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ORIGINAL PAPER

Biosynthesis of Levan by *Halomonas elongata* 153B: Optimization for Enhanced Production and Potential Biological Activities for Pharmaceutical Field

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Abstract
Halophilic organisms are a novel attractive option as cell factories for the production of industrially valuable bioproducts. *Halomonas elongata* is the cell factory of choice for ectoine production, but its levan production has not been well researched. Based on this scientific motivation, in this study, we evaluated the chemical and biological properties of levan produced by the halophilic extremophile *Halomonas elongata* 153B (HeL). First, the central composite design was used to determine the optimal process variables for maximum levan biosynthesis. Then, the levan produced from HeL was purified, quantified, and chemically characterized with FTIR, ^{1}H-NMR, and GPC analyses. This was followed by antioxidant, anti-inflammatory, antibiofilm, and antimicrobial activity tests to assess its biological activities as well as a cytotoxcity assay. Maximum levan yields of 5.13 ± 0.38 g/L were achieved after dialysis at the optimum levels of process variables. The ^{1}H-NMR spectrum of HeL revealed characteristic signals. It showed a strong antioxidant activity of 67.88% and the best radical scavenger. At a concentration of 400 µg/mL, HeL showed the most anti-inflammatory efficacy. Also, at all indicated concentrations (250, 500, 750, and 1000 µg/mL) HeL, acted against biofilms formed by *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 11778, *Candida albicans* ATCC 10231. Furthermore, HeL displayed antimicrobial activities against all strains tested. Finally, HeL showed high Cell viability in all dosages and no cytotoxicity was observed. In light of these results, HeL may have high potential in the medical, pharmaceutical and dermo-cosmetics industries.

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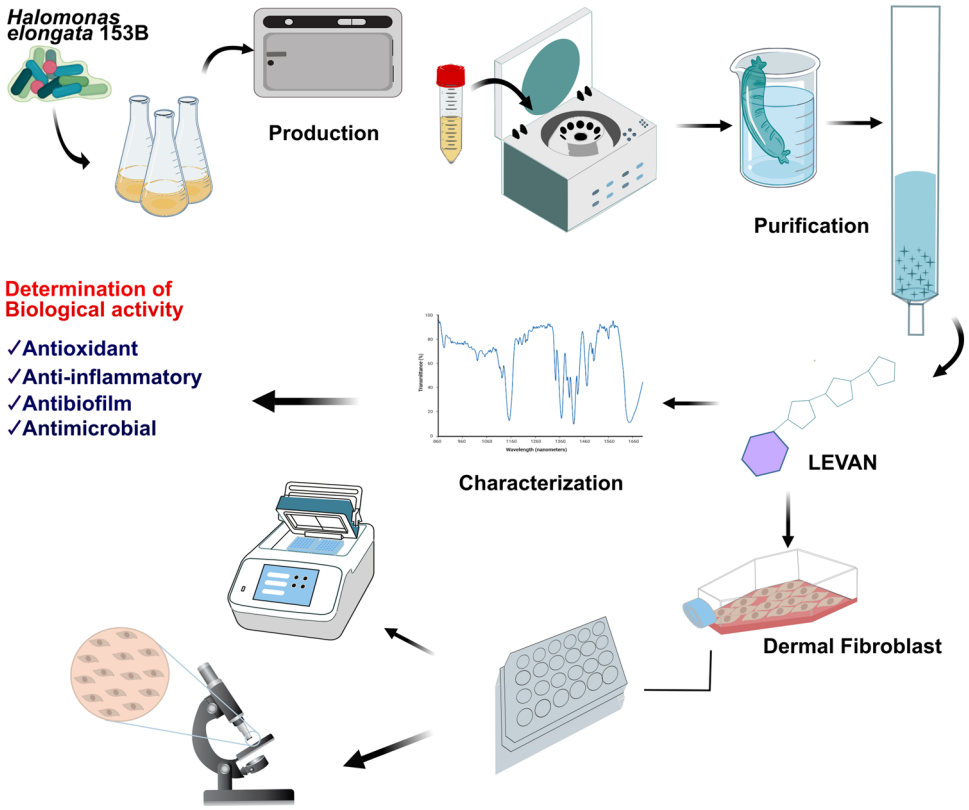
Graphical Abstract



Keywords Halophilic · Levan · *Halomonas elongata* · Response surface methodology · Biological activity · Cytotoxicity

Introduction

Today, considerable emphasis is placed on microbes isolated from uncommon and extreme settings, particularly their abilities to create a variety of useful compounds such as exopolysaccharides (EPS). Halophilic microorganisms isolated from extreme environments have specific advantages for biotechnological production use, such as their absence of pathogenicity, fast growth and readily accessible nutritional requirements, which admit them to be cultured in relatively inexpensive media with high salt content and high pH, preventing microbial contaminations. These bacteria are also natural producers of exoenzymes, exopolysaccharides, and else industrially useful compounds [1–4].

Levan, one of the most important EPSs, is formed of fructose molecules with a glucose residue at the end linked by β-2,6 glycosidic linkages and is generated with the process of a secreted levansucrase (EC 2.4.1.10) that directly transforms sucrose into the polymer [5] by microorganisms such as *Zymomonas* [6, 7] *Erwinia* [8] *Bacillus* [9] *Lactobacillus* [10] *Acetobacter* [11] *Gluconobacter* [12] *Streptococcus* [13] and *Pseudomonas* [14, 15]. Levan distinguishes itself from other water-soluble, biocompatible, and film-forming biopolymers due to an unusual mix of properties such as low intrinsic viscosity, health benefits, high adhesive strength, and the capacity to gel. Thanks to these unique features, it was applied to many application areas. Some of the research related to medical applications has been on levan-based thin films to heal damaged tissue. For the example, the case of levan-phosphonate and chitosan combined layer by layer to form films on glass slides. These modified slides were determined to have a remarkable coat shear strength [16]. Also, levan polymer utilized with matrix-assisted pulsed laser evaporation (MAPLE) was used to make films for drug delivery, microsensors and tissue regeneration [17]. As a prebiotic, levan can promote proliferation of beneficial bacteria in the colon and inhibit harmful microorganisms. Several studies have revealed various roles of levan or levan-type fructooligosaccharides on probiotic bacteria and complex intestinal microbiota [18–21]. In a study, the effect of levan was tested in rats fed a diet rich in cholesterol and dissolved levan was added to the drinking water of test animals. In levan-treated rats, increased HDL cholesterol levels and lower LDL cholesterol levels demonstrated the

beneficial effect [22]. It is of great importance to reveal the bioactive properties of biopolymers as well as their chemical characterization. In this way, it is possible to have a general idea about their potential applications. For example, the ability of a biopolymer to inhibit or eradicate a biofilm allows its use in different areas. Biofilm formation is a crucial step in the pathogenesis of many subacute and chronic bacterial infections. Eradication of biofilm is difficult with conventional antimicrobial agents as they have several antimicrobial resistance mechanisms [58]. To fight resistance to common antibiotics, new approaches to prevent bacterial growth and biofilm formation are needed. Natural antimicrobial substances, such as biopolymers, are therefore relevant alternatives for use as surface coatings to prevent and inhibit biofilm formation of bacteria due to their more efficient mechanisms. Even though it has been known for a long time, new efforts to link its distinctive features with high-value applications have reignited interest in this polysaccharide, making it a scientific and industrial focus [23]. Levan is a natural polymer with a variety of characteristics and high-value industrial applications. However, owing to the low productivities of the cost-effective deployed microbial systems, this polymer is not yet cost-effective. Integrative techniques, such as the simultaneous creation of high-value microbial products from low-cost substrates, are an essential cost-cutting alternative production optimization tool in this context [24]. However, multiple factors such as agitation, sterilization, aeration, temperature maintenance, incubation time and substrate cost, etc. lead to the high production cost of bioprocessing of various compounds. Bioprocess optimization can be time-consuming and challenging in general. To design tests, create models, and anticipate the best conditions that be concluded a certain response, statistical methods such as response surface methodology (RSM) are commonly utilized. RSM is generally used in biotechnology to improve fermentation medium conditions and other process parameters that are critical for the generation of a variety of microbial metabolites [25–28]. This approach has been used to successfully improve the yield of microbial polysaccharides as well as pullulan from *Aureobasidium pullulans* [29, 30] glucan from *Leuconostoc dextranicum* [31] or levan from *Zymomonas mobilis* [6, 7, 32] and *Pseudomonas fluorescens* [14]. Species belonging to *Halomonas* genus are gram-negative, aerobic, moderately halophilic bacteria thrives at saline and hypersaline environments and also that synthesize high-value products such as levan, polyhydroxybutyrates (PHB) and ectoin etc. [33].

