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IMPROVEMENT OF SELECTED STRAINS THROUGH GAMMA IRRADIATION FOR ENHANCED LIPOLYTIC POTENTIAL

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Abstract

The purpose of the present investigation was to enhance the production of industrially important enzyme lipase by subjecting the wild lipase producing fungal strains i.e. *Aspergillus niger, Rhizopus microsporus* and *Penicillium atrovenetum* to various doses of gamma irradiation (20, 40, 60, 80, 100, 120, 140 and 160 Gy). The isolation and lipolytic activity of selected mutant derived strains is described in this paper. Among all the mutants tested, MBL-5 obtained at 140Gy of *Aspergillus niger* strain showed highest extracellular lipase activity (13.75 ± 0.15 U mL^{-1}) while MBL-1 *Rhizopus microsporus* @ 20Gy showed the lowest activity i.e., 1.06 ± 0.11 U mL^{-1}. A range of pH 3, 5, 7, 9 and 11 was used to check the lipolytic potential of various mutants alongwith their wild type. It was observed that MBL-5 (*Aspergillus niger*) and MBL-2 (*Rhizopus microsporus*) showed enhanced extracellular lipase activity at pH 11 while MBL-3 (*Penicillium atrovenetum*) showed the highest extracellular lipase activity 22.53 ± 0.21 U mL^{-1} at pH 9. It indicates a possible role for the MBL-2, MBL-3 and MBL-5 mutant strains in the detergent industry for the development of eco-friendly technologies.

Introduction

Lipase enzyme occurs widely in nature but on commercial scale microbial lipases are significant. Lipase producing fungi are 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). Due to these synthetic properties it can be used widely in various fields of biochemical and organic conversions (Hsu *et al.,* 2002; Poonam *et al.,* 2005). In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries (Gunstone; Gulati *et al.,* 2005; 1999). Due to the rapid growth of microbial enzymes, ease of genetic manipulation in inexpensive media, these enzymes are considered more useful than plant or animal derived enzymes (Sharma *et al.,* 2001; Iftikhar & Hussain, 2002; Iftikhar *et al.,* 2003; 2007; 2008; Helen & Oliveira, 2009). In recent years in the field of biotechnology microbial lipases act as a versatile tool (Jaeger & Reetz, 1998; Lima *et al*., 2003).

In the last few decades, the exponential increase in the application of lipases in various fields 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; Iftikhar *et al.,* 2010; Iftikhar *et al.,* 2010). The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Rowlands, 1984). Irradiation by gamma ray may cause some mutations to the genes of cells through the DNA repair mechanisms within cells (Thacker, 1999; Ellaiah *et al.,* 2002; Iftikhar *et al.,* 2010). After

the 250 Gy of gamma irradation, the C/G base repair substitutions were the main type of gamma ray induced mutations in *E. coli* and the spontaneous mutational hot spots at position 620-632 in the lacI gene reduced (Wijker & Lafleur, 1998). Microbial strains for the overproduction and improvement of industrial products has been the hallmark of all commercial fermentation processes. The lipase of *Humicola lanuginosa* DSM 3819 was reported a suitable additive in detergents because of its thermostability and high activity at alkaline pH (Huage, 1987). Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Karanam *et al*., 2008). The aim of the present study was to enhance lipase productivity of the fungal strains by subjecting it to improvement by natural selection and random mutagenesis. Screening of microorganisms for selecting suitable strains is an important preliminary step in the production of desired metabolites.

Materials and Methods

Microorganism: Various strains like *Rhizopus microsporus, Penicillium atrovenetum* and *Aspergillus niger* were obtained from Biotechnology & Mycology Research Laboratory, Department of Botany, GC University Faisalabad. Identification of strains was carried out by Microscope (MEIJI Model: ML2100) and various measurements were taken for the selected strain (Iftikhar *et al.,* 2010).

