CHAPTER – I

INTRODUCTION

Solid waste is an inevitable by-product of human activities. In the past, this was not a major problem because almost everything was recycled and reused and whatever remained was taken care of by nature. However, the introduction of new materials and changing consumption patterns, especially in urban areas, have resulted in increasing volumes of waste and, as a result, breakdown of traditional system of waste management has taken place. In Nepal, as in many other developing countries, these changes have taken place rapidly over the past few decades, while the government and the people have failed to realize their serious implications and the urgent need to address them. As a result, many cities in Nepal are now suffering from the adverse impacts of unmanaged waste. The problem is acute, particularly in large cities like those within Kathmandu Metropolis where improper management of waste has led to environmental pollution, public health hazards, and adverse effects on an urban economy that depends heavily on tourism.

Various studies carried out since 1976 indicate that around two-thirds of the municipal solid waste generated in Kathmandu Metropolitan City is organic; this has remained relatively unchanged over the years (IUCN, 1992; Udas, 2004; ICIMOD, 2007). Of the rest inorganic components, majority can be recycled and reused with a little amount requiring land-filling. This indicates that rapid degradation of these organic components of the waste may help in the adequate management of the solid waste. Biological degradation is quite preferable to chemical degradation because of high efficiency of degrading enzymes with lesser adverse effects as compared to the chemicals used for degradation. Microbes produce degradative enzymes which degrade organic fractions of the waste considerably reducing its volume.

Majority of the organic fraction of the waste constitutes of starch, lipid, protein, cellulose, hemicellulose, lignin etc. Almost all of these components can be degraded via specific enzymes. Protein degradation initiates and continues in the presence of proteases. Various proteolytic microorganisms are capable of producing the enzyme protease. Proteases are the single class of enzymes which occupy a pivotal position with respect to their application in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptides bonds in other proteins. These enzymes are primarily used in the detergent, dairy and pharmaceutical industry and these may be alkaline, neutral or acidic in nature (Gupta, 2004).

Besides these, protease enzymes are being employed in many different ways for welfare of mankind. Proteases are the most important group of industrial enzymes. Commercially these can be used for cheese making, as batting agent in leather industries, recovering silver from photographic films, fruit-juice manufacturing, and production of liquid and powder detergents. These are responsible for the complex process involved in the normal physiology of the cell as well as in abnormal physiological conditions. Their involvement in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. The vast diversity of protease, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological application (Rao et al, 1998).

This study aims to explore useful microbial flora and their implications in solid waste management. Isolates exhibiting higher degree of activity can be exploited for solid waste management either in their wild or improved form. Such endogenous technologies are cost-effective and environment friendly. This may lead to formulate indigenous technology that can be applied to keep the city clean and healthier. This will also help to create engineered

processes to push the materials, for which natural cyclic processes are very slow or hardly exist, along the natural cycles, thus reducing environmental deterioration.

CHAPTER – II

OBJECTIVES

2.1 General objective:

To study proteolytic activity of bacteria from municipal solid waste of Kathmandu and their enzyme kinetics.

2.2 Specific objectives:

i. To screen proteolytic bacteria from solid wastes.
ii. To extract the protease enzymes produced by the bacterial isolates.
iii. To purify the enzymes partially.
iv. To study the kinetics of purified enzymes.

CHAPTER – III

LITERATURE REVIEW

3.1 Solid waste

Waste is a by-product of human activity. It contains the same material as are found in useful products, but it only differs from useful products by its lack of value (Goen, 1998). Waste may be solid or aqueous depending upon their composition. Solid wastes account for an increasing proportion of the waste streams generated by modern urban societies. Solid waste is one of the most visible environmental nuisances and major causes of pollution in the urban areas of Kathmandu Valley with consequences beyond the urban limits (ICIMOD, 2007). While part of this material will be made up of glass, plastics etc., a considerable proportion will be decomposable solid organic material such as paper, food wastes, sewage wastes, wastes from large scale poultry and pig farm etc (Smith, 1996).

Disposal of solid waste materials currently relies principally on landfilling (i.e. burial) in loose and compacted form and creates environmental problems such as leaching of nutrients and heavy metals into groundwater resources. Other forms of solid waste disposal around the world include incineration and landfill after baling of the wastes. These methods, however, create environmental problems of their own such as air pollution and long – term stockpiles of waste which remain essentially unchanged years after disposal thus limiting future uses of the land (Goen, 1998).

In addition, the impacts of inadequate and improper management of waste are many: direct and indirect adverse impacts on health and well-being, pollution of water either directly by solid waste dumping or indirectly via leachate and air impacts on cultural sites, impacts on aquatic life and the ecosystem, bad aesthetics and others. Children are particularly susceptible to respiratory problems caused by dust from dump sites but also to diarrhea, skin and eye infections, and other diseases. The major child – killing diseases in developing

countries are related to unsanitary conditions such as those associated with uncollected solid waste (IUCN, 1992).

For the general public the main risks to health are indirect and arise from the breeding of vectors like flies and mosquitoes and the attraction of rodents and stray animals to waste. These animals become infected with diseases that are then passed on to human (IUCN, 1992). Health care costs impose an enormous drain on the economy. At the operational level, the municipalities are the main bodies responsible for solid waste management on a daily basis. They spend significant amounts (typically in the range of 20 to 25% of their total expenditure) on solid waste management and have established separate sections within their institutions to deal with waste (ICIMOD, 2007). The large bill for remedial action is budgetary burden. If uncontrolled these costs will handicap future generation, as they face cumulative budgetary costs, health risks and environmental deterioration.

The types of the wastes as described by IUCN are given in the tables below:

Table 3.1 Solid waste types associated with various source classification.

Sources	Types of solid waste
Domestic	Garbage, rubbish, ashes, sewage, some special waste.
Commercial	Garbage, rubbish, ashes, sewage, some special waste, street wastes.
Industries	Construction, demolition waste, special waste, industrial waste, sewage, rubbish, street waste.
Agriculture	Garbage, rubbish, special waste, sewage, ashes.
Institutions	Special waste, street wastes, sewage, garbage, ashes.
Nature	Street waste, dead animals.

Source: IUCN, 1992

Table 3.2 Waste material by kind and composition

Kind	Composition
Garbage	Wastes from preparation, cooking and serving of food; market wastes; and wastes from handling, storage, and sale of products.
Rubbish	Combustibles :- paper, cartons, boxes, barrels, wood, excelsior, tree branches, yard trimmings, wood furniture, bedding rags, temple offerings. Non – combustibles :- hair, metal, feathers, bones, cans, metal furniture, dirt, glass, crockery, minerals, plastic, rubber, pots.
Ashes	Residue from fires used for cooking, heating, funerals and on-site incineration.
Street wastes	Sweepings, dirt, leaves, catch basin dirt, contents of litter receptacles.
Dead animals	Cats, dogs, cows, goats, pigs etc, which die naturally or accidentally.
Industrial wastes	Food-processing wastes, lumber and metal scrap, hides, old machine parts, waste from slaughter – houses etc.
Demolition wastes	Lumber, pipes, brick, masonry and construction materials from demolished buildings and other structures.
Construction wastes	Lumber scrap, pipes, bricks, and other construction materials.
Special wastes	Hazardous solids, pathological wastes, batteries.
Sewage	Solids from septic tanks, excrement.

Source: IUCN, 1992

The growing volume of waste materials is particularly acute in developing countries, where improved waste management technologies have not yet been widely adopted. Each year millions of tons of a vast variety of wastes generated daily by residents, industries, commercial establishments and institutions cause severe consequences, the most tragic of which are the millions of child and adult fatalities from disease caused by the improper disposal of these wastes (Munasinghe, 1994).

Compared to other countries, Nepal still generates very little waste and most of what is generated is not hazardous and easily recyclable (MOPE, 2001). A few studies prepared on waste generation in Kathmandu city indicate that the per capita waste generation is low compared to most other countries and that about two-thirds of the waste materials are organic (Lohani and Thanh, 1978; GTZ, 1988; Khanal, 1993; Rai, 1990; IUCN, 1992; Mishra and Kayastha, 1998; RESTUC, 1999). Therefore, the increasing volume of waste being generated would not be a problem if waste was viewed as a resource and managed properly.

According to a survey, the solid waste in Kathmandu Metropolitan City in 2001 constituted 69% organic fraction, 9% plastic, 9% paper, 1% metal, 3% glass, 1% rubber, 2% construction material, 3% clothe and only 3% of other materials (Udas, 2004). This data clearly shows that major fraction of the wastes generated in Kathmandu is of degradative type. The degradation of these fractions is enhanced by the hydrolytic enzymes. Since, the trend of adding these enzymes for the degradation of such fractions is not in practice; mostly the enzymes are produced by the indigenous microorganisms of the waste.

3.2 Enzymes:

Enzymes are single-chain or multiple-chain proteins that act as biological catalysts with the ability to promote specific chemical reactions under the mild conditions prevailing in most

living organisms. Enzymes have three distinctive characteristics – high specificity (the ability to promote a particular chemical reaction on a single or a small number of structurally related substrates), high reaction rate (the ability to enhance the reaction rate by factors as high as 10^{12} relative to the non-enzyme catalyzed reaction) and high capacity for regulation (the ability to modify their catalytic activity in response to changing cellular and physiological demands) (Wilson and Walker, 2000).

The term 'enzyme' was coined in 1878 by Frederick Wilhelm Kuhne to designate the 'biological catalysts' that had previously been called 'ferments' (Jain et al, 2005). With the exception of a few catalytic RNAs, all known enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. Many enzymes require non-protein co-enzymes or co-factors for their catalytic function (Nelson and Cox, 2005).

3.3 Classification of enzymes

Enzymes are classified by the reaction they catalyze. Many enzymes have been named by adding the suffix "-ase" to the name of their substrate or to a word or phrase describing their activity. For example, urease for the enzyme that hydrolyzes urea. Other enzymes were named by their discoverers for a broad function, before the specific reaction catalyzed was known. For example, an enzyme known to act in the digestion of foods was named pepsin, from the Greek *pepsis,* "digestion", and lysozyme was named for its ability to lyse bacterial cell walls.

Because of ambiguities in the nomenclature of enzymes and the ever increasing number of newly discovered enzymes, biochemists, by international agreement, have adopted a system for naming and classifying enzymes. This system divides enzymes into six classes, each

with subclasses, based on the type of reaction catalyzed (Table 3.3). Each enzyme is assigned a four-part classification number called Enzyme Commission number.

Table 3.3 International classification of enzymes

No.	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms).
2	Transferases	Group transfer reactions.
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water).
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups.
5	Isomerases	Transfer of groups within molecules to yield isomeric forms.
6	Ligases	Formation of C–C, C–S, C–O, and C–N bonds by condensation reactions coupled to ATP cleavage.

Source: Nelson and Cox, 2005

3.4 Protease

A protease is any enzyme that conducts proteolysis, i.e. begins protein catabolism by hydrolysis of the peptide bonds that link amino acid together in the polypeptide chain, which form a molecule of protein. Proteases occur naturally in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly – regulated cascades like the blood clotting cascade, the complement system, apoptosis pathways etc. They can either break specific peptide bonds

(limited proteolysis), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (unlimited proteolysis). The activity can be a destructive change abolishing a protein's function or digesting it to its principal components; it can be an activation of a function, or it can be a signal in signaling pathway. Proteases determine the life-time of other proteins like hormones, antibodies or other enzymes playing important physiological role (Wikipedia, 2009). Proteases account for about 60% of the total worldwide sale of enzymes. About 500 tons of protease is now produced per year (Chandran et al, 2006).

3.5Classification of protease

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (Jain, 2005). Currently, proteases are classified on the basis of three major criteria: Type of reaction catalyzed; Chemical nature of the catalytic site; and Evolutionary relationship with reference to structure (Barett, 1994).

