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Isolation of a Novel Alkaline Lipase Producing Fungus *Aspergillus fumigatus* MTCC 9657 from Aged and Crude Rice Bran Oil and Quantification by HPTLC

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ABSTRACT

Screening methods for the isolation of alkaline lipase producing fungi (acylglycerol hydrolases) from aged and crude rice bran oil and quantification of the Free Fatty Acid liberated (FFA) by the enzyme using HPTLC is discussed. Screening using rhodamine B detected lipase production as an orange halo around microbial colonies under UV light at 350 nm. Chromogenic plates incorporated with a lipidic substrate along with phenol red showed a sharp change in color from pink to yellow due to the presence of liberated fatty acids which was also an indication of lipase production. Media having pH in the range of 8.5 to 10.5 along with tributyrin substrate was used for screening an alkaline lipase producing isolate. The hydrolysis of tween opacity medium and production of an opaque halo in alkaline tributyrin agar plates confirmed the production of alkaline lipase by the isolated organism. The isolated fungus was identified as *Aspergillus fumigatus* MTCC 9657 from IMTECH, Chandigarh. There is no much information of alkaline lipase production by *A. fumigatus* isolated from aged rice bran oil. Positional specificity of the isolated lipase was determined to be non specific by TLC and FTIR. Quantification of lipase activity of *A. fumigatus* was carried out with the aid of High Performance Thin-layer Chromatography (HPTLC) which can detect even micro quantities of FFA produced and are rapid, efficient and easy-to use for simultaneous analysis.

Key words: Alkaline lipase, HPTLC, *Aspergillus fumigatus*, rice bran oil, TLC, FFA

INTRODUCTION

Lipases (glycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes produced by plants, animals and microorganisms belonging to sub class 1 of hydrolytic enzymes class 3 (Pahoja and Sethar, 2002). In comparison to animal or plant lipase, extracellular microbial lipase can be produced relatively inexpensively by fermentation and in large quantities (Macrae and Hammond, 1985). Microbial lipase can also be produced using agricultural residues like sugarcane bagasse, wheat bran and rice bran by SSF which is highly economical (Babu and Rao, 2007a). Because of the versatile reaction properties of lipase, they have been widely used in industrial applications, such as in food, chemical, pharmaceutical and detergent industries (Gupta *et al*., 2007; Park *et al*., 2005). Microbial lipases are widely used in the processing of fats and oils, degreasing formulations,

food processing, the synthesis of fine chemicals and pharmaceuticals, production of cosmetics, paper manufacture, waste management, biosensors etc. (Rubin and Dennis, 1997; Kazlauskas and Bornscheuer, 1998). Lipase can be used to accelerate the degradation of fatty waste (Masse *et al.*, 2001) and polyurethane (Takamoto *et al.*, 2001). Lipases are selected for each application based on its substrate specificity such as fatty acid alcohol, position and stereo specificity as well as temperature and pH stability (Saxena *et al.*, 2003).

Great awareness in industry has been developed for the utilization of extracellular enzymes produced under high pH. Attempts to isolate alkalophilic bacteria from soil and water samples, which are capable of producing alkaline lipases, started few decades ago. Watanabe *et al.* (1977) conducted an extensive screening for alkaline lipase-producing microorganisms from soil and water samples. Bhushan *et al.* (1994) found a lipase produced from an alkaliphilic *Candida* species in solid-state fermentation. Many strains of alkaliphilic bacteria have been isolated from diverse environments including neutral soil, soda lakes, soda soils, soda deserts and animal manure (Horikoshi, 1999; Kumar and Takagi, 1999). Emanuilova *et al.* (1993) and Khyami-Horani (1996) have reported the isolation of thermostable alkaline lipases from different bacilli. Alkaline thermostable lipases are used as additives in detergents, with promising applications in the detergent industry (Newmark, 1988). For example, lipase added in pre-wash soaking agents and detergent powders needs to be stable under alkaline pH and to function in the presence of surfactants (Treves *et al.*, 1984). However, as the applications increase, the availability of a lipase possessing satisfactory operating characteristics is a limiting factor. As each industrial application may require specific properties of the biocatalysts, there is still an increasing interest in finding new lipases that could create novel applications (Gupta *et al.*, 2004). Only a very few fungal lipases with optimum activity under alkaline conditions have been reported so far. In this study, we describe the various isolation techniques for alkaline lipase producing fungus from aged and crude rice bran oil. There is no much information of alkaline lipase production by *Aspergillus fumigatus* isolated from aged rice bran oil.

