



Production of Xanthanases by *Paenibacillus* spp.: Complete Xanthan Degradation and Possible Applications

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Background: A number of microorganisms and their enzymes have been reported as xanthan depolymerizers. *Paenibacillus* species are well-known polysaccharide hydrolyzing bacteria. However, *Paenibacillus alginolyticus* and *Paenibacillus* sp. XD are the only species in the genus which are now known to degrade xanthan.

Objectives: Complete biodegradation of the xanthan exopolysaccharide is a rarely found capability among microorganisms. The aim of this study is to survey xanthanase producing bacteria with an appropriate bioactivity for the biopolymer degradation under different environmental conditions.

Materials and Methods: The bacteria were isolated based on viscosity reduction of the xanthan solution. Bacterial isolates were identified using rep-PCR (repetitive element-based genomic fingerprinting) and 16S rDNA sequencing. Xanthanases were characterized by measuring their activity at different temperatures, pH values, and NaCl concentrations. Degradation of other polysaccharides and xanthan degradation products were investigated based on the screening plate method and TLC (thin-layer chromatography), respectively.

Results: Six isolates from different *Paenibacillus* species with a complete xanthan degrading capability were isolated from Urmia Lake. Phylogenetic analysis placed these strains within the genus *Paenibacillus* with the closest relatives that were found to be *P. nanensis*, *P. phyllosphaerae*, *P. agaridevorans*, *P. agarixedens*, and *P. taohuashanense*. These isolates displayed different levels of the xanthan biodegradation activity in temperatures ranging from 15 to 55 °C and pH values from 4 to 11. Xanthanolytic activity was generally prevented in presence of NaCl (> 0.1 mol.L⁻¹). Furthermore, the isolated *Paenibacillus* spp. could degrade several other polysaccharides including xylan, CMC (carboxymethyl cellulose), starch, alginate, and pectin.

Conclusion: Novel strains of the six different *Paenibacillus* species that were introduced in the present study are able to produce xanthanases with interesting characteristics. In light of the results from this study, special applications, particularly in healthcare, medicine, and the environment is hereby proposed for these enzymes.

Keywords: Bacterial enzymes, Biodegradation, *Paenibacillus* spp., Xanthan lyase, Xanthanase

1. Background

Xanthan is a bacterial slime polysaccharide, produced by different species of the genus *Xanthomonas*. Xanthan has a cellulosic backbone and a linear chain consisting of the sequence of mannosyl-glucuronyl-mannose attached at the C-3 position on the alternate glucosyl residue of the backbone. The proximal and distal mannosyl residues of the side chain are frequently acetylated and pyruvylated, respectively. Resistance to heat, stability in acidic and alkaline

media, and the superior rheological properties make it possible to use this indigestible biopolymer in food, pharmaceuticals, environment, petroleum and chemical industries (1). This microbial biopolymer is not easily degraded by microorganisms. Since the 1960s and shortly after industrial production of xanthan gum, a number of microorganisms and their enzymes have been reported as xanthan depolymerizers. Until now, the xanthan degrading bacteria that were reported in the literature were

found to belong to the 10 different genera capable of complete or partial degradation of the xanthan macromolecules: a *Bacillus* sp. (2), *Corynebacterium* sp. (NCBI 11535) (3), *Bacillus* sp. K11 (4) and *Bacillus* GL1 (5), *Paenibacillus alginolyticus* XL-1 (6), *Cellulomonas* sp. LX (7), *Microbacterium* sp. XT11 (8), isolates of *Brevibacillus thermoruber*, *Anoxybacillus flavithermus* and *Anoxybacillus rupiensis* (9), and *Enterobacter* sp. nov. LB37 (10). Furthermore, xanthan degradation by bacterial consortia and mixed cultures has also been reported (11-13).

Xanthanase is a name collectively used for a group of enzymes invading xanthan backbone and its side chains, eventually resulting in the complete xanthan degradation. Xanthan lyase, one of these enzymes, truncates side chains of xanthan molecules by removal of their distal mannose residues.

Most of the xanthan degrading bacteria have been isolated from the soil. Other environments such as hot springs (9) and activated sludge (14) provide the opportunity to explore novel xanthan degrading species. The present study has focused on bacterial xanthanases, a group of inadequately studied polysaccharide degrading enzymes, in soil and sludge of Urmia Lake, Iran.

