WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 12, 269-278. Review Article ISSN 2277– 7105

A REVIEW ON DIFFERENT SCREENING METHODS TO IDENTIFY AND ISOLATE POTENT LIPASE PRODUCERS

[1]Arun Kumar Sharma, [1]Vinay Sharma* and [2]Jyoti Saxena

[1]Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India.

[2]Department of Biochemical Engineering, Bipin Tripathi Kumaon Institute of Technology, Dwarahat, Uttrakhand.

Article Received on
22 Sept. 2016,

Revised on 12 Oct. 2016,
Accepted on 01 Nov. 2016

DOI: 10.20959/wjpr201612-7404

***Corresponding Author**
Vinay Sharma
Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India.

ABSTRACT

Lipases belonging to the class Hydrolases catalyze hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol. Lipases found promising application in various industries such as detergent, agrochemical, paper, chemical processing, dairy, pharmaceuticals, oleochemical, cosmetics, polymer synthesis, synthesis of surfactants and personal care products. Presently, the demand of the industries for novel sources of lipases with diverse catalytic features promotes the isolation and screening of new lipolytic microbial strains. Present review is focused on various methods of screening available for identification and isolation of lipolytic microorganisms from natural sources such as soil.

KEYWORDS: Lipases, triacylglycerol, screening, lipolytic strains, isolation.

INTRODUCTION

Lipases are broadly distributed in nature and capable of hydrolyzing fat into its components. Lipases can be extracted from plants, animal and microbes but microorganisms are the preferred sources of lipases because microbes can be easily cultivated at industrial scale for mass production of lipases, microbial lipases are more stable and possess desired properties. Recently the attention has given mostly to microbial lipases due to their capability to take part in a variety of reactions such as inter-esterification, hydrolysis, esterification, alcholysis, aminolysis, acidolysis, etc. These enzymes are also known to have numerous industrial applications viz., detergents, medical, dairy, biodiesel, pharmaceutical, textile, oleo-chemical, food, fat, cosmetic and perfume industries.[1] In view of the significance of lipases, there is

always necessity to design rapid screening techniques for identification of novel lipase producing microorganisms. Therefore, the present review chiefly focuses on a variety of screening methods (primary and secondary screening) that are presently available for the isolation and identification of lipolytic microorganisms.

METHODS FOR THE SCREENING OF LIPASE PRODUCING MICROORGANISMS

QUALITATIVE METHODS

Agar media containing substrates or indicator dyes can be utilized for identification of lipolytic microorganisms. They include two different types of assay: gel diffusion assays which are based on the addition of lipidic substrates into the media and assays which are based on the addition of a coloured dye into the media.

Gel diffusion assay using different substrates (lipidic)

These methods are mostly utilized for the selection of lipolytic microbes based on the capability of the microbes to cleave the lipids added into agar media to form a clear zone of lipolysis.[2] In these methods, lipidic substrate is added during the preparation of production medium and then microbial culture is inoculated into the production medium. Lipase producers can be identified by observing a clear halo zone around the colonies of microbes at the end of incubation.[3] In certain other methods lipase production is confirmed by the formation of turbid zone or white crystals around colonies in agar media (Fig. 1b).[4] The intensity of activity of enzyme can be determined by measuring the diameter of clear zones around the lipolytic colonies. The lipidic substrates such as triolein, tributyrin, olive oil, Tween 20, Tween 80, etc can be used in these methods. Screening of lipase producers using agar medium containing tributyrin is a broadly used method.[5] Agar medium containing olive oil may be a preferred choice for identification of lipase producers.[6]

Salihu *et al.* (2011)[7] reported screening of lipolytic fungi using Tween 20 as a substrate. Production of lipase in Tween-20 agar medium was indicated by the development of a white precipitate of calcium monolaurate around the colonies (Lipase hydrolyze the tween-20 and liberate the fatty acids which bind with calcium and calcium salt of fatty acid is visible around the colonies as insoluble precipitate) (Fig. 1b). Tween-20 contains the esters of lauric acid, while tween-80 contains esters of oleic acid.[8] Niyonzima and More (2013)[9] and Mahmoud *et al.* (2015)[10] reported screening using Tween-80 agar medium, whereas other investigators[11], [12], [13], [14], [15] have reported screening of lipolytic microorganisms using

