



PROSPECTIVE *IN-SILICO* APPROACH IN BIOREMEDIATION OF PETROLEUM HYDROCARBON: SUCCESS SO FAR

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Abstract: Bioremediation has the potential to reduce contaminated environment inexpensively yet effectively. But, the lack of information about the factors controlling the growth and metabolism in microorganisms in polluted environment often limits its implementation. However rapid advances in the understanding of bioremediation are on the horizon. With advances in biotechnology, bioremediation has become one of the most rapidly developing fields of environmental restoration, utilizing microorganisms to reduce the concentration and toxicity of various chemical pollutants, such as petroleum hydrocarbons. In this mini-review, the current state of the field is described and the role of synthetic biology in biotechnology in short and medium term is discussed. A number of bioremediation strategies have been developed to treat contaminated wastes and sites. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: the amenability of the pollutant to biological transformation to less toxic products, the bioavailability of the contaminant to microorganisms and the opportunity for bioprocess optimization. By the recent advances on *in-silico* dimensions of bioremediation, it seems that the synthetic biology software will soon drive the wet-lab implementation at molecular level.

Key words: Bioremediation, Crude oil, Environment, Pollution.

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INTRODUCTION

It is estimated that approximately five million tons of crude oil and refined oil enters the environment each year as a result of anthropogenic sources such as oil spills (Hinchee and Kitte, 1995). Past analysis of reported oil spills indicated that most of the oil comes from tankers, barges and other vessels as well as from land pipeline spills. Extensive changes in marine, as well as terrestrial ecosystems resulting since the first major commercial oil spill the Torrey Canyon spill which occurred on March 18, 1967 in England, the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999), the Prestige spill (2002), the Mumbai-Uran pipeline spill (2011), etc. have recently increased the attention of environmentalists, chemists, biotechnologists and engineers (Lü *et al.*, 2011, Tazaki *et al.*, 2004). Shipping accidents have a serious impact on the surrounding environment. The consequences include serious, widespread and long-term damage to marine ecosystems, terrestrial life, human health and natural resources. Hydrocarbon components have

Saxena et al., 2013; Prospective *in-silico* approach in bioremediation of petroleum hydrocarbon: success so far been known to belong to the family of carcinogens and neurotoxic organic pollutants (Das and Chandran, 2011).

Minor oil spills and oil contamination from non-point source discharges (e.g., urban runoff and boat bilge) are no less threats to public health and the environment, although they have received much less attention in the past. According to recent *National Water Quality Inventory* reports, non-point Source pollution remains the Nation's largest source of water quality problems (U.S. EPA, 2012). It is the main reason that approximately 40 % of surveyed rivers, lakes, and estuaries are not clean enough to meet basic uses such as fishing or swimming. Conventional oil spill countermeasures include various physical, chemical, and biological methods. Commonly used physical methods include booming and skimming, manual removal (wiping), mechanical removal, water flushing, sediment relocation, and tilling (Rosenberg and Ron, 1996).

Currently accepted Physical disposal methods of incineration or burial in secure landfills can become prohibitively expensive when amounts of contaminants are large. Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive (Atlas and Bartha, 1977, Das and Chandran, 2011). Biodegradation of oils is by no means a simple subject since, in nature; hydrocarbon decomposition involves interactions of complex physical, chemical and biological processes, the rates of, which are inter-dependent (Harayama et al., 1999). Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms (Hinchee and Kite, 1995). Many microorganisms are capable of degrading the crude oil present in the soil, it has been found beneficial to employ mix culture opposed to pure cultures in bioremediation as it shows the synergistic interactions (Ellis et al., 2008). Bacteria for hydrocarbon decomposition are commercially available as freeze dried bacteria, which can be used for bioremediation after propagation to a minimum of 2×10^8 CFU/ml (Atlas and Bartha, 1977). Bacteria that can degrade petroleum products are- *Pseudomonas*, *Aeromonas*, *Moraxella*, *Beijerinckia*, *F. lavobacteria*, *Chlorobacteria*, *Nocardia*, *Corynebacteria*, *Acinetobacter*, *Mycobacteria*, *Modococci*, *Streptomyces*, *Bacilli*, *Arthrobacter*, *Aeromonas*, *Cyanobacteria*, etc. (Milloli et al., 2009). Researchers now have the ability to cultivate microorganisms that are important in bioremediation and can evaluate their physiology using a combination of genome-enabled experimental and modeling techniques (Nair, 2007). Bioinformatics based on proteomics and genomics, offer remarkable promise as tools to address longstanding questions regarding the molecular mechanisms involved in the control of mineralization pathways. During mineralization, transcript structures and their expression have been studied using high-throughput transcriptomics techniques with microarrays. Generally however, transcripts have no ability to operate any physiological response; rather, they must be translated into proteins with significant functional impact. Towards the establishment of functional proteomics, the current advances in protein microarrays play a central role in the proteomics approach. Exploring the differential expression of a wide variety of proteins and screening the entire genome for proteins that interact with particular mineralization regulatory factors would help us to gain insights in to bioremediation (Fulekar, 2008). The objective of this document is to understand and establish a computational assessment of bioremediation strategy by a comprehensive review of the actual use of bioremediation in real world cases.