Improvement of strain through mutagenesis: In the present study *Rhizopus microsporus, Penicillium atrovenetum* and *Aspergillus niger* was subjected to the γ-irradiation by Mark IV irradiator (Co^{60}) at Nuclear Institute of Agricultural and Biology, Faisalabad for enhancement of lipases production. Five mL of cell suspension was transferred in each vial, sealed with paraffin exposed to gamma irradiator. Different test doses of gamma radiation were selected which were 10, 20, 40, 60, 80, 100, 120, 140 and 160 Gy. The treated cell suspension (0.1 mL) after different time intervals was transferred to PDA plates (4%) having 1% oxgall as colony restrictor and incubated at 30^{o}C for 3-5 days. The kill curves were prepared and time of exposure was optimized for the mutation of various fungal cells for hyderproduction of lipases (Iftikhar *et al.,* 2010). The selected mutants were assigned the code (MBL) for the convenience of strain and used for further studies in parallel with wild strain.

Selection of mutants: In order to restrict the formation of fungal colonies, oxgall (0.1-1.0 % w/v) was asked finally, on the basis of results, oxgall (1%) was used in PDA medium to obtain the best results.

Plate screening method: The basal medium used for selection of mutant was potato-dextrose-agar supplemented with 2% glucose as a carbon source with 1% oxgall as colony restrictor. After 2-3 days of incubation at 30^{o}C in dark, the size of clearing zone was determined. The colonies showing bigger zones were further sub-cultured. A few colonies were obtained showing larger clearance zones than wild type (Khattab & Bazaraa, 2005).

Isolation of mutants by selective marker: For selection of resistance to catabolite repression, 2-deoxy-D-glucose was used at 1 mg ml^{-1} (Fiedurek *et al*., 1987; Gromada & Fiedurek, 1997). The mutant cells were allowed to grow in PDA at 30^{o}C for 4-8 days. The colonies that appeared as background growth were picked and subjected to the lipase identification.

Identification of specific mutant: For the identification of specific mutants following two tests were performed.

Qualitative screening of mutants/enzyme diffusion zone test: Lipase positive strain was identified on Luria-Bertani-tributyrin agar plates g/L (Tryptone, 10; NaCl, 5; Yeast extract, 5; Tributyrin, 0.5; Agar, 20; pH 7). The medium was prepared by adding all constituents and then autoclaved and dispensed into sterile Petri dishes. The plates were inoculated and incubated at 30^{o}C for a period of 3-5 days. The development of a clear zone is an indication of lipolytic activity and its area is a measure of the extent of activity. At regular intervals of 24 h incubation, each plate was examined and measurements on the area of the cleared zone were taken to monitor lipolytic activity (Lee & Rhee, 1993). The strain showing the greatest diffusion areas (mm) were further studied.

Analytical test: The larger zone producing strains were scratched, dissolved into buffer, filtered and then the reaction for extracellular lipase activity was determined spectrophotometrically.

Production of extracellular lipases

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 g/L (KH_2PO_4, 0.5; NH_4NO_3, 0.2; $(NH_4)_2SO_4$, 0.4; $MgSO_4.7H_2O$, 0.02; Peptone, 0.1; Trisodium citrate, 0.5; Yeast Extract, 0.2; Glucose 50% w/v; pH 5.5) containing glass bead in 1 liter cotton wool plugged conical flask was sterilized at 15 lb in^2 pressure (121°C) for 15 min. One milliliter of spore suspension (containing 4.63 x 10^7 spores) was aseptically transferred to the flask. The flask was incubated at 30°C in an incubator shaker at 200 rpm for 24 h **(**Iftikhar *et al.,* 2010).

Shake flask fermentation: The selected mutant strains along with wild strain were screened for checking their lipolytic potential through submerged fermentation using the medium g/L (Soybean meal, 10; Olive oil, 20.0; Glucose, 10.0; K_2HPO_4, 2.0; $NaNO_3$, 0.5; $MgSO_4.7H_2O$, 0.5; pH. 7.0). 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^{o}C for 15 min. and cooled at room temperature. One ml of inoculum as 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 (Iftikhar *et al.,* 2010).

Extracellular lipase assay

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.