The synthesis of levan by a halophilic bacterium, *Halomonas elongata* 153B, isolated from a hypersaline environment, was examined in this work. In the study conducted by our group, the abilities of 12 halophilic strains to produce levan were screened and it was shown that the *H. elongata* 153B used in our study produced the high-yield levan [34]. This study was planned with the intent of optimizing the levan production of *H. elongata* 153B. The effects of main parameters such as initial sucrose concentration, agitation rate, and incubation time on levan production were evaluated with Central Composite design, which is an RSM method. Furthermore, biological activities such as antioxidant, anti-inflammatory, antimicrobial and antibiofilm effects of levan produced under optimum conditions with halophilic isolate were determined. This study firstly reports levan production by *H. elongata* 153B and its important biological activities and structural characterization.

Materials and Methods

Bacterial Strain, Culture Medium, and Cultivation Conditions

The microorganism for levan production used in this study was a halophilic bacterium *H. elongata* 153B (KF668257.1) isolated from Fadlum saltern (Sivas, Turkey) [35]. Production medium (pH 7) for levan was composed of (per liter): 1 g $(NH_4)_2SO_4$; 137.2 g NaCl; 0.1 g $MgSO_4{\cdot}7H_2O$; 7 g K_2HPO_4; 2 g KH_2PO_4; 0.32 g; 0.5 g peptone; 50 g sucrose [36]. Sterilization was performed at 110 °C for 25 min. The flasks were inoculated and then incubated for 72 h at 37 °C at 180 rpm with a working capacity of 50 mL.

Analytical Methods

Biomass Determination

A UV/Vis spectrophotometer (Thermo Scientific Multiskan Sky) was used to evaluate microbial cell development at 600 nm wavelength. The concentration of biomass from the point of dry cell weight (DCW) was specified using gravimetric methods. To do so, collected fermentation broth samples were centrifuged at 10,000 rpm for 15 min to separate cells, and the recovered cells were then washed twice with distilled water. The washed cells were then dispersed in distilled water, change to pre-weighed containers, and air-dried at 50 °C in a laboratory oven until a constant cell dry weight was attained.

Isolation and Purification of Levan

To separate cells, collected samples from the fermentation broth were centrifuged at 10,000 rpm for 15 min. Then, an equivalent volume of ice-cold ethanol to the cell-free supernatant containing soluble forms of levan was added. The solution was kept in at 20 °C overnight and centrifuged for 15 min at 10,000 rpm. The polymer pellets were then dissolved in hot dH_2O and dialyzed for 3 days at

4 °C against numerous runs of distilled water to remove salt and other contaminants (by dialysis tubing, cellulose membrane, molecular weight cutoff = 14.000 Da). Further purification of dialyzed polymer solution to remove residual proteins was performed by passing with a DEAE-Sepharose CL-6B weak anion-exchange column and then lyophilized. The dry weight of the levan sample was stored for further experiments.

Residual Sugar Determination and Quantification of Levan

The remaining supernatant, after the extraction of levan and sucrose used in fermentation from the fermentation medium was analyzed using the phenol–sulfuric acid method to determine the total residual sugar content (glucose, fructose, and sucrose) [37]. A spectrophotometer (Thermo Scientific Genesys 10S UV–VIS) was used to measure absorbance at 490 nm, and the total sugar content was evaluated using the standard sucrose calibration curve ($R^2 = 0.99$).

The levan solution obtained after dialysis was mixed with 0.1 N HCl at a ratio of 1:1 in capped glass tubes. These tubes were incubated in a water bath at 100 °C for 1 h, allowing the levan to be completely hydrolyzed to fructose [38]. The fructose concentration in the hydrolyzed samples was equal to the levan concentration and was determined by the dinitrosalicylic acid (DNS) method [39]. Absorbance was measured at 540 nm, and the amount of levan was determined using the standard fructose calibration curve ($R^2 = 0.99$).

Experimental Design and Statistical Analysis

Design Expert Statistical Software (Release 13.0.1.0) was used to do the statistical analysis of the data. For levan production, central composite design (CCD) was used to optimize the three almost important variables (sucrose concentration, agitation rate, and incubation period). Literature research guided the amounts of the factors that were used [5, 40]. The levels of the process variables were initial sucrose concentration 75–150 g/L, agitation rate 100–200 rpm, and incubation time 24–72 h. For the examination of three components at three levels, twenty trials were done using a face central composite statistical design ($\alpha = 1$). The equation given below was used to calculate the coded value of the actual level of each factor [41]. All tests were repeated three times, with the mean levan yield (g/L) serving as the response (Y). The data were subjected to quadratic regression analysis to develop an empirical model that links the measured response to the independent variables. The following second-order polynomial equation was used to define the system' s behavior.

$$coded\ value = \frac{actual\ level - (high\ level + low\ level)/2}{(high\ level - low\ level)/2}$$

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3,$$

where X_1, X_2, and X_3 represent the levels of the factors and β_0, β_1, …, β_{23} represents coefficient estimates with β_0 being the constant of the equation. By the analyzing the regression equation and examining the response surface contour plots, the optimal amounts of all three independent variables were established. A 3D Surface was also built to allow visual examination of the trend of maximum responses as well as the interactive impacts of the significant variables on the response.

Chemical Characterization

The structural integrity of the levan polymer was investigated by Fourier Transform-Infrared Spectroscopy (FTIR-Perkin Elmer Spectrum Two) in the range of 400–4000 wavenumbers (cm^{-1}). ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) spectra of the obtained pure levan polymer were collected on a JEOL ECZ 500R spectrometer at the standard probe temperature. The operational frequencies for the ^{1}H nucleus were 500.13 MHz. The molecular weights of the levan polymer were measured using multi-angle laser light scattering–gel permeation chromatography (MALLS–GPC), as detailed in [42].

Biological Activities of *H. elongata* 153B Levan

Biological activity tests are a guide in understanding whether biopolymers have a potential in different industries such as pharmaceuticals or cosmetics. In this context, in vitro antioxidant activity test, in vitro anti-inflammatory activity test, in vitro antibiofilm activity test (inhibition potential of biofilm formation and eradication potential of biofilm formation), in vitro antimicrobial activity test, in vitro cytotoxicity assay were performed on HeL polymer.