COMPOSITION OF PETROLEUM HYDROCARBONS

Petroleum is defined as any mixture of natural gas, condensate, and crude oil. Crude oil which is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of the

Saxena et al., 2013; Prospective *in-silico* approach in bioremediation of petroleum hydrocarbon: success so far elements hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom. It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than 1% (v/v), including phosphorus and heavy metals such as vanadium and nickel. The most water-soluble components of diesel, such as benzene, toluene, ethylbenzene and xylene isomers are usually termed as BTEX (Okoh, 2006).

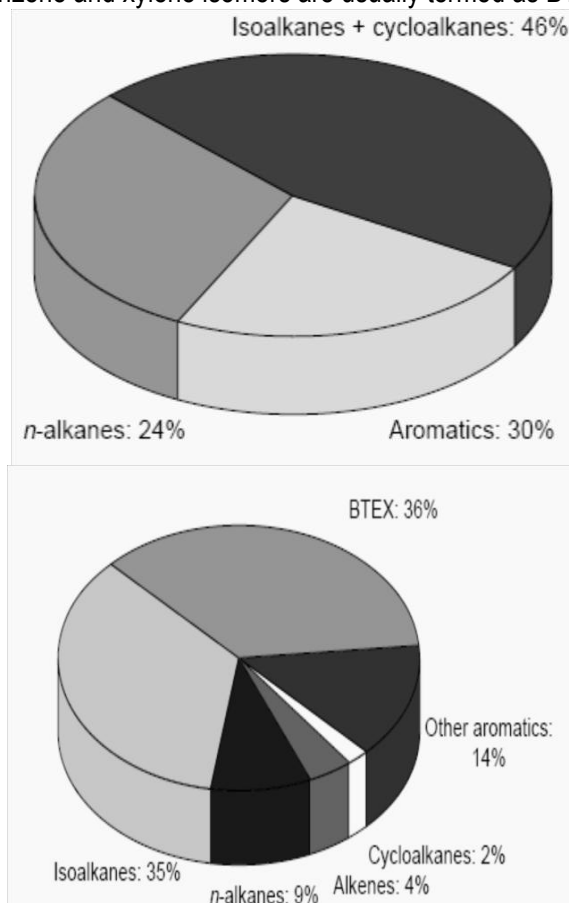
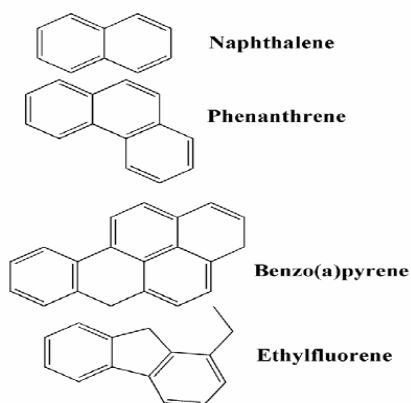


Figure 1. Composition of commercial gasoline and commercial diesel oil respectively (Marchal et al., 2004).

