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Biopolymer-Levan Characterization in *Bacillus* Species Isolated from Traditionally Fermented Soybeans (Thua Nao)

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Cite This: *ACS Omega* 2025, 10, 1677−1687

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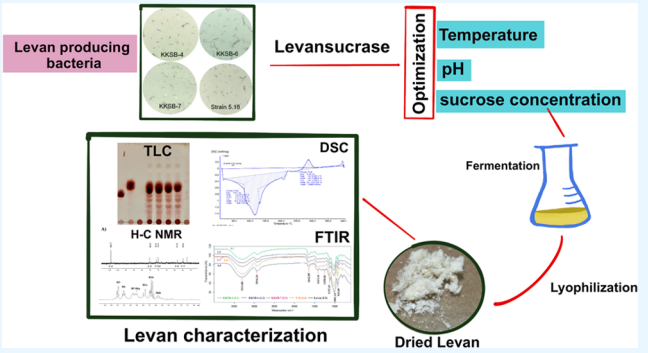
ABSTRACT: Bacterial levans are biopolymers composed of fructose units linked by β-2,6 glycosidic bonds that are degradable, nontoxic and flexible, representing a green technology with significant applications across various industries. Fermented soybeans are a common source of bacteria-producing polysaccharides. In this study, *Bacillus siamensis* KKSB4, *Bacillus velezensis* KKSB6 and *Bacillus amyloliquefaciens* KKSB7 isolated from traditionally fermented soybean (Thua-nao), along with *B. velezensis* strain 5.18 isolated from Jerusalem artichoke were investigated for their levansucrase activity and subsequent levan production. *B. siamensis* KKSB4, *B. amyloliquefaciens* KKSB7 and *B. velezensis* strain 5.18 exhibited the highest enzyme activity at 30% sucrose, whereas *B. velezensis* KKSB6 demonstrated optimal activity at 20% sucrose. Characterization of the levans using Fourier transform infrared spectroscopy (FTIR) revealed the peak at 3310.02, 1122.42, 1011.14, and 923.89 cm^{-1}, corresponding to the O−H group in fructose, C−O−H stretching, C−O−C stretching vibrations, and pyranose ring structures, respectively. ^{13}C NMR spectrum of levan aligned with the reference spectrum of *B. siamensis*, showing proton shifts at H3 (4.19 ppm), H4 (4.10 ppm), H5(3.96 ppm), H6a (3.89 ppm), H1a (3.76 ppm), H1b (3.67 ppm), and H6b (3.56 ppm). All strains produced high molecular weight levans (10^7−10^8 Da) with thermal stability, exhibiting melting temperatures exceeding 200 °C. The levans also demonstrated high water and oil holding capacities of over 100%, attributed to their large molecular complexes. Additionally, they exhibited antioxidant activity by scavenging DPPH radicals and oxidizing ABTS, particularly *B. siamensis* KKSB4 and *B. velezensis* KKSB6, although low FRAP activity was observed. Furthermore, the levans were produced by *B. siamensis* KKSB4 and *B. velezensis* KKSB6 promoted the growth of *Streptococcus thermophilus* DKT-3, suggesting its potential prebiotic properties. This work highlights *B. siamensis* KKSB4 and *B. velezensis* KKSB6 is an outstanding candidate for levan production with diverse functional properties.



1. INTRODUCTION

Biopolymers have gained significant attention due to their benefits in various fields, including the food and beverage industries, cosmetics, medicine, pharmaceuticals, and bioplastics. Levan is a biohomopolysaccharide composed of fructose units linked by β-(2 → 6) glycosidic bonds, forming a highly branched polysaccharide. The molecular weight of levan varies depending on the producing microorganisms such as bacteria, fungi, yeast, and microalgae. Levan is a free extracellular polysaccharide secreted by these microorganisms, which aids in adhesion, colonization, and protection against environmental stresses.[1] Due to its unique structural properties, levan can be processed into a variety of forms, such as biodegradable films, hydrogels, sponges, and nanocapsules for the controlled release of substances.[2] The structural variation of levan is determined by the number of D-fructo-furanosyl units, referred to as the degree of polymerization (DP), leading to a wide molecular weight range from 10^4 to 10^8 Da,[1] which directly influences its functional properties. Within the *Bacillus* genus, several species have been identified as potential levan producers, including *Bacillus subtilis* NATTO, *B. subtilis* AF17, *Bacillus licheniformis*, *B. amyloliquefaciens*, *B. licheniformis* BKAG21, *B. siamensis*, *Bacillus lentus*, *Bacillus aryabhattai*, and *Bacillus cereus*.[3−5] These species not only exhibit levan production but also demonstrate valuable bioactivities, such as antioxidant, antibacterial, antibiofilm, and anticancer proper-

Received: October 22, 2024
Revised: December 21, 2024
Accepted: December 25, 2024
Published: January 3, 2025

ties, further broadening the potential applications of levan-based products.

Levansucrase is a major enzyme responsible for levan synthesis in *Bacillus* species, utilizing sucrose as a substrate. As a member of fructosyltransferases family, levansucrase catalyzes sucrose hydrolysis and transfructosylation, releasing glucose as a byproduct.[6] This enzyme has been improved for high yield of levan production either by optimizing fermentation parameters in native strains or through overexpression in robust host systems.[7] The activity of microbial levansucrases varies depending on sucrose concentrations, which influences whether fructooligosaccharides or fructans (levans) are produced.

Levan-producing bacteria have been isolated from various sources for use in levan production across different substrates. For example, *Gluconobacter* isolated from sugar cane mills produced levan from sugar cane mills as a substrate,[8] while *B. subtilis* MTCC 441 produced levan using sugar cane juice as a substrate.[9] To achieve high yields and stability at a low cost, industrial byproducts have been employed for levan production. However, alternative complex media, such as sugar cane molasses or syrup or sugar beet molasses, had yielded variable results with production rates slightly higher or lower depending on the medium and sucrose concentration. Moreover, a critical factor in successful levan production is levansucrase biosynthesis and its secretion pathway, which are influenced by the levansucrase operon.[10] The potential strains demonstrated levan yields, such as, *B. subtilis* var. *natto* which produced 41.44 g L^{-1} of levan from 250 g L^{-1} sucrose,[3] and *B. licheniformis* NS032 which achieved 52 g L^{-1} from 200 g L^{-1} sucrose.[9] The molecular weight of levan, whether high (HMW) or low (LMW), depends on the affinity of levansucrase for fructosyl residues during transfer or elongation processes which are influenced by sucrose concentration.[11,12]

This study aims to identify new levan-producing strains from traditionally fermented soybeans, known as Thua-nao in Northern Thailand. The focus was on isolating *Bacillus* species, recognized as safe strains, for potential industrial applications such as food supplements. Sucrose concentration was investigated to determine the optimal conditions for levan production. The physicochemical and biochemical properties of the levans were characterized. This characterization aims to provide valuable insights into the specific properties of the levans, which will guide their applications in various industrial processes and future studies.

