JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

LEVAN PRODUCTION POTENTIALS FROM DIFFERENT HYPERSALINE ENVIRONMENTS IN TURKEY

Hakan Çakmak[1], Pınar Aytar Çelik[2,3], Seval Çınar[4], Emir Zafer Hoşgün[5], M. Burçin Mutlu[4] , Ahmet Çabuk[6]*

Address(es):
[1]Department of Biotechnology and Biosafety, Eskişehir Osmangazi University, Eskişehir, Turkey.
[2]Department of Biomedical Engineering, Eskişehir Osmangazi University, Eskişehir, Turkey.
[3]Horse Breeding Vocational School, Eskişehir Osmangazi University, Eskişehir, Turkey.
[4]Department of Biology, Eskişehir Technical University, Eskişehir, Turkey.
[5]Department of Chemical Department, Eskişehir Technical University, Eskişehir, Turkey.
[6]Department of Biology, Eskişehir Osmangazi University, Eskişehir, Turkey.

*Corresponding author: pinaraytar@gmail.com

doi: 10.15414/jmbfs.2020.10.1.61-64

ARTICLE INFO

Received 9. 12. 2019
Revised 12. 3. 2020
Accepted 12. 3. 2020
Published 1. 8. 2020

Regular article

OPEN ACCESS

ABSTRACT

12 halophilic strains from different hypersaline environments such as solar salterns in Tuzlagözü (Sivas), Fadlum (Sivas), Kemah (Erzincan), a hypersaline spring water in Pülümür (Tunceli) and a saline lake in Delice (Kırıkkale) belonging to Turkey, were investigated in terms of levan production. After incubation and ethyl alcohol treatment, dialysis process was operated for partial purification. Levan amounts in our samples after hydrolysis were calculated based on the amount of sugar obtained by acid hydrolysis of standard levan. Sugar amount of samples were determined using by high performance liquid chromatography system (HPLC). ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) spectra of the levan sample and standard were recorded. The results obtained by HPLC analysis showed that *Chromohalobacter canadensis* strain 85B had highest production potential as 234.67 mg levan/g biomass. The chemical shifts of ^{1}H-NMR spectrum of the extracted levan also showed high similarity to those of pure levan isolated from *Erwinia herbicola.* Furthermore, *Marinobacter* sp. 163Y strain also is also capable with regard to levan production. In this study, this strain could yield 230.80 mg/g levan and this potential was first reported in the literature.

Keywords: halophilic, levan, screening

INTRODUCTION

Microbial exopolysaccharides (EPS) are one of the major groups of biopolymers and they have unique functions; therefore many biopolymers are already being produced commercially on large scales. As a result, EPSs have increased attraction because of industrial and medical applications **(Rehm, 2010).** Among of microbial polysaccharides; pullulan, xanthan, dextran, levan have long been the focus of interest owing to extraordinary characteristics **(Iyer *et al.*, 2006)**.
The one of significant EPSs, levan is a β(2-6)-linked fructose homopolysaccharide that is extracellularly generated from sugar-based substrates by various microorganisms such as species of *Zymomonas*, *Aerobacter*, *Erwinia*, *Bacillus*, *Acetobacter*, *Azotobacter*, *Corynebacterium*, *Mycobacterium*, *Gluconobacter*, *Streptococcus*, and *Pseudomonas*. Levan's distinguishing properties including water solubility, film-forming ability, high solubility in oil, strong adhesivity, compatibility with salts and surfactants, low viscosity, heat stability, acid-alkaline stability and high holding capacity for water and chemicals, and good biocompatibility, make this molecule attractive **(Kang *et al.*, 2009; Kazak *et al.*, 2010; Nakaponga *et al.*, 2013)**. Thereby it has large potential uses as stabilizer, emulsifier, thickener, encapsulating agent, osmoregulator, food and feed additive, cryoprotector in various sectors, plasma substitute, prolongator of drug activity, source of prebiotic fibre, antitumor, antidiabetic and antihyperlipidemic agent in medicine **(Kang *et al.*, 2009; Freitas *et al.*, 2011; Sezer *et al.*, 2011; Jathore *et al.*, 2012; Esawy *et al.*, 2013)**. This polymer is not still cost-effective polymer due to low productivities of the commercially employed microbial systems. Thereby microbial systems having high level levan production capacity are siginificant industrially. In this context, halophilic isolates as well as *Halomonas maura, H.ventosae, H.cerina* have been considered as outstanding producers **(Arias *et al.*, 2003; Mata *et al.*, 2006; Gonzalez-Domenech, 2008; Poli *et al.*, 2009)**. Because of the fact that the genus of *Halomonas* is the best extremophilic levan producer, research trend has been focused to combine the benefits of osmoadaptation and halophilicity to favour a cost-effective and eco-friendly levan production procedure, minimize contamination risk and gain novel and valuable characteristics for exopolysaccharide **(Poli *et al.*, 2009)**.
The target of this work was to define the yields of levan for some isolates from different salterns of Turkey. For this purpose, several halophilic isolates were screened quantitatively in terms of levan exopolysaccharide production and characterized.

