GENERAL INTRODUCTION
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'Earth' is the home we all share and should pass on to our future generations as their legacy and 'life' is a delicate phenomenon on earth, and is supported by the environment. The quality of life is directly related to the quality of the environment in which we live and hence anything that hurt the environment or the nature can be termed harmful to life as a whole.

People have made much major harm on the world's living ecosystems. As a result, now the ecological problems grow faster than the rate of population and the growth of the world economy. Man's indiscriminate industrialization as part of a wider modernization process, has in fact, given way to a highly polluted, toxic world. Slowly but surely, industrialization and economic growth have both become major threats to our environment. Little do we realize to what extent we have changed this planet.

The development of civilization and the advance of technology have been instrumental in getting the ecosystem in complete disarray. The rising urbanization, wherein the rural characteristics of a town or area are completely wiped out, has left life in peril. As a result, environmental pollution has been on the upswing and has reached alarming proportions so that today, man's very survival on the globe is at risk.

The introduction of contaminants into an environment of whatever predetermined proportions, which can cause instability, disorder, impairment or discomfort to the physical systems or living organisms therein is generally termed as environment pollution. Pollutants or the elements of pollution are mainly foreign
substances/energies or contaminants or materials which are present in the environment and which turn toxic when they exceed their natural permitted levels.

Sometimes the term pollutant is extended to include any substance whose presence within a system at unnaturally high concentration can endanger the stability of that system. Over the years, environmental pollution has multiplied thousand folds, mainly due to the hazardously growing industries. Environmental degradation due to pollution indirectly affects human health through reduction of food quality and loss of safe drinking water supplies. Various complex compounds are daily injected into the environment and the growing population coupled with the unplanned development and urbanization keep on escalating the generation of waste tremendously. This situation is posing monumental management problems to the civic authorities of all nations especially those of the developing countries. Since this poses a huge question mark on the life and the future of the forthcoming generations, degradation studies are of utmost significance today.

In India, urbanization and growing populations have accelerated problems with the collection and disposal of both solid and liquid wastes. Every year the import of packaged consumer goods adds to the growing amount of non-biodegradable waste. Industrial units, which are coming up in large numbers, sometimes bring about more adverse effects on the biotic community of the environment than the benefits they can offer. When water environment gets polluted through the discharge of industrial and urban effluents it gradually leads to the depletion of aquatic life by destroying the essential supplies of food and oxygen and changing the pH of the solution.
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Manmade chemicals, many of which are very toxic, can be difficult to recycle and are expensive to destroy. Most wastes, hazardous or not, are simply dumped together at the nearest available government owned land (Wright and Welbourn, 2002). Eventually, solid wastes that accumulate in the environment pose numerous threats to humans and the environment, by way of the poisonous gases generated from the excessive amount of biodegradable material in the waste and also through 'leachate' which is the liquid that drains or 'leaches' from a landfill or in the requirement of the site for the disposal of waste. The water seeping into a landfill becomes contaminated once it comes in contact with the decomposing solid waste, and when it subsequently flows out of the waste material it is termed leachate.

Leachate varies widely in composition depending upon the type of waste and the time of its disposal and can cause contamination of ground water as well as surface water. In addition, the gases emitted during this process usually bring about extremely unpleasant odours, which can easily have harmful effects on the nearby inhabitants. It also poses a potential risk to the ground water quality (Weber et al., 2002). Studies also point to the fact that many substances that raise toxic and biocumulative effects remain in the environment for many years to come.

The elimination of a wide range of pollutants and wastes from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact. Simple treatments to reduce environmental pollution, on a wider scale, might have had greater advantage in earlier centuries when physical survival was often the highest imperative, when human population and
densities were of lower significance, when technologies were simpler and their byproducts were more benign. The case is no longer the same now. The existing methods of treatment and disposal of these contaminants seem to be unsustainable. Hence, the impact of pollution on the environment has been on the focus for sometime now and efforts to rinse out the earth have turned out to be a major area of study.

**Organic pollutants**

Organic pollutants comprise a potentially large group of pollutants, particularly in urban environments. Even at low levels, some of them can be dreadfully hazardous to human health. Many of these organic compounds are resistant to degradation through chemical, biological, and photolytic processes. Because of this, they have been observed to persist in the environment and are capable of long-range transportation, bioaccumulation in human and animal tissue, biomagnifying in food chains, and have caused significant impacts on human health and the environment. Some of the chemical characteristics of these include low water solubility, high lipid solubility, semi-volatility and high molecular masses.

Organic chemicals enter the environment in a variety of ways and in a variety of forms. The source of the chemicals, their mode of release and the means by which they are transported may differ. Organic compound may be released into the environment as solvents and bye products of a variety of manufacturing processes. Some of the waste from industrial sources may be exceptionally dangerous. The foreign, exogenous compounds termed as xenobiotics, have multiplied many thousand folds by man’s industrialized and mechanized activities (Wright and Welbourn, 2002). These xenobiotics, mostly of industrial
origin, are relatively new substances mainly formed from synthetic chemicals and are very difficult to categorize. So the biodegradation of these compounds is likely to require a process of evolution of novel enzymes and catabolic pathways. Toxic xenobiotic that are not recognised by the microbial enzymes thus may persist in the environment and thereby forming a potential threat to the ecosystem and public health.

Size, shape and the features like chlorination affect their fate, transport, bioaccumulation and toxicity in complex interactive ways (Wright and Welbourn, 2002). They may be synthetic organochlorides such as plastics and pesticides, or naturally occurring organic chemicals such as polyaromatic hydrocarbons (PAHs) and some fractions of crude oil and coal. One of the primary problems arising from the use of persistent, lipophilic organochlorine insecticides has been the problem of bioconcentration and biomagnification, collectively called bioaccumulation (Cockerham and Shane, 1994).

Poly aromatic hydrocarbons (PAHs), are the molecules which consist of three or more fused aromatic rings in various structural configurations (Blumer, 1976). They are formed during the incomplete combustion of organic material, and are therefore present in relatively high concentrations in products of fossil fuel refining (Nishioka et al., 1986; Wang et al., 1990). The environmental fate of polycyclic aromatic hydrocarbons (PAHs) is a matter to be concerned due to its ubiquitous distribution and their deleterious effects on human health. They have a high potential for biomagnification through trophic transfers due to their lipophilic nature (Clements et al., 1994; Kelly and Cerniglia, 1995). The biodegradation of PAHs by microorganisms is the subject of many excellent reviews (Shuttleworth and Cerniglia,
1995). It depends on the number of aromatic rings, and molecule topology or the pattern of ring linkage.

Polychlorinated Biphenyls (PCB’s) which may enter the environment by industrial accidents, weathering of PCB containing products or leaking from landfills (Clayton and Clayton, 1981) are also harmful to the environment. Due to their lipophilic and hydrophobic nature, they tend to bioaccumulate in living tissues and in the food chain (George et al., 1988). Accidental release of organic compounds as in the case of leaking of underground storage tanks on the soil surface or subsurface lead to the direct introduction of contaminants into the environment (Khan and Anjaneyalu, 2005).

Landfilling or incineration were the traditional methods for the removal of the polluted materials and their subsequent disposal. But the available space for making landfills and incinerators is declining. One of the greatest limitations to traditional cleanup methods is the fact that despite of their higher cost, they do not always ensure that contaminants are completely destroyed (Khan and Anjaneyalu, 2005).

However, it is believed that microorganisms are capable of degrading almost all the different complex and resistant xenobiotics found on the earth to a certain extent. Due to the enormous number of genera of microorganisms, numerous enzymatic pathways have been developed to metabolize organic compounds by dehalogenation, hydrolysis, oxidation, reduction, conjugation and methylation (Cockerham and Shane, 1994).

Many organic materials, in general, such as plant and animal matter and other substances originating from living organisms, or artificial materials that are similar to plant and animal matter can be recycled by way of the process of biodegradation. Interest in the
microbial biodegradation of pollutants has intensified in recent years as mankind strives to find sustainable ways to cleanup contaminated environments.

**Biodegradation of organic compounds**

Biological processes play a major role in the removal of contaminants and they take advantage of the astonishing catabolic versatility of microorganisms to degrade such compounds. Biodegradation is a natural phenomenon of reprocessing wastes by breaking down organic contaminants into smaller compounds with the help of microorganisms. When there is a complete decomposition of the compound into its elemental form, it is termed as a ‘complete biodegradation’ and if the complex molecule is only converted into lesser complex molecules, it is partial biodegradation (or biotransformation).

According to Alexander (1981), microorganisms were able to degrade many chemicals rapidly and thereby eliminate them from the environment. But certain compounds are there which degrade slowly, or partially and later accumulate in the environment and occasionally exhibit toxicity.

The biodegradability of certain substance depends largely on its molecular weight, molecular form and its crystallinity. The rate of degradation of a compound is influenced by these properties as well as its ambient conditions, such as whether the compound is in solution or adsorbed to any particulates. The biodegradability of a substance is inversely proportional to its molecular weight, that is, as the weight increases, the biodegradability shows a decrease. That is why small and simple molecular substances known as monomers, dimers, and repeating units break down easily, whereas complex forms like polymers that have larger molecular masses take much more time to
degrade. Apart from these three major factors, some other aspects that affect biodegradation are temperature, pH, redox potential, access to nutrients, oxygen supply, biomass of the degrader, tussle among the microbial communities and the nature and concentration of the substrate.

Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanism by which harmful pollutants are eliminated from the environment. The biodegradation of hydrocarbons in the environment is a complex process, whose quantitative and qualitative aspects depend on the nature and amount of the hydrocarbons present, the ambient and seasonal environmental conditions and the composition of the autochthonous microbial community (Leahy and Colwell, 1990). The microbial degradation of long chain (≥C₁₂) alkanes are considered to be a difficult task as they are having solubility less than 0.01 mg/l (Bell, 1973).

According to Maila, et al. (2005), in a hydrocarbon contaminated soil, biological removal of hydrocarbons differs with soil layer. The hydrocarbon degrading bacteria were found to be decreasing with soil depth. The sub surface soil environment, though devoid of sufficient nutrients, oxygen and other factors, harbours an array of microorganisms that play an important role in decomposition and the recycling of nutrients (Krumholz, 1998). According to Atlas (1981) and Leahy and Colwell (1990), the number and relative abundance of hydrocarbon degrading bacteria in the bacterial communities increases significantly in the presence of readily available hydrocarbons. The possibilities of using processes based on the microbial biodegradation of hydrocarbons for removal of pollutants
from the environment and upgrading oil refinery products have been suggested by van Hamme et al. (2003).

Dominant groups of microorganisms and the degradative pathways associated with polymer degradation are often determined by the environmental conditions. When $O_2$ is available, aerobic microorganisms are mostly responsible for destruction of complex materials. With microbial biomass, $CO_2$ and $H_2O$ are the final products. The availability of oxygen in soil is dependent on rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged and the presence of utilizable substrates which can lead to oxygen depletion (Bossert and Bartha, 1984). In contrast, under anoxic conditions, anaerobic consortia of microorganisms are responsible for polymer deterioration. The primary product will be microbial biomass, $CO_2$, $CH_4$, and $H_2O$ under methanogenic conditions (Gu and Mitchell, 2001).

