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Enhanced production and immunomodulatory activity of levan from the acetic acid bacterium, *Tanticharoenia sakaeratensis*

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ABSTRACT

Levan is a fructose polymer with β-(2 → 6) glycosidic linkages. It is produced by several microorganisms, and due to its potential biotechnological and industrial applications, various levan-producing bacteria with different levels of production efficiencies have been reported. We investigated the levan-producing ability of the acetic acid bacterium, *Tanticharoenia sakaeratensis*. The exopolysaccharides produced by the bacterium under a sucrose environment were characterized as levan by FT-IR, and ^{1}H and ^{13}C NMR. The molecular weight of levan thus produced range from 1.0×10^{5}–6.8×10^{5} Da. The maximum yield of levan from *T. sakaeratensis* is 24.7 $g \cdot L^{-1}$ in a liquid medium containing 20% (w/v) sucrose and incubated at 37 °C, 250 RPM for 35 h. The levan produced by *T. sakaeratensis* can promote nitric oxide production in RAW264.7 macrophage cells in a concentration-dependent manner, suggesting it has immunomodulatory effects. Our study reveals that *T. sakaeratensis* can be potentially employed as a new source of levan for industrial applications.

1. Introduction

Exopolysaccharides (EPS) are a type of polymer synthesized and secreted by various microorganisms. Well-known examples of bacterial EPS that have been used in the food and drug industries are levan, dextran, curdlan, and hyaluronan. Levan is a fructose homopolymer naturally produced by certain plants and microorganisms. Microorganisms use levan to enhance survival under stress conditions and it can also be utilized for energy storage under starvation conditions [1]. The degree of polymerization of microbial levan varies from 40 to more than 100,000 units, which is higher than that of plant-derived levan [2]. Levan is synthesized by levansucrase (E.C.2.4.1.10) that catalyzes hydrolytic and transfructosylation activities, using sucrose as its substrate. Thus far, levan displays several interesting biological activities, including antioxidant, anti-inflammatory, and anti-cholesterol activities. It has also been shown to display biocompatibility, bioadhesivity, low intrinsic viscosity, and low toxicity [1,3]. In addition, levan-based nanostructured systems for drug delivery, biomedical, and foods industries have gained considerable attention [4]. Levan is, therefore, a polymer with high potential for applications in various industrial sectors. As a consequence, searching for new levan-producing bacterial strains is a constant undertaking.

Acetic acid bacteria are gram negative, strictly aerobic, with ellipsoidal to rod shapes. They belong to the family *Acetobacteraceae* and their major characteristic is their ability to oxidize alcohols, sugars, and sugar alcohols to acid products. Several acetic acid bacteria have been recognized for their ability to secrete levan extracellularly in a sucrose-based environment. Examples include *Asaia bogorensis* [5], *Kozakia baliensis* DSM14400, *Neoasaia chiangmaiensis* NBRC101099, *Gluconobacter frateurii* TMW2.767 [6], *Gluconacetobacter xylinus* I-2281 [7], and *Gluconacetobacter diazotrophicus* [8]. Among these strains, *K. baliensis* produces markedly high molecular weights of levan [6].

In this study, we explored the levan-producing ability of *Tanticharoenia sakaeratensis*, a bacterium isolated from soil collected in Thailand and recently characterized as a novel, osmotolerant acetic acid bacterium by Yukphan et al. [9]. We characterized the structure and molecular weight of levan from *T. sakaeratensis* and determined the cultural conditions required to obtain its maximum yield. Fermentation time, agitation rate, and sucrose concentration were optimized by varying one factor at a time. Moreover, the effect of levan from *T. sakaeratensis* on nitric oxide (NO) production in RAW264.7 macrophages cells was examined for possible immunomodulatory activity.

2. Materials and methods

2.1. Microorganism, cell culture, and chemical reagents

T. sakaeratensis was obtained from the Thailand Bioresource Research Center (TBRC; cat#TBRC22). Nutrient broth (NB) and nutrient

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agar (NA) were purchased from HiMedia (India). Sucrose was purchased from Sigma-Aldrich (U.S.A.). All the other reagents used in this study, unless otherwise indicated, were purchased from Carlo Erba (France).

For cell culture, fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco-Invitrogen (U.S.A.). Sodium nitrite, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-(1-naphthyl)-ethylenediamine, lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4), and sulfanilamide were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (U.K.) and polymyxin B sulfate was purchased from Fluka BioChemika (Switzerland).

2.2. Bacterial cultivation for EPS production

T. sakaeratensis was grown on NA (HiMedia) (5 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ NaCl, 1.5 $g \cdot L^{-1}$ meat extract, and 1.5 $g \cdot L^{-1}$ yeast extract, and 15 $g \cdot L^{-1}$ agar) at 37 °C for 24 h. A single colony was then inoculated into NB (5 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ NaCl, 1.5 $g \cdot L^{-1}$ meat extract, and 1.5 $g \cdot L^{-1}$ yeast extract) and further grown in an incubator shaker at 37 °C, 180 RPM for 24 h. Afterwards, 5% (v/v) of bacterial culture was transferred into NB medium containing 10% (w/v) sucrose and grown for another 60 h. The supernatant was collected by centrifugation at 3500 ×*g* for 20 min and then used for the succeeding experiments.

