavy metals from the water environment (Rajamani et al., 2007).

Table 10.2

Microalgae	Target gene	Genome editing tools	Delivery system	References
HygR, mGFP, FKB12, and Gluc	CRISPR/Cas9	Electroporation	Jiang et al. (2014)	<i>C. reinhardtii</i> CC503
ZEP and <i>CpFTSY</i>	CRISPR/Cas9	Electroporation	Baek et al. (2016)	<i>C. reinhardtii</i> CC-4349
MAA7, <i>CpSRP43</i>	CRISPR/Cas9	Electroporation	Shin et al. (2016)	<i>C. reinhardtii</i> CC-124

and <i>ChlM</i>				
Nitrate reductase gene	CRISPR/Cas9	Electroporation	Wang et al. (2016)	<i>N. oceanica</i> IMET1
<i>CpSRP54</i> gene	CRISPR/Cas9	Bombardment	Nymark et al. (2016)	<i>P. tricornutum</i> CCMP2561
ZnCys TF	Cas9 editor line	Electroporation	Ajjawi et al. (2017)	<i>N. gaditana</i> CCMP1894
Zeaxanthin epoxidase gene	CRISPR-Cas9	Glass beads	Baek et al. (2018)	<i>C. reinhardtii</i> CC-4349
<i>FTSY</i>	CRISPR-Cas9	Electroporation	Yoshimitsu et al. (2018)	<i>Coccomyxa</i> sp. strain KJ
<i>apt9</i>	CRISPR-Cas9	Glass beads	Guzmán-Zapata et al. (2019)	<i>C. reinhardtii</i> (CC-125)
<i>UGPase</i>	TALEN	Bombardment	Daboussi et al. (2014)	<i>P. tricornutum</i> CCMP2561
<i>COP3</i> gene/ <i>aphVII</i> gene	ZFN-mediated	Glass beads	Sizova et al. (2013)	<i>C. strain</i> CC-4350

apt9, gene encoding an adenine phosphoribosyl transferase; *ChlM*, Mg-protoporphyrin IX S-adenosyl methionine O-methyl transferase; *CpFTSY*, signal recognition particle receptor protein, chloroplast; *CpSRP43*, chloroplast signal recognition particle 43; *CpSRP54*, chloroplast signal recognition particle 54; *FKB12*, peptidyl-prolyl cis–trans isomerase; *HygR*, hygromycin resistance; *MAA7*, beta subunit of tryptophan synthase; *UGPase*, UDP-glucose pyrophosphorylase gene; *ZFN*, zinc-finger nuclease; *ZEP*, zeaxanthin epoxidase.

11.1. The biology of metal phycoremediation by microalgae

Microalgal cells have the ability to prevent both nutrient deficiency as well as nutrient toxicity. Because of this, microalgae can incorporate metals from their environment at optimal concentrations. Balzano et al. (2020) documented that microalgae are involved in metal removal through two main processes which include (1) active sequestration and transport by specific

ligands and (2) passive adsorption onto microalgae.

It was confirmed that a broad range of organic ligands, also recognized as transporters, facilitate or mediate the transport of extra heavy metals across the cytosol toward vacuoles, mitochondria, and chloroplasts. Several transporters exist across the membranes of vacuoles and plasma that allow metals to be transported from the outside of vacuoles and environment, respectively, toward the cytoplasm. Conversely other transporters, which exist across vacuole and mitochondrial membranes, are able to reduce the cytoplasmic metal concentrations (Balzano et al., 2020).

Currently, there has been an increasing attention in utilizing microalgae for phycoremediation of metals. It was reported that the polypeptides phytochelatins (PCs) and metallothioneins (MTs) were involved in metal binding by the sulfide groups of the cysteine residues that exist in the above-mentioned polypeptides. There is a chemical affinity between metals with positive charges and the sulfide groups of the cysteine residues with negative charges which leads to formation of heavy metal-MT and heavy metal-PC complexes (Satoh et al., 1999).

Recently, microalgal genome sequencing has provided significant information about potential proteins as well as biosynthetic pathways involved in heavy metal detoxification. This includes compounds biosynthesized by microalgae to reduce the oxidative stress induced by heavy metals along with polypeptides which directly chelate heavy metals such as metallothioneins (MTs) and phytochelatins (PCs). Here, we describe the recent knowledge about microalgal metal binding proteins and their role in phycoremediation (Balzano et al., 2020).

The mechanisms of metal tolerance and accumulation in microalgae are diverse and include intracellular metal binding peptides and proteins (e.g., phytochelatins), metal binding to the cell wall, metal transporter activity, and oxidative stress tolerance.

Microalgae have the ability to produce phytochelatins that are polypeptides with the potential of binding to heavy metals. These polypeptides contain the specific structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ where n ranges from 2 to 11 (Ahner et al., 1995). The γ link is involved in the carboxyl group of the glutamate and the amino group of the cysteine residues. PCs are produced enzymatically when microalgae are exposed to high concentrations of heavy metals. The enzyme (phytochelatin synthase) slices the terminal glycine

from one glutathione and connects the γ -Glu-Cys to the amino terminal of another glutathione to produce an oligomer and this oligomer chain can be elongated gradually by connecting other γ -Glu-Cys moieties (Ahner et al., 1995).

Green algae cell wall contains heteropolysaccharides which offer sulfate and carboxyl groups for sequestration of metal ions (Mehta & Gaur, 2005). The results of previous studies showed the heterogeneity of metal binding sites and indicated that the binding of some metals (e.g., cadmium) is reversible at different pHs. For instance, the most effective pHs for binding and release Cd from cell walls are pH 8 and pH 2, respectively, while there is no association between other metals (such as gold) binding and their release with pH. The results of spectroscopic analysis of the metal binding groups evidenced that several functional groups are involved in metal binding. The Fourier Transform Infrared (FTIR) spectroscopy studies designated that carboxylate groups participate in the pH-dependent binding of Cd, while X-ray fine structure spectroscopy investigations specified that both sulfate and carboxylate groups bind Cd. Actually *Chlamydomonas* cell wall glycoproteins contain from 1% to 4% (g/g) sulfate, so these sulfated oligosaccharides may have a role in metal binding. Those findings indicated that microalgal cell wall plays a significant role in buffering the toxic effect of heavy metals and engineering of microalgal cell wall proteins can improve their capacity for metal binding (Rajamani et al., 2007).

On the other hand, it has been documented that there are many functional groups on the microalgal cell wall such as carboxyl, phosphoryl, sulfydryl and amino with a high affinity for metals. As these functional groups confer negative charge to the cell wall, they are able to adsorb metal ions in wastewater, which are normally in cationic form. These functional groups are related to a variety of cell wall components such as polysaccharides, peptidoglycan, proteins, teichoic acids, and teichouronic acid. Among various cell wall constituents, most of the metal binding sites are occupied by proteins and polysaccharides. Once metals are in the cell, they may bind to intracellular components or precipitate (Mehta & Gaur, 2005).

11.2. Genetic engineering of microalgae for metal phycore-mediation

Due to the high genetic, enzymatic and chemical diversity of microalgae as well as their rapid growth with sunlight and inorganic nutrients, they have

received increased attention for genetic engineering manipulation to be applied for reducing the heavy metal pollution in water bodies (Balzano et al., 2020).

The first genetic manipulation attempt for increasing heavy metal uptake in microalgae was done in 1999. Cai et al. (1999) expressed a foreign class-II metallothionein in *C. reinhardtii* and the resulting transgenic strain showed significantly higher cell growth in a toxic Cd concentration (40 μ M). According to their results, at low Cd concentrations (5 μ M) the metal binding capacity of the MT-II expressing cells was amplified two-fold compared to the *C. reinhardtii* wild type. While at higher concentration of Cd (40 μ M) which was inducer for phytochelatin synthesis the Cd binding capacity of transformed and wild-type cells was little different. However, the growth rate of MT-II transformants in 40 μ M Cd was significantly higher than wild-type cells. These results demonstrated that with induction of phytochelatin production, expressed foreign MT-II in the cytoplasm reduces heavy metal toxicity. This reduced toxicity of high Cd levels was not due to increased binding Cd to MT-II. Cai et al. (1999) proposed two possible mechanisms for that, the first mechanism was the binding of cytoplasmic Cd by MT-II may decrease the oxidative stress probably due to the reduction of the free glutathione pool and the second one was decreasing the effective Cd concentration at cytoplasmic positions that were sensitive to Cd inhibition. Moreover, higher growth rate of Mt-II transformed cells at a toxic Cd concentration indicted higher potential of transgenic cells for using Cd and its removal from the medium than the wild-type. These findings were promising for further manipulation of *Chlamydomonas* to enhance the recovery of toxic trace metals (Cai et al., 1999).

Later, in 2008, the cDNA of the metallothionein-like (MT-like) gene from *Festuca rubra* cv. Merlin was cloned into the chloroplastic genome of *C. reinhardtii* by Han et al. (2008). The obtained transgenic strains expressing MT-like gene were able to grow in high toxic concentrations (40–100 μ M) of Cd. Their findings also indicated the improvement in Cd²⁺ binding capacity and Cd²⁺ tolerance of the transgenic *C. reinhardtii* compared to its wild-type counterpart (Han et al., 2008). The manipulation of gene encoding metal tolerance protein (MTP) in *C. reinhardtii* was also examined by Ibuot et al. (2017) to test its role in improving Cd and Mn tolerance as well as metal transport capability (Ibuot et al., 2017). The

results showed that overexpression of the *CrMTP4* gene (a member of the Mn-CDF clade of the cation diffusion facilitator family of metal transporters) led to both increase in tolerance to Cd toxicity and increased Cd accumulation while no improvement in tolerance to Mn was seen (Ibuot et al., 2017).

Furthermore, Rajamani et al. (2007) succeeded to increase the capability of heavy metal binding in *Chlamydomonas* through the expression of synthetic genes that encode the metal binding proteins attached to the plasma membrane exposed to the periplasmic space. In their investigation, various metallothionein-II polymers were fused to the C-terminal of a low CO₂-induced plasma membrane protein and expressed in transgenic *Chlamydomonas*. The results of that study demonstrated that the created transgenic cells could grow at wild-type rates even in the presence of toxic concentrations of Cd. In addition, transgenic *Chlamydomonas* expressing the above-mentioned metal binding fusion proteins exhibited fivefold more Cd accumulation compared with the wild-type cells. Therefore, Rajamani et al. (2007) concluded that heavy metal tolerance and accumulation could be modified via selective expression of transgenes in the periplasmic space of microalgae.

In other study, He et al. (2011) investigated about the application of a combined technique to remove Hg from model and polluted sediments. For that purpose, they used a combination of ultrasound with transgenic *C. reinhardtii* biomass (2AMT-2) expressing a plasma membrane-anchored metallothionein polymer for Hg removal from sediments over a wide pH range. The findings of their study exhibited effectiveness of transgenic *C. reinhardtii* biomass (2AMT-2) for recovery of Hg released from sonicated sediments.

On the other hand, there are a few interesting investigations on the expression of foreign genes encoding enzymes involved in the biosynthesis pathways of molecules (with small molecular weight) which could be protective against metal mediated damage. For example, due to the important role of proline in the detoxification of heavy toxic metals in many plants and algae, Siripornadulsil et al. (2002) decided to genetically manipulate an enzymatic reaction in the proline biosynthetic pathway in *C. reinhardtii*. For this purpose, they introduced moth bean (*Vigna aconitifolia*) gene encoding Δ^1 -pyrroline-5-carboxylate synthase (P5CS), an enzyme which catalyzes the

rate-limiting step in proline synthesis, in *C. reinhardtii*. Then, they compared the effect of moth bean *P5CS* gene expression in Cd detoxification and improvement salt stress between transgenic *C. reinhardtii* and wild-type cells. Their findings indicated that the transgenic *C. reinhardtii* carrying moth bean *P5CS* gene had 80% increased free-proline levels compared to its wild-type, faster growth in poisonous Cd concentrations (100μM) and 4-fold higher binding ability to Cd concentrations compared to the wild-type cells (Siripornadulsil et al., 2002). Furthermore, their results suggested that an increase in free-proline protected Cd-stressed transgenic *C. reinhardtii* by reducing Cd-induced free radical damage and by maintaining a more reducing environment (higher GSH levels) in the cell. With increasing GSH levels in transgenic *C. reinhardtii* expressing moth bean *P5CS* gene, phytochelatin synthesis and sequestration of Cd increased too because GSH-heavy metal adducts are the substrates for phytochelatin synthesis (Siripornadulsil et al., 2002). In Table 10.3 genetic engineering of microalgae for metal bioremediation is gathered.

Table 10.3

Metal/toxic elements	Algal strain	Detoxification mechanisms	References
<i>C. reinhardtii</i>	Expressing metallothionein	Cai et al. (1999)	Cd
<i>C. reinhardtii</i>	Overexpression of CrMTP4 gene as a metal tolerance protein (MTP)	Ibuot et al. (2017)	Cd and Mn
<i>C. reinhardtii</i>	Overexpression of the HSN3 gene	Zheng et al. (2013)	Ni, Zn, Fe, Cu, Mn, and Mg
<i>C. reinhardtii</i>	Expressing of P5CS gene/effect of prolin as ligands	Siripornadulsil et al. (2002)	Cd

Cd, cadmium; Cu, copper; Fe, iron; Mg, magnesium; Mn, manganese; Ni, nickel; Zn, zinc.

Moreover, Zheng et al. (2013) examined the overexpression of the *C. reinhardtii* *HSN3* gene (which encodes for

phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase and its effect on the fourth step of the histidine biosynthesis pathway in higher plants) to understand the role of this gene in controlling the algal adaptation to nickel poisonousness. Their findings indicated that overexpression of *HISN3* in *C. reinhardtii* led to high tolerance to Ni stress, 48.3%–57.4% increase in cell population (due to improvement of Chl accumulation and photosynthesis efficiency) and moderate histidine accumulation compared to the wild type. Furthermore, more Ni and other metals including Zn, Fe, Cu, Mn and Mg were accumulated in *HISN3*-transformed cells than in the wild-types. All these promising results indicated the possibility of using genetic engineering to increase the algal resistance to the toxicity of heavy metals and improve their potential for phycoremediation of environments contaminated with these toxic metals (Zheng et al., 2013).