The antioxidant activity of HeL was measured in vitro by DPPH radical scavenging using a modified approach published by Domżał-Kędzia et al. [43]. Purple-colored free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is known to react with antioxidant compounds to form a colorless product of 1,1-diphenyl-2-picrylhydarzine (from dark purple to light yellow) and the reaction was analyzed by absorbance decrease in 517 nm [44]. DPPH was dissolved in methanol to obtain a 1 mM concentration. HeL test solutions were prepared by dissolving in water and diluted in methanol to obtain (serial dilution between 500 and 25 µg/mL). Each well received a 50 µL mM DPPH solution and was incubated

for 30 min in the darkness room temperature. A mixture of DPPH and methanol was used as control, mixtures of samples and methanol were used as blank and ascorbic acid in similar concentrations with samples used as a positive control. Inhibition (%) was calculated using the following equation (Eq. 1).

$$Inhibition(\%) = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} \times 100 \quad (1)$$

Anti-inflammatory activity of HeL was determined by the modified inhibition of the protein denaturation method [45, 46]. Varying concentrations of levan solution were combined with BSA solution and the pH of the reaction media was set to 6.3 with 1 N HCl. After cooling, 2.5 mL of PBS was added, and the samples were incubated at 37 °C for 30 min and 57 °C for 3 min. The absorbances of samples were measured at 416 nm and inhibition (%) was calculated using the following Eq. 2, diclofenac sodium as a standard.

$$Inhibition(\%) = 100 - \frac{Absorbance\ of\ the\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (2)$$

Antibiofilm activity of HeL was determined with minimum biofilm eradication concentration (MBEC) and minimum biofilm inhibition concentration (MBIC) using test microorganisms such as *Pseudomonas aeruginosa* ATCC 11778, *Candida albicans* ATCC 10231, *Bacillus subtilis* NRS-744, *Staphylococcus aureus* ATCC 6538, *Klebsiella pneumoniae* NRRLB 4420 and *Escherichia coli* ATCC 25922, *Enterococcus feacalis* ATCC 51289.

The effect of HeL on biofilm formation of each representative strain, was analyzed using the modified microdilution method of [47, 48]. In a 96-well microtiter plate with a flat bottom, diluted quantities of HeL ranging from 250 to 1000 µg/mL were prepared. As positive, non-treated, and blank controls were utilized penicillin-fluconazole antibiotics, phosphate-buffered saline, and medium alone, respectively. Except in the well with medium alone, an equal volume of the tested microorganisms (1×10^6 CFU/mL) was added and mixed with the agents (the blank control). Behind a 24 h incubation at 37 °C, supernatants were discarded, and three times washed with phosphate-buffered saline. A 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium-bromide (MTT) test was used to measure biofilm development. The number of bacteria that survived was investigated by taking into account their ability to convert a yellow tetrazolium salt to a purple formazan product at 570 nm. The OD readings suggested a high number of microorganisms surviving in the biofilm and Eq. 3 was used to calculate the percentage of inhibition. The biofilm inhibition concentration ($MBIC_{50}$) was determined as the concentrations that inhibited biofilm formation by 50%.

$$Biofilm\ inbition(\%) = \left[1 - \left(Abs570_{sample} - Abs570_{non-treated\ control}\right)\right] \times 100 \quad (3)$$

The antibiofilm activity of HeL in the biofilm was also analysed using the minimum biofilm eradication concentration (MBEC) assay [47, 48]. Briefly, 200 µL (1×10^6 CFU/mL) of each strain, was inoculated into each well of the flatbottom 96-well microtiter plate and incubated for 24 h under proper circumstances at 37 °C. For biofilm formation, the medium was then stained and the well attentively washed three times with sterile phosphate-buffered saline to cast out non-adherent cells. HeL was then added to the biofilms in dilutions serially from 250 to 1000 µg/mL and incubated for 24 h in appropriate conditions at 37 °C. At the end-point of the treatment of the biofilms with HeL, the adherent bacteria were washed three times with sterile phosphate-buffered saline. The numbers of surviving bacteria were established by an MTT assay. The amount of sample that could eradicate at least 50% of the biofilm formation can be considered as minimum biofilm eradication concentration ($MBEC_{50}$). Penicillin-fluconazole, phosphate-buffered saline, and the medium alone were used as the positive, nontreated, and blank controls, respectively. The percentage eradication was calculated using Eq. 3.

Antimicrobial activity of HeL was determined against *Listeria monocytogenes* ATCC 1911, *S. aureus* ATCC 25923, *K. pneumoniaea* NRRLB 4420, *P. aeruginosa* ATCC 11778, *E. feacalis* ATCC 51289, *E. coli* ATCC 35218, *B. subtilis* NRS-744, *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600 and *C. albicans* ATCC 10231. The Clinical and Laboratory Standards Institute (CLSI) disc diffusion assay for test microorganism was employed [49]. Bacteria in Mueller Hinton broth at 37 °C until turbidity equal to 0.5 Mc Farland, *Candida* sp. in Sabouraud Dextrose Broth at 30 °C were incubated for 24 h. Sterile discs on which 10 µL of levan solution is dropped in increasing concentrations were placed in the inoculated Petri dishes by taking 0.1 mL of the culture of appropriate turbidity and spreading it on Mueller Hinton Agar and Sabouraud Dextrose Agar solid media. Inhibition zone diameters (mm) formed after incubation were measured and evaluated in comparison with the control group. Penicillin G (10 mg/mL), amikacin (30 mg/mL), and fluconazole (10 mg/mL) as positive controls; dH_2O were used as negative control.

In vitro cytotoxicity of purified HeL was tested with Human Dermal Fibroblast Cell Line (PCS-201-012) at different concentrations (0, 50, 100, 250, 500, 750 and 1000 µg/mL) for 24, 48 and 72 h. The viability of the cells was explored with an MTT assay [50]. Concisely, cells at the 70–80% confluency were trypsinized and seeded onto a 96-well plate at the cell/well density of 1×10^4. Following overnight attachment, the medium (DMEM complete with 10% FBS and 1% penicillin–streptomycin) (Sigma-Aldrich®, Germany) was chanced with an experimental medium that contained HeL incubated for 24, 48, and 72 h

at 37 °C in humidified air containing 5% CO_2. Following the incubation period, the MTT(1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan, Thiazolyl blue formazan (Sigma-Aldrich®, Germany) reagent was mixed to the wells and incubated for 2 h at 37 °C in 5% CO_2. Following incubation, the MTT reagent was withdrawn from the cells, DMSO was added to each well, and the plate was shaken at room temperature for 5 min at a low speed. Absorbance values were measured with Gen5 Biotek Microplate Reader (BioTek, Epoch, ABD) at a wavelength of 570 nm and cell viability was calculated. Untreated cells (cells in wells with no sample other than DMEM medium) were utilized as a control and were determined to be 100% viable. All studies were carried out in triplicate.

All data were expressed as mean ± SD and in vitro cytotoxicity test data were statistically analyzed using GraphPad Prism 9 by One-Way ANOVA and Tukey' s multiple comparisons tests. The data were presented as a mean with a 95% confidence interval (CI). An p-value of less than 0.05 was deemed statistically significant.

Result and Discussion

Optimization by Response Surface Methodology

The RSM was utilized to identify the optimum levels of the process parameters; initial sucrose concentration, incubation period, and agitation rate. As is known from the literature, these parameters had significant effects on levan production by halophilic bacteria [5, 51, 52]. The amounts of the process variables determined by single-factor tests were as follows: initial sucrose concentration 75–150 g/L, agitation rate 100–200 rpm, and incubation time 24–72 h. Levan yield was measured after each fermentation, and the findings are shown in Table 1. ANOVA was used to statistically analyze the RSM data based on levan yield (Table 2). The study establishes the model' s utility and the need for a more complicated model with a better match. Table 2 showed that R^2 was 0.98, suggesting that the fitted model described 98 percent of the variability in maximum levan production. The F test for regression was significant at 5% ($p < 0.05$), showing that the model can clarify the variation in maximal levan production with the planned levels of the components. As shown in Table 2, the lack of fit (0.13) was not significant at the 5% level ($p > 0.05$), indicating that the experimental data fit the model well. Regression analysis of the experimental data

