Media Optimization Studies for Enhanced Production of Serratiopeptidase from Bacillus Licheniformis (NCIM 2042)

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Research Article

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ABSTRACT :

Production of an anti inflammatory Serratiopeptidase by fermentation with *Serratia marcescens* NCIM 2919 was studied to ascertain optimal nutritional conditions for large scale production. To study biosynthesis and production of Serratiopeptidase by isolated *Bacillus Licheniformis (NCIM 2042)* different growth media were studied. The modified fermentation medium concentration of Serratiopeptidase was found to be 22.85 IU/ml at temperature 35 °C, pH 6.5 after 24 hours duration. Before optimization of fermentation medium concentration of Serratiopeptidase was found 16.52 IU/ml at temperature 30 °C and pH 7.0 after 48 hours duration.

Keywords: Bacillus Licheniformis (NCIM 2042), Serratiopeptidase.

INTRODUCTION:

Serratiopeptidase is an anti-inflammatory enzyme widely used in the treatment regime of various diseases. It gives anti-inflammatory action by breaking down abnormal exudates and protein and by promoting the absorption of the decomposed product through the blood and lymphatic vessels. It also acts as an analgesic by inhibiting the release of pain inducing amines.

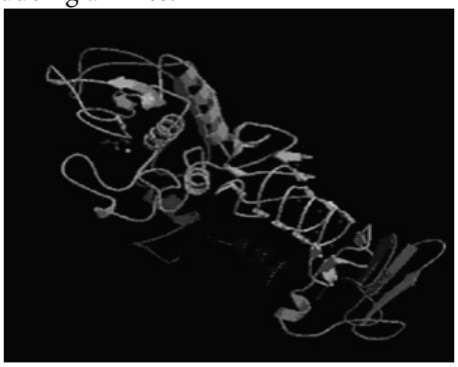

Fig. 1 : Crystal Structure of Serratiopeptidase

In the proposed investigation, *Serratia marcescens* NCIM 2919 strain was screened for the production of Serratiopeptidase enzyme. Further, media optimization studies were carried out for enhanced production of serratiopeptidase from the strain. Activity of serratiopeptidase enzyme is usually checked by 20 min hydrolysis of casein substrate at 370C and pH 9.0 . It is used in Inflammation, Sinusitis & Bronchitis, Infection & Atherosclerosis, Carpel Tunnel Syndrome and Dermatology.

MATERIAL & METHODS:

Procurement of Serratiopeptidase producing bacteria

The BACILLUS LICHENIFORMIS (NCIM 2042) strain was obtained from NCIM, Pune. It was subcultered and maintained in the basal medium with following composition.

Sr. no	Components	Quantity
1.	Beef extract	10 gm
2.	Sodium chloride	5.0 gm
3.	Peptone	10.0 gm
4.	Distilled water	1 L
5.	Agar	20.0 gm
6.	pH	7.0 to 7.5

Total Protein Estimation by Folin- Lowry Method

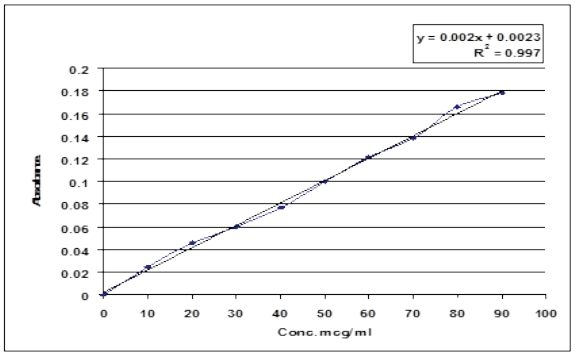

Fig. No. 2: Total protein estimation by Folin-Lowry Method

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Folin-Lowry method was used for the estimation of extracellular protein. The method developed by Lowry *et al* is sensitive enough to give a moderately constant value of protein estimation and hence largely followed. Protein content of enzyme extract is usually determined by this method. The blue colour developed by the reduction of the phosphomolybdic – phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry's method . The intensity of the colour depends on the amount of these aromatic amino acids present in the solution and thus varies with different proteins.