Dry cell mass determination: The mycelium was filtered through Whatman GF/C circles 47mmΦ (Cat#1822 047), England. Ten mL of the sample was poured through filter paper placed on filter assembly attached with diaphragm vacuum pump (ULVA Sinku kiko DA-60D, Japan) and placed in an oven at 80^{o}C. After 12 h gravimetrically dry cell mass was determined (Colen *et al*., 2006).

Results and Discussion

Three fungal strains were obtained and identified as *Aspergillus niger, Penicillium atrovenetum* and *Rhizopus microsporus*. Various doses of γ-radiations were used to induce mutation in the cells of wild strains in order to improve the extracellular biosynthesis of lipases. It was found that extracellular lipases production ranges between 2 U mL^{-1} to 7.8 U mL^{-1}. Various strains exhibited different results in terms of enzyme production presumably because the enzyme activity was associated with the cell growth (Cardenas *et al.,* 2001; Gutarra *et al.,* 2007).

Gamma irradiation treated strains of *Aspergillus niger:* A range of doses (20 - 160 Gy) of γ-radiations at an interval of 20 Gy were applied to induce mutation in cells of wild strain of *Aspergillus niger* (Tables 1-2). It was found that at dosage of 140 Gy, MBL-5 showed maximum extracellular lipases production (13.75±1.5 U mL^{-1} with 0.24 g/20mL DCM). MBL5 gave the maximum % increase of extracellular lipases (311 %) which is greater than previously reported activity (127%) by *Aspergillus* sp., (Ellaiah *et al.,* 2002). It might be due to the fact that products of the reactions caused by ionizing radiations damage bases and, to lesser extent, damage sugars (Zhiqiang, 2005).

Gamma irradiation treated mutants of *Rhizopus microsporus:* In order to increase the production of extracellular lipases, cells of *R. microsporus* was prepared and irradiated by different doses of γ-radiations (20-160 Gy), with an interval of 20 Gy because the continuous exposure by γ-irradiation resulted in complete damage and death of cells. Mutants were screened for the production of extracellular lipases through submerged fermentation (Table 3). Of the 32 mutant strains isolated MBL-4 at 80 Gy gave maximum enzyme production (10.25 ± 0.27 UmL^{-1}). After 120 Gy, complete death of cells occurred. Similar work has also been reported by Bapiraju *et al.,* (2004).

Gamma irradiation treated mutants of *Penicillium atrovenetum:* The wild culture of *Penicillium atrovenetum* was subjected to gamma irradiation with ranges of 20 - 160 Gy at an interval of 20 Gy because with gradual increase in dose intensity of gamma irradiation the number of deaths and damage were increased. Result indicated that after 60 Gy, 100% killing of cells occurred (Table 4). MBL-3 exhibited maximum extracellular lipase activity (6.75 ± 0.15 UmL^{-1}) at 60 Gy (Fig. 1a-c). Gamma radiations also reported as an effective mutagenic agent for improvement of extracellular lipase production by *Penicillium citrinum* (Pimentel *et al.,* 1994). An increase of 81% increased lipase activity has also been reported by *Penicillium expansum* by gamma irradiation but in present study results showed 114% higher lipase activity than the parent strain at 60 Gy (Sheng *et al.,* 2000).

Table 1. Production of extracellular lipases by *Aspergillus niger* and its mutant derivatives in shake flasks*.

