LEVAN PRODUCTION POTENTIALS FROM DIFFERENT HYPERSALINE ENVIRONMENTS IN TURKEY

Hakan Çakmak¹, Pınar Aytaç Çelik²,³, Seval Çınar⁴, Emir Zafer Hossgün⁵, M. Burçin Mutla⁶, Ahmet Çabuk⁶

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

*Corresponding author: pinaraytar@gmail.com

doi: 10.15414/jmbfs.2020.10.1.61-64

ARTICLE INFO

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, EPS have increased attraction because of industrial and medical applications (Rehm, 2010). Among of microbial polysaccharides; pullulan, xanthan, dextran, levan have long been of interest owing to extraordinary characteristics (Iyer et al., 2006).

The one of significant EPSs, levan is a β(2-6-1) linked fructose homopolysaccharide that is extracellularly generated from sugar-based substrates by various microorganisms such as species of Zymomonas, Aerobacter, Erwinia, Bacillus, Acetobacter, Azotobacter, Cornomonas, Mycobacterium, Gluconobacter, Streptococcus, and Pseudomonas. Levan’s distinguishing properties including water solubility, film-forming ability, high solubility in oil, strong adhesiveness, 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, osmoregulator, food and feed additive, cryoprotector in various sectors, plasma substitute, prolongator of drug activity, source of prebiotic fibre, antitumor, osmoregulator, food and feed additive, cryoprotector in various sectors, plasma substitute, prolongator of drug activity, source of prebiotic fibre, antitumor.

Keywords: halophilic, levan, screening

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 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).

The chemical shifts of 1H-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 1H-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.

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.

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 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%, 25%, 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, K2HPO4 0.3, MgSO4.7H2O 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(AGATTTGATCATGCTCAC-3') and 1492R(5'-GTTACCTTGTAGACTTC-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
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     | Pülimir hypersaline spring water, Tunceli | 12% MGM |
| Halomonas ventosae strain T2PU  | 1306 bp         | KJ161487.1     | Pülimir hypersaline spring water, Tunceli | 12% MGM |
| Halomonas sp. T7PU              | 1309 bp         | KJ161489.1     | Pülimir 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      | KF958241.1     | Fadum saltern, Sivas    | 23% MGM |
| Halomonas alkaliphila strain 305B | 1373 bp        | KF976353.1     | Tuzlagözü saltern, Sivas | 12% MGM |
| Idiomarina sp. 30BE             | 1465 bp         | KF976287.1     | Tuzlagözü saltern, Sivas | 18% MGM |
| Halomonas elongata strain 153B  | 1395 bp         | KF668257.1     | Fadum 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 KH₂PO₄; 2 g KH₂PO₃; 1 g (NH₄)₂SO₄; 0.1 g MgSO₄·7H₂O; 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 H₂SO₄. After acid hydrolysis of levan samples, the pH of the mixture was neutralized by the addition of NaOH.

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 (1H) 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 coworkers 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 smyrnensis strain isolated from İzmir province located in the Aegean Region of Turkey (Polli et al., 2013; Ates 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.
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).

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