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Research Article

OPTIMISATION STUDIES IN THE PRODUCTION AND PURIFICATION OF SERRATIOPEPTIDASE FROM *Serratia marscecens* UV MUTANT SM3

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ABSTRACT

Objective: Serratiopeptidase (SRP) is a proteolylic enzyme with wide medical applications. *Serratia marcescens* strain was UV mutated and maximum SRP producing UV mutant SM3 was selected by its caseinolytic property.

Methods: Batch production studies were carried out by varying carbon and nitrogen sources. The carbon sources used were maltose, starch, glucose and lactose. The nitrogen sources used were soya bean meal, tryptone, peptone and yeast extract. The initial pH of the medium containing maltose and tryptone was varied from 4 to 9. Purification of SRP was done by ammonium sulphate precipitation with various saturation conditions from 30% to 80%.

Results: The production medium containing tryptone, with maltose as carbon source gave 36,415EU/mg at 68th hr. The production medium containing maltose as carbon source gave 32,575EU/mg at 68th hr. which is far greater than the maximum yield already reported The media with initial pH 7 gave an SRP production of 37,060EU/mg. At 40% saturation, the specific enzyme activity of the pellet obtained was found to be 63,623EU/mg. The dialysed sample gave specific enzyme activity of 1,90,451EU/mg. The characterization of the enzyme was done by SDS PAGE.

Conclusion: From the results it is concluded that the production of serratiopeptidase enzyme is maximum from the UV mutated strain of *Serratia marcescens*.

Keywords: Serratiopeptidase, *Serratia marcescens*, UV mutation, Batch production studies, Optimization, SDS PAGE.

INTRODUCTION

Serratiopeptidase or serrapeptase or serralysin is a proteolytic enzyme that has been widely used successfully for pain and inflammation [1, 2] due to arthritis [3], trauma, surgery, sinusitis, bronchitis [4], carpal tunnel and painful swelling of the breasts. There is some preliminary indication that it may be useful for atherosclerosis. This enzyme is absorbed through the intestines and transported directly into the bloodstream.

SRP is known to be produced extracellular in submerged and solid-state fermentation by the bacterium *S. marcescens*. Research on the production, purification, and characterization of SRP from different strains of *S. marcescens* is already well documented. Production of SRP by *S. marcescens* is greatly affected by the constituents of the culture media, especially the organic nitrogen source and various studies have reported [5, 6] high quantities of metalloprotease [7] when using these types of media.

There are no reports in the production of SRP by using mutant strain of S. marcescens. Even though many studies were carried out in the production and characterization of SRP from S.marcescens, optimization in purification was not yet documented. The present study aims to increase the productivity of SRP in batch strategy by optimizing the media composition [8] and to increase the purity of the enzyme by doing optimization in purification aspect [9]. Three different strategies were used for batch fermentation, by optimizing carbon source first and nitrogen source followed by initial pH of the optimized media.

MATERIALS AND METHODS

Enzyme Standard

Commercially available serratiopeptidase enzyme tablets marketed by Glaxo Smithkline India limited with the brand name of Bidazen 5mg, equivalent of 10,000EU was used for standard enzyme studies. All chemicals and media were brought from Himedia chemicals India.

Microorganism

MTCC strain no 7298 *Serratia marcescens* bought from IMTECH Chandigarh and retrieved by prescribed procedure on Nutrient agar (without glucose) as minimal medium. [10, 11].

SRP Assay Method

Serratiopeptidase enzyme assay was carried out as described by Salmone and Wodzinsiki [12] with minor modification by SRP caseinolytic property. 0.75 ml consisting of 1.0% (w/v) casein in100 mM Tris/HCl, 1 mM $MgCl_2$, and 2 mM PMSF at pH 8.0. 0.1 ml of the assay sample (cell-free centrifuged supernatant, appropriately diluted with 50 mM Tris/HCl, pH 8 buffer) was added, and the mixture was incubated at 40°C. After 30 min, the reaction was quenched with 0.5 ml of 10% (w/v) TCA to precipitate the unhydrolyzed casein. After 15 min at 25°C, the reaction mixture was centrifuged at 10,000 ×g for 10 min and the supernatant tyrosine concentration were determined at 660 nm by Lowry's method [13] .One unit of enzyme activity (EU) was defined as the amount of enzyme that produced an increase in absorbance of 0.1 at 660 nm under the conditions of the assay. SRP standard was determined using commercially available tablet [14].

UV Mutation

The MTCC strain of *Serratia marcescens* was used for mutation [15, 16]. *S. marcescens* was first grown on the nutrient agar plate for 24 hr. Cells were scraped and suspended in sterile saline, and then diluted to a concentration range of 10^5–10^7 cells/ml. Under sterile condition, the diluted cells were exposed to ultra violet rays of 15 W ultra violet lamp for 20 s with mild agitation at a distance of 30 cm to give a survival rate of about 15%.