12. Conclusions and outlook

In recent years, microalgal-based wastewater treatment as an eco-friendly and cost-effective alternative method to conventional wastewater treatments as well as the use of the resulting microalgal biomass for the production of high value compounds like renewable biofuels has attracted much attention. However, there are some challenges such as slow growth rate and low lipid yield in the native microalgal species which need to be overcome before using them as an alternative source for phycoremediation and biofuel production. As it has been exposed in this chapter, genetic manipulation of microalgal metabolic/biosynthetic pathways can improve some characteristics of native strains including their potential for heavy metal removal, carbon fixation, light utilization, biomass harvesting and product extraction. GE microalgal strains were able to produce high levels of biomass and endogenous carbon storage compounds like TAGs and starch for enhanced biofuel production. Access to more genome sequences of new microalgal strains with potential for biodegradation of toxic pollutants as well as using high-throughput approaches, such as genomics, proteomics, and metabolomics, might facilitate the identification of new genes and pathways that could serve as targets for genetic modification. In addition, the appearance of new pollutants and screening the microalgal strains with ability to degrade them as well as identification of novel genes involved in the degradation of such pollutants are research gaps that need to be addressed in future studies. Nevertheless, the influence of transgenic microalgae on human health and environment should be carefully monitored and determined for biosafety regulation of genetically manipulated microalgae.

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Chapter 11: Heavy metals in the environment: toxicity to microbial remediation

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Abstract

Several indiscriminate anthropogenic activities have led to the contamination of terrestrial as well as aquatic environments with heavy metals such as zinc, lead, copper, cadmium, nickel, and selenite that are proven harmful to living organisms including both plants and animals. Heavy metals can pass through the food chain and possess detrimental effects on the existing biota. The nonbiodegradability of these heavy metals contributes to their persistence in the environment. Employing microorganisms and their enzymes in the biotransformation of these heavy metals into less toxic forms is one of the best approaches for their bioremediation. The current chapter aims to discuss the heavy metals toxicity in the ecosystem and the role of microorganisms in their bioremediation. The chapter also aims to focus on the genetic basis and molecular mechanisms behind the process.

Keywords

Anthropogenic activities; Biotransformation; Heavy metals; Nonbiodegradability

1. Introduction

The human population is increasing at an alarming rate. To meet the demands of the growing population, several anthropogenic activities such as urbanization, technological expansion, resource overutilization, environmentally questionable agricultural practices, and fast-growing industrialization are in action. These practices are either directly or indirectly possessing adverse effects on the environment. Among all the pollutants that are contaminating the environment, heavy metals are one of them.

Heavy metals constitute an ill-defined group of toxic chemical elements that are characterized by high atomic weight and high density. Apart from well-characterized “heavy” metals, nowadays, some metalloids and light metals such as arsenic, selenium, and aluminum are also included in this category because of their toxicities (Briffa et al., 2020; Tchounwou et al., 2012). Some common heavy metals whose densities are higher than 5g/cm^3 are titanium, vanadium, iron, cobalt, nickel, chromium, manganese, arsenic, molybdenum, tin, cadmium, silver, mercury, lead, zinc, and many more (Briffa et al., 2020). Heavy metals have a tendency to form covalent bonds with other compounds, which results in the formation of lipophilic compounds that can bind with the macromolecules of the cell and impose toxic effects. For example, they can bind to the cellular proteins and bring about some changes in their structure, or lead to their denaturation. Since heavy metals are nonbiodegradable, they can be broken down easily into nontoxic forms. Due to their nonbiodegradability, they can accumulate in the body of living organisms and cause severe bio-physiological consequences. They can pass through one organism to another by food chains. Because of these issues, heavy metal remediation is a serious concern worldwide.

For remediation of heavy metals contaminated environments, several methods exist. The physical and chemical methods for the remediation of heavy metal contamination are not cost-effective and environmentally friendly. Also, these methods are not effective when the concentration of the metal is low. Microbial remediation is an emerging alternative technology in terms of simplicity, work efficiency, cost-efficiency, and environmental sustainability. In microbial remediation, several microorganisms such as bacteria, yeast, fungi, and algae are used for the biotransformation of toxic heavy metals in their nontoxic forms.

The current chapter highlights the sources and toxicities of heavy metals

in the environment. It also aims to focus on the mechanism and genetic basis of bioremediation of heavy metals by microorganisms.

2. Sources of heavy metal contamination

The prime source of heavy metals is nature. Heavy metals are present in the earth's crust since the very beginning, that is, since the formation of the earth. But an increase in the use of heavy metals has led to the contamination of soil, air, and water with metallic pollutants. The sources of heavy metal contamination can either be natural or anthropogenic (Briffa et al., 2020). The natural sources include volcanic eruptions, weathering of metal-containing rocks, metal-corrosion, soil erosion, etc., whereas anthropogenic actions that lead to heavy metal contamination include mining, smelting, metal-based manufacturing industries, manufacture, and use of heavy-metal containing agricultural products such as metallo-pesticides, phosphate fertilizers, etc. (Ali et al., 2019). Fig. 11.1 shows the environmental sources of heavy metals and their cycling.

2.1. Sources of heavy metals in soil

As mentioned earlier, heavy metals are found naturally in the soil by the process of weathering of metal-containing rocks. But the amount of heavy metals released in the soil by the process of weathering is considered rarely toxic (Dixit et al., 2015). 95% of the earth's crust is composed of igneous rocks and the rest 5% are sedimentary rocks. Basal igneous rocks are rich in some heavy metals such as copper, cadmium, nickel, and cobalt, whereas shales are abundant in metals such as lead, copper, zinc, cadmium, and manganese. These heavy metals loaded in rocks and shales can be released into the soil by different natural processes such as weathering, leaching, erosion, volcanic eruption, etc. These processes are the part of earth's natural biogeochemical cycle. But an increase in different anthropogenic actions has disturbed and accelerated this slowly occurring natural cycle leading to the accumulation of heavy metals in the soil contributing to its contamination (Dixit et al., 2015). Several anthropogenic activities contribute to the increased heavy metal contamination in the soil. Firstly, heavy metals are generated comparatively faster through man-made activities than the natural geochemical cycles. Secondly, the frequency of transfer of heavy metals from mining sites to different local environments such as agroecosystems is quite high. Moreover, the heavy metal concentration in the jettisoned products is higher than that in the soil. Lastly, the mobility of the heavy metals released from man-made sources is found to be higher than that of natural sources, thus rendering them more bioavailable (Wuana &

Okieimen, 2011).

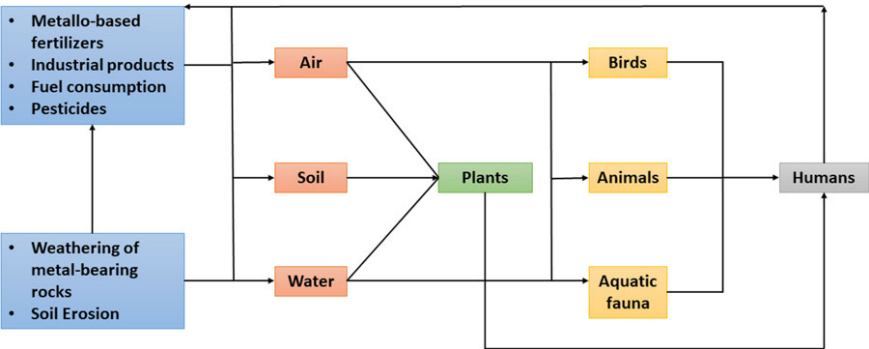


Figure 11.1 Environmental sources of heavy metals and their cycling in the nature.

One of the common anthropogenic sources of heavy metal contamination in agricultural fields is fertilizers. Fertilizers are mainly used to restore an elemental deficiency in the soil. Fertilizers containing nitrogen, phosphorus, and potassium are usually added to the soil to enhance the growth of the crops. But these fertilizers contain some heavy metal impurities such as copper, cadmium, lead, and arsenic, which on continuous use can lead to their accumulation in the soil and contribute to heavy metal contamination (Atafar et al., 2008). Pesticides such as insecticides and fungicides also contain elements such as copper, mercury, manganese, lead, and zinc. The application of such pesticides in the agricultural fields can also increase the risk of exposure of the agroecosystem to heavy metal contamination (Wuana & Okieimen, 2011). It has also been found that the application of wastewater in irrigation of the farming sites has also increased the concentration of heavy metals in the soil. Although, the heavy metal concentration in the wastewater is quite low, their continuous use in agriculture contributes to the heavy metal accumulation in the soil. Other major sources of heavy metal contamination in soil are mining, smelting, electroplating industries, fossil fuel power plants, biosolids, etc. Sources of different heavy metal contamination are shown in Table 11.1.

Table 11.1

Heavy metal	Sources	References
Weathering of Arsenic-containing	Garelick et al.	Arsenic

bedrocks, contamination of arsenic-rich geothermal fluids with surface water, mining, smelting, arsenopyrite, coal burning, arsenic-containing pesticides, wood-preserving arsenicals.	(2008)	
Mining, smelting, refining, batteries, alloys, pigments, sewage sludge.	Tchounwou et al. (2012)	Cadmium
Industrial establishments, welding, wastewater release, wood-preserved.	Wang et al. (2006)	Chromium
Erosion of parent rocks, volcanic eruptions, burning of fossil fuels, mining, lead-acid batteries, synthetic fertilizers, manufacturing industries.	Tchounwou et al. (2012)	Lead
Microbial methylation of inorganic mercury (methylmercury), electrical industries, wood preservatives, pharmaceutical preservatives.	Tchounwou et al. (2003), Dopp et al. (2004)	Mercury
Mining, combustion of coal, burning of wastes, steel processing, drinking water stored in metal tanks for long time	Wuana and Okiemen (2011)	Zinc
Steel industries, metal plating industries, fossil fuel combustion, mining, electroplating	Khodadoust et al. (2004)	Nickel

2.2. Sources of heavy metals in air

Heavy metals can enter the atmosphere via numerous sources, but they are mainly present in the form of particulate matter in the air. The main contributing sources are transports, factories, mineral dust, and power plants. Other sources may include volcanic eruptions and the combustion of fossil fuels. It has been found that pollen is a good indicator of air pollution. In a

study, pollen grains exposed to air in Nagpur, India have shown the presence of a high concentration of metals such as calcium, aluminum, and iron than the unexposed pollens (Kalbande et al., 2007). Several other studies have also shown the prevalence of one or more heavy metals in the atmosphere.

2.3. Sources of heavy metals in water

Water can be contaminated with heavy metals via multiple sources. Untreated wastewater contains a high concentration of heavy metals. Discharge of untreated wastewater and dumping of industrial wastes are the major sources for the contamination of water bodies with heavy metals. Heavy metals present on the surface get carried away by the flowing water and ends up in the water bodies. Other anthropogenic activities such as mining also contribute to the contamination of groundwater. Moreover, several studies have reported the contamination of drinking water with heavy metals. Sources for the drinking water heavy metal contamination were found to be corruptions of pipes, water tanks, plumbing systems, inappropriate disposal of liquid wastes in the deep wells, etc., agricultural activities near water sources, leaching of heavy metals from the soil, mining, and many more (Chowdhury et al., 2016).

3. Negative effects of the heavy metal contamination on environment

Fast urbanization and industrialization have posed adverse effects on the environment. Several anthropogenic activities have increased the release of heavy metals in the natural environments that have directly or indirectly given rise to the pollution of soil, water, and air. Heavy metals not only contaminate the environment but are also toxic to several living organisms including humans. They are considered more hazardous due to their environmental persistence, bioaccumulation, and toxicity (Ali et al., 2019). The impacts of heavy metal contamination on soil, plants, and human beings are discussed in this section.

3.1. Impacts on the soil

Soil is the major sink for heavy metal contamination. The adverse effects of heavy metal contamination in the soil are well-documented in various pieces of literature. Contamination of soil by heavy metals leads to an alteration and disturbance in the activities of the soil's microbial communities. By affecting the soil's microbial communities, the heavy metal pollution also shifts the enzymatic activities of the soil (Huang et al., 2009). Heavy metals get to indulge in the metabolic activities of the microorganisms and decrease their activities.

However, long-term exposure to heavy metals can bring about their tolerance in various microorganisms which can further assist in their bioremediation (Xie et al., 2016). The presence of a high concentration of heavy metals in the soil not only affects its microbiota but also induces changes in its physical and chemical properties. Several soil characteristics such as pH, density, solubility, porosity, relative surface area, clay content, ion concentration, and types, and amount of organic matter present in the soil are also affected by the heavy metal contamination (Briffa et al., 2020; Friedlová, 2010; Marques et al., 2009). Furthermore, the availability of one heavy metal element has also been found to be affected by the presence of another in the soil. For example, in a study, it was found that in the presence of cadmium, the inhibitory actions of manganese on the mineralized carbon were antagonized. Contrary, the toxicity of zinc in spring barley was found to be increased by the presence of copper (Chibuike & Obiora, 2014).

3.2. Impacts on plants

In general, heavy metals are required by the plants in certain levels to up-keep their proper physiological functioning. Hence, plants have a tolerance to the metal up till it is under its threshold level. But when heavy metals are supplied above the tolerance levels, they impose toxicological effects on plants. As heavy metals cannot be degraded further, when their concentrations exceed the optimal levels inside the plants, they directly or indirectly harm them.

There are several direct effects of heavy metal toxicity on plants. For instance, increased heavy metal concentration can lead to the inhibition of enzymes present in the cytoplasm of the plant cell. Moreover, oxidative stress can be created that can further result in the damage of cellular structures (Jadia & Fulekar, 2009). The indirect effect of increased heavy metal concentration includes toxic effects on the soil microbiota that can further lead to detrimental effects on plant growth. Furthermore, essential nutrients required by the plants can be replaced at the sites of cation exchange (Chibuike & Obiora, 2014). Table 11.2 summarizes the toxic effects of different heavy metals on various plant species.

