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Pilot scale microbial production and optimization of *Serratia peptidase* from *Serratia marcescens*

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Abstract
Serratia peptidase is active proteolytic enzyme which has the potential of cleaving peptide bond. Present investigation deals about the Microbial production of serratia peptidase using *Serratia marcescens* in small scale fermentor. Batch fermentor has been run continuously throughout the night to analyze the production of protein as well as kinetics. Culture broth was maintained at 150rpm for 72 hrs. Protein sample was isolated by centrifuging at 3000rpm for 10mints. The result revealed that *Serratia marcescens* showed the enormous production of protein in fed batch fermentor compared to the small scale level. Different substrates were been used for the production of enzyme. Among all cysteine showed the better activity as 2 units/ml of enzyme. Enzymatic assay of *Serratia peptidase* was done at different time interval of crude broth. Enzyme activity showed that maximum at 40ºC for 72hrs. It was observed that 0.65 units/ml of enzyme. Fed batch pilot scale production of *Serratia peptidase* was done at 0.5%cystein and 700rpm for 48hrs of run time.

Keywords: *Serratia marcescens*, *Serratia peptidase,* casein.

INTRODUCTION

The natural Chelation-Anti-Inflammatory *Serratia peptidase* has had wide clinical use - spanning over twenty-five years throughout Europe and Asia - as a viable alternative to salicylates, ibuprofen and the more potent NSAIDs. Unlike these drugs, *Serratia peptidase* is a naturally occurring, physiological agent with no inhibitory effects on prostaglandins and is devoid of gastrointestinal side effects. Uses: 1. Cardiovascular Disease 2. Arthritis 3.Rheumatoid Arthritis 4.Lung Problems 5.Eye Problems 6.Running Nose and sinusitis problems 7.Sports Injuries 8. Inflammation of any kind *Serratia peptidase* is a proteolytic enzyme isolated from the micro-organism *Serratia marcescens*. This enzyme is naturally processed commercially today through fermentation and was discovered in the silkworm intestine. This immunologically active enzyme is completely bound to the alpha 2 macroglobulin in biological fluids. Histological studies reveal powerful anti-inflammatory effects of this naturally occurring enzyme. *Serratia peptidase* digests non-living tissue, blood clots, cysts, and arterial plaque and inflammation in all forms.

NSAIDs are among the most widely prescribed drugs for rheumatoid arthritis and other inflammatory joint conditions. Their effects are mediated through inhibition of the biosynthesis of prostaglandins. They work by irreversibly blocking cyclooxygenase, the enzyme which catalyses the reactions of arachidonic acid to endoperoxide compounds. The neurological and gastrointestinal side effects of these agents have been reviewed in considerable detail. All of the NSAIDs, with the exception of Cytotec, inhibit prostaglandin El, a local hormone responsible for gastric mucosa cyto protection. A common side effect from these medications is gastric ulcers. More serious adverse reactions such as blood dyscrasias, kidney damage and cardiovascular effects have been noted. Most physicians rotate among the ten most widely prescribed NSAIDs, as soon as one causes side effects or stops working.

The search for a physiologic agent that offers anti-inflammatory properties without causing side effects may have ended with the discovery of the *Serratia peptidase* (SP) enzyme. SP is an anti-inflammatory, proteolytic enzyme isolated from the microorganism, *Serratia*El5. This enzyme is naturally present in the silkworm intestine and is processed commercially today through fermentation. The silkworm has a symbiotic relationship with the *Serratia* microorganisms in its intestines. The enzymes secreted by the bacteria in silkworm intestines have a specific affinity to a vital tissue and have no detrimental effect on the host's living cells. By dissolving a small hole in the silkworm's protective cocoon (avital tissue), the winged creature is able to emerge and fly away. The discovery of this unique biological phenomenon led researchers to study clinical applications of the SP enzyme in man.

MATERIALS AND METHODS

Screening For *Serratia peptidase* Enzyme Production

Casein Hydrolysis

Skimmed milk agar was prepared as follows: Dissolve the ingredients, adjust the pH to 7.2, distribute in 200ml portions into 250ml conical flasks and sterilize by autoclaving at 15lb pressure for 15minutes. Don't autoclave skimmed milk powder. Pasteurize the skimmed milk at 70°C for 15-20 min. Cool and mix with the autoclaved and cooled medium containing peptone and agar. Pour the autoclaved and cooled (45-50^{0}C) medium into sterile Petri plates (20ml each) and allow these to solidify. Label 3 skim milk agar plates with name of bacterial organism to be inoculated and the fourth plate as control. Make a single line streak inoculation from each culture into its labeled Petri plate across the surface of the medium.

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Incubate the plates (inoculated & uninoculated) for 24-48 hours at 30^0C.

Production Media

Nutrient broth was prepared and inoculates the 1% of *serratia marcescens* mother culture. Culture broth was maintained in 150rpm for 24hrs for preparation of preproduction media. 2% of the preproduction media are taken for the main production media with different substrate concentration.