Mutant Selection

Mutants were selected on the basis of zone of proteolysis on skim milk agar plates [17, 18]; three strains were selected UV mutant SM1, SM2 and SM3. Strains were compared with parent strain for serratiopeptidase enzyme activity in shake flask culture. High enzyme producing strain at 24th hour was selected for further studies.

Production Medium

In batch production, the medium reported by Pansuriya and Singhal [19] was used, which contained maltose 45 g/l, soybean meal 65 g/l, KH_2PO_4 8.0 g/l, and NaCl 5.0 g/l, pH 7.0. The medium was sterilized in an autoclave for 15 min at 121°C (500ml flask with 150ml

medium, 2%inoculum size 200rpm orbital shaker). This was used as production medium of SRP.

Batch Fermentation Strategy

All batch experiments were carried out with 250ml shaker flask with initial volume of 100ml medium before inoculation (250ml flask with 100ml medium, 2% inoculum size, 200rpm orbital shaker). Inoculum used was 24 hr seed culture grown in nutrient broth. The carbon source in the production media was varied. The different carbon sources used were maltose, starch, glucose and lactose. After optimizing carbon source, the nitrogen sources were varied. Tryptone, soybean meal, peptone and yeast extract were used as nitrogen sources with optimized carbon source (maltose). The initial pH conditions were varied from 4 to 9 in the optimized media.

Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was carried out by differing the concentration of ammonium sulphate from 30% to 80% saturation. Maximum yield of enzyme phase of fermentation broth was harvested and centrifuged at 10,000rpm for 20 min at 4°c and cell free supernatant was collected. The salt was added slowly to the supernatant, with gentle stirring, according to the saturation concentration. After standing for 4 h at 4°C, the precipitates were collected by centrifugation at 11,000rpm for 20 min, dissolved in about 500 ml of cooled distilled water [20,21].

Dialysis

Dialysis was done for the sample treated with ammonium sulphate at 40% saturation. Dialysis was carried out for 24 h with 1 l of distilled water and finally twice with 1 l of 10 mM Tris hydrochloride buffer (pH 8.3).

SDS PAGE

SDS PAGE was performed by 12% gel [22]. Each 10µl of 48hr crude sample, ammonium sulphate sample and dialysed sample were loaded in separate lanes and compared with standard protein marker brought from Genei India limited were run using Genei power pack system.

RESULTS AND DISCUSSION

UV mutant SM3 showed higher proteolytic activity of 1575.3 EU/ml and results were shown in Table 1. Batch production was carried out using different carbon sources and maltose was found to be best carbon source (Chart 1). The production medium containing maltose as carbon source gave 32,575EU/mg at 68th hr (Chart 2), which is far greater than the maximum yield already reported. While varying nitrogen sources, tryptone produces maximum amount of SRP (Chart 3). The production medium containing tryptone, with maltose as carbon source gave 36,415EU/mg at 68th hr (Chart 4). pH 7 was found to be optimized condition for maximum yield of SRP (Chart 5) and the optimized media with initial pH 7 gave an SRP production of 37,060EU/mg (Chart 6).

When compared with other batch production documented earlier [4,19], maximum SRP production of 11,580 EU/ml with an agitation of 400 rpm and aeration of 0.075 vvm, in comparison with our mutant strain, we have obtained higher yield of 32,275 EU/mg in 68th hr by batch mode in shaker flask study. Scaling up our study with aeration and agitation can give higher yield of SRP activity.

Ammonium sulphate precipitation

Ammonium sulphate was added slowly to the supernatant, with gentle stirring, from 30 to 80% saturation. The maximum specific enzyme activity of the pellet was found to be 63,623 EU/mg at 40% saturation (Chart 7).

Dialysis

Dialysis was carried out for the sample purified by ammonium sulphate precipitation at 40%saturation. Dialysed samples gave specific enzyme activity of 1,90,451 EU/mg (Table 2).

Table 1: Mutant Selection

Strain	Specific Enzyme Activity (EU/ml)
SM 1	1396.87
SM 2	1444.04
SM 3	1575.3
Parent strain	1107.3

SDS PAGE

Gel was prepared as prescribed by Hames B.D [17], SRP was characterised by SDS PAGE using 48hr crude sample, 40% saturated ammonium sulphate sample and dialysed sample along with standard protein marker as shown in the figure 1.

