

Optimization of lipase synthesis by *Mucor racemosus* - Production in a triple impeller bioreactor

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ABSTRACT

Fungal cultures were screened for their ability to produce extracellular lipase through different incubation periods, in spite of some *Aspergillus niger* gave highest lipolytic activities, *Mucor racemosus* was selected in this study due to its non-pathogenicity. Optimum lipase activity was obtained after 3 days incubation period at 35 °C using submerged culture technique. Using a modified complex medium (3% peptone; 0.2% KH₂PO₄; 0.05% KCl; 0.05% MgSO₄·7H₂O; 1% glucose; 1% olive oil and 0.01 M barium acetate as additives; pH was adjusted to 7.0 using 0.2 M phosphate buffer) stimulated *M. racemosus* lipase by 114.3%. The production of lipolytic enzyme by *M. racemosus* was up-scaled to 5 L stirred-tank bioreactor. Under optimized conditions, lipase and specific enzyme activities were improved by 2 folds (1211 U/mL and 28 U/mg protein) as compared to shake flask studies.

Keywords: lipase, *Mucor racemosus*, culture conditions, fermentation, bioreactor

INTRODUCTION

Lipases (Triacylglycerol acylhydrolases; EC 3.1.1.3) are versatile and ubiquitous biocatalysts with a wide range of application. Microbial lipases are commercially important because of their unique properties and the ease of their bulk extracellular production, compared to lipases from other natural sources (Jaeger and Eggert, 2002). The interest in microbial lipase production has increased in the last decades, because of its large potential in industrial applications as additives for foods (flavor modification), fine chemicals (synthesis of esters), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oils and fats in foods), leather (removal of lipids from animal skins) and medical (blood triglyceride assay) (Davranov, 1994; Pandey *et al.*, 1999; Burkert *et al.*, 2004; Kumar *et al.*, 2005).

Lipase production is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculum size (Kim *et al.*, 1996; Gupta *et al.*, 2004). Fungal lipases are typically produced in submerged cultures using complex culture media whose ingredients include vitamins, organic salts, amino acids and complex sources of organic compounds, such as yeast extract, peptone, soy meal and corn-steep liquor (Sarkar *et al.*, 1998; Chahinian *et al.*, 2000; Sharma *et al.*, 2001; Lin and Ko, 2005). Fungal lipases have a pH optimum in the neutral or slightly acid range. Elwan *et al.* (1986b) studied lipases from *Aspergillus sydowi* and found that, the optimum pH value varied greatly with variation in the buffer system used. Also temperature is considered

one of the most important factors and it varied greatly with different strains, for example, optimal temperature for *Acinetobacter radioresistens* was 30 °C when cultivated on 2.5 L tank fermentor (Li *et al.*, 2005), 37 °C for *Burkholderia multivorans* (Gupta *et al.*, 2007), 55 °C for thermophilic bacterium *Bacillus coagulans* (Kumar *et al.*, 2005). Several researchers found that, aeration increased lipase accumulation (Sugiura *et al.*, 1977; Gupta *et al.*, 2004). But the extent of aeration optimal is not the same for different producers.

The extensive application of lipolytic enzymes as industrial biocatalysts requires the development of large-scale processes for their production. Recently attention was directed to the production of lipases under large scale fermentations and at different aeration and agitation rates (Tan *et al.*, 2003, Gupta *et al.*, 2007).

Despite the presence of a large number of already discovered and characterized lipolytic enzymes, the search for new lipase producers remains important due to the wide range of practical industrial application. The aim of the present work was to determine the optimum parameters favorable for the growth and lipase production of *Mucor racemosus* using shake flasks studies. Then process variables have been used in a 5 L bioreactor for the fermentation process to obtain high lipase production.

