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Production, Characterization and Enhancement of Biopolymer Levan from *Lactobacillus fermentum* SHN1

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Abstract

Levan is a highly water-soluble biopolymer that has extensive applications in the pharmaceutical, personal care, food, and industrial fields. *Lactobacillus fermentum* SHN1 was isolated from milk and dairy products as an exopolysaccharide (levan) producer and identified according to morphological, biochemical tests and using the *16S rRNA* gene sequencing. The sequence alignment in the gene bank indicated that the isolate has a high percentage of similarity (100%) to the recovered sequence of *Lactobacillus fermentum* isolate. The produced biopolymer was characterized as levan by FTIR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. The effect of various nutritional and physical factors including pH, carbon source, sucrose concentration, nitrogen source, inoculum size, and incubation period, on the synthesis of levan by *L. fermentum* SHN1 was studied. The findings indicated that the optimal carbon source for the production of levan was 100 g/L sucrose, yielded of 25.95 g/L. Additionally, a combination of peptone + yeast extract at a ratio of 2:0.5 was identified as the most effective nitrogen source for levan production, giving in a yield of 24.21 g/L. *L. fermentum* SHN1 produces the greatest quantity of levan, 23.89 g/L, under a pH of 6.5 and an inoculum size of 1%, resulting in a yield of 25 g/L. The most favorable period for incubation was 48 hours, resulting in a production yield of 24.87 g/L.

Keywords: Biopolymer, Exopolysaccharides, *Lactobacillus fermentum*, Levan

1. Introduction

Levan is known as β -2, 6-linked fructose homopolymer; it has existed in many plants and microbial products. Levan generated by bacteria is considerably larger than that of plants, with numerous branches and molecular weights ranging from 2 to 100 million Daltons (Keith *et al*., 1991; Arvidson *et al*., 2006). Levan is a unique microbial exo-polysaccharide that has numerous industrial applications (Panteli´c *et al*., 2020; Lonˇcarevi´c *et al*., 2019). It is a water-soluble biopolymer consisting of fructose monomeric units that repeat, with a terminal group consisting of D-glucosyl residue. Levan possesses a number of advantageous qualities, including flexibility, biodegradability, biocompatibility, antibacterial activity, antioxidant activity, stimulation the immune system and the anti-inflammatory effects (Srikanth *et al*., 2015a; Costa e*t al*., 2021).

Levan is produced by the extracellular enzyme levansucrase (EC 2.4.1.10) in response to the presence of sucrose as a carbon source (substrate). This enzyme degrades sucrose, constructing levan polymer from the fructose residues (Poli *et al*., 2009). The unique levan polymer is distinguished by its viscosity, capacity to dissolve in water, resistance to acid, alkali and heat, as well as its biological properties and capacity for film formation (Bekers *et al*., 2005). Numerous bacterial species possess the capacity to produce levan, such as *Zymomonas, Azotobacter, Mycobacterium, Erwinia, Corynebacterium, Pseudomonas*, *Bacillus, Brachybacterium phenoliresistens, Bacillus subtilis Lactobacillus, Bacillus amyloliquefaciens and* others (Poli *et al*., 2009; Teixeira *et al*., 2010; Al-Halbosiy *et al*., 2018; Ngampuak *et al*., 2023; Al- Mousawi and Abd- Aljabar, 2018; Sánchez-León et *al*., 2023). In recent decades, wide ranges of microbial extracellular polymers (EPSs) have been identified and thoroughly studied, including their composition, structure, manufacturing, and functional capacities. The heightened fascination with microbial-produced polysaccharides stems from the increasing need for natural polymers in various fields such as food, medicine, and industry in recent times (Costerton *et al*., 1987; Mohammed *et al*., 2021).

Compared to levan produced from plants, microbial levan offers a wider range of applications. It is utilized in food, medicine, aquaculture, and personal care applications (Owner *et al*., 2016).

The objective of this study was the isolation and characterization of a newly bacterial isolate from natural milk and dairy products. The selected *L. fermentum* SHN1 was tested for the production of levan at the production medium, the effect of optimal conditions, carbon sources, nitrogen sources, initial pH, sucrose concentration, inoculum size and incubation time. FTIR, ^{1}H-NMR and ^{13}C-NMR analyses were conducted for levan identification and characterization.

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2. Material and methods

2.1. Isolation of the bacterial isolates

A total of 115 samples from different natural milk and dairy products were collected from local markets in Baghdad province, Iraq. A small amount of each sample was suspended in 10 ml of sterile distilled water. One milliliter from the homogenized suspension was added to 9 ml of sterilized De Man Rogosa-Sharp-broth (MRS broth) (HIMEDIA, INDIA) and incubated anaerobically (via candle jar) for 24hr at 37°C. After incubation, the suspension was serially diluted in a sterile normal saline (NaCl) 0.85% from 10^{-1} to 10^{-5} and streaked on MRS agar plates in duplicates. The plates were incubated anaerobically for 48hr at 37°C. The process was carried out several times to obtain single colonies. Then, the purified colonies were sub-cultured on MRS slants and stored at 4°C as stock cultures. The pure isolates were initially tested for their Gram staining, cell morphology, oxidase and catalase reaction (Ahmed, 2013; Al-Maliki, 2020; Mohsin *et al*., 2020).

