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Isolation and molecular identification of extracellular lipase-producing bacillus species from soil

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ABSTRACT

Microbial lipases are the most valuable Biocatalysts with Industrial applications that show a large variety of enzymatic properties among microbial populations. This study aimed to isolate extracellular lipase-producing Bacilli and their molecular identification in the soils of western Mazandaran, Iran. A total of 50 soil samples with bacterial lipolytic potential were collected from the depth of 10-15 cm of soils in the rural areas of western Mazandaran, Iran. The samples were directly cultured on tributyrin agar medium with Lipidic glycerol butyrate substrate after heat treatment and preparation of serial dilutions. DNA was isolated from bacillus isolates with a clear zone. The molecular identification of isolates was carried out by using 16S rDNA Real Time PCR, High Resolution Melting (HRM), direct sequencing, and sequence alignment in BLAST. The primary properties of lipase enzymes produced by isolates were determined using supernatant of medium culture by spectrophotometer measurement of enzymatic activity at different temperatures, different concentrations of substrate, and relative molecular weight determination of enzymes by SDS-PAGE. Total of 5 gram-positive bacilli was isolated. After identification of biochemical and morphological characteristics, sequence alignment of their 16S rDNAs, the strains were identified as B. megaterium 37-1, Bacillus safensis 1-1, Bacillus pumilus KN-Lip2, Bacillus subtilis KN-Lip3, and Lysinibacillus fusiformis KN-Lip4 in Genbank database. The highest lipase activity of each strain was determined as 626.1069 u/ml and 422.5517 u/ml for B. megaterium 37-1 and B. safensis 1-1, respectively. The relative molecular weight of lipases produced by both strains was about 55 KDa. V_{max} and k_m parameters were calculated by Michaeilis-Menthen hyperbola and Lineweaver-Burk plots. The V_{max} value of lipase enzyme produced by B. megaterium 37-1 at 37°C was higher than that produced by B. safensis 1-1. The k_m values of B. megaterium 37-1 were higher and lower than that of B. safensis 1-1 at 37°C and 55°C, respectively. In this study, lipolytic bacillus isolates were collected and identified from soil of various environments in the northern part of Iran. Among 5 new strains found in this study, B. megaterium 37-1 showed a higher enzymatic activity in compare to other strains.

Keywords: Bacillus strains, 16S rRNA; Lipase activity, kinetic study.

INTRODUCTION

Lipases can be naturally extracted from plants and animals. However, for the commercial purposes, they are mostly produced by microorganisms [4,13,20]. Several Bacillus spp. have been reported as the main sources of lipolytic

enzymes. Lipase-producing bacteria have been found in different environments such as oil contaminated soils, dairy industry wastes, oil seeds, decaying foods, organic materials, and hot springs [21,24].

Microbial lipases are widely used for biotechnological applications in dairy, detergents, textile industries, production of surfactants, and oil processing industries. Organic solvent resistant lipases are used in industries to hydrolyze water insoluble esters. Lipases are also used in pharmaceutical industries to separate racemic solutions and produce pharmaceutical uni-enantiomere. They are chirally important in efficacy of many drugs [3,9].

Bacterial lipases are classified into eight family based on their amino acids homology and molecular weights of proteins. The first family is also classified into six sub-families [4,13].

In this study, *B. megaterium* 37-1 and *B. safensis* 1-1 which had high level of lipase production among five isolated bacterial strains were selected and their enzymatic activities were determined.

MATERIALS AND METHODS

A total of 50 soil samples with the bacterial lipolytic potential were collected from the depth of 10-15cm of soils in the rural areas of western Mazandaran, Iran. Plants and particles with a diameter more than 2 mm were removed using a 2 mm sieve. Samples were dried at room temperature for 24 hours and kept at 4°C until use [16]. For suspension preparation, 1 g of each sample was added to 9 ml normal saline and left at room temperature for 30 min to form homogenous solution. The suspension was heat treated at 80°C for 3 min, to isolate bacteria spores [?????????]. Then, serial dilutions from 10^{-1} to 10^{-9} were made in physiologic serum. 1ml of each dilution was cultured on tributyrin agar medium with Lipidic glycerol butyrate substrate. After about 24- 48 h incubation at 37°C, bacteria were selected and re-cultured from colonies separate and clear rings [2,8].

Identification of isolated bacteria:
DIS-DIF-ENTERO kit based tests were used to determine biochemical properties of isolates. Tests were repeated two times. Gram staining method was also used to determine morphological properties and slides were examined by light microscopy.