Depending on their site of action, proteases are grossly subdivided into two major groups, i.e, exopeptidases and endopeptidases (Smith, 1986).

3.5.1.1 Exopeptidases

Exopeptidases cleave the peptide bonds proximal to the amino or carboxy termini of the substrate. Based on this site of action, these are further classified as aminopeptidases and carboxypeptidases respectively (Smith, 1986).

3.5.1.1.1 Aminopeptidases

This enzyme acts at a free N-terminus of the polypeptide chain and liberate a single amino acid residue or a dipeptide or a tripeptide. Aminopeptidases occur in a wide variety of

microbial species including bacteria and fungi (Watson, 1976). Aminopeptidases by *Bacillus licheniformis* has a molecular weight of 34 and its activity is enhanced by CO^{2+} ions (Khatiwada, 2004).

3.5.1.1.2 Carboxypeptidases

This enzyme acts at C-terminus of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups; serine carboxypeptidases, metallo-carboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzyme (Rao et al, 1998).

3.5.1.2 Endopeptidases

Endopeptidases cleave the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini (Smith, 1986). The endopeptidases are divided into four subgroups based on their catalytic mechanism.

3.5.1.2.1 Serine proteases

Serine proteases are characterized by the presence of serine group in their active site. These are numerous and widespread among viruses, bacteria and eukaryotes, suggesting that these are vital to the organisms. Serine proteases are recognized by their irreversible inhibition by 3, 4 – dichloroisocoumarian (3, 4 DCI); L – 3 carboxytrans 2, 3 – epoxypropyl – leucyl amido (4 – guanidine) butane, di-isopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl – L – lysine chloromethyl ketone (TLCK). Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11 (Rao et al, 1998). Serine proteases are further classified into serine alkaline proteases and subtilisins.

3.5.1.2.1.1 Serine alkaline proteases

Serine alkaline proteases are produced by several bacteria, yeasts and molds. They hydrolyze a peptide bond which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. The optimal pH of alkaline proteases is around pH 10. Alkaline serine proteases are produced by several bacteria such as *Arthrobacter* spp*, Streptomyces* spp *and Flavobacterium* spp (Boguslawski et al, 1983).

3.5.1.2.1.2 Subtilisins

Subtilisins of Bacillus origin represent the second largest family of serine proteses. There are two different types of alkaline proteases, subtilisin Carlsberg produced by *Bacillus licheniformis* and subtilisin Novo produced by *B. amyloliquefaciens*. They both have optimal temperature 60^0C and pH 10 with broad substrate specificity. Proteases of the subtilisin group are used in the pharmaceutical industry for the treatment of burns and wounds (Rao and Narasu, 2007).

3.5.1.2.2 Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Aspartic protease have been grouped into three families, namely, retropepsin, and enzymes from pararetroviruses (Barett, 1995). Most aspartic proteases show maximal activity at low pH (pH 3 to 4). The aspartic proteases are inhibited by pepstatin (Fitzgerald et al, 1990).

3.5.1.2.3 Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Cysteine proteases have neutral pH

optima, although a few of them like lysosomal proteases are maximally active at acidic pH (Khatiwada, 2004).

3.5.1.2.4 Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, haemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs et al, 1985). Metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing and may be useful in the treatment of disease such as cancer and arthritis (Khatiwada, 2004).

Besides these, other two classes of protease named as threonine proteases and glutamic acid proteases were also described after 1995 and 2004 respectively (Wikipedia, 2009).

Depending upon the pH of their optimal activity, proteases are classified into acid proteases, alkaline proteases and neutral proteases.

3.5.2.1 Acid proteases

These proteases are acid resistant and optimum pH for activity is around 2 – 3. For example, protease produced by *Aspergillus niger*. Acid proteases have application in meat tenderization, in the production of fermented foods and also in acidic cleaning compositions (Rao et al, 2007).

3.5.2.2 Alkaline proteases

Most important producers of these proteases are *Bacillus licheniformis, B. amyloliquefaciens, B. firmus, B. megaterium* and *B. pumilus*. Besides these, alkaline

proteases are produced by a wide variety of microorganisms like *Bacillus subtilis, Aspergillus oryzae, Streptomyces cellulasae, Aeromonas hydrophila*. Alkaline proteases are mostly used in detergent industry (Gupta, 2004). Alkaline proteases have numerous applications in food industries, silver recovery from x – ray films and several bioremediation processes. The silk degumming efficiency of an alkaline protease from *Bacillus sp.* RGR – 14 was also reported (Khatiwada, 2004).

3.5.2.3 Neutral proteases

These proteases have optimum pH 7.0 and mostly produced by *Bacillus subtilis, B. cereus, B. megaterium, Aspergillus oryzae, A. sojae* etc. Neutral proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies (Rao and Narasu, 2007).

3.6 Sources of proteases

Since proteases are physiologically necessary for living organisms, they occur naturally in all organisms. These enzymes are synthesized not only by microorganisms but also by higher plants and animals. However, microbes are considered preferred sources of protease due to their broad biochemical diversity and susceptibility to genetic manipulation. Microorganisms degrade proteins and utilize the degradation products as nutrients for their growth. Mahmoud et al (2008) reported that proteases account for about 60% of the total amount of the enzymes sale in the world with 2/3 of this amount being of microbial origin.

Among the proteolytic organisms most are aerobic bacteria, actinomycetes and fungi as well as certain facultative and strict anaerobes (Alexander, 1983). Among them, bacteria involved in proteolysis are the member of genera *Achromobacter, Acinetobacter, Aeromonas, Bacillus, Clostridium, Enterobacter, Escherichia, Lactobacillus, Leuconostoc, Micrococcus, Pseudomonas, Proteus, Serratia* and *Vibrio* (Alexander, 1983; Pelczar et al,

1993). Most of species of *Bacillus* have been shown to bear proteolytic activity. *Bacillus ceregulans, B. firmus, B. sphaericus, B. subtilis, B. megaterium, B. licheniformis, B. coagulans, B. brevis* and *B. pantothenticus* isolated from leather factory and slaughtering house effluents have also shown high proteolytic activity (Shrestha, 1995). The thermophilic bacteria *B. stearothermophilus, B. circulans, B. coagulans, B. licheniformis* are also having higher proteolytic activity (Shrestha, 1996).

Besides these, proteases are also produced by a wide diversity of plants and animals. On the basis of sources, proteases can be divided into plant proteases, animal proteases and microbial proteases (Chandran et al, 2006).

3.6.1 Plant proteases

The widely known proteases of plant origin are papain, bromelain, keratinases and ficin. Papain is a traditional plant protease and has a long history of use (Schechler and Berger, 1967). It is extracted from the latex of *Carica papaya* fruits and active between pH 5 to 9 and is stable upto 80^0C to 90^0C in the presence of substrates. It is used in brewing industry as a stabilizer for chill-proof beer because it removes small amounts of protein that cause turbidity in chilled beer (Jain et al, 2005).

Another plant protease bromelain is prepared from the stem and juice of pineapples. The enzyme is characterized as a cysteine protease and is active from pH 5 to 9 and inactivated at 70^0C. Either bromelain or papain can be used to treat the meat prior to its cooking so as to tenderize it by hydrolyzing peptide bonds because hydroxyprolyl residues create bonds in collagen helices, which contribute to the tough and rubbery texture often associated with cooked meat (Jain et al, 2005). Similarly, keratinases are also produced from plants and degrade hair, wool so as to prevent clogging of wastewater system (Rao et al, 1998).

The major factors that limit the use of plants as protease source are availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process (Chandran et al, 2006).

3.6.2 Animal proteases

The most familiar protease of animal origin is pancreatic trypsin, chymotrypsin, pepsin and reninns (Boyer, 1971). Trypsin is a serine protease and hydrolyzes peptide bonds in which the carbonyl groups are contributed by lysine and arginine residues. Trypsin has limited applications in the food industry. Chymotrypsin is found in animal pancreatic extract and it is very expensive enzyme. It is used only for diagnostic and analytical application. Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates. It is an aspartic protease and resembles human immunodeficiency virus type I (HIV – 1) protease, responsible for the mutation of the virus. It exhibits optimum activity between pH 1.0 and 2.0, while the optimal pH of the stomach is 2.0 to 4.0. It is inactivated above pH 6.0. Rennin (rennet) is pepsin like protease that is produced as an inactive precursor, prorennin, in the stomachs of all nursing mammals. It is used extensively in the dairy industry to produce a stable curd with good flavor. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in casein to generate insoluble para-casein C-terminal glycopeptides.

Besides these, animal proteases have a wide application. In the manufacture of leather, the hide is made free from hair by employing pancreatic enzyme which hydrolyzes the proteins of hair follicles, thus freeing the hair so that it may be easily scraped off from the hide. Pepsin is used to digest gelatin in the process of recovering silver from photographic films. Proteolytic enzymes from pig pancreas are used to alleviate skin diseases, bed sores and sloughing wounds. These enzymes act by destroying proteolytic enzymes of man, which prevent the healing of such wounds. The enzymes commonly used for wound debridement

are the proteases such as streptodornase, ficin and trypsin. But the production of animal proteases depends on the availability of livestock for slaughter, which is governed by political and agricultural policies (Chandran et al, 2006).

3.6.3 Microbial proteases

The inability of the plant and animal proteases to meet the current global needs has led to an increased interest in microbial protease. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbes serve as a preferred source of these enzymes because of their rapid growth, limited space requirement for cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties desirable for their various applications (Rao et al, 1998). Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Chandran et al, 2006).

Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. In bacteria, serine- and metallo-proteases are the principal classes of proteases found in several species such as *Bacillus subtilis, B. amyloliquefaciens, Pseudomonas sp., Lysobacter enzymogenes* and *Escherichia coli*. Identification and characterization of microbial proteases are prerequisites for understanding there role in the pathogenesis of infectious diseases as well as to improve their application in biotechnology. Furthermore, they find application in the food, pharmaceutic and detergent industries and are important tools in studying the structure of proteins and polypeptides (Khatiwada, 2004).

3.6.3.1 Bacteria

Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range

(pH 5 to 8) and have relatively low thermo tolerance, which is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Bacterial alkaline proteases are characterized by their high activity at alkaline pH (around pH 10) and their broad substrate specificity. Their optimal temperature is around 60^0C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao et al, 1998). The protease obtained from *Pseudomonas aeruginosa* PD100 was found to have potential applications in waste treatment, leather industry and detergent industry (Najafi et al, 2005).

3.6.3.2 Fungi

Fungi elaborate a wider variety of enzymes than do bacteria, for instance, *Aspergillus oryzae* produces acid, neutral and alkaline protease. The fungal proteases are active over a wide range of pH (pH 4.0 to 11.0) and exhibit broad substrate specificity. In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal and bacterial proteases in reducing the bitterness of food protein hydrolysates (Rao et al, 1998). Extracellular proteases of many animal and plant parasitic fungi have also been implicated in the penetration and digestion of host tissues. For instance, the protease obtained from a strain of *Arthrobotrys oligospora* could immobilize nematodes in vitro (Minglian et al, 2004)

3.6.3.3 Actinomycetes

Protease enzyme is also produced by Actinomycetes. Proteolytic enzymes of *Streptomyces* spp. or *Actinomycetes* spp. have been studied mainly in relation to the bactreriolytic action of the cultures of these bacteria. The presence of proteolytic enzyme in *Streptomyces* cultures and partial purification of a protease from *S. griseus* were reported in 1949 and 1955 (Boyer et al, 1960). The enzyme was at first concentrated by ammonium sulphate

(Boyer et al, 1960). Upton and Fogarty (1977) reported presence and purification of thermostable amylase and protease of *Thermomonopora viridans*, a thermophilic actinomyces, isolated from peat by other workers. Some examples of important protease producing actinomycetes include *S. hygroscopicus, S. thermoviolaceus, Thermoactinomyces thalpophilus and Thermomonospora viridis* (Shrestha, 1995).