MATERIAL AND METHODS

Isolation of microorganisms

The halophilic strains examined in this study were previously obtained from different hypersaline environments in Turkey solar salterns in Tuzlagözü (Sivas), Fadlum (Sivas), Kemah (Erzincan), hypersaline spring water in Pülümür (Tunceli) and a saline lake in Delice (Kırıkkale) **(Çınar *et al.*, 2016)**. Detailed information on the sources of the isolates is shown in Table 1. Isolations of the strains were performed on modified growth medium (MGM) and R2A medium. MGM was supplemented with 0.5% peptone, 0.1% yeast extract and 12%, 18%, 23%, 25% total salt concentration **(Dyall-Smith, 2009)**. R2A medium [g/L: yeast extract 0.5, protease peptone 0.5, casamino acids 0.5, glucose 0.5, soluble starch 0.5, Na-pyruvate 0.3, K_2HPO_4 0.3, $MgSO_4x7H_2O$ 0.05] was supplemented with 20% NaCl. Genomic DNAs of the isolates were extracted by boiling. Polymerase chain reaction (PCR) amplification products of the 16S rRNA genes were obtained from DNA samples of the isolates. 27F(5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R(5′-GGTTACCTTGTTACGACTT-3′) were used as PCR primers specific for Bacteria domain (Lane et al., 1985). The PCR conditions were used for amplification: a cycle of 94 °C for 3 min, 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 2 min; finally an extension step of 7 min at 72 °C **(Mutlu *et al.*, 2008)**. After PCR products belonging to 16S rRNA genes were purified with Wizard® SV Gel and PCR Clean-Up System (Promega), the purified products were sequenced by using CEQ DTCS Kit (Dye Terminator Cycle Sequencing Quick Start Kit, Beckman Coulter) and CEQ™ 8000 DNA sequencer (Beckman Coulter). All sequences were compared with the sequences available in database

of GenBank (using BLAST program) and Ribosomal Database Project (RDP). The sequences were submitted to GenBank database.

Table 1 Halophilic bacteria isolated from different salterns

Species	Sequence length	Accession no	Isolation field	Medium
Marinobacter sp. 163Y	1501 bp	KP795380.1	Tuzlagözü saltern, Sivas	R2A
Halomonas caseinilytica strain KB2	1419 bp	KF668253.1	Kemah saltern, Erzincan	18% MGM
Halomonas variabilis strain T1PU	1359 bp	KJ161496.1	Pülümür hypersaline spring water, Tunceli	12% MGM
Halomonas ventosae strain T2PU	1306 bp	KJ161487.1	Pülümür hypersaline spring water, Tunceli	12% MGM
Halomonas sp. T7PU	1309 bp	KJ161489.1	Pülümür hypersaline spring water, Tunceli	12% MGM
Halomonas sp. K15	1494 bp	KP795384.1	Kemah saltern, Erzincan	18% MGM
Halomonas organivorans strain DB4	1419 bp	KF668255.1	Delice saline lake, Kırıkkale	18% MGM
Halomonas sp. DB5	1348 bp	KY099605.1	Delice saline lake, Kırıkkale	12% MGM
Chromohalobacter canadensis strain 85B	1408 bp	KF668261.1	Fadlum saltern, Sivas	23% MGM
Halomonas alkaliphila strain 305B	1373 bp	KF985241.1	Tuzlagözü saltern, Sivas	12% MGM
Idiomarina sp. 30BE	1465 bp	KF976353.1	Tuzlagözü saltern, Sivas	18% MGM
Halomonas elongata strain 153B	1395 bp	KF668257.1	Fadlum saltern, Sivas	25% MGM

Levan Production

12 isolates were screened for levan production on the basal medium (pH 7) consisted of (per liter): 137.2 g NaCl; 50 g sucrose; 7 g K_2HPO_4; 2 g KH_2PO_4; 1 g $(NH_4)_2SO_4$; 0.1 g $MgSO_4.7H_2O$, 0.32 g; 0.5 g peptone **(Küçükaşik *et al.*, 2011)**. Sterilization was carried out at 110 °C for 25 min. After inoculation, the flasks were incubated for 72 h at 37 °C and 180 r.p.m. and the working volume was 50 mL. All the experiments were performed in duplicate.