TBA medium. Lipolytic activity in TBA plates is expressed as development of zone of hydrolysis (halo zone) around microbial colonies. Formation of clear zones around the colonies indicates the production of extracellular lipase, which hydrolyze the tributyrin (glycerol tributyrate), thus the opacity of the medium around such colonies cannot be retained (Fig. 1a). Opaqueness in the close vicinity of colonies, indicate that these colonies did not produce extracellular lipase for degradation of tributyrin.

Kiran *et al.* (2008)[16] reported that out of 57 bacterial isolates, 37 exhibited halo zones around their colonies in TBA medium plates, indicating extracellular lipase production. Kumar *et al.* (2012)[17] reported isolation of *Bacillus* sp. strain DVL2 as lipase producer from common city garbage of Haryana state using the tributyrin as substrate. Similarly, Veerapagu *et al.* (2013)[13] reported that twenty isolates exhibited halo zone amongst the two hundred bacterial colonies screened for extracellular lipase production on TBA medium plates. Sharma *et al.* (2016)[18] recently reported that among 31 fungal isolates tested for extracellular lipase production in TBA plates, 12 isolates exhibited zone of hydrolysis. Lipolytic activities of these positive isolates were further confirmed by secondary screening in submerged fermentation.

Gel diffusion assay with indicator dyes

The specific dyes like victoria blue, nile blue sulfate, night blue, thymopthalein, methylene blue and phenol red can be used as indicator dyes and added into solid agar medium. Lipase production in these media is confirmed by observing the change in colour as a consequence of pH change that occurred due to the free fatty acids liberated by the action of lipase on triacylglycerols.[19] Kouker and Jaeger (1987)[20] reported screening of lipolytic microorganisms using rhodamine-B as fluorescent dye and olive oil as substrate. Lipase production in these plates was indicated by the development of orange fluorescence around microbial colonies which was visualized upon exposure to UV light (Fig. 1c). The orange fluorescence was developed due to interaction of rhodamine-B with free fatty acids released by the action of lipase on triacylglycerols.[21] Other workers.[17], [22], [23], [24] also reported the use of rhodamine-B agar medium for screening of lipase producing microorganisms. Agar medium containing olive oil emulsion and bromocresol purple indicator forming yellow ring around the colonies were advocated by Zhou *et al.* (2012)[25] for screening of lipolytic fungi.

