Current Trends in Biotechnology and Pharmacy
Vol. 6 (1) 55-65 January 2012, ISSN 0973-8916 (Print), 2230-7303 (Online)

Proteolytic Enzyme Production by Isolated *Serratia* sp RSPB11: Role of Environmental Parameters

P. Lakshmi Bhargavi and R. S. Prakasham*
Bioengineering and Environmental Centre, Indian Institute of Chemical Technology
Hyderabad – 500 607, India
*For Correspondence - prakasam@iict.res.in

Abstract

An effective proteolytic enzyme producing microbial strain has been isolated from marine habitats and evaluated its extracellular protease production properties with respect to different fermentative physiological parameters. The strain has been identified based on biochemical tests according to Bergey's Manual of Systematic Bacteriology as *Serratia* sp and designated as RSPB11. This strain has potential to hydrolyze chitin, gelatin and casein revealing its industrial potential for production of multi-enzyme complex. Since the isolated strain belongs to *Serratia* genus, the protease produced by this strain is considered as serralysin and which is not inhibited by PMSF suggesting the enzyme belongs to other than serine type of protease. Further analysis denoted that this enzyme belongs to metalloprotease which is confirmed based on negatively regulation of caseinolytic (proteolytic) activity by EDTA. The maximized enzyme production occurred at medium initially adjusted to pH 7.0 incubated at 33°C under aerated environment. Analysis of the pH profile before and after fermentation depicted that irrespective of initial medium pH, it is shifted to pH 9.0 after fermentation suggesting the enzyme produced is alkaline in nature.

Keywords: Enzyme, Fermentation, Isolation, Protease, *Serratia* sp.

Introduction

Proteases/ proteinases are a group of hydrolytic enzymes (catalyse the reaction of hydrolysis of bonds with the participation of a water molecule) which are specific for digestion of proteins in to peptides and amino acids. Proteases differ in their ability to hydrolyze various peptide bonds hence, specificity associated with each enzyme differs based on catalytic site. 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), functional group present at active site (serine/ cysteine/ aspartic/ metalloproteases) of proteolytic activity with respect to functional group present at the active site. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases.

Most commercially available proteases belong to the class serine proteases, produced

by organisms belonging to the genus *Bacillus, Apergillus* 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. Most of pharma related metalloproteases are dependent on zinc (1) and a few uses cobalt, iron, manganese, etc (2).

Serratiapeptidase is a type of metallo-protease (EC 3.4.24.40) originally isolated from digestive system of silkworm (3). Subsequently, this enzyme also reported from different bacterial strains including *Pseudomonas* auri *Serratia marcescens*, *Proteus mirabilis* and *Escherichia freundii* (4). Functionally this enzyme breaks peptide bond of non-terminal amino acids under alkaline environment hence also referred as alkaline-endopeptidase. Serrapeptidase differs from other metalloend-opeptidases in the catalytic reaction, where the enzyme preferentially cleaves the peptide bonds associated with hydrophobic residues (5).

Initially this peptidase production was noticed during infection by *Serratia* sp. as well as other bacterial strains such as *Pseudomonas aeruginosa* or *Erwinia chrysanthemi*. The physiological function of serratia peptidase in these microbial strains is yet to be identified, however the enzyme seems to play a significant role in nutrient digestion/uptake in these bacterial strains (6). Maeda and coworkers reported that this protease plays a critical role in pathogenesis of *Serratia marcescens* (4). Interestingly, this enzyme production is mostly reported from clinical isolates (4, 7) however, reports from marine microbial strain YS-80-122, *Pseudomonas* sp., also noticed in the literature (8, 9).

Much attention has been focused on alkaline metalloprotease production by species of *Serratia* especially *Serratia marcescens* due to its potential for higher enzyme yields compared to literature reports and has been used as an anti-inflammatory agent all over the world (10). Alkaline metalloprotease production from different microbes has been evaluated by various researchers with respect to enzyme regulation and excretion mechanisms (11, 12), characterization and purification (10, 13), and genetic analysis of these enzymes (14, 15). Efforts also have been made to improve the economics of the bioprocess by using non conventional media components such as whey powder or squid pen powder (16-18). At present, Serratiapeptidase, a major alkaline metalloprotease is commercially produced with a SMP-overproducing mutant of *S. marcescens* ATCC-21074 (19).