MATERIALS AND METHODS

Microorganisms

The following microbial strains were screened for lipase activity: *Aspergillus niger* oil (isolated from oil), *A. niger*

102H, *A. niger* 16H, *A. niger* 7H, *A. carneus*, *A. flavus*, *A. fumari*, *A. fumigatus*, *A. niveus*, *A. terreus* 1H, *A. terreus* 2H, *A. terreus* 3H, *A. wentii*, *Botrytis* sp., *Cunninghamella* sp., *Fusarium. clomydo*, *F. solani*, *Fusarium* sp., *Mucor hiemalis*, *M. pusillus*, *M. racemosus*, *M. roxii* NRRL1894, *Penicillium cyclopium*, *P. duclauxi*, *P. funiculosum*, *Rhizoctonia* sp., *Rhizopus* sp., *Sclerotium* sp., *Trichoderma viride*. Microbial cultures were obtained from Microbiology Research Centre, Ain Shams University (MIRCEN); Botany Department, Faculty of Girls, Ain Shams University (BDFGASU); National Centre for Radiation Research and Technology (NCRRT); National Research Centre (NRC); Cairo, Egypt and the culture collection of Northern Regional Research Laboratory (NRRL), USA. The organisms were subcultured on potato dextrose agar for 7 days at 30 °C. The spore crop of each slant was scrapped in 5 mL sterile water. A spore suspension of about 8.75×10^7 /mL was used as a source of inoculum.

Lipase production

Five different cultivation media were evaluated for the production of lipase enzyme. The composition of the different fermentation media can be summarized as follows:

Medium I: 3.0% peptone; 0.2% KH_2PO_4 ; 0.05% KCl; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1% olive oil: glucose (0.5:0.5), pH 6.6 (Akhtar *et al.*, 1980).

Medium II: 0.1% NH_4NO_3 ; 0.2% KH_2PO_4 ; 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0% sucrose and 1.0% olive oil (Pal *et al.*, 1978).

Medium III: 1.2% NaH_2PO_4 ; 0.2% KH_2PO_4 ; 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.025% CaCl_2 ; 0.0005% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0015% $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$; 0.003% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 2% corn oil and 1% peptone, pH 5.5 (Hatzinikolaou *et al.*, 1996).

Medium IV: 1.0% peptone; 6.48% soybean waste; 0.2% KH_2PO_4 ; 0.05% KCl; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5% Arabic gum, pH 6.0 (Abdel-Aal, 1996).

Medium V: 3% peptone; 7.0% cotton seed waste; 0.2% KH_2PO_4 ; 0.05% KCl; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5% Arabic gum, pH 6 (Ahmed, 2000).

After preparation, the pH values of the prepared media were initially adjusted (5.5-6.6) before being sterilized by autoclaving for 15 min at a pressure of 1.5 lb/inch². Unless otherwise stated, the tested microorganisms were grown in 250 mL flasks containing 50 mL of the cultivation media and incubated at 30 °C on a reciprocal shaker (200 rpm) for 2, 3 and 4 days incubation periods. At the end of the cultivation, the biomass was harvested by filtration through a dried, pre-weighted filter paper and thoroughly washed with distilled H₂O, then dried in oven at 70 °C until complete dryness and then reweighed. The level of extracellular lipase was assayed according to lipase activity in the supernatant.

Determination of lipase activity

Lipase activity was determined according to the method described by Parry *et al.* (1966) using an emulsion of 10%

olive oil in 10% gum Arabic. The emulsion produced by treating the mixture of olive oil and gum Arabic solution in a top drive homogenizer for 10 min. The reaction mixture contained 3 mL of substrate (emulsion), 2.5 mL of deionized water, 1 mL of 0.2 M Tris-HCl buffer (pH 7.5) and 1.0 mL lipase sample. The reaction mixture was then supplemented with 10 mL ethanol. The reaction was carried out at 37 °C for 2 h in shaking water bath. The amount of oleic acid produced was determined by titrating the hydrolysis products with 0.05 N NaOH using thymolphthalein indicator. The control sample was prepared and treated similarly using boiled enzyme samples. The lipase activity values were calculated as the average of three parallel determinations displaying a variation coefficient lower than 5%. The amount of enzyme catalyzing the formation of one micro-equivalent (micromole) of oleic acid in 2 h at 37 °C and pH 7.5 was taken as one lipase activity unit.

1 U/mL = formation of U mol free fatty acids/1 mL enzyme solution.

The protein determination was carried out by the method of Lowry *et al.* (1951).