Parent /mutant strain *A. niger*	20Gy	40Gy	60Gy	80Gy	100Gy	120Gy	140Gy	160Gy
	Extracellular lipase activity (U mL^{-1})							
Wild	2.24 ± 0.23							
MBL1	4.33 ± 0.97	4.38 ± 0.12	3.29 ± 0.02	3.64 ± 0.38	3.41 ± 0.33	2.16 ± 0.288	3.33 ± 0.09	4.16 ± 0.09
MBL2	6.93 ± 0.06	2.49 ± 0.38	3.19 ± 0.19	3.25 ± 0.29	2.83 ± 0.19	1.44 ± 0.49	7.000 ± 0.17	5.99 ± 0.19
MBL3	1.38 ± 0.58	5.20 ± 0.17	4.00 ± 0.28	2.33 ± 0.19	2.74 ± 0.46	2.40 ± 0.45	2.91 ± 0.14	4.83 ± 0.47
MBL4	5.40 ± 0.55	2.08 ± 0.14	3.37 ± 0.17	2.52 ± 0.10	2.33 ± 0.09	3.24 ± 0.54	1.60 ± 0.20	4.58 ± 0.24
MBL5	2.83 ± 0.17	1.91 ± 0.23	4.83 ± 0.86	NP*	2.30 ± 0.02	3.11 ± 0.74	13.75 ± 0.15	NP*
MBL6	2.62 ± 0.33	2.41 ± 0.04	NP*	NP*	2.99 ± 0.38	0.92 ± 0.16	NP*	NP*
MBL7	2.98 ± 0.09	3.16 ± 0.86	NP*	NP*	NP*	0.80 ± 0.15	NP*	NP*
MBL8	4.08 ± 0.21	5.41 ± 0.04	NP*	NP*	NP*	0.94 ± 0.24	NP*	NP*
MBL9	3.24 ± 0.04	NP*	NP*	NP*	NP*	0.68 ± 0.02	NP*	NP*
MBL10	3.69 ± 0.59	NP*	NP*	NP*	NP*	5.35 ± 0.33	NP*	NP*
MBL11	2.81 ± 0.15	NP*	NP*	NP*	NP*	NP*	NP*	NP*
MBL12	3.26 ± 0.71	NP*	NP*	NP*	NP*	NP*	NP*	NP*
	Dry cell mass (g/20mL)							
Wild	1.52 ± 0.10							
MBL1	2.20 ± 0.26	1.42 ± 0.00	0.43 ± 0.00	0.49 ± 0.05	0.49 ± 0.00	0.29 ± 0.03	0.36 ± 0.05	0.76 ± 0.27
MBL2	1.16 ± 0.30	0.61 ± 0.06	0.48 ± 0.02	0.98 ± 0.12	0.43 ± 0.07	0.59 ± 0.12	1.89 ± 0.47	1.64 ± 0.31
MBL3	0.88 ± 0.30	1.18 ± 0.16	0.59 ± 0.02	0.60 ± 0.05	0.55 ± 0.02	0.62 ± 0.06	0.33 ± 0.00	1.01 ± 0.16
MBL4	1.36 ± 0.28	1.17 ± 0.01	0.38 ± 0.01	0.47 ± 0.00	0.20 ± 0.02	0.67 ± 0.02	0.41 ± 0.05	1.48 ± 0.13
MBL5	0.94 ± 0.14	1.44 ± 0.48	0.90 ± 0.09	NP*	0.34 ± 0.00	1.50 ± 0.61	0.24 ± 0.14	NP
MBL6	0.91 ± 0.45	1.33 ± 0.49	NP*	NP*	0.69 ± 0.09	0.96 ± 0.30	NP*	NP*
MBL7	0.96 ± 0.04	0.58 ± 0.09	NP*	NP*	NP	0.60 ± 0.21	NP*	NP*
MBL8	0.63 ± 0.01	1.05 ± 0.22	NP*	NP*	NP	0.74 ± 0.18	NP*	NP*
MBL9	0.63 ± 0.07	NP*	NP*	NP*	NP	0.71 ± 0.08	NP*	NP*
MBL10	0.74 ± 0.20	NP*	NP*	NP*	NP	0.34 ± 0.04	NP*	NP*
MBL11	0.74 ± 0.11	NP*	NP*	NP*	NP	NP*	NP*	NP*
MBL12	0.54 ± 0.07	NP*	NP*	NP*	NP	NP*	NP*	NP*

*Initial pH 7.0, incubation temperature 30°C, agitation rate 200 rpm

*(NP indicated non-production of lipase and death occurred)

Table 2. Production of extracellular lipases by *Rhizopus microsporus* and its mutant derivatives in shake flasks*.

