MOLECULAR STUDIES ON LIPASE OF PSEUDOMONAS FLUORESCENS LP1 AND ITS APPLICATION IN BIODIESEL PRODUCTION

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ABSTRACT

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Microbial lipases are commercially significant and used for biodiesel production. Vegetable oil (m) ethyl esters, commonly referred to as "biodiesel", are alternative to diesel fuels. In the present study, extracellular lipase of *Pseudomonas fluorescens* Lp1 isolated from edible oil contaminated soil has been investigated at molecular level and utilized for biodiesel production. The production of extracellular lipase from Pseudomonas fluorescens Lp1 was carried out in the production medium with olive oil (1%) as the carbon and inducer for the enzyme production. Lipase gene, *lipA* gene was amplified, sequenced and predicted using bioinformatics tools. For biodiesel production, the crude lipase of *Pseudomonas fluorescens* Lp1 was immobilized onto calcium alginate beads for transesterification of olive oil and waste cooking oil into biodiesel. The transesterification product of Fatty acid methyl ester (FAME) was analyzed by thin layer chromatography. The results of the investigation revealed the potential of lipase of *Pseudomonas fluorescens* Lp1 utilized in biodiesel production.

Keywords: *Pseudomonas*, Biodiesel, Lipase gene, Sequencing

I. INTRODUCTION

Lipases (triacylglycerol acyl ester hydrolases; EC 3.1.1.3) are biocatalysts that hydrolyse long chain triglycerides at the water/oil interphase to yield free fatty acids, monoglycerides, diglycerides and glycerols (1). Lipases are widely distributed in nature and found in many species of plants, animals, bacteria, yeast and fungi. Although their wide distribution, the enzymes from microorganisms are most interesting because of their potential application in various industries ranging from laundry detergent to stereo specific biocatalyst (2). The *Pseudomonas lipases* constitute a major group; they have been reported from *P. aeruginosa*; *P. fluorescens, P. glumae* and other *Pseudomonas* sp. (3, 4).

Vegetable oil and animal fat (m) ethyl esters, commonly referred to as "biodiesel", are prominent candidates as alternative Diesel fuels (5). Biodiesel can be processed from any type of vegetable oils and animal fats:

1. food grade vegetable oils, such as soybean, canola, palm, sunflower and peanut;
2. animal fats, such as lard, tallow, chicken fat and fish oils and
3. used cooking oils from restaurants.

Biodiesel is technically competitive with or offers technical advantages compared to conventional petroleum Diesel fuel. Besides being a renewable and domestic resource, biodiesel reduces most emissions while engine performance and fuel economy are nearly identical to those of conventional fuels. Alternative fuels and propulsion systems have the potential to solve many of the current social problems and concerns, from air pollution and global warming to other environmental improvements and sustainability issues (6, 7). Immobilization provides convenient handling of the enzyme, provides for its facile separation from the product, thereby minimizing or eliminating protein contamination of the product. Immobilization also facilitates the efficient recovery and reuse of costly enzymes, and enables their use in continuous, fixed-bed operation. A further benefit is often enhanced stability under storage and operational conditions (8).

Since the bacterial lipases owe many applications in various fields, there is a need of isolating new strains that produce lipase in higher levels. Hence the current study is focused on isolation, screening and identification of such lipase bacteria from soil contaminated soils. In order to apply the organism in

biotechnological field, there is a need to study the organism at the molecular level. Hence the molecular study which includes lipase gene amplification, sequencing of the gene and protein sequence prediction was performed. The lipase from *Pseudomonas fluorescens* Lp1 was immobilized on calcium alginate for esterification of waste cooking oil and olive oil for biodiesel production.

