

Full Paper

Strain improvement of *Aspergillus niger* for enhanced lipase production

John Geraldine Sandana Mala, Numbi R. Kamini, and Rengarajulu Puvanakrishnan*

Department of Biotechnology, Central Leather Research Institute,
Adyar, Chennai 600 020, India

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The enhancement of lipase production from *Aspergillus niger* was attempted by ultraviolet (UV) and nitrous acid mutagenesis, and the mutants were selected on media containing bile salts. Nitrous acid mutants exhibited increased efficiency for lipase production when compared with UV mutants in submerged fermentation. The hyperproducing UV and nitrous acid mutants were further subjected to a second step of mutagenesis to devise an economical and ecofriendly technique for lipase production by the effective use of hydrocarbons. One percent kerosene was found to be optimal for lipase production, and one of the mutant strains NAI1 exhibited 2.53 times more increased lipase activity than the parental strain did. This investigation indicates a possible role for the *A. niger* mutant strains in the biodegradation of oil-polluted environments for the development of ecofriendly technologies.

Key Words—*Aspergillus niger*; bile salts; hydrocarbons; lipase; mutagenesis; nitrous acid; UV

Introduction

Lipases (triacylglycerol acylhydrolases 3.1.1.3) are ubiquitous enzymes that catalyze diverse reactions and display a wide range of potential industrial applications (Soberon-Chavez and Palmeros, 1994). Microorganisms are preferred sources of lipases because of their easy availability in large quantities, their specificity, and their low cost of production; moreover, their fungal strains are widely recognized as the best lipase producers (Yadav, 1994). The industrial potential of fungal enzymes has stimulated research in the development of methods to improve strains, since the production level of enzymes in naturally occurring strains is sometimes low for commercial exploitation. Strain improvement by induced mutagenesis has been devel-

oped with rational selection procedures for an efficient screening of the mutants (Gromada and Fiedurek, 1997). In recent years, attempts have been made for the overproduction of microbial lipases by induced mutagenesis and by recombinant DNA technology (Gilbert, 1993). Enhanced lipase production by the use of hydrocarbons has been reported by Chen et al. (1993) and Takahashi et al. (1963a,b), and this property could be exploited as a novel technique of bioremediation for the restoration of oil-polluted environments. The effective bioremediation of petroleum contaminants requires a mixture of populations, and fungi have been known to metabolize the aromatic petroleum contaminants (Korda et al., 1997). In this study, the induction of mutant strains from *Aspergillus niger* by ultraviolet (UV) and nitrous acid mutagenesis for enhanced lipase production is described, and the possibility of the application of these mutants for an effective use of various hydrocarbons has been investigated to achieve an ecofriendly process.

* Address reprint requests to: Dr. Rengarajulu Puvanakrishnan, Department of Biotechnology, Central Leather Research Institute, Adyar, Chennai 600 020, India.
E-mail: puvanakrishnan@yahoo.com

Materials and Methods

Organism. An *A. niger* strain, isolated in our laboratory by Kamini et al. (1997) and identified at IMTECH, Chandigarh, India, was maintained on Czapek Dox agar (CDA) slants at 4°C.

Selection media. Medium A for the selection and isolation of mutants to enhance lipase production by the first mutation step contained 0.2% NaNO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.1% K₂HPO₄, 2.0% sucrose, 0.5% (v/v) tributyrin, 0.5% bile salts, and 2.0% agar. Medium B for the selection and isolation of mutants for lipase production from hydrocarbons by the second mutation step contained 0.2% NaNO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, 0.1% K₂HPO₄, 2.0% sucrose, 0.5% (v/v) liquid paraffin, 0.5% bile salts, and 2.0% agar. Medium C for lipase production contained the optimized constituents of the production medium as described by Kamini et al. (1997).

Selection of mutants. Different concentrations of bile salts (0.25–1.0%, w/v) were added to the CDA medium containing 0.5% (v/v) tributyrin (Medium A). The spore suspension was plated, and lipase activity was recorded after 72 h of incubation by measuring the diameter of halozone, and the mutants were selected based on the increase in diameter of the halozone.

UV mutagenesis. The spore suspension [4.3×10^8 colony-forming units (CFU)/ml] was adequately diluted with sterile water and spread on the CDA plates (Medium A). The agar plates were irradiated with UV light for 2–12 min at a distance of 30 cm by using a germicidal lamp to give a survival ratio of cells of 0.01–0.1%. After 4 days, the strains showing halozone were picked up and maintained on CDA slants at 4°C and assayed for lipase activity.