2.2. Detection of levan production

2.3. Primary screening of levan production on solid media

The isolated bacterial colonies were transferred to a modified levan screening solid medium composed of g/L: 5.5 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 100 Sucrose, 2 Peptone, 0.5 yeast extract, and 15 agar-agar (Abou-Taleb *et al*., 2015).

The cultured plates were kept anaerobically at 37°C for 48 hr and the isolates with a slimy mucoid appearance were identified as levan producer (Nasir *et al*., 2015).

2.4. Secondary screening of levan production in liquid media

The selected bacterial colonies recorded as levan producer were grown on levan screening broth as previously mentioned (without using agar agar) and incubated anaerobically at 37°C in a shaker incubator for 48 hr. For levan recovery, the broth was centrifuged at 10000 rpm for 10 minutes, and twice the volume of chilled absolute ethanol was added to the supernatant. The mixture was cooled at 4°C for 48 hr., and the precipitated levan polymer was collected using a cooling centrifuge at 8000 rpm for 10 min. The pellet was collected and dried at 50°C for 2-3 days to estimate levan dry weight (Semjonovs *et al*., 2016).

2.5. Molecular identification (genotypic identification)

The most productive isolate was identified by 16s rRNA gene sequencing. Genomic DNA was extracted from the bacterial growth according to the protocol of ABIO. The 16s rRNA from the genome was amplified by the use of the universal bacterial primer (27F: 5-GAGAGTTTGATCCTGGCTAG-3 and 1492R: 5-CTACGGCTACCTTGTTACGA-3. PCR products were sent to Macrogen Corporation in Korea for Sanger sequencing utilizing an automated DNA sequencer (ABI3730XL). The resulting sequences were compared using Basic Local Alignment Search Tool (BLAST) network services at the gene bank databases of the National Center for Biotechnology Information (NCBI) and the accession number was obtained. The Phylogenetic tree was constructed through the alignment of nucleotide sequences using the neighbor-joining algorithm in MEGA 11 software.

2.6. Determination the optimum conditions for levan production

For levan production, various parameters were studied to standardize the fermentation conditions. In each experiment, levan dry weight was determined.

2.6.1. Effect of pH

In order to evaluate the impact of pH values on levan production, the pH of the media was adjusted to various levels (4, 5, 6, 6.5, 7, and 8).

2.6.2. Effect of Carbon source

This experiment was carried out by using different carbon sources which include: Sucrose, fructose, maltose, lactose, glucose and mannose used as alternatives.

2.6.3. Effect of sucrose concentration

To determine the effect of sucrose concentration on levan production, the experiment achieved using six different concentrations of sucrose were used (25, 50, 75, 100, 125, 150) g/L.

2.6.4. Effect of Nitrogen source

The experiment was conducted through the use of six organic and inorganic sources of nitrogen (peptone, yeast extract, KNO_3, NH_4Cl and (peptone + yeast extract together) to investigate their impact on levan production.

2.6.5. Effect of inoculum size

Production of levan by the selected isolate was determined by inoculating the culture medium with 7 different inoculum size (0.5, 1, 2, 4, 8, 10, 15, 20) % of inoculum ($1.5x10^8$ CFU/ml, OD600 = 0.5).

2.6.6. Effect of incubation time

The incubation period required for levan production was evaluated. The selected isolate was incubated anaerobically at 37°C for 12, 24, 36, 48, 60, 72 and 96 hr. separately. Post fermentation, the culture was centrifuged (10000 rpm for 20 min), and the pellets were washed twice with distilled water then dried at 40 °C to determine the biomass (cell dry weight).

2.7. Production and purification of levan

Levan production was carried out by culturing the selected isolate in 250 ml Erlenmeyer flasks containing fifty milliliter of the media used for levan production (5.5 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 100 Sucrose, 2 Peptone, 0.5 yeast extract) gram per liter which autoclaved and then inoculated with 1% ($1.5x10^8$ CFU/ml, OD_{600} = 0.5 on McFarland) of the bacterial inoculum. The flasks were incubated at 37°C for 48hr in a shaker incubator at 120 rpm with N_2 gas flashing to obtain anaerobic conditions.

