Full Length Research Paper

Enhancement of Extracellular Lipase Production by Strain Improvement of Fungus *Aspergillus niger* LPF-5

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Received 07 June 2016; Accepted 16 August 2016

**Abstract.** Twelve fungal isolates belonging to different genera were quantitatively screened for extracellular lipase production in SmF (Submerged fermentation). Isolate LPF-5 demonstrated higher lipase activity and it was identified as *Aspergillus niger* based on morphology of its Petri plate culture, microscopic study of its slide culture and with the help of relevant literatures. Further an effort was made to increase the production of extracellular lipase by subjecting the most potent lipolytic fungal strain *A. niger* LPF-5 to the strain improvement by induced mutagenesis using UV radiations and nitrous acid. Among the all tested mutagens, nitrous acid was found to be the best mutagen (incubation time of 15 minutes) for inducing the favorable mutation in fungal strain *A. niger* LPF-5 which enhanced the lipase activity up to 30% (105.19 ± 0.91 U mL⁻¹ min⁻¹) when compared to lipase activity (81.00 ± 0.30 U mL⁻¹ min⁻¹) of wild strain while the lipase activity of the best UV mutant (*A. niger* UV3), obtained after an incubation of 6 minutes was 94.30 ± 0.54 U mL⁻¹ min⁻¹, which was 16.41% higher as compared to wild strain of *A. niger*. These results indicate that UV light and nitrous acid both were effective mutagens but nitrous acid was more potent mutagen than UV light.

**Keywords:** *Aspergillus niger*, extracellular lipase, lipolytic strains, mutagen, strain improvement, UV radiations,

1. INTRODUCTION

Lipases (EC 3.1.1.3, triacylclycerol acylhydrolases) catalyze the cleavage of ester linkage of triacylglycerols to liberate the monoacylglycerols, diacylglycerols, free fatty acids and glycerol on an interface of lipid-water (Fig. 1). The attention in extracellular lipases has developed over a period because of their outstanding catalytic activities and their significant applications in a variety of industries (Sharma et al., 2001).

![Fig. 1: Hydrolysis of triacylglycerol catalyzed by lipases.](image)

Fungal lipases catalyze enantio selective reactions. Hence, they have applications in several areas of biotechnology. Lipases are broadly used for hydrolysis, biotransformations, synthesis as well as separations in different industries such as paper, chemical, waste water treatment, flavor and food industries, and in medical diagnostic, scientific researches, chemical analysis and therapeutics (Toscano et al., 2011). Among the lipolytic microorganisms, filamentous fungi associated with the
diverse species of genera *Trichoderma, Rhizopus, Aspergillus* and *Penicillium* were reported as the potent lipase producers (Musa and Adebayo-Tayo, 2012).

A number of previous studies devoted to the optimization of the nutrient medium ingredients and the culture conditions for various enzyme productions in different organisms. But only some considered the possibilities for increasing the enzyme production by strain improvement (Bapiraju et al., 2004; Toscano et al., 2011). The amount of the enzymes secreted by wild strains is low, therefore overproduction of the enzymes requires improvement of strain and optimization of culture medium. The strain improvement is the process of improvement and alteration of microbial strains at genetic level in order to increase the production of metabolites for biotechnological applications (Gonzalez et al., 2003; Pathak et al., 2015). The major benefits of improved strains as industrial producers of extracellular enzymes are as follows: (i) reduction in overall cost of fermentation process; (ii) increase in productivity and (iii) improved strains may exhibit certain specialized desirable features. The desirable improved strains can be developed by random mutagenesis so that hyperproducer strains are successfully selected out and further used for improvement (Pathak et al., 2015).

UV irradiation was reported by Irfan et al. (2011) as an excellent mutagen for the development of strains like *A. niger* for highest productivity of a variety of enzymes. In last few years, efforts have been made for the excess production of microbial enzymes by induced mutagenesis (Ravindranath and Lakshmi, 2014). Toscano et al. (2011) reported that the extracellular production of lipase was increased by a mutant derivative of *A. niger* as compared to parent strain. This mutant strain was obtained by UV light.