2.3. Isolation and purification of EPS from T. sakaeratensis

The EPS isolation method was modified from Srikanth et al. [3]. In Brief, the supernatant was boiled to evaporate half of its volume and then centrifuged at 12,000 ×*g* for 60 min. The pH of the supernatant was adjusted to 10 with 1 M potassium hydroxide and then mixed with two volumes of cold absolute ethanol. The mixture was kept at −20 °C overnight. The precipitated EPS was collected by centrifugation at 3500 ×*g* for 20 min and then washed twice with 80% (v/v) ethanol. The EPS pellet was resuspended in water and dialyzed (MW cut off: 14 kDa, diameter 33 mm) against distilled water at 4 °C for 3 days. The concentrated EPS was then lyophilized and stored at 4 °C.

2.4. Structural identification of EPS from T. sakaeratensis

2.4.1. Detection EPS from T. sakaeratensis by TLC

EPS produced from *T. sakaeratensis* was detected by TLC silica gel 60 F254 (Merck Millipore) with a solvent system consisting of 1-butanol: acetic acid: water (3:3:2, v/v/v). The plate was sprayed with an orcinol-sulfuric acid solution (20 mg orcinol dissolved in 8 mL of absolute ethanol and 1 mL of H_2SO_4) and subsequently heated at 120 °C for 10 min [10]. To visualize ketose-containing polysaccharides, the TLC plate was sprayed with a urea-HCl solution (0.5 g of urea dissolved in 4 mL of 4 M HCl and 4 mL of absolute ethanol) [11]. EPS was also treated with 1 M hydrochloric acid at 100 °C for 20 min to determine its monosaccharide composition.

2.4.2. FT-IR and ^{1}H and ^{13}C NMR spectroscopy

The functional groups that make up levan structure were identified using an ATR-FTIR spectrometer (PerkinElmer-Frontier) operating at 250 scans per sample at a wave number range of 4000–400 cm^{-1} and a resolution of 4 cm^{-1}. For NMR analysis, the ^{1}H and ^{13}C NMR of D_2O-dissolved samples were measured with a Bruker AVANCE Ultrashield 400 MHz spectrometer.

2.4.3. Molecular weight determination

The average molecular weight (MW) of levan from *T. sakaeratensis* was analyzed by gel permeation chromatography (GPC) using a Waters 600 system controller and pump equipped with the TSKgel series of columns and a refractive index (RI) detector (Waters model 2414). The column was eluted with ultrapure water at a flow rate of 1 mL/min at 30 °C. Different MWs of a pullulan standard (0.18, 0.5, 6.2, 10, 48.8, 113, 348 and 805 kDa) were used to construct a calibration curve.

2.5. Optimization of levan production from T. sakaeratensis

To achieve high levan productivity, various fermentative factors, including incubation time, and sucrose concentration were studied. All experiments were performed in triplicate, unless otherwise indicated. Data are presented as mean ± S.D. (standard deviation).

2.5.1. Effect of fermentation time

A single colony of *T. sakaeratensis* was inoculated into NB medium without sucrose and grown for 24 h. Afterwards, 5% (v/v) of inoculated cultures were transferred into 50 mL of NB medium containing 10% (w/v) sucrose in 250-mL Erlenmeyer flasks and grown in an incubator shaker at 37 °C, 180 RPM for the following fermentation times: 0, 20, 28, 35, 43, 52, 72, and 92 h. Bacterial culture samples were collected at different time points, centrifuged, and the cell pellets were dried at 70 °C to determine their dry biomass weight ($g \cdot L^{-1}$). To estimate the yield of levan, the supernatant was boiled to evaporate half of its volume and subsequently centrifuged at 10,000 ×*g* for 40 min. The pH of the supernatant was adjusted to 10 with 1 M potassium hydroxide, and then added with two volumes of cold absolute ethanol and kept at −20 °C overnight to precipitate levan. The levan pellet was collected, dialyzed against water, and lyophilized.

2.5.2. Effect of agitation rate

The cultures were inoculated in NB medium containing 10% (w/v) sucrose and then grown in an incubator shaker at 37 °C with the following agitation rates: 100, 150, 180, 200, and 250 RPM for suitable fermentation time. Cells were collected and the amount of biomass and levan production was measured as described above.

2.5.3. Effect of sucrose concentration

The cultures were inoculated in NB medium containing 5%, 10%, 15%, 20%, 25%, 30%, and 40% (w/v) sucrose. Bacterial cells were grown in an incubator shaker at 37 °C with the optimum agitation rate and fermentation time. Cells were collected and then biomass and levan production were measured as described above.

2.6. Cell culture

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC), U.S.A. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, and incubated in a humidified incubator at 37 °C with 5% CO_2.

