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Isolation, Optimization, and Molecular Characterization of a Lipase Producing Bacterium from Oil Contaminated Soils

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ABSTRACT: Lipases have many applications in biotechnology, thanks to their ability of acylglycerides hydrolysis. They alsp possess the unique feature of acting at the lipid-water interface, which distinguishes them from esterases. Commercially useful lipases are produced by microorganisms with the extracellular lipase being produced by many bacteria including Pseudomonas. The greatest production of lipase takes place under optimum conditions such as appropriate temperature, suitable carbon, nitrogen sources, etc. This study tries to collect lipase-producing bacteria from the soil of oil-extraction factories and identify isolated bacteria, while creating optimum conditions for lipase production by bacteria. Having collected three soil samples from an oil extraction factory, lipase-producing bacteria have been identified, based on biochemical and morphological tests. Finally the optimal conditions for lipase production as well as molecular analysis has been evaluated. During the study, among the different bacteria, the strain to produce highest lipase has been selected. It has been found out that the optimal conditions for lipase production by this strain is as follows: 48 hours of incubation; incubation temperature of 37 °C; pH of 7; agitation speed of 150 rpm; peptone extract as the nitrogen source; and olive oil as a carbon source. A lipase-producing bacterium has been identified based on morphological, physiological, and biochemical characteristics as well as 16S rRNA analysis, identified as Pseudomonas spp. (accession number: KY288051).

Keywords: KY288051, Oil contamination, Pseudomonas spp., 16S rRNA.

INTRODUCTION

Lipases are industrially important enzymes that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol (Angkawidjaja and Kanaya, 2006; Gilham and Lehner, 2005) at lipid-water interfaces, involving interfacial adsorption and subsequent catalysis (Mohan et al., 2008). These unique enzymes are widely found in plants, animals, and microbials, being used increasingly in food industry, detergents, cosmetics, and pharmaceuticals (Grbavčić et al., 2007; Gupta et al., 2007; Park et al., 2005). Lipase is produced by many microorganisms and eukaryotes, but the most commercially useful are the ones, created by microorganisms. In the industry, due to exclusive properties, special attention has been paid to microbial lipase (Griebeler et al., 2011). Microbial enzymes are also more stable than the

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enzymes in plants and animals with easier and safer production (Hasan et al., 2006). Many microorganisms, such as bacteria, yeasts, and fungi have been known as producers of extracellular lipases, which are produced by many different species of bacteria, like Bacillus and Pseudomonas (Kiran et al., 2008; Wang et al., 2009). Over 160 strains of microorganisms were screened for exogenous lipolytic activity. The highest lipase activity was found in Streptomyces sp., Bacillus sp., and Pseudomonas sp. (Haba et al., 2000). Many industrial microbial lipases are derived from fungi and bacteria. Since the fungi can secrete enzymes into their environments, purification of this enzyme is easier. In modern processes, fungal genes are expressed in bacterial systems. Lipase-producing microorganisms can be found in various habitats such as industrial waste, oil plants, petroleum contaminated soil, oil seeds, and rotted food (Veerapagu et al., 2013; Golani et al., 2016). Medium with soyabean meal-0.77% (w/v); $(NH_4)_2SO_4$-0.1 M; KH_2PO_4-0.05 M; rice bran oil-2% (v/v); $CaCl_2$-0.05 M; PEG 6000-0.05% (w/v); NaCl-1% (w/v); inoculum-1% (v/v); pH 3.0; incubation temperature 35 °C and incubation period of five days have been identified as optimal conditions for maximal lipase production (Basheer et al., 2011). Enzyme production is strongly influenced by the age of the culture. Tributyrin and olive oil have proven to be the best sources of carbon for lipase-producing strains (Golani et al., 2016).

