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EXPERIMENTAL ARTICLES

Isolation and Characterization of Some Moderately Halophilic Bacteria with Lipase Activity[1]

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Abstract—Lipases are an important class of enzymes which catalyze the hydrolysis of long chain triglycerides and constitute the most prominent group of biocatalysts for biotechnological applications. There are a number of lipases, produced by some halophilic microorganisms. In this study, some lipase producing bacteria from the Maharla salt lake located in south of Iran were isolated. All isolates were screened for true lipase activity on plates containing olive oil. The lipase activity was measured using titrimetric methods. Among thirty three isolates, thirteen strains demonstrating orange zone around colonies under UV light, were selected for identification using the molecular methods and some morphological characteristics. The bacterium *Bacillus vallismortis* BCCS 007 with 3.41 ± 0.14 U/mL lipase activity was selected as the highest lipase producing isolate. This is the first report of isolation and molecular identification of lipase producing bacteria from the Maharla lake.

Keywords: lipase, *Bacillus vallismortis*, halophilic bacteria,the Maharla Salt Lake.

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Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) display the capability to catalyze the hydrolysis of triglycerides to diacylglycerides, monoglycerides and fatty acids, under aqueous conditions [1, 2]. They also have esterolytic activity on distinct substrates. Due to their multifaceted attributes, they are gaining importance for biotechnology. These enzymes are ubiquitous in nature and are widely distributed in plants, animals, and microorganisms such as bacteria, yeasts, and fungi [3–5]. Microbial lipases have immense applications in various fields, such as food, pharmaceutical, cosmetic, agrochemical, feedstock, detergent, textile, biodiesel and oil processing industries, in synthesis of fine chemicals and new polymeric materials, as well as in waste water treatment [1, 4, 6, 7]. Besides, recently the process of lipid modification using lipases has attracted significant attention. Different types of reactions they can catalyze in the absence of cofactors, a high stability in organic solvents and the ability to catalyze specific reactions with chemo-, regio-, and enantioselectivity, make them potentially the enzymes for biotechnological process [7, 8].

Most industrial enzymatic conversions may be inhibited by concentrated salt solutions and high temperatures; thus, microbial enzymes that display optimal activity at a wide range of temperature, pH and ionic strength, would be considered as useful biocatalysts in industrial process [6, 10, 11].

Moderately halophilic bacteria which can grow optimally in media containing 3–15% NaCl, are a valuable source of such enzymes. Therefore, screening and isolation of moderate and extreme halophiles from hypersaline environments, able to produce these enzymes would be useful for further industrial applications [6, 11].

It has been shown that the most efficient lipase-producing bacteria belong to various species of *Bacillus* genera, such as *Bacillus cereus* C71, *B. thermoleovorans* ID-1, *B. coagulans* BTS-3, *Geobacillus* sp. TW1 [4], *Bacillus* sp. strain L2, *B. sphaericus* 205y, *B. bogoriensis* sp. nov., *B. salarius* sp. nov. and *B. sphaericus* JS1 and other *Bacillus* species [1–5, 12–19]. *Bacillus* species are taxonomically very diverse and have been isolated from different saline habitats such as salterns, estuarine water, salt lakes, salty foods, sea ice and deep-sea hydrothermal vents [18].

The aim of this study is to isolate and identify some lipase-producing halophilic bacteria from the Maharla, a hypersaline lake in south of Iran, using 16S rRNA as a molecular marker. Their lipase activities were also determined.

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MATERIAL AND METHODS

Site description and sample collection. The Maharla is the most important saltwater lake in Fars province located in south of Iran. This lake with an area of nearly 30,000 acres is located in 35 kilometers from south of the city of Shiraz, with temperature variations between 9.5 and 36°C throughout the year. Lake water salinity changes between 30 to 320 g/l with the pH range of 5–6. Samples were collected at different locations and were kept in sterile plastic containers.

Screening and isolation of strains for lipolytic activities. The moderately halophilic lipase-producing bacteria were isolated through direct method using the selective medium containing (% w/v): agar 1.5, tributyrin (glyceryl tributyrate) 1, tween 80 1, peptone 0.5, yeast extract 0.3, NaCl 7, pH 7.5 at 37°C. Lipase-producing bacteria made a clear zone on tributyrin agar plates. All isolates also were screened for true lipase activity on plates containing (% w/v) Rhodamine B 0.001, nutrient broth 0.8, NaCl 7, agar 1, and olive oil 3, in distilled water, pH 7.5 [20]. Plates were incubated at 37°C for 48 h, and lipase production was identified by an orange halo around colonies, visible under UV light at 350 nm.

