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Research Article

ISOLATION AND DEVELOPMENT OF A SOIL FUNGAL STRAIN WITH HIGH LIPOLYTIC ACTIVITY BY MUTATION

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ABSTRACT

A fungal strain TL-12 was isolated from one hundred fifty fungal isolates obtained from oily soil samples. Initially fourteen isolates exhibited lipase activity and a promising strain TL-12 with extra-cellular lipase activity was identified as *Aspergillus* species and selected for further development. This strain was improved by physical and chemical mutagenesis to obtain a consistently high producer of extra-cellular lipase. UV, EMS and NTG methods were employed sequentially for the enhancement of extra cellular lipase production. Higher lipase producing UV mutants were subjected to EMS and then the higher lipase producing EMS mutants were further treated with NTG to obtain the high lipase yielding strain. The lipase activity increased up to 175%, 340% and 575% respectively, by UV, EMS and NTG treatments compared to the parent strain. The resulting improved strain labeled as Aspergillus TL-12(3) bears high potential for industrial applications. Lipase produced by this strain has a novel catalyzing activity to cleave the amide bond of penicillin G (benzyl penicillin) side chain to produce 6 amino penicillinic acid.

Keywords: Lipase, Mutation, UV, EMS, NTG, Extra cellular lipase.

INTRODUCTION

Lipases are glycerol ester hydrolases (EC 3.1.1.3) widely distributed in bacteria, yeasts and fungi[1-3]. The interest in lipases has grown over a period due to their excellent catalytic properties and their industrial applications[4]. Fungal lipases find application in many areas of biotechnology due to their ability to catalyze enantio selective reactions[5]. Lipases are most widely used enzymes in organic synthesis due to their wide substrate specificity[6]. Strain improvement is one of the ways to increase metabolites[7]. Lipases of fungal origin are obtained from *Candida rugosa*[8] *Aspergillus niger*[9, 10] and *Geotrichum* sp[11]. Mutations and screening of viable mutants is an effective and economic way to improve productivity[12]. UV and EMS treatment increases the lipase production of wild strain[13]. *Aspergillus* strain was improved by UV, NTG mtations[14]. The treatment of UV, HNO_2 and NTG increased the lipase production of *Aspergillus japonicus* [15].

In this work, we report the isolation of a lipase producing soil isolate named as *Aspergillus* TL-12 taken from a vegetable oil spillage area at an oil trading centre in Hyderabad. The aim of the present work is to enhance the lipolytic potential of the isolated strain through physical and chemical mutagenesis for the enhanced production of extracellular lipases. The study explores the increased production of lipase by mutagenesis with UV, EMS and NTG treatment.

MATERIALS AND METHODS

Chemicals

Ethyl methane sulfonate (EMS), N-methyl-N-nitro-N-nitroso guanidine(NTG) and dowex resin was procured from M/s Sigma, Benzyl Penicillin, and 6 amino penicillinic acid (6APA) from M/s Hi

Media. All other chemicals used in this study were of laboratory grade.

Media for primary screening of lipase producing microorganisms

PDA Petri plates with 2% Potato starch, 2% dextrose, 1% agar and 1% tributyrin were prepared (pH6.5) for screening the lipase producing organism.

Media for secondary screening of lipase producing microorganisms

Screening of lipase producing microorganism was done using basal medium containing 2% soybean meal, 1% peptone, 2% potato starch, 0.2 % K_2HPO_4, 0.1 % $MgSO_4.7\ H_2O$, and 0.5 % $CaCO_3$[16].

Pure culture maintenance

Pure cultures of the selected isolates were maintained on PDA agar slants and sub cultured every fortnight.

Spore suspension preparation

Added 5 ml of 0.8% sterile saline to five day old culture slant and the aseptically scraped portion was washed with another 5 ml of saline. Thus collected spore suspension was filtered through sterile cotton to remove the mycelial debris. The spore suspension was adjusted to 10^8spores/ml in a Neubeur haemacytometer (Marienfeld, Germany) for inoculation and mutation experiments.