MATERIALS AND METHODS

Microorganisms used and substrates for isolation: Microorganisms isolated from oil mill waste and aged rice bran oil from Trivandrum, a Southern city in India, were analyzed for lipolytic activity. Out of the 26 microbes isolated, a fungus designated as RF_5 was selected on the basis of utilization of different substrates like tributyrin, olive oil, tween 20 and tween 80, combination of tween 80 with $CaCl_2.H_2O$ and tween 80 with methyl red.

Screening of lipase producing microorganisms using agar plates

Qualitative estimation: plate assay: Screening of lipolytic organisms was made after streaking 0.1 mL from enriched cultures on nutrient agar plates containing 1.0% (v/v) tributyrin (Loba Chemie) supplemented with 0.1% yeast extract, 0.01% $CaCl_2{\bullet}H_2O$ and 1.5% Na_2CO_3. Media having pH in the range of 8.5 to 10.5 was used to isolate organisms producing alkaline lipase. The specific pH in the media was adjusted by varying the concentration of Na_2CO_3 from 0.25 to 1.5% (Ghanem *et al.*, 2000).

Screening by tween 80: A method for primary plate assay to determine lipase activity was done with Tween 80 as substrate. Modified agar medium (Schoofs *et al.*, 1997) was prepared with 10.0 g of Peptone, 5.0 g of NaCl, 0.1 g of $CaCl_2{\bullet}H_2O$, 15.0 g of agar and 10 g Tween 80 in

1, 000 mL of distilled water. The hydrolysis of the Tween opacity medium is associated with the lipolytic enzymes produced by microorganisms.

Screening using rhodamine B: Agar plates containing Rhodamine B 0.001% (w/v), nutrient broth 0.8% (w/v), NaCl 0.4% (w/v), agar 1% (w/v) and olive oil 2% was prepared in distilled water, adjusted the to pH 6.5 (Kouker and Jaeger, 1987). The assay plates were incubated at 55°C for 18 h and lipase production was identified as an orange halo around colonies under UV light at 350 nm.

Screening using phenol red: Chromogenic substrate plates were prepared by incorporating phenol red (0.01%) along with 1% lipidic substrate (tributyrin/olive oil), 10 mM $CaCl_2$ and 2% agar. The pH was adjusted to 7.3-7.4 by using 0.1 N NaOH (Singh *et al*., 2006).

Production of lipase in liquid media: Media used for alkaline lipase production consisted of peptone (2 g 100^{-1} mL), yeast extract (0.5 g 100^{-1} mL), NaCl (0.5 g 100^{-1} mL), $Na_2 CO_3$ (0.025 g 100^{-1} mL) and olive oil (1 g 100^{-1} mL). Cultivation was carried out in 250 mL Erlenmeyer flasks. The medium (100 mL) was inoculated with 2% inoculum. Fermentation was carried for 7 days in an orbital shaker (120 rpm) at 30°C. Samples were collected at 24 h interval and centrifuged at 10,000 rpm for 10 min. The filtrate was considered as crude enzymatic extract and was used for enzyme assay.

Lipase assay: Lipase activity was determined by incubating a reaction mixture containing 5 mL of olive oil emulsion (Macedo *et al*., 1997), 5 mL of 0.1 M Tris HCl buffer, pH 8.5 and 1.0 mL of the culture filtrate (lipase crude extract) at 30°C for 20 min, with shaking of 120 rpm. After incubation, the reaction was stopped by the addition of 10 ml of acetone and the liberated free fatty acids were titrated with 0.05 N NaOH in the presence of phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acids per min.

