

Screening and Isolation of Lipase Producing Fungi from Marine Water Obtained from Machilipatnam Costal Region

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ABSTRACT

The marine environment is highly susceptible to pollution by oil spillages and hence become an importance source for screening potential lipolytic microorganisms capable of degrading hydrocarbons. In addition to their salt tolerance, enzymes from halophiles can withstand and carry out reactions very much efficiently under extreme operational conditions encountered in industrial processes. In the present investigation, an extracellular lipase producing fungal organisms were screened and isolated from marine water samples collected from Manginapudi beach at Machilipatnam, Krishna district, A.P. India. Among 15 species of fungi isolated, 5 dominant fungi were selected to study their lipase producing activity. The extracellular lipase secreted in to the minimal salt broth was assayed both by qualitative screening method such as phenol red agar plate method and quantitative titrimetric method. Of the 5 dominant fungal isolates, *Engyodontium* sp. was found to produce maximum lipase, 7.2 U/mL compared to other fungal members. Since there is growing demand for lipases, rapid and persistent screening for microorganisms will open new insights for novel enzymes to be used for various synthetic processes and as well provides faster ways to solve many environmental problems. The present study gains its importance in view of this.

Keywords: Marine environment, screening, lipolytic microorganisms, extracellular lipase, *Engyodontium* sp.

INTRODUCTION

Hydrocarbon contamination is one of the major environmental problems today. Accidental release of petroleum products by shipping activities and release of oil spills from various petro chemical industries are of major concern for pollution of marine water ecosystems¹. Oil spills in marine waters may occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. This contributes to organic pollution in marine environment and these products are classified as hazardous wastes due to their mutagenic, cytotoxic and carcinogenic effects on human^{2,3}. The vast diversity of marine microorganisms play crucial role in degradation of these hydrocarbons that are released due to various human activities.

Oceanic waters occupy more than 70% of our earth's surface. They are prime sources in that they contribute a unique habitat for the growth of a wide variety of microorganisms. Marine microbes are considered as important entities in marine environments due to their performance in biogeochemical processes⁴. Marine environments are also repositories of a diverse genetic make ups and can provide new insights and understanding of many secondary metabolites. Several researchers directed their research towards exploitation of marine microbes in the production of bioactive products⁵. Many of the pharmaceutically important compounds were also isolated from marine life. Marine microbes are also

important sources of various industrially importance enzymes⁶. The prevailing extreme environmental conditions could be the reason for differences between the enzymes produced by marine microbes^{7,8,9,10}.

Among diverse microbial species, fungi are an important category of organisms in the marine world. A myriad of potential enzymes with novel physiological characteristics can be produced by fungi owing to their unique capacities⁶ which could be due to their characteristic properties that include their ability to adjust to harsh marine environments like changing temperature, nutrients, and salinity etc. To overcome the above said conditions, they have developed unique secondary metabolic pathways compared with terrestrial organisms. Among fungi, marine filamentous fungi have proved for their ability to produce many pharmacologically important secondary metabolites which is also proved by recent studies. Hence fungi in general and marine fungi in particular are considered as potential candidates for the screening and isolation of industrially important enzymes especially lipases.

The use of enzyme-mediated processes can be traced to ancient civilizations. Today, nearly 4000 enzymes are known, and of these, about 200 are in commercial use. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly¹¹. The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60 % of the total world supply of industrial enzymes is produced

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in Europe. At least 75 % of all industrial enzymes (including lipases) are hydrolytic in action. The global market for industrial enzymes is estimated at \$4.61 billion in 2016 and is expected to rise at a compound annual growth rate (CAGR) of 5.8 % from 2017 to 2022¹². Lipases are considered to be the third largest group based on total sales volume after proteases and carbohydrases. Lipases have the ability to bring about hydrolytic and synthetic reactions in both aqueous and non-aqueous media, hence have multifold applications in different industrial processes as important biocatalysts^{13,14,15}. They have shown their tremendous applications in industries such as oleo-chemical, organic synthesis, detergent formulation, nutrition, dairy, textiles, leather and paper^{16,17}. The most traditional application of lipases has been flavor development in cheese which has now been extended to a variety of other dairy products and confectionaries like butter, milk-chocolate and sweets which is achieved by selective hydrolysis of triglycerides to release free fatty acids that act as flavor precursors¹⁸. Marine fungi with their salt-tolerant enzymes have been used for bioremediation of environmental pollutants¹⁹. The potentiality of marine-derived fungi to grow on relatively rather simple and inexpensive substrates, and ability to produce enzymes with different physiological characteristics can place them at the forefront of contemporary commercial applications²⁰. Considering the industrial importance of lipases and the vast marine biodiversity coupled to the potential of marine fungi in the production of many industrially important enzymes, the current study is undertaken to isolate marine fungal isolates capable of producing lipases.

MATERIALS AND METHODS

Sample Collection

All the fungal strains tested for lipase activity were isolated from marine water sample obtained from Manginapudi beach at Machilipatnam, Krishna district, Andhra Pradesh, India. The marine water was collected in a sterile container and stored at 4 °C when not used immediately.

