African Journal of Biotechnology Vol. 6(5), pp. 564-568, 5, March 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Identification of potential fungal strain(s) for the production of inducible, extracellular and alkalophilic lipase

J. Savitha*, S. Srividya, R. Jagat, P. Payal, S. Priyanki, G. W. Rashmi, K. T. Roshini and Y. M. Shantala

Department of Microbiology, Sri Bhagawan Mahaveer Jain College, Center for Post-Graduate Studies, 18/3, 9th Main, Jayanagar 3rd Block, Bangalore 560 011, India.

Accepted 23 January, 2007

In the present study, a total of 32 fungal organisms belonging to 3 different genera were isolated from various sources of which 4 (three *Aspergillus* sp. and one *Mucor* sp.) were found to be positive for lipase production. Rhodamine B rapid screening technique was used to determine the lipase activity. *Mucor* sp. was found to exhibit a maximum fluorescence zone at 350 nm. Among the various types of triglycerides used as the carbon source, sunflower oil was found to be the most effective in inducing lipase in *Mucor* sp. The lipase in *Mucor* sp. was found to be inducible, alkalophilic and thermostable.

Key words: Alkalophilic lipase, extracellular, inducible, *Mucor* sp, Rhodamine B.

INTRODUCTION

During the last twenty years, the industrial use of enzymes has rapidly increased. Lipases have gained importance to a certain extent over proteases and amylases especially in the area of organic synthesis. Lipases are a class of water insoluble enzymes that catalyse the hydrolysis of ester bonds in lipid substrates, such as triacylglycerols (TAGs) releasing long chain fatty acids. Apart from hydrolysis, they bring about a wide range of conversion reactions that include interesterifications, alcoholysis and aminolysis. The unique characteristics of lipases include substrate specificity, stereospecificity, regiospecificity, and the ability to catalyse a heterogeneous reaction at the interface of water soluble and water insoluble systems (Macrae and Hammond, 1985).

Lipases are produced by plants, animals, bacteria and molds. Plant enzymes are not used commercially while animal, bacterial and mold enzymes are used extensively. Fungal lipases are derived mainly from *Candida* and *Aspergillus* sp. (Savitha and Ratledge, 1992) and *Geotrichum* sp. (Ginalska et al., 2004). Typical substrates are vegetable oil, animal fat, fish oil, olive oil, butter oil (milk fat) and synthetic TAG such as triolein. Among the desirable characteristic that commercially important lipases should exhibit are, alkali tolerance and thermostability. Most lipases reported so far are active at neutral pH.

The present study is aimed at screening and identifying potential fungal strain(s) for the production of inducible, extracellular and alkalophilic lipase.

MATERIALS AND METHODS

Isolation of fungi from soil

Soil samples were collected from different areas in and around Bangalore City. One gram of soil was collected using a sterile spatula in a sterile Petri plate. The soil was brought to the laboratory and processed immediately. The fungal organisms present in the soil were isolated using soil dilution method (Booth, 1971) and the cultures were maintained on Sabouraud's Dextrose agar and Czapek Dox agar media.

*Corresponding authors E-mail: drsvtj@yahoo.co.in. Tel.: +91-080-41210691. Fax: +91-080-41210692.

Rapid screening of fungi for the production of inducible extracellular lipase

This method involves measurement of fluorescence caused by the fatty acid released due to the action of lipase on olive oil. A quantitative fluorescence lipase assay is based on the interaction of Rhodamine B with fatty acid released during the enzyme hydrolysis of olive oil (Kouker and Jaeger, 1987).

The fungal isolates were inoculated on media of the following composition (g/L) Nutrient broth (HiMedia), 8.0; sodium chloride, 4.0; agar, 10.0. The medium was adjusted to pH 7.0, autoclaved and cooled to 60ºC. Olive oil (31.25 ml) and 10 ml of Rhodamine B solution (0.001%, w/v) was added with vigorous stirring and emulsified by mixing for 1 min. The medium was allowed to stand for 10 min at 60ºC to reduce foaming. 20 ml of the medium was poured into sterile petriplates (Kouker and Jaeger, 1987).