Table 1 Central composite design matrix with experimental response

Run	Sucrose concentration (g/L)	Agitation rate (rpm)	Incubation time (h)	Levan yield (g/L)	Biomass (g/50 mL)	Residual sugar (g/L)
1	112.5	200	48	4.87 ± 0.00	0.20 ± 0.03	45.32 ± 0.06
2	150	200	72	1.01 ± 0.00	0.62 ± 0.04	57.99 ± 0.04
3	112.5	150	48	3.59 ± 0.01	0.37 ± 0.02	43.54 ± 0.00
4	112.5	100	48	2.99 ± 0.01	0.11 ± 0.01	40.78 ± 0.02
5	75	100	72	4.53 ± 0.00	0.12 ± 0.01	6.83 ± 0.04
6	150	100	24	0.34 ± 0.04	0.03 ± 0.00	103.03 ± 0.00
7	112.5	150	72	2.98 ± 0.00	0.63 ± 0.08	20.40 ± 0.01
8	112.5	150	48	3.14 ± 0.00	0.54 ± 0.02	41.48 ± 0.01
9	112.5	150	48	3.62 ± 0.01	0.53 ± 0.01	42.65 ± 0.02
10	75	200	24	3.86 ± 0.02	0.14 ± 0.04	28.04 ± 0.00
11	112.5	150	48	3.38 ± 0.00	0.59 ± 0.04	44.80 ± 0.02
12	75	200	72	5.35 ± 0.01	0.39 ± 0.01	12.06 ± 0.00
13	112.5	150	24	1.84 ± 0.02	0.05 ± 0.05	65.54 ± 0.01
14	112.5	150	48	3.32 ± 0.02	0.45 ± 0.08	44.85 ± 0.03
15	150	150	48	1.03 ± 0.01	0.87 ± 0.01	82.83 ± 0.01
16	75	100	24	1.72 ± 0.04	0.49 ± 0.02	24.35 ± 0.04
17	150	200	24	1.05 ± 0.06	0.13 ± 0.00	99.03 ± 0.03
18	150	100	72	1.07 ± 0.04	0.07 ± 0.02	57.95 ± 0.04
19	75	150	48	3.97 ± 0.02	0.78 ± 0.08	7.82 ± 0.00
20	112.5	150	48	3.36 ± 0.04	0.55 ± 0.06	40.64 ± 0.02

Table 2 Analysis of variance (ANOVA) for central composite design

Source	DF[a]	SS[b]	MS[c]	F	p
MODEL (quadratic)	9	38.52	4.28	67.52	< 0.0001
Sucrose concentration	1	22.29	22.29	351.67	< 0.0001
Agitation rate	1	3.01	3.01	47.55	< 0.0001
Incubation time	1	3.76	3.76	59.28	< 0.0001
Sucrose concentration*Agitation rate	1	0.67	0.67	10.52	0.0088
Sucrose concentration*Incubation time	1	1.63	1.63	25.70	0.0005
Agitation rate*Incubation time	1	0.55	0.55	8.61	0.0149
Sucrose concentration*Sucrose concentration	1	1.68	1.68	26.52	0.0004
Agitation rate*Agitation rate	1	1.16	1.16	18.23	0.0016
Incubation time*Incubation time	1	2.09	2.09	32.90	0.0002
Residual error	10	0.63	0.06		
Lack of fit	5	0.47	0.09	2.97	0.1301
Pure error	5	0.16	0.03		
Total	19	39.15			

[a]Degress of freedom

[b]Sum of squares

[c]Mean square

revealed that agitation rate and incubation period had positive linear impacts on maximal levan generation, whereas beginning sucrose content had a negative linear influence ($p < 0.05$) (Table 3). Probability (p) values were utilized to assess the importance of each of the coefficients; the lower the p-value, the stronger the association with the corresponding coefficient. Incubation time had the greatest impact on levan production among the three independent factors evaluated, as indicated by the highest linear coefficient (0.61), followed by agitation rate (0.55). The initial sucrose concentration factor had a significant negative quadratic effect on levan production, meaning that as the level of these parameters enhanced, levan production increased and reduced when the level of these parameters increased over specified values. The interaction between initial sucrose concentration and the agitation rate was found to be significant, whereas the interactions between initial sucrose concentration and incubation duration were determined to be inconsequential, as indicated by p values greater than 0.05. As a result, these inconsequential terms were removed from the model' s polynomial equation. The results of the CCD design were fitted in a second-order polynomial equation using quadratic regression analysis (ANOVA) on the experimental data:

$$Y = +3.35 - 1.49X_1 + 0.55X_2 + 0.61X_3 - 0.78X_1^2 + 0.47X_2^2 - 0.87X_3^2 - 0.29X_1X_2 - 0.45X_1X_3 - 0.26X_2X_3$$

To investigate the impact of factors and their interactions on levan yield, response surfaces were plotted using Design Expert Statistical Software. Figures 1, 2, 3 show the effects of initial sucrose concentration, agitation rate, and incubation duration on levan formation. 3D response surface plots were created to provide a better understanding

Table 3 Estimated regression coefficients for levan yield

Factors	Coefficient estimate	df	Standard error	p value
Intercept	3.35	1	0.08	
Sucrose concentration	− 1.49	1	0.08	< 0.0001
Agitation rate	0.55	1	0.08	< 0.0001
Incubation time	0.61	1	0.08	< 0.0001
Sucrose concentration*Agitation rate	− 0.29	1	0.09	0.0088
Sucrose concentration*Incubation time	− 0.45	1	0.09	0.0005
Agitation rate*Incubation time	− 0.26	1	0.09	0.0149
Sucrose concentration*Sucrose concentration	− 0.78	1	0.15	0.0004
Agitation rate*Agitation rate	0.47	1	0.15	0.0016
Incubation time*Incubation time	− 0.87	1	0.15	0.0002

of the interplay between the aforementioned components. Each response surface plot displayed the function of any two parameters, with the third element set to zero. The shape of the corresponding contour plots reflects whether or not the independent variables reciprocal interactions are significant. The contour plots' elliptical shape shows that the interactions between the independent variables are substantial. The greatest value anticipated by the surface was contained within the contour diagram' s smallest ellipse [41]. Figure 1 shows a 3D figure (a) and its associated contour plot (b) demonstrating the effects of initial sucrose concentration and agitation rate on maximal levan production while the incubation time was held constant at its middle value (48 h). Figure 2 shows a 3D figure (a) and its associated contour plot (b) demonstrating the effects of initial sucrose concentration and incubation time on maximum levan generation while the agitation rate was held constant at its middle level (150 rpm). Figure 3 shows a 3D plot (a) and its associated contour plot (a) demonstrating the effects of agitation rate and incubation duration on maximal levan

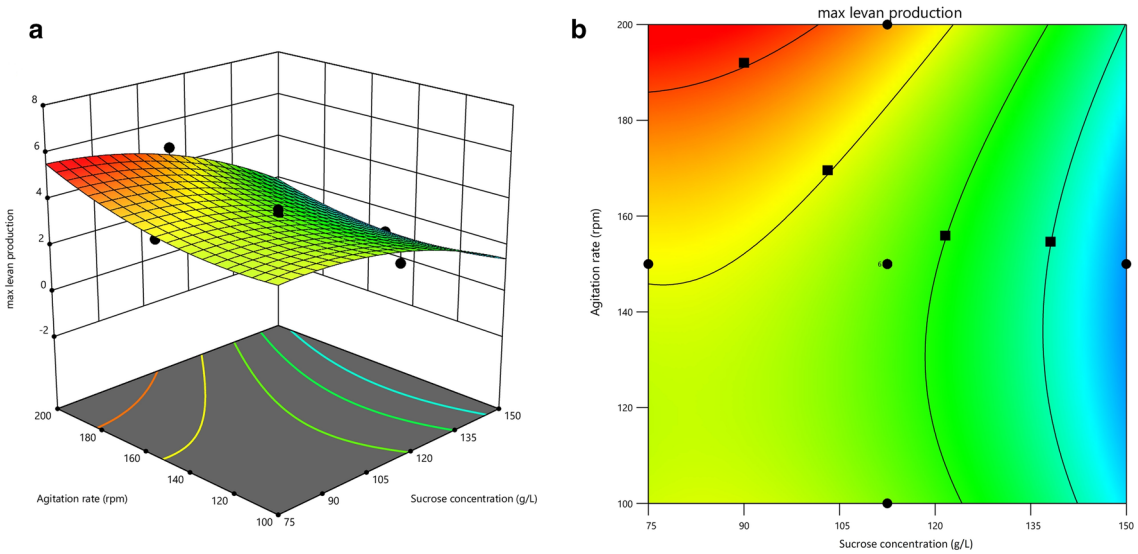


Fig. 1 Response surface plot (**a**) and contour plot (**b**) of the combined effects of initial sucrose concentration and agitation rate on max levan yield from *H. elongata* 153B under constant incubation time

