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OPTIMIZATION OF THE PRODUCTION OF EXTRACELLULAR PROTEASE FROM *STREPTOMYCES INDIAENSIS*

AJOY KUMAR P[1*] AND SATHYAPRABHA G[2]

1: Ph.D Research Scholar, Department of Microbiology, Marudupandiyar College (Affiliated to Bharathidasan University), Thanjavur – 613 403

2: Assistant Professor, Department of Microbiology, Marudupandiyar College (Affiliated to Bharathidasan University), Thanjavur – 613 403

***Corresponding Author: Supriya Dubey: E Mail: ajoykumarppp@gmail.com**

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ABSTRACT

The objective of present study was to screen and isolate protease producing actinomycetes bacteria from soil samples collected from Muthupet lagoon forest, Tamil Nadu, India. Total six actinomycetes colonies showed clear zone around the colony indicating protease activity. Among these, ISA1 isolate produced highest protease activity and was identified as *Streptomyces indiaensis* (ISA1**)** by morphological, biochemical and molecular test. Moreover, various physiological characters were studied like pH and temperature. The protease showed maximum activity at pH 6 and the temperature for maximum protease activity was found to be 35°C. In optimization, various nutrient supplementations were used such as carbon sources and Nitrogen sources. Highest protease productivity was observed in Maltose. At the same highest protease productivity in peptone as a nitrogen source. These properties suggest that protease from *Streptomyces indiaensis* (ISA1**)** could find potential application in various industries.

Keywords: Protease, Actinomycetes, *Streptomyces indiaensis,* Standard Fermented Medium and Orange Peels powder Fermented Medium

1. INTRODUCTION

Protease is one of the most important industrial enzymes, occupying nearly 60% of enzyme sales which obtained from microbial, Plant and animal sources

[1]. Extra cellular protease finds numerous applications in industrial processes like detergents, leather tanning, dairy meat tenderization, bakery, brewery, photographic industry etc. **[2]**. Although a variety of proteolytic fungi and bacteria are know, only few provide high activities with commericial success **[3]**. Furthermore, chemical analysis showed that it is composed of approximately 40% protein and is rich in other organic and inorganic components **[4]** suggesting it as a good candidate for culture media. The selection of medium components for the optimal production of extracellular protease along with some biochemical properties of the enzyme **[5]**, a formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent (thioglycolate), that enhances hair degradation and helps in clearing pipes clogged with hair-containing deposits, is currently available in market. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases.

Taguchi *et al.,* demonstrate the *streptomycetes* also contain a large amount of multitude of genes for protease enzyme synthesis **[6]**. Actinomycetes, Gram-positive filamentous bacteria, can degrade various macromolecules in soil **[7]**. Among actinomycetes, *Streptomyces* species are the most industrially useful because of their capacity of producing numerous secondary metabolites, particularly antibiotics. Similarly, these bacteria offer a second industrial interesting use by producing large amounts of proteolytic enzymes, with different substrate specificities **[8]**. *Streptomyces* spp. that produce proteases include *S. clavuligerus, S. griseus, S. rimouses, S. thermoviolaceus*, and *S. thermovulgaris* **[9]**. Some of these proteases, like the serine proteases of *Streptomyces griseus* **[10, 11]** and *Streptomyces fradiae* **[12]**, have been characterized structurally and enzymatically. There have also been many descriptions of isolation and partial characterization of alkaline protease activities from other members of the genus *Streptomyces* like *Streptomyces clavuligerus*, *Streptomyces gulbargensis*, *Streptomyces viridifaceins*, and *Streptomyces* sp. **[13, 14]**. Based on the above fact in this study protease producing organisms were isolated from soil based on the proteolytic screening techniques. Then the protease productivity was estimated and optimized using various physiochemical parameters.

