COMPARATIVE STUDIES ON THE LIPOLYTIC POTENTIAL OF WILD AND MUTANT STRAINS OF *RHIZOPUS OLIGOSPOROUS* VAR. *MICROSPOROUS* IIB-63 ISOLATED FROM LIPID RICH HABITATS

TEHREEMA IFTIKHAR*[2, 1], MUBASHIR NIAZ[@] AND IKRAM UL HAQ[1]

[1]*Institute of Industrial Biotechnology, GC University, Lahore*
[2]*Laboratory of Biotechnology, Department of Botany, GC University, Faisalabad, Pakistan*
Corresponding author: pakaim2001@yahoo.com/tehreema@gmail.com
92-42-36854508 (Res), 92-41-9201488 (Off)

Abstract

This is a comparative study for the optimization of cultural conditions for both wild and mutant strains of *R. microsporous* var. *oligosporus.* Out of all the 7 different culture media evaluated, M5 gave highest units of extracellular lipases 3.16 ± 0.02^a U mL^{-1} (W) and 10.99 ± 0.02^a U mL^{-1} (M). The effect of incubation temperature (15-45°C), initial pH (4.0 -10.0), inoculum size (0.5-3.5 mL) and volume of the medium (25-150 mL) on the production of extracellular lipase by both wild and mutant strains was investigated in shake flask. The rate of fermentation was also studied and found that maximum extracellular lipase was obtained after an incubation of 48 h by both wild and mutant strains. In the presence of soybean meal (0.4%) the maximum lipolytic activity was approximately 1.27 (W) & 1.42 (M) times higher than that in the absence of additive. Different additional carbon sources were added to basal medium with the aim of improving extracellular lipases production. Tween 80 showed considerable increase in lipases production by both wild (5.52 ± 0.005^a U mL^{-1}) and mutant (19.13 ± 0.005^a U mL^{-1}) strains as compared to others. Maximum extracellular activity of lipase 5.85 ± 0.01^a U mL^{-1} (W) & 28.32 ± 0.01^a U mL^{-1} (M) was obtained when 0.8% of casein was added in the fermentation medium as an organic nitrogen source. Maximum extracellular production of lipases 8.31 ± 0.01^a U mL^{-1} (W) and 34.34 ± 0.01^a U mL^{-1} (M) was observed when 0.8% $(NH_4)_2C_2O_4$ was added in the substrate as an additional inorganic nitrogen source.

Introduction

Enzymes are considered as nature's catalysts. Lipase (triacyl glycerol acyl-hydrolases, EC 3.1.1.3) catalyses hydrolysis of long chain acyl glycerol at an oil water interface. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of micro organisms or inexpensive media (Iftikhar *et al.,* 2010b; Saxena *et al*., 1999; Sharma *et al*., 2001; Iftikhar *et al*., 2007; Iftikhar *et al*., 2008; Helen & Oliveira, 2009). Gopinath *et al*., (2005) reported about 34 wild fungal species associated with edible oil mill wastes which were isolated by the serial dilution technique. This study also confirmed that the isolated fungi present on a wide range of substrates in the ambient environment and these results could also provide basic data for further investigations on fungal extracellular enzymes (Griebeler *et al*., 2009). *Rhizopus* species is among the most well known lipase producers and its enzyme is suitable for use in many industrial applications (Toshihiko *et al*., 1989; Belarbi *et al*., 2000, Iftikhar & Hussain, 2002; Iftikhar *et al*., 2003). The exponential increase in the application of lipases in various fields in the last few decades demands extension in both qualitative

improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low (Haq *et al*., 2009). The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection of microorganisms (Bapiraju *et al*., 2004). UV and NTG (N-methyl-N'-nitro-N-nitroso guanidine) was further reported as effective mutagenic agents for strain improvement of *Rhizopus* sp., for productivity of biomedically important enzyme lipase (Iftikhar *et al*., 2010[a]). Gromada & Fiedurek (1997) have also developed strain improvement by induced mutagenesis with rational selection procedures for an efficient screening of the mutants. Triton X-100 (0.01-0.1%) and oxgall 0.2% was also used by various workers in order to restrict the fungal colonies (Gadgil *et al*., 1995; Khattab & Bazaraa, 2005). The aim of the present work was to compare the lipolytic potential of wild and mutant derivatives of *R. microsporous* under submerged fermentation conditions.

Materials and Methods

Inoculum preparation: Spore inoculum: In the present study, 5-7 days old culture was used. The spore suspension was prepared in 10 mL sterilized solution of 0.005% Monoxol.O.T. (Di-Octyl ester of sodium sulphosuccinic acid). Sterilized inoculating needle was used to scratch the spores.

Vegetative inoculum: Hundred milliliter of Vogel medium containing glass bead in 1 liter cotton wool plugged conical flask was sterilized at 15 p.s.i pressure (121°C) for 15 min. One milliliter of spore suspension (containing 4.63×10^7 spores) was aseptically transferred to the flask (Iftikhar *et al*., 2010[b]). The flask was incubated at 30°C in an incubator shaker at 200 rpm for 24 h.

Spore count: Inoculum size was measured by measuring the density of spores as reported by Iftikhar *et al*., (2010[b]).

Buffer preparation: Buffers of various pH were prepared in the composition as reported by Iftikhar *et al*., (2010[b]).