Assay of serratiopeptidase

Serratiopeptidase is highly active in it's proteolytic activity, especially in caseinolytic and fibrinolytic activities. In comparison with known alkaline or neutral proteases like Chymotrypsin, Bromelin or Pronase Serratiopeptidase posses a stronger caseinolytic activity. The assay of serratiopeptidase is based on its caseinolytic activity.

Chemicals

Sodium borate – HCL buffer (pH 9.0)

Dissolve 19.0 gm sodium borate in 900ml-distilled water. Adjust the pH 9.0 with 1N HCL. Make up the volume to 1000ml with distilled water and check the pH.

Casein Solution

Hammer stein Casein 1.2gm (dried) in 100ml of sodium borate HCL buffer of pH 9.0. Dissolve and keep it on boiling water bath for 1-2 min. (Just to make the solution clear). Cool, filter through cotton and make up the volume to 200ml with buffer.

TCA

Dissolve 18 gm of Trichloroacetic acid + 30gm of sodium acetate (anhydrous) + 20 ml glacial acetic acid. Make up the volume to 1000ml with distilled water.

Sodium Carbonate 6% Solution

6 gm of anhydrous sodium carbonate dissolved in distilled water and make up the volume to 100 ml with distilled water.

0.2 N HCL

Dried Tyrosine

Folin's reagent (Twice Diluted)

Dissolve 1 ml of Folin's Reagent + 2 ml of distilled water and make up the volume to 3 ml.

5% Ammonium Sulphate solution

10 gm of Ammonium sulphate dissolved in distilled water and volume made to 200ml with distilled water.

Enzyme Dilution:

100mg →100 ml (A) with ammonium sulphate solution (Mix well. Keep it for 5 minutes)

1 ml (A) → 200ml (B) with sodium borate HCL buffer(Ph 9.0)

1 ml (B) used for the analysis.

TYROSINE STANDARD SOLUTION

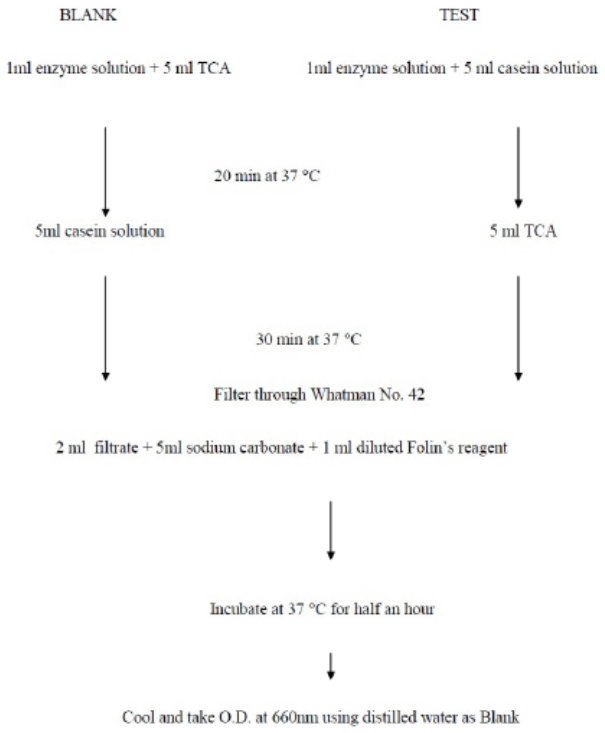
160 mg tyrosine (dried) →100 ml of 0.2 N HCL (A)

10ml (A) →1000ml (B) with 0.2 N HCL

2ml of solution (B) is taken for color development

FOR TYROSINE

Procedure



2 ml of tyrosine standard solution + 5ml of 6 % sodium carbonate + 1ml of diluted folin's reagent. Incubate at 37 ^{0}C half an hour and take the absorbance at 660 nm using water as blank.