2. MATERIALS AND METHODS

The soil samples were collected from six different locations of Muthupet lagoon forest at Southen end of the Cauvery river delta on the Bay of Bengal,

Tamil Nadu, India. Sampling was done using dredged and scuba diving method. Soil samples were collected within an area of approximately 500 m^2 in each location. The samples were taken from the surface of soil at different places and pooled together. The collected samples were placed in a cool box for transporting and stored at 4°C. About 250 g of soil was used as the test sample for each of the soil series. The physico-chemical properties of soil such as EC, pH, bulk density (g/cc), organic carbon (%), nitrogen, phosphorus, potassium, zinc, copper, manganese and iron content were analyzed **[15, 16]**.

Isolation of actinomycetes was carried out by serial dilution of the soil, followed by spread plate technique **[17]**. Starch Casein agar medium and Kuster's agar medium were used for the isolation and enumeration of actinomycetes. The medium was supplemented with 50mg/liter of Cyclohexamide (Sigma Aldrich) to avoid fungal contamination. Using the conventional serial dilution spread plate technique, 10^{-3} to 10^{-7} dilutions were poured over the Starch Casein agar medium and Kuster's agar medium in separate plates and spread out using a sterile rod. These plates were incubated for 7 days at 28ºC and the actinomycetes colonies were counted. The isolated organisms were streaked on gelatin-enriched nutrient agar medium (Gelatin 4%, pH 7.2) after 24 hrs of incubation at 37 ºC. The plates were flooded with 10% mercuric chloride reagent. A zone of clearance around the colony indicated production of proteases. In the proteolytic strain was identified based on their cultural morphological and biochemical characteristics **[18]**.

The protease productions were analysed using two different medium such as Standard Fermented Medium (SFM) and Orange Peels powder Fermented Medium (OPPFM)**.** The SFM containing the following ingredients Bacteriological peptone – 6 g**;** Magnesium sulfate ($MgSO_47H_2O$) – 0.5 g**;** *Potassium chloride* (KCl) – 0.5 g**;** Starch - 1 g and Distilled Water – 1000 ml. OPPFM containing such as Bacteriological peptone – 6 g; Magnesium sulfate ($MgSO_47H_2O$) – 0.5 g**;** Potassium chloride (KCl) – 0.5 g**;** Starch - 1 g**;** Orange Peels powder - 1 g and Distilled Water – 1000 ml. The test organisms were inoculated in to above medium separately and maintained at 37 ºC for 48 hours in a continuous shaker incubator (120rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10,000 rpm at 4ºC, and the clear supernatant was used as a enzyme source. The crude enzyme was assayed for proteolytic activity and used for further studies.

Crude enzyme was precipitated with ammonium sulfate at 60% saturation. All

subsequent steps were carried out at 4ºC. The collected precipitates were dissolved in 10ml of 0.1M pH 7.8 Tris-HCL buffer. The solution was applied on DEAE colums and eluted with 20mM pH 7.8 Tris-HCL buffer at a flow rate of 0.5ml/min. The protein content was determined using the method **[19]**. The reaction mixture consists of 0.5ml of purified enzyme, 0.5ml Tris-HCL buffer (50mM, pH8) and 1ml of 1% casein in Tris- HCL buffer of same pH. The mixture was incubated for 30 min at 55ºC and the reaction was stopped by the addition of 4 ml of 5% trichloroacetic acid (TCA). After 60 min, the solution was centrifuged at 4ºC for 15 min at 3000 rpm. 1 ml of supernatant was mixed with 5 ml of 0.4M Na_2Co_3 followed by the addition of 0.5 ml of phenol reagent and absorbance was read at 660 nm.

Effect of pH on growth and protease production was studied by adjusting pH of basal media ranging from 4-8. Effect of inoculums on protease production was studied by incubating the medium with inoculum ranging from 1-5 percent. Influence of temperature of growth and protease production was studied by incubating the culture media at temperature ranging from 25ºC-45ºC. Protease production using from different carbon supplementation such as glucose, fructose, starch, maltose and lactose. Effect of inoculums on protease production was studied by incubating the medium with inoculums ranging from 1-5 percent. Various nitrogen sources used for the optimization studies such as ammonium sulphate, ammonium chloride, urea, yeast extract and peptone. After 48 hrs of incubation, the culture was centrifuged at 10,000 rpm for 10 min at 4ºC to separate the clear supernatant. The supernatant was analyzed for protease activity by casein assay.

