Production and Characterization of Protease from *Serratia marcescens*

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Abstract

Extracellular protease produced by Serratia marcescens *isolated from the gut of termite* (Centrocestus formosanus) *was investigated in this study. The effect of temperature, pH, carbon and nitrogen sources on protease production was examined. The extracellular protease was separated from the culture supernatant of the organism through precipitation with ammonium sulfate. The protease was purified by Sephadex G-150 gel filtration and diethylaminoethanol (DEAE) - Sephadex A-50 ion exchange chromatography. The effect of temperature, pH, and salts on the proteolytic activity of the enzyme was also investigated. Optimal protease production was obtained after about 12-hour incubation. The optimum temperature and pH for protease production was found to be 40°C and pH 7, respectively. Protease production was highest when rice bran and casein were used as the carbon and nitrogen sources, respectively. The native molecular weight of the protease was estimated to be 56 kDA. The optimal temperature for hydrolysis of casein was 50°C and the optimal pH was 8. The protease was found to be inhibited by ethylenediaminetetraacetic acid (EDTA), NaCl, $CaCl_2$, $FeCl_2$ and $HgCl_2$. Proteolytic activity was however enhanced by KCl. The maximum velocity V_{max} and K_m values were 40 mg/min/mL and 0.91 mg/mL, respectively.*

Keywords: *Protease, inhibitors, rice bran, purification, termite.*

1. Introduction

Proteases are responsible for the hydrolysis of proteins to oligopeptides and amino acids (Schlegel 1993). They are excreted by both bacteria and fungi. They are the most important classes of enzymes and expressed throughout the animal and plant kingdom as well as viruses and bacteria. Proteases occur naturally in all organisms where they are involved in a machine of physiological reactions ranging from simple digestion of fold proteins to highly regulated cascades like the blood clotting cascade. Microorganisms represent an excellent source of enzyme owing to their broad biochemical diversity and their susceptibility to genetic manipulation.

Microbial proteases possess almost all the characteristic desired for their biotechnological application. Proteases are classified into six broad groups according to the character and catalytic active site and condition of action (Dubey *et al*. 2007). They include serine, threonine, cysteine, aspartate, glutamic acid and metalloprotease. According to Gupta *et al*. (2002) proteases represents one of the three large groups of industrial enzymes and find application in detergents, leather, food, pharmaceutical industries and bioremediation processes. The largest application of proteases particularly the alkaline proteases has probably been in the laundry detergent where they enhance the removal of the removal of protein based stains from clothing (Banerjee *et al*. 1999; Josephine *et al*. 2012). The various environmental conditions affecting the metabolic processes in microorganisms include pH, temperature, substrate, inoculums size, etc. In order to

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achieve maximum production or yield, these factors must be determined. The effect of different carbon and nitrogen sources has been reported by several authors (Giesecke *et al*. 1991; Ferrero *et al*. 1996; Rajkumar *et al*. 2011). Several agricultural residues have also been investigated as a possible substrate in the production of alkaline proteases (Gessesse 1999; Aikat and Bhattacharyya 2002; Johnvesly *et al*. 2002; Rajkumar *et al*. 2011). Due to the versatile requirement for proteases, attempts are made continually to isolate newer sources of protease producing microorganisms with a potential for industrial application. This work describes some of the factors that affect protease biosynthesis in *Serratia marcescens* isolated from the gut of termite (*Centrocestus formosanus).* Some of the catalytic properties of industrial interest were also determined.