CALCULATION:

Serratiopeptidase Units/mg =(A1-A2/A3-A4)×176×(1/20)

Conversion coefficient = 176

[Total volume of enzyme reaction mix (11ml)/ Volume of filtrate× Content of tyrosine in 2ml of Tyrosine std solution (32µg)]

20 - Reaction time in minutes

A1 - Absorbance of test solution

A2 - Absorbance of blank solution

A3 - Absorbance of tyrosine standard solution

A4 - Absorbance of 0.2 N HCL

Optimization of serratiopeptidase production by conventional method

Media optimization is an essential prerequisite to get higher productivity using a microbial strain. The production potential for a strain not only depends on the genetic nature, but also on nutrients supply and suitable culture conditions. So it is important to know the suitable nutrients and culture condition required for achieving higher productivity.

Further, it is proposed to study the production of Serratiopeptidase to develop repeated batch fermentation with suitable production medium, which is more convenient. Hence, it is proposed to study the effect of various nutritional and culture factors on Serratiopeptidase production.

Effect of various factors on Serratiopeptidase production in a production medium

An ideal production medium has to be designed to achieve optimum yields of the desired product. The ideal production medium must meet as many criteria as possible. Thus, it is proposed to study Effect of Incubation duration, Ini-

tial pH of the medium, Incubation temperature, Carbon sources , Inorganic Salts, Organic Nitrogen Sources and Inorganic Nitrogen Sources for the production of Serratiopeptidase.

Inoculum preparation

Five ml of sterile water was added to 24 hr fresh slants of the isolated strain BACILLUS LICHENIFORMIS (NCIM 2042). The slants were scrapped into sterile water and the resulted cell suspension was used as inoculum.

Shake flask Fermentation

Five ml of the inoculum prepared was transferred in the 50 ml basal media and incubated at 37 ^{0}C for 24 Hrs., on rotary shaker. At the end of the fermentation, 5ml broth was centrifuged at 500 rpm for 10 min and the supernatant was assayed for the presence of Serratiopeptidase. The process was carried out in triplicate and the average of all the three values was considered.

Effect of incubation duration on Serratiopeptidase production

The duration of incubation plays an important role in the production of microbial metabolite. To study the optimal incubation period for maximal Serratiopeptidase production, the inoculum prepared was transferred in 50 ml of basal media and the flasks were incubated at 37 0C for 24 hr. The samples were withdrawn periodically at 24 hr for 72 Hrs. and assayed for Serratiopeptidase activity as described earlier. The process was carried out in triplicate and the average of all the three values was considered.

Effect of initial pH on Serratiopeptidase production

The initial pH of the medium is important to study for optimization of the media foe Serratiopeptidase production since initial pH of the production media affects the enzyme reactivity. The Serratiopeptidase production medium was adjusted at various levels of pH (6.5, 7, 7.5, 8, 8.5, and 9.0). General procedure as mentioned earlier was followed for Serratiopeptidase production and for assay. The process was carried out in triplicate and the average of all the three values was considered.

Effect of Incubation temperature on Serratiopeptidase production

To study the effect of incubation temperature on maximal production of Serratiopeptidase, the Earlinmear (EM) flasks were inoculated and incubated at various temperatures such as 25 0C, 30 0C, 35 0C, 37 0C, 40 0C and 45 0C. The general procedure mentioned earlier was followed for Serratiopeptidase production and assay. The process was carried out in triplicate and the average of all the three values was considered.

Effect of various carbon sources on Serratiopeptidase production

Effect of various carbohydrates on Serratiopeptidase production was studied by using carbohydrates at 10 g/l level. The carbohydrates employed in this investigation were glucose, sucrose, xylose, glycerin and dextrose. The ingredients were weighed and transferred into 100 ml EM flasks, 50 ml of basal media was added to each flask, kept on rotary shaker for 15 min at 110 rpm. According to the results observed for Incubation Temperature, pH of initial media and Incubation duration the values for these parameters were maintained for further studies. The process was carried out in triplicate and the average of all the three values was considered.

