Original Article

Journal of Microbiology and Related Research
Volume 2 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/jmrr.2395.6623.2216.4

The Role of Protease as Detergents and Disinfectants in Instrument Cleaning and Reprocessing

Muzaheed*, Sanjay Rathod*

Received on 03.11.2016, **Accepted on** 12.11.2016

Author Affiliation
Department of studies and Research,
Gulbarga University,
Gulbarga.-585106.

Reprint Request
Muzaheed,
Assistant professor
Department of Clinical Studies,
College of Applied Medical Science,
University of Dammam,
Dammam 34212, Saudi Arabia.
E-mail: mujaheedmail80@gmail.com

Abstract

Preventing infections in patients undergoing surgical procedures is a primary goal for all members of the healthcare team. This is especially important in today's dynamic healthcare environment, in the face of newly recognized pathogens, well-known microorganisms that have become resistant to treatment modalities, and the economic pressures to reduce. Healthcare-associated infections. A key infection control practice for reducing the likelihood of a surgical site infection is proper reprocessing of surgical instruments. In its 2008 Guideline for Disinfection and Sterilization in Healthcare Facilities, the Centers for Disease Control and Prevention (CDC) notes that failure to properly disinfect and sterilize equipment carries not only the risk associated with breach of host barriers, but also the risk for person-to-person transmission as well as transmission of environmental pathogens; furthermore, thorough cleaning is required before disinfection and sterilization because inorganic and organic materials that remain on the surfaces of instruments interfere with the effectiveness of these processes. 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. 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* KLM1 KLM2, KLM3 and KLM4 Of these KLM4 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.

Keywords: Protease; Zymography; *Bacillus Cereus KLM4.*

© Red Flower Publication Pvt. Ltd.

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". When selecting detergents and other cleaning agents for use in healthcare facilities, it is also important to remember that the agent should be compatible with the medical device to be cleaned, and also with the materials used in the cleaning equipment itself.16 For example, the chemicals should not cause corrosion in any type of automated cleaner (e.g., ultrasonic cleaner, washer disinfectors, or washer sterilizers); and they should not promote electrolytic action between the equipment and the items being cleaned. In addition, any chemical should be easily removable from the item by rinsing it with readily available water with specific properties so that the item does not retain residual chemicals in amounts that could potentially be harmful to patients, damage the device itself, or create other hazardous situations. 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.

Materials and Methods

During the present study of investigation the slaughterhouse drainage samples were collected and samples were transported to the laboratory in sterile peptone broth. 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°C. About 100 isolates, were exhibited clear zone (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. 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). Further, the isolates that exhibited which have given more than 2 mm zone of lysis were chosen and designated KLM1, KLM2, KLM3 and KLM4. Amongst the four isolates KLM4 exhibited more zone of lysis and as such was chosen to characterization through morphological, microscopical and physiological characters. Blood agar media was chosen for detailed morphological study of fibrinolytic enzyme releasing organism to characterize them. To study the nature and structure of the isolate the standard methods described by Collins and Lyne (1995) were used. The microscopic observations of the isolates were used as per standard method described by Collins and Lyne (1995). All the biochemical examinations were carried out as described by Collins and Lyne (1995).

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.

Gelatin Hydrolysis

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

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.

Fermentation Studies

Production of protease from *B. cereus* KLM4 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°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).

Recovery of Protease Enzyme

After completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4°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.

Partial Purification of Protease

The culture fluid was centrifuged at 12000 rpm 30 min at 4°C. The supernatant was adjusted to 3.5% saturation with ammonium sulphate. The precipitate formed by standing overnight at 4°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).

Enzyme Assay

One ml of 1% casein solution was inoculated with 1 ml of appropriately diluted enzyme for 20 min at 30°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 mg tyrosine in 1 min at 60°C.

Zymography of Partially Purified Enzyme

The zymography partially purified enzyme as carried out as per the method described by Beaton (1997) (Plate 1).

Composition of Commercial Detergent

Following is the composition of the commercial detergent used in the present study (Table 1).

