“A study on various aspects of bioremediation with microbes as a tool of effective waste management”

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Declaration

I, Humaira Nur declare that this thesis and the work entitled "A study on various aspects of bioremediation with microbes as a tool of effective waste management" submitted to the Department of Mathematics and Natural Sciences (MNS), BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology is a record of work carried out by me under my supervisor.

I further declare that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

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DEDICATED TO MY PARENTS

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Contents

Abstract

The study was evaluating and assessing current waste management techniques and their impact on the environment. While the most used techniques produce detrimental outcomes, bioremediation is the most environment friendly approach. After reviewing multiple scientific research and review papers, it was seen that the microbial bioremediation is the more effective and efficient. It was also seen that microorganisms such as yeast and *Pseudomonas species* derive favorable outcomes. Apart from this, this review also included techniques of different bioremediation, their set up, usefulness, drawbacks and future prospects.

1. Introduction

Most of the processes going around the world lead to formation of waste that has to be removed. Over hundreds of years, much wastage has been accumulated on the earth. According to a World Bank report, in 2012, an estimated amount of 1.3 billion tons of municipal solid waste (MSW) was generated over the world. Moreover, it was also stated that the waste is expected to reach 2.2 billion tons per year (Hoornweg & Bhata-Tata, 2012). In the work of Mavropoulos (2015), it is mentioned that underdeveloped countries, one of the most common waste disposal strategies is open dumping. This is also known as landfilling. Landfills lead several problems that involve leaching of the waste into near water bodies, release of toxic gases, odor and other pollutions. Along with that, aftermath of this activity resulted in a myriad of diseases. The landfill site harbors many pathogenic microbes and vectors for diseases that can be spread to nearby vicinity (Vrijheid, 2000).

A more detrimental waste management scenario is waste burning. According to a study by Wiedinmyer et al. (2014), it was estimated that 41% of MSW were burned. The smoke that gets released from burning MSW can be spread to further localities from the burning point. Moreover, the burning process is a rather slow one, which allows gradual buildup of the pollutants (Lundin et al., 2013). Furthermore, burning waste also leads to the emission of Green House gases which have multifold adversities on human and environment. The most severe outcome of the release of Green House gas is global warming. Apart from increasing the temperature of the earth itself, global warming also catalysts the process of ice caps melting, change in ocean current and many more devastating outcomes (Zein & Chehayeb, 2015).

Another common waste management process is incineration. This process is similar to burning, but it is done in a more controlled manner. Moreover, it is WHO recommended method for managing hospital waste. However, incineration results in the generation of fly ash which is an environmental nuisance. Additionally, maintenance of incinerator is particularly hard in developing countries. The machines are often broken down and that leads to the release of harmful toxic byproducts (Berber, 2017).

While there are many ways to create noisome waste, environment friendly remedial process are only a handful. Bioremediation is one such technique that removes or at least degrades waste product with the aid of natural resources. This can be referred as the method of adding secondary treatment to a contaminated source to accelerate natural biodegradation process (OTA, 1991). It involves an array of techniques which involve biological sources to manage the waste while lowering environmental and biological hazards. One of the most convenient bioremediation techniques is the use of microbes for waste management.

Interest in bioremediation of polluted soil and water has increased in the last two decades primarily because it was recognized that organisms such as microbes were able to degrade toxic xenobiotic compounds which were earlier believed to be resistant to the natural biological processes occurring in the soil. Microbial activity in soils accounts for most of the degradation of organic contaminants. However, information about chemical and physical mechanisms can also be useful to identification of significant transformation pathways for these compounds (Singh et al. 2009).

Apparently, taking into consideration site of application, bioremediation techniques can be categorized as: ex situ or in situ. Pollutant nature, depth and degree of pollution, type of environment, location, cost, and environmental policies are some of the selection criteria that are considered when choosing any bioremediation technique (Frutos et al. 2012; Smith et al. 2015). Apart from selection criteria, performance criteria (oxygen and nutrient concentrations, temperature, pH, and other abiotic factors) that determine the success of bioremediation processes are also given major considerations prior to bioremediation project.

Although bioremediation techniques are diverse, most studies on bioremediation are focused on hydrocarbons on account of frequent pollution of soil and ground water with this particular type of pollutant (Frutos et al. 2010; Sui and Li 2011; Kim et al. 2014; Firmino et al. 2015). Besides, it is possible that other remediation techniques (Pavel and Gavrilescu 2008), which might as well be more economical, and efficient to apply during remediation, are considered when remediation of sites polluted with pollutants aside from hydrocarbons are involved. Furthermore, given the nature of activities leading to crude oil pollution, it is likely that pollution of the environment with pollutants excluding hydrocarbons can easily be prevented and controlled.

