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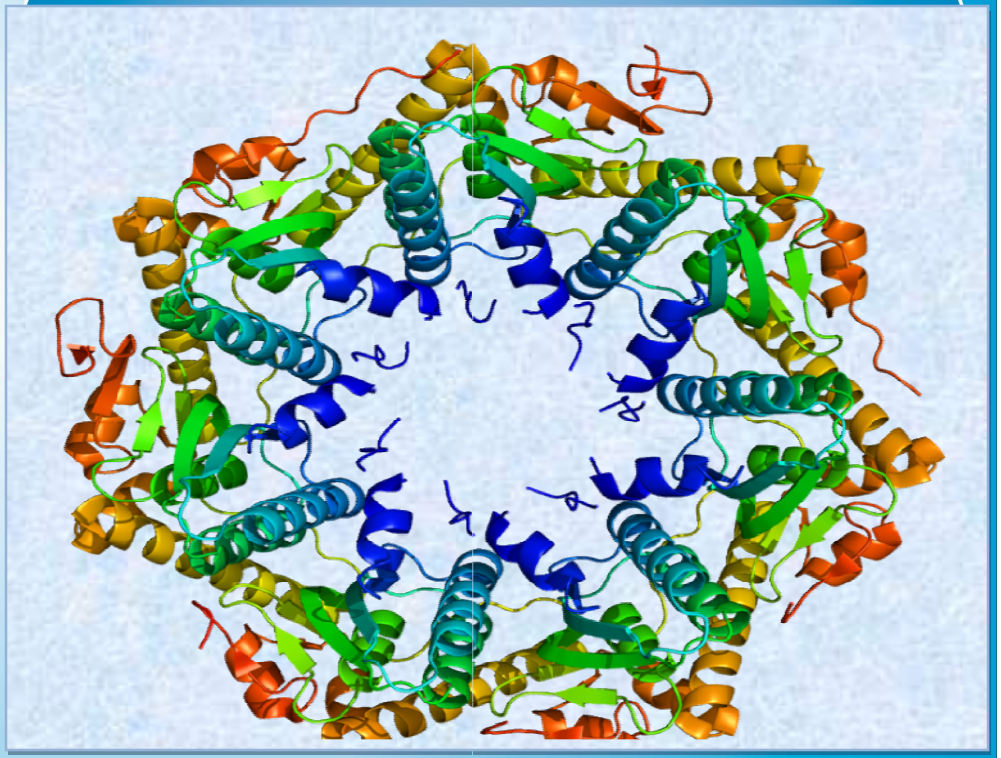
BACTERIAL PROTEASE – A BIODETERGENT AGAINST SURGICAL INSTRUMENTS

First Edition

Dr. Jai Shanker Pillai H P,
Dr. Sanjay Rathod,
Dr. Muzaheed Abdul Rasheed,
Dr. Madhuri. R. Basutkar



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(TNSRO) awarded him "**Har Gobind Khorana Young Scientist Award; 2011-12".** Owing to his significant contribution, he was awarded as "Distinguish Fellow of Bose Science Society' in the year 2012-13.

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Dr. Madhuri. R. Basutkar KSET, Ph.D, currently working as faculty In the Department of Microbiology, Gulbarga University, Kalaburagi Karanataka, India. She received her doctorate degree in Environmental Microbiology in the year 2019, from Gulbarga University Kalaburagi Karanataka. Her area of interest is Environmental Microbiology and she carried her research work on the Biodegradation of polycyclic aromatic reactive dyes by Microorganisms. In the year 2021, she cleared KSET competitive exam i.e. Karanataka Sate Eligibility Test for appointment of Assistant Professors. She also had done her M.sc in YOGA, programme from Annamalai University, Chidambaram, Tamil Nadu in 2020.

CONTENTS

1. INTRODUCTION

Surgery is a complex process performed by employing various surgical instruments. During surgery, the surgical instruments invariably come in contact with blood of the patients. If such instruments are not properly washed, it leads to contamination and foul smelling due to microbial degradation of blood finally paving way to transmission of diseases to other patients and health care personnel. Hence, in order to prepare surgical instruments and other medical devices for reuse, they must be cleaned with proper solutions. Cleaning not only avoids the transmission of diseases, it also forms an important aspect for the maintenance of hygiene and safety of surgical instruments.

Usually the surgical instruments are washed or cleaned by sterilization or by using chemical steriliants. However, sterilization cannot be used for thermo sensitive surgical instruments; similarly chemical steriliants can not remove the microbes that usually trapped behind the bioburden that is encrusted on or within surgical instruments. Therefore, this has spurred researchers to expand their efforts to identify new technologies and products that employ novel cleaning solution for the removal of bioburden from the surgical instruments.

One of such alternative steps is the use of “Biodetergents or Biocleaners”. Therefore in the present study an attempt has been made to extract enzyme from microorganisms, which can act against the bioburden laden on surgical instruments.

1.1. Aim and Objectives of the study:

The present work has been undertaken with the following aim and objectives

1. Collection of Samples
2. Isolation of bacterial species from the collected samples for the production of protease.
3. Morphological, Microscopic and Biochemical characterization of the obtained isolates
4. Selection of the obtained bacterial isolates for the hemolytic activity
5. Confirmation of haemolytic activity by cultivating on fibrin plates.
6. Production of protease enzyme from isolates.
7. Partial purification and assay of protease enzyme.
8. Characterization of protease enzyme.
9. Evaluation of partially purified enzyme for washing performance.
10. Evaluation of activity of protease enzyme with other detergents.

2. REVIEW OF LITERATURE

In surgery, cleaning and hygiene are very important and it is imperative that all surgery instruments and handling equipments be clean and hygienic. To achieve this goal, the cleaning system should include a specific sequence of cleaning agents like detergents. For many years, sodium tripolyphosphate (STPP) has been considered to be one of the most versatile and preferred builder of choice for detergents (Kandler, 1987). Since their advent, synthetic detergents are being increasingly used in bulk quantities worldwide. In India, synthetic detergents were introduced three decade ago (Ganesh Kumar 2003). The production of these synthetic detergents in 1980-90 varied between 12 and 14 lakh tonnes/annum and increased to 18.48 lakh tonnes in 1995. It was further projected that the production would increase to 25.50 lakh tonnes by 2000 AD indicating an extremely fast growth (The Economic Time, 1996). The detergents that are marketed at present in India have undergone wide transformations and have assumed various forms, viz., powders, liquids, bars/cakes, etc. Surprisingly more recently in western countries, pastes and gels have also emerged and have gained widespread popularity. Due to their ease in handling, most of these cater to the lucrative domestic market (Ganesh Kumar, 2003).

However, during the past few years, there has been serious public concern about the ecological problems arising from the use of such synthetic detergents on a large scale. Owing to the fact that these synthetic detergents are corrosive, toxic and exhibit a slow rate of biodegradation, their extensive usage leads to the formation of lumps, creating unhygienic conditions in the environment. However, the latest cleaning technologies include enzyme – containing formulations and zeolite based detergents. Of these, the enzyme detergents are proving extremely useful in keeping a check on environmental pollution. They offer option to the synthetic detergents with regard to their biodegradability, low toxicity, non-corrosiveness, environment – friendliness, enhanced cleaning properties as well as increased efficiency and stability in different formulations. In this context, these are also referred to as "Green Chemicals" (Ganesh Kumar, 2003).

2.1 Developments in enzyme – based detergents:

Enzymes have been used since biblical times. The cleaning of medical instruments with enzymes based detergents has been a practice for more than a decade. In 1913, Otto Rohm's patent indicated the use of pancreatic enzymes as washing aids for laundry cleaning and the product was marketed as a presoak detergent under the brand name "Burnus" (Ganesh Kumar, 2003). The first ever detergent containing the bacterial enzyme was introduced in to the market in 1956 under the trade name "Bio-40" (Gupta, 2002). Unfortunately, detergent proteases faced a setback in the early 1970s due to

unfavorable publicity when some workers developed allergic reactions while handling of these enzymes. This problem was solved by the introduction of dust free encapsulated products. Today detergent enzymes account for 89% in the world and Novo Nordisk and Genecor International are the major suppliers of detergents enzymes, supplying up to 95% of the global market.