Purification of Levan

After the incubation period, the polymer medium was transferred to the tubes and then centrifuged at 10.000 r.p.m. for 25 min. The supernatant obtained at the end of the centrifugation was treated with an equal volume of ethyl alcohol and left at -18 °C overnight. Then the alcohol-treated solutions were centrifuged at 10.000 r.p.m for 25 min and the supernatants were discarded. Pellets were dissolved by adding boiling water. For dialysis, Sigma D9777 coded membrane was used. The warmed solution was transferred to the dialysis membrane prepared overnight with distilled water. The membrane was then placed in a container containing 250 ml of distilled water. Dialysis process was continued to 48 hours. After the dialysis, the biopolymer transferred to centrifuge tubes was kept overnight at -80 °C and lyophilized.

Quantification of Levan

Levan amounts in the samples were calculated based on the amount of sugar obtained by acid hydrolysis of standard levan from *Erwinia herbicola* (Sigma Aldrich).

The levan produced in this study and the commercial levan samples were subjected to acid hydrolysis for 60 min at 100 °C using 6M H_2SO_4. After acid hydrolysis of levan samples, the pH of the mixture was neutralized by the addition of NaOH. Sugar amounts of samples were determined using HPLC system.
The HPLC system (Agilent 1100, Germany) was equipped with a Bio-Rad Aminex HPX-87P (USA) column (300 mm×7.8 mm) and a refractive index detector. The analytical column was operated at 80 °C with 0.2-µm filtered HPLC grade water as the mobile phase. The mobile phase flow rate was 0.6 mL/min.

NMR Analysis

All liquid state proton (^{1}H) NMR spectra of the levan produced in this study and commercial levan obtained from *Erwinia herbicola* were recorded on a JEOL ECZ 500R spectrometer at usual probe temperature. The operating frequencies were 500.13 MHz for 1H nucleus.

RESULTS AND DISCUSSION

Screening of Levan Yields

Twelve isolates obtained from various places in Turkey were investigated in respect to exopolysaccharide production. These isolates were grown in polymer medium including 13.7% NaCl. The strains of the *Halomonas*, *Marinobacter*, *Idiomarina* and *Chromohalobacter* were obtained and screened for the production levels of exopolysaccharides (EPS) (Table 2).

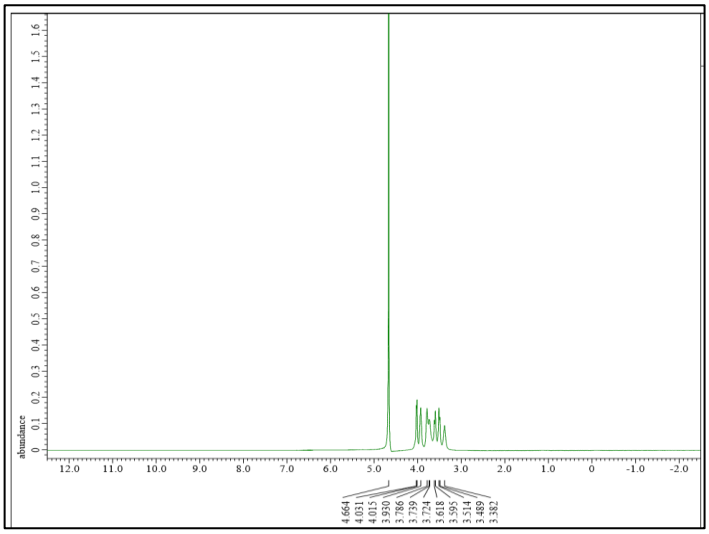
Table 2 Levan yield of the isolates

Strains	Levan yield (mg Levan/g)
Halomonas ventosae strain T2PU	185.50
Halomonas sp. DB5	195.59
Halomonas variabilis strain T1PU	199.14
Halomonas sp. K15	208.27
Halomonas alkaliphila strain 305B	209.92
Halomonas sp. T7PU	211.97
Halomonas organivorans strain DB4	214.81
Idiomarina sp. 30BE	216.33
Halomonas caseinilytica strain KB2	223.90
Halomonas elongata strain 153B	226.00
Marinobacter sp. 163Y	230.80
Chromohalobacter canadensis strain 85B	234.67

