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Isolation, Identification and Evaluation of Enzyme Production Capacity of CGTase Producing Bacteria from Lakes Salda and Van/Turkey

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Abstract

The aim of this study was isolation of CGTase positive microorganisms from lakes Salda and Van, both being extreme environments unique in character with high alkalinity pH>9.7. Four bacterial strains designated as SD5, SG2, SG3 and V3 were isolated by cultivation on selective Horikoshi agar and confirmed as CGTase producers. Microscopic inspection showed the four isolates to be Gram positive rods. Strains were identified as *Bacillus* based on DNA sequences of 16S rDNA region. Only the V3 and SG2 isolates could be resolved at species level identified as *B. agaradhaerens* and *B. patagoniensis*, respectively. Growth and CGTase production capacity of the strains were evaluated under shake flask conditions. All isolates achieved extracellular CGTase levels of 6-8 U/mL within 24 h of incubation. Growth curves of *Bacillus* sp. SG3, *Bacillus* sp. SD5, *B. agaradhaerens* V3 showed the bacteria to reach a plateau phase within 24-30 h however, extended fermentation up to 168 h showed *B. patagoniensis* SG2 to continue the growth cycle until the end of this period with higher CGTase activity observed. These four strains isolated which have thus been confirmed as CGTase positive bacteria can potentially be exploited as genetic sources for the synthesis of CGTase in industrial scale.

Key words: Bacillus sp., bacterial identification, cyclodextrin glycosyltransferase, alkaliphilic

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Salda ve Van Göllerinden SGTaz Üreten Alkalifilik Bakterilerin İzolasyonu, Tanılanması ve Enzim Üretme Kapasitelerinin Değerlendirilmesi

Özet

Bu çalışmanın amacı, CGTase pozitif mikroorganizmaların Salda ve Van göllerinden izolasyonudur. Her iki göl de yüksek seviyede bazik (pH> 9,7) özellikte benzersiz ekstrem ortamlardır. Çalışmada SD5, SG2, SG3 ve V3 olarak kodlanan dört bakteri suşu, seçici Horikoshi katı besiyeri üzerinde yetiştirilerek izole edilmiş ve CGTase üreticileri olarak doğrulanmıştır. Mikroskobik inceleme, dört izolatın Gram pozitif çubuk şeklinde bakteriler olduğunu göstermiştir. Suşlar, 16S rDNA bölgesinin DNA dizilerine dayanarak *Bacillus* cinsi bakteriler olarak tanılanmıştır. Bunlardan sadece V3 ve SG2 izolatları tür seviyesinde, sırasıyla *B. agaradhaerens* ve *B. patagoniensis* olarak tanılanabilmiştir. Suşların büyüme ve CGTase üretim kapasiteleri çalkalamalı inkübatör koşullarında değerlendirilmiştir. Tüm izolatların, 24 saatlik inkübasyon sonrasında hücre dışı ortamda 6-8 U/mL CGTase üretebildiği tespit edilmiştir. *Bacillus* sp. SG3, *Bacillus* sp. SD5 ve *B. agaradhaerens* V3 suşlarının büyüme eğrileri 24-30 saat içerisinde durağan faza ulaşmış ancak 168 saate kadar uzatılmış fermentasyon süresince *B. patagoniensis* SG2'nin yavaş ancak istikrarlı şekilde çoğaldığı ve CGTase aktivitesinin yükselerek devam ettiği gözlemlenmiştir. Hücredışı CGTase salgılama kapasitesi olduğu doğrulanan bu dört bakteri suşu gelecekte CGTase'in endüstriyel ölçekte üretilebilmesi için yürütülecek araştırmalarda potansiyel gen kaynakları olarak değerlendirilebilecektir.

