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Phylogenic Characterization of Lipase Producing *Bacillus* Strains Isolated from Persian Gulf Sediments

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Abstract: Lipase production in some native lipolytic strains (BB-1and BB-2) isolated from sediments of Qeshm Island, located in Persian Gulf, were detected in a medium consisting of Tween 80, Rhodamine B-Olive oil. Lipase activity was measured in the cell free supernatant of culture grown in a medium containing: pepton, yeast extract and olive oil with PH=7, at 37°C and 140 rpm for six days. Maximum lipase activity was identified in 3rd day. Morphological and biochemical characteristics identified precisely the strain of the bacteria as *Bacillus* sp. (BB-1 and BB-2). Moreover, analytical results of 16srRNA gene sequence of the isolated bacteria showed that the strains BB-1 and BB-2 belong to *Bacillus sp. G2DM-33* and *virigibacillus salarius,* respectively.

Key words: *Bacillus* sp, Lipase, Sediments, Rhodamine B.

INTRODUCTION

THE biota of marine microorganisms has developed unique metabolic and physiological functions that not only ensure survival in extreme habitats, but also offer a potential for the production of novel enzymes for potential exploitation. Out of the large number of species examined, only a fraction of marine bacteria have been isolated and cultured.

Among them, alkaliphilic *Bacillus* strains are of considerable importance in biotechnological applications (Asha Devi *et al*.,2008). Lipases (Triacylglycerol Acylhydrolase, EC3.1.1.3) are versatile and ubiquitous biocatalysts with a wide range of applications such as additives in food processing (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), diagnostics, cosmetics (removal of lipids) pharmaceuticals (digestion oil and fats in foods), leather (removal of lipids from animal skins), and medical (blood triglyceride assay) industries (Ginalska *et al*., 2006; Morabbi Heravi *et al*., 2008; Bayoumi *et al*., 2007).

These enzymes are active in the interface of aqueous and non aqueous phases, which distinguish them from esterase (Morabbi Heravi *et al*., 2008). Most of the lipases exhibit a high activity on lipids with fatty acid residues of C8 to C18 chain length (Prazeres *et al*., 2006). Lipases are produced by many microorganisms and higher eukaryotes. Enzyme producer microorganisms include bacteria, fungi, yeasts and actinomycetes (Ginalska *et al*., 2006). Microbial lipases are commercially important because of their unique properties and the ease of bulk extracellular production compared to lipases from other natural sources.

Among the lipase producer bacteria several species of *Bacillus* such as *B. subtilis, B. pumilus, B.licheniformis, B. thermoleovorans, B.stearothermophilus,* and *B. sphaericus* posse lipase are suitable for biotechnological applications.

Lipase production is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculums size (Morabbi Heravi *et al*., 2008; Gulati *et al*., 2005). One of the main habitats of a microorganism is the marine environment. Although gram-positive bacteria have been cultivated from seawater, marine invertebrates, and other sample types and marine sediments, including deep-see sediments are the primary oceanic habitats from which they have been recovered. Among the marine microorganisms, lipase producer bacteria can be found (Gontang *et al*., 2007).

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MATERIALS AND METHODS

The study was conducted in 2010 at the Microbiology Department of Shahid Chamran University, Ahwaz, Iran.

Sampling:
Some sediment samples were collected at 5 m, and 10 m depths, within Persian Gulf seawater at the site Qeshm Island (North of Larak Island). Water PH, salinity, and temperature were 8.4, 39.5 and 30°C, respectively.

Isolation of Microorganisms:
Collected samples from sediments of Qeshm Island seawater cultured in a broth medium called marine broth, incubated in 30°C for three days and then were transferred in a marine agar medium so that the colonies appeared. After subculturing each of the colonies, isolation was achieved (Figure 1).

Screening of Lipolytic Strains:
Each of the isolated strains cultured on a medium consisted of Tween80, Rhodamine B, Olive oil, nutrient broth, yeast extract and agar with PH=7 at 37°C for four days. The lipolytic strains were visualized as orange colonies under UV ray, but the non-lipolytic strains revealed pink colonies (Figure 2).