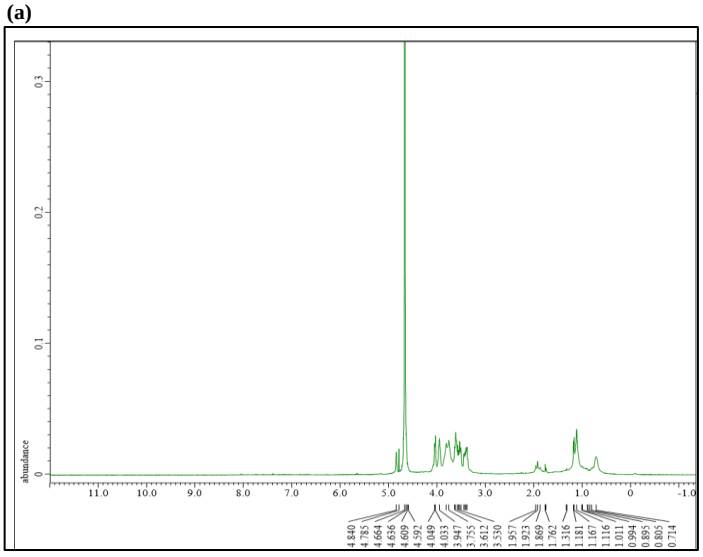
In our study, the results obtained by HPLC analysis showed that *Chromohalobacter canadensis* strain 85B had highest production potential as 234.67 mg levan/g biomass. According to the study of Radchenkova and colleagues, *Chromohalobacter canadensis* strain 28 isolated from Pomorie salterns could be used as extracellular polymer substance (EPS) producer **(Radchenkova *et al.*, 2018)**. Chemical analysis of the purified polymer indicated that this compound included EPS fraction (14.3% w/w) and protein fraction (72% w/w) including polyglutamic acid (PGA) (75.7% w/w). EPS fraction analysis indicated the following sugar composition (% w/w): glucosamine 36.7, glucose 32.3, rhamnose 25.4, xylose 1.7, and unidentified sugar 3.9. Although Radchenkova and co-workers mentioned that a strain of *Chromohalobacter canadensis* could produce extracellular polymer substance, but what type of EPS it produced was specified and was not investigated. In our study, this is the first report for halophilic bacterium such as *Chromohalobacter canadensis* able to synthesize directly a levan polymer.
Apart from *Chromohalobacter canadensis* 85B strain, other halophilic bacteria were found to produce levan in the literature. Firstly, a higher yield of EPS was produced by *Halomonas smrynensis* strain isolated from İzmir province located in the Aegean Region of Turkey **(Poli *et al.*, 2013; Ateş *et al.*, 2013)**. The same strain could produce levan as 8.84 g/L based on the spectrophotometric measurement **(Sarilmiser *et al.*, 2015)**. In another study, *Halomonas* and *Chromohalobacter* strains were compared with regard to levan production and *Chromohalobacter japonicus* strains were reported to be more potential levan producers than others **(Nasir *et al.*, 2015)**. Hussainy and coworkers demonstrated the levan production potential of Chromohalobacter *salexigens* strains and chemically modified into two derivatives, sulphated and carboxymethylated levan. Three types of biological activities were assayed for levan and its derivatives; anti-tumor activity, fibrinolytic activity and prebiotic activity **(Hussainy *et al.*, 2015)**.
Marinobacter sp. 163Y strain also is also promising in terms of levan production. In this study, this strain could produce 230.80 mg/g levan. This potential was first reported in the literature.

NMR Analysis

The chemical shifts of proton NMR spectra of the extracted levan from *Chromohalobacter canadensis* 85B in this study also indicated high similarity to those of levan isolated from *Erwinia herbicola* (Fig 1a-b). Profile differences may be due to the different bacteria from which the polymer is produced.



