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Isolation, Purification, and Characterization of Serratiopeptidase Enzyme from *Serratia marcescens*

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Abstract:- Serratiopeptidase is a proteolytic enzyme that is derived from a member of Enterobacteriaceae. *Serratia marcescens* is a gram-negative bacteria identified characteristically, which produces a red pigment called prodigiosin. Serratiopeptidase is a multi-functional proteolytic enzyme that dissolves non-living tissues such as fibrin, blood clots, inflammation in all forms without harming living tissues. In this study, the organism was isolated from the diseased silkworm's pupa by using Luria- Bertani (LB) agar media. The enzyme production can be enhanced by applying different physical and chemical parameters. *Serratia marcescens* was subjected to production such that in order to obtain the maximum level of cell-free supernatant Serratiopeptidase enzyme with all the optimized conditions. The enzyme was subjected to purification by four methods such as salt precipitation, dialysis, ion-exchange chromatography and gel filtration. When subjected to enzyme kinetics, Serratiopeptidase was active at temperature 35^{0}C, pH-9 with 8 minutes of the incubation period. The molecular weight of serratiopeptidase by SDS-PAGE was found to be between 50-55kDa.

Keywords:- *Serratiopeptidase, Serratia marcescens, Silkworm pupa, Luria – Bertani, Ion-exchange chromatography, gel filtration.*

I. INTRODUCTION

Serratia marcescens is a Gram-negative bacterium, classified under the large family of Enterobacteriaceae[1]. As a human pathogen, *S. marcescens* is involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections and wound infections. *S. marcescens* is a motile organism and can grow in temperature ranging from 30^o-40°C and in pH levels ranging from 5 to 9. It is differentiated from other gram-negative bacteria by its ability to perform casein hydrolysis, which allows it to produce extracellular metalloproteinases which are believed to function in a cell to extracellular matrix interactions. Some strains of *S. marcescens* are capable of producing prodigiosin pigment, which ranges in color from dark red to pale pink, counting on the age of the colonies.

Serratiopeptidase, also known as Serrapeptase or Serratia peptidase, is a proteolytic enzyme isolated from the non-pathogenic Enterobacteriaceae *Serratia marcescens*[2]. This enzyme is naturally present in the silkworm intestine. The enzyme in its natural form is utilized by the silkworm to dissolve the cocoons and emerge as moths outside.

This enzyme was discovered by German physician Dr. Hans Niper, he discovered it as a "miracle enzyme" for its ability to treat arterial blockage in patients full of arterial coronary disease[3]. The controlled fermentation of *Serratia sp.* secrets serratiopeptidase enzyme within the highly selective medium. Enzyme purification was achieved by ammonium sulfate fractionation and DEAE-cellulose chromatography.

Serrapeptidase is a 50-55kDa, alkaline metalloprotease that works to activate the Hageman factor-kallikrein-kinin systems of mammals and directly degrades or inhibits IgG and IgA immune factors as well as the regulatory proteins α-2-macroglobulin, α-2-antiplasmin, and antithrombin III[4]. The recovery process involves various steps of filtration, concentration and steps to form enzymes useful for pharmaceutical applications.

Serratiopeptidase possesses benefits in Circulatory disorders and Systemic yeast Infection, Fibromyalgia-Arthritis- Chronic Joint Pain, Chronic Pain Inflammation, Autoimmune Diseases, Bladder Infections, Fibrocystic Breast Disease, Chronic Fatigue and Clogged Arteries-Fibroids Spider Veins -Viral Infection.

II. MATERIALS AND METHODS

Collection of sample: Dead and Diseased pupa of silkworm of Bombyx mori Breed were collected (in an air-locked box with some mulberry leaves) from Kolar and Ramanagara District, Karnataka, India.

The isolated microorganism was identified by gram staining, biochemical test and screening [5]. The screening was done to check for a clear transparent zone around the streak to know that casein is hydrolyzed. The stock culture was maintained in LB agar media and allowed to grow at 37^{0}C overnight before being used.