2. MATERIALS AND METHODS

2.1. Isolation and Identification of Levan-Producing Bacterial Strains. Levan-producing bacteria were isolated from Thua nao (Thai fermented soybean), which was fermented for 6 days, using the spread plate technique on Luria−Bertani (LB) agar supplemented with 1% sucrose and incubated at 30 °C for 24 h. Bacteria were selected to determine the levansucrase activity. The potential bacteria were identified as *Bacillus siamensis* KKSB4 (PP731977), *Bacillus velezensis* KKSB6 (PP731824), *Bacillus amyloliquefaciens* KKSB7 (PP732219); and one strain was an endophyte of Jerusalem artichoke, named *B. velezensis* strain 5.18 (MH973231). These bacteria were cultured in LB broth supplemented with 1% sucrose and incubated at 30 °C, 3 g for 24 h. The cell suspension was adjusted with sterile distilled water to achieve an optical density of 0.65 at 600 nm (A_{600}) which was used as the inoculum for subsequent experiments.

2.2. Optimization of Levansucrase Production. The inoculum (6.9×10^8 CFU/mL) was transferred into LB broth with varying sucrose concentrations at 0, 5, 10, 15, 20, and 30% (w/v). The culture was incubated at 30 °C with shaking for 3 g to determine the optimal sucrose concentration. Subsequently, the optimal sucrose concentration was used to investigate the effects of the temperature and pH on levansucrase production. The bacterial culture was incubated at 30, 37, and 40 °C and the initial pH of the medium was adjusted to 6.0, 6.5, 7.0, 7.5, and 8.0. The culture was collected at 0, 12, 24, 36, and 48 h and then centrifuged at 2800*g* for 10 min at 4 °C to obtain the crude enzyme (cell-free culture). Levansucrase activity in the supernatant was assayed, while the bacterial growth was monitored by measuring the optical density (A_{600}) using a spectrophotometer.

2.3. Determination of Levansucrase Activity. The 0.5 mL aliquot of cell-free culture was mixed with 0.5 mL of 1% sucrose solution (as a substrate) in acetate buffer (pH 6.0). The reaction mixture was incubated at 30 °C for 10 min, followed by the addition of 0.5 mL of DNS reagent to terminate the reaction. The mixture was then boiled for 10 min and subsequently cooled in an ice bath. After the mixture was cooled, the total volume was brought up to 5 mL with distilled water. The amount of reducing sugar produced was determined using 3,5-dinitrosalicylic acid (DNS) method[13] at 540 nm by a spectrophotometer. Glucose was used as a standard for the calibration curve. The negative control was conducted using a cell-free culture without the substrate, and the reducing sugar value obtained was subtracted from the corresponding sample. One unit of levansucrase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per min under the assay conditions. The highest enzyme activity was defined as 100% to calculate the relative activity.

2.4. Levan Production and Purification. The bacterial isolates were cultured under their respective optimized conditions for levan production *B. amyloliquefaciens* KKSB7 and *B. velezensis* 5.18 were grown in LB broth containing 30% sucrose, pH 8.0, and incubated at 40 °C. *B. siamensis* KKBS4 was cultured in LB broth with 30% sucrose, pH 7.5 at 37 °C, while *B. velezensis* KKBS6 was cultured in LB broth with 20% sucrose, pH 6.0, and incubated at 37 °C. After 48 h of incubation, the supernatant was collected by centrifugation at 7168*g*, 10 min. Levan was precipitated from the supernatants by adding chilled ethanol at a ratio of 3:1 (ethanol and supernatant) and centrifuged at 2800*g* for 10 min. The precipitated levan was then freeze-dried to obtain a powdered form as crude levan.

For purification, the dried levan was dissolved in distilled water and extracted with a mixture of *n*-butanol and chloroform (1:5, v/v) to remove proteins, with the levan collected from the upper aqueous phase. After that, the levan was washed 4 times with distilled water and transferred into a dialysis bag (molecular weight cutoff: 8000 Da). The levan was dialyzed in distilled water for 48 h to achieve purification and collected as purified levan.

2.5. Characterization of Levan. *2.5.1. Thin Layer Chromatography (TLC).* Levan precipitated from the cell-free culture was analyzed using TLC on silica gel 60 F254 plates (Merck). The mobile phase consisted of butanol/acetic acid/water in a ratio of 3:1:2 (v/v/v), which was used to separate the components. After development, the TLC plates were air-dried and then stained with a sulfuric acid solution

https://doi.org/10.1021/acsomega.4c09641
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(1:9 H_2SO_4 and ethanol). The sugar compounds were visualized by heating the plates at 50 °C for 5 min.

2.5.2. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis. FTIR spectroscopy (Bruker TENSOR27) was employed to identify the functional groups present in the levan. Spectral data were collected using FTIR spectrophotometer in transmittance mode over the range of 4000 to 400 cm^{-1}.

2.5.3. Polymer Identification by ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) Analysis. NMR spectroscopy was utilized to elucidate the chemical structure of the polysaccharide. Ten milligrams of purified levan were dissolved in 1 mL of deuterium monoxide (D_2O). The ^1H and ^{13}C spectra were recorded at 60 °C using an NMR spectrometer (Bruker Ascend-400).

2.5.4. Thermal Properties of Levan Polymer. The thermal properties of levan were analyzed by using differential scanning calorimetry (DSC) with a NETZSCH DSC214 instrument. Ten milligrams of levan were sealed in an aluminum pan and the sample was subjected to a heating and cooling cycle from 10 to 300 °C at a rate of 10 °C min^{-1}.

2.5.5. Molecular Weight Determination. The molecular weight of purified levan was measured by using a Zetasizer Nano ZS (Malvern) analyzer. Levan samples were prepared in sterile reverse-osmosis water at concentrations of 1, 2, and 3 mg mL^{-1}. Measurements were conducted in triplicate, with a levan standard from *Erwinia herbicola* (Sigma-Aldrich) used as a reference.

2.6. Determination of Levan Properties.[14] *2.6.1. Water Holding Capacity.* Dry weight of levan (500 mg) was dissolved in 10 mL of deionized water and heated at 40 °C for 10 min. The mixture was then centrifuged at 18,928*g* for 60 min to obtain the levan pellet, which had absorbed water. Excess water was removed using filter paper. The weight of the levan pellet was recorded, and the water holding capacity was calculated using the formula:

$$\text{water holding capacity}(\%) = \frac{\text{weight of levan pellet} - \text{initial weight of levan}}{\text{initial weight of levan}} \times 100$$

2.6.2. Water Solubility Index. The water solubility index was determined by dissolving 500 mg of dry levan in 10 mL of deionized water followed by stirring at 30 °C for 1 h. The sample was centrifuged at 7168*g* for 15 min to separate the supernatant and pellet. Both fractions were dried at 70 °C for 4 h to measure the dry weight of solids from the supernatant and the dry weight of the pellet. The water solubility index was calculated using the formula

$$\text{water solubility index}(\%) = \frac{\text{dry weight of solid in supernatant}}{\text{initial weight of levan}} \times 100$$

