

Production of Protease by Actinomycetes under Submerged Fermentation

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Abstract: Actinomycetes were the most effective as well as biotechnological valuable prokaryotes and were liable for secondary metabolites production like antibiotics, organic acids, vitamins and enzymes. They were present in both terrestrial as well as marine environment. Marine actinomycetes were the important sources of enzymes isolation industrially. Present study deals with the collection of samples from 2 different places along the coast of Bay of Bengal near Visakhapatnam, India. 30 isolates were procured from the samples and all the samples were screened for enzyme activity as well as antibacterial activity. The isolate B22 showed enzyme activity against protease. Fermentation conditions viz., incubation time, incubation temperature, initial pH and inoculum age were optimized for maximizing the enzyme production by B22 strain. With the employed optimized conditions, B22 isolate showed the maximum protease activity of 46.13U/mL.

Keywords: Actinomycetes, Enzymes, Protease and Submerged fermentation

1. INTRODUCTION

Actinomycetes are aerobic, gram-positive bacteria having high (G+C) of about 55% in their DNA [1]. They have been an origin of a numerous useful products including pharmaceuticals, agrochemicals, vitamins and enzymes for use in a number of industrial applications. Actinomycetes are present in marine and terrestrial ecosystem [2]. Actinomycetes from terrestrial ecosystem have been exhausted and isolation of new strain from it is difficult, where as marine resource act as a home for millions of microorganisms.

Enzymes are biological catalysts that increase the rate of chemical reactions. The enzymes may act as molecules are called substrates, and the enzyme converts the substrate as products.

Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. They are diner in their specific activity, substrate specificity, pH, temperature optima, active site and catalytic mechanisms. Based on the site of action on the substrate, these are classified into endo-proteases and exo-proteases. Endo-proteases acts at the inner region of the polypeptide chain whereas, Exo-proteases acts at the end of the polypeptide chain. Based on the pH, these are classified into acid proteases, neutral proteases and alkaline proteases [3]. They have wide applications in detergent industry, food industry such as in dairy industry, baking industry, in manufacturing of soya products and in debittering of protein hydrolysates and pharmaceutical industry [4].

Submerged fermentation utilises free flowing liquid substrate, such as broth. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly, hence need to be constantly replaced or supplemented with nutrients. This fermentation technique is best suited for microorganisms that require high moisture content [5]. An additional advantage of this technique is that purification of products is easier. SMF is primarily used in the extraction of secondary metabolites that need to be used in liquid form.

Present study deals with the isolation of actinomycetes from marine sediment samples along the coast of Bay of Bengal near RK beach, Visakhapatnam, India and to optimize the process parameters for the maximum production of protease enzyme.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of two marine sediment samples from Bay of Bengal (Muthyalapalem and Dewis beach, Visakhapatnam) were collected and set aside in sterile containers for the systematic screening of actinomycetes. About 50g of each sample was collected from different regions at a depth of 20cm.

2.2 Isolation of Actinomycetes

Marine sediment samples were set aside at 4°C until isolation. Actinomycetes are isolated by planting out the samples in proper dilutions. About 5g of each of above sample was taken in a 250ml conical flask containing 100ml of sterile distilled water kept on an orbital shaker for 24 hours. The sample suspension was diluted serially up to 10⁻¹⁰ folds. Isolation was carried out on starch casein agar (SCA) plates by pour plate method seeded with sample suspension of 1ml each and incubated at 28°C for 14days [6]. After 14days, actinomycetes colonies were isolated from different plates. The actinomycetes colonies, which appeared were transferred and incubated at 28°C for 14days and maintained on SCA slants and pure cultures at 4°C [7].

2.3 Primary screening for Protease activity

The Actinomycetes isolates were streaked on Starch Casein Agar (SCA) medium and incubated at 28°C for 5 days. After 5 days the plates were observed for clear zones around the actinomycetes colonies which indicate protease activity [8].