Determination of glucose

Glucose was determined according to the method of Passing and Beblox (1983). A 10 µL of sample + 1 mL glucose oxidase kits (GOD) was incubated at 37 °C for 15 min. The produced color was measured at 520 nm. A standard curve was prepared using a pure glucose standard solution.

Toxicity test of *Mucor racemosus*

One hundred mL of YES medium (2% yeast extract and 15% sucrose) were inoculated with spore suspension of *M. racemosus* strain, incubated in dark for 14 days at 25 °C. Extraction of mycotoxins was carried out according to Munimbazi and Bullerman (1998). Aflatoxins (B₁, B₂, G₁ and G₂) were determined by HPLC according to the method of Deabes *et al.* (2007).

Response of some additives

In the present experiment, the lipase activities were investigated when *M. racemosus* was cultivated on the basal medium at its optimal fermentation parameters and fortified with inducible additives including representative of trace elements, fatty acids, amino acids and vitamins. The modified basal medium contained (% w/v): peptone, 3; glucose, 1.0; olive oil, 1.0; KH_2PO_4 , 0.2; KCl, 0.05; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05, pH of the medium adjusted by phosphate buffer (pH 7). The fermentation medium was incubated for 3 days at 35 °C using shaking technique. Relative activity % = activity of sample /activity of control (without additives) x 100.

Lipase production in 5 L stirred tank bioreactor

M. racemosus lipase was produced using in 5 L-stirred tank bioreactor Bio Flow 3000 (New Brunswick Scientific, NJ, USA). The bioreactor vessel contained 3.5 L working volume of the optimized fermentation medium: 3% peptone; 2% (glucose: olive oil 1:1); 0.2% KH₂SO₄; 0.05% KCl; 0.05% MgSO₄·7H₂O; 0.01 M barium acetate and 4% spore suspension (8.75 x 10⁷ spores).

The bioreactor was equipped with a three 4-bladed rushton turbine impeller diameter (di), 65 mm; tank diameter (dt), 135 mm, dt:di, 1: 0.48. The optimized medium was sterilized at 121 °C for 30 min. Glucose and phosphate buffer solutions (pH 7.0) were sterilized separately and were mixed aseptically with other components of the medium in the bioreactor upon inoculation. The medium was inoculated with 4% (v/v) spore suspension (8.75 x 10⁷/mL) and fermentation was carried out at 35 °C at pH 7.0. The impeller speed was adjusted to 300 rpm. The aeration was adjusted at 1 v/v/m. Samples were taken from the bioreactor every 12 h and analyzed for lipase activity, protein concentration, residual sugar and biomass estimation.

RESULTS AND DISCUSSION

Screening of lipase activity

The production of extracellular lipase varied not only with the type of the culture used but also by its species at different cultivation periods. Therefore among 29 different fungal species, 26 species of these cultures manifested large variation in its capacity to produce lipases (Table 1). We choose *M. racemosus* although its activity is less than that of *A. niger* (oil), because it is not pathogenic and more safe than the other *Aspergilli*. Toxicity test was performed to confirm this selection, the result is negative and aflatoxins not detect.

There is literature review full with production of lipases by different species of *Mucor* (Hiol *et al.*, 2000; Abbas *et al.*, 2002). According to our knowledge there is no reference concerning *M. racemosus* lipases. This finding with the toxicity test result justified its selection as a potent organism for lipases production.

Optimization of lipase production by *M. racemosus*

Five different media were used for lipase production by *M. racemosus*. The results in Figure 1 showed that medium I gave the maximum production (337.6 U/mL) with specific enzyme activity 7.3 U/mg. Medium IV shows moderate production of lipase (295.0 U/mL). Some authors stated that, lipase production greatly affected by the composition of the medium (Lin and Ko, 2005; Rodriguez *et al.*, 2006). Sarkar *et al.* (1998) stated that lipase production is enhanced in complex culture medium than in a simple one.