To determine the ability of fungi to utilize different oils as a sole source of carbon, Rhodamine B agar containing different oils (1%, v/v) was prepared. To determine the ability of the fungi to grow at alkaline pH, the fungi were inoculated on Rhodamine B agar adjusted to different pH (6.0, 7.0, 8.0, 9.0, 9.5 and 10.0). For inoculation, fungal discs (grown for 48 h) were placed on the agar surface and the plates were incubated at room temperature for 48 h. Lipase production is detected by irradiating the plates with UV light at 350 nm. Fungal colonies that have lipolytic activity showed orange fluorescent halo.

Inoculum preparation

After sporulation, 25 ml of sterile distilled water with 0.1% (v/v) Triton X-100 was added to the 48 h pre-grown culture. One ml of this was layered on to the slants and shaken gently; this was then collected in sterile conical flasks and used immediately. After incubation, the culture was filtered and the filtrate was used for quantitative estimation of lipase.

Culture and growth conditions

Mucor sp. was grown on medium of following composition (g/L) KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; yeast extract, 1.0; $CaCl_2.2H_2O$, 0.1; $FeCl_2.4H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.0001; di-ammonium tartarate, 1.5; pH 6.0. Glucose (1%, w/v) or a triglycerol (1%, w/v) was used as a sole source of carbon.

Assay of lipase activity-colorimetric assay

Lipase activity was determined with p-nitrophenyl palmitate (pNPP) by the method reported by Licia et al. (2006). The substrate for this reaction was composed of solution A and solution B. Solution A contained 40 mg of pNPP dissolved in 12 ml isopropanol. Solution B contained 0.1 g of gum arabic and 0.4 ml of triton X-100 dissolved in 90 ml of water. The substrate solution was prepared by adding 1 ml of solution A to 19 ml of solution B drop wise with constant stirring to obtain an emulsion that remains stable for 2 h. The assay mixture contained 1 ml of the substrate, 0.5 ml of buffer (glycine-NaOH, pH 11, 0.5 M), 0.1 ml of enzyme (the filtrate) and the volume was made up to 3 ml with distilled water. This was incubated at 40ºC for 45 min. The enzyme activity was stopped by adding 0.2 ml of isopropanol. The absorbance was measured at 410 nm against substrate free blank. The standard graph was prepared by using para-nitrophenol (0.4 to 4 µmoles). One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p nitrophenol per min under the assay conditions described (Maia et al., 1999).

Effect of pH on lipase activity

The optimum pH was determined by measuring the hydrolysis of pNPP in the pH range 5 - 10. The buffers used were: Citrate buffer - pH 5.0, 5.5; Na_2PO_4 - pH 6.0, 6.5, 7.0, 7.5; Tris-HCl pH 8.0, 8.5; glycine-NaOH- pH 9.0, 9.5, 10.0. To determine the pH stability, 0.1 ml of the enzyme was incubated with different buffers at 45ºC for 30 min and assayed for lipase activity.

Effect of temperature on lipase activity

The temperature optima of the enzyme were determined with pNPP by incubating the assay system in the temperature range of 30 - 80ºC using glycine-NaOH, pH 11.0 (0.5 M). To ascertain the stability, 0.5 ml of glycine-NaOH buffer (0.5 M, pH 11) and 0.1 ml of enzyme were incubated at different temperatures (30 to 50ºC) for 60 min and assayed for lipase activity.

RESULTS AND DISCUSSION

Isolation of fungi from soil

Totally 32 fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Mucor* were isolated.

Rapid screening of fungi for the production of inducible extracellular lipase

The 32 isolates were subjected to rapid screening by using Rhodamine B method (Kouker and Jaeger, 1987). Out of the 32 isolates three *Aspergillus* sp. and a *Mucor* sp. showed a zone of fluorescence at 350 nm (Figure 1). Among the four isolates *Mucor* sp. was found to produce a maximum (2.2 cm) zone of fluorescence (Figure 2). The four isolates were grown on Rhodamine agar containing each of the following carbon sources – glucose, olive oil, coconut oil, palm oil, sunflower oil, sesame oil, groundnut oil, rice bran oil and soybean oil (Table 1a).