II. MATERIALS AND METHODS

Microorganism and lipase production

Pseudomonas fluorescens **Lp1,** isolated from edible oil contaminated soil showed good lipolytic activity on Tween agar (Peptone - 10 g; NaCl - 5 g; $CaCl_2$ - 0.01 g; Agar -20 g; Tween - 10 ml; Distilled water - 1000 ml; pH - 7.5) was used in this study and cultivated in production medium composed of peptone (0.5%), yeast extract (0.5%), NaCl (0.05%), $CaCl_2$ (0.005%), and olive oil (1.0%, emulsified with gum acacia 0.5% w/v), pH 8.5. The production broth was inoculated with seed culture (3.0% v/v) and incubated at 40$C under shaking (150 rpm, 48 hrs) conditions. Culture broth was harvested by centrifugation (10,000g, 10 min at 4$C). The enzyme activity was determined in the cell-free broth thereof. Lipase activity was assayed by the photometric method of (9).

Isolation of genomic DNA and amplification of lipase (*lipA*) gene

Genomic DNA from *Pseudomonas fluorescens Lp1 was isolated by Cetyltrimethyl Ammonium Bromide (CTAB) method (10). The primers for amplification of lipase gene was designed through NCBI Primer - BLAST online tool based on the template of preexisting complete sequences of Pseudomonas fluorescens* lipase gene. The primers used were: 5'-TCCCACAGGGGGAGATTTGCAA-3'-forward primer and 5'- GATGTTGTCGGTGGCCGATTCC-3' - reverse primer. The polymerase chain reaction (PCR) amplification was carried out (Eppendorf Master cycler, thermocycler) with the cycling conditions as follows: Initial denaturation for 94°C for 2 minutes single cycle; 30 cycles of denaturation at 94°C for 1 minute; Annealing of primers at 51°C for 35 seconds, extension at 72°C for 90 seconds and single step final extension at 72°C for 3 mins.

Gene sequencing

The purification of the amplified product was carried out using QIAQuick (Qiagen) spin column. The direct gene sequencing was carried out by the method of (11) using DTCS quick start Dye terminator kit (Beckman Coulter). The removal of unbound dye and nucleotides from cycle sequenced product was carried out using DyeEx spin columns (Qiagen). The purified samples were sequenced in CEQ8000 auto analyzer, Beckman Coulter Inc.USA.

Sequence analysis

The obtained *lipA* gene nucleotide sequence was subjected to BLAST (Basic Local Alignment Research tool) analysis with online tool with the preexisting sequence available in NCBI/Genbank to confirm the sequence. The sequence was submitted in GenBank, NCBI through 'Sequin' submission tool and accession numbers was obtained. The *lipA* nucleotide sequence of *Pseudomonas fluorescens* Lp1 was aligned with lipase sequences of various *Pseudomonas* spp. obtained from GenBank, NCBI using Clustal-X Ver.2.0 aligning tool. The phylogenetic analysis was carried out using MEGA (Molecular Evolutionary Genetic Analysis) Ver.4.0 (12).The obtained lipase gene sequence was subjected to translation and open reading frame (ORF) prediction analysis by ORF finder online tool (http://www.ncbi.nlm.nih.gov/projects/gorf/).

Immobilization by Entrapment

Immobilization with calcium alginate is an entrapment method in which the enzyme solution was mixed with 2% sodium alginate in the ratio of 1:1 (v/v).The mixture was slowly dropped into 10 ml 0.1M $CaCl_2$ under continuous stirring using a Pasteur pipette. This resulted in the polymerization of the alginate to form calcium alginate gel beads of about 3-5 mm diameter were formed within 3 to 5 min. Gel beads with entrapped enzyme were collected by filtration through a sieve. The total weight of the gel beads obtained was measured, and this was taken as the amount of beads having the total activity of the 1 ml enzyme used for immobilization (if 1ml enzyme solution was added with 1 ml of sodium alginate). The beads were kept in $CaCl_2$ solution until used. The activity of bound enzyme and unbound lipase remaining in the calcium chloride solution used for immobilization was measured.