Moreover, the dependence on petroleum and other related products as major sources of energy seems to havecontributed to increased pollution resulting from this classof pollutant (Gomez and Sartaj 2013; Khudur et al. 2015).

Microorganisms (bacteria and yeasts) are subjects of many bioremediation studies, due to their ability of assimilating hydrocarbons. Until now there have been described at least 100 microbial species belonging to 30 genera from which 22 genera of bacteria and approximately 14 genera of yeasts (Atlas, 1992). The way that bacteria act in the biodegradation processes is relatively well known, while there are still many questions concerning the way yeasts participate in the same processes. The yeast species described in literature as being able to use hydrocarbons as carbon sources belong especially to the genera *Candida* (Mauersberger et al., 1996),*Clavispora, Debaryomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Sporidiobolus, Sporobolomyces, Stephanoascus, Trichosporon*and*Yarrowia* (Barth &Gaillardin, 1996)

Among the microbes used for this purpose, *Pseudomonas* species and yeast have shown remarkable outcomes (Wasi et al., 2013).

This review deals with overviewing several bioremediation techniques and assessing their potentials. Additionally, it also focuses on the use of microbes as a tool for bioremediation and comparison of its impact to other methods.

2. Methodology

This study was secondary research study that involved bibliographical analysis by using electronic search engines. Google was the search engine used for obtaining electronic study materials and Google scholar was specifically used for obtaining scientific articles. The search was with using the following key words "Waste management", "Sustainability", "Bioremediation", "Microbes", "Phytoremediation", "*Pseudomonas*spp", "Genetic engineering" "Oil spill" and many more. Only the resources from the past 30 years (1988-2018) were included in the study. After entering the key words, relevant resources were retrieved. These were then studied carefully to obtain necessary information. The most significant information was collected and compiled to articulate the review assessment. All the used literary sources have been cited and referenced.

The schematic diagram for the study process is given below:

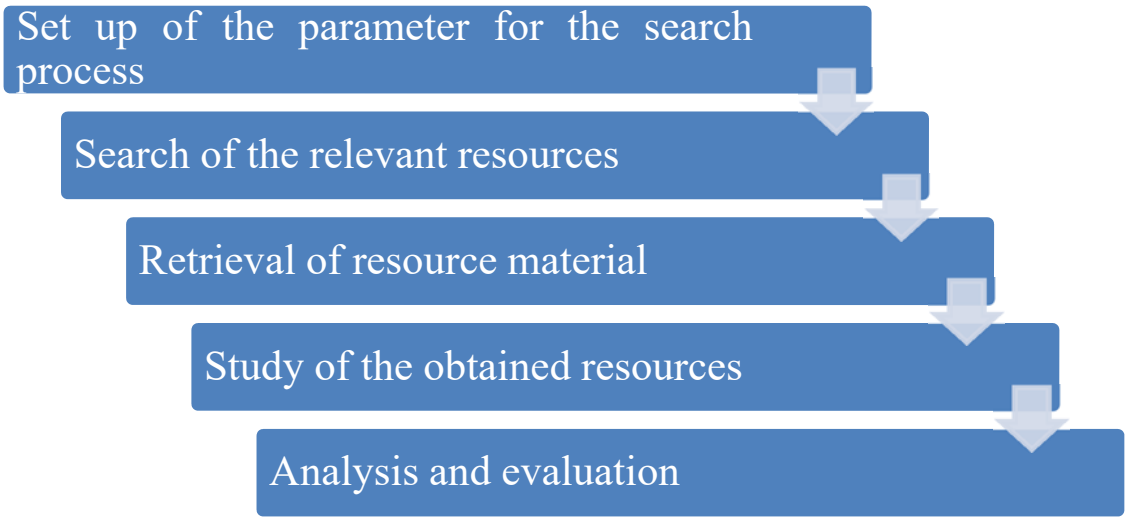

Figure 1: Schematic diagram of the study

3. Bioremediation

As mentioned before, bioremediation refers to the process of addition of biological components which mostly includes microbes to degrade waste products (Glazer and Nikaido 1995). Some of the key features of this technique are its low cost and sustainability. The term bioremediation includes biodegradation, phytoremediation, composting, mycoremediation, rhizofiltration and biostimulation (Azubuike et al., 2016). According to Alexander (1999) bioremediation relies largely on the enzymatic activities of living organisms, usually microbes, to catalyze the destruction of pollutants and transformation of pollutants to less harmful forms. It is done in two main approaches, in one electron acceptor is added and in another electron donor is added (EPA, 2013). Other major categories through which bioremediation can be divided was mentioned in the publication by Azubuike et al (2016). They mention there are 3 ways bioremediation process can be classified, *Ex situ*, *in situ* and Permeable Reactive Barrier (PRB).