Since alkalophilic lipase has a variety of applications in industry, a screening was done by using Rhodamine agar adjusted to different pH containing olive oil as a sole source of carbon. *Mucor* sp. was found to grow up to pH 10 indicating that the lipase is alkali tolerant (Table 1b). To study the effect of different triglycerides on lipase production by *Mucor* sp., the fungus was grown in medium containing triglycerides (Table 2) of 8 to 16 carbons. Lipase was found to be produced only in the presence of different oils indicating the inducible nature of the enzyme. Sunflower and coconut oil were the most effective in inducing enzyme production (Table 2).

Effect of pH on lipase activity

The optimal activity for pNPP hydrolysis was maximum at pH 10 in glycine-NaOH buffer (Figure 3). These data are in agreement with that of alkaline lipase reported for *Fusarium solani* FS1 (Maia et al., 1999) which has pH op-

Table 1a: Growth of the four isolates on Rhodamine agar containing different oils.

Oils	*Aspergillus* sp. 1	*Aspergillus* sp. 2	*Aspergillus* sp. 3	*Mucor* sp.
Sunflower	+	+	+	++++
Olive	++	++	++	++++
Palm	+++	++	++	++++
Rice bran	+	+	+	++++
Coconut	+	+	+	+++
Groundnut	++	++	++	++++
Sesame	++	++	++	+++
Soybean	+	+	+	+++

Growth: + Average; ++ Good; +++ Very Good.

Table 1b. Growth of the four isolates on Rhodamine agar at different pH.

pH	*Aspergillus* sp. 1	*Aspergillus* sp. 2	*Aspergillus* sp. 3	*Mucor* sp.
6	++	++	++	+++
7	++	++	++	+++
8	+	++	++	++
9	+	+	+	++
9.5	+	+	+	++
10	+	+	+	++

Growth: + Average; ++ Good; +++ Very Good.

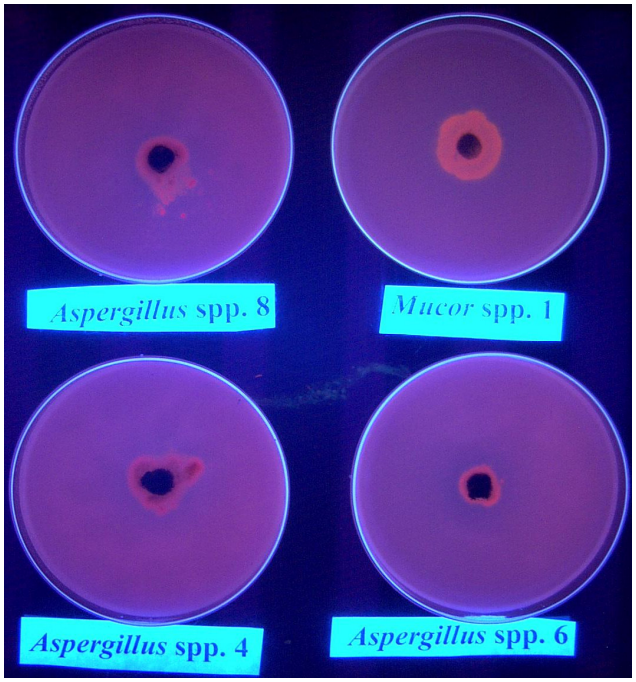

Figure 1. Growth of the four potential fungal isolates on Rhodamine agar containing olive oil as substrate.

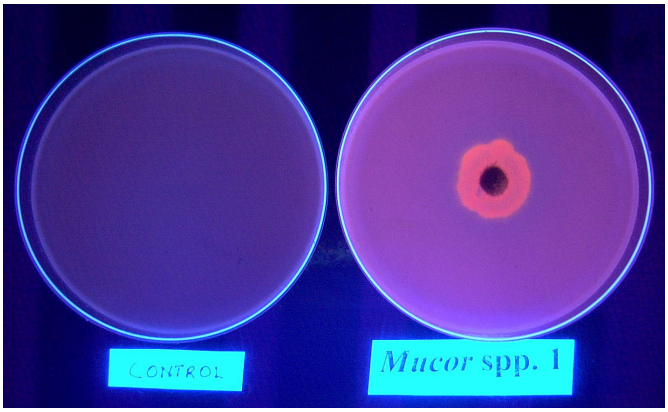

Figure 2. Growth of *Mucor* sp. on Rhodamine agar.