Nitrous acid mutagenesis. One milliliter of the spore suspension containing 4.3×10^8 CFU/ml was incubated at 30°C in a shaker at 175 rpm for 2 h to permit hydration. The spores were harvested by centrifugation at 10,000 rpm for 10 min and resuspended in 0.1 ml of sodium nitrite (5 mg/ml), which generated the mutagenic agent, nitrous acid in 0.9 ml acetate buffer. The suspension was incubated at room temperature (28–32°C) for different time intervals from 15–120 min. After incubation, the suspension was centrifuged at 10,000 rpm, and the pellet was washed two times with 100 µl phosphate buffer (pH 7.0) and finally resuspended in phosphate buffer. The samples were ade-

quately diluted and plated on the selective agar plates (Medium A) as mentioned earlier.

To enhance the lipase production by using hydrocarbons as substrates, the hyperproducing UV and nitrous acid mutant strains were further subjected to a second step of UV and nitrous acid mutations, as described above, and plated on Medium B containing 0.5% (v/v) liquid paraffin.

Fermentation conditions. An amount of 0.3 ml of the spore suspension containing 4.3×10^8 CFU/ml was added to a series of 250 ml Erlenmeyer flasks containing 50 ml of the optimized production medium (Kamini et al., 1997). The flasks were incubated on a rotary shaker at 175 rpm at 30°C and removed at 48 h. The mycelia were separated by filtration and used for intracellular activity, and the filtrate was used for extracellular activity.

Lipase assay. The extracellular lipase activity was assayed by the method of Yamada et al. (1962a) with slight modifications as described by Kamini et al. (1997). The intracellular activity was assayed by the method of Chen et al. (1993). To 1 g of the wet biomass, 10 ml of distilled water was added and sonicated to disrupt the cell wall. The contents were filtered and the clear filtrate was assayed for intracellular lipase activity. The lipase activity is expressed as the amount of enzyme releasing 1 µmol of free fatty acid per minute under the standard assay conditions.

Effect of hydrocarbons on lipase production. An amount of 1% (v/v) of different hydrocarbons, viz., liquid paraffin, gasoline, kerosene, and diesel oil, was added to the production medium (Medium C) instead of olive oil, and the fermentation was carried out. Samples were assayed for extracellular lipase activity at 48 h.

Cumulative effect of kerosene and olive oil concentrations. Kerosene and olive oil at different concentrations [0.5% and 1.0% (v/v)] were used as inducers and the fermentation was carried out. Samples were assayed at 48 h for extracellular and intracellular lipase activities.

Results

Isolation of mutants

In the presence of bile salts, the parental strain exhibited slight morphological changes. The growth characteristics were similar, though the cell size was reduced with increasing concentrations of bile salts. The

halozone was not distinct in the presence of bile salts because of an inhibition of lipase activity compared with the control. However, at 0.25% (w/v) bile salt concentration, the fungus showed similar morphological characteristics as that of the control, and at 1% (w/v) bile salt concentration, the fungus showed a decrease in colony size compared with the control. Therefore, the addition of 0.5% (w/v) bile salts was selected for the isolation of mutant strains. The mutants isolated from the selective agar plates containing Mediums A and B by a measurement of the increase in halozone were further confirmed for the production of lipase by submerged fermentation in Medium C.

Selection of mutants

In the first step of UV and nitrous acid mutation, 80 strains showing halozones on Medium A were isolated. Among these strains, 6 from UV mutation and 15 from nitrous acid mutation were selected as better lipase producing strains by lipase production tests in Medium C containing olive oil. In the second step of mutation, the mutant strains UVI and NAI were used as the initial strains for UV irradiation and nitrous acid mutagenesis, respectively. Among the 10 mutant strains isolated on Medium B, 3 from UV mutation and 8 from nitrous acid mutation exhibited increased lipase production.

Evaluation of UV and nitrous acid mutagenesis

The characteristics of UV and nitrous acid mutagenesis are evaluated in Table 1. A percent kill of 99.95% at 4 min was obtained for UV mutagenesis, but it was slightly lower for nitrous acid mutagenesis with an exposure time of 60 min. The mutants UVI and NAI showed 14.9 and 33.9% increases in lipase production; UVII and NAII mutant strains showed 20.7 and 39.1% increased enzyme production when compared with the parental strain (Table 1). No significant difference was found, however, in the intracellular activities between the UV and nitrous acid mutants. Enhanced lipase production was observed with nitrous acid mutants exhibiting 19.0 and 18.4% increased activity compared with the UV mutants.

Effect of hydrocarbons

The mutants obtained from the UV and nitrous acid mutagenesis were inspected for lipase production in the presence of various hydrocarbons that could be used as cheaper substrate alternates instead of olive

Table 1. Evaluation of UV and nitrous acid mutagenesis.