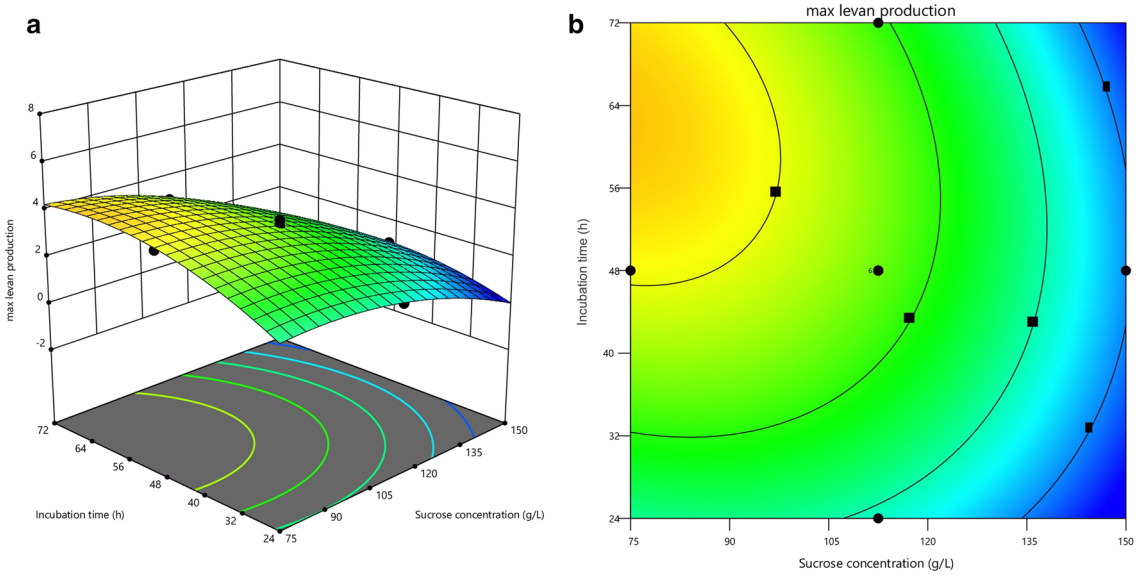


Fig. 2 Response surface plot (**a**) and contour plot (**b**) of the combined effects of initial sucrose concentration and incubation time on max levan yield from *H. elongata* 153B under constant agitation rate

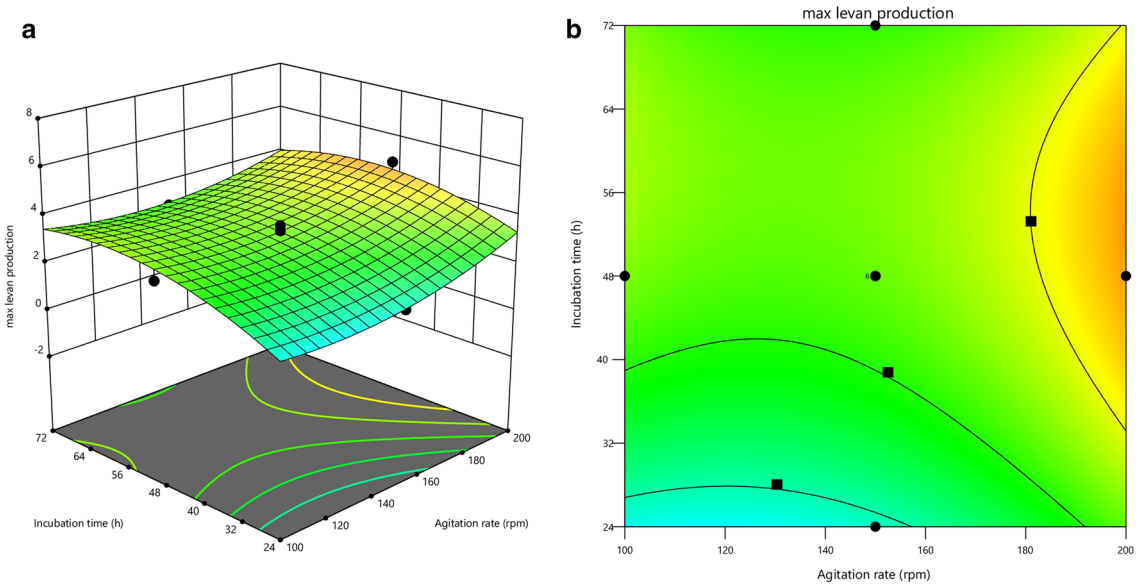


Fig. 3 Response surface plot (**a**) and contour plot (**b**) of the combined effects of agitation rate and incubation time on max levan yield from *H. elongata* 153B under constant initial sucrose concentration

production while the initial sucrose concentration was held constant at 112.5 g/L. A second-order polynomial model was used to calculate the values of these variables to find the maximal levan production corresponding to the optimum levels of initial sucrose concentration, agitation rate, and incubation period. The fitting of the experimental data to Eq. (3) allowed the levels of initial sucrose concentration ($X_1 = 75$ g/L), agitation rate ($X_2 = 200$ rpm) and incubation period ($X_3 = 72$ h) to be determined, yielding a maximum levan production of 5.13 ± 0.38 g/L.

The model equation' s suitability for predicting the optimum response value was tested using RSM-based optimum conditions (75 g/L, 200 rpm and 72 h). When the independent variables' optimum values were incorporated into the regression equation, maximum levan production was predicted as 5.48 g/L, whereas experiments at optimum conditions yielded 5.13 ± 0.38 g/L. As a result, the predicted value from the fitted equation and the observed value agreed well. The study conducted by Permatasari et al. [52] reported that levan yield 188 g/L produced by halophilic bacterium *Bacillus licheniformis* BK2. Such a high yield was obtained because the levan was measured after ethanol precipitation without dialysis. However, in our study, the levan yield is the value obtained after dialysis following ethanol precipitation, and possible impurities were removed, it is the amount of partially purified levan. In particular, the purity of biopolymers to be used in pharmaceutical and medical studies should be high and efficiency should be evaluated within this scope.

According to Kekez et al. [2], high levan yield was obtained for various sucrose concentrations (60–400 g/L), but using such a large amount of carbon sources at an industrial scale will not be feasible. Levan yield was 47.60 g/L in low sucrose concentration (60–200 g/L) and 99.20 g/L in the high sucrose concentration (300–400 g/L). In our study, the sucrose concentration, which was found to be 75 g/L after the optimization study, is considered reasonable from this point of view.

In the study conducted by Kucukasik et al. [36] after dialysis the levan production yield of the halophilic bacterial strain *Halomonas* sp. AAD6 was determined as 12.40 g/L.

Chemical Characterization

The chemical structure and associated properties of microbial EPSs are known to be highly influenced by process parameters and growth medium composition [53]. In well-designed EPS studies, biochemical characterization of the final product should be made after the approach to increase production efficiency through optimization of the fermentation medium. Several characterization experiments were also performed on levan polymer synthesized under optimal conditions in this work.

