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Lipolytic bacterial strains mediated transesterification of non-edible plant oils for generation of high quality biodiesel

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Biodiesel is one of the best alternative to depleting fossil fuels for transport sector. However, biodiesel production via lipase mediated transesterification has limitation of high costing microbial enzymes. In order to overcome this limitation, a process of sequential treatment of oil industry wastewater using isolated lipolytic bacterial strains and biodiesel production from non-edible plant oils was studied. In this study, efficient lipase producing bacteria were isolated and evaluated for production of biodiesel from mustard, soybean, jatropha and taramira oils utilizing methanol for the transesterification of oils and bioremediation. Selected strains were then identified, using 16s rRNA sequencing. Further, *Bacillus subtilis* strain Q1 KX712301 was optimized for biodiesel production from non-edible taramira oil via Plackett–Burman and central composite design. Highest volumetric yield of biodiesel obtained was 102% at optimized parameters. Finally, a sequential bioremediation of vegetable oil contaminated wastewater and then microbial production of biodiesel from non-edible taramira oil was carried out using efficient lipase producer *B. subtilis* strain Q1 at optimized conditions. During sequential process, complete chemical oxigen demand reduction of oil containing wastewater and theoretical volumetric yield of biodiesel was achieved. Gas chromatography/mass spectrometry chromatogram revealed that the total fatty acid methyl ester content of the produced biodiesel was >98% which is in accordance with the biodiesel quality standards specified by both ASTM and EU-14103.

[**Keywords:** Lipolytic bacteria; Lipases; Biodiesel; Enzymatic transesterification; Bioremediation; Sequential biodiesel production and bioremediation]

One of the major problems currently faced by world is nonrenewable fossil fuel depletion. World energy demand is increasing by 30% per year (1). In addition to this, available fossil fuels are also related to many environmental problems which include carcinogenic effect of diesel engine exhaust emission and GHG emission (2). To overcome these problems biodiesel seems to be a promising option. The two major drawbacks in biodiesel production are use of edible oils as feedstock and its chemical synthesis. World's 95% of biodiesel is generated using edible oil as feedstock which causes an imbalance to food supply (3). Using food resource for energy production cannot be implemented in developing countries where these countries have to increasingly deal with food crisis. To eradicate this upsetting situation researchers are proposing alternative oil sources also known as greener oil resources that are actually non-edible oils for biodiesel production (4).

Taramira, Jatropha and Mustard oils used in this study are ideal feed stocks for biodiesel production as they are non-edible and these plants can be grown on land not fit for the growth of food crops. Taramira oil is obtained from *Eruca sativa* plant belonging to Brassicaceae family (5). It can grow in barren lands with low soil fertility and rainfall. The anti-nutritional factors of taramira oil include its strictly pungent smell, skin irritation and high amount of erucic acid (40%) which not only renders it unpalatable but is also related to cardiac problems like cardialipidosis (6). *E. sativa* is native plant of Pakistan with the ability to grow in areas that are arid and salinity stricken. Seeds of this plant produce copious amounts of oil which can be converted into biodiesel. In Pakistan a lot of land is uncultivable due to salinity and a vast area constitute of dessert. This plant is well suited to be grown in bulk over these areas. Moreover, the people of these areas are mostly unemployed so cultivating taramira as an energy crop can open source of income for them. To the best of our knowledge we are reporting biocatalysis of taramira oil to produce biodiesel for the first time. Jatropha oil is obtained from *J. curcas* plant that belongs to Euphorbiaceae family. *Jatropha curcas* plant is desiccation and salinity resistant and can be grown on land unsuitable for growth of edible plants. Moreover, jatropha oil is non-edible due to presence of phorbol esters. Its biological toxic effects include alteration of cell morphology, promotion of tumor growth, mutagenesis of lymphocytes and induction of platelet aggregation (7). Mustard oil is obtained from the oil

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containing seeds of *Brassica campestris* L. which also belongs to the family Brassicaceae. It is high in erucic acid which as mentioned earlier is linked to cardiac diseases like myocardial lesions (8).

Biodiesel is produced by means of transesterification reaction (9). Kinetic study of transesterification process shows that it is a reversible reaction (10). The triglycerides (i.e., oils used for feedstock) are stepwise converted into diglycerides then monoglycerides and are finally converted to glycerol. In each of this step, one mole of ester (biodiesel) is released. This reaction is reversible but the reaction equilibrium is bent towards the formation of fatty acid methyl esters and glycerol (11). The transesterification reaction is reported to be pseudo-first order reaction (12). Various studies have been conducted in order to determine variables that effect the yield of biodiesel during transesterification of oils (13). Most commonly studied variables that effect biodiesel yield include activation energy, equilibrium constants, reaction rates, temperature, type and amount of catalysts used, molar ratios of oil to alcohol and presence of impurities in the feedstock like free fatty acids and moisture content (14–16). One of the study conducted by Marchetti et al. (17) gives comparative analysis of the kinetics of different catalyst (acid, base and lipase) used for biodiesel production and their effect on biodiesel yield. The study shows that lipase mediated transesterification is carried out at low temperature with high yield of biodiesel where moisture and free fatty acids in the feedstock does not affect the reaction and recovery of glycerol is easy but the cost of production is relatively high. The process of transesterification and production of biodiesel is a classic example of kinetically controlled synthesis (18). As explained by Rodrigues et al. (19), the kinetically controlled synthesis process is dependent upon an activated acyl donor like ester as in the case of biodiesel production, in order to reach the maximum transient yields. Transient yield on the other hand is also dependent upon simultaneously catalyzed three different types of reactions by the enzyme that are: target product formation, activated acyl donor hydrolysis and target product hydrolysis. The performance of enzyme in the kinetically controlled system depends upon various factors that include nucleophile adsorption on enzyme active center, reaction inhibitions, enzyme specificity towards product and active acyl donor (20).