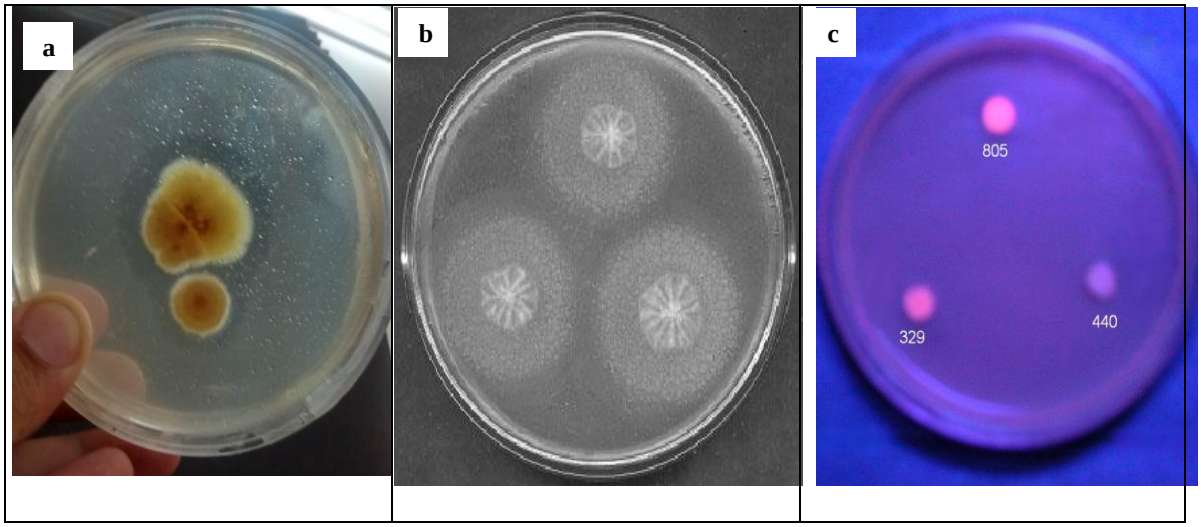


Fig.-1: Agar plate screening for lipases: (a): Zone of hydrolysis by fungal isolate in tributyrin agar medium[18]; (b): Formation of white precipitate by bacterial isolate in tween-20 agar medium[26]; (c): Formation of orange fluorescence in rhodamine-B-agar medium[26].

QUANTITATIVE METHODS

Activity of lipase can be estimated using different quantitative methods such as titrimetric, colorimetric, fluorescence and chromatographic methods.

Titrimetric methods

In these methods, titrimetric estimation of free fatty acids liberated by the catalytic activity of lipases on triacylglycerols is carried out. The substrates utilized in these methods include olive oil, triolein and tributyrin. Phenolphthalein or thymolphthalein indicators are used for titrimetric method. The most frequently used titrant is sodium hydroxide.[19] There are many reports[11], [14], [27], [28], [29], [30] of estimation of lipase activity by titrimetric method. Mukhtar *et al.* (2015)[30] reported quantitative screening of seven fungal isolates using this method. Highest activity (5.12 U ml^{-1}) was obtained by *A. niger* as compared to other isolates.

Colorimetric methods

They are more sensitive, simple and rapid as compared to volumetric methods. Copper soap method is one of the colorimetric method, in which the free fatty acids that are liberated by the catalytic activity of the lipase forms blue colour soaps of cupric complexes which can then be separated into an organic solvent followed by spectrophotometric estimation.[31], [32] There are many reports[13], [33], [34] of estimation of lipase activity by copper soap method.

Colorimetric methods using *p*-nitrophenyl esters

In these methods, synthetic lipidic substrates such as *p*-nitrophenyl esters of the long chain fatty acids are used which upon catalysis by lipase, hydrolyze into yellow coloured product (*p*-nitrophenol). The absorbance of *p*-nitrophenol is determined at 405-410 nm.[35] The synthetic substrates used for lipase assay are as follows: *p*NP-caprylate, *p*NP-propionate, *p*NP-palmitate, *p*NP-laurate, *p*NP-valerate and *p*NP-stearate.[36] Naphthyl esters can also be used as substrates which upon enzymatic hydrolysis convert into naphthol, which forms a red coloured complex with diazonuim salts. The absorbance of red colour complex is measured at 560 nm. Some commonly used naphthyl esters were naphthyl propionate, naphthyl caprylate and naphthylacetate. [37] Lipase assay using *p*NP-caprylate[38], *p*NP-palmitate[10], [39], [40], [41],[42], *p*NP-acetate[43] and *p*NP-propionate[36] as substrates has also been demonstrated. Abd-Elhakeem *et al.* (2013)[44] reported assay of lipase using phenyl acetate as substrate which upon enzymatic hydrolysis converts into phenol and liberated phenol can be determined by folin ciocalteu reagent.

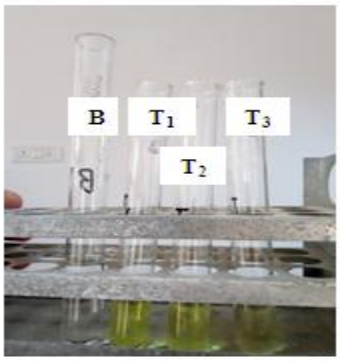
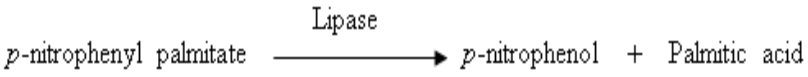


Fig.-2: Formation of yellow color product (*p*-nitrophenol) after action of lipase on *p*-nitrophenyl esters.



