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Original Research Article

In Silico analysis of Lipase gene (*Lip A*) of wild and mutated strains of *Pseudomonas* sp. isolated from oil contaminated soil

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A B S T R A C T

Extracellular lipase producing wild strain *Pseudomonas* sp. Lp1 isolated from edible oil contaminated soil was subjected to physical mutagenesis by ultraviolet radiation and chemical mutagenesis by Nitrous acid and Ethyl Methane Sulphonate to enhance the lipase production abilities. Among 18 mutants, high lipase yielding strain *Pseudomonas* sp. Lp6 and wild strain *Pseudomonas* sp. Lp1 were subjected to further molecular study. In order to analyse the lipase gene, *lipA* gene was PCR amplified, sequenced and predicted. The obtained sequences were subjected to BLAST (Basic Local Alignment Research tool) analysis for similarity search with the preexisting sequence available in NCBI/Genbank to confirm the sequences. The BLAST result showed 100% identity with complete coding sequences of *lipA* gene which have been already submitted in the GenBank/NCBI. The sequenced *lipA* gene amplified was 981 base pairs in size, the Open reading Frame (ORF) started from 81th base pair with methionine as start codon. The total ORF was 900 bps in size coding for 300 amino acids. There was no termination codon within the ORF so the sequence was considered as partial sequence. The mutational analysis was done by aligning the nucleic acid and amino acid sequences of wild and mutated strain using Clustal-X Ver.2 aligning tool and viewed by Bioedit Ver 7.0. The results showed the occurrence of random point mutation in five places which has made both transition and transversion changes in the base pairs. In the nucleic acid mutation, the transition occurred in the nucleotide positions of 202, 373 and 673 and the respective amino acid transcription also changed. Transversion occurred in the nucleotide positions of 82 and 793. In this present study, the transition mutation has occurred in higher level.

K e y w o r d s

Lipase, Mutation, *Pseudomonas*, strain improvement, *LipA* gene

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 (Saxena et al,1999). 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 (Yadav et al, 2004). The *Pseudomonas* lipases constitute a major group; they have been reported from *P. aeruginosa*; *P. fluorescens*, *P. glumae* and other *Pseudomonas* sp. (Ihara et al,1991; Kumura et al 1998). 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. Strain improvement is an essential part of process development for fermentation products. Developed strains can reduce the costs with increased productivity and can possess some specialized desirable characteristics and such improved strains can be obtained by mutation (Rowlands et al,1984). The increase in demand of lipases in various fields made the researchers to improve the production abilities of the strains by mutation and selection (Bapiraju et al, 2004; Karanam et al, 2008).

The aim of the present study is to enhance lipase productivity of the wild strain *Pseudomonas* sp. Lp1 isolated from edible oil contaminated soil by subjecting it to improvement by mutagenesis through physical mutagenesis by ultraviolet radiation and chemical mutagenesis by HNO_2 and Ethyl Methane Sulphonate. The lipase gene of both wild and mutated strain was sequenced to obtain comparison of mutation of lipase gene and lipase coding protein sequences of wild and mutated strains.

Materials and Methods

Microorganism and lipase production

Pseudomonas sp. Lp1, a wild strain, 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 Winkler & Stuckmann (1979).

Physical mutagenesis

Over night broth culture of *Pseudomonas* sp. Lp1 was transferred to a sterile Petridish and subjected to different dosage of Ultra Violet (UV) rays irradiation using Germicidal lamp emitting UV rays (254 nm) at the distance of 15 cm for various periods such as 5 min, 10 min, 15 min, 20 min, 25 min and 30 minutes. One ml of unexposed culture was kept as control. The exposed cultures were stored overnight at dark condition for photo-reactivation. Next day the culture was plated on the nutrient agar medium and incubated at 40^oC for 24 - 48 hrs. Colonies on the plates were observed and strains from each plate was tested for lipase activity and selected based on lipase yield.

