2. MATERIALS AND METHODS

2.1 STUDY AREA

In the present study, soil samples were collected from Nanjil milk plant wich is situated in mulagumoodu,kalkulam taluk, kanyakumari district, Tamil nadu,South India (Elevation about 350 meters(mean sea level) (fig 1 and 2). This milk plant is a ISO(9001-2000) certified firm Meticulous milk testing is done at each levels of milk processing. Quality controls including Microbiological lab and product development are done in Nanjl milk plant. Samples were serially diluted using distilled water and spread plated on the surface of casein agar plates (nutrient agar with 1% casein) and incubated at 30^{o}C for 48hrs (Naidu and Devi, 2005).

Composition of the Nutrient agar Casein medium

Ingredients	**g / L**
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Casein	10.00
Agar	15.00
Distilled water	1600ml
Final pH (at 25°C)	7.4±0.2

2.2 Screening for proteolytic activity

The isolated bacterial strains were purified on casein agar plates and they were inoculated into casein broth (i.e.) nutrient agar with 1% casein. After the incubation period culture filtrates were separated by centrifuging at 8000 rpm for 15min. The culture filtrate thus prepared was used for the qualitative protein production assay. Water agar medium of Carrim *et al.,* 2006 (1.8g agar in 100ml of dist. water) supplemented with 1% casein was poured in to petri plates and after solidification 8 mm diameter wells were made using a cork borer. 100µl of culture filtrates were inoculated each one in a separate well and a same volume of uninoculated broth was poured in a separate well as control. After 24 hrs of incubation, enzyme activity was visualized as a clear zone on addition of 1% mercuric chloride solution in 1N HCl. The diameter of zone formed was determined for all the positive strains.

2.3 Identification of the strains

Potential isolates were identified according to Bergey's manual of determinative bacteriology (Buchanan *et al.,* 1974) and were stored on agar slant with casein for further study.

2.4 Optimization

The cultural factors such as inoculm concentration, agitation, pH, temperature, salinity, carbon and nitrogen sources which are expected to affect the production of protease by the selected strain were optimized by selecting one parameter at a time. Unless otherwise mentioned, nutrient broth medium was used throughout the optimization study.

2.4.1 Inoculum concentration

Based on screening the most potent protease production strain was identified as *Bacillus cereus* and it was designated as *B. cereus*. It was inoculated on nutrient agar slant and incubated at 30 ºC. From the slant, a loopful of culture was inoculated into nutrient broth supplemented with 1% casein. This was treated as the pre-inoculum or mother culture. The inoculated, pre- inoculum culture flask was incubated in a rotary shaker at 150 rpm at 30ºC for 18 - 24 hrs. 1-3% of inoculum was tested to determine the optimum concentration.

2.4.2 Static and shaken condition

Effect of agitation on growth and protease production by un-agitated (Static) culture and the culture agitated at different levels viz., 50, 75, 100, 125, 150, 175 and 200 rpm was determined. Relative activity was estimated as mentioned previously.

2.4.3 pH

Different pH ranging from 6-11 (at an interval of pH 1) were maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6hrs until 48 hrs.

2.4.4 Temperature

Different temperatures such as 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 40 ^{o}C and 45^{o}C at an interval of 5^{o}C were tested for growth and enzyme activity and assessed for every 6hrs up to 48 hrs.

2.4.5 NaCl concentration

Different salinity ranging from 0 – 3.5% (at the interval of 0.5%) were maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48 hrs.

2.4.6 Carbon sources

Different carbon sources such as glucose, maltose, Fructose, sucrose and starch were added in the medium in separate tubes at the concentration of 1% and incubated. Growth and enzyme activity were assessed for every 6hrs up to 48hrs.

2.4.7 Concentration of ideal carbon source

Different concentration of glucose as carbon source (0.5 – 2.5%) was maintained in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

2.4.8 Nitrogen sources

Different nitrogen sources such as beef extract, yeast extract, peptone, ammonium nitrate, Ammonium sulphate and potassium nitrate were added in the medium separately at the concentration of 0.5% in the medium and incubated. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

2.4.9 Concentration of ideal nitrogen source

Different concentration of yeast extract as nitrogen source (0.1-1.0%) was maintained in the medium and incubated at 35^{o}C. Growth and enzyme activity were assessed for every 6 hrs up to 48hrs.