Parent /mutant strain	20Gy	40Gy	60Gy	80Gy	100Gy	120Gy	140Gy	160Gy
R. microsporus	**Extracellular lipase activity (U mL^{-1})**							
Wild	2.21 ± 0.19							
MBL1	1.06 ± 0.11	2.08 ± 0.14	14.33 ± 0.29	3.64 ± 0.48	4.08 ± 0.53	2.16 ± 0.28	NP	NP
MBL2	1.71 ± 0.19	4.58 ± 0.04	6.50 ± 0.28	9.53 ± 0.02	3.00 ± 0.28	2.33 ± 0.28	NP	NP
MBL3	1.12 ± 0.06	2.99 ± 0.48	9.40 ± 0.04	4.08 ± 0.33	1.58 ± 0.53	6.58 ± 0.72	NP	NP
MBL4	2.56 ± 0.77	3.08 ± 0.43	8.53 ± 0.03	10.25 ± 0.27	NP	NP	NP	NP
MBL5	1.95 ± 0.31	1.16 ± 0.28	9.25 ± 0.14	4.25 ± 0.43	NP	NP	NP	NP
MBL6	2.10 ± 0.14	2.58 ± 0.43	NP	3.38 ± 0.56	NP	NP	NP	NP
MBL7	2.21 ± 0.05	NP	NP	4.58 ± 0.33	NP	NP	NP	NP
MBL8	2.10 ± 0.14	NP	NP	NP	NP	NP	NP	NP
	Dry cell mass (g/20 mL)							
Wild	0.67 ± 0.07							
MBL1	1.64 ± 0.38	0.56 ± 0.14	0.92 ± 0.29	0.43 ± 0.09	0.51 ± 0.03	NP*	NP*	1.92 ± 0.58
MBL2	0.89 ± 0.12	0.51 ± 0.03	1.28 ± 0.71	0.78 ± 0.29	0.55 ± 0.06	NP*	NP*	0.48 ± 0.00
MBL3	0.52 ± 0.12	1.22 ± 0.69	0.42 ± 0.01	0.68 ± 0.06	0.37 ± 0.07	NP*	NP*	0.9 ± 0.31
MBL4	0.55 ± 0.07	0.58 ± 0.11	1.09 ± 0.42	0.27 ± 0.03	NP*	NP*	NP*	NP*
MBL5	0.68 ± 0.03	0.59 ± 0.07	0.30 ±0.00	0.57 ± 0.14	NP*	NP*	NP*	NP*
MBL6	0.52 ± 0.00	0.31 ± 0.04	NP*	0.38 ± 0.03	NP*	NP*	NP*	NP*
MBL7	0.85 ± 0.08	NP*	NP*	0.32 ± 0.01	NP*	NP*	NP*	NP*
MBL8	0.74 ± 0.03	NP*	NP*	NP*	NP*	NP*	NP*	NP*

*Initial pH 7.0, incubation temperature 30°C, agitation rate 200 rpm

*(NP indicated non-production of lipase and death occurred)

Table 3. Production of extracellular lipases by *Penicillium atrovenetum* and its mutant derivatives in shake flasks*.

Parent /mutant strain	Extracelluar			Dry cell mass		
P. atrovenetum	**20Gy**	**40Gy**	**60Gy**	**20Gy**	**40Gy**	**60Gy**
Wild	1.75 ± 0.02			2.04 ± 0.04		
MBL1	4.00 ± 0.57	3.94 ± 0.55	6.23 ± 2.26	0.98 ± 0.05	0.52 ± 0.00	0.95 ± 0.28
MBL2	3.97 ± 0.94	1.33 ± 0.16	5.83 ± 0.67	0.59 ± 0.08	0.81 ± 0.07	0.88 ± 0.18
MBL3	3.99 ± 1.24	1.20 ± 0.23	6.75 ± 0.15	0.94 ± 0.21	1.52 ± 0.70	1.60 ± 0.23
MBL4	3.16 ± 0.19	1.45 ± 0.11	NP*	1.11 ± 0.25	1.43 ± 0.38	NP*
MBL5	3.91 ± 0.62	1.46 ± 0.15	NP*	0.81 ± 0.13	1.40 ± 0.22	NP*
MBL6	4.48 ± 0.08	NP*	NP*	1.81 ± 0.04	NP*	NP*
MBL7	3.58 ± 0.53	NP*	NP*	1.78 ± 0.04	NP*	NP*
MBL8	4.58 ± 0.33	NP*	NP*	0.64 ± 0.11	NP*	NP*
MBL9	3.99 ± 0.19	NP*	NP*	1.12 ± 0.32	NP*	NP*
MBL10	3.16 ± 0.28	NP*	NP*	0.71 ± 0.06	NP*	NP*