Analysis of biochemical and biocatalytic properties of alkaline metalloprotease produced by different microbial strains revealed variation in specificity of action, metal component, optimal pH, temperature, etc (10) which influences biotechnological application specificity. This, in addition to the high price for this enzyme in the market are some of the powerful appeals that lead to search for new protease producing sources and subsequent bioprocess development. Microbial enzyme production is highly influenced by media components like carbon and nitrogen sources besides several other factors such as aeration/agitation, pH and temperature, salinity and incubation time (20, 21, 22). Hence, the present study focused on isolation of a bacterial strain with high protease production property, growth characterization, and enzyme production properties in addition to identification. The data indicated that the isolate belongs to *Serratia* sp and has higher enzyme titers compared to literature reports therefore this

isolate could be effective strain for industrial production. This strain's protease production is highly regulated by chelating agents, incubation temperature, hydrogen ion concentration in the medium, and incubation temperature.

Materials and Methods

***Screening of chitinase and protease producing microbes*:** Soil samples collected from marine environment (contaminated with sewage from fish processing plants) located near Bapatla, Andhra Pradesh, India were used in this study. A serial dilution method has been followed after enrichment technique for isolation of microbial strains. In brief, one gm of soil sample was added to sterile 100 ml media containing mineral salts ($(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 0.02 g, NaCl 0.5g, $MgSO_4$ 0.05g) and 5gm dried shrimp as well as crab shell powder followed by incubation for 48h at 30°C. After serial dilution, the obtained samples were spread over chitin agar plate (Colloidal Chitin 1.5 g, mineral salts, agar 20 g, distilled water 50 ml, sea water 50 ml, pH 7.0) and incubated for 5 days at 30°C. A clear- zone forming bacteria were selected and inoculated on 2% casein and 1% gelatin agar plates at pH 7.0 for identification of protease production. After incubation for 2 days at room temperature, clear hydrolytic zone forming bacterial strains were selected for further studies and maintained on agar slants.

***Biochemical and phenotypic characterization*:** Selective isolate was identified through its biochemical and physiological properties according to Bergey's Manual of Systematic Bacteriology (23).

***Scanning Electron Microscope analysis (SEM)*:** SEM was used to investigate the morphology of isolated strain. The sample for SEM was prepared by transferring the microbial strain to a clean eppendorff tube containing approximately 1.5 ml of 3.5% glutaraldehyde solution. Then, culture was incubated for 4 h at room temperature followed by wash with phosphate buffer (100 mM, pH 7.2). The culture is then dehydrated using alcohol gradient from 10 to 100%. The dehydrated samples were then air dried and fixed on the stubs using double adhesive tape. A thin layer of gold was coated over the sample using HUS-5GB Hitachi vacuum evaporator for 90 sec. These samples were then observed under scanning electron microscopy (Hitachi S- 3000N, Japan) at various magnifications at acceleration voltage of 10.0 KV.

Production media and culture conditions : One of the bacterial isolate designated as RSPB11 was maintained regularly on nutrient agar slants and used in this study. For enzyme production, yeast extract – peptone media consisting (%, g/100ml) of yeast extract – 1.0, Peptone – 1.0, Dextrose - 0.2, $MgSO_4$ - 0.02, KH_2PO_4 - 0.02, NaCl -0.02 at pH 7.0 was used. Inoculum (OD_{600nm}~1.5) was developed by growing the isolate in nutrient broth for 18h. For production of enzyme, 1.0% inoculum was added to 50ml production medium in 250ml conical flasks and then incubated at 30°C for 3-4 days. Samples withdrawn at specific time intervals were centrifuged at 10,000 rpm for 10 min and the supernatant has been used as enzyme source for assay. All the culture conditions were same unless otherwise mentioned.

***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 in the presence of 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 TCA was added to the enzyme solution, followed by

substrate addition. After incubating at room temperature for 30min both test and blank solutions were centrifuged at 10,000g for 10min. To the 0.4ml supernatant, 1.0ml 50mM Na_2CO_3 and 0.2ml Folin-ciocalteau reagent was added, the reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 660nm. 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.

***Optimization of culture conditions for the bacterial growth and the protease production by isolated strain*:** To select the optimum pH, temperature, aeration and agitation, enzyme production was investigated at different pH environments (pH 5.0-9.0), at different temperatures (27°C-40°C), aeration conditions with respect to volume of media in 250ml conical flasks (25ml-125ml) and speed of agitation from static to 200 rpm, respectively in separate flasks. The samples were collected every 24 h for 72h to measure the enzyme activity.

