I. INTRODUCTION

Water bodies such as lakes and rivers are natural resources to satisfy man's domestic, industrial and agricultural needs and part of this natural environment is proving for his recreational and aesthetic needs. A regular and plentiful supply of clean water is essential for the survival and health of most living organisms. Water is regarded as polluted when it is changed in its quality or composition directly or indirectly by mankind's activities becoming less suitable for drinking.

Water is basic and primary need of all vital processes. Due to increasing demands, population exploitation, industrial revolution, the water bodies are now being used as receptacles of sewage, sullage and industrial wastes. Water the elixir of life is becoming more and more unfit and dearer to mankind due to unwise use, neglect and mismanagement.

Sterile water is rarely found in nature. Water even obtained from deep wells and from springs usually contains a few bacteria. There are many cases where cities use lake both for the disposal of sewage and as a source of domestic purpose. Water contamination can be a very serious problem in the spread of infections in many developing countries where public health and other authorities are not able to control water contamination. Water constitutes a good habitat for a diverse variety of microorganisms. The proper disposal of sewage in a community is undoubtedly the most important aspect of water sanitation. The waste water from bathing and washing as well as body excreta contains much organic materials rich in pathogenic and non-pathogenic bacteria. Any water coming in contact with this sewage is easily contaminated by pathogens.
Water is not only essential to life but is the predominant inorganic constituent of living matter. Water serves as the second natural medium for the growth of microorganisms and stands next to soil. Growth of microorganisms in water mainly depends on the amount of available material nutrients and the dissolved oxygen present in it. It has been observed that when the amount of organic matter increases in water, the number of microorganisms also increases but upto a certain limit. The number of bacteria and other microbes will always be higher in rivers passing by thickly populated cities than of the villages because persons living in cities are continuously disposing sewage water and other waste products in rivers which contain a very high amount of mineral nutrient, a medium for their growth. Moreover the pH, temperature range and inorganic phosphate content as well as the situation of the lake and river also support the growth and cause a dense population of microorganisms. These organisms form a heavy bloom under these conditions. The possible factors responsible for limiting the growth and density of microorganisms are the available amounts of zinc, copper and the poor quantity of nitrate, nitrogen etc. It has been noticed that the excess of calcium is harmful for the luxuriant growth of microorganisms, specially to algae in general. However, inspite of enormous quantity of the substance that exists, only a small proportion of it is actually usable by human beings. They use water in the home, industry, agriculture and recreation.
Generally, "sanitation" means the "science of safeguarding health". According to National Sanitation Foundation of the USA, "Sanitation is a way of life, quality of living that is expressed in the clean environment".

In India water pollution is becoming a serious problem. To protect water from being contaminated, Parliament in 1974 passed the Water Act (Prevention and Control of Pollution). Sonzogni et al., (1980) studied the effect of land use upon pollution export to the great lakes and reported that loads of suspended solids, nitrogen and phosphate from urban area were 10-100 times greater than loads from equivalent area of undisturbed land. It has generally been reported that the urban run off have degrading effects and also potentially harmful pollutants (Whiting and Clifford, 1983).

Chloride is one of the major inorganic anion in water and waste water. Chloride concentration is higher in waste water than in raw water because sodium chloride is a common article of diet and passes unchanged through the digestive system. Chloride does not precipitate and sediments and cannot be removed biologically during treatment of the waste. The high concentration of chloride in fresh water is assigned to human sewage (Moyle, 1949) causing undesirable taste of water and beverages (Dhanaselvan and Lakshmanaperumalsamy, 1991).

Organic matter is normally present in polluted rivers and lakes as a result of drainage and the decay of algae and leaf litter of riparian vegetation. The zone of organic pollution is characterised by low Dissolved
Oxygen (DO) concentration and higher oxygen demand. Even when conditions are especially severe directly downstream of the discharge, the zone may be almost devoid of all living beings except, for certain bacteria which tolerate the extremely low dissolved oxygen and high toxicity of effluents (Dhanaselvan and Lakshmnapurumalsamy, 1991).