2.6.3. Oil Holding Capacity. The oil holding capacity of levan was evaluated by dissolving 500 mg of dry levan in 10 mL of soybean oil, followed by vortex mixing. The mixture was allowed to stand at room temperature for 1 h to facilitate absorption. Subsequently, the sample was centrifuged at 7168*g* for 20 min to remove unabsorbed oil. The weight of the oil-absorbed levan pellet was recorded, and the oil holding capacity was calculated using the following formula

$$\text{oil holding capacity}(\%) = \frac{\text{weight of levan pellet} - \text{initial weight of levan}}{\text{initial weight of levan}} \times 100$$

2.6.4. Antioxidant Activity.[15] *2.6.4.1. DPPH Assay.* The antioxidant activity of levan was evaluated using a 2,2-diphenyl-1-picryldrazyl (DPPH, Sigma-Aldrich) free radical scavenging assay. A 0.23 mM DPPH solution was freshly prepared in methanol. For the assay, 0.5 mL of DPPH solution was mixed with 0.5 mL of levan solution and incubated at 37 °C in dark conditions for 2 h. After incubation, the mixture was centrifuged at 2800*g* for 5 min and the absorbance of the supernatant was measured at 517 nm using a spectrophotometer (GENESIS 10S UV−vis). DPPH without levan was used as a negative control, while ascorbic acid (400 mg L^{-1}) served as a positive control. The antioxidant activity of levan was expressed as its ability to inhibit DPPH free radicals, calculated using the following equation

$$\%\ \text{inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.6.4.2. ABTS Assay. The antioxidant capacity of levan was further determined using 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) radical cation decolorization assay. $ABTS^{\bullet+}$ was generated by mixing 7 mM ABTS with 2.43 mM ammonium persulfate in a 1:1 ratio, followed by incubation in the dark for 16 h. Before use, the $ABTS^{\bullet+}$ solution was diluted with deionized water to an absorbance of 0.7 at 734 nm. Then, 0.1 mL of levan solution was added to 1.7 mL of $ABTS^{\bullet+}$ solution and incubated at 37 °C for 2 h. The mixture was centrifuged at 2800*g* for 5 min and the absorbance of the supernatant was measured at 734 nm. $ABTS^{\bullet+}$ alone served as the control and the scavenging capacity of levan was compared with ascorbic acid (400 mg L^{-1}) as a positive control. The scavenging activity was calculated by using the following equation

$$\text{scavenging activity}\ (\%) = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.6.4.3. FRAP Assay. The ferric reducing antioxidant power (FRAP) assay was performed to assess the ferric ion-reducing ability of levan. FRAP assay was based on the reduction of ferric-tripridyltriazine complex $[Fe^{3+}\text{-}(TPTZ)]^{3+}$ to the ferrous form $[Fe^{2+}\text{-}(TPTZ)]^{2+}$, which forms an intense blue complex. For the assay, 0.1 mL of levan solution was mixed with 0.9 mL of FRAP reagent, which consisted of a mixture of 10 mM TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine, Sigma-Aldrich) in 40 mM HCl, 20 mM $FeCl_3$, and 0.3 M sodium acetate buffer (pH 3.6) in a ratio of 1:1:10. The mixture was incubated at 37 °C for 30 min, followed by centrifugation at 2800 g for 5 min. The absorbance of the supernatant was measured at 595 nm, and the FRAP value was compared to an equivalent concentration of ascorbic acid (400 mg L^{-1}).

2.6.5. Prebiotic Properties.[16] Prebiotic activity score (PAS) evaluates the ability of carbohydrates to promote the growth of probiotics without supporting the growth of other intestinal bacteria. A probiotic strain, *Streptococcus thermophiles* DKT-3 was cultured in MRS broth without glucose and supplemented with levan as the carbon source. In contrast, *Escherichia coli* was

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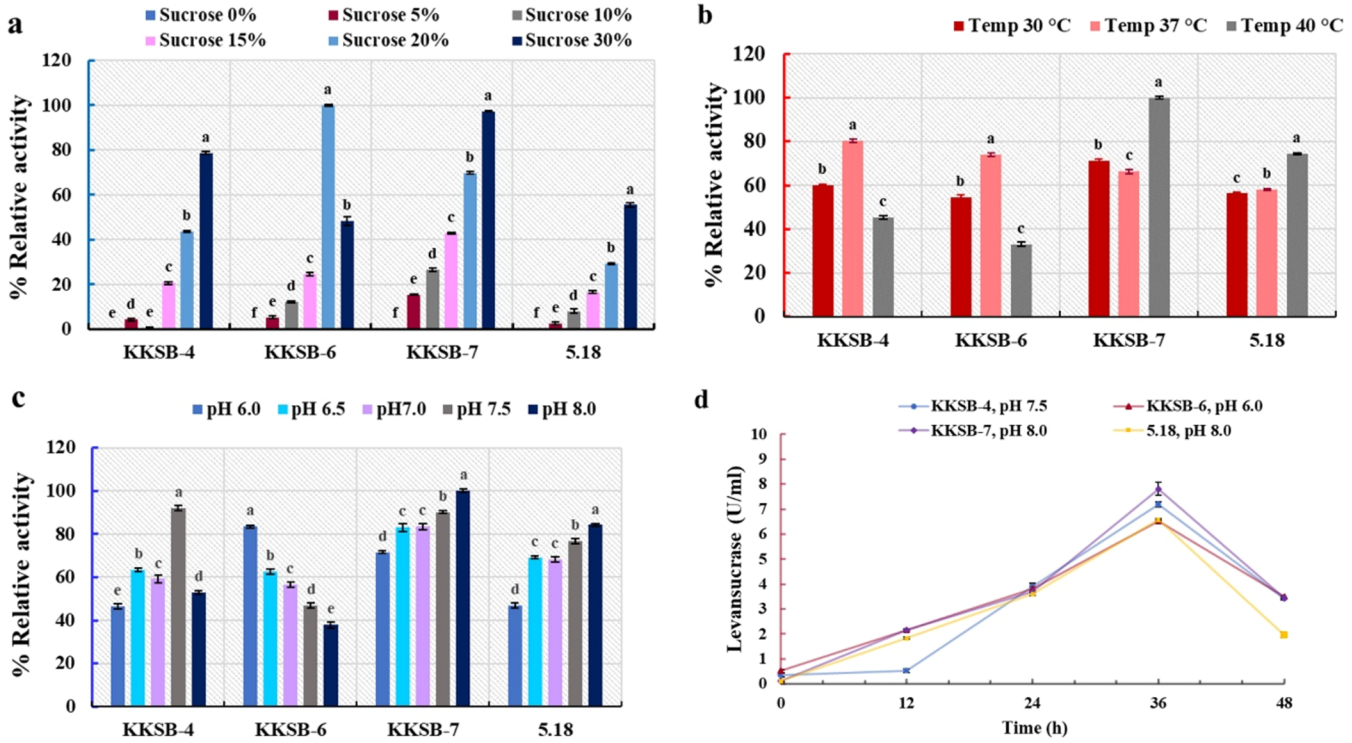

