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Halotolerant Amylase Production by a Novel Bacterial Strain, *Rheinheimera aquimaris*

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Abstract: In this study, amylase production capability of halophilic bacteria isolated from Maharloo hypersaline lake in the South of Shiraz, Iran was examined. In a primary screening program, 50 colonies were isolated being capable of using starch as a sole carbon source. To determine the amylase activity, starch digestion was measured using the iodometric methods. Among them, 13 strains with more amylase activity were identified by biochemical and morphological characterization and 16S rRNA gene sequence as a molecular marker. In this study, the bacterium *Rheinheimera aquimaris* BCCS 026 is reported to have the highest capability for production of amylase (61 U mL⁻¹) that make it noticeable for further studies.

Key words: Amylase, halophile, bacteria, *Rheinheimera aquimaris*

INTRODUCTION

Starch (C₆H₁₀O₅)_n is a major carbohydrate reserve in plants, consisting of a large number of glucose monosaccharide units joined together by glycosidic bonds. All the seeds of plants and tubers contain starch, which is predominantly present as amylose and amylopectin. Depending on the plant, starch generally contains 20 to 25% amylose and 80 to 75% amylopectin. Starch can be hydrolyzed into simpler carbohydrates by acids, amylolytic enzymes, or a combination of both. Nowadays, interest has been drawn on enzymes because they possess a high level of selectivity, work under mild reaction condition, are easy to dispose off and are environmentally friendly (Utong *et al.*, 2006). Amylases (E.C. 3.2.1.1) are enzymes which hydrolyze starch molecules to give diverse products including dextrans and progressively smaller polymers composed of glucose units (Chena *et al.*, 2005). These enzymes constitute a class of industrial enzymes, which alone form approximately 25% of the enzyme market covering many industrial processes such as sugar, textile, paper, brewing, distilling industries and pharmaceuticals (Saxena *et al.*, 2007).

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Amylases can be derived from several sources, such as plants, animals, bacteria and fungi. Because of the short growth period, biochemical diversity and the ease with which enzyme concentrations might be increased by environmental and genetic manipulation, the enzymes from microbial sources generally meet industrial demands (Oliveira *et al.*, 2007; Mishra and Behera, 2008). The majority of enzymes used to date have been obtained from mesophilic microorganisms. The applications of these enzymes are restricted because of their limited stability to extreme temperature, pH and ionic strength (Utong *et al.*, 2006). Therefore, efforts were made on the enzymes of thermophilic and halophilic bacteria, which could be used in many harsh industrial processes where the concentrated salt solution and high temperatures used would inhibit many enzymatic conversions (Saxena *et al.*, 2007; Amoozegar *et al.*, 2003). In this study, amylase production capability of halophilic bacteria isolated from Maharloo hypersaline lake in the South of Shiraz, Iran was examined. The amylase producing-bacteria were identified by biochemical and morphological characterization and 16S rRNA gene sequence as a molecular marker. Molecular markers are used as tools for estimating the phylogenetic relationships of different kinds of organisms. The genes encoding ribosomal RNAs and ribosomal proteins have been highly conserved throughout evolution and have diverged more slowly than other chromosomal genes. Comparison of the nucleotide sequence of 16S ribosomal RNA shows evolutionary relationships between diverse organisms.

MATERIALS AND METHODS

Isolation and Screening of Halophilic Amylase Producing Bacteria

In February 2008, soil and water samples were collected from different parts of the Maharloo salt lake in the South of Shiraz, Iran. The samples were serially diluted in sterile saline and the dilutions from 10^{-1} to 10^{-7} were spread on to nutrient agar containing 7% NaCl. After incubation at 37°C for 24 h, the colonies were transferred by replica plating on to saline starch agar containing: peptone 0.05%, KCl 0.01%, $MgSO_4 \cdot 7H_2O$ 0.05%, $(NH_4)_2SO_4$ 0.01%, NaH_2PO_4 0.01%, starch 2% (Mishra and Behera, 2008), agar 1.7 and 10% NaCl. After 48 h of incubation at 37°C, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v) (Amoozegar *et al.*, 2003). Amylolytic colonies were determined by colorless zone formation.

Enzyme Production

The amylase positive bacterial isolates were inoculated in 250 mL Erlenmeyer flasks containing 50 mL saline starch broth and incubated in a shaker incubator (150 rpm, 37°C) for 48 h.

Amylase Activity Assay

After removal of the cells by centrifugation at 4500 g for 10 min at 4°C, the supernatant was used to assay amylase activity as the enzyme solution. To determine the amylase activity, starch digestion was measured using the iodometric method (Amoozegar *et al.*, 2003).

Identification of Bacterial Isolates

Different morphological, cultural and physiological characteristics of the amylase producing bacterial isolates were studied for identification purposes and the obtained results were compared with standard description of Bergey's Manual of Determinative Bacteriology (Sharmin *et al.*, 2005).