2. Materials and Methods

2.1 Organism and Culture Conditions

The test organism, *Serratia marcescens*, is a local isolate from the gut of termite *Cenrocestus formosanus*. It was cultured on nutrient agar to obtain the pure culture of this organism. The organism was grown in a basal medium containing (g/L) K_2HPO_4, 1.5; $MgSO_4$, 0.05; $NaCl_2$, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2$, 0.2; $FeSO_4$, 0.2; sucrose, 0.5; yeast extract, 0.5 and caesin, 1.0. The inocula for the experiments were prepared by growing the organism in nutrient broth (NB, Oxoid) at 35°C for 18 hours on a rotary shaker (Gallenkamp). Sterilized medium (500 ml) in 1,000-ml conical flasks was inoculated with 10 ml of inocula (1.0×10^3 cells/ml). The flask was incubated at 30°C on a rotary shaker (150 rpm) for 48 hours. Samples were drawn from the flask at intervals of 6 hours for a period of 48 hours. The samples drawn were centrifuged at 5,000 × g for 30 minutes at 4°C. Cell free supernatant corresponding to growth phase was used as the crude enzyme for assay and further analysis.

2.2 Protease Assay

The protease activity was determined in a reaction mixture consisting of 1 mL of substrate solution (1.0% casein in Tris-HCl buffer, pH 7.0) and 1 mL of the enzyme solution. The reaction mixture was incubated for 60 minutes at 60°C. The proteins were precipitated by adding 2mL of 0.5% TCA and free amino acids released by protease from casein hydrolysis were estimated by the Lowry method (Lowry *et al*. 1951). The protease activity was defined as mol of tyrosine released per minute per ml of the enzyme.

2.3 Protein Assay

Protein was determined by the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at an absorbance of 280 nm.

2.4 Effect of Carbon and Nitrogen Sources on Protease Production

To test the effect of carbon and nitrogen sources on protease production, the basal medium was amended using rice bran, wheat bran and corn starch, respectively, as the carbon sources and ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, skimmed milk, yeast extract and casein, respectively, as the nitrogen source. The sources were added at a concentration of 1% (w/v).

2.5 Effect of Initial pH of Basal Medium on Protease Production

The effect of initial pH on protease production was determined by measuring activity in basal medium having pH ranging from 3.0 to 9.0.

2.6 Purification and Characterization of Protease

All chromatography procedures were carried out at 4°C except where stated otherwise.

2.7 Ammonium Sulphate Fractionation

Fifty mL of crude enzyme was precipitated (fractional) with $(NH_4)_2SO_4$ (analytical grade, BDH, Merck KGaA,

Darmstadt, Germany) at 70% (w/v) saturation. The precipitate was centrifuged at 10,000 rpm for 10 minutes. The precipitate was re-dissolved in Tris-HCl buffer (pH 7.8) and dialyzed against several volumes of the same buffer for 24 hours at 4°C using acetylated cellophane tubing prepared from Visking dialysis tube (Gallenkamp) as described by Tariq *et al*. (2011).

2.8 Sephadex G-150 Gel Filtration Chromatography

The acid-treated and ammonium sulphate fractionated protein solutions were loaded separately onto a column (1.5cm × 75cm) of Sephadex G-150 previously equilibrated with 15 mM sodium acetate buffer, pH 4.7. The column was eluted at a flow rate of 20 mL/h with the same buffer. Five mL fractions were collected and subsequently assayed for protease activity. For determination of molecular weight by gel filtration the standards used were: gamma globulin, 15 kDa; alpha chymotrypsinogen, 25.7 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa and creatine phosphokinase, 81 kDa (Sigma-Aldrich Co. LLC, Gillingham, Dorset, England, UK).

2.9 Effect of pH on protease activity

The effect of pH on the enzyme activity was determined in substrates (1%) casein having pH ranging from 5.0 to 9.0. The substrate was prepared using 0.05 M of different buffer system (Glycine-HCl, pH 3.0; acetate buffer, 4.0 and 5.0; phosphate buffer pH 6.0 to 7.0; Tris-HCl, pH 8.0 to 9.0). The enzyme activity was determined at 40°C.

2.10 Effect of Temperature on Protease Activity

Protease activity was assayed by incubating the enzyme reaction mixture at different temperatures, 30°C to 80°C for 1 hour. The thermal stability at 50°C to 80°C was also determined. Samples were taken at 5-minute intervals and analyzed for protease activity.

