



International Journal of **Biological Chemistry**

ISSN 1819-155X



Academic
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A Short Review on Various Screening Methods to Isolate Potential Lipase Producers: Lipases-the Present and Future Enzymes of Biotech Industry

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ABSTRACT

The increased demand for microbial originated Industrial enzymes especially lipases which have received least priority till last one and half decades and gained lots of importance in recent days owing to their applications in wide variety of fields such as food, dairy, pharmaceutical, detergent, textile, oleo-chemical, perfume and cosmetic industries etc., has lead to the identification of novel enzymes from new sources with unique properties. The present review mainly focuses on various currently available screening methods for identification of lipase producers.

Key words: Industrial enzymes, lipases, screening methods, lipase producers, novel enzymes

INTRODUCTION

Enzymes, the biological catalysts are considered as nature's gifts. They function as protein robots inside the cells and speed up the biological processes without undergoing any change. Because of their ability to tolerate and grow at extreme temperatures, pH, organic solvent tolerance, enantio selectivity, high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, rapid growth on inexpensive media, diversity in catalytic activities and broad substrate specificity, microbial enzymes are gaining much attention than enzymes obtained from plant and animal sources (Chaturvedi *et al.*, 2010; Shu *et al.*, 2010). Microbial originated enzymes are more stable than their plant and animal counterparts and their production is also easier, safer and convenient (Wiseman, 1995). Of the microbes, fungi are more preferable as lipase sources because of their ability to secrete enzymes in to media which facilitates their easier extraction from fermentation broth (Wiseman, 1995).

Lipases (Glycerol ester hydrolases EC 3.1.1.3) are widely distributed in nature and catalyze the hydrolysis of mono, di and tri acyl glycerols to liberate free fatty acids and glycerol (Sheldon, 1993). They are members of the α/β hydrolase fold super-family and can act at both aqueous and non aqueous interface which makes them different from esterases (Verger, 1997; Schmid and Verger, 1998). Recently lipases gained much attention because of their ability to participate in a wide variety of reactions like hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification aminolysis etc., and these multifaceted enzymes are also having roles in several industrial applications viz., dairy, detergents, medical and pharmaceutical, fat and oleo-chemical, food, cosmetic and perfume industries (Macrae and Hammond, 1985; Bjorkling *et al.*, 1991; Ghosh *et al.*, 1996).

Better screening methods allow one to screen potential lipase producing microbes which further helps in the elucidation of the three dimensional structure of the lipases that in turn can provide

some clues for understanding mechanism of action of the lipases viz., their hydrolytic activity, interfacial activation and stereo selectivity (Kazlauskas and Bornscheuer, 1998) and also helps in designing and engineering lipases for specific purposes. Apart from determination of the three dimensional structure, purification also allows the establishment of structure functional relationships and also contributes to the better understanding of kinetic mechanisms like hydrolysis, synthesis and group exchange of esters (Ghosh *et al.*, 1996; Taipa *et al.*, 1992).

Since last one and half decade significant number of different lipases have been discovered and commercialized (Hasan *et al.*, 2006), but still there is an increased demand for the isolation of new lipases which exhibit high activity and more stability in non-aqueous media, which are essential prerequisites for biodiesel production through transesterification (Xiao *et al.*, 2009; Bajaj *et al.*, 2010; Bisen *et al.*, 2010), regiospecificity for the modification of oils and fats to produce specific-structured lipids (Xu, 2000; Hayes, 2004), stability under alkaline conditions and in the presence of surfactants for use in detergent formulations (Hasan *et al.*, 2010) etc.

Considering the importance of lipases, there is every need to develop rapid screening methods for discovering novel and potential enzymes that can be effectively used for various industrial applications, for studying the enzyme mechanisms and as well for the determination of 3 dimensional structure which can further open the channels for studying structure-function relationships, altering the properties of the enzymes for better usage, for studies related to catalytic mechanisms and substrate specificity etc. Thus the current review mainly focuses on the various screening strategies that are currently available for the isolation of novel lipase producers.

SCREENING METHODS FOR THE ISOLATION OF POTENTIAL LIPOLYTIC MICROORGANISMS

Qualitative methods: Agar media with added substrates or indicator dyes can be used for screening lipolytic organisms. They include-gel diffusion assays based on the incorporation of lipidic substrates in to the media and assays based on the incorporation of a colored dye in to the media.