Positional specificity determination of lipase by lipid hydrolysis: The positional specificity of the lipase was determined and compared with the lipase from a non-specific lipase from *Candida rugosa*, 1, 3-specific lipase from *Rhizopus arrhizus*. Ten milligrams of pure tripalmitin and 5 mL of 0.1M Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$ were emulsified by sonication and separated into three equal portions. Then, the enzyme solution of lipase as well as 0.1 M Tris-HCl, pH 8.0, which served as a blank were added into the above three tubes, respectively. The mixtures were incubated at 37°C in a shaking water bath for 3 h.

After hydrolysis the fatty acid formed was extracted using hexane and separated into lipid classes. The lipid classes TG, DG, MG, FFA, Phospholipids (PL) and Glycolipids (GL) were separated on a TLC plate (20×20 cm) using the solvent system n hexane/diethyl ether/acetic acid (80:20:1 by vol). The separated bands were visualized by placing the plate in an iodine chamber. Lipid bands were identified with respect to the Rf values of lipid standards (Deepa *et al*., 2000).

FT IR experiments: A simple and fast infrared spectroscopy method was used for the characterization of 1, 3-specific and non-specific lipases. The method is based on the analysis of specific infrared bands that show the functional groups. The universal ATR spectra were recorded between 4000 and 650 cm^{-1} on a Perkin Elmer Series 100 FTIR Spectrometer.

HPTLC quantification of FFA produced by the enzyme: Quantification of lipids was carried out with the aid of high performance thin-layer chromatography (Camag, Muttenz, Switzerland) on HPTLC $60F_{254}$ plates (Merck, Darmstadt, Germany). A reaction mixture containing 5 mL of olive oil emulsion, 5 mL of 0.1 M Tris HCl buffer, pH 8.5 and 1.0 mL of the culture filtrate (lipase crude extract) was incubated at 30°C for 20 min, with shaking of 120 rpm. After hydrolysis the fatty acid formed was extracted using hexane. Olive oil spiked with standard oleic acid was dissolved in hexane (1 μg μL^{-1}) and loaded on 10×10 cm plates with increasing concentration from 1-7 μg in seven tracks as shown in Fig. 5. Then the plate was developed in a CAMAG glass twin trough chamber containing the solvent system hexane diethyl ether and acetic acid in the ratio of 80:20:1. In the same manner the lipid extracted after the hydrolysis using the enzyme was analyzed.

After the chromatographic development the plates were dipped in methanol HCl (5%) reagent followed by incubation at 110°C for 30 min. The lipid fractions were visualized by charring and plates were scanned from the origin to the solvent front by using Camag TLC II scanning densitometer. The measurement was performed in reflectance mode at 550 nm and using the concentrations of the FFA and relative peak area a calibration curve was obtained. From the calibration curve the FFA concentrations in the sample was calculated.

RESULTS AND DISCUSSION

Screening of lipase producing microorganisms: Isolation of lipase producing microorganisms on tributyrin agar plates was done on the basis of production of an opaque halo. The ability to grow in alkaline media isolated alkaline lipase producing *Aspergillus fumigatus* which was tolerant to pH 9.5.

Tweens (fatty acid esters of polyoxyethylene sorbitan) have been the most widely used substrates for the detection of lipolytic microorganisms in agar media (Emanuilova *et al*., 1993). Screening using tween agar plates showed precipitation around the organism. The method is based on the precipitation, as the calcium salt, of the fatty acid released by hydrolysis of tween. Liberated fatty acids bind with the calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the inoculation site. Similar results were obtained using tween 20 and 60 as substrates. Using this technique, primary screening of lipolytic microorganisms can be conducted using the formation of zones of intensification around the colonies and mycelia.