2. Objectives

The aim of this research is to study xanthan degrading microorganisms and their xanthanase enzymes. Urmia Lake, with a salinity ranging between 120 to 280 g.L⁻¹, is a unique life reservoirs. The discovery of native strains with novel enzymatic features provides new prospects for the applications of the polysaccharide degradation in different fields of study.

3. Materials and Methods

3.1. Bacterial Strains and Phenotypic Characterization

Naturally sun-dried benthic sludge and soil samples were collected from Urmia Lake in West Azerbaijan Province, Iran. One gram aliquots of the samples were transferred into 500-mL conical flasks containing 100 mL Xanthan Selective (XS) broth as the enrichment culture. XS broth was formulated using a mineral salts solution (MSS) containing 500 mg.L⁻¹ CaCl₂, 50 mg.L⁻¹ K₂HPO₄, 800 mg.L⁻¹ NaCl, 25 mg.L⁻¹ MgSO₄.7H₂O, and 700 mg.L⁻¹ KNO₃; pH 7.0 supplemented with 0.5% (w.v⁻¹) xanthan as the sole source of carbon and energy. The enrichment cultures were incubated in shaking incubator at 30 °C and 150 rev.min⁻¹. After reduction of the viscosity which indicates xanthan degradation, a 10-fold serial dilution of enrichment culture was spread on Xanthan

Yeast (XY) plates and incubated at 30 °C overnight (7). XY agar was formulated using MSS containing (w.v⁻¹) xanthan (0.3%), yeast extract (0.05%), and agar (1.5%). Colonies with the halo zone were selected and purified. These bacterial isolates were cultured in the XY broth at 30 °C and 150 rev.min⁻¹. Final viscosity of each culture was measured by Brookfield viscometric system using spindle No. 3 under 60 rev.min⁻¹ rotation at an ambient temperature.

Those cultures with a remarkable reduction in the viscosity were selected for further experiments. Phenotypic characteristics including Gram reaction, sporulation capability plus catalase, and oxidase tests were determined using the standard procedures.

In addition to the isolated strains, three type species of *Paenibacillus* spp. including *Paenibacillus agaridevorans* (IBRC-M 10934), *Paenibacillus nanensis* (DSM 22867), and *Paenibacillus phyllosphaerae* (IBRC-M 10879) were examined for their xanthanolytic activity.

3.2. Genomic Study

Total genomic DNA of the isolates was extracted using the cetyltrimethylammonium bromide (CTAB) method (15). Rep-PCR was carried out to assess the diversity of the isolates and to allow the selection of one representative strain from each of the discriminated species using a previously described procedure and appropriate primers, REP1R-I (16) and REP2-I (17).

The DNA fingerprints of the strains obtained by rep-PCR were compared by visual inspection of the banding patterns. The amplification products were transformed into digits of binary matrices and analyzed using NTSYS-pc, version 2.02e. Jaccard's similarity coefficients were calculated by the SIMQUAL program and these data were subjected to the UPGMA cluster analysis using the SAHN program.

The amplified 16S rDNA sequences (18) were compared with the reference sequences obtained from EzTaxon server using the BLAST algorithm (19). Multiple alignments were performed using the Clustal W program (20). The phylogenetic tree was constructed based on the neighbor joining method (21) using the software package MEGA 5.0 (22). The reliability of the tree topology was evaluated by bootstrap analysis (23) based on 1000 replications.

3.3. Xanthan Degrading Enzymes

3.3.1. Crude Enzyme Preparation

The supernatant of the cultures grown in the XY broth was used as the crude enzyme for further analyses using the procedure of Liu *et al.* (7).

3.3.2. Xanthanase Characterization

Xanthanase in the crude enzyme extract was measured using 0.5% xanthan in different buffer solutions (20 mmol.L⁻¹) at 15-50 °C, and sodium chloride concentrations ≤1 mol.L⁻¹. Equal volumes of the culture's supernatant and the xanthan solutions (each 100 µL) were mixed and incubated at the above temperatures for 15 min. The reducing sugars were measured using di-nitrosalicylic acid (DNS) method (24, 25).

To determine the optimal pH of enzyme activity, the experiments were carried out at 30 °C in the pH range of 4 to 11, while citrate buffer was used to adjust pH from 4 to 6, phosphate buffer from 6 to 7, tris buffer from 7 to 9, and carbonate-bicarbonate from 10 to 11.