2.2 Strains employed for protease enzyme detergent production:

Proteases are ubiquitous in nature and being derived from various kinds of microorganisms such as bacteria, fungi and protozoa. Amongst the bacteria, several species belonging to the genus *Bacillus* such as *Bacillus substilis* (Adinarayana, 2003), *Bacillus lentus (*Gupta, 1999) and *Bacillus licheniformis* (Oberoi, 2001) are frequently employed. Among the fungi *Aspergillus flavous* (Mulimani, 2002) has been recorded as a commercial strain for the production of protease enzyme. Insect proteases, especially from lipidopetrans viz., *Spiologsomo oblique*, have been used to supplement, though in sparing cases, the common detergents (Adil Anwar, 2000).

2.3 Classification of proteases:

Microbial proteases are classified into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme i.e., metallo – (EC, 3, 4,

24), asparatic – (EC, 3, 4, 23), cystaine or sulphydryl – (EC – 3, 4, 22), or serine type (EC, 3, 4, 21) (Kalisz, 1988; Rao, *et al.*, 1998).

2.4 Application of proteases:

2.4.1 In laundry:

The microbial enzymes, which have found application in laundry, are the proteases, amylase and lipases. More recently, the cellulases have also been employed in the detergent industry. The proteases hydrolyse the proteinaceous residues of blood, egg, grass and sweat to form soluble peptides, which are subsequently easily removed by detergent suds.

The washing performance of the enzyme detergent depends on many factors to achieve better results. These are detergent composition and dosage, pH, buffer capacity, water hardness, washing time, temperature, mechanical handling, soiling agents, textile types, to name a few. In addition, the specificity of the enzyme is another most important parameter. As a general opinion, it is considered that a detergent enzyme should have as specificity as possible. For example, a protease should be capable of degrading as many proteins as possible. However, a reasonably good wash performance can be achieved by a specific protease, in comparison to a non-specific protease. As the hydrolysis proceeds, small peptide fragments are formed by the action of an unspecific protease, which are rather difficult to remove as they are not very much soluble in detergent solutions. On the other hand, larger protein or peptide fragments are formed on hydrolysis with a specific protease due to the

breakdown of very few peptide bonds, which can be easily removed during the washing process (Anonymous, 1992). Presently, use of dual enzymes in detergent formulations is practiced; wherein the enzymatic hydrolysis and degradation can be broadened considerably in comparison to a single enzyme approach. Recently, workers of the Genencor International Inc., USA have developed enzymes called Endoglycosidiase which deglycosylate biopolymers like glycoprotiens, which are widely distributed in living organisms. They employed rDNA technology to develop Endo-b-N-acetyl glucosaminidase H (Endo H) as a cleaning agent. Endo H has a unique property to remove bacteria (*Staphylococci* and *E. coli*) from glass and cloth surfaces in buffer and detergent solutions (Rubingh, 1997).

At present, most of the advanced countries like Japan, United States, and some European countries almost invariably used the detergent incorporated with enzymes. Interestingly, in Japan, all detergent brands contain enzymes. In India, of few premium detergent brands presently available in market like Aerial (Procter and Gamble India Ltd), Surf Ultra, Rin Biolites, Revel Plus (Hindustan Lever Ltd.,) and Zymo (Henkel) contain enzymes in their formulations.

2.4.2 In dishwashing:

Enzymes have been successfully used in laundry detergents for many years as an aid to remove tough stains. However, the interest in using enzymes in automatic dishwashing detergents (ADDs) has increased recently. Both laundry and dishwashing detergents share similar function such as removal of stain due to egg, milk and starch-based soiling, etc. the performance of the enzymes in the ADDs are strongly influenced by the ADD formulation and the condition of the automatic dishwashing. At present, protease and amylases are the only two enzymes, which have found major application in dishwashing detergent (Lad, 1992). Enzyme-based dishwashing detergents are less abrasive in function and thus are suitable for use on delicate chinaware; they also prevent the erosion of designs and colour. This application was first exploited in Japan where the use of richly decorated chinaware and wooden kitchen utensils is wide spread.

Enzymatic ADDs has gained widespread usage since the last decade. In the past 2-3 years, ADDs with enzymes were launched in several European countries, Viz., Austria, Germany, Switzerland, Denmark and United Kingdoms. In Japan, all major ADD brands contain enzymes, whereas only one brand in the US market currently contains enzymes.

2.4.3 In textile Industry:

Currently, in the textile industry, there is a widespread demand for faded jeans. This involves subjecting such cloths to amylases – a process commonly referred to as biowashing or bioleaching, an alternative to the term, enzyme-fade. This allows elegant softness and unique shades to be given to the cloth, which overcomes the traditional methods of bleaching by sodium hypochlorite or tumbling which pumice stones and offers better safety as well as economy.

2.4.4 In food and dairy Industries:

With the better understanding of such enzymes, more and more areas of application are emerging, such as in dairy, food and beverage industries. The use of enzymes in these industries in the cleaning operations helps in creating the required hygienic conditions in such plants. Probably, the use of enzyme-based detergents in – place cleaning of membranes of ultra filtration (UF) and reverse Osmosis (RO) equipments proves promising and forms one of the most important aspect of modern dairy and food industries. The UF and RO membranes are put a variety of uses including concentration, clarification and/or sterilization of liquid foods like skim milk, whey, egg white, fruit juices, and beverages (Dalgaarol *et al.*, 1991).

Depending on the type of application, precise formulations are made; for instance, proteases are used for fouled dairy filters, a – amylases and b-glucanases in yeast and cereal, and cellulases and pectinases for wines and fruit juices. The enzyme detergent preparations presently marketed for cleaning of membrane systems are Teerg-a-zyme (alconox, Inc, New York, USA) and Ultrasil 53 (Henkel KgaA, Dusseldorf, Germany). These enzyme-based cleaners rely very much on the proteases to cleave and solubilize the protein foulant. The use of alkaline proteases from *Bacillus* sp. strain MK5-6 has also proved successful (Merin *et al.*, 1990). Pilot scale evaluation of the enzymes at plant level operations for UF membrane cleaning indicated the enzyme preparation to be highly effective and restored 100% flux in comparison to Terg-a-zyme, a commercial preparation which resulted in only 80% restoration of the flux. The use of proteases and lipases to degrade and solubilize protein and fat foulants has also proved beneficial.

2.5 Status of the enzyme industry:

Generally, the commercial preparation of various enzymes is employed in the formulation of different types of enzyme – based detergents. The proteases in particular, represent about 60% of all the industrial enzymes sales in the world (Godfrey and Reichelt, 1983). Of these, proteases used primarily as detergent additives, represent a major fraction. In 1992, proteases worth of $ 56 million were transacted in US alone (IB Market Forecast, 1992). However,

in the year 1994, the total market of industrial enzymes (Fig 2.1) accounted for around $400 million, of which enzymes worth $ 112 million were used for detergent production. As far as the Indian scenario is concerned, the picture is rather different where the respective figures for enzymes transacted and finding their way into the detergent industry for the corresponding year were Rs. 500 million and Rs. 20 million, only. Nevertheless, there is expected to be an upward trend in the use of enzymes in the domestic market and by the turn of the decade, the total value is likely to reach Rs. 3000 million or more (Hodgson, 1994).

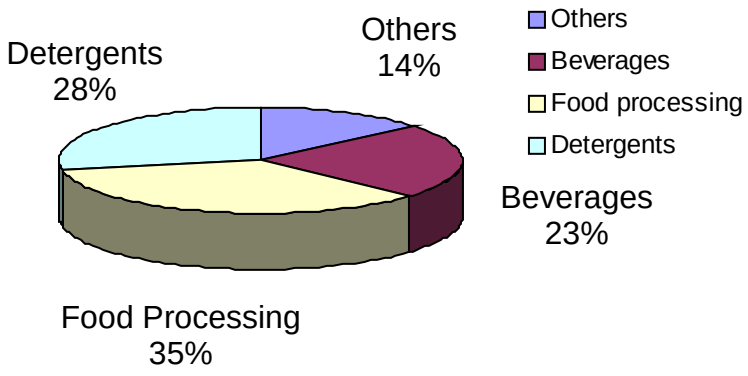

Figure 2.1 Global enzyme market

Presently, two companies actively engaged in India in the production and handling of a variety of industrial enzymes are Advanced Biochemicals Ltd. and Bicon (India) Ltd., located in Thane (Maharastra) and Bangalore, respectively. Both these companies share the bracket in the area and have an annual turnover of around Rs. 100 million each. The remaining amount of enzyme requirement of the country is catered by a number of other small units of manufactures and still a sizeable amount of its being met through imports. Further, many world premier industries are involved in the manufacture and trade of detergents enzymes (Table 2.1.).