Anahtar kelimeler: Bacillus sp., bakteriyel tanılama, siklodekstrin glikosiltransferaz, alkalifilik

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1. Introduction

CGTase (EC 2.4.1.19) has the systematic name 1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase and is also persists in literature as cyclodextrin glycosyltransferase, cyclomaltodextrin glucanotransferase or cyclomaltodextrin glycosyltransferase. CGTase is a hexosyltransferase that can catalyze four different types of reactions, namely, cyclization, disproportionation, coupling, and hydrolysis. In the coupling reaction a cyclodextrin ring is cleaved and transferred to an acceptor maltooligosaccharide substrate, and in the disproportion reaction a linear maltooligosaccharide is cleaved and the reducing sugar end is transferred to an acceptor maltooligosaccharide substrate. In cyclization, which is the main reaction of CGTases, transglycosylation reaction transforms α-1,4-glucans (i.e. starch) to cyclic non-reducing oligosaccharides, composed of six, seven, or eight glucose units, respectively named, α-, β-, and γ-cyclodextrins (CD) [1, 2]. CD are used for the encapsulation of a wide range of molecules or molecular parts, particularly those having hydrophobic properties. Encapsulation provides added stability, solubility, reactivity and bio- availability to the included molecule. Thus, CD have found numerous applications in many fields including agriculture, food, chemical and pharmaceutical industries [3, 4].

Microorganisms have constituted the main source for the production of CGTases, many of which have been isolated from extreme alkaliphilic environments [5, 6]. Alkaliphilic microorganisms can be isolated from alkaline environments, such as soda lakes, soda deserts, alkaline soils and carbonate springs and require pH values of 9 or more for growth. These environments owe their high basic pH values to the presence of high concentrations of sodium carbonate salts. Alkaliphiles have attracted much interest because of their ability to produce extracellular enzymes that are active and stable at high pH values [7]. For the synthesis of CD, it is important that the CGTase used is functional at alkaline conditions. The use of alkaline CGTase has markedly reduced production costs for CD and opened new markets for CD use in foodstuffs, chemicals, and pharmaceuticals [8].

Lake Salda (37°33′N; 29°41′E) is a mid-size crater lake located in a closed basin within Yeşilova (Burdur province) in south-western Anatolia at an altitude of 1180 m above sea level. Salda lake water has been reported to be rich in magnesium ions and has pH 9.5-9.6 [9]. Russel et al. [9] have in their study emphasized the unique character of Lake Salda in establishing an analogy of this crater lake with a crater formation on Mars. Lake Van is the largest lake in Turkey (3755 km^2) and 3rd largest soda lake in the world with on average 2.17% salinity and pH 9.7-9.8 [10, 11]. Previous studies have investigated the archeal and bacterial diversity of Salda and Van Lakes [10, 12, 13, 14]. Aygan and Arikan [15] and Aygan et al. [16] have studied the alfa-amylase and endoglucanase activities of Bacterial isolates from Van lake.

Extreme environments contain natural niches harboring organisms that have adapted to the prevailing conditions and thus, novel microorganisms isolated from these environments have evolved to express proteins which are able to function at the same extreme conditions. These novel isolates are therefore potential sources of proteins or genes encoding proteins that may be of interest to industrial producers of enzymes. The objective of this study is the isolation and identification of CGTase producing bacteria from these two alkaline environments in Turkey, and the determination and comparison of their enzyme production capacity.

2. Materials and methods

2.1. Collection of environmental samples and strain isolation

A total of 20 mud samples were collected from 5 different locations around Lake Salda. Additionally, 7 mud samples from 2 locations at the periphery of Lake van were obtained. Sampling locations are indicated in Figure 1. These samples were transported and stored at 4 °C until use. Samples were briefly shaken and left to settle and 100 µL sample of water was plated on modified Horikoshi medium II [17] containing 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$-$7H_2O$, 1% Na_2CO_3, 1.5% agar, 0.03% phenolphthalein and 0.01 %methyl orange (pH 10.3). Plates were incubated at 37 °C for 4 days. Strains were selected based on the appearance of yellowish zones at colony periphery and sub-cultured by streaking on the same medium. Strains were also cultivated in Horikoshi broth containing 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1% Na_2CO_3 prepared as described by [18]. Culture samples were diluted with a 50% aliquot of 50% glycerol-water solution and stored at -80 °C.

The color, morphology, size, surface characteristics of the colonies were observed. Gram staining was performed on the isolated strains and they were observed under a light microscope (Leica DM750, Wetzlar, Germany) at 1000x magnification in oil immersion for the shape and size of the cells.