2.6.1. Measurement of cell viability by the MTT assay

The MTT assay was performed as previously described [12] to determine the cytotoxicity of levan against RAW264.7 cells. Briefly, RAW264.7 macrophage cells were cultured in a 24-well plate in triplicate at a density of 1.5×10^5 cells per well, and then incubated at 37 °C for 18 h. The cells were subsequently treated with phenol red-free DMEM containing 0–1,000 μg/mL of levan with and without 5 μg/mL of polymyxin B for 24 h. Then, the medium was removed and 0.1 mg/mL of MTT solution was added into each well and further incubated for 2 h. After MTT removal, DMSO was added to dissolve violet formazan crystals in each well. The formazan solution was transferred to a microplate and the absorbance was measured at a wavelength of 550 nm using a microplate reader. All experiments were performed at

least 3 times with triplicate samples. The percentage of cell viability was calculated using the equation shown below:

$$\text{Cell viability}\ (\%) = (A_{sample}/A_{control}) \times 100$$

where A_{sample} and $A_{control}$ refer to absorbance values of sample and untreated cells, respectively.

2.6.2. Determination of nitric oxide (NO) production

Nitrite concentrations were determined by the Griess reaction and used to indirectly measure NO production as previously described [12]. A total of 1.5×10^5 cells were plated into each well of a 24-well plate and incubated at 37 °C for 18 h. The cells were treated with phenol red-free DMEM containing 0–1,000 μg/mL of levan from *T. sakaeratensis* with and without 5 μg/mL of polymyxin B for 24 h. The conditioned medium was then centrifuged at 13,700 ×*g* for 5 min at 4 °C. Afterwards, 100 μL of supernatant was mixed with 100 μL of Griess reagent to determine the amount of nitrite. The absorbance was measured at 546 nm using a microplate reader. Different concentrations of sodium nitrite (0–50 μM) were used to generate a standard curve and LPS was used as a positive control. All experiments were performed at least 3 times with triplicate samples. The percentage of NO production was calculated using the following equation:

$$\text{Nitric oxide production}\ (\%) = (\text{Nitrite}_{sample}/\text{Nitrite}_{control}) \times 100$$

where Nitrite_{sample} and $\text{Nitrite}_{control}$ refer to the amount of nitrite in sample and untreated cells, respectively.

2.7. Statistical analysis

Data are presented as mean ± S.D. (standard deviation). Differences between means were analyzed by one-way ANOVA with post-hoc Tukey HSD using GraphPad Prism 5. NS, not significantly different; *, **, and *** represent significant differences at *P* values of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results and discussion

3.1. Detection EPS from T. sakaeratensis by TLC

The results of TLC analysis of EPS from *T. sakaeratensis* are shown in Fig. 1. As seen in lane 9 of Fig. 1(A), EPS was detected at the origin of the TLC plate sprayed with orcinol-H_2SO_4. The other TLC plate shown in Fig. 1(B) was sprayed with a urea-HCl solution to specifically detect ketose-containing sugars [11]. The blue-gray spot of fructose, sucrose, standard levan, and EPS were detected as seen in lanes 3, 5, 7, and 9. Furthermore, after acid hydrolysis, the standard levan and EPS spots disappeared while the intensity of the fructose spot increased as shown in lanes 8 and 10 of Fig. 1(B). These results suggest that EPS from *T. sakaeratensis* is a fructan. It is noted that the spot of monosaccharide from HCl hydrolysis at high temperature developed at a lower position compared to the untreated monosaccharide (lane 1 vs 2, lane 3 vs 4). This is possibly due to disparity in structure between the product from acid treatment and the original form of aldose or ketose. Moreover, two spots were detected in fructose-containing samples after acid hydrolysis (lane 4, 6, 8 and 10) while only a single spot found in HCl treated-glucose (lane 2). The second spot in fructose-containing samples might be a side product generated from ketose degradation in acidic condition [13]. Since dehydration of ketose to furan derivative occurs faster than that of aldose, it is possible that less side product observed from glucose (aldose) dehydration. However, this assumption requires further investigation.

3.2. FT-IR spectroscopy

The structure of EPS produced by *T. sakaeratensis* was further analyzed by FT-IR. Fig. 2 reveals the functional groups in fructan. A strong

