

EXTRACTION AND ACTIVITY STUDIES OF INDUSTRIALLY IMPORTANT ENZYMES FROM MARINE *FUSARIUM SPECIES* ISOLATED FROM MACHILIPATNAM SEA WATER, (A.P), INDIA.Suseela Lanka¹, Muralidhar Pydipally² and J. Naveena Lavanya Latha^{1*}¹Department of Biotechnology, Krishna University, Machilipatnam, 521001, Andhra Pradesh, India.²Vimta Labs, Cherlapally, Hyderabad, 500 007, Telangana, India.***Corresponding Author: Dr. J. Naveena Lavanya Latha**

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ABSTRACT

Background: The Industrial application of microbial enzymes to large-scale organic synthesis is currently attracting much attention and has been uniquely developed especially in Japan. The discovery of new microbial enzymes through extensive and persistent screening has brought about many new and simple routes for synthetic processes. The applications of these enzymes in so-called hybrid processes of enzymatic and chemical reactions provide one possible way to solve environmental problems. So the current study is aimed at isolating a potential enzyme producing fungus from marine water samples of Machilipatnam coastal region. **Materials and Methods:** Screening for hydrolytic fungi from marine water samples led to the isolation of 10 different fungal strains. The strains were screened qualitatively by the formation of clearance zones in the petriplates and quantitatively by the enzyme assays. **Results:** Of the strains isolated, *Fusarium* sp. was found to be the best organism and on the basis of clearance zone on the specific media, the fungal species was found to produce multiple industrially important enzymes i.e. proteases, amylases and lipases. Then it was selected for further studies to estimate the enzyme activity and better amylase activity was identified. **Conclusions:** The halophytic fungi were found to be good sources of hydrolytic enzymes viz. proteases, amylases and lipases. **Main conclusion:** Compared to the culture collection strains, the environmental isolates can produce potential industrial enzymes for various biotechnological applications. The *Fusarium* sp. was found to be the best choice because of its ability to produce multiple industrial enzymes with reasonably good enzymatic activities. The highest amylase producing activity of the strain can be exploited in different industries that use amylase.

KEYWORDS: *Fusarium* sp., Protease, Amylase, Lipase, Enzyme activity.**INTRODUCTION**

Enzymes have played an important role in many aspects of life since the dawn of time. Civilizations have used enzymes for thousands of years without understanding what they were or how they work. Enzymes are natural protein molecules that act as highly efficient catalysts in biochemical reactions, i.e. they help a chemical reaction take place quickly and efficiently. Enzymes not only work efficiently and rapidly, they are also biodegradable.

They are highly efficient in increasing the reaction rate of biochemical processes that otherwise proceed very slowly, or in some cases, not at all. They are categorized according to the compounds they act upon. Some of the most common include; proteases which break down proteins, cellulases which break down cellulose, lipases which split fats (lipids) into glycerol and fatty acids, and amylases which break down starch into simple sugars. They play a diversified role in many aspects of everyday life including aiding in digestion, the production of food

and several industrial applications.^[11, 12, 18, 28] They have a great number of usages in food, pharmaceutical, textile, paper, leather and other industries.^[8]

The microbial cell, i.e., a bacterium, yeast, or mold, is the key instrument in many enzyme production processes. In recent years, the most significant development in the field of synthetic chemistry has been the application of biological systems to chemical reactions. Reactions catalyzed by enzymes or enzyme systems display far greater specificities than more conventional forms of organic reactions.^[13,29] Accordingly, the industrial use of enzymes have been developed rapidly.^[24] However, in many cases, the substrates in industrial processes are artificial compounds and enzymes known to catalyze suitable reactions for such processes are still unknown. Therefore, screening for novel enzymes that are capable of catalyzing new reactions is constantly needed. One of the most efficient and successful means of finding new

enzymes is to screen large numbers of microorganisms, because of their characteristic diversity and versatility.^[19, 25] By using the tools of modern biotechnology, the enzyme industry has developed safe host organism systems for the production of many enzymes that could not otherwise be produced. Considering the industrial importance of microbial enzymes, the present study was aimed at isolation of fungal strains capable of producing different industrially important enzymes. The investigation led to the identification of multiple enzymes producing *Fusarium* strain.