3.6.3.4 Viruses

Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal disease such as AIDS and cancer. Serine, aspartic and cysteine peptidases are found in various viruses (Rawlings and Barrett, 1993). An extensive literature is available on the expression, purification and enzymatic analysis of retroviral aspartic protease and its mutants (Samal et al, 1989).

3.7 Purification and characterization of proteases

A better understanding of the function of enzyme could be determined by purification of enzyme. The primary objective in purification is the removal of excessive amount of water in the cell free extract. A direct method is concentration of extract using solvents or salts with a high affinity which results in the precipitation of protein. Acetone is the most widely used solvent for the partial purification of enzyme. Similarly, in practice, ammonium sulphate is the salt commonly used since it is highly water-soluble, relatively cheap and available at high purity. Furthermore, it has no adverse effects upon enzyme activity. In addition to this, purification can be achieved by chromatographic and electrophoretic procedures. Column techniques have the advantage that the resulting fractions are amenable to quantitation using general analytical methods. Gel permeation chromatography separates proteins based on their size while ion exchange chromatography use substituted cellulose ion exchangers, diethylaminoethyl (DEAE) cellulose and carboxymethyl cellulose (CM).

Electrophoretic separation of protease can be achieved by difference in size and charge (Chandran et al, 2006).

Ammonium sulphate purification is an extremely useful and universally applicable purification step and is often employed at later stages of purification simply to concentrate the protein from dilute solution after procedures such as gel filtration. The solubility of proteins varies according to the ionic strength and hence according to the salt concentration of the solution. At low concentrations of salt, the solubility of the protein increases with salt concentration, an effect called 'salting-in'. However, as the salt concentration is increased still further, the solubility of the protein begins to decrease. At sufficiently high ionic strength the protein solubility will have decreased to the point where the protein will be almost completely precipitated from solution, an effect called 'salting-out'. Since proteins differ markedly in their solubility at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein. Indeed, enzyme purification schemes almost invariably include such a step.

Characterization of proteases can be carried out by determining the properties and kinetic parameters of the enzyme (Chandran et al, 2006). Protease enzymes show different responses towards changes in temperature, pH and substrate concentration which directly influences the reaction velocity of enzymes. The responses give us valuable clues about how enzymes function in living cells (Champe et al, 1994).

3.7.1 Effect of temperature

Being proteinaceous in nature, the enzymes are sensitive to heat. According to Jain et al (2005), the effect of temperature on enzymatic reactions usually shows two distinct effects.

- An increase in rate of reaction with increase in temperature until a maximum rate is achieved.

- A region at high temperature in which the rate decreases with increase in temperature due to the thermal inactivation of the enzyme.

The rate of an enzyme action increases with rise in temperature, the rate being frequently increased 2 to 3 times for a rise in temperature of 10^0C. But at higher temperatures, the rate decreases rapidly. Above 60^0C, many enzymes coagulate and thus become inactivated, because there occurs an irreversible change in their chemical structure. Because enzymes are globular proteins, most are thermolabile and begin to denature at temperatures between 45^0C and 50^0C. Denaturation of enzyme is indicated by loss of activity. At low temperatures, the catalytic activity of the enzyme predominates, although some thermal denaturation takes place during this period. Decreasing temperatures to near or below 0^0C although inactivate the enzyme but this is a reversible type of change and the enzyme regains its catalyzing power upon increasing the temperature to optimum. At higher temperature, although the catalytic activity of the enzyme increases, yet its denaturation predominates. Henceforth, all the enzyme is denatured in a very short time. The enhanced enzyme activity with the rise in temperature is due to the fact that the energy of molecule becomes greater, which, in turn, enhances the inherent reactivity of the molecules and the frequency of their collisions. It is because of the high rate of enzyme destruction at increasing temperatures that an enzyme is stable for weeks at 0^0C, for days at 10^0C, for hours at 30^0C but for fraction of seconds at 70^0C (Jain et al, 2005).

The effect of temperature upon the proteases from *Bacillus* spp. was when studied by incubating at different temperature from 25^0C to 70^0C, an optimum temperature for maximum activity was found to be at 55^0C (Khamviwath et al, 1992). *Bacillus subtilis* isolated from a hot spring showed proteolytic activity; the partially purified enzyme was stable at 60^0C for 30 minutes (Kembhavi et al, 1993). A heat stable proteinase produced by Bacillus strain displayed maximum activity at 60^0C. This is significantly different from the

optimal fermentation conditions, which confirmed the thermostable nature of enzyme (Elfadaly et al, 1993).

3.7.2 Effect of pH

Enzymes have an optimum pH (or pH range) at which their activity is maximal; at higher or lower pH, activity decreases. This is because amino acid side chains in the active site may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization, and elsewhere in the protein, ionized side chains may play an essential role in the interactions that maintain protein structure. However, in the closely packed environment of a protein, the pK_a value of an amino acid side chain can be significantly altered from its value in the free amino acid (Nelson and Cox, 2005).

In most cases the change in enzymatic activity at different pH levels is caused by changes in the ionization of the enzyme, substrate or the enzyme – substrate complex. The effect of pH on an enzyme reaction is crucial for most enzyme activities, since substrate binding and catalysis are dependent on the charge distribution of substrate and enzyme molecules. Some enzymes are active over a broad range of pH values, although most are active only over a relatively narrow range. The pH optimum varies for different enzymes. In addition, enzyme stability is affected by pH and typically the stability range tends to be much greater than the activity range. Enzymes are not stable at all pH levels, therefore it is important to determine the pH range of enzyme activity (Jain et al, 2005).

Optima of many bacterial enzymes are between pH 6.0 to 8.0, and those of yeasts and molds between pH 3.0 to 5.0. The influence of pH on enzyme action has been attributed to its effect on ionization of the enzyme and of the substrate. The activity of proteases from *Thermus* spp. strain Rt 41A against azocasein was observed at pH 8.0. Similarly, the enzyme was stable for at least 4 hours at 70^0C in the range of pH 5.0 to 10.0 (Peek et al,

1992). The optimum pH for the proteases obtained from alkalophilic *Bacillus* spp. was found to be 11.0 (Khamviwath et al, 1992). Similarly in another study, alkalophilic *Bacillus* spp. produced protease which gives maximum activities in the range of pH 10.5 – 11.5 and the enzyme was stable between pH 5.0 and 11.5 (Fujiwara and Yamamoto, 1987). An alkaline proteinase produced by alkalophilic *Bacillus pumilus* displayed optimum activity in the range of pH 10.0 – 10.5 at 45^0C (Qiu et al, 1990). A thermostable alkaline protease from *Thermoactinomyces* spp. performed its maximum activity at pH 11.5 (Tsachiya et al, 1992). Protease from *Bacillus* spp. EA-1 gave a narrow pH profile, the optimum activity at pH 9.0 and the enzyme was stable in the range of pH 8.0 to 10.0 (Salleh, 1994). Proteases from *Bacillus subtilis* has optimum enzyme activity at pH 6.5 to 7.5 at 40^0C.

3.7.3 Effect of substrate concentration

The effect of substrate concentration on the initial velocity of an enzyme reaction is very important for the practical use of enzymes as well as to a fundamental understanding of enzymatic reactions.

The rate of enzyme catalyzed reaction increases with substrate concentration until a maximum velocity (V_{max}) is reached. The rate of velocity of a reaction (V) is the number of substrate molecules converted to product per unit time and is usually expressed as millimole per litre per minute.

If the activity of an enzyme is determined over a range of substrate concentrations a curve similar to the rectangular hyperbola of Fig. 3.1 is often obtained. At low substrate concentrations, reaction velocity varies linearly with substrate concentration but at higher concentrations of the substrate the reaction velocity tends to be constant (Plummer, 1987).

Leonor Michaelis and Maud Menten in 1913, while studying the hydrolysis of sucrose catalyzed by the enzyme invertase, proposed this theory. The theory proposed a simple model that accounts for most of the features of enzyme-catalyzed reaction (Jain, 2005). The enzyme-catalyzed reactions may be symbolically represented as follows:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

where,

'S' is the substrate

'E' is the enzyme

'P' is the product

'ES' is the enzyme – substrate complex

K_1, K_{-1} and K_2 are rate constants.

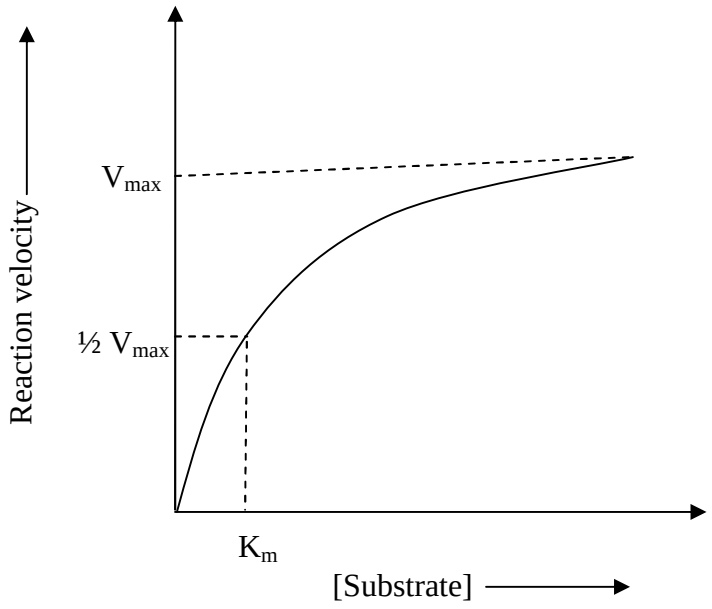


Fig 3.1 Effect of substrate concentration on the rate of enzyme activity

According to this model, Michaelis – Menten equation describes how reaction velocity varies with substrate concentration.

$$V_0 = \frac{V_{max}\,[S]}{K_m + [S]}$$

The Michaelis constant (K_m) values of the enzymes differ widely. However, for most of the enzymes, the general range is between (10^{-1} to 10^{-6} M). The K_m value depends on the particular substrate and on the temperature and ionic concentration.

3.8 Applications of proteases

Proteases have a large variety of applications, mainly in the detergent and food industries. It is also extensively used in the pharmaceutical industry. Protease for food and detergent industries are prepared in bulk quantities and used as crude preparation, while those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Rao et al, 1998).

3.8.1 Detergents

Proteases are one of the standard ingredients of all kinds of detergents used in laundry and accounts for approximately 25% of the total worldwide sales of enzymes. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name B10-40 (Rao et al, 1998). Due to the present energy crisis and the awareness for energy conservation, it is desirable to use proteases that are active at lower temperature. All detergent protease currently used in the market are serine proteases produced by *Bacillus* strains (Khatiwada, 2004).

3.8.2 Leather industry

The conventional methods of leather processing involve hazardous chemicals like sodium sulfide, which create problems of pollution and effluent disposal. This can be greatly reduced by the use of protease and it also helps for improving leather quality. Currently, trypsin is used in combination with other *Bacillus* and *Aspergillus* protease for bating. Hence, proteases are extensively used in the leather industry, particularly in bating hides to remove unwanted interfibrillar materials (Khatiwada, 2004).

3.8.3 Food industry

There is a great application of protease in food industry for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization (Rao et al, 1998). In cheese making, the primary function of proteases is to hydrolyze the specific peptide bond to generate para-casein and macro peptides. Chymosin is preferred due to its high specificity for casein, and now three recombinant chymosin product are available for use in cheese making (Pradhan, 1997). Similarly in baking industry, proteases are used to modify wheat gluten by limited proteolysis, which facilitates to prepare wider range of products. Besides, proteases are used to recover proteins from parts of animals and fish which would otherwise go to waste after butchering (Chaplin, 1990).