The second problem linked to biodiesel production is its chemical synthesis using an acid or a base as a catalyst. The major drawbacks of this method are excessive energy requirement, soap formation and generation of acid/alkali based environmentally hazardous wastewater (21). Biocatalysis of oil for biodiesel production using enzymes as catalysts overcomes all these problems (22). Lipases are the enzymes which are required for transesterification of oil and its conversion to biodiesel (23). Different approaches are currently being used for lipase mediated transesterification of oils in order to produce biodiesel. One of them is immobilization (24). Immobilization is a technical process in which the enzymes are fixed within or on the surface of a solid support thereby providing a heterogeneous enzyme system. The solid support provides enzyme with stability whilst maintaining its activity (25). An ideal immobilization will be the one which provides maximum enzyme turnover rate along with maintaining the enzyme activity for a longer duration of time (26). Out of many advantages provisioned by immobilization some are enhanced performance in terms of activity of enzyme in organic solvents, pH tolerance, functional and heat stability, the ability to reuse enzyme and an easier separation of the biocatalyst from the product formed (27). A lot of supports have been reported for immobilization of lipase in order to produce biodiesel which give high biodiesel yield. Tacias-Pascacio et al. (28) showed that enzyme activity and biodiesel yield greatly depends upon the support selected for immobilization. A good support can greatly enhance the yield of biodiesel (29). Another approach that can be used for biodiesel production via lipases is whole cell approach (13). In this study whole cell approach is used for biodiesel production. This approach is cost effective and enzyme activity loss and enzyme active site conformational changes can be avoided by this approach. Moreover, with whole cell approach the mass diffusion limitation of substrate can also be overcome (30). This approach is also well suited for initial approximation of biodiesel production using newly isolated lipase producing microorganism before proceeding towards more advanced and expensive approaches like immobilization. Growth of lipase producing bacteria at large-scale is however, not economical, therefore lipase producing strains can be exploited for bioremediation which results in production of large number of bacterial cells (sludge). The biomass produced can then be utilized during whole cell approach for biodiesel production. The major drawback of bioremediation is sludge formation due to biomass growth (the treatment of this sludge takes up about 30% of the total cost of bioremediation) (31). The utilization of sludge produced for biodiesel production will not only replace the treatment of sludge but will also reduce cost of lipase producing bacterial strain's biomass production or enzyme purification. Therefore, bioremediation of wastewater treatment followed by biomass harvesting can be practical and economical approaches to lipase mediated biodiesel production.

In this study, efficient lipase producing bacterial strains were isolated from oil containing wastewater of vegetable oil industry and were sequentially employed for bioremediation of vegetable oil industry wastewater and for biodiesel production from vegetable and plant oils that are not fit for human consumption. Optimization of different parameters for biodiesel production was also carried out using statistical tools, i.e., Placket–Burmann design and central composite design. The quality of biodiesel produced was analyzed by gas chromatography/mass spectrometry (GCMS). To the best of our knowledge this study reports for the first time biocatalysis of taramira and mustard oil using whole cell approach and the sequential process for the treatment of oil industry wastewater and production of biodiesel via enzymatic transesterification.

MATERIALS AND METHODS

Sample collection and storage Oil containing wastewater was collected from a local vegetable oil and ghee industry in Hattar, Pakistan and stored at 4 °C in laboratory according to the standard protocols of American Public Health Association (APHA) (32).

Physicochemical parameter analysis of wastewater sample Three parameters namely pH, oil and grease content and chemical oxygen demand of the wastewater sample were analyzed.

Isolation and screening of efficient lipase producing bacteria Lipase producing bacterial strains were isolated from the wastewater collected from local vegetable oil industry. Tween-20 agar (g/L: Peptone 10, NaCl 5, $CaCl_2.2H_2O$ 0.1, Agar 20, Tween 20 1% and pH 7.5) was used as screening media. About 100 µl of 10^{-1} to 10^{-9} serial dilution of wastewater sample made in sterile distill water was spread on Tween-20 agar plates. After 24-h incubation at 37 °C bacterial colonies which gave largest white hallows were selected and further inoculated for 24-h at 37 °C on phenol-red olive oil agar (w/v: phenol-red 0.01%, olive oil 0.1%, $CaCl_2$ 0.1% and agar 2% and pH 7.5) (33) and rhodamine B olive oil agar (34) (w/v: nutrient broth 0.8%, NaCl 0.4%, rhodamine B 0.001%, olive oil 2%, yeast extract 0.05% and agar 2% and pH 7.5) to confirm efficient lipase production.

Identification of selected isolates For the identification of isolated bacterial strains both biochemical and molecular identification procedures were carried out.

Selected bacterial isolates were subjected to biochemical testing for identification, in accordance with the literature (35).

For the purpose of molecular identification of selected bacterial isolates, their DNA was extracted first via cetyltrimethylammonium bromide method (36). 16S ribosomal RNA sequencing of the extracted DNA for molecular identification of isolates was commercially performed by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc., Seoul, Korea) using Sanger method.

Lipase production through submerged fermentation and lipase assay About 5% of 24-h fresh culture enriched in nutrient broth was inoculated in 50 ml of lipase production media (w/v: peptone 0.2%, KH_2PO_4 0.1%, NaCl 0.25%,

$MgSO_4 \cdot 7H_2O$ 0.04%, $CaCl_2 \cdot 2H_2O$ 0.04%, olive oil 2% and Tween-20 1–2 drops) contained in 250 ml fermentation reactor and incubated at 37 °C and 150 rpm. After 24 and 48 h of fermentation, 5 ml of media was taken out from the reactor and centrifuged at 10,000 rpm for 10 min. Supernatant was subjected to lipase assay. Lipase activity was determined using the method described by Kumar et al. (37) with a few modifications. *p*-Nitrophenyl laurate solution (20 mM) was prepared in 1:1 isopropanol and acetonitrile. Added 75 µl of *p*-nitrophenyl laurate solution to 25 µl of supernatant solution and made final volume of the solution 3 ml by adding tris–HCl buffer at pH 7. This solution mixture was incubated at 37 °C for 30 min. After 30 min the solution was chilled at −20 °C for 10 min to stop enzyme activity and centrifuged for 5 min at 10,000 rpm to remove the precipitates formed. The absorbance of released PNP was then noted at 410 nm. The unknown concentration of PNP released was measured from standard curve. Negative control containing heat inactivated enzyme was used as blank. Standard curve for PNP was developed by making 10–100 µM solution of PNP in tris–HCl buffer at pH 7 and recorded absorbance at 410 nm. One unit of enzyme activity was defined as enzyme catalysing the release of 1 µM of para-nitro phenol per minute per ml at 37 °C and pH 7.