The effect of waste discharge in a water system is determined by the oxygen balance of the system. Non-polluted surface water are generally fully saturated with dissolved oxygen. The input of oxygen demanding waste causes severe reduction in the dissolved oxygen concentration of the receiving water body due to oxidation of organic substances by microorganisms. The dissolved oxygen concentration further decline due to degradation of chemical substances which are not oxidised by the microbes. Key (1960) estimated 6% of streams in England having water with a dry weather flow of atleast 1 million gal / day which were grossly polluted (5 day BOD was 12 mg/L⁻¹ or more) and 21% were of doubtful or poor quality (BOD: 3-12 mg/L⁻¹).

Nitrogen and phosphorous are major components of run off and domestic sewage. In majority of lakes, phosphorous and nitrogen appear to be the limiting factor, controlling the aquatic plant growth. It is reported that 3.5 kg of phosphorous / capita / year of which approximately 60% is from the phosphate builders (used in synthetic detergents). Domestic waste contains 5-20 mg P/L⁻¹, polluted lakes 0.01-0.04 mg P/L⁻¹ and eutrophic lakes 0.03-1.5 mg P/L⁻¹. Pace (1986) stated that nitrogen and phosphate are two important elements responsible for eutrophication in water bodies. Swamy and Rao

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reported nitrate as an indicator of the extent of pollution of urban
ground water from domestic waste at Vishakhapatnam, India.

Natural microorganisms have played crucial role in the
breakdown of many industrial waste products in the environment since the
industrial revolution. Genetic manipulation of these microorganisms to
enable them to break down synthetic products is quite recent.
Unfortunately many industries discharge part or all of their waste into the
municipal sewage system making the treatment of domestic wastes difficult
and highly expensive. Microorganisms are being used to speed up the
biodegradation of organic halogen compounds, metals, organic and
inorganic acids.

The first object of modern sewage disposal is the oxidation of
putrescible organic matter. Bacteria are the agents in the process of
sewage purification. The sewage from a disease hospital, should be freed
from infectious material as a factor of safety. Sewage discharged into a
water body adapted for bathing should be so treated as to protect those
using the water.

Bacteria decompose dead plants and animals and convert
sewage and other wastes to usable and inoffensive substances. Although
the undesirable smell of the sewage is caused by microorganisms, the
smell can also disappear as a result of the action of microbial enzyme of
other aerobic and anaerobic microorganisms.

Microorganisms represent a significant percentage of the total
biomass of the earth. As a result of high rates of their growth and metabolic
activity and their adaptability, they occupy an important trophic level in the
proper functioning of the ecosystem. Microorganisms influence man in
several ways, they are ubiquitous in our environment (i.e.) they are found in soil, mud, water, air, food products etc. Biosphere contains a variety of microorganisms that proliferate in extreme environment.

The microbial world is characterized by an incredible metabolism and physiological versatility that permits them to inhabit hostile ecological niches and to exploit compounds unpalatable to higher organisms. Their metabolic versatility has led to the notion of microbial infallibility and their ability to degrade and to grow at the expense of any organic material is also the basis of the recycling of recalibrant organic matter in the biosphere.

A great quantity of organic compounds like proteins, carbohydrates, lipids and nucleic acids etc. are metabolised and recycled by microbes very easily. Many constituents of the organic matter present in aquatic environment serve as an energy and nutrient source of metabolism for heterotrophic microorganisms. The microbes play an important role in lake metabolism through their role in decomposition, nutrient release and recycling. Microbes are primarily responsible for trapping nutrients which shows lake eutrophication.

The diversity of the microorganisms and their activities vary from causing disease in human to others which produce various useful products like enzymes, amino acids, vitamins, alcohol etc. Commercially important microbial enzymes are usually extracellular and marketed in crude form. The metabolic equipment possessed by microbes could be exploited in obtaining some valuable products of daily use. The cheap raw materials available in nature as a waste may be converted into useful
commercial products by the activity of microbes. Microbes serve as a dual purpose (i) they are good agents of disposal of the wastes and (ii) the resultant products of their breakdown are useful as commercial products.

Microorganisms possess several characteristics which make them most ideal organisms for industrial processes. They produce a wide variety of enzymes making array of chemical conversions possible. The organism acts as a mini chemical factory, having efficiency of operating large amount of single product which can be isolated and purified. Among the microorganisms, bacteria are the most predominant alkalophiles which can be commercially exploited for the production of various alkaline enzymes for use in degradation of industrial effluents containing organic matter such as starch, cellulose, proteins, detergents, pharmaceuticals, food and leather industries.