Shake flask fermentation

Cultures were grown in 100 ml Erlenmeyer flasks filled to 20 ml of the volume with media. Shake flask fermentation was carried out at 28ºC, pH 6.0 and at agitation speed of 200 rpm for 72 hours. The culture was centrifuged at 10,000 rpm for 20 min to separate the fungal mycelium from the broth.

Isolation and screening of lipase producing strain

Spread plate technique was adopted for isolation of lipase producing microorganisms from the soil samples. One gram of each of the collected soil sample was added to 99 ml sterile distilled water and was mixed for 30 min in an orbital shaker. The stock solution thus prepared was then serially diluted in sterile distilled water until a dilution of 10^{-10} was achieved. From each dilution 0.5 ml of dilute was plated on PDA Petri plates and incubated at 28ºC for 5 days and the zones of hydrolysis were observed. Based on hydrolysis zones, selected colonies were inoculated in broth media for secondary screening of lipolytic organisms. After 72 hours of fermentation, broth samples from the shake flask were tested for zone of hydrolysis and lipase activity. Select isolates showing hydrolytic activity were labeled as TL (1 to14). Isolate TL-12 showing highest lipolytic activity of 6 U/ml with 1.8 Cm hydrolytic zone diameter (Table 1) was selected for further strain development by mutation experiments.

Isolation and selection of mutants

UV mutation

UV mutation method based on Bapiraju et al., [17] was modified and used for the present study. Three aliquots of 5 ml spore suspension (~10^8cells/ml) were dispensed aseptically in to separate 80mm Petri plates. An UV chamber containing 40W germicidal lamp emitting 2540-2550ºA of radiation was used for exposing the Petri plates containing TL-12 spores for 30 minutes at a distance of 8, 15 and 25 cm. The Petri plates were kept in dark overnight to avoid photo reactivation. Next, the spores were serially diluted and poured on primary screening media (PDA) plates. Selection of mutated colonies was done from plates with low survival and showing better hydrolytic zones than control after 72 hours. Portions from these mutant colonies were grown in broth media and after 72 hours, the lipase activity was monitored by zone of hydrolysis and lipase assay. The best lipase activity producing UV mutant colony was selected for EMS treatment.

EMS mutation

One ml of spore suspension (~10^8cells/ml) from the best UV mutant colony was taken in an Eppendorf (1.5 ml) tube, centrifuged and the resulting pellet was washed thrice with sterile distilled water and re-suspended in 1ml of sterile distilled water. To this, 100µl EMS was added and incubated at 30ºC under shaking (150 rpm). Samples of 100µl were collected at intervals of 15, 30, 45 and 60 min. The samples were centrifuged to remove the EMS and washed with sterile distilled water thrice. The resulting pellet was re-suspended in 1 ml of distilled water. Varying dilutions of the suspension were spread on PDA plates and incubated at 30ºC for 72 hrs. The PDA Plates were observed for 5 days. Mutants obtained from spore suspensions were treated with EMS for 30 min and were selected

for inoculated in broth media. Lipase activity of the broth was monitored after 72 hours by the hydrolytic zones and assay. The best mutant in this study showing highest lipase activity was taken for further development of strain by NTG treatment.

NTG mutation

One ml of spore suspension (~10^8cells/ml) from the best EMS mutant colony was taken in an Eppendorf (1.5 ml) tube, centrifuged and the resulting pellet was washed thrice with sterile distilled water and re-suspended in 1ml of sterile distilled water. To this, 100µl NTG solution (5mg of NTG dissolved in 0.1ml dimethyl sulphoxide) was added and incubated in a rotary shaker at 30ºC, 150 rpm for 90 minutes. Samples of 100µl were collected at intervals of 15, 45, 60 and 90 min. The samples were centrifuged to remove the NTG and washed with sterile distilled water thrice. The resulting pellet was re-suspended in 1 ml of distilled water. Varying dilutions of the suspension were spread on PDA plates and incubated at 30ºC and observed for 5 days. Best survival mutants based on hydrolytic zones obtained from spore suspensions treated with NTG for 45 min were selected and inoculated in broth media. Lipase activity of the broth was monitored after 72 hours by the hydrolytic zones and assay. The best mutant obtained from these studies labeled as TL-12 (3) was used for further studies.

