

Characterization of Lipase from *Bacillus subtilis* I-4 and Its Potential Use in Oil Contaminated Wastewater

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ABSTRACT

A lipase producing bacterium was isolated from oil contaminated effluents of various industries from Sheikhpura Road, Pakistan, and, on the basis of biochemical and 16S rRNA ribotyping, was identified as *Bacillus subtilis*. The optimum temperature and pH for the growth of the culture were 37°C and 7.0, respectively. *B. subtilis* I-4 had a lag phase of 4 h in LB medium while this phase prolonged to 6 h in oil containing medium. The optimum temperature and pH for the enzyme activity were 50°C and 7.0, respectively. Maximum lipase activity was found in the presence of Ca ions. Olive oil and Tween 80 induced lipase gene in the bacterium while concentration of oil greater than 2% retarded the growth of the organism. In addition to lipase *B. subtilis* I-4 also produced alkane hydroxylase and biosurfactant which could make this bacterium potential candidate for lipase production as well as bioremediation of oil-contaminated wastewater.

Key words: *Bacillus subtilis* I-4, lipase, alkane hydroxylase

INTRODUCTION

Lipases are glycerol ester hydrolases that have the ability to act on acylglycerols to liberate fatty acids and glycerol. They are water-soluble enzymes that act in catalyzing the hydrolysis of ester bonds in water-insoluble lipid substrates (Guo and Xu 2005). Lipases are ubiquitously produced by the plants (Bhardwaj et al. 2001; Belguith et al. 2009); animals (Carriere et al. 1994); and microorganisms (Olempska-Beer et al. 2006; Ramesh et al. 2013). Microbial lipases are the preferred potent source due to several industrial potentials (Hasan et al. 2006; Riaz et al. 2010; Veerapagu et al. 2013). They are present in many bacteria, fungi, plants, and animals (Rabbani et al. 2013; Sugahara et al. 2014). A considerable number of lipase producing microorganisms such as *Pseudomonas fragi*, *Staphylococcus aureus*, and *Burkholderia glumae* have been described

(Ghanem et al. 2000; Gupta et al. 2004; Sangeetha et al. 2014).

The advent of enzymology represents an important breakthrough in the biotechnology industry, with the worldwide usage of enzymes (Kirk et al. 2002). Recombinant lipase has been developed for use in applications such as baking, laundry detergents and even as biocatalysts (Guo and Xu 2005) in alternative energy strategies to convert the vegetable oil into fuel (Gupta et al. 2004), etc. Biological wastewater treatment could be effective by lipase-producing microorganisms because lipid residues are converted to carbon dioxide, water and biomass (Lefebvre et al. 1998).

The present study was concerned with the isolation and characterization of *B. subtilis* I-4 from oil contaminated environment and lipase production and bioremediation of oil contaminated wastewater.

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MATERIAL AND METHODS

Samples Collection and Enrichment

Six samples were collected separately from the waste effluents of ICI polyester, Sufi banaspati ghee and Paper mill from Sheikhpura (Pakistan). The temperature and pH of samples at their sites were also noted. The samples were transferred to the laboratory and were spread on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract and 1.5% agar) plates and incubated at 37°C for 24 h on the same day.

Samples were enriched using the method of Hasanuzzaman et al. (2004) by taking 1.0 mL of sample and inoculated in 100 mL sterile minimal salt (MS) medium containing FeSO₄.7H₂O 1.5 mg, KH₂PO₄ 0.47 g, MgSO₄.7H₂O 0.1 g, CaCl₂.2H₂O 0.001 g, Na₂HPO₄ 0.0119 g, NH₄NO₃ 0.4 g, MnSO₄.4H₂O 0.001 g in 100 mL distilled water and containing 1% olive oil as a sole source of carbon. Same samples were inoculated in another minimal salt medium containing (NH₄)₂SO₄ 0.5%, K₂HPO₄ 0.05%, MgSO₄.7H₂O 0.03%. All the samples were incubated at 25°C at 180 rpm for two weeks. Turbidity in the flasks was observed regularly.

Screening of Lipase Producing Bacteria

For selection purpose, 1.0 mL culture broth from the flasks was taken and spread on Peptone Yeast Agar medium (peptone 10 g, yeast extract 10 g, agar 15 g, olive oil 10 mL, Tween80 3.0 mL; final volume 1000 mL with distilled H₂O) containing emulsified olive oil and were incubated at 37°C for 24 h. Colonies showing the zone of lipolysis around them were selected and were further purified on L-agar plates

Selection of Most Efficient Lipase Producing Bacteria

Following screening tests were carried out to select the most efficient bacterial isolates for lipase production.