Radial diffusion method

Lipase activity can be estimated by tributyrin clearing zone technique, in which wells of 1.0 cm diameter are developed in TBA plates using gel puncture and 0.1 ml of culture broth containing enzyme are added into these wells, followed by incubation at 30 ºC for 24 h. Lipase activity in these plates is reflected by the formation of zone of clearance around wells. Diameter of these halo zones are measured and compared with standards.[29]

Fluorescence assay

These methods involve the use of fluorescent compounds (fluorescent labeled triacylglycerol) which upon enzymatic hydrolysis convert into fluorescent fatty acids. Amount of lipase in the sample is determined by measuring the fluorescence in the reaction mixture.[19] The alkayl groups of triacylglycerols can be conjugated with fluorescent groups such as pyrenyl.[45]

Chromatographic methods

Chromatographic techniques can be used for quantification of free fatty acids liberated from the triacylglycerol (TAG) by the catalytic activity of lipase. The thin layer chromatography (TLC) is a simple quantitative technique utilized for the detection of liberated fatty acids by the catalytic action of lipase on triacylglycerol. TLC can be followed by autoradiographic analysis of the fatty acid bands using radio labeled triacylglycerols.[46]

ACKNOWLEDGEMENTS

We are highly obliged to Professor Aditya Shastri, Vice-Chancellor, Banasthali University, Rajasthan for providing essential research facilities.

REFERENCES

1. Ghosh PK, Saxena RK, Gupta R, Yadav RP, Davidson S. Microbial lipases: Production and applications. Sci Prog, 1996; 79(2): 119-57.
2. Stead D. Microbial lipases: Their characteristics, role in food spoilage and industrial uses. J Dairy Res, 1986; 53(3): 481-505.
3. Thompson CA, Delaquis PJ, Mazza G. Detection and measurement of microbial lipase activity: A review. Crit Rev Food Sci Nutr, 1999; 39(2): 165-87.
4. Shelley AW, Deeth HC, Macrae IC. Review of methods of enumeration detection and isolation of lipolytic microorganisms with special reference to dairy applications. J Microbiol Methods, 1987; 6(3): 123-37.
5. Roberts IM. Hydrolysis of 4-methylumbelliferyl butyrate: A convenient and sensitive fluorescent assay for lipase activity. Lipids, 1985; 20(4): 243-47.
6. Hube B, Stehr F, Bossenz M, Mazur A, Kretschmar M, Schafer W. Secreted lipases of *Candida albicans*: Cloning, characterization and expression analysis of a new gene family with at least ten members. Arch Microbiol, 2000; 174(5): 362-74.
7. Salihu A, Alam Z, Karim MIA, Salleh HM. Suitability of using palm oil mill effluent as a medium for lipase production. Afr J Biotechnol, 2011; 10(11): 2044-52.