Lipase activity

Radial diffusion method

Fermented broth samples (200 µl) were added to 1% tributyrin agar plates. Wells (1.0 cm diameter) were made by gel puncture and the hydrolytic zones were measured after 24 hours incubation at 37ºC.

Determination of lipase activity

Lipase assay based on Trietz at al[18] was modified and release of free fatty acids was measured. One ml olive oil and 2ml of 200mM tris buffer, (pH 7.4) and 5ml distilled water were taken in a 50 ml volumetric flask. Then 1ml broth was added while mixing and incubated at 37ºC for 30 min. After completion of incubation, the reaction was stopped with addition of 5 ml methanol-acetone mixture (1:1). The above reaction was carried with blank (excluding sample). The blank (B) and test samples (T) were titrated against 0.05M sodium hydroxide solution.

One unit of lipase activity was defined as the amount of enzyme liberating 1µmol of fatty acid per min under the experimental conditions. Lipase activity was calculated with following formula

$$\text{Lipase activity} = \frac{(T-B) \times 1000 \times 0.05}{30}$$

Identification of strain

Morphology of the TL-12 isolate during vegetative (fig. 1) and reproductive (fig. 2) stages was seen under light microscope. Morphological analysis was carried out according to the hand book of soil fungi from Andhra Pradesh[19].

Hydrolysis of benzyl penicillin by lipase

Preparation of Immobilized lipase

10 ml broth (34 U/ml) from sub-culture of TL-12(3) was precipitated with 3 fold addition of cold acetone, followed by stirring for 15 min, standing it for 30 min and centrifuging at 5000 rpm. The precipitate was washed twice with cold acetone and air dried. Acetone dried crude lipase was dissolved in 10 ml of 50 mM phosphate buffer pH 7 and 250 mg of anion exchange resin (Dowex® 1×8 200-400 mesh) was added. Then the mixture was stirred at 4 °C for 24 hours for binding of lipase on resin. The resin was washed with cold water twice and centrifuged at 5000 rpm for 10 min. The resulting pellet was used for the hydrolysis of benzyl penicillin.

Hydrolysis of benzyl penicillin

Benzyl penicillin 250 mg was taken in to a 50 ml round bottom flask and 10 ml of 50mM phosphate buffer (pH 8) was added. 250 mg of lipase immobilized resin (2 U/mg resin) was added to this and magnetically stirred at 25°C for 24 hours. The pH of the reaction mixture was maintained by addition of 1N sodium hydroxide solution till the pH stabilized. Hydrolysis of benzyl penicillin to 6 amino penicillinic acid was detected by thin layer chromatography[20]. After 24 hours reaction sample of mixture was taken out and tested for the conversion of benzyl penicillin to 6 amino penicillinic acid on TLC plate using n-butanol: glacial acetic acid: water (3:1:1) and the results were confirmed by HPLC[21].

HPLC method

Standard 6 APA sample 2.5 mg/ml was filtered through 0.4 µ filter paper and then injected in HPLC system (Agilent technologies, 1100 series)

and peak area was recorded. Mobile phase was prepared with water, acetonitrile, acetic acid (64.5:35:0.5); column 250×4.6 mm, 5µ with packing material chemsil ODS, C-18: detector wavelength 254 mm injection volume 10 µl, column temperature 25± 2 °C, flow rate 1.0 ml/min, similarly 24 hour reaction mixture and starting material benzyl penicillin were injected and the chromatograms were recorded.