Polycyclic Aromatic Hydrocarbons



Aliphatic Hydrocarbons

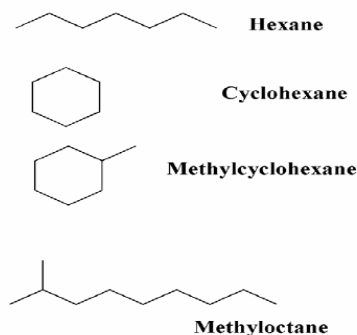
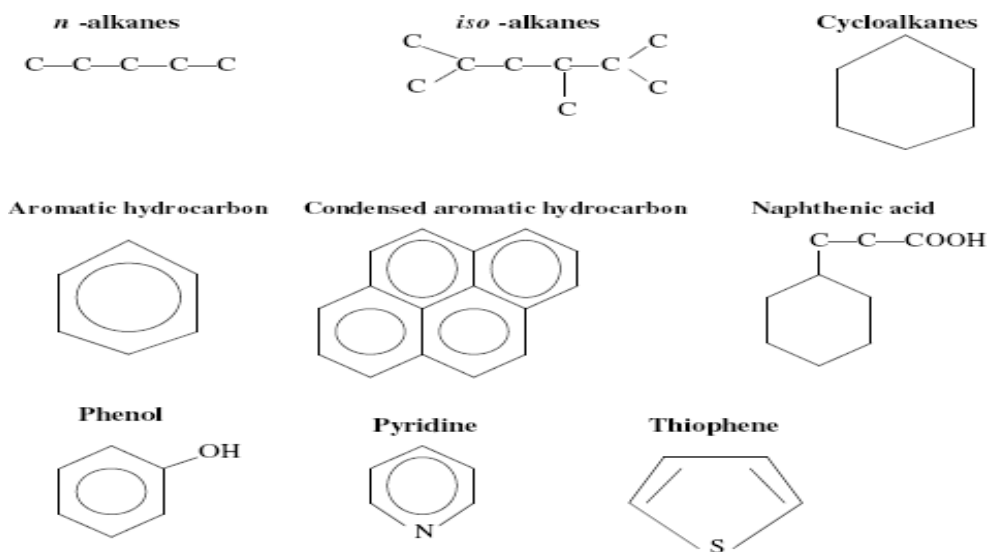


Figure 2. Molecular Structure of Several Aromatic and Aliphatic Hydrocarbons Found in Petroleum (Okoh, 2006).



Gasoline and diesel oil generally contain low amounts of alkenes. They are both composed of four main structural classes of hydrocarbons:

- *n*-alkanes or *n*-paraffins (linear saturated hydrocarbons);
- Isoalkanes or isoparaffins (branched saturated hydrocarbons);
- Cycloalkanes or naphthenes (saturated cyclic alkanes);
- Aromatics (Marchal *et al.*, 2004).

The main recalcitrant hydrocarbons in gasoline belong to the class of methylated alkanes. In particular, about 50% of initial trimethylalkanes resist to microbial degradation. Gas chromatographic identification indicate that the molecular recalcitrant structures are either branched hydrocarbons with a quaternary carbon or hydrocarbons with methyl groups on consecutive carbon atoms (Marchal *et al.*, 2004). Concerning diesel oil degradation, the major problem is the analytical complexity related to the huge number of components. Some other branched alkanes such as farnesane, pristane and phytane are also detected (Milloli, *et al.*, 2009). Nevertheless, the major fractions of the crude oil is still not characterized because the majority of the components cannot be resolved and appear as a hump or unresolved complex mixture (UCM) on chromatograms (Marchal *et al.*, 2004).

TOXICITY OF PETROLEUM HYDROCARBONS

The hydrocarbon such as toluene, phenol, benzene, xylene, hexane and other aliphatic compound community occurs as intermediates or by product in the synthesis of solvents, dyes and explosives (Mittal and Singh, 2009). There are chemicals responsible for causing widespread environmental pollution which is very harmful for environment due to their toxic, carcinogenic and mutagenic nature. Among these hydrocarbons toluene is most toxic aromatic compound even at low concentration and it enters the environment in the form of industrial discharge from petroleum refining, plastics, resins and pharmaceutical industrial effluents and oil spills (Taso *et al.*, 1998). The toxicity of petroleum hydrocarbons to microorganisms, plants, animals and humans is well established (NIOSH, 1990). The toxic effects of hydrocarbons on terrestrial higher plants and their use as weed killers have been ascribed to the oil dissolving the lipid portion of the cytoplasmic membrane, thus allowing cell contents to escape (Chaîneau *et al.*, 2005). Low concentration of toluene causes tiredness, confusion, weakness, drunken actions, memory loss, nausea, loss of appetite, hearing loss and loss of vision (Stdeicher, 1981). At low doses, petrol vapor is irritating to the eyes, respiratory tract and skin. Exposure to higher concentrations of vapor may produce CNS effects such as staggered gait, slurred speech and confusion. Very high concentrations may result in rapid unconsciousness and death due to respiratory failure (Machle, 1991). It is seen that petrol does not have significant mutagenic activity in humans (Loury *et al.*, 1986). However, it is assigned the Risk Phrase R45 ("may cause cancer") under the Chemical Hazard Information and Packaging for Supply (CHIPS) Regulations (Dement *et al.*, 1997). It is also known that petrol is not classified under CHIPS regulations as a reproductive or developmental hazard (McKee, 2000). The principal adverse effect arising from the ingestion of diesel is chemical pneumonitis (secondary to aspiration of vomitus) (Risher and Rhodes, 1995). But it is well established that benzene and/or its metabolites cause chromosomal aberrations in the peripheral blood lymphocytes of chronically exposed humans (Bogadi-Soare, 1997, Sasiadek, 1992).