2.11 Effect of Substrate Concentration on Protease Activity

The effect of substrate concentration [*S*] on the rate of enzyme action was studied using [*S*] values of 2.0 mg/ml to 10.0 mg/ml. The Lineweaver-Burk plot was made (Lineweaver and Burk 1934). Both V_{max} and K_m of the enzyme were calculated.

2.12 Effect of Heavy Metals on Enzyme Activity

A stock solution of 0.01 M of $HgCl_2$ and EDTA (ethylene diaminetetra acetic acid) were prepared. Two mL of each salt solution was mixed with 2 mL of substrate solution. The substrate/chemical mixture was incubated at room temperature for 5 minutes before it was used in enzyme assay.

2.13 Effect of Cations

A stock solution of 0.01 M of each salt was prepared. The effects of some salts/cations (NaCl, KCl, $CaCl_2$, and $FeCl_2$) on enzyme activity was also determined. The substrate/ salts mixture was also incubated before it was used for enzyme assay.

3. Results

Protease production at various incubation periods is shown in Fig. 1. The production was at its peak after about 12 hours of incubation. There was a decline after about 18 hours and this continued till about 30 hours of cultivation. Figure 2 shows the effect of various nitrogen sources on protease production by *Serratia marcescens*. Protease production was least when potassium nitrate was used as the nitrogen source, while the highest protease yield was obtained in the presence of casein. *Serratia marcescens* also had a high protease yield in basal medium amended with rice bran. Wheat bran and corn starch also gave fairly good protease yield (Fig. 3). The effect of temperature on protease production by *Serratia marcescens* is shown in Fig. 4.

The optimum temperature for protease production by the test isolate was found to be 40°C. The optimum pH for protease production by the test isolate was found to be 7. High protease yield was also obtained at pH 6, 8 and 9 (Fig. 5).

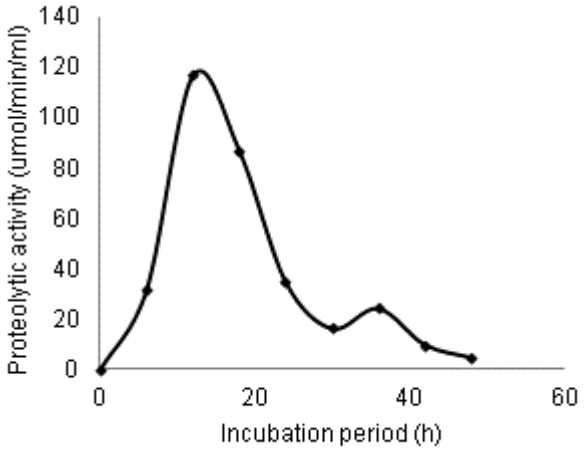


Fig. 1. Time course of *Serratia marcescens* extracellular protease production and activity.

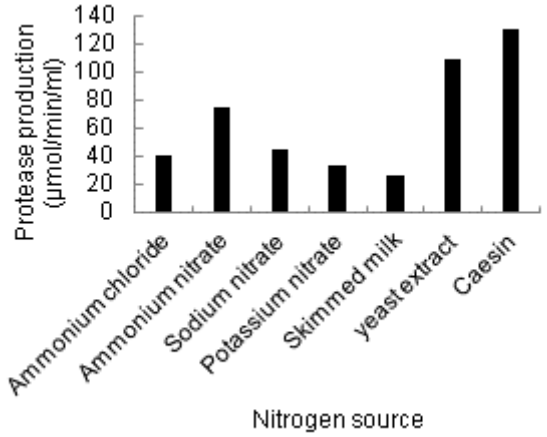


Fig. 2. Effect of nitrogen sources on protease production by *Serratia marcescens*.