Protein is one of the structural components of the plant material, while decomposing proteins, microorganisms are known to produce proteolytic enzymes for hydrolysis of proteins into polypeptides and amino acids (Hankin and Anagnostakis, 1976) based upon the size of the molecules that they can attack or preferably attack, proteolytic enzymes are called (a) proteinases (b) peptidases. Certain peptidases can degrade proteins. The distinction between proteinases and peptidases is not very realistic. The term protease refers to a mixture of proteolytic enzymes which may include proteinases and peptidases.

Enzymes are biocatalysts found in living organisms which increase the rate of biochemical reactions. Their presence enables the life
supporting chemical reactions and cell multiplication to proceed faster and with a specificity which includes the formation of by-products. Enzymes possess the properties of proteins. Over 700 enzymes are known and 100 have been crystallized. Enzymes are globular proteins with MW ranging from 10,000 to millions. Microorganisms like fungi, bacteria etc. produce enzymes which catalyse the protein molecules. Enzymes of bacteria may be divided into two groups, depending upon whether they are secreted into the surrounding medium or remain confined within the cell. The enzymes that belong to the first group are known as extracellular enzymes which are capable of producing their specific actions in the complete absence of living cell. The latter group are called intracellular enzymes or endoenzymes as they are produced within the cell.

In nature microbial mechanisms have always been responsible for the degradation of biodegradable waste matter. Proteases are enzymes produced by microorganisms which split peptide bonds. Proteases are either endo or exo peptidases. They are distinguished by their specificities. These enzymes with specificities determined by the carboxy or amine terminal of the polypeptide chains are known as exo peptidases. The endopeptidases are also called proteinases which hydrolyse the peptide bonds within polypeptide chain. Proteases regulate intracellular phenomena. Most proteins in living cells do not last as long as the cells themselves. Proteases and proteolysis play vital role in biology of all cells (Wolf, 1992). Action of proteases is divided into:
(i) **Limited proteolysis**, in which they split only a small number of peptide bonds of a target protein.

(ii) **Non-limited**, are those which show complete degradation of a target protein.

Proteins catalyse the splitting of peptide bonds. They are present in all living organisms in which they display many physio-logical functions. Bacteria synthesize a large variety of proteases. Many bacteria secrete proteases into the extracellular medium, some of which are toxins or act as virulence factors, while others have low specificity and break down proteins into small peptides and amino acids which can be transported and utilized.

Bacteria (prokaryotes) were the only form of life on earth for about two billion years, during which time they evolved into a global super organism and developed a remarkable mechanism for the creation and exchange of genetic material. Today the bacterial world can be viewed as a fascinating global entity unified by its solidarity and molecular communication. Bacteria constitute an entirely original and major domain of life. They are clearly distinct from other living beings.

Proteases / proteinases have been regarded as degradative enzymes which are capable of cleaving the peptide bonds of proteins by attacking at sites adjacent to the amino acids containing aromatic groups, thereby producing small peptides and amino acids. The role of these enzymes is to digest nutrient proteins or to participate in the turn over of
cellular proteins (North, 1982). Limited proteolysis has a key role in a wide range of cellular processes (Holzer and Tschensche, 1979).

Proteolytic enzymes are classified according to the reactions they catalyse. The most common and accepted classification is that proposed by Bergman which is as follows:

(1) **Peptidases or exo peptidases:**

Hydrolyse peptide bonds adjacent to terminal amino or terminal carboxylic acids. Example being amino peptidase, carboxy peptidase, dipeptidase etc.

(2) **Proteinase or Endopeptidases:**

These attack both centrally located and terminal peptide bonds. Examples being pepsinases, trypsinases, chymotrypsinases, etc. Proteases have been popular research objects and there is an extensive literature on its production and properties.

Microbial proteases can be divided into three groups based on pH range in which their activity is greatest; namely acid, neutral or alkaline proteases and are briefly summarised below:

(1) **Acid protease:**

These are mainly of fungal origin exhibiting maximum stability at pH 2.5 - 4.5 and having a molecular weight of around 35,000 Daltons. These are low in basic amino acid content and have low isoelectric points, but can hydrolyse a wide range of peptide bonds. These are not inhibited by diisopropylfluorophosphate, ethylene diamine tetraacetic acid or orthophenanthroline. These are much like animal enzymes and can be split
into two sub groups – pepsin like and renin like acid proteases. Aspartic acid is present in their active centres.

