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Genetic modification of *Aspergillus niger* by induced mutagenesis for lipase enzyme production

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ABSTRACT

The present investigation was carried out to enhance the production of lipase enzyme by genetic modification of the fungi Aspergillus niger by induced mutagenesis (UV irradiation and Ethidium bromide). Aspergillus niger strain was isolated from enriched soil sample and strains were further screened for the production of lipase using Rhodamine plate method. Strain improvement was achieved in presence of Ethidium bromide with concentration of 200μgcm^{-3} in combination with treatment under UV for 300s which was proved to be effective for enhanced lipase production. The results also indicates that chemical mutagenesis in combination with physical mutagenesis proved to be effective for stain improvement in A. niger. The selected mutant can be considered as a prospective candidate for the production of extracellular lipase for various industrial applications.

Keywords: Lipase, Aspergillus niger, induced mutagenesis, UV irradiation, Ethidium bromide.

INTRODUCTION

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) belonging to the class serine hydrolases catalyze hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol (Das *et al.*, 2016). Lipases are ubiquitous in nature produced by animals, plants and microorganisms. However due to specificity of action, microbial lipase have been the focus of attention especially for extracellular lipases (Sheikh Abdul Hamid *et al.*, 2003; Yuan *et al.*, 2010).

Lipases have shown its potential in various industries such as food and dairy, detergent, cosmetic, leather, paper and pulp, biodiesel and pharmaceutical (Mohammed Rabbani *et al.*, 2013, Hasan *et al.*, 2007). With increasing demand for lipases in various fields in the last few

decades, serious attention towards qualitative improvement and quantitative enhancement methods are required. Quantitative enhancement includes strain improvement and medium optimization for the overproduction of the enzyme, as the quantities produced by culture strains are low (Sita Kumari Karanum *et al*., 2008). Genetic modification studies have contributed a great deal to understand the thermostability and active site mechanism for industrial improvement (Sonal Sareen Pathak *et al*., 2015). Most of the wild strains which have potential use in industrial fermentation processes are subjected to industrial strain improvement to make fermentation economical (Mishra L *et al*., 2014).

The objective of the present investigation is to isolate and enhance the lipase production by induced mutagenesis (UV irradiation and Ethidium bromide) of filamentous fungi *Aspergillus niger*.

MATERIALS AND METHODS

Sample collection and Isolation of lipase producing microorganisms

Soil samples were collected from oil contaminated site at a depth of 2-5 cm in sterile plastic bags. The soil sample was enriched for the lipase production by mixing the soil with Neem oil seed cake. Daily sprinkling of the mixture of tween 80 and olive oil over a period of 10-15 days was followed (Manjula *et al*., 2017).

For isolation of microorganisms, 10g of samples were suspended in 90 mL of sterile physiological saline solution. The solution was subjected to stirring for 30 min at 120 rpm to break the soil clumps and allowed to settle down. The supernatant was decanted and a ten-fold serial dilution method in normal saline was used to dilute the samples. Sterile Martin Rose Bengal Agar (MRBA) plate was inoculated with 100 µL of each dilution tube and incubated at 28 °C for 3-4 days. A loop of each fungal colony observed in the agar plate was sub-cultured on Potato Dextrose Agar (PDA) plate and checked for growth separately.

Morphological identification of fungal isolates

The texture and colour of the fungal colonies were observed. Fungal nature of the colonies was confirmed by lacto-phenol cotton blue staining.

Screening of lipase producing microorganisms

Rhodamine B plate assay was used for screening of lipolytic activity of isolated strains (Kouker G *et al*., 1987). The fungal isolates were inoculated on media containing 0.8% w/v nutrient broth, 0.4% w/v NaCl, 500 µL of 0.01% Rhodamine B solution, 1% w/v agar and 7.5% v/v neem oil, adjusted to pH 7.0. The plates were then incubated at 37°C for 24 h. Lipase production is detected by irradiating the plates with UV light at 350 nm. Fungal colonies that have lipolytic activity showed orange fluorescent halo.

Induced Mutagenesis

Conditions for UV Mutagenesis

The protocol described by Karanam *et* al., 2008 was adopted. The spore suspension containing 1.5 x 10^5 conidia cm^{-3}, obtained from 7 days old culture of parental strain was subjected for UV treatment. The experiment was carried out in a UV Illuminator fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A°, at a distance of 16 cm away from the center of the Germicidal lamp (UV light source). The exposure time was 180s and 300s. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation. It was then serially diluted in phosphate buffer and plated on MRBA medium. The inoculated petri dishes were incubated at 30° C for 7 days. The survived fungal colonies were isolated as pure cultures on MRBA plates and stored at 4^oC for further use.