Table 11.2

Heavy metal	Level	Plant	Toxic effect	References
220ppm CdSO ₄	<i>Pisum sativum</i>	Reduction in plants' growth, minimized leaves, reduction in seed germination index, browning of root tips, and chlorosis.	Baruah et al. (2019)	Cadmium
	<i>Solanum lycopersicum</i>			
	<i>Triticum aestivum</i>			
	Reduced root	Pandey et al.	180µm CdCl ₂	

<i>Catharanthus roseus</i>	growth, nutrient absorption, chlorophyll content, and total biomass.	(2007)		
<i>Zea mays</i> L.	Substantial reduction in growth of seedlings, root growth put to an end.	Wang et al. (2007)	10^{-4} M $\text{CdCl}_2 \cdot 2.5 \text{H}_2\text{O}$	
21.34mg/g DM	<i>Fagopyrum tataricum</i>	Reduction in root and shoot length.	Pirzadah et al. (2020)	Lead
<i>Triticum aestivum</i>	Reduced germination, biomass, and elongation of roots and leaves; reduction in α -amylase activity and enhancement in activity of esterases.	Lamhamdi et al. (2011)	Upto 3mM	
Upto 3mM Mercuric nitrate solution	<i>Melia azedarach</i>	Reduction growth rate, dry weight of root-shoots, height, chlorophyll content, and leaf thickness, number of leaves	Hamim et al. (2019)	Mercury
	<i>Ricinus</i>			

	<i>communis</i>			
	<i>Jatropha curcas</i>			
	<i>Reutialis trisperma</i>			
<i>Vigna radiata</i>	Growth suppression in nodules and seedlings, reduction in photosynthetic pigments and leghemoglobin content, decrease in leaf area.	Mondal et al. (2015)	Upto 1.5ppm of mercury salt solution	
Upto 60mg/kg	<i>Helianthus annuus</i> L	Reduction in germination rates and dry weight; decreased root and shoot lengths.	Fozia et al. (2008)	Chromium
<i>Triticum aestivum</i> L.	Reduction in photosynthetic and transpirational rates.	Subrahmanyam (2008)	Upto 0.25mM $\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$	
Upto 150mg/L	<i>Brassica napus</i> L.	Reduction in shoot fresh and dry weight, decrease in leaf water potential and osmotic potential,	Ali et al. (2009)	Nickel
<i>Catharanthus roseus</i>	Decrease in	Arefifard et al. (2014)	Upto 50mM NiCl_2	

	photosynthesis, reduction in chlorophyll pigments content, increase in proline levels.			
As(III)- 50 and 100µM; As(V)- 100 and 500µM	<i>Oryza sativa</i> L.	Decrease in germination rates, inhibition of growth in terms of root/shoot length and root/shoot fresh.	Shri et al. (2009)	Arsenic

3.3. Heavy metal toxicity in humans

In addition to possessing impacts on the soil, water, and plants, heavy metals also affect human health. In any case, the presence of heavy metals in excess amounts can prove toxic to the environment. Heavy metals can cause serious health problems in humans by affecting vital organs and organ systems such as the nervous system, respiratory system, liver, kidney, and many more (Li et al., 2014). A high level of heavy metals in the human body can impair the synthesis of proteins, inhibit enzyme activity, damage the nucleic acids (both DNA and RNA), and bring about changes in the permeability of the plasma membrane of the cell properties. These metals can interact with the DNA and nuclear proteins, and further lead to oxidative deterioration in bio-macromolecules (Fig. 11.2) (Briffa et al., 2020). Some heavy metals also lead to the exhibition of apoptotic or carcinogenic pathways. Environmental pollutants including heavy metals such as arsenic, cadmium, chromium, mercury and nickel are known to be carcinogenic (Malik et al., 2021, 2022, Kishore et al., 2022). It has been reported that exposure to any of these heavy metals can disrupt the expression of tumor suppressor genes, impair the DNA repair mechanisms, and damage the cell's metabolisms by the formation of reactive chemical species (Kim, Choi,

et al. 2015; Kim, Kim, et al., 2015). Toxicities caused by different heavy metals can be removed through several emerging trends from industrial waste water (Malik et al., 2022). The toxicities derived from heavy metals are summarized in Table 11.3.

4. Technologies used for the heavy metal remediation

For the removal of heavy metals from the sites of contamination, several technologies have been employed. The treatment methods devised for the heavy metal remediation can be classified on the basis of the location of the treatment. When the contaminated soil or water is treated at its original place, then it is termed as in situ treatment whereas, when they are treated by moving them from the original location to different places such as laboratories and treatment plants, then it is called *ex situ* treatment. The heavy metal remediation technologies can further be classified into three categories: physical remediation, chemical remediation, and biological remediation.

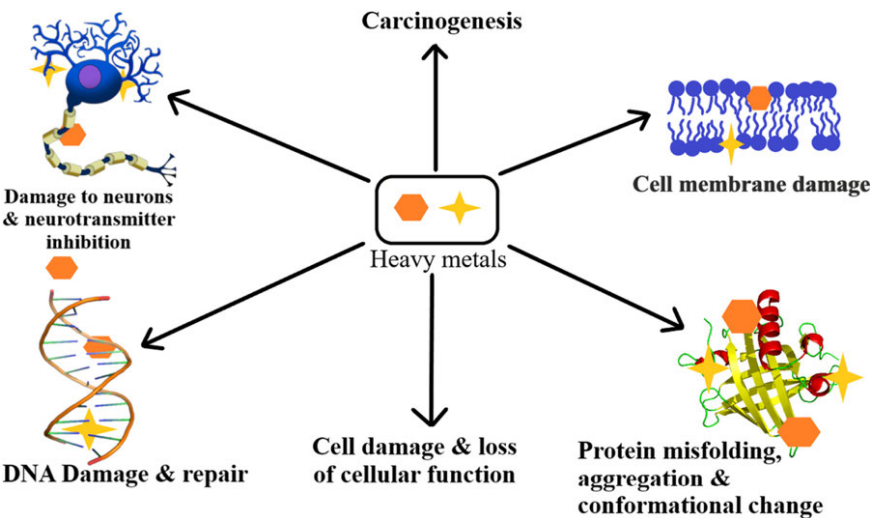


Figure 11.2 Major toxic effects of heavy metals.

Table 11.3

Heavy metal	Effects on humans	References
Anemia, miscarriage, premature birth or low-weight neonates, lead nephropathy, reduced sperm count, neural injury.	Wani et al. (2015)	Lead
Cardiomyopathy, angina, anemia, leukemia, pulmonary	Rice et al. (2014)	Mercury

diseases, digestive disturbances, kidney damage and injuries, neuronal problems, effects on nervous system, impairment of endocrine functions, reduced fertility, erectile dysfunction, ovarian dysfunction, menstrual disorders such as dysmenorrhea, oligomenorrhea, etc., premature births, miscarriage.		
Skeletal demineralization, osteomalacia, osteoporosis, Itai-Itai disease, renal dysfunction, nephrotoxicity, decreased sperm count, volume, and density, increased immature sperm formation, decreased libido, defect in oogenesis, increased spontaneous abortions, cardiovascular diseases, neurotoxicity, prostate, pancreas, and renal cancers,	Rafati Rahimzadeh et al. (2017)	Cadmium
Digestive tract irritation, pulmonary irritation, anemia, increased chances of cancers, miscarriage, cardio-vascular problems, DNA damage.	Briffa et al. (2020)	Arsenic
Lung fibrosis, cardiovascular diseases, respiratory tract cancer, contact dermatitis, reproductive toxicity.	Genchi et al. (2020)	Nickel
Pulmonary irritation, dermatitis, lung, nasal, and sinus cancers, teeth abnormalities.	Achmad et al. (2017)	Chromium

Neurotoxicity, alteration in cardiovascular functioning, increased infant mortality.	O'Neal and Zheng (2015)	Manganese
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4.1. Physical remediation

In physical remediation, the soil damage is reversed or stopped by employing physical technologies such as soil replacement, thermal desorption, separation, and containment techniques (Yao et al., 2012). In the soil replacement method, the contaminated soil is blended or covered with a generous amount of noncontaminated soil. In this way, the concentration of the contaminant gets reduced in the soil. Furthermore, several techniques such as capping, landfilling, and encapsulation are employed. This method is suitable for soils that contain a large amount of heavy metals in a small area. However, these methods are not cost-effective (Yao et al., 2012). The basis for thermal desorption is the volatility of the contaminants. In this method, the pollutant is volatilized from the soil by heating. Heavy metals with high vapor pressure such as mercury can be effectively removed by this method; however, the soil quality may be affected to a great extent (Li et al., 2019).

4.2. Chemical remediation

Chemical remediation involves both *ex situ* and *in situ* techniques for remediation. For example, soil washing is an *ex situ* chemical remediation technique that involves washing the soil with suitable washing solutions to leach out the contaminants from the soil. This technique is highly effective in the remediation of highly contaminated soil. However, it is not cost-effective. An *in situ* technique for soil remediation is soil flushing. It is a comparatively low invasive technique. But in this technique, the chances of liquid and semiliquid waste generation are high (Wuana & Okiemen, 2011). In both these techniques, washing agents such as water, surfactants, chelating agents, etc., are widely used to desorb the contaminants from the soil. For the removal of heavy metals from the soil, Ethyldiaminetetraacetic Acid (EDTA) has been widely used as an effective chelating agent (Leřtan et al., 2008).

4.3. Biological remediation

Biological remediation or bioremediation is a state-of-the-art technique for

the removal of heavy metals from polluted environments by employing biological approaches. Bioremediation includes phytoremediation and microbial remediation. Bioremediation is a cost-effective and eco-friendly technique as compared to conventional expensive and low-effective physical and chemical remediation methods.

In phytoremediation, plants are used to reduce to concentration and harmful effects of toxic contaminants from the soil (Ali et al., 2013; Malik et al., 2022). This method is suitable when the contaminants are spread over a wide area and are present in the range of root zones of the plants (Chibuike & Obiora, 2014). The strategies employed in phytoremediation are: phytoextraction, phytostabilization, rhizofiltration, phytovolatilization, and phytodegradation (Salt et al., 1998). In phytoextraction, heavy metals are transferred from the soil to the part of the plant above the ground. In this way, the concentration of the heavy metal is reduced in the soil. In phytostabilization, plants are used to reduce the heavy metals' mobility and bioavailability in the environment. In rhizofiltration, contaminants are removed from the polluted water by the means of plant roots. In phytovolatilization, heavy metals are absorbed by the plants are then converted into their volatile forms and are further released into the atmosphere. Phytodegradation involves the use of plants' metabolic processes and plant-associated microbes to break down or transform the heavy metal contaminants from the soil (Li et al., 2019; Singh Sidhu, 2016).

Another bioremediation method for the removal of heavy metals is microbial remediation. Microbial remediation makes use of different microorganisms in order to transform the pollutants into nontoxic products. Microbial remediation of heavy metals is discussed in detail in the following sections.

5. Microorganisms involved in the bioremediation of heavy metals

In nature, microorganisms are found all over the place and play an important role in the elemental biogeochemical cycles of transformations of metal between insoluble and soluble species. The interactions between the metals and the microbe can have both beneficial and harmful results. Apart from the chemistry of metals involved and microbial nature, these transformations are also dependent on other environmental factors such as temperature, pH, humic colloidal substances, moisture, presence of different ions, and different living organisms with their competitors which plays a crucial role in the formation of biofilm and microbial colonization (Gadd, 2010). Diverse approaches have been progressed by the microbes to overcome the toxic effects of metals, utilizing resistance, accumulation, or, more importantly, by lowering their toxicity and bioavailability through the mechanism of transformation and biomethylation (Gupta et al., 2016).

Microorganisms uptake the heavy metals mainly through adsorption which is a passive process or through bioaccumulation which is an active process. Some of the microorganisms such as fungi, bacteria, and algae have been utilized to wash up the environments contaminated by metal along with the applications of the consortium, metal-resistant strains in single and the immobilized form have given effective results for the mechanism of remediation of heavy metals (Kim, Choi, et al. 2015; Kim, Kim, et al., 2015).

5.1. Bacterial bioremediation

Microbial biomass has shown various biosorption capabilities, which also differ notably among microbes. Microbial cells must have the ability to adapt to the alteration of chemical, physical, and configuration of the bioreactor in order to increase the biosorption. However, the ability of biosorption shown by each microbial cell depends on its experimental conditions and pretreatment (Ayangbenro & Babalola, 2017). Bacteria are one of the important bio sorbents present in the environment because of their size, ubiquity, and their ability to grow under the controlled conditions, and flexibility to environmental conditions (Srivastava et al., 2015). The presence of bacteria in the environment is ubiquitous. The biomass of bacteria can be living or nonliving and are found in various shapes, such as filamentous (*Actinomyces*), rods (*Bacillus*), spiral (*Vibrio cholera*), and cocci

(*Streptococcus*). The mechanism of biosorption followed by the bacteria is an efficient and inexpensive technique for the removal of pollutants including nonbiodegradable waste, such as heavy metals, from the wastewater. The mechanisms for remediation and resistance from metal ions have been developed and adapted by the bacterial species for their survival (Mustapha & Halimoon, 2015). Research has been done widely on the bioremediation of heavy metal ions by bacterial agents. Bacterial biomass carries out the rapid elimination of metals such as Cd, Zn, Cu, Cr, and Pb (Ozer & Ozer, 2003). The great biosorption ability shown by the bacteria is due to potentially active chemisorption sites present on their cell wall and their high surface-to-volume ratios (Mosa et al., 2016).

In bacteria, the cell wall is the primary physical contact that links the bacterial biomass and the metal ions. The metal binding capacity within or on the cell wall of bacterial species takes place because of the overall negative charge due to the anionic functional groups (such as teichoic acids and peptidoglycan) in Gram-negative bacteria and (hydroxyl, amino, sulfate, and carboxyl) in Gram-positive bacteria. The removal of heavy metals through the cells of dead biomass is extracellular. Functional groups present on the cell wall such as amine, phosphonate, hydroxyl, and carboxyl are mainly responsible for these interactions. The amino groups have shown effective removal of Cr through electrostatic interactions and chelation and the carboxyl group has the ability to bind with the Cd on the surface through complexation (Kapahi & Sachdeva, 2019). Before using the bacterial species for bioremediation they should be exposed to the contaminants for the enzymatic induction. To initiate the enzymatic expression there is a requirement of the minimum concentration of the contaminants, which is necessary for the process (Adenipekun & Lawal, 2012).