In the present investigation we reported the production of extracellular lipase and identification of hyperproducer fungal isolate LPF-5 obtained from soil of oil mills. Further, lipase production by the potent lipolytic fungal strain (*A. niger* LPF-5) was enhanced by strain improvement using physical (UV light) and chemical mutagen (nitrous acid).

2. MATERIALS AND METHODS

2.1. Microorganisms

Twelve fungal strains belonging to different genera, isolated from soil of the five different oil mills associated with the production of groundnut, mustard and taramira oil, were used for extracellular lipase production in SmF. Isolated twelve fungal strains were maintained at 4 °C in slants containing Potato Dextrose Agar (PDA) medium.

2.2. Production of extracellular lipase in SmF

Spore suspensions were made from 7 days old slant of fungal isolates using sterile distilled water. One mL of above spore suspension was added in 100 mL of culture medium containing (g L⁻¹): bacteriological peptone 20.0, olive oil 1%, MgSO₄·7H₂O 0.6, KH₂PO₄ 1.0 and NH₄NO₃ 1.0, pH 7.0. The flasks were kept at 28 °C, 150 rpm for 6 days. At the end of incubation, the contents of each flask containing culture broth were filtered via Whatmann’s No. 1. filter paper. Centrifugation of filtrates was done at 4 °C, 8000 rpm for 10 minutes, in order to obtain the supernatant containing lipase (Sharma et al., 2015).

2.3. Lipase assay

Activity of lipase was measured by the protocol as described previously by Joshi et al. (2006). One unit (U) of enzyme activity was equivalent to how many micromoles (µM) of p-nitrophenol liberated by hydrolysis of ester linkage of pNPP by one mL of soluble enzyme at 35 °C in one minute of reaction.

2.4. Identification of the hyperproducer fungal strain

Identification of the hyperproducer fungal isolate LPF-5 was done by direct observation in Petri plate culture, microscopic observations in slide culture and with the help of authentic manual (Raper and Fennell, 1965). For visual observation, the isolate was point inoculated in Petri plates containing PDA medium and allowed to grow. For the micro-morphological examination, slides were prepared from the fresh Petri plate culture of isolate LPF-5 using LPCB (Lacto phenol cotton blue) stain as described by Aneja (1996).

2.5. Strain improvement through random mutagenesis

In the present investigation fungal isolate (LPF-5) was identified as *Aspergillus niger* and treated with mutagens (physical and chemical) to improve the yield of lipase.

2.5.1. Physical mutagenesis by UV light

Physical mutagenesis was done by ultraviolet (UV) radiations. UV rays are efficient mutagenic agents utilized for strain improvement and for increasing the efficiency of lipase activity (Bapiraju et al., 2004).
Two mL of sterile distilled water was transferred into 6 days old slant culture of *A. niger* LPF-5 and slant tube was vigorously shaken to liberate the spores in water, adjusted to $10^8$ spores mL$^{-1}$, transferred into sterile petri plates and then exposed to the UV radiations for 2, 4, 6, 8, 10, 20, 30 and 40 minutes of duration. The distance was kept at 10 cm away from the centre of UV light source for the exposure of fungal spores. Each of the UV exposed suspension of spores was kept overnight at 4 ºC in dark to prevent photo reactivation. After overnight incubation 0.1 mL of each UV treated spore suspension was kept aseptically onto PDA Petri plates. Sterile spreader was used for uniform spreading of suspension and the plates were placed for 5 days at 28 ºC. The distance was kept at 10 cm away from the centre of UV light source for the exposure of fungal spores. Each of the UV exposed suspension of spores was kept overnight at 4 ºC in dark to prevent photo reactivation. After overnight incubation 0.1 mL of each UV treated spore suspension was poured aseptically onto PDA Petri plates.