Conditions for Chemical Mutagenesis

The protocol described by Iftikhar *et al*., 2010 with modifications was followed. A stock of 0.5 mg mL^{-1} Ethidium bromide was prepared. A culture of fungal spores (4.63 X 10^7 spores mL^{-1}) in 9 mL of Vogel's medium was added with concentrations of 100µg cm^{-3} and 200µg cm^{-3} of ethidium bromide solution and incubated for 120s. It was then centrifuged three times at 10,000 rpm for 15 min., to remove the traces of mutagen. The experiment also included samples which were exposed to combined treatment of UV for time intervals of 180s and 300s and ethidium bromide for 120s. 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.

Lipase enzyme extraction

The pure culture was grown on MRBA broth to obtain Mycelia mat for further process using different neem oil concentrations (500 µl and 1000 µl). After 7 days of incubation the mycelia mat (*Aspergillus niger*) in the flask was subsequently filtered by using muslin cloth. The filtered mat was homogenised with 20mM phosphate buffer and centrifugation was carried out at 10000rpm for 20min. Further the culture filtrate was used as the crude enzyme source.

Enzyme assay/ Lipase assay (titrimetric method)

The lipase activity was determined using modified titrimetric method (Manjula *et al*., 2017, Borkar *et al*., 2009). The assay mixture composed of neem oil containing 1% (v/v) tween 80 solution, 4ml of 0.1M sodium phosphate buffer (pH 7.0), 500µl of 2% calcium chloride and 1ml of enzyme filtrate. The reaction mixture was incubated at 37°C in water bath for 20min with frequent shaking for every 5-10 min. The reaction was terminated by addition of 20ml of acetone: ethanol mixture (1:1 v/v). The reaction mixture was titrated against 0.1N Sodium hydroxide. The lipase activity was calculated using the following formula:

$$\text{Lipase Activity} = \frac{\text{(Test – Control)} \times \text{Normality of NaOH}}{\text{Incubation time (min)}} \times 100$$

= ....U/ml/min.

Unit Activity:

One unit of lipase activity was defined as the amount of enzyme liberating one micro mole of fatty acid per minute under standard assay conditions.

RESULTS AND DISCUSSION

Isolation and Screening of fungal strain

Aspergillus niger strain was isolated from enriched soil sample. The strain was characterised based on their macro and micro-morphological characteristics (Fig. 1& 2). The microscopic examination carried out by lactophenol cotton blue staining procedure revealed the morphological feature of mycelia and spore characteristics. Black coloured *Aspergillus* colony possessed black conidia and black coloured spores were observed on the surface of the colony.

Strains were further screened for the production of lipase using Rhodamine plate method (Fig. 3). *Aspergillus* species exhibited extracellular lipase activity with the formation of orange fluorescent halos around the fungal colonies when it was observed under the UV light (Fig.3). In Penicillin species also Rhodamine assay was carried out to screen the lipase- producing organisms and similar results were observed (Manjula et al., 2017). Rhodamine fluorescence-based assay was used to screen 32 fungal species from diverse sources, resulted in the effective screening of fungi species (Savitha et al., 2007). The possible explanation for the formation of fluorescent products generated from trioleoylglycerol hydrolysis has been described in which rhodamine B is used in the presence of uranyl ions, yielding orange fluorescent complexes with an excitation wavelength of 350 nm. The mechanism suggested is a complex formation between cationic rhodamine B and the uranyl-fatty acid ion (MacKenzie et al., 1967). According to Kouker G et al. (1987) a conceivable mechanism may be the generation of excited dimers of rhodamine which fluoresce at longer wavelengths than the excited monomer (excimer fluorescence).

Enzyme assay/ Lipase assay (titrimetric method)

The lipase activity was carried out by estimating the free fatty acid content in blank and in the test after the incubation of neem oil substrate with enzyme by titrimetric method. To study the effect of neem oil on lipase production by *Aspergillus* sp., the fungus was grown in medium containing different concentrations of neem oil (Table 1). Lipase was found to be produced only in the presence of different oils indicating the inducible nature of the enzyme (Savitha *et al*., 2007).

The titrimetric method for determination of the enzyme lipase activity yielded as follows:

The crude extracellular lipase activity was determined as 1.80 and 2.54 U/ml/min by titrimetric method which acts as control. These values are in agreement with previous study made by Manjula *et al*., 2017 in *Penicillin sp*.