3. RESULTS AND DISCUSSION

Physico-chemical properties of collected soil, was shown in **Table 1**. The pH of the soil was 5.84 and Bulk density was 1.32 (g/cc). The organic carbon content of the soil range was 0.16% and different level of N, P and K (67.2, 6.5 and 85 Kg/acre) content were noted in soil compared to general nutrient status of the soils **[20]**, at the same time high level Fe were recorded (49.52 ppm). In this study protease producing organisms were isolated from soil based on the proteolytic screening techniques. Then the protease productivity was estimated and optimized using various physiochemical parameters. After incubation, the plates contain more than 98 cfu *Actinomycetes sp.* colonies were observed. Among 98 colonies, only six *Actinomycetes* colonies screened for protease producing ability which were named as ISA1- ISA6 **(Table 2)**. Bergey's manual of systemic bacteriology based on

the cultural, morphological and biochemical characteristics results comparison the maximum enzyme producing isolate ISA1 were confirmed as *Streptomyces indiaensis.* Many bacteria produce alkaline protease and screened for various applications. Several workers used various screening plate media for the determination of alkaline protease **[21, 22]**. The **Table 3** results revealed the maximum protease productivity were noted in OPPFM (106.84±1.97 IU/ml) compared than SFM (72.05±1.49 IU/ml).

Purification of *Streptomyces indiaensis* (ISA1**)** was carried out in three successive steps as shown in **Table 4**. The crude extract contained 2261 mg protein; maximum activity was obtained by precipitation of 60% saturation of ammonium sulfate. The major fraction with fibrinolytic activity was applied to the DEAE-Sepharose FF column, which generated one single peak showing fibrinolytic activity in the eluate. The major active fraction was pooled, concentrated, and further purified using gel filtration via a Sephadex G-50 column, and a single peak with a high specific activity was acquired. Overall, 104.31 fold purification and recovery of 9.69 % activity (yield) were obtained after completion of the purification steps. The specific activity of the final enzyme preparation was 109.52 U/mg protein. The purified enzyme was designated as *Streptomyces indiaensis* (ISA1) in OPP fermentation medium **[23]**.

In this study maximum protease productivity were noted in pH 6 of both fermentation medium. At the same OPP fermentation medium highest level produced compared than SFM. The similar results were observed by Kohlmann *et al.,* the maximum protease production was obtained between pH 6 and 7 **[24]**. The optimal pH for proteolytic activity was determined to be 6 (Figure - 1), which is similar to the optimum observed for *Streptomyces pulvereceus* MTCC 8374 (6.0–9.0) **[25]**. Similarly, Sepahy and Jabalameli **[26]** reported the maximum growth and protease production by *Bacillus sp*. at pH 8, which is in agreement with our finding on the relationship between the growth of bacteria and their production of protease enzyme. Most of the *Geobacillus sp.* reported have their growth at a pH range of 6–9 for the production of protease **[27]**. This study highest productivity noted 35°C (Figure – 2). The same results were reported **[28]** the optimal temperature of protease was determined to be around 40°C. The related results were observed by Kohlman *et al.,* the maximum protease production was found to be 37°C **[23]**. Dissanayaka and Rathnayake **[29]** reported the optimum growth of some *Geobacillus sp*. between 30 and 70°C with maximum activity at 60°C. Additionally, Chen *et al.*

[30] reported a thermophile *G. caldoproteolyticus* isolated from sewage with the capacity to grow at 35 to 65°C.