Table–2.1: Commercial protease enzymes used in detergent formulations

Trade Names	Source organism	Optimum pH	Optimum temperature (^{O}C)	Manufacture
protease/ Subtilisins				
Alculase	*Bacillus, Licheniformis*	8-9	60	Navo Nordisk Dagsvnerd, Denmark.
Savinase	*Alkalophilic Bacillus sp*	9-11	55	Nova Nordisk Denmark
Espernse	*Alkalophilic Bacillus Sp*	9-11	60	Nova Nordisk Denmark
Maxacal	*Alkalophilic Bacillus Sp*	11	60	Gist – brecades, Delft, The Netherlands
Maxatase	*Alkalophilic Bacillus Sp*	9.5-10	60	Grist – Hrecades, The Netherlands
Opticlean	*Alkalophilic Bacillus Sp*	10-11	50-60	Solbay Enzymes GmbH, Harmover, Germany
Optimase	*Alkalophilic Bacillus Sp*	9-10	60-65	Solvay, Germany

Protosol	*Alkalophilic Bacillus Sp*	10	50	Advanced Biochemicals Ltd., Thana India
Alkaline Protease' Wuxi'	*Alkalophilic Bacillus Sp*	10-11	40-50	Wuxi Synder Bioproducts Ltd., China
Proleather	*Alkalophilic Bacillus Sp*	10-11	60	Amano Pharmaceuticals Ltd., Nagoya, Japan
Protease P	*Aspergillus sp*	8	40	Amano, Japan
Duruzym	*Protein engineered variant of savilance* ^{TM}	10-10.5	50	Novo – Nordisk, Denmark
Naxupem	*Bleach – resistant protein engineered variant of Alkalophilic Bacillus sp*	11-12	60	Solvay, Germany
Purafect	*Recombinant enzyme Donor- B. Lentus expressed in, Bacillus sp.*	10	40-65	Genencor International Inc, Rochester, USA

These enzymes are mostly derived from either submerged or solid state fermentation process by using different microorganisms, in particular, various strains of alkalophilic *Bacillus* sp. or genetically engineered organisms. Another alkaline protease preparation has been developed by Central Leather Research Institute, Madras under the trade name "Clarizyme". The enzyme was produced by *Aspergillus flavus* isolate using Solid State Fermentation (SSF).

2.6 Selection and evaluation of protease performance in detergents:

One of the important parameters for selection of detergent proteases is the pH value. It is known that detergent proteases perform best when the pH value of the detergent solution in which it works is approximately the same as the pH value for the enzyme. However, there are many more parameters involved in the selection of a good detergent protease, such as compatibility with surfactants, perfumes and bleaches (Gupta *et al.,* 1999; Kumar *et al.,* 1998), good activity at relevant washing pH and temperature (Aehle *et al.,* 1993; Oberoi *et al.,* 2001), compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Showell, 1999). Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases, which are effective over a wide temperature range (Oberoi *et al.,* 2001). In addition, the current consumer demands and increased use of synthetic fibers that can not tolerate high temperatures, have changed washing habits towards the use of low washing temperatures (Horan and Tamiya, 1997; Kitayama, 1992; Niclsen *et al.,* 1981). This has pushed enzyme manufacturers

to look for novel enzymes that can act under low temperatures. Novo Nordisk Bioindustry in Japan has developed a detergent protease called "Kannase", which retains high efficiency even at very low temperatures (10-20^{o}C). Further, a good detergent enzyme should also be stable in the presence of oxidizing agents and bleaches. In general, the majority of the commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. Hence, the latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability. Bleach and oxidation stability has been introduced through SDM and protein engineering by the replacement of certain amino acid residues (Estell *et al.*, 1985; Wolff *et al.*, 1996).

The evaluation of detergent proteases is mainly dependent upon parameters such as the pH and ionic strength of the detergent solution, the washing temperature and pH, mechanical handling, level of soiling and the type of textile. In the case of laundry detergents, protease performance is evaluated by using soiled test fabrics and the efficiency is measured either visually or by measuring the reflectance of light under standard conditions (Durham *et al.*, 1987; Masse and Tilburg 1983; Niclsen *et al.*, 1981; Wolff *et al.*, 1996). ΔR is the measure of efficiency and is defined as the difference in reflectance between fabric treated with and without enzyme. In a recent study by our group, the wash performance analysis of a SDS-stable alkaline protease

from *Bacillus* sp. RGR-14 (Oberoi *et al.*, 2001) was studied using a reflect meter. Wash performance analysis of RGR-14 protease on grass and blood action with detergent proved best, with a maximum reflectance change of 46% and 34% for grass and bloodstain removal, respectively, at 45^{o}C. Stain removal was also effective after protease treatment at 25^{o}C and 60^{o}C.

2.7 Detergents:

2.7.1 Meaning of Detergents:

A detergent is a mixture of substances primarily used for laundry and dish washing. The core components of most modern detergent are: surfactants, builder and co builder, bleach and bleach activator, and special additives such as fluorescent brightener, filler, corrosive inhibitor, antifoaming agent and enzymes. A detergent may contain more than one type of surfactant.

2.7.2 Surfactants:

These are surface-active agents with wetting, detergent and emulsifying properties. A simple classification of surfactants is based on the nature of the hydrophobic group. Four classes of surfactants are given in Table 2.2.

Table 2.2: Classes of surfactants.

Type	Composition	Example	Structure
Anionic	Sulfonated alcohols (C_{12}-C_{18}). Detergency is vested in anion part.	Sodium lauryl sulfate Sodium alkyl benzene sulfonate.	$C_{12}H_{22}OSO_2\ O^-NA^+$ R CH (CH_3) C_6H_4 SO_3^- NA^+
Cationic	Quaternary ammonium salts. Detergency is due to cation.	Hexadecyltriemethyl ammonium bromide. Generally used as germicides and fabric softeners.	$C_{16}H_{33}N^+$ $(CH_3)_3$ Br^-
Non-ionic	Condensation product of long chain alcohol and 7-8 ethylene oxide units.	n-dodecylocaethylene glycomonoether ethoxylate.	H_3C $(CH_2)_{10}$ $CH_2(OCH_2HC_2)_8$ OH H-bonding to many 'O' atoms makes the polyether end of the molecule water soluble.
Zwitterionic /Amphoteric	Contain both acidic and basic groups. Most common amphoterics are N-alkyl betaines	Laurylamdio propyl demethyl betaine (used in shampoos, skin cleaners).	$C_{12}H_{25}CON^+(CH_3)_2CH_2$ COOH

A useful index for choosing surfactants for various applications is the hydrophilic-lipophilic balance (relative percentage of hydrophilic to lipophilic groups in the surfactant molecules) and its range for application of surfactants as detergents is 13-16. All surfactant molecules have a water-soluble (hydrophilic) and a water-resistant (hydrophobic) part. At critical micelle concentration (10-4 – 10-2M), individual surfactant molecules spontaneously aggregate into micelles. The hydrophobic ends of surfactant molecules attach to oil and grease particles to form swollen micelles, which dissolve in water by

the attraction of the hydrophilic ends to the surrounding water. The cleaning power of detergents is provided by their surface-active properties.

2.7.3 Builders:

The builder in detergent is the second major component, which at lower concentration enhances the effect of the surfactant by deactivating calcium, and magnesium ions, which would otherwise use up surfactant molecules. Builders are thus water softeners and work by complexation (sodium tripolyphosphate STP), precipitation (sodium carbonate) or ion-exchange (Zeolites). Both organic (STP and sodium citrate) and inorganic (natural and synthetic) materials were used as builders earlier.

The most common builders used today are synthetic zeolites. Zeolites are solid ion exchangers that trap the divalent ions inside the solid particles and are used for the production of biodegradable detergents. The chronology of the development of detergent builders is given in Table 2.3

Table –2.3: Chronological development of detergent builders.