Olive Oil Hydrolysis Agar

L-agar medium (Trypton 10.0 g, NaCl 5.0 g, yeast extract 5.0 g, agar 15.0 g) containing 1% olive oil was streaked with the isolates and incubated at 37°C for 48 h until the zones of hydrolysis became visible.

Lipase Chromogenic Plate Assay

Bacterial isolates were grown in LB medium containing 1% olive oil for 48 h. A modified

method from Amara et al. (2009) was used and chromogenic substrate plates were prepared by using phenol red (0.01%) along with 2% olive oil as substrate, 4% Tween 80, 20 mM CaCl₂. The pH was adjusted to 7.3–7.4 by using 0.1 M NaOH, and mixture was added to the same quantity of 4% melted agar (50°C) to give orange-reddish color. Holes were punched into the medium with the help of Pasteur pipette. Culture supernatants were poured into the holes and incubated at 37°C for 30 min. Zones of yellow color around the wells indicate lipase activity by the isolates.

Lipase Quantification Medium

Bacterial isolates were inoculated in 50 mL LB-broth and incubated at 37°C for 2-3 days on a rotary shaker. Then, 1.0 mL cell supernatant was taken after centrifugation at 6,000 rpm for 5 min. LQ-Agar medium with 1% olive oil were taken. LQ-Agar medium (glucose 10 g, peptone 10 g, yeast extract 3.0 g, agar 15 g, olive oil 10 mL, Tween80 3.0 mL, NH₄Cl 1.5g, K₂HPO₄ 2.0 g, MgSO₄.7H₂O 0.5 g, NaH₂PO₄ 1.0 g; final volume made to 1000 mL with distilled water) with 1% emulsified olive oil was taken and then a sterile pasture pipette was used to make wells in the LQ-Agar medium. About 60 µL of the culture supernatant was loaded on each well and labeled accordingly. Plates were left at room temperature for about 2 h and then were incubated at 37°C for 24 h. The formation of clear zones of lipolysis indicated lipase production of by the isolate and measured in millimeters.

Morphological, Biochemical and Molecular Characterization

The isolate was Gram stained. For the biochemical characterization the isolate was tested for catalase activity, oxidase activity, Voges-Proskauer test, and utilization of different sugars following Benson (1994). For molecular characterization, genomic DNA was extracted as described by Carozzi et al. (1991). The 16S rRNA gene was amplified by the PCR using 16S rRNA primers (RS-1; 5'-AAACTC-AAATGAATTGACGG-3', and RS-3; 5'-ACGGGCGGTGTGTAC-3') (Rehman et al. 2007). PCR was performed by initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR product of 0.5 kb was purified with a Fermentas purification kit (#K0513) and the

amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. Nucleotide sequence similarities were determined using BLAST (NCBI database; <http://www.ncbi.nlm.nih.gov/BLAST>).

Bacterial Growth Curves

The strain was grown in LB broth and 1% olive oil under similar conditions of temperature and pH. The media (100 mL) were dispensed in 250 mL flasks and autoclaved at 121°C for 15 min. After inoculating with 100 µL of log phase growing cells of bacterial isolate, the flasks were incubated at 37°C for 24 h. After each 4 h, optical density of each flask was taken at 600 nm with the help of a spectrophotometer.

Enzyme Assay

Lipase assay was performed as described by Bussamara et al. (2010) with slight modifications. The reaction was initialized by the addition of 0.1 mL of cell-free culture supernatant to 0.9 mL of substrate solution containing 3.0 mg of pNPP/mL of isopropanol and 9.0 mL of solution B 50 mM Tris-Cl (pH-8.0) 200 mL, Tween80 0.2 mL, Triton X-100 0.8 mL. The mixture was then incubated at 37°C for 30 min after maintaining its pH at 8.0. The increase in absorbance was measured at 410 nm with reference to an enzyme free control.

Effect of Temperatures, pH and Metal Ions on Enzyme Activity

Optimum temperature for enzyme stability was determined by incubating the culture supernatants at different temperatures, i.e., 30, 40, 50, 70 and 90°C for 1 h and then the enzyme assays were performed as described by Bussamara et al. (2010). Optimum pH for enzyme stability was determined by incubating the culture supernatants at different pH, i.e., 5.0, 6.0, 7.0, 8.0 and 9.0 for 1 h and then enzyme assays were performed. Lipase activity was also determined in the presence of metal ions by incubating the culture supernatants separately in different flasks with 1mM solution of each salt, i.e., ZnCl₂, MgCl₂, CuSO₄ and CaCl₂ at 37°C for 1 h.

Effect of Tween80, Ethanol, Oil and Glucose on Bacterial Growth

The bacterial isolate was inoculated in phenol red broth (Phenol red 1.0 g, yeast extract 5.0 g,

tryptone 10 g, distilled water 1L) along with 1% olive oil, 0.1% Tween 80, 0.1% ethanol and 0.1% glucose and then incubated at 37°C for 24 h. Effect of different concentrations of olive oil (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) was also checked on the bacterial growth after incubated at 37°C for 24 h.