2.4 Assay for Protease activity

The reaction mixture containing 500µL of 1% casein, 300µL of 0.2M phosphate buffer (pH 7.0) and 200µL of enzyme extract were incubated at 60°C for 10min and arrested by the addition of 1mL of 10% TCA. The solution was centrifuged at 8000rpm for 15min and to the supernatant, 5mL of 0.4M sodium carbonate, 1mL of 10⁻³ diluted FC reagent was added and incubated at room temperature for 30min and then the absorbance was read at 660nm using UV-visible spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberated 1µg of tyrosine from substrate (casein) per min under assay conditions.

$$\text{Protease activity (U/mL)} = \frac{\mu \text{ mole of tyrosine equivalent released} * \text{Total volume of assay (mL)}}{\text{Time of assay (min)} * \text{Volume of assay (mL)} * \text{Volume in spectro (mL)}}$$

The stock and working solutions of tyrosine were prepared and the absorbance was measured at 660nm. A standard graph was drawn between the optical density at 660nm and concentration of tyrosine (U/mL) [9].

2.5 Optimization Studies

To improve the enzyme yield in the production of protease various parameters were studied and optimized. The effect of incubation time on enzyme production was determined by incubating the inoculated flasks for 1-10days and activities were estimated for every 24hr. The effect of temperature was studied by incubating from 26°C, 28°C, 30°C, 32°C and 34°C. Effect of initial pH was studied by adjusting pH from 5 to 9 by varying one unit. Influence of inoculums age studied from 4th day to 8th day.

3.RESULTS AND DISCUSSION

3.1 Isolation of Actinomycetes

A total of 30 actinomycetes were isolated from the two marine sediment samples and were designated as A and B. The no. of isolates obtained was shown in Table.1. The isolation plates were shown in Fig.1.

Table No.1: No. of isolates obtained from marine sediment samples

SAMPLE	ISOLATE
A	5
B	25

The B22 isolate was shown in slant in Fig.2.

3.2 Primary screening for Protease activity

The actinomycetes isolates were streaked on casein agar medium and incubated at 28°C for 5 days. After 5 days the plates were observed for clear zones around the actinomycetes colonies. B22 has shown a clear zone around the colony indicating protease activity shown in Fig.3.

3.3 Assay of Protease activity

Isolate B22 was subjected to submerged fermentation in 50 ml of production medium was inoculated with 4% inoculums prepared from a 7th day old culture. After 7 days, the fermentation broth was centrifuged at 10,000 rpm for 20 minutes at 8°C and clear culture filtrate obtained after centrifugation was assayed for protease activity. Isolate B22 showed protease activity of 3.33 U/ml.

3.4 Optimization of process parameters

3.4.1 Effect of Incubation Time

The effect of incubation time on protease production was studied by carrying out the fermentation at different incubation times ranging from 1 day to 10 days.

Protease production by B22 has increased gradually up to day 5 and then decreased which was shown in Fig.4.

This decrease in protease production after 5th day might be due to a reduction in the available nutrients and accumulation of toxic products of metabolism in the production medium. Debananda [10] reported the similar results that *Nocardopsis prasina* HA-4 achieved maximal protease production in 5 days.

3.4.2 Effect of Incubation Temperature

The effect of incubation temperature on protease was studied carrying out the fermentation at different temperatures viz., 26°C, 28°C, 30°C, 32°C, and 34°C.

B22 has shown maximum protease production at a temperature of 30°C. From the result we can observe that temperature had a significant impact on protease production which was shown in Fig.5. Generally, with increase in temperature, protease activity increases up to certain extent. Later, it reaches a maximum value and then decreases further.

Similar results obtained for Alissara Reungsang [11] reported that maximum protease production by *B.megaterium* were achieved ranging at 30°C to 45° C.

3.4.3 Effect of Initial pH

To study the effect of initial pH on protease production, pH of the production medium was adjusted to different pH values of 5.0, 6.0, 7.0, 8.0 and 9.0 and then fermentation was carried out.