Composition of Different Production Media:

Casein as Substrate

Tryptone - 3g
Yeast extract - 5g
Glucose - 5g
Casein - 5g
Glycerol - 1g
Distilled water - 1000ml
pH - 7

Cysteine as Substrate

Tryptone - 15g
Yeast extract - 5g
L-cysteine - 0.2g
NaCl - 1g
Disodium phosphate anhydrous- 0.3g
Sodium bicarbonate - 2g
Sodium acetate anhydrous - 12g
Sucrose - 5g
Distilled water - 1000ml
pH - 7

Enzyme Assay

Serratia peptidase activity was assayed by the modified method of Keay et al[2]. In brief, 0.2 ml of the enzyme was mixed thoroughly with 0.5 ml of 0.5% of casein solution. The mixture was incubated at 37°C for 10 min. Then the reaction was terminated by the addition of 2 ml of 110 mM Trichloroacetic acid and the mixture was again incubated for 30 min at 37°C. The incubated solution was filtered through Whatman No. 1 filter paper. Then 2 ml from the filtrate with 5 ml of 500 mM Na_2CO_3 and 0.5 ml of Folin's phenol reagent was added and mixed thoroughly. Again the mixture was incubated at 37°C for 30 min and the final solution was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine in 30 min at 37°C.

Protein Assay

Protein concentration was measured by the method of Lowry et al[6] using bovine serum albumin (Sigma, USA) as the standard. In column chromatography elution, the amount of protein was measured in terms of the absorbance at 660 nm. The specific activity was expressed as the enzyme activity per mg of protein.

Unit Definition

One unit will hydrolyze casein to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per minute at pH 7.5 at 37°C (color by Folin & Ciocalteu's reagent).

Final Assay Concentration

In a 6.00 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v)casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate, and 0.1 - 0.2 unit protease.

RESULTS

In small scale we have got higher results when compare to large scale. If we continue further to scale up process, the production will be comparatively low. To overcome this we have to do alternative methods such as Media Optimization, changing the pH, changing various carbon source, nitrogen source & percentage of the inoculum. We can also perform immobilization process for improving the enzyme efficiency.

Table 1. Estimation of Protein By Lowry's Method

Sample	Vol of BSA(ml)	Vol of Sample (ml)	Vol of Water (ml)	Reagents (ml)		FC (ml)		OD	Concentration (Ug/ml)
STD 1	0.2		0.8	5		0.5		0.103	20
STD 2	0.4		0.6	5	10	0.5	30	0.256	40
STD 3	0.6		0.4	5	min	0.5	min	0.344	60
STD 4	0.8		0.2	5		0.5		0.423	80
STD 5	1		0	5		0.5		0.561	100
T1	2	0.1	0.8	5		0.5		1.269	Un1
T2	2	0.1	0.8	5		0.5		1.405	Un2

Table 2. Estimation of Protein By Lowry's Method

Sample	Vol of BSA (ml)	Vol of Sample (ml)	Vol of Water (ml)	Reagents (ml)		FC (ml)		OD	Concentration (Ug/ml)
STD 1	0.2		0.8	5		0.5		0.103	20
STD 2	0.4		0.6	5	10	0.5	30	0.256	40
STD 3	0.6		0.4	5	Min	0.5	min	0.344	60
STD 4	0.8		0.2	5		0.5		0.423	80
STD 5	1		0	5		0.5		0.561	100
T1	2	0.2	0.8	5		0.5		1.152	Un1
T2	2	0.2	0.8	5		0.5		1.255	Un2
Pdn media	2	0.2	0.8	5		0.5		1.066	Un3

Table 3. Enzyme Activity

Fermentor Reading	Enzyme Activity(µg/ml)		Specific Enzyme Activity(µg/ml)	
	Filterate (mg/ml)	Non-Filterate (mg/ml)	Filterate (mg/ml)	Non-Filterate (mg/ml)
T1(5.30 pm)	0	0	0	0
T2(7.30 pm)	0.16	0.14	0.28	0.68
T3(9.30 pm)	0.32	0.25	0.37	0.91
T4(11.30 pm)	0.47	0.35	0.43	1.12s
T5(7.00 am)	0.61	0.44	0.78	1.89
T6(9.00 am)	0.61	0.44	0.78	1.89
T7(11.00 am)	0.42	0.38	0.99	2.38
T8(1.00 pm)	0.34	0.23	1.89	4.54