*Initial pH 7.0, incubation temperature 30^{o}C, agitation rate 200 rpm

*(NP indicated non-production of lipase and death occurred)

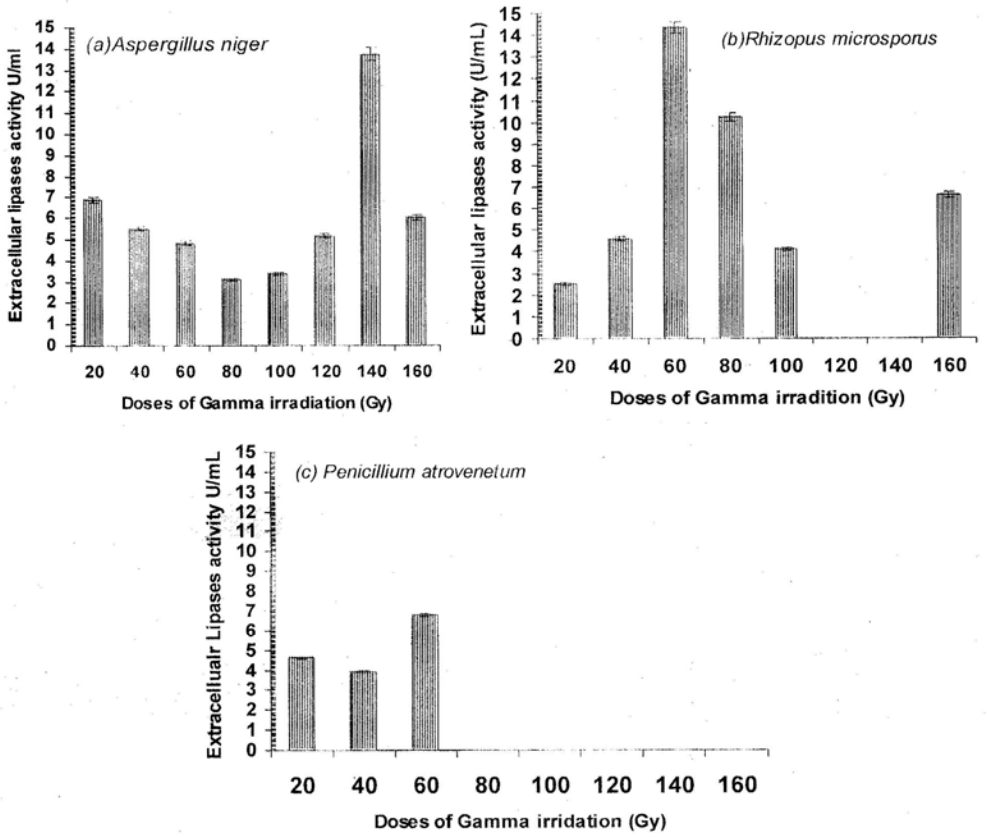
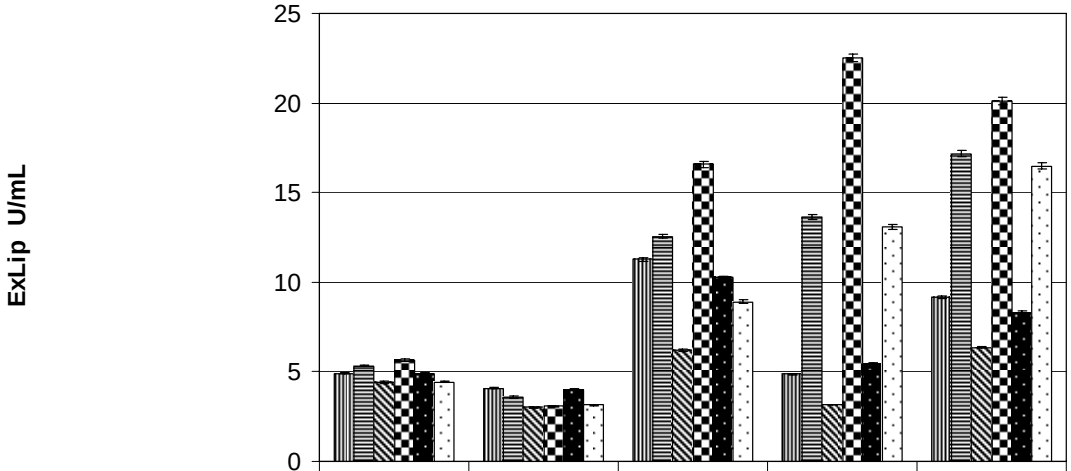