MICROORGANISMS CONVENTIONALLY USED IN BIOREMEDIATION: AN ASSET

For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure risks for clean-up personnel, or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation the public considers it more acceptable than other technologies. Most bioremediation systems are run under aerobic conditions, but running a system under anaerobic conditions may permit microbial organisms. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater, and soil habitats. Typical bacterial group known for their capacity to degrade hydrocarbons include *Pseudomonas*, *Marinobacter*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia*, and *Gordonia* group. Molds belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium* and the yeasts *Candida*, *Yarrowia*, and *Pichia* have been implicated in hydrocarbon degradation (Okoh, 2006).

Many bacterial catabolic pathways are specified by conjugative plasmids (Frantz and Chakrabarty, 1986). These plasmids are readily transferred laterally into new host bacteria, thereby enhancing the metabolic potential of other members of an ecosystem. Conjugative plasmids are thus important agents of genetic changes and evolution in bacteria, and could be picked up from or brought together in different organisms as groups of genes, which through mutations and recombination can specify new metabolic functions (Lessie and Gaffney, 1986). For example, five aerobic toluene degradative pathways are characterized in pseudomonads. The best characterized of

Saxena et al., 2013; Prospective *in-silico* approach in bioremediation of petroleum hydrocarbon: success so far these pathways is encoded by the TOL plasmid (pWW0) of *Pseudomonas. putida* PaW1 (Worsey et al., 1991), which converts toluene to benzyl alcohol, benzaldehyde, benzoate, and catechol, which further undergoes *meta* cleavage by an extradiol dioxygenase, or catechol 2,3- dioxygenase (C230). *Pseudomonas putida* F1 metabolizes toluene to 3- methylcatechol, which undergoes *meta*-cleavage by a C230 (Whited and Gibson, 1991). The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation. One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction.

There are the two main approaches to oil spill bioremediation:

- (a) Bioaugmentation, in which known oil degrading bacteria are added to supplement the existing microbial population, and
- (b) Biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates (Das and Chandran, 2011).

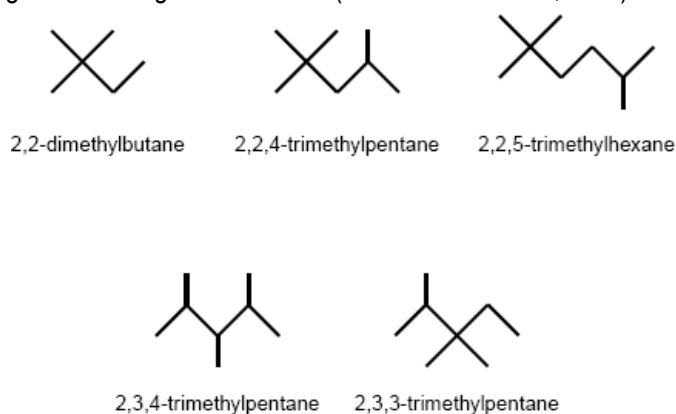


Figure 3. Main recalcitrant molecular structures of gasoline (Marchal et al., 2004)