The increasing global demand for oils raises waste production, which in turns renders environmental pollution with waste oils one of the major problems in today's industrial world which will lead to environmental destruction. As a result, easy and affordable methods should be employed to minimize the amount of waste. There have been numerous studies on the use of lipase for disposal of oily waste water, though these enzymes are commonly unstable in environmental conditions. Hence, the use of bacteria, compatible with soil and wastewater and capable of producing enzymes in the soil and oils waste, is a good choice to treat this type of wastewater (Treichel et al., 2010). Lipases affect a variety of substrates, including natural oils, fatty acids esters, and synthetic triglycerides. Many agro-industrial residues can be used as potential substrates to produce enzymes. Resistant to solvents, they can be used in a wide range of biotechnological processes, among which the new examples, such as biopolymer synthesis, production of biodiesel, and treatment of wastewater containing oils, has been successfully completed (Shelatkar et al., 2016). The high potential of lipases in food technology applications and related issues reflect the need to develop new affordable technologies to increase production, while enhancing the scale and purity of these enzymes. The use of lipases has been expanded greatly with new programs being studied in the food industry, continuously. There are various ongoing scientific investigations in the field of developing enzymatic hydrolysis processes to precede traditional biological treatment. Lipases properties have improved by both protein and genetic engineering. Innovations in enzyme stabilization plays an important role in using this enzyme as a biocatalyst in effective food processing technology. Apart from food industry, lipases are being employed in various industries such as production of biodiesel, bio-polymer, detergent, pulp and paper, health products, and pesticides. Properties and application of lipase as a catalyst for reactions with commercial potential may greatly expand industrial biotechnology (Couto and Sanromán, 2006). Lipases are the most important group of biocatalysts for biotechnological applications. In order to optimize these enzymes for utilization in the industry, lipase gene manipulation seems to

be necessary. The objective of this research is to screen, isolate, and identify lipase-producing bacteria from the soil of oil extraction factory and to classify the studied bacteria, based on 16S rRNA gene sequences.

MATERIALS AND METHODS

Three soil samples were collected from an oil-extraction factory in Karaj (35°83´ N, 50°96´ W), Iran, at a distance of about 5 meters, to be stored at 4°C for testing.

Five grams of the soil samples, in sterile conditions, were added to 95 ml of medium containing 5.0% peptone, 3.0% yeast extract, and 2% olive oil and vegetable oil. It was then stirred at 100 rpm for 24 hours at 35°C. This culture was diluted up to 6 times to different concentrations and were cultured in Tween 80 medium. Tween culture medium contained 1% Tween 80, 1.5% agar, 5.0% sodium chloride, and 1.0% calcium chloride. The colonies with clear zones were separated from the culture medium, transferred to new plates, and stored at refrigerator temperature (Shukla and Gupta, 2007).

Lipase activity was measured with an olive oil emulsion ratio of 1:3 and 2% polyvinyl alcohol, into which 4 ml of Tris buffer, with a pH of 8, and 1 ml of 1.0 M calcium chloride was added. After 40 hours, the culture medium, which contained 2% olive oil and had a pH of 7, was centrifuged in 6000 rpm for 20 minutes. Afterwards, 1 ml of the culture medium's supernatant, containing the enzyme lipase, was added to 5 ml of oil emulsion and shaked in 100 rpm for 20 min at 35°C. Later, 15 ml of acetone-alcohol mixture was added to stop lipase activity. Control samples contained reaction mixture and 1 ml inactivated enzyme at high temperatures. The amount of fatty acids was titrated with both Phenolphthalein reagent and 0.05 M sodium hydroxide. The amount of free fatty acid per minute was considered as a unit of lipase activity (U/ml) (Colen et al., 2006).

Tween-agar mixture medium, which contained Tween 80 and had a pH of 7, was used. Due to the presence of calcium chloride, hydrated in the medium, Tween 80 was decomposed by lipase as a result of its combination with calcium chloride, leading to the production of calcium oleate, appearing as a white precipitate around the colonies of bacteria. Enzymatic activity was evaluated based on the diameter of the white zone around colonies. Here, the incubation time ranged between 8 and 104 hours; the temperature between 17 and 45°C; the pH rate, between 5 and 9; and the agitation speed between 25 and 200 rpm, with soybean, peptone, yeast extract, triptone, and sodium nitrate as nitrogen sources and palm, olive, sunflower, coconut, and corn oils as carbon ones.

In order to identify positive lipase bacteria, such tests as Gram stain reaction, Vogues-Proskauer, oxidase, catalase, citrate, nitrate reductase, and urease were performed. Furthermore, biochemical properties were studied at 37 ° C.

Genomic DNA extraction was performed via phenol-chloroform method (Mohan et al., 2008), its purity and concentration being evaluated with a spectrophotometer (Eppendorf, Germany).

Polymerase Chain Reaction (PCR) was employed to amplify bacterial 16S rRNA gene. The master mix for the PCR was prepared as follows: 3µl of 10x PCR buffer, 1µl of 25mM $MgCl_2$, 3µl of 10mM dNTP mix, 0.5µl of Taq DNA Polymerase, 12.5µl of MilliQ water, and 1µl of each of the forward and reverse primers. Finally, 3µl of each DNA template was added in the corresponding tubes to make up the final reaction volume of 25µl.