For the chemical characterization of HeL, FT-IR analysis was carried out. Figure 4 depicts the distinctive peaks of HeL and commercial levan produced by *Erwinia herbicola* (Sigma-Aldrich L8647). The spectra revealed a similarity between the sample and pure and commercial levan, confirming the biopolymer' s identity as a levan-type polysaccharide. The spectrum of experimental levan sample was well-matched with the characteristic peaks

for commercial levan FT-IR spectra. The existence of fructose residue was observed from the spectrum at the bands around 3400 cm^{-1}, 2900 and 2950 cm^{-1} were the vibrations of OH stretching of fructofuranose residues and $–CH_2OH$ group and C–H stretching of fructofuranose residues, respectively. The new peak formation around 1554 cm^{-1} in the IR spectrum of *H. elongata* 153B levan can be explained by N–O stretching. C–H vibrations combined with aromatic skeletal vibrations produced bands in the 1430–1200 cm^{-1} range. Glycosidic linkage of C–O–C vibrations of fructofuranose rings and glycosidic linkages at 1120–1020 and 950 cm^{-1} were reported by Poli et al. [40], Erkorkmaz et al. [42], Erginer et al. [54]. Besides the FT-IR analysis, the chemical shifts were obtained and presented as ppm. Figure 5 shows proton NMR spectra characteristic peaks of HeL and commercial levan produced by *Erwinia herbicola* (Sigma-Aldrich L8647). The ^{1}H-NMR spectrum of HeL revealed characteristic signals. In the ^{1}H-NMR spectrum, all of the proton signals were seen between 3.37 and 4.77 ppm. Proton signals from *E. herbicola* levan were detected in the ^{1}H-NMR spectrum between 3.38 and 4.66 ppm. The chemical shifts of proton NMR spectra of the HeL also signified a high similarity to those of *E. herbicola* levan. The purified levan had the weighted average molecular weight (M_w) of $3.26 \times 10^6 \pm 0.01$ g/mol, the number average molecular

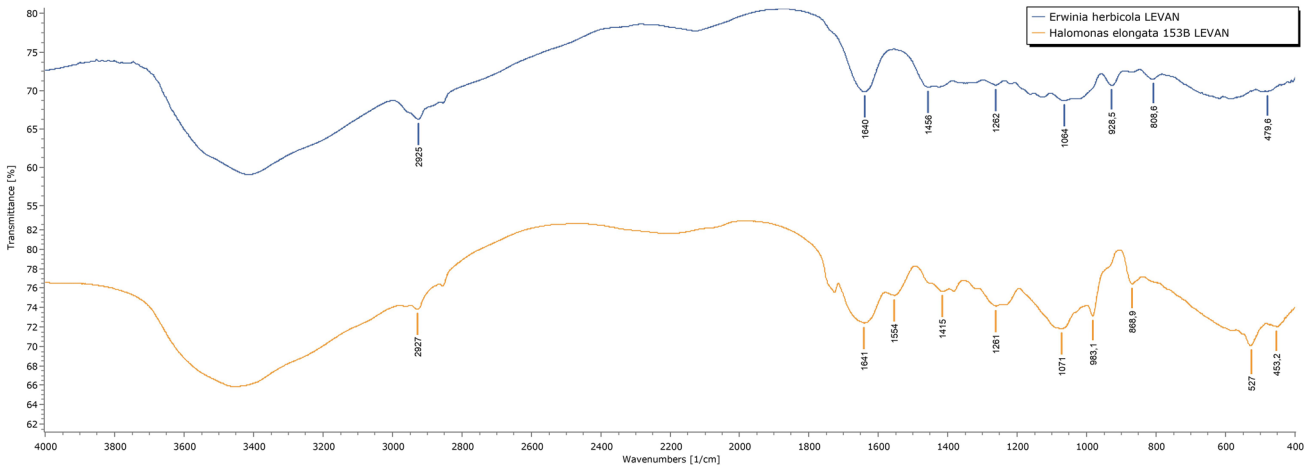


Fig. 4 FT-IR spectra of *H. elongata* 153B levan and commercial *Erwinia herbicola* levan

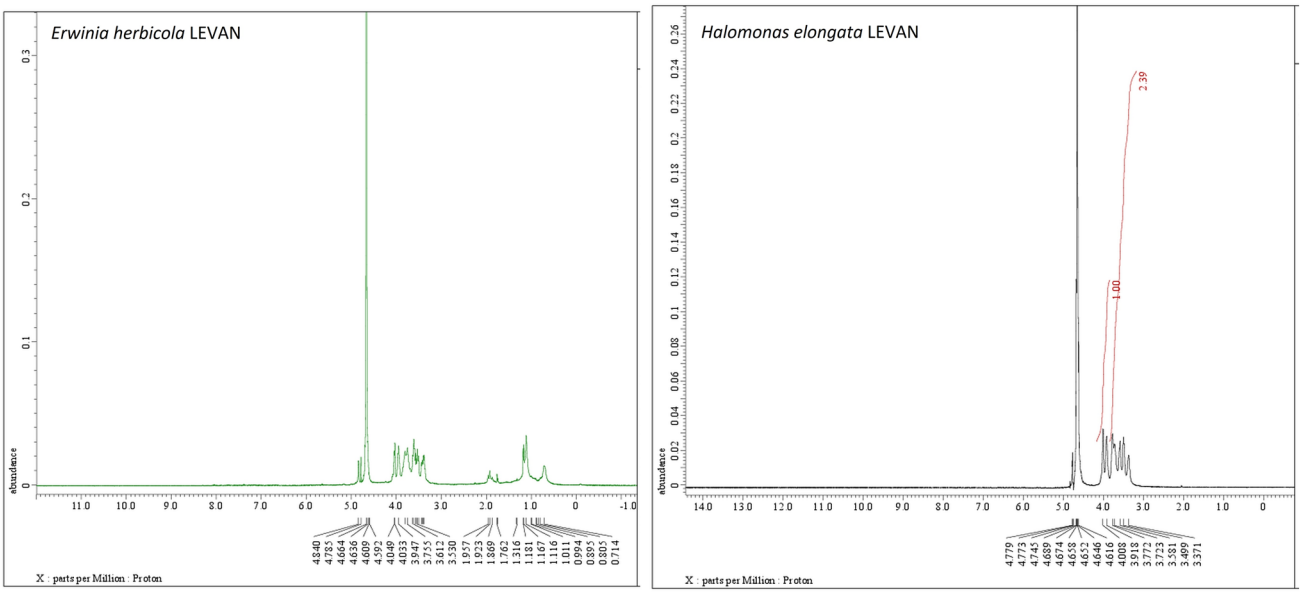


Fig. 5 NMR spectra of commercial levan from *Erwinia herbicola* and *H. elongata* 153B levan

weight (M_n) $1.86 \times 10^6 \pm 0.01$ g/mol, and polydispersity (M_w/M_n) values were found to be 1.76 ± 0.01. These results agree with previous studies by Kazak-Sarilmiser et al. [5] and Poli et al. [40] where the molecular size of levan was reported as greater. According to Kazak-Sarilmiser et al. [5] the weighted average molecular weight (M_w), number average molecular weight (M_n), and polydispersity (M_w/M_n) values for levan recovered from boric acid containing medium produced by *Halomonas smyrnensis* AAD6T were $1.48 \times 10^6 \pm 0.1$ g/mol, $6.35 \times 10^5 \pm 0.1$ g/mol, and 2.34 ± 0.2, respectively, whereas for levan recovered from boric acid-free medium, those values were $9.86 \times 10^6 \pm 0.1$ g/mol, $9.13 \times 10^6 \pm 0.1$ g/mol and 1.08 ± 0.1 in respective order. Poli et al. [40] reported that levan produced by *H. smyrnensis* $AAD6^T$ the weight average molecular weight (M_w) was found to be $1 \times 10^6 \pm 0.1$ g/mol.

