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MUTATION INDUCED ENHANCED BIOSYNTHESIS OF LIPASES BY *RHIZOPUS OLIGOSPORUS* VAR. *MICROSPORUS*

TEHREEMA IFTIKHAR[1,2*], MUBASHIR NIAZ[2], SYED QAISER ABBAS[2], MUHAMMAD ANJUM ZIA[3], IFFAT ASHRAF[5], KYE JOON LEE[4] AND IKRAM-UL-HAQ[1]

[1]*Department of Botany, Institute of Biotechnology, Govt. College University, Lahore, Pakistan*
[2]*Department of Botany, GC University Faisalabad, Pakistan.*
[3]*Department of Biochemistry, University of Agriculture, Faisalabad*
[4]*Laboratory of Biotechnology, Seoul National University, Seoul, South Korea*
[5]*Department of Environmental Sciences, GC University, Faisalabad*
[*]*Corresponding author: pakaim2001@yahoo.com*

Abstract

The present study describes the isolation, identification and screening of fugal strain *Rhizopus oligosporus* (var. *microsporus*) for the production of extracellular lipases. One hundred and sixty seven cultures of fungi were isolated from different environments such as soil, air, milk, pickle, oily bread, decayed fruits and vegetables by serial dilution method. The strains were initially selected qualitatively on Tween 80-Agar plates and were shifted to the slants of PDA for maintenance and storage at 4°C. Quantitative screening for extracellular lipase production by isolated strains was carried out in shake flasks and the most potent strain producing 3.20 ± 0.003 U mL^{-1} of enzyme was selected. The strain was then identified on the basis of standard morphological measurements and was assigned the code IIB-63. The selected strain was then subjected to physical (UV and Gamma radiations) and chemical mutagenic (MNNG/NTG, NA, EtBr) treatments in order to improve its lipolytic potential. During the treatment, mutants were qualitatively and quantitatively selected and IIB-63 NTG-7 was found to be the mutant showing highest lipases production (10.37 ± 0.06^a U mL^{-1}) with a zone size of 12.3 mm on Luria-Bertani-tributyrin agar plates. This mutant showed an overall 325% increase in activity over its parent strain for the production of extracellular lipase.

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 (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. BTS-24 for productivity of biomedically important enzyme lipase. 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).

Enzyme production has gained a recent take off the world over and there is still a lot of potential boost in this area of recent industrial production. It is likely that a number of traditional mechanical industrial processes will be shifted to bioprocesses. Furthermore, with the advancement of knowledge, enzyme technology will explore new areas of employment in human life but the production potential of enzymes is not uniform globally and it favors more to the developed world. At present, this part of the world has negligible share of enzyme production. The production process for lipases has not been commercialized in Pakistan but country is importing large amount of lipases for industrial application.There is a shifting trend of industrial set up in Pakistan and it is likely that building hi-tech industry will be the major focus of Pakistani entrepreneur in coming days. There is currently interest to permit its production locally. The present piece of work was designed by taking this futuristic view and will provide a base for evaluating the unexplored fungal genetic resource of the region for enzyme production and its probable utility for industrial employment. For this purpose, a deoxyglucose-resistant mutant derivative of a locally isolated strain of *Rhizopus oligosporus* var. *microsporus* was developed. The aim of the present work as to enhance the lipolytic potential of locally isolated *R. oligosporus* through physical and chemical mutagenesis for the production of extracellular lipases through shake flask.

Material and Methods

Isolation of lipolytic fungi*:* Lipase producing fungal strains were isolated from various habitats such as water, air, milk, rotten fruits/vegetables, bread roasted in oil, pickle and oil mill waste by serial dilution technique (Akano & Atanda, 1990). Tween 80-agar plates were used for the isolation of lipolytic fungi.

Screening of lipolytic fungi: Isolated strains were screened for the biosynthesis of extracellular lipases by shake flask fermentation. The screening was carried out in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium (Soybean meal 10.0; 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). The strain showing the highest activity was selected for further studies and was given the code IIB-63.
Identification of selected strain: Microscope (MEIJI Model: ML2100) was calibrated and various measurements were taken for the identification of selected strain.

Improvement of strain through mutagenesis: In the present study *Rhizopus oligosporus* var. *microsporus* IIB-63 was subjected to various physical and chemical mutagens to improve the yield of lipases.

Physical mutagenesis: Ultraviolet and gamma radiations were used and compared for physical mutagenesis.

Mutagenesis using UV lamp: Ultraviolet rays are effective mutagenic agents used for strain improvement and for enhanced lipase productivity (Bapiraju *et al*., 2004). Five ml cell suspension of *Rhizopus oligosporus* var. *microsporus* (4.63×10^{-7} spores mL^{-1}) was treated with UV lamp (emitting the energy of 2.6×10^6 $J/m^2/s$) for 30 to 270 min., at an interval of 30 min. The exposure was carried out at distance of 20 cm of its radiation from the center of germicidal lamp. The treated spore suspension (0.1 mL) after different time intervals was transferred to PDA plates (4%) having 1% oxgall as colony restrictor and incubated at 30°C for 3-5 days. The kill curve was prepared and time of exposure was optimized for the mutation of *Rhizopus oligosporus* IIB-63 for hyderproduction of lipases.