Ex situ mainly refers to transportation of the waste from the waste generation site to another site. The facilities for transportation, cost of the setup and outcome determine whether this technique will be applied or not (Philip & Atlas, 2005). On the other hand, *in situ* means performing the remediation in the site of waste occurrence. Oil spills, aquatic wastes, heavy metal contaminations are often treated with *in situ* techniques (Folch et al., 2013). Phytoremediation is another example of *in situ* bioremediation where plants are used for containing the waste (Roy et al., 2015). One of type of *in situ* technique is Permeable Reactive Barrier (PRB), which is a rather recent technique in bioremediation which is used to treat waste water. It involves putting a barrier in the flowing waste water, which will prevent entrance of large components and take those for remediation (Thiruvenkatachari, 2008).

3.1 Plants in bioremediation

As already mentioned, plants can be used for bioremediation as well. This process is called phytoremediation. The term means a collection of plant based technologies that involve use of either naturally occurring or genetically engineered plants for cleaning contaminated

environments (Flathman&lanza, 1998). These include phytoextraction, where the plants are used to extract heavy metals, phytostabilization a process that includes stabilization of the contaminated soil and phytovolatatization where the plants are used for extracting and releasing metals extracted from the contaminated site (Vasabi et al., 2010). Rhizofiltration is another type of phytoremediation where heavy metals from contaminated are extracted with the roots of aquatic plants. The plants used in different phytoremediation are poplar, weeping willow, sunflower, bean, spinach and other aquatic plants (Abdullahi, 2015).

3.2 Microbes in bioremediation

Microbial organisms are the most effective biological agents in bioremediation. Their vast number of enzymes helps them to degrade numerous substances. Microorganisms that carry out biodegradation in many different environments are identified as active members of microbial consortiums. These microorganisms include: *Acinethobacter, Actinobacter, Acaligenes, Arthrobacter, Bacillins, Berijerinckia, Flavobacterium, Methylosinus, Mycrobacterium, Mycococcus, Nitrosomonas, Nocardia, Penicillium, Phanerochaete, Pseudomonas, Rhizoctomia, Serratio, Trametes* and*Xanthofacter* (Singh et al., 2014).

Microorganisms individually cannot mineralize most hazardous compounds. Complete mineralization results in a sequential degradation by a consortium of microorganisms and involves synergism and cometabolism actions. Natural communities of microorganisms in various habitats have an amazing physiological versatility, they are able to metabolize and often mineralize an enormous number of organic molecules. Certain communities of bacteria and fungi metabolize multitude molecules that can be degraded is not known but thousands are known to be destroyed as a result of microbial activity in one environment or another. Most bioremediation systems are run under aerobic conditions, but running a system under anaerobic conditions (Colberg and Young, 1995) may permit microbial organisms to degrade otherwise recalcitrant molecules.

3.3 Microbial enzymes in bioremediation

The enzymes involved in bioremediation are the following types: oxidoreducates, laccases, peroxidases, hydrolytic enzymes and cellulases (Karigar& Rao, 2011). Oxidoreductases include oxygenases, deoxygenases and monooxygenases. These enzymes help in the degradation of phenolic compounds (Gianfreda et al., 1999). In a study by Rubiller et al. (2008), it was mentioned that many fungal species are considered to be suitable for the removal of chlorinated phenolic compounds from the contaminated environments. The activity of fungi is mainly due to the action of extracellular oxidoreductase enzymes, like laccase, manganese peroxidase, and lignin peroxidase, which are released from fungal mycelium into their nearby environment. Their filamentous nature aids them to reach the soil pollutants more effectively than bacteria.

In another study, the activity of catechol dehydrogenase is mentioned. The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the environment. They are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products (Silva et al., 2012). The enzymes laccases are can be produced as extracellular or intracellular enzymes. These are capable of catalyzing the oxidation of ortho and paradiphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines as well as some inorganic ions (Mai et al., 2000).

The activity of laccases is mentioned in the work by Prakash &Manjnath (2011), where they mentioned that liipases can be extracted from bacteria, plant, actinomycetes, and animal cell. However, among these microbial lipases are more versatile because of their potent application in industries. These enzymes can catalyze various reactions such as hydrolysis, interesterification, esterification, alcoholysis and aminolysis. The actions of hydrolytic enzymes involve degradation of many polymers (Vasileva-Tonkova&Galabova, 2003). Another major group of enzymes involved in microbial bioremediation are cellulases. These help to degrade cellulose to glucose (Adriano-Anaya, 2005).