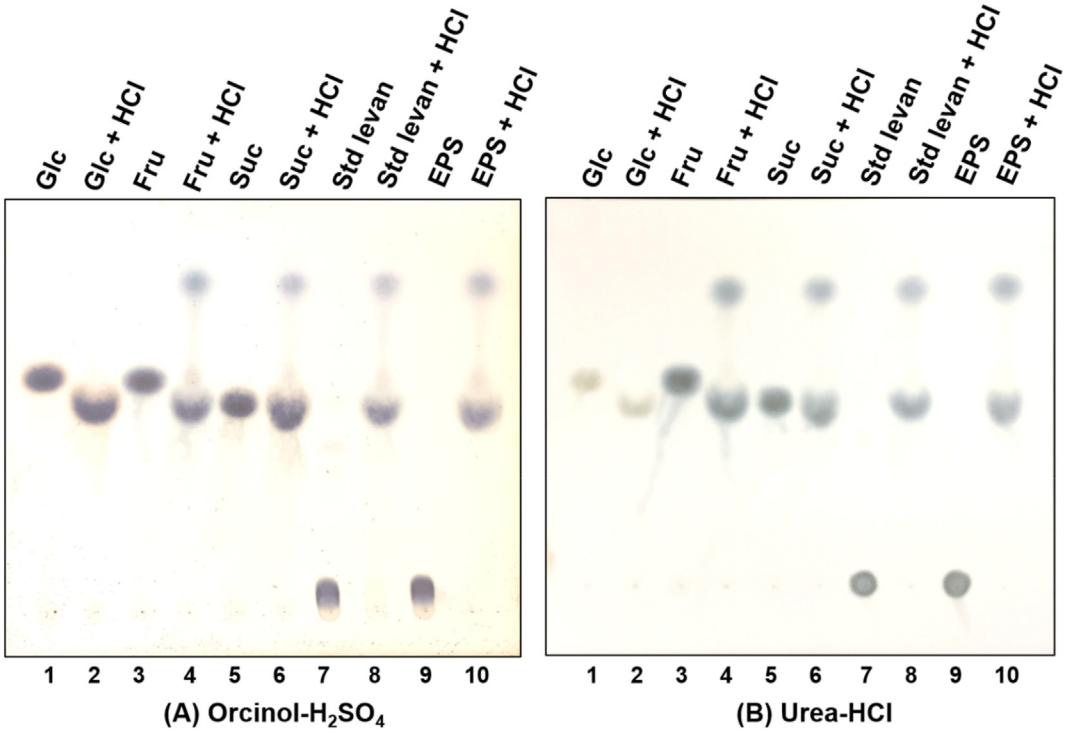

Fig. 1. TLC analysis of EPS from *T. sakaeratensis*. TLC plates were stained with orcinol-sulfuric acid (A) and urea-HCl (B). Lanes 1–6: 2 μg of standard sugars: glucose (glc), fructose (fru) and sucrose (suc) with and without acid hydrolysis; Lanes 7–8: 2 μg of standard levan from *Z. mobilis* with and without acid hydrolysis; Lanes 9–10: 2 μg of EPS from *T. sakaeratensis* with and without acid hydrolysis.

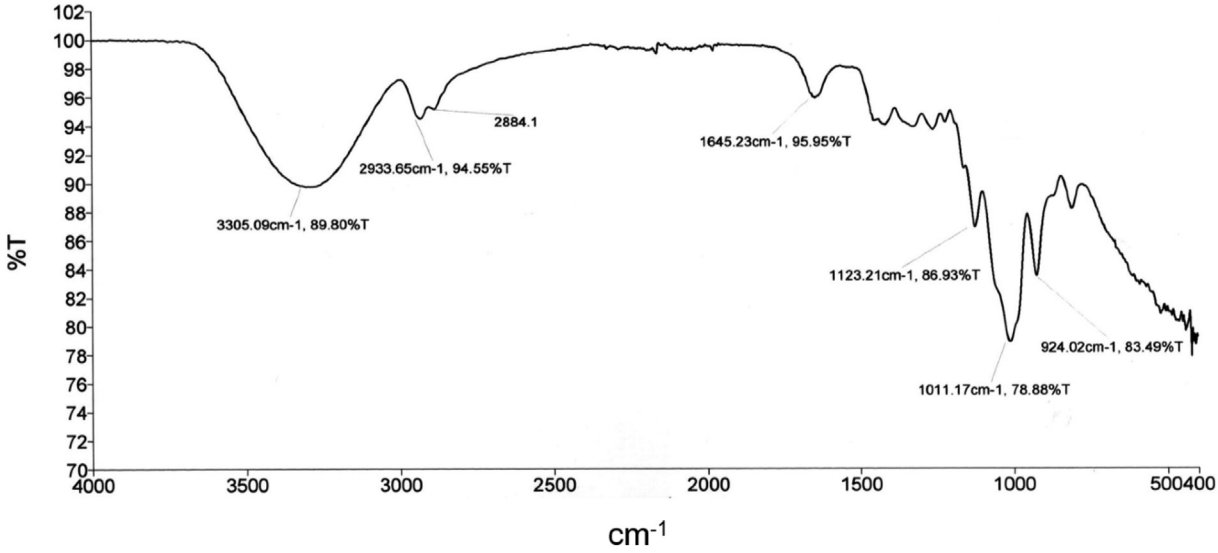

Fig. 2. The FT-IR spectrum of EPS produced from *T. sakaeratensis*.

band of O—H stretching is present at 3305 cm^{-1}, and C—H stretching vibrations are at approximately 2884 and 2933 cm^{-1}. These results suggest the existence of a fructose molecule [14]. Moreover, the band at approximately 1645 cm^{-1} indicates the C═O stretching vibration [3]. The stretching vibrations from glycosidic linkages (C—O—C) and the C—OH side groups are also observed between 1123 and 1011 cm^{-1} [15,16]. The band at approximately 924 cm^{-1} exhibits the stretching vibration of a pyran ring.

