Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(7); 928-932
doi: 10.25258/phyto.v9i07.11157

ISSN: 0975-4873

Research Article

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

Suseela Lanka[1*], Tabitha Trinkle B[2]

[1]*Department of Biotechnology, Krishna University, Machilipatnam, 521001, Andhra Pradesh, India.*
[2]*Department of Biochemistry, Krishna University Dr. MRAR PG Centre, Nuzvid, 521 201, Andhra Pradesh, India.*

Received 10th May, 17; Revised 16th June, 17, Accepted 12th July, 17; Available Online 25th July, 2017

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 ecosystems[1]. 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 processes[4]. 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 products[5]. Many of the pharmaceutically important compounds were also isolated from marine life. Marine microbes are also important sources of various industrially importance enzymes[6]. 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 capacities[6] 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 spectacularly[11]. 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

**Author for Correspondence: susheelalankaku@gmail.com*

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 2022[12]. 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 precursors[18]. Marine fungi with their salt-tolerant enzymes have been used for bioremediation of environmental pollutants[19]. 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 applications[20].

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 modification[21]. It contained (g L^{-1}), KNO_3 (2.5), KH_2PO_4 (1), $MgSO_4$ (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 serial 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 (10^6 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 10^6 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 ºC for a period of 96 hrs at 120 rpm. The flasks were harvested by filtering the contents through Whattman 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%), $CaCl_2$ 10 Mm and agar (2%). The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH[23]. 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 ºC for 10 min followed by incubation of reaction tubes at 40 ºC 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

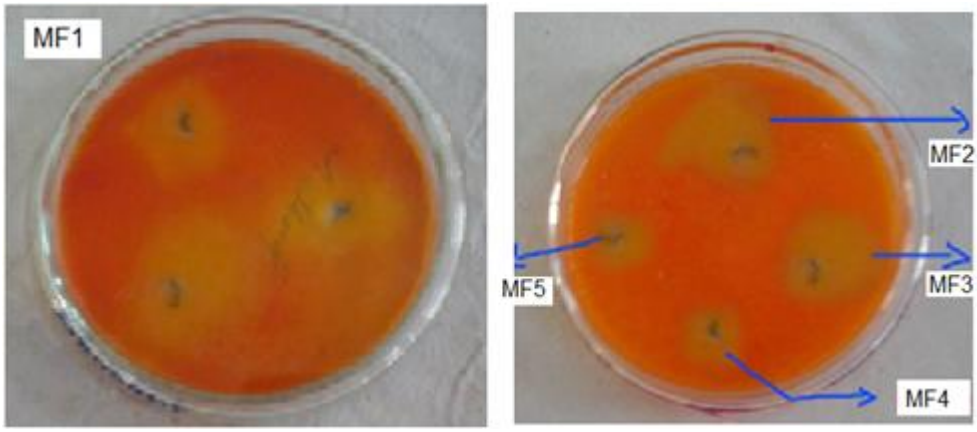

Figure 1: Fungal isolates and their corresponding zones on phenol red agar plate.
MF1-Zones produced by crude enzyme extracts of *Penicillium* sp., MF2, 3, 4 & 5- Zones produced by crude enzyme extracts of *Fusarium* sp., *Engyodontium* sp., *Trycophyton* sp., and *Aspergillus* sp.

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

S. No	Fungal Isolates
MF 1	*Penicillium* sp.
MF 2	*Fusarium* sp.
MF 3	*Engyodonitum* sp.
MF 4	*Trichophyton* sp.
MF 5	*Aspergillus* sp.

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

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

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

S.No	Fungal Isolates	Lipase activity (U/mL)
MF 1	*Penicillium* sp.	6.5
MF 2	*Fusarium* sp.	5.8
MF 3	*Engyodontium* sp.	7.2
MF 4	*Trichophyton* sp.	6.8
MF 5	*Aspergillus* 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 bioconversion[25]. 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 conditions[26].

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 Ramana[30]. 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.