Screening using rhodamine B: Agar plates containing olive oil and rhodamine B appear opaque and are pink colored. Lipase production is monitored by irradiating plates with UV light at 350 nm. After 16 h of incubation microbial colonies began to show an orange fluorescence; with continuing incubation time orange fluorescent halos were formed around the colonies of lipase producing strains (Fig. 1). After 48 h of incubation the fluorescence spread all around the plate. Hydrolysed substrate reacting with rhodamine B causes the formation of orange fluorescent halos around microbial colonies visible upon UV irradiation (Olusesan *et al*., 2009). The molecular mechanism underlying the formation of fluorescent products was reported to be unknown by Kouker and Jaeger (1987). Hou and Johnston (1992) suggested that the mono or diglycerides and fatty acid liberated into the medium by the enzyme complexed with rhodamine B to form dimers which can be visualized as fluorescent halos. Screening using tween and rhodamine B for detection of Lipase activity was employed for bacterial species like Bacillus also (Heravi *et al*., 2008). The rhodamine B plate method is insensitive to pH changes and allows reisolation of organisms which show no inhibition of growth or change of physiological properties. This method is effective in screening lipase producing organism and is practiced widely (Uttatree *et al*., 2010).

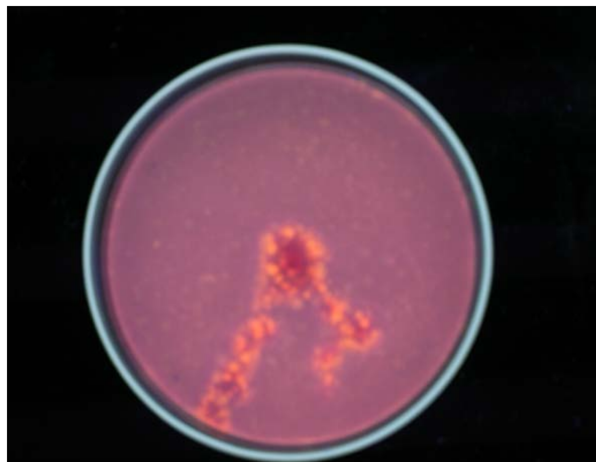

Fig. 1: Lipase activity in rhodamine B plate

Fig. 2: Detection of lipase in phenol red plate

Screening using phenol red: The free fatty acids liberated by the lipolytic organisms lower the pH resulting in the change of colour indicating the formation of lipase. Phenol red has an end point at pH 7.3-7.4 where it is pink and a slight decrease in pH (7.0-7.1) turns it yellow. The chromogenic substrate was kept at pH 7.3. As soon as hydrolysis initiates, the dye becomes yellow, indicating lipolysis. The principle behind the procedure is the lowering of pH due to release of fatty acids on lipolysis (Singh *et al.*, 2006). The yellow zone around the filter paper disc dipped in culture filtrate confirmed the isolation of lipase producing fungus. (Fig. 2) The present method could also successfully differentiate between esterase and lipase by using tributyrin and oil, respectively, as substrates. Esterase gave positive results (yellow on pink background) only on tributyrin plates and lipase was positive for both the substrates. This highly sensitive protocol is used for the detection and differentiation of even low level of lipases and esterases in plates.

Identification of the fungal isolate: The microbial type culture collection unit of Institute of Microbial Technology, Chandigarh, India identified the organism as *Aspergillus fumigatus* MTCC 9657 and it was deposited.

Lipase assay: By titrimetric assay lipase activity of 7 U mL^{-1} was estimated using olive oil emulsion as substrate on fourth day. It has been reported earlier that lipases require oil-water interface for their enzymatic action (Shukla *et al.*, 2007) and maximum lipase activity was obtained on fourth day by other fungal lipases in submerged fermentation (Babu and Rao, 2007b). Titrimetry is extremely laborious and time consuming. Therefore Lipase activity was determined by quantifying the amount of FFA liberated by the enzyme activity on specific oil substrates. The amount of FFA liberated was visualized by TLC and quantified by HPTLC. In comparison to titrimetry, even micro level of the FFA can be estimated by HPTLC.