Builder (all builders as sodium salts)	Year
Silicate + Carbonate	1907
Phosphate containing detergents	
Diphosphate	1993
Triphosphate	1946
Zeolite A + triphosphate	1976
Phosphate free detergents	
Zeolite A + Carbonate + Polycarboxylates	1983
Zeolite A + Amorphous or	1994
Crystalline discilicates + Polycarboxylates	
Zeolite P + Carbonate + Citrate or Polycarboxylate	1994
Zeolite X + Carbonate + Citrate or Polycarboxylate	1997
Zeolite AX (Zeolite X (80%) + Zeolite A (20%)	Recent development

Zeolites are hydrated crystalline aluminum silicates of natural or synthetic origin with pore size of molecular dimension (0.3-1nm) and have a three – dimensional framework composed of tetrahedral AlO4-5 and SiO4-4 units that are linked by oxygen atoms. The tetrahedral atoms (Al and Si) are connected by bent 'O' bridge. One such structure called sodalite cage or β-cage consists of eight 6 sided or 6 oxygen rings or six rings and six 4 rings. The more common zeolites available for detergents of commercial importance are type A, P, X and AX.

During the last decade, the trend towards compact detergents increased the demand for zeolite builder. Zeolite A is now a builder leading to compact and super compact detergents. The compact detergents emerged in 1992 and super compact detergents in 1998 which are characterized not only by particularly low dosage and thus high surfactant content, but also by an increase in bulk density (Regular powder, 500-600 g/l, super compact, 800-900 g/l and tablets, 1000-13000 g/l). Tablets represent the latest development in laundry detergents, which also require highly absorptive zeolite builders.

A typical composition of powdered traditional and compact detergent formulation is given in Table 2.4.

Table 2.4: Typical composition of powdered traditional and compact detergent formulation.

Constituent	**Composition (weight %)**	
	Traditional	**Compact**
Surfactants	10-15	10-25
Builders (phospate or zeolites)	28-55	28-48
Bleach	10-25	10-20
Bleach activator	1-2	3-8
Fillers (sodium sulphate)	5-30	None
Corrosion inhibitors	2-6	2-6
Enzymes	0.3-0.8	0.5-2.0

Fluorescent brighteners	0.1-0.3	0.1-0.3
Foam controlling agents	0.1-4.0	0.1-2.0
Perfume	Trace	Trace
Water	To 100%	To 100%

2.7.4 Co-builder:

Organic polymers (polycarboxylates) are added as co-builders to make the zeolite work more effectively. Co-builders include poly (sodium carboxylates) consisting of neutral or ionic allyl glycosides monomer and malic acid or itaconic acid comonomer and poly (ally-β-D-glucofuranosiduromic acid-co-maleic acid). Sodium carbonate is added to raise the pH as the zeolite works better at high pH.

2.7.5 Fabric softeners:

Fabric softeners provide secondary washing benefits. They imparts softness by controlling static electricity. The active ingredients are cationic surfactants such as di-n-alkyldimethylammonium chloride (n-alkyl = n-C_{14}, n-C_{16} and n–C_{18}), $[CH_3\ (CH_2)_{15}\}_2\ N+\ (CH_3)_2\ CL$ or the more readily biodegradable esterquat $[CH_3\ (CH_2)_{15}\ C(O)O\ CH_2\ CH_2]_2\ N+\ (CH_3)\ (CH_2CH_2OH)\ (CH_3SO_4^-)$.

These compounds adsorb on cotton and cationic head groups bind to the carboxylate anions on the surface of the cloth. The alkyl chains form a fatty monolayer, which lubricates the cotton fabric and thus prevents friction damage to the fibers, thereby giving a pleasant feel. Softness also reduces water retention after spinning and thus less energy is needed to dry the clothes.

2.8 Advantages of Enzymatic Detergents over Non-enzymatic Detergents:

Enzyme detergents require low working temperature and low mechanical energy; are cost-effective, eco-friendly, less toxic, non-corrosive and have enhanced stability in different formulations, as compared to the non-enzymatic detergents.

3. MATERIALS AND METHODS

3.1 Collection of the samples:

During the present study of investigation the slaughterhouse drainage samples were collected and samples were transported to the laboratory in sterile peptone broth.

3.2 Isolation of bacterial species from the collected samples for the production of protease

About 1 ml of sample was mixed thoroughly in 100 ml of saline, the suspension was serially diluted, and 0.1 ml of each of the sample was inoculated on blood agar plates and incubated at 37^{o}C.

3.3 Selection of the obtained bacterial isolates for the hemolytic activity

About 100 isolates, were exhibited clear zone (Plate 3.1) near the vicinity of the colony, visible by naked eyes were chosen and preserved on blood agar slants for further determination of haemolytic activity, morphological, microscopical and physiological characters.

3.4 Determination of haemolytic or fibrinolytic activity:

Though the blood agar media gives a clear picture of haemolytic (fibrinolytic) enzymes releasing organisms, fibrin plates were used to confirm the isolates. The fibrinolytic activities of selected colonies were determined by

the plasminogen fibrin plate method as described by Astrup and Mullertz (1952).

3.4.1 Preparation of fibrin plates and inoculation:

1% Agar – buffer solution is poured into plates and cooled to 39-41^{o}C. Then 7 ml of fibrinogen and 0.2 ml of merthiolate solution is added, pH is adjusted to 7.4 and thoroughly mixed with agar, 7 ml of thrombin solution is also added. The mixture is allowed to solidify and inoculated with the above haemolytic bacteria.

3.4.2 Selection of fibrinolytic (proteolytic) enzyme releasing bacteria depending on their capacity of zone formation:

The fibrinolytic activity of 100 isolates was tested as above by the fibrin plate method. The isolates that gave clear zone of lysis on fibrin plates (Plate 3.2) were selected and the zone of lysis was measured in mm. Further, the isolates that exhibited which have given more than 2 mm zone of lysis were chosen and designated JMS1, JMS2, JMS3 and JMS4. Amongst the four isolates JMS4 exhibited more zone of lysis and as such was chosen to characterization through morphological, microscopical and physiological characters.

3.5 The media used for characterization and identification:

Blood agar media was chosen for detailed morphological study of fibrinolytic enzyme releasing organism to characterize them.

3.6 Examination of colony characters:

To study the nature and structure of the isolate the standard methods described by Collins and Lyne (1995) were used.

3.7 Microscopic observations:

The microscopic observations of the isolates were used as per standard method described by Collins and Lyne (1995).

3.8 Biochemical examinations:

All the biochemical examinations were carried out as described by Collins and Lyne (1995).

3.8.1 Glucose fermentation:

10 ml of glucose-nutrient broth with bromothymol blue indicator was inoculated with isolates obtained from different sources and incubated at 37^{o}C for 24 hours. The tubes were observed for the colour change to yellow, which indicated the production of acid.

3.8.2 Catalase reaction test:

10 ml of trypticase soya broth was inoculated with culture and incubated at 37^{o}C for 24 hours. The test tubes were tested for presence of catalase by adding 3-4 drops hydrogen peroxide.

3.8.3 Casein hydrolysis:

The milk agar containing casein as protein source was used to demonstrate the secretion of exoenzymes. The medium inoculated with

isolates and incubated. Following incubation for 24 hours, plates were examined for clear zone near the vicinity of the colony.

3.8.4 Gelatin hydrolysis:

To 10ml of gelatin medium, a loopful of isolates to be tested were inoculated and incubated for 37^{o}C for 48 hours. The extent of liquefaction was noted after keeping the tubes at 4^{o}C for 30 minutes.

3. 8.5 Oxidase test:

The ability of each isolate to produce oxidase can be determined by the addition of test reagent P-amino dimethylamine oxalate to colonies grown on agar plate medium. The addition of the reagent turns the colonies black colouration, represents the positive test.

3.8.6 Acid from Mannitol Salt Agar:

Mannitol salt agar plates were inoculated with strains and incubated for 37^{o}C for 24 hours. The plates were examined for yellow colouration near the vicinity of the colonies for positive reaction.

3.8.7 Litmus milk test:

To 10ml of milk incorporated with litmus was inoculated with isolates to be tested and incubated for 37^{o}C 48 hours. Formation of purple colour was observed for positive test.