Table 1: Screening of fungi for lipase production

Microorganisms	Lipase activity (U/mL)		
	2 days	3 days	4 days
<i>Aspergillus niger</i> (oil)	535.0	455.0	435.0
<i>A. niger</i> 102 H	180.0	145.0	175.0
<i>A. niger</i> 16 H	275.0	239.7	153.0
<i>A. niger</i> 7 H	270.0	210.0	175.0
<i>A. carneus</i>	50.0	32.9	80.0
<i>A. flavus</i>	25.0	95.0	0.0
<i>A. fumari</i>	30.0	85.0	45.0
<i>A. fumigates</i>	32.9	40.0	20.0
<i>A. niveus</i>	0.0	80.0	40.0
<i>A. terreus</i> 1H	120.0	225.0	15.0
<i>A. terreus</i> 2H	175.0	215.0	135.0
<i>A. terreus</i> 3H	0.0	10.0	0.0
<i>A.wentii</i>	265.0	193.0	20.4
<i>Botrytis</i> sp.	80.0	20.4	40.8
<i>Cunninghamella</i> sp.	0.0	15.0	0.0
<i>Fusarium clamydo</i>	0.0	0.0	0.0
<i>Fusarium solani</i>	0.0	0.0	0.0
<i>Fusarium</i> sp.	0.0	0.0	0.0
<i>Mucor hiemalis</i>	95.0	185.0	250.0
<i>M. pusillus</i>	100.0	240.0	50.0
<i>M. racemosus</i>	220.0	337.6	175.0
<i>M. roxii</i> NRRL1894	72.0	130.0	47.0
<i>Penicillium cyclopium</i>	45.0	96.9	70.0
<i>P. duclauxi</i>	25.0	75.0	0.0
<i>P.funiculosum</i>	19.2	185.1	71.4
<i>Rhizoctonia</i> sp.	90.0	80.0	40.8
<i>Rhizopus</i> sp.	25.0	90.0	35.0
<i>Sclerotium</i> sp.	50.0	35.0	0.0
<i>Trichoderma viride.</i>	0.0	15.0	0.0

*Production medium used: medium I (see materials and methods)

The physiological studies were started by studying the effect of inoculum size. The lipase activity of the experimental organism was gradually increased by increasing the inoculum size (2-4 mL/100 mL medium) to reach the maximum at 4 mL (8.75 x 10⁷ spores/mL) and then decreased gradually by increasing of the inoculum size (data not shown). The fungal activity was tested using surface and the submerged culture techniques each was investigated under different incubation periods (1-5 days).

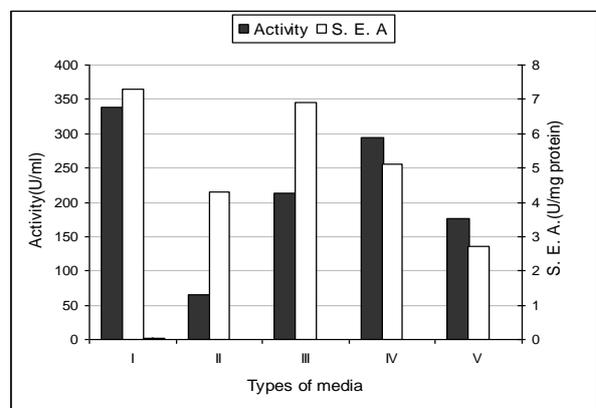


Figure 1: Production of *Mucor racemosus* lipase using different media

The submerged (shacked culture) technique affords high lipase production with the highest lipolytic activities (337.6 U/mL) after 3 days of incubation. After 48 h the productivity in submerged technique (6.7 U/mL.h) was higher than that of the static with about 42 fold. This may be attributed to the fact that submerged technique allowed better emulsification of the lipids (olive oil) and also good aeration (Table 2). The maximal production of lipase and S. E. A. (395.2 U/mL and 8.3 U/mg protein) were obtained at 35 °C (Figure 2). These results agreed with Silva *et al.* (2005) who reported that the best temperature in the basal medium for *Metarhizium anisopliae* lipase production was 32 °C. There was a sharp decrease in lipase activity (89.8 U/mL) at 45 °C and this may be attributed to the denaturation of enzyme.