Table 2: Purification

Purification steps	Volume in ml	Total recovery		Specific activity U/mg
		Protein in mg	SRP activity in EU	
Cell free supernatant	100	43.17	1600.16	37060
40% $(NH_4)_2SO_4$ precipitation	15	19.94	1268.64	63623
Dialysis	5	6.39	1217.52	190451

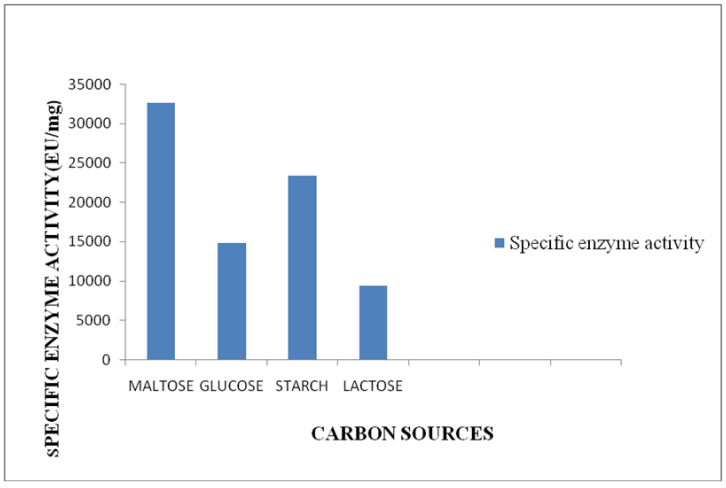

Fig. 1: Different Carbon Sources with Specific Enzyme Activity

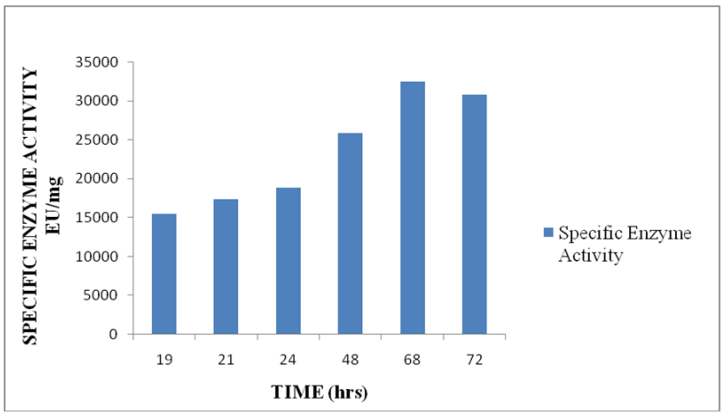

Fig. 2: Specific Enzyme Activity for Maltose

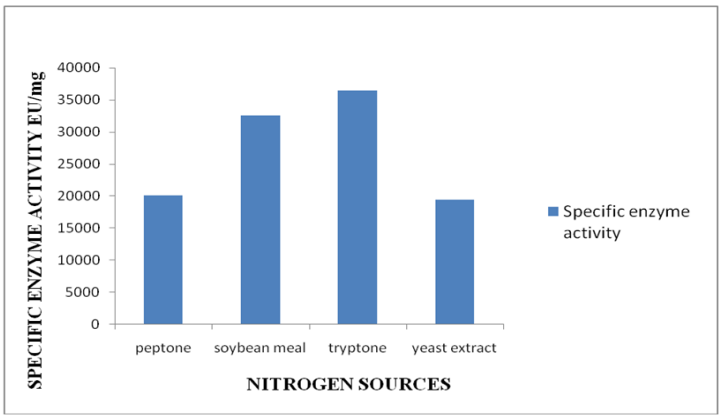

Fig. 3: Different Nitrogen Sources with Specific Enzyme Activity

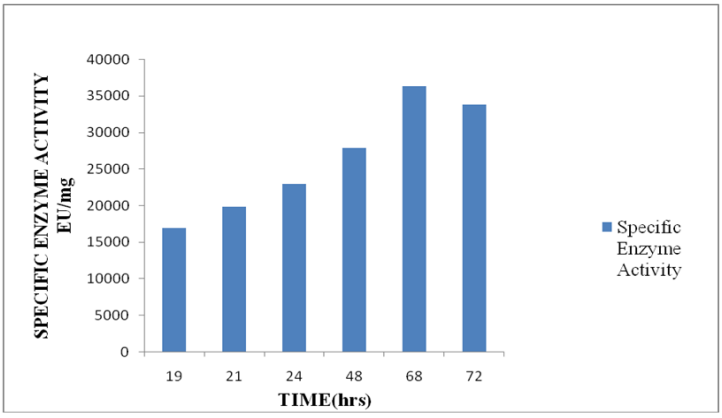