RESULTS AND DISCUSSION

Screening and identification of lipid hydrolyzing microorganisms

After screening of the soil microorganisms on tributyrin containing PDA plates (Fig 1), fourteen isolates were selected and tested for extracellular lipase activity by zone of hydrolysis and assaying the enzyme units (Table 1). Among the fourteen isolates, TL-12 has shown highest lipase activity and it was selected for morphological characterization under microscopy. The observations indicated that the strain belongs to *Aspergillus* Sp. (Fig. 2 & 3)

Strain development

UV irradiation

Aspergillus sp.TL-12 after exposure at various distances to UV irradiation and culturing on PDA plates has shown zero percent spore germination at 8 cm. The spores exposed at 15 cm distance have shown 1% germination while those at 23 cm have shown 5%. Among the minimum survivals found from 15 cm distance exposure, colonies showing bigger hydrolytic zones were selected (Table 2). Mutant UV-4 has shown highest hydrolytic zone and lipase activity among these UV mutated colonies. Hence, the UV-4 mutant was selected for the further EMS chemical mutagen treatment.

EMS treatment

Among the EMS treated mutants grown on PDA plates, six colonies were selected based on hydrolytic zone observation and proceeded for shake flask fermentation. EMS-3 mutant has shown highest zone of tributyrin hydrolysis and lipase activity in broth after 72 hours (Table 3). Hence the EMS-3 mutant was selected for the further NTG chemical mutagen treatment.

NTG treatment

EMS-3 mutant treated with NTG for 45 minutes was observed to be suitable for obtaining the minimal surviving isolates compared to 15 and 30 minute treatment. No germination was observed in the spores treated for 60 and 90 minutes with NTG. Among the 45 minutes NTG treated mutants grown on PDA plates, six colonies were selected based on hydrolytic zone observation and proceeded for shake flask fermentation. NTG-5 mutant has shown highest zone of tributyrin hydrolysis and lipase activity (34.5 U/ml) in the broth after 72 hours (Table 4).

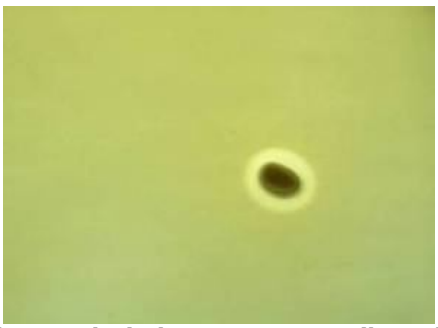

Fig.1: Hydrolytic zone on PDA tributyrin plate; Isolate TL-12 at the primary screening stage

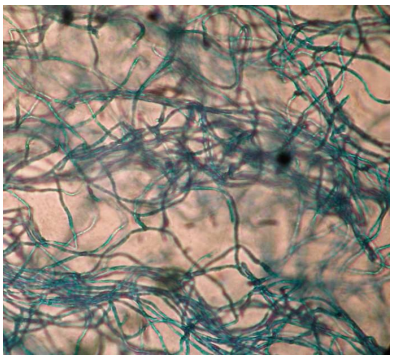

Fig. 2: *Aspergillus* sp. TL-12 Vegetative stage

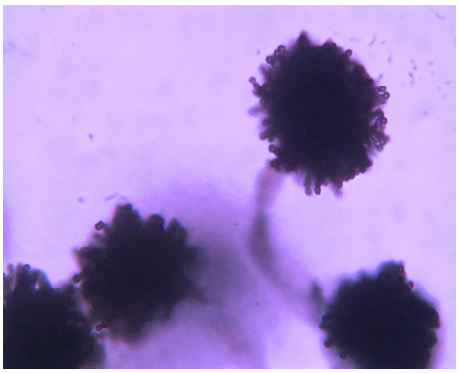

Fig. 3: *Aspergillus* sp. TL-12 Reproductive stage

Fig. 4: Hydrolytic zone on PDA tributyrin plate; Isolate TL-12 (3) at the final development stage

Biotransformation of benzyl penicillin

This Aspergillus Sp. TL-12(3) lipase has a novel catalyzing activity to cleave the amide bond of penicillin G (benzyl penicillin) side chain (Fig 5). Biotransformation of benzyl penicillin was observed by TLC. The TLC plate under UV shown Rf:0.6 and reaction shown 6 amino penicillinic acid with the Rf:0.2, which is matching standard. The 24 hour reaction was confirmed by analytical HPLC (fig 6 and 7)

Ellaiah et al (2002) found 200% increase in lipase production by Aspergillus niger mutant from UV and NTG treatment[22]. Sita kumatri et al achieved 276% improvement in lipase strain by *Aspergillus japnicus* by mutating with UV, HNO2 and NTG[15]. Bapiraju et al.2004 increased lipase by 232% by treating *Rhizopus* sp. with UV and NTG mutations[17]. Sadana Mala et al reported 2.53 fold increase by using UV and nitrous acid for mutation of *Aspergillus niger*[23]. In our study NTG 5 has shown 34.5U/ml lipase activity which is 169% higher than control and 575% more than mother strain.