Results and Discussion

***Isolation and screening of protease producing microbes*:** Various soil samples collected from marine environment were used for isolation of effective protease producing microbe. Chitin in the exoskeleton of shrimp and crab shells is associated intimately with proteins therefore for the isolation of chitionolytic and proteolytic bacteria, chitin utilization in the screening media offers a great advantage (18, 25). Therefore, enriched microbial population from the shrimp and crab shell media has been spread over chitin agar plate and incubated for 5 days until the visibility of hydrolytic zones. Selected microbes from the chitin agar plates were again spot inoculated on casein agar plate containing 1mM PMSF (serine protease inhibitor) and gelatin agar plate. After 2 days colonies showing maximum proteolytic zones were picked and purified over nutrient agar plates. Among the 10 potent chitinase producers only 3 microbes showed high casein hydrolytic zones, revealing that these isolated strains were not serine protease producers. This is concluded based on the fact that these colonies are showing the proteolytic activity in presence of PMSF which binds specifically to the active site serine residue in a serine protease leading to inactivation of serine proteases (26). As our protease of interest is metalloprotease due to their pharmaceutical importance, addition of PMSF in the casein agar plate during the screening of microbes helped in differentiating serine protease producers from other class of protease producers. All three

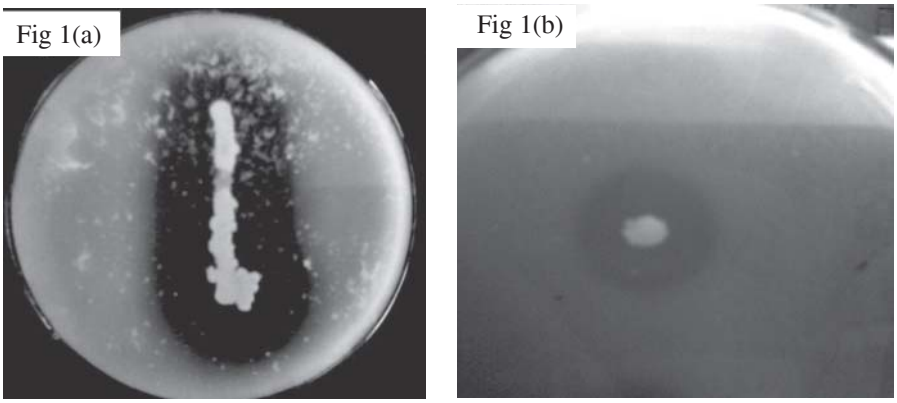

Fig 1. Petri plates showing the marine isolate with a potential to produce extracellular protease (a) and chitinase (b)

isolates were named and sub cultured in nutrient broth. Based on the larger hydrolytic zones on chitin (Fig 1a), casein (Fig 1b) agar plates and protease assay, one isolate designated as RSPB11 was selected for further studies.

***Biochemical, phenotypic and morphological characteristics of the RSPB11*:** Biochemical and physiological properties of RSPB11 was identified according to Bergey's Manual of Systematic Bacteriology (2). Table 1 indicates the isolated strain physical properties and Table 2 denote biochemical characterization tests and their results. Colonies of RSPB11 on nutrient agar plates were white, round with smooth and glossy surface as well as slightly opaque in nature. The isolate is characterized as aerobic, gram negative and rod shaped bacteria. Fig 2 shows the scanning electron microscope picture of the isolate and cells were visulaized as single, short rod shaped bacteria. Physiological tests show that the cells could survive and grow in the medium pH ranging from 5.0 to 11.0 and under saline conditions of 3.0% NaCl. From the biochemical tests, it was concluded that this isolate RSPB11 belong to Enterobacteriace family, and it is a member of *Serratia* genus. This genus is characterized with ten species (strains) distributed in two sub species (27).

