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Non-Recombinant Mutagenesis of *Bacillus mojavensis CUIE1819* for Hyper Production of Lipase and Treatment of Polluted Lakes

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Abstract

Microorganisms that degrade oil contribute significantly to the bioremediation of polluted lakes. Many microorganisms synthesize lipases, which are commercially significant. In the present study microorganisms producing extracellular lipase were isolated from various polluted lakes in Bangalore by using tributyrin agar. A lipase assay was done to determine the most efficient lipase-producing organism, which was then named *Bacillus mojavensis* CUIE1819 based on 16srRNA sequencing. After UV irradiation, the selected immobilized organisms were used to treat the lake water samples.

Keywords: Microbial Lipase, screening, production, Lipase assay, oil degradability, mutagenesis

Introduction

Microorganisms, because of their great plasticity and physiological flexibility, are ubiquitous. They have developed the ability to adapt and to survive in different environmental conditions, even in the most inhospitable conditions making them almost invincible and the underlying reason being the efficient enzyme system. Their ability to produce extracellular enzymes is of great significance for their survival adaptability in any niche. Many microorganisms, with the help of their diverse extracellular enzyme mechanisms, have found ways to degrade toxic chemicals and compounds into safe, tolerable, and useful products, and thus play a major role in bioprospecting.

A study, titled 'Wetlands: Treasures of Bangalore (Abused, Polluted, Encroached and Vanishing)', conducted and led by Professor Ramachandra T V from the Energy and Wetlands Research Group, Centre for Ecological Sciences, IISc, Bangalore, surveyed 105 lakes in the city and found that only four lakes seemed to be in a good condition while 25 lakes were in a very bad state fully covered with macrophytes or dumped with solid or liquid wastes and with little or no water. Sustainedflow of untreated sewage, the release of untreated industrial effluents, and the dumping of solid waste and building debris were found to be the main causes of lake pollution. (1)

Microorganisms that are living in such lakes may have developed the ability to sustain themselves in such an environment by releasing certain proteins or enzymes that help to degrade the substances that inhibit their growth. By isolating such organisms, we may be able to find ways to obtain such enzymes/ proteins or couture a mixture of organisms to degrade the harmful substances and revive the lake back to its ecological health. (2)

The effluents rich in oils released into

the lakes can also be degraded by enzymes such as lipases that are extracellularly released by microorganisms to prevent the decrease in the dissolved oxygen content and maintain a sustainable environment.

Isolation of extracellular lipase producing organism has always gained a lot of importance due to its various biotechnological importance and application in production such as detergents, textile, and dairy industries; oil processing; production of surfactants; synthesis of chiral pharmaceuticals. There is an interest to find new lipases that could create novel applications as each industrial application may require specific properties of the enzyme (3).

Bacterial lipases are mostly inducible enzymes, requiring some form of oil, fatty acids, fatty acid alcohol, or fatty acid ester for induction. Triacyl glycerols, the main substrates of lipases, are uncharged lipids. The triacylglycerols with long-chain fatty acids are esterified with glycerol and are insoluble in water, although those with short-chain fatty acids are sparingly soluble in water. Biosynthesis of lipase by microorganisms was found to be enhanced by the optimization of culture condition factors such as temperature, pH, aeration, medium composition, etc. (4-11)

Mutation of a producer strain and selection of a hyper producing phenotype has proved successful for strain improvement and yield of enzymes and primary and secondary metabolites (12). Although product yield may be improved by optimizing the culture conditions, productivity is ultimately controlled by the genome. Thus, to improve productivity, the organism's genome must be modified and this may be achieved in two ways viz. mutation or recombination. Recent advances in molecular biology have increased the number of methods by which genetic variation can be generated, the mutation is still the most important means of quickly and efficiently creating genetic variation in a population. Mutation represents an invaluable tool for biotechnological innovations and plays an important role in strain improvement of microorganisms used in the industry. Increased yield of fermentative lipase through mutagenesis followed by the subsequent selection of high yielding strain is found to be reported as a significant technique in the fermentation process. (13-16).