The biodegradation depends upon different factors. Rate of degradation are generally observed to decrease with decreasing temperature; this is believed to be a result primarily of decreased rates of enzymatic activity, or the “Q10” effect (Atlas and Bartha, 1972; Gibbs et al., 1975). The degradation of hydrocarbons also depends on the water activity. According to Dibble and Bartha (1979), the optimal rates of biodegradation is at 30-90% water saturation. In aquatic environments, salinity and pressure may also affect biodegradation rates while moisture and pH may limit biodegradation in soils. Adaptation by prior exposure of microbial communities to hydrocarbons increases hydrocarbon degradation rates. Adaptation is brought about by selective enrichment of hydrocarbon-utilizing microorganisms and amplification of the pool of hydrocarbon-
catabolizing genes (Spain et al., 1980). Climate and seasonal changes would be expected to select different populations of hydrocarbon utilizing microorganisms that are adapted to ambient temperatures (Leahy and Colwell, 1990). According to Dott et al. (1995), microorganisms could degrade a significant portion of the hydrocarbon mixture when the environmental conditions were optimized.

According to Britton (1984), the individual organisms can metabolize only a limited range of hydrocarbon substrates. So a mixed population with a variety of microorganisms are required to degrade complex mixtures of hydrocarbons such as crude oil (Bossert and Bartha, 1984).

**Microorganisms involved in biodegradation**

Bioremediation or the cleaning up of environment is made possible by the activities of aerobic and anaerobic heterotrophic microbes. They act as scavengers and reduce pollution load in a natural ecosystem by way of their metabolic versatility that make them capable of breaking down complex organic compounds. These organisms have evolved a self mechanism to regulate and protect themselves from the chemicals and are also able to bring about many chemical and physical changes in these chemicals.

Both bacteria and fungi are relatively plentiful in soil and members of both the groups contribute to the biodegradation of hydrocarbons (Bossert and Bartha, 1984). There are several microorganisms that are able to grow on liquid n-alkane as the sole source of carbon and energy. *Mycobacteria, Pseudomonas, Nocardia, Arthrobacter, Corynebacterium Achromobacter, Acinetobacter, Bacillus, Flavobacterium* and *Micrococcus* were the predominant bacterial species (Leahy Colwell, 1990) and *Candida, Torulopsis,*
Rhodotonula, Pichia and Debaromyces were the filamentous fungi that could grow on n-alkane. Hydrocarbon degrading Aspergillus and Penicillium spp. have been frequently isolated from both environments (Leahy and Colwell, 1990). Trichoderma and Mortierella spp. are the most common soil isolates while Aureobasidium, Candida, Rhodotorula, sporobolomyces spp. are the common marine isolates (Leahy and Colwell, 1990). Kirk and Gordon (1988) reported Corolospora, Dendryphiella, Lulworthia and Varicosporina, the marine organisms to be hydrocarbon degrading.

Strains of the genus Acinetobacter, capable of utilizing alkanes with C chain lengths ranging from 10 to 44 have been described by Makula et al. (1975) and Sakai et al. (1994). Bacteria that degraded PCB’s were Pseudomonas, Alcaligenes, Arthrobacter and Acinetobacter (Cockerham and Shane, 1994). The name of the bacterium, Gordonia alkanivorans which was a rubber degrading strain was proposed due to its ability to use alkanes as a carbon source (Kummer et al., 1999).

Enzymes involved in biodegradation

Because of the lack of water solubility and large size of the polymer molecules the microorganisms are unable to transport the polymeric materials directly into the cells where most biochemical processes takes place. So they are forced to produce certain enzymes which could penetrate the polymer and degrade them into smaller transportable molecules. These enzyme systems play an effective role in the successful metabolism and detoxification of the pollutants that get in to the environment.

The enzymes may be extra-cellular or intra-cellular and their mode of action may differ. Exoenzymes from the organisms, first
break down the complex polymers giving short chains that are small enough to permeate through the cell walls to be utilized as carbon and energy sources.

Enzymatic degradation occurs by a catalytic process. It depends on the substrate due to its specificity. The enzyme can be rendered inactive (denatured) very quickly by varying the pH, temperature, or solvent. Some enzymes require other enzymes (coenzymes) to be present in order to be effective, in some cases forming association complexes in which the coenzyme acts as a donor or acceptor for a specific group.

Hydroxylases or Oxygenases are the enzymes mainly involved in biological oxidation. Oxygenases enzymes are of two types - Dioxygenase and Monoxygenase. Hydroxylases are called Monooxynases when they catalyse the insertion of a single oxygen atom in the substrate as part of a hydroxyl group. The Monooxynases require a second reduced substrate which simultaneously undergoes oxidation.

The dioxygenases catalyze the insertion of a whole oxygen molecule into the substrate. Sometimes the outcome is a dihydroxy derivative, but more often the oxygen atoms are incorporated as a part of carbonyl (CO-) or a carboxyl (-COO-) grouping.

Another type of enzyme in the biological oxidation is Oxidase, where the oxygen molecules function as a hydrogen acceptor instead of incorporating themselves into the substrate.

The initial steps in the catabolism of cyclic (Perry, 1979), aliphatic (Singer and Finnerty, 1984) and aromatic (Cerniglia, 1984) hydrocarbons by bacteria and fungi involve the oxidation of the
substrate by oxygenases, for which the molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment.

Two species of pseudomonads, *P. aeruginosa* and *P. mendocina* which utilize C$_{10}$ acyclic isoprenoids were shown to contain the inducible enzyme geranyl-coenzyme A carboxylase, one of the unique enzymes in the isoprenol degradative pathway known to occur in *P. citronellolis* (Cantwell *et al.*, 1978). Actinomycete cellulosases and hemicellulases exhibit a strong preference for neutral to alkaline pH conditions (McCarthy 1987). A thermophilic actinomycete *Microbipora bispora* appears to be a strong cellulolytic strain (Yablonsky *et al.*, 1988, Ball and McCarthy, 1988). The two fungal enzymes isolated from *Candida tropicalis*, named alk I and alk II, that metabolize alkanes have been assigned to a new family designated as P$_{450}$ LII (Sanglard *et al.*, 1987; Sanglard and Loper, 1989). Like bacteria but not fungi, B-xylosidase activity appears to be cell associated and multimeric in actinomycetes (Bachmann and McCarthy, 1991). Extracellular peroxidase activity is responsible for lignin depolymerisation in *Phanerochaete chrysorium* (McCarthy and Williams, 1992).

There are several bacterial enzymes reported to be responsible for the aerobic degradation of alkanes such as dioxygenase (Maeng *et al.*, 1996), monooxygenase (Hamamura *et al.*, 2001) and cytochrome P450 (Maier *et al.*, 2001). Among the hemicellulose degrading enzymes the most important one is endoxylanase and multiple forms of this enzyme are secreted by microorganisms (McCarthy and Williams, 1992). The degradation of a complex and highly interactive polymeric substrate such as lignocellulose demands the cooperative
activity of a range of hydrolytic and oxidative enzymes (McCarthy and Williams, 1992).

*Nocardia* strain CF8 which grow on alkanes ranging from C\textsubscript{2} to C\textsubscript{16} was found to possess a copper-containing monooxygenases which was usually present in ammonia oxidizers and methanotrophs (Hamamura and Arp, 2000). It was suggested that, Laccase enzyme isolated from the white rot basidiomycete *Trametes trogii* which was able to degrade nitrobenzene and anthracene and it was suggested that it could be implicated in the degradation of these xenobiotic compounds (Levin et al., 2003). Biphenyl dioxygenase (BPDO) catalyzes the aerobic transformation of biphenyl and various polychlorinated biphenyls (PCBs). (Gomez-Gil et al., 2007).

**Mechanism of biodegradation**

Some microorganisms have the naturally occurring microbial catabolic diversity to degrade, transform or accumulate a wide range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pharmaceutical substances, radio nuclides and metals. Major methodological breakthroughs in microbial biodegradation have enabled detailed genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms and have also provided unprecedented insights into key biodegradative pathways as well as the ability of microorganisms to adapt to the changing environmental conditions.

 Mostly, hydrocarbons are hard to dissolve in water and thus their uptake is a difficult task for the microorganisms. According to Fewson (1988), recalcitrance of a compound escalate with the rise in branching and polymerization. The biodegradation of saturated
hydrocarbon depends on the availability of molecular oxygen, while anaerobic metabolism of unsaturated hydrocarbons is subjected to the hydration of double bond.

According to Britton (1984) and Singer and Finnerty (1984), the initial attack on alkanes occur by enzymes that have a strict requirement for molecular oxygen i.e. monooxygenases or dioxygenases. In the first case, (Equation 1) one atom of O$_2$ is incorporated into the alkane, yielding a primary alcohol. The other is reduced to H$_2$O, with the reduced form of nicotinamide dinucleotide phosphate (NADPH) serving as electron donor.

\[ \text{R-CH}_2\text{-CH}_3 + \text{O}_2 + \text{NaDPh} \rightarrow \text{R-CH}_2\text{-CH}_2\text{-OH} + \text{NADP+H}_2\text{O} \]

In the second case, (Equation 2) both atoms of O$_2$ are transferred to the alkane, yielding a labile hydroperoxide intermediate that is subsequently reduced by NADPH$_2$ to an alcohol and H$_2$O (Equation 3) (Atlas and Bhartha, 1998).

\[ \text{R-CH}_2\text{-CH}_3 + \text{O}_2 \rightarrow \text{R-CH}_2\text{-CH}_2\text{-OOH} \]

\[ \text{R-CH}_2\text{-COOH-OOH + NaDPh} \rightarrow \text{R-CH}_2\text{-CH}_2\text{-OH} + \text{NADP+H}_2\text{O} \]

The oxidation of n-alkanes by dioxygenases to the corresponding hydroperoxides followed by reduction to the corresponding alkane -1-ol is reported by Watkinson and Morgan (1990) which is less common.

Most frequently, the initial attack is directed at the terminal methyl group, forming a primary alcohol that is further oxidized to an aldehyde and fatty acid. Occasionally, both terminal methyl groups are oxidized in this manner, resulting in the formation of a dicarboxylic acid. Once a fatty acid is formed, further catabolism occurs by the β-oxidation sequence (Atlas and Bhartha, 1998).
Lead Better and Foster (1959) also showed that the initial oxidation is usually accompanied by the incorporation of molecular oxygen. The initial hydroxylation to the corresponding alcohol is normally followed by two NAD linked dehydrogenation steps to the corresponding acid after which it undergoes the β-oxidation pathway.

\[
\text{CH}_3 (\text{CH}_2)_n \text{CH}_2 \text{OH} \rightarrow \text{CH}_3 (\text{CH}_2)_n \text{CHO} \rightarrow \text{CH}_3 (\text{CH}_2)_n \text{COOH}
\]

Although oxidation on C₁ is the usual mechanism for alkane utilization, diterminal α, ω-oxidation has also been reported to give the corresponding dicarboxylic acid. Another possibility is oxidation in the 2nd position to give the corresponding secondary alcohol and ketone.

According to Britton (1984), the alkenes can be metabolized through four major pathway (1) through oxygenase attack on the terminal methyl group to form the corresponding aldehyde and acid, (2) through oxidation across the double bond to form the epoxide, (3) through subterminal oxygenase attack to produce the corresponding alcohol and acid and (4) through epoxidation across the double bond to form the diol.

The best-characterized system for alkane degradation is the Alk system of Pseudomonas putida GPo1 (van Bailen et al., 1994), sequentially converting alkanes to the corresponding alcohols, aldehydes, carboxylic acids, and acyl-coenzyme A's (CoAs), which then enter the β-oxidation pathway. Most of these systems catalyze the degradation of relatively short-chain alkanes, and very little is known about enzymes involved in the degradation of long chain alkanes.
Analytical methods used in the study of biodegradation

The biodegradation and the analysis of the biodegradation products can be monitored by the application of many analytical techniques. The most important techniques include Gas chromatography (GC), Mass spectroscopy (MS), Fourier Transform Infrared spectroscopy (FT-IR) and TOC analysis.