Screening of lipase producing *Mycoflora*

For the isolation of lipase producing microorganisms, minimal salt agar medium was prepared by adding olive oil as a sole carbon source with a slight modification²¹. It contained (g L⁻¹), KNO₃ (2.5), KH₂PO₄ (1), MgSO₄ (0.5), NaCl (5), Agar (12) and olive oil (15) with a pH of 8. The olive oil emulsified in the Tween 20 should be sterilized separately and then added to the sterilized medium in the laminar air flow before pouring into the petridishes. The marine water was serially diluted to 10-fold and approximately 100 µl of serially diluted sample was spread on lipase production medium and the plates were incubated at 20 °C for a period of 5-7 days and checked every day for growth for lipase producing species and for monitoring the problem of contamination.

Potato dextrose agar medium

The developed colonies were purified and transferred to the Potato dextrose agar slants for storage.

Partial identification of fungal isolates

The lipase producing halophilic fungi were identified based on morphological characteristics and microscopic observation of fungal spores using lactophenol cotton blue staining. For morphological characterization, the shape, size, arrangement and development of conidiophores, phialides and conidiospores were studied using the taxonomic tools of Hoog *et al.*²².

Lipase production

Lipase activity was studied by growing the fungi in 45 ml of minimal salt broth in 250ml Erlenmeyer flasks. Mature spore suspensions (10⁶ spores/ml) were prepared by gently washing the surface of the agar slant with 5 ml of sterile saline and the suspension was then adjusted to 10⁶ spores/ml in a Neubauer haemocytometer. 5 ml of diluted spore suspension was then added to the mineral salt broth. The flasks were incubated at 28 °C for a period of 96 hrs at 120 rpm. The flasks were harvested by filtering the contents through Whatman filter paper no.1. The filtrates were collected in pre-sterilized culture filtrate bottles and termed as crude enzyme.

Qualitative screening method- Phenol Red Agar method

Determination of lipase activity was done with the help of phenol red agar plate method. The medium contained phenol red (0.01%), olive oil (1%), CaCl₂ 10 Mm and agar (2%). The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH²³. After sterilization, the medium was poured into petriplates and after solidification, circular wells of 4mm were punched in the center of the petriplate by the help of corkborer. The well was filled with 100 µl of culture filtrate (crude enzyme) and the plates were incubated at 40 °C for a period of 15 min. The circular zone appeared around the well was measured mm as lipase activity.

Quantitative titrimetric method

The lipase activity was assayed using the method of Yadav *et al.*,²⁴ using olive oil as a substrate. Briefly, the method involves pre incubation of the reaction mixture (5ml of olive oil emulsion in 0.1 M 20 ml phosphate buffer) at 37 °C for 10 min followed by incubation of reaction tubes at 40 °C for 30 min with shaking at 120 rpm by adding 1ml of the test samples. The reaction was terminated by the addition of 15 ml of acetone-ethanol (1:1). The free fatty acids released were then titrated against 0.05 N NaOH after the addition of few drops of phenolphthalein indicator. For each reaction the control (heat inactivated enzyme) and the test (active enzyme) were maintained separately. All the tests were conducted in triplicates and mean activities were conducted adding the enzyme just before titration. One unit of lipase activity was defined as the amount of enzyme which produces 1µmol of fatty acids per minute under assay conditions.

RESULTS AND DISCUSSION

The marine water samples were collected from Manginapudi beach at Machilipatnam, Krishna District, Andhra Pradesh, India. Of the 10 genera and 15 species of fungi isolated from marine water, 5 dominant fungi were selected to further study their enzyme activity (Table 1). They were named as MF 1 to MF 5 indicating their origin i.e. Marine Fungi. The potentiality of these 5 different fungi in lipase production was studied by using minimal

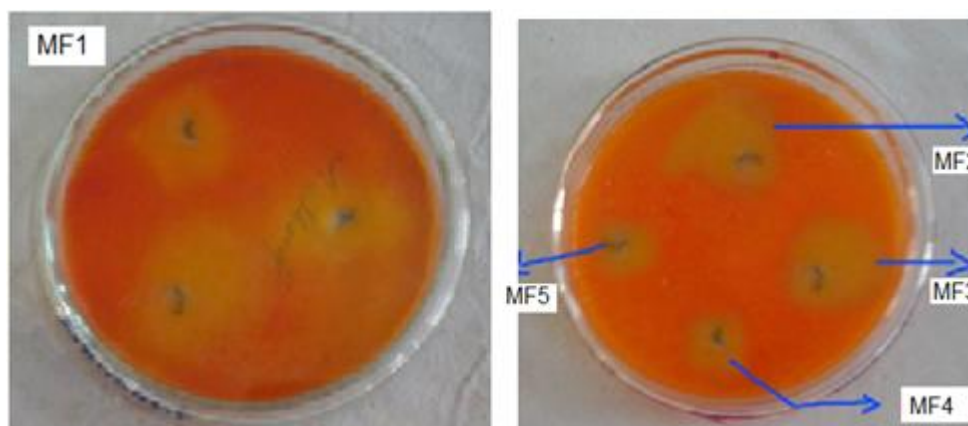


Figure 1: Fungal isolates and their corresponding zones on phenol red agar plate.