Oil toxicity test Two of the oils used, i.e., jatropha oil and taramira oil, are reported to have toxic effect on bacteria. So, in order to determine whether these oils inhibit the growth of selected bacterial isolates or not, oil toxicity test was performed. Olive oil and cooking oil were used as reference oils. About 50 ml of media (w/v: peptone 0.2%, KH_2PO_4 0.1%, NaCl 0.25%, $MgSO_4 \cdot 7H_2O$ 0.04%, $CaCl_2 \cdot 2H_2O$ 0.04%, respective oil 2% and Tween-20 1–2 drops) was taken in 250 ml Erlenmeyer flask and inoculated with 5% of 24-h fresh culture enriched in nutrient broth. After inoculation flasks were incubated at 37 °C and 150 rpm. Growth optical densities were recorded after 24, 48 and 72 h at 600 nm wavelength. Negative controls were run along each experimental set.

Methanol sensitivity test Methanol was added to autoclaved nutrient broth in 5%, 10% and 15% concentration. About 5% of 24 h fresh culture enriched in nutrient broth was added to nutrient broth containing different concentration of methanol and incubated at 37 °C and 150 rpm. Growth optical densities were observed after 24, 48 and 72 h of incubation at 600 nm wavelength.

Treatment of vegetable oil industry wastewater using isolated lipolytic bacterial strains Bioremediation was carried out in a batch reactor. The COD of the sample wastewater was also increased by addition of oil of same origin in the ratio of 1 ml/100 ml of the sample. Minimal salt media (MSM) (g/L: K_2HPO_4 1.12, KH_2PO_4 0.48, NaCl 5, $MgSO_4 \cdot 7H_2O$ 0.1, $(NH_4)_2SO_4$2 and EDTA 0.001) and autoclaved wastewater were mixed in 1:1 ratio in 250 ml reactor. About 5% 24-h fresh cultures enriched in nutrient broth were inoculated into the MSM-wastewater mixture and its initial COD was measured. It was then incubated at 37 °C and 150 RPM. Each day sample was taken out from the reactor for COD measurement. The bioremediation reaction was carried out for a total of eight days. Less than 2% of COD reduction occurred from day 6 to day 8 (72 h) so the degradation reaction was discontinued.

Biodiesel production from different oils using lipolytic isolated strains Isolates were further evaluated for the production of biodiesel using whole-cell approach. Cell mass was first generated by growing the strains in LB broth (g/L: peptone 10, NaCl 5 and yeast extract 5) and then centrifuging LB broth to attain cell pellet. About 1 ml of harvested cells from LB broth, were added to respective oils (taramira, jatropha, mustard and soybean oil) and mobilized in the oil layer. Methanol was added afterwards. The oil:methanol molar ratio was kept to be 1:6. The reactors were tightly closed to avoid methanol evaporation. *n*-Hexane was added as emulsifier. Reaction mixture was then incubated at 37 °C and 150 rpm for 48 h. After 48 h, reactor contents were shifted to separatory funnel which was kept static for 24 h to allow the layers to separate. After complete settling the upper clear yellow layer of biodiesel was collected in screw cap glass vials. Biodiesel produced was purified via distillation to remove any excess methanol if present. Percentage volumetric yield of produced biodiesel was calculated using Eq. 1:

$$\text{Volumetric yield of biodiesel (\%)} = \frac{\text{Amount of biodiesel produced (ml)}}{\text{Initial amount of oil used (ml)}} \times 100 \quad (1)$$

Optimization of parameters for biodiesel production using Plackett–Burman design For the purpose of optimizing different parameters to attain highest percentage volumetric yield of biodiesel with one selected isolate and taramira oil, Plackett–Burman design was used through Stat-Ease Design Expert Software version 7.0. Five factors, namely temperature, agitation speed, oil:methanol ratio, *n*-hexane concentration with respect to oil and inoculum size were selected for optimization. Incubation time was kept 48 h.

Optimization of significant variables by response surface methodology using central composite design The factors that appeared to be significant by Plackett–Burman design were then optimized by central composite design. Two factors molar ratio and temperature were optimized and central composite design was used through Stat-Ease Design expert software version 7.0.

Validation of Plackett–Burman and central composite design In order to validate Plackett–Burman and central composite design numerical validation test keeping yield as goal through the design expert software was performed and the predicted yields were compared with the experimental yields with the achievement of desired ratio of both the yields ≤ 1.

Sequential bioremediation of oil contaminated wastewater and biodiesel production from non-edible taramira oil Bioremediation process was carried out in continuous reactor for sequential process. For continuous bioremediation, the selected bacterial strain was first enriched in nutrient broth for 24 h. 100 ml 1:1 sterile MSM-wastewater mixture was taken in 250 ml bioremediation reactor and its initial COD was measured. Selected strain inoculum (5%) was then added to this mixture and incubated at 37 °C and 150 rpm. After every 24 h, 33.3 ml of incubated mixture was removed from the reactor and 33.3 ml of mixture was added. Flow rate, organic loading rate and organic removal rate were calculated from Eqs. 2–4, respectively:

$$\text{Flow rate} = \frac{\text{Volume of the reactor}}{\text{Retention time}} \quad (2)$$

$$\text{Organic removal rate} = \frac{\text{COD}_{\text{initial}} \times \text{Flow rate}}{\text{Volume of reactor}} \quad (3)$$

$$\text{Organic removal rate} = \frac{\Delta\text{COD} \times \text{Flow rate}}{\text{Volume of reactor}} \quad (4)$$

where ΔCOD is the difference in the COD of the influent and effluent. All of the MSM-wastewater mixture present in the reactor after 3 days was centrifuged at 4000 rpm for 15 min. Pellet (active cells) obtained was stored at 4 °C in normal saline till further use for transesterification. Biodiesel production by methanolysis of taramira oil through Q1 strain was further performed as described above.

Reuse of Biomass After carrying out the sequential experiment of bioremediation of oil industry wastewater and production of biodiesel from taramira oil at optimized conditions, the cell biomass was harvested from the reaction mixture and reused for biodiesel production at the same conditions.

Analysis Oil and grease content of the sample was estimated by partition-gravimetric method (32).

For the purpose of COD measurement, 2 ml of filtered sample, 3 ml of digestion solution ($K_2Cr_2O_7$ 10.216 g/L, H_2SO_4 167 ml/L and $HgSO_4$ 33.3 g/L) and 2.5 ml of H_2SO_4 were mixed in 10 ml glass vial and were kept in digester for 2 h at 150 °C. After 2 h glass vials were cooled to room temperature and COD was measured using Spectroquant Prove by Merck, Germany.