Shake flask fermentation: The selected mutant strain of *Rhizopus oligosporus* IIB-63NTG-7 (150 min) along with wild strain were screened for checking their lipolytic potential through submerged fermentation (Iftikhar *et al.,* 2010[a]). Fifty mL of fermentation medium was transferred to each cotton wool plugged Erlenmeyer flask. The flasks were sterilized in autoclave at 15 lb / inch[2] pressure at 121°C for 15 min and cooled at room temperature. One ml of inoculum was aseptically transferred to each flask. The flasks was placed in the orbital shaking incubator (Model: JEIO TEC SI-4000R, Korea), for incubation at 30°C with shaking speed of 200 rpm. After specific incubation time the content of the flasks was used for the estimation of enzyme. All the experiments were carried out in triplicate.

Spectrophotometric assay of lipases: After specific time interval lipase activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate (*p*-NPP) as substrate according to the method of Krieger *et al*., (1999).

One unit of enzyme activity is defined as the amount of enzyme that released 1 μmole *p*-nitrophenol per minute.

Results and Discussion

Screening for fermentation media: Keeping in view the economic considerations involved in fermentation process, 7 different culture media were evaluated for the synthesis of extracellular lipases by wild and mutant strains of *R. oligosporus* (Table 1). Out of all the media, M5 (g L^{-1} Peptone: 20, Glucose: 10, K$_2$HPO$_4$:2.0, MgSO$_4$.7H$_2$O: 0.12, NH$_4$Cl: 1.0, Yeast Extract: 2.5, pH: 7.0) yielded highest units of extracellular lipases 3.16 ± 0.02^a U mL^{-1} (W) and 10.99 ± 0.02^a U mL^{-1} (M). The rest of the culture media gave lesser production of enzyme by both wild type in the order M2>M7>M3>M4>M1>M6 and for the mutant the order was found to be M1>M2>M3>M4>M7>M6, respectively. The production of enzyme was found to be significantly different ($p \leq 0.05$) in media M5 from the rest of the media, so was selected for further studies. It might be due to the reason that glucose and peptone are easily metabolizable carbon and nitrogen sources, respectively, while K$_2$HPO$_4$ as potassium and phosphorous source. Thus these results are in line with the findings that lipase production was influenced by the type and concentration of carbon and nitrogen sources (Lotti *et al.*, 1998; Chahinian *et al.*, 2000; Jianghua *et al.*, 2000; Elibol & Ozer, 2001).

Rate of lipase production: The effect of incubation period on the extracellular lipase production by wild type and mutant strains of *R. oligosporus* was optimized. The fermentation was carried out for 64 hours and production of enzyme was calculated after every 8 hours. There was a positive correlation of enzyme production with time till 48 hours of incubation (3.22 ± 0.01^a U mL^{-1}, W & 11.02 ± 0.005^a U mL^{-1}, M). However, the trend reversed afterwards and enzyme production was lowered (Fig. 1). It is evident from the results that time course of enzyme production plays a very critical role in enzyme synthesis (Sztajar & Maliszewska, 1988). It might be due the growth phase, organisms entered. According to Prescott & Dunn (1987) the accumulation of lipase by the fungal strain was found to be optimum in the stationary phase of growth but it mainly depends on the composition of the medium and other factors. Further increase in the incubation period resulted decrease in the production of lipase by both wild type and mutant strains of *R. oligosporus*. It might be due to the exhaustion of the nutrients and production of metabolic byproducts (inhibitors) in the fermenting medium (Martinez *et al.,* 1993). The accumulation of these byproducts resulted in decreased production of lipase by fungal strain (Korn & Fujio, 1997). The findings are also in agreement with that of Kumar *et al.*, (2005). Hence incubation period of 48 hours was taken to be optimum and maintained throughout the course of this study.

Effect of incubation temperature: The effect of incubation temperature on the production of extracellular lipases by both wild and mutant cultures of *R. oligosporus* was investigated and the results are presented in Table 2. Lipase activity at different incubation temperatures *i.e.,* 15, 20, 25, 30, 35, 40 and 45°C was evaluated (Table 2). Both wild type (3.25 ± 0.01^a U mL^{-1}) and mutant (11.28 ± 0.01^a UmL^{-1}) strains gave maximum extracellular lipase activity at 30°C. With gradual rise of incubation temperature from 15°C lipase activity increased but after 30°C, it started decreasing and reached minimum (0.67 ± 0.01^f U mL^{-1}, W & 2.01 ± 0.01^f U mL^{-1}, M) at 45°C. It might be due to the fact that high temperature has inhibitory

effect on the growth of microorganism as well as productivity of enzymes (Maia *et al.*, 2001; Lima *et al.*, 2003). Other workers Burkert *et al.*, (2004), Kumar *et al.*, (2005), Singh *et al.*, (2006), Kiran *et al.*, (2008) also reported that 30°C to be optimum for lipase production. However, most of the fungal species show maximum growth and lipases production at temperature ranged from 25-30°C (Elwan *et al.*, 1986; Nadj *et al.,* 1992; Korn & Fujio, 1997). Therefore, incubation temperature of 30 ± 2°C was taken to be optimum and maintained for the rest of the study.