3.3.3. Enzyme Assay

The total xanthanase activity was measured using 0.5% xanthan in an appropriate buffer, optimum temperature without additional NaCl. Xanthan lyase activity was determined by measuring the increase in absorbance at 235 nm (5, 6). Crude enzyme (100 µL) was added to 500 µL xanthan solution (0.05% xanthan in 20 mmol.L⁻¹ tris-HCl buffer pH 7.0), mixed quickly, and the related absorbance at 235 nm wavelength was recorded continuously for 1 min at 30 °C. One unit of xanthanase corresponds to the amount of enzyme required to release 1 µmol of reducing sugar per minute. One unit of xanthan lyase was defined as the amount of enzyme required to produce an increase of 1.0 unit in the absorbance at 235 nm per min.

3.3.4. Depolymerization Products

To determine the degradation products, filter sterilized

crude extracellular enzyme(s) and xanthan solution (0.5% xanthan in 20 mmol.L⁻¹ sodium phosphate buffer pH 7.0) (1:4 v.v⁻¹) were mixed and incubated at 30 °C for 72 h. The products were analyzed by TLC on silica gel plates (26).

3.4. Polysaccharase Activity

Plate screening method based on complex formation between polysaccharide and dye: congo red, lugol's iodine, and ruthenium red were used to determine polysaccharide degrading activity among the selected strains of the *Paenibacillus* species using CMC, xylan, starch, pectin or alginate as the substrate (6, 27-31).

3.5. Statistical Analysis

Data from the characterized xanthanases at different temperatures, pH values, and NaCl concentrations were analyzed statistically using Minitab 16 software. Statistical analyses were performed by Student's t-test, one-way ANOVA and differences were considered to be statistically significant when p was >0.05.

4. Results

4.1. Xanthan Degrading Bacteria

Based on the intensive reduction in the viscosity of the broth culture, only ten out of seventy-six isolates were found to have the potential to degrade xanthan completely. Isolates were Gram positive or Gram variable, spore-forming rods, catalase, and oxidase positive.

A rep-PCR fingerprint was generated from REP primers and a dendrogram obtained using rep-PCR fingerprints as shown in Figure 1. It was assumed

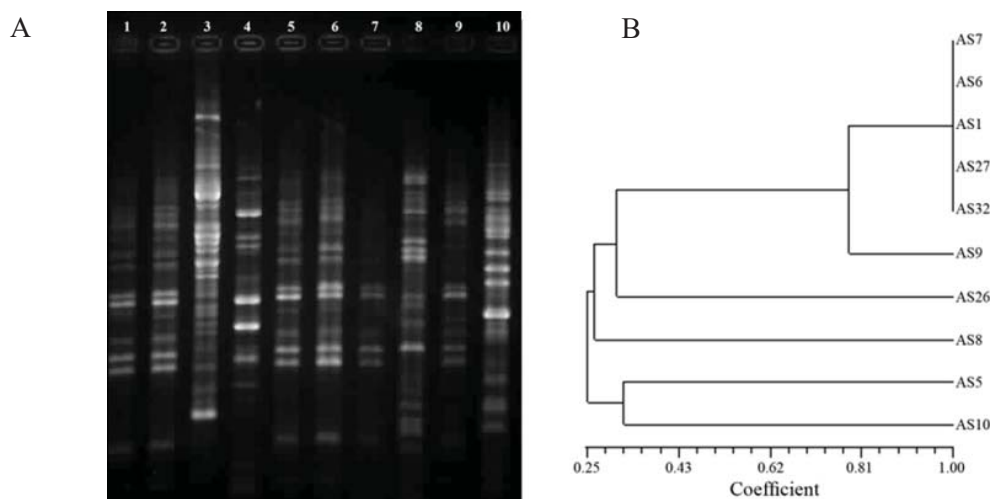


Figure 1. Gel electrophoretic image of the rep-PCR fingerprints generated by using REP primers (A): Lanes: 1 (AS7); 2 (AS6); 3 (AS5); 4 (AS26); 5 (AS1); 6 (AS27); 7 (AS32); 8 (AS8); 9 (AS9); 10 (AS10). The related dendrogram was obtained by applying UPGMA clustering method using NTSYS-pc version 2.02c (B)