The tested fungus *M. racemosus* was separately cultivated on buffered medium using different buffer at different pH (Table 3). Maximum lipase activity (485.0 U/mL) and S. E. A. (10.1 U/mg protein) were obtained by 0.2 M phosphate buffer at pH 7.0. As a general, good lipase production was obtained in all buffered medium (pH 4-7) after 3 days incubation. Low lipase production was obtained at alkaline pH. These results agree those obtained by many investigators, Abdel-Fatah and Hammad (2002) claimed that increasing pH over 6 decrease lipase produced by the tested filamentous fungi. In addition, Elwan *et al.* (1986a) reported that the amount of lipase produced by *Penicillium crysogenum* was markedly reduced over pH 7.0. Therefore, we can say that the optimum pH values were greatly dependent on the culture conditions as well as the type of the buffer used. Also lipase activity using optimum buffered pH (485.0 U/mL) was higher than that using optimal initial pH (395.2 U/mL, data not shown) with 1.23 fold. This is in agreement with Ammar (1983) and Elwan *et al.* (1986 a). Silva *et al.* (2005) used buffered medium with 0.1 M sodium phosphate buffer in the following pH: 5.7, 6.3 and 8.0. They observed that, the best lipase production by *M. anisopliae* was obtained when the medium was buffered with pH 5.7. Minning *et al.* (2001) used 100 mM potassium phosphate buffer pH 6.0 in the culture medium for high level production of *Rhizopus oryzae* lipase. *M. racemosus* extracellular lipase was highly affected by the nature of the carbon source used. Lowest lipase activity obtained using glucose as a sole carbon source (Table 4). Olive oil as a sole carbon source increased lipase activity (377.40 U/mL) but maximal yields of lipase by using mixture of glucose:olive oil (0.5:5 wt%). These indicated that *M. racemosus* lipase is inductive enzyme and olive oil was effective in inducing enzyme formation and the organism needed glucose and olive oil both together for high lipases production. These results were confirmed with Destain *et al.* (2005) who reported that olive oil in combination with glucose was used as the carbon source and inducer for the production of lipase. Also Maia *et al.* (2001) and Li *et al.* (2005) concluded that the production of microbial lipases generally needs a fat-related carbon source. In attempts to optimize the concentration of both glucose and olive oil show that increasing glucose and olive oil concentration up to 1% gave maximum lipolytic activity

556.0 U/mL (Figure 3 and 4). Higher levels of glucose and olive oil gave a deleterious effect on lipase production in spite of biomass stimulation in olive oil treatment.

Table 2: Effect of cultivation methods and incubation period on lipase activity and productivity by *M. racemosus*

ime (h)	Shaken		Static	
	Lipase activity U/mL	Productivity U/mL/h	Lipase activity U/mL	Productivity U/mL/h
24	92.02	3.83	0.0	0.00
48	322.05	6.71	27.0	0.16
72	337.60	4.68	0.0	0.00
96	260.70	2.71	0.0	0.00
120	199.40	1.66	0.0	0.00

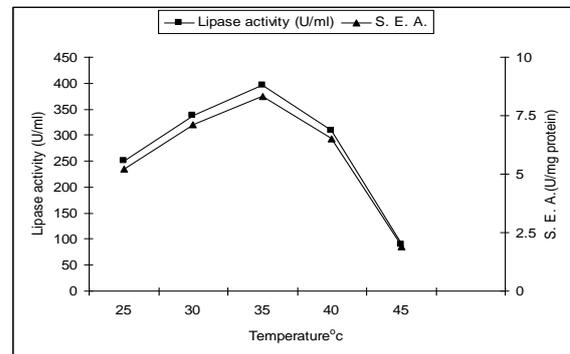


Figure 2: Effect of incubation temperature on lipase production by *Mucor racemosus*

Table 3: Effect of different buffered pH medium on lipase activity and S. E. A. by *M. racemosus*

Buffer Type	Final pH	Lipase activity (U/mL)	S.E.A. (U/mg protein)	Relative activity (%)
Citrate-phosphate buffer				
	4.0	235.8	4.90	59.66
	5.0	248.6	5.20	62.90
	6.0	255.0	5.31	64.52
	7.0	121.0	2.50	30.62
Phosphate buffer				
	6.5	379.1	7.90	95.93
	7.0	485.0	10.10	122.72
	7.5	405.0	8.44	102.48
	8.0	282.7	5.90	71.53
Tris-buffer				
	7.5	266.7	5.50	67.48
	8.0	240.0	5.00	60.73
	8.5	186.3	3.90	47.14
	9.0	115.6	2.40	29.25