Gel diffusion assays using various lipid substrates incorporated in to the media: These methods are mainly used for screening lipolytic microbes based on the ability of the microbes to break down the lipids incorporated in to solid media to form a clear zone of lipolysis (Lawrence *et al.*, 1967; Dring and Fox, 1983; Stead, 1986). This method minimizes the cost of screening and as well protects the microorganisms from the inhibitory effects of various indicator dyes (Thompson *et al.*, 1999). These methods involve preparation of growth medium with lipidic substrate followed by the inoculation of the microbial culture. Lipase activity can be identified as a clear zone around the colonies after incubation (Thompson *et al.*, 1999). In some other methods we can directly observe the formation of either clear or turbid zones around the colonies or the production of crystals on the agar surface which indicates the presence of lipolytic activity (Shelley *et al.*, 1987). The level of enzyme activity can be evaluated by measuring the diameter of the halo around the colonies. The lipidic substrates used in these methods include tributyrin, triolein, Tween 20, Tween 80, olive oil etc.

Screening lipase producers using tributyrin agar plates is a widely used method but this method cannot distinguish between true lipases and esterases (Roberts, 1985). Olive oil incorporated in to agar medium became a good choice for screening lipase-positive colonies (Hube *et al.*, 2000; Kim *et al.*, 2001; Martinez and Soberon-Chavez, 2001).



Fig. 1: Screening by using Tweens as substrates, the Tween 20 agar plate showing the zone of precipitation around the well due to the formation of insoluble calcium salts by the released free fatty acids due to the action of the lipase enzyme added to the well, which forms complexes with the calcium salts present in the medium

Sierra (1957) developed a cup plate method for determination of lipase activity using Tween 20 as a substrate (Fig. 1). Salihu *et al.* (2011) screened different fungal species using Tween 20 as a substrate. The lipase activity in this method can be detected due to occurrence of a zone of clearance and subsequent formation of white precipitate of calcium monolaurate around the colony (Cardenas *et al.*, 2001). Tween 80 as a lipidic substrate in to the growth medium was used by Schoofs *et al.* (1997), Akano and Atanda (1990) and Gopinath *et al.* (2005).

Gel-diffusion assays using indicator dyes: These methods involve use of various indicator dyes alone or in combination with various substrates to screen the lipolytic microorganisms (Thompson *et al.*, 1999). The specific dyes like victoria blue (Lawrence *et al.*, 1967), Nile blue sulfate (Christen and Marshall, 1984), methylene blue, night blue, phenol red, thymophthalein etc can be used as indicator dyes. The basic principle behind these methods involve formation of a clear and distinguishable color changes as a result of pH change that occur due to the release of free fatty acids from triacylglycerols during lipolysis. But the major drawback of using these substrates is that they are not suitable to detect true lipases as they are also hydrolyzed by esterases too and in addition they also inhibits the growth of the microbes.

Screening of lipolytic microbes using indicator dye such as phenol red is also in wide practice. The basic principle in this method involves lowering of the pH of the medium due to liberation of free fatty acids by the lipolytic organisms resulting in the change of the color (from pink to yellow) of the indicator dye (Fig. 2) (Singh *et al.*, 2006). The main advantage with this method is, it could successfully differentiate between esterases and lipases by using tributyrin and oil, respectively as substrates. Esterases give positive results (yellow on pink back ground) only on tributyrin plates where as lipases give positive results for both the substrates. This is highly sensitive and can be used to detect and differentiate even low level of lipases and esterases in plates.

Use of fluorescence dye such as Rhodamine B for screening lipolytic organisms by Kouker and Jaeger (1987) using olive oil as substrate is also in wide practice (Fig. 3). The basic principle in this method involves interaction of hydrolyzed substrates with Rhodamine B resulting in the formation

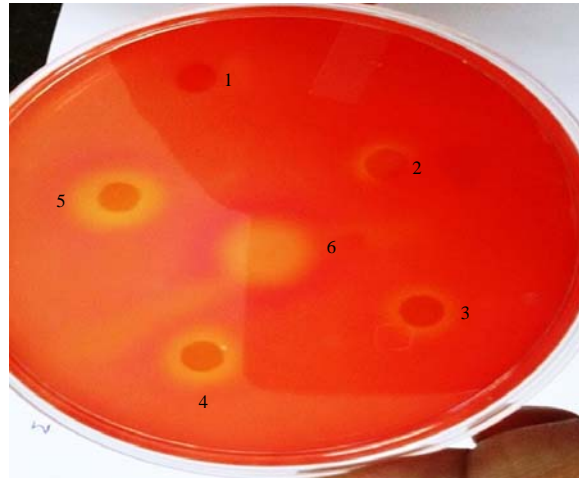


Fig. 2: Screening by phenol red agar plates containing chromogenic substrates. A phenol red olive oil agar medium plate displaying yellow colored zones around the wells due to the free fatty acids liberated by the lipolytic organisms which lowers the pH of the medium there by changing the color of phenol red to yellow, indicating the presence of lipase