B22 has shown maximum protease production at an initial pH 5.0 which was shown in Fig.6.

Muhammud [12] stated that the growth and other metabolic activities of the microorganisms for extracellular products depend upon the environmental pH.

3.4.4 Effect of Inoculum Age

The effect of inoculum age on protease production was studied by varying the age of inoculum from 4th day to 8th day.

B22 has shown maximum protease production with a 5th day old culture which was shown in the Fig.7. The health and morphological form of the inoculum is very important in fermentation process. The age of inoculum influences the pattern of growth and product formation by microorganism.

Ravishankar [13] and Roja [14] have reported maximum protease production with a 4th day old culture using *Aspergillus flavus* AS2.

4. FIGURES

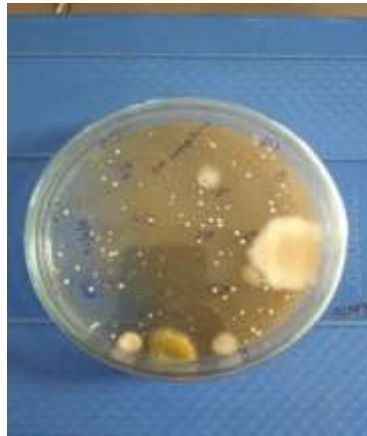


Fig.1: Actinomycetes obtained from marine sample

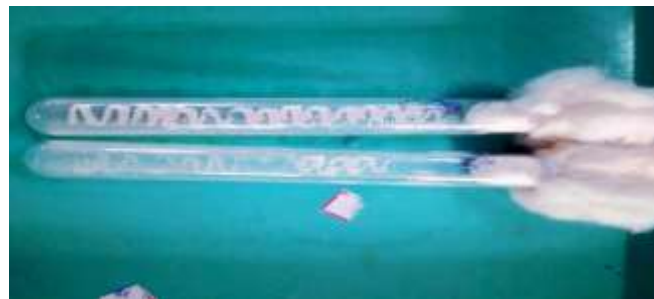


Fig.2: B22 strain shown in slants



Fig.3: Primary screening for protease activity showing clear zone around B22 strain