Effect of various inorganic salts on Serratiopeptidase production

The influence of various inorganic and organic nitrogen sources on Serratiopeptidase production was studied by incorporating each nitrogen source at 10 g/l level in to the basal medium. The inorganic salts sources employed in the study were zinc chloride, potassium sulphate, potassium chloride, copper sulphate, sodium citrate, di-sodium hydrogen phosphate, potassium di-hydrogen phosphate, sodium nitrate, sodium bicarbonate, sodium acetate. The process was carried out in triplicate and the average of all the three values was considered.

Effect of various Organic Nitrogen sources on Serratiopeptidase production

The organic nitrogen sources were tryptone, meat extract, yeast extract, peptone, beef extract, casein and gelatin at a concentration of 10g/L. The fermentation conditions were same as described earlier. The process was carried out in triplicate and the average of all the three values was considered.

Effect of various Inorganic Nitrogen sources on Serratiopeptidase production

The various ammonium salts used as a source of nitrogen were ammonium sulphate, ammonium chloride, ammonium Thiocyanate, ammonium acetate, ammonium bromide and ammonium oxalate. The fermentation conditions were same as described above. The results are shown in the figure. The process was carried out in triplicate and the average of all the three values was considered.

RESULTS

Effect of incubation duration on Serratiopeptidase production

To study the optimal incubation duration for maximal Serratiopeptidase production, the fermentation samples were withdrawn periodically at interval of 24 hr fermentation for 72 Hrs and assayed as described earlier. The assay readings were found to be 20.98 IU/ml, 17.95 IU/ml and 16.96 IU/ml for 24 Hrs, 48Hrs and 72 Hrs respectively.

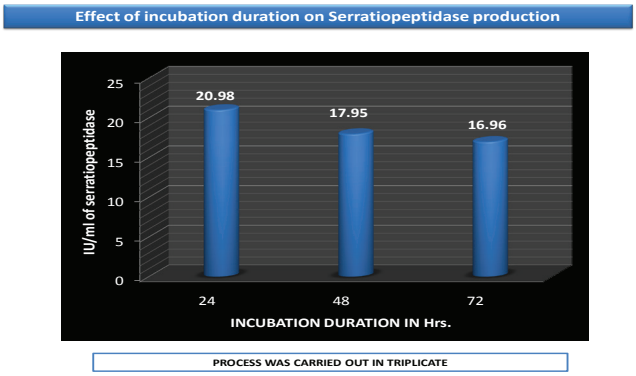


Fig no.3. Effect of incubation duration on Serratiopeptidase production

Effect of initial pH on Serratiopeptidase production

Different initial pH values (6-9) of media were used to study their effect on the Serratiopeptidase production.

The yield of Serratiopeptidase was found to be 15.92 IU/ml, 16.50 IU/ml, 22.53 U/ml, 13.99 IU/ml, 14.9 IU/ml and 13.69 IU/ml for the initial pH values of 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 respectively.

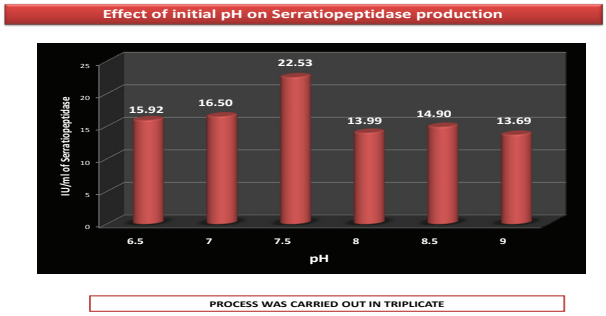


Fig. no. 4:Effect of initial pH of media on Serratiopeptidase production

Effect of Incubation temperature on Serratiopeptidase production

To study the effect of incubation temperature on the growth and Serratiopeptidase production, different temperature ranges (25 ^{0}C, 30 ^{0}C, 35 ^{0}C, 37 ^{0}C, 40 ^{0}C and 45 ^{0}C) were used. The yield of Serratiopeptidase was observed to be 16.08 IU/ml, 17.25 IU/ml, 19.39 IU/ml, 14.51 IU/ml, 14.82 IU/ml and 12.84 IU/ml for the incubation temperature of 25 ^{0}C, 30 ^{0}C, 35 ^{0}C, 37 ^{0}C, 40 ^{0}C and 45 ^{0}C respectively.