Positional specificity determination of lipase by hydrolysis: The lipase hydrolysis products 1, 3 or 1, 2-diglycerides, monoglycerides and free fatty acids were analyzed by TLC. Figure 3 is a typical TLC plate with lipase hydrolysis products. Lipases that produced 1, 2-diglycerides, monoglycerides and free fatty acids but not 1, 3 diglycerides were classified as 1, 3-positional specific enzymes. On the other hand, lipases that produced all of the above mentioned products were classified as random specific enzymes.

The position of the triglyceride was observed at the top of the TLC plate close to the solvent front and near the origin monoglycerides and above those diglycerides and at the middle FFA was observed. In between TG and DG, FFA was seen. From the intensity of the FFA spots compared to the control the production of the FFA can be observed.

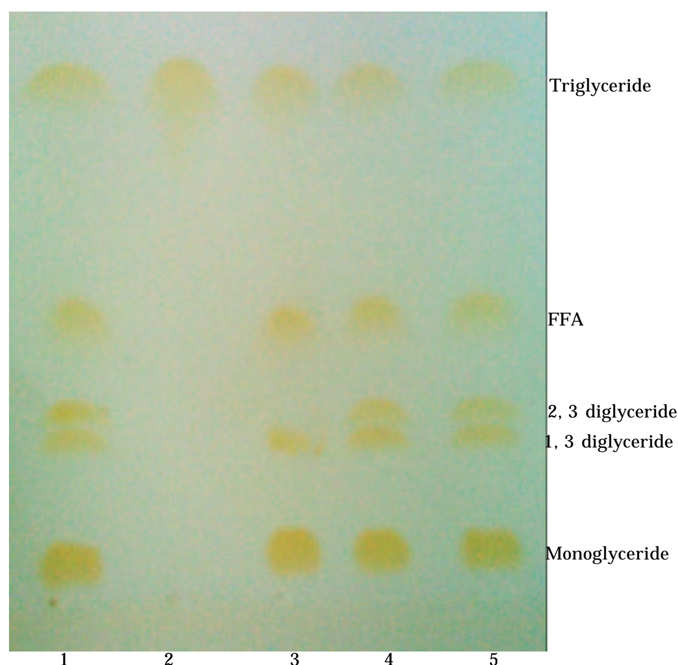

Fig. 3: TLC plates showing separation of Triglyceride (TG) and free fatty acids by 1, 3 specific and non specific lipase, lane 1: Mixture of lipid standards; lane 2: Standard triolein; lane 3: 1, 3-specific lipase from *Rhizopus arrhizus*; lane 4: Non-specific lipase from *Candida rugosa* and lane 5: Lipase from *A. fumigatus*

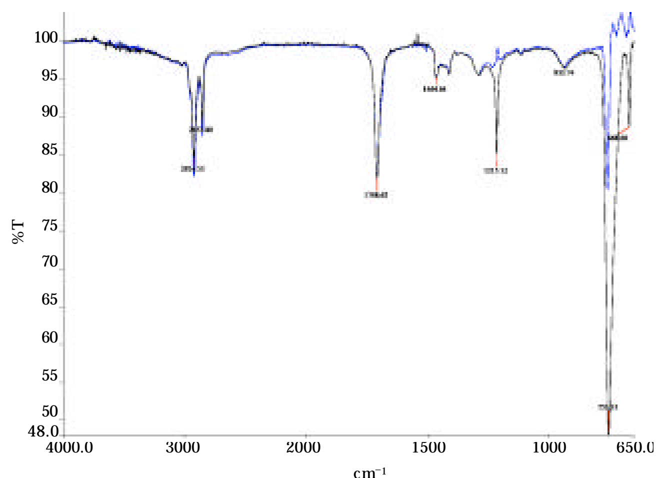

Fig. 4: FT IR spectra of 1, 3 specific and non specific lipase

A. fumigatus was found to hydrolyse all the three positions of the triacylglycerol and such enzymes are non specific enzymes. There are reports of lipase cleaving the ester bonds at the 1 and 3 positions of the triacylglycerol molecule isolated from *A. niger* (Variketta *et al*., 2000). The isolated *A. fumigatus* differed from other species by producing non specific lipase.