tima at pH 8.6 in Tris-HCl buffer at 25ºC. No activity was observed at pH 11 however, pH 7 showed moderate activity (Figure 3). The pH stability of the enzyme was determined by the activity retained at different pH (5 -11) after 30 min of incubation. The pH stability curve showed that the enzyme is stable under an alkaline pH range (Table 3). The enzyme retained 77% of its initial activity for 30 min at pH 10 with a marked decrease in activity beyond this range. The stability data shows a decline in lipase activity below pH 7 however, 54% activity was retained at this pH. This result is in accordance with the result provided by Maia et al. (1999), who reported that

Table 2. Lipolytic activity of *Mucor* sp. on different oils.

Oils	Mycelial dry weight (mg)	Activity in units (U)*
Sunflower	111	12.1
Olive	67	11
Palm	244	8
Rice Bran	69	11
Coconut	87	12.1
Groundnut	189	8
Sesame	54	11
Soybean	63	9
Glucose	250	0

*One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min.

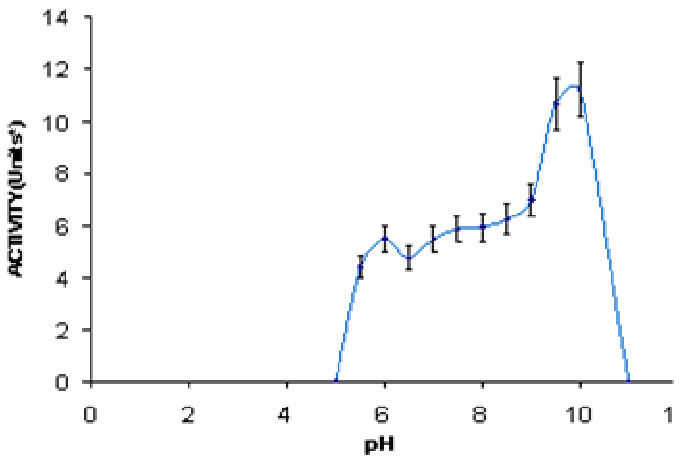

Figure 3. Effect of pH on lipase activity. One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min.

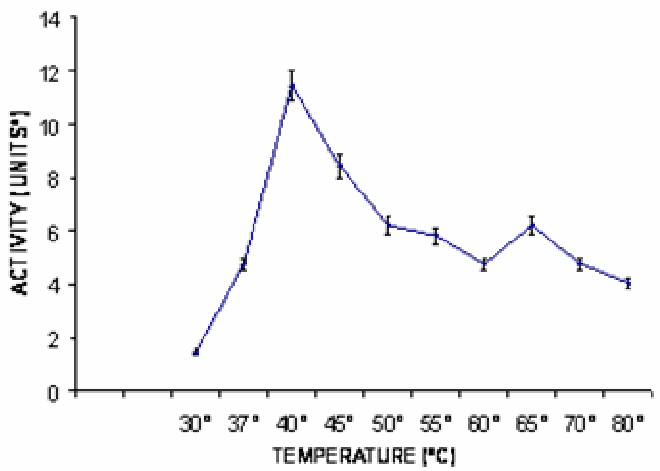

Figure 4. Effect of temperature on lipase activity. One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min.

Table 3. pH stability of *Mucor* sp. lipolytic activity.

pH	Activity in units (U)*	Relative activity (%)
7	3	54
8	3	65
9	5	73
10	11	77

*One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per min.

lipase from *F. solani* FS1 maintained around 80% of its initial activity when incubated for one hour at alkaline pH (7.2 - 8.6), with a decrease in lipase activity at pH beyond this range. It was also reported that the enzyme was more stable at pH 7.2 in Tris-maleate buffer, despite its optimum activity at pH 8.6 in Tris-HCl buffer (Maia et al, 1999).