The bio conversion of benzyl penicillin to 6 APA was the reaction mixture with in 15 min and complete conversion was attained after 24 hours.

Application

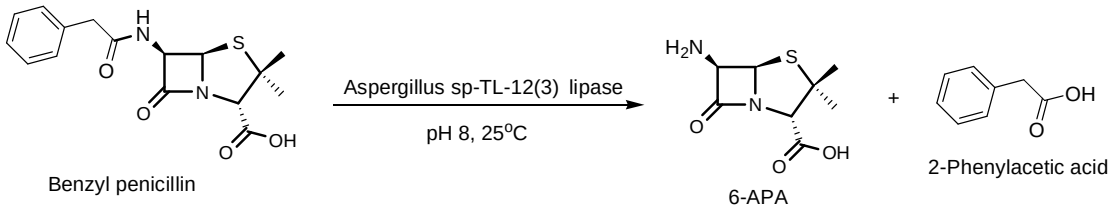

Fig. 5: *Aspergillus sp.* TL-12(3) lipase catalyzed biotransformation of benzyl penicillin to 6 amino penicillinic acid

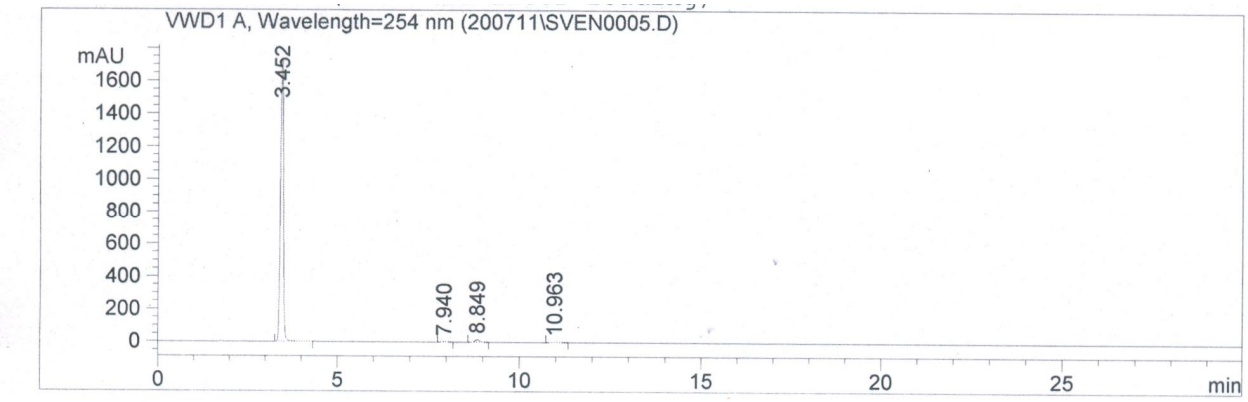

Fig. 6: Analytical HPLC of Benzyl penicillin

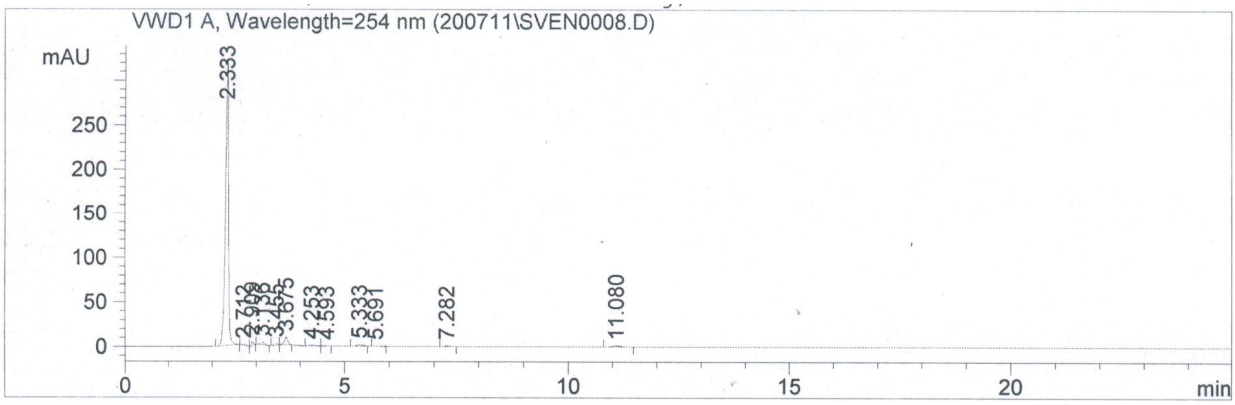

Fig. 7: Analytical HPLC of Benzyl penicillin biotransformation to 6APA with Aspergillus sp.TL-12(3) lipase.

Table 1: Soil isolates which are producing hydrolytic activity

Soil isolates	Zone dia cm(72 h)	Lipase activity (U / ml) 72 hours
TL-1	1.2	0.2
TL-2	1.3	0.5
TL-3	1.3	0.2
TL-4	1.5	1.2
TL-5	1.4	1.0
TL-6	1.2	0.3
TL-7	1.3	0.5
TL-8	1.4	1.0
TL-9	1.3	1.1
TL-10	1.6	1.7
TL-11	1.6	1.5
TL-12	**1.8**	**6.0**
TL-13	1.4	1.2
TL-14	1.5	1.5

Table 2: Hydrolytic zone diameter and lipolytic activity of mutants produced by ultra violet treatment of *Aspergillus* sp. TL-12 isolate

UV mutants	Hydrolytic zone(cm)	Hydrolytic activity (U / ml)	% of improvement
TL-12	1.8	6.0	100
UV 1	2.0	7.5	125
UV 2	2.3	9.0	150
UV 3	2.2	9.0	150
UV 4	**2.5**	**10.5**	**175**
UV 5	2.4	9.9	165
UV 6	2.3	9.0	150