Fig. 1a-c. Comparison of selected potent mutants of various fungal strains after Gamma (γ) irradiation treatment in shake flask*

*Initial pH 7.0, incubation temperature 30°C, agitation rate 200 rpm



	3	5	7	9	11
A.nigerW	4.91	4.08	11.27	4.86	9.15
A.nigerMBL5	5.33	3.58	12.56	13.63	17.15
P.atrovenatum W	4.41	3	6.22	3.17	6.37
P.atrovenatum MBL3	5.66	3.08	16.59	22.53	20.09
R. microsporous W	4.89	4.02	10.23	5.47	8.29
R. microsporous MBL2	4.44	3.16	8.9	13.08	16.49

Fig. 2. Effect of pH on the production of extracellular lipases by various potent mutants and their wild strains in shake flask*.

*Incubation temperature 30°C, agitation rate 200 rpm

Effect of pH on hyperproducers of fungal strains: 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 lipase was investigated (Fig. 2). The initial pH of the fermentation medium was adjusted from 3 - 11 in shake flasks. The hyperproducer of *Aspergillus niger* (MBL-5 at 140 Gy) showed maximum lipase activity ($17.15\pm0.26 UmL^{-1}$) at pH 11 with 0.34±0.01 g/20mL of Dry Cell Mass. While hyperproducer of *Penicillium atrovenetum* (MBL-3) exhibited maximum extracellular lipases activity ($22.53\pm0.1\ UmL^{-1}$) at pH 9 with dry cell mass of 1.39±0.1 g/20mL. The selected mutant of *Rhizopus microsporus* (MBL-2) also showed high lipase activity at pH 11 i.e., 16.49±0.25 U/mL, with 0.25 g/20mL of DCM. 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 biosynthesis. It might be due to that productivity of the enzyme by fungal culture was very pH specific (Gombert, 1999).

Conclusion

It is suggested that the high yielding fungal mutant strains of the *Aspergillus niger* (MBL-5 at 140 Gy), *Rhizopus microsporus* MBL-4 (80 Gy) and *Penicillium atrovenetum* MBL-3 (60 Gy) can be exploited commercially for large-scale industrial production of lipase. It is evident from the results that lipases of *Rhizopus microsporus, Penicillium atrovenetum* and *Aspergillus niger* can be used in the formation of detergent in different industries for the development of eco-friendly technologies (Gerday *et al.,* 2000; Bapiraju *et al.,* 2004; Joseph *et al.,* 2006). From the data it is clear that in acidic condition, all the strains showed poor lipases production while there is little effect on dry cell mass formation. Nadkarni (1971) also observed maximum activity at pH > 7 for *Penicillium fragi* and at pH 9 for *Penicillium aeruginosa* whereas increase in the acidity of media reduced lipase activity.

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