Extensive Journal of Applied Sciences
Available online at www.ejasj.com

EFFECT OF NUTRITIONAL FACTORS ON LIPASE BIOSYNTHESIS BY ASPERGILLUS NIGER IN SOLID STATE FERMENTATION

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ABSTRACT: Among the various fungal strains screened for lipase production, Rhizopus arrhizus NCIM 877, 878, 879 and Asp.ergillus niger NCIM 1207 produced significant quantities of enzyme when grown in synthetic oil based (SOB) medium under submerged conditions. Rhizopus strains showed major intracellular activity while A. niger NCIM 1207 produced mainly extracellular activity. Lipase production in A. niger NCIM 1207 was studied using both submerged fermentation (SmF) and solid state fermentation (SSF). De novo biosynthesis of lipase occurred only in the presence of lipid substrate and was completely repressed by glucose. The highest yields of enzyme were obtained in SSF using wheat bran as solid substrate in combination with olive oil as lipid substrate. Maximum lipase activity (630 IU/g dry solid substrate) was recovered when fermented wheat bran was extracted with NaCl (1%) supplemented with Triton X-100 (0.5%). The pH and temperature optima for lipase were 2.5 and 45 8C, respectively. The enzyme also exhibited high activity (75%) at extremely acidic pH of 1.5. Lipase activity (63%) was retained when enzyme was incubated in a buffer of 2.5 for 24 h at room temperature. The enzyme retained 63% of its original activity on incubation at 70 8C for 5 h. This organism, being GRAS cleared, can be used for large-scale production of enzyme for commercial purpose.

***Keywords*:** lipase biosynthesis,aspergillus,nutrltional factors.

INTRODUCTION

Lipases (glycerol ester hydrolyses EC 3.1.1.3) catalyze the hydrolysis of acyl glycerols to fatty acids, di-acyl glycerols, mono-acyl glycerols and glycerol. Under certain conditions, they also catalyze the synthesis of esters by transesterification, thioesterification and aminolysis. Lipases occur widely in bacteria (Nahara et al., 1989) yeasts (Rapp and Backhaus, 1992; Dalmou et al., 2000) and fungi (Jaeger et al., 1998; Ferreira et al., 1999). In recent years, research in microbial lipases has increased because of their practical applications in industry, in the hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, resolution of racemic mixtures or addition in detergents (Maleata, 1996). Fungi are widely recognized as the best lipase sources and are used preferably for industrial applications, especially in the food industry. Aspergillus Niger is among the most well-known lipase produces and its enzyme is suitable for use in many industrial applications (Macris et al., 1996; Fu et al., 1995). Most research concentrates on extra cellular lipases that are produced by wide variety of organisms (Rapp and Backhaus, 1992). Studies on conditions for the production of extra- cellular lipases by Aspergillus Niger show variations among different strains but the requirement of lipid carbon source is essential for enzyme production. The technique of solid state fermentation (SSF) Involves the growth and metabolism of microorganisms on moist solids without any free-flowing water. This technique has many advantages over submerged fer- mentation (SmF) including economy of space needed for fermentation, simplicity of fermentation media, no requirement of complex machinery, equipment and control systems, compactness of fermentation vessel owing to lower water volume, superior yields,less energy demand, lower capital and recurring expenditure (Lonsane et al.,1895;Satyanarayana,1994). Additionally, such a system finds greater applications in solid waste management, biomass energy conservation and in the production of secondary metabolites. However, SSF has some limitations such as limited choice of microorganisms capable of growth under reduced moisture conditions, controlling and monitoring of parameters such as temperature, pH, humidity, air flow (Lonsane et al.,1895; Nahara et al., 1982). Although, almost all litera- ture on SSF refers to fungal systems, there are very few reports on lipase production in SSF by A. niger to date (Olama and El-Sabaeny, 1993; Kamini et al., 1998).

Lipases active at highly acidic pH have not been reported so far from microbial sources. The objective of the study was acidic lipase production by A. niger NCIM 1207 by SSF and characterization of the enzyme with regard to thermostability, pH stability and opti- mum temperature and pH conditions for reaction.

MATERIAL AND METHODS

2.1. Materials

Malt extract, yeast extract, bacto-peptone were ob- tained from Difco Chemical Co. Detroit, USA. Celite 545 was obtained from Fluka Chemie, AG Switzerland. n -Butanol (water content B 0.1%), laboratory grade reagent, was procured from May and Baker Ltd., UK. Butter oil, wheat bran, rice polish, DORB, soybean, maize, olive oil, sunflower oil, coconut oil, til oil, have been purchased from local supplier. All other chemicals were of analytical grade and procured from B.D.H., UK.