3.9 Fermentation studies:

3.9.1 Production of proteolytic enzymes from isolate *Bacillus cereus* JMS4

Production of protease from *B. cereus* JMS4 was carried out in a medium containing the following composition of (g/lt) Peptone 5.0; yeast extract 5.0; skimmed milk 250.ml; agar12.0 and maintained at 37^{o}C for 48 hours in a shaker incubator (140 rpm). The pH of the medium adjusted with 1N NaOH or 1N HCl. Samples were drawn every 24 hr. Protease activity was estimated as per Adinarayana (2003).

3.9.2 Recovery of protease enzyme:

After completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4^{o}C and clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies and it also showed a clear zone of lysis on blood agar (Plate 3.3).

3.10 Partial purification of protease:

The culture fluid was centrifuged at 12000 rpm 30 min at 4^{o}C. The supernatant was adjusted to 3.5% saturation with ammonium sulphate. The precipitate formed by standing overnight at 4^{o}C was removed by centrifugation. The supernatant was adjusted to 80% saturation with solid ammonium sulphate and allowed to stand overnight. The precipitate was collected by centrifugation, dissolved in small volume of sodium borate-sodium hydroxide buffer (pH 11.0) and dialyzed (Feng, 2000).

3.11 Enzyme assay:

One ml of 1% casein solution was inoculated with 1 ml of appropriately diluted enzyme for 20 min at 30^{o}C. Reaction was stopped by addition of 4 ml of 5% trichloracetic acid. The tubes centrifuged after that 3000-x g for 10 min and the degraded products were measured by modified Lowry's method (Sandeep Kaur *et al.,* 2001). The absorbance was measured at 280 nm. A standard curve was generated using solutions of 0-50 mg tyrosine. One unit of protease activity was defined as the amount of enzyme, which liberated 1 μg tyrosine in 1 min at 60^{o}C.

3.12 Zymography of partially purified enzyme:

The zymography partially purified enzyme as carried out as per the method described by Beaton (1997) (Plate 3.4).

3.13 Composition of commercial detergent:

Following is the composition of the commercial detergent used in the present study (Table 3.1).

Table – 3.1: Composition of Enzyme Detergent:

Constituent	Composition (%)
Sodium tripolyphosphate (water softener, loosens dirt)	38.0
Sodium alkane sulphonate (surfactant)	25.0
Sodium perborate tetrahydrate (oxidizing agent)	25.0
Soap (Sodium alkane carboxylates)	3.0
Sodium sulphate (filter, water softener)	2.5
Sodium carboxymethyl cellulose (dirt suspending agent)	1.6
Sodium metasilicate (binder, loosens dirt)	1.0
Bacillus protease (3% active)	0.8
Fluorescent brighteners	0.3
Foam – controlling agents	Trace
Perfume	Trace
Water	to 100%

3.14 Evaluation of partially purified enzyme for washing performance:

Application of protease (5000 μ/ml) as a detergent additive was studied on white cotton cloth pieces (4x4 cm) stained with blood. The stained cloth pieces were taken in separate trays. The following sets were prepared and studied.

1. Tray with distilled water (100 ml) + Blood stained cloth
2. Tray with distilled water (100 ml) + Blood stained cloth + 1 ml of commercial detergent (7 mg/ml).
3. Tray with distilled water (100 ml) + Blood stained cloth + commercial detergent + partially purified enzyme.
4. Tray with distilled water (100 ml) + Blood stained cloth + partially purified enzyme.

The above trays were incubated at 60^{o}C for 30 minutes. At regular intervals of 5 minutes, cloth pieces were taken out from each set, rinsed with water, and dried, and visual examination (Plate 3.5) of various pieces was carried out. Untreated cloth pieces stained with blood were taken as control (Adinarayana, 2003).

3.15 Preparation of the enzyme detergent:

To the above-referred commercial detergent composition, the crude enzyme obtained was added at the rate of 7-ml/kg detergents. Thus prepared enzyme detergent has been presently designated as ***MICRODET*** (Plate 3.6)**.**

3.16 Evaluation of washing performance of the enzyme detergent *Microdet* on surgical instruments:

Various blood stained surgical instruments were taken and subjected to washing for 20 minutes period at 60^{o}C by commercial detergent and also the

microdet. Visual observation was made for the extent of bloodstain removal from the instruments after 20 minutes (Plate3.7).

3.17 Comparative evaluation of washing performance of *Microdet* with commercial detergents.

Washing performance of the microdet was compared with the capacity of the commercial branded detergents like Doctor, Surf Excel, Rin Supreme, Rin Shakti, Super Nirma, Henko, Nirma, Active Wheel, 555, Tide, 501, Double Dog, Aeriel, Trishul, Hipolene and Fena (Plate 3.8).

3.18 Compatibility of enzyme with commercial branded detergents:

Various commercial detergents, including Surf Excel, Surf, Ariel, Nirma, 501 Bar Soap, Snow White, Rin Shakthi, Dettol Liquid Soap and Tide (7mg ml-1) were incubated with 5000 μ/ml units of partially purified enzyme at 40^{o}C for 5 hours. Aliquots withdrawn at intervals of 60 min and the residual activity were determined under standard assay condition. Enzymes samples incubated in the absence of detergents served as control (Kamal Kumar, 2004).

3.19 Characterization of partially purified enzyme:

3.19.1 Effect pH on the activity of partially purified enzyme:

Effect of pH on the stability of the protease was studied by incubating the enzyme with buffer's covering the range of 5.5 –11.0, different buffers (0.1m) used were potassium phosphate (pH 5.5 – 7.5), Tris-HCl (pH 8.0-8.5) and sodium bicarbonate (pH 9-11.0) 85 (IU) of enzyme samples were added to

different buffers, to make the final volume of 0.1ml. After incubation at 40^{o}C for 1 hour, residual activity in each sample was determined by casinolytic assay and compared with the control sample kept at pH 7.0 (Sangita *et al.*, 1993).

3.19.2 Effect of temperature on the activity of partially purified enzymes:

The enzyme samples were incubated at various temperatures ranging from 0-70^{o}C, at pH 7.5. After 1 hour, residual activities were determined and compared with control kept at 4^{o}C at pH 7.5.

4. RESULTS

4.1 Morphological Characters of isolated strains:

The results on colony morphology of the isolates obtained from slaughterhouse drainage on blood agar are presented in Table 4.1.

The perusal of results indicated that the size of the colony obtained from slaughterhouse drainage measured 4 mm. The shape of the colony obtained is irregular and round. All the colonies isolated from slaughterhouse drainage are convex, smooth feathery, creamy and β-haemolytic.

4.2 Microscopic characters of isolated strains:

The results on microscopic characters of the isolates obtained from slaughterhouse drainage are presented in Table 4.2.

The perusal of the results indicated that the colonies from slaughterhouse drainage have shown gram positive, non-motile, rod shaped, cells in chains and free and presence of endospores.

4.3 Biochemical reaction of isolated strains:

Results on the biochemical reactions of the isolates studied from slaughterhouse drainage are presented in Table 4.3

Table – 4.1: Colony characters of isolated strains on blood agar:

Sl. No.	Bacterial strain	Source of isolation	Colony characters
1.	JMS1	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
2.	JMS2	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
3.	JMS3	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
4.	JMS4	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic

Table – 4.2: Microscopic characters of isolated strains:

Sl. No.	Strain type	Mobility	Gram reaction	Shape	Arrangement	Endospore
1.	JMS1	Motile	Gram +ve	Rods	Free	Present
2.	JMS2	Motile	Gram +ve	Rods	Free	Present
3.	JMS3	Motile	Gram +ve	Rods	Free	Present
4.	JMS4	Motile	Gram +ve	Rods	Free	Present

Table – 4.3: Biochemical reaction of isolated strains:

Sl. No.	Strain type	Catalyse	Glucose fermentation	Acid from mannitol	Growth in 6.5% NaCl	Acid from glycerol	Endspore	Oxidase	Heaemolysis	L co
1.	JMS1	+	-	-	-	-	+	-	β	
2.	JMS2	+	-	-	-	-	+	-	β	
3.	JMS3	+	-	-	-	-	+	-	β	
4.	JMS4	+	-	-	-	-	+	-	β	

The data presented in Table 4.3 indicated catalase, β-haemolysis and endospore positive, whereas glucose fermentation, acid from mannitol growth in 6.5% NaCl, oxidase and litmus coagulation negative.