Some of the heavy metals have been tested for bioremediation using bacterial species such as *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Micrococcus* sp., and *Enterobacter*. The stability and the survival of bacteria become better when they are in mixed cultures. Thus, for the biosorption of metals, the consortia of cultures are metabolically more superior and are more appropriate for the purpose of field application (Tarekegn et al., 2020). At 16mg/L of metal ion concentration De et al. (2008) notified a 78% reduction of Cr with the help of a bacterial consortium of *Arthrobacter* sp. and *Acinetobacter* sp. Abioye and his colleagues investigated the biosorption of

metals such as Cr, Pb, and Cd in the tannery effluent using a bacterial consortium of *Aspergillus niger*, *Bacillus subtilis*, and *Penicillium* sp. (Abioye et al., 2018).

5.2. Fungal bioremediation

In the natural environment, fungi are mainly famous for their ubiquitous presence, and in industrial applications, there is extensive exploitation of fungi (Abdi & Kazemi, 2015). The adaptation of fungi in terms of ecology, morphology, and metabolism takes place according to the environmental conditions and under natural conditions, they are also responsible for processes including nutrient cycle and decomposition (Archana & Jaitly, 2015). It has been reported that fungi have the ability to survive and withstand under stress conditions of pH, moisture, nutrients, etc. Mycoremediation is a technique that involves the utilization of fungus (dead or alive) for the elimination of contaminants from various segments of the environment. Mycoremediation by fungus is a cost-effective process and does not leave any type of harmful wastes in the environment. Therefore, it poses a complete solution for the removal of pollutants due to the full mineralization of pollutants present in nature. The achievement of mycoremediation depends mainly on the usage and identification of a suitable species of fungi for the selected heavy metals and other types of contaminants (Kapahi & Sachdeva, 2019). Fungi because of their recovery and magnificent capacities for metal uptake are widely used as biosorbents for the elimination of toxic and harmful metals. Most research and studies have shown that in the adhesion of inorganic chemicals a significant role is played by an active and lifeless cell of fungus (Tarekegn et al., 2020). They have the ability to gather the heavy metals on the fruit bodies in an effective manner, thus making them unavailable and lowering the concentration of heavy metals present in the media. In the future, the presence the contaminants or any other type of heavy metals in the media depends upon the chemical behavior shown by the elements, the life of the fungi, and the presence or absence of fungi in the media after separation (Ogbo & Okhuoya, 2011). Damodaran and their workers reported that *Saccharomyces cerevisiae* can separate up to 65%–79% of Cd and Pb from the tannery effluent (Kapahi & Sachdeva, 2019). Srivastava and Thakur (Srivastava & Thakur, 2006) also reported that *Aspergillus* sp. are efficiently used for the elimination of Cr from the tannery wastewater, it eliminates 65% of the Cr from the tannery effluent, compared to 85% of Cr from the

synthetic medium at pH 6 in the bioreactor system. The dead fungal biomass of *Rhizopus oryzae*, *Penicillium chrysogenum*, *Saccharomyces cerevisiae*, and *A. niger* have been reported to be used to convert the harmful and toxic Cr(VI) to nontoxic or less toxic Cr (III) (Park et al., 2005). *Candida sphaerica* has been reported to produce biosurfactants with the elimination efficiencies of 79%, 90%, and 95% for Pb, Zn, and Fe, respectively (Luna et al., 2016).

In recent years biosurfactants have gained more interest because of their biodegradability nature, diversity, and low toxicity. Mulligan and his workers assessed the viability of using rhamnolipid, sophorolipid, and surfaction for the elimination of heavy metals such as Zn and Cu (Mulligan et al., 2001). A single wash with 4% of sophorolipid has the ability to eliminate 60% of Zn and 25% of Cu, whereas 0.5% of rhamnolipid has the ability to eliminate 18% of Zn and 65% of Cu. Some strains of yeast, including *S. cerevisiae*, *Pichai guilliermondii*, *Rhodotorula pilimanae*, *Rhodotorula mucilage*, and *Hansenula polymorpha* have been used in the bioconversion of Cr (VI) to Cr (III) (Chatterjee et al., 2012).

The phylum basidiomycetes of fungi include mushrooms, wood-decaying species both brown-and white-rot fungi and other types of fungi. Throughout history, mushrooms because of their high medicinal and nutritional properties have played a crucial role in the human diet. Apart from this that they are used as a food, they are also used for mycoremediation because of their potential to uptake the heavy metals. The ability of metal uptake by mushrooms depends on the age of mycelia, fructification, and contact time (Raj et al., 2011). Some of the mushrooms that are of edible wild varieties can gather heavy metals over their background concentrations. In the fruiting body of the mushroom, the disproportional scattering of heavy metals takes place. Several white-rot fungi such as *Termitomyces clypeatus* and *Pleurotus ostreatus* have been reported to degrade or remove the steady pollutants (Kapahi & Sachdeva, 2019).

5.3. Algal bioremediation

In marine ecosystems, a large number of various species of algae are found. Algae are autotrophic organisms and therefore the requirement of nutrients is low and produces a large amount of biomass as compared to other microbial biosorbents (Abbas et al., 2014). Among the three groups of algae; that is, Chlorophyta (red), Phaeophyta (brown), and Rhodophyta

(green) brown algae have been found to show better biosorption capacity (phycoremediation) than the other one. Phycoremediation is the use of different types of cyanobacteria and algae for the remediation of heavy metal ions, either through degradation or removal of toxicants. The utilization of algal biomass in the bioremediation of heavy metals present in the tannery effluent is done via adsorption or through integration into the cells (Tarekegn et al., 2020; Malik et al., 2022). In algae, the biosorption of metal ions differs with the structure and kind of algal biomass, chemical constitution, and charge of the heavy metal ion. In in-situ remediation various algae, in column or batch, as combination or in single, in live or dead forms have been used (Kapahi & Sachdeva, 2019). Algae have different chemical moieties present on their surface, including carboxyl, amide, phosphate, and hydroxyl, which act as binding sites for metals (Abbas et al., 2014). Sodium, calcium, and magnesium ions present in the cell wall of algae get replaced with heavy metals through ion exchange. The dead cells of *Chlorella vulgaris* have been reported to remove, and ions from the aqueous solution under different conditions of biosorbent dosage, contact time, and pH (Abbas et al., 2014) and details of microbes involved in bioremediation of heavy metals are described in Table 11.4.

6. Mechanism of microbial remediation of heavy metals

In the highly contaminated region, microorganisms possess high adaptation and resistance to heavy metals. The presence of an extracellular polymeric substance on the cell mass of biomass can attach to the heavy metals through mechanisms like microprecipitation of metals or proton exchange (Comte et al., 2008). The surfaces of biomass have a negative charge due to the presence of phosphoryl, carboxyl, sulfur, and amino groups, which act as metal sinks or potential ion-exchange sites. Bioremediation processes take place via various mechanisms, including adsorption, precipitation, ion exchange, redox process, electrostatic attraction, and complexation. The immobilization/mobilization of metals by microorganisms can be initiated by redox reactions; and therefore, impact the process of bioremediation. Heavy metals such as Cr, As, Hg and Pb undergo the cycles of oxidation and reduction. The process of bioremediation is facilitated by converting an element from its stationary and insoluble form in the sediments into its soluble and mobile phase. The deleterious impacts of mobilization can also be seen when toxic metals are released and redistributed from their solid phase from the sediments into the solution phase. This enhances the bioavailability of microbes and heavy metals can reach the metabolic systems of microbes (Fomina & Gadd, 2014).

Table 11.4

Groups of microbes	Bioremediation species	Metals	References
<i>Enterobacter cloacae</i>	Hg	Al-Garni et al. (2010)	Bacteria
Zn	Salehizadeh and Shojaosadati (2003)	<i>Bacillus firmus</i>	
Cu, Ni	Kumar et al. (2011)	<i>Flavobacterium sp.</i>	
Cr	Bhattacharya et al. (2014)	<i>Acinetobacter sp.</i>	
Pb	Kumar et al. (2011)	<i>Staphlococcus sp.</i>	
<i>Aspergillus niger</i>	Ni	Taştan et al. (2010)	Fungi
Cr	Benazir et al. (2010)	<i>Saccharomyces cerevisiae</i>	

Hg	Muneer et al. (2013)	<i>Cndidapara psilosis</i>	
Ni	Taştan et al. (2010)	<i>Aspergillus sp.</i>	
Cr	Congeevaram et al. (2007)	<i>Aspergillus sp.</i>	
<i>Nostoc</i> sp.	Pb	Kumaran et al. (2011)	Algae
Cr	Tarekegn et al. (2020)	<i>Spirogyra sp.</i>	
Cd	Kumaran et al. (2011)	<i>Nostoc</i> sp.	
Cr	Tarekegn et al. (2020)	<i>Spirulina sp.</i>	
Cu	Tarekegn et al. (2020)	<i>Spirogyra sp.</i>	

Bacteria have the ability to reduce the Hg(II) to its more volatile and elemental form of Hg (0). The solubility of ions such as As (V) and Fe(III) can be increased through microbial reduction by reducing them to As(III) and Fe(II), respectively, and can also facilitate the leaching from the soil. Researches and studies have also reported bacterial from various natural aquifers that can transform As (Kapahi & Sachdeva, 2019). The mechanism of biomethylation of heavy metals is an important process in water and soil and can modify the volatility, mobility, and toxicity of heavy metals. Biomethylation also serves as an effective means of detoxification as it can remove the volatile methylated species from the cells (Bolan et al., 2014). Alkyl arsines and dimethylmercury, which are the methylated products of As and Hg are evaporated, and volatile are vanished from the soil. In soil, the fraction of organic matter also serves as a methyl donor. Therefore, the microbial decomposition of the organic matter is another indirect mechanism of mobilization of metals, which stimulates the release of these ions. *Schizophyllum commune* has been reported to remove heavy metals together with the dissolved organic matter. Elimination of metabolites like amino acid and carboxylic acid is a crucial mechanism of chelating ions of metals (Wengel et al., 2006).

The immobilization of metals is performed by the microbes and they act

as a sink for the metals by adopting various mechanisms such as bioaccumulation, biosorption, intra/intercellular precipitation (as oxalates of Co, Zn, Ni, Cd, and Cu), and bioconversion operation in various ways. The removal of elements can be performed easily by immobilization from its aqueous phase in wastewater or groundwater (Kapahi & Sachdeva, 2019). *Methanothermobacter thermautotrophicus* has been reported to reduce Cr(VI) to Cr(III) and immobilize them in oxide-/hydroxide forms (Singh et al., 2015). *Shewanella* sp. and *Bacillus cereus* have also been reported for immobilization and bacterial reduction for Cr(VI) (Chen et al., 2012; Middleton et al., 2003). The structures of cells, including cell walls and plasma membrane, mainly act as barriers and they check the entry of metal ions into the cells (Kapahi & Sachdeva, 2019).

6.1. Bioaccumulation

The mechanism of bioaccumulation takes place when the rate of adsorption of the contaminant is greater than the loss rate. Therefore, the contaminants persist to be contained and gathered inside the organisms (Chojnacka, 2010). It is a toxicokinetic process that affects the sensitivity of the living organisms toward the chemical. Organisms have the ability to resist the concentrations of these chemicals up to a certain level, beyond that concentration these chemicals become toxic and kill the organisms. High variability is seen in the sensitivity of organisms toward the chemicals, which mainly depends on the chemical involved and the types of organisms. The organisms performing bioaccumulation should have a tolerance level that ranges from one or more contaminants to the higher levels. In addition to this, they may also demonstrate the superior capabilities of biotransformation, changing the toxic form of chemical to its nontoxic form, which permits the organisms to lower the toxicity level of the contaminants while keeping it confined (Mishra & Malik, 2013).

Metal bioaccumulation may be economically and practically beneficial if they lead to metals that are of high concentration. The process of bioaccumulation is compared with biosorption, features by the fact that the recovery and removal of metals from the cells are connected with the necessity of transformation of cellular structure. In some cycles, this comes out as a lack of possibility in the application of biomass (Sklodowska, 2000). Many species of environmental bacteria have the feature to show the phenomenon of accumulating large amounts of metals in the cell wall, in cells

themselves, or in places that are bounded from the cytoplasm.

The quantity of this deposit could be even 6% of the dry cell mass and, taking into consideration in the water and soil environment, this phenomenon can lead to the temporary decrease in the concentrations of heavy metals in the environment (Kisielowska et al., 2010).

6.2. Biosorption

Biosorption mechanism is the classification of all the processes, throughout which alive or dead biomass eliminates the heavy metals or any other type of pollutants from the solutions. Biosorption taking place with the involvement of microorganisms can be conducted through surface adsorption concerning the accumulation of heavy metals on the surface of the cells and linking the metals with the extracellular polymers (Tarekegn et al., 2020). The other method depends on the infiltration of metals to the middle part of the cell or we can say that intracellular accumulation takes place. It occurs frequently when the process of biosorption is taking place as the first phase of the following intracellular gathering and the surface adsorption process occurs at a very fast rate - during a couple of minutes, they may lead to the accumulation of heavy metals or may play a dominant role in the metal linking in the middle region of the cell in an extended time (Kisielowska et al., 2010).

In microorganisms, the occurrence of sorption properties is a result of their outer cell shield. Metals are linked through the active groups of compounds present in the surface layers of the cells. Most commonly, this is a type of reaction of transfer of ion between the metal cations and the active groups that are gifted by the negative potential of the outer cell structures and the microorganisms that belongs to different systematic units feature through the presence of different chemical groups in the outer cell structure, which are active in metal linking (Tarekegn et al., 2020).

The practical usage and application of the biosorption process for the recovery and removal of heavy metals is mainly the outcome of the reversibility of this process. The process of desorption allows the removal and recovery of the metals (that are beneficial in the case of heavy metals that are valuable such as Cu, Au, and Zn). The solutions of weak mineral acid (such as 0.1M HCl) or chelating compounds (such as 10mM of EDTA) are applied during the process of desorption of metals that are linked by microorganisms (Sklodowska, 2000). Metal ions like Ni^{2+} , Cu^{2+} , Zn^{2+} , Cr^{3+} ,

and Cd^{2+} are effectively linked to the microbial biomass in the pH range of 5–7. The liberation of metals from the sorbents can occur if the value of pH decreases to 2, however, the metal ions like and have the ability to stay at this pH in the biosorbents (Kisielowska et al., 2010).