Mutagenesis using gamma radiations: Five mL cell suspension of *Rhizopus oligosporus* (4.63×10^7 spores mL^{-1}) was treated with gamma radiation. Five mL of cell suspension was transferred in each vial, sealed with plastic cover and paraffin. These vials were exposed to gamma irradiator. Different test doses of gamma radiation were selected which were 20, 40, 60, 80, 100, 120 and 140 k Rad. The treated spore suspension (0.1 mL) after different time intervals was transferred to PDA plates (4%) having 1% oxgall as colony restrictor and incubated at 30°C for 3-5 days. The kill curve was prepared and time of exposure was optimized for the mutation of *Rhizopus oligosporus* IIB-63 for hyderproduction of lipases.

Chemical mutagenesis: Nitrous acid, Ethidium bromide and NTG/MNNG were used and compared for chemical mutagenesis.

Mutagenesis using nitrous acid: Cell culture prepared in Vogel's medium was subjected to nitrous acid (0.1M sodium nitrite in phosphate buffer, pH 5.0) treatment at interval of 30, 60, 90, 120, 150 and 180 min., by incubating the mixture at 30°C. Treated cells were washed thrice at 10,000 rpm to remove the traces of mutagen. After suspending the cells in saline, these were plated on PDA plates having 1% oxgall and placed at 30°C for 3-5 days for the preparation of kill curve. After growth, mutants forming larger zones were picked up and transferred to PDA slants and were further tested quantitatively.

Mutagenesis using Ethidium bromide: A stock of 0.5 mg mL^{-1} Ethidium bromide was prepared and 1mL of Ethidium bromide solution was added to 9 mL of Vogel's medium containing culture of fungal spores (4.63×10^7 spores mL^{-1}). After specific time interval of 30, 60, 90, 120, 150 and 180 min., of incubation, it was centrifuged three times at 10,000 rpm for 15 min., to remove the traces of mutagen. Cells were plated on PDA plates for the preparation of Kill curve at 30°C for 30-5 days.

Mutagenesis using MNNG/NTG: To prepare the stock solution, 0.15 mg of N-methyl-N'-nitro-N-nitrosoguandine (MNNG/NTG) was dissolved per mL of buffer saline. One mL of MNNG stock solution and 9 mL of Vogel's medium containing spores of *Rhizopus oligosporus* (4.63×10^7 spores mL^{-1}) were added in flask and kept in water bath (Memmert) at 30°C. After intervals of 30, 60, 90, 120, 150 and 180 min., 1 mL sample was withdrawn and centrifuged thrice for 15 min., at 10,000 rpm (Eppendorf Centrifuge, 5415D) to remove the traces of mutagen from cell suspension. Treated cells were plated and kill curve was prepared in order to optimize the time for the induction of mutation.

Selection of mutants

Selection of colony restrictor: 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.

Selection of 3 log kill mutant dose by kill curve: After treating spores with five different mutagens, hundred fold serial dilutions of mutated spores (putative mutagen treated) were prepared to give approximately 30 colonies or less per plate. In a dark room, the spore dilutions (0.1mL) were spread onto PDA media containing 1% oxgall as colony restrictor. Non- irradiated spores were also plated as control. All processes were carried out in strict aseptic conditions in a laminar air flow. The plates were covered with aluminum foil, placed in an incubator (SANYO Model: MIR-553), set at 30°C for 3-7 days or till colony formation. More than thousand colonies were screened for selection of each putative mutant and few mutants were isolated on PDA plates to study their enzyme activities. The best mutant was selected from a number of variants (Petruccioli *et al*., 1999).

Calculation of colony forming units

The colony forming units were calculated as follows:-

$$\text{C.F.U. mL}^{-1} = \frac{\text{Number of colonies on agar plate}}{\text{Amount plated (0.1 mL)}} \times \frac{1}{\text{Dilution factor}}$$

Screening procedure

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°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 @ 1 mg ml^{-1} (Fiedurek *et al*., 1987; Gromada & Fiedurek, 1997). The mutant spores were allowed to grow in PDA at 30°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 of *Rhizopus oligosporus* var. *microsporus* following two tests were performed.

Qualitative screening of mutants/enzyme diffusion zone test: Lipase positive strain was identified on Luria-Bertani-tributyrin agar plates. 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°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

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 using the medium given in unless mentioned otherwise. 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.

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.