Partial Purification and SDS-PAGE Analysis of Lipase

The bacterial cultures supernatants were harvested by centrifugation at 6000 × g at 4°C for 10 min and concentrated by the addition of solid ammonium sulfate (60%). The precipitate was harvested by centrifuging at 6000 × g for 10 min and dialyzed (Cellu-Sep membrane cat no 5-5050-34; pore size 34 nm for mw of protein 50,000) against 10 mM sodium tartrate buffer (pH-5.5) for overnight. The dialyzed sample was centrifuged at 6000 × g for 1.5 h in centricon Ultracel YM-100 membrane (100,000 NMWL) to remove and concentrate the protein having molecular weight lower than 100 kDa. The protein solution above the centricon was discarded and flow through was taken, having proteins less than 100 kDa. This protein solution was further used in Ultracel YM-20 membrane (20,000 NMWL) to remove the protein having MW 20, or less than 20 kDa. The protein solution in the upper chamber of the centricon was further concentrated in concentrator (Eppendorf concentrator, 5301) at 4°C and was finally analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as a standard.

Thin Layer Chromatography (TLC)

The TLC plate was dried after loading the samples, developed with Hexane-Diethyl Ether-Acetic Acid (80:30:1) solvent system and finally was stained by spraying it with 30% H₂SO₄ and 0.2% ninhydrin reagents separately. The plates were dried under a hot air drier and were observed for the appearance of color bands. The developed and air dried plated were also visualized using UV light at 366 nm and 254 nm.

Biosurfactant Assay

Drop collapse test

The assay was performed on a glass surface coated with oil and a 5.0 µL sample droplet was added to

the center of a well and observed after 1 min. The droplet was observed for its shape.

Tube Emulsification Test

In tube emulsification test 2.0 mL petrol and 2.0 mL bacterial culture (16 h grown) were taken and the mixture was vortexed for 10 min. The mixture was observed for layer formation.

Alkane Hydroxylase Assay

B. subtilis I-4 was inoculated in MS media containing 1% kerosene oil and 1% petrol and incubated at 37°C for 48 h at 130 rpm.

Statistical Analysis

All the experiments were repeated two or more times and the results were averaged.

RESULTS AND DISCUSSION

Screening and Identification of Lipolytic Bacteria

Among the 16 bacterial isolates, only the most potential lipase producer (I-4) was selected for further studies on the basis of different screening tests (Table 1). The temperature and pH of the sample were 30°C and 7.5, respectively from where the isolate I-4 was isolated.

Table 1 - Screening tests performed for the selection of most efficient lipase producing bacteria.

No.	Isolates	OHA (mm)	EAL (EU/ml/hr)	LCA (mm)	LQM (mm)
1	ICI	9	500	8	4
2	ICI-1	8	0	2	2
3	ICI-2	10	0	0	3
4	ICI-3	5	400	4	1
5	SF-1	2	100	0	0
6	SF-2	3	400	0	2
7	SF-3	2	0	6	0
8	MS-1	8	300	10	2
9	MS-2	7	200	6	2
10	MS-3	14	800	18	7
11	I-2	6	0	10	2
12	I-4	19	1800	20	10
13	SB-6	2	200	11	0
14	SH-2	5	400	8	1
15	SH-4	8	300	0	3
16	SH-6	4	200	2	4

OHA: Olive oil hydrolysis agar; EAL: Enzymatic assay of lipase; LCA: Lipase chromogenic plate assay; LQM: Lipase quantitation medium.

Olive oil hydrolysis agar was used to analyze the diameter of zones of lipolysis. Zone of 19 mm was produced on OHA medium after 48 h of incubation at 37°C by *B. subtilis* I-4. The isolate showed the zones of 20 and 10 mm on LCA and LQM, respectively (Table 1) and was able to produce lipase (1800 EU/mL). The biochemical tests performed for the isolate are shown in Table 2. The partial 16S rRNA gene was sequenced and the (500 bp) analysis clearly demonstrated that strain I-4 was a member of the genus *Bacillus* and exhibited maximum similarity (Maximum query coverage 100%; identity 92%) with the 16 S rRNA sequence of *Bacillus subtilis*. This sequence data has been submitted to the GenBank databases under accession no. JN546608.

Table 2 - Biochemical tests for the identification of most efficient lipase producing bacterium.