2.2. Methods

2.2.1. Microorganisms and Growth Media

The cultures were obtained from the National Collec- tion of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and were maintained on MYG medium (malt extract 0.5%, yeast extract 0.25%, glucose 1.0% and agar 2.0%). The rich MGYP medium contained (w/v) malt extract 0.3%, yeast extract 0.3%, bacto-peptone 0.5% and glucose 2.0%. Aspergillus minimal medium (AMM) contained (w/v) NaNO3 0.05%, MgSO4 7H2O 0.05%, KCl 0.05%, KH2PO4 0.2%, yeast extract 0.1%, bacto-peptone 0.5%. Synthetic oil based (SOB) medium is AMM with 1% olive oil. The pH of the medium was adjusted to 5.5 with NaOH prior to sterilization.

2.2.2. Lipase Production in SmF

The culture was grown in 500 ml Erlenmeyer flasks containing 100 ml MGYP or SOB medium. The flasks were inoculated with spores (approximately 106/ml) from a 7 days old culture on MYG slope and incubated at 30 8C on a rotary shaker (150 180 rpm) for 72 h. The mycelium was harvested by filtration and the culture filtrate was used as a source of extra cellular enzyme.

2.2.3. Preparation of Dry Mycelium

The mycelium harvested by filtration was washed with distilled water twice to remove traces of medium and then washed with chilled acetone. The acetone treated mycelium was vacuum dried for 6 h to remove acetone and water. This vacuum dried mycelium was used for determining cell bound (intracellular) lipase activity.

2.2.4. Lipase Production in SSF

Erlenmeyer flasks (500 ml) containing 10 g of wheat bran (or any other agricultural residue) moistened with 24 ml SOB medium with 1 ml of oil were sterilized at 121 8C for 30 min. After cooling, the flasks were inoculated with spore suspension (1 ml) containing 106 spores from 7 days old culture grown on MYG slope. The contents of each flask were mixed thoroughly with inoculating needle for uniform distribution of fungal spores in the medium. The flasks were incubated at 30 C. A flask was harvested, its contents were extracted for enzyme and lipase activity was assayed every 24 h for the period of 6 days.

2.2.5. Enzyme Extraction

After SSF 100 ml of aqueous solution of NaCl (1%) was added to each flask and the mixture shaken on a rotary shaker (180 rpm) for 2 h at room temperature for extraction of enzyme from the fermented Koji (Al-Asheh and Duvnjak, 1994; Ebune et al., 1995). At the end of extraction, the suspension was squeezed through a double layer muslin cloth and it was centrifuged at 5000g for 20 min at 4 C. The clear supernatant obtained was used as the extracellular enzyme.

2.2.6. Celite Adsorption of Extra cellular Enzyme

A standard procedure based on Colman and Macrae (Coleman et al., 1973) was used to immobilize the extracellular enzyme. Celite 545 (1.0 g) was added to 20 ml of culture filtrate with mixing. Ice-cold acetone (25 ml) was then added over a period of 5 min while stirring with a magnetic stirrer and the suspension was stirred for an additional 30 min at 0 8C, then filtered and air dried. The celite adsorbed preparation (1.2 g) contained approximately 200 mg (925) of water and was used as a source of extracellular enzyme.

2.2.7. Spectrophotometric Assay using pnpp Substrate

The spectrophotometric method (Vonderwulbeche et al., 1992) with slight modifications was used for rapid and routine measurement of lipase activity using p-nitrophenylpalmitate (pNPP) as the substrate. The substrate

solution was prepared by adding solution A (40 mg of pNPP in 12 ml of propane-2-ol) to 9.5 ml of solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 in 90 ml of distilled water) drop wise with intense stirring. The emulsion obtained remained stable for at least 2 h. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of suitable buffer (0.5 M) and 0.1 ml of suitably diluted enzyme. The assay mixture was incubated at 50 C for 30 min and the p -nitrophenol released was measured at 410 nm in Spectronic-117 spectrophotometer. One unit of activity was expressed as mmol of p -nitrophenol released per minute under the assay conditions.