Table 1. Screening of fermentation media for the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Synthetic media	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
M1	1.04 ± 0.005[e]	6.18 ± 0.01[b]
M2	2.03 ± 0.01[b]	5.01 ± 0.01[c]
M3	1.31 ± 0.02[d]	4.05 ± 0.02[d]
M4	1.06 ± 0.01[e]	3.24 ± 0.01[e]
M5	3.16 ± 0.02[a]	10.99 ± 0.02[a]
M6	0.96 ± 0.01[f]	2.97 ± 0.01[f]
M7	1.64 ± 0.01[c]	3.21± 0.005[e]

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at *P*≤0.05
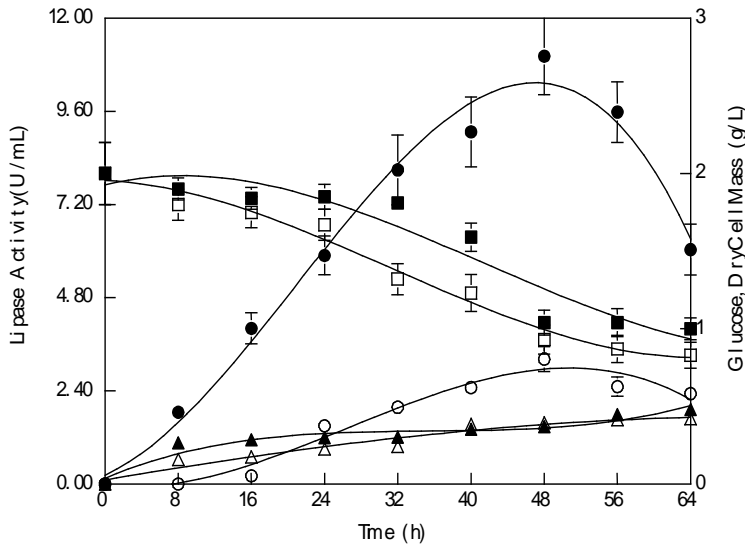*Initial pH: 7.0, incubation temperature 30°C, fermentation period 48h.

Fig. 1. Rate of fermentation for the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.
*Initial pH 7.0, incubation temperature 30°C, agitation rate 200 rpm
○ Lipase activity (Wild Type) UmL^{-1}
● Lipase Activity (Mutant) UmL^{-1}
△ Dry Cell Mass (Wild Type) gL^{-1s}
▲ Dry Cell Mass (Mutant) gL^{-1}
□ Glucose (Wild Type) gL^{-1}
■ Glucose (Mutant) gL^{-1}

Table 2. Effect of incubation temperature on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Temperature (ºC)	Extracellular lipase activity (U mL^{-1})	
	Wild	**Mutant**
15	0.50 ± 0.005^f	1.50 ± 0.005^g
20	1.08 ± 0.005^e	5.46 ± 0.05^d
25	2.68 ± 0.05^b	8.04 ± 0.005^b
30	3.25 ± 0.01^a	11.28 ± 0.005^a
35	2.33 ± 0.01^c	6.99 ± 0.01^c
40	1.82 ± 0.005^d	3.24 ± 0.01^e
45	0.67 ± 0.01^f	2.01 ± 0.01^f

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 7.0, fermentation period 48h, agitation rate 200 rpm

Table 3. Effect of inoculum size on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Inoculum size (mL)	No. of spores	Extracellular lipase activity (U mL^{-1})	
		Wild	**Mutant**
0.5	2.31×10^7	1.08 ± 0.01^f	3.99 ± 0.04^d
1.0	4.63×10^7	3.39 ± 0.05^a	11.37 ± 0.15^a
1.5	6.95×10^7	2.38 ± 0.09^b	7.14 ± 0.09^b
2.0	9.25×10^7	1.67 ± 0.03^c	5.08 ± 0.04^c
2.5	11.56×10^7	1.33 ± 0.04^d	3.98 ± 0.03^d
3.0	13.87×10^7	1.23 ± 0.04^e	3.24 ± 0.01^e
3.5	16.18×10^7	0.90 ± 0.15^g	2.70 ± 0.05^f

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 7.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30ºC

Effect of inoculum size: Effect of size of inoculum on the biosynthesis of extracellular lipases by wild and mutant strain of *R. oligosporus* was evaluated. Table 3 shows the effect of different inoculum levels on the production of lipases. The size of inoculum increased from 0.5-3.5 mL with an interval of 0.5 mL. The inoculum size of 1mL (4.63 x 10^7 spores) yielded maximum extracellular lipase activities both by wild (3.39 ± 0.05^a UmL^{-1}) and mutant (11.37 ± 0.15^a UmL^{-1}) strains. It might be due to adequate amount of mycelium produced, which synthesize optimum level of enzyme. As the dose of inoculum increased there was gradual decrease of lipase activity. It might be due to the reason that fungus consumed majority of the substrate for growth and metabolic processes, hence enzyme synthesis decreased. There was a non-significant difference of enzyme production at lower doses of inoculum that might be due to the fact that the growth of the fungus was not proper and the time required to reach the stationary phase of growth was increased. Therefore, the secretion of lipases by the fungal mycelium was greatly reduced at low levels of inoculum (Yasser *et al*., 2002). Our findings are in agreement with Singh *et al*., (2006). Hence 1.0 ml of inoculum was optimized for maximum lipases production.