- *Production:*

For the production of Serratiopeptidase enzyme LB broth was used which contains Tryptone(10g/l), sodium chloride (10g/l), yeast extract (0.6g/l), pH-9. The medium was sterilized in an autoclave at 121^{0}C for 15lbs before adding the bacterial inoculum. (500ml flask with 200ml medium,4% of inoculum size kept in an orbital shaker for 48hours at 35^{0}C). After the incubation time for 48hours, the LB broth was centrifuged at 6000rpm for 10minutes. The

supernatant was collected in a beaker for the purification process and the pellet was discarded.[6]

➢ *Purification of enzyme*

Salt precipitation (Ammonium sulfate): The collected supernatant in the beaker was taken for the purification step. From this, 2ml of supernatant was collected in an Eppendorf tube and labeled as crude. The remaining supernatant was measured and poured in a beaker to this magnetic bead was added. The beaker is kept on a stirrer with an ice pack placed in the bowl. 70% saturated ammonium sulfate was used for purification. 66g of ammonium sulfate was added pinch by pinch in 150ml of obtained supernatant in ice-cold condition for 3-4hours. Later salt suspension was stored in 4^0C overnight.[7],[8]

Dialysis: Dialysis is carried out using the cellulose acetate membrane. The salt suspension was centrifuged at 10000rpm for 10minutes to remove small molecules from crude enzyme and pellet was collected using 10mM Tris HCL(10ml) and poured into dialysis bag and kept in magnetic stirrer for 2-3 hours by changing the distilled water at every interval of time. Dialysis is the process of separation of substance in the solution by means of their unequal diffusion through the semipermeable membrane. The solution was placed in a dialysis tube to remove the low molecular weight proteins[9]

Ion-exchange chromatography: The column was filled by DEAE(Diethyl aminoethyl- anion exchange) cellulose gel. It was left to settle down and 7 different illusions using Tris-HCL, NaCl and autoclaved water were prepared. Dialysis sample was added to the upper layer of the sample and elution buffer was added one by one and collected in their respective tubes.[10]

Gel filtration: The column was filled with Sephadex G-75 gel and allowed to settle down. Selected elution buffer and phosphate buffer was added to the column and collected in 25 different Eppendorf tubes. (i.e., 1ml each in 25 Eppendorf tubes).

Protease enzyme assay standard: To determine the enzyme activity, where one unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine per mL per minute. The amount of protein in enzyme extract was measured before and after purification graph is shown in Fig 1[11],[12]

Estimation of Protein by Lowry's method: The aliquots of BSA from 0.2-1.0ml of BA working standard in 5 test tube and makeup to 1ml using distilled water, the test tube with 1ml distilled water serve as blank, add 5ml of alkaline copper reagent (Reagent C) and incubate for 10 minutes. After incubation add 0.5ml of FC reagent and incubate for 30 minutes in dark condition, measure the absorbance at 660nm. The graph is shown in Fig 2[13]

Proteases Enzyme assay:0.1 M Potassium Phosphate buffer (a. KH_2PO_4: 25ml of this is prepared b. K_2HPO_4: 25ml of this is prepared. add a to b dropwise to make pH-7),0.654% casein was added to the phosphate buffer, TCA(6.1N) was prepared, Na_2CO_3 (0.5M) was prepared, Folin- Ciocalteau. 5ml of phosphate buffer without substrate was added to test tube and name as Blank, 5ml of substrate buffer was added to all other tubes, all the tubes were incubated, 5minutes at 37^0C for equilibrium, 0.1ml of enzyme supernatant was added, tubes were incubated at 37^0C for 10minutes, 0.5ml of 6.1N TCA and 0.5ml of phosphate buffer was added to blank and incubated for 30minutes at 37^0C, The samples were filtered and 2ml of the filtrate was transferred to another set of test tubes, 5ml of Na_2CO_3 was added and 0.5ml of Folin-Ciocaltaue reagent was added to all the test tubes and was incubated in dark for 30minutes. The absorbance was measured at 660nm.[14]

➢ *Calculation of enzyme activity*

After the standard graph is obtained, the concentration of tyrosine is calculated in µg/ml, this value is converted to µmoles/ml using the following formula:

$$\frac{amount\ of\ enzyme\ in\ \mu g \times Molecular\ Weight\ of\ Tyrosine \times Volume}{1000}$$

The activity of the enzyme is calculated by the formula and results are tabulated in table 2:

$$\frac{\mu\ mole\ of\ L-tyrosine\ liberated \times Total\ volume\ of\ assay \times Dilution\ factor}{Volume\ of\ enzyme \times Volume\ of\ assay \times incubation\ time}$$

After finding the concentration of protein in the enzyme sample, the specific activity, fold purification, percentage yield of each purified sample was determined using the following formula and results are tabulated in Table 2

$$\textbf{\textit{Specific activity}} = \frac{\textbf{Enzyme activity in units/ml}}{\textbf{Protein concentration of samplen mg/ml}}$$

$$\textbf{\textit{Fold of purification}} = \frac{\textbf{Specific activity of sample}}{\textbf{Specific activity of crude sample}}$$

$$\textbf{\textit{Percentage yield}} = \frac{\textbf{Enzyme activity of sample}}{\textbf{Enzyme activity of crude sample}}$$

➢ *Characterization*

Characterization is the study of the chemical reactions that are catalyzed by enzymes. Characterization was performed using purified enzymes and carried out with protease assay[15]

- *Effect of pH on Enzyme activity*

10ml of sodium acetate was prepared and pH 5 and pH 6 were set, 10ml of phosphate buffer with pH 7 and pH 8 was prepared and 10ml of glycine buffer with pH 9 and pH 10 was prepared. After this casein was added to all buffer and 100μl enzyme was added. The enzyme assay was carried out to determine the enzymatic activity and the results are shown in Fig 3.

