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# Isolation and Phylogenetic Analysis of Some Lipase Producing Bacteria from a Saline Lake

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**Lipases are a member of enzymes which catalyze the breakdown of triglycerides to glycerol and free fatty acids. In this experiment we isolated lipase production capability of halophilic bacteria form Maharloo hyper saline lake located in south of Iran. In addition, phylogenetic analysis of isolates was done. The screening and isolation of lipase producing bacteria were done on selective media and enzyme activities were assayed according to the tetrimetric method. Isolated strains were identified based on 16S rDNA genes, using universal primers. Phylogenetic tree were constructed with the neighbor-joining method and aligned using MEGA software version 4.0. Thirteen lipase producing bacteria were screened and isolated. Characterization of these potential isolates by 16S rRNA gene analysis found them related to *Bacillus* and *Staphylococcus* genera. Phylogenetic tree displayed about 99-100% relationship between bacteria. *Bacillus* sp. BCCS A21 was found as the highest lipase producing strain with 16.5 U/mL of supernatant activity. All isolates were able to grow comfortably in the media containing 0-7% of salt. *Bacillus* sp. BCCS A21 with the high ability of lipase production on salty media could be applied in such industrial processes.**

**Key words:** Lipase, Maharloo, *Staphylococcus*, *Bacillus,* isolation.

Enzymes are considered as useful catalysts in industrial process[1-2]. They are widely distributed in many species of animals, plants and microorganisms such as bacteria, fungi, yeasts and etc[2-3]. Enzyme production by microorganisms are often more useful because of the short growth period, the high yields enzyme production, widely catalytic activities and their simple features to manipulate[4]. Microbial habitats can be found in wide various geographic conditions such as salinity, high and low temperature, pH, light intensity, pressure, oxygen and nutrient concentrations[5]. Among them halotolerant and halophilic microorganisms can grow in ecological area with high salinity concentrations like saline soils, salt lakes and salted foods. They may produce compounds (such as such as extracellular, hydrolytic enzymes) with diverse potential usage in industrial process[6-7]. Therefore, isolation of such microorganisms from harsh saline regions to produce enzymes would be useful for further industrial applications[2, 8].

Lipases are represent hydrolases group, which hydrolysis of triacylglycerols to free fatty acids and glycerol[2, 6, 9]. They have widely use in various fields such as detergents, dairy foods, bakery foods, food dressings, health foods, meat and fish, fats and oils, chemicals, pharmaceuticals,

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cosmetics, leather, paper, cleaning industries[9]. Many investigations have been reported the isolation of bacteria with lipolytic activities such as *Bacillus pumilus* BCCS 002, *Bacillus endophyticus* BCCS 003, *Staphylococcus epidermidis* BCCS 009[2], *Bacillus circulans* [10] *Staphylococcus lipolyticus* sp. nov.[11] *Spongiibacter marinus* gen. nov., sp. nov.[12]. It seems that; attempts to find enzyme producing microorganisms with different features have been continued yet.

In this work, we studied the isolation, molecular identification and phylogenetic analysis of halophilic bacteria from the Maharloo hypersaline lake in south of Iran, and their ability to produce lipolytic enzymes. was studied.

## MATERIALS AND METHODS

### Screening and isolation of lipase producing microorganisms

The lipase producing microorganisms were isolated in May 2012 and May 2013 from five different points (water and sand) of Maharloo hypersaline lake (this lake with an area of nearly 30,000 acres is located in 35 kilometers from south of the city of Shiraz, Iran). Microorganisms were screened based on the formation of a clear zone on tributyrin agar plates with the following composition (gr/L); yeast extract 3, peptone 5, tributyrin (glyceryl tributyrate) 10, tween (80) 10 and agar 15, 75% lake water and 25% distilled water, pH 7.5 at 37 ºC[2].

### Preserving of Microorganisms

The selected isolates were stored at 37ºC in the 40% glycerol solution for further studies.

### Microorganisms Growth at 0–15% concentration of NaCl

Isolated strains growth conditions were investigated at various concentration of NaCl ranging from 0–15% (w/v) on nutrient broth medium using 96 wells plates.

### Lipase production medium

Isolates were grown in a 250 mL Erlenmeyer flask containing 50 mL lipase production medium with composition of (gr/L); yeast extract 5, peptone 5, $CaCl_2$ 0.05, and olive oil (10, emulsified with gum acacia 5), 75% lake water and 25% distilled water, pH 7 at 37ºC (140 rpm, 48 h). After incubation, each production medium was centrifuged at 4000 rpm for 10 min at 4 °C and the supernatants were used as crude enzyme[2].

### Lipase assay

Lipase activities were determined according to the tetrimetric method, as described by Ghasemi, *et al.* (2011)[2]. One unit of lipase activity was defined as the amount of enzyme releasing one µmol free fatty acids per mL, per minute under the assay conditions.