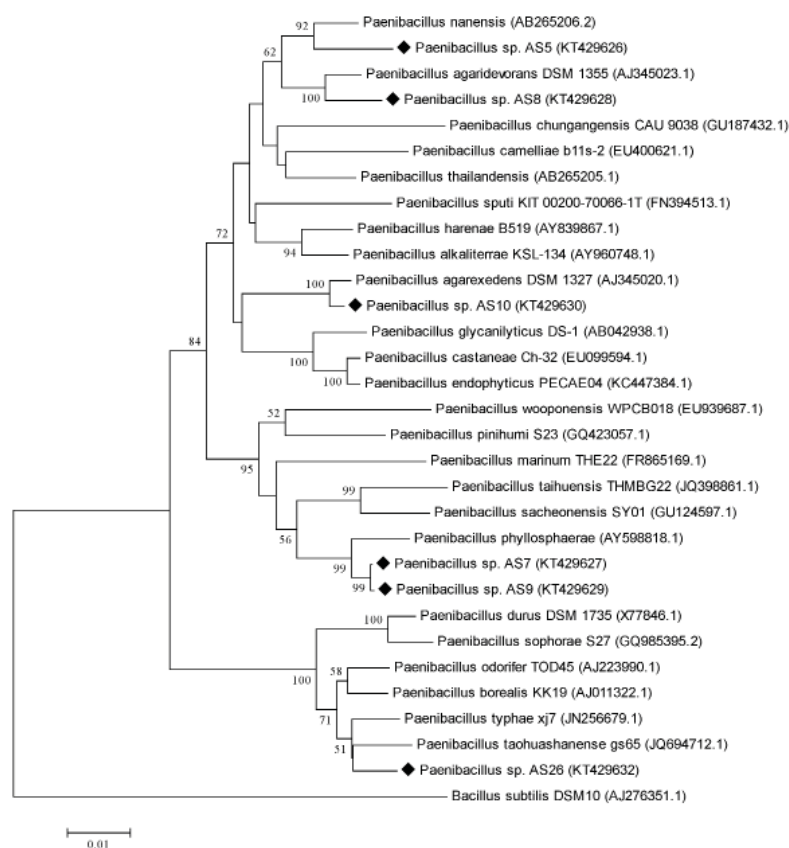
Table 1. The similarity levels of 16S rDNA sequences of xanthan degrading strains of the *Paenibacillus* spp. presented in this study with those of the closest type species

Query: Accession No.	Closest match : Accession No.	Similarity (%)
<i>Paenibacillus</i> sp. AS 5: KT429626	<i>P. nanensis</i> MX2-3 ^(T) : AB265206	97.63
<i>Paenibacillus</i> sp. AS 7 : KT429627	<i>P. phyllosphaerae</i> PALXIL04 ^(T) : AY598818	98.04
<i>Paenibacillus</i> sp. AS 8: KT429628	<i>P. agaridevorans</i> DSM 1355 ^(T) : AJ345023	98.12
<i>Paenibacillus</i> sp. AS 9: KT429629	<i>P. phyllosphaerae</i> PALXIL04 ^(T) : AY598818	97.34
<i>Paenibacillus</i> sp. AS 10: KT429630	<i>P. agarexedens</i> DSM 1327 ^(T) : AJ345020	99.11
<i>Paenibacillus</i> sp. AS 26: KT429632	<i>P. taohuashanense</i> gs65 ^(T) : JQ694712	97.61

that all isolates that share the same REP pattern were clonally related to a defined taxon. Six REP groups were designated and one representative strain per REP pattern was selected for further studies.

The 16S rDNA sequences of the selected strains were compared to those available in the EZtaxon

database and phylogenetic analysis placed all of these selected strains within the genus *Paenibacillus*. The similarities were ranging from 97.34% to 99.11% (Table 1). The phylogenetic tree was constructed by the neighbor-joining method as shown in Figure 2.

**Figure 2.** A neighbor-joining phylogenetic tree showing taxonomic relationships between selected strains and the related species belonging to the genus *Paenibacillus* based on 16S rRNA gene sequences. *Bacillus subtilis* DSM 10 was used as an out-group. Bootstrap analyses were performed with 1000 replications, only values $\geq 50\%$ are shown. (Bar: 0.01 substitutions per nucleotide position).