*Relative activity % = activity of sample /activity of control X100
Control = (395.2 U/mL at optimal pH)

Table 4: Effect of glucose and olive oil as a carbon sources

Carbon source 1% (w/v)	Lipase activity(U/mL)
Glucose	267.75
Olive oil	377.40
Glucose + Olive oil (control)	485.00

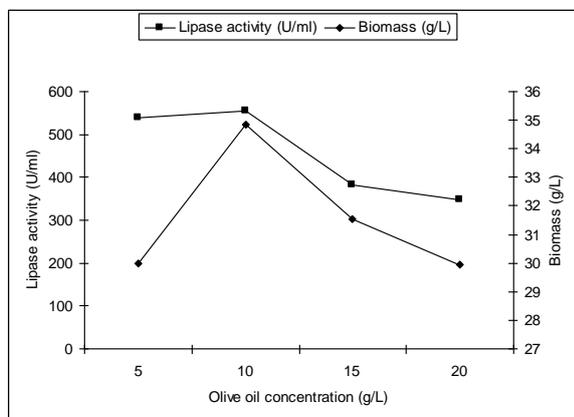


Figure 3: Effect of different concentration of glucose on biomass and lipase activity (concentration of olive oil 0.5%)

Our results were confirmed by Lin and Ko (2005) who reported that higher levels of glucose in the medium displayed an inhibitory effect on *Antrodia cinnamomea* lipase production. Furthermore, lipase activities of many other fungi such as *Aspergillus wentii* (Chander *et al.*, 1980); *Mucor hiemalis* (Akhtar *et al.*, 1980) and *Bukholderia* (Rathi *et al.*, 2001) are stimulated by the addition of glucose to the production media. The lower production of lipase by increasing olive oil concentrations may be due to the inhibition effect of the fatty acid (oleic acid) in the culture medium as result of olive oil hydrolysis (Akhtar *et al.*, 1980).

The most suitable nitrogen for lipase production by the experimental fungus proved to be peptone (Figure 5). All the tested nitrogen sources (organic and inorganic) caused a decrease in lipase production. Freire *et al.* (1997) and Rodriguez *et al.* (2006) found peptone to give the best results for lipase production. Freire *et al.* (1997) assumed that peptone contains certain co-factors and amino acids which match physiological requirement for lipase biosynthesis.

Response of some additives

Results of this investigation was illustrated in Table 5. On the tested trace elements as acetate, the enzyme was favorably affected, stimulated (114.3 & 100.8%) by Ba²⁺ and K⁺ and inhibited by Na⁺ on the tested trace elements as sulphates, also the enzyme was favorably affected, stimulated (111.2 & 106.1%) by Li⁺ and Na⁺ but inhibited (86.8-76.6%) by Zn²⁺, Fe⁺⁺. Sugiura (1984) and

Iwai and Tsujisaka (1984) recorded that the mechanism of which the metal ions influence the lipase production is

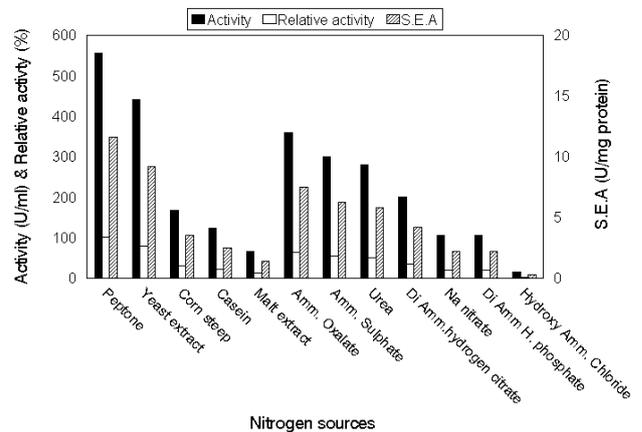


Figure 5: Effect of different nitrogen sources on lipase production by *M. racemosus*