Results and Discussion

Isolaton and screening of fungal lipolytic strains: One hundred and sixty seven cultures of fungi were isolated from different environments such as soil, water, air, milk, pickle, oily bread, decayed fruits and vegetables by serial dilution method. The fungal colonies appearing on the Tween80-agar plates were picked and shifted to the slants of potato dextrose agar (Table 1). The isolates were screened quantitatively through submerged fermentation (data not given). It was found that production of lipases ranged from 0.02 ± 0.001 U mL^{-1} to 3.20 ± 0.003 U mL^{-1}. Out of all the strains tested, culture IIB-63, isolated from pickle gave maximum production (3.20 ± 0.003 UmL^{-1}) of extracellular lipases. Other strains did not exhibit considerable lipase activity, presumably because the enzyme activity was associated with the cell growth (Cardenas *et al*., 2001; Gutarra *et al*., 2007). Therefore, it was selected for further studies.

Identification of IIB-63 (hyperproducer of lipase): Keeping in view the measurements below and literature consulted (Mirza *et al*., 1979), IIB63 was found to be the member of Zygomycota named "*Rhizopus oligosporus* var. *microsporus"* (Saito) Schipper & Stalpers, (1984) that was confirmed by Prof. Dr. Syed Qaiser Abbas (Fungal taxonomist) at the Laboratory of Mycology and Biotechnology, Department of Botany, GC University, Faisalabad Pakistan. *Rhizopus* species is also reported among the most well known lipase producers (Belarbi *et al*., 2000 and Haq *et al*., 2002). Micrographs of strain IIB-63 are presented in Plates 1-4. The microscopic characteristics are given below.

Table 1. Number of fungal isolate from various sources.

Collection areas	Number of strains picked
Normal environment/ altered or enriched environment	
Air	20
Milk	15
Decayed fruits	20
Pickle	20
Decayed vegetables	10
Butter	23
Bread roasted in oil	26
Contaminated soil of oil industry	10
Water	23
Total	**167**

Sporangiophore: Attachment of sporangiophore = Opposite to rhizoids; Length of sporangiophore = 210µm; Width of sporangiophore =10 µm; Thickness of the wall of sporangiophore = 1-2 µm.

Sporangium: Diameter of sporangium = 60 µm; Diameter of columella = 40 µm; Length of sporangium = 60 µm; Shape of sporangium= Elongated.

Spore: Shape of spore = Globose, rough boundary, Size of spore= 4 µm.

Rhizoids: Average length = 30-40 µm; Width= 5 µm

Strain improvement

Physical mutagenesis

Screening of UV treated mutants: In order to increase the production of extracellular lipases, different mutants were prepared by irradiating the cells of *R. oligosporus* (IIB-63 strain) with different doses of UV irradiation (30 to 270 min) each differing in exposure time of 30 minutes in a semi-darkened area. It was found that dosage of UV for 240 min., produced 80.52% killing ($5X10^2$CFU mL^{-1}) as 3 log kill of fungal spores (Fig. 1). Further exposure to UV irradiation resulted in complete death of cells. UV treated cells were plated on the tributyrin selection plates to get approximately 30 colonies per plate. Ten mutants were further screened for the production of extracellular lipases through zone size (mm) and submerged fermentation. Out of the total ten isolates, IIB-63UV-10 gave maximum enzyme production (5.08 ± 0.005^a U mL^{-1}) with a zone diameter of 7.3 mm (Table 2), while a relatively less activity was recorded in other isolates. Strain # IIB-63UV-5 showed minimum extracellular lipase activity (0.73 ± 0.005^f U mL^{-1}). A % increase in activity of all the mutants is given in Table 2. UV-10 gave the maximum production of extracellular lipases with 158.75 % increase of activity among all mutants. On the basis of the findings of Table 4.3, UV-10 was selected for further studies and assigned the code IIB-63UV-10. Bapiraju *et al.,* 2004 also reported ultraviolet rays as potent mutagenic agents for strain improvement.

Table 2. Activity of extracellular lipase produced by various potent mutants of *Rhizopus oligosporus* in shake flask*.