***Optimization of process parameters for protease production*:** Protease production by the isolate, *Serratia* sp RSPB11, was noticed at 12 hours of growth and enzyme production reached to the maximum level after 48h of cultivation. Growth of the isolate, monitored by taking the absorbance of media against blank at 600nm at specific time intervals shows a gradual increase in biomass production. Protease production was also noticed in correlation with biomass production (Fig 3) but a notable protease activity has been observed after 24h. This data suggested that the protease production by this strain is

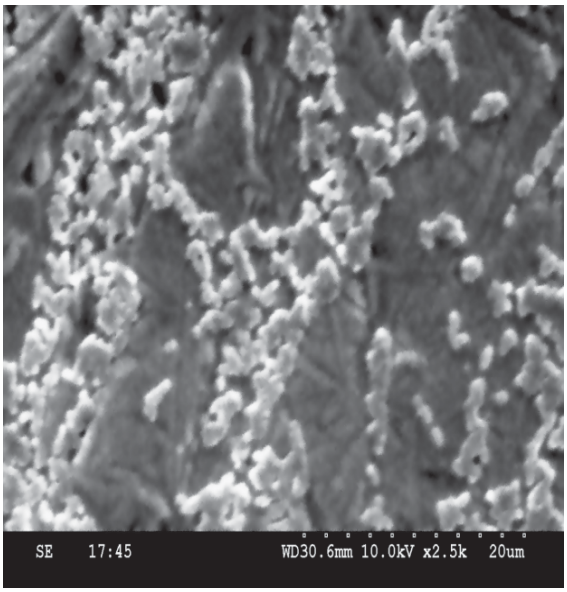

Fig 2. Scanning electron micrograph of marine isolate *Serratia* sp. RSPB11

growth associated. This is further supported by the fact that a constant protease activity was observed during stationary phase. The results further suggested that improved level of protease production could be possible with high active biomass production.

The impact of incubation time as well as other physical parameters like initial pH, temperature, and agitation on protease production by isolated *Serratia* sp RSPB11 was investigated. Initial pH of the media was adjusted with either 1M HCl or 1M NaOH for attaining desired pH conditions. From the results it is clear that initial pH of 7.0 supported the growth of bacterium, where a maximum biomass and enzyme activity (5230U/ml) was noted (Fig 4). Analysis of the pH of the fermentation medium at specific time intervals indicated a gradual rise in pH and reached alkaline condition (pH ~ 9.0) after 48h (data not shown) indicating that this enzyme is an alkaline protease. Media adjusted with different pH conditions (pH 5.0, 6.0, 8.0,

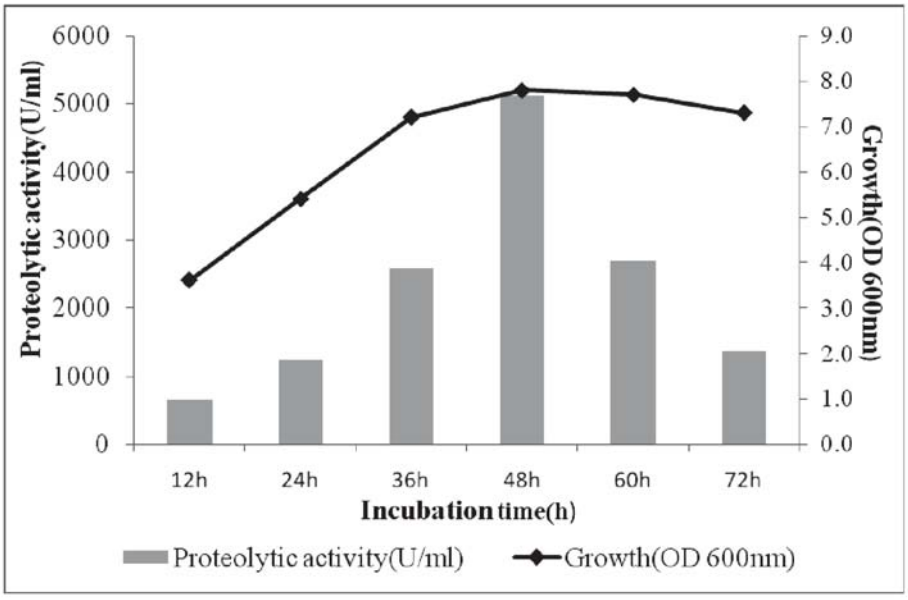

Fig 3. Effect of incubation time on growth and protease production by the marine isolate *Serratia* sp. RSPB11