Fig.4: Effect of Incubation Time

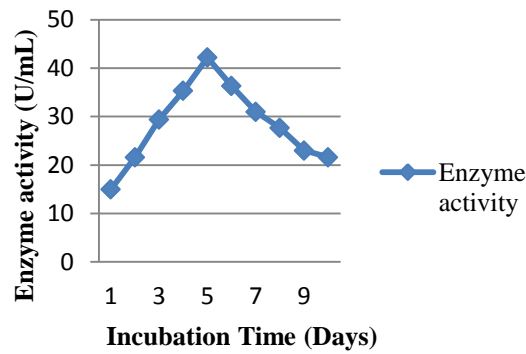


Fig.5: Effect of Incubation Temperature

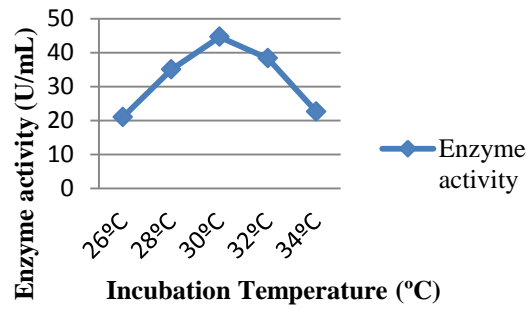


Fig.6: Effect of Initial pH

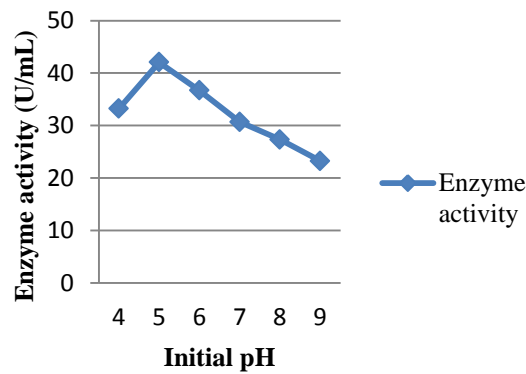
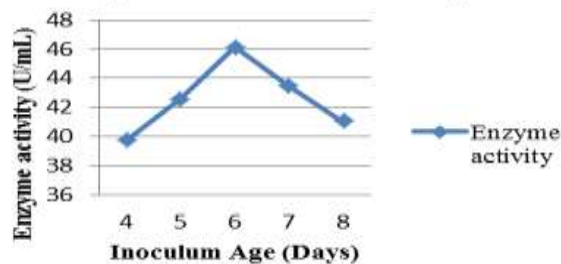


Fig.7: Effect of Inoculum Age



5. CONCLUSION

The diversity of life in the marine environment is extraordinary. Marine ecosystem is the one of the promising target for survey of bioactive actinomycetes. In the present study protease activity of novel actinomycetes strain isolated from marine sediments were determined. An attempt was made to optimize the fermentation conditions to obtain better yield of enzyme. The physiological parameters optimized are incubation time, temperature, initial pH and inoculum age. The maximum protease activity is 46.13U/mL, obtained for 5days at temperature 30°C, 5.0 pH of inoculums 6th day old culture. The isolated marine actinomycetes B22 showed moderate protease activity and is a potential candidate for industrial use.

6. REFERENCES

- [1] Ganesan P, Reegan AD, David RHA, Gandhi MR, Paulraj MG, Al-Dhabi NA, Ignacimuthu S. Antimicrobial activity of some actinomycetes from Western Ghats of Tamil Nadu, India. Alexandria J Med (2016).
- [2] Demain AL (1999) Pharmaceutically active secondary metabolites of microorganisms. Appl Microbiol Biotechnol 52: 455-463.
- [3] Rao VD, et al. (1998) Structure of type II beta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. Cell 94(6):829-39.
- [4] T. Janaki "Enzymes from actinomycetes- review", International Journal of ChemTech Research Vol.10 No.2, pp 176-182, 2017.
- [5] Pandey and soccol "Application of Tropical Agro-industrial Residues as Substrate for Submerged Fermentation Processes" 1998.
- [6] Ramesh S, Narayanasamy M (2009) Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World J Microbiol Biotechnol 25: 2103-2111.
- [7] Williams ST, Cross T (1971) Actinomycetes In: Booth C, editor. Methods in microbiology (Volume 4). Academic press, New York.
- [8] Hassan et al., 2012 "Anti-inflammatory activity of crude saponin extracts from five nigerian medicinal plants", 9(2):250-255 <http://dx.doi.org/10.4314/ajtcam.v9i2.10.250>.
- [9] R. Paranthaman, "Production of Protease from Rice Mill Wastes by Aspergillus niger in Solid State Fermentation" World Journal of Agricultural Sciences 5 (3): 308-312, 2009 ISSN 1817-3047 © IDOSI Publications, 2009.
- [10] Debananda S. Ningthoujam, "Screening, Identification of Best Producers and Optimization of Extracellular Proteases from Moderately Halophilic Alkali thermo tolerant Indigenous Actinomycetes", World Applied Sciences Journal 7 (7): 907-916, 2009 ISSN 1818-4952 © IDOSI Publications, 2009.
- [11] Alissara Reungsang "Biohydrogen production from cassava starch manufacturing wastewater", 2006.
- [12] Muhammad "Zinc, copper and iron levels of normal and low birth weight neonates and their respective mothers", J App Pharm 01(01): 37-42; October, 2009.
- [13] Ravishankar et al., 2012. In: Makkar, H.P.S, Biofuel co-products as livestock feed - opportunities and challenges, FAO, 423-446.
- [14] Z. Roja "Ergonomics risk analysis in construction operations", Agronomy Research 14(2), 15-12, 2016.