6.3. Biotransformation

The mechanism of biotransformation of heavy metals is the reaction including methylation, reduction, demethylation, and oxidation. In this process, the enzymatic systems of the microorganisms are involved. Practically useful maybe because of reactions of significantly valuable or toxic metal reduction, such as Gram-positive bacteria isolated from the polluted sewers can cause the reduction of a highly toxic form of chromium Cr(IV) to its less toxic form Cr(III) , which can be eliminated from the environment (Kisielowska et al., 2010). Any type of microscopic fungi or bacteria can conduct the process of reduction of metal ions (especially the metals that are more valuable as silver or gold) to its metallic form. In microorganisms, this reaction can occur on the cell surface, in vacuoles, and also in the extra-cellular environment, which is important in the recovery of metal (Tarekegn et al., 2020).

6.4. Biocrystallization and bioprecipitation

The crystallization and precipitation of heavy metal compounds may occur as a result of the activity of microorganisms, which leads to the transformation of heavy metal into their sparingly form, which reduces their toxicity at the same time. Several biocrystallization and bioprecipitation processes take part in some of the biogeochemical cycles, including mineralization of manganese and silver, formation of microfossils, and depositing of manganese and iron. Metal precipitation inside or on the surface of the result can be the outcome of not only the direct activity shown by enzyme but also the outcome of galactosis perform by secondary metabolites (Skłodowska, 2000).

6.5. Bioleaching

Biomining is a common term that involves both bioleaching as well as biomining. This process includes the mobilization of the ions of positive heavy metals from insoluble ores frequently through bio-oxidation, and complexation or biological dissolution (Jin et al., 2018). Secretion can be produced by the metabolism of microbes, like organic acid with low molecular weight,

which has the ability to dissolve the soil particles and heavy metals holding minerals of heavy metals. Chanmugathas and his colleague have reported that energy and nutrients can be effectively used by the microbes in organic acid secretion and leaching of Cd is promoted in nutritious conditions. In particular, the rate of leaching in the absence of nutrients was seen to be 9% and that with the presence of glucose and other types of nutrients was 36% (Chanmugathas & Bollag, 1988). In several research and studies, it has been found that some of the microbes, such as *Citrobacter* may have the ability to generate free inorganic phosphate, which leads to the formation of a metal phosphate coat that is insoluble and can capture a large amount of the toxic metals (Marchenko et al., 2015).

Mostly the prokaryotic microorganisms take part in the redox reactions and alter the valence of the heavy metals, thus changes their activity, which can affect their toxicity and mobility (Gavrilescu, 2004). For example, Hg^{2+} , once reduced to Hg^0 by aerobic bacteria, Hg^{2+} can vaporize, and can be reduced Hg^0 to by the smoke of tobacco. *Coryne bacterium* and other microbes can reduce the toxic and water-soluble form of chromium (Cr^{6+}) into poorly water-soluble and less toxic form of chromium (Cr^{3+}), and the dead *Bacillus licheniformis* Ro8 has the ability to reduce Pb^{2+} to Pb^0 (Jin et al., 2018).

7. Factors affecting microbial remediation of heavy metals

The tendency of the heavy metals to be inhibitory and stimulatory to the microorganisms mainly depends on the factor such as chemical forms of the metals, total concentrations of the metal ion, and some of the related factors, including redox potential. Several environmental factors such as pH, organic acids of lower molecular weight, temperature, and humic acids may change the transportation, valence state of the heavy metals, transformation, and the bioavailability of the heavy metals toward the microorganisms (Rasmussen et al., 2000).

Heavy metals have the tendency to form free ionic sites at the acidic pH levels, with the availability of more protons for saturation at the site of metal binding. At higher concentrations of hydrogen ions, the surface of adsorbed is more likely to be positively charged, thereby lowering the attraction between the metal cations and the adsorbent, hence increases the level of toxicity. The role of temperature is very significant in the process of adsorption of heavy metals. A rise in temperature may increase the rate of diffusion of adsorbent across the layer of the external boundary. The bioavailability of heavy metals can be improved when the solubility rate of the heavy metals increases with a rise in temperature (Bandowe et al., 2014). However, with a rise in the temperature, the actions of microorganisms can be increased within a suitable range, and it increases the metabolism of microbes and their enzymatic activity, which will accelerate the process of bioremediation. The rate of stability of the metal-microbe complex mainly depends on the configuration of the microbial cell wall, sorption sites, and the ionization of chemical moieties present on the cell wall. The result of the degradation process depends on the range of the environmental factors and the substrate (Tarekegn et al., 2020).

8. Genetic basis of heavy-metal biotransformation in microorganisms

In bacteria, many genetic systems are known for maintaining the intracellular homeostasis of the essential metal ions and for obtaining resistance against toxic metals. In various research and studies, microorganisms are been used to, precipitate, sequester, or change the oxidation state of various heavy metals. The enhancement in the biosorption ability and the affinity of bacterial cells toward heavy metals can also be done by the expression of metalloptides and metallothionein, and it is an effective technology for the expansion of biosorbents based on bacteria. Metallothioneines are small, cysteine-rich proteins that are synthesized under the circumstances of heavy metal stress that have been found in both eukaryotes and prokaryotes (Gupta et al., 2016). The only known locus of bacterial metallothioneins is designated as *smt*, which has been structurally characterized and cloned in *Synechococcus* strain PCC and *Synechococcus* strain PCC 6301 (Stetter, 1996; Sunit et al., 2015). The locus of *smt* contains two differently transcribed genes, *smtB*, and *smtA* which mediate resistance to cadmium and zinc in the strains of *Synechococcus* (Suni et al., 2015; Tabakka et al., 2005).

Gomes as his coworkers in 2013 have reported the isolation of 178 strains of *Escherichia coli* from agricultural, hospital, residential, and industrial wastewater and recreational waters at the city of Rio de Janeiro. The strains have been reported to possess a genetic resistance marker for mercury which marks it as an efficient alternative for the process of bioremediation. The work was done to find out the genetic and phenotypic characteristics that are associated with mercury resistance and diversity. The data of RAPD have discovered a high degree of polymorphism among the mercury-resistant strains of *E. coli* and expresses good discriminative effects and reproducibility (Gomes et al., 2013). To investigate the genetic variability, denaturing gradient gel electrophoresis (DGGE) and Random amplification of polymorphic DNA (RAPD-PCR) were used. The diversity was detected within the gene fragment of *merA* while denaturing gradient gel electrophoresis typing. This research and studies have discovered that there is a growth in the epidemiological studies based on HgR *E. coli* and maintain the evidence related to the nonclonal environment of *E. coli* mercury resistant strains circulating in rural and urban aquatic systems in the city of Rio

de Janeiro (Gupta et al., 2016).

Naik and his colleague in 2012 have investigated the role of bacterial metallothionein in the bacteria that are lead-resistant and isolate *Proteus penneri* strain GM10, *Proteus penneri* strain GM03, *Bacillus subtilis* strain GM02, *Providentia rettgeri* strain GM04, and *Pseudomonas aeruginosa* strain 4EA which were isolated from the soil that is polluted with the waste of car battery from Goa, India. All the isolated strains except *Pseudomonas aeruginosa* strain 4EA showed the presence of plasmid (Naik et al., 2012). The PCR amplification of *smtA* genes that are encoded with bacterial metallothionein and intracellular accumulation of 22 and 19mg of lead per gram of dry weight in *Proteus penneri* strain GM10 and *Salmonella choleraesuis* strain 4A correspondingly discovered the presence of metal-binding metallothionein and responsible for the resistance toward the lead (Gupta et al., 2016).

9. Conclusions

The current chapter summarized various sources of heavy metals in the environment including both natural and anthropogenic sources. Several natural process such as volcanic eruptions, weathering of metal-bearing rocks, soil erosions, etc., and anthropogenic activities such as industrial activities, mining, smelting, wastewater release, etc. contribute to the heavy metal contamination in the environment. The chapter also explicitly summarized the impacts of heavy metal pollution on the soil, water, and human. In human, heavy metal toxicity can cause several physiological complications such as cardiovascular disorders, neuronal and neurological disorders, renal dysfunctions, fertility dysfunctions such low sperm count and decreased libido, premature birth, as well as cancer. Moreover, the methods of microbial remediation of heavy metal contaminated environment have also been discussed in this chapter. In microbial remediation, processes such as bioaccumulation, biosorption, biotransformation, bio crystallization, bioprecipitation, and bioleaching are employed. Several factors such as pH, temperature, moisture content, and molecular weight of the contaminant affect the process of microbial remediation. At last, the genetic basis for the biotransformation of heavy metals by microorganisms has been discussed.

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Chapter 12: Role of bacteria in biological removal of environmental pollutants

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Abstract

The increase in the development rate has also enhanced the number of hazardous and baleful pollutants in the environment. The physico-chemical approaches for the remediation of these pollutants are not only expensive but they are also considered as less effective due to secondary treatment based disposal issue. Comparatively, the microbial approach for the removal of environmental pollutants is considered cost-effective, eco-friendly, and reliable. Several genera of bacteria have been reported to degrade various environmental pollutants including polyaromatic hydrocarbons, plastic wastes, heavy metals, pesticide residues, dyes, and many more. Moreover, genetically engineered bacteria are also found to be helpful in this process. This chapter aims to discuss the role of bacteria in the degradation of various harmful environmental pollutants and also describes the metabolic pathways for the degradation of pollutants along with the limitations of using bacteria for this purpose.

Keywords

Biodegradation; Metabolic pathways; Plastic wastes; Polyaromatic hydrocarbons

1. Introduction

Environmental pollution has reached a perturbing proportion comprehensively. Industrialization and urbanization along with the development in the economic sector have certainly led to the upsurge in the ingestion of energy and waste discharges. International public health problems have included this global environmental pollution that also has greenhouse gas emissions and acid deposition under it, along with water pollution and waste management. Environmental pollutants have many different antithetical effects on health and the most important ones are infant mortality, allergy, respiratory disorders, cardiovascular disorders, malignancies, endothelial dysfunction, etc (Kelishadi et al., 2009). There is also possible implication for chronic noncommunicable diseases, several studies have indicated that exposure to environmental particulate has somehow increased the risk of morbidity and mortality from many diseases, cancers, organ disturbances, and other chronic diseases (Kargarfard et al., 2011). Because of all such reasons, there is an important need in taking action against it and controlling pollution in several ways or else it will degrade the environment.

As environmental pollution is the introduction of detrimental materials instigated by pollutants into the environment, pollutants are both harmful to life and to the environment they are also labeled as wastewater that contaminates the water, air, or soil. There are different types of pollutants classified according to the type of pollution they cause. Some of the environmental pollutants and their hazards are:

Chlorofluorocarbons (CFC): They are destructive to the ozone layer, which is the protective layer. CFCs are also used in perfumes, sprays, and other canned products, and because they are comparably lighter than air so they go up to the atmosphere, and there the UV light removes the chlorine (Abbasi & Abbasi, 2011), the chlorine if comes in contact with the ozone layer of the earth they can cause some serious irreparable damages, which can eventually lead to the entrance of cosmic rays and cosmic rays are very harmful to human as it can cause cancer thus carcinogenic to human beings and can also lead to marine and terrestrial plant growth deterioration.

Lead: This element facily dissolves in water and then enters the cycle it can cause many devastating health problems like negating the enzymes work thus slowing down the normal functioning of the body, it also causes

nervous breakdown and when taken in large quantity it can have an adverse effect in the brain.

Methane: It is the gaseous form of fossil fuel, a major component of natural gas. When present alone it is harmless to the environment but as it is one of the greenhouse gases so when it reacts with CO_2 in presence of water vapor, it takes up and holds on to most of the radiation heat from the sun (Kelishadi & Poursafa, 2010). As it traps the excessive heat in the atmosphere thus it is one of the major contributing factors in causing global warming and climate change. Apart from the greenhouse gas, it is also released from cattle feed, as the demand of the meat industry is increasing the rearing of cattle has also increased significantly. Therefore, the release of methane increases the mean temperature of the earth and thus causes global warming.

Carbon monoxide: It is one of the most fatal pollutants of the air and is also produced in several instances wherever there is incomplete combustion, for example in vehicles the fuel in them has to undergo combustion to move the piston that will power the engine to run, same this theory applies to all full-powered equipment in industries. But then again, the space within the engine is compacted and sometimes it does not allow enough O_2 for complete combustion so the fuels, which are composed of high hydrocarbon concentrations do not combust fully and release carbon monoxide as its by-product (Coogan et al., 2012). CO obstructs the air and makes it difficult to breathe thus it is also considered as one of the most prominent causes of death related to suffocation in the home wherever firewood is used in enclosed spaces. It also plays a huge role in forming ground-level ozone.

Particulate matter (PM): They can be either in solid form or in liquid form in both organic particles and inorganic particles, the main reason for their release is also incomplete combustion, for example, pollen, dust, aerosol spray, etc. As they are tiny particulate thus can be easily inhaled and can cause respiratory problems like lung cancer and can prove harmful for people suffering from asthma attacks.

Sulfur dioxide: It is the main ingredient of acid rain that results in the corrosion of roofs and several health complications. In acid rain when oxidation takes place then weak sulfuric acid is formed, when it reacts with oxygen and other chemical oxides along with rainwater it becomes acid rain.

Several such environmental pollutants are Nitrogen oxides that cause

acid rain, plastics, mercury, ground-level ozone, nitrates associated with nitrate pollution causing eutrophication (Loux et al., 2011), contamination in water because of excessive amounts of nitrates washed out from animal waste, inorganic fertilizers, etc., phosphorus, uranium associated with radioactive pollution and its exposure can cause various cancers to humans, premature aging, death, fertility problems, etc., volatile organic compounds (VOCs) released from burning gasoline, wood, natural gas, and coal, polychlorinated biphenyl (PCBs), chemical fertilizers disturbs the ecological balance of the water bodies and harmful for humans if used for a longer time, over dumped landfills and visual pollutants.