Figure 1. Optimum conditions for levansucrase activity under various parameters at 36 h of fermentation: (a) effect of sucrose concentration, (b) effect of temperature, (c) effect of medium pH on enzyme activity, and (d) enzyme production of four bacterial strains in their optimum conditions. Different letters on each bar indicate significant differences within each strain at $p < 0.05$.

grown in nutrient broth (NB) with levan under the same conditions. The bacterial growth was determined using the plate count method at 0, 24, and 48 h. A positive control was set up by using media with glucose as the carbon source. The high PAS score indicates that the prebiotic substrate promotes the growth of probiotic strain when compared to growth in glucose, demonstrating its prebiotic potential. Conversely, the growth of *E. coli* in prebiotics should be minimal when compared with growth in glucose. The PAS score was calculated using the following equation

$$\mathrm{PAS} = \frac{(\log \mathrm{P48} - \log \mathrm{P0})\mathrm{prebiotic}}{(\log \mathrm{P48} - \log \mathrm{P0})\mathrm{glucose}} - \frac{(\log \mathrm{E24} - \log \mathrm{E0})\mathrm{prebiotic}}{(\log \mathrm{E24} - \log \mathrm{E0})\mathrm{glucose}}$$

When log *P* is the growth of probiotic strain in log CFU/mL value at 48 h (P48) and at 0 h (P0) when grown in MRS medium supplemented with either glucose or levan. Log *E* is the growth of *E. coli* in log CFU/mL values at 24 h (E24) and at 0 h (E0) when grown in NB medium supplemented with either glucose or levan.

2.7. Statistical Analysis. All experiments were performed in triplicate, with the results expressed as mean ± standard deviation. Comparisons among groups were conducted using analysis of variance (ANOVA) with STATISTIX 10 software, followed by the least significant difference (LSD) test at $P < 0.05$ for multiple comparisons.

3. RESULTS AND DISCUSSION

3.1. Optimal Conditions Differ in Different Strains for Levansucrase and Levan Production. Sucrose plays a role as a precursor for levan production through the action of levansucrase. Higher sucrose concentrations correlated with increased levansucrase production, as shown in Figure 1. The optimal sucrose concentration for levansucrase was 30% for KKSB-4, KKSB-7, and 5.18, while KKSB-6 produced levansucrase at a lower sucrose concentration of 20%. These bacteria are mesophilic, growing at 30−37 °C with the optimum growth at 37 °C. However, KKSB-7 and 5.18 demonstrated enzyme production at 40 °C. The strains also showed different responses to the initial pH of the medium; KKSB-7 and 5.18 produced high levansucrase under alkaline conditions (pH 8), while KKSB-6 displayed optimal pH at 6 and KKSB-4 showed high levansucrase at pH 7.5.

Numerous studies have shown that various *Bacillus* species produce levansucrase optimally at temperatures between 30−50 °C and pH levels of 6−7.5, with sucrose concentrations ranging from 20−50%, for example, *B. siamensis*,[17] *B. methylotrophicus*,[18] and *B. subtilis*.[19] In this study, *B. siamensis*, *B. velezensis*, and *B. amyloliquefaciens* followed similar patterns, with the sucrose concentration being a key determinant for levansucrase production. High sucrose levels induced enzyme secretion and enhanced transfructosylation activity, leading to increased levan formation. However, high sucrose also inhibited bacterial growth. At high sucrose concentration, low molecular weight levans are produced; as sucrose acts as an acceptor for the fructosyl-enzyme complexes, driving the synthesis of short-chain oligosaccharides.[17] In case of *B. subtilis*,[20] it produced low molecular weight levans (10^3−10^5 Da) when grown in media with high sucrose concentration (60%). In contrast, all four bacterial strains produced high molecular weight levan (over 10^6 Da) at low sucrose concentration (20−30%). Temperature also significantly influenced levan size with different species responding uniquely. For example, *B. licheniformis* RN-01 produced high

https://doi.org/10.1021/acsomega.4c09641
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molecular weight levan at 50 °C, but low molecular weight levan at 30 °C.[21] Conversely, *B. subtilis* C4 showed higher production of high molecular weight levan at 37 °C compared to 60 °C,[22] which aligns with the findings of this study.

Levan production was determined by using TLC to observe sucrose utilization and product formation during fermentation. A sucrose band was present at the beginning time for all strains, as it was the primary substrate in the medium (Figure 2). After fermentation, sucrose was degraded into glucose and fructose, with fructose subsequently used to form a polysaccharide chain represented by the bottom spots on the TLC plate. Levan production began in parallel with levansucrase production (Figure 1d). As levansucrase functions through two key steps: sucrose hydrolysis and fructose transfer.[17] The hydrolysis of sucrose produces glucose and fructose, after which transfructosylation facilitates the polymerization of fructose into levan, leaving excess glucose behind. The excess glucose inhibits levansucrase, reducing its hydrolytic activity; however, the enzyme continues transfructosylation, allowing the levan formation to proceed. At 48 h, the dark spots of levan were visible, alongside glucose and residual sucrose. This pattern indicated that the optimal harvest time for levan production was around 48 h.

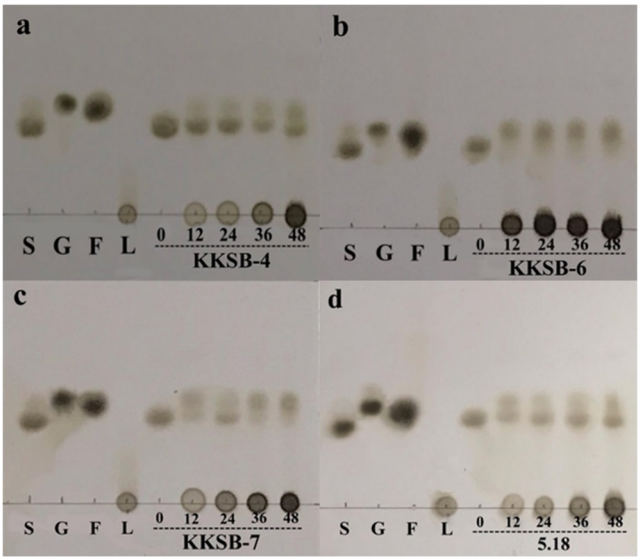

Figure 2. Thin-layer chromatography analysis of levan produced by strains (a) KKSB-4, (b) KKSB-6, (c) KKSB-7, and (d) 5.18 under optimal conditions at various times compared with sucrose (S), glucose (G), fructose (F), and a levan standard (L).