A Medium for Lipase Producing:
The basal medium for lipase production contained (g/l) peptone (30), yeast extract (10), olive oil (10) with PH 7. 0.1 ml of cell suspension in100 ml of the medium incubated at 37°C for 144 hours in orbital shaker in140 rpm. However, a little of the sample was tested for the rate of lipase-enzyme activity regularly every 24 hours. Samples were centrifuged at 10000 g for 10 minutes at 4°C. The settle cells were separated, and their enzyme-activity was measured by culture filter. Lipase activity was determined by titration of the free fatty acids were released by the enzyme action on an emulsion of olive oil (25% v/v) and Arabic gum (75% v/v) in 0.1M phosphate buffer (PH=7.0) (Freire at al.1997). The Olive oil emulsion (5.0 ml) was hydrolyzed with 1.0 ml enzyme solution in 4.0 ml of 0.1M phosphate buffer, PH=7.0 at 37°C and160 rpm for one hour. The reaction was stopped by addition of 15.0 ml ethanol (96%). The liberated oleic acid was calculated by titration with NaoH (0.05N). One unite of lipase activity was defined as the amount of enzyme required to release 1μmol of oleic acid per ml per hour (Kanlayarki and Boonpan, 2007).

Characterization of Lipase:
Lipase activity was determined at regularly different time intervals such as 24 hours, 48 hours, 72 hours, 96 hours and 120 hours of incubation. The optimum PH=7, temperature=37 °C and substrate concentration=25 ml olive oil for lipase activity was according to what was determined in a previous study (REF). However, the activity was assayed by the titrimetric method as explained above (Senthikumar and Selvakumar, 2008).

Fig. 1: Colonies of bacterial srtains (E4=BB-1 and D3=BB-2).

(a) (b)

Fig. 2: Screening of lipolytic strains. (a) BB-1(E4). (b) Right:BB-2(D3) and Left:negative sample.

Results:

Identification of Bacterial Strains:

Initial Identification:

The characters of the organisms were studied following the standard microbiological methods. Morphology, vegetative cell, and spore characters were observed. The physiological and biochemical characters such as catalase, starch hydrolysis, acid from glucose, manitol, xylose, arabinose, citrate utilization, casein hydrolysis, nitrate reduction, and gelatin hydrolysis were studied. However, a Morphological and biochemical characteristic used for proprietary identification of the isolated strains, and that was *Bacillus* sp. and called *BB-1* and *BB-2* (Figure 3).

Molecular Analysis by 16srRNA Sequencing:

DNA extraction of isolates was performed by using Fermentase DNA extraction kit provided by Gen Fanavaran (Tehran, Iran). Molecular identification of the selected isolates was performed by the amplification of 16srRNA with eubacterial universal primers (F: 5'CCGAATTCGTCGACAACA3', and R: 5'CCCGGGATCCAAGCTATC3') provided by Gen Fanavaran Com. DNA amplification was carried out in 50µL reaction mixtures containing 5µL PCR buffer, 1µL dNTP mixture, 2µL $Mgcl_2$, 0.75µL of each primer, 3µL of DNA, and 2µL Taq DNA polymerase. Amplification was performed in a thermocycler (Biorad-C1000, USA) using the following program. A 5 minutes denaturation period at 94°C was followed by 30 cycles each: 1 minute at 94°C, 40" at 61.3°C, and 2 minutes at 72°C with a final extention for 20 minutes at 72°C. All PCR chemicals were purchased from Cinagen (Tehran, Iran). The PCR product was purified from the agarose gel by gel purification kit (Bioneer, USA), and sequenced by Tag Copenhagen A/S Company, Korea (www.tagc.com), and their homology with other genomes (Gene Bank) were analyzed through Blast at http://www.ncbi.nih.gov/blast. Phylogenetic tree was drawn by Mega4 software. The results of blast showed that the strains E4 and D3 belong to *Bacillus sp. G2DM-33* and *virigibacillus salarius* respectively (Figure 4 and Figure 5).