In this study various carbon and nitrogen supplementation used for optimization production of protease using isolated *Streptomyces indiaensis* (ISA1). The maximum enzyme production recorded at lactose in both fermentation medium at the same highest value was noted in *Streptomyces indiaensis* (ISA1) **(Figure 3)**. Moreover, other carbohydrates have been reported by many investigators to cause an increase in the yield of alkaline protease. For example, Malathi and Chakraborty **[31]** stated in their work that maltose is a good source of carbon for the high production of protease enzyme. Tsuchiya *et al.* **[32]** also reported the use of maltose for high production of protease enzyme, while Phadatare *et al.* **[33]** and Ibrahim *et al.* **[34]** reported on high protease enzyme activity using sucrose and fructose respectively. Further, Yang *et al.* **[35]** published that *G. thermodenitrificans* SG-01 utilizes fructose to grow, while *G. thermodenitrifcans* SG-02 utilizes fructose and glucose for maximum growth and *G. denitrificans* SG-03 utilizes sucrose. In nitrogen source maximum enzyme productivity were recorded at peptone in both fermentation medium highest values noted **(Figure 4)**. This is in agreement with the findings of Khusro **[36]**, who reported that yeast extract played a crucial role in the production of the enzyme due to the presence of important elements and growth factors for microorganisms. Prakasham *et al.* **[37]** reported the use of a complex organic nitrogen compound resulting in an increase of protease amount secreted compared to that when using an inorganic nitrogen compound. Similarly, Qureshi *et al.* **[38]** reported an increase in the amount of protease produced by *B. subtilis* when using organic nitrogen sources from yeast extract.

Table 1: Physico-chemical properties of Mangrove Ecosystem soil

Physico-chemical properties	Observation
Texture	Sandy Clay Loam Soil
Bulk density (g/cc)	1.32
Lime Status	Nil
EC_{sc} ds^{-1}	0.27
Organic carbon (%)	0.16
pH	5.84
N (Kg/acre)	67.2
P_2O_5 (Kg/acre)	6.5
K (Kg/acre)	85
Fe (ppm)	49.52
Zn (ppm)	1.78
Mn (ppm)	2.33
Cu (ppm)	0.67

Table 2: Production of Protease Enzyme using isolated *Actinomycetes sp.*

Isolated *Actinomycetes sp.*	Enzyme Activity (U/ml)	
	SFM	OPPFM
ISA1	72.05±1.49	106.84±1.97
ISA2	12.60±1.37	81.89±1.32
ISA3	46.81±3.32	70.49±1.67
ISA4	27.68±2.23	24.00±1.02
ISA5	39.09±2.88	36.81±1.75
ISA6	19.79±1.78	19.26±1.49

Values are expressed Mean ± Standard Deviation; n=6
SFM - Standard Fermented Medium; OPPFM - Orange Peels powder Fermented Medium

Table 3: Purification of Protease by *Streptomyces indiaensis* (ISA1)

Purification steps	Volume (ml)	Enzyme Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	100	2374	2261	1.05	1	100
Ammonium sulfate precipitation	75	1175	78	15.06	14.35	49.49
Dialysis	45	347	3.3	105.15	100.15	14.62
DEAE-cellulose	15	230	2.1	109.52	104.31	9.69