3.4 *Pseudomonas* in bioremediation

One of the major species involved in bioremediation is *Pseudomonas* species. Matsumura et al., (1976) first reported aerobic degradation of hexaclorocyclohexane (HCH), a persistent pesticide, by a *Pseudomonas* strain. Later on, its degradation by a *P paucimobilis* was reported by Wada et al. (1989). The role of *Pseudomonas* species in the biodegradation of γ-HCH is also well established (Nawab et al. 2003; Wasi et al. 2011). Moreover, *P. putida* has been demonstrated to degrade phenols (Chung et al. 2004; Basha et al. 2010). Detoxification of the phenolics like pentachlorophenol by *Pseudomonas* sp. Bu34 has also been reported by Lee et al. (1998). Catechol degradation was clearly demonstrated by Kumar et al. (2005) using a *P. putida* strain. Moreover, O'Reilly and Crawford (1989) reported the degradation of p-cresol by an immobilized *Pseudomonas* sp.

3.5 Yeast in bioremediation

Yeast can be an effective tool for bioremediation. For years, *Saccharomyces cerevisiae* and its relative *Saccharomyces sensustricto* complex have been deemed highly useful utilized in the industrial sector (Vaughan-Martini & Martini, 1998). Apart from producing alcoholic beverages, namely beer, wine and cider, *S. cerevisiae* has its usefulness in the manufacture of renewable bio fuels and pharmaceuticals. Moreover, *S. cerevisiae* cells can be used for treating environmental pollution.

4. Facts that make Microbes as effective tools of Waste Management

4.1 Availability

Readily available microbial biomass can be used as a primary criterion in the selection of biomass to be used during the bioremediation process (Volesky 1990). S. cerevisiae cells can be found in large amounts because they are a by-product of large fermentation industries. S. cerevisiae biomass is the second major by-product (after spent grain) of the brewing industry; during fermentation, the yeast biomass increases three to six fold. In typical lager fermentation, approximately 2.6 kg of surplus yeast solids are produced per cubic meter of beer produced (Huige 2006). Other sources of S. cerevisiae biomass from fermentation industries are wine (including sparkling wine), distilled liquor and bio-ethanol production. Contrary to the biomass obtained from the pharmaceutical industries, yeast cells from fermentation industries are stable and are not subject to the drastic treatments associated with the recovery process of the primary product. For instance, fungal biomass used in the pharmaceutical industry is subject to treatment with solvents; these treatments can affect metal removal performance and cause concerns about its subsequent use (Volesky 1990).

4.2 Ability to remove heavy metals

The yeast biomass from brewing can accumulate a large range of metals, namely Ag (I), Cd(II), Cr(III), Cs(I), Cu(II), Ni(II), Pb(II), Sr(II) and Zn (II), at a variety of pHs (Avery & Tobin 1992; Chen & Wang 2008; Ferraz et al. 2004; Han et al. 2006; Machado et al. 2009; Marques et al. 1999; Soares et al. 2002; Zhao and Duncan 1997; Zouboulis et al. 2001).

4.3 Price

The biomass from brewing has received little attention as a profitable product. The hops used in beer production give the yeast a bitter taste. Spent brewer's yeast is generally sold, after heat inactivation, as an inexpensive product to the animal feed industry (Ferreira et al. 2010; Huige 2006). Therefore, the surplus yeast produced from fermentation industries can be obtained at a low price.

4.4 Safe organism

S. cerevisiae strains are described as "generally recognized as safe" organisms by the US Food and Drug Administration, which means that these cells can be freely manipulated without public concern; this fact increases the feasibility of using yeast biomass in bioremediation processes.

5. Model System

Yeast cells, particularly *S. cerevisiae*, are a suitable model for performing fundamental studies because they are eukaryotic cells that can be easily cultured and manipulated and have a completely sequenced genome (Goffeau et al. 1996). The use of omics technology can provide a wide range of knowledge about the mechanisms of metal accumulation and the impact of these metals on the cells. Additionally, it is possible to improve the bio sorption properties of heavy metals by genetic manipulation.

5.1 Flocculation characteristics

Traditionally, the brewing industry has used flocculent yeast strains. These strains can aggregate into multi-cellular masses (flocs) and settle rapidly in suspension media.

Together, these properties make yeast biomass from brewing a very promising weapon in the fight against heavy metal pollution.