Effect of the volume of the medium: In the present study, the different volumes of fermentation medium, *i.e.,* 25, 50, 75, 100, 125 and 150 mL were investigated in 250 mL Erlenmeyer flask for the production of extracellular lipases by both wild (IIB-63) and mutant strain (IIB-63 NTG-7) of *R. oligosporus* (Table 4). The maximum extracellular production of lipases by both wild ($3.71 \pm 0.005^a\,mL^{-1}$) and mutant ($11.49 \pm 0.005^a\,U\,mL^{-1}$) strains was observed when 20% volume (50mL/250mL flask) of the medium was used. At this volume of the medium, growth of the fungus was optimal due to sufficient oxygen supply, resulting in the maximum enzyme production. There was a gradual decrease of enzyme production with increase in the volume of fermentation medium (Table 4). It might be due to the improper agitation and inadequate aeration which consequently decreased enzyme production (Martinez *et al.,* 1993). At low level (25 mL) of the volume of the fermentation medium both wild ($1.66 \pm 0.005^e\,UmL^{-1}$) and mutant ($5.10 \pm 0.005^f\,UmL^{-1}$) strains showed insignificant production of enzyme. It might be due to the insufficient supply of nutrients for the growth of fungus and hence enzyme formation (Vanot *et al.*, 2002). Therefore, 50 mL of the fermentation medium was optimized for further studies.

Effect of initial pH of medium: The production of enzyme is very sensitive to the pH of fermentation medium, therefore, optimization of pH is necessary for maximum production of lipases. The effect of different initial pH of the fermentation medium on the production of extracellular lipases was investigated (Table 5). The initial pH of the fermentation medium was adjusted from 4.0 to 10.0 in shaking flasks. Production of enzyme by both wild ($4.01 \pm 0.005^a\,U\,mL^{-1}$) and mutant ($11.55 \pm 0.005^a\,U\,mL^{-1}$) strains of *R. oligosporus* found to be maximum at pH 8.0. It might be due to the reason that organism required slightly basic pH for its metabolic processes as well as for the production of lipases (Kiran *et al.,* 2008) whereas Peter (1995) has also reported good enzyme production in acidic range. As the pH of the medium changed, there was reduction in the enzyme formation. It might be due to that productivity of the enzyme by fungal culture was very specific to pH (Gombert, 1999). Therefore, pH 8.0 was selected for the production of lipase for further studies.

Effect of various agro-industrial byproducts: Various agro industrial by-products like cotton seed meal (CSM), soybean meal (SBM), wheat bran (WB), wheat flour (WF), sunflower meal (SFM), almond meal (AM), rice bran (RB) were tested for their effect on lipases production (Table 6). Inclusion of Soybean meal (SBM) at 0.4%, which is a byproduct of oil mills, was found to be best protein substrate for the induction of extracellular lipases production. In SBM the maximum lipase activity was approximately 1.27 (W) & 1.42 (M) times higher than that in the absence of additive (Table 7). The stimulatory effect of SBM might be due to the fact that it is a good source of different amino acids to the fungus in addition to other vitamins, protein, carbohydrate, fats, Ca, Mg, P, K and S for maximum enzyme formation (Nahas, 1988; Fadiloglu & Erkmen, 2002). On the other hand almond meal (AM) has also been reported as maximum producer of lipases by *Rhizopus oligosporus* (Iftikhar & Hussain, 2002). By increasing the concentration of SBM in the fermentation medium, the enzyme production was increased gradually but after 0.4% it showed a decreasing trend of enzyme production (Table 7). Therefore, 0.4% SBM was optimized for further studies.

Table 4. Effect of the volume of medium on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Volume of synthetic media (mL)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
25	1.66 ± 0.005^e	5.10 ± 0.005^f
50	3.71 ± 0.005^a	11.49 ± 0.005^a
75	3.40 ± 0.015^b	7.22 ± 0.005^c
100	3.21 ± 0.005^c	7.50 ± 0.005^b
125	2.62 ± 0.005^d	6.79 ± 0.017^d
150	1.74 ± 0.019^e	6.11 ± 0.005^e

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 7.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30ºC

Table 5. Effect of initial pH of the medium on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

pH	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
4	0.16 ± 0.005^f	2.49 ± 0.02^e
5	0.83 ± 0.02^e	3.01 ± 0.06^d
6	2.66 ± 0.005^c	5.98 ± 0.005^c
7	3.40 ± 0.08^b	7.00 ± 0.08^b
8	4.01 ± 0.005^a	11.55 ± 0.005^a
9	1.00 ± 0.06^d	5.48 ± 0.005^f
10	-	-

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Fermentation period 48h, agitation rate 200 rpm, incubation temperature 30ºC

Table 6. Effect of the addition of different agro-industrial by products on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Agro-industrial byproducts (1% w/v)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
CSM	1.32 ± 0.01^e	4.57 ± 0.005^e
WB	2.67 ± 0.01^b	6.08 ± 0.01^c
SBM	4.35 ± 0.004^a	14.98 ± 0.01^a
WF	1.02 ± 0.002^g	4.31 ± 0.01^f
SFM	2.09 ± 0.005^c	5.56 ± 0.005^d
AM	1.12 ± 0.003^f	7.99 ± 0.01^b
RB	1.86 ± 0.001^d	4.03 ± 0.011^g

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
CSM: cotton seed meal, SBM: Soybean meal, WB: Wheat Bran, WF: Wheat Flour, SFM: Sunflower meal, AM: Almond meal, RB: Rice Bran
*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30ºC

Table 7. Effect of the addition of different concentration of soybean meal on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Conc. of SBM (%)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
0.2	3.47 ± 0.006^e	12.97 ± 0.005^b
0.4	5.09 ± 0.008^a	16.43 ± 0.005^a
0.6	4.51 ± 0.003^b	12.38 ± 0.005^d
0.8	4.47 ± 0.003^c	11.98 ± 0.01^c
1.0	4.36 ± 0.04^d	12.99 ± 0.005^b

Each value is an average of three replicates ± denotes standard deviation among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$.