2.2.8. Lipase Catalyzed Transesterification of Butter Oil

Determination of lipase activity was based on the formation of butyl esters by transesterification of butter oil with butanol. The transesterification reaction was carried out in a 25 ml stoppered conical flask, which was shaken at 100 strokes per minute in a controlled temperature water bath at 35 C. The reaction mixture contained 50 mg vacuum dried mycelium or 500 mg celite adsorbed enzyme preparation, 250 mg butter oil and 5.5 g of water saturated butanol. Fifty ml of water/ buffer was added to the reaction mixture when the vacuum dried mycelium was used.

2.2.9. GLC Analysis

Analysis of esters was carried out by GLC using capillary column (Phillips, 0.25 um film of silicon OV1, 3.8 m 0.22 mm; injector and FI detector at 300 C). For samples, which contained incompletely solvolyzed or unchanged triglycerides, the temperature was set at 40 C for 3 min then rising at 3 C/min up to 320 C to elute unchanged triglycerides. Esters were identified by interpolation from standards. Analysis was normally carried out on 1 ml samples using added undecane (0.15 mg/ml) as an internal standard, which was prepared in n -hexane.

RESULTS AND DISCUSSION

3.RESULTS

3.1. Screening of Lipase Producing Microorganisms in SmF

SOB medium demonstrated that Rhizopus arrhizus strains produced highest levels of intracellular lipase while A. niger produced maximum extracellular lipase (data not shown). The use of carbohydrates such as glucose in SOB medium appeared to be beneficial for enzyme production in case of A. niger NCIM 1207 (Table 1). This could be attributed to rapid assimilation of easily metabolizable carbon source compared to lipid producing more biomass and lipase activity. The addi- tion of Triton X-100 to the fermentation medium resulted in enhanced levels (18 IU/ml) of enzyme production (Table 1). Since A. niger NCIM 1207 produced high levels of extracellular lipase active at pH 2.0, further studies on optimization of enzyme production in SSF and its characterization were carried out.

Table 1. Effect of carbon source on extracellular lipase production by A. niger NCIM 1207.

Carbon source	Biomass [a] (mg dry weight)	Lipase activity [b](IU/ml)
AMM + glucose (1%)	400 ± 40	0.02
AMM + olive oil (1%)	845 ± 45	3.5
AMM + glucose (1%) +olive oil (1%)	1100 ± 95	6.8
AMM[c] + glucose (1%) +olive oil (1%) +Triton X-100 (0.5%)	1060 ± 75	18

[a] The biomass was derived from 100 ml of the medium.

[b] The culture was grown in SmF as mentioned in Section 2. The activities were calculated after 5 days of incubation using pNPP assubstrate.

[c] Triton X-100 (0.5%) was added on 3rd day of fermentation and the activity was determined after 5th day of fermentation.

3.2. Optimization of Lipase Production in SSF

3.2.1. Time Course of Enzyme Production

The time course of lipase production using wheat bran as a substrate is given in Fig. 1. Maximum enzyme activity was found on the 5th day of fermentation. A prolonged incubation time beyond this period did not help to further increase the enzyme yield.

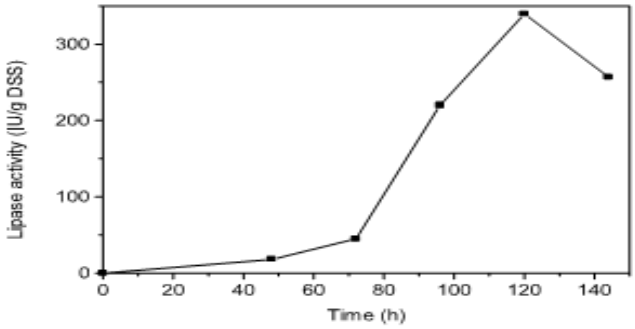


Fig 1. Time course of lipase production in SSF using wheat bran.

3.2.2. Effect of Moisture on Lipase Production

To check the influence of moisture on lipase activity during SSF, wheat bran (10 g) was moistened with different amounts of SOB medium and the results are shown in Fig. 2. Maximum enzyme yield was obtained when wheat bran was moistened with SOB medium in a 1:2.5 ratio. There was a decline in enzyme production above this ratio as the porosity of the medium is decreased.

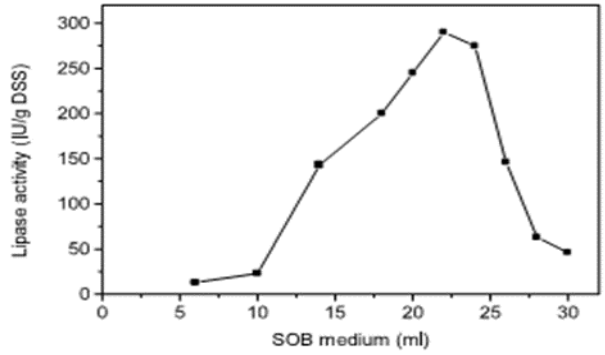


Fig 2. Effect of moisture level on lipase production in SSF.