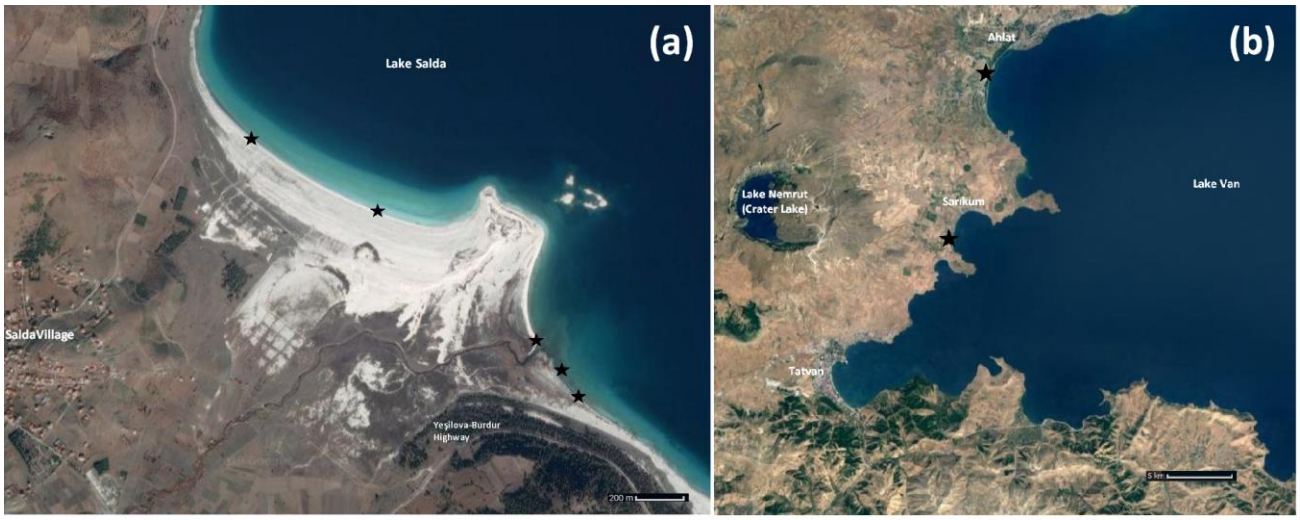

Figure 1. Satellite images of southeastern banks of lakes Salda (a) and Van (b). Stars indicate sampling locations. (Original images curtesy of Google Maps, https://maps.google.com)

2.2. Identification of the isolated strains

The color, morphology, size, surface characteristics of the colonies were observed. Gram staining was performed on the isolated strains and they were observed under a light microscope (Leica DM750, Wetzlar, Germany) at 1000x magnification in oil immersion for the shape and size of the cells. Along with these observations, bacterial identification was performed with 16S rDNA sequencing. For genomic DNA isolation bacteria were cultured in LB Lennox broth and isolation of DNA was performed using the MasterPure™ Gram Positive DNA Purification Kit (Epicentre® Biotechnologies, WI, ABD). DNA amplification was performed using the universal primer set 27F and 1492R to amplify an approximately 1400 bp fragment [19]. Sequencing was performed using Sanger dideoxy sequencing and the sequences obtained were analyzed comparing to sequences in the GenBank BLASTN database [20].
Multiple sequence alignments were performed using DNAMAN (DNAMAN 5.2.2, Lynnon Corporation, Quebec, Canada) and SnapGene (GSL Biotech LLC, USA) software. FinchTV (version 1.4.0, Geospiza, USA) and Sequencher 5.1 (Gene Codes Corporation, AnnArbor, USA) were used for inspecting the sequence chromatograms. Phylogenetic analyses were performed using the MEGA6 program [21] (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