Production of biodiesel catalyzed by the lipase

The enzymatic bioconversion of waste cooking oil and olive oil separately into biodiesel was conducted as follows. Oil (7.89 ml) and methanol were put in a glass-capped tube (30 ml). Then, immobilized lipase (1.0 g corresponding to 68.0 U of activity by pNpp spectrophotometric assay) was added to the reaction mixtures, and the conversion reaction was conducted for 48 hrs at 40°C with rotatory shaking (220 rpm). 0.33 ml each of methanol was added to the tube three times at 0, 12, and 24 h of reaction. After incubation 200 ìl of reaction mixture was mixed with 1 ml hexane for 2 min. This was centrifuged, and then 10 ìl of the upper layer was applied to a silica gel plate with the standards. As a developing solvent, hexane/ethyl acetate/acetic acid (90:10:1) was utilized, and methanol/sulfuric acid (1:1) was used as a color reagent. After spraying color reagent over the silica gel plate after development, the plate was heated at high temperature and analyzed.

III. RESULTS AND DISCUSSION

Among the total 32 bacterial isolates, 11 showed positive results towards lipase production by producing white precipitate around the colony. The isolate that showed comparatively more precipitation zone was considered as most potent strain and selected for the present study. The maximum lipase activity produced by the strain was about 68.0 U/ml was observed after 48 h of fermentation. Based on biochemical, cultural, and morphological characteristics, the isolate was identified as *Pseudomonas fluorescens* and designated as strain Lp1. Lipase-producing psychotropic bacteria belong to the genus *Pseudomonas* was isolated from soil (13 14, 15).

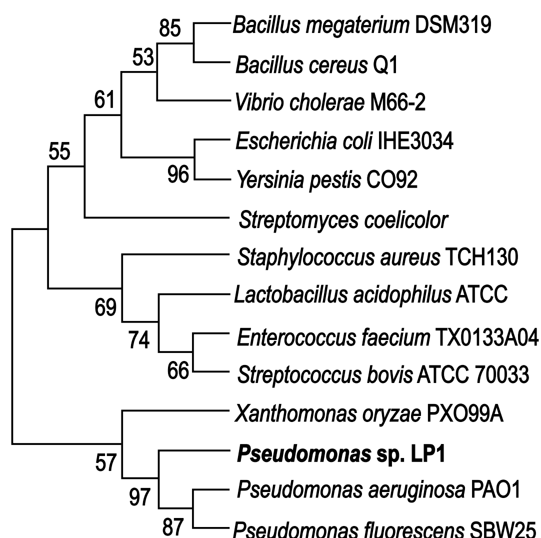

Fig. 1. Gene phylogeny of *Pseudomonas fluorescens* Lp1

FOOT NOTE

Bacillus cereus Q1(NC_011969), Bacillus megaterium DSM319 (CP0019820), Enterococcus faecium TX0133a04 (NZ_AEBC01000198), Escherichia coli IHE3034 (CP001969), Lactobacillus acidophilus ATCC 4796 (NZ_ACHN01000051), Pseudomonas aeruginosa PAO1 (NC_002516), Pseudomonas fluorescens SBW25 (NC_012660), Staphylococcus aureus TCH130 (NZ_ACHD01000004) Streptococcus bovis ATCC 700338 (NZ_AEEL01000009) Streptomyces coelicolor (AF009336) Vibrio cholerae M66-2 (NC_012578), Xanthomonas oryzae PXO99A (NC_010717) Yersinia pestis CO92 (NC_003143)

The DNA isolation was made from *Pseudomonas fluorescens* **Lp1 confirmed in agarose gel electrophoresis. The amplification of** *lipA* was made through PCR and the product in the agarose gel was around 1kb in size. The amplified products were sequenced and the sequence consisted of about 981 base pairs. The BLAST analysis showed 100% of identities with complete coding sequences of *lipA* gene and the gene phylogeny showed 97% similarity with lipase nucleotide sequences of *Pseudomonas* spp. (Figure.1) which have been already submitted in the GenBank and the accession number for *lipA* gene HQ 594465 and. The ORF finding result revealed that among the total 981 base pairs sequenced, the ORF started from 81th base pair with methionine as initiation codon (Figur.2). The total ORF showed around 900 base pairs in size, coding for 300 amino acids. There was no termination codon within the ORF, so the sequence was considered as a partial sequence of *lipA*. **The lipase gene of** *P.fluorescens* SIK W1 consisted of an open reading frame 1347 base pairs long commencing with an ATG start codon encoding a polypeptide of 499 amino acid residues and a TGA stop codon (16). Nucleotide sequence analysis of *P.fluorescens* HU380 revealed an open reading frame of 1854 bp encoding the lipase and its amino acids sequence deduced (17). Lipase gene of *Pseudomonas fluorescens* B52 was isolated directly from the genomic DNA of with the genome-walking method, an effective method for isolating lipase gene from bacteria. There