16S rRNA gene sequencing:
Overnight cultures were inoculated into BM Broth (contains the following per liter: pepton, 10g ; lactose, 5g ; Nacl, 3g ; Beef extract, 3g ; K_2PO_4, 2g. pH 7.2) and incubated in shaker incubator at 37°C for 48 hours. DNA was isolated using Phenol/Chloroform method (Sambrook et al, 1989) The following set of primers were used to amplify 16s rRNA gene: F: 5'AGAGTTTGATCCTGGCTCAG3' and R: 5'GACGGGCGGTGTGTACAA3' [22].

The PCR was performed in a volume of 50 µl including 200 ng of genomic DNA, 0.3 ρmol of each F and R primers, 2 unit *Taq* DNA Polymerase, 0.2 mM of dNTPs, 50 mM of KCl, 10 mM of Tris-HCl, and 2 mM of $MgCl_2$. The PCR cycling was carried out on iCycler thermal cycler (BioRad Laboratories, Hercules, California, USA). Cycling conditions was set up as follows: 95ºC for 5 min as initial denaturation; 95ºC for 40 sec, 55 ºC for 30 sec, and 72ºC for 40 sec for 30 cycles, and 72ºC for 5 min as final extension.

To categorize isolates, the PCR products were examined by High Resolution Melting (HRM) method (Corbett, RG6000). High Resolution Melting Real-Time PCR (HRM RT-PCR) was carried out as described before plus adding 10 µmol SYTO-9 fluorescent dye (Invitrogen) to the PCR tube. HRM was analyzed in 72°C- 90°C with a 0.1°C/S temperature shift and absorption in Green Channel. Isolates were categorized into four groups including A, B, C, D, three PCR products of each group were sequenced (Macrogen Company, South Korea) and aligned by BLAST (http://www.ncbi.nlm.nih.gov/nuccore) to determine the different strains.

SDS-PAGE:
Proteins were examined by precipitation with acetone. The bacteria cultured into tri-butyrin broth medium were centrifuged (Centurio Company, England) and supernatant including secretory proteins was transferred into a sterile conical flask. Cold 80% acetone was slowly added into the flask while it was shaken on ice. The flask was kept at 4°C until two distinct phases formed [15]. The solutions were mixed, and then centrifuged at 14000 rpm for 20 min. The pellet was dried, dissolved with 40 µl Tris (20 mM), and stored at -20°C until use.

The SDS-PAGE was used to distinct proteins. 15 µl of prepared sample was mixed with 10 µl of loading buffer (5x), 2 µl of reducing agent (Fermentas), 25 µl of ddH_2O, and was applied to the gel after boiling at 95°C for 5 min. The electrophoresis was carried out at 30 mA. The protein bands were stained with Coomassie brilliant blue G-250, and their molecular weights were determined by marker proteins (Fermentas; SM.431).

Culture conditions for lipase production:
A total of 50 ml Luria Bertony medium comprising glycerol-butyrate was added into a 250 ml conical flask. 30 µl of stored bacteria was inoculated into the medium and was cultured in shaker incubator (N-Biotek-inc. company, South Korea) (150 rpm) at 37°C, pH 7. After 48 hours, the medium was rapidly chilled at 4°C and centrifuged at 4000 rpm for 30 min at 4°C. The supernatant was used for further examination of enzyme activity [2,19].

Determination of lipase activity:
Lipase activity was determined by Colorimetric assay [18]. The substrate solution was prepared by adding 200 µl Tween to 40 mg ρ-nitrophenyl palmitate soluble in 10 ml DMSO. The enzyme solution was also prepared by adding 40 µl supernatant to 500 µl Tris-HCl (50 mM, pH 8). Enzyme activity was determined by adding different volumes of substrates including 0.4, 0.8, 2, and 4 mg/ml ρ-nitrophenyl palmitate (sigma). The activity for every concentration was measured at 37°C and 55°C, pH 7, 8, 9, and 10. The amount of produced ρ-nitrophenol was measured by spectrophotometry. The OD was recorded after 1, 2 and 3 min. The extinction coefficient and lypolytic activity were controlled by using 145.00 L mol/cm and substrate as blank, respectively. One unit of enzyme is the lipase activity that releases1µmol/min ρ-nitrophenol.