Characteristics	UV	Nitrous acid
Percent kill	99.95%	99.8%
Time of exposure	4 min	60 min
Extracellular lipase activity by UVI & NAI	40.0 U/ml	46.6 U/ml
Extracellular lipase activity by UVII & NAII	42.0 U/ml	48.4 U/ml
Intracellular lipase activity by UVI & NAI	17.4 U/ml	18.5 U/ml
Intracellular lipase activity by UVII & NAII	18.0 U/ml	20.0 U/ml

Medium for the production of lipase by parental and mutant strains: urea 0.5%; meat extract 1.0%; sucrose 0.5%; KCl 0.05%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; KH_2PO_4 0.1%; Na_2HPO_4 0.3%; olive oil 1.0% (v/v).

Culture conditions: fermentation time 48 h; temperature 30°C; agitation 175 rpm.

Lipase production by parental strain 34.8 U/ml.

UVI, hyperproducing mutant from I step of UV mutagenesis; NAI, hyperproducing mutant from I step of nitrous acid mutagenesis; UVII, hyperproducing mutant from II step of UV mutagenesis; NAII, hyperproducing mutant from II step of nitrous acid mutagenesis.

oil. All the mutant strains exhibited increased enzyme production in kerosene (Fig. 1). NAII showed a lipase activity 2.53 times higher than the parental strain. Nitrous acid mutants were found to enhance lipase production than the UV mutants in the presence of all the hydrocarbons. Diesel oil was also observed to be an effective substrate, and the lipase activities of 11.6 and 15.8 U/ml were obtained with NAI and NAII mutants, respectively, at 48 h. NAII also exhibited higher secretion ratios than other strains (data not shown), indicating increased permeability to use the hydrocarbons by this mutant strain.

Effect of the combination of kerosene and olive oil

The results in Table 2 indicate that an enhanced enzyme activity of 44.09 U/ml was achieved with 1% (v/v) olive oil and 0.5% (v/v) kerosene with parental strain in comparison with the lipase activity of 34.8 U/ml with only 1% (v/v) olive oil. However, negligible enzyme activity was observed at all concentrations in the mutant strain NAII.

Discussion

The strain improvement of microorganisms is an important requisite for enzyme production because the

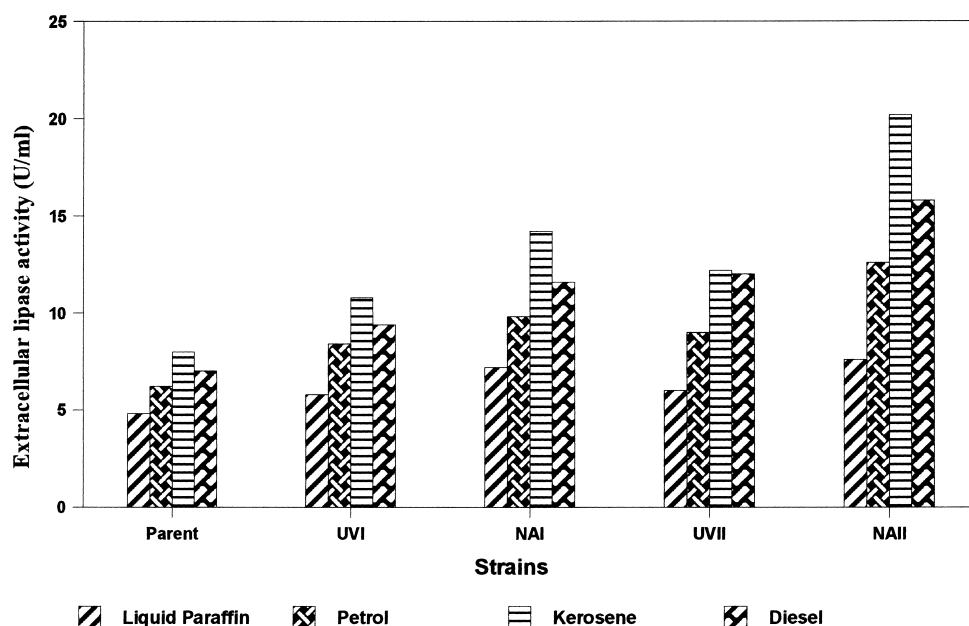


Fig. 1. Effect of hydrocarbons on lipase production by *Aspergillus niger*.

Medium for production of lipase: urea 0.5%; meat extract 1.0%; sucrose 0.5%; KCl 0.05%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; KH_2PO_4 0.1%; Na_2HPO_4 0.3%; hydrocarbon 1.0% (v/v). Culture conditions: fermentation time 48 h; temperature 30°C; agitation 175 rpm.

Table 2. Cumulative effect of olive oil and kerosene on lipase production by parental and NAII strains.