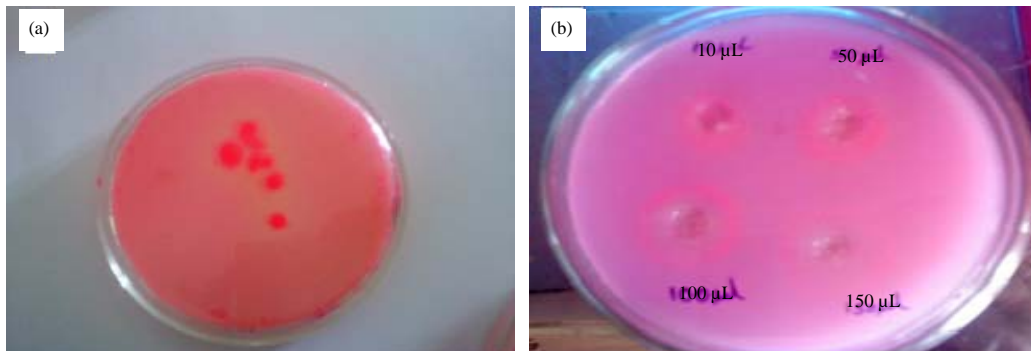


Fig. 3(a-b): Screening by rhodamine olive oil agar method, (a) Rhodamine olive oil agar plate displaying the orange fluorescence due to the growth of lipase producing fungi. The lipase produced by the fungi acts upon olive oil present in the medium and liberates free fatty acids, which form complexes with the Rhodamine dye and produces orange fluorescence and (b) Rhodamine olive oil agar plate displaying the orange fluorescence around the well due to the release of free fatty acids produced by the action of the extracellular lipase added to the wells, which form complexes with the Rhodamine dye and produces orange fluorescence

of orange fluorescent halos around microbial colonies which can be visible upon UV irradiation (Olusesan *et al.*, 2009). The main advantage of using Rhodamine B is, it does not inhibit the growth of test microorganism or change its physiological properties (Kouker and Jaeger, 1987; Thompson *et al.*, 1999). Hence this method is effective in screening lipase producing organisms and is practiced widely (Uttatree *et al.*, 2010). Screening using Tween and Rhodamine B for detection of lipase activity was employed for bacterial lipases like *Bacillus* by Heravi *et al.* (2008).

QUANTITATIVE SCREENING METHODS

Lipase activity can be estimated by using various quantitative methods which can be used for screening potential lipase producers. They include; colorimetric methods, titrimetric methods, spectrophotometric methods, fluorescence method, chromatographic methods (TLC/HPLC/GC-MS, HPTLC) and molecular screening methods etc.

Titrimetric methods: Volumetric methods are based on the titrimetric estimation of liberated free fatty acids from triacyl glycerols by the catalytic action of lipases. These methods mainly involve incubation of substrate with the enzyme followed by end point alkali titration of the released free fatty acids. These methods have been known since, 1932 (Cherry and Crandall Jr., 1932). The substrates used in these methods include Triolein, Tributyrin and Olive oil. The most commonly used alkali is NaOH. From then these methods were routinely employed for estimation of lipase activity more than half a century (Parry *et al.*, 1966).

Colorimetric methods: These methods like titrimetric methods are most widely used quantitative methods for screening lipase producers and are more sensitive than volumetric methods. One of these methods is Copper soap method, where the free fatty acids that are released by the action of the enzyme forms blue color soaps of cupric complexes which can then be extracted in to an organic solvent followed by spectrophotometric evaluation (Lowry and Tinsley, 1976; Duncombe, 1964). The initial colorimetric methods were subjected to several modifications that drastically reduced incubation times and by elimination of phase transfer steps (Yang and Biggs, 1971; Blain *et al.*, 1976; Lowry and Tinsley, 1976; Kim *et al.*, 1984; Kwon and Rhee, 1986). Lipase activity in these methods was measured by measuring the amount of free fatty acids released based on a standard curve of free fatty acid (oleic acid). One unit of lipase activity was defined as the amount of enzyme releasing 1 μm of fatty acid per min under standard assay conditions. Lipase activity (U mL^{-1}) = $\mu\text{m mL}^{-1} \text{min}^{-1}$.