Mutants	Zone size (mm)	Lipase activity (UmL^{-1})	% Increase in activity
Wild Strain/Control	4.5	3.20 ± 0.25	100
Effect of UV radiation			
IIB-63^{UV-1} (240 min)	3.2	2.92 ± 0.005^{d}	91.25
IIB-63^{UV-2} (240 min)	5.5	4.12 ± 0.38^{c}	141.09
IIB-63^{UV-3} (240 min)	2.1	1.67 ± 0.49^{e}	52.18
IIB-63^{UV-4} (240 min)	1.5	0.89 ± 0.005^{f}	27.81
IIB-63^{UV-5} (240 min)	1.2	0.73 ± 0.005^{f}	22.81
IIB-63^{UV-6} (240 min)	1.7	0.99 ± 0.005^{f}	30.93
IIB-63^{UV-7} (240 min)	1.8	1.76 ± 0.005^{e}	55.00
IIB-63^{UV-8} (240 min)	5.8	4.69 ± 0.005^{b}	155.00
IIB-63^{UV-9} (240 min)	4.8	3.99 ± 0.005^{c}	124.68
IIB-63^{UV-10} (240 min)	7.3	5.08 ± 0.005^{a}	158.75
Effect of gamma radiation			
IIB-63 $^{-1}$ (120 k Rad)	8.1	6.31 ± 0.49^{b}	197.18
IIB-63 $^{-2}$ (120 k Rad)	2.3	1.85 ± 0.20^{f}	57.81
IIB-63 $^{-3}$ (120 k Rad)	4.6	3.21 ± 0.58^{e}	100.31
IIB-63 $^{-4}$ (120 k Rad)	4.7	3.95 ± 0.05^{d}	123.44
IIB-63 $^{-5}$ (120 k Rad)	5.6	4.78 ± 0.02^{c}	149.37
IIB-63 $^{-6}$ (120 k Rad)	5.3	4.55 ± 0.005^{c}	142.19
IIB-63 $^{-7}$ (120 k Rad)	8.3	6.67 ± 0.01^{ab}	208.44
IIB-63 $^{-8}$ (120 k Rad)	8.5	6.88 ± 0.005^{a}	215.00
IIB-63 $^{-9}$ (120 k Rad)	5.9	4.98 ± 0.015^{c}	155.63
IIB-63 $^{-10}$(120 k Rad)	1.9	1.09 ± 0.005^{g}	34.06

Table 2. (Cont'd.).

Mutants	Zone size (mm)	Lipase activity (UmL^{-1})	% Increase in activity
Effect of Ethidium bromide			
IIB-63 $^{EtBr-1}$ (150 min)	4.9	3.99 ± 0.02^{i}	124.68
IIB-63 $^{EtBr-2}$ (150 min)	8.0	6.12 ± 0.01^{b}	191.25
IIB-63 $^{EtBr-3}$ (150 min)	7.2	5.06 ± 0.03^{f}	158.13
IIB-63 $^{EtBr-4}$ (150 min)	8.0	6.06 ± 0.005^{c}	189.38
IIB-63 $^{EtBr-5}$ (150 min)	7.4	5.47 ± 0.03^{e}	170.94
IIB-63 $^{EtBr-6}$ (150 min)	4.8	3.88 ± 0.006^{j}	121.25
IIB-63 $^{EtBr-7}$ (150 min)	8.6	6.99 ± 0.006^{a}	218.44
IIB-63 $^{EtBr-8}$ (150 min)	5.0	4.08 ± 0.01^{h}	127.50
IIB-63 $^{EtBr-9}$ (150 min)	7.0	5.01 ± 0.005^{g}	156.56
IIB-63 $^{EtBr-10}$ (150 min)	7.9	6.00 ± 0.01^{d}	187.50
Effect of Nitrous acid			
IIB-63^{NA-1} (150 min)	7.0	5.12 ± 0.0057^{e}	160.00
IIB-63^{NA-2} (150 min)	5.0	3.66 ± 0.006^{h}	114.38
IIB-63^{NA-3} (150 min)	8.4	6.88 ± 0.006^{b}	215.00
IIB-63^{NA-4} (150 min)	3.0	2.28 ± 0.05^{j}	71.25
IIB-63^{NA-5} (150 min)	7.3	5.23 ± 0.01^{d}	163.44
IIB-63^{NA-6} (150 min)	8.9	7.18 ± 0.01^{a}	224.38
IIB-63^{NA-7} (150 min)	7.8	5.75 ± 0.03^{c}	179.69
IIB-63^{NA-8} (150 min)	5.9	4.77 ± 0.02^{f}	149.06
IIB-63^{NA-9} (150 min)	3.4	2.37 ± 0.015^{i}	74.06
IIB-63^{NA-10} (150 min)	5.5	3.96 ± 0.006^{g}	123.75
Effect of MNNG/NTG			
IIB-63 $^{NTG-1}$ (150 min)	4.0	2.99 ± 0.006^{h}	93.44
IIB-63 $^{NTG-2}$ (150 min)	5.5	4.32 ± 0.006^{g}	135.00
IIB-63 $^{NTG-3}$(150 min)	3.1	2.27 ± 0.006^{i}	70.94
IIB-63 $^{NTG-4}$ (150 min)	8.3	6.75 ± 0.03^{d}	210.94
IIB-63 $^{NTG-5}$ (150 min)	8.2	6.55 ± 0.01^{e}	204.69
IIB-63 $^{NTG-6}$ (150 min)	7.8	7.04 ± 0.20^{b}	223.75
IIB-63 $^{NTG-7}$ (150 min)	12.3	10.37 ± 0.06^{a}	324.06
IIB-63 $^{NTG-8}$ (150 min)	8.4	6.55 ± 0.03^{e}	204.69
IIB-63 $^{NTG-9}$ (150 min)	8.6	6.93 ± 0.02^{c}	216.56
IIB-63 $^{NTG-10}$ (150 min)	6.0	4.98 ± 0.01^{f}	155.63

Each value is an average of three replicates ± denotes standard deviation among replicates.

