CHAPTER II

REVIEW OF LITERATURE
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The mad rat race among nations over the globe for development jeopardised the health of man itself. Progress in agriculture and industry is taken as a general criterion for the development of any country. This craze resulted into unlimited exploitation of every bit of natural resources. The splendid plentifulness of nature is a heritage that should be conserved for future generations and not be spoiled. Such activities of man had adverse effect on all forms of living organisms in the biosphere. Unlimited exploitation of nature by man disturbed the delicate ecological balance between living and non-living components of the biosphere. The unfavourable conditions created by man himself threatened the survival not only of man himself but also of other living organisms.

Next to air, water is the important constituent of life-support systems. Water is the most important natural resource. We depend on water for irrigation, industry, domestic needs, shipping, sanitation and disposal of waste. Most of our water bodies such as ponds, lakes, streams, rivers, seas and oceans have become polluted due to industrial growth, urbanisation and other man made problems.

Many rivers and ponds of the world receive heavy flux of sewage, domestic waste, industrial effluents, agricultural wastes etc. which contain substances varying from simple nutrients to highly toxic chemicals.

The addition of any substance to water or changing of its physical and chemical characteristics in any way interferes with its use for
legitimate purposes and is called water pollution. Normally water is never pure in a chemical sense. It contains impurities of various kinds – dissolved as well as suspended. These include dissolved gases (H₂S, CO₂, NH₃ and N₂), dissolved minerals (Ca, Mg, Na salts), suspended matter (clay, salt, sand) and even microbes.

Anirudhan et al., (1997) designed a new method to remove arsenate ions from aquatic systems by using copper impregnated coconut husk carbon. Nandan and Madhu (1996) reported the periodic variations in the physico-chemical characteristics of river Chambal at Kota and revealed that water quality remains constant except for seasonal variations in turbidity, temperature and nitrates. Bhattacharya et al., (1997), studied the physico-chemical characteristics of Gurupar Estuary, Mangalore which receives treated sewage which showed moderate polluted conditions. The value of water temperature, pH, salinity, biochemical oxygen demand and sulphide were low during south west monsoon and high during summer months. The physico-chemical analysis of Ghaggar water was assessed to reveal the quality of the water and it was observed that the sites receiving industrial and animal excretory wastes were found polluted. (Kaur et al., 1996).

Kumar et al., (1996) observed some aspects of water quality degradation along the north east coast of India. In addition to the characterisitic physico-chemical background parameters, water quality was monitored in terms of heavy metals and some biological determinants. Spatial and temporal changes in the concentration of these parameters
from inshore to offshore water, and their possible effects on the marine ecosystem had also been discussed.

Madhu and Anirudhan (1996) studied adsorption thermo dynamics of phosphate on sediments of tropical back water systems. The effect of phosphate concentrations, contact time, pH, diverse ions and temperature on adsorption of phosphate by sediments from retting and non-retting zones have been studied. The results observed was that the percentage of phosphate adsorbed increased with decrease in initial concentration of phosphate and increase in temperature.

Nagarathna and Hosmani (2002) correlated physico-chemical parameters and phytoplanklons in a polluted lake. The correlation matrix and cluster analysis indicated that water temperature, phenolphthalein alkalinity and pH have the greatest similarity. The cluster analysis serves as an important means in determining the pollution level of the lake.

Pandey et al., (2002) assessed the physico-chemical characteristics of sub surface water of Makrana District Nagpur. Study of variation range of different physico-chemical parameters in relation to fish productivity and health was studied by Chandrawati Jee (2002). Observations were recorded for assessing the conductive/normal range of various physico-chemical conditions of water and soil for the purpose of correlating it with fish growth and disease incidence. Fokmare and Musaddiq (2002) conducted a study on physico-chemical characteristics of Kapshi-lake and Purna river water in Akola District of Maharashtra.
The birth of microbiology occurred in 1674 when Anton Van Leeuwenhoek, an inquisitive Dutch drapery merchant, peered at a drop of lake water through a glass lens that he had carefully ground. What he observed through this simple magnifying glass was undoubtedly one of the most amazing sights that humans have never ever beheld; "the first glimpse of the world of microorganisms".

Bacteria belong to a group of living organisms known as microorganisms without distinction regarding whether they are plants or animals. They are so called because of their small size. All cells are small, but bacteria do not exist as parts of organisms. They exist as single cells. Those who are not familiar with the elementary rudiments of bacteriology have an erroneous concept of the role of bacteria in nature. Since the early development of bacteriology was concerned with a study of disease-producing organisms, the impression is generally held that the sole purpose of bacteria on this earth is to cause human ills. This belief is entirely erroneous. Only a few bacterial species are harmful to man. The great majority are not only harmless but absolutely necessary for the existence of living things. Life could not exist in the complete absence of bacteria.