Chemical mutagenesis

HNO_2 (Nitrous acid) mutagenesis was performed by adding 9 ml of *Pseudomonas* sp. Lp1 culture to 1 ml of stock solution (0.01M sodium nitrite) and kept at room temperature for various time periods as 10min, 20min, 30min, 40min, 50min and 60 minutes. After incubation, 0.5 ml of phosphate buffer was added to the culture

and neutralized with 0.5 ml of 0.1M sodium hydroxide. 0.1 ml of exposed suspension was plated on sterile nutrient agar plates and incubated at 40^{o}C for 24 - 48 hrs. The isolates from each plate was tested for lipase activity and selected based on lipase yield. The mutagenesis with Ethyl Methane Sulfonate (20 mM of Ethyl Methane Sulfonate - 2%, 4%, 6%, 8%, and 10%) was also performed as described.

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

Genomic DNA from the strains *Pseudomonas* sp. Lp1 and *Pseudomonas* sp. Lp6 was isolated by Cetyltrimethyl Ammonium Bromide (CTAB) method (Azadeh et al, 2009). 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'-TCCCACAGGGGGAGATTTGC AA-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 Sanger et al., (1977) 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 sequences were subjected to BLAST (Basic Local Alignment Research tool) analysis with online tool with the preexisting sequence available in NCBI/Genbank to confirm the sequences. The sequences were submitted in GenBank, NCBI through 'Sequin' submission tool and accession numbers were obtained. The *lipA* nucleotide sequence of *Pseudomonas* sp. 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 (Nei and Kumar,2000). The obtained lipase gene sequence was subjected to translation and Open Reading frame (ORF) prediction analysis using ORF finder online tool (http://www.ncbi.nlm.nih.gov/projects/gorf/). The wild and mutated gene and amino acid sequences were aligned using Clustal-X Ver.2.0 aligning tool to compare the sequences and locate the mutated positions and viewed by Bioedit ver.7.0.5 software.

Results and Discussion

Pseudomonas sp. Lp1, a wild strain isolated from soil contaminated with edible oil subjected to mutagenesis, yielded a total of 18 mutants such as *Pseudomonas* sp. Lp2 - Lp7 through UV, Lp8 - Lp13 from HNO_2 and Lp14 - Lp19 from Ethyl Methane Sulphonate. Among mutants, *Pseudomonas* sp.Lp6 of UV mutant produced lipase more

than the wild strain was selected and subjected to molecular studies along with the wild strain. *Pseudomonas* sp. Lp1 produced 68.0 U/ml of lipase after 48 hrs of fermentation whereas the mutant *Pseudomonas* sp. Lp6 produced 115.6 U/ml of lipase which was 1.7 folds greater lipase activity than the wild strain.

Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, γ-rays, UV rays, *etc.*, and chemical mutagens such as NTG, EMS, etc. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Parekh et al, 2004). UV light has been shown to be lethal and mutagenic in a variety of organisms, including fungi. The correlation between the quantity of energy absorbed by DNA and the observed biological effects (survival and mutation frequency) are illustrated in the wavelength region between 254 and 320 nm. A *Pseudomonas* mutant generated by UV, HNO_2 and NTG reported an increase in lipase production of 3.25-fold (Caob et al, 2000). A 200% increase in lipase yield by *Aspergillus niger* mutant from UV and NTG treatments (Ellaiah et al, 2002). A 276% increase in lipase production was achieved by strain improvement of *A. japonicus* by induced mutations employing UV, HNO_2 and NTG (Karanam et al, 2008). The UV and NTG were effective mutagenic agents for strain improvement of *Rhizopus* sp. BTS-24 for enhanced lipase productivity (Bapiraju et al, 2004).