### 2.5.2. Chemical mutagenesis using nitrous acid

Nitrous acid was used for chemical mutagenesis. Two mL suspension of spores was prepared from 6 days old slant culture of fungus *A. niger* LPF-5. 0.01M sodium nitrate solution was prepared in sodium acetate (CH$_3$COONa) buffer (0.05 M, pH 5.0). One mL suspension of spores containing $10^8$ spores mL$^{-1}$ was taken into Eppendorf vial and to this 0.1 mL of 0.01M sodium nitrate solution was added and placed in a shaker incubator. Samples of one mL spore suspension containing sodium nitrate were withdrawn at regular interval of 15, 30, 45, 60 minutes. Each of the samples was centrifuged to remove the supernatant containing nitrous acid and one mL of sterile distilled water was added into pellet of treated spores. Again tubes were centrifuged, supernatant was discarded and one mL of sterile distilled water was transferred into pellet in order to prepare the nitrous acid treated spore suspension. 0.1 mL of each nitrous acid treated suspension of spores was transferred onto the Petri plates containing PDA medium, followed by uniform spreading by sterile spreader and the plates were placed for 5 days at 28 ºC. Growth of the fungal isolates was detected at the end of incubation and slants were prepared in PDA media. Slants were used for the preparation of inoculum for estimation of extracellular lipase activity in submerged fermentation (Karanam and Medicherla, 2008).

### 2.5.3. Selection of the hyperproducer mutant by quantitative screening

Each of the UV and nitrous acid treated cultures of *A. niger* LPF-5 was used for extracellular lipase production in SmF. Lipase activity of each treated culture was compared with the lipase activity of the wild/parent strain (Iftikhar et al., 2015).

### 3. RESULTS AND DISCUSSIONS

#### 3.1. Quantitative screening of twelve fungal isolates for extracellular lipase production in SmF.

In the present study, production of lipase ranged from 2.41 ± 0.02 U mL$^{-1}$ min$^{-1}$ to 82.21 ± 0.90 U mL$^{-1}$ min$^{-1}$. Maximum lipase activity (82.21 ± 0.90 U mL$^{-1}$ min$^{-1}$) was achieved from isolate LPF-5 at 72 h of incubation among the all tested strains (Fig. 1). Therefore it was selected for further studies. This hyperproducer strain was isolated from groundnut and mustard oil contaminated soil of Todwal Udyog, Industrial Area, Newai. Among the three isolates of soil sample “S”, isolate LPF-12 exhibited the maximum activity (32.64 ± 0.58 U mL$^{-1}$ min$^{-1}$) after four days of incubation (Fig. 2).

![Fig. 1: Lipase activity from four fungal isolates (LPF-3, 5, 6 and 8) of soil sample “T”](image-url)
Isolate LPF-17 from soil sample “K” demonstrated highest activity (76.17 ± 0.59 U mL⁻¹ min⁻¹) after four days of incubation (Fig. 3). Isolate LPF-24 from soil sample “SM” and LPF-28 from soil sample “M” demonstrated 53.20 ± 1.09 U mL⁻¹ min⁻¹ and 71.33 ± 1.10 U mL⁻¹ min⁻¹ lipase activity, respectively (Fig. 4).

Similar to our results, Colla et al. (2010) reported screening of lipolytic fungi from diesel oil contaminated soil sample using SmF. Toscano et al.
(2011) reported quantitative screening of twelve fungal strains associated with different genera for lipase production. \textit{A. niger} revealed the maximum activity of lipase (7.5 U cm\(^{-3}\)) among the tested fungi. Rifaat et al. (2010) reported maximum lipase activity (16.0 U mL\(^{-1}\)) by \textit{Fusarium oxysporum} in SmF. Therefore it was selected for physicochemical studies. Samad et al. (1990) earlier reported highest activity of lipase (23 U mL\(^{-1}\) min\(^{-1}\)) by a new strain of \textit{Rhizopus rhizopodigormis} at 72 h of incubation. Shirazi et al. (1998) reported lipase production by seven strains of \textit{Saccharomyces cerevisiae} in SmF and maximum production of 4 U mL\(^{-1}\) min\(^{-1}\) was obtained by the strain DSM 1848 in the medium containing yeast extract and olive oil. Maia et al. (1999) reported highest lipase activity (9500 U L\(^{-1}\)) by \textit{F. solani} FS1 culture after 96 h of incubation in the fermentation broth containing 1% olive oil.