3.2. Physiochemical Characteristics of Bacterial Levan. Figure 3 presents the key infrared (IR) spectral features of bacterial levan, which confirms its molecular composition. A prominent peak at 3310.02 cm^{-1} corresponds to the O−H stretching vibration of the hydroxyl group in fructose, which is a key constituent of levan. A weaker peak at 2934.24 cm^{-1} represents the C−H stretching vibration, typically appeared in the 3000−2800 cm^{-1}. This vibration arises from the C−H bonds in the sugar molecules. The C−H bending vibration of the methyl group was indicated at 1413.41 cm^{-1}, while a peak at 1642.05 cm^{-1} was attributed to bound water or O−H−O stretching. The C−O−H stretching vibration appeared at 1122.42 cm^{-1} and the glycosidic linkage (C−O−C) stretching vibration, a defining feature of carbohydrates, showed at 1011.14 cm^{-1}. The peak at 923.89 cm^{-1} was associated with pyranose ring structures, which are typical of sugar molecules like levan. These spectral characteristics closely matched those of a levan standard from *E. herbicola* (Sigma).

Figure 4 presents the ^{13}C NMR spectrum of levan derived from the four bacterial strains, showing six carbon positions; the chemical shifts of these carbons were detailed in Table 1 with references of levan from other bacterial species. Their carbon positions were closely related to those of *B. siamensis*. The ^{1}H NMR spectrum confirmed the structure of levan with proton shifts at specific positions: H3 (4.19 ppm), H4 (4.10 ppm), H5(3.96 ppm), H6a (3.89 ppm), H1a (3.76 ppm), H1b (3.67 ppm), and H6b (3.56 ppm).

The molecular weight of bacterial levan typically ranges from 1×10^4 to 1×10^9 Da, which is higher than the molecular weight of plant-derived levan. In this study, the average molecular weight of levan produced by the bacterial strains was determined as follows: KKSB4 had a molecular weight of 6.1×10^7 Da (±8857), KKSB-6 produced levan with a molecular weight of 2.2×10^8 Da (±28,767), KKSB-7 exhibited a molecular weight of 8.5×10^7 Da (±15,933) and strain 5.18 produced levan with a molecular weight of 6.9×10^7 Da (±8,067). When compared to other species, *Bacillus paralicheniformis* produced levan with a molecular weight of 5.517×10^7 Da[23] and *B. aryabhattai* yielded levan with a

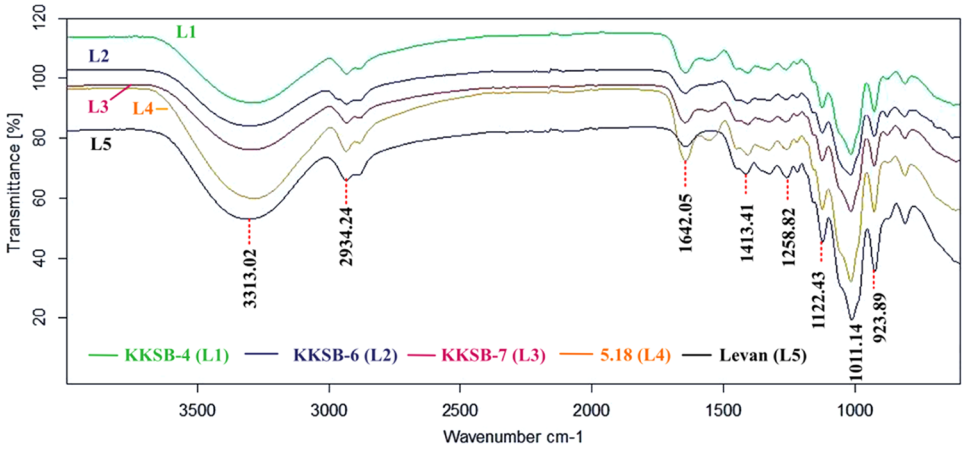

Figure 3. FTIR spectra of levan from 4 bacterial strains compared with levan standard representing the functional groups of polysaccharides.

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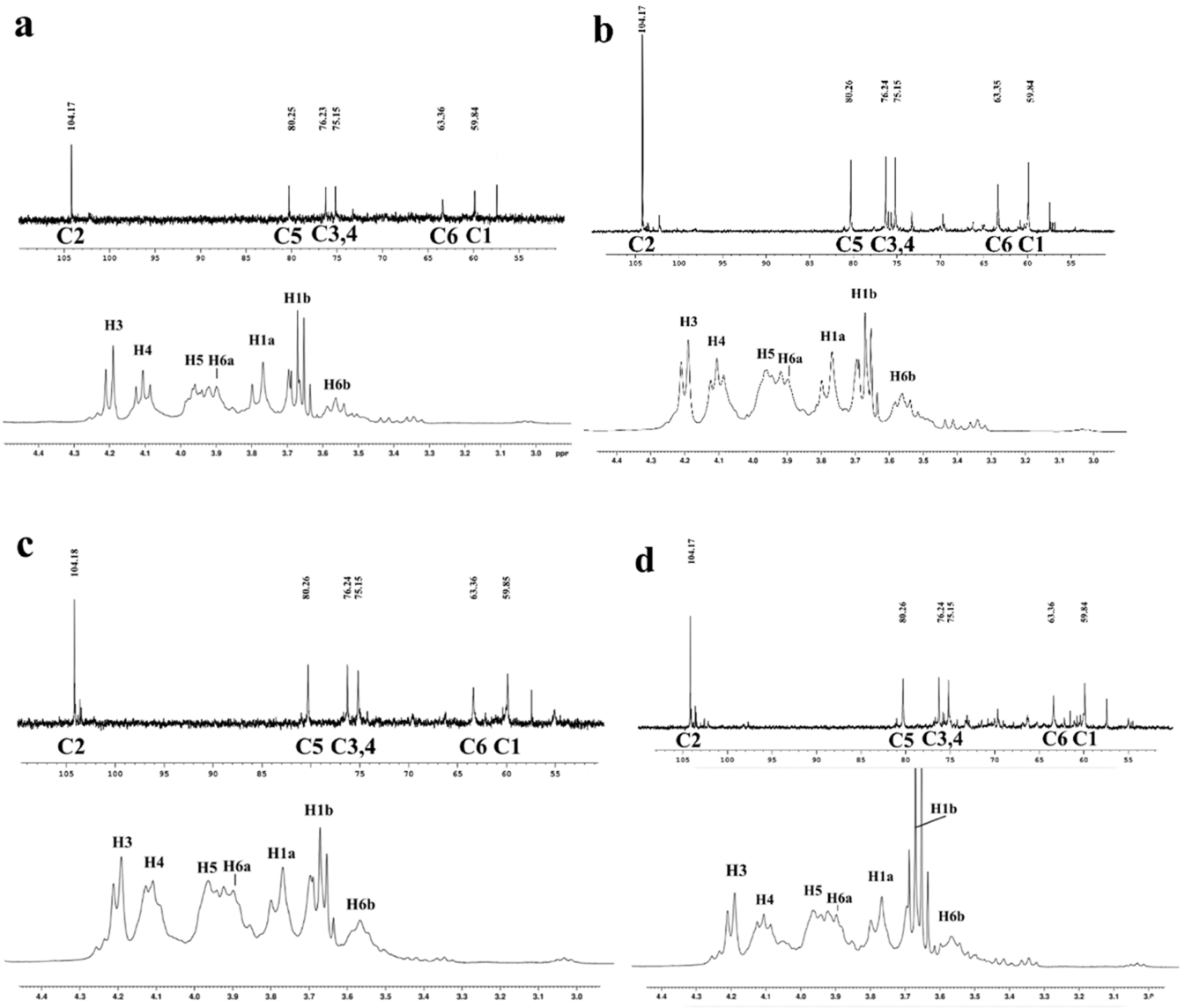

Figure 4. ^{13}C NMR and ^{1}H NMR spectrum of levan obtained from Thua-noa bacterial isolates including KKSB-4 (A), KKSB-6 (B), KKSB-7 (C), and 5.18 (D).