3.3. ^{1}H and ^{13}C NMR spectroscopy

The ^{1}H and ^{13}C NMR spectra of EPS from *T. sakaeratensis* are shown in Fig. 3A and B, respectively. The chemical shifts from the ^{1}H spectrum are at 4.07 ppm (H3), 3.99 ppm (H4), 3.84 ppm (H5), 3.78 ppm (H6a), 3.65 ppm (H1a), 3.58 ppm (H1b), and 3.45 ppm (H6b). All of the proton signals are between 3.40 and 4.10 ppm, suggesting that there are no anomeric protons (5.40 to 4.20 ppm) in this fructan [17]. The signals

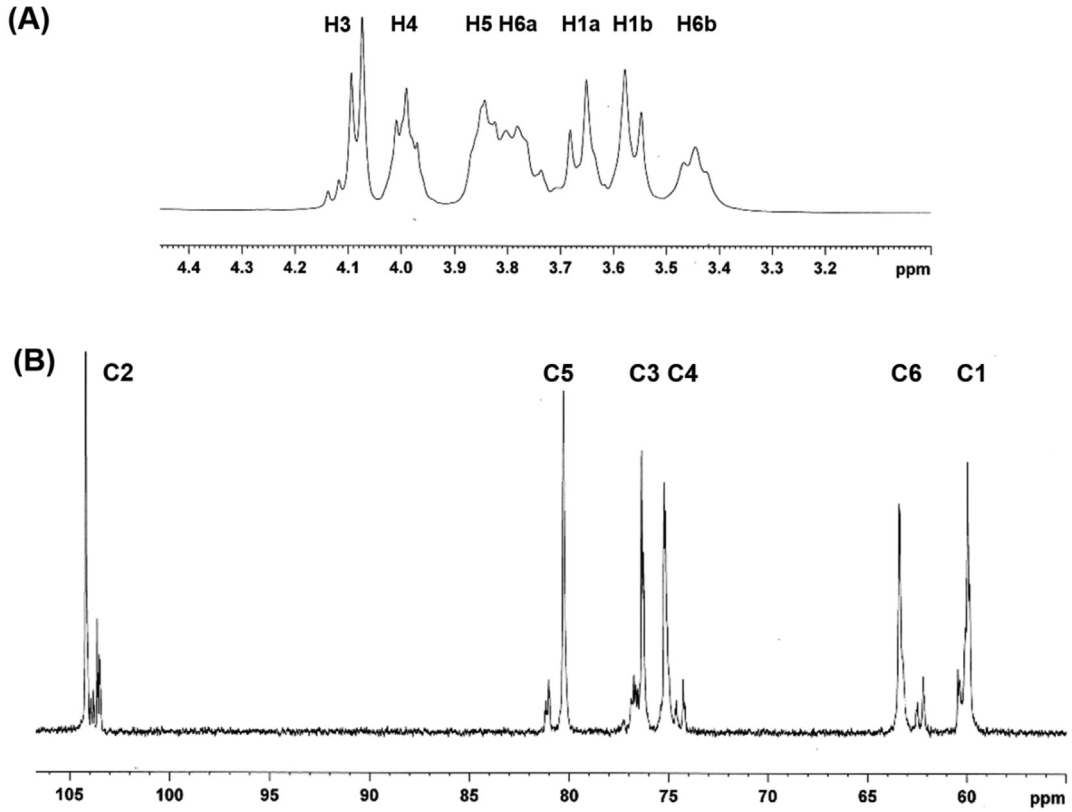

Fig. 3. NMR spectrum of EPS produced from *T. sakaeratensis*. (A) ^{1}H NMR (B) ^{13}C NMR.

Table 1
Previously reported ^{13}C NMR chemical shifts of levan from different bacterial strains.

Carbon atom	^{13}C NMR chemical shift (ppm) of reported levan					
	Tanticharoenia sakaeratensis	*Zymomonas mobilis*	*Bacillus megaterium* GJT321	*Brenneria goodwinii*	*Leuconostoc citreum* BD1707	*Pseudomonas fluorescens*
C-1	59.95	59.90	59.86	60.953	60.234	60.435
C-2	104.17	104.20	104.14	104.568	104.537	104.696
C-3	76.33	76.29	76.25	77.273	76.628	76.770
C-4	75.22	75.20	75.15	75.914	75.532	75.784
C-5	80.29	80.29	80.23	80.675	80.622	80.880
C-6	63.39	63.39	63.31	63.830	63.716	63.978
Reference	This study	Commercial standard levan	[30]	[31]	[17]	[15]

of chemical shifts in the ^{13}C NMR spectrum are at 104.17 ppm (C2), 80.29 ppm (C5), 76.33 ppm (C3), 75.20 ppm (C4), 63.39 ppm (C6), and 59.95 ppm (C1), corresponding to the resonance signals from beta-2,6 fructofuranose or levan, based on previous reports summarized in Table 1.