3.8.4 Pharmaceutical industry

The wider diversity and specificity of protease helps to develop effective therapeutic agents such as tumor dissemination and the invasion of parasites (Pradhan, 1997). Oral administration of protease has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Also oral administration of protease produces an anti – inflammatory response in burn patients and speeds up the healing process (Rao and Narasu, 2007). Clostridial collagenase or subtilisin is used in combination with broad spectrum antibiotics in the treatment of burns and wounds (Rao et al, 1998).

3.8.5 Other applications

Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in elucidation of structure – function relationship in the synthesis of peptides, and in the sequencing of proteins as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins (Rao et al, 1998).

The protease is also found effective in recycling process. The enzymatic method makes it possible to recycle not only silver but also polyethylene terephthalate (PET) film base as a raw material from x-ray films (Fujiwara et al, 1987). An interest has been arising in proteases as targets for developing therapeutic agents against relentlessly spreading diseases such as cancer, malaria and AIDS (Khatiwada, 2004).

CHAPTER – IV

MATERIALS AND METHODS

4.1 Materials

Different equipments and materials required for present study are mentioned in Appendix – I.

4.2 Methods

4.2.1 Study design and sampling sites selection

The study was carried out from September 2008 to May 2009 at the Research Laboratory of Central Department of Microbiology, Tribhuvan University, Kirtipur. A total of 20 different sampling sites were selected randomly from the four municipalities (Kathmandu Metropolitan, Lalitpur Sub-metropolitan, Bhaktapur Municipality and Madhyapur Thimi Municipality) in Kathmandu Valley (Table 5.1).

4.2.2 Sample collection and transportation

Triplicate of samples from the depth of 6 inches of the solid waste piles were collected from each sampling site with sterile forceps in wide-mouthed sterilized glass bottles. Sampling was done from September 2008 to February 2009. Temperature of the waste piles was measured at the site of collection of all the triplicate samples. The sample was transported to laboratory and pH was measured. The degradable fraction of the waste was segregated from the non – degradable fraction.

4.2.3 Laboratory processing

4.2.3.1 Primary screening of gelatin degrading bacteria

A series of ten-fold dilution of the sample upto 10^{-6} was prepared in sterile water. The samples from dilutions 10^{-5} and 10^{-6} were inoculated on mineral base agar with 1% gelatin (Manandhar, 1996) by spread plate technique (Pelczar et al, 1993). The plates were

incubated at 37^0C for 24 hours. The isolates were stabbed on gelatin agar and incubated at 37^0C for 24 hours. Zone of hydrolysis was observed by flooding freshly prepared mercuric chloride solution. The organisms showing hydrolysis were screened as proteolytic bacteria.

4.2.3.2 Identification of screened bacteria

Three isolates showing larger zone of hydrolysis in gelatin agar (with diameter more than 20 mm) were selected for secondary screening. The bacteria were identified on the basis of different morphological, cultural and biochemical tests as listed in Appendix – IV.

4.2.3.3 Enzyme extraction

The organisms were inoculated in mineral base broth with 1% gelatin and incubated in water bath shaker at 30^0C at 150 rpm for 24 hours to prepare pre-fermenter culture. Each culture was then transferred to production fermenter and incubated in water bath shaker at 30^0C at 150 rpm for 3 days. The broth culture was then centrifuged at 10000 rpm for 10 minutes in a cold centrifuge. The supernatant was collected as crude enzyme (Khatiwada, 2004).

4.2.3.4 Assay of enzyme activity

The crude enzyme from each isolate was loaded on the wells made on gelatin agar and incubated at 37^0C for 24 hours. The plates were then flooded with freshly prepared mercuric chloride solution to observe the zone of hydrolysis (Dingle et al, 1953).

4.2.3.5 Secondary screening of bacteria

The organisms were screened out on the basis of the size of zone of hydrolysis of gelatin given by the respective enzymes. The screened enzymes, revealing the diameter of zone of hydrolysis more than 15 mm, were then purified partially by acetone precipitation method.

4.2.3.6 Study of enzyme kinetics

4.2.3.6.1 Enzyme activity at different temperature

The purified enzyme extracted from each screened organism was incubated at different temperatures (4^0C, 15^0C, 26^0C, 37^0C, 45^0C, 60^0C, 70^0C, 80^0C) for 1 hour and 50 µl of each treated enzymes were loaded on the wells made on gelatin agar and incubated at 37^0C for 24 hours (Dangol, 1997). Zone of hydrolysis of the gelatin in each case was recorded.

4.2.3.6.2 Enzyme activity at different pH

The partially purified enzyme extracted from each screened organism was taken and 50 µl of each was loaded on the wells made on gelatin agar with different pH (4, 5, 6, 7, 8, 9) and incubated at 37^0C for 24 hours. Zone of hydrolysis of the gelatin in each case was recorded (Dangol, 1997).

4.2.3.7 Salt fractionation of the protease

The enzyme from the screened organism was processed for salt fractionation with ammonium sulphate. Fractions of the enzyme with 30%, 60% and 90% ammonium sulphate saturation were prepared (Reed et al, 1998). Then, 50 µl of each fraction was loaded on gelatin agar and zone of hydrolysis was determined. Enzyme activity of each fraction at different temperature and pH was also determined.

4.2.4 Statisitcal Analysis

The obtained data were analysed by ANOVA and paired samples t-test using statistical software (SPSS version 11.5). Dependent variable protease activity was compared with independent variables temperature and pH by one way ANOVA at 95% and 90% significance levels respectively. The effectiveness of ammonium sulphate fractionation for

the purification of protease was studied by paired samples t – test at 95% significance level. The results were then interpreted.

An outline of the design of the research work is given below.

Design

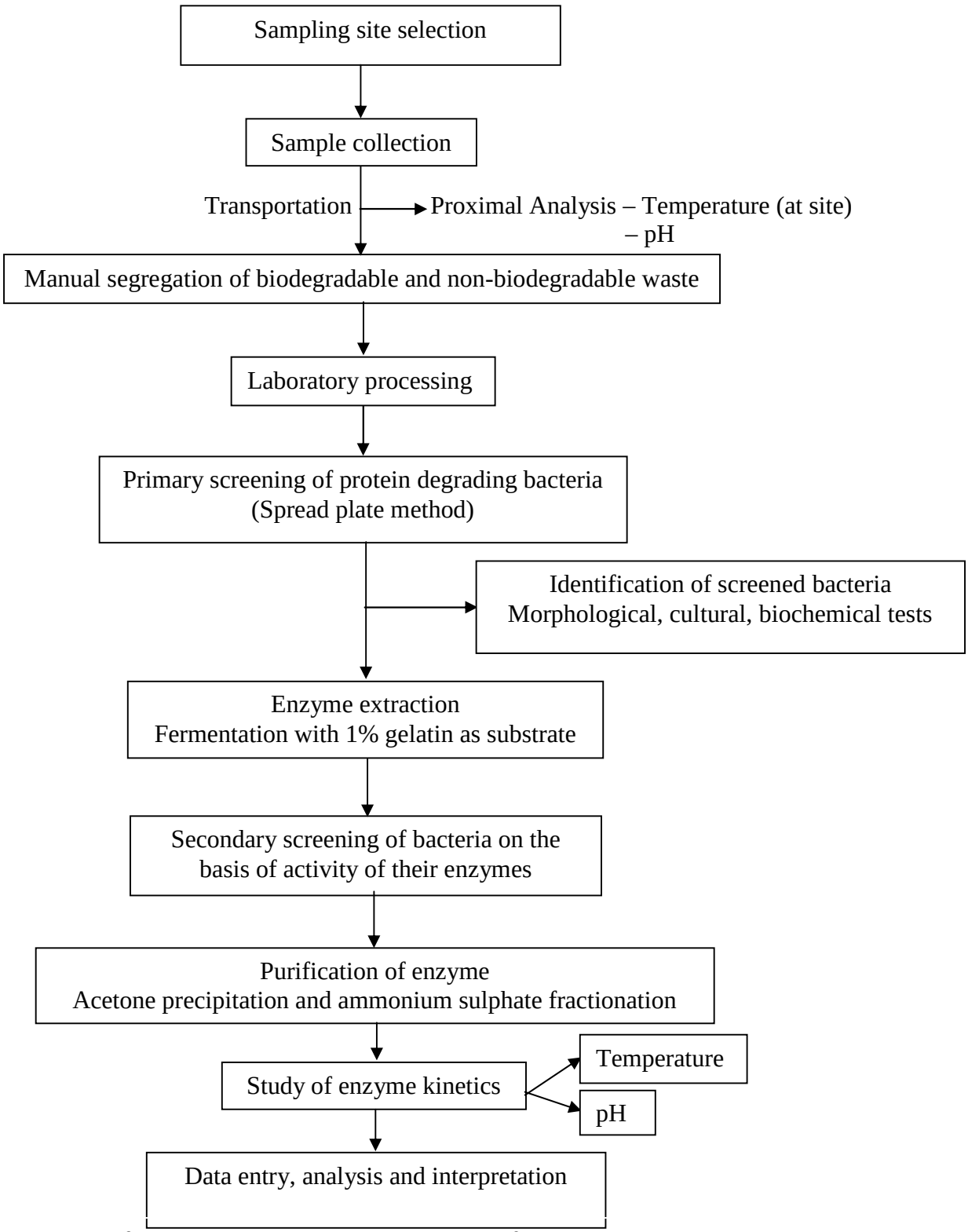


Fig. 4.1 Flow chart for the isolation and characterization of substrate degrading bacteria

CHAPTER – V

RESULTS

5.1 Distribution of sampling sites

Altogether 20 triplicate samples of solid waste were collected from different sites within Kathmandu valley from September 2008 to February 2009. The sampling sites were selected randomly and the major dumping sites of Kathmandu (Teku), Bhaktapur (Bhelukhel) and Lalitpur (Sanepa) were included (Table 5.1).

Table 5.1 Determination of pH and temperature of different solid waste samples

S. N.	Municipalities	Sampling site	Sample code	pH	Temperature (^{0}C)
1	Lalitpur	Satdobato	L1	7.0	18
2		Batuk Bhairab, Lagankhel	L2	7.0	38
3		Sanepa (dumping site)	L3	7.0	22
4		Pulchowk	L4	7.0	12
5		Dhobighat	L5	7.0	18
6	Kathmandu	Gopikrishna	K1	7.1	17
7		Teku (dumping site)	K2	7.0	25
8		Kalimati	K3	6.9	18
9		Minbhavan	K4	7.0	18
10		Bafal	K5	7.0	17
11		Bhelukhel (dumping site)	B1	7.0	34

12	Bhaktapur	Hanumante bridge	B2	7.0	41
13		Suryabinayak bus park	B3	7.0	28
14	Madhyapur Thimi	Pacho	B4	7.0	30
15		Manohara bridge, Jadibuti	B5	7.0	35
16	Kiritpur	Khashibazaar	R1	7.0	18
17		Tinkune	R2	7.0	43
18		Sundarbazaar	R3	7.0	16
19		Chikhu	R4	7.0	16
20		Kumari club	R5	7.1	18

5.2 Screening and identification of bacteria

A total of 113 isolates with distinct colony characteristics on mineral base agar containing 1% gelatin were obtained from these 20 triplicate samples, of which 22 exhibited gelatinase activity (Fig. 5.1).