Table 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%

Evaluation of Partially Purified Enzyme for Washing Performance

Application of protease (5000 m/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°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 2) of various pieces was carried out. Untreated cloth pieces stained with blood were taken as control (Adinarayana, 2003).

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).

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°C by commercial detergent and also the microdet. Visual observation was made for the extent of bloodstain removal from the instruments after 20 minutes (Plate 4).

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 5).

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 m/ml units of partially purified enzyme at 40°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).

Results

The results on colony morphology of the isolates obtained from slaughterhouse drainage on blood agar are presented in Table 2. 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 b-haemolytic. The results on microscopic characters of the isolates obtained from slaughterhouse drainage are presented in Table 3. 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. Results on the biochemical reactions of the isolates studied from slaughterhouse drainage are presented in Table 4. The data presented in Table 4 indicated catalase, b-haemolysis and endospore positive, whereas glucose fermentation, acid from mannitol growth in 6.5% NaCl, oxidase and litmus coagulation negative.

The results on zone of lysis of haemolytics isolated on fibrin plates are presented in Table 5. 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 KLM4.

Protease Production by Isolate KLM4

The results on the production of protease from isolate KLM4. 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.

Zymography

The results on zymography of isolate KLM4 are presented in Plate 1. 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.

Enzyme Partial Purification and Assay

The results are presented in Table 6. 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.

Evaluation of Partially Purified Enzyme for Washing Performance

The results are presented in Plate 2. 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.

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 4. 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.

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 5).

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. 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.

Table 2: Colony characters of isolated strains on blood agar

Sl. No.	Bacterial Strain	Source of Isolation	Colony Characters
1.	KLM1	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
2.	KLM2	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
3.	KLM3	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic
4.	KLM4	Drainage of slaughter house	4mm, irregular, convex, smooth, feathery, creamy and β - hemolytic

Table 3: Microscopic characters of isolated strains

Sl. No.	Strain type	Mobility	Gram reaction	Shape	Arrangement	Endospore
1.	KLM1	Motile	Gram +VE	Rods	Free	Present
2.	KLM2	Motile	Gram +VE	Rods	Free	Present
3.	KLM3	Motile	Gram +VE	Rods	Free	Present
4.	KLM4	Motile	Gram +VE	Rods	Free	Present

Table 4: Biochemical reaction of isolated strains

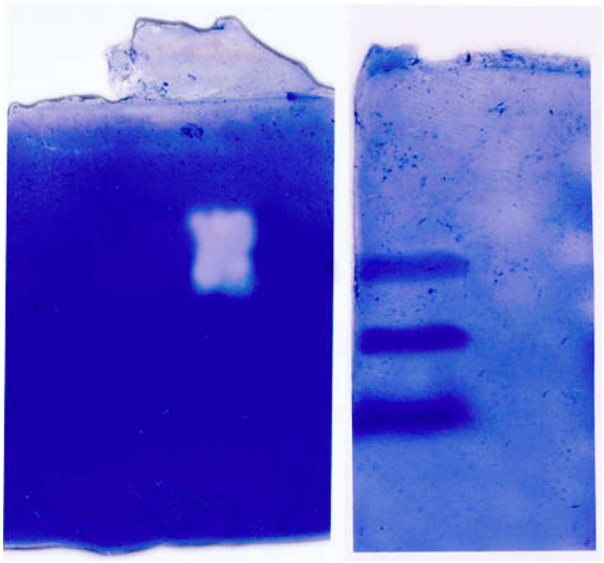
Sl. No.	Strain type	Catalyse	Glucose fermentation	Acid from mannitol	Growth in 6.5% NaCl	Acid from glycerol	Endspore	Oxidase	Heaemolysis	Litmus coagulate
1.	KLM1	+	-	-	-	-	+	-	β	-
2.	KLM2	+	-	-	-	-	+	-	β	-
3.	KLM3	+	-	-	-	-	+	-	β	-
4.	KLM4	+	-	-	-	-	+	-	β	-

Table 5: Diameter of Zone (in mm) formed by isolated strains on fibrin Plate.