2.3. CGTase activity assays

The four bacterial isolates were cultured in baffled Erlenmeyer flasks containing 100 mL of liquid media consisting of Horikoshi broth supplemented with 1% soluble starch. Fermentations were performed in duplicate flasks using a shaker incubator (Certomat IS, Sartorius Stedim, Germany) at 37 °C and 250 rpm. Optical density (OD) of the cell cultures were determined spectrophotometrically (Libra S50, Biochrom, UK) measuring the absorbance at 600 nm. Culture supernatants were obtained by centrifugation of 1 mL of samples at 5500 × *g* for 10 min using a microcentrifuge (Eppendorf, Germany). CGTase activity was determined based on the reduction of color intensity of phenolphthalein after complex formation with β-CD according to [18] with slight modifications. A 250 µL volume of reaction mixture containing 4% soluble starch and 50 mM K_2HPO_4 adjusted to pH 6 was mixed with 25 µL cell supernatant. The mixture was incubated at 50 °C for 10 min in a block heater (Thermo Scientific, USA). The reaction was stopped by adding 875 µL 30 mM NaOH solution and 125 µL of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution was added. The mixture was left to stand for 15 min at room temperature and the reduction in absorbance was measured spectrophotometrically at 550 nm against a blank prepared using sterile culture medium. Activity values were obtained using a standard curve obtained with 0-2.5 µmol of β-CD (C4767, Sigma) solubilized in 2.5 mL volume. One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol β-CD per min. OD and CGTase activity measurements were performed in duplicate and the mean values and standard deviations were calculated using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Strain isolation and identification

Three CGTase producing bacterial strains were isolated from Lake Salda and one strain from Lake Van. The isolates labeled as SD5, SG2, SG3 and V3 formed colonies on selective HM agar which had pale yellow zones

surrounding them. The colony morphologies of strains isolated from Lake Salda had some common properties that were not found in the Lake Van isolate (V3). Strains from Lake Salda formed medium to large sized flat elevation circular colonies with undulate margins. These were characteristically glossy in appearance and had a yellow tinge which was especially visible along the raised colony edges. The strain isolated from Lake Van on the other hand, had an irregular and filamentous form with a flat opaque surface, opaque and creamy in color. Observed under the microscope, the strains were all identified as Gram positive rod shaped bacteria. The macromorphology and Gram dyed cell micrographs are presented in Figure 2. Although not apparent in Figure 2, the SD5 strain was confirmed as a sporulating microorganism upon microscopic observation. The size and spread of the colonies are possible indications that these are motile bacteria, as are most of the species belonging to the genus Bacillus [22].