New colorimetric method using phenyl acetate as substrate: A new colorimetric method was recently developed by Abd-Elhakeem *et al.* (2013) which uses phenyl acetate as substrate and the liberated phenol by the action of the lipase on the substrate is estimated using Folin ciocalteu reagent.

Spectrophotometric methods using pNP naphthol and thiol esters: These methods mainly involve use of various synthetic lipidic substrates like p-nitrophenyl esters of the long chain fatty acids which upon enzyme hydrolysis transforms into yellow colored p-nitrophenol and is measured at 405-410 nm (Huggins and Lapides, 1947; Stuer *et al.*, 1986; Kojima *et al.*, 1994; Ushio *et al.*, 1996; Becker *et al.*, 1997; Liebeton *et al.*, 2001). One unit (U) of lipase activity was defined as the amount of enzyme that released 1 μm of p-nitrophenol min^{-1} under the standard assay conditions. The standard substrates used for enzyme assay include pNP-palmitate, pNP-valerate, pNP-caprylate, pNP-laurate and pNP-stearate. Naphthyl esters like naphthyl caprylate, naphthylacetate and naphthyl propionate can also be used as substrates which yields naphthol, where the red colored complex with diazonium salts is measured at 560 nm (Gandolfi *et al.*, 2000). In addition to these, thio esters can also be used as substrates where the thiols produced are coupled with the Ellman's reagent (5, 5'dithiobis 2-nitro benzoate, DTNB) to form yellow colored TNB anions, which are measured at 412 nm (Lombard *et al.*, 2001).

Spectrophotometric method using cis-parinaric acid: In this method a naturally occurring fatty acid like cis-parinaric acid (PnA) is used and the basic principle behind this method is reversible binding of fatty acids to bovine serum albumin which exhibits a different absorption maxima. The release of oleic acid from triolein is monitored over time by measuring the ratio of Optical Densities (OD) at 319.0 and 329.0 nm (Rogel *et al.*, 1989).

Fluorescence assay: Fluorescence methods for assaying lipases can be carried out employing fluorescent compounds which basically involves measurement of the fluorescence present in the released fluorescent fatty acids by the action of the lipases (Wilton, 1990, 1991). The fluorescent groups such as pyrenyl (Thuren *et al.*, 1987; Negre-Salvayre *et al.*, 1993) can be used to label the alkyl groups of triacylglycerols. Fluorogenic substrates such as 1-(3-*o*-alkyl-2,3-(3,2)-diacylglycerols were used by Duque *et al.* (1996). This technique does not require separation of substrate and reaction products. A non-fluorescent substrate such as 4-methylumbelliferyl oleate has also been employed to assay lipases, which mainly depends on the release of highly fluorescent 4-methylumbelliferone after lipase action (Jacks and Kircher, 1967). A highly sensitive assay using 4-methylumbelliferyl butyrate based on fluorescence was developed by Roberts (1985). This method is more sensitive and can detect as low as nano mole quantities. A Rhodamine B based quantitative fluorescence lipase assay has been described by Jette and Ziomek (1994) based on the interaction of the fluorescence dye with fatty acids released by the action of lipases.

Table 1 explains various qualitative and quantitative screening methods that were most widely employed for screening.

CHROMATOGRAPHIC PROCEDURES

TLC/HPLC/HPTLC/GC-MS/LC-MS for quantification of lipase activity: Chromatography also serves as one of the important tool for quantification of the lipids and as well free fatty acids liberated from the Tri Acyl Glycerols (TAGs). These include, TLC (Thin layer chromatography), High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC), Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS) etc. There were reports of usage of these techniques