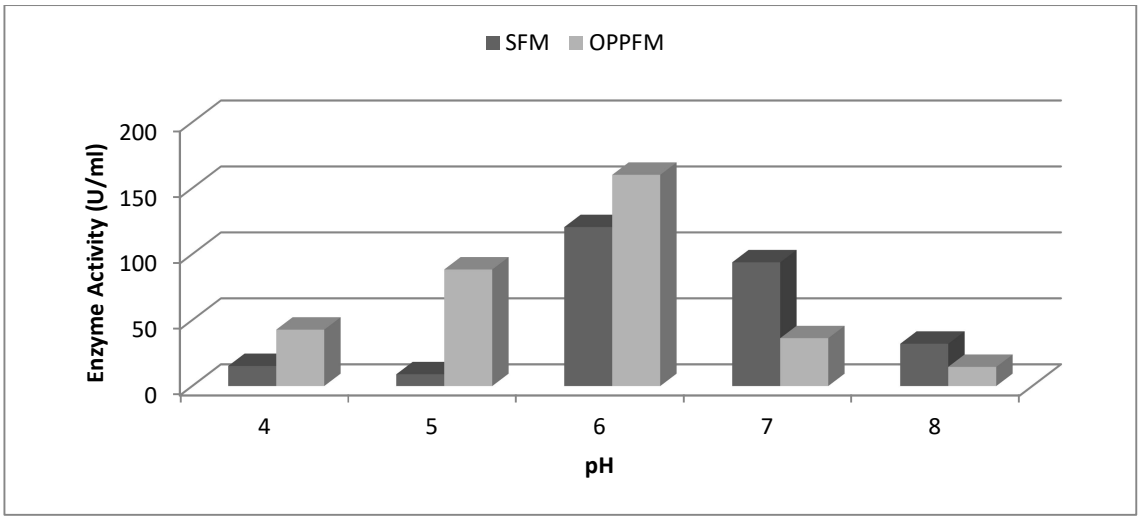

Figure 1: Effect of pH on Protease production by *Streptomyces indiaensis* (ISA1)

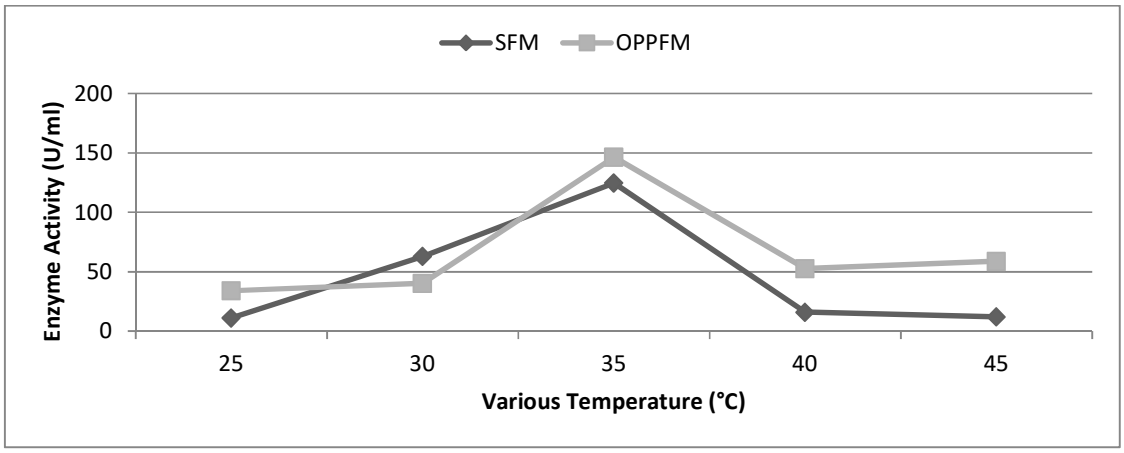

Figure 2: Effect of Temperature on protease production by *Streptomyces indiaensis* (ISA1)