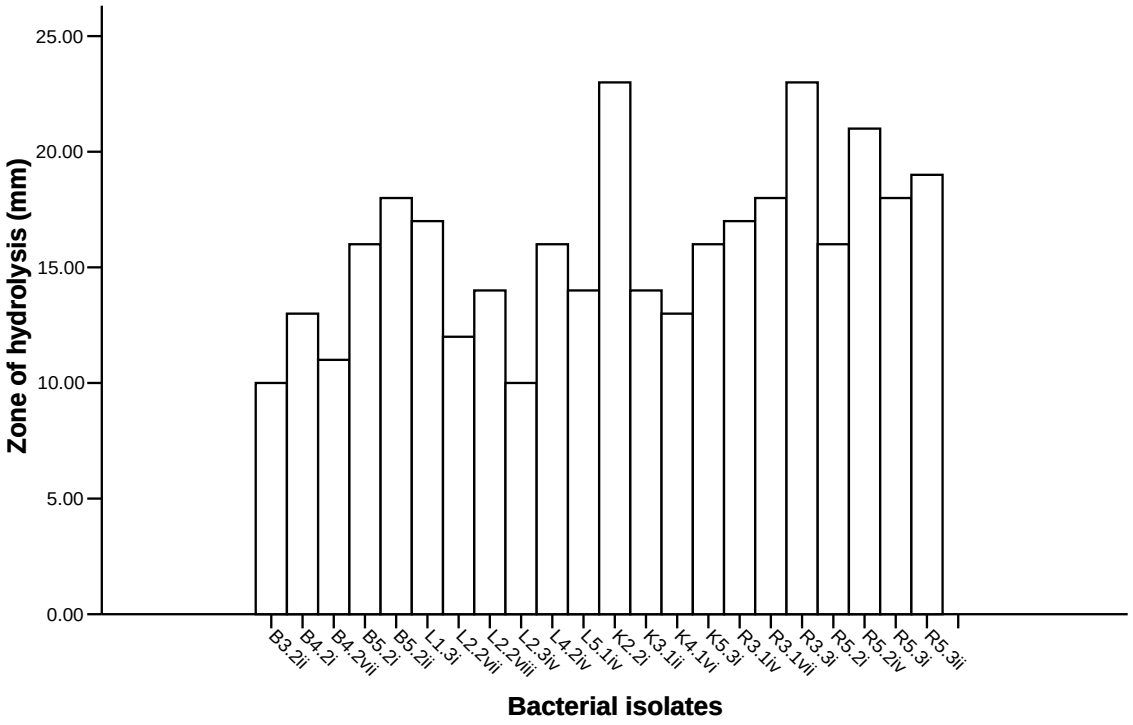


Fig 5.1 Primary screening of the isolates for extracellular protease on gelatin agar

Of these 22 gelatin degrading bacteria, 3 potent producers (Laboratory code: K2.2i, R3.3i and R5.2iv), with the diameter of zone of gelatin hydrolysis more than 20 mm, were selected (Table 5.2). The three organisms were attempted to identify on the basis of their morphological, cultural and biochemical characteristics (Appendix IV). The crude enzyme extracts from these organisms were also assayed for secondary screening. The crude enzymes from isolates K2.2i and R3.3i revealed zone of hydrolysis with diameter more than 15 mm in the gelatin agar media (Table 5.3).

Table 5.2 Identification of the potent producer of gelatinase

S.N	Organisms	Remarks

1	K2.2i	*Micrococcus* spp
2	R3.3i	Unidentified (Gram positive cocci)
3	R5.2iv	*Micrococcus* spp

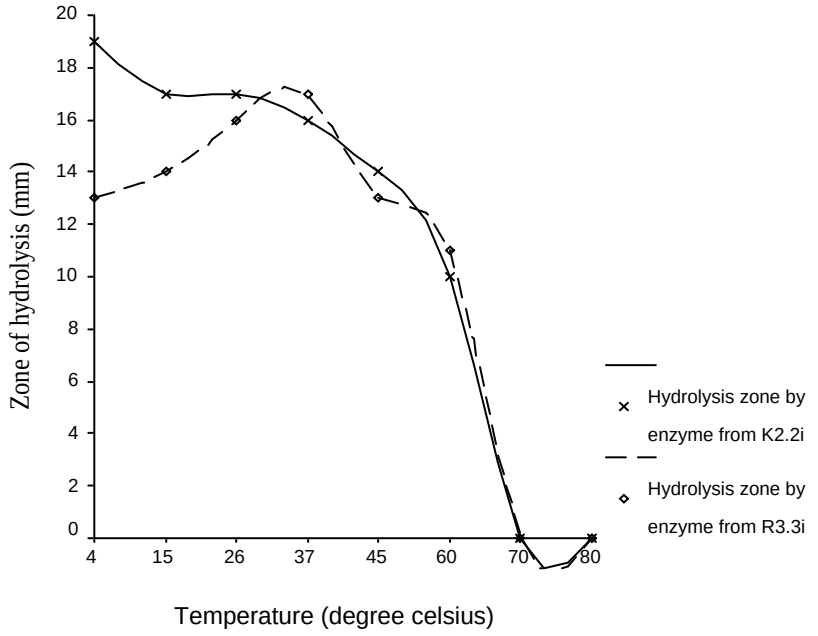
Table 5.3 Secondary screening of bacteria

S.N	Enzymes from the isolate	Zone of hydrolysis (mm)
1	**K2.2i**	**16**
2	**R3.3i**	**17**
3	R5.2iv	13

5.3 Kinetics of the partially purified enzymes

The enyzmes were purified partially by acetone precipitation method and then by ammonium sulphate fractionation. Their temperature and pH dependency were also studied. During ammonium sulphate fractionation, 30%, 60% and 90% salt fractions of enzyme were prepared. While assaying these fractions of each enzyme, 90% fraction of the enzyme from isolate R3.3i did not exhibit any activity.

Fig 5.2 Effect of temperature on the activity of acetone precipitated enzyme



The activity of the enzyme from the isolate K2.2i was high at cold temperatures (maximum at 4^0C) which gradually decreased with the increase in temperature. But the activity of the enzyme from the isolate R3.3i was high at mesophilic temperature (maximum at 37^0C). The enzymes from both the isolates were high active a wide range of temperature from 4^0C to 60^0C but both enzymes lost their activity at 70^0C and above (Fig 5.2). The effect of temperature on the activity of both the enzymes was statistically significant with P values of 0.045 and 0.014 for enzymes from R3.3i and K2.2i respectively (Appendix-VI).

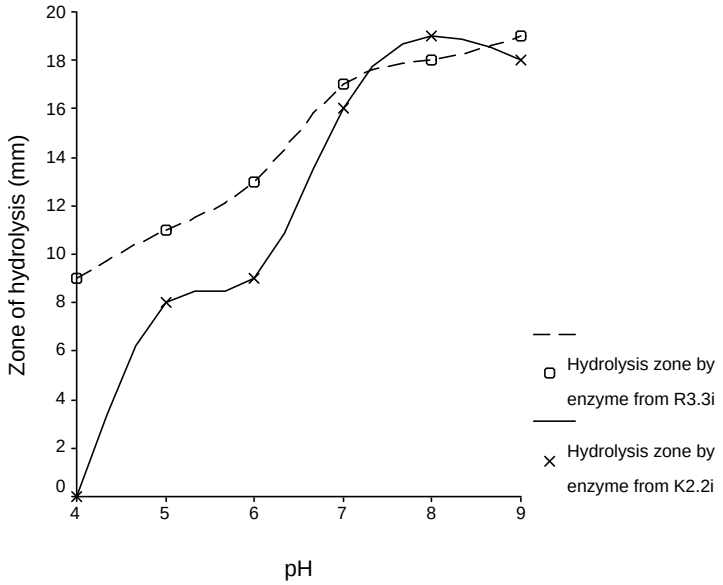
Table 5.4 Effect of temperature on the activity of salt fractionated enzyme

S.N	Temperature (^{0}C)	Zone of hydrolysis by enzyme from R3.3i	Zone of hydrolysis by enzyme from K2.2i

		30% fraction	60% fraction	30% fraction	60% fraction	90% fraction
1	4	15	**16**	20	**22**	17
2	15	16	**18**	20	**22**	16
3	26	19	**22**	18	**20**	14
4	37	20	**23**	18	**20**	12
5	45	18	**20**	16	**18**	11
6	60	14	**16**	10	**10**	8
7	70	0	**0**	0	**0**	0
8	80	0	**0**	0	**0**	0

The activity of the 60% fractionated enzyme was found to be highest in both the cases (Table 5.4). In case of both isolates, the activities of 30% and 60% fractionated enzymes were higher than those of their corresponding acetone precipitated fractions (Fig 5.2 and Table 5.4). The activities of acetone precipitated enzymes and 60% ammonium sulphate fractionated enzyme of both the isolates were compared by paired samples t – test. In both the cases, the activity of 60% ammonium sulphate fractionated enzymes were significantly high with P values of 0.005 and 0.014 for the enzymes from R3.3i and K2.2i respectively (Appendix – VI).

Fig 5.3 Effect of pH on the activity of partially purified enzyme



The enzyme from K2.2i was active at a pH range of 5 – 9 with maximum activity at pH 8 while that from R3.3i at the pH range of 4 – 9 with maximum activity at pH 9 (Fig 5.3). The effect of pH on the activity of both the enzymes was statistically significant with P values of 0.032 and 0.078 for enzymes from R3.3i and K2.2i respectively (Appendix-VI).

Table 5.5 Effect of pH on the activity of salt fractionated enzyme

S.N	pH	Zone of hydrolysis by enzyme from R3.3i		Zone of hydrolysis by enzyme from K2.2i		
		30% fraction	60% fraction	30% fraction	60% fraction	90% fraction
1	4	0	**11**	0	**0**	0
2	5	0	**12**	8	**8**	0
3	6	13	**15**	11	**12**	8
4	7	19	**21**	17	**20**	12
5	8	21	**23**	21	**22**	17

6	9	21	**24**	19	**20**	15

The activity of the 60% fractionated enzyme was highest in both the cases. Only the 60% fractionated enzyme from the isolate R3.3i showed activity at pH 4 and 5 (Table 5.5). In case of both isolates, the activities of 30% and 60% fractionated enzymes were higher than those of their corresponding acetone precipitated fractions (Fig 5.3 and Table 5.5). The activities of acetone precipitated enzymes and 60% ammonium sulphate fractionated enzyme of both the isolates were compared by paired samples t – test. In both the cases, the activity of 60% ammonium sulphate fractionated enzymes were significantly high with P values of 0.006 and 0.033 for the enzymes from R3.3i and K2.2i respectively (Appendix – VI).

CHAPTER – VI

DISCUSSION AND CONCLUSION

6.1 Discussion

Rapid and haphazard urbanization has led to increasing volumes of waste being generated in the urban areas of Nepal. Although the total amount of waste is still small compared to other countries, poor government response and weakness of institutions at the local level have resulted in the improper management of the waste. According to Central Bureau of Statistics (CBS) in 1997, the results of a survey of 3980 urban residents from all over Nepal; 59% considered unmanaged waste to be the main environmental problem in the cities. Changing consumption patterns and breakdown in the traditional systems of waste management also contribute to the problem of waste management in the urban Nepal, especially in the city of Kathmandu (MOPE, 2001). This has ultimately resulted environmental pollution, public health hazards and adverse effects on urban economy that depends heavily on tourism. Regarding the impacts of unmanaged solid waste, this problem is to be addressed seriously.

The best approach for improving the environmental performance of solid waste management systems, while keeping them economically affordable and socially acceptable, is via Integrated Solid Waste Management. When integrated together, composting, biogasification and material recycling can provide cost effective ways to manage municipal solid waste (Goen, 1998).

Households are the main sources of solid waste in Nepal. A few studies prepared on waste generation in Kathmandu city indicate that the per capita waste generation is low compared to most other countries and that about two-thirds of waste materials are organic. Lohani and Thanh(1978) estimated that per capita waste generation in Kathmandu was 0.25kg/day. GTZ (1988) estimated it to be 0.4kg/day and Khanal (1993) 0.46kg/day. Rai (1990)

estimated the rate to be 0.565kg/day. Misra and Kayastha (1998) estimated that the per capita waste generation rate of Nepalese cities varied from 0.25 to 0.5kg/day depending on the size of the city while RESTUC (1999) estimated the average amount to be 0.48kg/day. By 1999. about three millions urban residents in Nepal generated an approximately 426,500 tonnes of waste, 29% of this in Kathmandu alone (MOPE, 2001).

