ISOLATION OF PROTEASE PRODUCING MICROORGANISM FROM FOOD WASTE

Yasser Hussein Issa Mohammed

Department Of Biol-chemistry, Hajja University, Hajja, Yemen

Email: issayasser16@gmail.com

(Received **on Date: 6th September 2015** **Date of Acceptance: 14th October 2015**)

ABSTRACT

Proteases are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields. Proteases are also known as peptidyl –peptide hydrolases and are industrially useful enzymes which catalyze the hydrolysis of peptide bond from protein molecule. Proteases constitute 0-6%of the global industrial enzyme market, most of which are alkaline protease. They are commercially important and isolated from various living sources such as plants, animals, bacteria and fungi. Isolation and screening of protease producing bacteria five strains were isolated from food waste samples collected mess yard, hotel and canteen. strains were selected on the basis of zone of hydrolysis they exhibited on the agar plates and agar plate The selected strains were inoculated on different sterilized nutrient media and incubated at 37°C to get a standard colony. Substrate two types of wastes to be used as substrate food waste samples were collected from different habitats food waste areas. One gram of food waste sample was added to a glass tube containing 10 mL sterilized distilled water. The sample was serially diluted and streaked on agar plates. The plates were incubated for 24 h at 37°C and enzyme activity was observed. Positive colonies those degraded skim milk was studied for protease production. Isolation and Screening: 1 gm of waste was suspended in 10 ml of sterile water and serialdilution10-7 was selected and streaked on nutrient agar plate and incubated for 48 hrs at 37°C. Colonies were randomly selected and streaked on Skimmed Milk Agar plate to isolate protease producing bacteria. Two colonies produced clear zone on SMA plate and colony having higher zone was selected for experiment. Two screening broth such as: casein-yeast extract-lactose-mineral salt broth (casein-1.2%, yeast extract-0. %, K2HPO4-0.0%,CaCl2-0.0% , MgSO4-0.0% ,Lactose- 0.0%) and tryptone-yeast extract-dextrose broth (tryptone-1%, dextrose-0.1%, yeast extract-0.%) were used for final selection.

Keyword: Proteases, food waste, casein-yeast, metalloproteases, Zymography.

INTRODUCTION

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient conditions. One group of the enzymes that has been studied extensively is proteases or proteolytic enzymes due to their wide variety of application in various fields. Protease (peptidase or proteinase) is an enzyme that hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases differ in their ability to hydrolyze various peptide bonds. Hence, specificity associated with each enzyme differs based on catalytic site. Proteases are widely distributed in all living organisms: in plants (papaya,pineapple)in animals and mainly in microbes(bacteria, fungi and viruses). Proteases are involved in regulating practically every aspect of plant life cycles. Plant proteases are involved in seed germination, recycling damaged plant proteins, regulating aging processes in plants and modifying proteins to perform specific purposes in plant cells. Animal proteases play a very vital role in the metabolism of the organism. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Protease production is an inherent capacity of all microorganisms and a large number of microbes belonging to bacteria, fungi, yeast and actinomycete are known to produce proteases. Due to processing almost all the characteristics desired for their biotechnological applications, microbial proteases are preferred to the plant. Numerous proteinases are produced by microorganisms depending on the species of the producers or the strains even belonging to the same species. Several proteinases are also produced by the same strain under various cultural conditions. From economical point of view microbial proteases are the commercial enzymes. Microbial proteases account for approximately 40% of the total worldwide sales of enzymes. Proteases constitute one of the most important groups of industrial enzymes, accounting for more than 65% of the industrial enzyme market [1,2,7] of these; alkaline protease accounts for 30% of the world's total enzyme production and applications in detergent formulations alone make up to 89% of total sales. Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes. The vast diversity of proteases, in contrast to the specificity of their action has attracted

worldwide attention focused on exploiting their physiological and biotechnological applications [2,12,18]. Proteases are also having extensive applications in the development of environmental friendly technologies as well as in several bioremediation processes. Several classification systems currently available, provides rich and vast information about each and every identified protease. These schemes can be categorized under 4 major categories based on the characteristic features like: pH (acidic/neutral/ alkaline), peptide bond specificity (endo/exo peptidases) and functional group present at active site (serine / cysteine / aspartic / metalloproteases) of proteolytic activity [3,14]. Most commercially available proteases belong to the class serine proteases, produced by organisms belonging to the genus *Bacillus, Aspergillus* and *Streptomyces*. Alkaline proteases are more preferable at industrial scale compared other acidic proteases. Metalloproteases are those enzymes whose catalytic mechanism involves a metal which plays an important role in pathogenesis hence have advantage in health care sector. A Metallo protease These are proteolytic enzymes whose catalytic mechanism involves a metal. The enzymes typically require an essential metal ion (Ni^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+} and Co^{2+}) for functional activity [4]. Most metalloproteases are zinc-dependent, but some use cobalt. The metal ion is coordinated to the protein via three ligands. The ligands coordinating the metal ion can vary with histidine, glutamate, aspartate, lysine and arginine. The fourth coordination position is taken up by a labile water molecule. The introduction of metal-binding sites into proteins could induce specific and predictable conformational changes as well as allow the regulation of enzymatic activity.

The first "consensus sequence" for members of the metalloprotease family was based on homology found within the human fibroblast collagenase and the 11 amino acids flanking the zinc-binding site of the serratia protease, a bacterial metalloprotease that also shares strong homology with thermolysin at this site. Later, the primary sequence motif HEXXH was found in many zinc-containing proteases, including several eukaryotic zinc metalloproteases, and was suggested to be indicative of membership in the family [5,8,15].

Table 1: Examples of metalloproteases using specific metal ions

Metal ion	Example of metalloprotease
Zn^{2+}	Glutamate carboxypeptidase and glutamylaminopeptidase, Collagenase and

	Endorphins
Mg^{2+}	Inorganic diphosphatase,Phosophoadenylylsulphatase
Co^{2+}	Methylmalonyl coenzyme A mutase,methionine aminopeptidase,nitrile hydratase
Ni^{2+}	Urease
Ca^{2+}	Lactonase

Table 2: Examples of organisms with metalloproteases produced by them:

Metalloprotease Name	**Organism**
Thermolysin	*Bacillus thermoproteolyticus*
Elastase	*Pseudomonas aeruginosa*
Lysostaphin	*Staphylococcus* sp
Acidolysin	*Clostridium acetobutylicum*
Neutral metalloprotease	*Bacillus* sp
Vimelysin	*Vibrio* sp
Collagenase	*Clostridium histolyticum*
Immunoglobulin A (IgA) protease	*Streptococcus sanguis*
Metalloproteases A,B and C	*Erwinia chrysanthemi*

MATERIALS AND METHODS

Seed medium

25 ml of Nutrient broth was prepared in conical flask. Culture was taken from the slant and inoculated. The medium was incubated at 30°C for 18-24 h till the optical density of the medium reaches 0.8

at 600 nm. This culture was used for inoculation of the production medium.

Production medium and culture conditions

The medium used for enzyme production was YPD medium consisting (%, g/100ml) of Yeast extract – 1.0, Peptone – 1.0, Dextrose –1.0, $MgSO_4$ - 0.02, KH_2PO_4 - 0.05, NaCl - 0.25, pH 7.0 was used. Seed medium was used as the inoculum .1% inoculum was added to the production medium and incubated at 30°C for 2 days. The sample was collected, centrifuged at 10000 rpm for 10 minutes and the supernatant was used for the protease assay.