- *Effect of temperature on enzyme activity*

A phosphate buffer was prepared and set at optimum pH 9. Casein was added to all the test tubes with different temperatures. The temperature for incubation was set at 25^0C, 30^0C, 35^0C, 40^0C, 45^0C, 50^0C, 55^0C. 100μl enzyme was added and enzyme assay was performed. The results are shown in Fig 4.

- *Effect of incubation time on enzyme activity*

Phosphate buffer was prepared and set at optimum pH 9. Casein was added and pasteurized at 75-80^0C for 30minutes. The optimum conditions for incubation time were 4min, 8min, 12min, 16min, 20min, 24min and 28min respectively. 100μl enzyme was added and enzyme assay was performed. The results are shown in Fig 5.

- *Effect of substrate concentration on enzyme activity*

The substrate was casein taken with different concentrations i.e.,0.2%, 0.4%, 0.6%, 0.8% and 1.0% and added to the phosphate buffer and all the optimum conditions were maintained. 100μl of the enzyme was added and enzyme assay was performed. The results are shown in Fig 6.

➢ *Molecular weight determination by SDS-PAGE*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by preparing electrode buffer, separating gel buffer, stacking gel buffer, stock, freshly prepared ammonium persulphate (APS) and SDS, staining solution, destaining solution and SDS loading dye. First, 15% separating gel was prepared followed by stacking gel, then sonicated for 15 minutes, 0.050 ml APS, 0.100ml SDS, 0.010 ml TEMED were added into separating gel, then immediately poured into the space between the base plate and notch plate. Allowed it for solidification. Mixed 0.1 ml of each sample such as BSA, crude, salt, dialysis and ion exchange with 0.1 ml of loading dye. Kept it in a water bath for 10 minutes at 85°C Then stacking gel was added after mixing it with TEMED, and the comb was placed. After solidification, the comb was removed and samples were loaded and kept in electrophoretic apparatus of voltage 50V for 6 hours. Then the gel was kept in staining solution for 24 hours. After that, it was kept in the destaining solution. Finally, the molecular weight of the samples was determined.[16]

III. RESULTS AND DISCUSSION

➢ *Collection of sample:* The silkworms (Bombyx mori) were collected from Kolar and Ramanagara district, Karnataka. India. The enzyme was identified to be gram-negative bacteria.