Composting and/or biogasification can result in higher diversion levels because it can deal with a large part of the municipal solid waste stream (Goen, 1998). Thus in the context of agricultural country like Nepal where a market for compost can be demonstrated, composting plants may be a good option that should be promoted and supported.

Composting involves microbial degradation of organic fraction of waste. The organic fraction of waste includes all kinds of proteins, carbohydrates and fats in various forms. So highly efficient degradative microorganisms should be explored. This work has been done to explore highly efficient proteolytic indigenous bacteria for the degradation of protein portion of the waste. To carry out this work, street wastes from 20 different sampling sites of Kathmandu, Lalitpur, Bhaktapur, Madhyapur Thimi and Kirtipur were sampled randomly, one triplicate sample from each site. Locations from Kathmandu valley were selected since the Kathmandu valley, which has the capital city of Kathmandu along with four other municipal towns, Lalitpur, Bhaktapur, Kirtipur and Madhyapur thimi is the main urban area of Nepal. Haphazard and unplanned urban growth generally invites many environmental problems such as public space and river bank encroachment, air and water pollution, and solid waste generation. Among these the unmanaged solid waste seems to be the most visible problem (MOPE, 2001).

During sampling, temperature was recorded on the site of collection. The samples were transported to the laboratory as soon as possible and the degradative and the non –

degradative portions of the waste were segregated. In the laboratory, pH of the sample was also recorded. The temperature of the solid waste piles varied from 12^0C to 43^0C; the highest temperature being recorded was at Tinkune, Kirtipur (43^0C) and that at Hanumante bridge, Bhaktapur (41^0C). Such high temperatures in the solid waste piles of these places may be the evidence of composting process going on within these piles. This may happen only when the waste is piled up for a considerably long time (of months) without any turnover. However, mesophilic temperature of 25^0C was recorded from the pile at the major dumping site of Kathmandu (Teku). Continuous transfer of the wastes to landfill site immediately after collection may account for the mesophilic temperature of such a huge pile of waste at Teku. Similarly at the dumping site of Bhaktapur (Bhelukhel), the temperature of the pile was recorded to be 34^0C which also falls under mesophilic range. This may be due to the fact that these wastes were also not allowed to pile up for months without turnover but were processed for composting in a small scale and were used in the fields as fertilizer. Besides these dumping sites, the temperature at some other sites like Suryabinayak Bus Park, Pacho, Manohara bridge at Jadibuti, Batuk Bhairab at Lagankhel and Sanepa were also in the mesophilic range. The temperature of the solid waste piles of the rest of the sampling sites was in the psychrophilic range (12^0C to 18^0C).

Sampling was done during winter season from September to February and the temperature of each of the solid waste piles was higher than the ambient temperature. The samples were collected from the depth of about 6 inches in each of the solid waste piles. Seasonal variations, placement of waste, age of waste, depth, location of waste, and available moisture have significant effects on temperatures (Yesiller and Hanson, 2003). Kendall et al (2005) reported increasing temperature (1-15^0C) for newly placed wastes whereas steady elevated temperatures (50-60˚c) were observed in older wastes. The majority of temperature increase for wastes occurs under anaerobic conditions. Shrestha (1994) studied the physical parameters of solid waste collected from different places of Kathmandu and

reported that in both the winter and summer season temperature of waste was higher than that of atmospheric temperature due to exothermic reaction of microbial metabolism present. The result obtained in this study was in accordance with these literatures.

The pH of the collected samples after transport in the laboratory was recorded. The pH of almost all the samples was found to be around neutral range (6.9-7.1). This is similar to that reported by Shrestha (1994). Shrestha (1994) reported that the pH of the solid wastes varying from 5.88 to 8.35 during winter season and from 6.02 to 8.85 during summer season. The study of temperature of solid waste piles and the pH records enables us to understand the nature of waste, age of waste and the kinds of microorganisms proliferating on the waste.

Proteolytic bacteria from these 20 triplicate sets of samples were screened using mineral base agar supplemented with 1% gelatin. Solid waste harbours many kinds of bacteria. The bacteria from solid waste were isolated in media of pH 7 at 37^0C and screened for the highest proteolytic activity using gelatin as the substrate. It is possible that gelatin, as a high molecular weight protein, induces an increase in the protease production to degrade the substrate to an available form for the microorganism. The use of gelatin in the culture medium provided us a qualitative assay, a simple, inexpensive, straight forward method to assess the presence of proteolytic activity of a given colony. This method, for instance, allows to verify in an initial screening whether the bacteria secrete extracellular proteases. In summary, the simplicity of this method is its greatest advantage (Vermelho et al, 1996).

A total of 113 isolates were obtained from these samples, of which only 22 isolates were found proteolytic. This indicated that only about one – fifth of the organisms present in the solid waste were heterotrophs utilizing gelatin as the sole source of carbon while the majority were chemolithotrophs. Of these proteolytic bacteria, only 3 isolates (K2.2i,

R5.2iv and R3.3i) were found to be potent producers (with diameter of zone of gelatin hydrolysis more than 20 mm) of gelatinase enzyme. These three bacteria were identified on the basis of their morphological, biochemical and cultural characteristics as described in Bergey's Manual of Determinative Bacteriology. All the isolates were non – capsulated and non – sporulating gram positive cocci. The isolate K2.2i was oxidative; non – motile; mannose, sorbitol, sucrose and arabinose positive; indole negative and hydrolyzing tween 60. Similarly, the isolate R5.2iv was oxidative; non – motile; sucrose and arabinose positive; indole negative and hydrolyzing tween 60. Thus the isolates K2.2i and R5.2iv were found to be the strains of *Micrococcus* spp. while the isolate R3.3i was found to be fermentative; producing water soluble red pigment; highly motile; mannose, mannitol, sorbitol, sucrose and raffinose positive; indole negative and hydrolyzing tween 60 potentially. The isolate R3.3i remained unidentified on the basis of the tests performed.

The bacteria were selected on the basis of degree of hydrolysis given by their colony on nutrient agar supplemented with 1% gelatin. The colony of these three kinds of isolates exhibited a zone of hydrolysis exceeding 20 mm in diameter while the rest 110 bacteria developed the zone with comparatively lesser diameter. These three bacteria were cultivated in fermentation broth for the production of proteolytic enzyme. The crude enzyme was assayed for further screening. Of these 3 bacteria, the enzymes from two strains, K2.2i and R3.3i produced zone of hydrolysis greater than 15 mm in diameter and were selected for purification and characterization. All the three isolates were fairly good in producing the proteolytic enzyme in fermentation broth supplemented with 1% gelatin with more or less similar diameter of zone of hydrolysis when assayed in nutrient agar containing 1% gelatin. There was no standard protease producing bacteria available and the comparison was made among the screened bacteria themselves. The selection was also made on the same basis.

Since this work was aimed to explore the indigenous bacteria from solid waste with high potential to secrete extracellular protease which can be further used to manage the solid waste itself, the enzyme activity was interpreted in terms of zone of hydrolysis. Montville (1983), Walsh et al (2005) and Mahmoud et al (2008) have shown a linear relationship between the enzyme activity and zone of hydrolysis. Thus it can be considered that the enzyme revealing larger zone of hydrolysis has greater activity. The diameter of the zones was linearly proportional to the log of the enzyme activity applied over a range from 0.01 to greater than 100 IU/ml (Montville, 1983). According to Walsh et al (2005), a linear relationship between the diameter of the zone of hydrolyzed substrate and the log of the enzyme activity applied is observed over a broad activity range. The level of sensitivity exhibited by protease was excellent with linearity being maintained to activity value as low as 0.0003 NU/ml. (One Northrop unit of activity (NU) equates to the amount of enzyme that yields 40% hydrolysis of casein under assay conditions). Mahmoud et al (2008) have also verified relationship between protease activity and zone of hydrolysis.

The enzymes were partially purified by acetone precipitation method. Acetone being organic solvent is used to precipitate the enzyme of interest from solution thus, separating it from monosaccharides, oligosaccharides, nucleotides, free amino acids etc. and from many other proteins which remain in the solution (Palmer, 1995).

The enzymes from both the isolates K2.2i and R3.3i were active from 4^0C to 60^0C while no activity was observed at 70^0C and above. The enzyme from isolate K2.2i exhibited high activity at psychrophilic range of temperature while that from isolate R3.3i revealed high activity at mesophilic range of temperature with optimum temperature around 37^0C. Among the tested temperatures, the enzyme from isolate K2.2i revealed the highest activity at 4^0C but since the activity under 4^0C was not studied, 4^0C temperature may or may not be optimum for this enzyme. This indicated that the protease enzyme from isolate K2.2i could

be implicated in the environment with very cold season whereas that from R3.3i was appropriate for the places with ordinary environmental temperatures.

The effect of temperature upon the proteases from *Bacillus* spp. was when studied by incubating at different temperature from 25^0C to 70^0C, an optimum temperature for maximum activity was found to be at 55^0C (Khamviwath et al, 1992). Similarly, according to Elfadaly et al (1993), a heat stable proteinase produced by *Bacillus* strain displayed maximum activity at 60^0C. Chandran et al (2006) reported that the protease from *Penicillium* spp. was optimally active at 45^0C. According to Wery et al (2003), the extracellular protease activities were optimal between 30^0C and 45^0C. The thermostable protease obtained from hyperthermophilic *Bacillus* strain HUTBS71 was found to be optimum at 65^0C (Akel et al, 2009). According to Mahmoud et al (2008), protease enzyme from *Bacillus subtilis* B-6 showed maximum activity at 30^0C. The optimal temperature for catalytic activity of *Streptomyces microflavus* was 40^0C (Rifaat et al, 2006). The optimum temperature for the activity of alkaline protease produced by *Bacillus firmus* MTCC 7728 was 40^0C (Rao and Narasu, 2007). The extracellular protease from *Micrococcus* spp. MCC – 315 exhibited optimum activity at 37 to 40^0C (Prasad et al, 1986). Optimal condition for activity of protease from *Micrococcus* sp. INIA 528 on azocasein was 34^0C (Fernandez, 2008). All these literatures are evident of a variety of protease enzymes which act optimally at different temperatures ranging from psychrophilic to hyperthermophilic range.

Protease enzymes produced by various strains of microorganisms and also under various conditions have their own range of cardinal temperatures with specific optimum temperature for their maximum activity. The effect of temperature on these enzymes was found to be statistically significant when analyzed using one way ANOVA (Analysis of Variance), with P values of 0.045 and 0.014 for the enzymes from R3.3i and K2.2i respectively.

The effect of pH on the activity of the enzyme was studied by loading the enzyme in the wells made on agar media supplemented with 1% gelatin where pH was already maintained during the preparation of media. The media of pH 4, 5, 6, 7, 8 and 9 were prepared for the purpose. The enzyme from isolate K2.2i was found to be active from pH 5 to pH 9 while that from R3.3i was active from pH 4 to pH 9. Protease from the isolate K2.2i displayed maximum activity at pH 8. Since the effect of pH above 9 was not studied, it could not be concluded that whether the optimal pH for maximum activity of the enzyme from strain R3.3i was 9 or not. Whatever may be the optimum pH for these enzymes, both were found to be effectively active at alkalophilic pH.