In terms of oil degrading capacity, studies on yeasts able to use various petroleum components as sole carbon source, showed that their biodegrability decreases from n-alkanes > branched alkanes > low molecular weight aromatic hydrocarbons > cycloalkanes > high molecular weight aromatic and polar compounds.

There are four rules for yeasts with xenodegrating abilities: (a) aliphatic compounds are the first to be degraded; (b) alkanes with C10-C18 carbon chaines are preferentially assimilated; (c) unsaturated hydrocarbons are transformed with lower rates; (d) branchedalkanes are easier degraded than linear ones, but only when the branch is higher than C9.

The alkanes with long and medium carbon chains are biodegraded in yeast cells through the cytochrome P450 system, and those with less than C9 require biotin addition to the growth medium. Yarrowialipolytica and Candida maltosa are able to use mono-branched alkanes as sole carbon and energy source. These are incorporated in lipids, converted into soluble cellular

compounds (proteins, aminoacids), intermediate metabolites (dicarboxylic acids with β-methyl group) and partially oxidized to CO2.

Cycloalkanes are used only in small rates (5 to 10 %) compared to the n-alkanes, andonly when their concentration level is not toxic. Although less is known on theirdegradation, it seems that it does not involve cytochrome P450.Phenol and its derivate (resorcinol, chlorophenol, catechol, quinoline,hydroxiquinoline, nitrophenol and dinitrophenols) can be assimilated by Aureobasidium,Rhodotorula, Candida, Yarrowia and Trichosporon strains through β-ketoadipate pathway (Csutak et al., 2010).

Certain C. maltosa cells are able to biodegrade also 2-, 3- and 4-monochlorphenols. Studieson Trichosporon strains isolated from heavily oil-polluted soils, revealed their ability to grow on phenol and Diesel (Kaszycki et al., 2006). Yeasts cannot grow on polycyclic aromatic hydrocarbons (PAH) but are able to cooxidizebiphenyl, naphtalene and benzopyrene using the monooxigenase cytochrome P450pathway induced by the presence of n-alkanes. Studies on fungi and yeast (Candida,Rhodotorula, Trichosporon) communities from aquatic environments polluted with PAH,especially phenantren, revealed high degradation rates for Trichosporonpenicillatum (Macgillivray et al., 2001).

6. Scenario of Environmental Bioremediation

6.1 Tannery wastage

Tannery waste is one of the major polluting sources to the environment. The effluent from tanneries includes high level of chromium, cadmium, sulfuric acid, azo dyes, sulfide and many more (Nacheva et al., 2004). Management of this waste is a highly concerning issue. Microbes, especially *Pseudomonas* spp. have been used to treat tannery waste. *P. aeruginosa* were found to have activity with hydrocarbons in the study by Kiraye et al. (2016) and also can use carbon from other organic compounds as their energy source. Other species of Pseudomonas family like *P fluorescens* & *P ambigua* (Samanta et al., 2002). In a study by Akpomie & Ejechi (2016), it was seen *P. aeruginosa* when mixed with tannery waste could effectively degrade phenol, sulfide, tannin and chromium.

6.2 Oil spills

Transportation system on water has resulted in oil spills. Marine vehicles carrying large volume of oil or hydrocarbon often face accidents; these accidents can spill oil on the water bodies. Spilled oil can vastly hamper the aquatic eco system as it prevents entrance of sunlight and oxygen in the water. Mechanical removal of oil from water can be extremely difficult given the fact oil spreads everywhere. However, use of bioremediation can be very helpful in cleaning oil spill. Microbes like *Pseudomonas* have been playing significant role in clearing oil spill from marine lands. Certain microbes can use hydrocarbon as a carbon source (Thapa et al., 2011).

The specificity of the degradation process is related to the genetic potential of the particular microorganism to introduce molecular oxygen into hydrocarbon and to generate the intermediates that subsequently enter the general energy- yielding metabolic pathway of the cell. (Millioli et al., 2009). Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving toward it, while other microbes like fungi grow in a filamentous form

near the contaminantBacteria for hydrocarbon decomposition are commercially available as freeze dried bacteria, which can be used for bioremediation after propagation to a minimum of 2×108 CFU/ml. Bacterias that can degrade petroleum products are-*Pseudomonas, Aeromonas, Moraxella, Beijerinckia, F lavobacteria, chrobacteria, Nocardia ,Corynebacteria, Atinetobacter, Mycobactena, Modococci, Streptomyces, Bacilli, Arthrobacter, Aeromonas*and *Cyanobacteria* (Braddock, 1997).

In a study by Das &Mukherje (2006), it was seen that among the hydrocarbon degrading microbes *Pseudomonas* is the most effective one. Its large genome allows it to degrade a vast range of compound by producing many enzymes.