Analytical Methods:

Protease assay

Protease activity was assayed according to Anson method and was slightly modified (24). The reaction mixture contained 2.5 ml of 0.65% Hammerstein casein and 0.5 ml of appropriately diluted enzyme. Enzyme was diluted in 50 mM Glycine NaOH buffer pH 9.0. The reactants were incubated at 37°C for 10 min and the reaction was stopped by adding 2.5 ml of 110 mM trichloroacetic acid (TCA). A suitable blank was run simultaneously, in which 2.5 ml of TCA was added to the 0.5ml of diluted enzyme solution, followed by Casein substrate addition after incubation for 10 min. After incubation at room temperature for 30min both test and blank solutions were centrifuged at 10,000g for 10min. To the 0.4 ml supernatant, 1.0ml 50mM Na_2CO_3 and 0.2ml Folin-ciocalteau's reagent was added, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 660nm [2,6,21].

Protein Determination

The total protein content of the samples was determined according to the method described by Lowry et al., (1951). The protein assay mixture consisted of 200 µl of diluted extract of the enzyme, 1 ml of alkaline copper solution(2%Na_2CO_3 in 0.1 N NaOH:1.56% $CuSO_4$:2.37% Potassium sodium tartarate in ratio 100:1:1).The contents in tube were vortexed and incubated for 10 min. Then 200µl of freshly prepared diluted Folin-ciocalteau reagent (1:2) was added and mixed and kept in dark at room temperature for 30 min. Blue colour was developed and OD was measured at 600nm.Simultaneously a blank was set with 200µl distilled water,1ml alkaline copper solution and 100µl of Folinciocalteau reagent. The protein concentration in the reaction was determined based on the standard curve obtained with bovine serum albumin as standard

Dry cell weight

For the calculation of dry cell weight: Initial weight of the eppendorf was measured. One ml of culture was taken in the eppendorf and culture was centrifuged at 10,000rpm for 10 min at 4°C.The supernatant was discarded leaving the pellet. The pellet was then dried until there is no more moisture left. The weight of the

eppendorf with pellet was noted. The initial weight of the eppendorf was subtracted from the weight obtained to get dry cell weight.

Unit definition

One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions. Enzyme units were measured using slope obtained from tyrosine (0–1000 µg/ml) as standard.

Optimization of nutritional parameters for the bacterial growth and the protease production by isolated strain:

Effect of Carbon sources

The medium was prepared with composition same as YPD but the carbon source is replaced by different other carbon sources. The composition consists of Yeast extract – 1.0%, Peptone – 1.0%, $MgSO_4$ - 0.02%, KH_2PO_4 - 0.05%, NaCl -0.25% and carbon source-1%. The various carbon sources used include dextrose, fructose, galactose and maltose. The medium was prepared autoclaved and inoculated with seed medium containing the culture. The flasks were incubated at 30°C and 150rpm.The samples were collected for every 3h upto 72h.The optical density of the samples was measured at 600nm.The samples were then centrifuged at 10000rpm for 10 min and the supernatant was used as sample for protease assay to calculate the units of enzyme produced. The results were tabulated and graph was plotted.

Effect of Nitrogen sources

The medium was prepared with the composition: Nitrogen sources-2.0%, $MgSO_4$ - 0.02%, KH_2PO_4 - 0.05%, NaCl -0.25% and Dextrose-1%. Different nitrogen sources as Beef extract, chitin, casitone, malt extract, meat extract, peptone, tryptone, yeast extract, casein, skim milk, soyabean meal were used. The medium was autoclaved and inoculated with 1% inoculum (seed medium containing the culture). The flasks were incubated at 30°C and 150rpm. The samples were collected for every 24h upto 72h. The optical density of the samples was measured at 600 nm. The samples were centrifuged at 10000 rpm for 10 min and supernatant was used as sample for protease assay. The results were noted and observed. The four nitrogen sources that helped in producing more number of enzyme units were selected. The experiment was repeated those 4 Nitrogen sources but the samples were collected for every 3 h upto 72h. The protease assays were carried out with the supernatant of the samples. The results were tabulated and graph was plotted.

Purification of protease produced from *Serratia* sp

Enzyme production for purification

The production of protease was carried in the medium that has been statistically optimized. The medium composition is

Dextrose -0.5%, Casein-2%, KH_2PO_4-0.06%, $CaCl_2$-0.004%, $MgSO_4$-0.02%, NaCl-0.25%. A 1% inoculum was added from the seed medium. The flasks were incubated in a shaking incubator at 30^0C and 150 rpm. After 48hrs of fermentation the cells were separated from culture broth by centrifugation at 10000rpm for 10 min at 4^0C.

Ammonium Sulphate precipitation and Dialysis

The supernatant obtained after the centrifugation was collected in a flask and finely powdered Ammonium sulphate (20-80%) was added slowly to the clear supernatant with constant stirring for 2 hrs. The solution was centrifuged at 10000 rpm for 10 min at 4^0C and the supernatant and pellet was collected. The pellet was dissolved in 10ml of Glycine NaOH buffer pH 7.2. Then the supernatant and the pellet dissolved in buffer were used as samples for protease and protein estimations. The pellet dissolved in the Glycine NaOH buffer was put in dialysis membrane and packed with clips at the two ends and then dialyzed against the same buffer. The protease activity and the protein content were estimated for the dialyzed sample. The dialyzed sample was used for the further purification.

Gel filtration chromatography

Gel filtration was performed with water-cooled (5° C) columns of Sephadex G-75 equilibrated with 0.1 M phosphate buffer (pH 7.2). The dialyzed enzyme preparation was applied to the columns and eluted, in the upward flow mode, at a flow rate of 30ml per hour with the equilibrating buffer. Fractions (2 ml.) were collected and assayed for protease activity and protein was estimated using lowrys method at 600 nm. The unadsorbed protein fraction was eluted with the same buffer. The active fractions were collected and used for further purification.

SDS-PAGE

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed for determination of purity and molecular weight of the enzyme. SDS-PAGE was performed by using a BioRad Mini-Protean II gel apparatus. Samples of protein preparations and molecular mass standards (BioRad, Promega) were combined with sample buffer containing 1ml of 0.5M TRIS/HCl pH 6.8, 0.8ml glycerol (v/v), 1.6ml 10% (w/v) SDS, 0.4ml ß-mercaptoethanol (v/v), and 0.2ml 0.5% (w/v) bromophenol blue and heated at 100 °C for 5 min. The samples were cooled to 25 °C and loaded onto 10% (w/v), 0.75-mm-thick gels and electrophoresed. The molecular mass of purified protein samples was determined by comparing its mobility with those of various protein standards.

Zymography with casein

Casein zymography was performed using 10% polyacrylamide slab gels containing SDS and casein receipe (15mg/ml) in separating gel. The protease containing sample was applied to the gel in standard

SDS loading buffer containing 0.1% SDS but lacking 2-mercaptoethanol; it was not boiled before loading. After electrophoresis, the gels were soaked thrice for 20 min in 2.5% (v/v) Triton X-100 at room temperature to remove the SDS. The gels were stained with 0.1% Coomassie brilliant blue R-250 in methanol–glacial acetic acid–water (40:10:60) followed by destaining with methanol–glacial acetic acid–water (40:10:60). Enzyme activity was visualized by incubating the gel for 12 h in 50 mM Glycine–NaOH buffer at pH 11.0 at room temperature.

Characterization of protease enzyme

The properties of extra cellular protease produced by *Serratia* sp RSPB11 referred as protease were studied and the enzyme was characterized. All the experiments were done in triplicate.

Effect of pH on enzyme activity

To study the effect of pH on enzyme activity the caseinolysis in the buffers of different pHs was studied. The buffers used were Citrate buffer (pH 4.0-6.0), Sodium Phosphate buffer (7.0-8.0) Glycine - NaOH (pH 9.0-12.0).0.65% casein was dissolved in buffers with different pH ranging from 4.0-12.0.