3.2.3. Effect of Different Agricultural Residues on Lipase Production

Among the different agricultural residues tested for lipase production by A. niger NCIM 1207, wheat bran supported enzyme production far better than any other natural substrate (Table 2). Further optimization studies were carried out using wheat bran as the substrate.

Table 2. Production of lipase by A. niger NCIM 1207 in SSF using various Substrates and oils

Solid substrate and oil	Lipase production (IU/g DSS)
Wheat bran + olive oil	305 ± 27
Rice polish + olive oil	80 ± 5.0
DORB + olive oil	135 ± 20
Maize grains + olive oil	48 ± 1.2
Soybean seeds + olive oil	64 ± 1.5
Wheat bran + sarson ka oil	290 ± 5.0
Wheat bran + til oil	340 ± 24
Wheat bran + sunflower oil	125 ± 4.0
Wheat bran + castor oil	130 ± 4.0
Wheat bran + coconut oil	180 ± 5.0

3.2.4. Effect of Different Oils on Lipase Production

The effect of different oils on lipase production was studied. From the results (Table 2) it could be concluded that A. niger NCIM 1207 lipase required a lipid based carbon source for their production, although their role in lipase synthesis is poorly understood. All oils tested supported enzyme production however, til and olive oils gave maximum yields of lipase activity.

3.2.5. Extraction of Enzyme Using Different Salt Solutions With and Without Surfactants

Extraction of enzyme from fermented wheat bran was carried out with tap water, distilled water, 1% NaCl or 1% (NH4)2SO4. There was no marked difference in the recovery of enzyme but NaCl (1%) was found to be effective for extraction of enzyme (Table 3). The supplementation of NaCl with Triton X-100 as surfactant (0.5%) helped in increased recovery (twofold) of enzyme from the fermented wheat bran. The yield of lipase was as high as 630 IU/g of DSS.

Table 3. Effect of different salt solutions on extraction of lipase from fermented wheat bran

Salt solution	Lipase production (IU/g DSS)
Tap water	265 ± 24
Distilled water	230 ± 16
NaCl (1%)	310 ± 24
NaCl (2%)	260 ± 14
(NH4)2SO4 (1%)	270 ± 18
NaCl (1%) + Tween 80 (0.5%)	320 ± 20
NaCl (1%) + Triton X-100 (0.5%)	630 ± 24
NaCl (1%) + Triton X-100 (1.0%)	625 ± 27

3.3. Characterization of Enzyme in Aqueous Solutions

3.3.1. Effect of pH on Enzyme Activity

Lipase assay was performed from pH 1.5 to 4.5 using variety of buffers at 50 mMin reaction mixture at 45 8C for 20 min using pNPP as substrate. Buffer systems used were KCl/HCl buffer (pH 1.0/2.0), citrate/phosphate buffer (pH 2.5/5.0). The optimum activity of lipase was observed between 2.5 and 3.0 with sharp decline in activity above the pH 3.5. However, the enzyme was very activeeven at pH 1.5 with 75% residual activity (Fig. 3). The lipase was stable at a broad pH range from 2.5 (70% residual activity) to 9.0 (50% residual activity) after 24 h incubation at room temperature (Fig. 4).

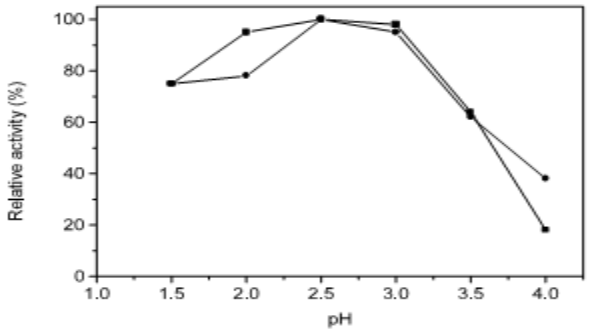
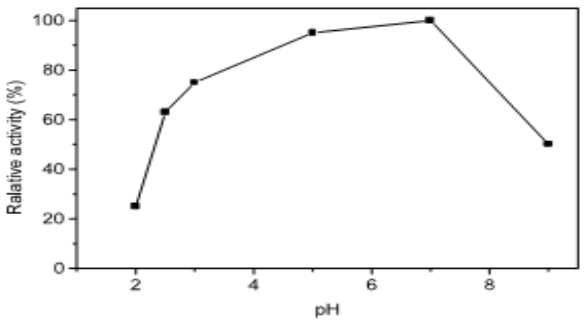


Fig 3. Effect of pH on lipase activity. ■----■, lipase activity in aqueous system (buffer); ●----●, trans-esterification activity in organic solvent.



butanol).