Immobilization of enzymes is a process of physical localization of enzymes in a defined surface to improve the enzymatic properties and enhance their operational performance without altering their catalytic activity(17). Many techniques are available to immobilize enzymes using natural and synthetic materials like adsorption, ionic bonding, entrapment, and covalent bonding. The use of immobilized enzymes at the industrial level is extensive as they show potential advantages over their counterparts like maintaining stability (even under harsh environmental conditions of temperature, pH, and organic solvents), reusability, and easy recovery of enzyme and thus lowering production cost. (18-22)

However, it is also important to look into the aspect of the bioremediation process of polluted water sources. With the ability of the lipase enzyme to break down lipids and glycerides, it can be used to treat lake water sources that are polluted by various industrial effluents that are rich in oil wastes. In this paper, we isolate and screen the extracellular lipase producing microorganisms from four different lake water sources (Bellandur, Varthur, Agara, and Madivala lakes) by obtaining water and soil samples and characterized by using lipase assay to obtain the best lipase-producing microorganism and identifying using 16srRNA sequencing. The identified organism was made to undergo non-recombinant UV- Mutagenesis for hyper production of the extracellular lipase and the best lipase microorganism is immobilized and used for the treatment of the polluted lake water.

Materials and Methods

Experimental chemicals

The chemicals and reagents used in this research were purchased from sigma USA and Hi media. The experiments were conducted in triplicates and the mean values were considered.

Collection and preparation of sample

Water and soil samples were collected in July from Agara, Bellandur, Madivala, and Varthur Lakes, Bangalore urban district, Bangalore, Karnataka, India. The pH and temperature of both water and soil were noted. The lake water samples were centrifuged and the obtained supernatant was preserved for further analysis. The samples were labeled and preserved at 8^0C.

Primary screening of lipase producing organism by plate assay

The 100µl of the serially diluted samples was placed on tributyrin agar media plates containing 5g of peptone; 3g of yeast extract; 10 ml of tributyrin, and 20 g of agar-agar per liter, followed by incubation for 48 h at 37 °C. After incubation, colonies that had a higher zone of clearance were used for further studies.

Secondary screening of lipase producing organism and optimisation of lipase production media

The four selected colonies were cultured in autoclaved tributyrin broth and incubated at 37^0C for 48 hours. Four different cultivation media were evaluated for the production of lipase. The composition of the different production media is summarized as follows: Medium (A) per 100ml media: 0.3g of yeast extract, 0.1g of peptone, 1ml of olive oil, 0.07g of K_2HPO_4, 0.03g of KH_2PO_4, 0.05g of $MgSO_4$, 0.01g of $MnCl_2$, 0.025g of $(NH_4)_2SO_4$ and 0.01g of $CaCl_2$. Medium (B) per 100ml media: 1ml Tween 80 which replaced olive oil in media (A). Medium (C) per 100ml media: 2g dextrose added to media (A). Medium (D) per 100ml media: 2g dextrose added to media (B). The pH of all the production media was adjusted to 6.5 using 0.5 HCl, before autoclaving at 121°C for 15 min. The enzyme production was carried out in 500 ml Erlenmeyer flasks containing 94 ml of production media that was inoculated with 6 ml of the overnight incubated culture. The flasks containing the different production media were incubated in a shaker incubator at 37^0C with constant agitation at 125rpm for 48 hours. The cells were pelleted out by centrifugation at 10000rpm 4°C for 10 minutes. The supernatant was taken and used to determine the lipase activity. The highest lipase activity expressing organism in the specific media was selected for further analysis.

Assay for lipolytic activity

For the standard, 0.5µg/ml of p-nitrophenol working standard was prepared. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of working standard was pipetted out into a series of labelled test tubes. It was then made up to 2ml using phosphate buffer of pH 6.5. The same set of test tubes was placed in the water bath at 60^0C for 30 minutes. 0.25ml of 0.1M Na_2CO_3 was added to each of the samples to stop the reaction. Using a UV-Vis spectrophotometer, the optical density of the reaction mixture was determined at 410 nm. The reaction mixture containing 0.2 ml of the enzyme extract, 1.8 ml of 0.05 M phosphate buffer (pH 6.5), and 0.01M p-NPP dissolved isopropanol was kept at 60^0C for 30 minutes in a water bath. To stop the reaction 0.25ml of 0.1M Na_2CO_3 was added. Using a UV-Vis spectrophotometer, the optical density of the reaction mixture was determined at 410 nm. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol of p-nitro phenol per min from p-nitro phenyl palmitate. From the lipase assay, the organism that produced a higher amount of lipase was selected for further studies.