Effect of pH on enzyme stability

For knowing the pH stability, the casein and enzyme containing buffers were incubated at 37°C for 1 h, 2 h and 3 h. The residual enzyme activity was determined using standard assay conditions. The enzyme activity of the sample was calculated before the incubation and considered as control.

Effect of Temperature on enzyme activity

The effect of temperature on the enzyme activity was studied by dissolving 0.65% casein in Glycine NaOH buffer pH 9.0.This casein solution is distributed into different tubes. For studying the effect of temperature on the enzyme, tubes were incubated at various temperatures ranging from 28 – 70°C for 10 minutes time intervals. The residual activity of the enzyme was determined and the tube that was incubated at 37°C was considered as control and relative activity is calculated with respect to it i.e., considering the activity at 37°C to be 100%.

Thermo stability of the enzyme

The reaction mixture with 0.65% casein dissolved in Glycine NaOH buffer, pH 9.0 and enzyme are incubated at different temperatures for period of 15 min, 30 min and 60 min. Then the assay was performed using the standard assay conditions to calculate the residual enzyme activity. The enzyme activity was also calculated before the incubation and that is taken as control.

RESULTS AND DISCUSSIONS

Isolation and screening of protease producing strain:

Based on the larger hydrolytic zones on chitin, casein agar plates and protease assay, one isolate designated as RSPB11 was selected for further studies.

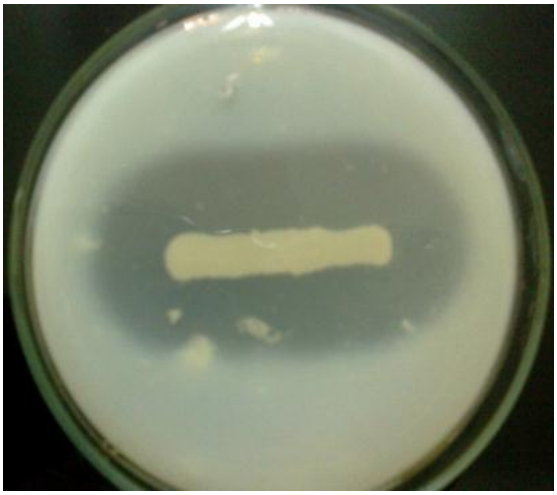


Fig 1: Petri plate showing the quadrant streaking of the isolate isolate Fig 2: Petriplate showing the organisms ability to hydrolyse casein

Multi enzyme complex

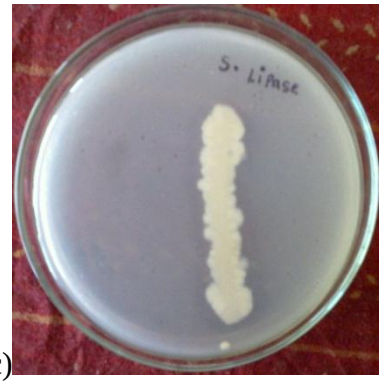
This strain has potential to hydrolyze chitin, gelatin, tributyrin and casein revealing its industrial potential for production of multi enzyme complex

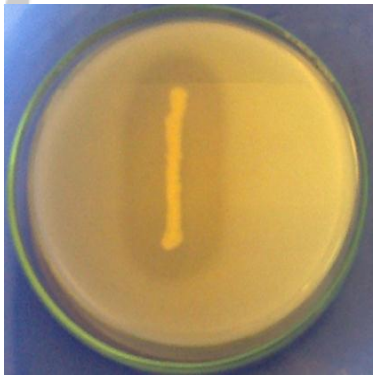
.

Petri plates showing the ability of the organism to hydrolyses different substrates a) skim milk b) casein c) tributyrin d) chitin

a) 

b) 

c) 

d) 

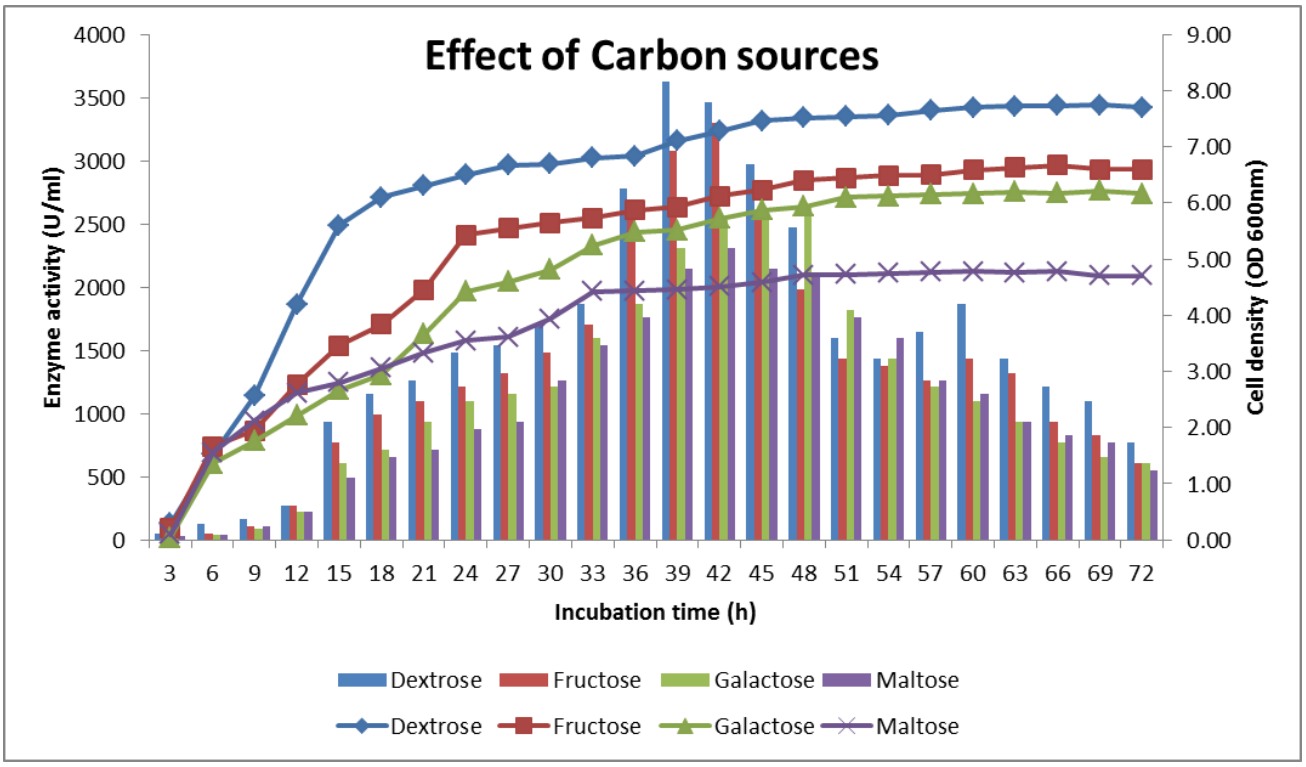
Optimization of culture conditions for the bacterial growth and the protease production by isolated strain:

Effect of carbon sources

To analyze the effect of the carbon sources on the growth and protease enzyme production a set of flasks with different carbon sources were taken. Protease production after 48h was estimated for each carbon source such as Arabinose (3730 U/ml), Cellulose (3900 U/ml), Chitin (3350 U/ml), Dextrose (4690 U/ml), Fructose (3975 U/ml), Galactose (4240 U/ml), Maltose (4400 U/ml), Mannose(3755 U/ml) , Starch (3055 U/ml), Sucrose (3265 U/ml) and Xylose (3870 U/ml). Different carbon sources have different effects on extracellular protease production based on its utilization by the particular microbe. Based on the results

dextrose, fructose, galactose and maltose were selected and further studied by sampling for every 3h. Dextrose at 1% level could support the protease production, where an enzyme activity of 5465 U/ml has been detected (fig 6), followed by fructose (5300 U/ml), maltose (4640 U/ml) and galactose (4310 U/ml) after 42h.Increased alkaline protease production was also reported by several other studies that used different sugars such as Sorbitol, Starch [21], Lactose (raja 2005) sucrose [32] and maltose [23] indicating that the best carbon source for enzyme production is different according to different microbes.