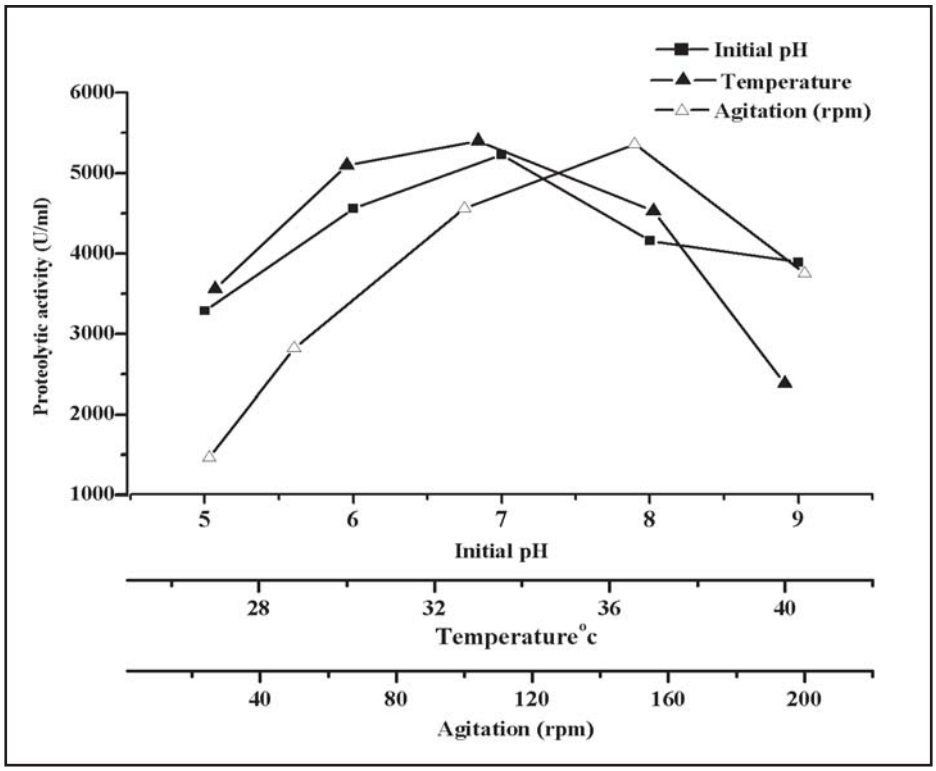

Fig 4. The effect of pH, temperature, and speed of agitation on production of protease by the marine isolate *Serratia* sp. RSPB11

9.0) also reached a final pH ~ 9.0 at the end of the fermentation, but the enzyme production was low at other than pH 7.0 conditions. This increase in pH value along with fermentation process hence this protease production bioprocess may be attributed to ammonia produced as a consequence of the aminoacids catabolism released by protein hydrolysis by produced protease enzyme as reported (28). pH dependent protease production by different microbial strains were also observed by several investigators, where the initial pH 6.0 (29) to 8.0 (30) could support the increase in biomass as well as production of protease. The above data also depict that higher yields may be effectively obtained by constant maintenance of pH of the medium during fermentation process as noticed by Venil, (31) and Panasuriya (32). Efforts are being made in this direction.

Among the physical factors, temperature is one of the most critical parameters that could affect the bioprocessing. To evaluate the same, media inoculated with *Serratia* sp RSPB11 has been incubated at different temperatures ranging from 27°C to 40°C. The enzyme production has been noticed in all tested temperature conditions suggesting at this temperature range the isolated *Serratia* sp. RSPB11 survives and produces metabolism linked protease production. Though protease production noticed all most all studied environments higher production was noticed at 33°C (5400U/ml). A very low enzyme activity of 2385U/ml observed at 40°C (Fig 4). Several studies revealed that temperature was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane (33). The optimum temperature of *Serratia* sp RSPB11 was slightly higher than *Serratia marcescens* NRRL B-23112 at 25°C (32), *Serratia* sp DT3 at 28°C (29), and lower than *Serratia marcescens* sp7 at 40°C (30), *Pseudomonas aeruginosa* MTCC 7926 metalloprotease at 40°C (13). A temperature range of 30-37°C has been employed in several works (17, 34).

In general, it is well known that all organisms and microbes vary in their aeration requirement. In particular, during aerated environment, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed has been found to influence the extent of mixing in the shake flasks and also affect the nutrient availability (35). In view of the above, speed of agitation was studied by incubating the flasks on orbital shakers at various rpm (25-200 rpm) conditions. The data depicted that fermentation processed at 25 rpm, a very low production (1460U/ml) of protease was noted and a maximum production of 5363U/ml observed at 150rpm. This data denote that this isolate needs a particular speed of agitation for achieving the maximum biomass along with the production of enzyme.