### 3.2. Identification of hyperproducer fungal isolate (LPF-5 strain)

Among the all evaluated fungal strains, isolate LPF-5 exhibited highest lipase activity in SmF therefore it was selected for identification by direct visual observation in Petri plate culture and microscopic observations in slide culture. Black coloration colony with black colored spores was observed in Petri plate culture of organism. The colony possessed a white border along its boundary (Fig. 5a, 5c and 5d); reverse: bright yellow pigment in the medium (Fig. 5b and 5e). For the micro-morphological examination, slides were prepared from the fresh Petri plate culture of isolate LPF-5 using LPCB stain and prepared slides were seen using microscope fitted out with camera. Structural characteristics of fungal sample were observed using different objective lenses (10X and 40X). Images were taken with the attached camera. Spherical conidia attached on the vesicle (conidial head) of the asexual reproductive structure called the conidiophores were seen (Fig. 5f). From the above described characteristics and with the help of literatures (Raper and Fennell, 1965; Gilman, 2001; Nagamani et al., 2006), the fungal isolate LPF-5 was identified as \textit{Aspergillus niger} and assigned the code LPF-5.

Iftikhar et al. (2014) reported that the fungal isolate MBL-1412 was found to be a potent hyperproducer of lipase and it was identified as \textit{Aspergillus} \(sp\). by morphological and microscopic examination. Nwuche et al. (2011) previously reported partial identification of fungal isolates of soil. Fungi of the following four genera \textit{Penicillium}, \textit{Mucor}, \textit{Aspergillus} and \textit{Trichoderma} were identified based on the plate morphology and microscopic study of slide culture. Pandey et al. (2015) also reported identification of lipase producing fungal isolates based on physiological characteristics. However, these days molecular identification is also being done.
Enhancement of Extracellular Lipase Production by Strain Improvement of Fungus *Aspergillus niger* LPF-5

3.3. Physical mutagenesis by UV light and selection of the potent mutant strain

In the present study, different mutants of fungus *A. niger* LPF-5 were prepared by irradiating the spores with different doses of UV light (2 to 40 minutes). Among the UV mutants, *A. niger* UV3 showed highest specific activity. Fig. 6 shows that *A. niger* UV3 (obtained after an incubation of 6 minutes) had maximum lipase activity of $94.30 \pm 0.54$ U mL$^{-1}$ min$^{-1}$. It was 16.41% higher as compared to wild strain of *A. niger*. Short term incubation of UV radiations increased the lipase activity, while the long term incubation (10, 20 and 30 minutes) decreased the activity. Lipase activity was increased by 2.98%, 7.46% and 16.41% when *A. niger* LPF-5 culture was incubated with UV light for 2, 4 and 6 minutes, respectively. It seems that exposure of UV light for short duration induced favourable mutations in parent strain. Further increase in incubation time, decreased the enzyme activity accordingly (Figure 6). No growth was observed in case of *A. niger* after 40 minutes incubation with UV light. It might be because the spores failed to germinate by prolonged exposure of UV light. Minimum activity of lipase ($27.80 \pm 0.42$ U mL$^{-1}$ min$^{-1}$) was found after 30 minutes of incubation.

The achieved impact of UV mutagenesis on activity of *A. niger* LPF-5 lipase was found similar to the report of Toscano et al. (2011) where selected mutant of *A. niger* (UV$_2$) demonstrated 19% more activity of lipase (8.85 U cm$^{-1}$) as compared to activity (7.50 U cm$^{-1}$) of wild strain. Karanam and Medicherla (2008) reported 27% higher lipase activity (9.42 U mL$^{-1}$ min$^{-1}$) of UV mutant (AUV$_3$) strain compared to parent strain (7.44 U mL$^{-1}$ min$^{-1}$) of *A. japonicus* MTCC 1975.

In agreement with our result, Prabakaran et al. (2009) reported that fungal isolates were cultured in PDA plates and exposed to UV irradiation for 3, 5, 10 and 15 minutes. Among the all tested exposure time, all the three isolates *A. fumigates*, *P. chrysogenum* and *V. terrestre* exhibited higher lipase activity after 5 minutes of exposure time.
3.4. Chemical mutagenesis using nitrous acid and selection of the potent mutant strain