Fig 4: Effect of carbon sources on the growth and production of protease

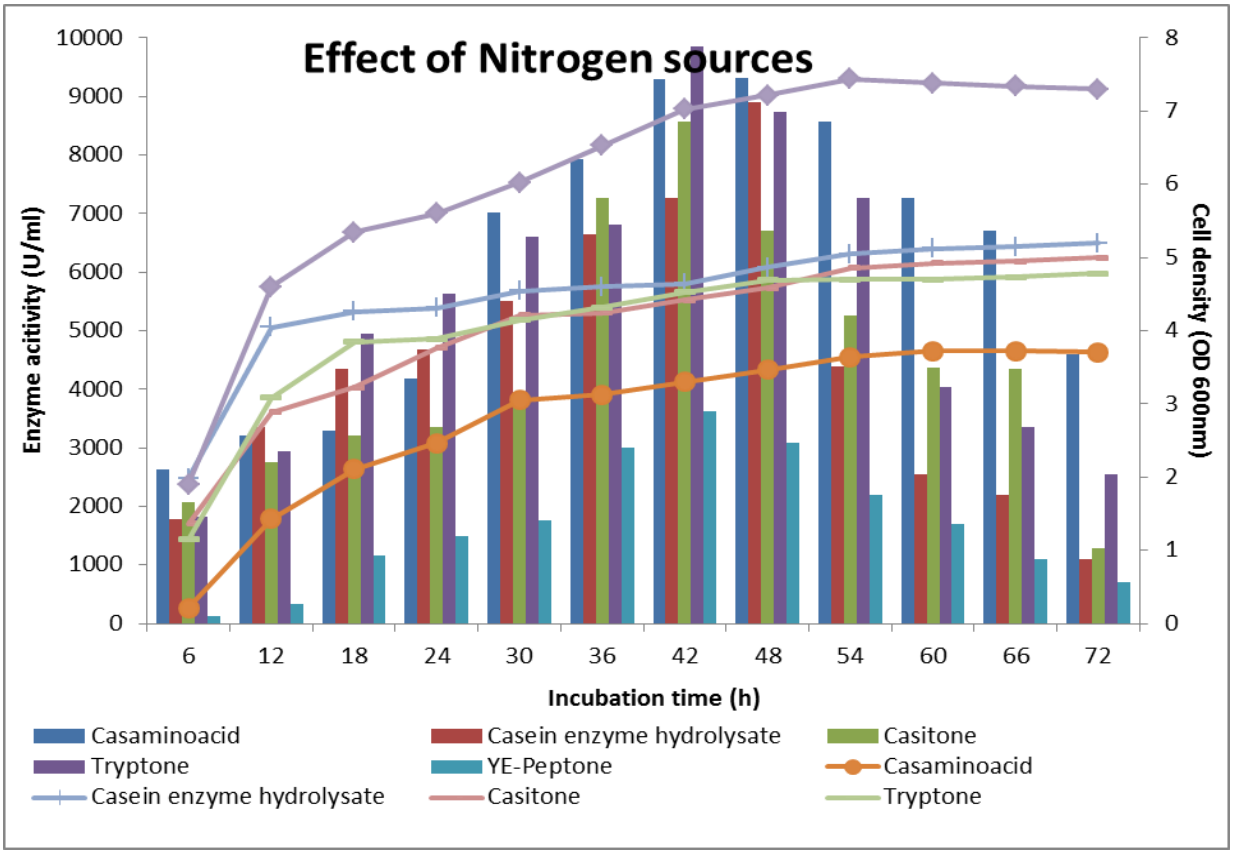


Effect of Nitrogen sources.

Nitrogen sources play a very important role in the metabolism of the cell and enzyme production. Alkaline protease production depends heavily on the availability of nitrogen sources in the medium, which has regulatory effects on enzyme synthesis. Although complex nitrogen sources were usually needed for proteases production, the requirement for a specific organic nitrogen supplement differs from organism to organism [25]. Various nitrogen sources as beef extract, chitin, casitone, malt extract, meat extract, peptone, tryptone, yeast extract, casein, skim milk, soyabean meal were initially used to check their effect on the production of the enzyme and it was found that they didn't have any regulatory effect. Then the high protein containing organic nitrogen sources Casaminoacids, Casein enzyme

hydrolysate, Casitone and Tryptone were selected for the optimization. It was observed from the study that there was no correlation between the growth of the organism and protease enzyme production indicating that protease enzyme production is non-growth associated. Similar effect was shown for *Bacillus sp.*[31] and other marine isolate [7] where growth was best supported by a combination of peptone and yeast extract, while the optimum protease production was with casaminoacid. The biomass yield was high but the enzyme production was low when a combination of yeast extract and peptone was used. Tryptone gave a maximum production of 9845 U/ml followed by Casaminoacids (9320 U/ml), Casein enzyme hydrolysate (8910 U/ml), Casitone (6710 U/ml) and Yeast extract-Peptone (5005 U/ml) after 48h.Tryptone was the best nitrogen source for protease production by *Serratia* sp. [35].

Fig 5: Effect of nitrogen sources on the growth and productionprotease



Purification of the protease enzyme

Ammonium sulphate precipitation method and Dialysis

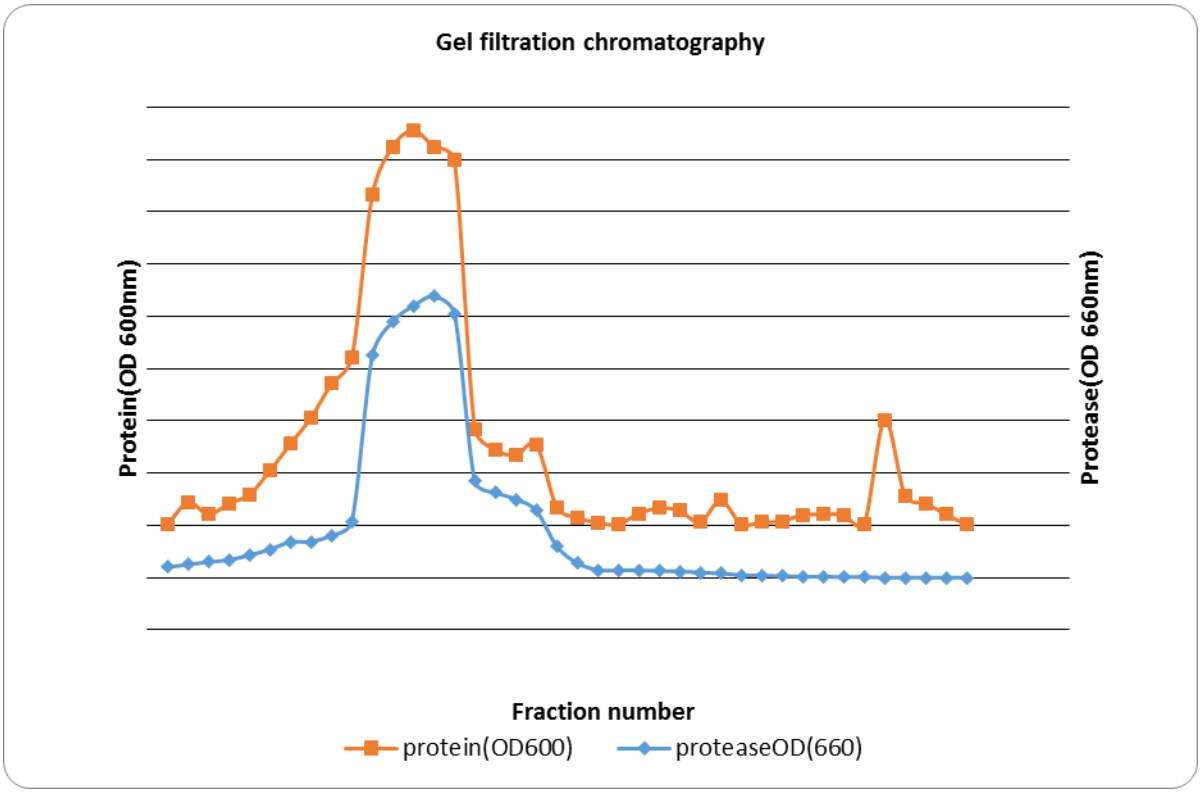
Initial step of purification was the concentration of proteins by precipitation using ammonium sulphate. The precipitation of protease was performed with 20%-80% of ammonium sulphate saturation. The precipitate was collected by centrifugation at 10,000 rpm for 15 min, The supernatant and pellet were assayed after each fraction .The protein and the protease in each fraction was determined. There was a maximum enzyme activity at 80%.