Bacterial 16S rRNA gene was amplified using the universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1107 R (5' AAGGTTACCTCACCGACTTC 3') primers (Lane, 1991). The thermal cycler was programmed as follows: 5 min at 94ºC,

30 cycles of 45s at 94ºC, 1 min at 58ºC, 1 min at 72ºC, and 10 min at 72ºC. The PCR product was sequenced for 16S rRNA (Macrogen, Korea). The sequencing results were compared, using the Basic Local Alignment Search Tool (BLAST) program on NCBI and 16S rRNA gene sequence homology was analyzed, using GenBank data. A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 5 program.

The difference between the treatments was determined based on a completely-randomized factorial design by means of analysis of variance (ANOVA). Data averages were compared according to Duncan test with both SPSS version 19 and EXCEL version 2013 (Chicago, USA).

RESULTS AND DISCUSSION

Having separated the prototypes of the three studied regions, nine colonies, suspected of producing lipase, were isolated from the soil samples of Regions B and C. In the soil sample from Region A, completely saturated with oil, no lipase-producing bacteria were found. The obtained strains were studied in a qualitative medium, containing olive oil and Tween 80, with their ability to produce enzymes assessed (Table 1).

Results show that the enzyme activity of B samples was more than the C ones; moreover, the highest enzymatic activity in each group belonged to bacteria No. 2 from B samples (7.1 U/ml) and bacteria No. 5 from C samples (3.2 U/ml). To determine the type of bacteria strains, biochemical tests were performed on bacteria numbers 2 and 5 (Table 2). Based on this experiment, strain No. 2 was Gram-negative rod-shaped while No. 5 was Gram-positive cocci. To determine the optimum conditions for lipase production, bacterial strain No. 2, having the largest halo and greatest lipase activity, was selected.

Both medium characteristics and the rate of bacterial growth have an influence on the incubation time for enzyme production. Results from this study show that at a constant temperature of 37 °C, by increasing incubation time up to 48 hours, the enzymatic activity rises to 23.7 U/ml then to decrease bacterial lipase activity (Figure 1).

Table 1. Enzymatic activity of strains isolated from soil.

Sample No.	1	2	3	4	5	6	7	8	9
Soil sample	B	B	B	B	C	C	C	C	C
Gram staining	+	-	+	+	+	-	-	+	+
Enzyme activity (U/ml)	4.7	7.1	6.2	5.3	3.2	1.1	2.6	2.5	1.7

Table 2. Biochemical and morphological tests on bacterial strains.

Test	Strain No. 2	Strain No. 5
Gram staining	-	+
Morphology	Rod-shaped	Coccus
Mobility	+	+
Catalase	+	+
Oxidase	+	-
Indole	-	-
Methyl red	-	-
Citrate	+	-
Urease	-	-

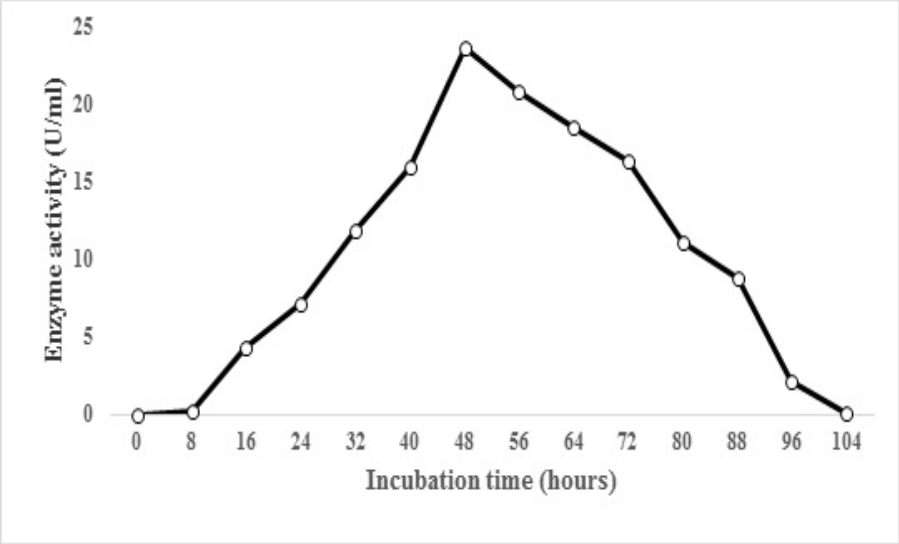

Fig. 1. Effect of incubation time on enzyme activity.