Effect of temperature on lipase activity

The optimal temperature for lipase activity on pNPP was found to be 40ºC. At this temperature lipase showed a three-fold increase in activity compared with that at 37ºC. A considerable decrease in the lipolytic activity was observed at temperatures beyond 40ºC (Figure 4). The optimal reaction temperature reported here is similar to that reported for *F. solani* FS1 (Maia et al., 1999) that showed maximum lipase activity at 45°C. The lipase produced by *Fusarium oxysporum, Fusarium* sp. line and *F. oxysporum f.* sp. *Vasinfectum,* showed optimum activity at 42 and 45ºC, respectively (Hoshino et al., 1992). The stability of the crude enzyme extract was determined by the activity retained at different temperatures (30 - 50°C) after 60 min of incubation. Maximal stability of the enzyme was observed at 30°C (Table 4). Incubation beyond one hour promoted inactivation of this enzyme. Lipase from *F. solani* FS1 was found to be more

Table 4. Temperature stability of *Mucor* sp. lipolytic activity.

Temperature (℃)	Activity in units (U)*	Relative activity (%)
30	11	100
40	13.2	92
50	5.72	52

*One lipase unit (U) is defined as the amount of enzyme that liberated 1 µmol p-nitrophenol per minute

stable in the temperature range of 25 - 30℃ after its thermal exposure of 1 h (Maia et al., 1999).

CONCLUSION

Due to the inducible, alkalophilic nature and stability of the enzyme, the *Mucor* sp. isolated from our present study can be exploited for the industrial production of alkalophilic lipase. The occurrence of an inducible, extracellular, alkalophilic lipase in fungi grown on vegetable oil is well documented in lipid biotechnology. A lipase that is stable at high alkaline conditions and high temperature is however rare, and in the present study we have isolated a fungal organism (*Mucor* sp.) which produces an inducible, extracellular, alkalophilic and thermostable lipase.There have been very few reports available so far with molds having alkalophilic and thermostable lipase (Licia et al., 2006; Ginalska et al., 2004). Therefore, the organism reported in this paper can be exploited for commercialization as lipase of these characteristics finds immense application as additives for washing powders. However, further work is clearly needed to increase the production of lipase in the wild strain isolated by us (*Mucor* sp.) as the activity is comparatively less than the strains used for commercial production.

ACKNOWLEDGEMENT

The authors wish to thank the Chairman, Jain Group of Institutions (JGI) for funding the project.

REFERENCES

Booth C (1971). Introduction to general methods. Methods in Microbiol. 4**,** (Ed C. Booth) Academic Press, London and New York, pp. 1-47.

Kouker G, Jaeger KE (1987) Specific and Sensitive Plate Assay for Bacterial Lipases. Appl. Environ. Microbiol 53(1): 211-213.

Ginalska G, Bancrez R, Kornillowicz-Kowalska T (2004) A thermostable lipase produced by a newly isolated *Geotrichum*-like strain, R59. J. Indus. Microbiol. Biotechnol. 31(4): 177-182.

Hoshino T, Sasaki T, Watanabe, Yuichi W, Nagasawa T, Yamane T (1992). Purification and some characteristics of extracellular lipase from *Fusarium oxysporum* f.sp. *lini*. Biosci. Biotechnol. Biochem. 56: 660-664.

Licia MP, Cintia MR, Mario DB, Guillermo RC (2006). Catalytic Properties of Lipase Extracts from *Aspergillus niger*. Food Technol. Biotechnol. 44(2): 247-252.

Macrae AR, Hammond RC (1985). Present and future Applications of Lipases. Biotechnol. Genet. Eng. Rev. 3: 193-217.

Maia M de MD, Morais MMC de, Morais Jr MA de, Melo EHM, de Lima Filho JL (1999). Production of extracellular lipase by the phytopathogenic fungus Fusarium solani FS1, Revista de Microbiologia. 30(4): 304-309.

Savitha J, Ratledge C (1991) An Inducible, Intracellular, Alkalophillic Lipase in *Aspergillus flavipes* grown on triglycerols, World J. Microbiol. Biotechnol. 8(2): 129-131.