Biochemical tests	<i>B. subtilis</i> I-4
Gram staining	+
Methyl red	-
Voges-Proskauer	-
Motility	+
Catalase	+
Oxidase	-
Citrate	+
Starch hydrolysis	+
Casein hydrolysis	+
Indole	-
Glucose	+
Mannitol	+
Inositol	+
Sorbitol	NA
Rhamnose	NA
Arabinose	+
Lactose	+
Sucrose	+
Xylose	+
Fructose	+
Melibiose	NA
Amygdalin	NA

+: positive; -: negative; NA: Not applicable

Effect of Olive Oil on the Bacterial Growth

Growth curves of the strain were analyzed in LB and 1% olive oil. LB medium was taken as control for the bacterial growth. Bacterial growth was slightly slowed down in the presence of 1% olive oil as compared to LB medium. The maximum cell density was obtained after 16 h of incubation at 37°C in both LB and 1% olive oil (Fig. 1).

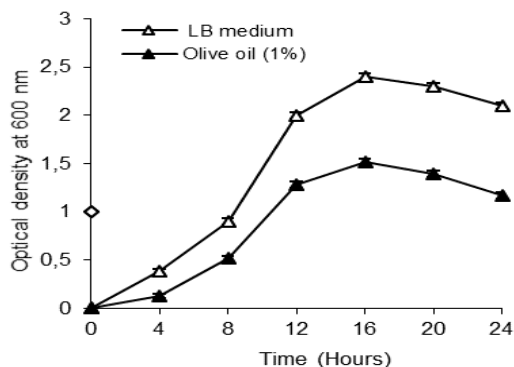


Figure 1 - Growth curves of *Bacillus subtilis* I-4 in the presence of LB and olive oil over a period of 24 h of incubation at 37°C.

Effect of pH and temperature on activity and stability of lipase

Bacillus subtilis I-4 showed optimum growth at 37°C and pH of 7.0; however, its growth was not retarded at 45°C. Largely, bacteria prefer pH around 7.0 for best growth and lipase production, such as in the case of *Bacillus* sp. (Sugihara et al. 1991), *Acinetobacter* sp. (Barbaro et al. 2001) and *Burkholderia* sp. (Rathi et al. 2001). However, maximum activity at higher pH (>7.0) has been observed in many cases (Wang et al. 1995; Khyami-Horani 1996; Dong et al. 1999; Sharma et al. 2002). In the present study, lipase from *B. subtilis* not only showed stability at higher temperatures but also showed maximum activity at 50°C (Fig. 2A); the optimum pH for enzyme

stability was 7.0 (Fig. 2B). An extracellular lipase isolated from *B. megaterium* AKG-1 showed optimum activity at 55°C and pH 7.0 (Sekhon et al. 2005).

The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism. For example, the best temperature for the growth and lipase production in the case of *Bacillus* sp. RSJ1 was 50°C (Sharma et al. 2002). Lipase produced by the *Bacillus* sp. MPTK 912 had the optimum pH of 8.0 and the optimum temperature was 35°C (Mukeshkumar et al. 2012). Sinchaikul et al. (2001) reported that the purified lipase had an optimum pH of 8.5 and showed maximal activity at 55°C. Shabtai and Daya-Mishne (1992) reported that the enzyme hydrolyzed a variety of fatty acid esters and had an optimum pH of about 7.0. The enzyme retained its full activity at 20 to 55°C.

Divalent cations stimulate, or inhibit enzyme production in microorganisms. The effect of different metal ions on lipase activity was also determined through enzyme assay using 1mM concentrations of different salts. Lipase from *B. subtilis* I-4 was activated by Ca^{2+} , while Mg^{2+} , Zn^{2+} , and Cu^{2+} ions inhibited its activity (Fig. 2c). It has been reported that lipase was inhibited by Co^{2+} , Ni^{2+} , Fe^{2+} , Fe^{3+} and EDTA, and activated by Ca^{2+} , Li^{+} and SDS (Matsumae et al. 1993). Rathi et al. (2001) observed stimulation in lipase production from *Burkholderia* sp. in the presence of Ca^{2+} and Mg^{2+} .