Biodegradation is demarcated as the biologically catalyzed reduction in the complexity of chemical compounds, it is the process by which living microorganisms decompose organic matter into smaller compounds (Marinescu et al., 2009). After the completion of biodegradation, the method is named mineralization, nevertheless, most of the time any change in the substrate mediated biologically is termed as biodegradation thus being aware of microorganisms used in the process is necessary to get an understanding of biodegradation (Bennet et al., 2002). The transformation of the substance takes place through the metabolic process or enzymatic process and is based on 2 processes namely growth and cometabolism. In growth, an organic pollutant is used as the only source of carbon and energy and it causes complete degradation or mineralization of organic pollutants, while cometabolism is the metabolism of an organic compound in the presence of a growth substrate used as major carbon and energy source (Fritsche & Hofrichter, 2000). Various microorganisms such as fungi, bacteria, and yeast are involved in the biological degradation process. The reports related to the involvement of algae and protozoa are insufficient and are very less, the final product of the degradation is carbon dioxide (Pramila et al., 2012). As it is known that organic material can be degraded aerobically in presence of oxygen as well as anaerobically that is in absence of oxygen (Cameotra & Bollag, 2003), biodegradable materials are most commonly organic materials like plant and animals' matter and other such materials that are originated from living organisms or even artificial materials that are close enough to materials of plant and animals to be put to use by microorganisms. Certain microorganisms have bewildering, naturally occurring microbial catabolism ability and can decompose or break down or

transform or amass a huge number of compounds that include hydrocarbons, for example, oil, PCBs (polychlorinated biphenyls), polyaromatic hydrocarbons (PAHs), radionuclides and metals (Leitão, 2009). The term biodegradation is usually utilized in relevancy ecology and waste management and mainly relates to the restoration of the environment or environmental remedy (bioremediation). The process of bioremediation is divided into 3 phases or 3 levels, the first level of this process is through natural attenuation. In natural attenuation, contaminants are reduced by native microorganisms without the help of any human intensification, after this, the second level is biostimulation. When nutrients and oxygen are applied to the systems there only biostimulation is put to use to improve the effectiveness of the systems and to fast-track the process of biodegradation. As a final point, at the time of bioaugmentation microorganisms are added to the systems, these additional or supplement organisms should be more effective than the natural flora in decomposing or breaking down the target pollutants (Diez, 2010). The implemented recycling technology requires microorganisms to be able to quickly adapt to and effectively utilize the target pollutants under specific conditions within a reasonable period (Seo et al., 2009). Many factors can affect the use of pollutants as substrates or being metabolized by microorganisms like the genetic potential and specific environmental factors for instance pH, temperature and nitrogen availability also the available phosphorus sources after that it also seems to determine the rate and degree of deterioration. As a result, the use of genetically modified microorganisms or genetically engineered microorganisms (GEM) in bioremediation has attracted widespread attention, GEM seems to have a high degradation capacity and is generally verified successfully for degradation or breaking down of various pollutants under well-defined conditions. These environmental concerns along with the ecological distresses and regulatory constraints are major obstacles that come in way of testing GEM in this field (Joutey et al., 2013). Even though we are cognisant that biodegradation is usually used in ecology, biomedicine, and waste management and when used in the natural environment it is called bioremediation and now it is commonly associated with environmentally friendly products.

2. Microorganisms involved in the biodegradation of pollutants

As described above biodegradation is associated with environmental bioremediation, so it can also be stated that biodegradation is a way of nature to recycle waste or break down its organic matter into nutrients that will be reusable and can be used by several other organisms. In the microbiological sense, “biodegradation” refers to the degradation or the breakdown of all organic materials through a wide variety of life forms, consisting primarily of bacteria, yeasts and fungi, and possibly other organisms. There are some biodegradable pollutants or organic compounds that in the last 10 years have been synthesized, which are highly toxic and are released into the environment for application for a longer period, fuels, PCBs (polychlorinated biphenyls), polycyclic aromatic hydrocarbons (PAHs), pesticides and dyes are such compounds. Radionuclides and metals are other synthetic chemicals that are very resistant to biodegradation by native flora as compared to the usual naturally occurring organic compounds that are readily broken down when introduced into the environment.

Hydrocarbons are organic compounds whose structure is composed of hydrogen and carbon and are linearly connected, branched, or cyclic molecules. They can be observed as either aromatic or aliphatic hydrocarbons. Benzene comes under aromatic hydrocarbons whereas aliphatic hydrocarbons are observed to have three forms namely alkanes, alkenes, and alkynes (Kafilzadeh et al., 2011).

Polycyclic aromatic hydrocarbons (PAHs) are one the important pollutants and it comes under the class of hydrophobic organic contaminants (HOCs) that are widely found in air, soil, and sediments, industrial production is one of the chief cause of PAH pollution. As more and more data on their toxicity, environmental persistence, and prevalence are available, their research has attracted people's interest for more than 2 decades (Sayara et al., 2011). PAHs can be absorbed from soils and sediments rich in organic matter, accumulated in fish and other aquatic organisms, and spread to humans through the consumption of seafood. On the one hand, the biodegradation of PAHs can be seen as part of the normal process of the carbon cycle, on the other hand, it can be seen as the process of removing man-made pollutants from the environment. The use of microorganisms to bioremediate the environment contaminated by PAH seems to be an

attractive technology for the remediation of contaminated parts.

Polychlorinated biphenyls (PCBs) are mixtures of synthetic organic chemicals. PCBs were primarily used in tons of industries and have several commercial applications that also include electrical, heat transfer, and hydraulic equipment as plasticizers in paints, plastics, and rubber products. They are also used in pigments, dyes, and carbonless copy paper. These wide ranges of applications are possible because they are nonflammable, chemically stable, have a high boiling point, and also have high insulating properties. But PCBs are toxic and can act as endocrine disrupters and can cause cancer (Seeger et al., 2010).

Pesticides, dyes, radionuclides, and heavy metals are such major pollutants that cause some serious harmful effects on the environment and also on humans and can be resolved through biodegradation.

2.1. Degradation caused by bacteria

There are several reports on the degradation of environmental pollutants by different bacteria. There are many reports on the decomposition of environmental pollutants by various bacteria. It is even known that certain bacteria only feed on hydrocarbons (Yakimov et al., 2007). Bacteria that can decompose hydrocarbons are called hydrocarbon-decomposing bacteria. This is the case with the nitrate-reducing bacterial strain *Pseudomonas* sp. and *Brevibacterium* sp. isolated from oil-contaminated soil (Grishchenkov et al., 2000). Data provided by several researchers suggest that anaerobic biodegradation may cause greater concern. 25 genera of bacteria that can decompose hydrocarbons have been isolated from the marine environment. In addition to that, some researchers have also contributed to isolating around 80 bacteria strains that belonged to 10 genera namely *Bacillus*, *Staphylococcus*, *Streptococcus*, *Shigella*, *Corynebacterium*, *Alcaligenes*, *Acinetobacter*, *Escherichia*, *Klebsiella*, and *Enterobacter*. *Bacillus* was considered to be the best hydrocarbon-degrading bacteria. Bacterial strains capable of degrading aromatic hydrocarbons have been isolated several times, mainly from soil bacteria, usually, Gram-negative bacteria, most of which belong to the genus *Pseudomonas*, along with it the biodegradable pathways have also been reported in bacteria of the genus *Mycobacterium*, *Corynebacterium*, *Aeromonas*, *Rhodococcus*, and *Bacillus*. Albeit several bacteria can metabolize organic pollutants however a single bacterium does not have the enzymatic capability to break down or decompose all or most of the organic

compounds in contaminated soil. Factually mixed microbial communities have the greatest biodegradability potential because genetic information from more than one organism is required to decompose the complex mixture of organic compounds in polluted areas (Maszenan et al., 2011). Both anaerobes and aerobes can bio-transform PCBs. PCBs when chlorinated in a higher amount are reduced and dehalogenation by anaerobic microorganisms whereas biphenyls, which are chlorinated in a lower amount are oxidized by aerobes (Seeger et al., 2001). Several pieces of research on bacterial strains have been extensively reported that can degrade or decompose or break down azo dyes under both aerobic and anaerobic conditions (Dos Santos et al., 2007), after several studies it has been reported that microbial decolorization of azo dyes are much more effective under anaerobic conditions whereas in such conditions it has been found that it leads to the formation of aromatic amine, which is mutagenic as well as toxic to humans, which subsequently requires oxidative or aerobic stage for their degradation. In this case, the method of anaerobic/aerobic biological treatment of textile dye wastewater using a combination of microorganisms is very common. Biological degradation of heavy metals is not possible but they can be transformed from one organic complex or one oxidation state to another (Khalid et al., 2010), and along with all these bacteria are also useful in the bioremediation of heavy metals. Microbes have developed the ability to defend against heavy metal toxicity through a variety of mechanisms, including adsorption, absorption, methylation, oxidation, and reduction, when bacteria use metals as terminal electron acceptors for aerobic respiration, the reduction of metals can occur through dissimilatory reduction of metals (Fernández et al., 2012), microbial methylation plays an important role in the bioremediation of heavy metals because methylated compounds are usually volatile. Sulfate-reducing bacteria are anaerobic heterotrophic bacteria, which use various organic substrates with SO_4^{2-} as the terminal electron acceptor. Heavy metal ions can be captured by the cell structure and then absorbed at the binding site of the cell structure. This absorption method has nothing to do with the biological metabolism cycle and is called biological absorption or passive absorption. Heavy metals can also enter the cell through the cell membrane through the metabolic cycle of the cell. This method of absorbing metals is called active absorption.

2.2. Degradation caused by fungi

Microfungi have been described as a very important and interesting group of eukaryotic aerobic microorganisms, ranging from single-celled yeast to mycelial mold, yeasts mainly grow in the form of single cells or pseudomycelium, while molds usually grow in the form of true hyphae. Fungi just like bacteria can metabolize the dissolved organic matter and thus are an important part of degrading microbiota and are also responsible for the degradation of carbon in the biosphere but unlike bacteria, they can grow in low moisture areas and also in low pH solutions that help them in degradation of organic matter. Fungi have extracellular multi-enzyme complexes, which are more efficient, especially in decomposing natural polymer compounds, because of their hyphae system, they can also quickly settle and penetrate the substrate, and transport and redistribute nutrients in the hyphae (Matavulj & Molitoris, 2009). Mycorrhiza is the symbiotic relationship between fungi and the roots of vascular plants and in mycorrhizal association, the fungus colonizes intracellularly in arbuscular mycorrhizal fungi (AMF), or colonizes the roots of the host plant outside the cell in ectomycorrhizal fungi and are an important factor of soil life and soil chemistry. The bioremediation of mycorrhiza is called mycorrhizal repair or mainly mycorrhizoremediation (Khan, 2006), as fungi are prone to degradation, that is why they are very important for the recovery of highly flammable polymers (such as lignin) and the removal of hazardous waste from the environment.

2.3. Degradation capacities of algae and protozoa

Although algae and protozoa are important members of microbial communities in aquatic and terrestrial ecosystems, only a few reports are suggesting that they are involved in the biodegradation of hydrocarbons. From several studies conducted, it can be assumed that the algae, *Prototheca zopfii*, may have used crude oil and mixed hydrocarbon substrates in the separation, and showed strong degradation of normal and isoalkanes and hydrocarbons, nine species of cyanobacteria, five species of green algae, one species of red algae, and one species of brown algae were also observed. Although algae and protozoa are important members of microbial communities in aquatic and terrestrial ecosystems, they are reported to be involved in the biodegradation of hydrocarbons. Naphthalene can be oxidized by alga and 2 diatoms and in contrast, protozoa do not use hydrocarbons, despite this, protozoan populations have been shown to greatly reduce the number of bacteria that can be used to remove hydrocarbons, so

their presence in biodegradable systems is not always useful. Protozoa that grow on yeast and hydrocarbon-using bacteria do not directly use crude oil, in general, the limited data available does not seem to indicate that algae and protozoa have important ecological significance in the decomposition of hydrocarbons in the environment. Some studies have shown that some fresh algae (such as green algae, chlorella, salmonella, and cryptococcus) can absorb and decompose polycyclic aromatic hydrocarbons (Elisangela et al., 2009). The interaction between pesticides and algae shows that algae can not only bioaccumulate pesticides, but also biotransform some of the environmental pollutants, the use of dyes as carbon and nitrogen sources can decompose azo dyes, but this depends on the chemical structure of the dye and it was found that this decomposition is an inducible catabolic process, Common type *C. Vulgaris*, *Lyngbya gerlerimi*, *Nostoc lincki*, *Oscillatoria rubescens*, *Elkatothrix Viridis* and *Volvox aureus* can fade and remove methyl red, orange II, FN-3G, alkaline cations and basic fuchsin (El-Sheekh et al., 2009). *Chlorella*, *Anabaena aequalis*, *Westiellopsis prolifica*, *Stigeoclonium lenue*, *Synechococcus* sp. have the ability to tolerate heavy metals whereas *Chlorella*, *Anabaena*, and marine algae species are quite often used in the removal of heavy metals nevertheless the operational conditions somehow limit its practical application of such types of organisms (Dwivedi, 2012). Metals are absorbed by the adsorption of algae and metal chelation of unicellular algae has been studied and along with that the biological absorption of heavy metals by brown algae has also been studied for a long time this includes the absorption of heavy metals by many cell wall components (such as alginate and fucoidan). Most research in this field is conducted on marine algae and soil algae. According to reports, in continuous culture, *Echinospirium* microalgae or *S. incrassatulus* eliminates Cr (VI), Cd (II), and Cu (II) from the cultures (Pena-Castro et al., 2004). Protozoa are the main grazer of decaying bacteria that look for organic pollutants. Therefore, the interaction between protozoa and decaying bacteria has a direct impact on the results of bacterial decomposition. Several researchers have created a food chain model to study the biodegradation of benzene and toluene by the flagellar protozoan *Heteromita globosa* or xenogeneic weeds, they found that during the logarithmic growth of the flagella population, the rate at which bacteria decompose these benzene and toluene has increased by 8.5 times. Protozoa *Infusorians* can accelerate the

biodegradation of foreign substances (such as PAHs) in the environment, for example, the decomposition rate of naphthalene can be increased four times than before and there are several possible hypotheses regarding the mechanism by which protozoa promote the biodegradation of organic pollutants and that mainly includes the following six parts: (a) Nutrient mineralization, which increases nutrient circulation, (b) Bacterial activation, which controls numbers, rubs aging cells or removes active ingredients, (c) Selective grazing, which reduces competition for resources and space, thus encouraging the growth of bacteria that cause degradation, (d) Physical changes will Increase the oxygen content and surface area of the damaged material, (e) Direct Decomposition can release specific enzymes involved in decomposition, and (f) Symbolic metabolism provides energy and carbon resources for bacteria in the degradation process (Chen et al., 2007).

