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Strain Improvement and Characterization of Lipase Produced by *Pseudomonas Sp.*

Research Article

M.B. Sulochana*, Arunasri R, Parameshwar A.B and Mohan Reddy K

Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga 585106, Karnataka, India

***Corresponding author:** M.B. Sulochana, Department of Post Graduate Studies and Research in Biotechnology, Gulbarga University, Gulbarga 585106, Karnataka, India, E-mail: mbsulochana@rediffmail.com

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Abstract

The improvement of microbial strains offers the greatest opportunity for the cost reduction without significant capital investment. The purpose of the present investigation is to enhance the production by subjecting the indigenous lipase producing strain *Pseudomonas aeruginosa RAS-4 to* strain improvement by using UV light as a physical mutagen and immobilization. Strain improvement studies showed that mutation was the best compared to immobilization technique showing 108% higher than the parent strain using UV light as a mutagenic agent. The molecular weight of lipase was determined to be 42 kDa. Localization of the lipase specific gene was carried out by plasmid curing and acridine orange proved to be the best curing agent revealing that the lipase gene is associated with the genomic DNA.

Keywords: Strain improvement, UV, immobilization

Introduction

Lipase enzyme plays a vital role in many food, dairy, leather, paper, pharmaceutical, detergent, textile and cosmetic industries. Lipases are secreted by microorganisms like bacteria, yeasts, molds and a few protozoa. The production of lipase by microorganisms depends largely on the species, strains and culture conditions. Microbial lipases are diverse in their enzymatic properties, substrate specificity and are usually more thermo stable than animal or plant lipases. Research on lipase production has intensified in recent years due to its potential application in various industrial processes. There are several reports on isolation of lipase producing microorganisms and the effect of nutritional factors on their growth and lipase production [1] determined the lipase activity by a rhodamine triglyceride-agarose assay. Several species of Pseudomonas have been reported to produce this enzyme [2]. However, no reports are available on the production of lipase by mutated species of *Pseudomonas*. Therefore, the present study has been undertaken to study the efficacy of lipase production after inducing mutation in *Pseudomonas* sp. and also by immobilization technique. Quantitative enhancement could be obtained by strain improvement as the quantities produced by wild strains are low [3]. Improvement of the commercial applicability of microbial strains has been practiced for centuries [4]. Industrial strain improvement plays a central role in the commercial development of microbial fermentation processes. Meanwhile, strain improvement of the enzyme producer offers the greatest opportunity for cost effective production without significant capital outlay [5].

Materials and Methods

Collection of samples

Sterilized polythene bags were used for the aseptic collection of soil samples contaminated with oil and grease from different areas of oil mills of Gulbarga and Raichur regions. Samples were brought to laboratory and processed within 4 hours.

Isolation of the isolates

Pseudomonas Cetramide agar was used as a selective media for the isolation of Pseudomonas Identification of the colonies was done by the presence of bluish green pigmentation -pyocyanin [6].

Selective medium for the screening of lipase

Tributyrin agar containing 1.3% (w/v) nutrient broth was used

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as selective medium for the screening of lipase producing strains [7] and further screening was done for the rapid detection of lipase on Rhodamine B-Olive oil agar plates containing Rhodamine B .001% (W/V), agar 2%, nutrient broth 1.5% and olive oil 2.5% in distilled water (pH-7). Lipase production was monitored by irradiating plates with conventional UV light at 350 nm [8].

Growth and Enzyme profile

Growth and enzyme profile of the enzyme was carried out using basal medium which consisted of Peptone - 10g, Beef extract - 3g, NaCl - 5g, Olive oil 1%, prepared in 1000 ml distilled water with pH – 8, temperature – 35°C and incubated for 36 h at 150 rpm. After incubation the samples were drawn and centrifuged at 1000 rpm for 10 min at 4°C. Cells were separated and culture filtrate was used for the assay of enzyme activity. The production of lipase and the growth was estimated at regular intervals of 4 h up to 48 h.

Enzyme assay

Lipase activity was measured according to [9], with little modification using p-nitrophenol palmitate (PNPP). One volume of 16.5mM PNPP (2-propanol) and 9 volumes of 50 mM Tris HCl buffer (pH-8) containing 0.4% Triton X-100 and 0.1% gum Arabic. The reaction was started by adding appropriate amount of enzyme solution of 100 µl and absorbance produced due to p-nitrophenol was determined at 410nm.