FT IR experiments: The spectra of hexane extracts obtained from 1, 3 specific *Rhizopus arrhizus* and *A. fumigatus* nonspecific lipase in media are shown in Fig. 4. An increase in intensity around 1215.12 is seen in nonspecific lipase, which is characteristic of amines and ester. 1, 3 specific lipase showed no such peak. The major protein absorption bands due to the peptide group vibrations occur in the 1900-1200 cm^{-1} spectral regions which is seen in both 1,3 specific and nonspecific lipase (Antonino *et al*., 2005).

Peak around 2924 and 1708 cm^{-1} is mainly due to the production of free fatty acids. When triacylglycerol is hydrolyzed by 1, 3 specific lipase, the area peak at 1708 and 1215 cm^{-1} (in the positions 2 and 3 of the triacylglycerol analogue) decreases. This can be accomplished because only the triacylglycerol in position 3 is hydrolyzed by 1, 3 specific lipase (Ricardo *et al*., 2000). Lipases that hydrolysed all the three positions were classified as non specific enzymes and the lipase from *A. fumigatus* was found to be a non specific enzyme. The IR method allows the positional specificity determination in the lipase reaction by the analysis of specific infrared bands. Similar findings were reported by Rahman *et al*. (2005) with their *Pseudomonas* sp. organic solvent-tolerant lipase. Another thermostable lipase from *Bacillus* sp. also hydrolyzed ester bonds non-specifically (Nawani and Kaur, 2000).

HPTLC quantification of FFA produced by the enzyme: Nowadays, HPTLC is becoming a routine analytical technique by virtue of its advantages of low operating cost, high sample throughput and need for minimum sample clean-up. A further major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase, unlike HPLC, thus lowering time and cost per analysis (Abou-Donia *et al*., 2008).

The separation of the triglyceride, diglyceride, monoglyceride and FFA were observed by charring. A calibration graph was plotted using HPTLC profile of oleic acid standard (Fig. 5). From

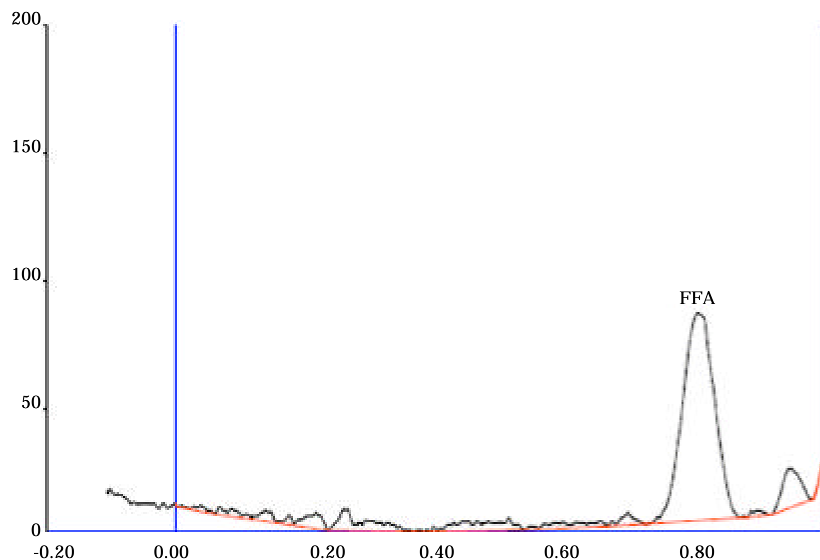

Fig. 5: HPTLC profile of oleic acid standard

Table 1: Concentration and percentage of free fatty acids formed

Samples pH	Volume (µL)	Total area	Concentration (µg)	FFA (%)
Control	10	-	-	-
	20	-	-	-
7.0	10	350	1.02	0.94
	20	630	1.80	
7.5	10	525	1.50	1.23
	20	770	2.20	
8.0	10	626	1.78	1.74
	20	1201	3.45	
8.5	10	1120	3.20	3.00
	20	2030	5.80	
9.0	10	1015	2.90	2.63
	20	1750	5.00	
9.5	10	420	1.20	1.40
	20	1050	3.00	