Gene submission:
The 16S rRNA genes of the isolates were submitted in Gene bank including gi including gi: 394790521 for *B. megaterium*, gi: 394848418 for B. Safensis.

Kinetic study
Hyper32 software was used to calculate V_{max} and k_m kinetic indices by drawing Michaelis-Menten Hyperbola curves. Lineweaver-Burk plots were also drawn to overcome Michaelis-Menten Hyperbola defects, as it is a linear plot contrary to Michaelis-Menten Hyperbola. Synthetic parameters were calculated based on released ρ-nitrophenol at different concentrations of substrates at 37°C and 55°C.

RESULTS

Five bacterial isolates with higher lipolytic activity were isolated from soil, morphologically were gram-positive, rod shaped with lateral and terminal spore. The biochemical properties of the strains are shown in Table 1.

Comparison between submitted 16S rRNA gene sequences in this study and those recorded on Genbank showed that isolate 1-1 had %99 similarity to *Bacillus safensis*, isolate 1-2 had %100 similarity to *Bacillus pumilus* EG_1, isolate 2-2 had %100 similarity to *Bacillus amyloliquefaciens*, isolate 3-9 had %100 similarity to *Lycinibacillus fosiformis*, and isolate 37-1 had %99 similarity to *Bacillus megaterium* 37-1. The results were consistent with those obtained by biochemical tests.

The relative purification of lipase was carried out by microbial culture supernatant and precipitation with acetone. Glycerol was the only source of carbon for bacterial growth, which is usable only if extra-cellular lipase is available in the broth medium. A 55 kDa band coincident with lipase molecular weight was observed by SDS-PAGE of *B. safensis* 1-1 and *B. megaterium* 37-1 (Figure 1).

Table 1: Biochemical properties of 5 strains isolated from soil by DS-DIF-ENTRO Kit

Isolate / Test	37-1	1-1	1-2	2-2	9-3
Catalase	+	+	+	+	+
Citrate	+	+	+	+	+
Malonate	+	+	+	+	+
Esculine	+	+	+	+	-
Lysine	-	+	-	+	+
Arginine	-	-	-	+	+
Ornitine	-	+	+	+	+
Hydrogen sulfid	-	-	-	-	-
Phenylalanine	-	-	-	-	-
Vp	-	-	-	-	-
Indole	-	-	-	-	-
Glocose	+/-	-	-	-	-
B-galactosidase	+	+	+	+	-
Lactose	-	-	-	-	-
Mannitol	-	-	-	-	-
Sucrose	-	-	-	-	-
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Arabinose	-	-	-	-	-
Maltose	-	-	-	-	-
Adonitol	-	-	-	-	-
Trehalose	-	-	-	-	-
Ramnose	-	-	-	-	-
Dulsitol	-	-	-	-	-
Urease	+	+	+	+	+

