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Short Communication

Mutation induced enhanced biosynthesis of lipase

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The purpose of the present investigation is to enhance production of biomedically important enzyme lipase by subjecting the indigenous lipase producing strain *Rhizopus* sp. BTS-24 to improvement by natural selection and random mutagenesis (UV and N-methyl-N'-nitro-N-nitroso guanidine, NTG). The isolation of mutants and the lipolytic activity of selected mutants were described. The best natural selectant $BTNS_{12}$ showed 110% higher lipase activity than the wild strain (BTS-24). The lipase yield of the best UV mutant $BTUV_3$ was 164% higher than the parent strain ($BTNS_{12}$) and 180% times higher than the wild strain (BTS-24). Also, the lipase yield of the best NTG mutant $BTNT_2$ was 133 % higher than the parent strain ($BTUV_3$) and 232% higher than the wild strain (BTS-24). The results indicated that UV and NTG were effective mutagenic agents for strain improvement of *Rhizopus sp.* BTS-24 for enhanced lipase productivity.

Key words: Lipase, Rhizopus, UV, NTG.

INTRODUCTION

Lipases EC (3-1-1- 3) or triacylglycerol hydrolases catalyze hydrolysis of the ester bonds of triacylglycerols (Kohno et al., 1994). These enzymes have been found in many species of animals, plants and microorganisms. In particular, lipases from fungi are important in industrial applications (Essamri et al., 1998), and have been widely used for biotechnological applications in dairy industry, oil processing, production of surfactants and preparation of enantiomerically pure pharmaceuticals (Hiol et al., 2000).

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. The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection.

The aim of the present investigation is to enhance lipase productivity of the wild strain *Rhizopus sp.* BTS-24 by subjecting it to improvement by natural selection and random mutagenesis (UV irradiation and NTG (N-methyl-N'-nitro-N-nitroso guanidine) treatment).

MATERIALS AND METHODS

Microorganism

Rhizopus sp. BTS-24, a wild strain, isolated from coconut oil mill waste showing good lipolytic activity was used in this study. The strain was grown on potato dextrose agar (Waksman , 1957) slants at 28ºC for one week and stored in the refrigerator at 4-7ºC until further use.

Natural Selection

The wild strain Rhizopus sp. BTS-24 was subjected to natural selection. The organism grown on potato dextrose agar (PDA) slants was scraped off into sterile phosphate buffer (0.02 M and pH 7.0) containing Tween 80 (1:5000) to give uniform suspension. The suspension was transferred into a sterile conical flask and

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thoroughly shaken for 30 minutes on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to remove vegetative mycelium from the suspension. The spore suspension was then serially diluted with phosphate buffer and the dilutions were used for plating.

One ml of the respective dilutions of spore suspension was added to the melted PDA medium at 40°C and after thorough mixing was poured into sterile petri dishes. The plates were incubated at 28°C for 7 days. Colonies were selected on the basis of the morphological variations including sporulation and the presence of aerial hyphae. The selected colonies were transferred onto PDA slants and were allowed to grow at 28°C for one week. A total of 15 isolates were selected and were designated as natural selectants ($BTNS_1$ to $BTNS_{15}$). These selectants along with the wild strain were tested for the production of lipase.

UV Irradiation

Further work on improving the yield was done by mutation and selection. High yielding natural selectant was subjected to UV irradiation. The spore suspension of the best natural selectant ($BTNS_{12}$) was prepared in phosphate buffer following the same method mentioned in natural selection. Four ml quantities of the spore suspension were pipetted aseptically into sterile petri dishes of 80 mm diameter having a flat bottom. The exposure to UV light was carried out in a "Dispensing – Cabinet" fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A^0. The exposure was carried out at distance of 20.0 cm away from the center of the Germicidal lamp (UV light source) with occasional shaking. The exposure times were 15, 30, 45, 60, 75 and 90 min. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in phosphate buffer and plated on PDA medium. The plates were incubated for 7 days at 28°C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. A total of 15 colonies (designated as $BTUV_1$ to $BTUV_{15}$) were selected from the plates showing less than 1% survival rate (45, 60 and 75 min UV exposure time) and tested for lipase production. Among the selectants, the best UV mutant strain was used for further studies.

NTG treatment

The best UV mutant ($BTUV_3$) was used for NTG treatment. The spore suspension was prepared in the same manner as described earlier. To a 9 ml of spore suspension, 1 ml of sterile solution of NTG (3 mg ml^{-1} in phosphate buffer) was added. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150 and 180 min and immediately centrifuged for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10 ml of sterile phosphate buffer. The samples were serially diluted in the same buffer and plated over PDA as mentioned earlier. A total of 15 colonies (designated as $BTNT_1$ to $BTNT_{15}$) were selected from the plates showing less than 1% survival rate (120 and 150 min NTG treated spore suspension) and tested for lipase production.

Growth and lipase production

The growth of the fungi cultivated on PDA slants was scrapped with 5 ml of sterile distilled water and transferred into 250 ml Erlenmeyer flask containing 45 ml of production medium (gingelly oil cake, 10 g/l; $(NH4)_2SO_4$, 5 g/l; Na_2HPO_4, 6 g/l; KH_2PO_4, 2 g/l; $MgSO_4$, 3 g/l and $CaCl_2$, 3 g/l with pH 5.0) in 250 ml Erlenmeyer flask. The flasks were incubated at 28^0C for 3 days on a rotary shaker (100 rpm). The culture broth was then filtered and the clear filtrate was used as the crude enzyme source.