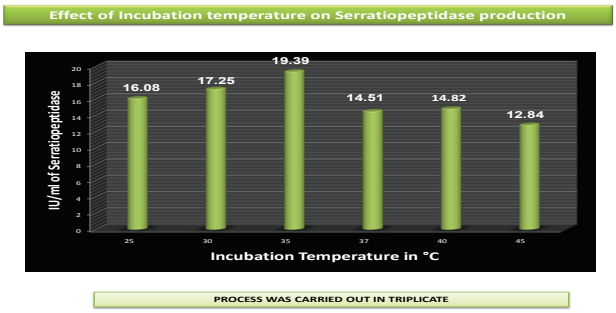


Fig no. 5: Effect of incubation temperature on Serratiopeptidase Production

Effect of various carbon sources on Serratiopeptidase production

The carbohydrates employed in this investigation were glucose, sucrose, fructose, xylose, glycerine, maltose and dextrose. The Serratiopeptidase yield was found to be 18.87 IU/ml for both Glycerine and Maltose. It was 10.98 IU/ml, 13.5 IU/ml, 18.3 IU/ml, 5.43 IU/ml and 5.31 IU/ml for glucose, fructose, xylose, sucrose and dextrose respectively. (Figure 6)

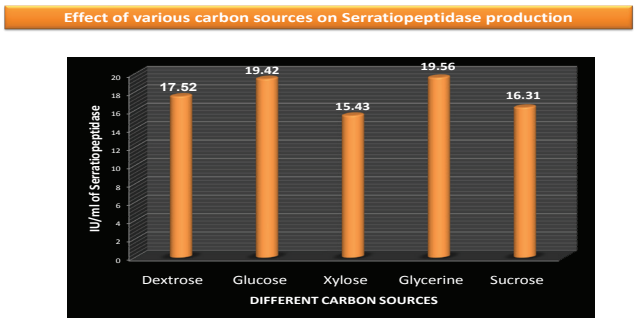


Fig. no. 6 : Effect of various carbon sources on the serratiopeptidase production

Effect of various inorganic salts on Serratiopeptidase production

The yield of Serratiopeptidase was found to be 12.07 IU/ml, 3.12 IU/ml, 7.98 IU/ml, 4.26 IU/ml, 22.14 IU/ml, 3.45 IU/ml, 1.74 IU/ml, 18.60 IU/ml, 14.91 IU/ml and 1.77 IU/ml for Copper Sulphate, Potassium Sulphate, Potassium Chloride, Potassium Dihydrogen Phosphate, Sodium Acetate, Sodium Citrate, Sodium Nitrate, Disodium Hydrogen Phosphate, Sodium Bicarbonate and Zinc Chloride respectively.

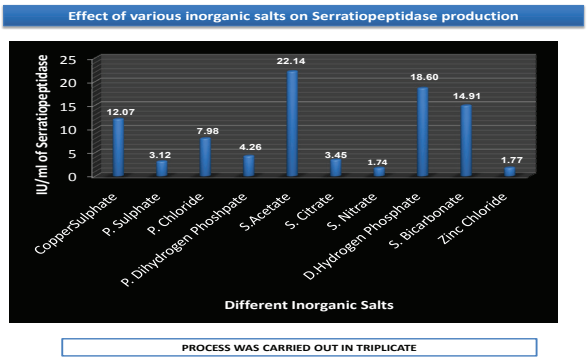


Fig. No. 7 : Effect of various inorganic salts on Serratiopeptidase production

Effect of various Organic Nitrogen sources on Serratiopeptidase production

The different Organic nitrogen sources used were Gelatin, Tryptone, Beef extract, Peptone, Yeast Extract, Meat Extract and Casein. The yield of Serratiopeptidase was found to be 16.59 IU/ml, 28.05 IU/ml, 24.03 IU/ml, 22.74 IU/ml, 25.73 IU/ml, 23.79 IU/ml and 21.33 IU/ml for them respectively.