It has been reported that *Staphylococcus* and *Trichoderma viride* produce the maximum lipase after 48 hours (Sirisha et al., 2010; Kashmiri et al., 2006), whereas Pseudomonas spp and *Bacillus coagulans* follow the same example after 72 hours (Tembhurkar et al., 2012; kumar et al., 2007),.

The study of incubation temperature (with a constant time of 24 hours) revealed that increasing the incubation temperature up to 37 ° C contributed to an increasing enzyme activity up to 7.1 U/ml; though, any further increase in the temperature reduced the bacterial enzyme activity (Figure 2).

The pH rate of the culture medium influences the amount of lipase production. Our experiments showed that the bacterium was capable of producing lipase while the pH was between 5.0 and pH 9.0, while it has been found that bacteria in tributyrin broth medium experience their maximum lipase production at pH 7.0 (7.4 U/ml) (Figure 3). The maximum lipase activity of *Staphylococcus aureus* has been reported to be at pH=7.0 and at a temperature of 37°C (Sirisha et al., 2010). In another research, a *Bacillus subtilis*, isolated from oil-contaminated wastewater, had its maximum lipase activity in 37°C (Iqbal and Rehman, 2015).

For better oxygenation, agitation was required for the microorganisms to produce lipase as there was no lipase production at stationary condition. While it ranged from 25 rpm to 150 rpm, agitation enhanced the lipase enzyme production with the optimum agitation speed for lipase production by the bacteria being 150 rpm (11.6 U/ml). Higher speeds decreased the lipase production (Figure 4).

Increasing lipase production was directly related to increased oxygen transfer rate as well as increased surface area of contact with the media components, which was possible in agitated condition. However, at higher agitation rates, both the growth and lipase production declined (Gulati et al., 2000; Elibol et al., 2000).

Among the soybeen, peptone, yeast extract, triptone, and sodium nitrate, the extract from peptone (15.8 U/ml) enhanced lipase production, whereas lipase production was low with sodium nitrate (Figure 5).

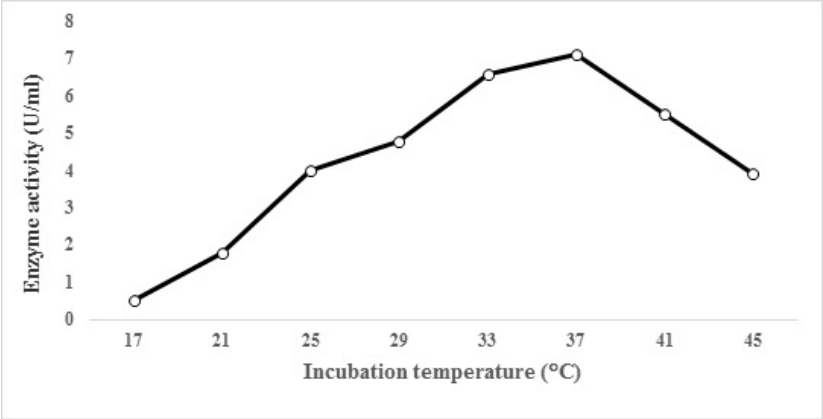

Fig. 2. Effect of incubation temperature on enzyme activity.

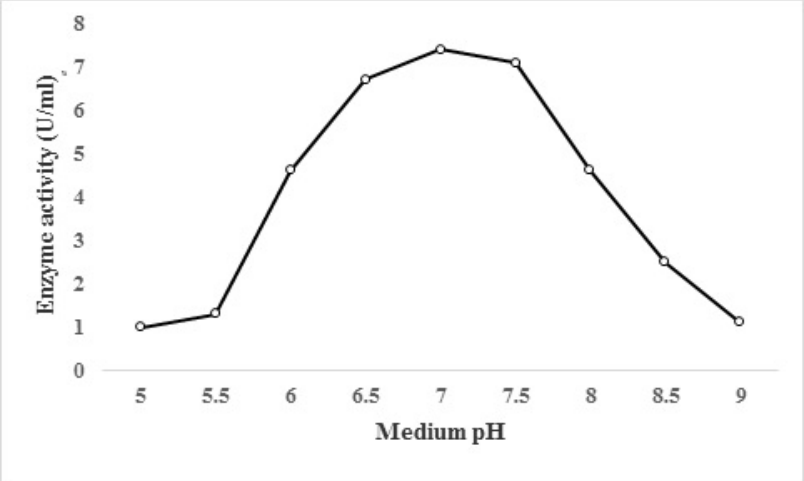

Fig. 3. Effect of medium pH on enzyme activity.