1. Gas Chromatography (GC)

In GC analysis, the components in a sample are separated to give a representative spectral output. The GC instrument vaporizes the sample, separates and analyzes the various components. Each component ideally produces a specific spectral peak that is noted on a paper chart or is recorded electronically. The time elapsed between injection and elution is called the "retention time." As the retention time varies in different compounds, it is easier to differentiate each compound. The size of the peaks is proportional to the quantity of the corresponding substances in the specimen analyzed.

2. Mass Spectroscopy (MS)

Mass spectroscopy is an analytical tool used for measuring the molecular mass of a sample. It also provides the structural information of the compound. This procedure is useful for the structural elucidation of organic compounds and for peptide or oligonucleotide sequencing.

3. Fourier Transform Infrared Spectroscopy (FTIR Analysis)

Fourier Transform Infrared Spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials, both organic and inorganic. It is used to identify unknown materials present in a specimen. The FTIR spectrum is equivalent to the "fingerprint" of the material.
The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies which are characteristic to that molecule. During FTIR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the infrared rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks. The resulting FTIR spectral pattern is then analyzed and matched with known signatures of identified materials in the FTIR library.

Infrared Spectroscopy measures adsorptions of vibrating molecules and yields information about molecular structure and structural interactions (Alben and Fiamingo, 1984).

4. Total Organic Carbon (TOC) Analysis

Total organic carbon (TOC) analyzers measure the amount of total organic carbon present in a liquid sample. In this technique, a liquid sample is initially introduced to an inorganic carbon (IC) removal stage, where acid is added to the sample. At this point, the IC is converted into carbon dioxide gas that is stripped out of the liquid by a sparge carrier gas. The remaining inorganic carbon-free sample is then oxidized and the carbon dioxide generated from the oxidation process is directly related to the TOC in the sample.

In the present study on biodegradation, gas chromatography (GC) has been used to separate and analyse the degradation products of latex. Mass spectroscopy (MS) has been used along with the GC to monitor the molecular weight of the products and Fourier Transform Infrared spectroscopy (FTIR) has been used to confirm the chemical nature of the products. Total organic carbon in medium after the biodegradation process was measured with the help of TOC Analyser.
Introduction

Biological treatment of waste water

The water quality of most of the main rivers is declining. Sewage discharge without sufficient treatment has become the main source of organic impurities in water (Jin et al., 2005). In recent years, environmental concerns coupled with stringent governmental regulation have prompted research on the environmental compatibility of industrial effluent.

Waste water treatment is the process by which impure and unhygienic water is cleansed by removing the contaminants from the water, mainly through reduction of biological oxygen demand and chemical oxygen demand, so that the treated water could meet the acceptable quality standards. Biological oxygen demand (BOD) is the measure of the amount of organic carbons that bacteria can oxidize. Chemical oxygen demand (COD) is the measure of oxygen requirement of a sample that is susceptible to oxidation by strong chemical oxidant. It is mainly the large amounts of waste discharged from industries, factories, etc with high levels of BOD and COD that cause contaminations in the water bodies. Most of the industrial waste generated in small cities come from small scale operations and these are disposed off along with city refuse.

The release of hydrocarbons into aquatic environments which contain low concentrations of inorganic nutrients often produces excessively high carbon/nitrogen or carbon/phosphorus ratios, or both which are unfavourable for microbial growth (Atlas, 1981). According to Boethling and Alexander (1979), the rates of uptake and mineralisation of many organic compounds by microbial populations in the aquatic environment are proportional to the concentration of the compound.
Usually waste water treatment takes place in three stages—viz. primary secondary and tertiary. In the primary treatment, suspended solids that might decrease the efficiency of subsequent treatment processes are removed from the waste water stream. This removal is achieved in settling tanks or basins, where the solids are drawn off from the bottom. In a secondary sewage treatment which mainly depends on the microbial activity, a smaller portion of the dissolved organic matter is mineralised and a larger portion is progressively converted into a solid mass which can be removed later. The combination of primary and secondary treatments usually reduces the original COD and BOD of the sewage by 80-90%. In the tertiary treatment, nitrates, phosphates and other pollutants, which may still remain in the water, are removed by using advanced techniques.

Biological waste water treatment which is carried out with the help of microbial activity is mainly of three types; aerobic, anaerobic and facultative. Aeration is essential for the aerobic process since it needs free oxygen. The rate of cleansing depends on the concentration of the dissolved oxygen. The anaerobic treatment process which takes place in the absence of oxygen consists of liquefaction and gasification of the organic compounds. In the facultative process, the organisms can grow in both aerobic and anaerobic conditions.

In suspended growth process, organic matter in the waste water gets converted to gases and the cells are maintained in suspension with liquid. The principal suspended growth treatment process is the activated sludge process. It is a system in which flocculated biological growth is continuously circulated and contacted with organic waste in the presence of oxygen. The process involves
an aeration step followed by a solid-liquid separation step from which the separated sludge is recycled back for admixture with the waste.

An extended aeration process is required when sufficient land area is not available. It is a modification of activated sludge process and consists of an aeration tank, a clarifier thickener and a recycling line. Oxidation ditch is a modified form of activated sludge process and is of extended aeration type. In this process, the raw waste is fed into the system without any pretreatment. For small flows of sewages and industrial wastes, it is proved to be an economical method.

Aerobic attached growth treatment, which is also called fixed film process, includes trickling filter, roughing filter, rotating biological contactor and fixed bed reactor.

A trickling filter or percolating filter consists of a circular structure containing a bed of sustainable crushed stones, crushed slag or any hard and insoluble medium. These packing beds provide the habitat for the biological filter which will oxidize the applied load of pollutants. Roughing filter have the same biological action as that of trickling filter. It is used to reduce the organic loading on down stream process and in seasonal nitrification application. Rotating biological contactor (RBC) consists of a set of disc of polystyrene or polyvinyl chloride on rotating shaft which is just above the liquid level in a half cylinder. Packed bed reactors consist of continuous reactions that are packed with a medium to which the microorganism can become attached.

In Anaerobic suspended growth treatment process, an initial breakdown to organic acids is followed by fermentation of the organic acids to methane and CO₂. It includes anaerobic digestion where there is the decomposition of the organic matter in the absence of molecular
oxygen. In the anaerobic contact process, untreated wastes are mixed with recycled sludge solids and then digested in a reactor sealed to the entry of air.

Stabilization ponds are the ponds in which the stabilization of waste is brought about by a combination of aerobic, anaerobic and facultative bacteria. The major types of the treatment process are aerated lagoons, anaerobic lagoons and waste stabilization ponds.

Natural rubber latex

Natural rubber is a polymer which have been found in the latex of over 895 species of plants belonging to 311 genera of 79 families. But it is synthesized in large amounts from the rubber tree, Havea brasiliensis belonging to the family Euphorbiaceae, for the commercial purpose.

At present, more than 9.5 million hectares in about 40 countries are devoted to rubber tree cultivation with a production about 6.5 million tons of dry rubber each year. The world supply of natural rubber is barely keeping up with a global demand for 12 million tons of natural rubber in 2020 (Wang, 2007).
Plate-1. Rubber plantations (*Hevea brasiliensis*)

The minor sources of natural rubber latex are *Manihot glaziovii*, *Ficus elastica*, *Parthenium argentatum* and *Taraxacum koksaghys* of which *Parthenium argentatum* is now gaining attention due to the production of guayule rubber (Narayan, 1992). Gutta percha is a trans isomer of the polyisoprene and is synthesized by much fewer plants of which the major one is *Palaquium gutta*. (Warneke *et al.*, 2007).

*Hevea brasiliensis*, commonly known as the rubber tree is a native of Brazil which was introduced to tropical Asia in 1876. The tree is now grown in the tropical regions of Asia, Africa and America. The rubber tree is sturdy, quick growing and tall. A warm, humid, equable climate (21°C-35°C) and a fairly distributed annual rainfall of not less than 200cm are necessary for the optimum growth of this plant. Natural rubber is an elastic hydrocarbon polymer that naturally occurs as a milky colloidal suspension, or latex, in the sap of a rubber tree.
Rubber tapping is a process of “controlled wounding” by which the rubber latex is gathered, where, thin shavings of bark are removed and the fluid that drains is collected into a container attached to it. Coconut shells and polythene cups are popularly used as containers in the plantations of India (Narayan, 1992).

Each time a rubber tapper must remove a thin layer of bark along a downward half spiral on the tree trunk. If done carefully and with skill, this tapping panel will yield latex for up to 5 years. Then the opposite side will be tapped allowing this side to heal over. The spiral allows the latex to run down to the collecting cup.

Plate-2. Tapping of natural rubber latex from *Hevea brasiliensis*
Introduction

Plate-3. Latex coming out from the rubber tree

Production of latex is confined to the latex vessels which exclusively occur in the phloem region. Natural rubber latex is a white or slight yellowish opaque liquid with a specific gravity of about 0.92. Latex is a negatively charged colloidal dispersion of rubber particles in an aqueous serum. The dominant particulate phase of freshly collected latex is the rubber hydrocarbon. The two main particulate phases contained in hevea latex are rubber particles and lutoid particles. Rubber particles are usually spherical but can also be oval or pear shaped and it constitute 30-45 % of the whole volume. They are strongly protected in suspension by a film of adsorbed protein and phospholipids. 10-20% volume of the latex constitutes lutoid particles. They are subcellular membrane bound bodies ranging in size from 2-5 μ. The membrane encloses a fluid serum referred to as lutoid serum. It is a stabilizer of rubber hydrocarbons. In young latex vessels, lutoids contain suspension of helical protein called microfibrils. There are certain other kinds of substances called frey wyssling particles. They are spherical, usually larger in size than rubber and yellow in colour. During the tapping, the lutoid is damaged and it releases a protein
named 'hevein' that present in the lutoids. It was found to be responsible for the coagulation of latex, (Gidrol et al., 1994) It forms a cross link between rubber particles resulting in coagulation of latex at the cut ends of latex vessels.

**Biosynthesis of natural rubber latex**

In *Hevea brasiliensis*, the synthesis of latex takes place within the latex vessels. Sugar is first broken down to acetyl coenzyme A (acetyl-S-CoA) by a series of reaction which occurs in virtually all cells. This substance which is a thiol ester of coenzyme A and acetic acid, is then converted to isopentenyl pyrophosphate by the reaction sequence outlined below (where phosphate is represented by OP and pyrophosphate by OPP).

\[
\begin{align*}
\text{Acetyl-CoA} & \rightarrow \text{Acetoacetyl-CoA} & \beta \text{ hydroxy-\beta-methylglutaryl-CoA} \\
\text{Mevalonic acid} & \rightarrow \text{Mevalonic acid} & \text{Mevalonic acid} \\
\text{5-Pyrophosphate} & \rightarrow \text{Mevalonic acid} & \text{5-Phosphate} \\
\text{Mevalonic acid} & \rightarrow \text{Isopentenyl Pyrophosphate} & \text{(1)}
\end{align*}
\]

The next step is the formation of isoprenoid compounds, the isomerization of isopentenyl pyrophosphate to dimethylallyl pyrophosphate (eq. 2).
Dimethylallyl pyrophosphate is a strong electrophilic reagent and can attack the terminal methylene group of isopentenyl pyrophosphate as shown below:

\[
\begin{align*}
\text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP} & \text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP} \\
\text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP} & \text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP} \\
\text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP} & \text{CH}_3\text{C} &= \text{CH}\text{CH}_2\text{OPP}
\end{align*}
\]

......(2)

The C\(_{10}\) compound so formed is also an electrophilic reagent and can react with another molecule of isopentenyl pyrophosphate in a similar fashion to give a C\(_{15}\) compound, which in turn, can form a C\(_{20}\) compound and so on.