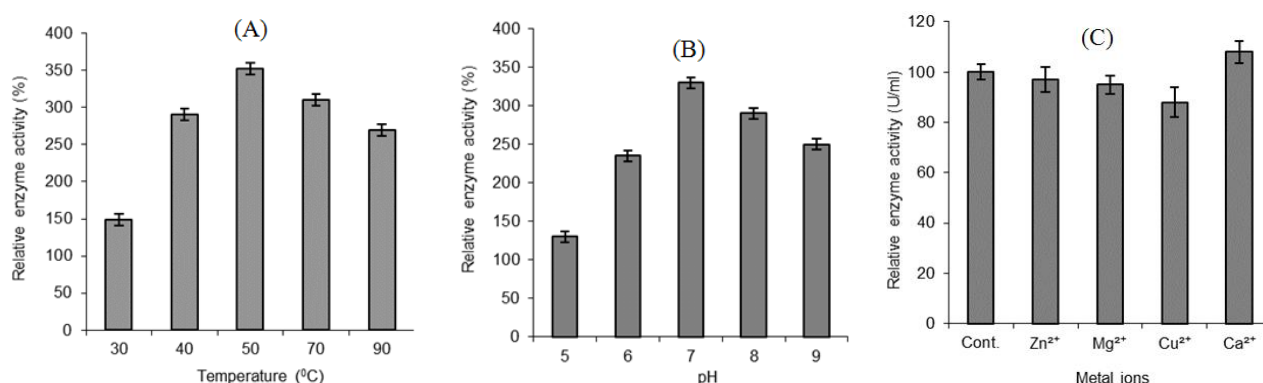


Figure 2 - Effect of temperature (A), pH (B) and metal ions (C) on lipase activity of *Bacillus subtilis* I-4.

Sharma et al. (2002) also reported stimulation in lipase production from *Bacillus* sp. RSJ1 in the presence of calcium chloride. However, most other

metal ion salts were inhibitory to lipase production. The enzyme was inhibited by Al^{3+} , Co^{2+} , Mn^{2+} , and Zn^{2+} ions while K^{+} , Fe^{3+} , Hg^{2+} ,

and Mg^{2+} ions enhanced the enzyme activity; Na^+ ions had no effect on enzyme activity (Kumar et al. 2005). Mukeshkumar et al. (2012) reported that Fe^{2+} , Mg^{2+} , Triton X-100, and Tween esters enhanced lipase production in *Bacillus* sp. MPTK 912.

Effect of Different Substrates on Lipase Production

Lipase production by the bacterial isolate in the presence of different substrates and oil concentrations was analyzed. Olive oil (1%) in broth cultures of isolate induced lipase production significantly as compared to 1% tributyrin. Increasing concentration of olive oil from 0.5 to 3% oil showed an increase in bacterial growth up to 1% and then a gradual decrease in growth with further increase in oil concentration was observed. At 3% oil, growth of the bacterial isolate was completely ceased (Fig. 3a). It could be due to a certain ability of the organism to use it as a carbon source and high concentration of such substrate

could become toxic for the organism. Addition of 0.1% surfactant, i.e., Tween 80 showed significant increase in lipase production. Isolate I-4 showed lipase activity by the yellowing of phenol red medium after 24 h of incubation. The addition of ethanol to the medium showed no positive effect on lipase production; in fact, its production was reduced (Fig. 3b). However, irrespective of inducing compounds added to the medium, both the activity of lipase and bacterial growth were increased when lipids were added to the medium, indicating that they not only acted as lipase inducers, but could also be utilized as energy and carbon sources. Tweens have been shown to stimulate the production of lipase in *Acinetobacter* and *Pseudomonas* species (Lin et al. 1995; Martinez and Nudel 2002). Olive oil was present in the growth medium as a carbon source. Glucose and peptone acted as the best carbon and nitrogen sources for lipase activity in the production medium (Mukeshkumar et al. 2012).

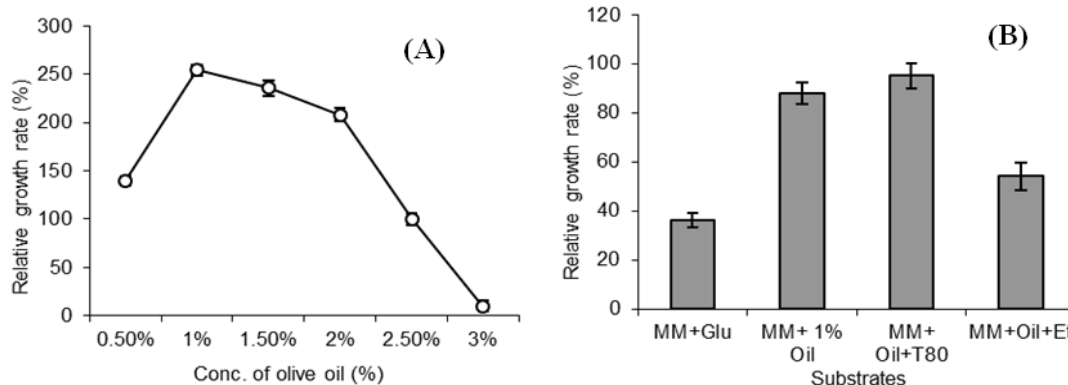


Figure 3 - Effect of olive oil concentration (A) and different substrates (B) on the growth of *B. subtilis* I-4.