6.3 Heavy metals

Many industrial processes have resulted in heavy metal contamination. Metals such as chromium, cadmium, nickel, arsenic and lead are often found in industrial waste. These can cause the following diseases: kidney damage, cancer, bone depletion and many more (Stassen et al., 1999; Nordberg et al., 2002; IARC, 1993).

Chromium has been widely used in various industries. Hexavalent chromium (Cr6+) is a priority toxic, mutagenic and carcinogenic chemical, whereas its reduced trivalent form (Cr3+) is much less toxic and insoluble. Hence, the basic process for chromium detoxification is the transformation of Cr6+ to Cr3+. A number of aerobic and anaerobic microorganisms are capable of reducing Cr6+. In the presence of oxygen, microbial reduction of Cr6+ is commonly catalyzed by soluble enzymes, except in *Pseudomonas maltophilia* O-2 and *Bacillus megaterium* TKW3, which utilize membrane-associated reductases. In a study by Abbas et al. (2014) it was found out that, *Pseudomonas sp.* can remove almost 70% of cadmium from samples in their log-phase. It was further reported in another study of mercury bioremediation that, Pseudomonas putida has the ability to reduce divalent mercury sulphides to Mercury metal, in this way mercury ions as well as sulphides can be bioremediated using these bacteria species (Essa et al. 2002).

Multiple metal tolerances in *P. fluorescens* and its biotechnological significance have been portrayed in multiple studies (Appanna and Hamel 1996; Appanna et al. 1996). Moreover, the role of phosphate in the culture medium of P. fluorescensATCC13525 was also ascertained on the extra cellular and intracellular accumulation of lead (Al-Atoukey et al. 1991). Lopez et al. (2000) demonstrated the effect of pH on the biosorption of nickel and other heavy metals by P. fluorescens 4F39. Shah and Thakur (2003) carried out the enzymatic dehalogenation of pentachlorophenol by *P. fluorescens* of the microbial community from tannery effluent. They have found that *P. fluorescens* also utilize pentachlorophenol as a carbon source. Utilization of petroleum hydrocarbons by *P. fluorescens* isolated from a petroleum contaminated soil was further reported by Barathi and Vasudevan (2001).

7. Recent Approaches

Recently, many other approaches are being taken to enhance bioremediation. Electro Kinetic-Bioremediation (Ek-Bio) is one of the techniques that can be conducted to remediate contaminated soil.

The application of electro kinetics helps to transport the ions where the electric field is applied across the soil. Positive ions will be attracted to the cathode and negative ions will be attracted to the anode. Meanwhile, bioremediation is the process of using bacteria to lessen the toxicity levels of mercury (Harbottle, 2013). Thus, the pollutant molecules and bacteria in the soil will be affected by the process of electro kinetic and bioremediation that had been applied. Electrokinetic separation is an emerging technology that relies on the application of a low-density, direct current through the soil to separate and extract heavy metal, radionuclides and organic contaminants from unsaturated soil, sludge and sediment. This technology can be applied to contaminant concentration ranging from a small amount of ppm to concentrations greater than 10,000 ppm (Gupta et al., 2012). However, it may not be effective for treating multiple contaminants that have significantly different concentrations (Azhar et al., 2016).

Advances in molecular biological analyses allow unprecedented microbial detection and are increasingly incorporated into bioremediation. Advances in next generation sequencing (NGS) have now placed metagenomics and metatranscriptomics within reach of environmental engineers. As NGS costs decrease, metagenomics and metatranscriptomics have become increasingly feasible options to rapidly scan sites for specific degradative functions and identify microorganisms important in pollutant degradation. The combination of genomics, transcriptomics, proteomics and metabolomics has provided a crucial insight into microbial communities and their mechanisms in bioremediation of polluted environment. These omic techniques are capable of revolutionizing biological treatment in environmental engineering by allowing highly sensitive characterization of previously uncultured microorganisms. Omics enables the discovery of novel microorganisms for use in bioaugmentation and supports systematic optimization of biostimulation strategies (Czaplicki &Gnucsh, 2016). The recent techniques that are used are as follows:

7.1 Recognizing different microbes

The use of molecular era innovations has also changed research in the field of bioremediation. Two main strategies were employed to characterize community diversity and function. The first main strategy is shared by denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment polymorphism (T-RFLP). This strategy examines microbial diversity by exploiting differences in a highly conserved gene such as the rRNA gene and then separating fragments with different sequences using a variety of methods. This approach has been shown to be powerful for focusing on community-level interactions in bacteria and archaea (16S rRNA) as well as fungi (18S rRNA). The second strategy consists of targeting a known gene using quantitative PCR (qPCR) to quantify organisms within a community who possess the target gene. A similar approach known as reverse transcription quantitative PCR (RT-qPCR) can be applied to quantify the transcription of a target gene in RNA studies (Colombo et al. 2011; van Herwijnen et al. 2006). T-RFLP has been another method commonly used in monitoring bioremediation.