2.5 Mass scale culture

2.5.1 Mass scale culture in shake flasks

2.5.1.1 Free cells

Mass scale protease production was done with free *Bacillus cereus* cells (as 1% inoculum) as well as cells immobilized in alginate beads in the protease production medium with optimized parameters such as 150 rpm, 35^{o}C, pH - 10, 0.5% NaCl, 1% glucose, 0.5% yeast extract. Mass scale was done in 2L conical flasks with 1.250L of the medium. Growth and protease activity were evaluation the end of 36hrs incubation.

Mass scale protease production was done with free *Bacillus cereus* cells as well as cells immobilized in alginate beads in the protease production medium with wheat, bran, rice bran, corn straw and molasses at a concentration of 1% optimized parameters taken in separate 2L flasks. Protease activity was assayed at the end of 36hrs of incubation. Growth was also estimated.

2.5.1.2 Immobilized cells

The alginate entrapment of *B. cereus* strain was performed according to the method of Johnsen and Flink (1986). Briefly, 3% sodium alginate solution was prepared under sterile conditions. Both alginate slurry and cell suspension [(0.03 g dry cell weight, (DCW)] were mixed and stirred for 10 min. The resultant slurry was added drop wise into 0.2 M calcium chloride solution with the aid of a syringe of 1.0 mm and left for curing at 4°C with mild stirring at 60 rpm for 1 hr. The beads were then washed for 3 to 4 times with sterile distilled water. When the beads were not used, they were preserved in 0.9% sodium chloride solution at 4°C. All operations were carried out aseptically in a laminar air flow unit.

The immobilized beads, prepared by using alginate along with the control were transferred into 1.250 L of optimized production medium in 2L of flasks. The flasks were incubated at 35 °C for 36 hrs in a shakes incubator kept at 150 rpm. Sample was withdrawn at the end or 36 hrs and estimated for growth protease activity.

2.5.2 Mass scale culture in Fermentor

The parameters optimized for shake flasks were used for mass scale production in a 3L lab scale fermentor and incubated at 150rpm agitation for 30 hrs. Which was decided based on trial runs. DO (Dissolved oxygen) was maintained at 60% using air compressor based on trial runs in the fermentor. At the end of fermentation period, the whole culture broth was centrifuged at 10,000 rpm for 15 min. to remove the cells and the clear supernatant was used as crude enzyme (Olajuyigbe and Ajela, 2005). Fermentor study was done separately for C and N sources as well as for the ideal cheaper substrate (i.e) 1% wheat bran. Growth was estimated spectrophotometrically.

2.6 Purification of protease

The protease from *Bacillus* sp. was purified as per the standard protein purification procedures which involves various steps such as centrifugation, ammonium sulphate precipitation, and ion exchange chromatography. Protease obtained through mass scale production in fermentor using selected C and N sources was used for this purpose.

2.6.1 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a convenient and effective method because of its high solubility, cheapness, lack of toxicity to most of the enzymes (Dixon and Webb, 1979). It forms dense solution enhancing protein collection, which may be precipitated by centrifugation.

Procedure

The aliquots of the extract was taken and pre-chilled at 4°C. The amount of ammonium sulphate required to give different saturation levels (40-60%) as per standard chart was added slowly to each aliquote while stirring (using a magnetic stirrer) (Green and Hughes, 1955). The aliquote stirried for overnight at 4°C and was centrifuged at 10,000 rpm for 30 min. The supernatant was removed and the precipitate was dissolved in buffer (e.g. 50mM Tris - HCl pH-8-10). The enzyme solution thus obtained was dialyzed in a dialysis membrane No.150 (Himedia) against the same buffer for 24 hrs with several intermittent buffer changes. The supernatant was assayed for total protein and enzyme activity. Based on the result 60% ammonium sulphate was used for the precipitation of mass scale culture filtrate.