Lipase Assay

The lipase activity in the culture filtrate was determined by titrimetry (olive oil substrate emulsion method) (Musantra, 1992). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µmole equivalent fatty acid/ml/min at 40 ^{0}C under the standard assay conditions. All the experiments were carried out in triplicate and the mean of the three was presented.

Table 1. Natural selectants and their lipase activity.

Natural selectant	Lipase activity (u/ml)
$BTNS_1$	7.45
$BTNS_2$	7.26
$BTNS_3$	7.22
$BTNS_4$	7.25
$BTNS_5$	8.24
$BTNS_6$	7.47
$BTNS_7$	7.53
$BTNS_8$	8.07
$BTNS_9$	7.53
$BTNS_{10}$	7.36
$BTNS_{11}$	7.25
$BTNS_{12}$	8.48
$BTNS_{13}$	Nil
$BTNS_{14}$	7.12
$BTNS_{15}$	7.44
Wild strain *Rhizopus sp.* BTS-24	7.69

RESULTS AND DISCUSSION

The lipase production by the natural selectants is shown in Table 1. $BTNS_{12}$ showed highest lipase production (8.48 U/ml), which was 110% higher than the wild strain (7.69 U/ml). Also, only two other natural selectants, $BTNS_5$ and $BTNS_8$ showed higher lipase activity than the wild strain. The natural selectant $BTNS_{12}$ was chosen for further strain improvement employing UV irradiation. Hopwood et al. (1985) suggested that 99.9% kill is best suited for strain improvement as the fewer survivors in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture. The plates having less

Table 2. UV mutants and their lipase activity.

UV mutant	Lipase activity (u/ml)
$BTUV_1$	12.42
$BTUV_2$	5.69
$BTUV_3$	13.88
$BTUV_4$	3.71
$BTUV_5$	7.45
$BTUV_6$	2.25
$BTUV_7$	6.22
$BTUV_8$	Nil
$BTUV_9$	12.45
$BTUV_{10}$	6.91
$BTUV_{11}$	5.37
$BTUV_{12}$	Nil
$BTUV_{13}$	1.24
$BTUV_{14}$	4.23
$BTUV_{15}$	9.12
Parent strain ($BTNS_{12}$)	8.46

than 1% survival rate (45, 60 and 75 min) were selected for the isolation of mutants. A total of 15 mutants were selected and tested for lipase production (Table 2). Four UV mutants ($BTUV_1$, $BTUV_3$, $BTUV_9$ and $BTUV_{15}$) showed higher lipase activity than the parent strain. Ellaiah et al. (2002) reported 156% fold increase in lipase yield of *Aspergillus niger* by UV mutagenic treatment whereas in the present investigation, the lipase yield of UV mutant $BTUV_3$ was 164% higher than the parent strain ($BTNS_{12}$) and 180% higher than the wild strain (BTS-24).

The UV mutant $BTUV_3$ was selected and was next subjected to further strain improvement by NTG treatment for six different time intervals (30, 60, 90, 120, 150 and 180 min). NTG is considered to be a very effective mutagen Adelberg et al.,1965; Cerda-Olmedo and Hanawalt ,1968). Its action has been attributed to its decomposition products (Cerda-Olmedo and Hanawalt, 1968). Plates having less than 1% survival rates (120 and 150 min) were selected for the isolation of mutants and the lipolytic activity of the selected NTG mutants is presented in Table 3. $BTNT_2$ lipase activity was 133% higher than the parent strain ($BTUV_3$) and 232% higher than the wild strain (BTS-24). Caob and Zhanga (2000) reported an increase in lipase production of 3.25–fold by using a *Pseudomonas* mutant of UV and NTG. Also, a 200% increase in lipase yield by *Aspergillus niger* mutant of UV and NTG was reported by Ellaiah et al. (2002). In the present investigation, a 232% increase in lipase production was achieved by strain improvement of indigenous isolate *Rhizopus sp.* BTS-24 by natural selection and induced mutations employing UV and NTG.

Improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the process with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of UV radiation (physical mutagen) and NTG (chemical mutagen) in strain improvement for enhanced lipase productivity was demonstrated in the present investigation. It is hoped that the high yielding mutant strain of the isolate *Rhizopus sp.* BTS-24 can be exploited commercially for large scale industrial production of lipase.

Table 3. NTG mutants and their lipase activity.

NTG mutant	Lipase activity (u/ml)
$BTNT_1$	12.35
$BTNT_2$	17.89
$BTNT_3$	8.14
$BTNT_4$	6.69
$BTNT_5$	17.21
$BTNT_6$	6.70
$BTNT_7$	Nil
$BTNT_8$	11.13
$BTNT_9$	13.27
$BTNT_{10}$	15.78
$BTNT_{11}$	7.75
$BTNT_{12}$	9.45
$BTNT_{13}$	9.48
$BTNT_{14}$	5.46
$BTNT_{15}$	5.87
Parent strain ($BTUV_3$)	13. 41

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