Precipitation using 80% ammonium sulphate yielded a recovery of 37.61%. This treatment resulted in increase of specific activity to 6833.33 mg compared to crude supernatant. So crude extracellular protein was precipitated by addition of 80% ammonium sulphate for further studies. The precipitated enzyme was dissolved in Phosphate buffer pH 7.0 and the sample was dialyzed. The protease activity and the protein were estimated for the dialyzed sample. The specific activity The specific activity has doubled when compared to that obtained by ammonium sulphate precipitation. . Dialysis using the obtained solution has yielded a recovery of 39.26% .The dialyzed sample was used for the further purification.

Gel filtration chromatography

A symmetrical peak of protease activity, accounting for approximately 95 per cent of the applied activity, was observed. During this method, 40 fractions were collected and the protein and protease in each fraction was determined. Maximum protease activity was observed in active fraction number 14 with 220501U/ml. The peak was well separated from small amounts of non proteolytic 660 nm.-absorbing material. The specific activity 18,375.15 (U/mg). This step resulted in 13.57-fold purification of the enzyme with 36.7% recovery. Active fractions were pooled and dialysed for further purification.

Fig 6: Sephadex G-75 gel filtration of dialysed protease sample of Serratia RSPB11.The column dimensions were 2.6 by 90 cm. Fractions (2 ml.) were assayed for protease activity at 660nm and absorbance at 600nm



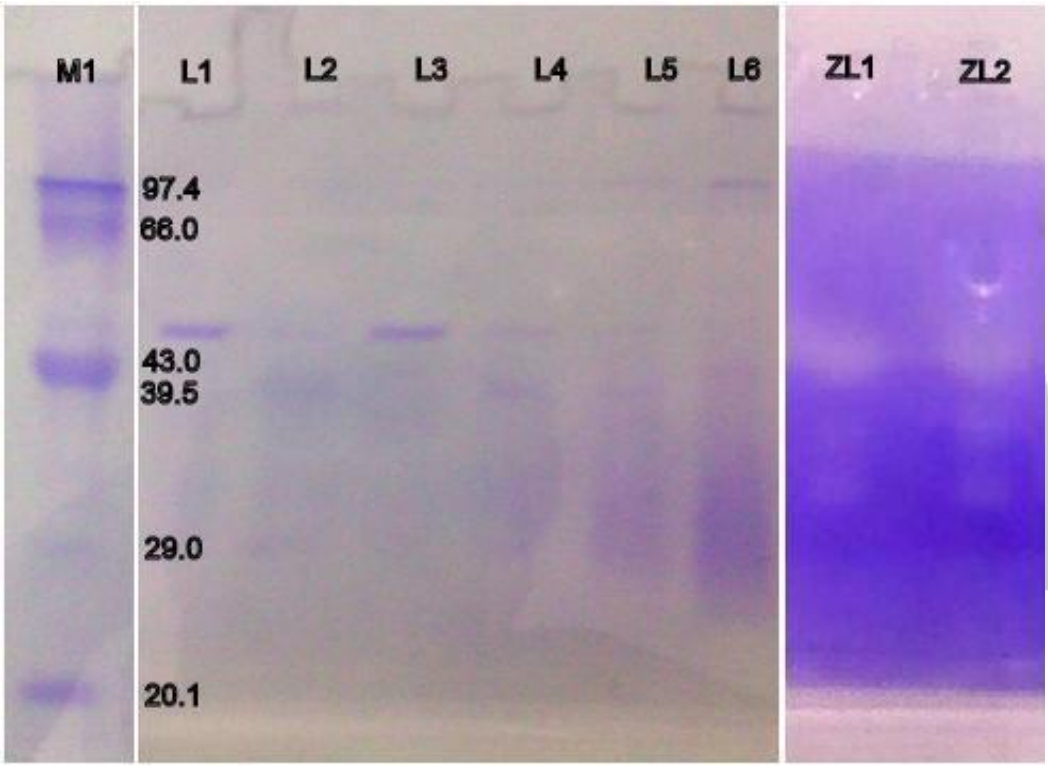
SDS-PAGE

The purity at each step of purification was then verified by SDS-PAGE (Fig. 12). This purified alkaline protease with a molecular mass of 50 kDa was detected.

Zymography with casein.

A clear zone against the dark Coomassie background indicates protease activity. It indicates that the loaded protease sample is able to utilize the casein confirms its purified nature.

Fig. 7: (a) SDS-PAGE analysis of the purified protease. Lane M1, molecular mass markers (in kDa): 97.4 –phosphorylase b; 66.0 – bovine serum albumin; 43.0 – ovalbumin;29.0 –carbonic anhydrase; 20.1 – soyabean trypsin inhibitor. L1- crude enzyme; L2- dialyzed sample L3,L4-ammonium sulphate precipitate; L5 -purified protease by Sephadex G-75, L6,L7- purified protease by ion exchange chromatography. (b) Native-PAGE , Lane M1, molecular mass markers (in kDa): ZL1,ZL2- zymography of purified protease.



Overall purification of the enzyme

The overall purification scheme showing the specific activity, the recovery and the purification effected at each purification step is summarized in Table 11. Precipitation using 80% ammonium sulphate yielded a recovery of 37.61%.this treatment resulted in increase of specific activity to 6833.33 mg compared to crude supernatant. Dialysis using the obtained solution has yielded a recovery of 39.26% .The specific activity has doubled when compared to that obtained by ammonium sulphate precipitation. After the final

purification step the enzyme was purified 25-fold with a specific activity of 23,450 U mg^{-1} and a recovery of 25.4% from the spent medium.