2.6.2 DEAE-cellulose Ion exchange chromatography

DEAE-cellulose was purchased from Sigma and activated as per manufacturer's instructions. The resin was packed into a C 10/20 column **(**AKTA prime, Amersham). Care was taken to avoid trapping of air bubbles. All the buffers used were filtered and degassed before each run. The column was pre-equilibrated with the 20 mM Tris-Cl buffer, pH 8.5. One ml of the enzyme sample was loaded onto the pre-equlibrated column. The column was then washed with the same buffer

(20 mM Tris-Cl buffer, pH 8.5) to remove the unbound proteins (indicated by zero absorbance at 280 nm). The bound protein was eluted by applying a linear gradient of 0-0.8 M NaCl in the same buffer at a flow rate of 0.5 ml/min. and monitored at 280 nm. 1 ml fractions were collected and were analyzed for protease activity. The active fractions were pooled, assayed for protease activity and protein content and used for further studies on characterization (Orhan *et al.,* 2005).

2.7 Characterisation

2.7.1 Determination of molecular weight of the enzyme by SDS-PAGE-(Laemmli, 1970)

Introduction

Sodium dodecyl polyacrrylamide gel electrophoresis (SDA-PAGE) is probably the most widely used technique for analysing mixtures of proteins with a high resolving power. With SDS, an ionic detergent, proteins loose their individual charges and a net negative charge is formed due to complexing of SDS with protein. The update of detergent is the same per unit mass (1.4g SDS per gm of protein) for all proteins and hence the mobility on electrophoresis is proportional to the molecular mass. Depending upon the size of the proteins to be separated, the concentration of acrylamide is selected to optimize the sieving effect. In addition, a stacking gel of low acrylamide concentration allows rapid movement of sample to the top of separating gel in the form of narrow zone of proteins. In this method the proteins are separated by SDS-PAGE electrophoresis and size of polypeptide chains of given protein is determined by comparing its electrophoresis mobility in SDS-PAGE gel with standard marker proteins of known molecular weight.

Gel Casting

Chemistry involved in gelling

Polyacrylamide gel results from the polymerization of acrylamide monomers into long chains and cross linkages are brought by N-N-methylene bisacrylamide. Polymerization of acrylamide is initiated by the addition of either ammonium per sulphate or riboflavin. Tetra methylene diamine (TEMED N-N-N-N) acts as an accelerator of polymerization. Effective pore size of polyacrylamide gel is greatly influenced by the total acrylamide concentration in the polymerization mixture. Buffer system in PAGE is designed in such a way that the protein is separated into individual polypeptide.

To study the homogeneity of the proteins, polyacrylamide gel electrophoresis was carried out for the separation of protein according to their electric charges. Most commonly the strong anionic detergent SDS is used in combination with a reducing agent and heated to disassociate the proteins before they are loaded on the gel. The amount of SDS bound is always proportional to the molecular weight of the poly peptides and is independent of their sequence. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (i.e.) SDS-PAGE is an excellent tool to identify and monitor proteins during purification and to access the homogeneity of the purified proteins.

Sample preparation and electrophoresis

The samples were mixed with equal amount of sample buffer and were boiled in a boiling water bath for 5 min. After that, the samples were loaded into the wells and allowed for electrophoresis at 50V initially. After the dye front had reached the

end of the stacking gel, the voltage was increased to 100V and proteins were allowed to migrate through resolving gel.

Procedure

The casting apparatus was assembled and the gel volume was determined using distilled water. Then the solution for the resolving gel of 15% concentration was prepared. The solution was poured into the gel plate and overlaid it with a layer of butanol: water (1:1) to prevent exposure of the gel to air, allowed to solidify and then the solution for the stacking gel was prepared. The butanol layer was removed and washed twice or thrice to remove traces of butanol. The components of the stacking gel (5%) were added; the gel solution was poured over the separating gel and allowed to solidify after placing the comb.

Sample loading

Protein marker and samples were loaded into the wells of the stacking gel. The electrophoresis buffer was added in to the top and bottom reservoir. The samples were electrophoresed at 100V until the tracking reached 0.5cm from the bottom of the plate. Power supply was turned off and the gel was kept in the staining solution.

Detection of proteins

Protein detection was done in many ways. Widely used method is Coomassie brilliant blue staining.

Coomassie brilliant blue staining

This is the standard method of protein detection. Easy visibility requires the order of 0.1-1mg of protein per band. After electrophoresis, the gel assembly was

removed and the glass plates were separated. The gel was immersed in 100 ml of coomassie brilliant blue staining solution (Merril, 1990) overnight with continuous shaking and then the gel was destained with the destaining solution. The protein bands were observed in the Gel-documentation system (DGelDAS, biotech, Yercaud).