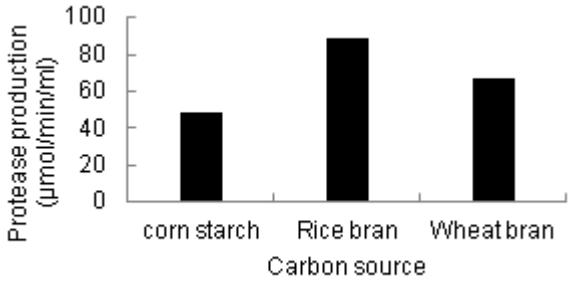


Fig. 3. Effect of carbon sources on protease production by *Serratia marcescens*.

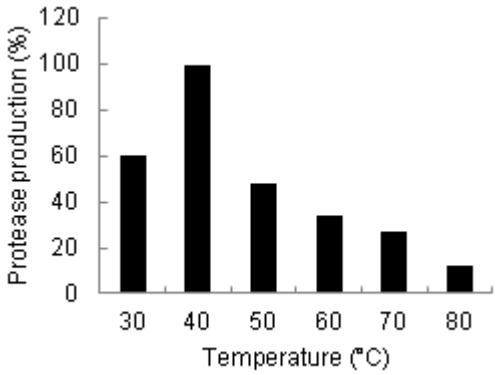


Fig. 4. Effect of temperature on protease production by *Serratia marcescens*.

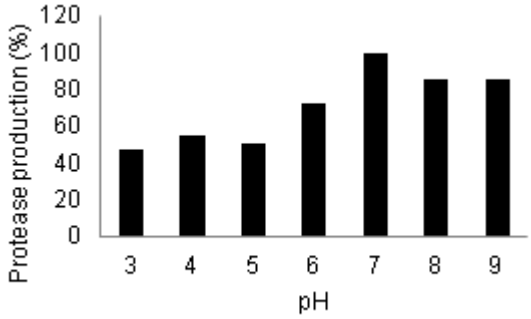


Fig. 5. Effect of pH on protease production by *Serratia marcescens*.

When the protein was adsorbed onto DEAE-Sephadex A-50 chromatography, the caseinolytic activity was recovered as a single sharp peak (Fig. 6). The sephadex G-150 gel filtration chromatography analysis of the molecular mass of the purified enzyme (Fig. 7) shows the greater proportion of proteolyitc activity eluting at 25 µmole/min/ml.

The summary of the purification process is shown in Table 1. After ammonium sulfate precipitation, a yield of 72.67% was obtained with a purification fold of 1.98. Ion exchange chromatography resulted in 30.03% yield with 3.31 purification fold while gel filtration gave a yield of 10.52% with 10.27 purification fold. The specific activity of the protease after ion exchange was 11.53, while gel filtration gave a partially purified protease of 35.70 µ/mg protein. From the linear semi logarithmic plot of V_e/V_o against relative molecular weight (Fig. 8), the native molecular weight was estimated to be about 56 kDa.