Molecular characterisation of the selected extracellular lipase producing microorganism

By sequencing and analysis of the 16SrRNA gene, the molecular identification of the selected strain was carried out. The genomic DNA was extracted with the commercial kit Qiagen and the partial 16S gene was amplified using the pair of primers 16S-F:AGAGTTTGATCCTGGCTCAG and 16S-R:AAGGAGGTGATCCAGCCGCA. The reaction mixture consisted of 50 µl 1× buffer of Taq DNA polymerase, 0.2 mM of each dNTP, 0.5 µMof each primer, 2 mM of magnesium chloride, 1 U of Taq DNA polymerase (Invitrogen), 50 ng of genomic DNA, and the final volume adjusted with ultrapure water. The program used for PCR was 94°C for 240 sec, followed by 35 cycles of 94°C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec, and finally, extension at 72°C for 360 sec.

The amplified product was cleaned and analyzed on BDT v3.1 cycle sequencer on ABI3730XL, Genetic Analyzer. Consensus sequence of 16SrDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI gene bank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program ClustalW. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

Improvement of strain by uv- mutagenesis

The selected organism was then cultured in the tributyrin broth in a rotary shaker incubator at 150rpm 37^{0}C for 48 hours. A stock culture containing 7×10^3 cells/ml was prepared by centrifuging the cultured media at 10000rpm for 10 minutes at 4^{0}C and the pellet was resuspended in 0.9% saline solution. In a sterile 80 mm diameter Petri dish, 20 ml of 7×10^3 cells/ml was pipetted aseptically. UV mutation was carried out in the biosafety cabinet. The exposure time was 10, 20, 30, 40, 50, and 60 minutes. To avoid photo reactivation of the mutants, the UV-exposed suspension was stored in the dark overnight under refrigerated conditions. It was then serially diluted in saline in such a way that by the end of 10^{-5} dilution only 1×10^3 cells were present and were spread-plated on tributyrin agar medium. (4) The plates were incubated for 48 hours at 37^{0}C and the number of colonies in each plate was counted assuming each colony to be formed of a single cell. Mutants for hyperproduction of lipase were detected visually by the intensity of the zone of clearance and were further selected based on the enzyme production in the liquid medium.

Production of lipase using selected mutants

The selected mutants were then cultured in 100 ml of optimized media, composed of 6ml of the inoculum, and incubated in the shaker at 150rpm, 37^{0}C for 48 hours. Lipase assay was carried out using p-NPP (23) for the wild and the other two mutant strains. The mutant that produced relatively higher lipase was selected for further work.

Analysis of the lake water samples

Prior to the treatment of lake water, the total protein, total carbohydrate, and free fatty acid content were measured by using standard protocols (23-24).

Immobilization of the organism

The wild and the mutant strains were cultured separately in the optimized media for 48 hours at 37^{0}C in a rotary shaker incubator at 150rpm. When the culture showed 7×10^3 cells, 2ml of the culture each of the wild and the selected mutant strains were taken and centrifuged at 4^{0}C, 10000 rpm for 10 minutes and the pellet was resuspended in saline solution to halt the growth. 100ml of 4% sodium alginate was prepared and heated to melt the sodium alginate and then cooled to 37^{0}C. Meanwhile, 250ml of 2% $CaCl_2$ was prepared and stored in

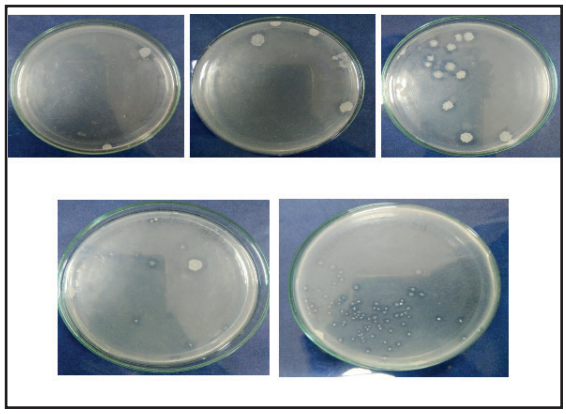
ice-cold condition until its use. 20g of dummy sodium alginate beads were prepared by adding it dropwise to ice-cold 2% $CaCl_2$.2ml of the wild strain was added to 100 ml of the 4% freshly melted sodium alginate at 37^0C and mixed well. 20g of this bead was prepared by adding dropwise to ice-cold 2% $CaCl_2$. Similarly, 2ml of the mutant strain was added to 100 ml of the 4% freshly melted sodium alginate at 37^0C and mixed well. 20g of this bead was prepared by adding dropwise to ice-cold 2% $CaCl_2$.