Phylogenetic analysis

The *Pseudomonas* sp. which showed maximum activity of lipase was characterized up to species level by molecular characterization. The strain was then subjected to 16S rRNA sequencing and was compared with the known sequences. The phylogenetic tree was constructed using the aligned sequences of similar species using Bioinformatics tool (www.ncbi.nim.nih/blast).

Strain improvement

The characterized strain of *Pseudomonas* sp. was subjected for strain improvement by mutational and immobilization studies. It *was* subjected to UV irradiation to induce mutations for the better yield of lipase according to the method of Saha and Bhattacharya [10]. The bacterial suspension of the parent strain was irradiated by using a 15 W Philips UV lamp for different time intervals ranging from 5 min to 30 min at a distance of 5cm to 20 cm away from the UV source. The irradiated suspension was protected from light by covering with black polythene bags in order to minimize any photo reactivation effects. This UV treated mutant isolates were then inoculated on tributyrin agar at 37°C for 24 h and the formation of zone around the colonies were confirmed as lipase production by the mutant strains.

Immobilization

Immobilization was carried out by entrapment of cells using Sodium alginate and calcium chloride [9]. Production of lipase by *Pseudomonas* sp. with the immobilized cells was carried out by using the immobilized beads into a 50 ml of production medium in 250 ml of Erlenmeyer's flask, for 24-48 hours in batch fermentation for the lipase production. For the evaluation of bead stability, immobilization was carried out with different concentrations of sodium alginate from 2- 5% and $CaCl_2$ solution from 0.1 M – 0.4 M. Then the beads were inoculated into the production medium for lipase production and the enzyme activity of free cells and immobilized cells were compared.

Determination of molecular weight of lipase

Molecular weight of lipase was determined by using SDS-PAGE, according to the method of Laemmli (1970). 100 µg of lipase was dissolved in 10 µl of sample buffer 1% SDS, 5% Mercaptoethanol, 0.003% Bromophenol blue and 10% Glycerol in 0.063 M Tris HCl pH 6.8 were boiled at 100°C for 3 min. 12.5% separating gel was poured and allowed it to solidify. 5% stacking gel was poured on separating gel and comb was inserted and allowed to solidify and the sample was electrophoresed with 0.025 M Tris HCl electrode buffer containing glycine and 0.1% SDS. After the electrophoresis the gel was stained with Coomaise Brilliant Blue R-250 and left overnight to visualize the protein bands

Zymogram

The native polyacrylamide gel electrophoresis (PAGE) of lipase was carried out as described by [11]. The electrophoresed gel was placed in petriplate containing 2% agar, 3% olive oil and 0.001% Rhodamine B and was incubated at 37°C for 3-4 h. The lipase activity was detected by observing zone of hydrolysis corresponding to the position of band showing lipase activity.

Determination of lethal dose of curing agents for *Pseudomonas*

Plasmid curing experiment was carried out in order to localize the lipase specific trait in the genome of the *Pseudomonas* sp. using ethidium bromide and acridine orange as curing agents. The lethal dose for *Pseudomonas* sp. was determined, in the nutrient broth containing acridine orange and simultaneously the experiment was run on nutrient agar plates impregnated with acridine orange. 0.1 ml of 20 h old *Pseudomonas* sp. culture was inoculated into 50 ml of sterilized nutrient broth (pH-7) supplemented with different concentrations of curing agents ranging from 1- 10 µg/ml were incubated at 37°C for 24 h and kept on an orbital rotatory shaker at a speed of 150 rpm. The concentration of curing agent at which the *Pseudomonas* sp. grew with 0.25-0.3 at OD- 660 after 24 h of incubation, this was selected as the concentration for the elimination of plasmid from *Pseudomonas* sp.

Results and Discussion

Isolation and characterization of the strain was carried out by following [4], *Pseudomonas* sp. is a gram negative, non-spore forming, non-motile. The microscopic and biochemical tests are as shown in Table 1.

Screening of lipase

The qualitative assay was done for screening of lipase on Tributyrin agar plates and was confirmed by the presence of clear zone of around the colonies as shown in Figure 1. Similar observation was reported by [12]. True lipase producers were screened on Rhodamine B olive oil; the presence of bright orange fluorescence halo around the colony indicated the lipolytic activity.

Citation: Sulochana MB, Arunasri R, Parameshwar AB, Mohan Reddy K. Strain Improvement and Characterization of Lipase Produced by *Pseudomonas Sp.*. J Cell Sci Molecul Biol. 2014;1(1): 101.