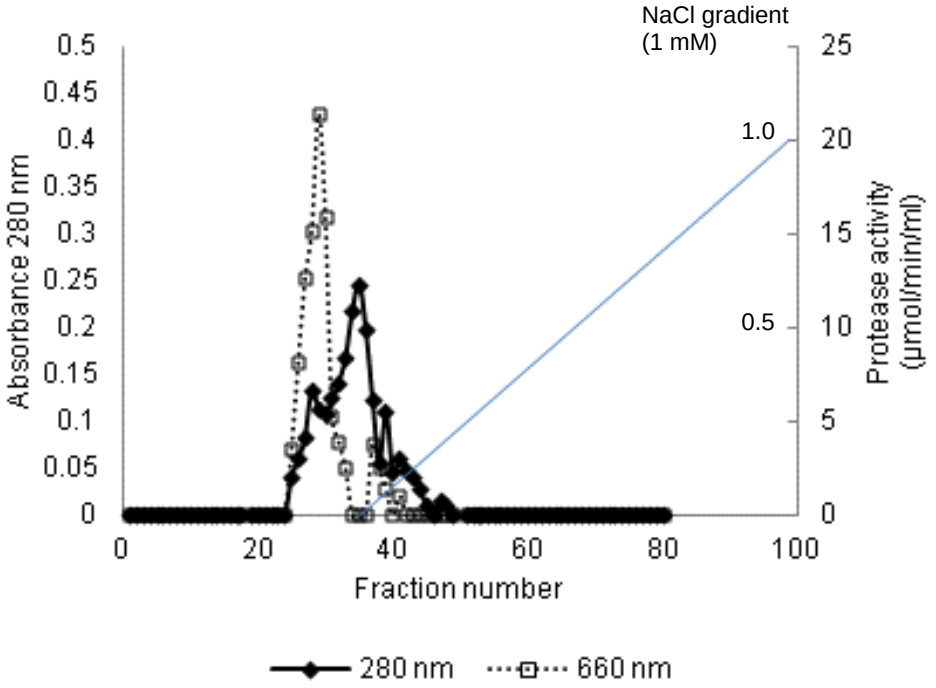


Fig. 6. Elution profile of *Serratia marcescens* extracellular protease from ion exchange (DEAE-Sephadex A-50) chromatograph.

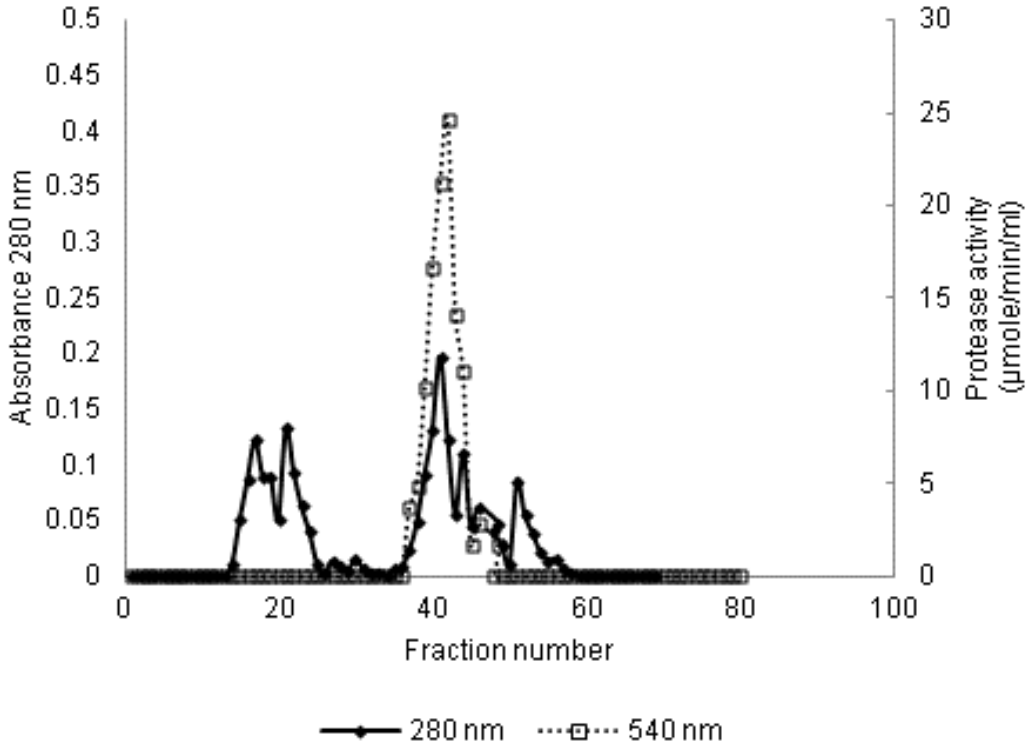
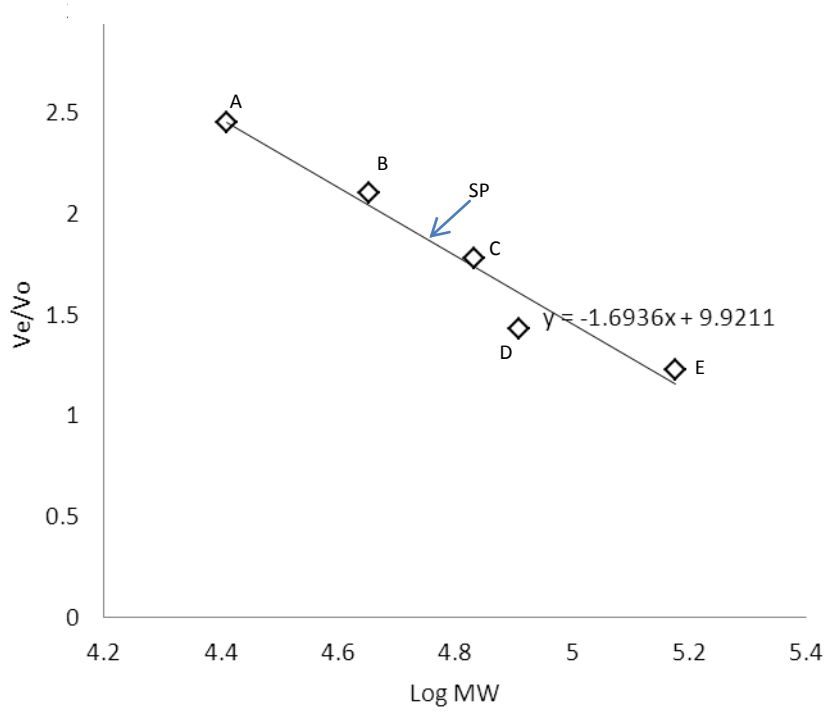