MATERIALS AND METHODS

Isolation of Fungi from Marine water

All fungal strains tested for enzyme activities were isolated from marine water from Machilipatnam coastal region located in Andhra Pradesh, India. Screening was done by serial dilution method, wherein PDA (potato dextrose agar) media was prepared, autoclaved and poured in sterile petriplates. 50 µl of marine water sample was diluted up to 10⁻⁵ dilutions and was spread on respective solidified PDA plates. The inoculated petriplates were incubated at 28°C for a period of 5 days. Ten different fungal isolates were differentiated on the basis of morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the fungal isolates were cultivated on czepakdox agar medium. The shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog et al.^[10]

Screening for extracellular enzymes

The fungal isolates obtained were then screened for production of extracellular enzymes viz, Amylases, Proteases and Lipases.

Screening for Amylase producers

Isolates were grown on starch agar medium containing soluble starch-2%, peptone-0.05%, KCl- 0.01% (w/v), MgSO₄·7H₂O-0.05% (w/v), (NH₄)₂SO₄-0.01%, NaH₂PO₄-0.01% (w/v).^[26] After seven days of incubation at 28°C, the culture plates were tested for Amylase activity. A zone of clearance observed around the fungal inoculums when Grams iodine solution is added was then selected for further biochemical investigations. Biochemical estimation for amylase was done by starch-iodine method for selected isolates showing high zone of clearance.^[1]

Screening for Protease producers

The isolates were screened for protease producing ability by using Casein Agar Medium^[3, 21] containing Casein-0.3%, KNO₃-0.3%, NaCl-0.2%, K₂HPO₄-0.2%, MgSO₄-0.005%, CaCl₂-0.002%, Yeast extract-0.1%, Agar-3% and Skin milk agar Medium composed of Skin milk powder-10%, NaCl-5%, and Agar-2 %. The protease activity was detected by the formation of a zone of clearance around the colony.

Screening for Lipase producers

The lipolytic activity of the fungal cultures was observed using Rhodamine plate assay and Modified lipase assay media. Fungal isolates were inoculated in the petriplates with Modified lipase assay medium^[2,4] (15 g Peptone, 5 g NaCl, 1g CaCl₂, 10 ml Tween 20, 15 g Agar and pH 7.0) and the culture filtrate was then used for enzyme assay. The lipase activity was detected due to occurrence of a zone of clearance around the colony and subsequent formation of white precipitate of calcium mono laurate around the colony.^[5,7] The lipolytic activity of the culture was observed, using Rhodamine plate assay^[14] with Rhodamine B- Olive oil Agar medium containing Olive oil 3% (v/v), Agar 2% (w/v), Rhodamine B 1% (v/v), Tris HCl buffer (pH 7), 50 mM CaCl₂ 1%. The Rhodamine agar medium was prepared in distilled water, autoclaved and cooled to 60°C. The cooled medium was added with 3% of olive oil previously sterilized at 160°C for 2 hrs. in hot air oven and 1% filter sterilized Rhodamine B (1 mg/ml). The contents were mixed well to dissolve and the medium was poured into petri dishes. Circular wells of 3 mm were punched in the agar plates and 10 µl of the culture supernatant was dispensed into each well. Lipase activity was identified as an orange fluorescent halo under UV light at 350 nm after 24 hrs. of incubation at 37°C.

The best enzyme producers were selected based on measurements of clearance zones and they were further quantified by enzyme assay methods.

Assay for Amylase activity

Amylase activity was determined as described by Okolo et al.,^[20] The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme extract. After 10 minutes of incubation at 50°C, the reducing sugars (glucose equivalents) liberated were estimated by the Dinitrosalicylic acid (DNS) method.^[16] The blank consists of 0.5 ml of 0.1 M acetate buffer (pH 5.0), 1.25 ml of 1% starch solution and 0.25 ml of distilled water. One unit of amylase activity is defined as the amount of enzyme releasing 1 µmol of glucose equivalent per minute under the assay conditions.

Assay for Protease Activity

Protease activity was assayed using Hayashi et al., modified method.^[9] The reaction mixture containing 1ml enzyme, 5 ml of 1% casein (50mm Tris, pH 8) substrate were incubated for 1 hr at 37°C. To the reaction mixture, 5 ml of TCA (10 mM) was added to stop the reaction and incubated for 30 min at 37°C. The reaction mixture was then filtered and 2 ml of filtrate was then added to 5 ml of 500 mM sodium carbonate solution and 1 ml of folin phenol reagent. This was incubated for 30 min at 37°C and the colour intensity was measured at 660 nm.