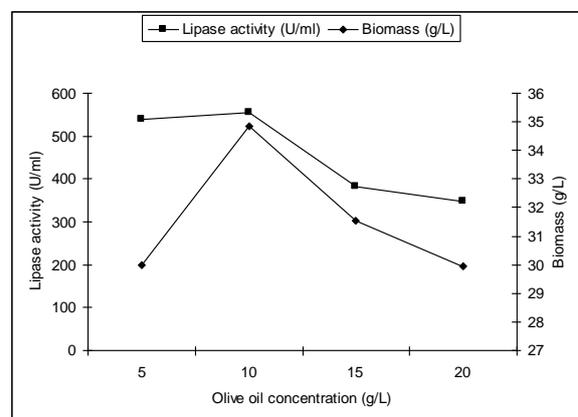


Figure 4: Effect of different concentration of olive oil on biomass and lipase activity (concentration of glucose 1%)

unknown. From our result we conclude that, the same ion exhibited different biochemical activities specific for each microorganism and it may be changed owing to the salt employed. Thus Na⁺ exerted an inhibitory (as carbonate and acetate) and activatory (as sulphate).

The tested fatty acids proved to be conducive for lipase production, both saturated (palmitic) and unsaturated (oleic acid) fatty acid caused increasing on lipase production at all concentrations (except 100 mg oleic). These results were agreed with Pokorny *et al.* (1994), Long *et al.* (1996), Dalmau *et al.* (2000) and Li *et al.* (2005) reported that lipase synthesis is known to be repressed by high concentration of oleic acid. Tween 20 and 80 had inhibitory effects (54.7-31.4%) on the lipolytic activities at all levels Table 5. These results were similar to those of Prazeres *et al.* (2006) who found that Tween 40, Tween 80 inhibited up to 30% of *Fusarium oxysporum* lipase activity. *M. racemosus* lipase was negatively

responded to the added amino acid. Glutamic acid had a maximum inhibitory effect (29.4%). These results agreed with Litchfield and Prescott (1970) and Fernandez *et al.* (1990) who showed that there were a repression of extracellular *Aeromonas proteolytica* and *Pseudomonas fluorescens* lipase, respectively by amino acids. On studying the addition of different concentrations of some vitamins to the fermentation medium we found that the optimum concentration (0.1, 0.1 and 0.75 mg/100 mL) of ascorbic acid, folic acid and becofort respectively giving stimulatory effects (108.10, 107.47 and 103.73% respectively). Minning *et al.* (2001) used biotin in the,

Table 5: Response of some additives on lipase activity

Concentration	Additives	Relative activity %
Metal salts (0.01 M)	Barium acetate	114.3
	Sodium acetate	44.7
	Potassium acetate	100.8
	Sodium carbonate	40.0
	Manganesesulphate	80.3
	Zinc sulphate	13.2
	Lithium sulphate	111.2
	Ferrous sulphate	23.4
	Ferric sulphate	19.3
Fatty acids (mg/100 mL)	Sodium sulphate	106.1
	Palmitic acid, 25mg	103.1
	Palmitic acid, 50mg	105.9
	Palmitic acid, 75mg	109.3
	Palmitic acid, 100mg	104.2
	Oleic acid, 25mg	110.8
	Oleic acid, 50 mg	111.8
	Oleic acid, 75mg	100.8
Oleic acid, 100mg	94.8	
Tweens (mg/100 mL)	Tween 20, 50mg	61.9
	Tween 20, 75mg	66.3
	Tween 20, 100mg	58.9
	Tween 80, 50mg	68.6
	Tween 80, 75mg	46.4
Amino acids (mg/100 mL)	Tween 80, 100mg	45.3
	Leucine, 1mg	96.5
	Tyrosine, 1mg	93.41
	Asparagine, 1mg	91.4
Vitamines (mg/100 mL)	Glutamic, 1mg	70.59
	Arjinine, 1mg	82.30
	Ascorbic acid, 0.1mg	108.10
	Ascorbic acid, 0.2mg	106.22
	Ascorbic acid, 0.3mg	88.83
	Folic acid, 0.05mg	93.73
	Folic acid, 0.1mg	107.47
	Folic acid, 0.15mg	91.83
	Becofort, 0.25mg	98.10
Becofort, 0.5mg	81.23	
Becofort, 0.75mg	103.73	

culture medium of *Pichia pastoris* lipase, also Lin and Ko (2005) and Xu and Chen, (2006) used vitamin B, at a concentration of 0.1% in the growth medium of *Antrodia cinnamomae*.