Fig. 7. Elution profile of *Serratia marcescens* extracellular protease from Sephadex G-150 gel filtration chromatography.

Table 1. Summary of purification of *Serratia marcescens* extracellular protease.

Fraction	Protease activity	Protein content	Total activity	Total protein	Specific activity	Yield (%)	Fold
Crude	162.82	46.87	16,282.0	4,687	3.47	100	1
Ammonium sulphate precipitation	182.05	26.4	11,833.2	1,716	6.89	72.67	1.98
Ion exchange	122.24	10.6	4,889.6	424	11.53	30.03	3.31
Gel filtration	114.26	3.2	1,713.9	48	35.70	10.52	10.27

Values represent the mean of duplicate determinations.



A - Alpha Chymotrypsinogen; B - Ovalbumin; C - Bovine serum Albumin;
D - Creatine Phosphokinase; and E - Gamma Globulin.

Fig. 8. Molecular mass determination of *Serratia marcescens* extracellular protease.

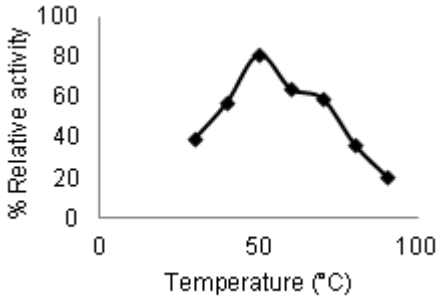


Fig. 9. Effect of temperature on *Serratia marcescens* extracellular protease activity.

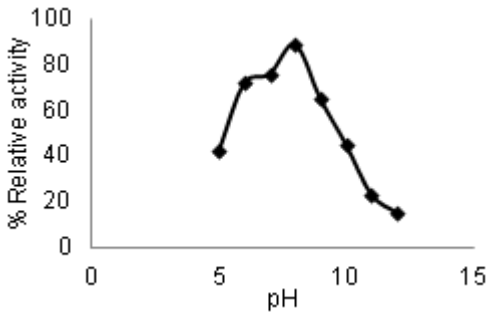


Fig. 10. Effect of pH on *Serratia marcescens* extracellular protease activity.