Maximum Enzyme Activity and Lipase Producing Conditions:

The isolated strains with positive test on the Rhodamine B agar plate were cultured in a broth medium consisting of pepton, yeast extract and olive oil as a carbon source (PH=7), 37°C and 160 rpm and different samples were tested during 24-hour intervals (Selva Mohan *et al* 2008;., Vargas *et al*., 2004). Maximum lipase activity showed in the 3th day (72 hours) in six-day incubation period (Figure 6).

Discussion:

Microorganisms play major roles in energy transformations and biochemical processes in exceedingly diverse habitats especially, in marine environments. Knowledge of both microbial diversity and microbial activity in the coastal area could be important for business and industry as well as in maintaining the health of near-shore ecosystems (Madigan *et al*. 2000). Studies in the past two decades show that heterotrophic bacteria not only function as decomposers (such as lipase producing bacteria), but also as a channel dissolved organic substances and inorganic nutrients to higher atrophic levels through the microbial food web (Azam, 1998; Abolhasani Sooraki *et al*., 2005).

The aim of this study was detecting the potential of the lipolytic-enzymes activity in the sediments of Persian Gulf seawater. Screening and isolation of microorganisms for lipolytic activity are relatively easy and are most frequently performed by using agar plates containing triglycerides. The screening system used in this study was based on a chromogenic substrate (Vargas *et al*., 2004). Two bacterial strains were isolated and identified as *Bacillus sp* according to the results of the morphological and biochemical assays. Tween 80, Rhodamine B-Olive oil plates were used and screened for production of lipase (Seinthikumar and Selvakumar, 2008). Colonies which showed orange fluorescence under UV irradiation were a lipase producer (Morabbi Heravi *et al*., 2008). Quantitative assay for lipase was carried out for Bacillus sp. (BB-1 and BB-2). The results showed that the production of lipase enzyme on the first day was 25.25 U/m for BB-1, 25.50 U/m for BB-2. They were increased on the third day to 26.37 U/m for BB-1, and 25.91 U/m for BB-2), but they decreased in subsequent days (Figure 4). The fall of enzyme activity after third day might be due to the absorption of the enzyme by the substrate or the proteolytic enzyme synthesis of Bacillus sp. (BB-1and BB-2) (Senthikumar and Selvakumar, 2008).

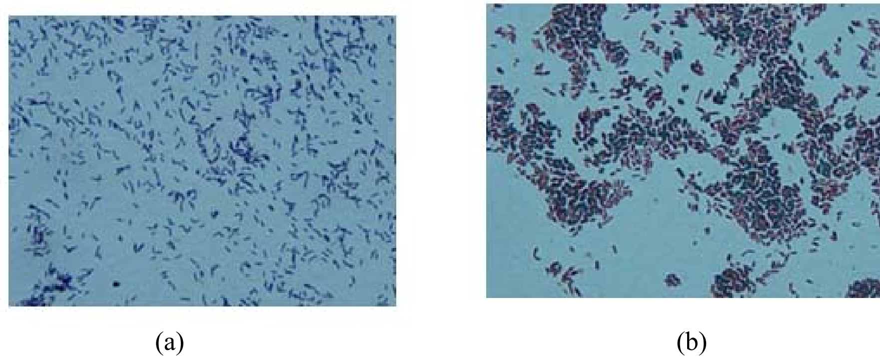


Fig. 3: Microscopic photos. (a) BB-1(E4). (b) BB-2(D3).