Colour	Red
Shape	Circular
Elevation	Flat
Opacity	Opaque

Table 1:- Colony Characterization

➢ *Protease enzyme standard assay*

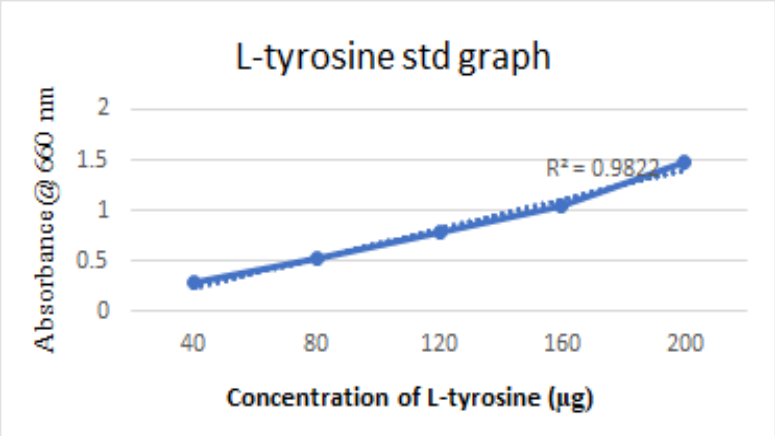

Fig 1:- The graph showing the standard of L-tyrosine

➢ *Protein estimation:*

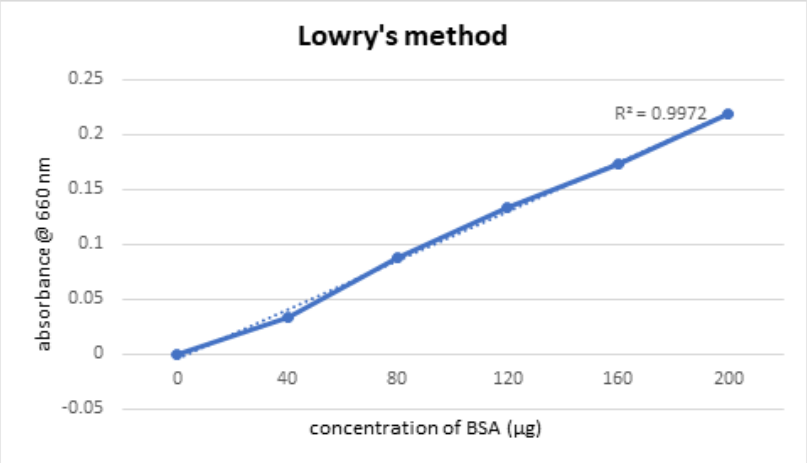

Fig 2:- The graph showing the standard of BSA

➢ *Characterization*:

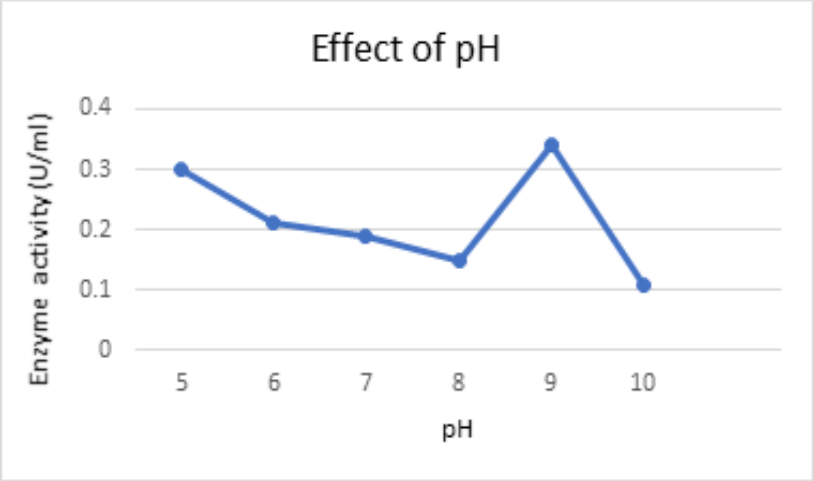

Fig 3:- Enzyme activity at various pH

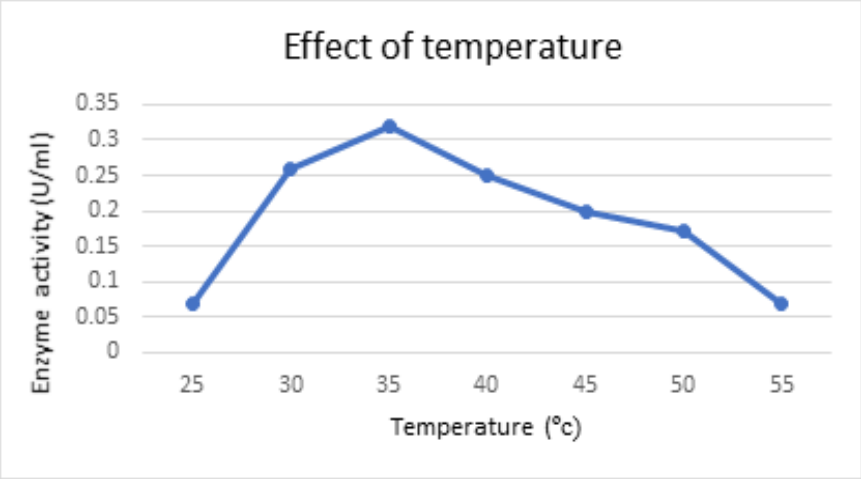

Fig 4:- Enzyme activity at different temperature

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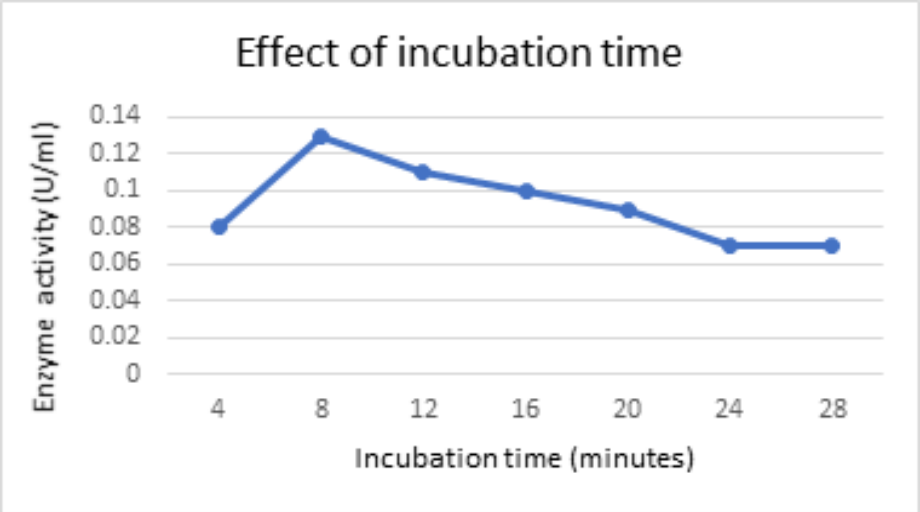

Fig 5:- Enzyme activity at different incubation time

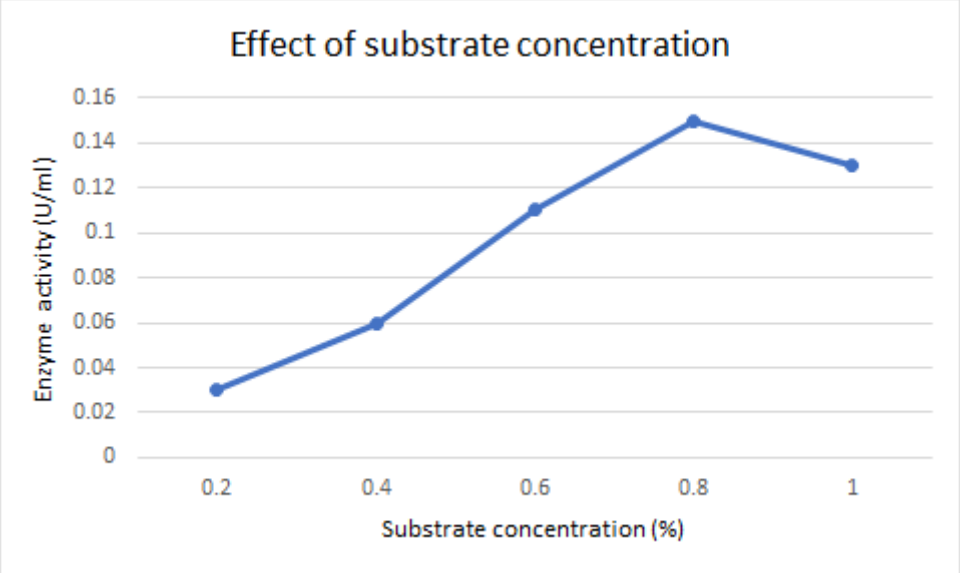

Fig 6:- Enzyme activity at a different substrate concentration

➢ *SDS-PAGE*

The molecular weight of the Serratiopeptidase enzyme was found to be 55kDa based on std. BSA marker.

Samples	Protein conc. (mg/ml)	Enzyme activity (U/ml)	Specific activity (U/ml)	Fold	Yield (%)
Crude	**3.46**	**0.089**	**0.025**	**1**	**100**
Salt precipitation	**4.28**	**0.082**	**0.019**	**0.21**	**92.1**
Dialysis	**3.12**	**0.070**	**0.022**	**0.24**	**78.6**
IEC	**1.97**	**0.046**	**0.023**	**0.5**	**51.6**
GFC	**1.72**	**0.034**	**0.019**	**0.21**	**38.2**

Table 2:- Enzyme purification and activity table

IV. CONCLUSION

The microorganism used for the production was the bacterial strain of *Serratia marcescens*. Serratiopeptidase is being used in many clinical studies against various diseases for its anti-bacterial, analgesic, anti-inflammatory and fibrinolytic effects. Although many bacteria produce serratiopeptidase, *Serratia marcescens* is the best producer of Serratiopeptidase. An attempt was made towards finding the best growth conditions for the successful cultivation of Serratiopeptidase Enzyme. The enzyme activity was checked at every stage of Purification. Characterization was done to study the optimum conditions for the enzyme activity. Achieving maximum enzyme activity at physiological temperature makes this enzyme extremely valuable in the chemotherapeutic treatment of pain and inflammations.

The apparent molecular weight of Serratiopeptidase by SDS-PAGE was found to be 55kDa. Furthermore, studies have to be made on the application of Serratiopeptidase for treating various diseases.

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