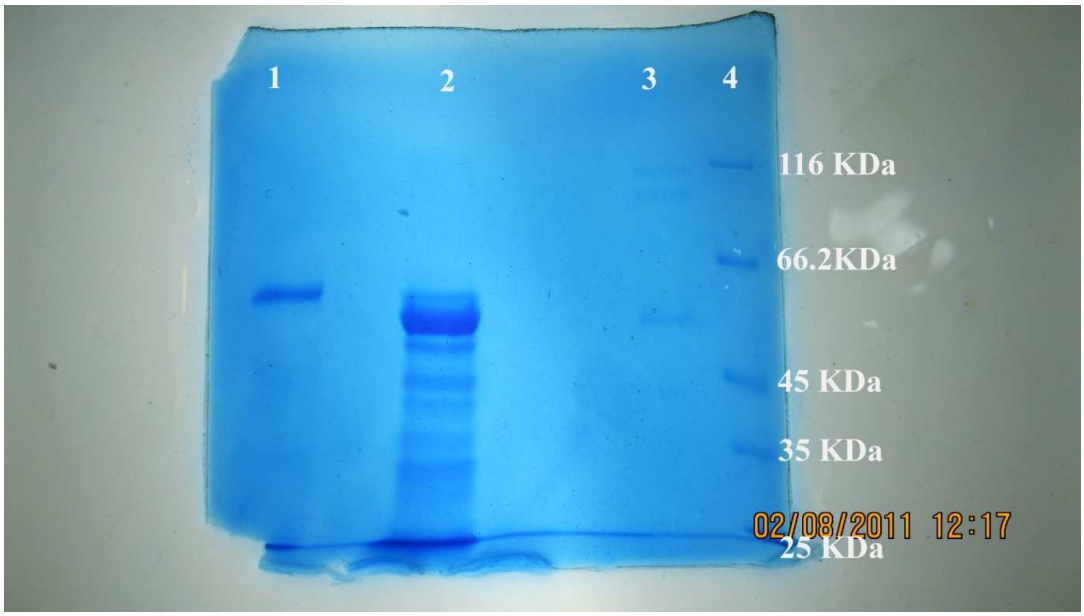


Fig NO.1 SDS-PAGE results of *B. safensis* 1-1 and *B. megaterium* 37-1, Lane 1: Negative control, Lane 2: *Bacillus megaterium 37-1*, Lane 3: *Bacillus safensis 1-1*, Lane 4: Protein marker (Fermentas; SM, 431)

Lipase Activity:

Lipase activity and synthetic indices were determined by spectrophotometry of released para-nitrophenol at 410 nm. The lipase activities of *B. safensis* and *B. megaterium* at different concentrations at 37°C and 55°C are given in Table 2.

Table 2: Lipase activity of *B. safensis* and *B. megaterium* at different concentrations at 37°C and 55°C. The tests were done p-nitrophenyl palmitate (sigma) as substrate according to Karadzic procedure (Karadzic et al., 2006) as mentioned in material and method

Lipase activities of different experiment as Units/ml					
Substrate mg/ml					
		0.4	0.8	2	4
Sample	Temperature	Specific activity p-nitrophenyl palmitate U/ml			
B. safensis	37°C	0.071	0.138	0.248	0.323
	55°C	0.091	0.193	0.256	0.422
B. megaterium	37°C	0.083	0.126	0.203	0.324
	55°C	0.096	0.194	0.473	0.626

The highest lipase activity for *B. safensis* was 0.422 U/ml which was recorded at 55°C and 4 mg/ml paranitrophenyl palmitate as substrate. The enzyme activity for *B. megaterium* was 0.626 U/ml at 55°C. The highest enzyme activity for both strains was recorded at pH 9 (Figure 2).

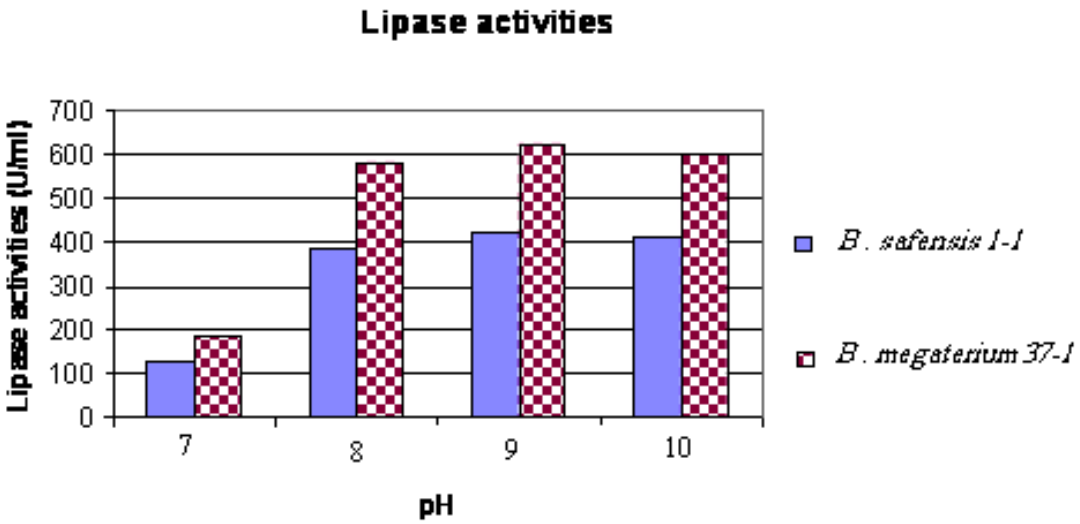
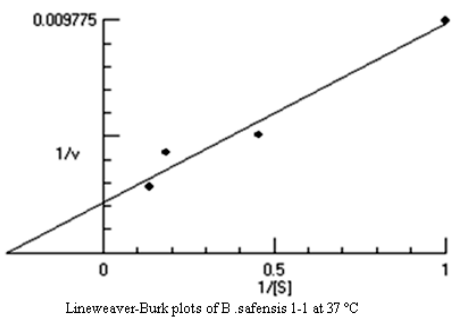
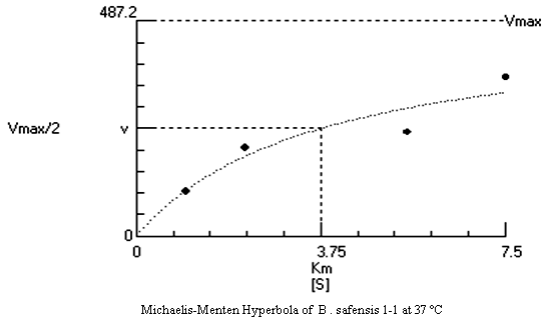