Analysis of 16S rRNA Gene Sequences

The purified amylase producing bacterial isolates were grown in saline nutrient broth (7% NaCl). After centrifugation at 4500 g, 10 min, at 4°C and twice washing with distilled water, the pellets were selected for PCR amplification. Bacterial DNA was extracted by heat extraction method. The Partial sequence of the 16S rRNA gene was amplified by PCR using the universal prokaryotic (16S rRNA) primers, 5'- ACGGGCGGTGTGTAC -3' and 5'-CAGCCGCGTAATAC-3', which amplify a ~800-bp region of the 16S rRNA gene. PCR was performed in a final volume of 50 µL containing PCR amplification buffer (1X), Taq DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 µM) and template DNA (4 ng). Amplification conditions were as the following: initial denaturation at 94°C for 5 min, 10 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 2 min, 20 cycles at 92°C for 30 sec, 50°C for 30 sec and 72°C for 2.5 min with a final extension of 72°C for 5 min. Taq polymerase was added to the reaction after the first denaturation step. The lower denaturation temperature (92°C) during the 20 cycle step was used to avoid loss of enzyme activity (Fiore *et al.*, 2000). The PCR products were electrophoresed in 1% (w/v) agarose gel containing ethidium bromide (1 µg mL⁻¹). A single ~800 bp DNA fragment was cut and extracted from the gel using a Core Bio Gel Extraction Kit. The sequences were determined by the CinnaGen Company and then published in National Centre for Biotechnology Information (NCBI) databases. The sequence similarity searches were done using the BLAST program that is available from the NCBI and GeneDoc software, version 2.6.002 (<http://www.nrbcs.org/gfx/genedoc>).

RESULTS

In a primary screening experiment, 50 colonies were isolated being capable of using starch as a sole carbon source and forming halos on saline starch agar medium. Thirteen strains from which the colonies formed large and clear halos were purified and tested for amylase activity assay. Among them, the bacterium *Rheinheimera aquimaris* BCCS 026 has shown the highest capability for production of amylase (61 U mL⁻¹). The PCR amplification of 16S rRNA gene revealed efficient amplification; a single band of amplified DNA product of ~800 bp was recorded. The DNA sequences were published in the NCBI databases under the specific accession numbers. The amount of amylase production (U mL⁻¹) and the lengths of the 16S rRNA region of the strains and their specific accession numbers are shown in Table 1. The result of PCR blasted with other sequenced bacteria in NCBI showed similarity to the 16S rRNA of other bacteria. Edited sequences were used as queries in BLASTN searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine the nearest identifiable match

Table 1: The amylase activity (U mL⁻¹ supernatant), lengths and specific accession numbers of the 16S rRNA region of halophilic bacteria

Bacterial strain	Accession No.	Length (bp)	Activity (U mL ⁻¹ supernatant)
<i>Bacillus endophyticus</i> BCCS 022	FJ426405	702	36
<i>Aeromonas veroni</i> BCCS 025	FJ429320	720	49
<i>Rheinheimera aquimaris</i> BCCS026	FJ429321	840	61
<i>Bacillus subtilis</i> BCCS 027	FJ517538	832	54
<i>Bacillus subtilis</i> BCCS 028	FJ517539	784	48
<i>Bacillus</i> sp. BCCS 032	FJ605152	880	58
<i>Bacillus</i> sp. BCCS 034	FJ607328	802	47
<i>Bacillus</i> sp. BCCS 033	FJ607327	796	55
<i>Rheinheimera</i> sp. BCCS 035	FJ619743	809	43
<i>Bacillus</i> sp. BCCS 037	FJ619745	802	49
<i>Halobacterium</i> sp. BCCS 030	FJ517540	683	52
<i>Bacillus</i> sp. BCCS 029	FJ517540	683	55
<i>Bacillus subtilis</i> BCCS 031	FJ554670	809	55

56 Gram-negative rods and 52 Gram-positive cocci. They found that 132 strains from Gram positive rods, 35 strains from Gram-negative rods and 26 strains from Gram positive cocci were amylase producer. These data are similar to our findings and reveal that, Gram positive bacteria are the dominant amylolytic strains in the saline environments. Meanwhile the bacterium *Rheinheimera aquimaris* BCCS 026 is introduced as a producer of amylase (61 U mL⁻¹) with the highest ranking among the studied bacteria for the first time in this investigation.

Soil is the main reservoir of the genus *Bacillus*. Endospore formation, universally found in the genus *Bacillus*, is thought to be a strategy for survival in the soil environment, wherein these bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of *Bacillus* in most habitats examined. In this investigation as previous studies, the most dominant isolated bacterial strains were *Bacillus*. Olajugbe and Ajele (2005) have isolated 25 bacterial isolates from soil, of which nine isolates were identified as *Bacillus*. They were *B. brevis*, *B. licheniformis*, *B. subtilis*, *B. macerans*, *B. mycoides*, *B. coagulans*, *B. polymyxa*, *B. cereus* and *B. megaterium* species. In another study, 29 isolates as *B. megaterium* and 24 isolates as *B. subtilis* out of 306 soil samples (Olajugbe and Ajele, 2005). These findings agree with the results of this study, indicating that *Bacillus* genera are widespread in soil habitats.