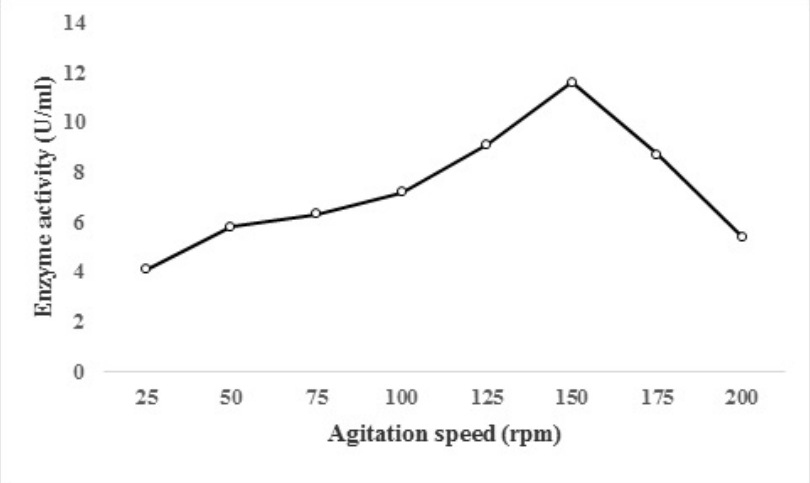

Fig. 4. Effect of agitation speed on enzyme activity.

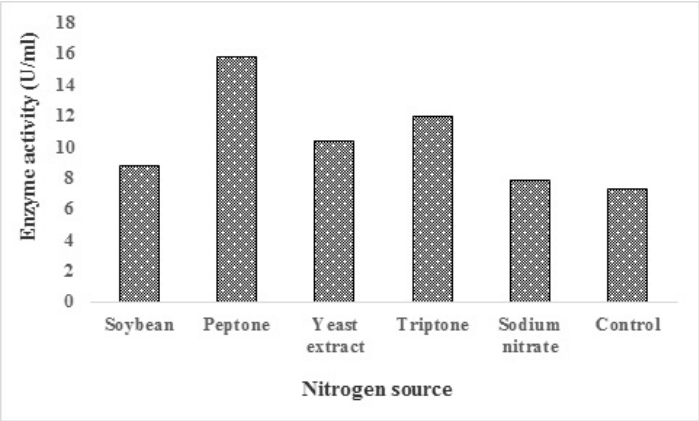

Fig. 5. Effect of different nitrogen sources on enzyme activity.

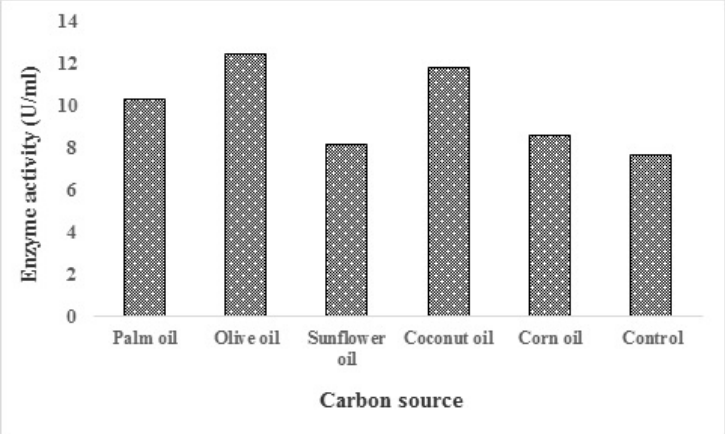

Fig. 6. Effect of different carbon sources on enzyme activity.

When organic nitrogen sources such as peptone and yeast extract were used, the bacteria, especially various thermophilic Bacillus sp. and various Pseudomonas, were able to produce high levels of lipase (Sharma et al., 2002; Bhattacharya et al., 2016). Among five different oils, olive oil as a carbon source had the highest effect on lipase activity (12.5 U/ml) (Figure 6). Vegetable oils such as olive, palm, corn, soybean, sunflower, and Coconut oils induce lipase production, comprising the sole source of carbon in the medium (Lima et al., 2003). Experimental results and the fitted models have revelead that olive oil is a better carbon source for lipase production, compared to glucose (Muralidhar et al., 2001). The best results in production of lipase have been obtained from the use of olive oil as the carbon source in the presence of nitrogen sources (Fadiloglu and Erkmen, 2002).