Biological Activities of *H. elongata* 153B Levan

In Vitro Antioxidant Activity

In vitro scavenging activity of HeL was evaluated using a non-enzymatic DPPH assay. DPPH scavenging activity of HeL was determined using optical color change of DPPH after being incubated with samples at dark for 30 min and % scavenging activity was calculated according to formula shared in the materials methods section. Concentration-dependent % inhibition results were shared in Fig. 6. Experiments were performed in triplicate and results were shared as mean ± SD.

Ascorbic acid, evaluated as a control, showed antioxidant activity between 85 and 140% for doses with the same concentration as the sample. The antioxidant activity of HeL varies between 19.67 and 67.88%. At 200 µg/mL concentration, HeL showed a strong antioxidant activity of 67.88% and the best radical scavenger.

HeL showed stronger antioxidant activity at a lower concentration than levan produced from *Bacillus velezensis* VTX20 [55], *Bacillus subtilis* AF17 [56], at the same concentration as from *Bacillus licheniformis* [57] and *B. subtilis* natto KB1 [43].

In Vitro Anti-inflammatory Activity

Inhibition of protein denaturation was used to test the anti-inflammatory efficacy of HeL. Figure 7 shows the percent inhibition findings as a function of concentration. At concentrations of 50–250 µg/mL, the normal medicine Diclofenac sodium exhibited 53.48–99.82% anti-inflammatory activity, while the HeL showed 38.41–82.67% anti-inflammatory activity at 50–400 µg/mL. At a concentration of 400 µg/mL, the HeL showed the most anti-inflammatory efficacy. Mummaleti et al. [46] found that levan generated by *B. subtilis* had anti-inflammatory action of 64.23% and 100% at 300 µg/mL and 500 µg/mL, respectively. Anti-inflammatory compounds are in high demand in the pharmaceutical sector, and levan generated by *H. elongata* 153B was discovered to exhibit considerable anti-inflammatory action in this investigation.

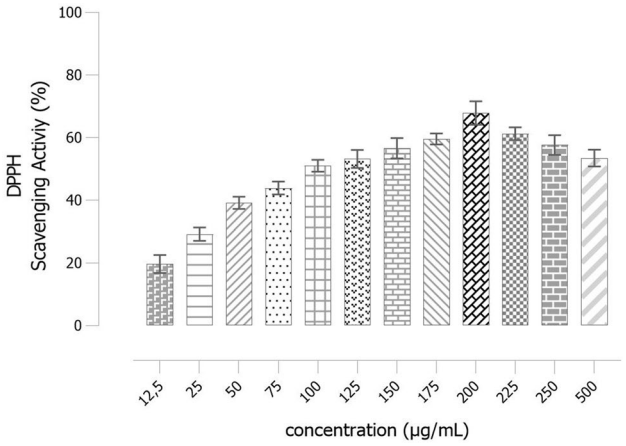


Fig. 6 Dose dependent DPPH scavenging activity (%) results of *H. elongata* 153B levan against ascorbic acid

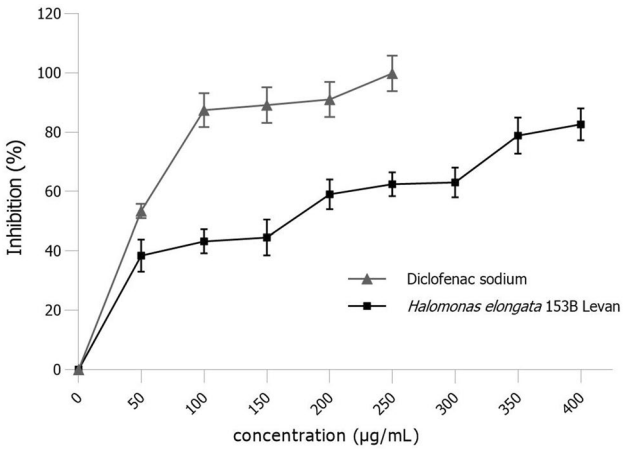


Fig. 7 Anti-inflammatory activity results of *H. elongata* 153B levan and standard Diclofenac sodium

In-Vitro Antibiofilm Activity

The inhibitory effects of levan concentrations on biofilm formation by *E. coli* ATCC 25922, *P. aeruginosa* ATCC 11778, *S. aureus* ATCC 6538, *C. albicans* ATCC 10231, *B. subtilis* NRS-744, *K. pneumoniae* NRRLB 4420, *E. faecalis* ATCC 51289 were investigated using the modified microdilution method (Fig. 8). HeL was determined to inhibit

biofilm formations at all concentrations of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 11778, *S. aureus* ATCC6538, *C. albicans* 10231, especially for *E. coli* ATCC 25922, *P. aeruginosa* ATCC 11778 and $MBIC_{50}$ is 750 µg/mL for *S. aureus* ATCC 6538.

The eradication effects of levan concentrations on biofilm formation by *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 11778, *C. albicans* ATCC 10231, *B. subtilis* NRS-744, *K. pneumoniae* NRRLB 4420, *E. faecalis* ATCC 51289 were investigated using the minimum biofilm eradication concentration (MBEC) assay. HeL, at all indicated concentrations (250, 500, 750, and 1000 µg/mL) acts against biofilms formed by *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 11778, *C. albicans* ATCC 10231. Low concentrations of levan do not affect biofilms *by B. subtilis* NRS-744, *K. pneumoniae* NRRLB 4420 and *E. faecalis* ATCC 51289, it destroys 8.14–13.68% depending on the increase in concentration (Fig. 9). $MBEC_{50}$ value was determined as 1000 µg/mL for *P. aeruginosa* ATCC 11,778 and *S. aureus* ATCC 6538. The biofilm eradication effect of HeL effect against microorganisms is shown in Fig. 9.

In-Vitro Antimicrobial Activity

The synthesized levan biopolymer by *H. elongata* 153B displayed antimicrobial activities against *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 2592, *K. pneumoniae* NRRLB 4420, *P. aeruginosa* ATCC 11778, *E. faecalis* ATCC 51289, *E. coli* ATCC 35218, *B. subtilis* NRS-744, *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600 and *C. albicans* ATCC 10231 as shown in Table 4. The maximum zone of inhibition was observed on *E. coli* ATCC 35218 of 1000 µg/mL. Previous research has found that levan has antibacterial efficacy against pathogenic bacteria such as *Escherichia* sp., *Staphylococcus* sp. and *Pseudomonas* sp. [15, 46, 57].

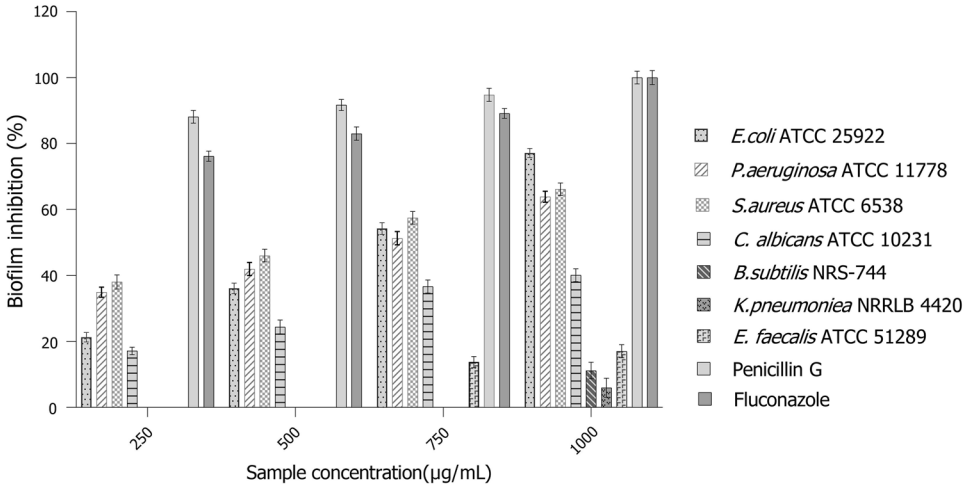


Fig. 8 Biofilm inhibition (%) results of *H. elongata* 153B levan and Penicillin G and Fluconazole (positive control)