**Physical properties of natural rubber:**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity</td>
<td>0.92</td>
</tr>
<tr>
<td>Refractive index</td>
<td>1.52</td>
</tr>
<tr>
<td>Coefficient of cubical expansion</td>
<td>0.00062/°C</td>
</tr>
<tr>
<td>Cohesive energy density</td>
<td>63.7 Cal/cc</td>
</tr>
<tr>
<td>Heat of combustion</td>
<td>10.7000.00 Cal/gm</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>0.00032 Cal/sec-cm(^2/°C)</td>
</tr>
<tr>
<td>Dielectric constant</td>
<td>2.37</td>
</tr>
<tr>
<td>Power factor (1000 cycles)</td>
<td>0.15 - 0.2</td>
</tr>
<tr>
<td>Volume resistivity</td>
<td>10(^{15})/ohm.cm.</td>
</tr>
<tr>
<td>Dielectric strength</td>
<td>1000 volts/Mil</td>
</tr>
</tbody>
</table>
Chemistry of Natural Rubber latex

Natural rubber is a high molecular weight polymeric substance with visco elastic properties. Rubber exhibits unique physical and chemical properties. Apart from a few natural product impurities, natural rubber is essentially a polymer of isoprene units, a hydrocarbon diene monomer. Synthetic rubber can be made as a polymer of isoprene or various other monomers. The material properties of natural rubber make it an elastomer and a thermoplastic.

The natural rubber is composed of a linear polymer chain which is highly cross linked to form a network. Chemically, the natural rubber is cis 1, 4 poly isoprene in which individual isoprene molecules are formed by 1, 4 addition. It is having a molecular formula \((\text{C}_5\text{H}_8)_n\). Each isoprene unit contains one double bond in the cis configuration. It has an average molecular mass about \(10^6\) Da. About 90% of the dry weight of natural rubber latex is the hydrocarbon and the rest is the non rubber contents like proteins, resins, fatty acids, sugars and minerals.

\[
\text{Isoprene unit} \quad \xrightarrow{\text{Polyisoprene}} \quad \text{Polyisoprene}
\]

The composition of the latex varies with respect to the clone, seasonal effects and the state of the soil (Narayan, 1992). The average composition of latex is as follows:

<table>
<thead>
<tr>
<th>Composition of natural rubber latex</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyisoprene</td>
<td>25-35%</td>
</tr>
<tr>
<td>Proteins</td>
<td>1-1.8%</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>1-2%</td>
</tr>
</tbody>
</table>
Neutral lipids - 0.4-1.1%
Polar lipids - 0.5-0.6%
Inorganic components - 0.4-0.6
Amino acids and amides - 0.4%
Water - 50-70%

Fresh latex, as it comes from the tree, is slightly alkaline or neutral. It turns acidic rapidly, due to bacterial action. The formation of organic acids neutralizes the negative charge on rubber particles and the latex gradually gets coagulated on storage. To prevent the coagulation, anti coagulants such as ammonia, sodium sulphite and formalin are used. Ammonia is the most popular latex preservative.

At high temperatures natural rubber is plastically ductile and useful for the production of elastomers. Raw rubber is soft and tacky; its tensile strength and abrasion resistance is low. When the linear polymer chain is cross linked with sulphur bridges by the process vulcanization, it acquires superior physical properties such as tensile strength, tear strength and heat build-up resistance.

When the natural rubber latex is ultra centrifuged, it separates into three layers. The upper white layer comprises the rubber particles. The middle aqueous serum includes amino acids, proteins, carbohydrates, organic acids, inorganic salts and nucleotidic materials. The bottom fraction consists mainly of lutoid particles, frey wyssling particles, mitochondria and other particulate components of normal plant cells having a density greater than that of serum.

It was reported that latex from Hevea brasiliensis is having antifungal properties. The strongest antifungal effect was obtained with
*Trichosporon cutaneum* and *Cryptococcus neoformans* (Giordani and Bue, 2002).

**Processing of natural rubber latex**

Raw latex that obtained from the rubber tree is transformed into several forms by processing for marketing. The different marketable forms are Processed latex and latex concentrates, Ribbed sheet rubber, Crepe rubber and Technically specified block rubber.

![Diagram of processing of natural rubber latex](image-url)
Rubber and non-rubber content in lattices vary according to season, soil and atmospheric conditions, clone, tapping system, etc. the quantity of rubber present in the latex is calculated from its dry rubber content (drc). It can be defined as the quantity in g of rubber present in 100g of latex. So the latex received in a processing factory is weighed and the dry rubber content is estimated as a preprocessing operation. The drc of the latex usually falls in the range of 30-40. The dry rubber content is estimated by standard laboratory method in which latex is coagulated, rolled to a thin sheet, drying and weighing or by the dipper method or with a metrolac which is a special type of hydrometer calibrated to read drc directly.

Preserved latex concentrates are generally marketed in two concentrations: latex between 36 and 56% dry rubber content (drc) and latex between 51 and 60% drc. Certain substances like preservatives which prevent the bacterial action, anti coagulant which prevent precoagulation, were added to the latex prior to processing. Ammonia is the widely used preservative while ammonia, sodium sulphite and formalin are used as anticoagulants.

Field latex preserved with a suitable preservative is termed as preserved field latex. The processing of field latex consists of adding the preservatives to the latex, bulking, settling and blending into consignments of suitable size for dispatch. The whole crop is treated in a large reception tank from which it is transferred to a bulking tank and kept undisturbed for a day. After the removal of sediments, the latex is thoroughly but gently mixed.

Preserved latex concentrates is having good market value as it is an important raw material for the manufacturing of several
products. Concentration of latex is mainly by the processes such as evaporation, creaming, centrifugation and electro decantation.

Evaporation method involves the removal of water only. Creaming is a process by which the latex is separated into two layers, an upper layer of concentrated latex and a lower layer of serum containing very little rubber by adding a creaming agent such as ammonium alginate or tamarind seed powder. The lower layer of serum (skim) is removed, leaving the latex concentrate which is then tested, packed and marketed.

Centrifugation is the process by which the preserved field latex was separated into two fractions, one containing concentrated latex 50%-60% dry rubber and the other containing 5-10% dry rubber by a suitable centrifuge machine. The rotating bowl of the centrifuge is fed continuously with latex which results in the continuous collection of concentrated latex that can be drawn out through an outlet at the centre and serum fraction (skim latex) near the circumference from where it can be withdrawn through another outlet. During centrifugation the centrifugal force replaces the gravitational force which bring about separation of rubber particles. The skim fraction is generally coagulated with sulphuric acid, creped, dried and marketed as skim rubber, which is a low grade rubber and not used in the manufacture of products which require good service properties (Radhakrishnapillai, 1980).

Processing of latex into ribbed sheet rubbers involves the coagulation of latex in suitable containers into thin slabs of coagulum and sheeted through a set of smooth rollers followed by a groove set, and dried to obtain ribbed sheet rubbers. Sheet rubbers are
classified into ribbed smoked sheets and the air dried sheets depending on the drying method.

Acetic acid or formic acid is generally used for coagulation of latex. Coagulum from latex often shows a tendency for surface darkening. To prevent this, once the latex sets into coagulum, a small quantity of sodium bisulphate (1.2gm/ kg drc) dissolved in water may be sprinkled over the surface of the coagulum. After coagulation the coagulum is removed from the pans or tank and thoroughly washed in the running water.

The sheets after two or three hours dripping in shade are put in the smoke house where the temperature is maintained between 40-60\(^\circ\) C. Sheets are dried gradually in the smoke house where by blisters are avoided.

Air dried sheets are light coloured sheet prepared in the same way like ribbed smoked sheets but dried in a sheet or tunnel in hot air instead of smoke. Sodium bisulphate which inhibits enzymatic decolouration and lightens the colour of the sheets is generally added into the latex.

When coagulated latex, or any form of field coagulum is passed several times through a minimum of 3 mills with heavy rolls, crinkly lace like rubber will be obtained which when air dried is called crepe rubber. There are different types of crepe rubbers according to the type of starting material.
<table>
<thead>
<tr>
<th>Type of crepe rubber</th>
<th>Starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-coagulated crepe</td>
<td>Field latex</td>
</tr>
<tr>
<td>Soil crepe</td>
<td>Pre coagulated thin crepe</td>
</tr>
<tr>
<td>Pale latex crepe</td>
<td>Fresh coagulum produced from natural rubber latex</td>
</tr>
<tr>
<td>Estate brown crepe</td>
<td>Cuplumps and other higher grades of field coagulum</td>
</tr>
<tr>
<td>Rilmilled crepe</td>
<td>Wet slab coagulum, unsmoked sheets and cuplumps</td>
</tr>
<tr>
<td>Smoked blanket crepe</td>
<td>Smoke rubber derived exclusively from ribbed smoked sheets or cuttings</td>
</tr>
<tr>
<td>Flat bark crepe</td>
<td>All types of low grade scrap including earth scrap</td>
</tr>
</tbody>
</table>

Latex which gets dried upon the tapping panel (tree lace) and the collection cups (shell scrap) and the latex that is overflowed on the ground (earth scrap) are also collected as scrap. For the safe storage and marketing they are processed into several forms.

Technically specified rubber (TSR) are largely block types made by adopting new methods of processing for size reduction, dewatering, dirt removal, drying and grading of which the first three processes are accomplished together. Drying of the crumbs, pellets or granules produced for the manufacture of block rubbers is carried out at a temperature of about 100°C, usually for 4-8 hours. The dried crumb, pellets or granules are pressed when they are warm (60°C) in a hydraulic press 30 to 50 tonne presses are generally used for the purpose. Bales not exceeding 50kg are prepared. Then they are tested for the dirt content, ash content, volatile matter, nitrogen content, initial wallace plasticity, plasticity retention index and colour. Based on the test results
the grading is made. The bales are then wrapped in polythene films and packed in HDPE bags and marketed.

The viscosity of natural rubber increases on storage and this phenomenon of storage hardening can be prevented by using either hydrazine hydrate or hydroxylamine hydrochloride where by retaining the original viscosity for long periods and such rubbers are termed constant viscosity rubbers. Addition of hydroxylamine at 0.15% produces low viscosity rubber (Mooney viscosity 60-65) while treatment of latex with 0.15% hydrazine hydrate produces a high constant viscosity rubber (Mooney viscosity 85-90).

The viscosity of rubber can be brought to a low range by the addition of a fixed quantity of plasticiser (a non staining naphthenic rubber process oil) to the viscosity stabilized rubber. Rubber, the viscosity of which is reduced to a low range is termed as low viscosity rubber. Both constant viscosity (CV) and low viscosity (LV) rubbers are being produced in India in limited quantities.

Oil is added to the latex as an emulsion and then coagulated with acid in the processing of latex into oil extended rubber. The coagulum so obtained is then processed as block rubbers.

The processing of tyre rubber is similar to the other forms of block rubbers. The principles involved in the new form of processed rubbers are reduction in the cost of rubber by blending lower grade scraps and latex rubber and keeping the mooney viscosity low by adding extender oils or plasticizers.

Rubber is purified inorder to remove the non rubber constituents by repeated centrifugation (Suchiva et al., 2000). Deproteinised natural rubber exhibit improved dynamic mechanical properties including heat
buildup and flex-cracking resistance. It is more suitable for medical applications than normal natural rubber as the latter contains proteins which are potential allergy causing compounds (Suchiva et al., 2000).