• Incubation period 48h, incubation temp 30^{o}C, agitation speed 200 rpm

Screening of gamma treated mutants: Various doses (20 k Rad to 140 k Rad) of γ-radiations were used to induce mutation in cells of wild strain and selected mutant of *R. oligosporus.* Initially a kill curve was prepared by using gamma radiation as mutagen. It was found that dosage of 120 k Rad produced 81.92% killing ($3X10^2$ CFU mL^{-1}) as 3log kill of fungal spores (Fig. 2). Of all the isolates tested IIB-63γ-8 gave good lipase activity (6.88 ± 0.005^a U mL^{-1}) with a colony zone size of 8.5 mm (Table 2). 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).

Chemical mutagenesis

Screening of Ethidium bromide treated mutants: For this purpose the aforesaid mutant of γ-radiations (IIB-63γ-8) and parent strain of *R. oligosporus* (IIB-63) were further subjected to Ethidium bromide treatments. The concentration of EtBr used was 500μg mL^{-1} for 180 min which produced 96.56 % killing and 3.44 % survival rate (Fig. 3). Among all mutants, EtBr-7 was found to be the best producer of extracellular lipases with an enzyme production of 6.99 ± 0.006^a U mL^{-1} showing 218.44% increase in activity. It was assigned the code of IIB-63EtBr-7. It is also evident from the results that Ethidium bromide did not show a significant difference in enzyme production as compared to gamma radiations. Therefore it can not be considered an effective mutagen for lipases production. Similar work has also been reported by Zhiqiang, (2005) for the over production of lipases.

Screening of nitorus acid treated mutants: The wild strain and selected mutants were further subjected to nitrous acid treatment at the dose of 0.1M for different time intervals ranging from 30-180 min and the kill curve was obtained (Fig. 4) which showed the 92.75% killing. Complete death of cells happened when the time increased from 150 min. Selective mutant strains were isolated on the basis of qualitative screening showing bigger halo-zones than wild strain on tributyrin selection plates. These mutants were further evaluated for lipases production by submerged fermentation (Table 2). Among all mutants tested, NA-6 derived from parent was found to be the best producer of lipases with an enzyme production of 7.18 ± 0.01^a UmL^{-1} showing 224.38 % increase in activity. It is evident from the results that nitrous acid mutants exhibited increased efficiency for lipase production when compared with UV mutants in submerged fermentation (Mala *et al*., 2001). It was further assigned the code of IIB-63NA-6.

Screening of MNNG/NTG treated mutants: The previously gathered cultures were further subjected to MNNG/NTG treatment. The cultures were treated with MNNG dose at 150 μg mL^{-1} for different time intervals ranging from 30-180 min., and a kill curve was initially formulated (Fig. 5). With gradual increase in treatment time the number of survivors was decreased. According to the results, a treatment time of 150 min., found to be optimum for inducing potent mutations after which 100 % killing occurred. The mutants were then evaluated qualitatively and quantitatively for extracellular lipases production (Table 2). There was maximum % increase in activity of IIB-63NTG-7 (324.06 %). Among all mutants tested, NTG-7 derived from wild type was found to be the most potent producer of lipase with an enzyme production of 10.37 ± 0.06^a U mL^{-1} with a zone size of 12.3 mm. NTG is previously also reported as an effective mutagen for strain improvement of *Rhizopus* spp. for enhanced lipase activity (Roblain *et al*., 1989; Bapiraju *et al*., 2004; Zhiqiang, 2005). The selected mutant was therefore assigned the code IIB-63NTG-7 and used for further studies in parallel with wild strain.

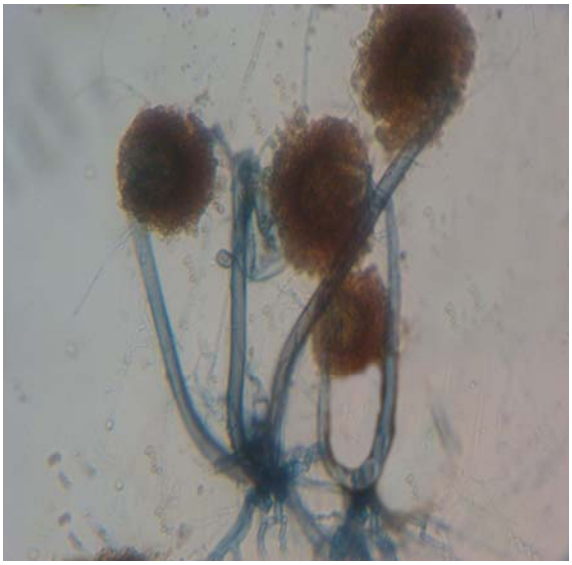


Plate 1. Two/ Three sporangiophore arising from a single point.

Plate 2. Single sporangiophore arising exactly opposite to the rhizoids.

