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Effective stimulating factors for microbial levan production by *Halomonas smyrnensis* AAD6^T

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Levan is a bioactive fructan polymer that is mainly associated with high-value applications where exceptionally high purity requirements call for well-defined cultivation conditions. In this study, microbial levan production by the halophilic extremophile *Halomonas smyrnensis* AAD6^T was investigated systematically. For this, different feeding strategies in fed-batch cultures were employed and fermentation profiles of both shaking and bioreactor cultures were analyzed. Initial carbon and nitrogen source concentrations, production pH, NaCl and nitrogen pulses, nitrogen and phosphorous limitations, trace elements and thiamine contents of the basal production medium were found to affect the levan yields at different extends. Boric acid was found to be the most effective stimulator of levan production by increasing the sucrose utilization three-fold and levan production up to five-fold. This significant improvement implied the important role of quorum sensing phenomenon and its regulatory impact on levan production mechanism. Levan produced by bioreactor cultures under conditions optimized within this study was found to retain its chemical structure. Moreover, its biocompatibility was assessed for a broad concentration range. Hence *H. smyrnensis* AAD6^T has been firmly established as an industrially important resource microorganism for high-quality levan production.

[**Key words:** Levan; Fructan; *Halomonas smyrnensis*; Exopolysaccharides; Boric acid; Quorum sensing]

Levan is a naturally occurring polymer that is composed of β-D-fructofuranose with β(2–6) linkages between fructose rings, and is synthesized by the action of a secreted levansucrase (EC 2.4.1.10) that directly converts sucrose into the polymer (1). As a homopolysaccharide with many distinguished properties like high solubility in oil and water, strong adhesivity, good biocompatibility and film-forming ability, it has great potential as a novel functional biopolymer in foods, feeds, cosmetics, pharmaceutical and chemical industries (2). In fact, a recent literature analysis on microbial exopolysaccharides attributed levan together with xanthan, curdlan and pullulan as the most promising polysaccharides for various industrial sectors (3). Levan has many potential uses as emulsifier, stabilizer and thickener, encapsulating agent, osmoregulator and cryoprotector in addition to its uses in medicine as plasma substitute, prolongator of drug activity, radio protector, antitumor and antihyperlipidemic agent (2,4,5). Recently, levan has also proven to be a promising biopolymer for laser direct writing (LDW) technologies in obtaining novel bioactive surfaces with novel functionality (6,7).

Due to its exceptionally high production costs, levan could never find its proper place in the polymer market and therefore, high-level levan producing microbial systems gain escalating industrial importance. Levan is produced as an exopolysaccharide (EPS) from sucrose-based substrates by a variety of microorganisms, including *Acetobacter*, *Aerobacter*, *Azotobacter*, *Bacillus*, *Corynebacterium*, *Erwinia*, *Gluconobacter*, *Mycobacterium*, *Pseudomonas*, *Streptococcus* and *Zymomonas*. In addition, halophilic bacterium *Halomonas* sp. has been reported as the first levan producer extremophile by our research group (8). Further research on the potential use of levan by *Halomonas* sp. as a bioflocculating agent (9), its suitability for peptide and protein based drug nanocarrier systems (5) were reported. With this microbial system, productivity levels were improved by use of cheap sucrose substitutes like molasses (10) as well as other cheap biomass resources (11) as fermentation substrate. Moreover, levan and aldehyde-activated levan were successfully deposited by matrix-assisted pulsed laser evaporation (MAPLE) resulting in uniform, homogeneous, nanostructured, biocompatible, thin films (6,7) and furthermore the feasibility of phosphonate-modified levan as adhesive multilayer films were demonstrated (12). In addition, the antioxidant potential of this levan polysaccharide in high glucose condition in the pancreatic INS-1E cells by demonstrating a correlation between reduction in oxidative stress and apoptosis with its treatment was reported for the first time by our research group (13). *In vitro* anti-cancer activity of this linear levan and its oxidized forms containing increasing amounts of aldehydes in their structure were also investigated in human lung adenocarcinoma A549, human liver hepatocellular carcinoma HepG2/C3A, human gastric adenocarcinoma AGS, and human breast adenocarcinoma MCF-7 cell lines for the first time by our research group. Anti-cancer activity was found to depend on the dose as well as on the cell type and this effect became more apparent with the increasing degree of oxidation (14). Ternary blend films of chitosan, PEO and levan were also prepared to evaluate their morphological, thermo-mechanical, surface and

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biological properties (15). Moreover, the generic metabolic model of *Halomonas smyrnensis* AAD6^T was reconstructed to elucidate the relationship between levan biosynthesis and other metabolic processes (16). Recently, the producer strain has been identified as a novel species of the genus *Halomonas* and named as *H. smyrnensis* AAD6^T (17) and its whole genome sequence has been announced (18).