SBM: Soybean meal

*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30°C

Table 8. Effect of the addition of different carbon sources on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Carbon sources (1%)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
Lactose	3.52 ± 0.005^c	8.69 ± 0.005^d
Maltose	3.14 ± 0.02^d	6.46 ± 0.26^e
Sucrose	2.37 ± 0.26^e	8.58 ± 0.02^d
Xylose	3.52 ± 0.01^c	11.50 ± 0.01^b
Dextrose	3.18 ± 0.005^d	9.63 ± 0.005^c
Glucose	4.18 ± 0.03^b	11.44 ± 0.03^b
Tween 80	5.52 ± 0.005^a	19.13 ± 0.005^a
Starch	4.20 ± 0.01^b	9.63 ± 0.01^c

Each value is an average of three replicates ± denotes standard deviation among replicates. Numbers followed by different letters differ significantly at $p \leq 0.05$

*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30°C

Effect of additional carbon sources: In the present course of study, effect of different carbon sources such as lactose, maltose, sucrose, xylose, dextrose, glucose, starch and Tween 80 were evaluated for the production of extracellular lipases by wild (IIB-63) and mutant (IIB-63NTG-7) strains of *R. oligosporus*. Of all the carbon sources, Tween 80 showed considerable increase in lipases production by both wild (5.52 ± 0.005^a U mL^{-1}) and mutant strains (19.13 ± 0.005^a U mL^{-1}) as compared to others (Table 8). Since it was miscible with water and did not generally inhibit fungal growth (Martinez *et al*., 1993). Tween 80 was also reported as the best carbon source for lipases production (Handelsman & Shoham, 1994; Dai and Xia, 2005; Singh *et al*., 2006) whereas Lianghua & Liming (2005) reported an inhibitory effect of Tween 80 on lipases production by *Bacillus coagulans*. Variation in the concentration of Tween 80 was also effective for lipase production (Table 9). Maximum enzyme activity by both wild (5.68 ± 0.01^a U mL^{-1}) and mutant strain (22.62 ± 0.01^a U mL^{-1}) was obtained at 0.6% concentration of Tween 80 as it

provided optimum amount of carbon for the production of extracellular lipases. Enzyme level however decreased with further increase in Tween 80 concentration. It might be due to the increase in fatty acid accumulation through hydrolysis of substrate, suppressing lipases synthesis. Sidhu *et al*., (1998) used 0.5% Tween 80 for the production of extracellular lipases. Espinosa *et al*., (1990) also reported that Tween 80 exerted a positive effect on enzyme production in a range from 0.02% to 2.00%. Therefore, 0.6% Tween 80 was optimized for further studies.

Effect of addition of different nitrogen sources: The effect of various organic and inorganic nitrogen sources on the production of extracellular lipases by wild and mutant strains of *R. oligosporus* was evaluated. (Tables 10-13). The effect of peptone, *p*-nitrophenol, casein, nutrient broth, urea, yeast extract and corn steep liquor @ 1% w/v added to the fermentation medium is presented in Table 10. The maximum extracellular lipases activity of wild (5.70 ± 0.04^a U mL^{-1}) and mutant (26.96 ± 0.04^a U mL^{-1}) strains were obtained when casein was added to the fermentation medium (Table 10). The data of Table 11 showed the effect of different amounts of casein on the production of extracellular lipases by wild and mutant strains of *R. oligosporus*. Casein at a level of 0.2, 0.4, 0.6, 0.8 and 1.0% was used for lipases production. Maximum extracellular activity of lipases [5.85 ± 0.01^a U mL^{-1} (W) & 28.32 ± 0.01^a U mL^{-1} (M)] was obtained when 0.8% of casein was added in the fermentation medium as an organic nitrogen source. This might be due to the fact that casein is a good source of different amino acids. The stimulatory effect of casein might be due to the supply of different amino acids to the fungus for maximum enzyme formation (Fickers *et al*., 2004). Where as Fadiloglu & Erkmen (2002) reported a stimulatory effect of peptone on lipase production. In contrast, Ginalska *et al*., (2007) reported that lipase production was suppressed when casein, peptone and yeast extract was replaced with urea in the fermentation medium. Varying quantities of different inorganic nitrogen sources were used for the study of their individual effect on enzyme production both for wild and mutant strains of *R. oligosporus* (IIB-63) and the results are presented in Table 12. The inorganic salts used were ammonium chloride [NH_4Cl], ammonium sulfate [$(NH_4)_2SO_4$], ammonium nitrate [NH_4NO_3], ammonium acetate [$NH_4CH_3COO^-$], ammonium ferro (II) sulfate dodeca hydrate [$(NH_4)_2 Fe(SO_4)_2.12H_2O$], hydroxyl ammonium chloride [$HONH_3Cl$], ammonium oxalate [$(NH_4)_2C_2O_4$] and ammonium molybdate [$(NH_4)_6MoO_{24}$]. Ammonium oxalate [$(NH_4)_2C_2O_4$] gave maximum extracellular lipase activity by both wild (7.89 ± 0.01^a U mL^{-1}) and mutant (32.26 ± 0.002^a U mL^{-1}) strains compared to other inorganic nitrogen sources. The data of Table 13 shows the effect of different amounts of $(NH_4)_2C_2O_4$ on the production of lipase by wild and mutant strains of *R. oligosporus*. Maximum extracellular production of lipases 8.31 ± 0.01^a U mL^{-1} (W) and 34.34 ± 0.01^a U mL^{-1} (M) were observed when 0.8% $(NH_4)_2C_2O_4$ was added in the substrate as an additional inorganic nitrogen source. However, the other nitrogen sources gave insignificant production of extracellular lipases because they were not easily metabolized by the fungi for its growth and enzyme formation (Gao & Breuil, 1995; Iftikhar *et al*., 2003). However, 0.8% casein and 0.8% ammonium oxalate were selected for the optimum production of extracellular lipases.