Table 3: A summary of the purification of alkaline protease from Serratia RSPB11

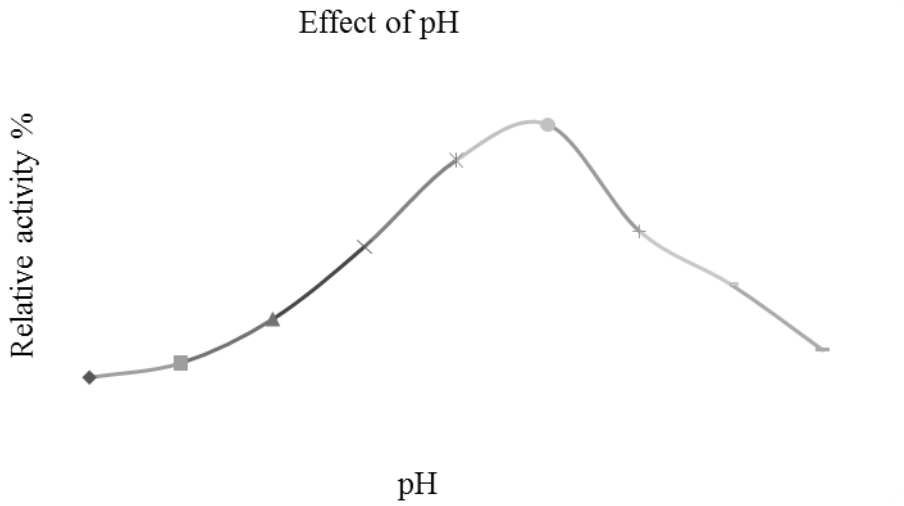
Sample	Total protein (mg)	Total enzyme activity (U)	Specific Activity (U/mg)	Purification Fold	Recovery
Crude	442.5	599500	1354.80	---	100%
Ammonium sulphate fraction (80%)	33	225500	6833.33	5.040	37.61
Pellet after dialysis	17.22	235400	13670.15	10.090	39.26%
Gelfitration chromatography	12	220501	18,375.15	13.5709	36.7%

Characterization of the enzyme protease

Effect of pH on enzyme activity and stability

Relative activities of protease in different pH buffers have been calculated and maximum activity showed by the protease in pH 9.0 has been considered as 100%. A very low activity (9%) noted at pH 4, followed by pH 5.0 (14.2%), 6.0 (30%) and 7.0 (56.1%) clearly shows that the enzyme is not either acidic or neutral protease. The optimum pH for caseinolytic activity was found to be pH 9.0 by protease enzyme confirms its alkaline nature. Optimum pH of proteases from different strains of *S.marcescens* varied from 8.5 to 10 [17,27] with an exception at neutral pH 7.0. *Serratia rubidaea* isolated from slaughter houses able to produce two types of metallo proteases where the optimal pH for the enzyme activities was 8.0 and 10.0 [16].

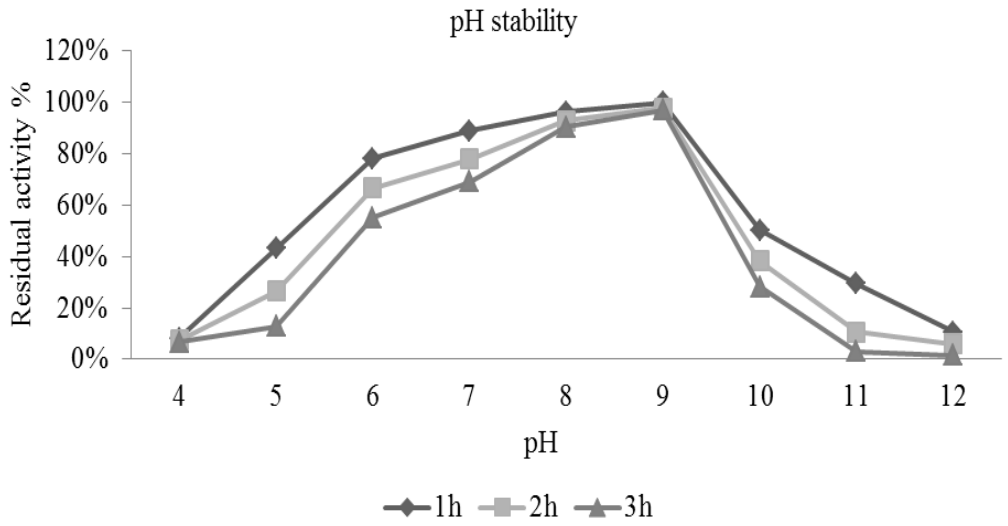
Fig 8: Effect of pH on the enzyme activity



Effect of pH on the stability of the enzyme

The tubes containing different pH buffers with 0.65% casein and enzyme in them are incubated at 37^{0}C for a period of 1h, 2h and 3h. The residual enzyme activity was calculated considering enzyme activity at pH 9.0 to be 100%.Protease showed greater than 50% activity at pH (6.0-10.0) after 1h incubation time. After 3 h there was a fall in the activity in higher pH (10.0-12.0) and also in the lower pH (4.0-5.0).The enzyme was comparatively stable in pH range of 6.0-9.0.The protease from supernatant of *Serratia marcescens* NRRL B-23112 retained more than 50% of its activity from pH 6.5 to 10.5 .*Serratia rubidea* [16]and *Bacillus cereus* TKU006 [33]were stable over a broad pH 5-9,pH 3-11respectively.

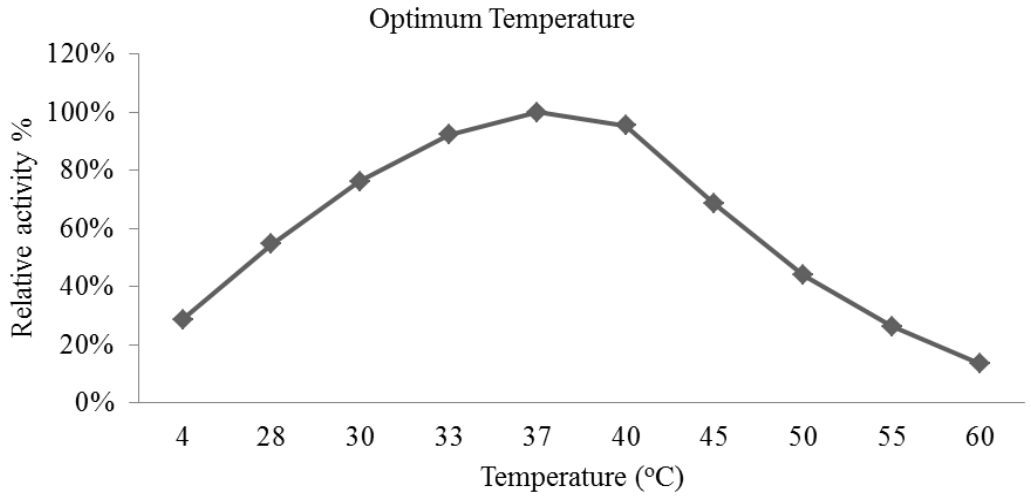
Fig:9: pH stability of the enzyme



Effect of temperature on enzyme activity and stability

Relative activities of protease at different temperatures have been calculated and maximum activity showed by the protease at 37°C has been considered as 100%. At 33°C and 40°C also notable relative activities of 92% and 96% suggests a broad range of temperature optima. A very low relative activities of 44%, 26.4%, 13.6% observed at 45°C, 50°C, 60°C depicts the fact that protease needs an optimum temperature for cleaving the substrate. Proteases from *S. marcescens* ATCC 25419 [27] and *S. marcescens* subsp. *Sakuensis* TKU019 [17,19] have reported optimum temperatures of 45°C and 50°C respectively.