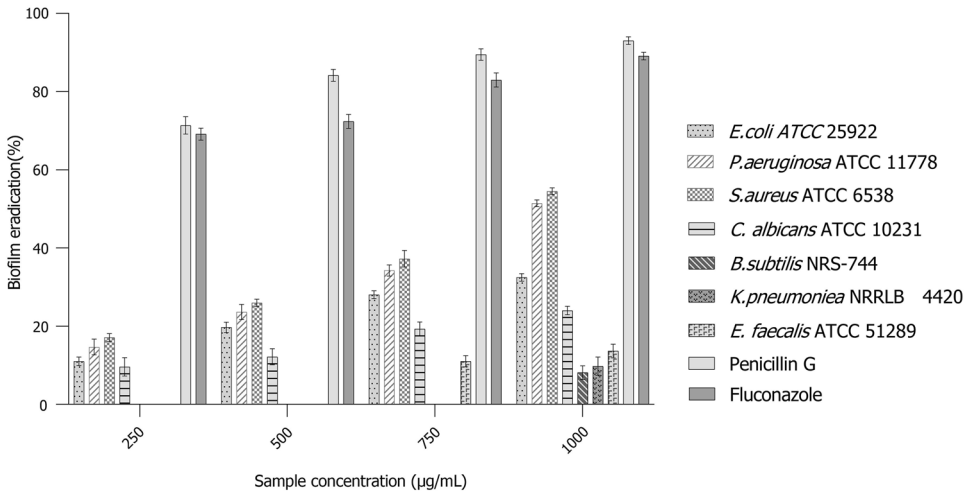


Fig. 9 Biofilm eradication (%) results of biofilm formation of by *H. elongata* 153B levan and Penicillin G and Fluconazole (positive control)

In Vitro Cytotoxicity Effects

Cultures of primary or immortalized human skin cells from epidermis or dermis such as keratinocytes and fibroblast are used generally for evaluation of bioactive ingredients or compounds and biopolymers via in vitro assays including viability, toxicity, and biocompatibility tests [58, 59]. The aim of this study was to examine the proliferative effect of levan polymer on monolayer cultures of cells from the epidermis and dermis of human skin. Figure 10 shows the cell viability of human dermal fibroblast cells after 24, 48, and 72 h of treatment with pure levan biopolymer generated by *H. elongata* 153B. In order to find an answer to the question of whether there is a time-dependent variation of the cytotoxic effect, a wide time interval was evaluated. Whereby MTT cell proliferation analysis at first 24 and 48 h for all doses showed higher viability than control and dose of 750 µg/mL showed the highest viability with the value of 111% for 24 h while the highest dose (750 µg/mL) showed the highest viability (116.6%) at the end of 48 h and 100 µg/mL showed the highest value at 72 h (103.2%). Cell viability was high in all dosages of the material, and no cytotoxicity was observed. These findings are consistent with those of Kim et al. [60] who discovered that levan from *Z. mobilis* and *B. subtilis* natto KB1 has no cytotoxicity in human fibroblast cell lines [43].

Table 4 Antimicrobial activity results of *H. elongata* 153B levan

Microorganisms	Inhibition zone (mm)							
	Sample concentration (µg/mL)					Controls		
	100	250	500	750	1000	PC_1	PC_2	NC
L.monocytogenes ATCC 19115	–	–	–	–	–	23	14	–
S. aureus ATCC 25923	–	9	10	11	13	30	12	–
K. pneumoniea NRRLB 4420	–	–	–	6	7	29	15	–
P. aeruginosa ATCC 11778	8	9	11	12	13	30	15	–
E. faecalis ATCC 51289	–	–	–	–	–	23	15	–
E. coli ATCC 35218	8	9	10	12	15	32	12	–
B. subtilis NRS-744	–	–	–	–	–	31	20	–
S. aureus ATCC 6538	9	10	12	13	14	43	16	–
E. coli ATCC 25922	–	–	8	11	14	30	16	–
P. aeruginosa ATCC 27853	–	–	8	10	11	22	14	–
S. aureus ATCC 12600	8	9	11	13	14	43	15	–
C. albicans ATCC 10231	–	–	9	10	12	30	–	–

Note: For *C. albicans* ATCC 10231 PC_1: Fluconazole (10 mg/mL) NC: H_2O; For other test microorganisms PC_1: Penicillin G (10 mg/mL) PC_2: Amikacin (30 mg/mL) NC: H_2O

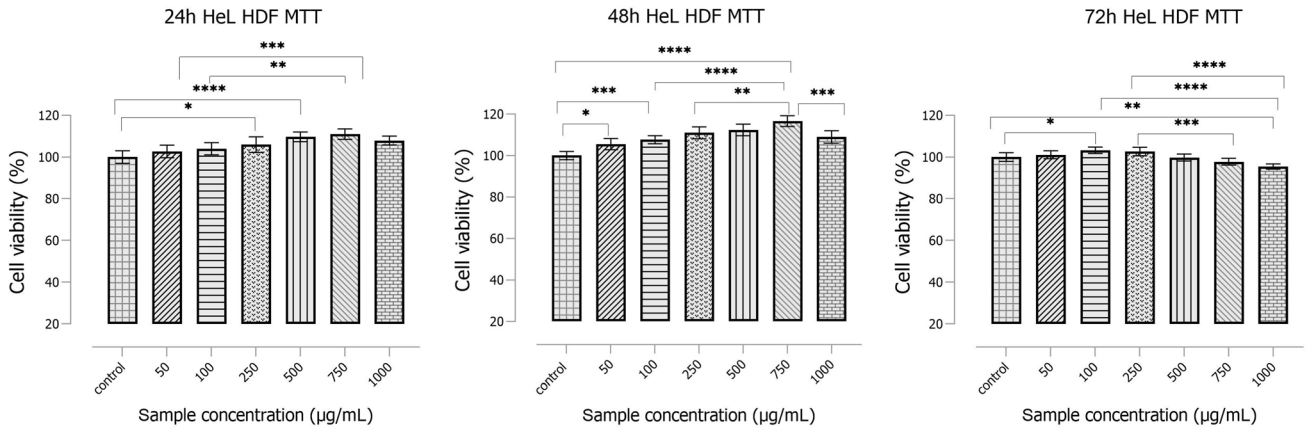


Fig. 10 Viability results of PCS-201-012 (Human Dermal Fibroblast Cell line) cells after being cultivated with *H. elongata* 153B levan

Conclusion

This is the first initiative to optimize *H. elongata* 153B fermentation parameters for maximum levan production employing Response Surface Methodology and characterize this biopolymer obtained. Levan was generated in batch culture using a synthetic medium containing sucrose from *H. elongata* 153B cells. Response surface methods were used to determine the linear and quadratic effects of these variables on levan production, and the model adopted satiably clarified the effects of the process variables. Using a second-order polynomial equation to match the experimental data, the optimum levels of initial sucrose concentration ($X_1 = 75$ g/L), agitation rate ($X_2 = 200$ rpm), and incubation time ($X_3 = 72$ h) were defined. The maximum levan production at the optimum experimental process parameters was 5.13 ± 0.38 g/L. Effective biological activities such as antioxidant, anti-inflammatory, antibiofilm, and antimicrobial effects of HeL for biomedical and pharmaceutical applications were revealed. It has also been found that this biopolymer is a biocompatible and does not have a toxic effect. Further studies will be concentrated the use of bioreactors for continuous production of the levan and its use in biomedical and pharmaceutical applications.

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Declarations

Competing Interests The authors have not disclosed any competing interests.

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