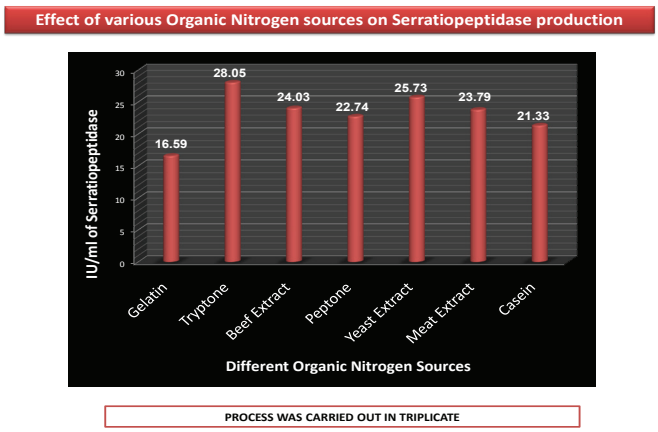


Fig No. 8: Effect of various organic nitrogen sources on Serratiopeptidase production

Effect of various Inorganic Nitrogen sources on Serratiopeptidase production

Different ammonium salts as nitrogen sources were studied including Ammonium Chloride, Ammonium Thiocyanate , Ammonium Oxalate, Ammonium Sulphate, , Ammonium Bromide , and Ammonium Acetate at 10-gm/L levels, for their effect on production of Serratiopeptidase. The yield of Serratiopeptidase was found to be 15.19 IU/ml, 12.46 IU/ml, 18.49 IU/ml, 19.24 IU/ml, 1.84 IU/ml and 2.83 IU/ml for Ammonium Chloride, Ammonium Thiocyanate, Ammonium Oxalate, Ammonium Sulphate, Ammonium Bromide and Ammonium Acetate respectively.

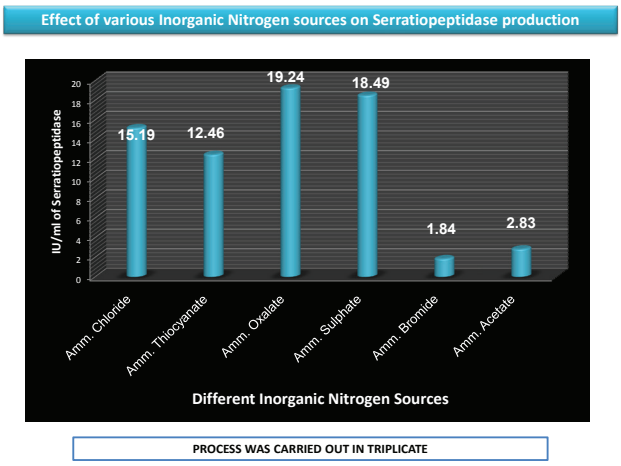


Fig No. 9: Effect of Different Inorganic Nitrogen Sources on Serratiopeptidase Production

DISCUSSION

Composition of Optimized media

SR. No.	COMPONENT	QUANTITY (gm/Litre)
1	Glycerine	10
2	Glucose	10
3	Disodium Hydrogen Phosphate	10
4	Sodium Acetate	10
5	Tryptone	10
6	Ammonium Oxalate	10
7	Ammonium Sulphate	10
8	pH	7.5

Composition of Optimized Media

The media and process parameters optimization for enhanced production of Protease from Bacillus Licheniformis NCIM 2042 was carried out. The media was optimized for different Carbon sources, Organic Nitrogen Sources, Inorganic Nitrogen Sources, Incubation duration, Initial pH of the medium and Incubation temperature. Maximum alkaline protease production was realised when the medium comprised of glucose, Peptone and ammonium sulphate as Carbon source, Organic Nitrogen Source, Inorganic Nitrogen Source respectively at pH 10 and 37^0 C temperature for 24 hrs. incubation.

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