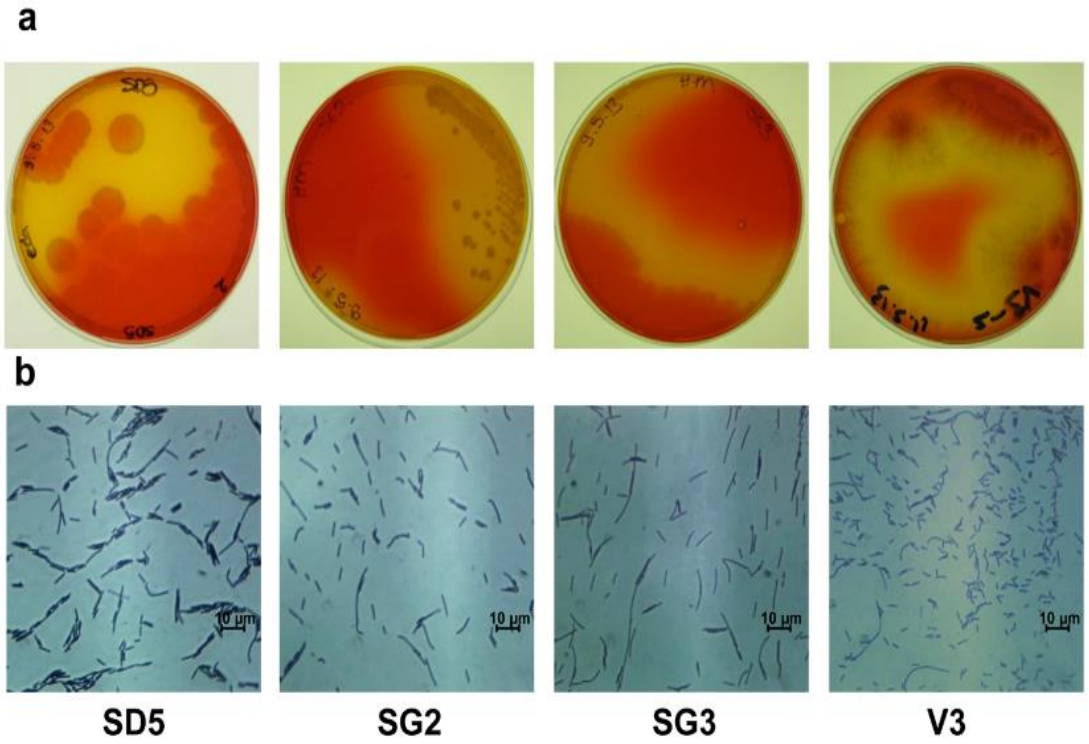

Figure 2. Colonies formed on Horikoshi agar incubated for 72 h (a) and micrographs of Gram stained cells obtained at 1000× using a light microscope (b)

The 16S rDNA of bacterial isolates was amplified, sequenced and analyzed. Sequences of 1082-1399 bp were obtained and submitted to GenBank. Accession numbers assigned were KU851858 for V3, KU851857 for SD5, KU686705 for SG2, KX037370 for SG3. The phylogenetic tree constructed using the sequence data is shown in Figure 3. All four isolates were identified as belonging to the genus *Bacillus*. Two of the isolates, SD3 and SG5, procured from different locations around Lake Salda were genetically more closely related, with sequences sharing >99% identity with 16S rDNA sequences of *B. oshimensis*, *B. lehensis* and *B. hunnanensis* strains made available on the NCBI Blast database. In another study performed in our laboratory, the CGTase gene of *Bacillus* sp. SD5 has been isolated and sequenced, yielding the information that the CGTase sequence has a maximum 98% identity to the CGTase cDNA of related *Bacillus* strains [23]. This finding provided a confirmation that *Bacillus* sp. SD5 was more closely related to the three mentioned strains. Hence, the taxonomy for the *Bacillus* sp. SD3 and SD5 could not be resolved on species level. On the other hand, *Bacillus* sp. SG2 was identified as *B. patagoniensis*. This species of bacteria was among those isolated and confirmed to be CGTase producer by Gomes et al. [24]. The isolate from Lake Van (V3) was identified as *B. agaradhaerens* and was identified as being distinctly different than the other three isolates obtained from Lake Salda. The CGTase production capacity of other novel isolates of *B. agaradhaerens* from different global locations have been demonstrated [25, 26, 27].