Bacterial enzymes are of great importance in today's sophisticated world. Most of the proteins synthesized by bacteria are enzymes which must be produced in a balanced array for proper functioning of the cells. Bacteria synthesize a large variety of proteolytic enzymes. Proteases have been regarded as degradative enzymes which are capable of cleaving the peptide bonds of proteins producing small peptides and amino acids.
Purva et al., (1998) studied thermostable alkaline protease from Alkalophilic *Bacillus* sp. IS-3. It was observed that the production of the enzyme was substantially increased when the type and concentration of carbon, nitrogen and metal ion sources of culture media were optimized. Enzyme yield as high as 128.2 UmL$^{-1}$ was obtained under optimal cultural conditions. Sen and Satyanarayana (1993) studied the optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. It was studied that among eleven media combinations used, the enzyme production was high in soyabean meal starch medium. Madan et al., (2000) experimented on the production of Alkaline protease by a UV-mutant of *B. polymyxa* and recorded good production of alkaline protease by the UV mutant strain on Reese medium supplemented with 0.5% casein and 0.25 % glucose. Kaur et al., (1998) characterized and purified alkaline protease from *B. polymyxa*. It was observed that the purified enzyme was significantly inhibited by EDTA, Cu$^{2+}$ and Hg$^{2+}$ and also noted that the UV-induced mutant of *B. polymyxa* produced 63% more protease compared to its parent.

Sinha and Satyanarayana (1991) highlighted that among six strains of thermophilic *Bacillus species* screened, *Bacillus licheniformis* N$_3$ was found to be a good producer of extracellular alkaline protease. The results of the experiment showed that oil cake supplements did not improve enzyme production. Joshi and Ball (1993) studied the extracellular enzyme production of facultative bacteria of CaCO$_3$ kilns near Jabalpur.
Partial purification and characterization of a thermostable alkaline protease of an alkalophilic *Bacillus* sp. NG-27 was studied by Sumandeep *et al.*, (1999) who observed that the enzyme was stable at 80°C for more than 1 hour. It exhibited a half life of 55 and 50 min at 90°C and 99°C, respectively and retained 65% of its activity. Chauhan and Gupta (2002) purified and characterized alkaline protease from *Bacillus* sp. The enzyme was inhibited by serine protease (PMSF) and showed enhanced activity in presence of EDTA and β mercaptoethanol. This suggested it to be thiol dependant serine hydrolase.

Human activities create environmental pollution. In addition to the inexorably expanding human population the industrial development needed to sustain this population which has been accompanied by a new quality of environmental pollution (Timmis *et al.*, 1994). Organic pollution which results from the discharge of biodegradable organic matter such as sewage and the effluent from the processing of biological materials (food, textiles and antibiotics) results in the increase of heterotrophic components of the ecosystem, in the concentration of plant nutrients (nitrogen and phosphorous) in the water, enhance the producer component of the ecosystem thus creating an imbalance known as *Eutrophication*. When eutrophication adversely affects the water quality or causes nuisance, it constitutes *pollution*.

The observable effects on the biota associated with organic pollution are the results of a number of interacting factors. The most important of which are (1) change in nutrient status, (2) change in concentration of the
dissolved oxygen and carbon dioxide, sewage and industrial effluents which are most common cause of organic pollution. Additional factors such as increase in suspended solids which accumulate on the river bed, toxicity due to ammonia, detergents and possibly toxic metals also cause pollution. The increased organic concentration results in an increase in the heterotrophic microbial populations, both planktonic and benthic.

The increased population and activity of decomposer community in response to the elevated nutrient level increases the demand of O₂. This may cause a depletion in the dissolved O₂ in the water to an extent depending upon the concentration of organic nutrient present, measured by the BOD. As a result of the decomposing activities the CO₂ concentration tends to increase, thus affecting the pH. Essentially, all practical biological sewage and waste water treatment processes have to satisfy a number of basic objectives with respect to their operation. These include:

1. effective bio-oxidation of the biodegradable carbonaceous and nitrogenous pollutants, such that a minimum quality of readily dewaterable biomass (sludge) is produced;
2. adsorption onto and absorption into the sludge, other soluble pollutants and entrapment of particulate pollutants within the microbial flacks or films; and
3. easy and complete separation of suspended biomass from the treatment effluents prior to discharge from the biotreatment process (Hamer, 1985).
Bio-remediation is needed because certain chemicals accumulate in the environment to levels that threaten human health or environmental quality. It was in fact in mid-1960s, when DDT accumulation changed the old belief (that microbes were able to degrade all compounds entering the environment) that serious efforts were begun to exploit the natural capacity of microorganisms to degrade complex compounds. In 1994 OECD workshop recognised that bio-remediation can have local, regional and global applications and that both indigenous and genetically-engineered microbes may play an important role.