3. Factors affecting biological removal of pollutants

Biodegradation involves the degradation, removal, modification, immobilization, or detoxification of various chemical substances and physical wastes in the environment by bacteria, fungi, and plants, microorganisms participate in enzymatic pathways that act as biocatalysts and promote biochemical reactions that destroy desired pollutants. Microorganisms can deal with pollutants only when they come into contact with various composite materials that can help them produce energy and nutrients to make more cells. The effectiveness of bioremediation depends on many factors. These include the chemical properties and concentration of pollutants, the physical and chemical properties of the environment, and their accessibility to microorganisms (El Fantroussi & Agathos, 2005). The main reason for the rate of decomposition depends on the fact that bacteria and pollutants do not come into contact with each other. In addition, microorganisms and pollutants are unevenly distributed in the environment due to many such factors, the control and optimization of the bioremediation process is a complex system, the presence of microbial populations capable of decomposing pollutants, the availability of pollutants in microbial populations, and environmental factors (soil type, temperature, pH, the presence of oxygen or other electron and nutrient receptors).

Due to their metabolic mechanism and ability to adapt to adverse environmental conditions, microorganisms can decompose a variety of organic pollutants. Therefore, microorganisms play an important role in restoring this part but whatsoever, but their effectiveness depends on many factors including the nature of the chemical and concentration of pollutants, the availability of microorganisms, and the physical and chemical properties of the environment. So, these are the factors that influence the process of the decomposition or breaking down of pollutants by microbes and are either related to microorganisms and their nutritional needs or also termed as biological factors or the environment aka environmental factors.

3.1. Biological factors

Biological factors affect the degrading or breaking down of organic compounds in the course of the competition among microorganisms in limited carbon sources along with the antagonistic interfacing amongst microorganisms, or the predation of microorganisms by protozoa and phages. The decomposition rate of impurities generally depends on the

concentration of impurities and the amount of “catalyst” present. The number of “catalysts” is the number of organisms that can metabolize pollutants, and the number of enzymes they each produce and the cells expressing certain enzymes can increase or decrease the rate of decomposition of pollutants. In addition, the level of specific enzymes in the metabolism of pollutants and their “affinity” with pollutants must be considered thus the availability of pollutants is very important then only this includes the most important biological factors: mutation, horizontal gene transfer, enzyme activity, interaction (competition, inheritance, and predation), self-growth until reaching critical biomass, population size, and composition (Naik & Duraphe, 2012).

Biological factors are also called the metabolic capacity of microorganisms. The biological factors that affect the microbial degradation of organic compounds include the direct inhibition of enzyme activity and proliferation in the process of microbial degradation. For example, this inhibition occurs when there is a limited amount of initial carbon competition between microorganisms, antagonistic interactions between microorganisms, or predation of microorganisms by protozoa and phages. The decomposition rate of impurities generally depends on the concentration of impurities and the amount of “catalyst” present. In this case, the number of “catalysts” is the number of organisms that can metabolize pollutants, and the number of enzymes produced by each cell, furthermore, the metabolic rate of pollutants depends to a large extent on the specific enzymes involved and their “affinity” for pollutants and the availability of pollutants. In addition, the metabolic rate of pollutants depends to a large extent on the specific enzymes involved and their “affinity” for pollutants, and the availability of pollutants. In addition, for the unrestricted growth of microorganisms, sufficient nutrients and oxygen must be provided in a useable form and in an inappropriate ratio, Other factors that affect the rate of biodegradation by controlling the rate of enzyme-catalyzed reactions are temperature, pH, and humidity. The biological enzymes involved in the degradation pathway have an optimal temperature, and they do not show the same metabolic rate or turnover at all temperatures.

In fact, every 10°C decrease in temperature reduces the biodegradation rate by about half (Singh et al., 2011). Biodegradation can also work out in an extensive pH range. However, in most aquatic and terrestrial systems, a

pH of 6.5–8.5 is usually the best choice for biodegradation. Water affects the metabolic rate of pollutants because it affects the type and number of soluble substances in the pollutants and the osmotic pressure and pH value of terrestrial and aquatic systems.

3.2. Environmental factors

The type of soil and the content of organic matter in the soil affect the possible absorption of organic compounds on the solid surface, absorption is a similar process in which pollutants enter most of the soil matrix. Both adsorption and absorption reduce the use of pollutants by most microorganisms and the metabolic rate of chemical substances decreases proportionally. The porosity changes of the unsaturated zone and the saturated zone in the aquifer matrix will affect the movement of liquid and the migration of pollutants in groundwater, the capability of the matrix to pass oxygen, methane, carbon dioxide and other gases in fine-particle deposits and fats will be reduced, making it easier to be saturated with water, this will affect the rate and type of biodegradation.

The redox potential of the soil is a measure of the electron density of the system and bioenergy is obtained through the oxidation of compounds, in which electrons are transferred to various highly oxidized compounds, which are called electron acceptors. Low electron density (E_h greater than 50mV) means oxidative aerobic conditions, and high electron density (E_h less than 50mV) means reductive anaerobic conditions.

The metabolic properties of microorganisms and the physical and chemical properties of selected pollutants determine the possible interactions in the process, the actual successful interaction between them somehow, depends on the environmental conditions at the point of interaction. The growth and activity of microorganisms are affected by pH, temperature, humidity, soil structure, water-solubility, nutrients, site characteristics, redox potential, and oxygen content, lack of trained human resources in this field, and the physical and chemical bioavailability of pollutants that includes contaminant concentration, types of pollutants, solubility, chemical structure, and toxicity. The factors listed above determine the decomposition kinetics (Adams et al., 2015). Biodegradation can occur in a wide pH range, but in most aquatic and terrestrial systems, a pH of 6.5–8.5 is usually the best choice for biodegradation. Water affects the metabolic rate of pollutants because it affects the type and amount of soluble substances available,

as well as the osmotic pressure and pH of terrestrial and terrestrial organisms along with the aquatic system.

3.2.1. Nutrient availability

The addition of nutrients regulates the nutrient balance necessary for microbial growth and reproduction, and also affects the speed and effectiveness of biodegradation. By optimizing the ratio of bacterial C:N:P, the balance of nutrients, especially because of the intake of essential nutrients (such as N and P) can improve the efficiency of biodegradation. To survive and continue their microbial activity, microorganisms need certain amounts of nutrients, such as carbon, nitrogen, and phosphorus and at low concentrations, the degree of decomposition of hydrocarbons is also limited so adding an appropriate amount of nutrients has been proved to be a beneficial strategy to increase the metabolic activity of microorganisms, thereby increasing the rate of biodegradation under cold conditions (Couto et al., 2014). Biodegradation in the aquatic environment is very less by the availability of nutrients just like the nutritional needs of other organisms, oleaginous microorganisms also need nutrients for optimal growth and development. These nutrients can be obtained in the natural environment, but the quantity is very small (Macaulay, 2015).

3.2.2. Temperature

Among the physical factors, temperature is considered to be the most important for determining the survival rate of microbes and determining the composition of hydrocarbons (Das & Chandran, 2011). In a cold environment just like in the Arctic, natural processes can cause oil to decompose very slowly, which puts more pressure on microorganisms to remove spills of oil or petroleum. The low water temperature in this area forces the transportation channels in the microbial cells to close or even freeze the entire cytoplasm, thereby making most lipophilic microorganisms metabolically inactivated. Biological enzymes that participate in the pathway of degradation have an optimal temperature and do not have the same metabolism rate or turnover at all temperatures. In addition, the decomposition or breaking down of the process of a certain compound requires a certain temperature. Temperature also greatly affects the physiological characteristics of microorganisms, and therefore speeds up or slows down the bioremediation process. The speed of microbial activity increases with increasing

temperature and reaches its highest level at the optimal temperature and it declines haphazardly with further increase or decrease in temperature and it will somehow stop after reaching a certain temperature.

3.2.3. Oxygen concentration

Different organisms need oxygen and some other organisms do not need oxygen depending upon their requirement they can better promote the rate of biodegradation according to their needs. Biodegradation occurs under aerobic and anaerobic conditions because oxygen is the gas requirement of most living organisms and the presence of oxygen can improve hydrocarbons in most cases and have the ability to enhance metabolism.

3.2.4. pH

The pH value of the compound, which represents the acidity, alkalinity, and alkalinity characteristics of the compound, has an influence on the metabolic activity of microorganisms and also increases or decreases the elimination process by measuring the pH of the soil and the potential microbial growth can be indicated (Asira, 2013). The results are poor, and the metabolic process is very sensitive to small changes in pH.

3.2.5. Toxicity of contaminants

Among high concentrations of toxic substances, certain pollutants may have a toxic effect on microorganisms and it slows down the speed of disinfection. The degree and mechanism of toxicity depend on the specific toxin, its concentration, and the exposed microorganisms. Some organic and inorganic compounds are toxic to certain life forms.

4. Application of bacteria in biological degradation of environmental pollutants

Different reports claim the degradation of different environmental pollutants by different bacterial species. In the context of biodegradation, bacteria are found to be very helpful microorganisms due to their well-known biosorbent properties that have proven advantageous in the treatment of polluted environments. They are known to have the ability to flourish under controlled as well as extreme environmental conditions. Moreover, bacteria are sometimes regarded as the engines for recycling the nutrients of the earth.

It is known that the rule for biodegradation of emerging pollutants is not generalized. Bacteria use different mechanisms to metabolize pollutants and use them as sources of carbon and energy. The reactions involved in the biotransformation of these pollutants employ different substrate-specific enzymes, and the bio-transformed product may enter the central metabolic pathway of the cell, for example, the Krebs cycle. The application of bacteria and the metabolic pathway involved in the biodegradation of different environmental pollutants has been discussed in this section.

4.1. Petroleum hydrocarbons

Petroleum is an important natural resource on which nearly the whole world relies for its fuel and various other demands. However, increased energy demands have also increased the consumption of petroleum sharply. But petroleum is considered one of the alarming pollutants all over the world due to its toxic effects on the environment as well as on living organisms (Xue et al., 2015). Petroleum hydrocarbon pollution is caused mainly by anthropogenic activities including industrial runoffs, the release of effluents, accidental spills and discharge, leakage, and various other activities of petroleum industries located either offshore or onshore (Varjani, 2017). Since petroleum hydrocarbons are persistent organic pollutants, their recovery and remediation are major issues of concern for scientists. To understand the scope for the remediation of petroleum hydrocarbons, we must learn about the composition of crude oil, its environmental fate, and its toxicity.

Crude is the naturally occurring raw oil that is further distilled to produce other products. Crude oil consists of mainly four fractions (Chandra et al., 2013):

- (a) saturates containing aliphatic hydrocarbons,
- (b) aromatics containing aromatic or ringed hydrocarbons,
- (c) resins, and
- (d) asphaltenes

Saturates consist of saturated/unsaturated linear/branched open-chained aliphatic hydrocarbons such as n-alkanes, isoalkanes, cyclic aliphatic compounds, terpenes, etc. Aromatics consist of monocyclic aromatics and polycyclic aromatic hydrocarbons (PAHs) that contain more than one aromatic ring. Resins consist of aromatic compounds with long alkyl chains that are soluble in n-pentane and n-hexane. They mainly act as peptizing agents in crude oil. The last fraction of asphaltenes consist of compounds that contain polar groups and are soluble in benzene or toluene. Due to their polycluster-like structures, they are difficult to be biodegraded (Chandra et al., 2013). These petroleum hydrocarbons when released into the environment by any means can cause the contamination of the sites including soil, groundwater, or oceans. It has also been found that some crude constituents such as benzene, toluene, ethylbenzene, xylene, and PAHs are carcinogenic and hemotoxic for humans (Chandra et al., 2013; Costa et al., 2012).

In petroleum oil-rich environments, various petroleum-degrading bacteria have been found. The microbial degradation of various petroleum hydrocarbon compounds has been found to involve oxidizing reactions. However, due to the presence of specific oxygenase enzymes in different bacterial species, their pathway for biodegradation generally differs. For example, some bacterial species have the ability to degrade aliphatic hydrocarbons, while others can degrade aromatics. Thus, the enzymes involved in these two cases will differ greatly. Table 12.1 lists different petroleum hydrocarbon-degrading bacteria and their specific compounds for biodegradation.

Biodegradation of petroleum hydrocarbons involves a series of enzymatic reactions. Hydrocarbons present in petroleum can be degraded by the metabolic activities of either a single bacterial species or a bacterial consortium belonging to either the same or different genera. However, in the biodegradation of hydrocarbons, a consortium of microorganisms has been proven to be more beneficial. Although the genes for the enzymes involved in the hydrocarbon-degradation pathway are usually located in the bacterial

plasmid, the genes for these enzymes are located in the chromosome of the *Acinetobacter* sp. (Varjani, 2017). A wide variety of bacteria have the ability to degrade simple hydrocarbon compounds such as n-alkanes, but very few bacterial species can degrade complex hydrocarbons such as PAHs, resins, and asphaltenes. Bacteria such as *Achromobacter insolitus* can degrade pol-yaromatics such as phenanthrenes but in a microbial consortium including *Sphingobacterium* sp. and *Bacillus cereus* (Janbandhu & Fulekar, 2011; Shah et al., 2020).

Bacteria can metabolize petroleum hydrocarbon in three possible ways: phototrophic, anoxygenic; chemotrophic, aerobic; and chemotrophic, anaer-obic (Varjani, 2017). Although the bacterial degradation of petroleum hydro-carbons is mostly aerobic in nature, some species exceptionally show anaer-obic degradation pathways. However, both aerobic and anaerobic hydro-carbon degradation pathways have some reactions like oxidation, reduction, hydroxylation, dehydrogenation, etc. in common.