Table 9. Effect of the addition of different concentration of Tween80 on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Conc. of Tween 80 (%)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
0.2	5.05 ± 0.04^e	12.63 ± 0.04^e
0.4	5.51 ± 0.01^b	14.19 ± 0.06^d
0.6	5.68 ± 0.01^a	22.62 ± 0.01^a
0.8	5.47 ± 0.01^c	19.07 ± 0.005^b
1.0	5.16 ± 0.01^d	18.99 ± 0.00^b

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30°C

Table 10. Effect of the addition of different organic nitrogen sources on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Nitrogen sources 1% (w/v)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
Peptone	2.67 ± 0.011^d	8.26 ± 0.005^c
p-Nitrophenol	0.33 ± 0.01^g	0.97 ± 0.012^g
Casein	5.70 ± 0.04^a	26.96 ± 0.04^a
Nutrient broth	2.18 ± 0.011^e	7.88 ± 0.011^d
Urea	2.80 ± 0.005^c	6.43 ± 0.011^e
Yeast extract	5.17 ± 0.014^b	19.25 ± 0.021^b
Corn steep liquor	1.83 ± 0.012^f	5.40 ± 0.013^f

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30°C

Table 11. Effect of the addition of different concentrations of the casein on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Conc. of casein (%)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
0.2	3.76 ± 0.012^e	11.06 ± 0.005^d
0.4	4.42 ± 0.004^d	13.04 ± 0.02^c
0.6	5.01 ± 0.003^c	14.75 ± 0.01^b
0.8	5.85 ± 0.005^a	28.32 ± 0.01^a
1.0	5.71 ± 0.402^b	25.99 ± 0.005^e

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \geq 0.05$
*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30°C

Table 12. Effect of the addition of different inorganic nitrogen sources on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Nitrogen sources (1% is it w/v or v/v)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
NH_4Cl	3.83 ± 0.003^{cd}	9.78 ± 0.01^{f}
$(NH_4)_2SO_4$	4.16 ± 0.001^{c}	13.19 ± 0.01^{c}
NH_4NO_3	3.16 ± 0.005^{d}	15.71 ± 0.005^{b}
NH_4CH_3COO	4.84 ± 0.012^{b}	12.34 ± 0.007^{d}
$(NH_4)_2Fe(SO_4)_2.12H_2O$	3.17 ± 0.013^{d}	8.08 ± 0.01^{g}
$(NH_4)_2 Fe(SO_4)_2.6H_2O$	3.84 ± 0.011^{cd}	9.79 ± 0.002^{e}
$HONH_3Cl$	1.34 ± 0.015^{i}	3.42 ± 0.004^{k}
$(NH_4)_6MoO_{24}$	3.02 ± 0.016^{d}	7.70 ± 0.001^{h}
$NaNO_3$	0.67 ± 0.021^{g}	6.99 ± 0.007^{i}
$AgNO_3$	1.16 ± 0.012^{f}	3.39 ± 0.01^{k}
KNO_3	1.83 ± 0.02^{e}	4.67 ± 0.002^{j}
$NaNO_2$	-	12.35 ± 0.01^{d}
$(NH_4)_2C_2O_4$	7.89 ± 0.01^{a}	32.26 ± 0.002^{a}

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 8.0, fermentation period 48h, agitation rate 200 rpm, incubation temperature 30ºC

Table 13. Effect of the addition of different concentrations of the $(NH_4)_2C_2O_4$ on the production of extracellular lipases by *Rhizopus oligosporus* IIB-63 and its mutant derivative in shake flasks*.

Conc. of $(NH_4)_2C_2O_4$ (%)	Extracellular lipase activity (U mL^{-1})	
	Wild	Mutant
0.2	6.71 ± 0.02^{c}	18.25 ± 0.04^{d}
0.4	5.88 ± 0.04^{d}	20.85 ± 0.02^{c}
0.6	6.74 ± 0.02^{c}	26.28 ± 0.02^{d}
0.8	8.31 ± 0.01^{a}	34.34 ± 0.01^{a}
1.0	7.85 ± 0.04^{b}	31.27 ± 0.04^{b}

Each value is an average of three replicates ± denotes standard deviation among replicates.
Numbers followed by different letters differ significantly at $p \leq 0.05$
*Initial pH 8.0, fermentation period 48 h, agitation rate 200 rpm, incubation temperature 30ºC

References

Bapiraju, K.V.V.S.N., P. Sujatha, P. Ellaiah and T. Ramana. 2004. Mutation induced enhanced biosynthesis of lipase. *African Journal of Biotechnology,* 3 (11): 618-621.

Belarbi, E.H., E. Molina and Y. Chisti. 2000. A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. *Enzyme Microb. Technol.,* 26: 516-529.

Burkert, J.F.M., F. Maugeri and M.I. Rodrigues. 2004. Optimization of extracellular lipase production by *Geotrichum* sp., using factorial design. *Bioresource Technol.,* 91(1): 77-84.