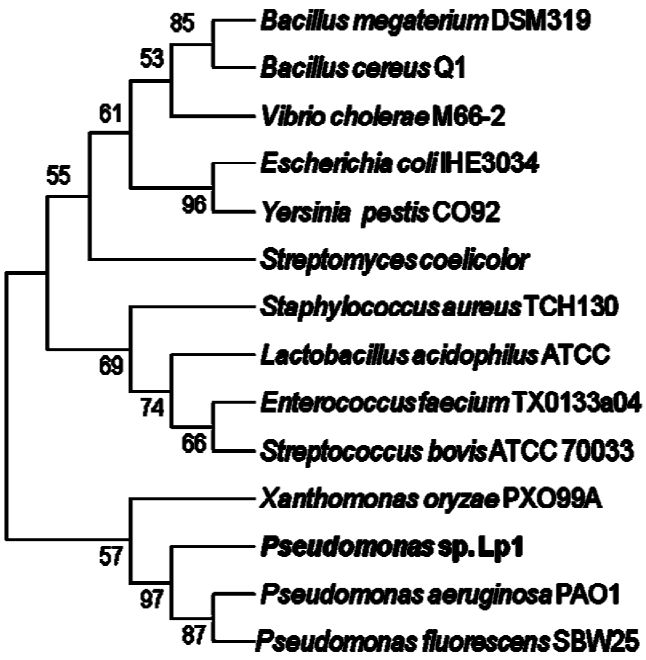
The DNA isolation was made from *Pseudomonas* sp. Lp1 and *Pseudomonas* sp. Lp6 and confirmed in agarose gel electrophoresis. The amplification of *lipA* was made through PCR and the product in the agarose gel was around 1kb in size. The amplified products were sequenced and the sequence consisted of 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**.** The accession numbers for *lipA* gene of wild and mutated strain were HQ 594465 and HQ 594466 respectively. The ORF finding result revealed that among the total 981 base pairs sequenced, the ORF started from 81^{th} 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 (Chung et al, 1991). Nucleotide sequence analysis of *P.fluorescens* HU380 revealed an open reading frame of 1854 bp encoding the lipase and its amino acids sequence deduced (Kojima et al, 2003). 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 (Jiang et al, 2003). 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 (Akatsuka et al, 1994). In the present study the mutational analysis was done by aligning the nucleic acid and amino acid sequences of wild and mutated strain. The results showed the occurrence of random point mutations in five places with both transition and transversion changes in the base pairs (Table.1). In the nucleic acid mutation, the transition has occurred in the nucleotide positions of 202, 373 and 673 and the respective amino acid transcription also has been changed. Transversion has occurred in the positions of 82 and 793. However, in this present study, the transition mutation has occurred in higher level (Figure 3).

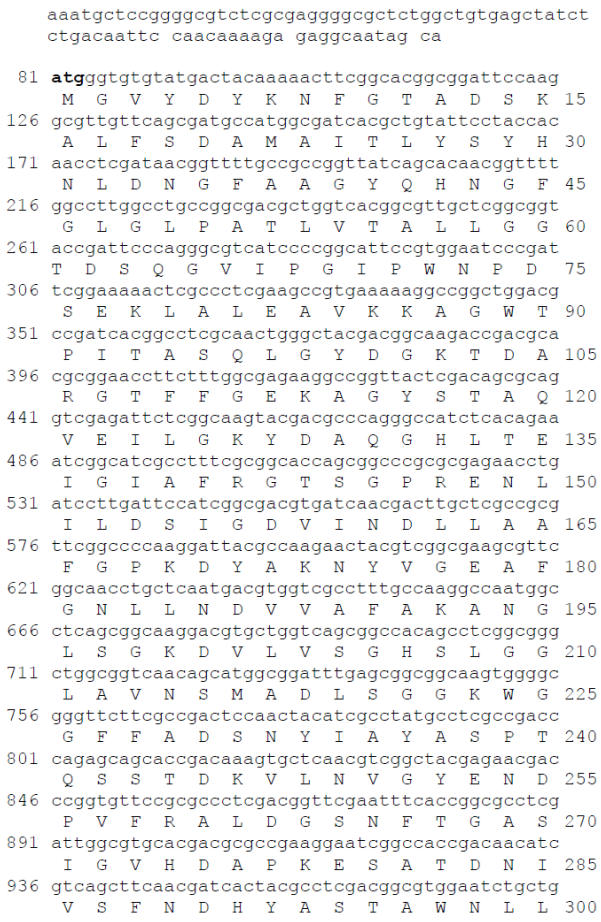
The MTase-deficient *E. coli* displayed increased G:C to A:T and A:T to G:C transitions (10- and 3-fold, respectively) and increased G:C to C:G, A:T to C:G, and A:T to T:A transversions (10-, 2.5-, and 1.7-fold, respectively) ada and ogt single mutants did not suffer elevated spontaneous mutation rates for any base substitution event, and the cloned ada and ogt genes each restored wild-type spontaneous mutation rates to the ada ogt MTase-deficient strains (William et al, 1994).