Growth and lipase production profile

Growth of the strain began after 4h and reached the maximum up to 48h and then gradually decreased. Simultaneously the production of enzyme started after 8h and reached the maximum at 32h with the enzyme activity of 6 U/ml and there after decreased as shown in Figure 2. These observations corroborate the findings on *B. subtilis* [13], *P. aeruginosa* [1] where maximum lipase activity was produced in the late exponential growth phase and then decreased as the growth decreased. The decline in the extracellular lipase may be due to the increase in oleic acid concentration.

Phylogenetic analysis

The 16S rRNA sequence of *Pseudomonas sp.* was compared with NCBI database and the phylogenetic tree was constructed which is as shown in Figure 3. This strain showed homology of 99.9% *with P. aeruginosa* K3 and was identified and designated as *P. aeruginosa* RAS-4.

Strain Improvement

A potent mutated bacterial strain was isolated and characterized from oil mills and was designated as *P. aeruginosa* RAS-4, which produced maximum enzyme activity of 12 U/ml. The lipolytic activity was screened by rapid plate method and the appearance of clear zone of hydrolysis on tributyrin and orange fluorescence on Rhodamine agar was observed around the colonies. [3] and [5] reported that the improvement of the microbial strain offers the greatest opportunity for the cost reduction without significant capital investment. Survival data of *Pseudomonas* sp. on UV treatment are similar to the results of [15] as shown in Table 2

Immobilization - Effect of sodium alginate concentration

To determine the optimum concentration of sodium alginate as

Table 1: Morphological and Biochemical characteristics of *Pseudomonas* sp.

Test	Result
Gram reaction	Gram –ve
Cell type	Rods
Motility	Motile
Spore	Non spore
Fermentation:	
Glucose	+ve
D-Ribose	+ve
Lactose	-ve
Sucrose	-ve
Starch	-ve
Mannitol	+ve
D-Xylose	-ve
D-Fructose	+ve
L-Arabinose	-ve
Hydrolysis:	
Gelatin liquefaction	+ve
Citrate utilization	+ve
Nitrate reduction	+ve
Catalase	+ve
Oxidase	+ve
Growth at 42.C	+ve

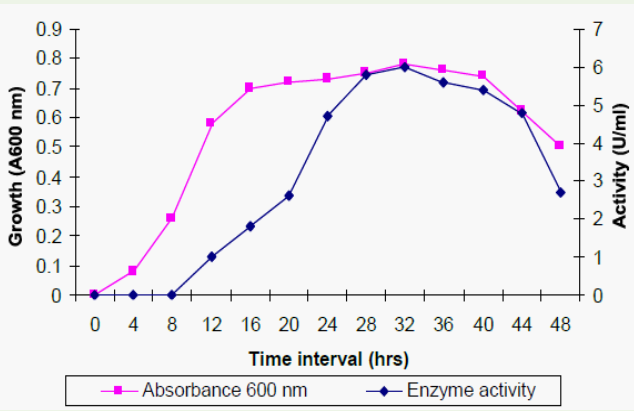

Figure 1: Growth and enzyme profile.

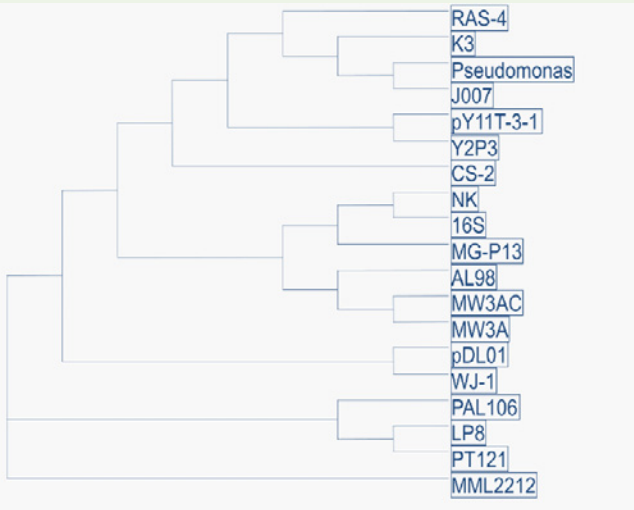

Figure 2: Phylogenetic tree analysis based on the partial sequencing of 16s rRNA.