Fig 4. Effect of pH on lipase stability. The enzyme was incubated in 0.05 M buffer systems (KCl - HCl buffer, pH 1.5, 2.0; citrate - phosphate buffer, pH 2.5 - 6.0; phosphate buffer, pH 7.0, borate buffer, pH 8.0 - 9.0) at room temperature and the residual activity was assayed after 24 h.

3.3.2. Effect of Temperature on Lipase Activity and Stability

Lipase activity was determined at different temperatures under standard assay conditions. The enzyme exhibited maximum activity at 45 8C with substantial activity between 30 and 55 C (Fig. 5). Studies on lipase stability at different temperatures revealed no loss of activity after 5 h incubation at 60 8C. The enzyme showed 40% of its original activity when incubated at 75 8C for 4 h (Fig. 6).

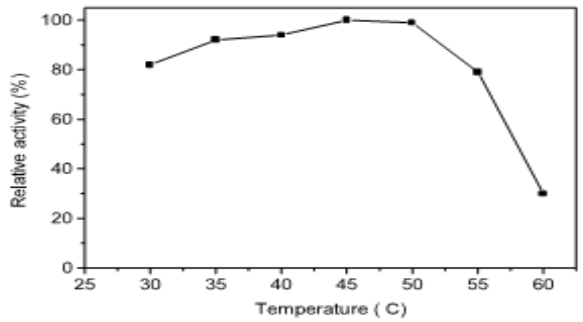


Fig. 5. Effect of temperature on lipase activity.

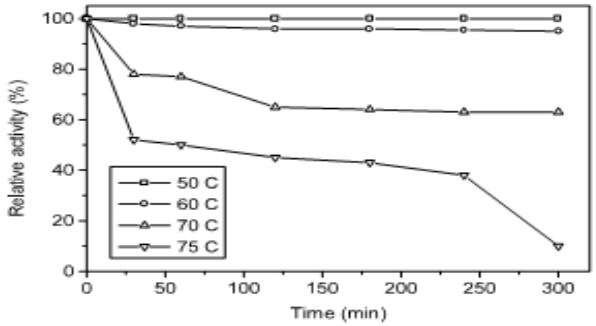


Fig 6. Effect of temperature on lipase stability. The enzyme solution was incubated at different temperatures and the residual activity was assayed every hour up to 5 h. □---□, 50 °C; ○---○, 60 °C; △---△, 70 °C; ▽---▽, 75 °C.

3.4. Characterization of Enzyme in Organic Solvent

3.4.1. Effect of Water on Transesterification Activity of Lipase

Transesterification reactions were carried out using vacuum dried mycelium. The reaction mixture contained 50 mg of vacuum dried mycelium, 250 mg butter oil, 5.5 g of water saturated butanol. Different amounts (0/800 ml) of citrate/phosphate buffer (100 mM, pH 2.5) were added to the mycelium. The reaction conditions were as described earlier for transesterification activity. In total reaction mixture of 6 ml, 200 ml of buffer (3- 4%) was necessary (Fig. 7). Increased yields were obtained when buffer was added to on vacuum dried mycelium.

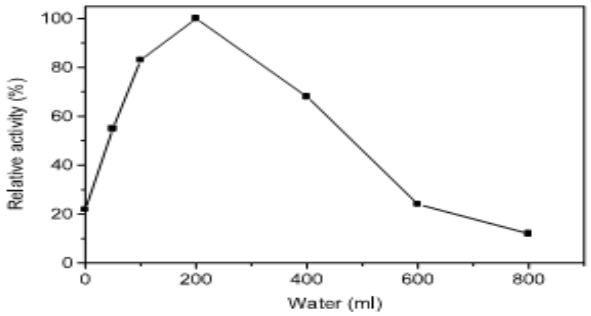


Fig 7. Effect of water on trans-esterification activity of lipase in butanol.