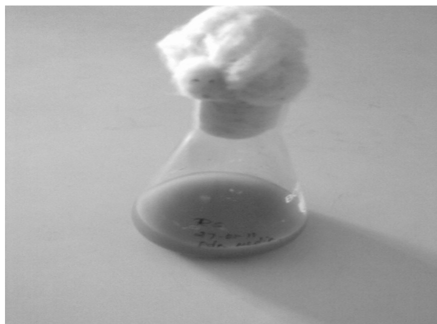


Fig1. Production Media For Casein

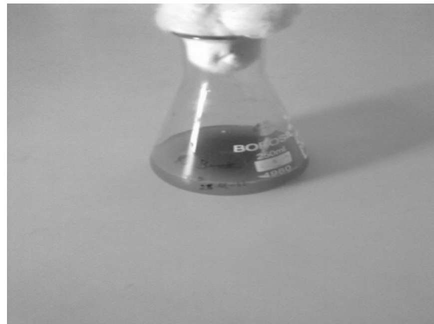


Fig 2. Casein

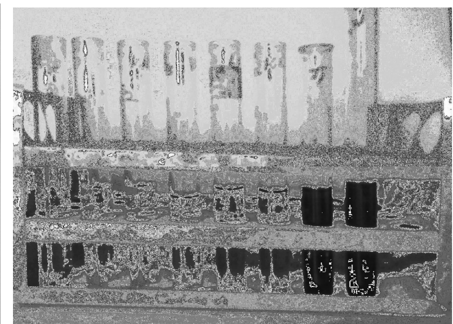
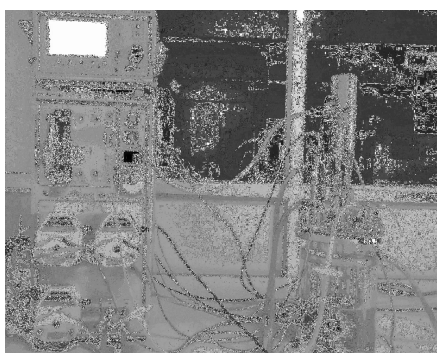


Fig 3. Estimation of Protein By Lowry's Method Fig


4. Large Scale Level

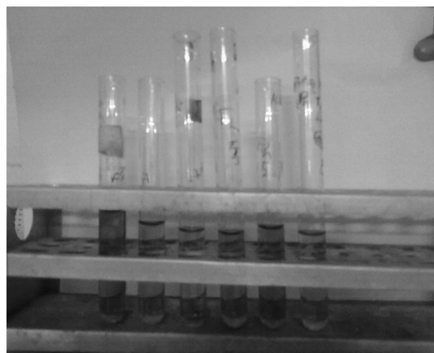


Fig 5. Enzymatic Assay

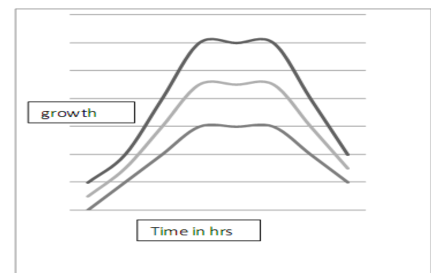


Fig 6. Growth Curve

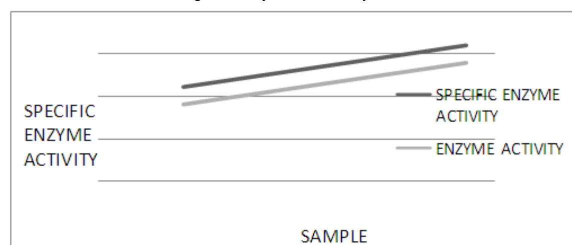


Fig 7. Enzyme Activity

DISCUSSION

Present work reveals the Casein as substrate for the production of *serratia peptidase* enzyme. Casein acts as protein source which leads to the production of proteolytic enzyme. It has clearly confirmed that *Serratia marcescens* can able to produce proteolytic enzyme by casein hydrolysis. Large scale production of enzyme is much useful for biomedical application in commercial aspects. In present result enzyme activity of both filtrate and non filtrate has been taken in different time interval. Fermentor conditions were optimized by setting the parameter in different ranges. Growth curve of *Serratia marcescens* in large scale in relatively low compared to the small scale level. It has been observed that 0.33 and 0.65. It was observed that twice times lower than the normal scale production. The result revealed that maximum activity of enzyme at temperature of 40ºC for two days. Maximum production was observed in the exponential phase of the microorganism during the growth. Casein assay shows the filtrate sample has the better enzyme activity and has the lower specific enzyme activity. It was noticed that maximum activity of 0.61Units/ml enzyme in filtrate solution. But in case of non-filtrate has shown better specific enzyme activity as 1.89 units/mg of solid. Enzyme activity was checked for the commercial available *serratia peptidase*. Compared to the previous result we had observed the less activity due to the environmental factors. Hereby it needs to optimize the concentration of media for large scale level.

CONCLUSION

Serratia peptidase is one of the important enzyme in medicinal field. It used to cure the different diseases like cardiovascular attack and heart diseases. Current study reveals that effective production of *Serratia peptidase* from *Serratia marcescens*. It has been observed the highest activity than previous result in large scale level. Characterization of enzyme was done and it was effective as 40ºC and pH 7.0. The activity of enzyme was low in large scale level than small scale level of production. Further it needs to optimize the substrate to increase the production of enzyme in large scale level.

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