4.4 Determination of fibrinolytic activity

The results on zone of lysis of haemolytics isolated on fibrin plates are presented in Table 4.4.

Perusal of the results indicated that the zone of lysis of isolates occurred in the range of 45.2 to 5.6 mm. The maximum zone lysis occurred is 5.6 mm by isolate JMS4

Table – 4.4: Diameter of Zone (in mm) formed by isolated strains on fibrin plate:

Sl. No.	Bacterial strain	Source of isolation	Zone of lysis (mm)
1.	JMS1	Slaughter house drainage	5.2
2.	JMS2	Slaughter house drainage	5.2
3.	JMS3	Slaughter house drainage	4.0
4.	JMS4	Slaughter house drainage	5.6

4.5 Protease production by isolate JMS4:

The results on the production of protease from isolate JMS4 are presented in Fig 4.1.

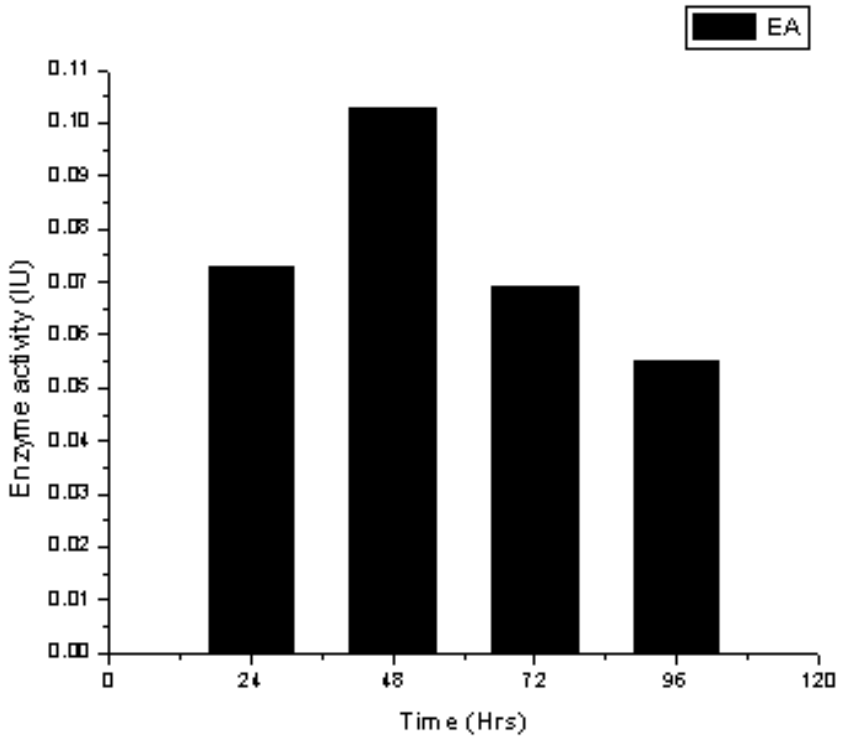

Fig. 4.1: Production of proteases by JMS4

The results revealed that the production of protease enzyme increased up to 48 hours of fermentation. Thereafter the decreased in the enzyme activity was observed. The maximum enzyme production was observed at 48 hours of fermentation.

4.6 Zymography:

The results on zymography of isolate JMS4 are presented in Plate 3.4.

The results revealed that the clear areas were interpreted as representing areas of protease activity and whole dark areas represent areas where protease activity is absent.

4.7 Enzyme partial purification and assay:

The results are presented in Table 4.5. It is observed that the crude enzyme presented an activity of 0.103 IU / ml / min while the partially purified enzyme showed an activity of 0.116 IU / ml / min.

Table - 4.5: Partial Purification and assay of Enzyme

Sl. No.	Purification steps	Total Enzyme activity (IU)	Total protein (mg)	Specific activity
1.	Crude enzyme	0.103	0.9	0.114
2.	Ammonium solution (70%)	0.116	0.68	0.170

4.8 Evaluation of partially purified enzyme for washing performance:

The results are presented in Plate 3.5. The results reveal that the blood stain on the cloth piece remained as it was even after 30 minutes rinsing in the controls and commercial detergent. Blood stain was totally removed from the cloth after rinsing it with a combination of detergent and enzyme for a period of 20 minutes, whereas it was removed after 25 minutes when rinsed with crude enzyme alone.

4.9 Evaluation of washing performance of the enzyme detergent *Microdet* on surgical instruments:

The results of the study to evaluate the washing performance of the enzyme detergent microdet on the surgical instruments are presented in Plates 3.7. After an incubation of 20 minutes, stains were not removed completely with detergents alone, while the combination of the enzyme with commercial detergent (Microdet) removed the bloodstains from the surgical instruments very effectively.

4.10 Comparative evaluation of washing performance of Microdet with commercial branded detergents:

Of all the branded detergents, Tide and Aerial removed bloodstains after 20 minutes rinsing on par with the microdet (i.e., commercial detergent supplemented with crude enzyme) (Plate 3.8).

4.11 Compatibility of crude enzyme with commercial branded detergents:

The results of the studies involving the compatibility of the crude enzyme obtained in the present study along with the commercial branded detergents are presented in Fig. 4.2. It was observed that the enzyme activity was retained at only 50% at the end of 5 hours when incubated with the commercial branded detergents, except in the presence of Tide and Aerial and also in our new enzyme wherein it was retained at slightly higher rate of 60.

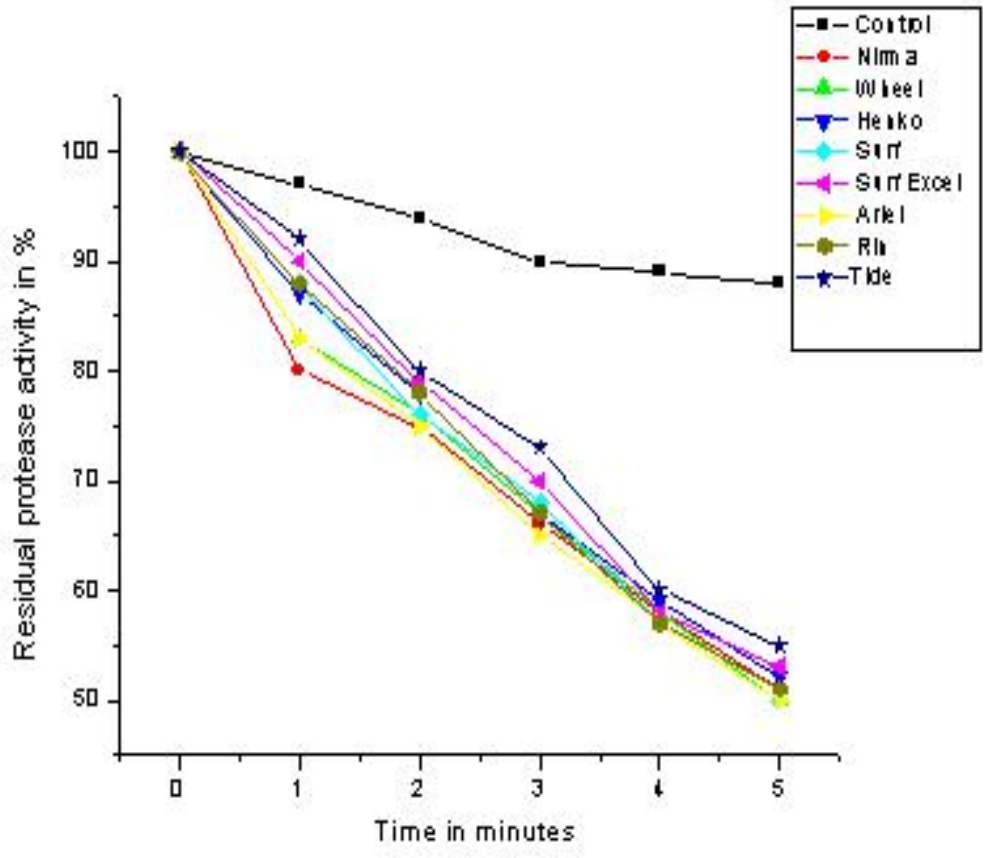

Fig. 4.2: Compatibility of crude enzyme with commercial branded detergents

4.12 Characterization of Enzyme:

4.12.1 Effect of temperature on enzyme activity:

The results of the studies on the effect of the temperature on enzyme activity are presented in Fig 4.3.