3.4. Molecular weight determination

The MWs of levan from *T. sakaeratensis* were analyzed by GPC. The majority of levan MWs are approximately 1×10^5–6.8×10^5 Da (Fig. S1; Table S1). Also, the MWs higher than 8.05×10^5 Da were detected with the lower percentage. Notably, several levan-producing acetic acid bacteria are known to produce relatively high MW levan. For example, *Gluconobacter frateurii* TMW 2.767 and *Gluconobacter cerinus* DSM 9533T produce levan in the MW range of 4×10^6–9.8×10^7 Da [6]. *Neoasaia chiangmaiensis* NBRC 101099 (1×10^8–5.75×10^8 Da) and *Kozakia baliensis* DSM 14400 (1×10^9–2×10^9 Da) are distinctive for producing levan of high MWs [6].

3.5. Optimization of levan production

Levan yields depend not only on the microbial strain but also on the cultural conditions. Different factors influence levan productivity. Here, we optimized incubation time, agitation speed, and sucrose concentration by varying one factor at a time. As shown in Fig. 4(A), levan productivity increased constantly until 35 h of fermentation, and then steadily decreased. Biomass did not change significantly from 20 to 92 h of fermentation. The maximum yield of levan from *T. sakaeratensis* was obtained during the stationary phase (unpublished data), which is similar to that of other bacterial species [3,18].

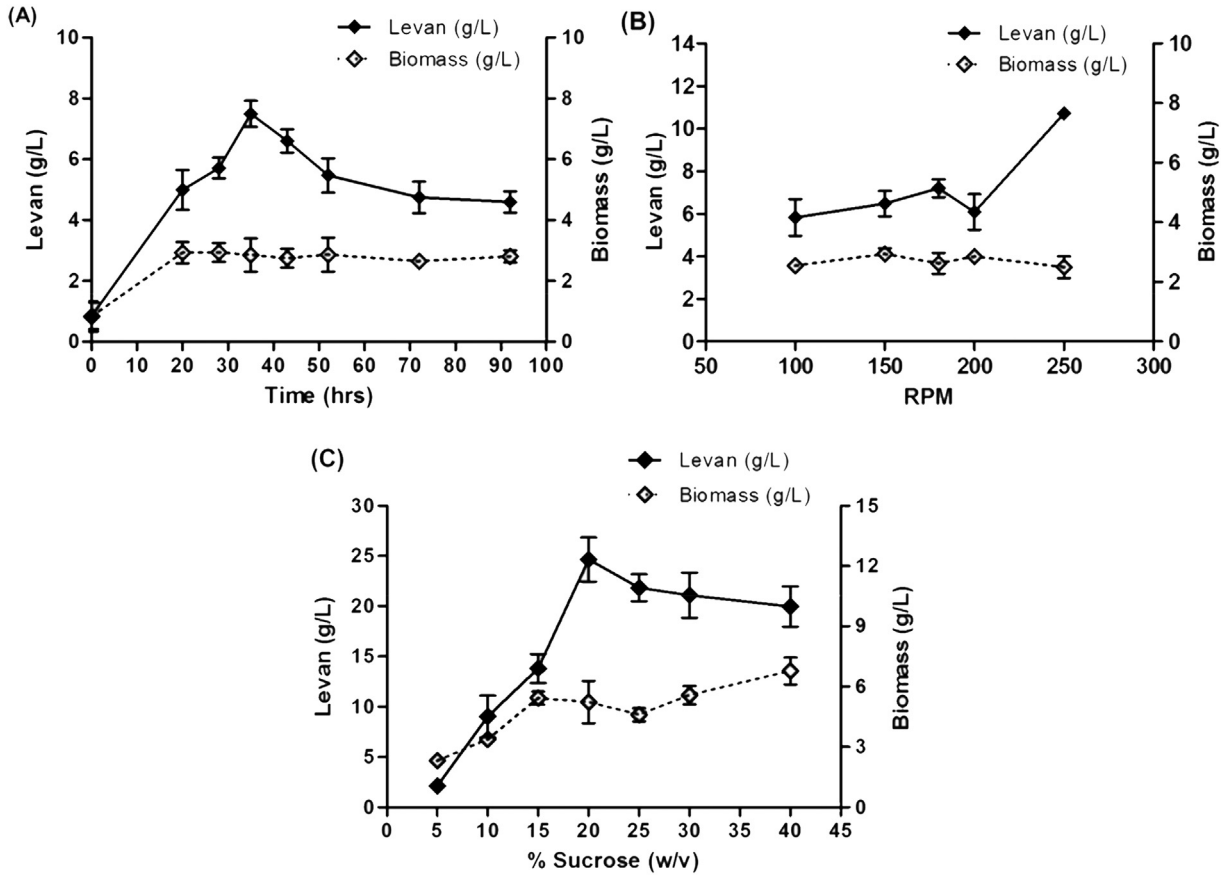

Fig. 4. Optimization of levan production from *T. sakaeratensis*. The amounts of levan ($g \cdot L^{-1}$) and biomass ($g \cdot L^{-1}$) produced under different cultural and nutritional conditions were examined. (A) Incubation time, (B) agitation rate, and (C) sucrose concentration. The data points represent the mean ± SD of triplicate samples.