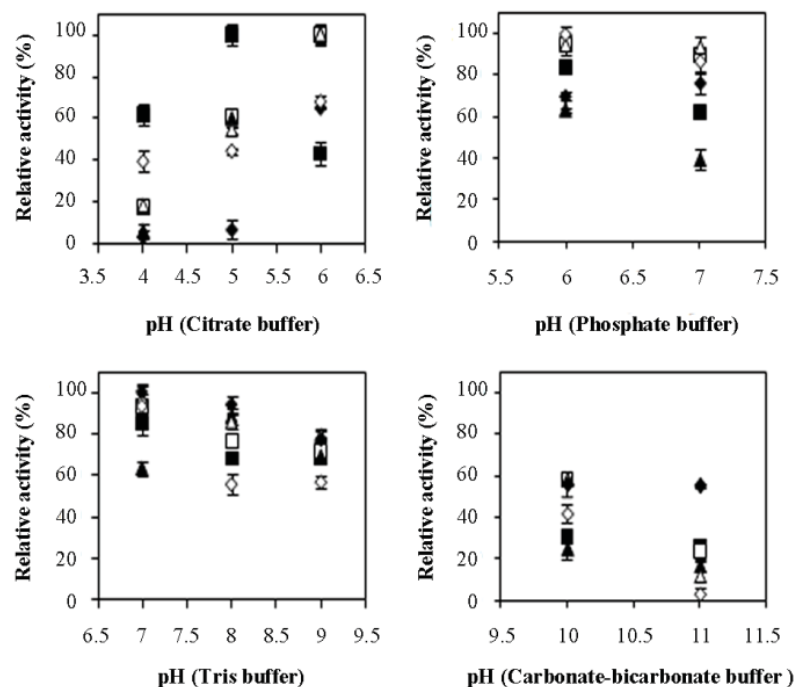


Figure 3. The effect of pH on the xanthanase activity of the selected strains of *Paenibacillus* spp. Xanthanase activity was assessed by measuring the release of reducing sugar using DNS method. Experiments were carried out at 30°C for 15 min in the following buffers with the concentration of 20 mmol.L⁻¹ Citrate, Phosphate, Tris, and Carbonate-bicarbonate for pH range of 4-6, 6-7, 7-9, and 10-11, respectively. AS5 (■); AS7 (□); AS8 (▲); AS9 (Δ); AS10 (◆); AS26 (◇).

4.2. Xanthanase Characterization

The xanthanolytic activity of the selected strains of the *Paenibacillus* spp. is depicted for the different pH values using different buffering solutions (Fig. 3). The strains exhibited maximum xanthanolytic activity within pH values ranging from 5 to 7. In a number of strains, xanthanases have maintained a remarkable portion of their activity toward acidophilic or alkalophilic conditions. Xanthanase from *Paenibacillus* sp. AS5 has kept about 60% of its activity at pH 4. However, *Paenibacillus* sp. AS7 has retained about 60% of xanthanase activity at pH 10 and showed 20% of its activity at pH 11.

The selected strains showed different maximum xanthanolytic activities at a range of temperature changes from 35 to 45 °C (Fig. 4a). The strain AS5 showed the greatest thermophilic activity at 45 °C in addition to maintaining 40% of this activity at 50 °C.

All strains showed their highest xanthanolytic activity in absence of additional salt (NaCl) except for the strain AS5 which maintained its maximum enzyme activity up to 0.1 mol.L⁻¹ NaCl (Fig. 4b).

The xanthanase activity of the selected strains of the *Paenibacillus* spp. was measured under the optimum temperature, pH condition and in absence of additional salt (NaCl). As well, their xanthan lyase activity was determined under constant conditions (30 °C and Tris