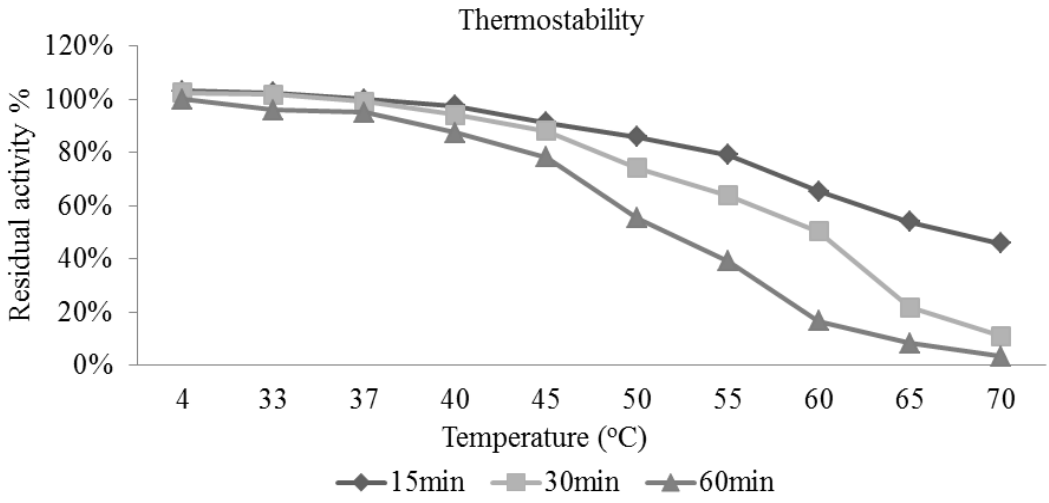
Fig 10: Effect of temperature on enzyme activity



Thermo stability of the enzyme protease

The tubes containing Glycine NaOH buffer pH 9.0, enzyme and 0.65% casein are incubated at different temperatures in varied range for period of 15 min, 30 min and 60 min. Enzyme activity before incubation was also calculated and considered to have 100% activity. Residual enzyme activity was calculated for the samples incubated at different temperatures. Loss of activity even after 1h at 4°C was not recorded and a lowest residual activity (3.2%) was noted at 70°C. A relative activity of more than 50% was achieved for incubation period of 30 min for a broad range of 33°C – 60°C.This shows that the enzyme is moderately thermo stable. There was a loss of activity as the incubation time was increased. Proteases from *S.marcescens*, *S. ureilytica*TKU013 was stable at 25°C – 50°C for a minimum incubation time and loss of activity rapidly increased above 60°C [27,17,32].

Fig11: Thermostability of the enzyme



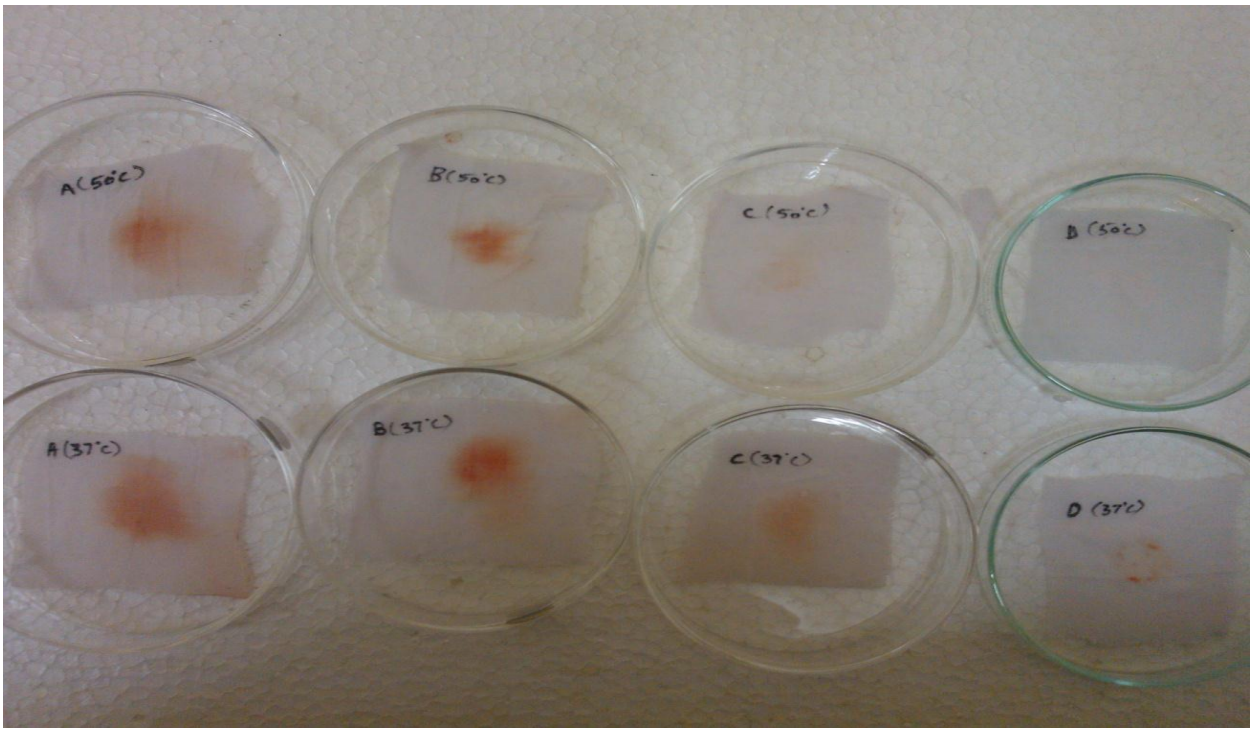
Application of purified alkaline protease isolated from Serratia RSPB11

Blood stain removal studies

Incubation of protease and combination of protease and detergent with blood stained cotton cloth piece at room temperature and 50 ^{0}C for 15 minutes showed removal of the stains. Rapid blood stain removal was noticed with the combination of water, detergent and alkaline protease than the supplementation of commercially available detergent. This indicates the role

of alkaline protease isolated from *Serratia* RSPB11 in industrial application especially in detergent.

Fig12: The purity at each step of purification was then verified by SDS-PAGE.



CONCLUSION

Much of the work was done on production and purification of proteases but most of them have worked on serine proteases. Less work has been done on the metalloprotease production especially protease or serrapeptase or Serratiopeptidase. Serrapeptase has a wide variety of applications especially in the medical sector as giving relief from pain, inflammation, arthritis, back problems, leg ulcers and digests nonliving tissues and blood clots etc. But due to the high cost of the enzyme in the market has appealed to search for a new source and subsequent bioprocess development.The conventional one variable at a time method was followed for the optimization of the nutritional parameters affecting the production of protease The best carbon source was dextrose and nitrogen source was casein. The concentration or levels of dextrose and casein in the medium were optimized.The enzyme was purified 10 fold using Ammonium sulphate precipitation followed by dialysis of the sample achieving a maximum specific activity of 13020U/mg.The dialysate was purified and achieved a specific activity of 18375U/mg

and 36.7% recovery by gel filtration chromatography. The active fractions were pooled and dialysed for further purification.Previously purified protease sample by gel filtration is subjected to ion exchange chromatography and attained a maximum specific activity of 23,450 u/mg. The sample was 17 fold purified and was further characterized. The characterization of the enzyme was done. The optimum pH for caseinolytic activity was found to be pH 9.0 by protease enzyme confirms its alkaline nature. The enzyme was stable in pH range of 6.0-9.0.The enzyme protease was stable at pH of 9.0 and there was 50% activity after incubation for 1 hr. The optimum temperature required for maximum activity of protease was found to be 37 oC. There was a broad range of temperature optimum ranging from 330C to 400C.The enzyme was stable at this temperature for an incubation period of 30 min. The purified alkaline protease has shown its proteolytic activity for the removal of blood stains proved its importance in detergent industries.

ACKNOWLEDGEMENTS

Yasser Eissa wish to acknowledge the financial assistance provide by University of Hajja, Yemen.