Chahinian, H., G. Vanot, A. Ibrik, N. Rugani, L. Sarda and L.C. Comeau. 2000. Production of extracellular lipases by *Penicillium cyclopium* purification and characterization of partial acylglycerol lipase. *Biosci. Biotechnol. Biochem.,* 64: 215-222.

Dai, D. and L. Xia. 2005. Enhanced production of *Penicillium expansum* PED-03 lipase through control of culture conditions and application of the crude enzyme in kinetic resolution of racemic Allethrolone. *Biotechnol Prog.,* 21(4):1165-8.

Elibol, M. and D. Ozer. 2001. Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. *Process Biochem.,* 36: 325-9.

Elwan, S.H., M.S. Ammar and S.M. Mohawed. 1986. Lipases from *Aspergillus sydowi. Zentralbl. Microniol.,* 141(3): 233-239.

Espinosa, E., S. Sanchez and A. Farres. 1990. Nutritional factor affecting lipase production. *Biotechol. Lett*., 12(3): 209-214.

Fadiloglu, S. and O. Erkmen. 2002. Effects of carbon and nitrogen sources on lipase production by *Candida rugosa. Turkish J. Eng. Env. Sci*., 26: 249-254.

Fickers, P., J.M. Nicaud, C. Gaillardin, J. Destain and P. Thonart. 2004. Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. *J. Appl. Microbiol.,* 96: 742-749.

Gadgil, N.J., H.F. Daginawala, T. Chakrabarti and P. Khanna. 1995. Enhanced cellulose production by a mutant of *Trichoderma resei. Enzyme Microbiol. Technol.,* 17: 924-946.

Gao, Y. and C. Breuil. 1995. Extracellular lipase production by sapwood staining fungus, *Ophiostoma piceae. World J. Microbial. Biotechnol*., 11(6): 638-642.

Ginalska, G., H.Y. Cho, N.S. Cho, R. Bancerz, T. K. Owicz, A. Leonowicz, S.J. Shin and S. Ohga. 2007. Effect of culture conditions on growth and Lipase production by a newly isolated strain, *Geotrichum–like R59 (Basidiomycetes). J. Fac. Agr., Kyushu Univ.*, 52(1): 29-34.

Gombert, D.M.G. 1999. Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. *Process Biochem*., 35(1-2): 85-90.

Gopinath, S.C.B., P. Anbu and A. Hilda. 2005. Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience*., 46: 119-126.

Griebeler, N., A.E. Polloni, D. Remonatto, F. Arbter, R. Vardanegaa and J.L. Cechet. 2009. Isolation and screening of lipase producing fungi with hydrolytic activity. *Food and Bioprocess Technology* (doi:10.1007/s11947-008-0176-5).

Gromada, A. and J. Fiedurek. 1997. Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase production. *J. Appl. Microbiol*., 82: 648-652.

Handelsman, T. and Y. Shoham. 1994. Production and characterization of an extracellular thermostable lipase from a thermophilic *Bacillus* sp. *J. Gen. Appl. Microbiol*., 40: 435-443.

Haq, I.U., S. Ali, A. Saleem and M.M. Javed. 2009. Mutagenesis of Bacillus lichniformis through ethyl methyl sulphonate for alpha amylase production. *Pak. J. Bot*., 41(3): 1489-1498.

Helen, T. and D.D. Oliveira. 2009. A review on microbial Lipase production. *Food Bioprocess Technol*., 1935-5130.

Iftikhar, T. and A. Hussain. 2002. Effect of nutrients on the extracellular lipase production by the mutant strain of *R. oligosporous* T^{uv}-31. *Biotech.,* 1(1): 15-20.

Iftikhar, T., I.U. Haq, and M.M. Javed, 2003. Optimization of cultural conditions for the production of lipase by submerged fermentation of *Rhizopus oligosporous* T^{uv} 31. *Pak J. Bot*., 35(4): 519-525.

Iftikhar, T., M. Niaz, M. Afzal, I.U. Haq and M.I. Rajoka. 2008. Maximization of intracellular lipase production in a lipase-overproducing mutant derivative of *Rhizopus oligosporus* DGM 31: A kinetic study. *Food Technol. Biotechnol.,* 46: 402-412.

Iftikhar, T., M. Niaz, S.Q. Abbas, M.A. Zia, I. Ashraf, K.J. Lee and I.U. Haq. 2010a. Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *microsporus. Pak. J. Bot.,* 42(2): 1235-1249.

Iftikhar, T., M. Niaz, M.A. Zia and I.U. Haq. 2010b. Production of extracellular lipases by *Rhizopus oligosporus* in a stirred fermentor. *Brazilian Journal of Microbiology*, 41(4): 1124-1132.

Iftikhar, T., M. Niaz, M.A. Zia, M.A. Qadeer, S.Q. Abbas, M.I. Rajoka and I.U. Haq. 2007. Screening of media and Kinetic studies for the production of lipase from a mutant strain of *Rhizopus oligosporus*", *Biotechnologies International Symposium "Which biotechnologies for south countries" Proceedings of Biotech World 2007*. Oran Algerie, 24-25 November 2007.

Jianghua, Li., W.U. Michen and W.U. Xianzhang. 2000. Fermentation technology of alkaline lipase produced by *Penicillium cyclopium* PG37. *Wuxi Qinggong Daxue Xuebao*., 19(2): 100-104.

Khattab, A.A. and W.A. Bazarra. 2005. Screening mutagenesis and protoplast fusion of *Aspergillus niger* for the enhancement of extracellular glucose oxidase production. *J. Ind. Microbiol.,* 36(3): 199-205.