The activity of lipase enzyme under UV treated for 180 sec and 300 sec respectively was found to be 5.28 and 6.30 U/ml/min respectively. Similarly, treatment with ethidium bromide at concentrations of 100µgcm^{-3} and 200µgcm^{-3} was found to be 6.34 and 6.45 U/ml/min respectively. The combined treatment of samples with ethidium bromide and UV mutagenesis yielded better results than compared with individual treatment. Ethidium bromide with concentration of 200µgcm^{-3} in combination with treatment under UV for 300s proved to be effective for enhanced lipase production.

Table 1: Lipase activity of *Aspergillus niger* under different concentrations of Neem oil

Sl.no	Volume of substrate (Neem oil) (mL)	Source of enzyme (Condition of mutagenesis)	Activity (U/mL/min)
1	0.5	-	1.80± 0.095
2	1.0	-	2.54±0.057
3	1.0	UV treated for 180s	5.28±0.057
4	1.0	UV treated for 300s	6.30±0.011
5	1.0	EtBr 100μgcm^{-3}	6.34±0.023
6	1.0	EtBr 200μgcm^{-3}	6.45±0.020
7	1.0	EtBr 100μgcm^{-3} & UV treated for 180s	6.60±0.023
8	1.0	EtBr 100μgcm^{-3} & UV treated for 300s	6.88±0.057
9	1.0	EtBr 200μgcm^{-3} & UV treated for 180s	7.49±0.057
10	1.0	EtBr 200μgcm^{-3} & UV treated 300s	7.85±0.037

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

Figure 1

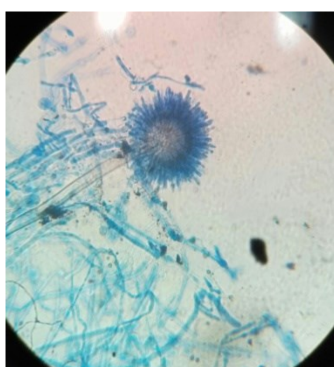

Figure 2

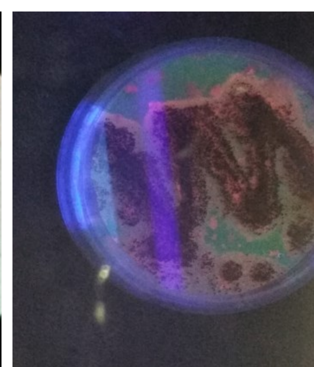

Figure 3

Figure 1: *Aspergillus niger* strain isolated from enriched soil and grown on MRBA media.

Figure 2: Microscopic image of *Aspergillus* sps under 40x magnification.

Figure 3: Pure culture of *Aspergillus* sps grown on rhodamine assay plate.

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes (Karanam *et al.*, 2008). The use of physical and chemical mutagenic agents such as UV, HNO_2, NTG *etc.*, for hyper-production of lipase enzyme has been reported by many workers (Bapiraju *et al.*, 2004; Karanam *et al.*, 2008; Toscano *et al.*, 2011). An increase in lipase production of 3.25-fold was reported in *Pseudomonas* mutant obtained by UV, HNO_2 and NTG (Caob and Zhanga., 2000). Lipase yield was increased to 200% in *Aspergillus niger* mutant from UV and NTG treatments (Elliah *et al.*, 2002). Karanam *et al* (2008) also reported an increase in lipase production by 276% by strain improvement of indigenous isolate *A. japonicus* by UV, HNO_2 and NTG. According to Toscano *et al* (2011), NMG concentration of 200 μg cm^{-3} for 4 h resulted in enhanced lipase activity of 15.5 Ucm^{-3}. Chand *et al* (2005) used a mixture of NTG, ethidium bromide and UV or a mixture of NTG and ethidium bromide to mutate the fungal strain and found that the resultant mutant strains showed more activity in cellulase production than the wild type strains.

The molecular mechanism of genetic modification is still unclear. There are reports on epigenetics being the basis of modification (Aghcheh *et al.*, 2015). Among the genes of *Aspergillus niger* where sense transcription dominated on straw but antisense predominates on glucose were several permeases, carbohydrate active enzymes, and a putative lipase (Delmas *et al.* 2012).

CONCLUSION

In industrial biotechnology, microorganisms have become a major source for the production of primary and secondary metabolites, as well as enzymes and recombinant proteins. Extensive strain improvement strategies were laid down initially by classical mutagenesis and today by advanced genetic manipulation techniques. In the present findings, *Aspergillus niger* developed as a tool for strain improvement for higher lipase production. The now emerging knowledge about how epigenetic mechanisms influence regulatory processes and the interplay of epigenetics and product formation by industrially used fungi needs to be investigated.

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Conflicts of interest: The authors stated that no conflicts of interest.

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