Table 1. Comparison of Chemical Shift (ppm) in ^{13}C NMR of Levan from Different Species of *Bacillus*

	chemical-shift (ppm)						
sources	C-1	C-2	C-3	C-4	C-5	C-6	refs
B. siamensis	59.91	104.20	76.29	75.19	80.28	63.38	28
Bacillus polimyxa	60.07	104.20	77.00	75.70	80.50	63.60	11
B. subtilis	60.10	104.40	76.50	75.40	80.50	63.60	29
B. licheniformis	62.90	106.90	79.30	78.10	83.00	66.10	24
B. siamensis KKSB4	59.84	104.17	76.23	75.15	80.25	63.36	this study
B. velezensis KKSB6	59.84	104.17	76.24	75.15	80.26	63.35	this study
B. amyloliquefaciens KKSB7	59.85	104.18	76.24	75.15	80.26	63.36	this study
B. velenzensis strain 5.18	59.84	104.17	76.24	75.15	80.26	63.36	this study

molecular weight of 5.3×10^7 Da.[24] These findings suggest that the molecular weight of levan can vary considerably among bacterial species, and all four strains demonstrated the potential to produce high-molecular-weight (HMW) polysaccharides (over 10^6 Da). This may be attributed to the high affinity of their levansucrase enzymes for transferring several fructosyl residues, promoting HMW formation during the elongation process. The results contrast with the hypothesis that LMW levan is favored for production at high sucrose concentrations.[12] Another contributing factor could be the levansucrase operon, a tricistronic operon that includes the *sacB* gene (levansucrase), the *levB* (*yveB*) gene (levanase), and the *yveA* gene (unknown function). High levanase activity has been associated with the production of LMH levan, no higher

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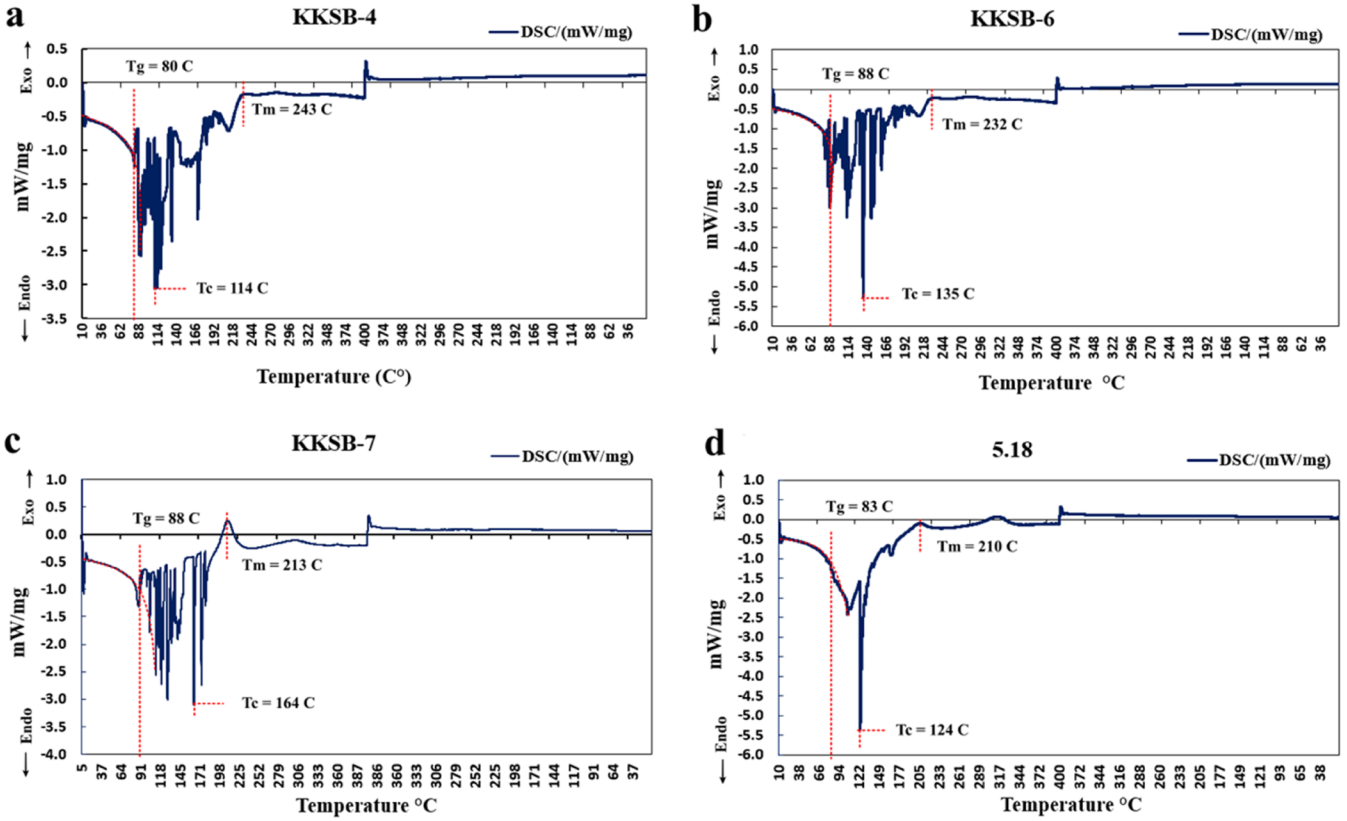

Figure 5. Thermal analysis by DSC technique of levan products from 4 bacterial strains: (a) KKSB-4, (b) KKSB-6, (c) KKSB-7, and (d) 5.18.