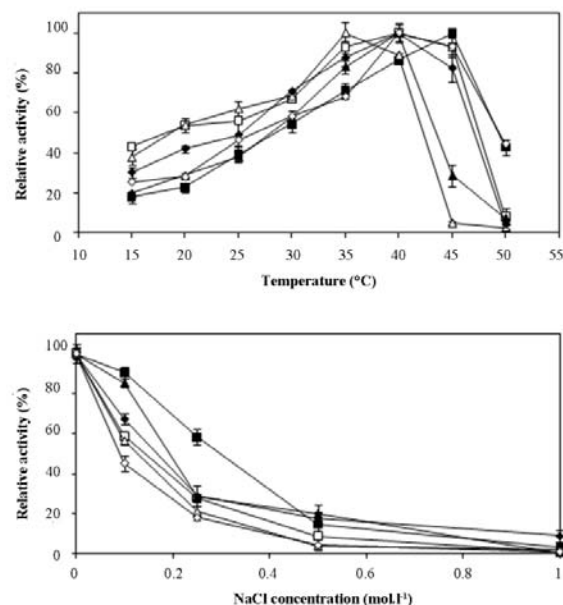


Figure 4. The effect of temperature and salinity on the xanthanase activity of the selected strains of *Paenibacillus* spp. Xanthanase activity was assessed by measuring the release of reducing sugar using DNS method. (5a): Experiments were carried out at various temperatures ranging from 15-55 °C. (5b): Reactions were carried out at different concentrations of salt ranging from 0 to 1 mol.L⁻¹ NaCl. AS5 (■); AS7 (□); AS8 (▲); AS9 (Δ); AS10 (◆); AS26 (◇).

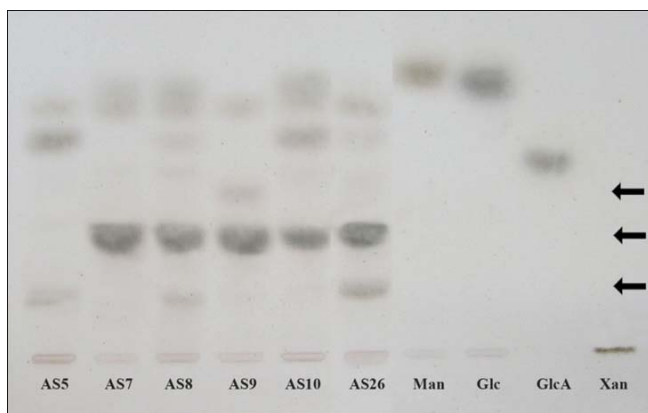


Figure 5. TLC analysis of xanthan depolymerization products which were produced by the strains of *Paenibacillus* species. The reaction was carried out at 30 °C and a reaction mixture which contained equal volumes of the crude enzyme and 0.5% xanthan solution in phosphate buffer pH 7. Arrows indicate xantho-oligosaccharides. Abbreviations: Man; D-mannose, Glc; D-glucose, GlcA; D-glucuronic acid, and Xan; Xanthan

buffer pH 7). Among the selected strains, *Paenibacillus* sp. AS7 has exhibited the highest xanthanase and xanthan lyase activity with 0.46 and 7.1 U.mL⁻¹, respectively. In comparison, other strains showed lower xanthanase activity: AS9 >AS5 >AS10 >AS8 >AS26 with 0.38, 0.26, 0.16, 0.12, and 0.1 U.mL⁻¹, correspondingly. *Paenibacillus* sp. strain AS8 showed the lowest xanthan lyase activity with 0.32 U.mL⁻¹.

According to TLC analysis, the polymer was degraded into oligosaccharides and simple sugars by all strains. It was evident from the variations in the pattern of the bands and their intensities that the xanthanase and their related activities were different among the examined *Paenibacilli* isolates (Fig. 5).

4.3. Biodegradation of other Polysaccharides

All the xanthanase producing strains of *Paenibacillus* spp. were assessed for their catalytic activity against polysaccharides other than xanthan through application of a number of biopolymers. The obtained results have revealed the following activities: CMCase, xylanase, amylase, pectin lyase, and alginate lyase activities. Among the three type species obtained from the culture collection, only *Paenibacillus agaridevorans* could degrade xanthan.

5. Discussion

From 1998 to 2010, the surface area of Urmia Lake has been drastically diminished from 5650 Km² to about 2005 Km², due to the construction of dams on rivers that flow into the lake and uncontrolled usage of ground water for

agricultural application. According to statistical models and time series, the lake level will be further reduced about 3 m in 2100 and expansion of parched land could become a major crisis in the region (32). Thus, extinction of a large number of unknown bacteria and loss of useful genomes is of great concern. In an ecological crisis such as this, study of the biodiversity and possible applications of microorganisms inhabiting the region has become a priority.