3.4.2. Effect pH on Transesterification Activity of Lipase

The transesterification reaction was carried out at different pH using 100 mM buffer systems (HCl/KCl buffer, pH 2.0; citrate/phosphate buffer, pH 2.5/7.5). The reaction mixture contained 50 mg of dry vacuum dried mycelium, 250 mg of butter oil, 5.5 g of water saturated butanol and 200 ul of buffer placed on mycelium. The reaction conditions were as described earlier for transesterification activity. The enzyme had the same optimum pH (2.5) in organic solvent and aqueous buffer (Fig. 3).

4. DISCUSSION

It can be concluded that lipase activity in Rhizopus strains is associated with the cell. A. niger NCIM 1207 produced mainly highest levels of extracellular lipase (630 IU/g of wheat bran). Previous reports on the physiology of lipase production showed that the mechanisms regulating biosynthesis vary widely in different microorganisms. Lipase production in Rhizopus [20], Rhodotorula [21], Aspergillus [22] seems to be constitutive and independent of the addition of lipid substrates to the fermentation medium. The present studies reported that A. niger NCIM

1207 produced mainly extracellular lipase in SmF conditions only in the presence of oil suggesting that lipase activity is induced by the presence of lipid substrates in the medium. Extracellular lipase production from different microorganisms has been reported on lipids [21, 22], carbohydrates [23] and also on a mixture of both [9]. In the present studies, the growth of A. niger NCIM 1207 in a medium containing both substrates resulted in production of enhanced levels of lipase which is in agreement with the report by Macris et al. [9]. The use of surfactants like Triton X-100 during SSF fermentation helps to increase enzyme production [16, 17, 24]. We also found that incorporation of Triton X-100 to fermentation medium resulted in a threefold increase in enzyme production in SmF. This effect could be attributed to increased permeability of cells. A similar increase in enzyme levels in SSF was observed when enzyme from Koji was extracted with NaCl (1%) supplemented with 0.5% Triton X-100. In the light of several advantages of SSF, lipase production from A. niger NCIM 1207 was attempted on various natural substrates among which wheat bran emerged as the most suitable with olive oil as lipid carbon source. Haq et al. [25] reported that Rhizopus oligosporus produced high levels of extracellular lipase by SSF using almond meal as the substrate. The universal suitability of wheat bran may be attributed to the presence of sufficient nutrients and is able to remain loose even in moist conditions, thereby providing a large surface area [26]. The moisture content in SSF is a crucial factor that determines the success of the process [26]. The moisture level in SSF has a great impact on the physical properties of the solid substrate [26, 27]. Moisture levels higher than optimum causes decreased porosity, lower oxygen transfer and alteration in wheat bran particle structure. Likewise, lower moisture than optimum decreases the solubility of the solid substrate, lowers the degree of swelling and produces a higher water tension. In the present work, maximum lipase production was obtained with wheat bran to SOB medium ratio of 1:2.5.

In general, lipases from A. niger strains are active between pH 4 and 7 and temperature between 40 and 55 8C [15, 28, 29]. Lipase from A. niger NCIM 1207 was active at pH 2.5 with 75 and 90% residual activity at extreme acidic pHs 1.5 and 2.0, respectively. This enzyme is also stable over a broad pH range from 2.5 to 9.0 up to 24 h at room temperature. The optimum temperature of lipase from A. niger NCIM 1207 is 50 8C and it is stable at 60 8C for 5 h. The addition of minimum amount of water (3/4%) is necessary for transesterification activity. The addition of water reduced the synthesis of esters and shifted the equilibrium of the reaction towards hydrolysis, producing fatty acids. Thus transesterification becomes dominant only when the availability of water is restricted [30]. It was also seen that this enzyme shows the same optimum pH in organic solvent. This phenomenon is called pH memory and is well documented in the literature [30].

CONCLUSIONS

In conclusion, a lipase producing strain of A. niger NCIM 1207 has been isolated, which produced mainly extracellular enzyme. Elevated enzyme levels (630 IU/g DSS) were obtained in SSF using wheat bran which, to the best of authors' knowledge, were among the highest reported in the literature from fungal sources. This lipase has some properties in common with lipases from other A. niger strains except the optimum pH (2.5) and the stability at highly acidic pH (2.5). In organic solvent, the lipase exhibits highest activity at pH 2.5, which is evident from transesterification activity. Such unique lipase, active and stable at extremely acidic pH (2.0), has not been reported so far. These results, together with the fact that this fungus is generally recognized as a safe (GRAS) microorganism in the food, beverage and pharmaceutical industry, makes this process worthy of future investigation.

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