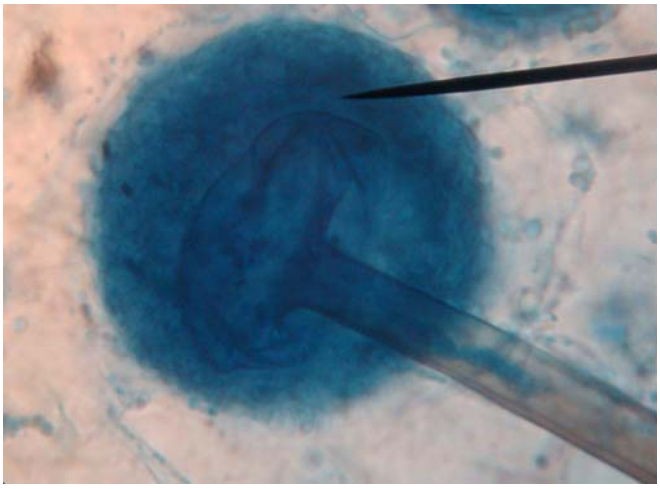


Plate 3. Microscopic image of a sporangium showing columella inside.

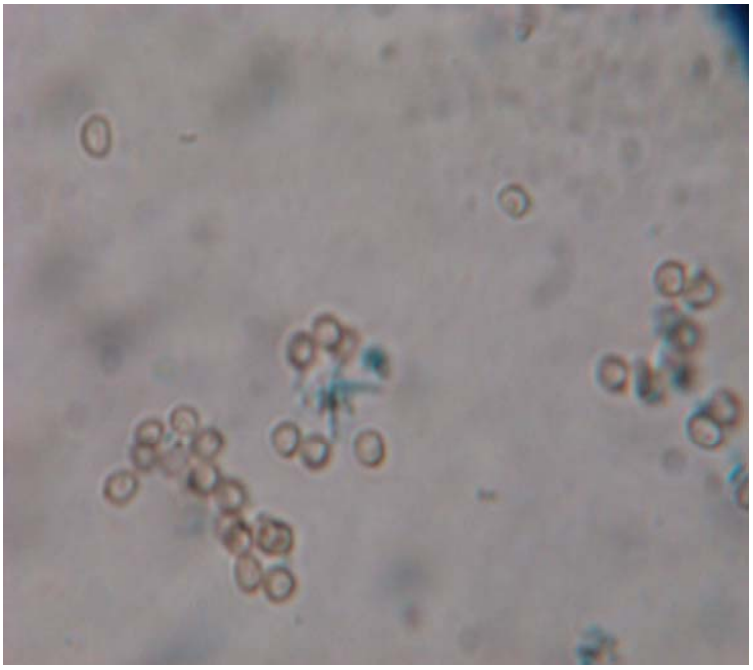
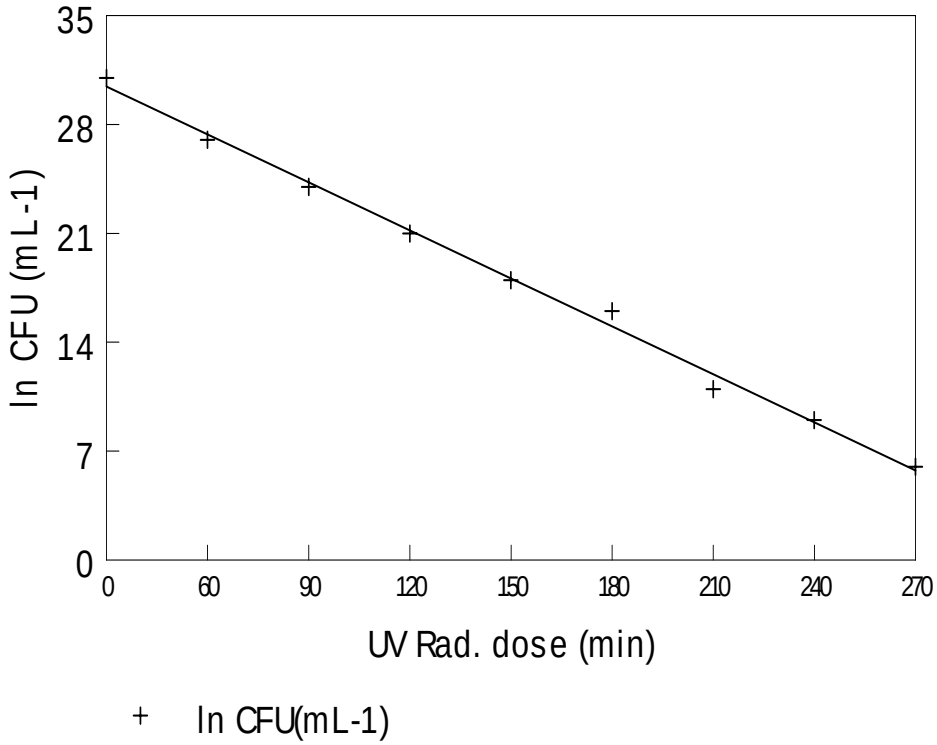
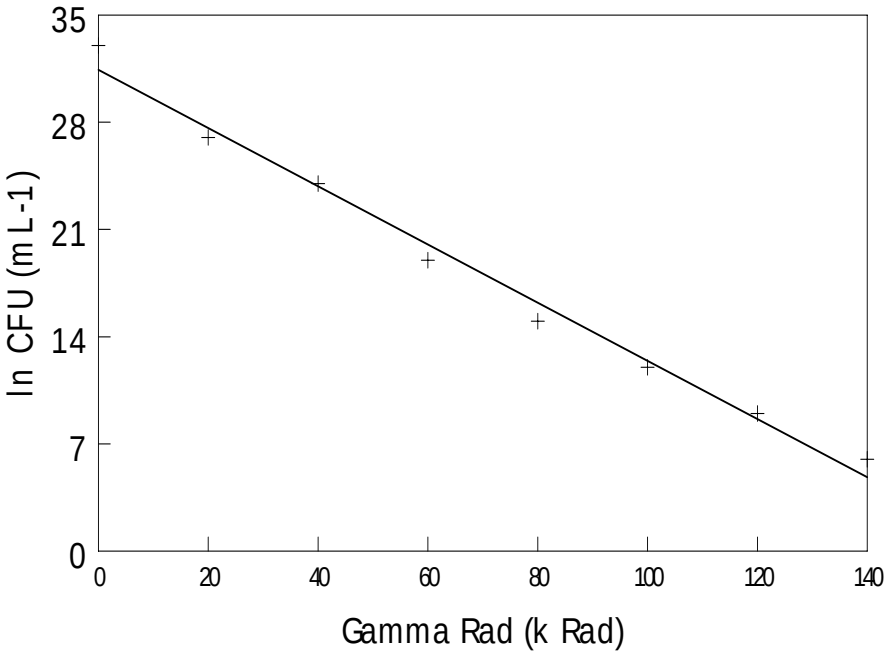