The genus *Bacillus* is well known as an enzyme producer and many industrial processes use species belonging to this genus for commercial production of enzymes (Sanchez-Porro *et al.*, 2003). The members of this genus are used for the synthesis of a very wide range of important medical, agricultural, pharmaceutical and other industrial products. These include a variety of antibiotics, enzymes, amino acids and sugars. In previous studies amylase production in several *Bacillus* species have been examined (Utong *et al.*, 2006, Saxena *et al.*, 2007; Mishra and Behera, 2008; Itkor *et al.*, 1989; Jana and Pati, 1997; Sugita *et al.*, 1996, 1997) and species of this genus are considered as an ideal host for the industrial production of bulk extracellular amylases. In contrast to *Bacillus* species, in this experiment we have introduced a novel bacteria *Rheinheimera aquimaris* for extracellular amylase production. The genus *Rheinheimera* was proposed by Brettar and his associates in 2002 (Yoon *et al.*, 2007). Phylogenetic analyses based on 16S rRNA gene sequences showed that the genus *Rheinheimera* belongs to the Gammaproteobacteria. The genus *Rheinheimera* previously comprised three recognized species: *Rheinheimera baltica*, *Rheinheimera pacifica* and *Rheinheimera perlucida*. Recently, Yoon *et al.* (2007) have introduced a novel species *Rheinheimera aquimaris* that was isolated from seawater of the East Sea in Korea. *Rheinheimera aquimaris* is Gram negative rod or cocci, non-spore forming and motile by means of single polar flagella. Growth occurs at wide temperature range (4 to 43°C) and in the absence or presence of NaCl up to 8% (w/v). It can hydrolyze Casein, L-tyrosine, starch, glycogen, Tweens 20, 40 and 60. These characteristics make *Rheinheimera aquimaris* a novel choice for production of industrial hydrolytic enzymes that are probably active in wide temperature ranges and ions concentrations.

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REFERENCES

- Amoozegar, M., F. Malekzadeh and K. Malik, 2003. Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. J. Microbiol. Methods, 52: 353-359.

- Chena, W., J. Changb, C. Chiua, S. Change, W. Chena and C. Jianga, 2005. *Caldimonas taiwanensis* sp. nov., a amylase producing bacterium isolated from a hot spring. Syst. Appl. Microbiol., 28: 415-420.
- Fiore, M.F., D.H. Moon, S.M. Tsai, H. Lee and J.T. Trevors, 2000. Miniprep DNA isolation from unicellular and filamentous cyanobacteria. J. Microbiol. Meth., 39: 159-169.
- Itkor, P., O. Shida, N. Tsukagoshi and S. Udaka, 1989. Screening for raw starch digesting bacteria. Agric. Biol. Chern., 53: 53-60.
- Jana, M. and B. Pati, 1997. Thermostable, salt-tolerant α -amylase from *Bacillus* sp. MD 124. J. Basic Microbiol., 37: 323-326.
- Mishra, S. and N. Behera, 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr. J. Biotechnol., 7: 3326-3331.
- Mohapatra, B., M. Bapuji and A. Sree, 2003. Production of industrial enzymes (amylase, carboxymethylcellulase and protease) by bacteria isolated from marine sedentary organisms. Acta Biotechnol., 23: 75-84.
- Olajugibe, F. and J. Ajele, 2005. Production dynamics of extracellular protease from *Bacillus* species. Afr. J. Biotechnol., 4: 776-779.
- Oliveira, A., L. Oliveira, J. Andrade and A. Junior, 2007. Rhizobial amylase production using various starchy substances as carbon substrates. Braz. J. Microbiol., 38: 208-216.
- Rohban, R., M. Amoozegar and A. Ventosa, 2008. Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. J. Ind. Microbiol. Biotechnol., 36: 333-340.
- Sanchez-Porro, C., S. Martin, E. Mellado and A. Ventosa, 2003. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J. Appl. Microbiol., 94: 295-300.
- Saxena, R., K. Dutt, L. Agarwal and P. Nayyar, 2007. A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. Bioresour. Technol., 98: 260-265.
- Sharmin, S., M. Towhid Hossain and M.N. Anwar, 2005. Isolation and characterization of a protease producing bacteria *Bacillus amovivorus* and optimization of some factors of culture conditions for protease production. J. Biological Sci., 5: 358-362.
- Sugita, H., J. Kawasaki, J. Kumazawa and Y. Deguchi, 1996. Production of amylase by the intestinal bacteria of Japanese coastal animals. Lett. Applied Microbiol., 23: 174-178.
- Sugita, H., J. Kawasaki and Y. Deguchi, 1997. Production of amylase by the intestinal microflora in cultured freshwater fish. Lett. Appl. Microbiol., 24: 105-108.
- Utong, J., F. Al-Quadani and H. Akel, 2006. Effect of various growth conditions on production of extracellular amylase from thermotolerant *Bacillus* species isolated from hot springs in Jordan. J. Boil. Sci., 6: 621-625.
- Yoon, J., S. Park, S. Kang and T. Oh, 2007. *Rheinheimera aquimaris* sp. nov., isolation from seawater of the East Sea in Korea. Int. J. Syst. Evol. Microbiol., 57: 1386-1390.