the calibration curve the FFA produced in the samples were estimated. The densitometric analysis showed that 0.94, 1.23, 1.74, 3.0, 2.63, 1.40% of FFA was produced at pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5, respectively (Table 1). The maximum activity of the enzyme is at pH 8.5. The other parameter studies well correlated with the results of the FFA produced by the enzyme at this pH. The HPTLC results showed a trend that the maximum FFA production was at pH 8.5 (Fig. 6). The HPTLC plate spiked with olive oil-FFA mixture showed intensification of spot with increasing concentration of FFA as evident from Fig. 7a. The HPTLC plates also showed intensification of spot at pH 8.5 due to the presence of more FFA as shown in Fig. 7b. The HPTLC method was found to be simple, precise, specific and accurate. Quantification of individual phospholipid compounds was performed by HPTLC in studies by More and Pandit (2010). This method was used by Vitro *et al.* (2000) for examining the fatty acid distribution in phosphatidylcholine during its enzyme-catalyzed hydrolysis.

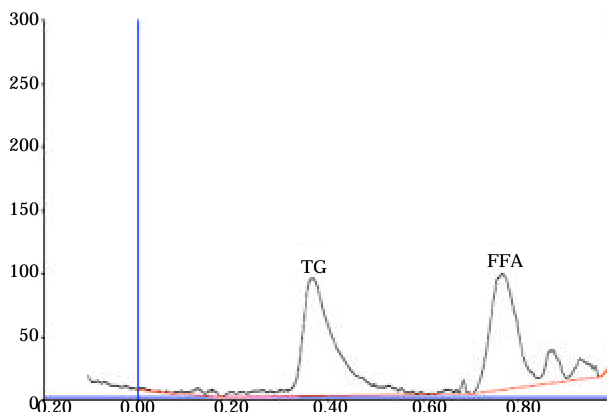

Fig. 6: HPTLC profile of olive oil after enzyme treatment at pH 8.5

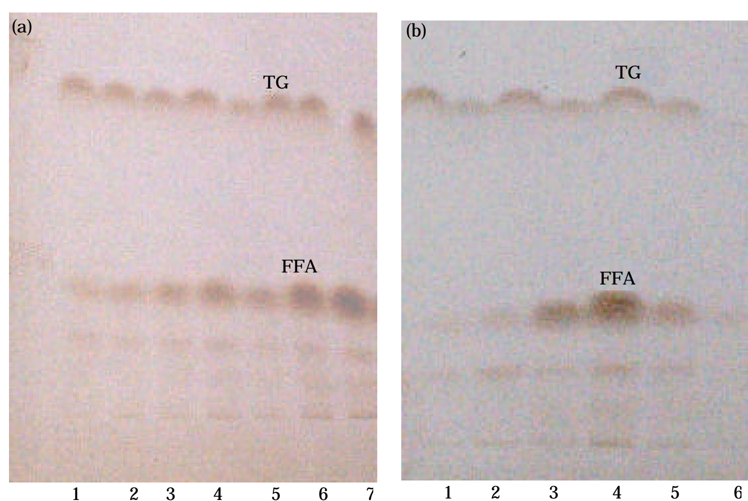

Fig. 7: HPTLC plates showing separation of Triglyceride (TG) and free fatty acids; a, olive oil spiked with standard oleic acid, lane 1-7: Increasing concentrations of standard oleic acid and b, fatty acid profile of enzyme treated olive oil at different pH, Lane 1-Control, 2-pH 7.5, 3-pH 8.0, 4-pH 8.5, 5- pH 9.0, 6- pH 9.5

CONCULUSION

Quantificatim of lipase activity of *A. fumigatus* by HPTLC was carried out in this paper which can detect even micro quantities of FFA produced and are rapid, efficient and easy to use for analysis.

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