Fourier transform infrared spectrometer (FTIR) was done by Tensor 27 (Bruker, Billerica, MA, USA). FTIR spectrophotometer was equipped with ZnSe ATR. Liquid sample was loaded in sample assembly designed for liquids and the analysis was performed. A total of 16 scans were carried out and the average was represented in the form of a spectrum showing different peaks at different ranges. The software used in the analysis was Opus65 (Bruker).

GCMS was performed on a capillary column, DB-5MS Agilent (30 m × 0.25 mm, 0.25 µm of film thickness). Helium was used as the carrier gas with flow rate of 1.5 ml/min. The column temperature was programmed from 50 to 300 °C at the rate of 10 °C/min. The temperature of both injector and detector was set at 250 °C. The mass spectrometer was set to scan in the range of m/z 50–550 with electron impact mode of ionization. Methyl acetate was used as internal standard.

RESULTS AND DISCUSSION

Physicochemical parameter analysis of vegetable oil industry wastewater The evaluated characteristics, i.e., pH, oil and grease content and COD of wastewater sample are given in Table 1.

The wastewater obtained from vegetable oil industry had only gone through filtration as a treatment for the removal of oil and grease present in it. As filtration does not completely remove all the oil and grease present in wastewater effectively, the amount of oil and grease in wastewater came out to be quite high which resulted in high COD of the wastewater. The pH of wastewater was however neutral. The COD of sample wastewater was further increased by the addition of cooking oil in the ratio of 1 ml/100 ml of wastewater. This COD increase also helped in assessing the ability of the isolated bacterial strains to cope up with high organic load.

Isolation and screening of lipolytic bacterial strains As wastewater contained oil as contaminant, this made it an excellent substrate for isolation of lipase producing oil degrading bacteria. For initial isolation Tween-20 agar was used. Countable range of colonies came only on plates spread with dilution 10^{-6} and 10^{-7}.

TABLE 1. Physicochemical characteristics of Wastewater collected from Vegetable Oil Industry.

Parameters	Original sample (mg/L)	After addition of extra oil (1 ml oil/100 ml sample) (mg/L)
Fats, oil and grease content	992	3105
COD	1046	3798
pH	7.2	7.2

CFU/ml for plate inoculated with 10^{-6} and 10^{-7} dilution and was calculated to be 1.12×10^9 and 5.5×10^9, respectively. Initially, 38 strains were identified to be lipase positive out of which 9 strains which gave the largest white hallows were selected and further screened. In order to confirm the lipase production, the selected isolates were further grown in media containing oil instead of Tween-20. Isolates were inoculated on phenol red olive oil agar and rhodamine B agar. Lipase producers converted red colored phenol-red olive oil agar plates to orange and gave fluorescent orange illumination under UV light for rhodamine B olive oil agar plates. On the basis of these tests, four isolated strains were selected.

Identification of isolates Both biochemical and molecular identification of four of the selected isolates was carried out. Results for biochemical testing of isolates are given in Table 2.

For molecular identification of isolates 16S rRNA sequencing was commercially performed by Macrogen, Korea. The received sequences were subjected to phylogenetic analysis. The phylogenetic analysis of the 16S rRNA sequence showed that three of the strains belonged to genus *Bacillus* and one of the strain belonged to genus *Pseudomonas*. The phylogenetic tree for strains Q1, Q5, Q6 and Q8 was constructed by using neighbor joining method and is shown in Fig. 1. The sequence data was further submitted into NCBI Gen Bank and accession numbers for each strain was acquired. The strains were designated as *B. subtilis* Q1 KX712301, *B. subtilis* Q5 KX712302, *B. subtilis* Q6 KX712303 and *Pseudomonas aeruginosa* Q8 KX712304.

Enzyme assay Lipase activity of isolated strains along with *Staphylococcus aureus* ATCC 6538 was assayed. *S. aureus* ATCC 6538 was used as a reference strain as it is a known lipase producer. All the strains showed appreciable lipase activity even without optimization and at conditions specified for enzyme assay, i.e., pH 7 and 37 °C (Fig. 2). *S. aureus* ATCC 6538 gave the highest crude enzyme activity at 24 h and highest crude enzyme activity at 48 h was given by Q5. The results of enzyme assay depicted that these strains can be further used for the purpose of both bioremediation and biodiesel production.

Oil toxicity test Jatropha oil contains phorbol esters which are responsible for its toxic nature (38). Considering the inhibitory effect of jatropha oil on certain microorganisms, the bacterial isolates were tested by carrying out jatropha oil toxicity test. The results attained however, depicted that none of the isolated strain was inhibited by the jatropha oil. In fact, isolates when grown in jatropha oil, gave highest growth OD as compared to taramira and reference oils used in this study. *S. aureus* ATCC 6358 and strain Q8 (*Pseudomonas* sp.) also did not show any inhibition with jatropha oil. These results were supported by the results reported by Devappa et al. (39) which showed *B. subtilis* to be least susceptible to concentrated phorbol esters extracted from jatropha oil whereas *Pseudomonas putida* and *S. aureus* depicted moderate susceptibility.

Taramira oil contains phytochemicals like tannins, phenolics, alkaloids, flavonoids, cardiac glycosides and saponins which are all known for their antimicrobial activity (40). All bacterial strains showed a general growth pattern in media containing taramira oil. Growth OD was low in first 24 h as compared to jatropha and reference oils used in the experiment but it kept on increasing as the incubation time increased from 24 to 72 h which showed adaptation of microorganisms towards taramira oil as a carbon source in media.

Methanol sensitivity test Methanol is an organic solvent which inhibits bacteria by dissolving in their cell membrane and then disturbing its permeability and integrity. Certain bacteria have developed mechanisms to resist the toxicity of these organic solvents by changing the cis/trans structure of their fatty acids, still most of the microorganisms are sensitive to methanol. Methanol is also responsible for the inhibition of enzymes by causing change in their 3D conformation through dehydration (41). Lipase in specific has been reported to be inhibited by methanol during transesterification reaction for production of biodiesel thus resulting in low yields of methyl esters (42,43). Methanol sensitivity test at low concentration of methanol was carried out on the bacterial isolates to check their methanol tolerance level. All the strains gave good growth with 5% concentration of methanol at 24, 48 and 72 h. At 10% concentration of methanol all strains showed reasonable growth after 72 h of incubation. With 15% methanol concentration an accountable growth OD was observed with strains Q1 and Q5 after 72 h, strain Q6 showed very little growth whereas Q8 and *S. aureus* ATCC 6538 did not show any growth even after 72 h of incubation.