8. Cardenas J, Alvarez E, De Castro Alvarez MS, Sanchez-Montero JM, Valmaseda M, Elson SW, Sinisterra JV. Screening and catalytic activity in organic synthesis of novel fungal and yeast lipase. J Mol Catal B: Enzym, 2001; 14(4-6): 111-23.
9. Niyonzima FN, More SS. Screening and Identification of a Novel Alkaline Lipase Producing Bacterium. Int J Pharm Biol Sci, 2013; 4(2): 1037-45.
10. Mahmoud GA, Koutb MMM, Morsy FM, Bagy MMK. Characterization of lipase enzyme produced by hydrocarbons utilizing fungus *Aspergillus terreus*. Eur J Biol Res, 2015; 5(3): 70-7.
11. Bhavani M, Chowdary GV, David M, Archana G. Screening, Isolation and Biochemical Characterization of Novel Lipase Producing Bacteria from Soil Samples. Int J Biol Eng, 2012; 2(2): 18-22.
12. Mukesh KDJ, Rejitha R, Devika S, Balakumaran MD, Rebecca AIN, Kalaichelvan PT. Production, optimization and purification of lipase from *Bacillus* sp. MPTK 912 isolated from oil mill effluent. Adv Appl Sci Res, 2012; 3(2): 930-38.
13. Veerapagu M, Narayanan AS, Ponmurugan K, Jeya KR. Screening, selection, identification, production and optimization of bacterial lipase from oil spilled soil. Asian J Pharm Clin Res, 2013; 6(3): 62-7.
14. Rai B, Shrestha A, Sharma S, Joshi J. Screening, Optimization and Process Scale up for Pilot Scale Production of Lipase by *Aspergillus niger*. Biomed Biotechnol, 2014; 2(3): 54-9.
15. Shukla BN, Desai PV. Isolation, Characterization and Optimization of Lipase Producing *Pseudomonas* sp. from Oil Contaminated Sites. Int J Curr Microbiol Appl Sci, 2016; 5(5): 902-09.
16. Kiran GS, Shanmughapriya S, Jayalakshmi J, Selvin J, Gandhimathi R, Sivaramakrishnan S, Arunkumar M, Thangavelu T, Natarajaseenivasan K. Optimization of extracellular psychrophilic alkaline lipase produced by marine *Pseudomonas* sp. (MSI057). Bioprocess Biosyst Eng, 2008; 31(5): 483-92.
17. Kumar D, Kumar L, Ngar S, Rain, C, Prashad R, Gupta VK. Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in *esterification* and resolution reactions. Arch Appl Sci Res, 2012; 4(4): 1763-70.
18. Sharma AK, Sharma V, Saxena J. Isolation and Screening of Extracellular Lipase Producing Fungi From Soil. Am J Pharm Health Res, 2016; 4(8): 38-50.

19. Lanka S, Latha JNL. A Short Review on Various Screening Methods to Isolate Potential Lipase Producers: Lipases-the Present and Future Enzymes of Biotech Industry. Int J Biol Chem, 2015; 9(5): 207-19.
20. Kouker G, Jaeger KE. Specific and sensitive plate assay for bacterial lipases. Appl Environ Microbiol, 1987; 53(1): 211-13.
21. Olusesan AT, Azura LK, Abubakar F, Hamid NSA, Radu S, Saari N. Phenotypic and molecular identification of a novel thermophilic *Anoxybacillus* species: A lipase-producing bacterium isolated from a Malaysian hotspring. World J Microbiol Biotechnol, 2009; 25(11): 1981-88.
22. Rifaat HM, El-Mahalawy AA, El-Menofy HA, Donia SA. Production optimization and partial purification of lipase from *Fusarium oxysporum*. J Appl Sci Environ Sanit, 2010; 5(1): 39-53.
23. Rajan A, Kumar SDR, Nair JA. Isolation of a novel alkaline lipase producing fungus *Aspergillus fumigatus* MTCC 9657 from aged and crude rice bran oil and quantification by HPTLC. Int J Biol Chem, 2011; 5(2): 116-26.
24. Prabhakar T, Bhogavalli PK, Vallem PR, Srirangam V. Studies on optimization of extracellular lipase from potential fungal strain(s) isolated from oil contaminated soil. J Microbiol Biotechnol Res, 2012; 2(3): 418-25.
25. Zhou J, Chen WW, Jia ZB, Huang GR, Hong Y, Tao JJ, Luo XB. Purification and Characterization of Lipase Produced by *Aspergillus oryzae* CJLU-31 Isolated from Waste Cooking Oily Soil. Am J Food Technol, 2012; 7(10): 596-608.
26. Carissimi M, Stopiglia CDO, De Souza TF, Corbellini VA, Scroferneker ML. Comparision of Lipolytic Activity of *Sporothrix schenckii* Strains Utilizing Olive Oil-Rhodamine B and Tween-80. Tecno-Logica, 2007; 11(1): 33-6.
27. Sundar WA, Kumaresapillai N. Isolation, Purification and Medium Optimization of Lipase Enzyme Producing Strains of *Aspergillus niger* Isolated from Natural Sources. Int J Pharm Pharm Sci, 2013; 5(2): 321-24.
28. Ghasemi M, Farahbakhsh A, Farahbakhsh A, Safari AA. Using Submerge Fermentation Method to Production of Extracellular Lipase by *Aspergillus niger*. Int J Biol Biomol Agric Food Biotechnol Eng, 2014; 8(9): 1020-23.
29. Ravindranath TP, Lakshmi NM. Isolation and Development of a Soil Fungal Strain with High Lipolytic Activity by Mutation. Int J Pharm Chem Biol Sci, 2014; 4(1): 1-8.
30. Mukhtar H, Hanif M, Rehman AU, Nawaz A, Haq, IU. Studies on the Lipase Production by *Aspergillus niger* through Solid State Fermentation. Pak J Bot, 2015; 47: 351-54.