T-RFLP was also generally applied to the highly conserved ribosomal RNA (rRNA) genes (Liu et al. 1997; Mills et al. 2003). The first step in T-RFLP analysis involves performing PCR amplification using a normal forward primer and a fluorescently-modified reverse primer. Following amplification, amplicons are digested using multiple restriction enzymes that each target a specific DNA location. The enzyme digestion step generates distinct, fluorescently-labelled DNA fragments of varying lengths because each amplicon has a different genetic sequence. Then, fragments are separated using capillary gel electrophoresis and fluorescence is detected, producing an electropherogram. In the electropherogram, each peak corresponds to a distinct microorganism within the community. Since the size of the peak can be correlated to a specific amount of a given microorganism within the community, T-RFLP is more quantitative than DGGE, although still not a fully quantitative method. A number of software packages have been developed to compare electropherograms between treatments and perform statistical analyses to quantify similarities. While no strain identification can be obtained from a particular terminal restriction fragment (T-RF), researchers have been able to associate T-RFs to microorganism sequences using ribotype databases. These ribotype databases have been

constructed by compiling a collection of microorganism sequences, their T-RFs generated using various combinations of restriction enzymes, and their identity so that a T-RF can be quickly matched and identified.

7.2 Genetic engineering

Scientists are currently looking into certain genetically engineered microorganisms to increase their ability to metabolize specific chemicals such as hydrocarbons and pesticides. The possibilities of using genetic engineering for improvement of bioremediation process had an early boost in the late 1980's. Recombinant DNA techniques have been studied intensively to improve the degradation of hazardous waste under laboratory condition. The genetically engineered microorganisms have higher degradative capacity and have been demonstrated successfully for the degradation of various pollutants under defined conditions. Genetic modification technology has resulted often in a wide variety of current and potential applications for use in the process of bioremediation. Bioremediation explores gene diversity and metabolic versatility of microorganisms (Fulekar, 2009).

The genetic architecture of these organisms makes them valuable in biodegradation, biotransformation, biosorption and bioaccumulation. The necessary blue print of gene encoding for biodegradative enzymes is present in chromosomal and extra-chromosomal DNA of such microbes. Recombinant DNA techniques facilitate to evolve the ability of an organism to metabolize a xenobiotic by detection of such degradative genes and transforming them into appropriate host via suitable vector under the tight control of appropriate promoters. It depends on susceptibility to alteration and exchange of genetic information. The recombinant DNA technology explores PCR, anti-sense RNA technique, site directed mutagenesis, electroporation and particle bombardment techniques.

The biotechnology armed with recombinant DNA technology is now fine tuning the bioremediation technology by improving pollutant– degrading microbes through strain improvement and genetic modification of specific regulatory and metabolic genes that are crucial in developing effective, safe and economical techniques for bioremediation. Bioremediation is

not effective only for the degradation of pollutants but it can also be used to clean unwanted substances from air, soil, water and raw materials form industrial waste. Bioremediation is not effective only for the degradation of pollutants but it can also be used to clean unwanted substances from air, soil, water and raw materials form industrial waste (Singh et al., 2013). Recent advances in molecular biology, biotechnology, and enzymology are the driving forces toward engineer-improved fungi and enzymes for mycoremediation.

The ease of genetic engineering, transportation, and scaling-up makes fungi the organisms of choice in bioremediation (Obire et al. 2008). A number of the genetic engineering approaches that have been developed have proven beneficial in adding the desired qualities in metabolic pathways or enzymes. Strain manipulation is becoming easier with the exponential expansion of molecular tool boxes and genome sequences. However, the best source is that of the genes of fungi, where mycotransformation is well understood. Specific gene alterations can be designed and controlled via metabolic engineering. Metabolic control is shared by enzymes. Mathematical modeling of metabolic control analysis can be used to make predictions as to how metabolic pathways will respond to manipulation. Fungal genes can be cloned to meet the objectives of mycoremediation. Fungal mutants that over secrete specific enzymes can be produced, and various processes using such mutants may be designed and scaled up in the treatment of wastes and wastewaters. Fungal protoplasts can be exploited to enhance processes related to mycoremediation. At present, efforts to increase flux through specific pathways have met with limited success. Potentially, the future of metabolic engineering is bright, but there is still a long way to go to understand this area of the metabolic network before the introduction of bioengineered yeast or fungi in the field of mycoremediation. Recent advances in biotechnology can open the door for the development of genes responsible for the mineralization of PCBs by fungi. Genes encoding Lignin peroxidase in 30 fungal species have been screened that may open new frontiers for the degradation of PCBs.