Growth was investigated qualitatively at various concentration of NaCl ranging from 1–15% (w/v) on nutrient agar. All purified isolates were preserved at –70°C in the 15% glycerol solution for long storage.

Production of lipase and lipase assay. For enzyme production, bacteria were cultivated in 250 ml Erlenmeyer flasks containing 50 ml medium composed of (% w/v): peptone 0.5, yeast extract 0.5, NaCl 7, $CaCl_2$ 0.005, and olive oil (1.0, emulsified with gum acacia 0.5), pH 7.5. The production broth (50 ml) was incubated at 37°C under shaking (150 rpm, 48 h) conditions [5]. Cells were separated from the cultivation medium after centrifugation at 4800 rpm for 20 min at 4°C and the supernatant was used as the source of extracellular crude enzyme.

Lipase activity was assayed by alkali titration using olive oil as substrate, as described by Saxena et al. with some modifications [9]. The assay mixture consisting of 2.5 ml of olive oil, 3.5 ml of phosphate buffer (0.1 M; pH 7.0) and 2 ml of the crude enzyme preparation was incubated for 20 min at 37°C. The reaction was terminated by adding 10 ml ethanol, and the amount of liberated fatty acids during incubation was tittered with 0.05 N KOH in the presence of phenolphthalein as an indicator. One unit of lipase activity was defined as the amount of enzyme that librated 1 moles of free fatty acids per ml per minute under the assay conditions.

Amplification and sequencing of 16S rRNA. Molecular identification of the selected lipase producing bacteria was performed based on the nucleotide sequences in 16S rRNA encoding gene. The genomic DNA content was extracted and then PCR procedure was applied using two sets of primers [21]. The PCR products were electrophoresed in a 1% (w/v) agarose gel using TBE buffer containing 1 μg/ml ethidium bromide. A single 800 bp DNA was cut and extracted from the gel using Core Bio Gel Extraction Kit. The sequence was determined by CinnaGen Company with the primers. The edited sequences were used as queries in BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST/), to determine the nearest identifiable match present in the complete GenBank nucleotide database.

The 16S rRNA gene sequences of the isolated strains were published in NCBI under the following accession numbers: FJ411178, FJ392547, FJ422562-FJ422568, FJ422570-FJ422573.

RESULTS

Isolation and Characterization of the bacterial strains. Several bacterial strains were isolated from the Maharla salt lake and were screened for the lipolytic activity. 33 isolates produced a clear zone on tributyrin agar plates. Among them, 13 isolates had orange halo around colonies detected under UV light at 350 nm. Primary morphological identification showed that of them 11 isolates were gram-positive rod-shaped and spore-forming bacteria and 2 isolates were gram-positive cocci (Table 1). The 16S rRNA gene sequence showed that the gram-positive, rod bacteria have above of 98–100% homology with the other *Bacillus* spp. and the cocci bacteria have above of 99–100% homology with the other *Staphylococcus* spp.

The halophilic properties of the isolates were qualitatively observed at different concentrations of NaCl. The growth was occurred in the range of 1–15% (w/v) NaCl. The *Staphylococcus* strains can grow at 1–7% NaCl, and the *Bacillus* strains at 1–10% (Table 2).

Production of lipase and lipase assay. The supernatants from the bacterial cultures were tested in triplet independent repeats for the lipolytic activity using the titrimetric method. Table 1 shows the average of extracellular lipolytic activities of the studied bacteria. Of these, the highest lipase activity was found in *B. vallismortis* BCCS 007.

DISCUSSION

The present study shows that when the tributyrin used as a carbon source, allows the growth and subsequent isolation of lipolytic bacteria. However, only some of isolates produce true lipases after growing on the olive oil as a substrate. It also reveals that the isolated bacteria demonstrated lipase activity at harsh conditions (7% NaCl) (Table 1). According to the classification of halophilic bacteria [22], all *Bacillus* obtained in this study, were moderate halophiles which exhibit good growth at NaCl concentrations 1–10%, while *Staphylococcus* strains grew only at 7% NaCl (Table 2). Members of the genus *Bacillus* form endospores; this capacity of contributes to their resis-

Table 1. Some physiologic characteristics, the lipase activity (U/mL), the accession numbers and the length base pairs of the DNA published sequences at the NCBI, for 13 strains of environmentally isolated bacteria from the thr Maharla Salt Lake. The highest lipase activity, 3.41 ± 0.14 (U/ml) for *B. vallismortis* BCCS 007, is shown in bold