In commercialization of microbial biopolymers, economic hurdles need to be overcome, especially for very expensive biopolymers like levan. Since *H. smyrnensis* AAD6^T is the only extremophile producing levan, it is to be expected that its biopolymer will also have some unique properties to adapt to extreme conditions. Therefore extensive research has been directed to combine the advantages of osmoadaptation and halophilicity to favor a cost-effective levan production process. Recently, about 10-fold increase in theoretical levan yields from *H. smyrnensis* AAD6^T cultures were reported by use of low cost substrates like sugar beet molasses and starch molasses as substitutes for sucrose (10). On the other hand, as a bioactive polymer, levan is mainly associated with high-value applications where exceptionally pure biopolymers are needed which in turn call for using good quality substrates and well-defined cultivation conditions (4). Under such defined conditions, consistency in both product quality and yield is ensured to a high extend by preventing the carryover of impurities and metabolic byproducts.

In the light of these facts, optimization studies were initiated to improve the formerly reported (8) levan yields obtained under semi-defined cultivation conditions. For this, effects of initial carbon and nitrogen source concentrations, production pH, NaCl and nitrogen pulses, nitrogen and phosphorous limitations, trace elements and thiamine contents of the basal production medium were systematically investigated. However, boric acid supplementation stood out as the most effective stimulator by increasing the levan yields up to five-fold. Chemical structure, molar-mass and branching properties of the levan polymer produced in this study were found to be identical to earlier reports. Viability tests with monkey kidney fibroblast cell line COS-7 ascertained its high biocompatibility.

MATERIALS AND METHODS

Microorganism, media and cultivation conditions Halophilic bacterium *H. smyrnensis* AAD6^T (17) isolated from Çamalti Saltern area (Aegean Region of Turkey) was used as microbial source for levan production in this study.

The basal medium consisted of 137.2 g/L NaCl; 10–300 g/L sucrose; 7 g/L K_2HPO_4; 2 g/L KH_2PO_4; 0.1 g/L $MgSO_4.7H_2O$; 1 g/L $(NH_4)_2SO_4$ and 0.5 g/L peptone. Trace element solution: $MnCl_2{\cdot}4H_2O$ 0.36 g/L, $ZnSO_4{\cdot}7H_2O$ 0.44 g/L, $FeSO_4{\cdot}7H_2O$ 2.3 g/L, $CuSO_4{\cdot}5H_2O$ 0.05 g/L was filter sterilized and added as 0.1% (v/v) to the medium. Filter sterilized thiamine solution was added at 0.8 mg/L final concentration. Boric acid solutions were autoclaved separately for 15 min and then added to final medium.

For the shake flask cultures, Certomat BS-1 orbital shaker was used with the set temperature and agitation rates of 37 °C and 180 rpm, respectively. The working volume was 100 mL in 500 mL Erlenmeyer flasks. Bioreactor cultivations were performed using BIOSTAT Q multi-bioreactor system with controlled environment of temperature (37 °C) and pH (pH 6–8). The pH was adjusted to pH 7 by using 1 M NaOH or with 1 M HCl. The working volume was 500 mL, aeration was provided at a rate of 0.1 vvm and agitation was set to 200 rpm. All the experiments were performed in duplicate.

Analytical methods Cell growth was monitored by measuring the optical densities at 660 nm using Lambda35 UV/Vis spectrophotometer (PerkinElmer, Massachusetts). Biomass concentration in terms of dry cell weight (DCW)/liter was determined gravimetrically using harvested cells which were washed with distilled water and then dried at 100 °C until constant cell dry weight was achieved. To determine the total carbohydrate concentration, levan samples were dissolved in ultra-pure distilled water (1% w/v). Carbohydrate content was determined using phenol/sulfuric acid method using glucose as standard (19). The maximum specific growth rate (μ_{max}) of the cultures was determined from the slope of the semi-log plot of biomass versus time in the exponential phase using linear regression. Protein concentration was determined by the Bradford test using Bovine Serum Albumin (BSA) as standard (20). Quantitative measurement of total uronic acid was performed by the method with glucuronic acid as the standard (21). Glucose concentrations were determined using enzymatic kits by Roche, Germany. Sucrose concentration analysis was performed by Agilent 1100 high performance liquid chromatography (HPLC) system with refractive index detector using the Zorbax Carbohydrate Analysis Column 4.6 × 250 mm (Agilent, USA). All the samples collected from fermentation broth were centrifuged at 10,000 rpm for 20 min to precipitate cells. The cell free samples were filtered through 0.2 μm filter prior to use and equal volume of 50:50 acetonitrile:water solution was added. The flow rate was 1.4 m/min, the mobile phase was 75:25 acetonitrile:water, and the temperature of the column was 30 °C.