2.7.2 Zymogram analysis

Casein zymography was carried out according to the method described by Kim *et al.,* 1998 with slight modifications. Casein (0.12% w/v) was dissolved in 20 mM Tris-Cl buffer (pH 8.5) and copolymerized with 10 % resolving gel. Samples were prepared by diluting the enzyme in zymogram buffer (0.125 M Tris-Cl, 2% SDS, 10% glycerol and 0.02% bromophenol blue, pH-6.8). The samples were then loaded into wells and electrophoresed at a constant current of 12 mA at 4ºC. After electrophoresis, the gel was incubated for 30 min. at room temperature in reactivation buffer (100 mM Tris-Cl buffer, pH 9) containing 2.5% (v/v) Triton X-100. The gel was then washed with distilled water to remove Triton X-100, incubated in reaction buffer (Tris-Cl, pH 9) for 30 min. at 37ºC, stained with Coomassie Brilliant Blue for 30 min. and destained as described above. The protease activity was detected as a clear colourless zone against dark blue background.

2.8 Stability studies

2.8.1 Effect of pH on protease stability

The effect of pH on protease activity was evaluated over a pH range 6-11, using different buffers such as sodium phosphate 0.1 M (pH 6-7), 0.1 M Tris- Cl (pH 8-10) and 0.1 M glycine NaOH (pH 11-12) in the reaction mixture. Sample was pre-inoculated for 3 hrs and the activity was expressed in terms of relative activity calculated as mentioned previously.

Note: Stability tests were done with purified enzyme.

2.8.2 Effect of temperature on protease stability

The temperature stability of the enzyme was determined by preincubating the enzyme at different temperatures (30-80ºC) for 3 hrs and then assaying the relative activity (%) under the standard assay conditions. Relative activity was estimated as mentioned previously.

2.8.3 Effect of NaCl concentration on protease stability

The effect of NaCl concentration on enzyme activity was assessed by carrying out the assay at different NaCl concentration ranging from 0-3% and incubated at 60^{o}C for 3 hrs. The percentage relative activity was calculated considering the maximum activity as 100%.

2.8.4 Effect of metal ions on protease stability

The influence of various metal ions on the purified enzyme was studied by incubating the enzyme in the presence of various metal ions $ZnCl_2$, $MnCl_2$, $CaCl_2$, $MgCl_2$, $CuSO_4$, $HgCl_2$, $AgNO_3$, $FeCl_2$, $CdCl_2$, and $NiCl_2$ at a final concentration of 1mM and 10mM at 60ºC for 1hr. The percentage relative activity was calculated by considering the activity of enzyme (in absence of metal ions) at 60ºC and pH-10 as 100% activity.

2.8.5 Effect of specific inhibitors on protease stability

Regarding protease inhibitors 5 mM concentration of PMSF (Phenyl methyl sulphonyl fluoride), DFP (diiosoproppyl fluorophosphate), EDTA (ethylene - diamine tetraacetic acid), and 1,10 phenanthroline were pre incubated with purified enzyme for 1hr. at 60^{o}C and enzyme assay was done to find the extend of inhibition.

2.8.6 Effect of oxidizing agent and surfactants on protease stability

To investigate the effect of oxidizing agent (H_2O_2) and surfactants (SDS and Tween-80) on the enzyme stability, the purified protease was pre-incubated with different concentrations of H_2O_2, SDS and Tween-80 (1 and 5%) for 3hrs. and then their activity was measured by standard procedure as mentioned earlier.

2.8.7 Effect of organic solvents on protease stability

The effect of various organic solvents such as ethanol, acetone, methanol and chloroform on purified protease activity was tested. The reaction mixture was prepared by pre incubating the purified enzyme with each solvents was added to 3 ml of purified protease solution and incubated at 60^{o}C for 3 hrs (Wan *et al.*, 2010). Different concentrations of the solvent from 5 to 25% at an interval of 5% were evaluated.