Bacterial strain	Morphology	Gram Staining	Spore forming	Tributyrin hydrolysis	Olive oil hydrolysis	Length (Base pair)	Lipase activity (U/mL)
B. pumilus BCCS 002	Rod	+	+	+	+	833	1.91 ± 0.38
B. endophyticus BCCS 003	Rod	+	+	+	+	831	2.41 ± 0.38
B. simplex BCCS 004	Rod	+	+	+	+	813	2.5 ± 0.25
B. subtilis BCCS 005	Rod	+	+	+	+	785	2.66 ± 0.14
B. amyloliquefaciens BCCS 006	Rod	+	+	+	+	563	2.83 ± 0.14
B. vallismortis BCCS 007	Rod	+	+	+	+	816	**3.41 ± 0.14**
B. aquimaris BCCS 008	Rod	+	+	+	+	810	2.66 ± 0.28
B. endophyticus BCCS 011	Rod	+	+	+	+	663	2.16 ± 0.28
B. subtilis BCCS 012	Rod	+	+	+	+	842	1.41 ± 0.28
B. pumilus BCCS 013	Rod	+	+	+	+	833	1.33 ± 0.14
B. pumilus BCCS 014	Rod	+	+	+	+	843	1.91 ± 0.28
S. epidermidis BCCS 009	Cocci	+	–	+	+	829	2.41 ± 0.28
S. epidermidis BCCS 015	Cocci	+	–	+	+	845	3.08 ± 0.14

Table 2. The feasibility of growing at different concentrations of NaCl (% w/v) for the 13 naturally isolated strains of bacteria

NaCl, %						Bacterial strain
1	3	5	7	10	15	
+	+	+	+	+	±	*B. pumilus* BCCS 002
+	+	+	+	+	±	*B. endophyticus* BCCS 003
+	+	+	+	+	+	*B. simplex* BCCS 004
+	+	+	+	+	±	*B. subtilis* BCCS 005
+	+	+	+	+	±	*B. amyloliquefaciens* BCCS 006
+	+	+	+	+	+	*B. vallismortis* BCCS 007
+	+	+	+	+	±	*B. aquimaris* BCCS 008
+	+	+	+	+	±	*B. endophyticus* BCCS 011
+	+	+	+	+	±	*B. subtilis* BCCS 012
+	+	+	+	+	±	*B. pumilus* BCCS 013
+	+	+	+	+	±	*B. pumilus* BCCS 014
+	+	+	+	–	–	*S. epidermidis* BCCS 015
+	+	+	+	–	–	*S. epidermidis* BCCS 009

Note: ±variable, + growth, – not growth.

tance to a broad range of physiological stresses. Several strains, such as *Bacillus pseudofirmus*, *B. cohnii*, *B. vedderi*, *B. agaradhaerens* [23] *Bacillus* sp. SD-B1, *Bacillus* sp. MO12, *B. licheniformis* strain GXN151, *B. licheniformis* CICC 10219, *Bacillus* sp. GSP63, *S. epidermidis, B. subtilis* WL-6 [22] and *B. pumilus* [24] were isolated from saline environments. The results of present studies suggest that *B. vallismortis* BCCS 007 with the highest activity (3.41 ± 0.14 U/mL) can be considered as a good candidate for the bioconversion of triglycerides to diacylglycerides, monoglycerides and fatty acids. There are reports of other researchers on the activities of this bacterium. Park et al. (2006) have proposed the efficacy of *B. vallismortis* strain EXTN-1 to induce systemic resistance in potato against PVX and PVY [25] and Kim et al. (2007) have reported the identification of a fibrinolytic enzyme by *B. vallismortis* [26]. In another investigation *B. vallismortis* with lipase activity was isolated from soil samples collected in Korea [27]. Therefore,

B. vallismortis BCCS 007 could be used as a potential strain for production of halophilic lipase. This bacterium may play an important role in the degradation of lipidic compounds in saline environment. *S. epidermidis* is widely studied because of its pathogenicity, but only in few works lipase production by *S. epidermidis* has been proposed [28, 29]. In the present work, we have shown that *S. epidermidis* strains are able to produce lipase in saline media. This study indicates the presence of halophilic lipases in the halophilic bacteria that could be applied in industrial processes where concentrated salt solutions would inhibit ordinary lipases. We also found that the Maharla Salt Lake is populated with diverse bacterial groups, which are a potential source of industrial enzymes for biotechnological applications.

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