### Identification of lipase producing strains

Lipase producing strains were identified based on 16S rDNA genes by PCR, using universal primers (forward primer 5´2 CAGCCGCGGTAATAC 32´ and reverse primer 5´ ACGGGCGGTGTGTAC 3´2 ) as described by Ghasemi, *et al.* (2011)[8].

### Phylogenetic analysis of bacteria

A phylogenetic study (based on 16S rDNA genes) of the selected strains was performed using the MEGA4 software version 14.0.0.162[13]. The branching pattern was designed based on the neighbor-joining method.

## RESULTS

### Study of isolation and identification of selected strains

More than 160 colonies were formed on tributyrin agar plates. Among them only 13 unique colonies were screened based on the formation of a clear zone on these plates, which had selected for further studies. All 13 isolates were identified based on 16S rDNA genes. After sequencing of 16S rDNA genes of selected strains, each edited sequence was compared as queries in BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the homologous sequences in the complete GenBank nucleotide database, separately. Results indicated that two strains have above 98-100% homology with *Bacillus* genus and other strains have above of 99–100% homology with the *Staphylococcus* genus. Identified strains were published in NCBI under specific accession numbers (Table 1).

### Study of phylogenetic analysis of bacteria:

The evolutionary history was inferred using the Neighbor-Joining method[14]. The bootstrap consensus tree inferred from 1000 replicates[15] is taken to represent the evolutionary history of the taxa analyzed[15]. Branches corresponding to partitions reproduced in less than

50% bootstrap replicates are collapsed. The percentage of replicate tree in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[15]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[16] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 554 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Fig. 1)[13].

**Study of NaCl concentrations on strains growth**

Halotolerant /halophilic properties of isolates were determined at 0-15% NaCl. Table 2 shows the results of strains growth. Results indicate that *Staphylococcus* genus can grow at 0 – 7% and *Bacillus* genus at 0 – 15% NaCl.

**Study of lipase production and lipase assay**

The supernatants as crude extracellular enzyme were used for lipase activity. Results in table 1 show the highest activity was obtained by *Bacillus* sp. BCCS A21 (16.5 U/mL) followed by

**Table 1.** Shows the lipase activity (U/mL), the accession numbers of the DNA published sequences at the NCBI

| Row | Accession number | Strains Name | Lipase activity (U/mL) |
|---|---|---|---|
| 1 | KF437341 | *Staphylococcus* sp. BCCS A17 | 4.5 |
| 2 | KF437340 | *Staphylococcus* sp. BCCS A18 | 4 |
| 3 | KF437339 | *Staphylococcus* sp. BCCS A19 | 1.5 |
| 4 | KF437338 | *Staphylococcus* sp. BCCS A20 | 2 |
| 5 | KF437337 | *Bacillus* sp. BCCS A21 | 16.5 |
| 6 | KF437336 | *Bacillus sp*. BCCS A22 | 15.5 |
| 7 | KF668505 | *Staphylococcus* sp. BCCS A23 | 4 |
| 8 | KF668506 | *Staphylococcus* sp. BCCS A24 | 9.5 |
| 9 | KF668507 | *Staphylococcus* sp. BCCS A25 | 14 |
| 10 | KF668508 | *Staphylococcus* sp. BCCS A26 | 3 |
| 11 | KF668509 | *Staphylococcus* sp. BCCS A27 | 4 |
| 12 | KF668510 | *Staphylococcus* sp. BCCS A28 | 9 |
| 13 | KF668511 | *Staphylococcus* sp. BCCS A29 | 5.5 |

**Table 2.** The feasibility of isolated growth at different concentrations of NaCl (% w/v)

| Strains Name | NaCl % | | | | | |
|---|---|---|---|---|---|---|
| | 0% | 3% | 5% | 7% | 10% | 15% |
| *Staphylococcus* sp. BCCS A17 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A18 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A19 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A20 | + | + | + | + | - | - |
| *Bacillus* sp. BCCS A21 | + | + | + | + | + | + |
| *Bacillus* sp. BCCS A22 | + | + | + | + | + | + |
| *Staphylococcus* sp. BCCS A23 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A24 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A25 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A26 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A27 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A28 | + | + | + | + | - | - |
| *Staphylococcus* sp. BCCS A29 | + | + | + | + | - | - |

Note: + growth, – not growth.