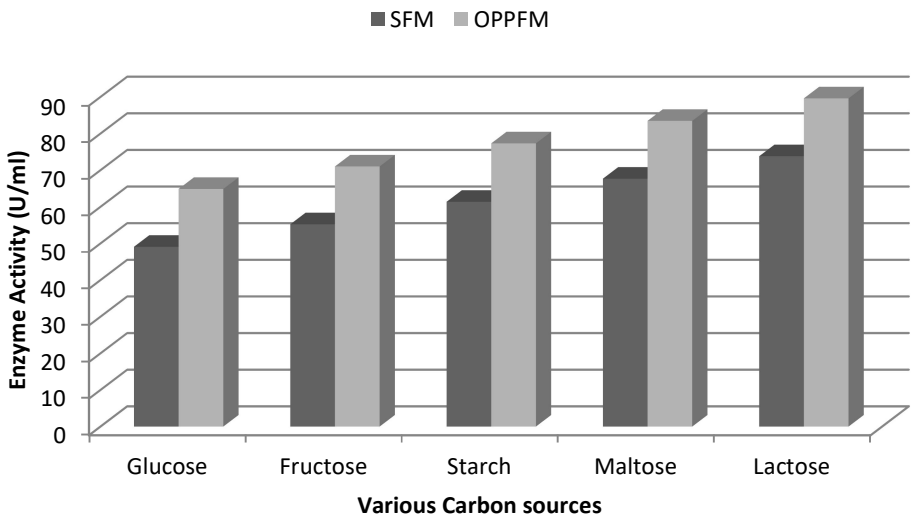

Figure 3: Effect of various carbon sources on protease production by *Streptomyces indiaensis* (ISA1)

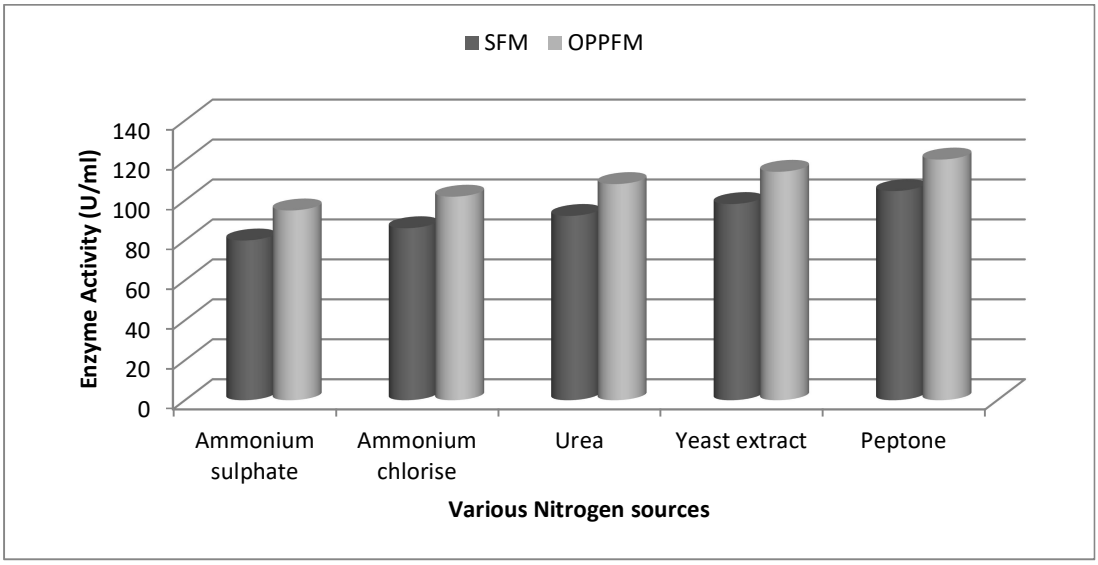

Figure 4: Effect of various nitrogen sources on protease production by *Streptomyces indiaensis* (ISA1)

4. CONCLUSION

In the present, study the actinomycetes colonies were isolated from soil sample and screened for protease production using Gelatin agar medium. The maximum protease producing organism was identified based on cultural, morphological and biochemical characteristics. The isolated colony confirmed as *Streptomyces indiaensis* (ISA1). Highest protease production was noted in both fermentation medium of SFM and OPPFM (72.05±1.49 and 106.84±1.97 U/ml). This strain used for the further optimization studies. In this optimization investigation, highest protease productivity

was observed in pH 6 and temperature 35°C. In optimization, various nutrient supplementations were used such as carbon sources and Nitrogen sources. Highest protease productivity was observed in Maltose. At the same highest protease productivity in peptone as a nitrogen source. Finally it was conclude that *Streptomyces indiaensis* (ISA1) strain produced high levels of Protease enzyme. Based on the Protease characteristics suitable for industrial use. The production process can be commercialized after optimization.

5. ACKNOWLEDGEMENTS

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