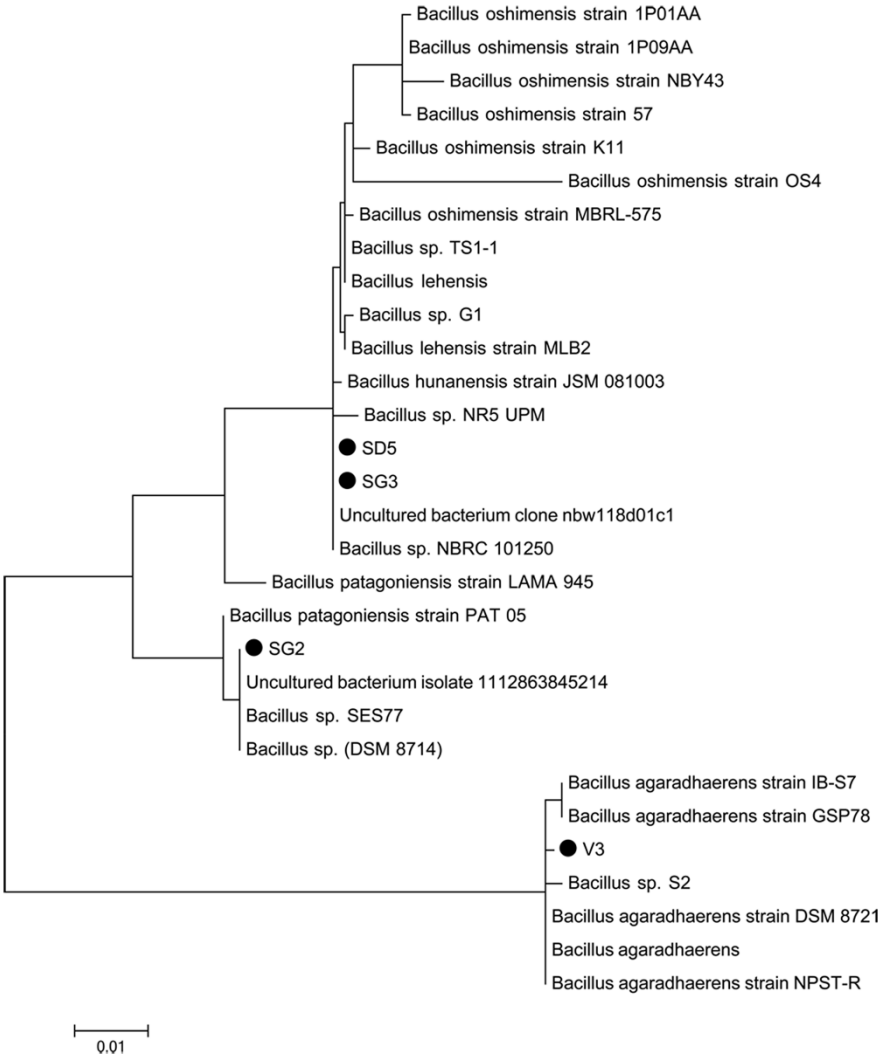

Figure 3. Phylogenetic tree showing genetic relatedness of isolated bacteria

3.2. Liquid culture and CGTase production

The CGTase production potential of cultures was further investigated with shake flask fermentation trials in 50 mL volume (Figure 4a). OD measurements indicated that all four strains exhibited logarithmic growth within the first 12 hours into incubation (Figure 4b). Cultures of strains SD5, SG3 and V3 proliferated at a higher rate reaching OD values four fold higher than strain SG2 by the 24 h into fermentation. The non-fastidious nature of SG2 was also evident from the colony morphology in Figure 2 where the colonies were small and covered a relatively less area then those of other strains. This observation may be attributable to the deficiency of the media used for a certain component or condition that the strain requires for optimum growth. Although the growth rate of *Bacillus* sp. SD5, SG3 and *Bacillus agaradhaerens* V3 seemed to be higher than *Bacillus patagoniensis* SG2, all four isolates reached CGTase activities in the range of 6-8 U/mL within 24 h. Fermentation was endured for 168 h to observe that the slow growing strain *Bacillus patagoniensis* SG2 increased in cell concentration, reaching a peak CGTase activity of 9.2 U/mL at the 96th hour (data not shown). Peak extracellular CGTase activities for the other strains were 8 U/mL at 24 h for *Bacillus* sp. SG3, 7.6 U/mL for *Bacillus* sp. SD5 at 84 h and 7.6 U/mL for *Bacillus* sp. V4 at 12 h. Among these four isolates *Bacillus* sp. SD5 was utilized as a genetic source for the heterologous expression of CGTase in *Pichia pastoris* [23].

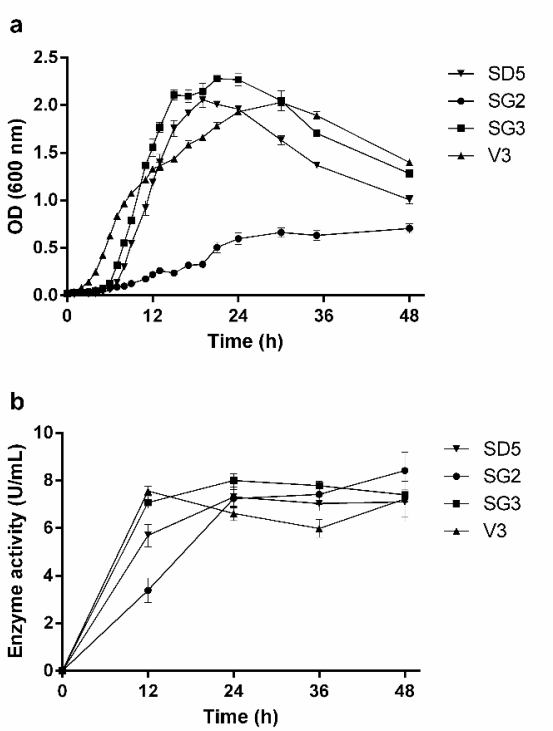

Figure 4. OD (a) and extracellular CGTase production b) of bacterial isolates from lakes Salda and Van cultured under shake flask conditions