Research projects are being modified to expand the range of microorganisms used for bio-remediation. There is a search for naturally occurring microbes that have better pollutant degradation kinetics which attack a wider range of pollutant compounds and do so over a wider range of microbial growth conditions. There is also search for microbes that could grow under extreme environmental conditions, such as tolerance to organic solvents, growth under extremely alkaline substrates or higher temperatures.

Researchers have also been using genetic engineering to develop new microbial strains with novel bio-degradative capabilities. For instance, modified microbes may be produced through genetic coding for the attack of complex chlorinated hydrocarbons such as dioxins which are non-degradable by naturally occurring microorganisms. Adding genes that code for enzymes which breakdown toxic chemicals by microbes, able to survive and grow in much disturbed and harsh environments would
greatly extent the range of compounds that might by treated with bio-
remediation. For example, in Japan a research team has already isolated
a species of *Pseudomonas* that can grow in solvents containing more
than 50% toluene, a condition that kills most organisms through disruption
of cell membrane (Aneeea, 2002). Adding appropriate genes for catabolic
enzymes to this strain has great potential for expanding the range of bio-
conversions into non-aqueous solvents.

The goal of biological treatment of waste water is to employ
microorganisms to completely oxidise organic components of the water
into carbon dioxide and water. As a result of oxidation, the effluent water
from the treatment plant contain only small amounts of incompletely
decomposed organic matter. Hence the water is no longer able to support
extensive growth of heterotrophic and autotrophic microorganisms.

The enriched microorganisms are cultured to degrade a
specific waste or waste mixtures and are useful when generic micro-
organisms are not effective or remain in low numbers despite physical
and chemical enhancement. The enzymatic systems of some generic
microorganisms may be inadequate for degrading a waste and therefore
addition of microorganisms specifically cultured on target waste as a
carbon energy source may greatly enhance the biodegradation activity
(Becker et al., 1985). The common groups of microorganisms responsible
for pollutant degradation are bacteria, fungi and yeasts.

Bough et al., (1972) reported the use of *Bacillus megaterium*
for the treatment of meat packing waste. Camhi and Rogers (1976)
described the use of *Pseudomonas denitrificans* for the treatment of sulphite liquor waste. Tanabe *et al.*, (1986, 1987) described the use of soft rot *Erwinia carotovora* and *Bacillus* sp. for the pre-treatment of pectic waste water from orange canning factory.

Rubio and Molina (1989) described the use of mixed culture of *Cellulomonas* sp. and *Bacillus subtilis* for the treatment of potato waste effluents. Ghosal and Bhowmik (1995) developed a biological method for removal of phenol from pharmaceutical industry waste water, by using mixed microorganisms like *Pseudomonas*, *Chlorella*, *Candida* triplicates. Shivaji and Shrikanth (1996) described the use of *Bacillus* and *Pseudomonas* sps. for the treatment of domestic waste water pollutants and Jain *et al.*, (1997) described the use of *Acinetobacter calcoaceticus* for treatment of pulp mill waste water. Microorganisms have also been isolated which can degrade different organic compounds like benzene (Dagley *et al.*, 1964), phenol (Feist and Hageman, 1969), naphthalene (Davis and Evan, 1964), salicylate (Chakrabarty, 1972), toluene (Chakrabarty, 1976), p- and m-hydroxy benzoates (Johnson and Stanier, 1971).

Microbes are also involved in the degradation of synthetic compounds like 2,4,D chlorinated phenols (Bollag *et al.*, 1968; Loos, 1975), 2,4,5-trichlorophenoxy acetic acid (Rosenberg and Alexander, 1980), atrazine (Kaufman and Kearney, 1970) and some PCBS (Ahmed and Focht, 1972).

Literature is also available on the enzymatic/microbial degradation of Xenobiotics, especially the most persistent chlorinated organic compounds. Recently, the microbial augmentation techniques
have been reported to be successful in removing chlorinated aliphatic and aromatic hydrocarbons during treatment of industrial effluents and polluted ground water without generating toxic end products (Kuhn et al., 1985; Boumer and Wright, 1988; Fathepure and Vogel, 1991).

Presently several companies are supplying selected bacteria known as bio-enhancers for introduction into the waste water treatment to enhance treatment efficiency. Bio-enhancers used for the benzene, toluene and xylene (BTX) degradation contain *Pseudomonas putida* and yeast. The bio-enhancers used in the sewage treatment contain *Bacillus*, *Pseudomonas*, *Nitrosomonas*, *Nitrobacter*, *Cellulomonas*, *Aerobacter* and *Rhodospseudomonas* commonly found in soils and waste water treatment plants (Chin et al., 1995). They also demonstrated that in sewage treatment, the addition of bio-enhancers, improve the treatment efficiency by removing BOD, COD, detergents, oil and gases. Bio-enhancers lowered the rate of sludge production and accumulation and also minimized odour problem at the sewage treatment plant.