(2) Neutral Protease:

These are wide spread both in bacteria and fungi having molecular weight in the range of 40,000 - 45,000 Daltons and several have been shown to be zinc containing metallo enzymes (McConn et al., 1964; Keay et al., 1970; and Latt et al., 1969). They are inhibited by orthophenanathroline and EDTA but not by diisopropylflurophosphate. They exhibit some specificity towards bonds adjacent to leucine or phenyl alanine amino groups. These are used for food processing and have been shown to be active components in dental plaque reaction eg. Bacillus subtilis and Bacillus thermoproteolyticus which produces neutral proteases montase and thermoase respectively.

(3) Alkaline Proteases :

These are frequent among bacteria and fungi. They resemble the animal enzymes, trypsin and chymotrypsin. The alkaline proteases have molecular weights in the range of 25,000 – 30,000 Daltons. The most studied are subtilisin carlsberg and subtilisin novo produced by various Bacilli (Keay et al., 1970). The alkaline proteases have found extensive usage in detergents, leather tanning and food industries. Of alkaline proteases is the thermostable protease of Streptomyces rectus (Mizisawa et al., 1964). “Enzyme thermolysin” produced by B. thermoproteolyticus can retain 90% activity after 30 minutes at 70°C and thus find extensive usage in food industries.
Another scheme of classification of proteases is based on the substrate / specific proteins which is being hydrolysed viz. Keratinase, collagenase, gelatinase, elastase, caseinase etc.

A satisfactory classification has been proposed by Hartley (1960) and Hase and Finkelstein (1993) characterizing the catalytic mechanisms. This also forms the bases for enzyme commission to classify proteases. The four major groups of proteases are classified on basis of active sites and sensitivity to various inhibitors. The scheme is as follows:

(a) Serine Proteases: (EC-3.4.21):

Serine is an essential amino acid of the active centre of these proteases; these are endoproteases and have alkaline pH optima. Serine proteases have low molecular mass ranging from 18,500 - 35,000 Daltons. The microbial serine proteases are usually very stable and have a strong proteolytic activity. Serine proteases are inhibited by diisopropylfluorophosphate or phenylmethylsulfonylfluoride (PMSF). Serine proteases are useful for washing of clothes stained with blood or other proteinaceous matter. Proteases improve the general washing efficiency as protein make dirt adhere to fabrics. These enzymes are prepared in granulated form for detergents and as dedusted powder for dehairing. Serine proteases are subdivided into four groups of trypsin like proteinases which are most active at pH 8 and are sensitive to specific trypsin inhibitor like diisopropylfluorophosphate (DIFP) and soyabean trypsin inhibitor (Jurasek et al., 1969). They are resistant to denaturing agents (50% ethanol and anionic detergents).
(b) Cysteine Proteases: (EC-3.4.22):

These are active in the acid range between pH 2 and pH 3 and also a type of thiol protease containing SH group. Inhibitors of these proteases are p-chloromercuribenzoate and indoacetamide. These proteases have limited industrial applications. They have molecular mass between 30,000–45,000 Daltons. The active sites of these enzymes are cysteine residues.

(c) Aspartic Proteases: (EC-3.4.23)

These are active in acidic pH and aspartic acid forms the active site. They have a molecular mass between 30,000–45,000 Daltons. Labelled inhibitors have been used directly to demonstrate the involvement of one or two aspartic acid residues (Kovaleva et al., 1972; Liu and Hatano, 1974; Nakamaru and Takahashi, 1978). Specific inhibitors of aspartic proteases are pepstatin.

(d) Metallo Proteases: (EC-3.4.24)

These are maximally active at neutrality having molecular mass between 19,000–37,000 Daltons and contain metal atoms as cofactors. The metallo proteases are endoproteases. Zn$^{2+}$ is essential for its activity and Ca$^{2+}$ is important for maintaining stability. Neutral metallo proteases are specific towards hydrophobic / bulky amino acid. Metallo proteases are produced by several Bacillus species like B. polymyx. B. amylo liquefaciens, B. cereus, while B. thermoproteolyticus produces a
remarkable protease "Thermolysin" with high thermostability upto 80°C. Metallo protease from Bacillus spp. have been developed industrially for use as rennet substitutes but it is abandoned due to excessive casein hydrolysis. At present these proteases are used for bating in tanneries to hydrolyse barley protein in breweries and for proteolysis in the food industry. They are inhibited by chelating agents like EDTA, phosphoramidate etc.