(a)



(b)

Figure 1 (a) NMR profile of commercial levan from *Erwinia herbicola* **(b)** NMR profile of produced levan from *Chromohalobacter canadensis* strain 85B

CONCLUSION

In this study, twelve halophilic strains isolated from different hypersaline fields in Turkey were investigated in terms of levan production abilities. *Chromohalobacter canadensis* 85B and *Marinobacter* sp. 163Y were first reported to synthesize a levan polymer in the literature. Proton NMR profiles of the obtained levan from *Chromohalobacter canadensis* 85B and commercial levan isolated from *Erwinia herbicola* indicated high similarity. Further research will be focused on advanced purification and characterization of levan from *Chromohalobacter canadensis* 85B.

Acknowledgements: The authors would like to thank Dr. Okan Zafer Yeşilel from Department of Chemistry at Eskisehir Osmangazi University for FTIR spectra of the biopolymer. This study was supported by Eskisehir Osmangazi University Scientific Research Projects Committee (Project No: BAP 201538A101).

REFERENCES

ARIAS, S., DEL MORAL A.E., FERRER, M.R., TALLON, R., QUESADA, E., BEJAR, V. (2003). Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology, *Extremophiles*, *7*, 319-326, https://doi.org/10.1007/s00792-003-0325-8.

ATES, O., ARGA, K.Y., ONER, E.T. (2013). The stimulatory effect of mannitol on levan biosynthesis: Lessons from metabolic systems analysis of *Halomonas smyrnensis* AAD6^T, *Biotechnology Progress*, *29*, 1386-1397, https://doi.org/10.1002/btpr.1823.

ÇINAR, S., MUTLU, M.B. (2016). Comparative analysis of prokaryotic diversity in solar salterns in eastern Anatolia (Turkey), Extremophiles, *20*, 589-601, https://doi.org/10.1007/s00792-016-0845-7.

DYALL-SMITH, M. (2009). The Halohandbook- Protocols for Haloarchaeal Genetics, Version 7.2, (https://haloarchaea.com/wp-content/uploads/2018/10/Halohandbook_2009_v7.3mds.pdf).

ESAWY, M.A., AMER, H., GAMAL-ELDEEN, A.M., ENSHASY, H.A.E., HELMY, W.A., ABO-ZEID, M.A., AWAD, G.E. (2013). Scaling up, characterization of levan and its inhibitory role in carcinogenesis initiation stage, *Carbohydrate Polymers*, *95*, 578-587, https://doi.org/10.1016/j.carbpol.2013.02.079.

FREITAS, F., ALVES, V.D., REIS, M.A. (2011). Advances in bacterial exopolysaccharides: from production to biotechnological applications, *Trends in Biotechnology*, *29*, 388–398, https://doi.org/10.1016/j.tibtech.2011.03.008.

GONZALEZ-DOMENECH, C.M., MARTINEZ-CHECA, F., QUESADA, E., BEJAR, V. (2008). *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium, *International Journal of Systematic and Evolutionary Microbiology*, *58*, 803-809, https://doi.org/10.1099/ijs.0.65322-0.

HUSSAINY, S.M., SHEREF, F.A., AMER, H., ELSAKHAWY, T.A. (2015). Biosynthesis of Applicable Levan by a New Levan Producing Moderately Halophilic Strain *Chromohalobacter salexigens* TA1 and its Biological Activities, *Current Science International*, *4*, 423-434, https://pdfs.semanticscholar.org/bf3c/88b2c1c4ff254180406354d4c46e496b18f5.pdf?_ga=2.97152351.1125870020.1583329465-922782084.1576485385.

IYER, A., MODY, K., JHA, B. (2006). Emulsifying properties of a marine bacterial exopolysaccharide, *Enzyme and Microbial Technology*, *38*, 220-222, https://doi.org/10.1016/j.enzmictec.2005.06.007.

JATHORE, N.R., BULE, M.V., TILAY, A.V., ANNAPURE U.S. (2012). Microbial levan from Pseudomonas fluorescens: Characterization and medium optimization for enhanced production, *Food Science and Biotechnology*, *21*, 1045-1053, https://doi.org/10.1007/s10068-012-0136-8.

KANG, S.A., JANG, K.H., SEO, J.W., KIM, K.H., KIM, Y.H., RAIRAKHWADA, D. et al. (2009). Levan: Applications and perspectives. In: Rehm B. Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives, Caister Academic Press, Inc., 145–161. Norkfolk, https://www.caister.com/biopolymers.