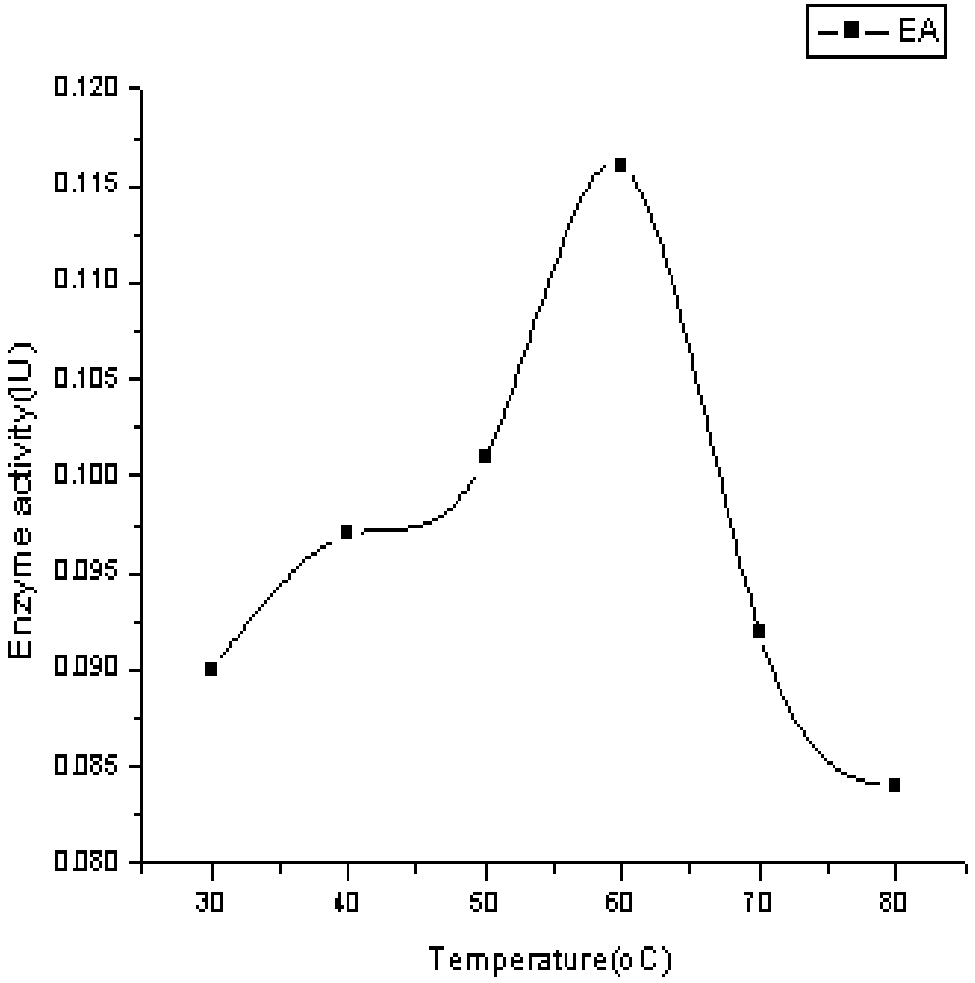

Fig. 4.3: Effect of temperature on enzyme activity

The temperature kinetics of the enzyme suggests that the enzyme activity is increasingly sharply from 30 to 60^{o}C, followed by a sudden decline after 60^{o}C. The enzyme from isolate JMS4 was found to be maximally active at 60^{o}C.

4.12.2 Effect of pH on enzymatic activity:

The results on the effect of pH on the enzyme activity are presented in Fig.4.4.

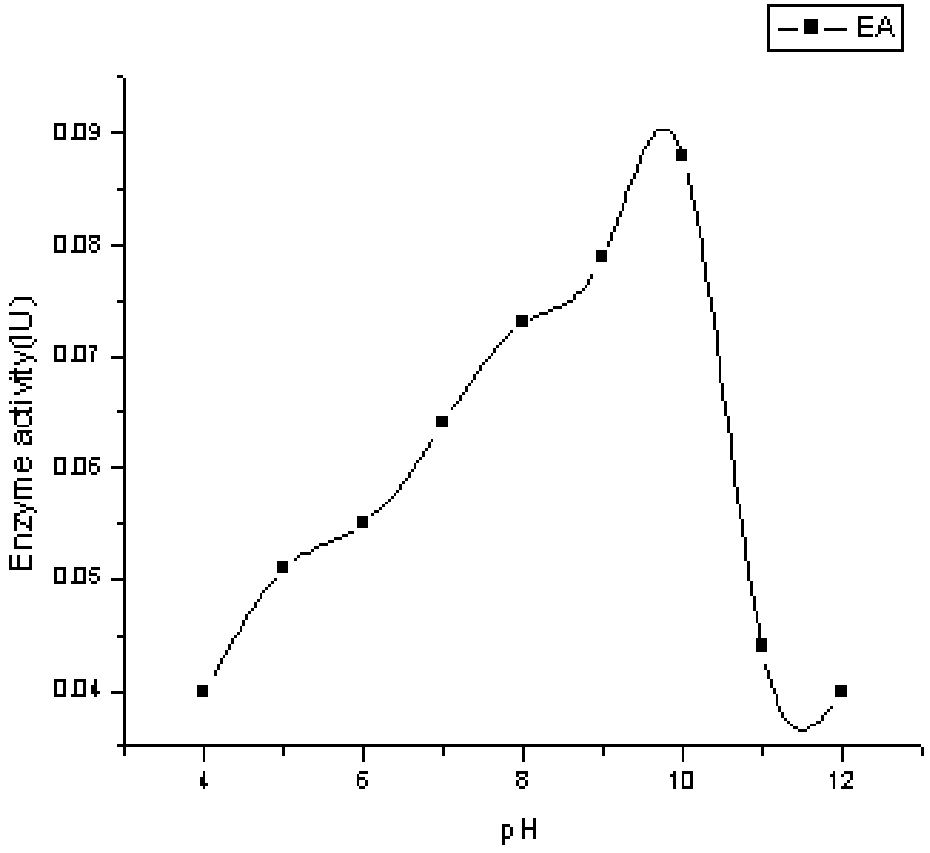

Fig. 4.4: Effect of pH on enzyme activity

The pH kinetics of the enzyme activity revealed that the enzyme activity is increased from pH 4 to 12. The optimum pH recorded was at pH 10 for maximum activity. The enzyme activity gradually declined at pH beyond pH 10.

5. DISCUSSION

Reusable medical instruments are required to be properly cleaned and disinfected between each use, cleaning is defined as to removal of all foreign material such as blood, protein, cellular debris, tissue, respiratory secretions, mucus, saliva, feces, etc., from objects. If the instrument is not clean, the organic soil can harbor embedded microorganisms, organic soil can prevent the penetration of the germicide and render the sterilization process ineffective (Martin and Reichelderfer, 1994).

A variety of enzyme product has been developed for use in so-called "biological or enzymatic detergents" to enhance the removal of bioburden from surgical instruments. They are three basic types enzymes used in detergents: proteases, amylases and lipases. Proteases are the most important type of enzyme to look for when choosing an enzymatic detergent for medical use because there is a high content of protein in most body fluids (including blood, tissue and mucous) which cannot be easily removed with regular detergents/surfactants and water, proteases break down protein into individual amino acids or short string of amino acid. Amino acid and peptides are much more soluble in water and will float away the surface of the instrument.

The incorporation of enzymes into a detergent for endoscope cleaning has several benefits in addition to enhancing overall cleaning performance. Clogging of endoscope channel is virtually eliminated with the appropriate enzymatic detergents. This reduces the need for costly routine maintenance and results in large saving. As a result, the quality of the images is improved. Properly formulated enzymatic detergents are non-corrosive and do not attack any metal surfaces on medical instruments and will effectively work in mild conditions without damaging valves, rubber gaskets or any surface of the flexible fiber optic endoscope or other medical instruments. Therefore, in the present study, an attempt is made to develop enzymatic detergent for cleaning of surgical instruments.