Proteolytic enzymes of microorganisms have been extensively studied by North (1982). Protozoans, bacteria and fungi are visualized as potent protease producers. Sharma and Satyanarayana (1980) studied the production of proteases by some molds. Optimization of saprophytic and pathogenic molds have been done at different pH and temperature (Dion, 1950). Proteolytic enzymes are produced by various bacteria like Serratia, Bacillus, Pseudomonas, Proteus etc. The enzymes associated with these microbes are actually mixtures of proteinases and peptidases. The proteinases are secreted in the fermentation medium during growth while peptidases are often liberated only on the autolysis of the cells. Animal flesh being a rich source of protein is vulnerable to degradation where the microorganisms include 99% bacteria.

**Industrial Applications of Protease Enzyme:**

(1) Among the commercial applications, proteolytic enzymes are used in the bating and dehauling of hides. Acid protease samples are supplied to local tanneries where they are processed to smooth, grainy and shining variety which is not possible through chemicals.
The leather technologists observed, facilitated decreasing of hides presumably through lipase present in acid protease, besides combining the steps of bating and pickling. This saves time, labour and utilities, offering improved pliability and quality of leather.

(2) Proteolytic enzymes are employed in the textile industry to remove proteinaceous sizing and in silk industry to liberate the silk fibres from the naturally occurring proteinaceous material in which they are embedded.

(3) Beverages targeted at children, convalescing patients, pregnant / lactating women and people allergic to milk proteins, find partially hydrolysed casein and soyabean meal as useful ingredients in their food. Initial trials using acid proteases to prepare partial protein hydrolysates have been very encouraging, as slower hydrolysis of these protein provide better control over the degree of hydrolysis.

(4) More than half the cheese produced world wide now employs fungal acid proteases instead of calf rennet (Lowe, 1992). Proteases from M. pusillus and M. michie gave excellent results in actual cheese making trials (Aunstrup, 1980). These enzymes are now used as milk coagulants.

(5) Bacterial proteases are used in chill proofing of beer, in the production of condensed fish solubles and as feed supplements.

(6) Proteases are being used for the hydrolysis of gelatin layer from spent photography and X-ray film, thus helping in waste recycling.
(7) Microbial serine proteases are usually very stable, have strong proteolytic activity with a low specificity. *Bacillus* proteases viz. subtilisin carlsberg and subtilisin novo have performed excellently in detergents and thus improve the general washing efficiency of fabrics. Blood, sweat and dirt adhered to proteinaceous substances on clothes are effectively cleaned by proteases. Granular forms of enzymes are used in detergents. High temperature wash employs thermostable protease.

(8) Dalev (1994) processed feathers by pretreatment with 0.3M NaOH solutions at 80°C, along with mechanical shearing. This was followed by enzyme alkaline protease B72 from *Bacillus subtilis*. The spray dried product was a heavy grey powder with a protein content of 795 g / kg. This was used as animal feed under the name of feather protein concentrate.

(9) Doubtless proteases were the first industrially applied enzyme used in the manufacture of cheese in which the initial step is the coagulation of milk by rennin followed by separation of curd and whey.

(10) The application of proteases in food industry is rapidly becoming one of their major uses. Pure proteins are quite tasteless while some of the amino acids and certain peptides produced by protein hydrolysis are highly flavourful. Even more important, is the softening, tenderizing, increased digestibility, increased solubility and shortened cooking time which results from partial digestion of many foods with proteolytic enzymes.
(11) A relatively new application of proteases is in the treatment of stock feeds. For example enzymic attack on barley can improve its nutritional value in poultry feeds. Similarly the enzymic digestion of whole fish powder can increase the nutritional value of animal feeds. An alternative use is to feed the enzyme to the stock along with the untreated fodder.

(12) Still relatively new is the application of proteases to the baking industry by the hydrolysis of wheat gluten by proteases leading to greatly improved dough. Similarly, in the field of "quick-cook" cereals, a preliminary treatment of the cereal with a protease may shorten the cooking period and render the cereal more digestible by infants.