Followed by genomic DNA extraction from strain No. 2, primers 27F and 1107R were used to amplify 16S rDNA sequences from bacterial genomic DNA. After the PCR and electrophoresis, a 1500 bp single band was observed (Figure 7).

PCR product was sequenced and blast (NCBI) searches were conducted on the DNA sequence to compare the different 16S rDNA sequences and determine the most closely related species. Phylogenetic tree was constructed using the neighbor-joining model (Figure 8).

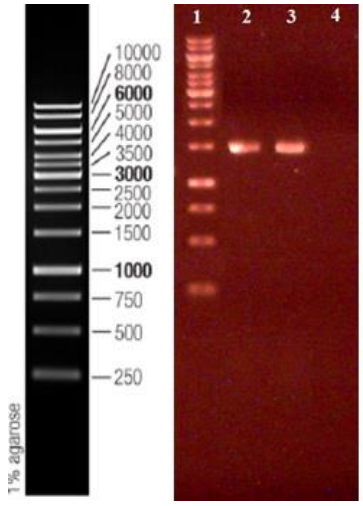

Fig. 7. PCR products of 16S rRNA gene on 1% agarose gel.

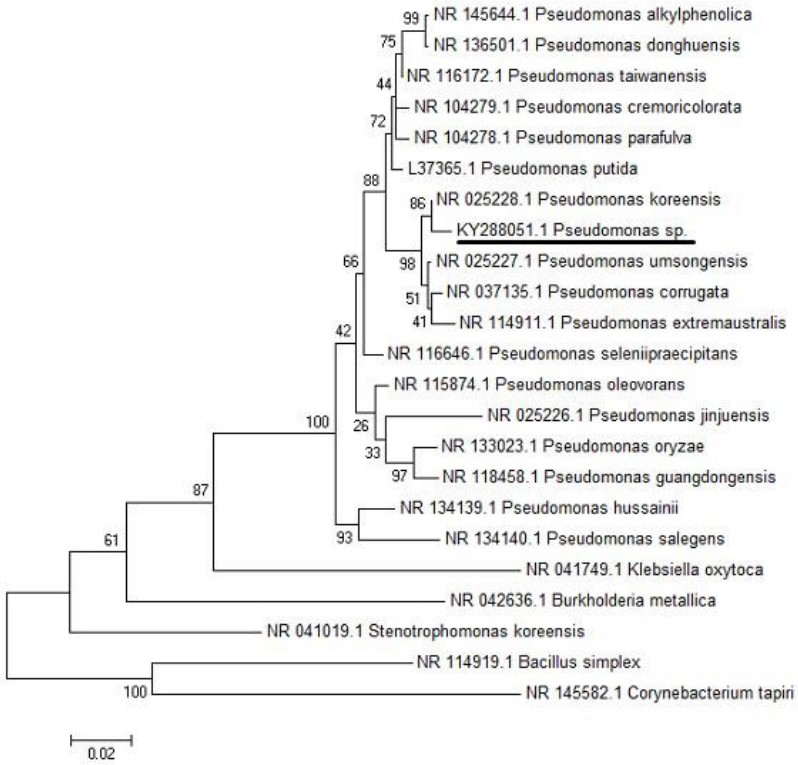

Fig. 8. Phylogenetic tree based on the neighbor-joining model of the MEGA 5 program.

Multiple sequence comparison and phylogenetic tree showed that the strain No. 2 belonged to the Pseudomonas spp. having 99% similarities with Pseudomonas 16S rDNA. The studied gene, named "IAU-3" 16S ribosomal RNA with accession No. KY288051.1, was submitted in GenBank.

CONCLUSION

Results show that lipase-producing bacteria can be obtained from oil-contaminated soil. Moreover, these bacteria in media containing-peptone and olive oil produce the maximum amount of lipase. Also, pH=7 is optimal for enzyme production. Biochemical

and morphological studies have shown that one of the best lipase-producing bacteria in oil-contaminated soil is Pseudomonas spp, while molecular studies have revealed that 16s rDNA can be a good indicator to identify and classify bacteria. Ribosomal RNA molecules play an important role in protein synthesis, being conserved in all living organisms, with their sequences used for phylogenetic classification of bacteria.

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