31. Duncombe WG. The colorimetric micro-determination of non-esterified fatty acids in plasma. Clin Chim Acta, 1964; 9(2): 122-25.
32. Lowry RR, Tinsley IJ. Rapid colorimetric determination of free fatty acids. J Am Oil Chem Soc, 1976; 53(7): 470-72.
33. Prabakaran M, Thennarasu V, Ayeswariya Mangala R, Bharathidasan R, Chandrakala N, Mohan N. Comparative studies on the enzyme activities of wild and mutant fungal strains isolated from sugarcane field. Indian J Sci Technol, 2009; 2(11): 46-9.
34. Namboodiri VM, Chattopadhyaya R. Purification and biochemical characterization of a novel thermostable lipase from *Aspergillus niger*. Lipids, 2000; 35(5): 495-502.
35. Becker P, Abu-Reesh I, Markossian S, Antranikian G, Markl H. Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. Appl Microbiol Biotechnol, 1997; 48(2): 184-90.
36. Sarkar D, Laha S. Optimization of extracellular lipase enzyme production from *Aspergillus niger* by submerged and solid state fermentation process. Int J Pharm Biol Sci, 2013; 4(4): 978-85.
37. Gandolfi R, Marinelli F, Lazzarini A, Molinari F. Cell-bound and extracellular carboxylesterases from *Streptomyces*: Hydrolytic and synthetic activities. J Appl Microbiol, 2000; 89(5): 870-75.
38. Pereira EO, Tsang A, McAllister TA, Menassa R. The production and characterization of a new active lipase from *Acremonium alcalophilum* using a plant bioreactor. Biotechnol Biofuels, 2013; 6: 1-10.
39. Joshi GK, Kumar S, Tripathi BN, Sharma V. Production of alkaline lipase by *Corynebacterium paurometabolum*, MTCC 6841 isolated from Lake Naukuchiatal, Uttaranchal State, India. Curr Microbiol, 2006; 52(5): 354-58.
40. Karanam SK, Medicherla NR. Enhanced lipase production by mutation induced *Aspergillus japonicus*. Afr J Biotechnol, 2008; 7(12): 2064-67.
41. Iftikhar T, Niaz M, Haq IU. Comparative studies on the lipolytic potential of wild and mutant strains of *Rhizopus oligosporous* var. *microsporous* IIB-63 isolated from lipid rich habitats. Pak J Bot, 2010; 42(6): 4285-98.
42. Xia JJ, Huang B, Nie ZY, Wang W. Production and characterization of alkaline extracellular lipase from newly isolated strain *Aspergillus awamori* HB-03. J Cent South Univ Technol, 2011; 18(5): 1425-33.

43. Paranjothi S, Sivakumar T. Isolation, Purification and Characterization of Lipase Enzyme from *Rhizopus japonicus*. Int J Curr Res Chem Pharm Sci, 2016; 3(1): 10-5.
44. Abd-Elhakeem MA, Elsayed AM, Alkhulaq TA. New Colorimetric Method for Lipases Activity Assay in Microbial Media. Am J Anal Chem, 2013; 4(9): 442-44.
45. Negre-Salvayre A, Dagan A, Gatt S, Salvayre R. Use of pyrenemethyl laurate for fluorescence-based determination of lipase activity in intact living lymphoblastoid cells and for the diagnosis of acid lipase deficiency. Biochem J, 1993; 294(3): 885-91.
46. Ruiz-Larrea MF, Galdiz-Valdovinos B, Rodriguez-Fernandez C. Kinetic study of hepatic triglyceride lipase from rat liver soluble fraction. Enzyme, 1981; 27(3): 215-19.