Partial characterization of protease produced by isolated Serratia sp. RSPB11 : To evaluate the nature of proteolytic enzyme produced by isolated *Serratia* sp. RSPB11, the enzyme activity studied in the presence of 1 mM EDTA to know whether this enzyme belongs to metalloprotease family. Use of 1mM EDTA in the reaction medium inhibited the proteolytic activity of the produced enzyme, indicting negative regulation of protease activity by this chelating agent. The result suggested that the enzyme produced by isolated *Serratia* sp. RSPB11 belongs to metalloprotease. This was further confirmed from the literature reports where protease activity was observed to be inhibited by a metalloprotease inhibitor like EDTA (36). To evaluate further the nature of enzyme, the enzyme activity was measured in terms of zone of inhibition in the presence of

PMSF (results not shown). This further confirmed that this enzyme is not belonging to serine type of protease as reported in other proteases (37).

The protease produced by isolated microbial strain is considered as one of the serralysin type of protein. This is confirmed based on the following observations- a) The isolated strain belongs to genus *Serratia*, b) The produced proteolytic enzyme is negatively regulated by EDTA, c) all serralysin type proteases are metalloproteases and not belongs to serine type of proteases, d) The proteolytic enzyme production is observed in presence of serine protease inhibitor, PMSF and e) The enzyme production is maximum at alkaline environment, hence this protease does not belongs to aspartate type.

Conclusion

In the present investigation, a serralysin type of protease producing microbial strain has been isolated using marine soil samples. The strain has been purified and characterized in terms of its biochemical and physiological growth properties. Based on biochemical tests, the isolate has been identified up to genus level and observed that this strain belongs to *Serratia* sp. Extracellular enzyme production properties were studied and observed that this strain produces more than one extracellular enzymes; such as chitinase and gelatinase depending up on the nutritional conditions. This strains ability towards protease production and since the strain belongs to *Serratia* sp. hence, the produced protease is considered as serralysin type of protease which is known for its commercial importance. In this context, the protease production was investigated further in terms of optimal requirements for physiological growth factors. The optimized protease production has been noticed at a physiological pH of 7.0 and at temperature 33°C as well as at 150 rpm therefore, these parameters are crucial for effective production yields.

Acknowledgement

One of the authors, P.Lakshmi Bhargavi is greatly indebted to Council of Scientific and Industrial Research, New Delhi for financial support in the form of Senior Research Fellowship.

References

1. Hooper, N.M. (1994). Families of zinc metalloproteases, FEBS Letters, 354 (1):1-6.
2. Kayoko, M.,Fukasawa, Toshiyuki, H., Yukio,O., and Junzo, H. (2011). Metal Preferences of Zinc-Binding Motif on Metalloproteases, Journal of Amino Acids, Article ID 574816:1-7.
3. Miyata, K., Tomoda, K., Isono, M. (1971). Serratia protease. Part III. Characteristics of the enzyme as a metalloenzyme. Agricultural and Biological Chemistry, 35: 460-467.
4. Maeda, H. (1996). Role of microbial proteases in pathogenesis. Microbiology and Immunology,40:685–699
5. Louis, D., Bernillon, J. and Wallach J.M. (1999). Use of a 49-peptide library for a qualitative and quantitative determination of pseudomonal serralysin specificity. International Journal of Biochemistry and Cell Biology, 12:1435-41.
6. Supuran, C.T., Scozzafava, A. and Clare B.W. (2002). Bacterial protease inhibitors. Medical Research Reviews, 22:329–72.
7. Matsumoto, K.,Maeda, H., Takata, K., Kamata, R. and Okamura, R. (1984). Purification and characterization of four

proteases from a clinical isolate of *Serratia marcescens* kums 3958. Journal of Bacteriology, 157:225–232.

8. Fang. W., Jianhua, H., Chengye, Y. and Mi, S. (2010). Cloning, Expression, and Identification of a Novel Extracellular Cold-Adapted Alkaline Protease Gene of the Marine Bacterium Strain YS-80-122, Applied Biochemistry and Biotechnology, 162:1497–1505.

9. Jean-Pierre, C., Ioan, P., Mostafa, B., Jozef, V.B. and Charles, G., (2000). Purification, physico-chemical characterization and sequence of a heat labile alkaline metalloprotease isolated from a psychrophilic *Pseudomonas* species, Biochimica Biophysica Acta 1479: 265-274.

10. 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. Process Biochemistry, 36: 501-515.