Fig NO. 2 Lipase activity of *B. safensis* 1-1 and *B. megaterium* 37-1 at different pH. p-nitrophenyl palmitate (sigma) was used as substrate according to the Karadzic procedure as mentioned in material and methods

The V_{max} and k_m indices for *B. safensis* 1-1 and *B. megaterium* 37-1 at 37°C and 55°C and different concentrations of substrate calculated by Michaelis-Menthen hyperbola and Lineweaver-Burk plots are given in Figure 3 and Table 3.



Lineweaver-Burk plots of B .safensis 1-1 at 37 °C



Michaelis-Menten Hyperbola of B . safensis 1-1 at 37 °C

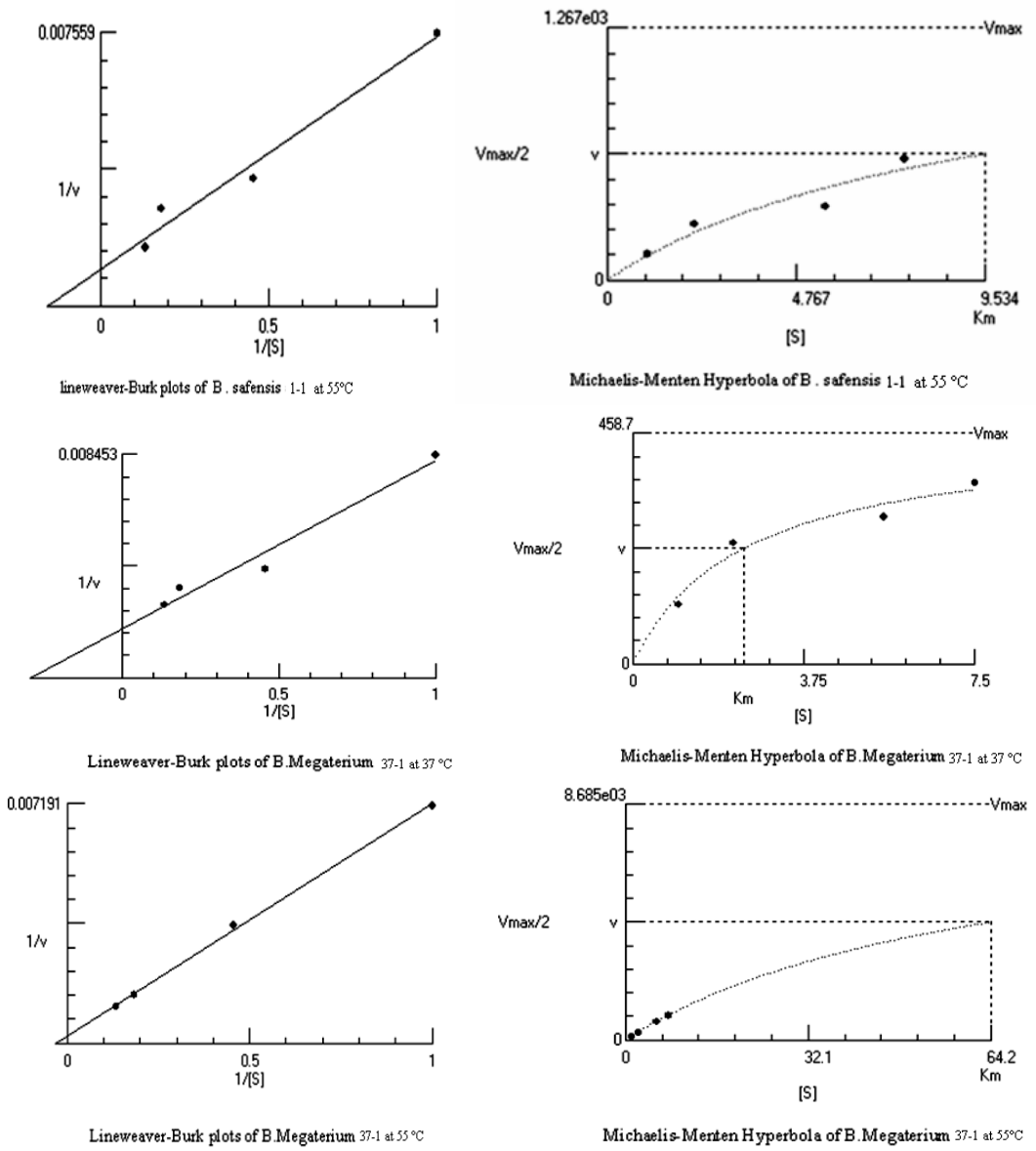


Fig NO.3 Michaelis-Menthen hyperbola and Lineweaver-Burk plots of lipases produced by *B. safensis* 1-1 and *B. megaterium* 37-1 at 37°C and 55°C. (Amara et al, 2009)

Table 3: V_{max} and k_m indices calculated for lipase activity of *B. safensis* 1-1 and *B. megaterium* 37-1 by Michaelis-Menthen and Lineweaver-Burk plots at different temperatures by Karadzic procedure, substrate and absorbance at 430 nm (Amara et al., 2009)

V_{max} and K_m Michaelis-Menthen and Lineweaver-Burk plots for lipase activities at different temperature					
		Hanes plot	Lineweaver-Burk	Hanes plot	Lineweaver-Burk
		37°C		55°C	
B. safensis 1-1	V_{max}	**482**	**469.4**	**1.019e03**	**988.2**
	K_m	**3.761**	**3.512**	**6.757**	**6.355**
B. megaterium37-1	V_{max}	**770.5**	**527.6**	**6.824e03**	**4.352e03**
	K_m	**6.518**	**3.471**	**49.18**	**30.3**

As shown in Table 3, V_{max} value of lipase enzyme produced by *B. megaterium* 37-1 was higher than the value of *B. safensis*1-1 at 37°C while k_m value of lipase enzyme produced by *B. megaterium* 37-1 was lower than the value of *B.*

*safensis*1-1. However, V_{max} and K_m values of *B. megaterium* 37-1 were respectively higher than that of *B. safensis* 1-1 at 55°C.

DISCUSSION