Table 1: Determination of lipase activity by various assay methods

Assay	Substrates used	Analyzed product	Basic principle involved behind the method
Qualitative assays			
Plate assays	Tweens, tributyrin, long chain fatty acid esters and tri acyl glycerols	Short chain fatty acids	Change of color of the dyes (phenol red etc) added to the medium or Halo based or by observing fluorescence after complexation of fatty acids with fluorescent dye Rhodamine B
Quantitative assays			
Titrimetry	Fats, oils, triacyl glycerols and methyl esters	Fatty acids	End point reactions (Neutralization reactions) by using pH indicator
Spectrophotometry	p-nitrophenyl fatty acid esters	p-nitrophenol	Estimation of released para nitro phenol or β naphthol by measuring absorbance at 410 nm
	β naphthol fatty acid esters	β naphthol	
Tweens	Tweens	Fatty acids	Measurement of turbidity produced by the precipitation of fatty acid with calcium/copper
Fluorescence assay			
Triglycerols with alkyl group substituted with a fluorescent group	The alkyl group of triglycerols substituted with a fluorescent group	Free fluorescent phenyl group 4-methyl umbelliferone	By the analysis of the fluorescent product due to shift in fluorescence wavelength after triacylglycerol hydrolysis

since 1980's (Mangold, 1984; Kates, 1986; Christie, 1987; Gunstone *et al.*, 1994). In case of Gas Chromatographic methods, the fatty acids liberated need to be converted to their corresponding methyl esters in order to render them volatile. The TLC also serves as a simple qualitative and as well quantitative technique for the identification of released fatty acids. For quantification, TLC can be followed by a densitometric or autoradiographic analysis (using radio labeled TAGs) of the fatty acid bands. These techniques offer high specificity and sensitivity and can be used to detect the released fatty acids as low as pico moles (Ruiz-Larrea *et al.*, 1981). The major problems concerned to these techniques include time-consumption and they are not continuous.

Released free fatty acids can also be quantitated by using High Performance Thin Layer Chromatography (HPTLC), a method that is more simple, precise, specific and accurate. Quantification of individual phospholipid compounds was performed by HPTLC in studies by More and Pandit (2010). The method was also used by Vitro *et al.* (2000) for determining the fatty acid distribution. Rajan *et al.* (2011) quantified the liberated free fatty acids produced by the action of alkaline lipase from *Aspergillus fumigates* MTCC 9657 using HPTLC.

A highly sensitive HPLC technique was developed for quantification of fatty acids released by pancreatic lipase by Maurich *et al.* (1991) using paranitro phenyl esters. From then onwards so many researchers employed HPLC for quantification of released fatty acids by the action of lipases. The LC-MS can also be employed to perform lipase assay (Hao *et al.*, 2007).

MOLECULAR METHODS FOR SCREENING OF LIPASES

Molecular methods are occupying prominent position in identifying true lipases. The production yields can be significantly enhanced by molecular methods compared to traditional methods (where production is chiefly by microbial cultivation). These methods include recombinant DNA technology, protein engineering, directed evolution (Jaeger *et al.*, 2001). Liu *et al.* (2010) and Syren *et al.* (2010) employed rational design method for screening of lipases. Meta genomics approach, a molecular genomics approach was employed for the screening of non cultivated microbes (Steele *et al.*, 2009; Lammler *et al.*, 2007; Rondon *et al.*, 2000; Henne *et al.*, 2000; Yun and Ryu, 2005). This approach is now being widely employed to search novel industrial enzymes because of the availability of genetic material of most of the organisms (Lorenz and Eck, 2005; Kakirde *et al.*, 2010; Kennedy *et al.*, 2008; Jiang *et al.*, 2010). This approach also overcomes the problem of cultivation of microbes as majority of the micro organisms are not to cultivation (Handelsman *et al.*, 1998). There were so many recent reports proving the novelty of this approach in the identification of true lipases (Jiang *et al.*, 2006; Lee *et al.*, 2006; Elend *et al.*, 2007; Jeon *et al.*, 2009; Liu *et al.*, 2009; Meilleur *et al.*, 2009; Glogauer *et al.*, 2011).

CONCLUSION

Considering the versatility of lipases owing to their wider applications, the lipases are becoming increasingly significant in various industrial applications like-pharmaceutical, synthesis of fine chemicals, oleochemical, detergent industries, cosmetics etc. The regio, chemo and enantio selectivity of lipases especially make these biocatalysts to use in various biotransformation reactions and as well in the resolution of racemic mixtures. This necessitates the screening and isolation of novel lipase producing microbes as an essential prerequisite. Hence, the current review is mainly focused on various screening methods that are available to isolate potential lipolytic microorganisms to serve the industrial needs.

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