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

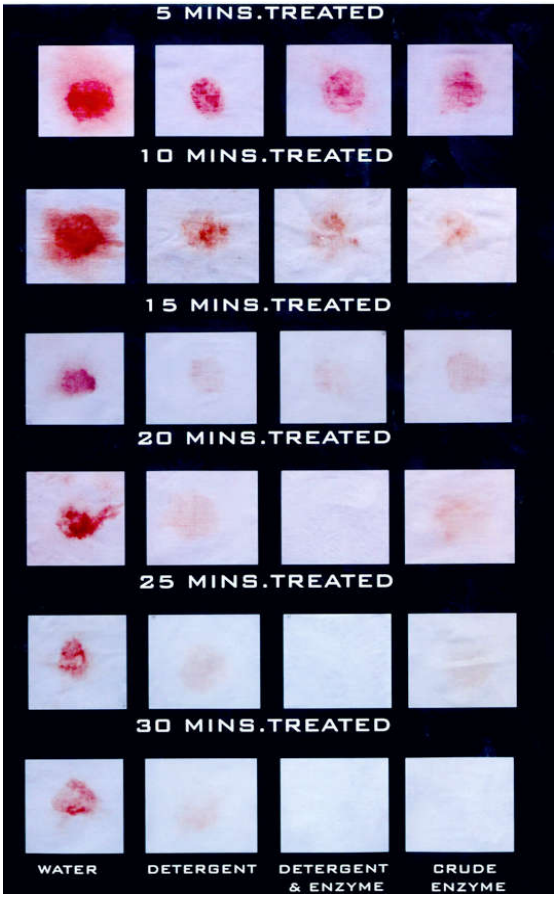
Table 6: Partial Purification and assay of Enzyme

Sl. No.	Bacterial strain	Source of Isolation	Zone of Lysis (mm)
1.	KLM1	Slaughter house drainage	5.2
2.	KLM2	Slaughter house drainage	5.2
3.	KLM3	Slaughter house drainage	4.0
4.	KLM4	Slaughter house drainage	5.6

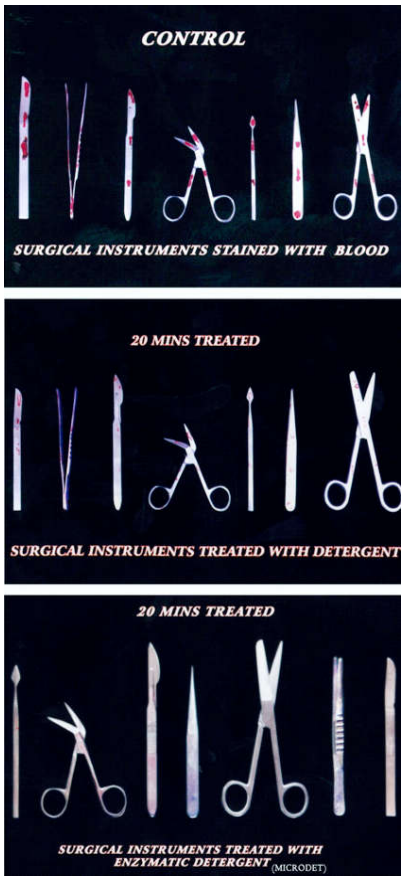


Platte. 1: Zymography of partially purified enzyme

Platte. 3: Microdet detergent



Platte. 2: Evaluation of partially purified enzyme for washing



Platte. 4: Evaluation of washing performance of the enzyme detergent micredet on surgical instrument