Lipases form an important group of enzymes in biotechnology with a wide range of applications in food, dairy, detergents, textile and some other industries, which are produced by microorganisms, and especially bacteria. *Bacillus*, *Pseudomonas*, and *Burkholderia* are the most important lipase producing bacteria [7,12]. However, many researches are on going to introduce new bacterial sources of lipases, and use lipases as the biocatalysts in different industries [10,18]. Amongst, the lipases of *B. subtilis* and *B. megaterium* are small extracellular enzymes and classified to the subfamily 1.4 of bacterial lipases, based on amino acid sequence similarity [15,19].The subfamily 1.4 lipases are mesophilic, have pH optima in a neutral to alkaline range (pH 7–11), and preferentially hydrolyze substrates with medium or short-chain fatty acids [6,17]

In this study, 5 Bacillus isolates were obtained from the superficial soils, and two strains with the largest lipase ring in the medium with lipidic glycerol butyrate as substrate were selected for further analysis for their enzyme activities and kinetic indices. The highest enzyme activity measured in *B.safensis* and *B. megatrium* were at 55°C under condition pH=9 and 4 mg substrate concentration. These results are similar to the study Morabi heravi et al., [14]. They were reported with cloning *B. pumilus* lipase gene strain F3 in *Escherichia coli* BL21 (DE3) that the most enzyme activity at the 0.11 U/ml rate incubated for 20 hours. Lima et al., [11] showed Lipase activity in *B. Megatrium* in 27 to 70°C range. Maximum enzyme activity was recorded at 55°C. On the other hand, Smibert and krieg [23] shown *B.safensis* has the ability to produce of lipase. Also were consistent with those reported for *Bacillus haludorans* [18] and S1/7 [18]. V_{max} indices for lipases produced by *B. megaterium* were higher than those recorded for *B. safensis* at both 37°C and 55°C. However, K_m indices recorded for lipases produced by *B. megaterium* were respectively lower and higher than those of *B. safensis* at 37°C and 55°C. These results show that the enzymes produced by *B. megaterium* and *B. safensis* have a higher efficiency at 37°C and 55°C, respectively.

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