There are a number of types of synthetic rubber with various physical and chemical characteristics. Among the most widely used are styrene-butadiene rubbers, ethylene-propylene rubbers, butyl rubbers, acrylic elastomer, and silicone rubbers. Like natural rubber all are polymers, synthetic rubbers are sourced from various hydrocarbons, which are blended and reacted under controlled conditions to form the polymers.

**Latex as a pollutant**

Latex is extensively used today in almost every manufacturing field, but the flip side is that when it is discarded, it throws up extremely grave pollution hazards. The raw rubber materials are converted to elastic rubber products by the process of vulcanization that leads to cross-links between the elastomeric chains (Chapman and Porter, 1988). The vulcanizate has a network structure insoluble in any organic solvent (Tsuchii et al., 1985). Low heat generation properties in service are of paramount importance, along with low rolling resistance. It has high resistance to tear and abrasion and is relatively easy to process. It has excellent dynamic properties with a low hysteresis loss and good low temperature properties.

Nearly 65% of all rubber is consumed for the automobile tyres and tubes in developed countries. Significant amounts are used in engineering components, belting, hose and footwear. The rubber is used in association with metal and steel in many of the engineering components. Natural rubber is also used in the manufacturing of hoses, footwear, condoms, battery boxes, foam mattresses, balloons, toys, etc.
Introduction

The natural rubber finds extensive use in soil stabilization in vibration absorption and in road making. Waste tyres are significant problem with increasing number of automobiles. About two million tonnes of used tyres are released each year within the European Union. As the disposal of used tyres which contain 35-40% natural rubber is a major problem, recycling is one of the popular methods for the treatment of waste tyre which cannot be considered as an economical method.

Plate- 4: Accumulation of rubber wastes

Used natural rubber products like medical gloves, household gloves, condoms and balloons are also creating environmental problems to some extent. Used rubber footwear is a household litter that also adds to the pollution.

The medical gloves which are mainly used on a disposable basis for a very brief time, the large scale release of balloons, mainly as part of promotional activities, have all led to criticism by environmentalists. It has been argued that once the balloons return to the ground they disfigure the landscape, they endanger wildlife and may introduce alien chemicals to the soil. There has been a growing interest in degradation of polymers as solid
disposal including the tyres has become a major problem in the world (Roy et al., 2006).

**Toxicity of latex**

Major production of the latex finds application in the automobile industry in the manufacturing of tyres. There were a range of toxic substances present in the tyres, but these materials are tightly bound in a stable matrix which may slowly released into the environment. It depends on the sensitivity of the receiving environment and the presence of plants, animals or humans that may be affected.

Crumb rubber asphalt concrete leachates which contain a mixture of organic and metallic contaminants exhibited moderate to high toxicity for algae and moderate toxicity for water fleas (Azizian et al., 2003). Benzothiazole and 2(3H)-benzothiazolone the organic compounds used in manufacturing of tyres and the metals mercury and aluminium used in processing are later leached out in potentially harmful concentrations. They all ultimately reach the environment and may remain there as recalcitrants (De Wever et al., 2001). The other contaminants like leaching of rubber products, fine particles of automobile tyres and antifreeze compounds may also reach the rivers by urban run off. They also alter the environment and may be toxic to the aquatic systems (Santodonato et al., 1976). Although there are several reports submitted on microbial breakdown of natural rubber, there are only few reports available on the mechanism involved in the biodegradation of natural rubber latex.

**Biodegradation of natural rubber latex**

With the growing awareness of the persistence of polymeric compounds, a number of investigators have turned their attention to the study of the degradability of polymers. It was important to know the
potential for microbial degradation from both the view point of prolongation of usage and of waste disposal problems.

Accumulation of the automobile waste is becoming a major problem even in the developed nations. Recycling and safe disposal of waste rubber products have become a global concern and it has initiated research in the area of biological methods to decompose recalcitrant rubber products.

Since polymers are extremely stable, their degradation cycles in the biosphere are limited. Natural rubber is relatively resistant to microbial decomposition compared to many other natural polymers (Haisey and Papadatos, 1995). The rate and extent of microbial degradation is influenced by the rubber formulations, the microorganism present and their interaction with the environment.

Organic impurities in the rubber could support microbial growth even if the rubber hydrocarbon itself were not metabolized. It is also possible that microorganisms using impurities as their carbon and energy sources could deteriorate the rubber as a result of co-metabolism without using the rubber hydrocarbon as source of energy (Shaposhnikov et al., 1952; Cundell and Mulcock, 1975).

Linos et al., (2000) had classified the rubber degrading bacteria into two groups according to the decomposition strategies. The first group, containing the organisms that grow only in direct contact to the rubber substrate can considerably disintegrate the material during cultivation. They do not form any halos but they show relatively strong growth on polyisoprene. The second group of organism forms clear zones or translucent halos around the bacterial colonies when it was cultivated on natural rubber dispersed in mineral agar. They metabolize the
polyisoprene by secretion of one or several enzymes. The representatives of this group show relatively weak growth on the rubber surface.

The electron microscopic studies have shown that hydrocarbons are accumulated in the microorganisms as intracytoplasmic inclusions. The glycolipids in the outer portion of the cell wall form micro emulsions of the hydrocarbons so that they may move across the cell membrane (Low et al., 1992).

An oxidative cleavage of the double bond in the polymer backbone for initiation of rubber degradation has been suggested by Tsuchii and Takeda (1990) Diketone derivatives of oligo (cis1,4-isoprene) were identified as metabolites of rubber degradation (Bode et al., 2000; Bode et al., 2001).

Biotechnological methods in general present promising prospects for finding solutions to the future demands of environmentally benign rubber waste management systems.

**Microorganisms in the biodegradation of latex**

According to the previous studies, actinomycetes were almost the only organism that considerably decomposed natural rubber using the hydrocarbon as the sole carbon source (Heisey and Papadatos, 1995; Jendrossek et al., 1997; Linos et al., 1999; Rifat and Yosery, 2004; Banh et al., 2005). Actinomycetes are adapted to grow on solid substrates. Their primary carbon sources in the soil are insoluble and polymeric natural materials, necessitating the secretion of a range of extracellular enzymes as their hyphae penetrate and colonise the substrate (McCarthy and Williams, 1992).

Actinomyces and Proactinomyces have been observed as rubber oxidizing microorganisms (Spence and van Niel, 1936). Corynebacterium,
Introduction

*Mycobacterium,* and *Nocardia* (CMN group) were the most potent organisms that comes under actinomycetes that could degrade the hydrocarbon chain (Arensoketter et al., 2001; Rose and Steinbuchel, 2005). Recently, several *Gordonia* species which also belong to the CMN group such as *Gordonia polyisoprenivorans* (Linos et al., 1999), *G. alkanivorans* (Kummer et al., 1999), *G.westfalica* (Linos et al., 2002) etc were reported to be with strong rubber decomposing properties.

Degradation of natural rubber latex by two gram negative bacteria viz. *Xanthomonas* sps. (Tsuchii and Takeda, 1990) and *Pseudomonas aeruginosa* (Linos et al., 2000) were reported in previous works. The latter was able to produce holes in polymer films when the polymer is used as the sole source of carbon.

**Enzymes in the biodegradation of latex**

The hydrophilic and polar nature of enzymes present obstacles to their complexing with certain types of polymers, many of which are insoluble in water and often water repellent. The problem of solubility exist in the natural rubber latex also.

Even though there were studies on the degradation of natural rubber latex, biochemical mechanism of natural rubber degradation is still unknown. According to previous reports, it is the oxygenase enzyme that is mainly involved in the rubber degradation. Since the double bonds of cis 1, 4, polyisoprene are cleaved by incorporation of oxygen, a monooxygenase or dioxygenase based reaction mechanism is possible. According to Linos and Steinbuchel (2001), there may be a common oxidative cleavage mechanism for all types of rubber degrading organisms and proposed a metal dependent dioxygenase mechanism.

According to Tsuchii and Takeda (1990), the extracellular enzyme produced by the *Xanthomonas* strain had degraded the cis 1,4 polyisoprene
by the oxidative cleavage of the isoprene double bond which resulted in the formation of a low molecular weight oligomers having molecular weight in between $10^3$ and $10^4$ Da. Recently, an enzyme called rubber oxygenase (RoxA) was purified from Xanthomonas sp. (Braaz et al., 2005).

**Treatment of waste water containing latex**

In India, rubber is produced mainly in three states - Karnataka, Tamilnadu and Kerala. The highest quantity of rubber is produced in Kerala which account to about 90% of the total production of the country. There are 218 rubber producing industries where rubber processing are done which delivers a huge quantity of waste water to the environment. The volume of effluent from rubber processing unit is 25 to 40 times greater than the volume of rubber that is produced and these effluents are extensively polluting the water bodies. In addition, some of the chemicals that remain in the sap after the coagulation of latex can also be toxic.

The ammonia which is present in the pre-vulcanized latex is also a toxic chemical. Even a short-time exposure to ammonia can cause irritations. At concentrations between 57 and 500 ppm, 83 to 92 % of the inhaled amount of ammonia is retained in the respiratory tract.

The process of making latex concentrate involves washing, centrifugation and de-ammoniation process, often from the field latex. So the effluent from a latex processing unit may contain excess field latex effluent, stabilizers like ammonia and sodium sulphite and formaldehyde.

Skim latex, containing 2.5-10% of rubber, is obtained along with the concentrated rubber latex, as an equal fraction in volume during centrifugation of the field rubber latex (Radhakrishna pillai, 1980). The skim latex serum contains high ammonia, sulphate, organic and inorganic substrates and is the major source of pollutants in latex concentrate factory. Protein and non-rubber constituents which have specific gravities
higher than that of rubber also migrate into skim fraction during centrifugation and not only reduce the quality of rubber but also affect the coagulation process. The usual method of recovery of skim rubber is by coagulation with sulphuric acid. In acid coagulation, the acid content of the coagulated rubber reduces its quality and shows some tendency to scorch (Naunton, 1961).

Chemical, physical and bacteriological properties of effluents from different types of rubber processing factories have shown that the substances contained in them are excellent substrates for the microbial proliferation generating high biological oxygen demand and objectionable odour.

**Physicochemical treatment**

Physical processes are based on the exploitation of the physical properties of the contaminants and are generally the simplest form of treatment. Physical methods comprise, screening, sedimentation, floatation and filtration. Chemical oxidation aims at the selective removal of the more bioresistant fractions and their conversion to readily biodegradable intermediates that can subsequently be treated biologically. It can break the large polymer molecules which is difficult to permeate the bacterial cell walls into smaller intermediate compounds that can enter the cells. Thus a chemical pretreatment is preferable prior to biological treatment (Mantzavinos and Psillakis, 2004). Chemical methods utilize the chemical properties of the effluent or of the added reagents. Commonly used chemical processes are precipitation, coagulation, and disinfection. Other physical and chemical methods such as air stripping, carbon adsorption, oxidation and reduction ion exchange and membrane processes like reverse osmosis and electrodialysis are also important in certain cases (Gonzalez, 1996). Trickling filtration is the technique widely
used for purification of sewage and industrial effluents which can be biochemically oxidized (Muthuraj et al., 1973). Coupling chemical pre-oxidation with biological post treatment is conceptually beneficial as it can lead to increased overall treatment efficiencies compared with efficiency of each individual stage (Mantzavinos and Psillakis, 2004).