Assay for Lipase Activity

Lipase assay was carried out by using copper soap method. Quantification of fatty acids released by lipase is

determined by reference to a standard curve prepared using oleic acid. 5ml of benzene and 1 ml of cupric acetate were taken into screw-cap test tubes and into a 50 ml Erlenmeyer flask with a stopper, 25 ml of olive oil taken was then pre incubated for 15 min with magnetic stirring in a water bath at 37°C. Sufficient amount of enzyme was added to initiate lipolysis of the emulsified substrate. The mixture is then immediately vortexed for 2 min to stop the reaction and to form the colored fatty acid cupric soaps. The mixture is then centrifuged at 1000 rpm for 5 min at room temperature to obtain the clear benzene upper phase.^[15] The absorbance is measured at 715 nm for the benzene layer of sample against reagent blank.

RESULTS AND DISCUSSION

Microbial enzymes are often more useful than enzymes derived from plants and animals because of the great variety of catalytic activities available, the high possible yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media.^[6] Microbial enzymes are also more stable and safer than their corresponding plant and animal enzymes and their production is more convenient.

The aim of the present work is to isolate a potential fungal species among 10 fungal isolates that were screened and isolated from the marine water using PDA medium. The fungal isolates were tested for their ability to produce three industrially important enzymes like amylases, proteases and lipases. As marine environment is highly diversified we have selected marine water for our investigation. The aim of the work is achieved by the isolation of a fungal strain which showed better enzyme producing activities both qualitatively by the formation of zones in the petriplates and quantitatively by the enzyme assays. The potential fungal strain was identified as *Fusarium* sp. based on morphological studies.

Screening of the fungal isolates for amylase production was carried out in starch agar plates followed by iodine test and observed for zone of hydrolysis. Figure A shows the amylase activity of the fungal isolate. Amylolytic activity of the crude enzyme extract was also determined quantitatively by the method of Okolo *et al.*,^[20] and it was found to be 136.2 U/mL (Table 1).

Proteolytic bacteria are widespread in nature and are able to grow under various growth conditions such as different temperatures, pH and ionic strength. Extracellular proteolytic activity of fungal isolate is determined by the partial hydrolysis of milk casein present in the skim milk agar medium, resulting in the formation of clear zones of different sizes with translucent and cream whitish in colour. The results were shown in the Figure B. The presence of halo tolerant protease from the halophilic fungi could be applied in industrial processes where the concentrated salt solution

used would inhibit ordinary proteases.^[17, 22, 23] The hydrolytic zone produced on the casein plate could be related to the amount of protease produced by the fungus.^[27] Protease assay was carried out by the method of Hayashi *et al.*,^[9] to estimate the proteolytic activity by the addition of crude enzyme to the substrate. Assay results were compared with the standard curve of tyrosine and the enzyme activity was found to be 81.6 U/mL (Table 1). Protease unit was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the defined assay conditions.

A rapid assay of lipase activity was done by using Rhodamine B-Olive oil plates to identify specific lipase producers.^[14] Lipase activity was detected as orange-red fluorescent halos upon UV irradiation and the results were shown in Figure C. The isolates which showed positive for lipase production were subjected to further screening based on quantitative estimation using copper soap method. Fatty acids liberated during hydrolysis of olive oil substrate by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λ_{max} 715 nm), yielding a blue colour.

Quantification of fatty acids released by lipase is determined by reference to a standard curve prepared using oleic acid and the enzyme activity was found to be 84.1 U/mL (Table 1).

Considering the above results a unique fungal strain has been isolated from the marine water that is capable of producing all the three industrially important enzymes *viz.*, amylases, proteases and lipases which are widely used commercially.

Figure A: Amylase activity was determined by a qualitative method, where the isolates were grown on starch agar medium and the culture plates were tested after seven days of incubation at 28 °C by the addition of Grams Iodine. Presence of amylase activity was confirmed by a zone of clearance around the fungal inoculums.

Figure B: Protease activity was determined by a qualitative method, where the isolates were grown on casein agar medium and skin milk agar Medium. The protease activity was confirmed by the formation of a zone of clearance around the colony.

Figure C: The lipolytic activity of the culture was observed using Rhodamine plate assay with Rhodamine B- Olive oil Agar medium. Circular wells of 3 mm were punched in the agar plates and 10 µl of the culture supernatant was dispensed into each well. Lipase activity was identified as an orange fluorescent halo under UV light at 350 nm after 24 hrs of incubation at 37 °C.