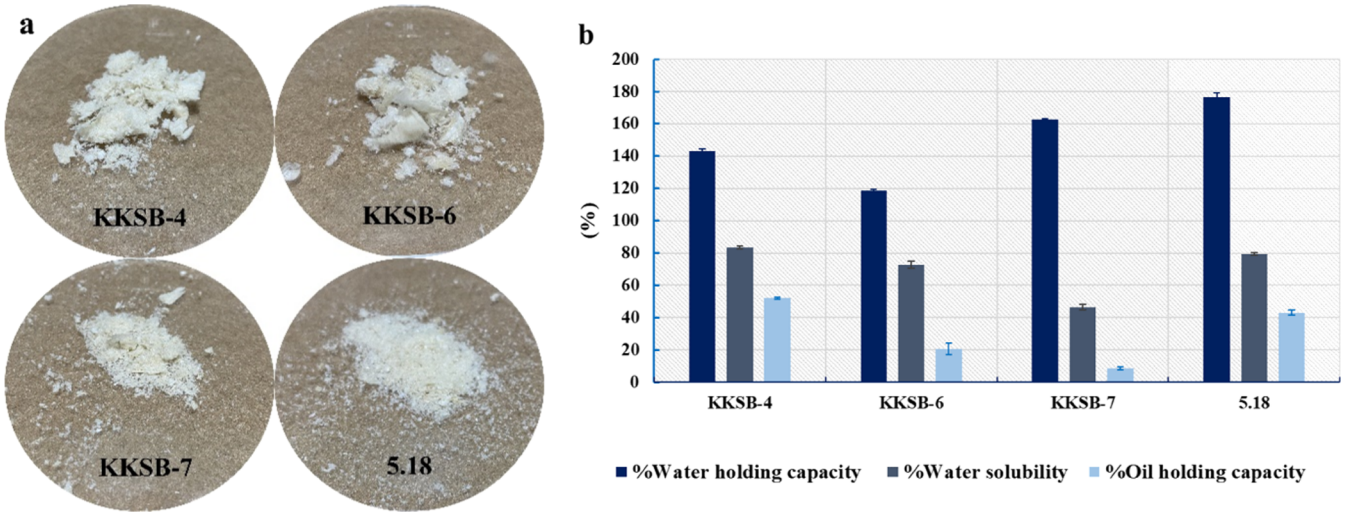

Figure 6. (a) Feature of lyophilized levans and (b) the levan capacities in water holding, water solubility, and oil holding of KKSB-4, KKSB-6, KKSB-7, and 5.18.

than 10^6 Da.[25] Further studies are needed to investigate the mechanisms of levan production in these bacteria, including functional analysis of the levansucrase gene.

The combined results from IR, NMR, and molecular weight analyses confirmed the structural integrity and consistency of the levan produced by the bacterial strains, identifying it as a levan-type fructan. High molecular weight levan has been shown to possess health benefits, such as lowering blood cholesterol levels and exhibiting antitumor activity. In contrast, low molecular weight levan is commonly used in drug encapsulation, providing a slow and sustained release of therapeutic agents.[26,27] The molecular weight of levan produced through microbial fermentation varies depending on the specific bacterial strains, culture composition, and fermentation conditions. Optimizing these parameters is essential to achieving a high yield of the desired levan form, whether for biomedical applications or other industrial uses.

The thermal properties of the polymer, including glass transition temperature (T_g), crystal transition temperature (T_c), and melting temperature (T_m), were evaluated to provide valuable insights into the structure and functionality of the biopolymer levan. The thermal characteristics of bacterial levan

https://doi.org/10.1021/acsomega.4c09641
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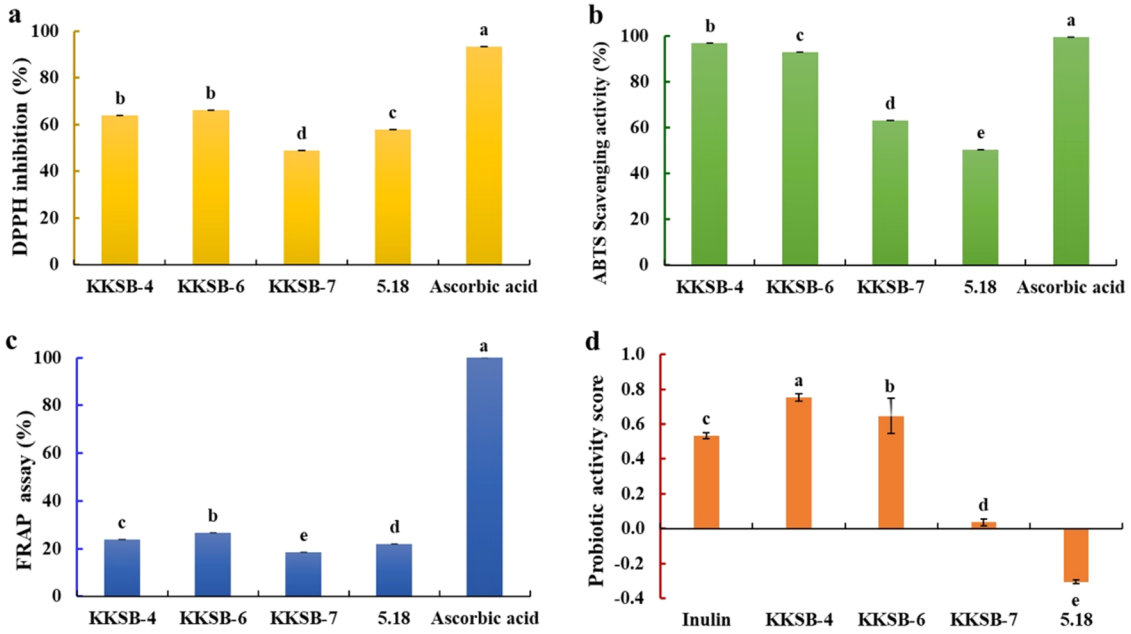

Figure 7. Properties of levan from four bacterial strains by investigation of antioxidant property with (a) DPPH inhibition, (b) ABTS scavenging activity, and (c) FRAP assay. Ascorbic acid concentration at 400 mg L^{-1} was used as a standard antioxidant. (d) Prebiotic activity score of levan compared with inulin. Bars labeled with different letters indicate significant differences between strains at $p < 0.05$.

were assessed using the DSC method, revealing T_g, T_c, and T_m values, as shown in Figure 5. T_g indicates the softening of the amorphous region within the polymer structure. The T_g values for the KKSB-6 and KKSB-7 strains were 88 °C, whereas KKSB-4 and strain 5.18 exhibited slightly lower T_g values of 80 and 83 °C, respectively. Similarly, the T_g of *Paenibacillus* sp. levan was recorded at 79.61 °C.[30] T_c reflects the point at which the crystalline part of the polymer remains solid until the melting temperature (T_m). Among the samples, levan from KKSB-7 displayed the highest T_c value (164 °C), while KKSB-4 exhibited the lowest T_c value (114 °C). Tm represents the temperature at which the polymer becomes a viscous fluid. The T_m values of KKSB-7 (213 °C) and strain 5.18 (210 °C) were slightly lower than the T_m values reported for *B. subtilis* AF17,[31] and *Paenibacillus* sp.,[30] which ranged from 214 to 218 °C. In contrast, the T_m values of KKSB-4 (243 °C) and KKSB-6 (232 °C) were higher and closer to those reported for levan from the halophilic bacterium *Halomonas* sp. AAD6, which had a T_m of 253 °C.[32]

The thermal properties of levan suggest its potential for use in industrial heat processing applications. In this study, the levans showed thermal stability between 114−164 °C with depolymerization occurring at 210−243 °C. These characteristics are consistent with those reported for levans produced by *B. megaterium*, *B. subtilis*, and *Microbacterium laevaniformans*, which have been shown to form semitransparent, cohesive, and adhesive films when combined with ethyl cellulose and glycerol through extrusion processes.[33] Thus, all bacterial levans are promising biopolymers for various biotechnological applications.