was an open reading frame (ORF) of 1854 base pairs, which encoded 617 amino acids (18). The *lipA* gene encoding an extracellular lipase was cloned from the wild-type strain of *Serratia marcescens* Sr41. Nucleotide sequencing showed a major open reading frame encoding a 64.9-kDa protein of 613 aminoacid residues; the deduced amino acid sequence contains a lipase consensus sequence, GXSXG (19).

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    aaatgctccggggcgtctcgcgaggggcgctctggctgtgagctatct
    ctgacaattc caacaaaaga gaggcaatag ca

 81 atgggtgtgtatgactacaaaaacttcggcacggcggattccaag
     M  G  V  Y  D  Y  K  N  F  G  T  A  D  S  K 15
126 gcgttgttcagcgatgccatggcgatcacgctgtattcctaccac
     A  L  F  S  D  A  M  A  I  T  L  Y  S  Y  H 30
171 aacctcgataacggttttgccgccggttatcagcacaacggtttt
     N  L  D  N  G  F  A  A  G  Y  Q  H  N  G  F 45
216 ggccttggcctgccggcgacgctggtcacggcgttgctcggcggt
     G  L  G  L  P  A  T  L  V  T  A  L  L  G  G 60
261 accgattcccagggcgtcatccccggcattccgtggaatcccgat
    T  D  S  Q  G  V  I  P  G  I  P  W  N  P  D  75
306 tcggaaaaactcgccctcgaagccgtgaaaaaggccggctggacg
     S  E  K  L  A  L  E  A  V  K  K  A  G  W  T 90
351 ccgatcacggcctcgcaactgggctacgacggcaagaccgacgca
     P  I  T  A  S  Q  L  G  Y  D  G  K  T  D  A 105
396 cgcggaaccttctttggcgagaaggccggttactcgacagcgcag
     R  G  T  F  F  G  E  K  A  G  Y  S  T  A  Q 120
441 gtcgagattctcggcaagtacgacgcccagggccatctcacagaa
     V  E  I  L  G  K  Y  D  A  Q  G  H  L  T  E 135
486 atcggcatcgcctttcgcggcaccagcggcccgcgcgagaacctg
     I  G  I  A  F  R  G  T  S  G  P  R  E  N  L 150
531 atccttgattccatcggcgacgtgatcaacgacttgctcgccgcg
     I  L  D  S  I  G  D  V  I  N  D  L  L  A  A 165
576 ttcggccccaaggattacgccaagaactacgtcggcgaagcgttc
     F  G  P  K  D  Y  A  K  N  Y  V  G  E  A  F 180
621 ggcaacctgctcaatgacgtggtcgcctttgccaaggccaatggc
     G  N  L  L  N  D  V  V  A  F  A  K  A  N  G 195
666 ctcagcggcaaggacgtgctggtcagcggccacagcctcggcggg
     L  S  G  K  D  V  L  V  S  G  H  S  L  G  G 210
711 ctggcggtcaacagcatggcggatttgagcggcggcaagtggggc
     L  A  V  N  S  M  A  D  L  S  G  G  K  W  G 225
756 gggttcttcgccgactccaactacatcgcctatgcctcgccgacc
     G  F  F  A  D  S  N  Y  I  A  Y  A  S  P  T 240
801 cagagcagcaccgacaaagtgctcaacgtcggctacgagaacgac
     Q  S  S  T  D  K  V  L  N  V  G  Y  E  N  D 255
846 ccggtgttccgcgccctcgacggttcgaatttcaccggcgcctcg
     P  V  F  R  A  L  D  G  S  N  F  T  G  A  S 270
891 attggcgtgcacgacgcgccgaaggaatcggccaccgacaacatc
     I  G  V  H  D  A  P  K  E  S  A  T  D  N  I 285
936 gtcagcttcaacgatcactacgcctcgacggcgtggaatctgctg
     V  S  F  N  D  H  Y  A  S  T  A  W  N  L  L 300
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Fig. 2. Open reading of frame of lipase gene of *Pseudomonas fluorescens* Lp1