**Enzyme Immobilization:**

While in commercial applications enzymes are used commonly in soluble to free form, this practice is very wasteful. In order to stop this wasteful practice and to reduce the amount of enzyme leaching the products, enzyme Immobilization is practiced (Laborsky, 1973).

In practice, immobilization of an enzyme is achieved by fixing it on the surface of a water insoluble material, by trapping it inside a matrix that is permeable to the enzyme substrate end products and by cross linking it with suitable agents to give insoluble particles. Proteases have been immobilized variously to hasten various reactions. Some of the examples are acid, alkaline and neutral proteases which have been immobilized in polyacrylamide gel by entrapment. Renin and Subtilisin have been immobilized by cross linking on gluteraldehyde and amino ethyl cellulose. Among 50 immobilised enzymes, as compared to their soluble
forms, Mellose (1971) found 30 to be more stable, 8 less stable and 12 to be showing no change. The shielding of the active sites/groups on the enzyme from reactive groups may have increased enzyme stability.

The Enzyme Industry (Market Position and Cost Factors):

World consumption of industrial extracellular microbial enzymes is difficult to evaluate but an estimate that worldwide sales of microbial enzymes amount to about $1 billion per year (Rao et al., 1998). Although this value is mostly for non-food and detergent industries, the medicinal and analytical applications are not included. Some of the commercial extracellular proteases and their trade names have been depicted below:

- **Bacillus subtilis**
  - Neutrase
  - Rapidermase
  - Proteinase 18
  - Protein

- **Bacillus licheniformis**
  - Alcalase
  - Optimase
  - Maxatase P.

- **Bacillus thermoproteolyticus**
  - Thermoase (N.P.)
  - Thermolysin

As each manufacturer has his own method of analysis and enzymes are sold on activity basis, enzyme costs are difficult to compare. Proteases are the single class of enzymes which occupy a pivotal position due to their wide application in detergent, pharmaceutical, brewing, leather,
food and agricultural industries. The current estimated value of the world wide sales of industrial enzymes is $1 billion and proteases account for about 60% of the total sales (Rao et al., 1998). The recent growth in the industrial and medical application of enzymes have resulted from greatly improved methods for their production, especially from plant and microbial sources. In no small degree has it also evolved a result of our deeper understanding of the chemical and physical properties of enzymes and of the kinetics of the total enzyme, substrate system, and specific reference to the role of activators and inhibitors. This fundamental knowledge of enzyme systems will inevitably lead to major growth in the industrial and medical applications of enzymes in the near future.

Since prehistoric times, humans have exploited microorganisms for their own use. By means of trial and error, people developed strains of microbes that were used in the production of beverages, food, textiles and antibiotics without knowing that microbes were the responsible agents. Expanded development of microbial screening and cultural techniques has brought us to a point where microbially produced products are a major part of our life.

The microbes play an important role in lake metabolism through their role in decomposition, nutrient release and cycling. The functional aspects of microbes, i.e. decomposition of organic mater is an important step in detritus formation and thereby forms a major link in the food chain of the aquatic system.
Keeping the above facts in consideration, a major integrated research work was undertaken for the study of a natural lake (Sagar lake) in the district of Sagar, where virtually speaking no microbiological work has been undertaken earlier. The work can be greatly extended by genetically manipulating the present isolates either by mutation or protoplast fusion. Similarly devising certain media contain cheap substrates and inducers, which can enhance enzyme production and thus help to reduce enzyme cost. This work will give an idea of biodiversity of microorganisms with special reference to proteolytic bacteria present in Sagar lake water.

The present work has, therefore been planned to study the proteolytic bacteria in the effluents of Daphrin Hospital into Sagar lake.

The main objectives are:

1. Selection and survey of study sites.
2. To study the physico-chemical characteristics of effluents of Daphrin Hospital into Sagar lake, i.e. pH, temperature, alkalinity, chloride, DO, BOD, COD, nitrate and phosphate.
4. Characterizing the protease potential of the bacterial isolates
   (i) Effect of incubation time on protease activity.
   (ii) Effect of pH on protease activity and stability.
   (iii) Effect of temperature on protease activity and stability.
   (iv) Effect of metal ions on protease activity.
   (v) Effect of inhibitors on protease activity.
5. Biodegradation ability of the proteolytic bacteria.