11. Bromke, B. J. and Hammel, J. M. (1978). Regulation of extracellular protease formation by *Serratia marcescens*. Canadian Journal of Microbiology, 25:47-52 .

12. Braun, V. and Schmitz, G. (1980). Excretion of a protease by *Serratia marcescens*. Archieves of Microbiology, 124: 55-61.

13. Ulhas, P. and Ambalal, C. (2009). Purification and characterization of solvent-tolerant, thermostable, alkaline metalloprotease from alkalophilic *Pseudomonas aeruginosa* MTCC 7926, Journal of Chemical Technology and Biotechnology, 84: 1255–1262.

14. Rick, G. W., Michelle, B., Carie, A. B. and Ifor, R. B. (2001). The *aprX–lipA* operon of *Pseudomonas fluorescens* B52: a molecular analysis of metalloprotease and lipase production, *Microbiology* 147: 345–354.

15. Kent, B., Marty, Christopher L. W. and Linda J. G. (2002). Characterization of a Cytotoxic Factor in Culture Filtrates of *Serratia marcescens*, Infection and Immunity, 70:1121-1128.

16. Romero, F., García, L.A. and Díaz, M. (1998). Protease production from whey at high concentrations by *Serratia marcescens*. Resource and Environmental Biotechnology, 2: 93-115.

17. Romero, F., Ustariz, J., Laca, A., Garcia L. A. and Diaz, M. (2008). Fermentation conditions increasing protease production by *Serratia marcescens* in fresh whey. Revista Tecnica de la Facultad de Ingenieria Universidad del Zulia, 31:79 – 89.

18. 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.

19. Kim, K.S., Park, K.S., Byun, S.M., Pan, J.G. and Shin, Y.C. (1995). Over production of *Serratia marcescens* metalloprotease (SMP) from the recombinant Serratia marcescens strains, Biotechnology Letters, 17:497-502.

20. Gupta, R., Beg, Q.K., Khan, S. and Chauhan, B. (2002). An Overview on Fermentation, downstream processing and

properties of microbial alkaline proteases, Applied Microbiology and Biotechnology, 60: 381-395.

21. Prakasham, R.S., Subba Rao, 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.

22. Mahalaxmi, Y., Sathish, T., Subba Rao, Ch. and Prakasham, R.S. (2010). Corn husk as a novel substrate for the production of rifamycin B by isolated *Amycolatopsis sp*. RSP 3 under SSF. Process Biochemistry, 45: 47–53.

23. Grimont, P.A.D. and Grimont, F. (1984). Genus VIII. *Serratia*. In: Krieg NR, Holt JG (eds) Bergey's Manual of systematic bacteriology, vol 1. Baltimore, Williams and Wilkins. 477-484.

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

25. Teng, W.L., Khor, E., Tan, T.K., Lim, L.Y. and Tana, S.C. (2001). Concurrent production of chitin from shrimp shells and fungi, Carbohydrate Research 332 305–316.

26. James, G.T. (1978). Inactivation of the protease inhibitor phenylmethyldulfonyl fluoride in buffers, Analytical Biochemistry 86 : 574.

27. Identification of Enterobacteriaceae, Issue no: 3 Issue date: 28.09.10 Issued by: Standards Unit, Department for Evaluations, Standards and Training. BSOP ID 16i3.

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

29. Nguyen, T.T. and 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.

30. Joseph, B. and Palaniyandi, S. (2011). Determination of Alkaline Protease Production In *Serratia Marcescens* Sp7 Using Agro Wastes As Substrate Medium, Optimization Of Production Parameters And Purification Of The Enzyme, World Academy of Science, Engineering and Technology, 74: 252-256.

31. Venil, C.K. and 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.

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

33. Rahman, R.N.Z.R., Geok, L.P., Basri M. and 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.

34. Rahman, R.N.Z.R., Geok, L.P., Basri M. and Salleh, A.B. (2005). An organic solvent-tolerant protease from

Pseudomonas aeruginosa strain K Nutritional factors affecting protease production. Enzyme and Microbial Technology 36: 749–757

35. Nascimento, W.C.A. and Martins, M.L.L. (2004). Production and properties of an extracellular from thermophilic *Bacillus* sp. Brazilian journal of microbiology, 35: 91-95.

36. Salamone, P.R. and Wodzinski, R.J. (1997). Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*, Applied microbiology and biotechnology, 48: 317-324.

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