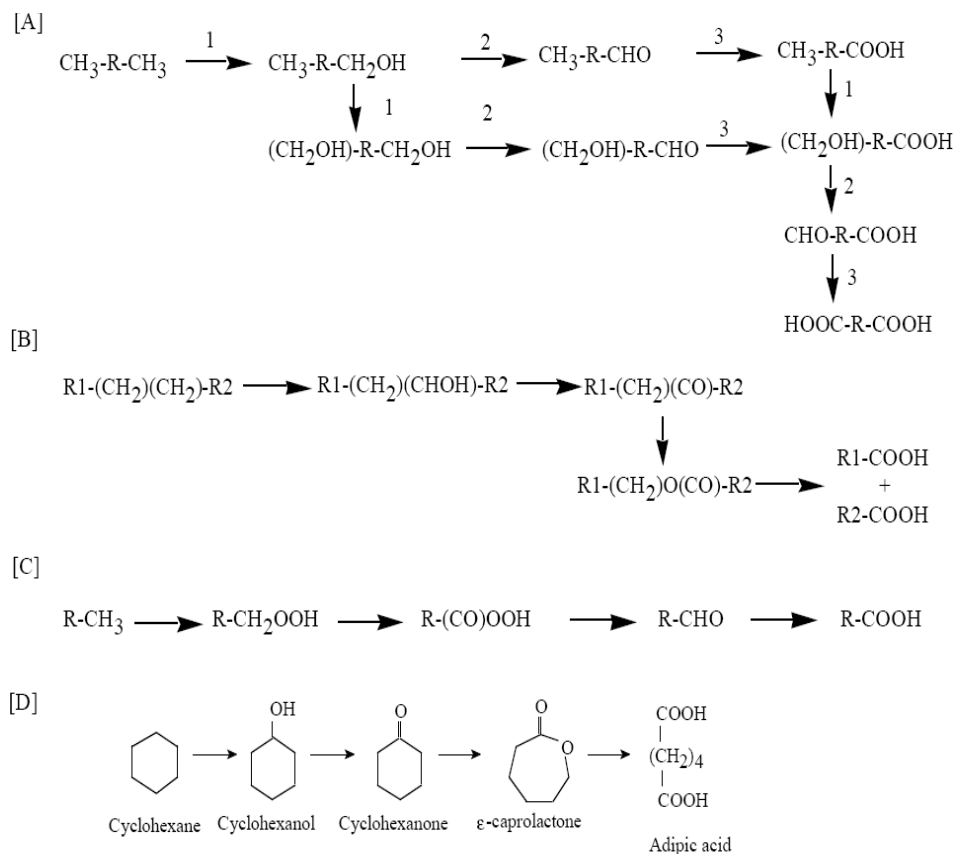


Figure 4. Alkane Degradative Pathways

- [A] Terminal oxidation of n-alkanes. α- and ω-hydroxylation is catalyzed by the same set of enzymes. With bacteria, steps 1, 2 and 3 are catalyzed by alkane monooxygenase, fatty alcohol dehydrogenase and fatty aldehyde dehydrogenase, respectively. With yeast, step 1 is catalyzed by P450 monooxygenase, while steps 2 and 3 are catalyzed either by fatty alcohol oxidase and fatty aldehyde dehydrogenase, respectively, or by the P450 monooxygenase involved in step 1;
- [B] Subterminal oxidation of n-alkanes;
- [C] n-Alkane degradation via alkyl hydroperoxides;
- [D] Degradation of cyclohexane (Harayama *et al.*, 1999).