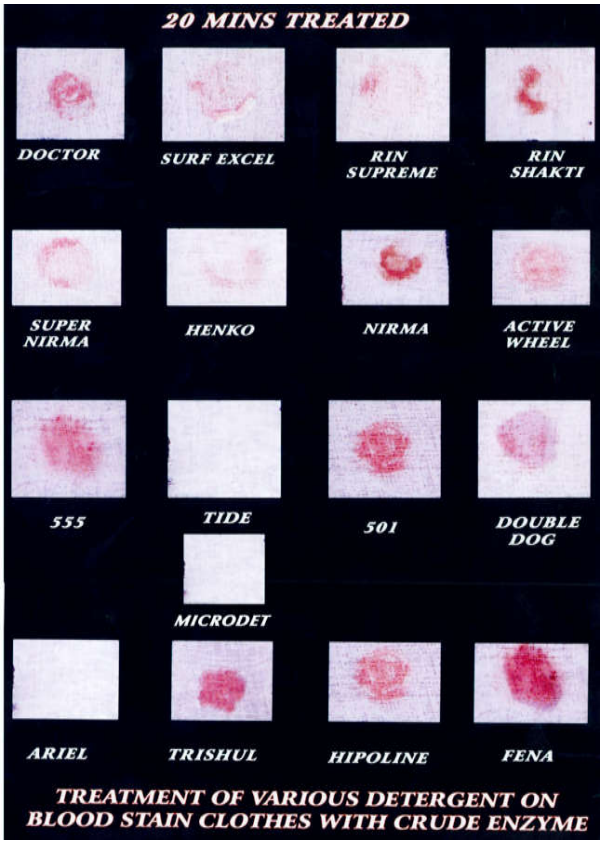


Plate 5: Comparative evaluation of washing performance of microdot with commercial detergent

Discussion

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* KLM1, KLM2, KLM3 and KLM4 Of these KLM4 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. 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* KLM4 for stability to pH and temperature. The results observed on the studies of the effect of temperature on enzyme activity. The maximum enzyme activity was observed at 60°C. Thereafter decrease in the activity was observed. The enzyme in the presence of its substrate was more active below the 60°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°C might be due to the stability effect of its substrate (Ahmed F. Abdel – Fattah 1983) The temperature optima of 60°C was also reported by Adinarayana (2003) for maximum enzyme activity.The results on the effect of pH on the enzyme activity. 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).

Summary and Conclusion

Based on various biochemical properties the protease isolated from *Bacillus cereus* KLM4 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* KLM4 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.

Acknowledgements

This work was supported in Gulbarga University, Gulbarga. Department of studies and Research. Department of Microbiology.

References

1. Astrup, T. and Mullertz, S., The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys. 1952; 40:346-351.
2. Adil Anwar and Mohammed Saleemuddin. Alkaline protease from Spilosoma oblique: Potential application in bio-formulations. Biotechnol. Appl. Biochem. 2000; 31:85-89.
3. Ahmed, F., Abdel-Fattah and Abdel Mohsen S. Ismail. Preparation and properties of fibrinolytic enzymes produced by Cochlinobolus lunatus. Unpublished, accepted for publication. 1983.
4. Beaton, N. C., Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dedecylsulfate and co-polymarized substrate. J. Food Protect, 1997; 42:584-590.
5. Collins, C. H., Patricia, M. Lyne, J. M., Grange Collins and Lyne's. Evaluation of fluorometric and Zymographic methods as activity assay for Stromelysins and gelatinases. Microbiological Methods seventh Edn. 1995.
6. Feng, Y. Y., Yang, W. B., Ong, S. L., Hu, I. Y., Ng, W. J. Fermentation of starch for enhanced alkaline protease production by constructing an alkalophilic Bacillus pumilis strain. Appl. Microbiol. Biotechnol. 2001; 57:153-160.
7. Kamal Kumar, B., Bala Krishnan Bala and Rele. Compatibility of alkaline xylanases from an alkaliphilic Bacillus NCL (87-6-10) with commercial detergents and proteases. Journal of Industrial Microbiology and Biotechnology, 2004; pp. 4-11.
8. Sandeep Kaur, Vohra, R. M., Mukesh Kapoor, Qasim Khalil Beg and Hoondal, G. S. Enhanced production and characterization of a highly thermostable alkaline protease from Bacillus sp. P-2. World Journal of Microbiology and Biotechnology, 2001; 17:125-129.
9. Sangita V. Phadatare, Vasanti V. Deshpande and Mandyam C. Srinivasan, 1993. High activity alkaline protease from Conidiobolus coronatus (NCL 86,8.20): Enzyme production and compatibility with commercial detergents. Enzyme and Microbial Technology, 1993; 15(1):72-76.