Like temperature, pH also have considerable effect upon the activity of any enzyme. Peek et al (1992) reported that protease from *Thermus* spp. strain Rt 41A was stable in the range of pH 5 to pH 10 with an optimum pH of 8. Similarly in another study, the enzyme from alkalophilic *Bacillus* spp. was stable between pH 5 and pH 11.5 with optimum activity in the range of pH 10.5 to 11.5 (Fujiwara and Yamamoto, 1987). According to Salleh (1994), protease from *Bacillus* spp. EA-1 gave a narrow pH profile, with a stability range of pH 8 to pH 10 and optimum activity at pH 9. Partial characterization of protease from *Penicillium sp* showed that the enzyme was quite stable at pH 6.0 – 8.0 with optimum value at pH 6.5 (Chandran et al, 2006). The extracellular protease activities tested were optimal at neutral pH (Wery et al, 2003). The extracellular protease from *Micrococcus* spp. MCC – 315 exhibited optimum activity at pH 10.6 (Prasad et al, 1986). Optimal condition for activity of protease from *Micrococcus* sp. INIA 528 on azocasein was at pH of 7 (Fernandez, 2008).

According to Juarez and Stinson (1999), the activity of an extracellular protease from *Streptococus gordonii* had a pH optimum between 7.0 and 9.0.When assayed above pH 9.5

or below pH 5.0, no proteolytic activity was observed, and the enzyme was not activated when the pH was adjusted to 7.0. The activity of thermophilic protease from hyperthermophilic *Bacillus* strain HUTBS71 was observed at pH range from 7.0 to 9.2 with an optimum pH 7.8 (Akel et al, 2009). Mahmoud et al (2008) reported that protease enzyme of *Bacillus subtilis* B-6 was found to show maximum activity at pH 7.2. The optimal pH for catalytic activity of protease from *Streptomyces microflavus* was pH 7 (Rifaat et al, 2006). The optimum pH for the activity of alkaline protease produced by *Bacillus firmus* MTCC 7728 was 9 (Rao and Narasu, 2007).

Thus pH has an important influence on the activity of any enzyme. That's why it becomes necessary to study the effect of a wide range of pH on the activity of an enzyme. This knowledge enables us to understand the nature of the enzyme and its suitability for its various applications. In this study, the enzymes were found to be alkalophilic but their stability range could not be predicted accurately because the effect of pH above 9 was not studied.

The enzymes from both the bacteria were found to be active at a wide range of temperature and pH. This predicts various important applications of these enzymes. The one this work is concerned is on their application in solid waste management. Both of these enzymes were active and working effectively in the temperature range of environment as well as upto 60^0C. Similarly these enzymes were working at a wide range of pH; from acidic to alkaline range. Because of these characteristics of these enzymes, their use in the management of piled up solid waste via protein degradation could be attempted. Besides, these bacteria were also found to be the potential producers of lipase enzyme indicated by the hydrolysis of tween 60. Thus these bacteria may also be used to extract the lipase enzyme. Since this work was performed to explore the possibilities of use of enzymes for

the management of solid waste problem, the effect of substrate concentration on the activity of enzyme was not studied.

Furthermore, both the enzymes were fractionated with ammonium sulphate crystals to prepare 30%, 60% and 90% fractions. Ammonium sulphate is the salt commonly used since it is highly water-soluble, relatively cheap and available at high purity. Also, it has no adverse effects upon enzyme activity. Salt fractionation of an enzyme is based on the principle that solubility of proteins varies according to the ionic strength and hence according to the salt concentration of the solution. That's why different amount of the salt used to prepare different fractions of the enzyme result in the precipitation of different proteins alongwith the enzyme of interest. That's why different salt fraction of an enzyme exhibit different degree of enzyme activity.

All the three fractions of both the enzymes were assayed at 37^0C and pH 7. The 60% fractions of both the enzymes displayed maximum activity. This was in accordance with many literatures. The 55 to 80% ammonium sulphate fraction could be heated at 50^0C for 10 minute without any loss of the enzymatic activity (Juarez and Stinson, 1999). Mahmoud et al (2008) reported that 60% ammonium sulphate fraction of protease enzyme of *Bacillus subtilis* B-6 showed maximum activity. The 60% ammonium sulphate fraction of protease from *Streptomyces microflavus* displayed maximum catalytic activity (Rifaat et al, 2006). The 90% fraction of enzyme from isolate R3.3i did not exhibit any enzymatic activity on the mineral base agar supplemented with 1% gelatin.

The activity of these fractions of the enzymes were studied under different temperatures (4^0C, 15^0C, 26^0C, 37^0C, 45^0C, 60^0C, 70^0C and 80^0C) and different pH (4, 5, 6, 7, 8 and 9). All the fractions of enzyme from isolate K2.2i and 30% and 60% fractions of enzyme from isolate R3.3i were found to be active from 4^0C to 60^0C (Table 5.2). The activity of 60%

fraction of both the enzymes under different temperatures and pH was found significantly high ($P<0.05$) than the activity of respective enzymes purified partially by acetone precipitation.

The activity of 30% and 60% fractions of enzyme from isolate K2.2i was highest at the temperature range of 4^0C to 15^0C while that of 90% fraction was highest at 4^0C. The activity of all the fractions of this enzyme went on decreasing order as the temperature increased. On the other hand, the activity of 30% and 60% fractions of the enzyme from isolate R3.3i first increased with the increasing temperature and then decreased with further increase in temperature. Both these fractions displayed maximum activity at 37^0C.

Regarding pH, the activity of these enzymes was more in alkalophilic range as shown in Table 5.3. All the fractions of enzyme from isolate K2.2i revealed maximum activity at pH 8. The 30% fraction of enzyme from isolate R3.3i showed maximum activity in the pH range of 8 to 9 while the 60% fraction showed maximum activity at pH 9. The activities of these enzymes were not studied at pH above 9. That's why it could not be concluded whether the activity of these enzymes from isolate R3.3i was maximum at pH 9 or at the pH above 9. The 90% fraction of enzyme from isolate R3.3i did not display any activity while assayed at 37^0C and pH 7. This may indicate either at 90% ammonium sulphate fraction, only the proteins other than the enzyme of interest was precipitated or this fraction of enzyme was not active at this temperature and pH. Many enzymes may show activity at extremes of pH and temperature without showing activity at this ordinary temperature and pH.

The activities of two most potent proteolytic enzymes obtained in this study were compared. The results revealed that both were active within a broad range of temperature (from 4^0C to 60^0C) and pH (from 4 to 9) as depicted in Fig. 5.2 and Fig. 5.3 respectively.

But the enzyme from isolate K2.2i displayed maximum activity at 4^0C and pH 8 while that from isolate R3.3i showed optimal activity at 37^0C and pH 9. When these enzymes were fractionated with ammonium sulphate and their activities were studied, every fraction of each of the enzymes displayed their activity in the pattern similar to those displayed by the respective acetone precipitated enzymes but the activities of each extract was in the decreasing order starting from 60% salt fraction followed by 30% salt fraction, acetone precipitated fraction and then by 90% salt fraction.

From the study of the temperature and pH condition of the piled up solid waste and the activity profile of the obtained enzymes, it can be predicted that both of these enzymes are suitable for their application in solid waste management, although many important aspects like the effect of various inhibitors possibly present in the solid waste, the effect of enormous amount of substrate present in the waste and the effect of various by – products produced during the decomposition of these wastes were not studied in this present work. The enzymes from isolate K2.2i can be applied in the management of solid waste in the cold regions or in the cold seasons whereas that from isolate R3.3i can be applied during warm weathers of mesophilic range of temperature; both under alkaline condition.

6.2 Conclusion

Protein degrading bacteria were isolated and screened from the municipal solid waste dumped in various locations in Kathmandu Valley. Proteolytic activity of the bacteria and their enzyme kinetics of the enzymes from isolates K2.2i and R3.3i were studied. From the study of temperature and pH condition of the piled up solid waste and the activity profile of the obtained enzymes, it can be concluded that both of these enzymes are suitable for their application in solid waste management via protein degradation. The enzyme from isolate

K2.2i can be implemented in cold regions or in cold seasons while that from isolate R3.3i during warm weathers of mesophilic range of temperature, both under alkaline conditions.

CHAPTER – VII

SUMMARY AND RECOMMENDATIONS

7.1 Summary

The rate of solid waste generation in Nepal is in considerably increasing trend with the rapid population growth and urbanization. This will overcome nature's ability to process these wastes through natural cycles. If untreated, this will result in piling up of solid wastes imparting many adverse impacts to the biotic as well as abiotic community existing on the earth. On the way to devise effective technologies to address the problem of unmanaged solid waste, the interests in alternating large-scale waste processing technologies continue to grow. The present study had been carried out as a small effort to lead the development of effective technology for the management of piled up solid waste in the future. This suggested the production of protease enzyme commercially utilizing easily available and cheap source municipal solid waste for its use in degradation of proteinous materials in the waste. The work has also targeted to study kinetics of enzyme at variable temperatures and pH.

- The study was conducted from September 2008 to May 2009 in the Central Department of Microbiology, Tribhuvan University, Kirtipur.
- Total 20 triplicate samples were collected from different solid waste piles of Kathmandu Valley including the major dumping sites.
- Highly proteolytic bacteria were isolated from the collected samples and identified.
- The most potent bacteria produced maximum proteolytic activity in agar media supplemented with 1% gelatin in 24 hours of incubation.
- Then, the laboratory production of protease was carried out using the same organism in mineral base broth with 1% gelatin.

- The culture after 4 days of continuous shaking in shaker at 30^0C was centrifuged to obtain cell free extract and subjected to acetone precipitation. Acetone treatment under chilled condition yielded fairly good amount of enzyme.
- The enzyme thus obtained was assayed at 37^0C using agar medium of pH 7. The assay was performed according to Dingle's cup plate method. From this assay, the enzymes from two isolates, K2.2i and R3.3i, were selected for further study.
- Then, the enzyme was subjected to ammonium sulfate fractionation at 30%, 60%, and 90% saturation. After enzyme production, extraction and purification was over, study of kinetics (in terms of temperature and pH) of extracted enzymes was also carried out.
- The activity of enzymes was studied under a range of incubation temperature from 4^0C to 80^0C and a range of medium pH from 4 to 9. It revealed that enzyme from isolate K2.2i displayed maximum activity at 4^0C and pH 8 while that from isolate R3.3i exhibited maximum activity at 37^0C and pH 9. Similarly different salt fractions of each enzyme also imparted the activity in the pattern similar to those of their respective acetone precipitated fraction.

7.2 Recommendations

Based on the findings of the study following recommendations are put forward:–

- The present study was confined only to municipal solid waste but further research should include all kinds of wastes including waste waster, sewage, industrial wastes, hospital waste, sludge etc.
- This study involved only the effect of temperature and pH on the activity of enzyme. Further research should be made studying the effect of various inhibitors possibly present in the targeted waste, effect of various activators, effect of substrate concentration etc. for full characterization of the enzyme and its effective implementation.

- This study was only focused on protease. Further study should be undertaken focusing on other hydrolyzing enzymes like amylase, lipase, cellulose etc. for the maximum degradation of each organic fraction of the solid waste.
- The study could be extended to field trial to detect the suitable conditions for effective management of solid waste.
- The research could be helpful to use the solid waste as raw material in producing valuable fertilizers for maximum crop yield by the implementation of the most easily available microorganisms. This can lead to dual benefit; one in the effective management of solid waste and the other in the yield of crop.
- Genetic manipulation using recombinant DNA technology can be used to increase potency and productivity of the protease. Furthermore, the structure-function and relationship of protease, coupled with gene-shuffling techniques, promises a fair chance of success, in the near future, in evolving proteases that were never made in nature and that would meet the requirements of the multitude of protease applications.