KAZAK, H., TOKSOY ÖNER, E., DEKKER, R.F.H. (2010). Extremophiles as Sources of Exopolysaccharides, In: Ito, R., Matsuo, Y. (Eds) Handbook of Carbohydrate Polymers: Development, Properties and Applications. Nova Science Publishers, Inc., New York, 605-619, http://www.novapublishers.org/catalog/product_info.php?products_id=11033.

KÜÇÜKAŞIK, F., KAZAK, H., GUNEY, D., FINORE, I., POLI, A., YENIGUN, O., NICOLAUS, B., TOKSOY ONER, E. (2011). Molasses as Fermentation Substrate for Levan production by Halomonas sp., *Applied Microbiology and Biotechnology*, *89*, 1729-40, https://doi.org/10.1007/s00253-010-3055-8.

MATA, J.A., BEJAR, V., LLAMAS, I., ARIAS, S., BRESSOLLIER, P., TALLON, R. (2006). Exopolysaccharides produced by the recently described halophilic bacteria *Halomonas ventosae* and *Halomonas anticariensis*, *Research in Microbiology*, *45*, 24-27, https://doi.org/10.1016/j.resmic.2006.06.004

MUTLU, B.M., MARTINEZ-GARCIA, M., SANTOS, F., PENA A., GUVEN, K., ANTON, J. (2008). Prokaryotic diversity in Tuz Lake, a hypersaline environment in Inland Turkey. *FEMS Microbiology Ecology*, *65*, 474-483, https://doi.org/10.1111/j.1574-6941.2008.00510.x

NAKAPONGA, S., PICHYANGKURAA, R., ITO, K., IIZUKAB, M., PONGSAWASDIA, P. (2013). High expression level of levansucrase from Bacillus licheniformis RN-01 and synthesis of levan nanoparticles, *International Journal of Biological Macromolecules*, *54*, 30–36, https://doi.org/10.1016/j.ijbiomac.2012.11.017

NASIR, D.Q., WAHYUNINGRUM, D., HERTADI, R. (2015). Screening and characterization of levan secreted by halophilic bacterium of Halomonas and Chromohalobacter genuses originated from bledug kuwu mud crater, *Procedia Chemistry*, *16*, 272-278, https://doi.org/10.1016/j.proche.2015.12.050

POLI, A., KAZAK, H., GÜRLEYENDAĞ, B., TOMMONARO, G., PIERETTI, G., TOKSOY ÖNER, E. NICOLAUS B. (2009). High level synthesis of levan by a novel Halomonas species growing on defined media, *Carbohydrate Polymers*, *78*, 651-657, https://doi.org/10.1016/j.carbpol.2009.05.031

POLI, A., NICOLAUS, B., DENIZCI, A.A., YAVUZTURK, B., KAZAN, D. (2013). *Halomonas smyrnensis* sp. nov., a moderately halophilic, exopolysaccharide-producing bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *63*, 10–18, https://doi.org/10.1099/ijs.0.037036-0

RADCHENKOVA, N., BOYADZHIEVA, I., ATANASOVA, N., POLI, A., FINORE, I., DIDONATO, P., NICOLAUS, B., PANCHEV, I., KUNCHEVA, M., KAMBOUROVA, M. (2018). Extracellular polymer substance synthesized by a halophilic bacterium Chromohalobacter canadensis 28, *Applied Microbiology and Biotechnology*, *102*, 4937-4949, https://doi.org/10.1007/s00253-018-8901-0

LANE, D.J., PACE, B., OLSEN, G.J., STAHL, D., SOGIN, M., PACE, N.R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses, *Proceedings of the National Academy of Sciences USA*, *82*, 6955–6959, https://doi.org/10.1073/pnas.82.20.6955

REHM, B.H. (2010). Bacterial polymers: biosynthesis, modifications and applications, *Nature Reviews Microbiology*, *8*, 578-592, https://doi.org/10.1038/nrmicro2354

SARILMISER, H.K., ATES, O., OZDEMIR, G., ARGA, K.Y., ONER, E.T. (2015). Effective stimulating factors for microbial levan production by *Halomonas smyrnensis* AAD6, *Journal of Bioscience and Bioengineering*, *119*, 455-463, https://doi.org/10.1016/j.jbiosc.2014.09.019

SEZER, A.D., KAZAK, H., TOKSOY ONER, E., AKBUĞA, J. (2011). Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristic, *Carbohydrate Polymers*, *84*, 358-363, https://doi.org/10.1016/j.carbpol.2010.11.046