Presumably, olive oil also functions as an inducer of the lipase operon. This hypothesis was tested by Boekema et al. (2007), who studied lipase production after the growth in PG medium supplemented with alternative carbon sources, i.e., glucose and sucrose, which functioned as a good or poor carbon source. Tween80 dramatically increased the extracellular lipase activity of strain PG1 in the presence of sucrose but much less in the presence of glucose as a carbon source. These results indicated that Tween 80, which, in contrast to Triton X-100, contained a fatty acyl ester bond, functioned as an inducer of the lipase operon and, furthermore, that the expression of this operon in

strain PG1 in the presence of glucose was prone to catabolite repression. Minakshichouhan and Dawande (2010) found that the enzymatic hydrolysis was highest with Tween 20, followed by Tween 80.

Thin Layer Chromatography

Bacterial isolate was analyzed for its ability to degrade the lipids through TLC as different components of lipid degradation could be visualized by different staining reagents. Fatty acids were visualized by 0.2% ninhydrin. *B. subtilis* I-4 showed pink bands when sprayed with ninhydrin, which were absent in the control,

indicating the degradation of lipids into its components. Lipid concentration decreased significantly in I-4 treated culture as compared to the control, when sprayed with H₂SO₄ (results not shown).

SDS-PAGE

The enzyme molecular mass was determined by the SDS-PAGE and induction of lipase in the presence and absence of olive oil was observed. Isolate I4 showed visible increase in protein production (data not shown), presumptively lipase, visible as band of nearly 53kDa on the gel (Fig. 4). Shabtai and Daya-Mishne (1992) reported that the approximate molecular weight of lipase was 40 kDa. The enzyme hydrolyzed a variety of fatty acid esters and had an optimum pH of about 7.0. Sinchaikul et al. (2001) reported the molecular mass of the lipase determined by the SDS-PAGE was approximately 43 kDa. The apparent molecular masses of LipA and LipB determined by the SDS-PAGE were 50 and 57 kDa, respectively (Salameh and Wiegel 2007). Kumar et al. (2005) reported the molecular weight of lipase 31 kDa on the SDS-PAGE for *B. coagulans* BTS-3. Kumar et al. (2012) reported a single band of 62.2 kDa lipase from *B. pumilus* RK31 on SDS-PAGE. Mukeshkumar et al. (2012) described that the enzyme was purified to homogeneity by Sephadex G-100 column chromatography and revealed molecular weight of 66 kDa.

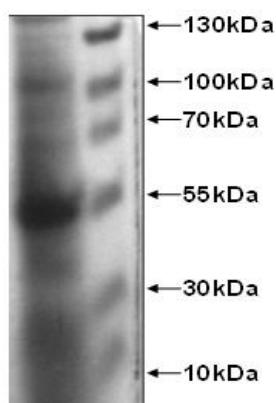
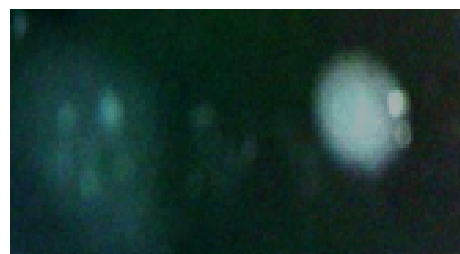


Figure 4 - SDS-PAGE of partially purified lipase of *B. subtilis* I-4. M, protein marker. The gel is 12% stained with Coomassie blue.

Biosurfactant Production

Bacillus subtilis I-4 was also tested for its ability to produce biosurfactants and emulsify immiscible

compounds. The culture showed positive slide drop collapse test as the drop of culture did not remain beaded when applied on a surface coated with oil (Fig. 5).

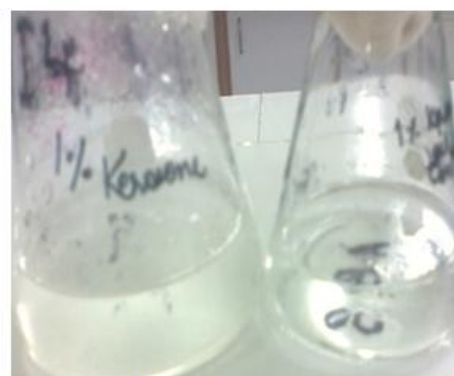


B. subtilis I-4 Control

Figure 5 - Drop collapse test of biosurfactant production, isolate I-4 showing collapse of drop on oil covered surface, and control showing no collapse of culture drop.

Alkane Hydroxylase

The ability of isolate to survive in the presence of hydrocarbons, including kerosene and petrol was analyzed. The culture was able to utilize kerosene oil and petrol when grown in minimal medium having 1% hydrocarbons as sole source of carbon (Fig. 6).



B. subtilis I-4 Control

Figure 6 - Growth of *B. subtilis* I-4 in 1% kerosene oil; turbidity indicates the growth in the medium after 48 h of incubation as compared to control showing no growth.