**Biological Treatment**

Biological treatment process utilize biochemical reactions to bring about a chemical change in the properties of a contamination of interest. The chemical properties are altered under the action of wide variety of microorganisms resulting the decomposition of the specific compound is not complete and low molecular weight compounds such as aldehydes, ketones and organic acids. As these compounds are usually of low toxicity to microbial or aquatic life they are further biodegraded under proper conditions.

Microbial treatment is given the most attention due to its environmental friendly approach and its ability to mineralize the toxic organic compound (Pripch and Duaglis, 2005). This process may be achieved aerobically in a number of ways. The most widely used aerobic process are trickling filters, rotating biological contactors (RBC), activated sludge process and their modified versions. The anaerobic process is used both in the treatment of specific waste water and in sludge conditioning (Belhateche, 1995).

The most common biological systems that use to treat rubber processing effluents are waste stabilization ponds and oxidation ditches. Microbial seeding from a chemostat has also been used in conjunction with conventional activated sludge treatment to improve the ability of a refinery waste water treatment plant to absorb intermittently high loads of hydrocarbons (Wong and Goldsmith, 1988).
1.2 REVIEW OF LITERATURE

In the past couple of decades, the use of microbial catalysts in the field of biodegradation of organic compounds has seen a tremendous growth. The microbial decomposition of waste is in fact the practical application of microbial metabolism for solving ecological problems. Solid wastes are decomposed by microorganisms in landfills and by composting, whereas liquid waste treatment uses microbes to degrade organic matter, thereby reducing the chemical oxygen demand (COD) as well as the biological oxygen demand (BOD).

Large numbers of microbes are found to be co-existing in almost all natural environments, particularly in soil, water, and sewage. The intensity of biodegradation is influenced by several factors such as nutrients, oxygen, pH, composition, concentration and bio-availability of the contaminants, their chemical and physical characteristics and the pollution history of the contaminated environment (Schinner and Margesin, 2001).

1.2.1. Biodegradation of organic compounds

Hydrocarbons are released into the environment mainly through petrochemical industries, plastic industries, pesticide industries, paint industries and the wood preservation industries. They may be aliphatic, aromatic or alicyclic of which the latter is more resistant to biodegradation (Leahy and Colwell, 1990).

It is true that these organic compounds on a long time exposure to the natural environment is subjected to microbial degradation. However, the hydrocarbons differ in their capacity to serve as microbial substrates. n- alkanes of the C_{10}-C_{22} range are the most readily and frequently utilized hydrocarbon substrates. The physical characteristics of
n-alkanes above \( \text{C}^{22} \) are not favourable for biodegradation because at physiological temperatures they are solids with extremely low water solubility (Bartha and Atlas, 1987).

Biodegradability of aliphatic hydrocarbons increases with decreasing saturation and increasing reactivity. Branching decreases biodegradability as it creates multiple carbon bonds that interfere with the biodegradation (Bossert and Bartha, 1984). Single substituent groups on benzene affect the degradation in the order (COOH or OH), \( \text{NH}_2 \), OCH\(_3\), \( \text{SO}_2\text{H} \), NO\(_2\) (increasing persistence). Meta substitution for halogens on phenol causes the greatest persistence. Ortho- and para-substitution of halogens has less effect. Increasing amounts of chlorination or bromination in a molecule increases its persistence.

Poly aromatic hydrocarbons (PAHs) released into the environment may originate from many sources, including gasoline and diesel fuel combustion (Lim et al., 1999 and Marrh et al., 1999) and are difficult to degrade. *Sphingomonas yanoikuyae* was identified to be an organism that could act on PAH (Khan et al., 1996). Biodegradability of PAHs depends on the number of fused rings, number and position of substituents of the rings and degree of ring saturation (Cerniglia, 1992).

Microorganisms are present in very large number in the environment. They can grow very fast and can change their phenotypic composition more readily than higher organisms. They could use any conceivable substrate and almost every energy source such as carbon, nitrogen, phosphorus and sulfur. In addition to these factors, the wide tolerance range of environmental factors and elements make the microorganism capable of executing their degradative activity.

Biodegradation, the microbially catalysed reduction in the complexity of chemicals depends mainly on the environmental factors
and microbial characteristics. The environmental factors affecting microbial activity include physical factors like pH, temperature, osmotic pressure, salinity, moisture and the chemical factors like nutrient status, oxygen concentrations, presence of toxic compounds, levels of carbon dioxide and other gases (Weston et al., 1988).

The metabolism of the organic compounds by microorganisms takes place through an oxidative or reductive process. In the aerobic oxidative metabolism, the enzymatic action of monooxygenases and dioxygenases results in the production of carbon dioxide and water as end products, where oxygen is the preferred electron acceptor as it gives the highest energy yield (Burns and Dick, 2002). In the absence of oxygen, bacteria utilize other electron acceptors like nitrate, manganese, iron, sulphate and carbon dioxide. Sometimes, the organisms view the xenobiotics itself as another electron acceptor choice.

Long chain aliphatic hydrocarbons are converted into fatty acids by oxidation of a terminal carbon to an alcohol, then an aldehyde, then to a carboxylic acid. The fatty acids thus generated are activated with CoA and simply fed into the normal pathway for fatty acid degradation - beta oxidation (Klug and Markovetz, 1971). These reactions yield 2-carbon acetyl-CoA units that can be consumed in the citric acid cycle.

Complex aromatic compounds are first converted to a "starting substrate" such as catechol. Formation of catechol may involve the introduction of oxygen atoms by a monooxygenase. A dioxygenase breaks open the aromatic ring of catechol, producing cis-muconate, an unsaturated dicoxylic acid. This product is then oxidized to acetyl-CoAs by the beta oxidation pathway. Substitution of aromatic rings (e.g. with -CH₃ or -Cl) interferes with the oxic degradation of the aromatic ring.
In anaerobic environments some low chlorinated compounds are considered to be very persistent. This is because the initial step in activation of these compounds requires incorporation of oxygen through mono- or dioxygenases. (Per)chlorate reduction is unique type of anaerobic respiration which produces the molecular oxygen in anaerobic environments. The oxygen formed may be incorporated into the anaerobically persistent compounds and activate it.

The process in which a complex community of microorganisms is established on a surface is known as microfouling or formation of biofilm. Biofilms, consisting of both microorganisms and their extracellular polysaccharides, are highly diverse and variable in both space and time (Gerkhe et al., 1998). Bacteria inhabiting biofilms usually produce one or more polysaccharides that provide a hydrated scaffolding to stabilize and reinforce the structure of the biofilm. It mediate cell-cell and cell-surface interactions and provide protection from biocides and antimicrobial agents (Jackson et al., 2004).

Degradability of a polymeric materials depends on their structures, presence of degradative microbial populations, and the environmental conditions that encourage microbial growth (Gu, 2003). More specifically, it depends on the material composition, molecular weights, atomic composition and the chemical bonds in the structure and the physical and chemical characteristics of the polymeric surfaces (Caldwell et al., 1997).

1.2.2. Microorganism capable of biodegradation

Microorganism especially bacteria live in all possible environments because of their size and few other characteristics which enable them to survive in any environment which is inhospitable to other living organisms.
*Pseudomonas* spp. is the predominant organism among the microbes that utilize hydrocarbons. *Mycobacterium, Nocardia,* some yeasts and molds constitute the other organism that make hydrocarbon as their food. *Rhodococcus* spp. was also reported to have the metabolic potential to degrade a variety of natural and man-made hydrocarbons (Larkin *et al.,* 1998).

There were several reports on the bacterial strains that degrade both aromatic and aliphatic hydrocarbons. Several environmental isolates such as *Arthrobacter* sp. (Efroymson and Alexander, 1991) and *Acinetobacter calcoaceticus* and *Alcaligenes odorans* (Lai and Khanna, 1996) were found to degrade both alkanes and naphthalene. *Rhodococcus* AD45 was reported to degrade 1,2- dichloroethylene, propylene, toluene and styrene, but with low affinities and oxidation rates than those for isoprenes (van Hylckama Vlieg, 1999). *Mycobacterium* sp. strain CH1 isolated from polycyclic aromatic hydrocarbon (PAH)-contaminated freshwater sediments, was found to be capable of mineralizing three- and four-ring PAHs including phenanthrene, pyrene, and fluoranthene. They could utilize phenanthrene, pyrene and a wide range of alkanes as a sole carbon and energy source (Churchill, *et al.,* 1999). Zhukov *et al.,* (2007) reported the consumption of aliphatic hydrocarbons by two bacterial strains- *Rhodococcus ruber* Ac-1513-D and *Rhodococcus erythropolis* Ac-1514-D which can grown on mixed *n*-alkanes as well as diesel fuel was studied.

A *Sphingomonas* sp. strain CHY-1 was isolated by Demaneche *et al.,* (2004) from PAH polluted soil and it could degrade chrysene, a four ring PAH highly resistant to biodegradation. *Sphingomonas chlorophenolica* was reported to be able to degrade pentachloro phenols as well as other chlorophenols (Yang *et al.,* 2005). A gram-negative
bacterial strain, *Pseudomonas nitroreducens* isolated from a drainage sediment is reported to have the capacity in utilizing alkylphenol polyethoxylates as a sole source of carbon and energy to grow (Chen et al., 2006). The three bacterial strains, *Pseudomonas spinosa*, *P. aeruginosa* and *Burkholderia cepacia*, were found to be efficient degraders of both alpha- and beta-endosulfan (Hussain et al., 2007). Recently, *Gordonia terreza* IIPNI, isolated from petroleum drilling sites, was able to catabolise pyridine and 4- methyl pyridine as its sole source of carbon and nitrogen (Stobdan et al., 2008).

Tsuchi et al. (1978), isolated *Acinetobacter* sp. 351 from soil and was found to degrade 30% of liquid polybutadiene in 3 days. The disrupted cells of *Chlorella vulgaris* was also found to have the ability to degrade long chain n-alkanes (Schröder and Rehm,1981). An alkane-degrading, sulfate-reducing bacterial strain, AK-01, has been reported to be isolated from a petroleum-contaminated sediment (So and Young, 1999). Margesin et al., (2003) reported the bacterial strains, *Pseudomonas putida*, *Acinetobacter* spp. and *Rhodococcus* spp. to be involved in the degradation of n- alkanes. Recently, two genes encoding AlkB-type alkane hydroxylase homologues, designated *alkMa* and *alkMb*, were isolated from *Acinetobacter* sp. strain DSM 17874 that have been shown to be involved in the degradation of *n*-alkanes with chain lengths of 10 to 20 carbon atoms. (Throne-Holst et al., 2006, Throne-Holst et al., 2007).

A selective white rot fungus, *Ceriporiopsis subvermispora* that produce free radicals from lipids was found to be able to degrade lignin (Jensen et al., 1996; Srebotnik et al., 1997). A *Bacillus* sp. isolated from the sludge of pulp and paper mill was reported to be able to degrade the kraft lignin, a by-product in paper production. Significant reduction in
The group of bacteria capable of degrading hydrocarbons are termed as hydrocarbonoclastic group. A gram-negative marine bacterium, *Marinobacter hydrocarbonoclasticus*, has been reported capable of n-alkane degradation (Lattuati *et al.*, 2001). Strains of *Bacillus*, *Arthrobacter* and *Pseudomonas* which were isolated from hydrocarbon spilled soils that possess the ability to consume petroleum hydrocarbons belong to this group (Parvateesam *et al.*, 2004).