Figure 9 shows the effect of temperature on protease activity. Optimum protease activity was obtained at 50°C. At temperatures above and below these points, there was reduction in protease activity. The effect of pH on protease activity is shown in Fig. 10. Protease was active in pH range of 6.0 to with optimum at pH 8.0. No protease activity was obtained below pH 5.0 and at pH above 12.0. The effect of various salts on protease activity is shown in Fig. 11. Proteolytic activity was inhibited by EDTA, NaCl, $CaCl_2$, $FeCl_2$, and $HgCl_2$. Protease activity was however enhanced by KCl. From the Lineweaver Burk plot (Fig. 12), the K_m value was 0.91 mg/mL, while V_{max} for the protease was 40 mg/min/mL.

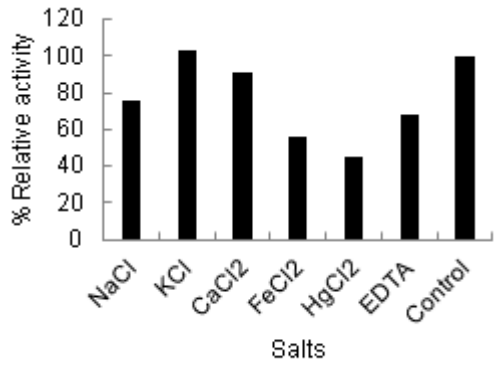


Fig. 11. Effect of salts on *Serratia marcescens* extracellular protease activity.

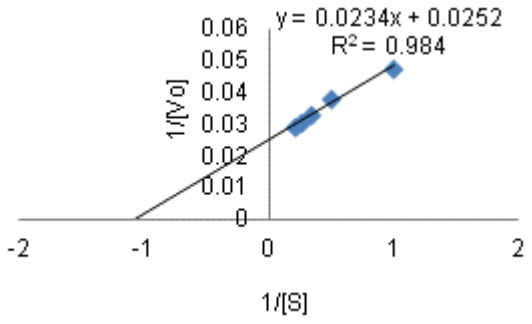


Fig. 12. Lineweaver-Burk plot of *Serratia marcescens* extracellular protease activity.

4. Discussion

In this work, protease was extracted from *Serratia marcescens* culture. Enzymatic activity of the protease was studied using casein as substrate. The optimal conditions for protease production were determined. The molecular mass of the protease was also determined using diethylaminoethanol (DEAE)-Sephadex A-50 and Sephadex G-150 gel filtration chromatography.

Proteolytic activity was first detected in *Serratia marcescens* culture supernatants towards the end of the exponential growth phase (after about 8 hours of incubation). This phenomenon of protease synthesis occurring at the end of the exponential growth phase is a common feature of many bacteria, including *Vibrio alginolyticus* (Long *et al*. 1981) and *Clostridium sporogenes* (Allison and Macfarlane 1990). Optimum protease production was obtained between 12 to 18 hours after incubation. This happens to fall within the lag phase of bacterial growth and is possibly responsible (Henriette *et al*. 1993). The composition of the culture medium had no effect on the onset of protease production. However, protease synthesis seemed to be regulated in some way, as it occurred only after active growth was about to cease. A gradual decrease in enzyme units was observed with increase in incubation period, clearly suggesting that the enzyme production is growth associated in nature.

The results indicated that the best nitrogen source for protease production by *Serratia marcescens* was casein. Several investigators have studied the effect of nitrogen sources on enzyme productivity. Marine *Pseudomonas* strain 1452 having the ability to produce extracellular protease uses casein, as the nitrogen and carbon source (Makino *et al*. 1981). Yeast extract also produced a large amount of protease. However, ammonium chloride, ammonium nitrate, sodium nitrate, skimmed milk, and potassium nitrate produced a poor protease yield when used as nitrogen source. This result is in accordance with that of Nguyen and Quyen (2011) who reported that optimum protease production was achieved when casein was used as substrate and that the production was low with ammonium nitrate and urea.