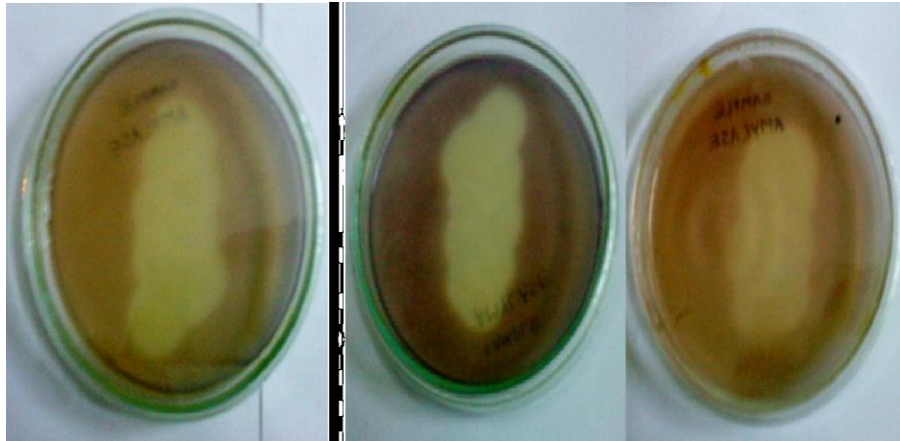


Figure: A. *Fusarium* sp. showing amylase activity

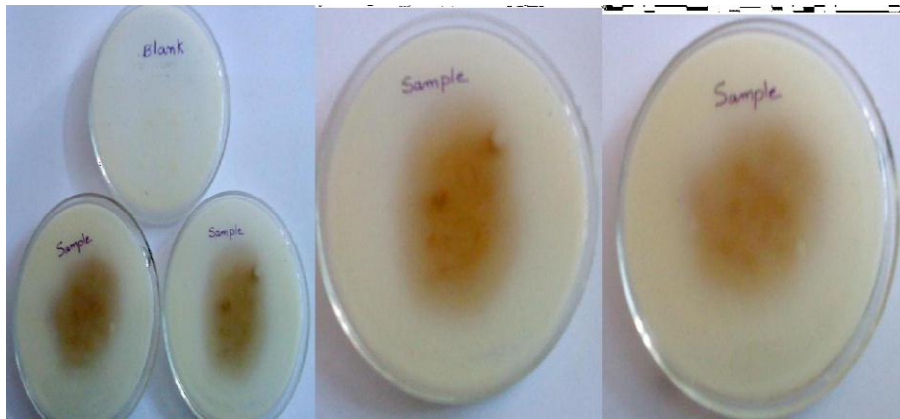


Figure B: *Fusarium* sp. showing protease activity

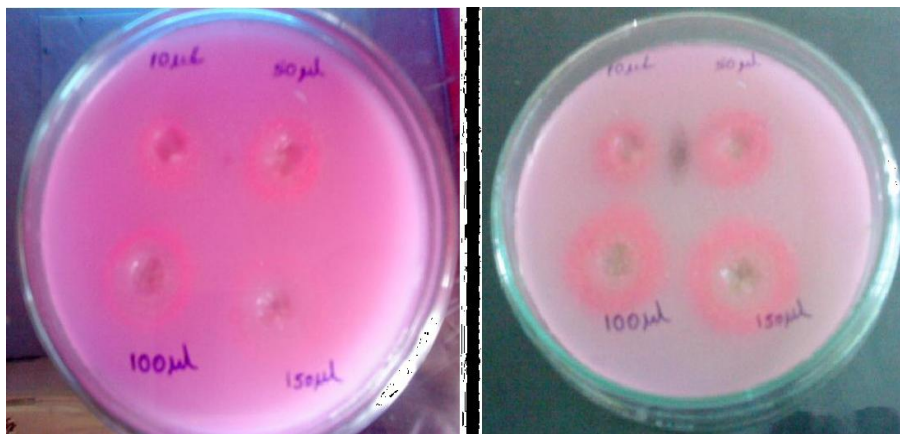


Figure C: *Fusarium* sp. showing lipase activity

Table: 1. Enzyme assay results:

S. No	Enzyme	Enzyme Activity (Units/mL)
1.	Amylase	136.2
2.	Protease	81.6
3.	Lipase	84.1

CONCLUSION

The Assay results show that the strain has highest amylase producing activity than the proteases and lipases. Further optimization and strain conformation studies have to be conducted to recover the highest yield from the fungal strain.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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