MF1-Zones produced by crude enzyme extracts of *Penicillium* sp., MF2, 3, 4 & 5- Zones produced by crude enzyme extracts of *Fusarium* sp., *Engyodontium* sp., *Trichophyton* sp., and *Aspergillus* sp.

Table 1: Dominant Fungal isolates obtained from marine water sample.

S. No	Fungal Isolates
MF 1	<i>Penicillium</i> sp.
MF 2	<i>Fusarium</i> sp.
MF 3	<i>Engyodontium</i> sp.
MF 4	<i>Trichophyton</i> sp.
MF 5	<i>Aspergillus</i> sp.

Table 2: Fungal isolates and their corresponding enzyme activity in mm.

S. No	Fungal Isolates	Enzyme activity (mm)
MF 1	<i>Penicillium</i> sp.	15
MF 2	<i>Fusarium</i> sp.	11
MF 3	<i>Engyodontium</i> sp.	18
MF 4	<i>Trichophyton</i> sp.	16
MF 5	<i>Aspergillus</i> sp.	10

Table 3: Fungal isolates and their corresponding enzyme activity in U/mL.

S.No	Fungal Isolates	Lipase activity (U/mL)
MF 1	<i>Penicillium</i> sp.	6.5
MF 2	<i>Fusarium</i> sp.	5.8
MF 3	<i>Engyodontium</i> sp.	7.2
MF 4	<i>Trichophyton</i> sp.	6.8
MF 5	<i>Aspergillus</i> sp.	5.6

salt agar medium containing olive oil as sole carbon source. Partial identification of fungal strains was done by visual observation of the morphology of fungal sp. in petridish culture and as well with microscopic studies using Lacto phenol cotton blue staining.

Growth and multiplication of microorganisms on any substrates is often considered as the first step towards its bioconversion²⁵. In the Present study 5 fungal sp. out of 15 fungal isolates showed good extra cellular lipase activity. The only organisms isolated were fungi. Bacteria did not grow on the isolation medium. This may be due to differential growth conditions, especially composition and as well the pH of the growth medium.

Lipase activity was determined by a qualitative phenol red agar plate method and a quantitative titrimetric assay was further done to estimate the amount of extracellular lipase produced. The culture filtrate obtained by growing *Penicillium* sp., *Fusarium* sp., *Engyodontium* sp., *Trichophyton* sp., and *Aspergillus* sp. was used for qualitative and quantitative estimation. The *Engyodontium* sp. showed highest lipase activity (7.2 U/mL) compared to other fungal species. Out of 5 fungi examined, 3 species exhibited high enzyme activity, probably because the enzyme activity was associated with growth of the cell and favorable environmental conditions²⁶.

The lipase activity was indicated by a change of colour of phenol red agar medium. The liberation of free fatty acids by the activity of lipase in the crude enzyme extract results in lowering of the pH of the medium. At low pH, the colour of phenol red changes from orange to yellow indicating lipolysis. The zones of hydrolysis in mm by these 5 dominant fungal sp. (MF 1 to MF 5) on phenol red agar plate were found to be 15mm, 11mm, 18mm, 16mm, 10mm respectively (Table 2).

Figure 1 show yellow colored zones due to lipolytic activity of lipase in crude enzyme extract which results in change of colour of phenol red to yellow.

The quantitative estimation of enzyme activity was done by titrimetric method. The lipase activity in U/mL by these 5 fungal isolates, MF 1 to MF 5 were found to be 6.5, 5.8, 7.2, 6.8, 5.6 U/mL respectively (Table 3). Thus, *Engyodontium* sp. was identified as the most potential strain, since it's crude enzyme extract showed maximum zone (18mm) on phenol red agar medium and maximum enzyme activity (7.2 U/mL) in titrimetric estimation.

All the test fungi were found to produce lipase activity in their cultivation fluid when tested by using minimal salt agar medium. A comparison of the data obtained indicated nearly similar pattern of lipase activity except in few cases. In present investigation, an effort has been made to study the lipase activity of 5 test fungi grown on minimal salt agar medium.

In the current study, maximum lipase activity was recorded in culture fluids of *Engyodontium* sp. Production of lipase by this species has been reported earlier by Jaouani et al.,²⁷.

But the lipase production was reasonably good and comparable in all the fungal isolates screened and isolated from marine water. Lipase production by marine *Fusarium* sp. was also reported by Suseela et al.,²⁸. Extracellular lipase from marine *Aspergillus awamori* BTMFW032 was reported by Soorej et al.,²⁹ and from *Aspergillus sydowii* by Bindiya and Ramana³⁰. Extracellular lipase production was also reported from marine *Penicillium* sp. by Smitha et al.,³¹. Overall, the results obtained during this investigation and those reported by other workers indicated that *Engyodontium* is one of the important genera for the production of lipase.

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