CHAPTER – VIII

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APPENDIX-I

Equipments and Media/Chemicals/Reagents

A. Equipments used during the study

1. Autoclave (Life)
2. Centrifuge (Eppendorf)
3. Hot air oven (NSW)
4. Incubator (Memment)
5. Refrigerator (Samsung)
6. Microwave (Intellowave)
7. Microscope
8. Weighing Machine
9. Water Distillation plant
10. Water bath shaker (Grant)
11. Thermometer
12. pH meter
13. Micropipette (Finn)
14. Cork borer
15. Glass wares (Borosil)
16. Inoculating loop
17. Bunsen burner

B. Media/Chemicals/Reagents

1. Agar-Agar Powder
2. Nutrient agar
3. Gelatin powder
4. Absolute alcohol
5. Acetone
6. Ammonium sulfate crystals
7. pH 7 buffer tablets
8. Dipotassium Hydrogen Phosphate
9. Magnesium Sulfate
10. Sodium Chloride
11. Calcium Carbonate
12. Mercuric chloride
13. Hydrochloric acid
14. Sodium Hydroxide
15. Gram's Iodine
16. Sodium Azide
17. Crystal violet
18. Safranin
19. Immersion oil

APPENDIX – II

Composition and preparation of media and reagents

A. Composition and preparation of different culture media

1. Nutrient Agar

Composition

Peptic digest of animal issue	5.00 gm
Beef extract	1.50 gm
Yeast extracts	1.50 gm
NaCl	5.00 gm
Agar	15.00 gm
Final pH at 25 ^{0}C	7.4 ± 0.2

Preparation: As directed by manufacturing company, 28mg of the media was dissolved in 1000ml of the distilled water. The media was then sterilized by autoclaving at 15lbs pressure (121 ^{0}C) for 15minutes. The sterilized media was then poured into sterilized petriplate then allowed to cool.

2. Gelatin Mineral Base Agar

Composition

Gelatin	10.00 gm
K_2HPO_4	1.00 gm
$MgSO_4.7H_2O$	0.50 gm
NaCl	trace
$CaCO_3$	2.00 gm
$(NH_4)_2SO_4$	1.00 gm
Agar- Agar	15.00 gm
Final pH	7.1 ± 0.2

Preparation: As listed in the composition accurate weight of components were dissolved in 1000 ml of the distilled water. The media was then sterilised by autocalving at 15 lbs pressure (121 ^{0}C) for 15 minutes. The sterilised media was then poured into sterilized petriplate and then allowed to cool.

3. Gelatin Mineral Base broth

Composition

Gelatin	10.00 gm
K_2HPO_4	1.00 gm

$MgSO_4.7H_2O$	0.50 gm
NaCl	trace
$CaCO_3$	2.00 gm
$(NH_4)_2SO_4$	1.00 gm
Final pH at 25 ^{0}C	7.1 ⨽ 0.2

Preparation: As listed in the composition accurate weight of components were dissolved in 1000 ml of the distilled water. The media was sterilised by autocalving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Sterilised media was inoculated and proceeded for fermentation.

4. Gelatin Agar (For Primary Screening)

Composition

Gelatin	10.00 gm
Nutrient agar	28.00 gm
Final pH at 25 ^{0}C	7.0± 0.2

Preparation: As listed in the composition accurate weight of components were dissolved in 1000 ml of the distilled water. The media was then sterilised by autocalving at 15 lbs pressure (121 ^{0}C) for 15 minutes. The sterilised media was then poured into sterilized petriplate and then allowed to cool.

5. Gelatin agar (For Protease assay)

Composition

Gelatin	10.00 gm
Sodium azide	0.50 gm
Nutrient agar	28.00 gm
Final pH at 25°c	7.0± 0.2

Preparation: As listed in the composition accurate weight of components were dissolved in 1000 ml of the distilled water. The media was then sterilised by autocalving at 15 lbs pressure (121 ^{0}C) for 15 minutes. The sterilised media was then poured into sterilized petriplate and then allowed to cool.

B. Composition and preparation of different biochemical media

1. Hugh and Leifson's Media

Composition

Peptic digest of animal tissue	2.00 gm
NaCl	5.00 gm
K_2PO_4	0.30 gm
Glucose	10.00 gm

BTB	0.05 gm
Agar	2.00 gm
Final pH at 25^0C	6.8 ⨽ 0.2

Preparation: As directed by manufacturing company, 19.40gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121 ^{0}C) for 15minutes.

2. SIM (Sulphide Indole Motility Agar)

Composition	
Peptic digest of animal	30.00 gm
Beef extract	3.00 gm
Peptonized iron	0.20 gm
NaS_2O_3	0.025 gm
Agar	3.00 gm
Final pH at 25°C	7.3 ⨽ 0.2

Preparation: As directed by manufacturing company, 36.23gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121 ^{0}C) for 15minutes.

3. Sugar fermentation tests (2%)

Composition	
Peptone	10.00 gm
NaCl	5.00 gm
Beef extract	1.00 gm
Phenol red	0.018 gm
Sugar	20gm
pH	7.4

Preparation: Above listed components except sugar were dissolved well in 1000 ml of distilled water. Required amount of respective sugars were added in separate tubes and the media were sterilized by autoclaving at 15 lbs pressure (121^{0}C) for 15 minutes.

C. Composition and preparation of different staining reagent

I.Gram Stain

(a) Crystal violet (Hucker's)

Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled Water (D/W)	80 ml

Crystal violet was dissolved in ethyl alcohol and the ammonium oxalate in D/W. Then the two solutions were mixed.

(b) Gram's Iodine

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled Water	300.0 ml

Iodine and potassium iodide was dissolved in D/W.

(c) Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.0 ml
Distilled Water	5.0 ml

(d) Safranin (Counter Stain)

Safranin (2.5% solution in 95% ethyl alcohol)	10.0ml
Distilled Water	100.0 ml

II. Spore stain

Malchite green	5.0 gm
Distilled water	100.0 ml

III. Capsule stain

(a) Crystale violet capsule stain:

Crystal violet(85% dye content)	1.00 gm
Distilled water	100.0 ml

1% solution of crystal violet stain was prepared.

(b) Nigrosin

Nigrosin (water soluble)	10.0 gm
Distilled water	100.0 ml

The stain is prepared by mixing nigrosin with water.

D. Biochemical Test Reagents

i. Catalase Reagent (For Catalase test)

To make 100ml:

Hydrogen peroxide	3ml
Distilled water	97ml

Preparation: 3ml hydrogen peroxide was added to 97ml distilled water and mixed well.

ii. Kovac's Reagent

To prepare 40ml reagent:

4-dimethyl aminobenzyldehyde	2gm
Isoamyl alcohol	30ml
Con. HCl	10ml

Preparation: 2gm of reagent was dissolved in 30ml of isoamyl alcohol in clean brown bottle. Then 10ml of concentrated HCl was added and mixed well.

iii. Oxidase Reagent (For Oxidase test)

To prepare 10ml:

Tetramethyl-*p*-phenylenediamine dihydrochloride	0.10 gm
Distilled water	10.00 ml

Preparation: The chemical was dissolved in water and mixed well.

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APPENDIX – III

Test procedures

A. Procedure for Gram Staining

1. A thin film of the material to be examined is prepared and dried.
2. The material on the slide is heat fixed and allowed to cool before staining.
3. The slide is flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide is rinsed with tap water, shaking of excess.
5. The slide is flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide is rinsed with tap water, shaking off excess.
7. The slide is flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide is flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide is blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

B. Procedure for Spore staining

1. A thin smear is prepared using sterile technique and air dried.
2. The smear is heat fixed and allowed to cool before staining.
3. The slide with smear is then flooded with malachite green stain and steamed over boiling water for 5 minutes. The stain is added if stain boils off.
4. The slide is removed from boiling water, allowed to cool and washed with water.
5. The smear is counterstained with safranin for 20-30 seconds and washed off with tap water.
6. The slide is blot dried with bibulous paper and examined under oil immersion at 1000X.

C. Procedures for Capsule staining

1. A heavy smear of the culture was prepared on a grease free clean slide and air dried.
2. The smear was flooded with nigrosin and let to stand for 5 minutes.
3. The smear was then washed with crystal violet solution for one minute.
4. The slide was dried and examined under microscope under oil immersion at 100X.

APPENDIX- IV

Identification charts:

Morphological characteristics:

Tests performed	Isolates' code		
	K2.2i	**R3.3i**	**R5.2iv**
Gram staining	Gram positive cocci in cluster	Gram positive cocci in short chain	Gram positive cocci in cluster
Catalase	Negative	Negative	Negative
Oxidase	Positive	Negative	Positive
Capsule staining	Capsule absent	Capsule absent	Capsule absent
Spore staining	Spore absent	Spore absent	Spore absent

Biochemical characteristics:

Tests performed	Isolates' code		
	K2.2i	**R3.3i**	**R5.2iv**
Oxidative-fermentative	Oxidative	Fermentative	Oxidative
Sulfur Indole Motility	Indole negative and non-motile	Indole negative and Motile	Indole negative and non-motile
Sugar fermentation tests			
Mannose	++	++	-
Mannitol	-	++	-
Sorbitol	++	+	-
Sucrose	++	+	++
Lactose	-	-	-
Arabinose	++	-	+
Raffinose	-	++	+

[NB: ++ : highly acidic ; + : moderately acidic and - : negative for sugar utilization.]

APPENDIX-V

Ammonium Sulfate Fractionation Chart

Table: Amount of ammonium sulphate crystals required for a particular percentage saturation

Final Concontration (%)	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Initial Concentration (%)	**Ammonium Sulphate added (gl^{-1})**								
0	107	166	229	295	366	442	523	611	707
10	54	111	171	236	305	379	458	545	636
20		56	115	177	305	316	392	476	565
30			57	119	244	253	328	408	495
40				59	122	190	262	340	424
50					61	127	197	272	353
60						63	131	204	283
70							66	136	212
80								68	141
90									71

APPENDIX-VI

Data analysis by SPSS 11.5 version.

One-way ANOVA for the effect of temperature on protease activity

		Sum of Squares	df	Mean Square	F	Sig.
Zone of hydrolysis by acetone precipitated enzyme from R3.3i	**Between Groups**	328.333	2	164.167	6.164	0.045
	Within Groups	133.167	5	26.633		
	Total	461.500	7			
Zone of hydrolysis by acetone precipitated enzyme from K2.2i	**Between Groups**	336.542	2	168.271	11.473	0.014
	Within Groups	73.333	5	14.667		
	Total	409.875	7			

Paired Samples t – Test for the effect of ammonium sulphate fractionation at different temperature

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of The Difference				
				Lower	Upper			
Pair 1	-3.8750	2.69590	0.95314	-6.1288	-1.6212	4.065	7	0.005
Pair 2	-2.3750	2.06588	0.73040	-4.1021	-0.6479	3.252	7	0.014

[NB: Pair 1: Zone of hydrolysis by acetone precipitated fraction and 60% ammonium sulphate fraction of enzyme from R3.3i
Pair 2: Zone of hydrolysis by acetone precipitated fraction and 60% ammonium sulphate fraction of enzyme from K2.2i]

One-way ANOVA for the effect of pH on protease activity

		Sum of Squares	df	Mean Square	F	Sig.
Zone of hydrolysis by acetone precipitated enzyme from R3.3i	**Between Groups**	75.000	2	37.500	13.235	0.032
	Within Groups	8.500	3	2.833		
	Total	83.500	5			
Zone of hydrolysis by acetone precipitated enzyme from K2.2i	**Between Groups**	220.167	2	110.083	6.717	0.078
	Within Groups	49.167	3	16.389		
	Total	269.333	5			

Paired Samples t – Test for the effect of ammonium sulphate fractionation at different pH

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of The Difference				
				Lower	Upper			
Pair 1	-3.1667	1.72240	0.70317	-4.9742	-1.3591	4.503	5	0.006
Pair 2	-2.0000	1.67332	0.68313	-3.7560	-0.2440	2.928	5	0.033

[NB: Pair 1: Zone of hydrolysis by acetone precipitated fraction and 60% ammonium sulphate fraction of enzyme from R3.3i

Pair 2: Zone of hydrolysis by acetone precipitated fraction and 60% ammonium sulphate fraction of enzyme from K2.2i]