Microbial enzymes responsible for degradation are generally specific for individual compounds or classes of compounds. There is no single organism or community that can destroy all organic wastes.

Many workers reported bio-degradation of industrial and domestic waste effluents by microorganisms. Shivaji and Shrikanth (1996) observed that with specific strains of *Bacillus* and *Pseudomonas* sp. there had been a gradual decrease in BOD in domestic waste water proving their efficiency in organic content removal. Manoharan and Subramanian
the food chain, water use, occupational exposure, etc. The use of waste water and sludge may be a potential risk to the exposed human population.

Waste water is treated to prevent any adverse effect on the receiving water body and to allow its re-use. The removal or inactivation of hazardous materials may involve a number of different physical, chemical and biological processes (Arthur, 1981; Shuckrow et al., 1981). Generic microorganisms occur naturally and are indigenous to the waste. The use of generic microorganisms is usually most effective when they can readily change the molecular structure of the target materials under physical and chemical conditions optimum for their activity (Shuckrow et al., 1981).

The most important method for treating or pretreating the organic waste water is the method of biological decomposition. Microbial activity is used in the detoxification and degradation of sewage and industrial waste. The wastes may also be pretreated physically or chemically to reduce toxic or inhibitory effects by microorganisms or to change waste materials to a form that promotes microbial activity. The most common forms of pretreatment are dilution with water, neutralization and precipitation (Becker et al., 1985).

Proteases are degradative enzymes capable of cleaving protein into peptides and amino acids. This enzyme digest nutrient protein or participate in the turn-over of cellular protein (North, 1982). Limited proteolysis has a major role in wide range of cellular processes (Holzer and Tschensche, 1979). The ability of proteolytic enzymes to carry out selective modification of proteins by limited cleavage, as in the activation
of hormones, means that some proteins have regulatory function. As a result of specific proteolytic processes and use of more selective substrates many proteases are being detected in all types of organisms. Protozoan, fungi and bacteria are potent protease producing microorganisms (Sharma and Satyanarayana, 1980).

*Xanthomonas alfalfa* produces proteases which liquefied gelatin and is caseinolytic in nature. Alkaline protease and peptidase have proved to be valuable reagents in the lab for clinical and industrial processes. Enzymes of alkalophilic bacteria are used in a number of fermentative processes for the breakdown of proteins in many food industries. Alkaline proteases are very useful in tanning industry in the manufacture of biodetergents (Aunstrup, 1980). Extracellular alkaline proteases have been isolated from alkalophilic *Bacillus strain* MKS 21 which are being used in the above industries (Tsuchinder et al., 1986).

Sharma and Satyanarayana (1980) studied the production of proteases by some molds. Optimisation of saprophytic and pathogenic molds have been done at different pH and temperature (Dion, 1950). North et al., (1983) studied increase in protease activity in cellular slime mold *Polyspendylium pallidium*, induced by bacteria containing aspartic and cysteine proteases.

Proteolytic enzymes are known to be produced by various bacteria like *Serratia, Bacillus, Pseudomonas, Proteus, Clostridium*, etc. The enzymes associated with these microbes are actually mixtures
of proteinases and peptidases. While the proteinases are usually secreted/excreted in the fermentation medium during growth, the peptidases are often liberated only on the autolysis of the cells. It is the proteinases which are of commercial interest.

The fungal proteases possess a wide pH activity range than bacterial or animal proteases, so the former has a wider and greater scope of usage. Species of *Aspergillus* and *Mucor* produce a good amount of protease and at sporulation, the various fungal proteolytic enzymes present in the medium, can be recovered. Solid/semi-solid state fermentations give better results with fungi, while submerged fermentation is better suited for bacteria. The synthesis of these enzymes are linked to particular phases of development, like strains of *Bacillus megaterium*, *B.subtilis* and *B.cereus* which produces protease during the log phase and stationary phase of growth respectively. The concentration of purely carbonaeous medium components should normally be kept at low levels (0.4 – 1.0 %) for optimum production. (Guntelberg, 1954).

It has been estimated that the commercial production of *Bacillus* protease was 500 tons in 1980. Amongst all the enzymes used world wide, bacterial proteases alone occupied 35% of the market share in terms of sales while microbial rennet and fungal proteases have 5% and 4% share respectively (Aunstrup, 1980). Rao *et al.*, (1998) estimated that the current value of the world wide sale of industrial enzymes is $ 1
billion and proteases account for about 60% of the total sale. The proper way to compare enzymes from different sources is by their performance in the intended applications. The cost of an enzyme depends not only on the process of its manufacture but also factors like customs, local trade patterns and competition. Developing new applications for the existing commercially available enzymes is rare but has emerged as a challenge for all industries producing consumer goods especially their research and development teams.