Fig. 4: Specific Enzyme Activity for Tryptone

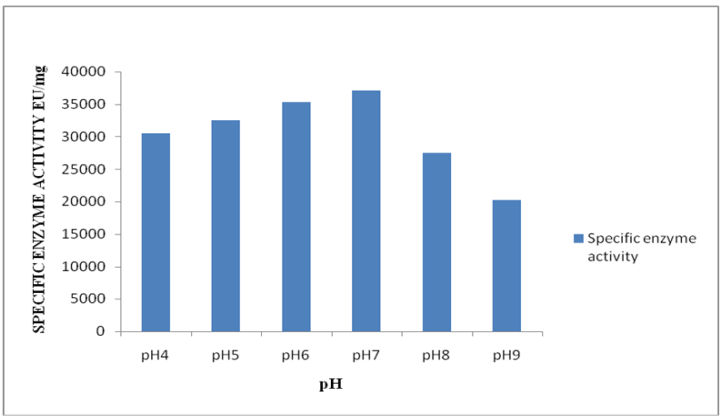

Fig. 5: pH with Specific Enzyme Activity

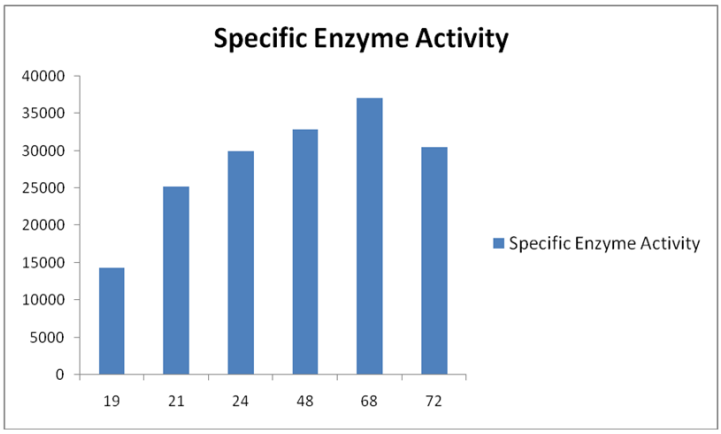

Fig. 6: Specific Enzyme Activity For pH 7

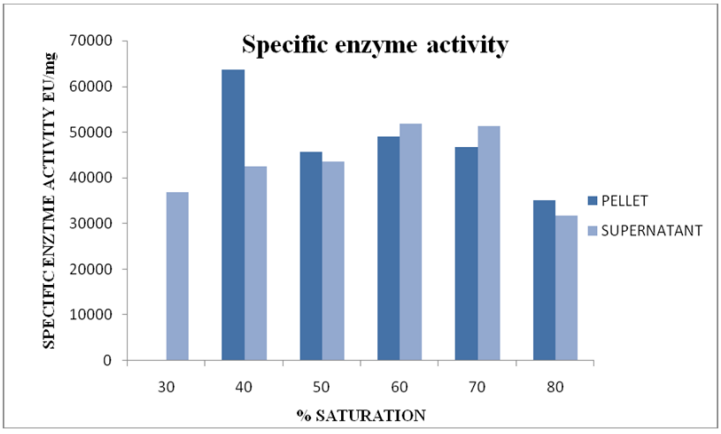

Fig. 7: % Ammonium Sulphate Saturation with Specific Enzyme Activity

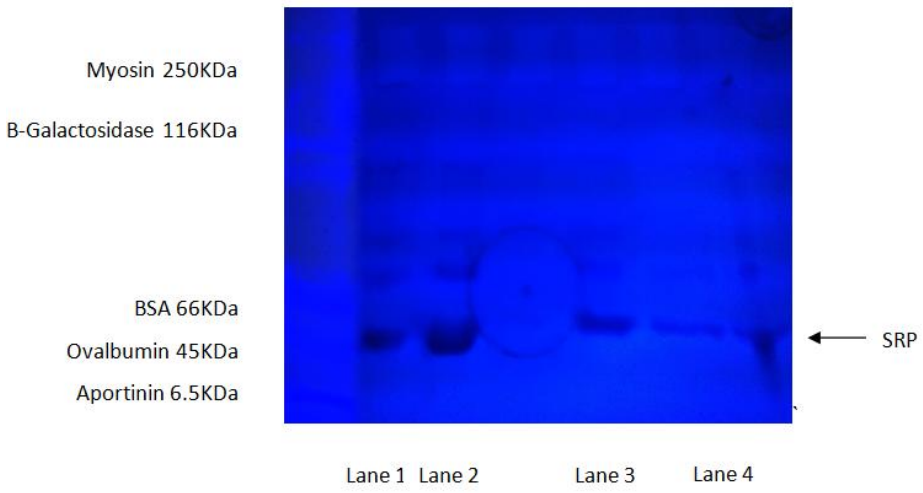

Fig. 8: SDS PAGE with CBB Staining

Lane 1- Standard protein marker, lane 2 - 48hr crude sample, lane 3 - ammonium sulphate sample, lane 4- dialysed sample.

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