Table 12.1

Type of hydrocarbon	Bacteria species	Hydrocarbon compound	References
<i>Pseudomonas aeruginosa</i>	Long chain alkanes (C ₁₂ –C ₃₈)	Muriel-Millán et al. (2019)	Aliphatics
n-alkanes (C ₆ –C ₄₀)	Wang et al. (2011)	<i>Dietzia</i> sp.	
n-alkanes (C ₁₃ –C ₁₇)	Zhukov et al. (2007)	<i>Rhodococcus ruber</i>	
Branched-chain and n-alkanes	Brown et al. (2016)	<i>Gordonia sihwensis</i>	
n-alkanes	Lattuati et al. (2002)	<i>Marinobacter</i> sp.	
<i>Mycobacterium cosmeticum</i>	Monoaromatic compounds (benzene, toluene, ethylbenzene, and o-xylene)	Zhang et al. (2013)	Aromatics

Monoaromatic compounds	Mukherjee et al. (2010)	<i>Pseudomonas aeruginosa</i>	
Mono/polyaromatic compounds	Ma et al. (2014)	<i>Achromobacter xylosoxidans</i>	
Polyaromatic compounds	Eskandari et al. (2017)	<i>Bacillus licheniformis</i>	
		<i>Bacillus mojavenensis</i>	
<i>Pseudomonas</i> sp.	Resins	Venkateswaran et al. (1995)	Resins
<i>Pseudomonas</i> sp.	Asphaltenes	Tavassoli et al. (2012) and Jahromi et al. (2014)	Asphaltenes
<i>Bacillus</i> sp.			
<i>Citrobacter</i> sp.			
<i>Lysibacillus</i> sp.			
<i>Enterobacter</i> sp.			
<i>Staphylococcus</i> sp.			

4.2. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls or PCBs are the mixtures of various synthetic organic compounds, that are considered as kinds of persistent organic pollutants in the environment. Although being an environmental pollutants, they have a wide range of applications due to their nonignitability, chemical stability, high electric insulation properties, and high boiling point. PCBs have their applications in the electrical industry, paint industry, paper industry, rubber industry, and many more. But the use of PCBs has many consequences. It has been found that PCBs can disrupt the endocrine system. Moreover, they are carcinogenic as environmental mutagens has drastic effect on human health (Seeger et al., 2010; Malik at al., 2021). Microbial remediation of PCBs is one of the best approaches for their degradation (Kishore at al., 2022).

PCBs can be bio-transformed by both aerobic and anaerobic bacterial species. PCBs that are highly or moderately chlorinated are put through

reductive dehalogenation that involves dechlorination on specific sites such as *para*- and *meta*-positions (Seeger et al., 2010). However, dechlorination at *ortho*-position has also been discussed in some pieces of literature. Anaerobic bacteria such as *Desulfitobacterium*, *Dehalococcoides*, *Desulfomonile*, *Dehalobacter*, *Sulfospirillum*, and *Desulforomonas* have been reported to participate in the reductive dehalogenation reaction in the biodegradation pathway of PCBs (Pieper & Seeger, 2008).

PCBs that are not chlorinated to much higher extents are oxidized by aerobic bacteria. Several aerobic bacteria that use biphenyl as a carbon and energy source such as strains of *Pseudomonas*, *Burkholderia*, *Acidovorax*, *Comamonas*, *Rhodococcus*, *Cupriavidus*, *Bacillus*, etc. have been isolated and well-characterized (Furukawa & Fujihara, 2008; Pieper & Seeger, 2008). *Burkholderia xenovorans* LB400 has the capacity to degrade diverse varieties of PCBs. It is hence considered the perfect bacterium for studying PCB biodegradation (Seeger et al., 2001). In the upper biphenyl pathway of PCBs degradation, the biphenyl is converted into 2-hydroxypenta-2,4-dienoate and benzoate by a series reactions involving enzymes named biphenyl dioxygenase (BphA), dihydrodiol dehydrogenase (BphB), 2,3 dihydroxybiphenyl 1,2 dioxygenase (BphC), and 2-hydroxy-6-phenyl-6-oxohexa-2,4, diene-oate hydrolase (HOPDA hydrolase or BphD) (Taguchi et al., 2007). The 2-hydroxypenta-2,4-dienoate and benzoate is further converted into acetyl-coA by the lower catabolic pathway. Acetyl-coA further enters the TCA cycle and gets involved in the central metabolism of the bacterium (Pieper & Seeger, 2008).

4.3. Pesticides

Pesticides are chemical used in agriculture to control or kill unwanted pests that can harm crops and reduce their productivity. Some pesticides are non-persistent whereas some are persistent in the environment. Because of their persistency, they are categorized under environmental pollutant as they can pass through the food chain and cause toxicities in living organisms. Micro-organisms such as bacteria and fungi have the ability to degrade pesticides (Malik et al., 2022).

In a study, three bacteria *Pseudomonas aeruginosa*, *Enterobacter ludwigii*, and *Enterobacter cloacae* isolated from domestic sludge showed the ability to remove the pesticide chlorpyrifos from the sludge in 3days. Further investigations showed that chlorpyrifos was uptaken by these bacteria in their

native state inside their cells. These bacteria also showed similar activities in the soil (Shabbir et al., 2018). Bacteria make the remediation of pesticides more effective. It has been reported that bacteria such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Brucella melitensis*, and *Bacillus subtilis* can perform the bioremediation of pesticide-containing soil by 92%, 89%, 87%, and 85% respectively. Without bacteria, the process shows an efficiency of 34% in a period of 30 days (Supreeth & Raju, 2017).

Pyraclostrobin is a fungicide that belongs to the strobilurin group of pesticides, which are considered the major environmental pollutants. In a study, a microbial consortium of *Bacillus* sp. CSA-13, *Paenibacillus alvei* CBMAI2221, *Bacillus* sp. CBMAI2222, *Bacillus safensis* CBMAI2220, and *Bacillus aryabhattai* CBMAI2223 were evaluated for the bioremediation of pyraclostrobin contamination in in-vitro as well in the soil. These bacteria were able to enhance the bioremediation process and reduced the pesticide concentration significantly. This shows that they can effectively be applied in the process of bioaugmentation (Birolli et al., 2020).

In microbial cells, the metabolism of pesticides involves three phases. In the first phase, the parent pesticide molecule is transformed into a water-soluble and comparatively less toxic intermediate through reactions such as oxidation, reduction, and hydrolysis. In the second phase, the water-solubility of the intermediate formed is further increased by conjugating it with other molecules such as amino acids and sugars. In the last phase, the products of phase-II reactions are transformed into secondary conjugates. The end products are usually nontoxic in nature. Enzymes such as hydrolases, oxygenases, peroxidases, etc. are involved in these phases (Van Eerd et al., 2003).

4.4. Plastics

Plastics are ubiquitously seen everywhere nowadays. The increasing use of plastics is a major issue of concern for the whole world. Plastics as pollutants have the lithosphere, hydrosphere, atmosphere, as well as biosphere. Bioremediation is mediated by various microbes as reported by Malik et al., 2022. Bacteria have been widely studied for the biodegradation of plastics. It has been shown that bacteria are proven useful in plastic degradation due to their natural ability to degrade long-chain fatty acids. Over 20 bacterial genera are reported to be involved in the degradation of polyethylenes. They include *Bacillus*, *Streptococcus*, *Staphylococcus*, *Acinetobacter*, *Pseudomonas*,

Klebsiella, *Rhodococcus*, *Ralstonia*, etc (Ghatge et al., 2020). Bacterial strains involved in the degradation of different types of plastics and their respective sources are listed in Table 12.2.

4.5. Dyes

Synthetic dyes constitute a large group of environmental pollutants that have wide roles in several industries. They are carcinogenic in nature and can cause severe harm to the environment and living organisms. The most common source for the release of dyes into the environment is wastewater. Bacteria have been found to be effective in the process of biodegradation of synthetic dyes. The bacterial cells cleave the structure of the original dyes into smaller fragments. Moreover, sometimes complete mineralization of dyes i.e., their conversion into carbon dioxide, biomass, and inorganic compounds is achieved by the bacterial cells (Kolekar & Kodam, 2011). *Pseudomonas* sp., *Escherichia coli*, *Bacillus subtilis*, *Enterococcus* sp., *Staphylococcus* sp., *Acinetobacter* sp., *Klebsiella* sp., *Clostridium* sp., etc. have been found to be effective and efficient bio-degraders of azo dyes (Sudha et al., 2014).

4.6. Bacterial constraints to biodegradability

The physiology of bacterial cells can limit their efficiency of biodegradation. This can usually happen when the substrates to be degraded are present in very low concentrations. Also, degradation reaction pathways linked to the growth of the bacteria can only move along when the ambient conditions are suitable to sustain the reaction kinetics and thermodynamics (Kolvenbach et al., 2014). There are many bacterial strains that are known to be effectively taking part in biodegradation but only in laboratory conditions. Their growth is limited in the environmental pH, oxygen concentration, soil contents, moisture levels, and nutrient bioavailability. Although many bacterial species can survive in extreme environments, most of them do not have the capacity to subsist in the environment of the world's outer to laboratory conditions (Karigar & Rao, 2011).

5. Genetically engineered bacteria for biodegradation of pollutants

The potential of genetically engineered bacteria in the biodegradation of pollutants has been explored by biotechnologists. They have shown their potential applications in the bioremediation of contaminated soil, wastewater, polluted groundwater, etc.

Table 12.2

Plastic	Microorganism	Source	References
<i>Rhodococcus ruber</i> C2o8	Disposal site soil	Orr et al. (20o4)	LDPE
Marine water	Sudhakar et al. (2o08)	<i>Bacillus sphericus</i> Alt	
		<i>Bacillus cereus</i> BF2o	
Waste dumping soil	Tribedi and Sil (2o12)	<i>Pseudomonas sp.</i> AKS2	
Marine water from Arabian sea	Harshvardhan and Jha (2o13)	<i>Bacillus subtilis</i> H1584	
Waxworm's gut	Yang et al. (2o14)	<i>Enterobacter asburiae</i> YT1	
		Bacillus sp. YP1	
Domestic sewage water	Sarmah and Rout (2o18)	<i>Phormidium lucidum</i>	
		<i>Oscillatoria subbrevis</i>	
Mediterranean sea	Delacuvellerie et al. (2o19)	<i>Alcanivorax borkumensis</i>	
<i>Achromobacter xylosoxidans</i>	Soil	Kowalczyk et al. (2o16)	HDPE
Dumpsites for plastic wastes	Balasubramanian et al. (2o1o)	<i>Arthrobacter sp.</i> GMB5	
		<i>Pseudomonas sp.</i> GMB7	

<i>Pseudomonas</i> sp. E4	Soil	Gyung Yoon et al. (2012)	LMWPE
<i>Serratia</i> <i>marcescens</i>	Soil	Azeko et al. (2015)	LLDPE

HDPE, high-density polyethylene; *LDPE*, low-density polyethylene; *LLDPE*, linear low-density polyethylene; *LMWPE*, low molecular weight polyethylene.

The identification of genes involved in the biodegradation pathways of some toxic pollutants such as toluene, chlorobenzene acids, etc. has been achieved. One separate plasmid is needed for the degradation of a specific compound. One plasmid cannot be involved in the degradation of different pollutants of different categories. The plasmids can be categorized into four divisions: (a) the plasmid degrading octane, hexane, and decane, OCT, (b) the plasmid degrading xylenes and toluenes, (c) plasmid decomposing camphor, CAM, and (d) the plasmid degrading naphthalene, NAH (Ramos et al., 1994). Bacterium *Pseudomonas putida*, which contained XYL and NAH plasmids was genetically modified by inserting a hybrid plasmid containing recombinant elements of OCT and CAM plasmids. This strain could now degrade octane, camphor, salicylic acid, and naphthalene. By genetic modifications, the bacterium could grow rapidly on crude oil due to its fast hydrocarbon-degrading capability and efficiency. Hence, this bacterium was termed a superbug or oil-eating bug (Sayler & Ripp, 2000).

For the removal of heavy metals from the environment, several genetically engineered bacteria have been developed. For example, genes encoding for outer membrane protein LamB and metallothionein from *Saccharomyces* and mammals were genetically engineered in *E. coli*. The recombinant strain showed 15–20 times more cadmium ion enrichment capacity than the original strain (Sousa et al., 1998). Similar other bacteria have also been genetically engineered for the biodegradation of other pollutants such as pesticides (Lan et al., 2014). Although genetically engineered bacteria are highly useful in the biodegradation of environmental pollutants, they must be released into the natural environments only after properly assessing and addressing their ecological risks and safety protocols (Fig. 12.1).

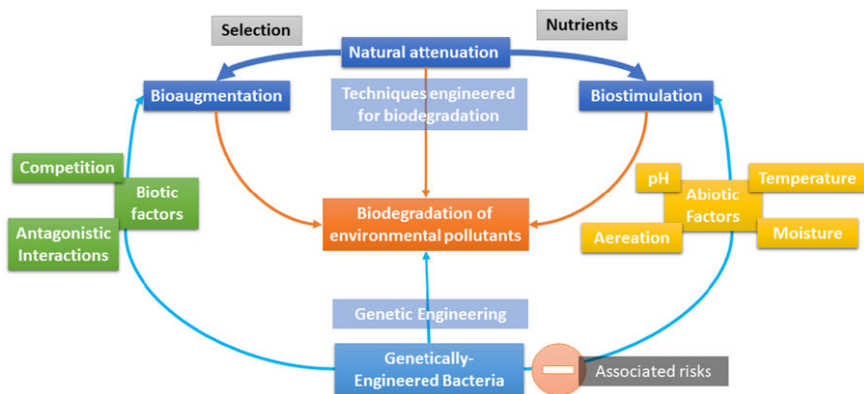


Figure 12.1 Biodegradation of environmental pollutants by utilizing the natural abilities of microorganisms and by genetically-engineered bacteria.

6. Conclusions

The current chapter summarized the role of bacteria in degrading various environmental pollutants such as pesticides, plastics, dyes, petroleum hydrocarbons, heavy metals, etc. Anthropogenic activities such as industrialization and urbanization have increased the amount and types of different pollutants in the environment. Many of these pollutants are persistent in the environment and take a very long time to be degraded naturally. Microorganisms such as bacteria have been naturally found to metabolize and hence bio-transform these pollutants into nontoxic forms. Employing bacteria in the biodegradation of environmental pollutants has proven to be beneficial to a large extent. Moreover, the efficiency of these bacteria has also been increased by genetic engineering. This chapter provides an opportunity to explore the role of different bacterial species in the biodegradation and removal of different pollutants from contaminated environments. Furthermore, it also concisely summarizes the basic mechanism for the pathway of biodegradation of some toxicologically relevant pollutants.

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