Certain carbohydrates were introduced as carbon sources into the production medium. The result obtained indicated that Rice bran was the best carbon source that induced the production of protease by *Serratia marcescens*

in production medium. However, wheat bran and corn starch also produced a high yield. In a study carried out by Nguyen and Quyen (2011), it was observed that enzyme production was maximal when potato starch or yeast extract was used as the carbon source and that protease production decreased in media containing another carbon source including sucrose, lactose and glucose by 8-26% in comparison to that with potato starch.

The optimum temperature of protease production was 40°C. Many investigators have studied the relation of temperatures and enzyme production. The optimum temperature for enzyme production was observed to range from 2°C to 70°C or more depending on the type of organism, the medium conditions and the type of enzyme. Secades *et al*. (2001), observed the same results that the optimum temperature for an extracellular protease produced by *Flavobacterium psychrophilum* was at temperatures between 25°C and 40°C. Jobin and Grenier (2003) investigated the production of proteases by *Streptococcus suis* serotype 2 and recorded that the optimum temperature for protease production ranged from 25°C to 42°C. Kumara swamy *et al*. (2012) reported an optimum temperature of 47°C for protease production by *Bacillus* spp.

The production medium was adjusted to different pH values using phosphate buffer of various pH. The present study showed that the optimum pH for protease production by *Serratia marcescens* was pH 7. Similarly, the optimal pH of protease activity produced by *Clostridium bifermentans* NCTC 2914 (National Collection of Type Cultures (NCTC), Public Health England, Porton Down, Salisbury, Wiltshire, England, UK) was 7 (Macfarlane and Macfarlane 1992). Moreover, investigating the production of proteases by *Streptococcus suis* serotype 2, the proteases were identified and characterized using chromogenic and fluorogenic assays and zymography. The optimum pH for all four proteases was between 6 and 8 (Jobin and Grenier 2003). An optimum pH of 11 reported by Johnvesly *et al*. (2002) when working on extra cellular thermo-stable protease produced by thermo-alkaliphilic *Bacillus* spp. JB-99 was contrary to other observations.

In general, the molecular masses of previously found proteases are rarely more than 50 kDa (Klingeberg *et al*. 1995). In this study, the purified protease turned out to be one polypeptide chain with a native molecular weight of 56 kDa (averages of the values obtained by DEAE-Sephadex A-50 chromatography) (Laemmli 1970). This is similar to the findings of Tariq *et al*. (2011). The characterization of the protease showed that the optimum pH for proteolytic activity was 8.0. This value is in accordance to those reported by Tariq *et al*. (2011) and Doddapaneni *et al*. (2007). Enzymatic activity was inhibited by NaCl, $FeCl_2$, $HgCl_2$ and EDTA (Tariq *et al*. 2011) and was activated by KCl thus; it can be classified as a metalloprotease (Aiyappa and Haris 1976). The optimum temperature was found to be 50°C. Enzyme activity was lost at temperature below and above 30°C and 90°C, respectively. This shows that the enzyme is active over a wide temperature range.

5. Conclusion

In conclusion, *Serratia marcescens* was able to produce protease enzyme on growth medium and showed optimum activity at pH 8.0 and temperature of 50°C. The best carbon and nitrogen sources were found to be rice bran and casein. The genetic manipulation of this organism is required to increase productivity and then it can be used for protease production on industrial scale. Further work should also be done on the use of rice bran for optimization of protease production by *Serratia marcescens* as it is a cheap and readily available substrate. However western blot analysis, electron microscopic studies and structural studies have to be performed to elucidate the exact nature of the protein.

6. References

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