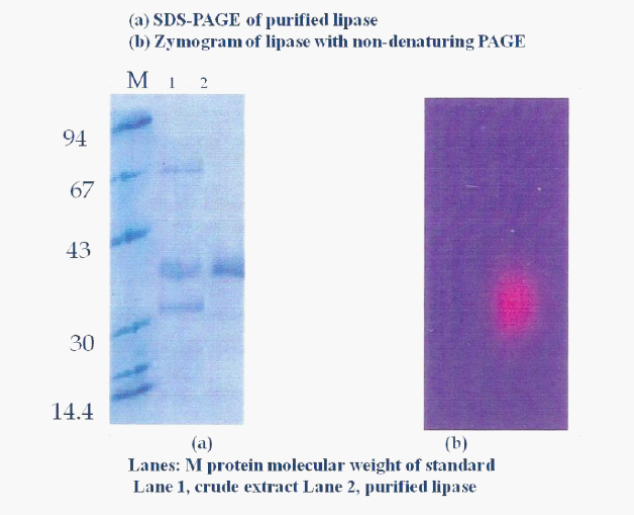

Figure 3: Zymogram *P. aeruginosa* RAS-4.

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Table 2: Survival data for *Pseudomonas* sp. on UV treatment.

Duration of UV light	No. of colonies formed	Survival percentage
0	4000	100
5	19	0.47
10	15	0.32
15	10	0.25
20	6	0.15
25	2	0.05
30	1	0.02

Table 3: Optimization of sodium alginate and Calcium Chloride.

Sl. No.	Sodium Alginate (%)	Beads strength	Sl. No.	Concentration of Calcium Chloride	Beads strength
1.	2	Very weak beads	1.	0.1	No beads
2.	3	Weak beads	2.	0.2	Weak beads
3.	4	Strong beads	3.	0.3	Strong beads
4.	5	Hard beads	4.	0.4	Hard beads

an immobilization matrix, sodium alginate of 2% to 5% were prepared and bacterial suspension was added to it. The beads thus formed were inoculated into the production medium. For the evaluation of bead stability, the immobilization procedure was carried out with different concentrations of $CaCl_2$ solution from 0.1 M – 0.4 M. The beads were then inoculated into the production medium for lipase production and the enzyme activity of free cells and immobilized cells were compared as shown in Table 3.

Determination of Molecular Weight and Zymogram

The molecular weight of the enzyme was determined by SDS-PAGE along with standard markers and the molecular weight of lipase was found to be 42 kDa as shown in Figure 4. The molecular weight of the homogenous lipase was determined about 42 kDa by SDS-PAGE for *Mucor sp*. [17]. The lipase produced by *Pseudomonas aeruginosa* YS -7 showed 40 kDa as reported by [18], a single band was observed in native gel under 350nm UV light that confirmed the homogencity of the lipase and showed olive hydrolysis properties during running the zymogram [19] detected 50 kDa purified lipase from *P. flurosens* by activity staining on olive oil and Rhodamine B similar finding were also reported by [20] from *Mucor meihei, Pseudomonas, Burkholderia multivorans* and *P. aeruginosa* on the gel by zymography.

Localization of Lipase Gene by Plasmid Curing

The localization of the lipase specific trait in *Pseudomonas aeruginosa* RAS-4 genome was made by indirect method of plasmid elimination experiment. 7ug/ml acridine orange was the best concentration and plasmid was eliminated at 8th generation. Based on these results acridine orange was found to be the best curing agent. The lipase activity was not altered when the plasmid of isolate RAS-4 was completely eliminated, based on all the above results it is confirmed that the lipase gene is located in chromosomal DNA of the isolate RAS-4 but not in plasmid. [21] Used acridine orange, ethidium bromide and SDS as a curing agent on phenotypic stability in *Pseudomonas putida* degrading E-carpolactum. Curing analysis of *P. aeruginosa* was carried out by ethidium bromide, acridine orange, novobiocin and SDS as per the reports of Edward Raja and Selvam [22].

Summary

A rapid plate method was done for screening of lipase on Tributyrin agar plates and was confirmed by the presence of clear zone of hydrolysis of 4.2cm on tributyrin and orange fluorescence on Rhodamine agar. 16S rRNA showed 99.9% homology with *Pseudomonas aeruginosa* K3 strain and consequently the isolate was identified and designated as *Pseudomonas aeruginosa* RAS-4. Strain improvement studies showed, mutation was the best when compared to the immobilization technique showing 108% higher yield than the parent strain using UV light as a mutagenic agent. The molecular weight of lipase from non-mutated was determined to be 42 kDa. L ocalization of lipase specific gene was carried out by plasmid curing and acridine orange proved to be the best curing agent revealing that the lipase gene is associated with the genomic DNA. Isolation of *Pseudomonas aeruginosa* RAS-4 a potential strain producing lipase is an important contribution, however, the mutant strain RAS-4 isolated from parent is comparatively efficient producer of lipase.

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