4. Conclusions

Wild type microbial isolation and characterization is a key step in research endeavors geared at biotechnological production. Furthermore, challenged with environmental threats, the identification and investigation of microbial communities are of paramount importance providing documentation of the existing microflora. In this context, this is the first report of isolation of bacteria which are capable of producing CGTase from two major soda lakes in Turkey. In doing so, four strains have been obtained which can be utilized for CGTase production as well as for exploitation as genetic sources for production of CGTase or other alkaliphilic enzymes. It is anticipated that this study has added to our understanding of the microbial communities that reside in these local environments and the necessity of protection of these ecosystems from the impact of human activity to conserve these unique microbial habitats.

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References

[1] Tonkova, A. (1998). Bacterial cyclodextrin glucanotransferase. *Enzyme and Microbial Technology*, 22(8), 678–686. https://doi:10.1016/S0141-0229(97)00263-9

[2] Van der Veen, B. A., Uitdehaag, J. C. M., Dijkstra, B. W., & Dijkhuizen, L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specifity. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1543(336), 336–360. https://doi:10.1016/S0167-4838(00)00233-8

[3] Del Valle, E. M. M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry*, 39(9), 1033–1046. https://doi:10.1016/S0032-9592(03)00258-9

[4] Szejtli, J. (2013). *Cyclodextrin technology - topics in inclusion science* (Vol. 1). Netherlands: Springer Science & Business Media. https://doi:10.1007/978-94-015-7797-7

[5] Biwer, A., Antranikian, G., & Heinzle, E. (2002). Enzymatic production of cyclodextrins. *Applied Microbiology and Biotechnology*, 59(6), 609–617. https://doi:10.1007/s00253-002-1057-x

[6] Han, R., Li, J., Shin, H.-D., Chen, R. R., Du, G., Liu, L., & Chen, J. (2014). Recent advances in discovery, heterologous expression, and molecular engineering of cyclodextrin glycosyltransferase for versatile applications. *Biotechnology Advances*, 32(2), 415–428. https://doi:10.1016/j.biotechadv.2013.12.004

[7] Atanasova, N., Petrova, P., Ivanova, V., Yankov, D., Vassileva, A., & Tonkova, A. (2008). Isolation of novel alkaliphilic bacillus strains for cyclodextrin glucanotransferase production. *Applied Biochemistry and Biotechnology*, 149(2), 155–167. https://doi:10.1007/s12010-007-8128-5

[8] Horikoshi, K. (2011). Introduction and history of alkaliphiles. In K. Horikoshi (Ed.), *Extremophiles handbook* (pp. 19–26). Japan: Springer. https://doi:10.1007/978-4-431-53898-1_1.1

[9] Russel, M. J., Ingham, J. K., Zedef, V., Maktav, D., Sunar, F., Hall, A. J., & Fallick, A. E. (1999). Search for signs of ancient life on Mars: expectations from hydromagnesite microbialites, Salda Lake, Turkey. *Journal of the Geological Society*, 156(5), 869–888. https://doi:10.1144/gsjgs.156.5.0869

[10] Berber, İ., & Yenidünya, E. (2005). Identification of alkaliphilic Bacillus species isolated from Lake Van and its surroundings by computerized analysis of extracellular protein profiles. *Turkish Journal of Biology*, *29*(3), 181–188.

[11] Kempe, S., Kazmierczak, J., Landmann, G., Konuk, T., Reimer, A., & Lipp, A. (1991). Largest known microbialites discovered in Lake Van, Turkey. *Nature*, 349(6310), 605–608. https://doi.org/10.1038/349605a0

[12] Berber, İ., & Berber, Ş. (2006). Numerical analysis of SDS-PAGE protein patterns of facultative alkaliphilic Bacillus species isolated from lake Van, Turkey. *Fresenius Environmental Bulletin*, 15(5), 409–416.