Figure.1 Taxonomic position of *Pseudomonas* sp. Lp1 lipase gene



Genbank accession details of comparative strains: *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)

Figure.2 Open reading of frame of lipase gene of *Pseudomonas* sp. Lp1

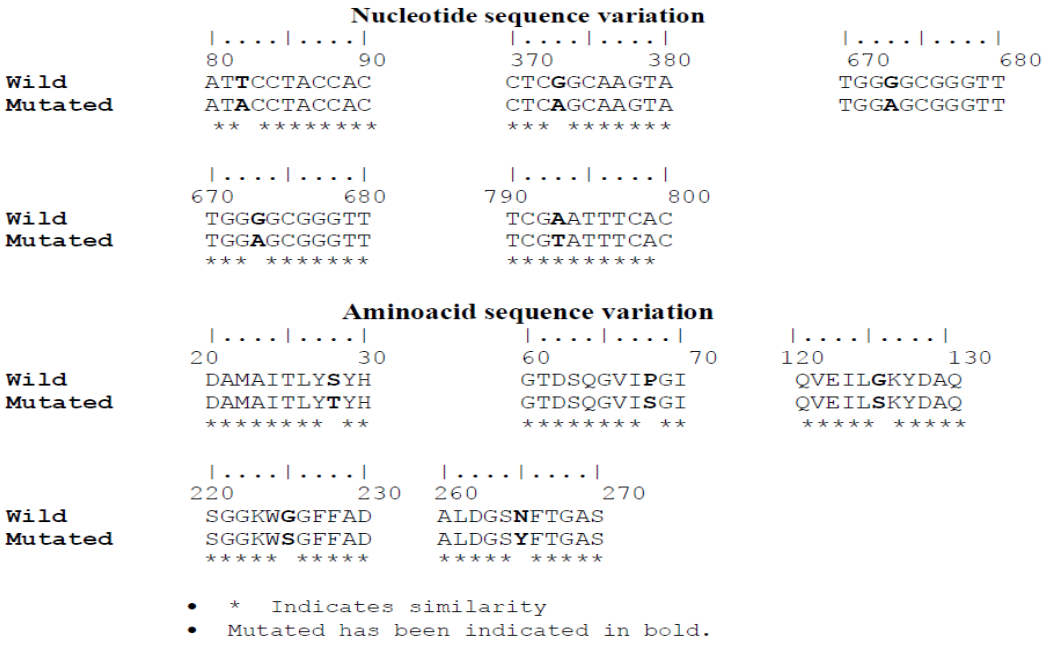


*****Left side numbers indicates the nucleotide count*
** Right side numbers indicates the amino acid count*

Table.1 Nucleotide and amino acid variations between *Pseudomonas* sp. Lp1 and *Pseudomonas* sp.Lp6 in *LipA* genes

Nucleic acid Base pair changes			**Amino acid changes**		
Nucleotide position	***Pseudomonas* sp. Lp1**	***Pseudomonas* sp. Lp6**	**Amino acid position**	***Pseudomonas* sp. Lp1**	***Pseudomonas* sp. Lp6**
82	T	A	28	S (Serine)	T (Threonine)
202	C	T	69	P (Proline)	S (Serine)
373	G	A	125	G (Glycine)	S (Serine)
673	G	A	225	G (Glycine)	S (Serine)
793	A	T	265	N (Asparagine)	Y (Tyrosine)

Figure.3 Comparison of lipase gene and aminoacid sequences of *Pseudomonas* sp. Lp1 and *Pseudomonas* sp.Lp6



A high lipase yielding mutant *Pseudomonas* sp.Lp6 was derived from wild *Pseudomonas* sp.Lp1 through UV mutagenesis. The lipase gene analysis showed 981 base pairs, the ORF started from 81th base pair with methionine codon. The total ORF showed around 900 base pairs in size coding for 300 amino acids. The mutational analysis revealed the occurrence of the transition in the nucleotide positions of 202, 373 and 673 and the transversion in the positions of 82 and 793 and respective amino acid transcription also has been changed. Further studies are aimed to scale up the lipase production with the mutant.

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