Isolation and purification of levan Samples collected from the fermentation broth were centrifuged at 10,000 rpm for 20 min to precipitate cells. The supernatant phases containing the soluble levan polymers were treated with an equal volume of ethanol, stirred vigorously and kept overnight at −18 °C. The precipitate from the ethanol dispersion was collected by centrifugation at 12,000 rpm for 30 min, re-dissolved in hot distilled water, dialyzed against several runs of distilled water for 3 days and then lyophilized. The dry weight of the levan samples were determined and used for the yield calculations.

Characterization of levan Fourier transform-infrared (FT-IR) spectroscopy spectra of levan samples were obtained with Nicolet 6700 FT-IR Spectrometer between 400 and 4000 wave numbers (1/cm). The molecular weights of the levan samples were determined by gel permeation chromatography (GPC). The chromatography system was equipped with serial connected two columns (Shodex Protein KW-304 and Waters Ultrahydrogel DNA 7.8 × 300 mm) and Ultrahydrogel guard column, and measurements were carried out at 25 °C. The mobile phase was 0.1 M solution of sodium nitrate ($NaNO_3$) in 2% acetic acid - water mixture with a flow rate of 1.0 ml/min. The sample concentration was adjusted to 0.5–2.0 mg/ml and all the samples were filtered through 0.2 μm filter before injection to the system. Branching of polymer samples were analyzed through the confirmation plot graph which identify the linear relationship between root-mean-square radius (RMS) and molar-mass (g/mol).

***In vitro* biological activity studies** To assess the biocompatibility, WST-1 Cell Proliferation and Viability Assay (Roche Applied Science, Germany) was performed with the monkey kidney fibroblast cell line COS-7 (catalog no. CRL-1651; American Type Culture Collection, Rockville, MD). Briefly, the cells were seeded into a 96 well plate at a density of 1.5 × 10^4 cells/well in a 100 μL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) and cultured overnight in an atmosphere of 5% CO_2 at 37 °C. After 24 h incubation, the culture medium was removed and replaced with a 100 μL fresh medium containing varying concentrations of levan (dissolved in serum- and antibiotic-free medium). After 24 h of levan treatment, WST-1 reagent (10 μL) was added directly to culture wells and incubated for 2 h. The absorbance was measured at 450 nm with a GloMax Multi+ Microplate Multimode Reader (Promega, USA). Untreated cells were used as control. The control cells were considered 100% viable. Statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons using the Prism analysis program (Graphpad, V 5.0).

RESULTS AND DISCUSSION

Microbial EPS production may or may not be associated with growth, and nutrient availability is an important factor in EPS synthesis for which a significant amount of carbon and electrons are channeled by the metabolism (22). In batch cultures, polysaccharide synthesis takes place when the medium is depleted with one or more nutrients, and it is often maximal in media with a high carbon/nitrogen ratio. However, generalizations should be avoided for the fermentation conditions due to the diversity of the nutritional and environmental requirements of the EPS producer strains (23). Optimum conditions for EPS synthesis are affected by limitations of nitrogen and phosphate sources, oxygen content, pH, temperature, proportion between the air and medium volume, agitation rate, inoculum size and also nutrients in the culture medium (24). Therefore, in this study, effective stimulating factors for microbial levan production by *H. smyrnensis* AAD6^T were investigated.

Previous studies established that levan production by *H. smyrnensis* AAD6^T was growth-associated (8) and therefore optimization of the growth conditions holds primary importance in achieving maximal levan yields. Besides, a partially growth-associated EPS production trend is typical of other EPS-producing strains, including *Xanthomonas* sp. during xanthan production (4).

Moreover, 1.844 g/L levan yield from the stationary phase bioreactor cultures using a defined media containing sucrose as