Table 2
Different strains of levan-producing acetic acid bacteria with different percentages of conversion yield.

Acetic acid bacteria	Sucrose ($g \cdot L^{-1}$)	Levan ($g \cdot L^{-1}$)	Fermentation time (h)	Conversion yield (%)	Ref
T. sakaeratensis	200	24.7	35	24.70	This study
Acetobacter xylinum NCIM 2526	60	13.2	122	44.00	[3]
Gluconacetobacter diazotrophicus PAL5	100	24.8	–	49.60	[24]
Gluconobacter nephelii P1464	220	26.4	48	24.00	[32]
Gluconobacter frateurii TMW 2.767	80	11.9	48	29.75	[33]
Gluconobacter cerinus DSM 9533	80	6.3	48	15.75	[33]
Neoasaia chiangmaiensis NBRC 101099	80	7.3	48	18.25	[33]
Kozakia baliensis DSM 14400	80	7.8	48	19.50	[33]

Different agitation speeds ranging from 100 to 250 RPM had no significant effect on the biomass yield (Fig. 4(B)). However, a considerable increase in levan productivity was observed at 250 RPM, suggesting that vigorous shaking is critical for levan formation in *T. sakaeratensis*. The effect of agitation is likely to be strain-dependent, as previous studies show that high agitation speed has no effect on, and even inhibit, levan production in *Bacillus subtilis* MTCC 441 and *Bacillus polymyxa* [19,20].

Sucrose serves as both a bacterial carbon source and a substrate for levansucrase, both of which influence the efficiency of levan synthesis. We investigated different sucrose concentrations for their impact on levan formation and biomass yield. The yield of levan increased steadily from 2.1 $g \cdot L^{-1}$ to 24.7 $g \cdot L^{-1}$ as the sucrose concentration increased from 50 $g \cdot L^{-1}$ to 200 $g \cdot L^{-1}$ (Fig. 4(C)). However, at sucrose concentrations greater than 200 $g \cdot L^{-1}$, levan productivity decreased while the biomass yield slightly increased. In *Acetobacter xylinum* NCIM2526, the optimum sucrose concentrations range from 50 $g \cdot L^{-1}$ to 60 $g \cdot L^{-1}$, and similar to our result, levan production decreases drastically at higher sucrose concentrations [3]. In the case of *T. sakaeratensis,* the decline in levan production is likely not due to osmotic stress, since this strain of bacteria was previously characterized as an osmotolerant species [9]. Moreover, high sucrose concentrations did not lower the production of *T. sakaeratensis* biomass. It is possible that the lower yield of levan is due to substrate inhibition of levansucrase. At high concentrations, sucrose is expected to accumulate within the periplasmic space of gram-negative bacteria, inhibiting levansucrase and thus reducing levan production [21]. Furthermore, it is known that glucose can inhibit levansucrase activity [21,22]. Glucose accretion under high concentration of sucrose substrate might occur and lower the yield of levan. However, further investigation is required to confirm this hypothesis.

After optimization, the maximum yield of levan from *T. sakaeratensis* was 24.7 ± 2.1 $g \cdot L^{-1}$. Other genera of levan-producing acetic acid bacteria include the following: *Acetobacter* [3], *Asaia* [5], *Gluconacetobacter* [23,24], *Komagataeibacter* [7], *Gluconobacter*, *Neoasaia*, and *Kozakia* [6]. The different conversion yield values (%) of levan based on the fructose in sucrose are summarized in Table 2. Here, we add genus *Tanticharoenia* of the family *Acetobacteraceae* as a new member of levan-producing bacteria.

3.6. Effects of levan on cell viability

Different concentrations of levan from *T. sakaeratensis* were evaluated for cytotoxicity against RAW264.7 cells by the MTT assay. Cells were treated with 31.25–1000 μg/mL of levan for 24 h, then the percentage of viable cells relative to the untreated control was determined. As shown in Fig. 5, cell viability levels in levan-treated and the control groups did not differ significantly. Therefore, levan treatment does not affect the proliferation of RAW264.7 macrophage cells.