Psychrotrophic bacterial strains belonging to *Pseudomonas* sps. were found to possess both the alk catabolic pathway for the degradation of alkane and the nah catabolic pathway for PAH biodegradation. They were able to degrade C₅ to C₁₂ n-alkanes, toluene and naphthalene at both 5 and 25°C (Whyte *et al.*, 1997). Some strains belonging to *Pseudomonas putida*, *Acinetobacter* spp. and *Rhodococcus* spp. were also isolated from cold conditions capable of degrading n-alkanes (Margesin *et al.*, 2003).

*Pseudomonas citronellolis* that was isolated from the soil by the enrichment culture with citronellol as the sole carbon source, was found to be degrader of the isoprenoids- citronellol and farsenol (Seubert, 1960). Zander *et al.* (1970), have reported that squalane was cyclized to form tetrahymenol by *Tetrahymena pyriformis* through a hydration reaction. The degradation of alkanes and acyclic isoprenoids has been reported for several microorganisms (Yamada *et al.*, 1975; Schroder and Rehm, 1981; Buhler and Schindler, 1984). Two mycobacterial isolates *M. fortuitum* and *M. ratisbonense* isolated from a sewage treatment plant were found to utilize squalane which is a multiply branched saturated hydrocarbon (Berekaa and Steinbuchel, 2000).
1.2.3. Microorganisms in the biodegradation of latex

There are million tons of natural rubber used for the production of automobile tyres, industrial parts, foot wears and other domestic goods. Rubber tyres currently contain 35-40% natural rubber (Tsuschii and Tokiwa, 2001). The consecutive usage of rubber is resulting in the accumulation of rubber wastes among which used tyres and the automobile inner tubes are the major ones.

The disposal of waste rubbers, especially the wear out automobile tyres is becoming a daunting problem. The amount of tyre waste is increasing year after year. These used rubber release toxic fumes when they burn (Michael, 1991). Medical gloves, household gloves, condoms and balloons are the other rubber products which are slow to degrad when they reach the environment. The helium filled latex rubber balloons that are released into the environment burst into tiny pieces which is harmful to animal life when they are ingested. Recycling of polymeric wastes was an environmental problem of great concern (Liu et al., 2000). Reuse and recycling of waste rubber material implied problems in most countries (Bredberg et al., 2001). Due to hydrophobic and resilience property, rubber composting was not considered as an appropriate method for rubber disposal (Roy et al., 2006). Identification of a potent rubber degrading strain could provide a biotechnological solution to these problems.

It was important to know the potential for the microbial degradation of polymers from both the view point of prolonged usage and of waste disposal problems. 20% by volume of solid waste was comprised of polymers, which were hard to degrade, generally due to its inert nature (Roy et al., 2006).
Though there were several previous works about the biodegradation of rubber only a few number of organisms were reported to be potent rubber degraders. Owing to its hydrophobicity, the polymer was resistant to an easy microbial degradation. Though the microbial degradation of natural rubber was reported to be possible, it was a very slow process (Tsuchi et al., 1985) and the growth of bacteria utilizing rubber as a sole carbon source was slow (Jendrossek et al., 1997). The incubation period of such organisms extended over weeks or even months. So it was a tedious process to obtain enough cell mass or degradation products of the polymers for further analysis.

The incubation period for the optimum conditions of natural rubber latex might vary according to the organism. Almost all the rubber degraders especially actinomycetes are slow growers. *Streptomyces* strain was reported to have 10-12 weeks of incubation (Bode et al., 2001; Rose et al., 2005). But, the bacteria like *Gordonia westfalica* which did not form clear zone grew fast and took only about 6 weeks for effective degradation (Brooker et al., 2004).

Most of the natural rubber degrading bacteria were identified as members of the group actinomycetes, a large group of mycelium forming gram positive bacteria (Heisey and Papadatos, 1995; Jendrossek et al., 1997; Linos et al., 1999; Arenskotter et al., 2001; Rifaat and Yosery, 2004; Banh et al., 2005). Of these, *Corynebacterium*, *Mycobacterium*, and *Nocardia* (CMN group) which showed the adhesive growth on the latex were found to be more powerful degraders (Tsuchi et al., 1985; Linos et al., 1999; Arenskotter et al., 2001; Rose and Steinbuchel, 2005). Several species of *Gordonia* which belonged to the CMN group such as *Gordonia polyisoprenivorans* (Linos et al., 1999), *G. alkanivorans*
(Kummer et al., 1999), G. westfalica (Linos et al., 2002), etc were reported to be with strong rubber decomposing properties.

Jendrossek et al. (1997), had isolated several strains of rubber degrading bacteria of which majority of them were found to be the members of actinomycetes, Streptomyces was the dominant one. The others were Micromonospora, Actinoplanes, Nocardia, Dactylosporangium and Actinomadura. They were found to produce clear zones on opaque rubber containing solid media. It indicated that the ability to utilize rubber latex was widely distributed among Actinomycetes. But the extent to which the hydrocarbon was degraded might vary.

Spence and van Niel (1936), introduced the clear zone technique for the first time, by emulsifying natural rubber latex in mineral agar resulting in an opaque medium. The microorganisms which were able to degrade natural rubber formed translucent halos around the colonies when they were grown in the latex media. Later this technique was used by Rook, (1955) and Borel et al., (1982) to isolate natural rubber degrading fungi and bacteria. But, it could not be considered as a dependable technique since all the rubber degrading bacteria did not form halos on such the latex overlay agar plates. Too little polyisoprene was locally available to allow formation of visible colonies by these organisms.

Linos, et al. (2000°), had classified the rubber degrading bacteria into two broad groups- the bacteria that form clear zones and that do not form clear zone in the latex agar medium where latex was supplied as the only carbon source. The formation of clear zone indicated the extracellular nature of the enzyme present in the organism. The only one gram negative bacteria belonging to this
group is a *Xanthomonas* strain which was able to cleave the hydrocarbon chain and resulted in the production of low molecular weight oligomers (Tsuchi *et al.*, 1990). Most of the bacteria that came under the first group belonged to Actinomycetes (Jendrossek *et al.*, 1997).

Though the bacteria, *Pseudomonas aeruginosa* AL98 could not produce any clear zone it was able to produce holes in the polymer films (Linos *et al.*, 2000b) and thus it can be classified into the second group. The bacteria that came under the second group was considered as more potent degraders of the rubber hydrocarbon. Though they did not produce clear zones, they were able to solubilise solid piece of natural rubber and to use the resulting emulsion as a carbon source. *Gordonia polyisoprenivorans* and *G. westfalica* belonged to this group (Linos *et al.*, 1999, Linos *et al.*, 2002). *Achromobacter* sp. which grew adhesively and in direct contact with the rubber substrate was able to colonize the rubber surface and led to disintegration of the material during cultivation, though it failed to form translucent halos on the medium. It showed a higher colonization efficiency on small or treated pieces of natural rubber latex gloves, while a lower colonization efficiency was recognized when grown on large or nontreated natural rubber latex gloves (Berekka *et al.*, 2005).

In previous studies, the degradation potential of the microorganism was determined by weight loss in percentage (Heisey and Papadatos, 1995), residual weight, recovery and loss of tensile properties (Ikram *et al.*, 2000). The degradation was found to be depending on the ability of microorganism to colonize on the polymer (Bode *et al.*, 2000), which results in the formation of keto group compounds (Linos *et al.*, 2000a; Linos *et al.*, 2000b). Extend of
degradation has been measured according to the decrease in molecular weight (Bode et al., 2000; Rose et al., 2005).

There are several organism that could grow on rubber latex. But majority of them depended on the impurities or the non rubber contents in it (Shaposhnikov et al., 1952). So it was essential to purify the latex for the degradation studies. If the organism was able to grow on the purified latex it indicated that it utilized the hydrocarbon chain for its growth.

A *Nocardia* strain when cultivated on natural rubber latex gloves was able to produce oligomers with molecular weights from $10^4$ to $10^5$ (Tsuchi et al., 1985). The raw natural rubber latex when treated with a crude extracellular extract of a *Xanthomonas* strain resulted in the accumulation of low molecular weight oligomers of $10^3$ and $10^4$ Da. (Tsuchi et al., 1990). But the studies of Tsuchi and Tokiwa, (1999) showed that it was not a potent strain to colonize and to decompose solid rubber. But there was no soluble rubber degrading enzymes detected and it showed that the degradation of the vulcanized rubber was dependent on the colonization of the polymer surface. The occurrence of carbonyl groups for each oligomer at both ends suggested that cleavage of polymeric chain by oxygenative attack at the double bonds.

*Pseudomonas citronellolis* was known for the ability to grow on linear terpenes and *Streptomyces coelicolar* was reported to be able to cleave the carbon back bone of synthetic poly (cis1,4 isoprene). They utilized the low molecular weight degradation products for growth (Bode et al., 2000).

A mega plasmid of 101kb was isolated from the rubber degrading bacteria *Gordonia westfalica* kb1 (Broker et al., 2004).

Biodegradation of the vulcanized rubber was also possible although it is even more difficult due to the linkages of the poly (cis 1,4 isoprene) chains which resulted in reduced water adsorption and gas permeability of the material (Seal and Morton, 1985). *Nocardia* sp. 835A. was able to cause scissions of polymeric chains in natural rubber vulcanizates (Tsuchi et al., 1985). Degradation of vulcanized rubber was reported by some other strains also (Tsuchi et al., 1985, Heisey and Papadatos, 1995 and Tsuchi et al., 1996). Most of the microorganism tested for devulcanisation were sensitive to rubber additives due to their toxicity. *Resinicium bicolor* was able to detoxify the rubber material after which it was degradable with the bacterial strain *Thiobacillus ferroxidans* (Bredberg et al., 2002). A white rot Basidiomycete, *Cerioporiopsis subvermispora* was reported to be able to degrade vulcanized natural rubber sheets on a wood medium (Sato et al., 2003).

There were many attempts to degrade the synthetic rubber also eventhough it was much more difficult (Tsuchi et al., 1985; Linos, et al., 2000; Bode et al., 2001; Braaz et al., 2004). The actinomycete *Nocardia* sp. was reported to grow well on the vulcanized as well as synthetic isoprene rubber (Tsuchi et al., 1985). *Acinetobacter calcoaceticus* and *Pseudomonas citronellolis* were also able to grow on the synthetic rubber (Bode et al., 2001).
1.2.4. Enzymes in the biodegradation of the latex

Enzymes are mostly involved in the chemical mode of polymer degradation, pertaining to the decomposition of polymers that are a part of organized living species. Organized species have evolved to the point where they have enzymes that can break down certain polymers in their digestive systems, which have become highly specific to their biological processes.

At least two categories of enzymes are actively involved in biological degradation of polymers: extracellular and intracellular depolymerases (Dio, 1990; Gu et al., 2000). Extracellular enzymes from the microorganisms first breakdown the complex polymers giving short chains or the oligomers. Thus, the new molecules formed as a result of depolymerisation are small enough to permeate through the cell walls to be utilized as carbon and energy sources.

Though there were several studies concerned with biodegradation of natural rubber, the basic molecular mechanism by which rubber was degraded was not known to the scientific community. The natural rubber and other natural polyisoprenoids are the only biopolymers whose cleavage by enzymes is not find out yet. Enzymes involved in rubber degradation, particularly enzyme catalyzed cleavage of the rubber back bone still remains as a paradox. Till now, there was only a single organism (Xanthomonas sp.) reported from which a rubber degrading enzyme was isolated. However, chemical analysis of degradation products formed due to incomplete biodegradation, revealed some information about the biochemistry of rubber degradation.