[13] Ozcan, B., Cokmus, C., Coleri, A., & Caliskan, M. (2006). Characterization of extremely halophilic Archaea isolated from saline environment in different parts of Turkey. *Microbiology*, 75(6), 739–746. https://doi:10.1134/s002626170606018x

[14] Poyraz, N., & Mutlu, M. B. (2017). Alkaliphilic bacterial diversity of Lake Van/Turkey. *Biological Diversity and Conservation*, 10(1), 92–103.

[15] Aygan, A., & Arikan, B. (2008). A new halo-alkaliphilic, thermostable endoglucanase from moderately halophilic Bacillus sp. C14 isolated from Van Soda Lake. *International Journal of Agriculture & Biology*, 10(4), 369–374.

[16] Aygan, A., Arikan, B., Korkmaz, H., Dinçer, S., & Çolak, Ö. (2008). Highly thermostable and alkaline α-amylase from a halotolerant-alkaliphilic Bacillus sp. AB68. *Brazilian Journal of Microbiology*, 39(3), 547–553. https://doi:10.1590/S1517-83822008000300027

[17] Park, C. S., Park, K. H., & Kim, S. H. (1989). Screening method for glucanotransferase using. *Agricultural and Biological Chemistry*, 53(4), 1167–1169.

[18] Kaneko, T., Kato, T., Nakamura, N., & Horikoshi, K. (1987). Spectrophotometric determination of cyclization activity of β-cyclodextrin-forming cyclomaltodextrin glucanotransferase. *Journal of the Japanese Society of Starch Science*, 34(1), 45–48.

[19] Lane, D. J. (1991) 16S/23S rRNA sequencing. In E. Stackebrandt & M.Goodfellow (Eds.), *Nucleic acid techniques in bacterial systematics* (pp. 115–175). Chichester, United Kingdom: John Wiley and Sons Ltd.).

[20] Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389–3402.

[21] Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725–2729. https://doi:10.1093/molbev/mst197

[22] Fraser, G., Hughes G. (1999) Swarming motility. Current Opinion in Microbiology, 2(6): 630-635. https://doi:10.1016/S1369-5274(99)00033-8

[23] Kabacaoğlu, E., & Karakaş Budak, B. (2017). Heterologous expression of β-γ-type cyclodextrin glycosyltransferase of newly isolated alkaliphilic Bacillus sp. SD5 in Pichia pastoris. *Starch/Staerke*, 69(9–10), 1600365. https://doi:10.1002/star.201600365

[24] Gomes, A. C. S. M., Dos Santos, S. R., Ribeiro, M. C., Cravo, P. V. L., Vieira, D. G., De Souza, K. M. C., & Amaral, A. C. (2018). Is there still room to explore cyclodextrin glycosyltransferase-producers in Brazilian biodiversity? *Anais Da Academia Brasileira de Ciências*, 90(2), 1473–1480. https://doi:10.1590/0001-3765201820170670

[25] Hao, J.-H., Huang, L.-P., Chen, X., Sun, J.-J., Liu, J.-Z., wang, W., & Sun, M. (2017). Identification, cloning and expression analysis of an alpha-CGTase produced by stain Y112. *Protein Expression and Purification*, 140, 8–15. https://doi:10.1016/J.PEP.2017.07.015

[26] Abdelnasser, S. S. I., Mohamed, A. E. T., & Ali, A. A. S. (2010). Characterization of immobilized alkaline cyclodextringlycosyltransferase from a newly isolated Bacillus agaradhaerens KSU-A11. *African Journal of Biotechnology*, 9(44), 7550–7559. https://doi:10.5897/AJB10.644

[27] Martins, R. F., & Hatti-Kaul, R. (2002). A new cyclodextrin glycosyltransferase from an alkaliphilic Bacillus agaradhaerens isolate: purification and characterisation. *Enzyme and Microbial Technology*, 30(1), 116–124. https://doi:10.1016/S0141-0229(01)00461-6

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