Lipase production by *M. racemosus* in 5 L-stirred tank bioreactor

In order to design a strategy to improve the large-scale production of *M. racemosus* lipases in this work lipolytic enzyme production in a stirred tank bioreactor has been investigated. The results presented in Figure 6 showed that a typical glucose consumption pattern was observed in which a highly faster glucose uptake has been seen. There was a sharp decrease in glucose concentrations (0.37 & 0.03 g/L after 12 and 24 h, respectively). Nevertheless, the lipase and specific enzyme activities were gradually increased until reaching their maximum values of 1211 U/mL and 28.0 U/mg protein after 48 h with a yield coefficient of about 58.5 U/g cells/L. Concomitantly, the initial dissolved oxygen (DO) level was rapidly decreased during the initial phase of the fermentation process until reached to 0.7% after 48 h. Then, it increased gradually until reached its maximum value at the end of the fermentation process and this was accompanied by an increase in the biomass (31.8 g/L). These higher biomass values might have been attributed to the accumulation of the byproducts. In general, when a complex medium is used, by-products are easily produced because the carbon or nitrogen content is not controlled at a suitable level (Ito *et al.*, 2001). Also dissolved oxygen has a significant effect on the lipase production. Thus, it can be proposed that *M. racemosus* fermentation being aerobic, oxygen is necessary to obtain optimum lipase activity (Puthli *et al.*, 2006).

From the aforementioned results, both maximum lipolytic activity and yield coefficient were obtained after 48 h. These values represented 2, 2.3- fold of those obtained using the shake-flask cultivation technique at their optimum conditions (635.7 U/mL; 8.8 U/mL/h) respectively.

The enhancement could be attributed to the balanced oxygenation and agitation in the fermentor during the exponential phase and early stationary phase to achieve appropriate cell growth and subsequent improved lipase activity. In the case of stirred bioreactors also, multiple bioreactors are now becoming important due to efficient gas distribution, higher gas phase residence time, increased gas hold-up, superior liquid flow (plug flow) characteristics and lower power consumption per impeller as compared to the single impeller systems resulting into a substantial savings in the operational costs (Puthli *et al.*, 2006).

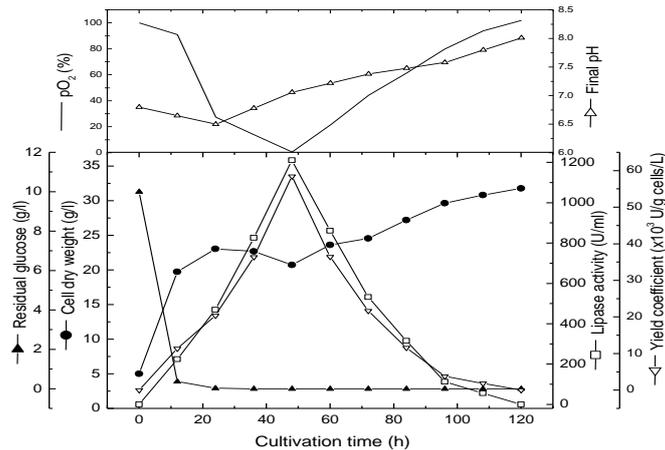


Figure 6: Production of *M. racemosus* lipase in 5 L stirred tank bioreactor

CONCLUSIONS

Lipase activity using 5 L stirred tank bioreactor (1211 U/mL) was improved by 2 folds as compared to shake flask studies (635 U/mL). Moreover, maximum lipase release was attained about 24 h earlier than in the shake flask experiments. Nevertheless, further research would be necessary to optimize the conditions allowing a stable production of *M. racemosus* lipolytic enzymes in larger scale bioreactors.

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