3.7. Effect of levan on nitric oxide (NO) production

Certain polysaccharides of plant and microbial origin can enhance the immune response by activating macrophages, leading to the generation of NO and the release of inflammatory cytokines [25]. To investigate the immunomodulatory effect of levan from *T. sakaeratensis*, we measured the amount of NO formed in RAW264.7 cells treated with various concentrations of levan. As NO is converted to the more stable nitrite, the concentration of nitrite detected by the Griess reaction serves as a proxy for NO. As shown in Fig. 6, treating cells with levan ranging from 62.5–1000 μg/mL significantly enhanced NO production in a

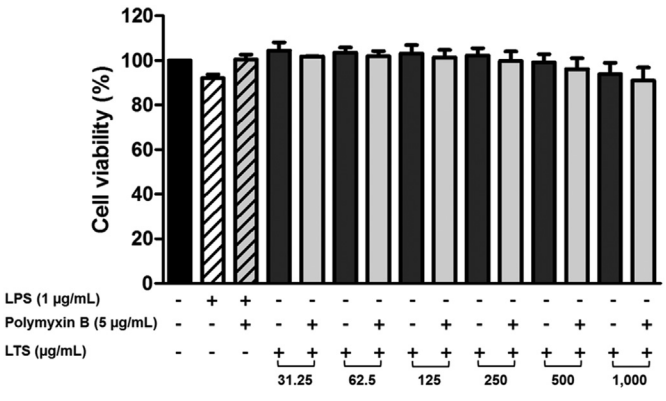

Fig. 5. Effects of levan from *T. sakaeratensis* on cell viability. RAW264.7 cells were treated with different concentrations of levan for 24 h and then assayed by MTT to measure cell viability. Bars represent the mean percentage of viable cells relative to the untreated control ± SD of three independent experiments with triplicate samples. LPS, lipopolysaccharides; LTS, levan from *T. sakaeratensis*.

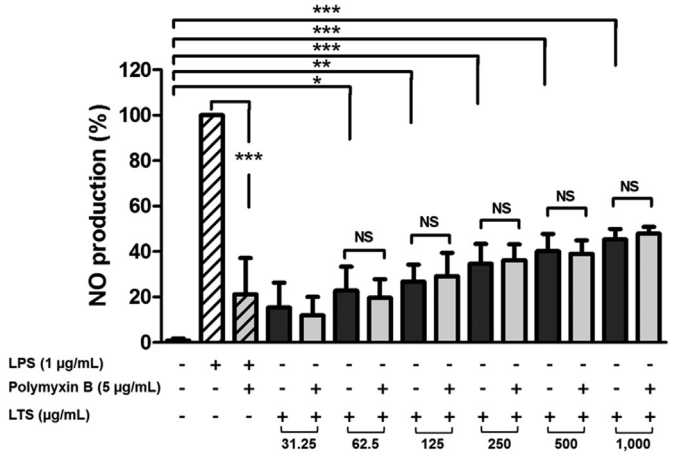

Fig. 6. Effect of levan on NO production. RAW264.7 cells were treated with various concentrations of levan from *T. sakaeratensis* (LTS) for 24 h with and without the presence of 5 μg/mL of polymyxin B. Cell treated with LPS (1 μg/mL) served as a positive control, and the produced NO was considered as 100%. The data represents the mean ± SD of three independent experiments with triplicate samples. NS, not significantly different; *, **, and *** represent significant differences at P values of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

dose-dependent manner. *T. sakaeratensis* is gram negative, thus its cell wall is composed of lipopolysaccharides (LPS). To rule out the possibility that increased NO production after levan treatment is due to activation via endotoxin contamination, cells were treated with polymyxin B, a cyclic amphipathic peptide that specifically binds to and neutralizes LPS activity [26]. While polymyxin B inhibited about 80% of NO production in LPS-treated cells, NO production in the polymyxin B and levan co-treatment remained at a level similar to that of the levan only-treated cells (Fig. 6). These results indicate that levan, and not LPS contamination, from *T. sakaeratensis* promotes the production of NO in RAW264.7 cells.

NO plays beneficial roles in the immune defense mechanism. For example, it has both anti-microbial and anti-tumor activities, although its overproduction has been implicated in inflammation and autoimmune diseases [27]. Our study reveals that levan from *T. sakaeratensis* may be immune-modulating by activating NO production, suggesting a potential for prophylactic applications. In line with this suggestion, levan from *B. subtilis* (natto) induces the production of TNF-α and IL-12 p40 in macrophages; and TLR4 expression may also be related to macrophage activation by levan [28]. Moreover, levan from *Z. mobilis* induces NO production in macrophage cells that can mediate cytotoxicity against tumor cells [29].

4. Conclusion

In the present study, EPS from the acetic acid bacterium, *T. sakaeratensis*, was structurally characterized and identified as levan. The MWs of *T. sakaeratensis* levan are in the range of 1×10^5–6.8×10^5 Da. The optimized culture conditions were determined, and utilized to obtain the maximum yield of levan. Moreover, *T. sakaeratensis* levan exhibits immunomodulatory effect by promoting the production of NO in RAW264.7 cells. *T. sakaeratensis* is a novel levan-producing bacterium that may be considered as a potential microbial source of levan that can be used for various applications.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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CRediT author statement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.07.001.

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