Treatment of oil and grease containing wastewater using batch reactor For the treatment of wastewater contaminated with oil, bioremediation was carried out in a batch reactor with both single bacterial cultures and mixed culture. The results showed that *B. subtilis* strain Q1 and consortia C2 (strain Q5, Q8 and *S. aureus* ATCC 6358) showed highest COD reduction of 90% in 8 days of incubation period. *B. subtilis* strain Q5 showed least COD reduction (75%) out of all the strains. Almost all bacterial strains and consortia showed their maximum COD reduction potential during first 4 days of incubation after which daily COD reduction was stabilized to 1 or 2% reduction. Consortia C1 (strain Q1, Q6 and *S. aureus* ATCC 6358) showed only 80% reduction of COD whereas *B. subtilis* strain Q1 alone showed highest COD reduction of 90%. It was may be due to the antimicrobial activity of both *Bacillus* strains Q1 and Q6 against *S. aureus* ATCC 6358 as *Bacillus* sp. are reported to produce antimicrobials against *S. aureus* when grown in media together. COD reduction efficiency of *B. subtilis* strain Q1 was in consistence with the results reported by Kanmani et al. (44) for *B. subtilis* COM6B which gave 92.7% removal efficiency with 15% oil and grease content in wastewater.

Biodiesel production and its FTIR analysis Four oils, those from jatropha, taramira, soybean and mustard plant seeds were selected for the production of biodiesel using lipase producing

TABLE 2. Results for biochemical testing of isolates.

Isolates	Catalase	Nitrate	Citrate utilization	TSI	Urease	Oxidase	Methyl red	Vogues-Proskauer	Identification
Q1	+	+	+	A/A	−	+	−	+	*Bacillus* sp.
Q5	+	+	+	A/A	−	+	−	+	*Bacillus* sp.
Q6	+	−	+	K/A	−	+	−	+	*Bacillus* sp.
Q8	+	−	+	K/K	+	+	+	+	*Pseudomonas* sp.

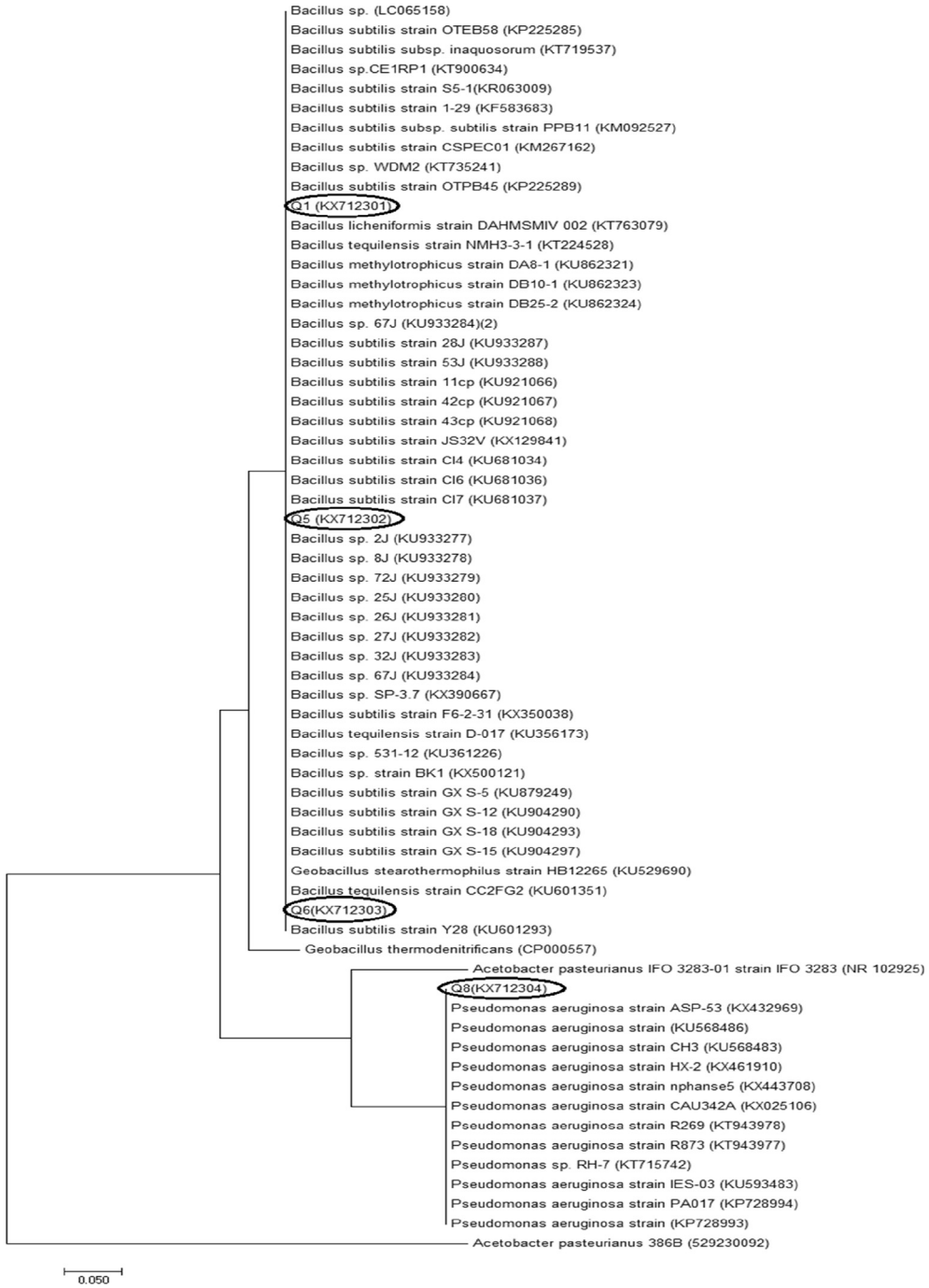

FIG. 1. Phylogenetic tree for partial 16S rRNA gene sequences from isolates Q1, Q5, Q6 and Q8 showing relationships between presented strains and related sequences.

bacterial strains. Production of biodiesel was then confirmed by performing its FTIR analysis. As biodiesel is mono alkyl ester of long chain fatty acids thus, its IR spectrum should contain peaks of ester groups which are observed at the frequency (cm^{-1}) range of 1735–1750. The infrared spectra of all 24 samples of biodiesel produced by all six bacterial strains from all the four oil gave peaks in the range of 1735–1750 cm^{-1} which showed the presence of esters in sample thus confirming the production of biodiesel.