Treatment of lake water using immobilized organisms

To the conical flask containing 100ml of lake water inoculated with 5g of dummy beads. The other conical flasks were inoculated with 5g of the immobilized wild strain followed by the addition of 5g of the immobilized mutant strain and incubated in a rotary shaker incubator at 37^0C for 48 hours. To check if the treatment procedure was carried out successfully, free fatty acid concentration was estimated by the titrimetric method.

Results and Discussion

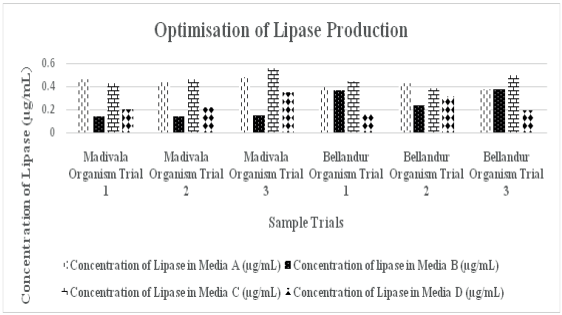
Screening by plate assay



The serially diluted water, soil sample, and direct water samples were placed on tributyrin agar media (TBA). After 48 hours of incubation period zone of clearance was observed (Fig. 1-3). The colonies obtained from the Madivala lake and Bellandur lake samples were used for the production of lipase.

Optimization of lipase production media and lipase assay using p-npp

Media (C) supplemented with dextrose as carbon, and olive oil an inducer of lipase showed the maximum concentration of lipase. Lipase assay using p-NPP as a substrate was conducted by spectrophotometric analysis using a UV-Vis spectrophotometer. In this study, the



organism isolated from Madivala lake produced the highest average concentration of lipase of 0.493µg/mL and thus was selected for further work. (Fig. 4)

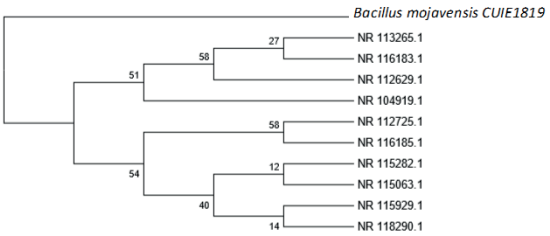


Fig. 5: Phylogenetic analysis

Fig. 4: Optimisation of lipase fermentation media using Madivala and Bellandur microorganism

Molecular characterisation of the selected lipolytic organism

The Madivala organism was cultured in tributyrin broth and pelleted and sent for sequencing and showed high similarity with *Bacillus mojavensis* based on nucleotide homology and phylogenetic analysis. (Fig. 5) The evolutionary history was inferred by using

the Maximum Likelihood method based on the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA7 using the bootstrap method and the phylogenetic tree was constructed using Neighbor-join and BioNJ to a matrix of pairwise distances that were estimated using the Maximum Composite Likelihood approach (MCL). The sequence was then submitted in NCBI as *Bacillus mojavensis CUIE1819* and the accession number was given as MK49204.

Strain improvement by uv- mutagenesis

The parental strain of *Bacillus mojavensis CUIE1819* was subjected to UV treatment for different time intervals i.e. 1o to 60 minutes. Of all the isolated obtained, 50 minutes and 60 minutes of mutation showed active mutant organism with lesser cell mass with a higher zone of clearance compared to the parent strain. For better analysis of the mutants, the mutants were cultured in the optimized media and after 48 hours of incubation in the rotary shaker incubator at 37^0C at 150rpm. After centrifugation of 2ml of the culture with triplets for both mutants and the parent strain (for comparison) and the pellet was suspended in saline solution and serially diluted from 10^{-1} to 10^{-3}