Strains	Olive oil + kerosene (%)	EL (U/ml)	IL (U/ml)
Parent	0.5+0.5	33.49	5.5
	0.5+1.0	33.92	6.64
	1.0+0.5	44.09	8.85
	1.0+1.0	37.73	4.93
NAII	0.5+0.5	33.9	7.48
	0.5+1.0	41.7	4.6
	1.0+0.5	48.03	5.43
	1.0+1.0	44.68	5.3

EL, extracellular lipase activity; IL, intracellular lipase activity.

Medium for the production of lipase: urea 0.5%; meat extract 1.0%; sucrose 0.5%; KCl 0.05%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%; KH_2PO_4 0.1%; Na_2HPO_4 0.3%; olive oil 0.5–1.0% (v/v); kerosene 0.5–1.0% (v/v).

Culture conditions: fermentation time 48 h; temperature 30°C; agitation 175 rpm.

Lipase production by parent strain in 1% (v/v) olive oil, 34.8 U/ml. Lipase production by NAII in 1% (v/v) olive oil, 48.4 U/ml.

NAII, hyperproducing mutant from II step of nitrous acid mutagenesis.

production levels of several proteins are quite low. Strains of *A. niger* have been subjected to different types of mutagenesis for enhancing the production of glucose oxidase (Fiedurek and Gromada, 1997), phytase (Chelius and Wodzinski, 1994), pectinase (Minjares-Carranco et al., 1997) and catalase (Fiedurek and Gromada, 1997). A novel approach has been made to enhance the lipase production from a potent strain of *A. niger*, which shows 35.4 U/ml of lipase activity in optimized production medium (Kamini et al., 1997).

The screening procedure for the selection of mutant strains for enhancing lipase production is carried out in the presence of bile salts, known to inhibit lipase activity, as reported by Naka and Nakamura (1992). Lipase activity is indicated by the formation of halozones around the colonies in media containing tributyrin (Lawrence et al., 1967). In the presence of bile salts and tributyrin, the formation of halozones will be inhibited for the parental strain, but the mutant colonies with enhanced lipase activities will produce halozones. This property will enable lipase producing mutants to be identified effectively. This forms the basis of the selection scheme for a rapid screening of mutant strains of *A. niger* with enhanced lipase production. An amount of 0.5% (v/v) bile salts is found to be suitable as the selective factor, since it has been reported that low

concentrations of bile salts are not totally inhibitory (Benzonana and Desnuelle, 1968).

The induction characteristics of UV and nitrous acid mutagenesis denote a higher efficiency of UV to induce mutagenesis when compared with nitrous acid, as indicated by the percentages of kill values. With another strain of *A. niger* for phytase production, 100% kill at 9.0 min is reported by Chelius and Wodzinski (1994). Our observations of maximum percent kill at an exposure time of 60 min (99.8%) at 5 mg/ml concentration of nitrous acid (Table 1) concur with an earlier finding by Markwell et al. (1989) for glucose oxidase production by *A. niger*. However, enhanced lipase production is observed with mutants produced by nitrous acid mutagenesis, and this could be due to the hydration of the spore suspension before nitrous acid exposure, resulting in increased permeability of the cell to cause effective induction. Moreover, the increase in the percentage of mutants obtained by nitrous acid mutagenesis further substantiates the effectiveness of the chemical mutagen to yield potent mutants.

The hyperproducing mutants are further subjected to a second step of UV and nitrous acid mutagenesis to use hydrocarbons for lipase production. Moreover, this study could enable the mutant strains for possible application in the restoration of an oil-free environment, which is a major aspect of bioremediation. Microbial degradation of petroleum hydrocarbons has been a subject of importance to environmentalists, as reported by Atlas (1991). The use of hydrocarbons has also been shown for the production of amino acids from kerosene (Yamada et al., 1962b) and for lipase production from kerosene (Chen et al., 1993), *n*-paraffins (Chen et al., 1992a), petroleum products (Chen et al., 1992b), and hexachlorocyclohexane (Appaiah and Karanth, 1995). Since then, studies have been taken up to carry out lipase production by using hydrocarbons by the mutant strains. The effect of a combination of olive oil and kerosene on lipase production was cumulative for the parental strain. However, the mutant strain NAll exhibited a negligible effect on lipase production in comparison with enzyme production with 1% (v/v) olive oil alone, presumably because of substrate inhibition. The enhancement of lipase production in the presence of hydrocarbons could be due to the influence of hydrophobicity of the cell surface and the productivity of biosurfactants such as glycolipids or lipopeptides (Georgiou et al., 1992). The increased en-

zyme production by the mutant strains is also attributed to the increased permeability of lipase and the lipase productivity per cell as calculated from the lipase secretion ratios (Chen et al., 1993). Moreover, the enhanced lipase production by the hyperproducing mutant strain, NAll, further confirms the efficiency of nitrous acid mutagenesis.

This study contributes to the development of potent mutants and to the establishment of a novel and economic technique of an application of these mutants in biodegradation, thereby reducing pollution hazards.

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