Optimization of parameters for biodiesel production from taramira oil from *B. subtilis* strain Q1 using Plackett–Burman design Strain Q1and taramira oil were selected for optimized biodiesel production. As our aim was to develop a sequential process of bioremediation and biodiesel production and strain Q1 gave highest COD reduction so it was selected for optimization. Five factors, namely temperature, agitation, inoculum size, oil:methanol ratio and *n*-hexane concentration with respect to oil were optimized. Experiments were performed according to conditions given in 15 runs by Plackett–Burman design and response (percentage volumetric yield of biodiesel) was recorded (Table 3).

Highest volumetric yield of biodiesel 102% was obtained with Run 14 when the reactor was incubated at 37 °C, oil:methanol ratio of 1:9, 300 rpm, inoculum size 30% and *n*-hexane 6%.

ANOVA analysis of the design gave F-value 20.92 which depicted that the experimental model generated by design was significant. F-test on each factor was also applied by the software to identify

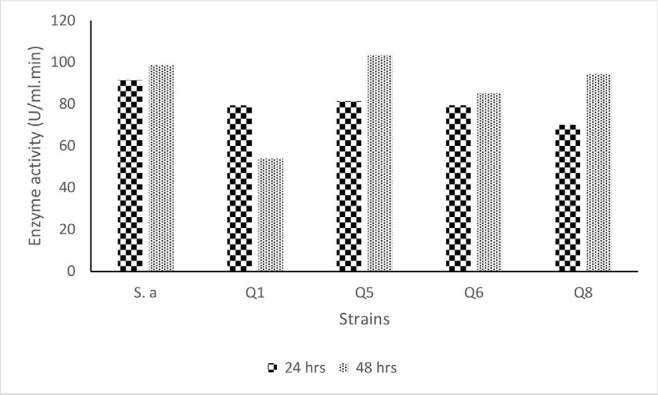

FIG. 2. Crude enzyme activity of lipase produced by selected lipolytic bacterial strains during 24 and 48 h.

significant factors. Values of Prob> F less than 0.0500 indicated that factors are significant. In this case, Molar ratio and temperature are significant factors with Prob> F value of 0.002 and 0.04, respectively (Fig. 3). Molar ratio affected the response, i.e., %age volumetric yield of biodiesel positively which means increasing the molar ratio increases the yield (Fig. 4A). However, in most of the reported enzymatic synthesis of biodiesel (19), an increase in oil:methanol molar ratio decreases the yield of biodiesel as methanol inhibits lipases. In this study, the non-occurrence of inhibition with increase of molar ratio may be due to the utilization of high inoculum size (from 10% to 30% with respect to amount of oil), due to which some of the cells remained protected from methanol till end or it may be due to direct use of cell pellet instead of immobilization, and then mobilizing the cell pellet in oil layer to protect it from sudden direct contact with methanol as it is added. Moreover, the isolated strains also showed tolerance to methanol at lower concentrations as depicted by the results of methanol sensitivity test. Temperature affects the yield negatively which means that increasing the temperature decreases the yield (Fig. 4B). The unexpected behavior of enzymes while carrying out catalysis in non-aqueous media like the biodiesel reaction carried out in this study has also been explained by Halling (45). The variation in the enzyme behavior can be due to partitioning of water or changes in solvation, substrate, organic ions, products or acids (45). This gives enzymatic synthesis of biodiesel an advantage over chemical

TABLE 3. Percentage volumetric yield of biodiesel in response to conditions specified by Plackett–Burman design for each run.

Run	Factor 1 A: molar ratio	Factor 2 B: agitation (rpm)	Factor 3 C: inoculum size (%)	Factor 4 D: temperature (°C)	Factor 5 E: *n*-hexane (%)	Volumetric yield of biodiesel (%)
1	9	150	10	37	10	100
2	9	300	10	37	6	100
3	9	150	30	55	6	84
4	3	150	10	37	6	60
5	6	225	20	46	8	98
6	3	150	30	37	10	98
7	9	150	30	55	10	94
8	3	150	10	55	6	0
9	6	225	20	46	8	100
10	3	300	30	37	10	60
11	9	300	10	55	10	98
12	6	225	20	46	8	98
13	3	300	30	55	6	0
14	9	300	30	37	6	102
15	3	300	10	55	10	0

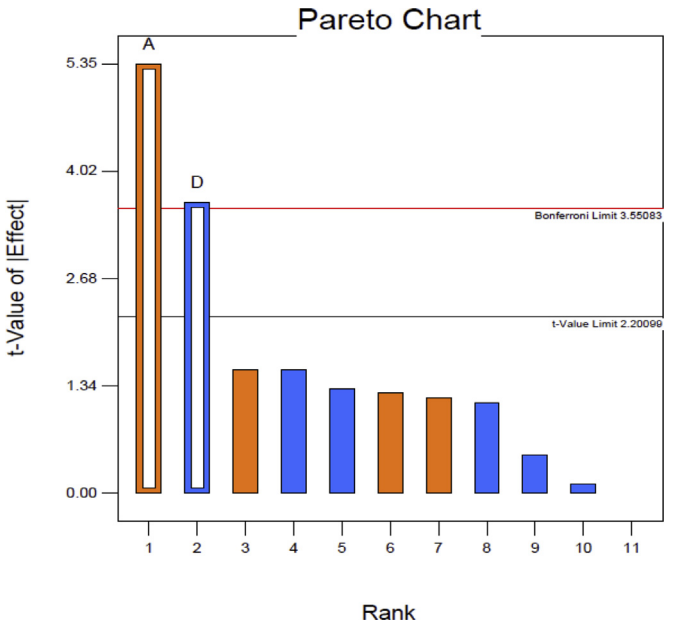

FIG. 3. Illustration of significant factors via Pareto chart, where A corresponds to molar ratio and D corresponds to temperature.

synthesis as chemical synthesis requires high temperature which means more energy to increase temperature which leads to higher cost. However, highest yield of biodiesel obtained in this study was at 37 °C (optimum growth temperature of strain Q1) which renders this process energy and cost efficient.