Strain	Dilution	Number of Colonies	Average Size of Zone of Clearance (mm)
Wild strain trial 1	10^{-1}	360	4
	10^{-2}	172	
	10^{-3}	3	
Wild strain trial 2	10^{-1}	156	6
	10^{-2}	79	
	10^{-3}	2	
50 minutes mutant trial 1	10^{-1}	0	NA
	10^{-2}	0	
	10^{-3}	0	
50 minutes mutant trial 2	10^{-1}	450	6
	10^{-2}	173	
	10^{-3}	60	
50 minutes mutant trial 3	10^{-1}	0	NA
	10^{-2}	1	
	10^{-3}	0	
60 minutes mutant trial 1	10^{-1}	0	NA
	10^{-2}	0	
	10^{-3}	0	
60 minutes mutant trial 2	10^{-1}	136	6
	10^{-2}	25	
	10^{-3}	6	
60 minutes mutant trial 3	10^{-1}	67	7
	10^{-2}	26	
	10^{-3}	2	

Production of lipase by mutant strains

The isolates were inoculated in 100mL of the optimized fermentation medium to check for lipase enzyme production (Fig. 6). The hyper enzyme-producing strains were selected for lake water treatment.

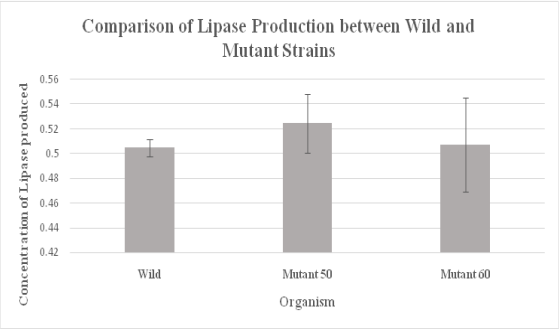


Fig. 6: Comparison of lipase production between wild and mutant strains

Lake water analysis for protein, carbohydrate and free fatty acid

Protein estimation

The amount of protein present in each of the water samples was estimated using Lowry's Method of Protein estimation using 0.01 mg/ml of BSA as the working/standard solution. (Table 2). The highest amount of protein was found to be present in Varthur lake followed by Bellandur and Madivala. Agara lake showed less protein.

Table 2: Amount of protein present in the lake water samples

Lake Sample	Concentration of protein (mg/ml)
Agara	0
Bellandur	0.014
Madivala	0.002
Varthur	0.079

Carbohydrate estimation

The amount of carbohydrate present in each water sample was estimated by the Anthrone method of Carbohydrate Estimation using 100µg/ml of glucose as the working/standard solution. (Table 3). Again, the highest amount of carbohydrate was found to be present in Varthur lake followed by Bellandur, Agara, and Madivala.

Free Fatty Estimation

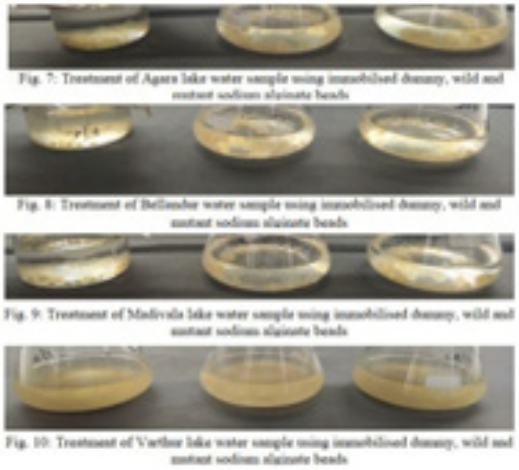
The amount of free fatty acid present in each of the samples before treatment was estimated titrimetrically against an alkali like 0.01N KOH.

Table 3. Amount of carbohydrates present in the lake water samples

Lake Sample	Concentration of carbohydrate (µg/ml)
Agara	4.179
Bellandur	16.886
Madivala	3.612
Varthur	67.385

Immobilization and treatment of lake water samples

5g each of the immobilized dummy, wild, and mutant strain beads that were prepared using 4% sodium alginate in 2% $CaCl_2$, were added to each of the 100ml of the four different lake water samples (Fig. 7-10) and incubated in a rotary shaker incubator at constant agitation of 150 rpm, at 37^0C for 48 hours. After 48 hours each of the samples was tested for fatty acid content by the titrimetric method using 0.01N KOH. (Fig. 11). From this we can infer that the lipase enzyme that was released by *Bacillus mojavensis CUIE1819* could degrade the lipids that were present in the Agara lake. However, in the case of Bellandur, Madivala, and Varthur lakes, no increase in fatty acid was observed may be due to the inhibitory action of certain compounds that may be present in the other lakes.