Plate 4. Microscopic image of spores.



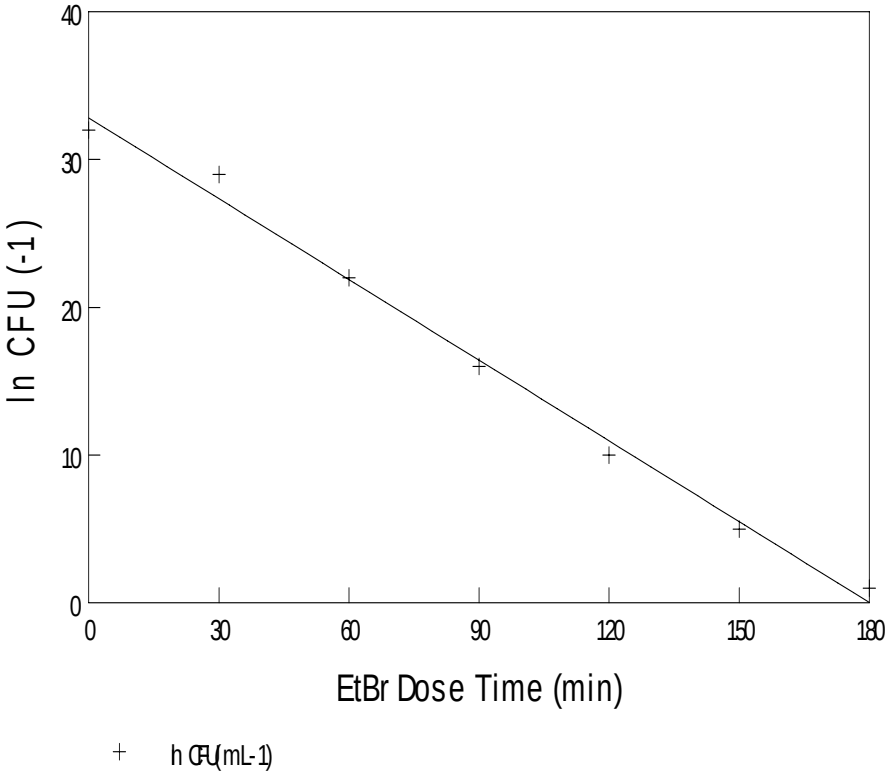
% of Survival = 6.21/31.88*100 = 19.48%, % of Killing = 100 – 19.48 = 80.52 %

Fig. 1. Dose plot of UV to formulate the kill curve.