3.3. Levan Properties in Water/Oil Holding Capacity and Water Solubility. The freeze-dried levans from the four bacterial strains (KKSB-4, KKSB-6, KKSB-7, and 5.18) appeared as white, rough powders for KKSB-4 and KKSB-6 (Figure 6a), similar to the levan produced by *Bacillus lentus* V8.[34] In contrast, the levans from KKSB-7 and 5.18 were fine powders. However, all levans were classified as high-molecular-weight polysaccharides.

Polysaccharides, including levan, can adsorb water within their molecular structure, which contributes to their texture and flexible and tender polymer characteristics. The levans from the four bacterial strains exhibited a high water adsorption capacity with over 100% of water holding capacity (Figure 6b). Previous studies have reported similar findings with *B. megateruim* and *B. subtilis* showing water holding capacity of 231 and 99%, respectively.[35,36] The high water holding capacity of levan was attributed to its porous structure which increases the surface area of the hydrophilic fructose units in the levan polymer.[37]

Levan is obtained by β-(2,6) bonds of fructose units; it is soluble in both oil and water. Water solubility is an important factor in its application as a food additive, e.g., as surfactants and stabilizers. The levan from KKSB-4, KKSB-6, and 5.18 exhibited a high water solubility index, whereas KKSB-7 had a lower water solubility index.

The oil holding capacity of levan is important for enhancing emulsion stability, flavor retention, palatability, fat absorption, and shelf life extension with potential hypolipidemic activity.[38] In this study, the oil holding capacity varied among levan from the different bacterial strains (Figure 6b). Previous studies have reported oil holding capacity of 36.10% for levan produced by *Bacillus mojavensis*[39] and 35.26% for levan from *B. subtilis*.[14] The oil holding capacity of levan was influenced by the source of polymer and the conditions under which the sample was prepared.[40]

3.4. Antioxidant and Prebiotic Properties of Levan. Ascorbic acid, a known antioxidant, was used as a positive control to compare the antioxidant activities of levan produced by the four bacterial strains. The DPPH inhibition assay revealed that levans from KKSB-6 and KKSB-4 exhibited strong antioxidant activity (Figure 7a), while the levans from KKSB-7 and 5.18 demonstrated around 50% DPPH inhibition. These results suggested that the levan from all four bacterial

https://doi.org/10.1021/acsomega.4c09641
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strains could reduce DPPH free radicals, indicating their potential as antioxidant agents for therapeutic and food applications. The ABTS assay, which measures the ability to scavenge oxidized ABTS radicals, showed that the levans from KKSB-4 and KKSB-6 had a high scavenging activity comparable to that of ascorbic acid (Figure 7b). In contrast, the levans from KKSB-7 and 5.18 showed over 50% scavenging activity. This indicated that levan from all four bacterial strains held strong potential as an antioxidant agent against ABTS radicals. The ferric ion-reducing antioxidant power (FRAP) assay indicated that the levans from all four bacterial strains had low FRAP activity compared to ascorbic acid, which demonstrated 100% antioxidant power at 400 mg L^{-1} (Figure 7c).

Most levans exhibit scavenging activity against DPPH radicals, as demonstrated by *B. subtilis* KB1 with 31% activity[35] and *B. megaterium* PFY-147 with 35% activity.[36] This activity is attributed to the presence of −OH functional groups in the levan structure, which donate electrons to free radicals and terminate free radical chain reactions. A similar mechanism underlies the interaction of levans with ABTS free radicals. To enhance the antioxidant activity, the incorporation of levan with other compounds, such as metal ions, could be explored. For FRAP assay, which is highly dependent on the presence of polyphenols, showed low activity for levan. This result is consistent with the absence of polyphenols in levan's structure, explaining its limited reducing power in this specific assay.

The probiotic activity score (PAS) of the levans, as shown in Figure 7d, revealed that levans from KKSB4 and KKSB6 promoted the growth of *Streptococcus thermophilus* DKT-3 better than inulin which is known for its high prebiotic activity, e.g., promoting the growth of *Lactobacillus casei* at score of 0.75.[41] This suggests that these levans possess a promising prebiotic potential. In contrast, the levans from KKSB7 and 5.18 did not promote growth of *S. thermophiles* DKT-3, indicating a lack of prebiotic properties in these strains. Prebiotic effects are commonly associated with fructooligosaccharides (FOS) or small molecular weight levans, which promote the growth of beneficial intestine bacteria (*Bifidobacterium* and *Lactobacillus*) by increasing short-chain fatty acid production, including propionic, butyric, and valeric acids. A small molecular weight levan (8000 Da) was from *B. amyloliquefaciens* JN4 indicated prebiotic property by regulation the adhesion capacity of *Lactobacillus reuteri* JN101.[42] In contrast, high molecular weight levans, such as those produced in this study, may cause steric hindrance, making them difficult to hydrolyze. Levan requires specific enzymes for degradation, e.g., levanase, β-(2,6)-fructan 6-levanbiohydrolase, or levan fructotransferase. Interestingly, *Lacticaseibacillus paracasei* had been shown to hydrolyze levan due to the presence of a cell wall anchored β−fructofuranosidase, which has a higher affinity for bacterial levan.[43] For *S. thermophilus* strains, no growth was observed on fructans[44] though some studies demonstrated a reduced generation time for *S. thermophilus* TA040 when cultured in skim milk with inulin[45] similar to the findings of this study. The complex structure of high molecular weight levan may affect its hydrolysis, thus influencing its prebiotic potential.

4. CONCLUSIONS

Fermented soybeans (Thua-nao) are rich in *Bacillus* species that contribute to the production of polysaccharides and amino acids, which enhances their flavor profile. The levan-producing *Bacillus* strains from Thua-nao demonstrated significant levansucrase activity. Optimal growth conditions and enzyme production varied between strains, with the sucrose concentration playing a crucial role. Most strains exhibited higher enzyme activity at elevated sucrose levels, except for KKSB6 that showed optimal activity at a lower sucrose concentration. All four bacterial strains efficiently utilized sucrose concentration between 200−300 g L^{-1} for levan production, generating high molecular weight levan (10^7−10^8 Da). These levans exhibited high water and oil holding capacities as well as thermal stability, making them promising candidates for applications in the food industry, such as surfactants, coatings, and encapsulation agents. Additionally, the antioxidant activity of the levans was confirmed; and KKSB4 and KKSB6 demonstrated prebiotic potential by promoting the growth of *S. thermophilus* DKT-3, a strain isolated from kefir milk. Future research will focus on optimizing the production of these *Bacillus* strains and their levansucrase enzymes as well as exploring the potential of levanase enzymes to produce fructooligosaccharides for enhancing their functional applications in various industries.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the Fundamental Fund of Khon Kaen University under the National Science, Research and Innovation Fund (NSRF).

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