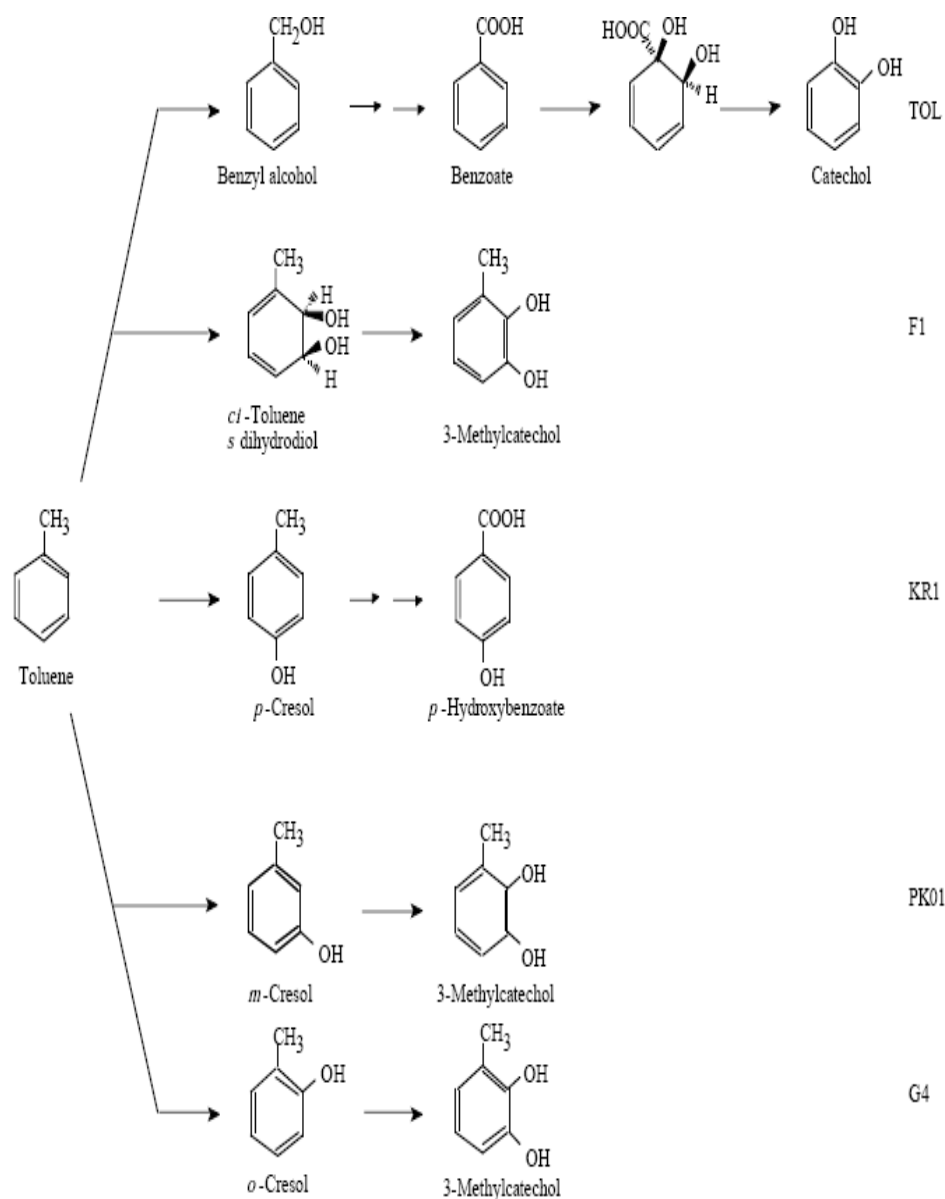


Figure 5. Divergent Pathways for the Aerobic Degradation of Toluene.

The pathways from the top to the bottom are found with *P. putida* (TOL), *P. putida* F1, *P. mendocina* KR1, *P. pickettii* PKO1, and *B. cepacia* G4, respectively (Harayama *et al.*, 1999).

The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment. Numerous scientific review articles have covered various factors that influence the rate of oil biodegradation. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 and 9. Nutrient availability, especially of nitrogen and phosphorus, seems to be the most limiting factor. It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of contaminants (Chaîneau *et al.*, 2005). Accepted values for a mixed microbial

Saxena et al., 2013; Prospective *in-silico* approach in bioremediation of petroleum hydrocarbon: success so far population in the soil are C: N, 10:1; and C: P, 100:1. Nitrogen and phosphorus can be supplied with common inorganic fertilizers with the N: P ratio at 16:1 when the optimum nitrogen fertilization for a sandy matrix is lower than 100 mg N kg dry soil weight. Urea formaldehyde was found to be the most satisfactory nitrogen source (Ferguson et al., 2003).

In contrast to the fact that aerobic microbial hydrocarbon metabolism has been extensively investigated, the same is not true about anaerobic hydrocarbon metabolism (Heider and Fuchs, 1997). The roles of bacteria that participate in these processes under anoxic/anaerobic conditions during biodegradation are not fully understood. Oxygen is not available in all environments where hydrocarbons occur (e.g. in deep sediments, flooded soils, eutrophic lagoons, stagnant fresh and ocean waters and in oil reservoirs). Several studies have investigated the question, whether or not the biodegradation of hydrocarbons is possible under anoxic conditions. It was not until the late 1980s that new groups of microorganisms were found to degrade hydrocarbons under strictly anoxic conditions. Studies have confirmed that these microorganisms activate organic compounds by special biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism (Riser-Roberts, 1992). N-alkanes, branched alkanes, cycloalkanes, and some alkenes have been shown to be degraded under anaerobic conditions. For example, unsubstituted, methyl-substituted, and ethyl-substituted cyclopentenes, cyclopentanes and cyclohexanes were consumed without a substantial lag in the presence of sulfate but rather less effectively under methanogenic conditions. Dimethyl-substituted cyclopentanes and cyclohexanes were biodegraded only in the presence of sulfate (Widel and Rabus, 2001).