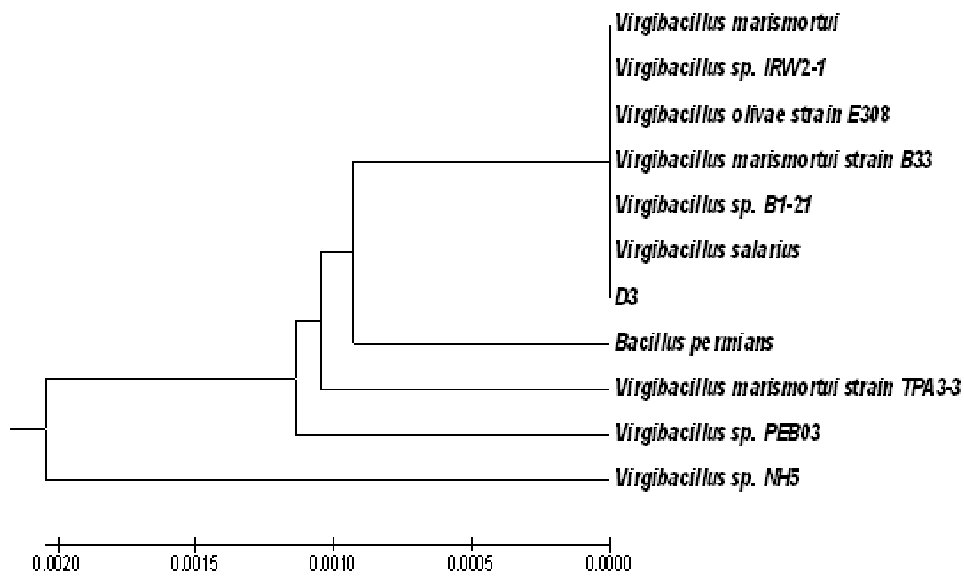


Fig. 4: Phylogenetic tree of strain D3(UPGMA phylogenetic method).

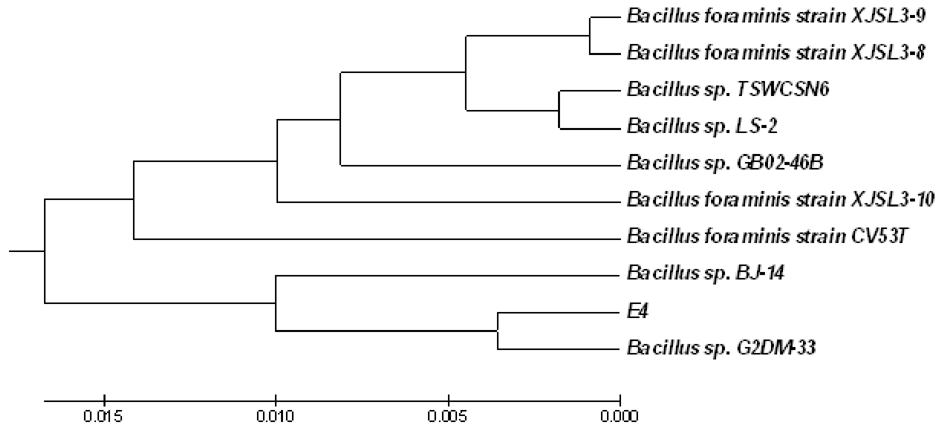
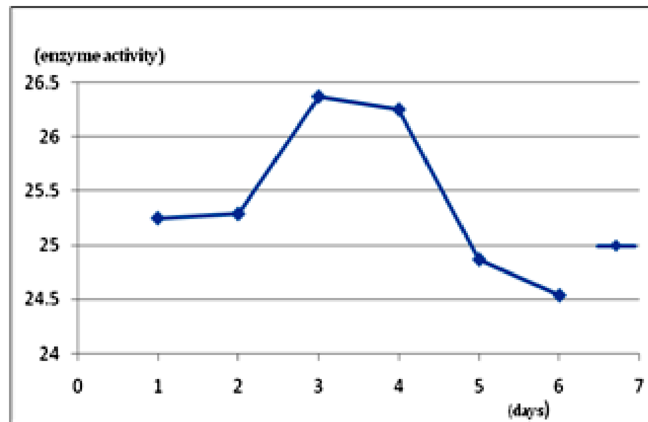
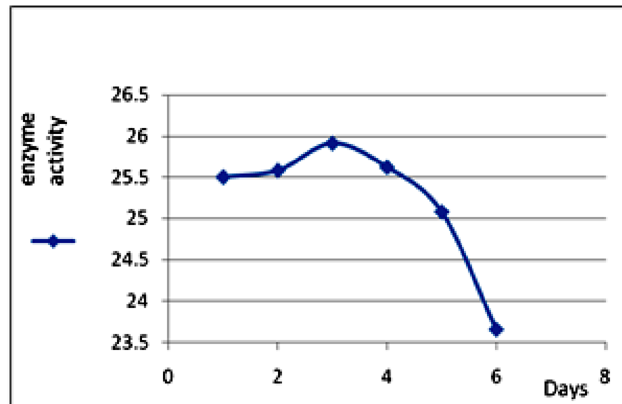