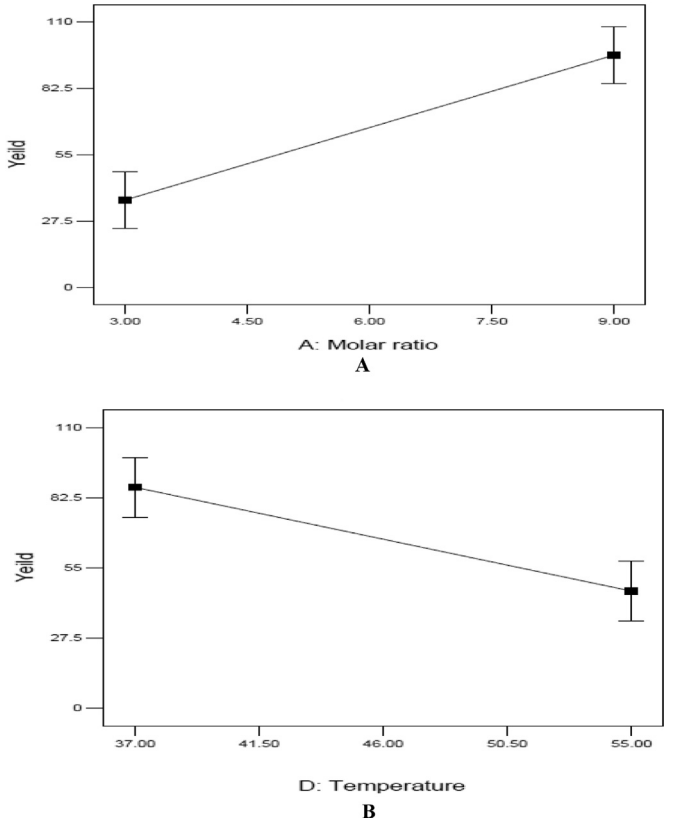

FIG. 4. Plot of volumetric yield (response) as a function of (A) molar ratio (significant factor) and (B) temperature (significant factor).

Final equation in terms of coded (significant) factors for volumetric yield of biodiesel as given by software is

$$\text{Yields} = 66.33 + 30.00 \times \text{A} - 20.33 \times \text{D} \quad (5)$$

where A is molar ratio and D is temperature.

Final equation in terms of actual (significant) factors for volumetric yield of biodiesel as given by software is:

$$\text{Yields} = 110.25926 + 10.00000 \times \text{Molar ratio} - 2.25926 \times \text{Temperature} \quad (6)$$

Optimization of significant variables using central composite design The two factors, temperature and molar ratio which came out to be significant in Plackett–Burman design were further optimized using central composite design and the interactive effect of both these variables on the yield of biodiesel was studied through response surface methodology. Experiments were performed in accordance with the 13 runs given by the design and response in terms of volumetric yield was recorded (Table 4). As Plackett–Burman design depicted that an increase in molar ratio and decrease in temperature results in high volumetric yield of biodiesel, so for central composite design, the range selected for molar ratio of oil:methanol was from 1:9 to 1:12 and for temperature 30–37 °C. ANOVA analysis of the design gave F-value 12.89 which depicted that the experimental model generated by design was significant. In the case of central composite design, molar ratio affected the response, i.e., %age volumetric yield of biodiesel negatively as opposed to Plackett–Burman design which means increasing the molar ratio decreases the yield. This means that further increase in methanol started to inhibit the microbial and enzymatic activity as described in the previous studies (19). In the case of temperature, the results opposite to Plackett–Burman design were observed again. This time temperature effected the volumetric yield positively which means that increasing the temperature increased the %age volumetric yield. This means that low temperatures slow down *B. subtilis* strain Q1. The highest volumetric yield of 100% was achieved at oil:methanol ratio of 1:9 and temperature 37°C. The interactive effect of both these factors on biodiesel yield are depicted in 3D surface and contour plots (Fig. 5A and B).

Final equation in terms of coded (significant) factors for volumetric yield of biodiesel as given by software is:

$$\text{Yield} = 79.62 + 13.77 \times \text{A} - 8.86 \times \text{B} \quad (7)$$

where A is temperature and B is molar ratio.

TABLE 4. Percentage volumetric yield of biodiesel in response to conditions specified by central composite design for each run.

Run	Factor 1A: temperature celsius	Factor 2B: molar ratio	Response yield %
1	33.50	8.38	91
2	38.45	10.50	94
3	28.55	10.50	55
4	30.00	9.00	76
5	37.00	9.00	100
6	33.50	10.50	85
7	33.50	10.50	85
8	33.50	10.50	86
9	33.50	10.50	85
10	33.50	12.62	84
11	37.00	12.00	73
12	30.00	12.00	42
13	33.50	10.50	79

Final equation in terms of actual (significant) factors for volumetric yield of biodiesel as given by software is:

$$\text{Yield} = 9.86066 + 3.93408 \times \text{Temperature} - 5.90829 \times \text{Molar ratio} \quad (8)$$

Both Plackett–Burman design and central composite design were validated and the results are shown in Table S1.

Sequential bioremediation of oil contaminated wastewater and biodiesel production from non-edible taramira oil using strain Q1 at optimized conditions For sequential bioremediation and biodiesel production, bioremediation study was conducted in a continuous reactor so that active cells can be obtained at the end of the reaction. *B. subtilis* strain Q1 was selected for sequential process as it achieved highest COD reduction. Complete COD reduction (100%) was achieved after 3 days.

Cells were then harvested from the continuous reaction and utilized for biodiesel production with taramira oil at optimized conditions. The %age volumetric yield of biodiesel came out to be 100%. The IR spectrum of produced biodiesel gave ester peak at 1745.28 cm^{-1}. The percentage volumetric yield of biodiesel attained this time was 2% less than the yield obtained with cells grown in LB broth at the same conditions. This may be due to inoculum used in sequential reaction may have included a higher percentage of dead biomass with cells which have achieved their death phase along with cells in log and stationary phase as continuous system contains cells present in all the phases at the same time. Moreover, the cell pellet may also have contained higher percentage of cell debris and suspended impurities present in wastewater along with viable lipase producing cells and hence, a lower percentage of viable cells may have led to decrease in percentage yield but still this decrease is very small and can be neglected. GCMS analysis of biodiesel produced was also carried out to ascertain the quality of biodiesel. GCMS chromatogram revealed that the total fatty acid methyl ester content of the produced biodiesel was >98% which is in accordance with the biodiesel quality standards specified by both ASTM and EU-14103. The fatty acid methyl esters present in biodiesel produced from taramira were erucic acid methyl esters (45.8%), palmitic acid methyl esters (10.5%), oleic acid methyl esters (29.8 %), linolenic acid (10.9%) methyl esters and stearic acid methyl esters (1.9%).