It is obvious that the degradation of petroleum and refined products proceeds much faster in the presence of oxygen than under anoxic conditions. Furthermore, aerobic microorganisms degrade a larger range of hydrocarbon compounds than anaerobic. Aerobic bioremediation processes are very effective in treating hydrocarbon contamination, however they are often expensive (e.g. hydrogen peroxide). For this reason anaerobic biodegradation provides cost-effective and advantageous *In-situ* bioremediation technology that can be used for the decontamination of soil, sediment, and ground water contaminated with petroleum hydrocarbons. It was observed that benzene, toluene, ethylbenzene, and xylene (BTEX) are degradable without oxygen in contaminated groundwater (Coates et al., 2002).

System biological (In-silico) approach in bioremediation

In-silico approach using variety of computational tools to predict novel biodegradation pathways for pollutant degradation permits to explore the potential of microorganisms in cleaning up the particular compound from the environment. However, given the wealth of novel pathways, it is necessary to evaluate their relative feasibility, particularly within the context of cellular environment (Jamini et al., 2012). Prediction methods such as Pathway Prediction System (PPS), META and others rely on databases of rules describing biotransformations that occur in cellular and environmental processes (Ellis et al., 2008). *Acinetobacter baylyi* is an innocuous soil bacterium that degrades pollutants and produces lipases, proteases, bioemulsifiers, cyanophycin, and biopolymers (NIOSH, 1990). *A. baylyi* is easily transformed and manipulated by homology-directed recombination, enabling straightforward metabolic engineering. Therefore, the GEM is accompanied by an extensive library of mutants, and was validated against wildtype growth phenotypes in 190 environments and gene essentiality data for nine environments (Durot et al., 2008).

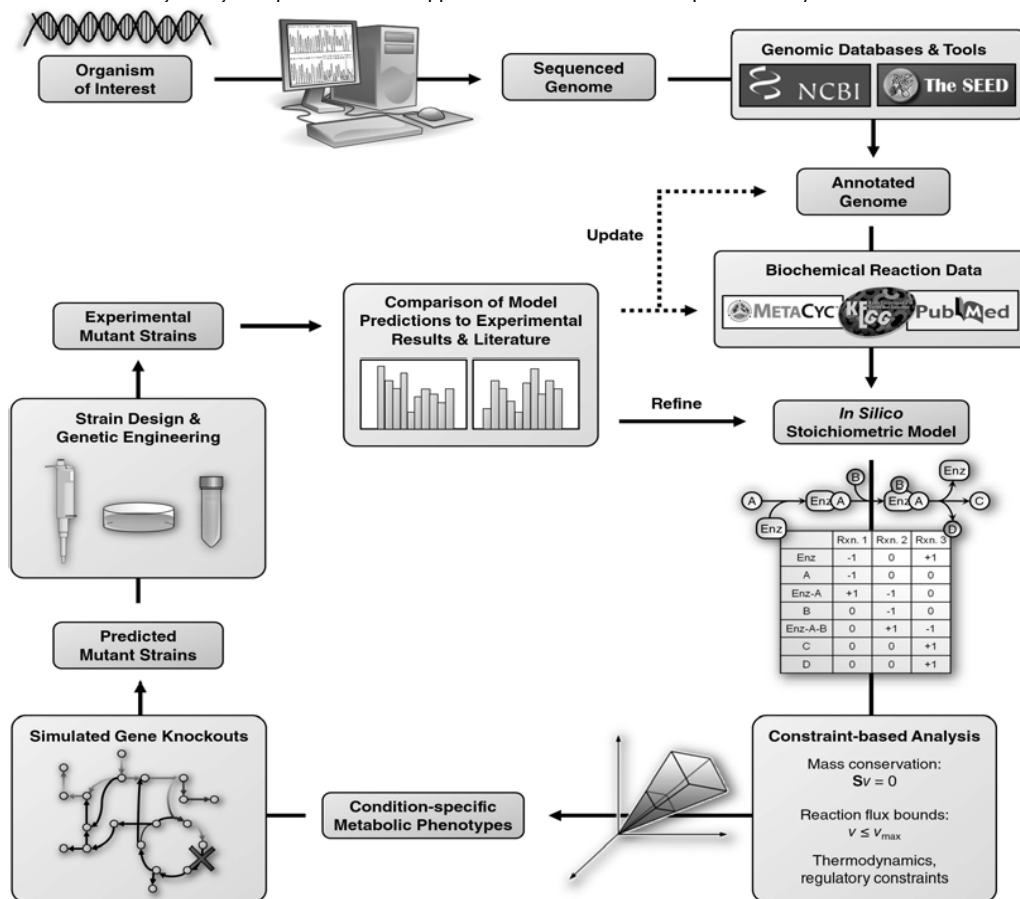


Figure 6. Outline of In-silico approach to develop novel Biodegradation Pathways (Milne *et al.*, 2010).