A crude extracellular enzyme isolated from Xanthomonas sp. was reported to be able to cleave the carbon back bone (Tsuchi and Takeda, 1990). From the rubber grown cultures of Xanthomonas and
Nocardia certain low molecular weights oligomers with aldehyde and keto end groups were isolated and identified (Tsuchi and Takeda, 1990; Tsuchi et al., 1985). Analysis of natural rubber degradation products produced by Streptomyces coelicolor and Streptomyces griseus after 70 days of growth on latex gloves revealed an oligomer pattern similar to that observed for Xanthomonas sp.. However products with different end groups were detected (Bode et al., 2000, Bode et al., 2001). Since all the studies were performed with undefined culture broth, it is not known whether the products identified were formed in one or more enzymatic steps.

No enzyme involved in the rubber degradation has been isolated in an active form. Recently, a gene of Xanthomonas sp. whose gene product could be involved in degradation was cloned (Jendrossek and Reinhardt, 2003).

Two genes encoding enzyme capable of poly cis1, 4 isoprene cleavage had been isolated independently from different bacteria. An enzyme Rox A, was identified from the bacterium Xanthomonas sp. (Braaz et al., 2004). It could cleave poly cis1,4 isoprene resulting in the formation of short chain isoprenoid intermediates. Lcp, a latex clearing protein was identified in Streptomyces sp. (Rose et al., 2005). Degradation in the presence of lcp resulted in the intermediates which had been identified as isoprenoids with approximately 20 isoprene units (Ibrahim et al., 2006).

RoxA was identified as a dioxygenase through the isotope labeling experiments (Braaz et al., 2005). 12-oxo-4,8-dimethyl tridec-4,8-diene-1-al (ODTD) was identified as the major degradation product formed by the action of RoxA. There were also some minor metabolites which differ from the major degradation products only in the number of
repetitive isoprene units between terminal functions, CHO-CH2- and-CH2-COCH3 (Braaz et al., 2004).

Intermediates formed as a result of the of the degradation of poly (cis1,4 isoprene) by RoxA and Lcp had been found to possess similar chemical structure, possessing one terminal ketone and one terminal aldehyde functional group (Braaz et al., 2004; Rose and Steinbuchel, 2005). But they were found to be two individually evolved enzyme systems for rubber cleavage as they did not share any sequence homologies. Till now, there is no protein identified in bacteria other than Xanthomonas sp. reported to be homologous to Rox A. But the presence of genes encoding lcp homologues had been shown in the genomes of several actinomycetes capable of rubber degradation.

Additives that were added during the manufacturing process of rubber products were also reported to promote or inhibit the biodegradation of rubber material (MacLaghlan et al., 1966; Gomez and Moir, 1979). The antioxidants extracted from synthetic polyisoprene, which was prepared for tyre production, showed some inhibitory effect on the growth of G. westfalica (Berekaa et al., 2000). It was also shown that extraction of latex gloves with organic solvents before incubation, enhanced the growth of some rubber-degrading strains (Berekaa et al., 2000).

Although these enzymes certainly had a different physiological function, the biochemically generated radicals were found to be capable of degrading polyisoprenoids. Fetzner (2002), postulated a reaction mechanism for cofactor-independent dioxygenases in which an amino acid residue (probably histidine) acted as a proton acceptor. A combination of both enzyme mechanisms might allow a new mechanism that did not require a fatty acid mediator.
An enzyme mediator systems for invitro oxidative degradation of polyisoprene were described based on free radical chain reactions of lipids using different known enzyme such as manganese peroxidase, laccase, horse radish peroxidase and lipoxygenase / linoleic acid (Enoki et al., 2003; Sato et al., 2003). Lipoxygenase from *Glycine max* (soybean) or peroxidase from *Amoracia rusticana* (horseradish) activated their substrates, linoleic acid and 1-hydroxybenzotriazole and was able to cleave cis-polyisoprene and trans-polyisoprene. This was resulted in a decrease in the molecular weight of the polymers (Enoki et al., 2003). Furthermore, examination of latex gloves treated with these enzyme mediator systems for 48 h revealed hole formation in the material, as detected by scanning electron microscopy. Non-vulcanized and vulcanized polyisoprene rubber can also be decomposed by controlling the free radical chain reactions of lipids using oxidative enzymes, manganese peroxidase, laccase and horse radish peroxidase (Sato et al., 2003).

Furthermore, most of the degradation products detected (Tsuchi and Takeda, 1990) contained aldehyde and keto groups. This could be explained by oxygenases like RoxA. Even enzyme mediator systems yielded invitro degradation products containing aldehyde or keto groups (Enoki et al., 2003; Sato et al., 2003).

It was interesting to note that, the degradation products which were having molecular masses close to $10^4$ Da, formed as a result of biodegradation as in the earlier reports, were getting accumulated in the medium before they were further metabolized. This indicated that there was an endocleavage rather than an exocleavage mechanism of rubber degradation. However, this observation was not consistent with random endocleavage of the polyisoprenoid chain (Rose and Steinbuchel, 2005).
Based on the intermediates (Tsuchi et al., 1985; Tsuchi and Takeda, 1990; Bode et al., 2000) isolated from several natural rubber grown bacterial cultures, a biochemical degradation route had been proposed for rubber degradation. The degradation of polymer backbone was assumed to be initiated by random oxidative cleavage of one double bond in the polymer chain (Braaz et al., 2004). The resulting low molecular mass oligomers were further degraded presumably by \( \beta \) - oxidation.

1.2.5. Microbial treatment of natural rubber latex centrifugation effluent

The rubber industry, which has shown tremendous growth in the last couple of decades, contributes in a major way to the contamination of the environment, especially that of water. The effluent from rubber factories constitute the waste water formed from the initial stages of processing that most often occur in or near the plantations.

Rubber factory effluent is one of the major liquid waste from natural product processing. It was estimated that about 100 million liters of effluent were discharged daily from rubber processing factories (Tang et al., 1999).

Effluent may differ in their physical and chemical properties depending upon the treatment given. The usual procedure of disposing effluent was by discharging it into nearby stream or river, is not really a safe procedure as far as their BOD, high mineral and nitrogen contents are concerned (Kulkarni et al., 1973\textsuperscript{a}). Such indiscriminate discharge of waste effluent into public waters is undesirable (Muthuraj et al., 1973). Land areas adjacent to industrial sites are prone to be adversely affected due to dumping of industrial materials (Sahai et al., 1983). It may also pollute the soil of adjacent crop fields by seepage and overflow (Sharma
et al., 1997). The rubber factory effluent at high concentrations showed an adverse effect on plant growth (Sharma et al., 1997).

The processing of concentrated latex consumes large volume of water and there is a waste discharge in large quantities, resulting in the production of highly polluting effluent, which if discharged untreated to water sources will have adverse impact on water quality (Muthuraj et al., 1973). The effluents discharged from a latex concentration unit are strongly acidic in nature (Madhu et al., 1991). This is because, skim rubber processing involves the recovery of 4-5% of dry rubber by cheap grade sulphuric acid coagulation that leaves the serum portion contaminated by the acid making the pH range of effluent into 2-3. The bulk of the contaminated serum together with the washings is eventually discharged into the effluent pond. This sulphate ion releases hydrogen sulphide gas while undergoing microbial degradation causing malodour and is found to be detrimental to the environment (Ibrahim et al., 1979).

According to Kulkarni et al. (1973b), skim effluent was richer than cuplump effluent in nitrogenous compounds with a very high BOD. The waste contained both organic and inorganic constituents which were potentially oxygen absorbing materials (Muthuraj et al., 1973). According to Tang et al. (1990), the rubber latex effluent was a polluting source that had a high biological oxygen demand (BOD) and contain high level of suspended soils. The average BOD value of the concentrated latex effluent was 4854mg/l and the corresponding average COD value was 11,568 mg/l. The high values of BOD and COD indicated the organic nature of the effluent (Madhu et al., 1991). Suspended and dissolved solid contents were also high. It contained high levels of nitrogen and sulphate. Ammonia which was present in the preserved latex, remained
in the skim latex and contributed the high amount of nitrogen to the effluent (Furumai et al., 2007).

The effluent subjected to the biological treatment first goes to a primary tank for the removal of suspended solids and later it was shifted for the secondary treatment. If sufficient land area is not available then the effluent after primary settling may be subjected to an extended aeration activated sludge type biological treatment process. A rubber trap equalization neutralization and clarification steps will reduce substantial quantity of pollutants particularly BOD and suspended solids. The primary treated effluent can be treated in a secondary / biological treatment unit. It is envisaged to render secondary treatment by adoption of extended aeration activated sludge process. The biological treated effluents should be settled in a secondary settling tank.

Activated sludge treatment can also be used for the treatment of latex effluent. It is a process where waste water is treated aerobically by a microbial consortium dominated by heterotrophic bacteria. They are flocculated in the mixed liquor under a supply of excessive oxygen which is necessary to form discrete clumps of micro organisms. Hall and Melcer (1983) reviewed a number of cases in which the adaptation of the activated sludge systems were used to biodegrade toxic industrial wastes. The ability of activated sludge system to acclimatize in order to degrade various toxic pollutants had been known for some time. (Painter, 1985).

Natural rubber latex centrifugation effluent was also subjected to aerobic treatment with Acinetobacter sp. BTJR-10 (Jayachandran et al., 1994). Immobilized cells of Acinetobacter BTGR-10 were packed into a packed bed reactor and was successfully applied for the continuous treatment of the effluent.
In an activated sludge, different group of organisms dominated during different time intervals. The efficiency of wastewater treatment by activated sludge was related to the bacterial population but also to the protozoa (Nicolau et al., 1997). These protozoa had an important role in maintaining a good balance in the biological ecosystem. They eliminated the excess bacteria and stimulated their growth and they promote flocculation (Gerardi, 1995). By consuming the free bacteria, they helped to decrease the turbidity of the effluent as well as its BOD and its suspended matter content (Curds et al., 1968).

An oxidation ditch of detention time of 2-3 days was also considered as an alternative for treating the effluents of the crumb rubber unit. A bioaugmented extended aeration reactor was used for the treatment of latex concentrate effluent with an average chemical oxygen demand (COD) of 10240 mg/l (Kadir et al., 1999). Later, a coupled method of bioaugmentation and extended aeration methods had reduced the pollutant load of latex. It was found to be more than 17% ± 2.6% efficient for SS, BOD and COD removal (Khadir et al., 2000).

50% to 60% reduction in the chemical oxygen demand (COD) of the latex effluent could be achieved by installing an activated sludge system, modifying feed supply equipment and updating the dissolved air flotation (DAF) system.

Treatment of waste water is essential for the break down of organic matter so that it will not cause pollution problems when the water is released into the environment. The treated effluent may be disposed into water bodies or on environmentally secure land or may be used for irrigation after ascertaining the proper NPK ratio in the effluent.
1.3. OBJECTIVES

The following objectives were considered for the present study:

1. **Isolation and screening** of an efficient bacterial strain capable of degrading natural rubber latex.

2. **Purification** and **identification** of selected strain.

3. **Optimisation** of the conditions for the maximum degradation of the latex by the organism.


5. Isolation of **enzyme** involved in biodegradation of latex from the selected strain.

6. **Purification** of enzyme by ammonium sulfate fractionation and ion exchange chromatography.

7. **Characterisation** of the enzyme

8. **Characterisation** of the physico-chemical and biological parameters of the latex centrifugation **effluent**.

9. Application of the **free cells of the selected strain in the treatment** of latex centrifugation effluent.

10. **Designing of an activated sludge bioreactor** system for the effective treatment of the latex centrifugation effluent.

11. **Treatment of the** latex centrifugation effluent with the designed bioreactor.