In the present study the slaughterhouse drainage sample collected was screened for presence of bacteria, which can utilize blood protein as their protein source. The samples were serially diluted on blood agar. From these plates, depending upon their morphological, microscopical and physiological characters, the isolates were suspected to be *Bacillus cereus.* The colonies were confirmed by cultivating on selective media.

On PEMBA (Polymyxin pyruvate egg-yolk mannitol bromothymol blue agar) media the isolated colonies were peacock blue in colour. This confirms the presence of *Bacillus cereus*. The isolates have been designated as *Bacillus cereus* JMS1, JMS2, JMS3 and JMS4. Of these JMS4 isolate was chosen as potential producer of protease enzyme depending upon its zone of lysis on

fibrin plates and studied further for production of protease enzyme and its compatibility for the use of enzymatic detergent preparation for the cleaning surgical instruments.

5.1 Production of protease:

Generally, proteases produced from microorganisms are constitutive or partially inducible in nature and under most culture conditions *Bacillus* species produce extracellular proteases during post exponential and stationary phases. In order to evaluate the stage of growth of bacteria for maximum production of proteases extracellularly the bacteria was grown in medium. The results indicated that the enzyme production (Fig 4.1) was more at 48 hours of fermentation thereafter the decreased enzyme production was observed. The results obtained were in good agreement with the results of Adinarayana *et al.*, (2003) and Panuwan Chantwannakul *et al.*, (2002).

5.2 Zymography:

Electrophoretic methods in particular gelatin and casein zymography are extensively used for the detections of proteolytic enzymes, because they display several advantages including their simplicity and high sensitivity. Zymography allow the identification of the enzyme of interest in a complex mixture of proteinases, and estimation of the molecular weight of latent and active forms of the enzyme due to the process of denaturation and renaturation that takes place within the gel. Zymography gives a measure of total potential

enzymatic activity but does not allow determination of the exact level of activity present in a sample.

The results of the zymography technique were presented in Plate 3.4. The results revealed that, the bacterial extract loaded on chromatography has ability to degrade casein present as a substrate in the zymogram, so that it leaves a clear zone of lysis on zymogram, indicated the presence of proteolytic enzymes in the extract. The similar findings were also reported by Mols et al, (2004), and Ping-Chung Liu *et al.*, (1997).

5.3 Enzyme purification and Assay:

A number of protease from different source have been purified and characterized by different methods. After separating the culture from the fermentation broth by filtration or centrifugation the culture supernatant is concentrated by means ultrafiltration (Kang *et al.,* 1999; Smacchi *et al.,* 1999), salting out by solid ammonium sulphate (Kumur, 2002) or solvent extraction methods using acetone (Kumar *et al.,* 1999) and ethanol (El-Shanshoury *et al.,* 1995).

In our studies the protease has extracted by solid ammonium sulphate and results are presented in Table 4.5. The concentrated protease exhibited more (0.176/IU) activity when compared to crude enzyme extract (0.103 I/U). Our results are in good agreement with the results reported by Sandeep Kaur (2001), Ping-Chung Liu (1997) and Adinarayana (2003).

5.4. Evaluation of partially purified enzyme for washing:

The results on the washing performance partially purified enzyme are presented in plate 3.5. The results revealed that the detergent added with enzyme of *Bacillus cereus* JMS4 removed bloodstains from cloth piece within 20 minutes. Whereas, Crude enzyme and detergent remove blood stains 25 and 30 minutes respectively. The result clearly indicates that the performance of removal of bloodstain from the objectives is effective best at in presence of enzyme and detergents.

5.5. Evaluation of washing performance of the enzyme detergent Microdet on surgical instruments:

The results on washing performance of Microdet on surgical instruments are presented in Plate 3.7. The results revealed that Microdet removed all the bloodstains present on the surgical instruments within 20 minutes; it may be due the presence of proteolytic enzymes of strain *Bacillus cereus* JMS4 in the detergent formulation.

5.6. Comparative evaluation of washing performance of Microdet with commercial detergents:

The results on the washing performance of microdet with commercial branded detergents are presented in Plate 3.8. The data revealed that Microdet, Tide and Arieal removed the bloodstain within 20 minutes, whereas other detergents like Doctor, Surf excel, Rin Supreme, Rins hakthi, Super Nirma, Henko, Nirma, Active Wheel, 555, Tide, 501, Double Dog, Ariel, Trishul, Hiplene and Fena have removed blood stain after 20 minutes. The results of

Arieal and Tide were same as that of Microdet. This may by be due to presence of cationic and non-ionic components in their formulations along with proteolytic enzymes.

5.7. Compatibility of crude enzyme with commercial branded detergents:

An ideal detergent enzyme should be stable at high pH and temperature, withstand oxidizing and chelating agents, and be effective at a low enzyme level (0.4% to 0.8%) in a detergent solution. It should also have broad substrate specificity. Therefore, in order to assess the utility and compatibility of the bacillus cereus JMS4 enzyme with the commonly used detergents, its properties such as stability was determined. The results on the compatibility of enzyme activity with commercial available detergents are presented in Fig 4.2. The data revealed that the enzyme from isolate *Bacillus cereus* JMS4 has retained activity more than 50% indicating that in can be used as an additive for perpetration of detergent solutions. The maximum stability has been occurred in commercial detergent Tide (Proctor and Gamble) contains cationic and non-ionic components in its formulation, along with proteolytic enzymes. Hence, the protease from *Bacillus cereus* JMS4 appeared to have the potential to be used as an additive in detergent containing similar formulations. Similar observations have been made in the case of alkaline proteases from *Tritirachium album* (Samal, 1990), *Spilosoma Obliqua* (Adil Anwar, 2000),

Conidiobolus coronatus (Phadetare *et al.,* 1993) and Kunamueni Adinarayana (2003).

5.8 Characterization of protease:

The protease used in a detergent formulation should have a high level of activity over a broad range of pH and temperature. One of the drawbacks of enzyme recovered from thermopiles is non-stability to pH and temperature. Thus its desirable to search for new protease with novel properties like thermo and alkaline stable.

In the present study we examined the efficiency of an enzyme, recovered from *Bacillus cereus* JMS4 for stability to pH and temperature.

The results observed on the studies of the effect of temperature on enzyme activity are presented in Fig 4.3. The maximum enzyme activity was observed at 60^{o}C. Thereafter decrease in the activity was observed. The enzyme in the presence of its substrate was more active below the 60^{o}C. It's known that temperature increase the reaction velocity and also affects the rate of enzyme activity. At high temperature its adverse effect become significance as the reaction proceeds Thus the residual enzyme activity detected less at temperature higher than 60^{o}C might be due to the stability effect of its substrate (Ahmed F. Abdel –Fattah 1983) The temperature optima of 60^{o}C was also reported by Adinarayana (2003) for maximum enzyme activity.

The results on the effect of pH on the enzyme activity are presented in Fig 4.4. The observation revealed that the optimum pH recorded was at pH 10. Indicated that the enzyme even active at alkaline pH, which is most desirable quality for the enzyme used in detergent formulation (Ruchi Oberio *et al.,* 2001; Sangita, 1993 and Adil Anwar, 2004). The similar optimal ranges were also found and reported Adinarayana (2003) Kamal Kumar (2004) and Sandeep Kaur (2001).

Optimal ranges were also found and reported Adinarayana (2003) Kamal Kumar (2004) and Sandeep Kaur (2001).

6. SUMMARY AND CONCLUSION

Based on various biochemical properties the protease isolated from *Bacillus cereus* JMS4 is thermostable protease. It is stable at alkaline pH at high temperatures, and in presence of commercial and local detergents. These properties indicate the possibilities for use of the protease in the manufacture of surgical cleaning detergent industry. Therefore, the enzyme obtained from *Bacillus cereus* JMS4 has been used in the preparation **Microdet.** The Microdet under the present study showed promising results in the removal of bloodstains from the surgical instruments. However, the economics of its production for commercial exploitation has to be worked out.

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