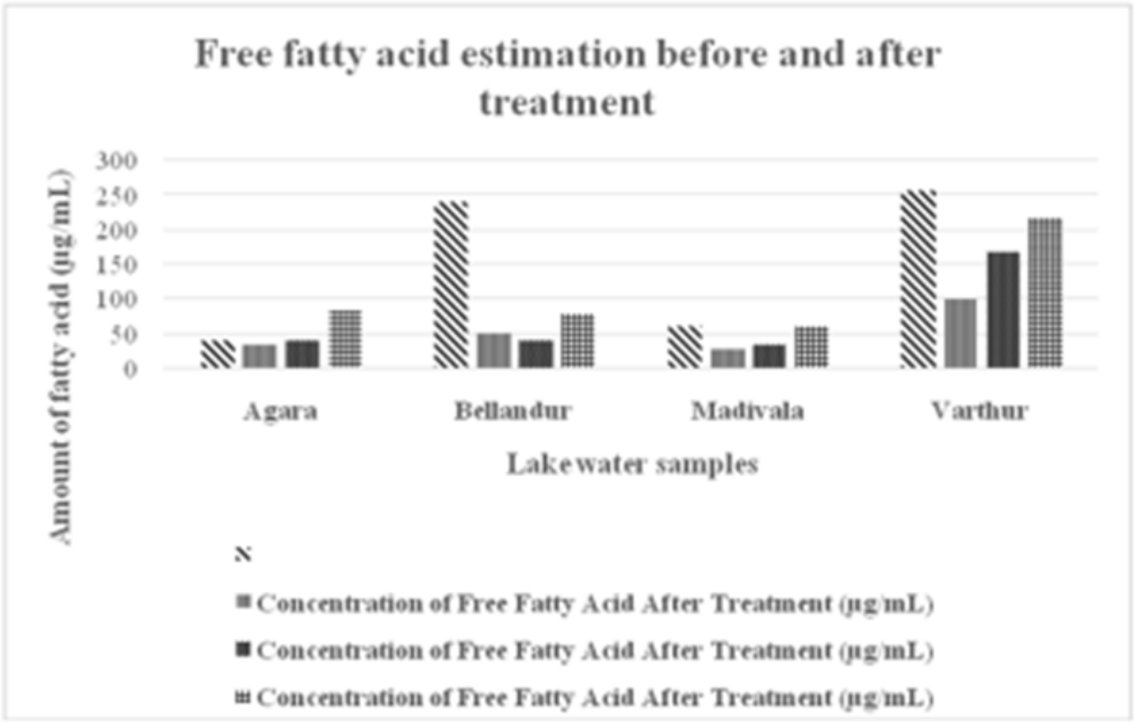


Fig. 11: Estimation of free fatty acid before and after treatment with the immobilized organism

Conclusion

Lipase producing bacteria were isolated from four different lakes: Agara, Bellandur, Madivala, and Varthur. Based on the zone of clearance, two organisms were selected to determine the highest lipase producing organism by using four different media, supplemented with dextrose as the sole carbon source and olive oil as the lipase inducer. The highest lipase producing organism was determined spectrophotometrically by lipase assay using p-NPP as substrate. After 16srRNA sequencing, the organism was found to be *Bacillus mojavensis CUIE1819* (MK49204) and was mutated by UV irradiation to check for hyper lipase production in the optimized media. The mutant that was exposed to UV for 50 minutes was seen to produce a higher amount of lipase compared to the parental/wild strain.

Water analysis of the different lake water samples was done for protein, carbohydrate, and free fatty acid using Lowry, Anthrone, and titrimetric estimation using KOH respectively. Both wild and mutant strains were immobilized in 4% sodium alginate and added to 100 mL of the lake water sample to treat it. After 48 hours of incubation in the rotary shaker incubator at 150rpm, 37^0C, free fatty acid estimation was done to see if there was an increase in the free fatty acid content. Agara lake water sample showed an increase in the free fatty acid level. However, the other lake water samples showed negative results. This could be due to the presence of certain lipase enzyme inhibitors. It is also seen that the Agara lake water sample is much cleaner compared to the other lakes. Therefore, there are chances of the absence of the lipase enzyme inhibitors and so the treatment was successful.

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Conflicts of Interest

The authors declare no conflict of interest.

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