REFERENCES

1. Sarma PM, Bhattacharya D, Krishnan S. Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium *Leclercia adecaroxylata*. Applied and Environmental Microbiology 2004; 70: 3163-3166.
2. Rahman KSM, Rahman T, Lakshmanaperumalsamy P, Banat IM. Occurrence of crude oil degrading bacteria in gasoline and diesel station soils. Journal of Basic Microbiology 2002; 42: 286-293.
3. Margesin R, Labbe D, Schinner F, Greer CW, Whyte LG. Characterization of hydrocarbon degrading microbial populations in contaminated and pristine Alpine soils. Applied and Environmental Microbiology 2003; 69: 3085-3092.
4. Sowell SM, Norbeck AD, Lipton MS, Nicora CD, Callister SJ, Smith RD, et al. Proteomic analysis of stationary phase in the marine bacterium "*Candidatus Pelagibacterubique.*" Applied and Environmental Microbiology 2008; 74: 4091–4100.
5. Mayer AMS, Rodriguez AD, Taglialatela-Scafati O, Fusetani N. Marine Pharmacology in 2009-2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. Marine Drugs 2013; 11(7): 2510–2573.
6. Velmurugan N, Lee YS. *Marine Fungi and Fungal-like Organisms* (*Marine and Freshwater Botany*) "Enzymes from marine fungi: current research and future prospects," ed. Jones EBG. Berlin: Walterde Gruyter, 441–474.
7. Pang K, Chow RKK, Chan C, Vrijmoed LLP. Diversity and physiology of marine lignicolous fungi in arctic waters: a preliminary account. Polar Research 2011; 30: 1–5.
8. Intriago P. Marine microorganisms: perspectives for getting involved in cellulosic ethanol. AMB Express 2012; 2(1): 46.
9. Passarini MRZ, Santos C, Lima N, Berlinck RGS, Sette LD. Filamentous fungi from the Atlantic marine sponge *Dragmacidon reticulatum*. Archives of Microbiology 2013; 195: 99–111.
10. Rämä T, Norden J, Davey ML, Mathiassen GH, Spatafora JW, Kauserud H. Fungi ahoy! Diversity on marine wooden substrata in the high North. Fungal Ecology 2014; 8: 46–58.
11. Godfrey T, West S. Introduction to industrial enzymology in Industrial enzymology. Edn 2, In Godfrey T & West S. (Eds.), New York: Stockton Press 1996; pp.1-8.
12. Industrial enzymes report by Markets and Markets, 2016.
13. Saxena RK, Davidson WS, Sheoran A, Giri B. Purification and characterization of an alkaline thermostable lipase from *Aspergillus carneus*. Process Biochemistry 2003b; 39: 239-247.
14. Naqvi SH, Khan MY, Rafiq M, Dahot MU. Screening of lipase producing fungi and catalytic activity from molasses culture medium. Sindh University Research Journal 2012; 44: 105-112.
15. Thota P, Bhogavalli PK, Rao VP, Sreerangam V. Screening and identification of potential fungal strains for the production of extracellular lipase from soil. Plant Sciences Feed 2012; 2: 79-84.
16. Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson WS. Microbial lipases: production and applications. Science Progress 1996; 79: 119-157.
17. Sharma R, Chisti Y, Banerjee UC. Production, purification, characterization, and applications of lipases. Biotechnology Advances 2001; 19: 627-662.
18. Sharma S, Kanwar SS. Organic solvent tolerant lipases and applications. The Scientific World Journal 2014; Volume 2014, Article ID 625258, 1-15.
19. Passarini MRZ, Rodrigues MVN, DaSilva M, Sette LD. Marine-derived filamentous fungi and their potential application for polycyclic aromatic hydrocarbon bioremediation. Marine Pollution Bulletin 2011; 62: 364–370.
20. Rafaella C. Bonugli-Santos, Maria R. dos Santos Vasconcelos, Michel R. Z. Passarini, Gabriela A. L. Vieira, Viviane C. P. Lopes, Pedro H. Mainardi, Juliana A. dos Santos, Lidia de Azevedo Duarte, Igor V. R. Otero, Aline M. daSilva Yoshida, Valker A. Feitosa, Adalberto Pessoa Jr, Lara D. Sette. Marine-derived fungi: diversity of enzymes and biotechnological applications. Frontiers in Microbiology 2015; 6: 1-15.
21. Suseela L, Muralidhar P, Naveena Lavanya Latha J. Optimization of Process Variables for Extracellular Lipase Production from *Emericella nidulans* NFCCI 3643 Isolated from Palm Oil Mill Effluent (POME) Dump Sites Using OFAT Method. Research Journal of Microbiology 2015; 10(2): 38-53.
22. Hoog GS, Guarro J. Explanatory chapters and keys to the genera, Edn 2, in: Hoog GS, Guarro J, Gene J, Figueras MJ (Eds.), Atlas of Clinical Fungi. Centraal bureau voor schimmel cultures. Spain Press., Netherlands and Universitat Rovira i Virgili. pp.361-1008.
23. Singh R, Gupta N, Goswami VK, Gupta R. A simple activity staining protocol for lipases and esterases. Applied Microbiology and Biotechnology 2006; 70: 679-682.

24. Yadav RP, Saxena RK, Guptha R, Davison S. Lipase by *Aspergillus* and *Penicillium* species. International Journal of Food Microbiology 1993; 19(3): 217-227.
25. Molla AH, Fakhru L, Razi A, Abd-aziz S, Hanafi, MM, Roychoudhury PK, Alam MZ. A potential resource for bioconversion of domestic waste water sludge. Bioresource Technology 2002; 85: 263 -272.
26. Gutarra MLE, Godoy MG, Castilho LR, Freire DMG. Inoculum strategies for *Penicillum simlicissimum* lipase production by solid -state fermentation using a residue from the babassu oil industry. Journal of Chemical Technology and Biotechnoly 2007; 82: 313-318.
27. Jaouani A, Neifar Md, Prigione V, Ayari A, Sbissi I, Amor SB, Tekaya SB, Varese GC, Cherif A, Gtari M. Diversity and Enzymatic Profiling of Halotolerant Micromycetes from Sebkha El Melah, a Saharan Salt Flat in Southern Tunisia. BioMed Research International 2014; Article ID 439197, 11 pages.
28. Suseela L, Muralidhar P, Naveena Lavanya Latha J. Extraction and activity studies of industrially important enzymes from marine *Fusarium* species isolated from Machilipatnam sea water, (A. P), India. European Journal of Pharmaceutical and Medical Research 2016; 3(12): 254-258.
29. Soorej M. Basheer, Sreeja Chellappan, Beena PS, Rajeev K. Sukumaran, Elyas KK, Chandrasekaran M. Lipase from marine *Aspergillus awamori* BTMFW032: Production, partial purification and application in oil effluent. New Biotechnology 2011; 28(6): 627-38.
30. Bindiya P, Ramana T. Optimization of lipase production from an indigenously isolated marine *Aspergillus sydowii* of Bay of Bengal. Journal of Biochemical Technology 2012; 3(5): S203-S211.
31. Smitha SL, Neil Scolastin Correya, Rosamma Philip. Marine fungi as a potential source of enzymes and antibiotics. International Journal of Research in Marine Sciences 2014; 3(1): 5-10.