A great future lies in successful genetic splicing and bringing together pathway fragments with a view to constructing an entirely new white-rot fungus that can utilize PCBs as the sole source of carbon (Harbhajan 2006). The first complete eukaryotic genome belongs to the yeast *Saccharomyces cerevisiae* (Dujon 1996). The genome sequence has laid a strong foundation for work in the disciplines of agriculture, industry, medicine, and remediation. In a paper for fungal

comparative genomics, the Fungal Genome Initiative (FGI) Steering Committee identified a coherent set of 44 fungi as immediate targets for sequencing (Birren et al. 2003). Several projects have released information on the genome sequences of the yeasts *Schizosaccharomycespombe* and Candida albicans and the filamentous fungi *Aspergillus nidulans, Aspergillus fumigatus, Neurosporacrassa*, and *Coprinuscinereus.* The 13.8 million base pair genome of S. pombe consists of 4,940 protein coding genes, including mitochondrial genome and genes (Wood

et al. 2002). Ten thousand genes are predicted in the 40-Mb genome in the sequence of the first filamentous fungus, N. crassa (Galagan et al. 2003). The 30 million base pair genome of the first basidiomycete, Phanerochaetechrysosporium strain RP78, has been sequenced using a whole-genome shotgun approach (Martinez et al. 2006). The genome reveals genes encoding oxidases, peroxidases, and hydrolytic enzymes involved in wood decay. This opens up new horizons related to the process of biodegradation of lignin and organopullutants and in the area of mycoremediation. Recently, yeast has been engineered with a binding affinity to cellulose (Nam et al. 2002). Genes encoding the cellulose binding domain (CBD) from cellobiohydrolase I (CBHI) and cellobiohydrolase II (CBHII) of *Trichodermareesei*have been expressed on the cell surface of *Saccharomyces cerevisiae*.

Unlike bacteria, the role of biotechnological innovations related to biodegradation by fungi is relatively less well understood. Moreover, bacteria and fungi exhibit different mechanisms in the biodegradation of pollutants such as pesticides. Significant progress has been achieved in molecular biology related to fungi, especially related to the extraction of genetic material (RNA and DNA), gene cloning, and genetic engineering of fungi.

8. Limitations

Bioremediation is limited to those compounds that are biodegradable. Not all compounds are susceptible to rapid and complete degradation. There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound. Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants. It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations. Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases. Bioremediation often takes longer than other treatment options, such as excavation and removal of soil or incineration. Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation (Vidali, 2001).

Conclusion and Future Perspectives

Among the top ten biotechnologies for improving human health, bioremediation is recognized as one of the technologies (Eapen et al. 2007). The application of molecular-biology-based techniques in bioremediation is being increasingly used and has provided useful information for improving of bioremediation strategies. Furthermore, environmental metagenomics data from soil and sea can be a useful source of genes. Combinational approaches such as genome shuffling are also useful for generating new genes or modifying enzyme activities to allow efficient bioremediation (Kawahigashi 2009). This new biotechnology approach will open exciting new vistas for enhancing bioremediation programs in the coming years.

Whereas bioremediation using transgenic bacteria seems presently to be in the doldrums, phytoremediation using transgenic plants could offer some new answers to environmental cleanup of toxic wastes. New genetic method risk-mitigation may help ensure that neither the transgenic plants, nor the transgenes they contain, will escape into the environment (Davison 2005). The potential of engineered phytoremediation plants should be demonstrated in field trials, some of which have emerged in the last few years. The ecological impact and underlying economics of phytoremediation with transgenic should be carefully evaluated and weighted against known disadvantages of conventional remediation techniques or risks of having the recalcitrant heavy metal or metalloid species in our environment (Kotrba et al. 2009).

In addition, the combination of plants for removing or degrading toxic pollutants and rhizospheric microorganisms for enhancing the availability of hydrophobic compounds can break down many types of toxic foreign chemicals, including herbicides. In view of the importance of mycorrhizal (macro) fungi in plant growth and particularly in the mobilization and cycling of elements in the soil, the colonization of contaminated soils with the suitable fungal species would be beneficial to promote bioavailability of the environmental pollutants. Gadd (2007) further demonstrated suitability of genetic engineering approach in constructing fungi with improved metalloresistance.

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