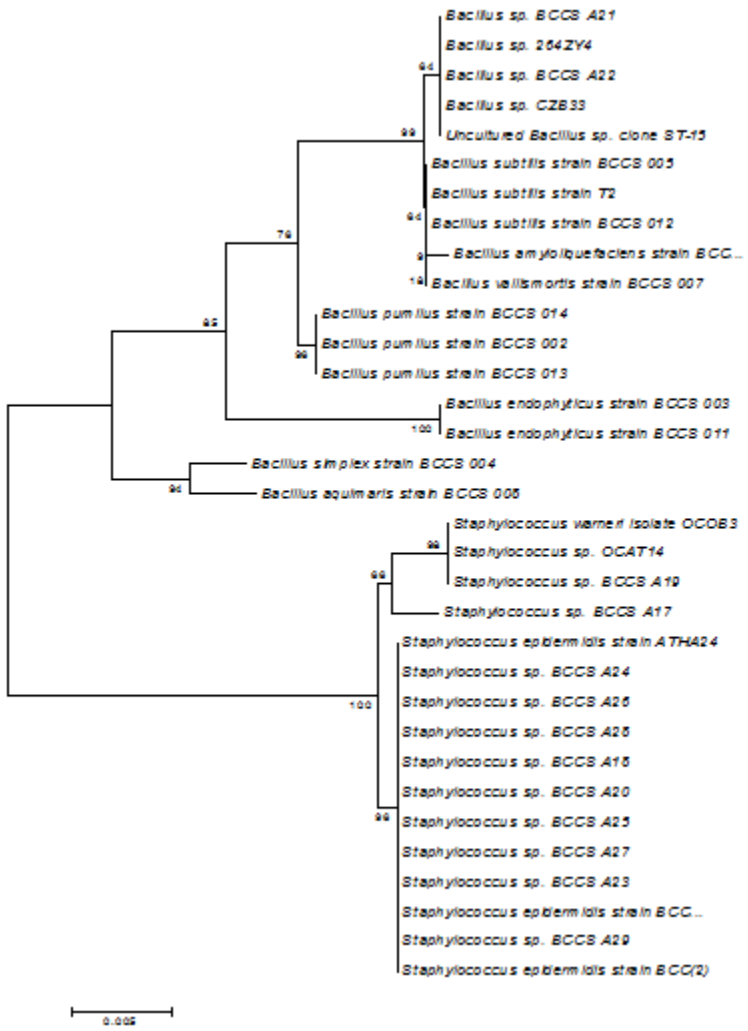


**Fig. 1.** Phylogenetic position of strains based on16S rDNA sequences. The sequences were aligned using the MEGA4 software. The branching pattern was based on the neighbor-joining method

*Bacillus* sp. BCCS A22 (15.5 U/mL) and *Staphylococcus* sp. BCCS A19 (1.5 U/mL) was showed the lowest enzyme activity under the defined conditions.

## DISCUSSION

In this study we have studied the isolation lipase producing microorganisms from Maharloo saline lake located in south of Shiraz, Iran. 13 strains were isolated. All microorganisms are belonged in two groups of bacteria informally known as *Bacillus and Staphylococcus.* Table 2 shows results of halotolerant /halophilic properties of isolates, all bacteria can grow at 0-7% NaCl, and only the *Bacillus* species can grow up to 15% NaCl, and it indicates that the genus of *Bacillus* are more resistance. It seems that endospore forming by these genera allows them to resistance to harsh condition[2]. Lipase activity of these isolates were done (table 1), results show *Bacillus* sp. BCCS A21 (16.5 U/mL) with the highest activity along the isolates. Halophilic bacteria as a source of novel hydrolytic enzymes have been studied like; *Bacillus, Paenibacillus, Halobacterium, Aeromonas, Salicola, Halovibrio, Halomonas,*

*Oceanobacillus, Thalassobacillus, Halobacillus, Virgibacillus, Gracilibacillus, Salinicoccus* and *Piscibacillus* genera[2, 7-8]. In addition, Joseph, *et al*. (2006)[17], Ghasemi, *et al*. (2011)[2] and Esakkiraj, *et al*. (2010)[18] have been studied the lipase production by *Staphylococcus* species. Therefore, these findings confirm that searching for new bacteria with more various activities is expected.

Figure 1 shows the evolutionary relationships of 33 taxa, which selected from NCBI (Gene Bank databases) (13 strains in this study, 13 strains from previous study and 7 strains from other investigations), based on 16S rDNA genes. The phylogenetic tree clearly shows the relation rate between strains. It indicates the sequences of *Bacillus* sp. BCCS A21 and *Bacillus* sp. BCCS A22 have 99% similarity with *Bacillus subtilis* strain BCCS 005 and the other present bacteria in this branch. Other isolated stains (the *Staphylococcus* genus*)* also show 100% similarity. Compare with our findings (isolation of lipase producing bacteria along two years) and previous study of lipase producing bacteria from the Maharloo saline lake by Ghasemi, *et al*. (2011)[2], results indicats that the biodiversity of lipase producing bacteria in this lake includes only two genera of bacteria (*Bacillus* and *Staphylococcus)*. Therefore, the populations of other microorganisms with different features may consist widespread diversity. This work represents the *Bacillus* sp. BCCS A21 with lipase activity on saline conditions may degrade lipidic compounds in marine water as well, and also could be used in different industries, where salt is included.

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