Table 3: Hydrolytic zone diameter and lipolytic activity of mutants produced by EMS treatment of *Aspergillus* sp. TL-12/UV4 mutant

EMS mutants	Hydrolytic zone(cm)	Hydrolytic activity (U / ml)	Comparison with control strain (%)	Comparison with mother strain (%)
UV4(Control)	2.0	10.5	100	175
EMS1	2.4	15.0	142	250
EMS2	2.5	15.6	148	260
EMS3	**2.8**	**20.4**	**194**	**340**
EMS4	2.5	12.0	114	200
EMS5	2.5	12.3	117	205
EMS6	2.3	10.5	100	175

Table 4: Hydrolytic zone diameter and lipolytic activity of mutants produced by NTG treatment of *Aspergillus* sp. TL-12/EMS3 mutant

NTG mutants	Hydrolytic zone(cm)	Hydrolytic activity(U/ ml)	Comparison with control strain (%)	Comparison with mother strain (%)
EMS3(Control)	2.8	20.4	100	340
NTG 1	2.8	21.0	103	350
NTG 2	2.8	21.0	103	350
NTG 3	2.9	22.5	110	375
NTG 4	3.0	27.0	132	450
NTG 5	**3.2**	**34.5**	**169**	**575**
NTG 6	3.0	21.0	103	350

CONCLUSION

The extracellular lipase producing *Aspergillus* sp. TL12 was isolated from the oily soil. The isolated strain was mutated with UV, EMS and NTG treatment. The best lipase producing NTG 5 mutant (34.5 U/ml) was obtained which is labeled as *Aspergillus* sp.TL-12(3). We successfully increased 5.75 fold over the initial activity of the wild soil isolate.

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