" Left side numbers shows the nucletide count
" Right side numbers shows aminoacid count

Physical entrapment in a polymeric gel microsphere is by far the most commonly used technique for enzyme immobilization and true successes may be limited to the problems associated to the mass transfer resistance imposed by the fact that the substrate has to diffuse to the reaction site and mechanical stability of the carriers in bioreactors (20). Lipase immobilization by entrapment is based on low porosity of matrix which at the same time retains enzyme within the carrier and provides substrate/or products diffusion (21). In the present study the entrapment of lipase was established in calcium alginate beads of 3- 5 mm diameter size. The activity of bound enzyme and unbound lipase remaining in the calcium chloride solution used for immobilization was measured to give the efficiency of immobilization which was found to be 48 %.

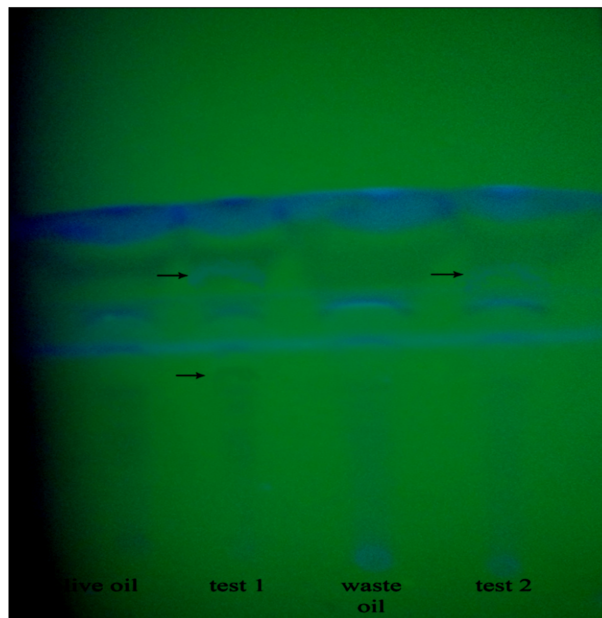

Fig. 3. Thin layer chromatography showing bands of biodiesel production under UV light

FOOT NOTE

Lane 1: Control olive oil; Lane 2: Test 1;
Lane 3: Control waste oil; Lane 4: Test 2

The biodiesel production was analyzed by thin layer chromatography. The bands developed on the chromatogram (Figure.3) indicated the production of fatty acid methyl esters through by transesterification process. Olive oil (Test 1) results in the formation of two bands whereas waste oil (Test 2) resulted in the formation of single band indicated the production of biodiesel. This GC method was developed especially for the confirmation of the components in biodiesel produced from waste cooking oil at the catalysis of lipase. Peak identification was achieved by comparison with chromatograms of standard substances. Biodiesel production with 51.5% and 60% yields were achieved by free and immobilized lipase respectively. Immobilized lipases were known to give better transterification activities in the organic solvents (22). This might be due to larger surface area of the immobilized lipase. However, free lipases were known

to have mass transfer problem since these form aggregates in low water media. Methanol-tolerant lipase of *Photobacterium lipolyticum* lipase (M37) M37 has been shown to be a suitable enzyme for use in the biodiesel production process (23). The transesterification activity of the immobilized lipase from *P. aeruginosa* LX1 indicated that it is a potential biocatalyst for biodiesel production (24).The study revealed the ability of *Pseudomonas fluorescens* **Lp1in biodiesel production in immobilized state.**

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