Fig. 5: Phylogenetic tree of strain E4(UPGMA phylogenetic method).



(a)



(b)

Fig. 6: (a) E4 enzyme activity. (b) D3 enzyme activity.

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REFRENCES

Abolhasani Sooraki, A., Roayaei M. Ardakani, and H. Kermanian, 2005. Total culturable bacteria and bacterial diversity in northern intertidal areas of Persian Gulf. Enviromental science, 9: 35-42.

Asha Devi, N.K., R. Balakrishnan, R. Gopal and S. Padmavathy, 2008. Bacillus clausii *MB9* from the east coast regions of India: Isolation, biochemical characterization and antimicrobial potentials. Current science, 95(5): 627-636.

Bayoumi, R.A., S.S. El-louboudy, N.M. Sidkey and M.A. Abd-El-Rahman, 2007. Production, purification and characterization of thermoalkalophilic lipase for application in bio-detergent industry. Journal of applied sciences research, 3(2): 1752-1765.

Ginalska, G., H.Y. Cho, N.S. Cho, R. Bancerz, T. Korilowicz, A. Leonowicz, S.J. Shin, and S.H. Ohga, 2007. Effect of culture conditions on growth and lipase production by a newly isolated strain, *Geotrichum*-like R59 (Basidiomycetes). Journal of faculty of Agriculture, Kyushu Univ., 52(1): 29-34.

Gontang, E.R., W. Fenical and P.R. Jensen, 2007. Phylogenetic Diversity of Gram-Positive Bacteria Cultured From Marine Sediment. Applied and environmental microbiology, 3272-3282.

Gulati, R., J. Isar, V. Kumar, A.K. Prasad, V.S. Parmar and R.K. Saxena, 2005. Production of a novel alkaline lipase by *Fusarium globulosum* using neem oil, and its applications. Pure Applied Chemistry, 77(1): 251-262.

Kanlayakri, W. and A. Boonpan, 2007. Screening of halophilic lipase-producing bacteria and characterization of enzyme for fish sauce quality improvement. Kasetsart J. (Nat. Sci.), 41: 576-585.

Morabbi Heravi, K., F. Eftekhar, B. Yakhchali and F. Tabandeh, 2008. Isolation and Identification of a Lipase Producing *Bacillus* sp. from Soil. Pakistan Journal of Biological sciences, 11(5): 740-745.

Prazeres, J.N., Bortollotti J.A. Cruz and G.M. Pastore, 2006. Characterization of Alkaline Lipase from *Fusarium oxysporum* and the Effect of Different Surfactants on the Enzyme Activity. Brazilian Journal of Microbiology, 37: 505-509.

Selva Mohan, T., A. Palavesam and G. Immanvel, 2008. Isolation and characterization of lipase-producing *Bacillus* strains from oil mill waste. African Journal of Biotechnology, 7(15): 2728-2735.

Senthikumar, R. and R.G. Selvakuma, 2008. Isolation and Characterization of an Extracellular Lipase Producing *Bacillus* sp. SS-1 from Slaughterhouse soil. Advanced Biotech, 24-25.

Vargas, V.A., O.D. Delgado, R. Hatti-Kaul and B. Mattiasson, 2004. Lipase-producing Microorganisms from a Kenyan Alkaline Soda Lake. Biotechnology Letters, 26: 81-86.