A few halotolerant and halophilic plants and animals such as *Dunaliella salina* and *Artemia urmiana* grow in the hypersaline lake. Yet, a greater diversity of animals and plants exists around the lake within heterologous habitats from low salt soils to those of saturated salinity. According to the model introduced by Nankai *et al.* (26) the complete xanthan degradation demands the presence of a complex of five different enzymes collectively called xanthanase. Complete xanthan degrading bacteria are rare. Here, we introduced a number of *Paenibacillus* spp. as dominant xanthan degrading bacteria in the Urmia Lake.

Thus far, only two out of 182 species have been reported as xanthan degrading *Paenibacillus* spp. *Paenibacillus alginolyticus* XL-1 degrades the xanthan molecule side chains and apparently leaves the backbone intact (6). In another research by Muchová *et al.* (14) it was shown that extracellular depolymerase(s) of the *Paenibacillus* sp. XD could cause an evident viscosity drop in the xanthan solution.

In this research, selected strains have shown greater than 97% similarities in 16S rDNA sequences, however, the final classification of the strains requires DNA-DNA hybridization before being recognized as the novel taxa (33).

The genus *Paenibacillus* has been reported to hydrolyze a variety of carbohydrates such as starch, hemicelluloses, cellulose, CMC, chitin, pectin, curdlan, pustulan, and pullulan (34, 36-38). However, apparently, none of the species in the genus have been examined for their xanthanolytic activity. Degradation of the xanthan by *P. agaridevorans* indicates that the xanthanolytic activity of type species in the genus should thoroughly be evaluated and their saccharolytic activity demands to be assessed.

Xanthanase complexes exhibit differences in their properties such as optimal pH and temperature of the activity and stability, as well as salt tolerance. While xanthanase complex of a halotolerant strain of *Bacillus* sp. strain K11 has shown a remarkable salt tolerance in the presence of 40 g.L⁻¹ NaCl, the xanthanase from *Bacillus* sp. strain 13-4 appears to be sensitive to the presence of salt (4). In addition, the presence of the salt has improved the tolerance of the mixed culture

to the temperatures up to 65 °C (12). In the present study, *Paenibacillus* spp. has shown the greatest xanthanase activity in absence of NaCl, but one of the strains (i.e., the strain AS5) has maintained the partial activity in presence of the salt. Halophilic xanthanases will be of great interest in the future researches. As previously reported, the optimum pH and temperature for xanthanase activity of a mixed culture were 5-6 and 35-45 °C, respectively (11). However, the optimum temperature for xanthan degradation by a mixed culture as reported by Cadmus *et al.* (12) was found to be 50 °C. In a published European patent numbered as 0 030 393, xanthanase complex remained active at a temperature between 25 °C and 70 °C, preferably 30 °C, and a pH between 5 and 7, preferably pH 5.6. The bacterial strains subjected to the examination by Cadmus *et al.* (4) were found to have maximum xanthanase activity at a temperature of 42 °C and a pH of 5.4. Also, no xanthanolytic activity was observed below pH 4 or above pH 8. Moreover, the optimal pH and temperature for the xanthan degrading reaction in *Cellulomonas* sp. LX was 6 and 40 °C, respectively (7). In the present study, the optimum temperature of xanthanase enzymes show a shift toward 35-45 °C. In addition, xanthanase complex from AS5 strain shows a partial activity at 55 °C. The optimum xanthanase activity of the strains was observed at the pH 5-8. The alkalophilicity of the xanthanase from the strain AS10 is a novel finding, plus the fact that the xanthanase from the strains AS5 and AS7 have maintained their partial activity at higher pH values up to 11.

Xanthanase has found applications in the oil industry such as reduction of the viscosity of xanthan containing fluids and removal of the xanthan formation damage (4, 12, 13, 35). Xantho-oligosaccharides could induce phytoalexin accumulation in soybean cotyledon tissues, indicating their role as elicitor-active compounds (7). However, the applicability of the xantho-oligosaccharides in the management of plant diseases caused by *X. campestris* pv. *campestris* has been disputed (36).

Moreover, xantho-oligosaccharides, much like many other oligosaccharides, may find applications as prebiotics in food and feed. Similar to as chitooligosaccharides, they can be used as feedstock for production of certain pharmaceuticals. Furthermore, as an occupational hazardous material, xanthan dust induces allergy and its inhalation is health threatening to those people who work in small xanthan consuming industries. Healthcare management in such plants may demand xanthanase for complete removal of xanthan to prevent indoor pollution of air and surfaces.

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