2.9 APPLICATIONS

2.9.1 Antimicrobial activities of purified protease of *B. cereus* DF 101

2.9.1.1 Antibacterial activity

Bacterial human pathogens viz., *E. coli* (ETEC)*, L. monocytogenes, S. aureus* (MRSA)*, S. typhi, S. paratyphi, P. aeruginosa, V. cholerae* (O1)*, K. pneumoniae, K. oxytoca* and *P. mirabilis* were obtained from CMC, Vellore and NICED, Calcutta. Only human pathogenic strains, with high virulence potency were used in the present study.

Bacterial inoculum preparation

Bacterial inoculum was prepared by inoculating a loopful of test organisms in 5ml of nutrient broth and incubated at 37ºC for 3-5 hrs till a moderate turbidity was developed. The turbidity was matched with 0.5 McFarland standards and then used for the determination of antibacterial activity.

Well diffusion method

To test the antibacterial activity, well diffusion method was used. Sterile Muller Hinton agar plates were prepared and the inoculum of test microorganisms given above was spread uniformly in each plate as pure culture. Wells were made by using a sterile borer having a diameter of 6mm. About 50µl of enzyme solution (250U) was loaded in the well. The plates were kept at 4^{o}C for 1 hr for the diffusion of the test solution and then placed in the incubator for 24hrs at 37^{o}C. Each test was done in triplicates. Inhibition of bacterial growth was evaluated from the zone of inhibition formed.

Composition of Nutrient broth

Ingredients	**g / L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Distilled water	1000ml
pH	7.4±0.2

Composition of Mueller Hinton Agar

Ingredients	**g / L**
Beef, infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Distilled water	1000 ml
pH	7.3±0.1

2.9.1.2 Antifungal Activity

In the present study *Candida albicans* was the only human pathogen used. However five different fungal plant pathogen were evaluated. The fungal plant pathogen cultures were collected from CAS Botany, University of Madras, Chennai and Tamil Nadu Agricultural University, (TNAU), Coimbatore. The test fungal isolates were sub-cultured and maintained on Potato dextrose agar slants and stored in a refrigerator at 4ºC, until use.

Colletotrichum musae, Aspergillus niger, Alternaria alternata, Rhizoctonia solani, were the phyto pathogens used in the present study.

Note: Antifungal activity was tested in Potato dextrose agar.

Composition of Potato Dextrose Agar

Ingredients	**g/ L**
Potato Infusion from	200.0
Dextrose	20.0
Agar	15.0

Distilled water	1000 ml
pH	5.6 ± 0.2

2.9.2 Detergent additive

2.9.2.1 Wash performance

Human blood was used to stain the cotton fabric and it was air dried. Purified protease was used to check the wash performance as crude enzyme may contain other ingredients which may influence.

The stained cloth pieces were taken in separate flasks. In 3 flasks, the treatment was given in the following combinations.

1. 100 ml detergent solution (7mg/ml) + stained cloth piece
2. 100 ml detergent solution (7mg/ml) + 1000µl of 100U enzyme solution + stained cloth piece
3. 100 ml of tap water + 1000 µl of 100U of enzyme solution + stained cloth piece

They were incubated in a shaker incubator at 60^{o}C for 30 min. Later the cotton fabric was taken out, rinsed in tap water, dried and examined.

2.9.2.2 Compatibility with commercial detergents

The suitability of the protease as a detergent additive was determined by testing its stability in the presence of commercial detergents including surf excel,ariel, tide, and power. Detergents were diluted in sterile tap water to give a final concentration of 7 mg/ml to simulate washing conditions. The partially purified enzyme was then added to different detergent solutions and incubated at 60°C for 3 hrs. The enzyme without adding any detergent and incubated under the same

conditions was taken as the control. Enzyme activity was measured at 60°C, pH 10.0 and compared with the control.

2.9.3 Protein recovery from bone wastes

The effect of protease enzyme on protein recovery was done by incubating the smashed bones of goat, chicken and fish. Repeatedly washed goat (1g), chicken(1g) and fish(0.5g) bones were incubated in 20ml of buffer (50mM Tris Hcl pH 8.0) at 60ºC with 50U/ml of enzyme for 2 hrs. Control (i.e) set without enzyme (Bone+ buffer) and with enzyme (Bone+ enzyme + buffer) were used. The protein release from bones was observed by determining the protein in the samples withdrawn from the reaction mixture at the end of 2hrs. The protein concentration was estimated according to the method of Lowry *et al.,* 1951.