The integrated approach of using the knowledge on enzymology and bioinformatics such as protein–ligand docking tools offers a rapid means of identifying new potential targets for bioremediation. These *In-silico* approaches are being evaluated in other fields of science such as medicine and drug discovery. But studies related to screening targets for a pollutant for bioremediation are limited. Ligninolytic enzymes play a crucial role in the global carbon cycle. The ligninolytic system is an extracellular enzymatic complex that includes peroxidases, laccases and oxidases responsible for the production of extracellular hydrogen peroxide (H_2O_2) (Prasad *et al.*, 2011). Those enzyme systems exhibit differential characteristics depending on the species, strains and culture conditions (Kirk and Farrell, 1987). The fungi absorb nutrients available in the ambient when the molecules are small, and when they are bigger the fungi uses their enzymes (Esposito and de Azevedo, 2004). The enzymes responsible for lignin degradation are mainly: lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper containing phenoloxidase, known as laccase. The potential application of ligninolytic enzymes in biotechnology has stimulated their investigation (Vikineswary *et al.*, 2006) and the understanding of physiological mechanisms regulating enzyme synthesis in lignocellulose bioconversion could be useful for improving the technological process of edible and medicinal mushroom production (Songulashvili *et al.*, 2007). Ligninolytic enzymes have a potential in several industrial and biotechnological processes within a wide variety of organic and inorganic specific substrates (Esposito and de Azevedo, 2004; Rodríguez and Toca, 2006). Consequently, the aim of this review is to highlight the potential industrial and biotechnological applications of ligninolytic enzymes.

In a study, Selected ligninolytic enzymes (Lip, Mnp, laccase) and PAH ligands—

Saxena et al., 2013; Prospective *in-silico* approach in bioremediation of petroleum hydrocarbon: success so far naphthalene, acenaphthene, fluorene, anthracene, and pyrene—were docked using FRED in Open Eye Software (Rosenberg and Ron, 1996). The *In-silico* results revealed that the pyrene ligand docked with all three enzymes. The order of interaction of various PAHs with various enzymes decreased in the order of pyrene> anthracene> fluorene> acenaphthene> naphthalene. Among the five PAH ligands, maximum interaction occurred between pyrene and all three ligninolytic enzymes. These results lend support to our experimental observation showing that pyrene was degraded to the maximum extent by species such as *C. versicolor* and *P. ostreatus* (Arun et al., 2008).

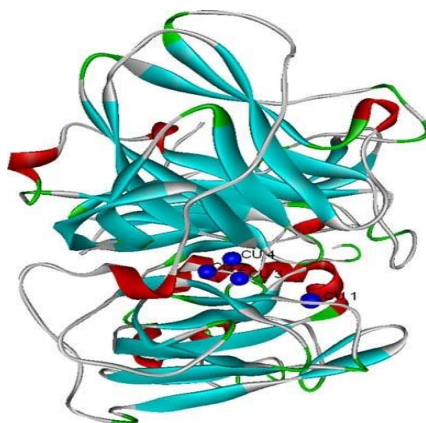


Figure 7. Three dimensional model of *Pycnoporus cinnabarinus* laccase in ribbon display mode showing α - helices in red, β -sheets in cyan, β -turns in green and copper atoms in blue colour (Prasad et al., 2011).

CONCLUSION

A number of reasons have been identified as challenges posed to the microorganisms working in contaminated sites. Such potential limitations to the biological treatment include: poor bioavailability of the chemicals, presence of other toxic compounds, inadequate supply of nutrients and insufficient biochemical potential for effective biodegradation. The next decade will belong to understanding molecular mechanism and cellular manipulation using the integration of bioinformatics. Bioinformatics has wide application in bioremediation for the structure determination and pathways of biodegradation of xenobiotics. In order to fully utilize the data generated from the various 'omics' tools, better annotation of the genes, pathways, and metabolites are needed.. This will require co-ordination from scientists to share data, and database managers to update, maintain, and provide quality control. Taken together, these tools will allow for accurate interpretation of the 'omics' data, leading to generation of judicious predictive models and strategies for successful implementation of bioremediation applications in the near future.

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