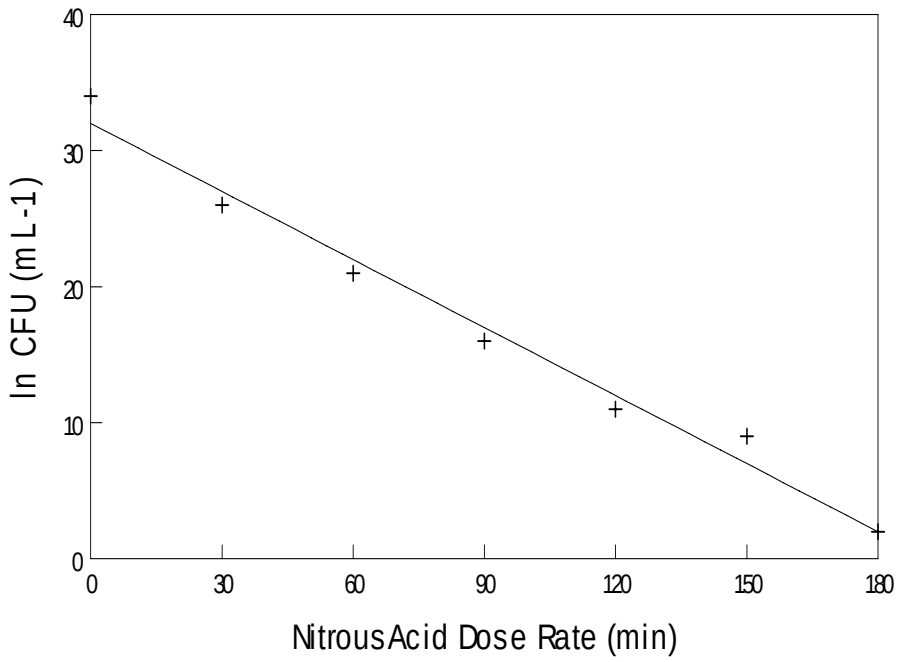
% of Survival = 5.70/31.54*100 = 18.07 %, % of Killing = 100- 18.07 = 81.92 %

Fig. 2. Dose plot of gamma radiation to formulate the kill curve.



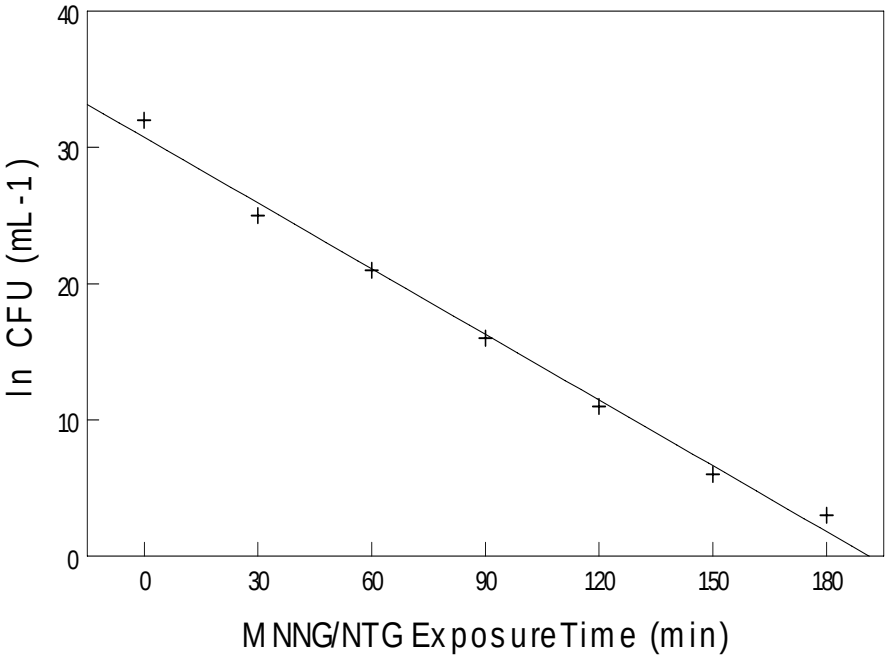
% of Survival = 1.09/31.73*100 = 3.44 %, % of Killing = 100- 3.44 = 96.56 %

Fig. 3. Dose plot of ethidium bromide used for formulating the kill curve.



% of Survival = 2.30/31.73*100 = 7.25 %, % of Killing = 100 – 7.25 = 92.75 %

Fig. 4. Dose plot of nitrous acid used for formulating the kill curve.



% of Survival = 2.9/ 31.87*100 = 9.09 %, % of Killing = 100 – 9.09 = 90.90 %

Fig. 5. Dose plot of MNNG/NTG to formulate the kill curve.

Selection of 3 log kill mutant dose by kill curve: After each mutagenic treatment, serial dilutions were made of the suspensions in such that 0.1mL was plated on PDA plates. The number of colonies was restricted to 30 or less per plate for the formulation 3 log kill curve. 1% Oxgall as colony restrictor was optimized for the clear selection of the colonies on selection medium.

Isolation of mutants by selective marker: 2-de-oxy-D-glucose is glucose analogue used for detecting resistant mutants of lipase. Each mutagen treated spores were spread on PDA plates having 2-de-oxy-D-glucose (1 mg mL^{-1}) as selective marker. A few colonies were selected on the basis of larger clearance zones than wild strain and were subjected to enzyme diffusion zone test to select the best one. Similar work has also been reported for the selection of mutants (Fiedurek *et al.,* 1987).

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