CONCLUSION

In the present study, lipase producing *B. subtilis* I-4 was isolated from oil contaminated industrial effluents and produce 1800 EU/mL lipase. The

optimum temperature and pH for the growth of the culture were 37°C and 7.0, respectively. *B. subtilis* I-4 had a lag phase of 4 h in LB medium while this phase prolonged to 6 h in oil containing medium. The optimum temperature and pH for the enzyme activity were 50°C and 7.0, respectively. Maximum lipase activity was in the presence of Ca ions. Olive oil and Tween 80 induced lipase gene in the bacterium while concentration of oil higher than 2% retarded the growth of the organism. In addition to lipase, *B. subtilis* I-4 also produced alkaline hydroxylase and biosurfactant, which could make this bacterium potential candidate for lipase production as well as bioremediation of oil-contaminated wastewater.

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REFERENCES

- Amara AA, Salem SR, Shabeb MSA. The possibility to use bacterial protease and lipase as biodetergent. *Global J Biotechnol Biochem.* 2009; 4(2): 104-114.
- Barbaro SE, Trevors JT, Inniss WE. Effect of low temperature, cold shock, and various carbon sources on esterase and lipase activities and exopolysaccharide production by a psychrotrophic *Acinetobacter* sp. *Can J Microbiol.* 2001; 47: 194-205.
- Belguith H, Fattouch S, Jridi T, Ben Hamida J. Immunopurification and characterization of a rape (*Brassica napus* L.) seedling lipase. *Afr J Biotechnol.* 2009; 12(21): 3224-3234.
- Benson HJ. *Microbiological applications*. Laboratory manual in general microbiology. Wan C. Brown Publishers, Dubuque, 1994.
- Bhardwaj K, Raju A, Rajasekharan R. Identification, purification and characterization of a thermally stable lipase from rice bran. A new member of the phospholipase family. *Plant Physiol.* 2001; 127: 1728-1738.
- Boekema BKHL, Beselin A, Breuer M, Hauer B, Koster M, Rosenau F, et al. Hexadecane and Tween80 stimulate lipase production in *Burkholderia glumae* by different mechanisms. *Appl Environ Microbiol.* 2007; 73(12): 3838-3844.
- Bussamara R, Fuentesfria AM, Oliveira ES, Broetto L, Simcikova M, Valente P, et al. Isolation of a lipase-secreting yeast for enzyme production in a pilot-plant scale batch fermentation. *Bioresource Technol.* 2010; 101(1): 268-275.
- Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG. Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. *Appl Environ Microbiol.* 1991; 57: 3057-306.
- Carriere F, Thirstrup K, Hjorth S. Cloning of the classical guinea pig pancreatic lipase and comparison with the lipase related protein2. *FEBS Lett.* 1994; 388: 63-68.
- Dong H, Gao S, Han S, Cao S. Purification and characterization of a *Pseudomonas* sp. lipase and its properties in non-aqueous media. *Appl Microbiol Biotechnol.* 1999; 30: 251-256.
- Ghanem EH, Al-Sayeed HA, Saleh KM. Analkalophilic thermostable lipase produced by a new isolate of *Bacillus alcalophilus*. *World J Microbiol Biotechnol.* 2000; 16: 459-464.
- Guo Z, Xu X. New opportunity for enzymatic modification of fats and oils with industrial potentials. *Org Biomol Chem.* 2005; 3(14): 2615-2619.
- Gupta R, Gupta N, Rathi P. Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol Biotechnol.* 2004; 64(6): 763-781.
- Hasan F, Shah AA, Hameed A. Industrial applications of microbial lipases. *Enzyme Microb Technol.* 2006; 39: 235-251.
- Hasanuzzaman M, Kathryn M, Umadhay-Briones SM, Morita N, Nodasaka Y, Yumoto I, et al. Isolation, identification, and characterization of a novel, oil-degrading bacterium, *Pseudomonas aeruginosa* T1. *Curr Microbiol.* 2004; 49: 108-114.
- Khyami-Horani H. Thermotolerant strain of *Bacillus licheniformis* producing lipase. *World J Microbiol Biotechnol.* 1996; 12: 399-401.
- Kirk O, Borchert TV, Fuglsang CC. Industrial enzyme applications. *Curr Opin Biotechnol.* 2002; 13: 345-351.
- Kumar R, Sharma A, Kumar A, Singh D. Lipase from *Bacillus pumilus* RK31: Production, purification and some properties. *World Appl Sci J.* 2012; 16(7): 940-948.
- Kumar S, Kikon K, Upadhyay A, Kanwar SS, Gupta R. Production, purification, and characterization of lipase from thermophilic and alkaliphilic *Bacillus coagulans* BTS-3. *Protein Expr Purif.* 2005; 41: 38-44.

- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227: 680-685.
- Lefebvre X, Paul E, Mauret M, Baptiste P, Capdeville B. Kinetic characterization of saponified domestic lipid residues aerobic biodegradation. *Water Res*. 1998; 32(10): 3031-3038.
- Lin S-F, Chiou C-M, Tsai Y-C. Effect of triton X-100 on alkaline lipase production by *Pseudomonas pseudoalcaligenes* F-111. *Biotechnol Lett*. 1995; 17: 959-962.
- Martinez DA, Nudel BC. The improvement of lipase secretion and stability by addition of inert compounds into *Acinetobacter calcoaceticus* cultures. *Can J Microbiol*. 2002; 48: 1056-1061.
- Matsumae H, Furui M, Shibatani T. Lipase-catalyzed asymmetric hydrolysis of 3-phenylglycidic acid ester, the key intermediate in the synthesis of diltiazem hydrochloride. *J Ferment Bioeng*. 1993; 75: 93-98.
- Minakshichouhan M, Dawande AY. Partial purification, characterization of lipase produced from *P. aeruginosa*. *Asiatic J Biotech Res*. 2010; 1: 29-34.
- Mukeshkumar DJ, Rejitha R, Devika S, Balakumaran MD, Immaculate NRA, Kalaichelvan PT. Production, optimization and purification of lipase from *Bacillus* sp. MPTK 912 isolated from oil mill effluent. *Adv Appl Sci Res*. 2012; 3(2): 930-938.
- Olempska-Beer ZS, Merker RI, Ditto MD. Food processing enzymes from recombinant microorganisms-a review. *Reg Toxicol Pharmacol*. 2006; 45: 144-158.
- Rabbani, M, Bagherinejad MR, Sadeghi HM, Shariat ZS, Etemadifar Z, Moazen F, et al. Isolation and characterization of novel thermophilic lipase-secreting bacteria. *Braz J Microbiol*. 2013; 44: 1113-1119.
- Ramesh S, Kumar R, Devi A, Balakrishnan, K. Isolation of a lipase producing bacteria for enzyme synthesis in shake flask cultivation. *Int J Curr Microbiol App Sci*. 2014; 3(3): 712-719.
- Riaz M, Shah AA, Hameed A, Hasan F. characterization of lipase produced by *Bacillus* sp. FH5 in immobilized and free state. *Ann Microbiol*. 2010; 60: 169-175.
- Rathi P, Saxena RK, Gupta R. A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. *Process Biochem*. 2001; 37: 187-192.
- Rehman A, Ali A, Muneer B, Shakoori AR. Resistance and biosorption of mercury by bacteria isolated from industrial effluents. *Pakistan J Zool*. 2007; 39: 137-146.
- Salameh MA, Wiegel J. Purification and characterization of two highly thermophilic alkaline lipases from *Thermosyntropha lipolytica*. *Appl Environ Microbiol*. 2007; 73 7725-7731.
- Sangeetha R; Arulpandi I, Geetha, A. Molecular characterization of a proteolysis-resistant lipase from *Bacillus pumilus* SG2. *Braz J Microbiol*. 2014; 45: 389-393.
- Sekhon A, Dahiya N, Tiwari RP, Hoondal GS. Properties of a thermostable extracellular lipase from *Bacillus megaterium* AKG-1. *J Basic Microbiol*. 2005; 45(2): 147-154
- Shabtai Y, Daya-Mishne N. Production, purification, and properties of a lipase from a bacterium (*Pseudomonas aeruginosa* YS-7) capable of growing in water-restricted environments. *Appl Environ Microbiol*. 1992; 58(1): 174-180.
- Sharma R, Soni SK, Vohra RM, Jolly RS, Gupta LK, Gupta JK. Production of extracellular alkaline lipase from a *Bacillus* sp. RSJ1 and its application in ester hydrolysis. *Ind J Microbiol*. 2002; 42: 49-54.
- Sinchaikul S, Sookkheo B, Phutrakul S, Pan FM, Chen ST. Optimization of a thermostable lipase from *Bacillus stearothermophilus* P1: overexpression, purification, and characterization. *Protein Expr Purif*. 2001; 22(3): 388-398.
- Sugihara A, Tani T, Tominaga Y. Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *J Biochem*. 1991; 109: 211-216.
- Sugahara VH, Varea GS. Immobilization of *Beauveria bassiana* lipase on silica gel by physical adsorption. *Braz Arch Biol Technol*. 2014; 57: 842-850.
- Veerapagu M, Sankara DR, Narayanan A, Ponmurugan K, Jeya KR. Screening selection identification production and optimization of bacterial lipase from oil spilled soil. *Asian J Pharm Clin Res*. 2013; 6: 62-67.
- Wang Y, Srivastava KC, Shen G-J, Wang HY. Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus* strain A30-1 (ATCC 53841). *J Ferment Bioeng*. 1995; 79: 433-438.

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