In the present investigation, production of lipase was enhanced by subjecting wild strain of A. niger LPF-5 to chemical mutagenesis using nitrous acid. Fig. 7 depicts that the best nitrous acid mutant (HN1) was obtained after an incubation of 15 minutes. The lipase activity (105.19 ± 0.91 U mL$^{-1}$ min$^{-1}$) of best nitrous acid mutant was 30% higher than parent strain (81.00 ± 0.30 U mL$^{-1}$ min$^{-1}$) and 11.50% higher than best UV mutant (A. niger UV3) (94.30 ± 0.54 U mL$^{-1}$ min$^{-1}$). When the spore suspension of the fungus was incubated with 0.01 M solution of sodium nitrate for short duration (15 and 30 minutes), the lipase activity was increased as compared to wild strain. It indicated that short term incubation with nitrous acid incorporated favourable mutations in parent strain. Lipase activity was increased to 89.47 ± 6.42 U mL$^{-1}$ min$^{-1}$ after an incubation of 30 minutes, while it decreased by prolonged incubation with nitrous acid (45 and 60 minutes). It was decreased to 67.70 ± 0.36 U mL$^{-1}$ min$^{-1}$ at 45 minutes of incubation and 47.15 ± 0.24 U mL$^{-1}$ min$^{-1}$ at 60 minutes of incubation in comparison with parent strain (81.00 ± 0.30 U mL$^{-1}$ min$^{-1}$) (Fig. 7).

Similar to our results, Iftikhar et al. (2015) reported increased production of lipase in SmF than parent strain by subjecting lipolytic strain A. niger (MBL-33) to strain improvement using nitrous acid for 30 to 180 minutes. In our study nitrous acid was found more potent mutagenic agent than physical mutagen. Nitrous acid increased efficiency of lipase activity of parent strain by 30% while UV irradiation increased efficiency of lipase activity by only 16.41%. Karanam and Medicherla (2008) incubated spores of A. japonicus MTCC 1975 with nitrous acid for 10 to 60 minutes in order to enhance lipase production. The best HNO$_2$ mutant (AHN$_3$) exhibited 77% more lipase activity than wild strain (7.44 U mL$^{-1}$ min$^{-1}$) and 39% higher activity than UV mutant (AUV$_3$) (9.47 U mL$^{-1}$ min$^{-1}$). In another study, Ravindranath and Lakshmi (2014) worked on two stage mutagenesis, where hyperproducer of Aspergillus sp. was treated with UV followed by EMS (ethyl methane sulphonate). The best UV mutant (UV4) exhibited 75% higher lipase activity (10.5 U mL$^{-1}$) than wild strain (6.0 U mL$^{-1}$) and lipase activity (20.4 U mL$^{-1}$) of best EMS mutant was 94% higher than UV mutant (UV4). Caob and Zhanga (2000) previously reported an enhancement in the efficiency of lipase activity by 3.25-fold from a Pseudomonas mutant created by UV, nitrous acid and N-methyl-N-nitro-N-nitrosoguanidine.

4. CONCLUSION

Twelve fungal strains belonging to diverse genera were used for quantitative screening for evaluating their lipolytic potential in SmF. Based on the results of quantitative screening, isolate LPF-5 was found hyperproducer of lipase and identified as A. niger based on visual observation of Petri plate culture and microscopic observation of slide culture. Further lipase production was increased by subjecting hyperproducer fungus A. niger LPF-5 to strain improvement by induced mutagenesis using UV radiations and nitrous acid. In our study nitrous acid was found more potent mutagenic agent than the physical mutagen. Nitrous acid increased efficiency of lipase activity up to 30% (105.19 ± 0.91 U mL$^{-1}$ min$^{-1}$) while UV irradiation increased efficiency of lipase activity up to 16.41% (94.30 ± 0.54 U mL$^{-1}$ min$^{-1}$) as compared to lipase activity (81.00 ± 0.30 U mL$^{-1}$ min$^{-1}$) of parent strain. It is hoped that the mutant derivative of high yielding novel fungal isolate A. niger LPF-5 can be exploited for production of extracellular lipase at industrial level.

ACKNOWLEDGEMENTS

We are highly obliged to Professor Aditya Shastri, Vice-Chancellor, Banasthali University, Rajasthan for providing essential research facilities.

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Enhancement of Extracellular Lipase Production by Strain Improvement of Fungus Aspergillus niger LPF-5

Sharma et al.


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