REFERENCES

Banik RM, Prakash M (2004) Laundry detergent compatibility of the alkaline protease from *Bacillus cereus* .Microbiol Res 159:135-140

Prakasham, R.S., SubbaRao, Ch., Rao, R.S., Lakshmi, G.S. and Sarma, P.N. (2007).L-Asparaginase production by isolated *Staphylococcus* sp. – 6A: design of experiment considering interaction effect for process parameter optimization. Journal of Applied Microbiology, 102:1382–1391.

Pansuriya, R.C., and Rekha, S. S., (2010). Evolutionary Operation (EVOP) to Optimize Whey-Independent Serratiopeptidase Production from *Serratia marcescens* NRRL B-23112, Journal of Microbiology and Biotechnology, 20: 950–957

Benjamin, K. Simpson ,Food Biochemistry and Food Processing.

Claudia c. hase and Richard a. finkelstein, Bacterial Extracellular Zinc-Containing Metalloproteases, microbiological reviews, Dec. 2003, p. 823-837

Claudia c. hase and Richard a. finkelstein, Bacterial Extracellular Zinc-Containing Metalloproteases, microbiological reviews, Dec. 2003, p. 823-837

Roychoudhury S, Parulekar SJ, Weigand WA. Cell Growth and a-Amylase Production Characteristics of *Bacillus amyloliquefaciens*. Biotechnol Bioeng. 1988;33:197–206. doi: 10.1002/bit.260330209

R. B. Raja, D. J. Mukesh Kumar, M. D. Balakumaran, P. T. Kalaichelvan, A. Pandey, A. Singh (2011). Isolation, Production & Application of Extracellular Phytase By *Serratia marcescens. Asian J. Exp. Biol. Sci.*Vol 2(4): 663 – 666.

Romero, F., Garcia, L.A., Salas, J., Diaz, M. and Quiros, L. (2001). Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. ProcessBiochemistry, 36: 501-515.

Rahman, R.N.Z.R., Geok, L.P., Basri M, Salleh, A.B., (2005). Physical factors affecting the production of organic solvent-tolerant protease by 30 Pseudomonas aeruginosa strain K. Resource Technology, 96: 429-436.

Nguyen, T.T., Quyen, D.T., (2011). Over production of an extracellular protease from Serratia sp.DT3 just using soybean powder, World journal of agricultural sciences 7(1):29-36.

P. **Lakshmi Bhargavi and R. S. Prakasham** (2012) Proteolytic Enzyme Production by Isolated Serratia sp RSPB11: Role of Environmental Parameters. Current Trends in Biotechnology and Pharmacy Vol. 6 (1) 55-65.

Lakshmi Narasu Mangamoori, Kiran Kumar Doddapaneni, Radhika Tatineni, Ravi Nagaraj Vellanki, Bharat Gandu, Nagender Reddy Panyala, Balumaddileti Chakali(2007), Purification and characterization of two novel extra cellular proteases from *Serratia rubidaea*. *Process Biochemistry* 42:1229–1236.

Jiang Jiaxin, Huang Guangrong, Dai Dehui and Hu Weilian (2008) Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with astatistical method. *African Journal of Biotechnology* Vol. 7 (8), pp. 1115-1122

Vsenil, C.K., Lakshmanperumalsamy, P., (2009). Application of response surface methodology in medium optimization for protease production by the new strain of *Serratia marcescens* sb08, Polish journal of microbiology 58:117-124.

C. M. Cabral, A. Cherqui,† A. Pereira, and N. Simo˜es* .,(2004). Purification and Characterization of Two Distinct Metalloproteases Secreted by the Entomopathogenic Bacterium *Photorhabdus* sp. Strain Az29.Applied and environmental microbiology, July 2004, p. 3831–3838 Vol. 70.

P. Charles, V. Devanathan, Periasamy Anbu, M. N. Ponnuswamy, P. T. Kalaichelvan and Byung-Ki Hur., (2008). Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. Journal of Basic Microbiology 2008, *48*, 347–352.

Arnold S. Kreger and Olwcn K. Griffin., (1975). Cornea-damaging proteases of

*Serratia marcescens** Volume 14 Niimhur Investigative Ophthalmology.

A Bayoudh, N Gharsallah, M Chamkha, A Dhouib, S Ammar and M Nasri.,(2000). Purification and characterization of an alkaline protease from Pseudomonas aeruginosa MN1. Journal of Industrial Microbiology & Biotechnology 24, 291–295.

Ishtiaq Ahmed, Muhammad Anjum Zia and Hafiz Muhammad Nasir Iqbal., (2011). Purification and Kinetic parameters characterization of an Alkaline Protease from Bacillus subtilis through Submerged Fermentation Technique. Pakistan World Applied Sciences Journal 12 (6): 751-757.

M. Kalpana Devi, A. Rasheedha Banu, G.R. Gnanaprabhal, B.V. Pradeep and M. Palaniswamy., (2008). Purification, characterization of alkaline protease enzyme from native isolate Aspergillus Niger and its compatibility with commercial detergents. Indian Journal of Science and Technology http://www.indjst.org Vol.1 No 7 "Fungal alkaline protease"

Malay Mandal, Sudip Das, Tapati Chakraborti, Amritlal Mandal and Sajal Chakraborti., (2003). Identification, purification and partial characterization of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in bovine pulmonary artery smooth muscle. Molecular and Cellular Biochemistry 254**:** 145–155.

Ch. Subba Rao, T. Sathish, P. Ravichandra, R.S. Prakasham *. , (2009). Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. Process Biochemistry 44 ., 262–268.

Wang, S.L., Chao, C.H., Liang, T.Z. and Chen, C.C. (2009). Purification and Characterization of Protease and Chitinase from Bacillus cereus TKU006 and Conversion of Marine Wastes by These Enzymes, Marine biotechnology11:334-344

Charles Gerday, Jean-Pierre Chessa, Ioan Petrescu, Mostafa Bentahira, Jozef Van Beeumen (2000). Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species. *Biochimicaet BiophysicaActa 1479:265-274.*

Egwin, Evans C (PhD). (2011). Partial Characterization of Protease activity from *Rhynchophorus palmarum* (Palm Weevil) African Journal of Food Science and Technology (ISSN: 2141-5455) Vol. 2(6) pp. 140-145.

Anson, M.L. (1938). Estimation of Pepsin, Papain and Cathepsin with haemogloblin. Journal of General Physiology, 22: 79-89.

Lowry, O. H., Rosebrough, N. J. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193: 265-275.

Wang SL, Kao TY, Wang CL, Yen YH, Chern MK, Chen YH. A solvent stable metalloprotease produced by *Bacillus* sp. TKU004 and its application in the deproteinization of squid pen for β-chitin preparation. Enzyme Microb Tech. 2006;39: 724–731. doi: 10.1016/j.enzmictec.2005.12.007

Daatsellar MCC, Harder W. Some aspects of the regulation of the production of extracellular proteolytic enzyme by a marine bacterium. Arch Microbiol 1974;101:21–34

Li-Qun Jin, Zhi-Qiang Liu, Yu-GuoZheng and Yin-Chu Shen , Identification and characterization of Serratia *marcescens* ZJB-09104, a nitrile-converting bacterium, World Journal of Microbiology and Biotechnology Volume 26, Number 5 (2010), 817-823, DOI: 10.1007/s11274-009-0238-5

Wang, S.L., Chao, C.H., Liang, T.Z. and Chen, C.C. (2009). Purification and Characterization of Protease and Chitinase from Bacillus cereus TKU006 and Conversion of Marine Wastes by These Enzymes, Marine Biotechnology, 11:334–344.