Reuse of biomass The reused biomass gave 44% yield of biodiesel which depicted that the cells retained their potential to produce biodiesel after first cycle of biodiesel production. There could be multiple reasons behind the low yield of biodiesel during reuse some of which include: lipases tend to lose their activity by continuous exposure to short chain alcohol like methanol and the transesterification reaction results in the formation of glycerol as a byproduct. This glycerol can deposit on the surface of enzyme or the cellular biomass thus leading to reduced activity of enzyme and low yield of biodiesel (24). The strategies to overcome this inability to reuse biomass include the treatment of the enzymes or biomass with ultrasound to remove the deposited glycerol or to use immobilization approach with extremely hydrophobic supports. The lipase mediated transesterification also requires to have water in the reaction mixture for lipase to keep its stable conformation which is required for proper activity of enzyme. Lipase is an interfacial enzyme that operates in the interface of water and oil layer to perform transesterification, but access water can led to unwanted side reactions like hydrolysis. This water borne hindrance can be overcome by utilization of molecular sieves for water expulsion.

Although the reuse of biomass is economically beneficial in direct production of biodiesel from oil via lipase producing cell

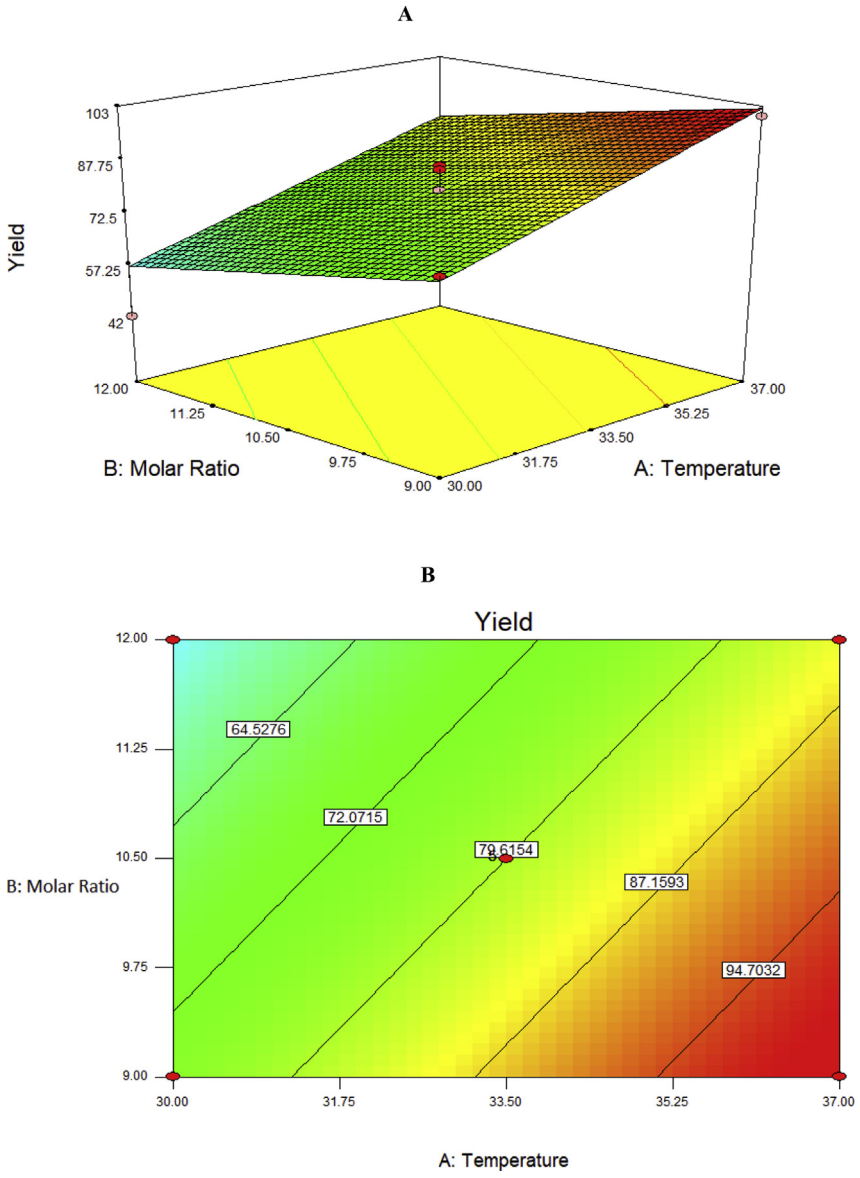

FIG. 5. (A) Three-dimensional response surface plot for biodiesel yield showing the interactive effects of the temperature and molar ratio. (B) Illustration of contour between temperature and molar ratio in terms of yield.

biomass, in the case of proposed method (sequential bioremediation and biodiesel production), large amount of biomass continues to be produced in bioremediation phase, so biomass reuse is not required as such.

This research findings conclude that the isolated lipase producer strains were promising for both bioremediation of vegetable oil industry effluents and for biodiesel production from non-edible plant oils. The desirable characteristics depicted by *B. subtilis* strain Q1 were suggestive of the fact that it can be exploited for a large scale sequential system for wastewater treatment and biodiesel production. The biodiesel production process optimization, that was a part of this study, could help in further selection of more variables and their levels to enhance the isolates efficiency for both bioremediation and biodiesel production. Biodiesel production at low temperature offered biocatalytic synthesis an advantage over chemical synthesis, as biodiesel production at low temperature makes the process both cost and energy efficient. All the results affirm that the isolated strains can find promising applications in both of these arenas. In future, immobilization studies for the lipase produced by *B. subtilis* strain Q1 can also be carried out to enhance stability of the enzyme at extreme reaction conditions and get the advantage of enzyme reusability. Some of the problems that still exist with enzymatic transesterification include deposition of the reaction byproducts like glycerin or free fatty acids on cell surface in case of whole cells or on enzyme surface effectively inhibit enzyme activity. Different approaches to overcome these problems can also be probed in future like the use of combi-lipases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.11.001.

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