Kiran, G.S., S. Shanmughapriya, J. Jayalakshmi, J. Selvin, R. Gandhimathi, S. Sivaramakrishnan, M. Arunkumar, T. Thangavelu, K. Natarajaseenivasan. 2008. *Biop. Biosys. Eng.,* (Epub ahead of print).

Korn, M.S. and Y. Fujio. 1997. Effect on the degree of maceration of soybean fermented by *Rhizopus* strains. *J. Fac. Agric*., 41(3-4): 231-237.

Krieger, N., M.A Taipa, E.H.M. Melo, J.L. Lima M.R. Aires-Baros and J.M.S. Cabral .1999. Purification of *Penicilum citrinum* lipase by chromatographic processes. *Bioprocess Eng*., 20:59-65.

Kumar, S, K. Kikon, A. Upadhyay, S.S. Kanwar and R. Gupta. 2005. Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expr. Purif.,* 41(1): 38-44.

Lianghua, T. and X. Liming. 2005. Purification and partial characterization of a lipase from *Bacillus coagulans* ZJU318. *Appl Biochem. Biotechnol.,* 125(2): 139-46.

Lima, V.M.G., N. Krieger, M.I.M. Sarquis, D.A. Mitchell, L.P. Ramos and J.D. Fontana. 2003. Production of Lipase by *Penicillium aurantiogriseu*m, *Food Technol. Biotechnol.,* 41(2): 105-110.

Lotti, M., S. Monticelli, J.L. Montesinos, S. Brocca, F. Valero and J. Lafuente. 1998. Physiological control on the expression and secretion of *Candida rugosa* lipase. *Chem. Phys. Lipids,* 93: 143-148.

Maia, M.M.D., A. Heasley, M.M.C.D. Morais, E.H.M. Melo, M.A. Morais W.M. Ledingham and J.L. Lima. 2001. Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation. *Bioresour. Technol.,* 76(1): 23-27.

Martinez, C.P., P. Christen and A. Ferrers. 1993. Optimization of conditions by factorial design for the production of lipase by *Rhizopus delemar. J. Fac. Quim*., 76(2): 94-97.

Martinez, C.P., P. Christen and A. Ferrers. 1993. Optimization of conditions by factorial design for the production of lipase by *Rhizopus delemar. J. Fac. Quim*., 76(2): 94-97.

Martinez, C.P., P. Christen and A. Ferrers. 1993. Optimization of conditions by factorial design for the production of lipase by *Rhizopus delemar. J. Fac. Quim*., 76(2): 94-97.

Nadj, D., S. Petrovic, B. Lajos, A.B. Stancic and B. Jozef. 1992. Biosynthesis of extracellular lipase by *Geotrichum candidum* and its characterization. *ACTA Biol. Iugosl. B. Mikrobial*., 29(1): 65-73.

Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporous* under various growth conditions. *J. Gen. Microbiol*., 134(1): 227-233.

Peter, R. 1995. Production, regulation and some properties of lipase activity from *Fusarium oxysporium. Enzyme and Microbial. Technol*., 17: 283-883.

Prescott, S. and A. Dunn. 1987. *Industrial Microbiology*. 4th Ed. *CBS Publishers and Distributors, New Delhi, India*., pp. 550-565.

Saxena, R.K., P.K. Ghosh, R. Gupta, W.S. Davidson, S. Bradoo and R. Gulati. 1999. Microbial lipases: Potential biocatalysts for the future industry. *Curr. Sci.,* 77(1):101-115.

Sharma, R., Y. Chisti and U. Banerjee. 2001. Production, purification, characterization and applications of lipases, *Biotechnol Adv.,* 19: 627-662.

Sidhu, P., R. Sharma, S.K. Soni and J.K. Gupta. 1998. Production of extracellular alkaline lipase by a new thermophillic *Bacillus* sp. *Folia Microbiol*., 43(1): 51-54.

Singh, S., G. Kaur, A.K. Chakraborti, R.K. Jain and U. C. Banerjee. 2006. Study of the experimental conditions for the lipase production by a newly isolated strain of *Pseudomonas aeruginosa* for the enantioselective hydrolysis of (±)-methyl trans-3(4-methoxyphenyl) glycidate. *Bioprocess Biosyst. Eng*., 28: 341-348.

Snedecor, G.W. and W.G. Cochran. 1980. Statistical Methods. 7th Edition, *Ames, Iowa State University Press.* ISBN 0-81381560-6.

Sztajar, H. and I. Maliszewska. 1988. The effect of culture conditions on lipolytic productivity of microorganism. *Biotech. Lett*., 10(3): 199-204.

Toshihiko, K., T. Mari, T. Ishii, Y. Iloth, K. Kirimura and S. Usami. 1989. Production of lipase by *Rhizopus oligosporous* a newly isolated fungus. *J. Hokoku Waseda*., 50(125): 61-66.

Vanot, G., D. Valerie, M.C. Guilhem, T.L.R. Phan and L.C. Comeau. 2002. Maximizing production of *Penicillium cyclopium* partial acylglycerol lipase. *Appl. Microbiol. Biotechnol*., 60(4):417-9.

Yasser, R., A.N. Abdel-Fattah, A. Soliman, A. Ahmed, S.A. Gaballa, Sabry and I.E. Ahmed. 2002. Lipase production from a novel thermophilic *Bacillus* sp.: application of plackett-burman design for evaluating culture conditions affecting enzyme sormation. *ACTA Microbiol. Polonica*, 51(4): 353-366.

(Received for publication 14 April 2008)