The culture was centrifuged at 10000 rpm for 10 min to remove the bacterial cells. The supernatant containing levan was collected and mixed with twice the volume of absolute iced ethanol, then cooled at 4°C for 48 hrs. The precipitated levan polymer was collected using a cooling centrifuge at 8000 rpm and dried at 50°C for 2-3 days. After that, dialysis technique was used for the partial purification process. The collected levan pellet was dissolved in a minimum amount of demineralized water,

then dialyzed using a membrane with a cutoff of 10 Kilo Dalton (KD) against deionized water (dH_2O) for a duration of 3 days, with daily water replacement. After that, levan was collected by precipitation using double volume of chilled absolute ethanol. The precipitated polymer was dried in an oven at 50°C for2-3 days to determine the levan dry weight for further analysis (Zhang *et al*., 2014)

2.8. Characterization of levan

2.8.1. Fourier Transform-Infrared spectroscopy (FTIR):

The functional groups present in the partially purified levan polymer were analyzed using Shimadzue FTIR spectrophotometer (Shimadzu- Japan) using KBr pellets. The spectrum was recorded at a wave range of (4000 to 400) cm^{-1}. Furthermore, the FTIR spectra of the standard levan from *Erwinia herbicola* sigma- Aldrich were applied to compare the spectra of the current study with standard levan.

2.8.2. Nuclear Magnetic Resonance (NMR)

The spectra of ^{1}H and ^{13}C NMR were measured and recorded using UXNMR, a Bruker instrument. Levan was solubilized in dimethyl sulfoxide (DMSO) for both analyses. The ^{1}H NMR spectrum was conducted at a frequency of 400 MHz, whereas the ^{13}C NMR spectrum was conducted at a frequency of 100 MHz. The chemical changes were determined and reported in parts per million (ppm). Also, ^{1}H and ^{13}C NMR spectra of the standard levan from *E. herbicola* sigma- Aldrich were detected for comparison.

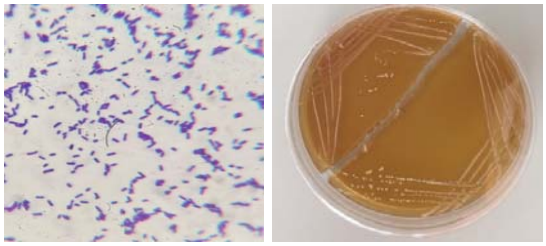
2.9. Statistical analysis

The effect of different parameters on levan production were tested statistically using *T-test*, one-way ANOVA and Tukey's test to assess the data's significance at $p < 0.05$. The average mean values were reported along with standard deviation. The softwares used for the analysis are (R Studio and the figures by Origin software).

3. Results and discussion

3.1. Isolation of Lactobacillus Isolates:

A total number of 27 isolates (23%) have been collected from various sources of dairy products (yogurt, cheese, butter, labneh, milk, cream, and whey) from local markets were belong to the genus *Lactobacillus*. All of these isolates were gram-positive, cocci, organized singly, pairs or short chains, non-motile, oxidase and catalase negative (Jameel and Haider, 2021; Jeyagowri *et al*., 2023). These isolates were identified using morphological, microscopical, and biochemical tests. Their morphological characteristics showed that they belong to the genus *Lactobacillus* by their small (2-5mm), creamy, smooth round, little sticks colonies and opaque without pigment on MRS agar as shown in Figure (1) (Taye *et al*., 2021; Hussein and Luti, 2023).



A B

Figure 1: Cells and colony morphology of *Lactobacillus spp*. A- Bacterial cells under a microscope using oil emersion lens (100×) B- Colonies growing on MRS agar.

3.2. Primary screening on solid media

Levan production had been determined in all 27 isolates of *Lactobacillus* spp. The identification and evaluation of levan production were documented based on the observation of mucoid and viscous colonies on the surface of the levan screening medium. The data presented in Table (1) indicated that 15 isolates only had a viscous mucoid appearance which were subsequently employed in a secondary screening process.

The screening for levan was conducted using sucrose-rich media as a substrate which induces the enzymatic activity of levansucrase enzyme (also known as fructosyltransferase) (Nasir *et al*., 2015). Its main function is to facilitate the development of ß-(2,6)-levan by breaking down sucrose into its constituent fructose and glucose units. Furthermore, it participates in the synthesis of fructooligosaccharides (FOS) (Vieira *et al*., 2021). Moreover, the morphological findings indicated a viscous and mucoid appearance, consistent with (Nasir *et al*., 2015; Alshammery and Alaubydi, 2020; Hamada *et al*., 2022). The identification of EPS in lactic acid bacteria is accomplished through the observation of the slimy and mucoid characteristics of the colonies on a solid medium (Tsveteslava and Ivanov, 2016; Chun-lei *et al*., 2014). In their study, Ahmed *et al*. (2022) successfully isolated a strain capable of making levan, which was identified as *Lactobacillus reutri*. They noticed that when sucrose was present, the colonies exhibited a slimy mucoid look, indicating the formation of extracellular polymeric substances (EPS) from sucrose. Also, Mamay *et al*. (2015) observed that the presence of levan was identified based on the slimy appearance of the colonies on solid media.

Table 1: Primary screening for levan synthesis by *Lactobacillus* spp. isolates on solid medium

No.	Bacterial isolates	mucoid growth	No. of isolates
1	Lactobacillus spp.	+++	LA3
2	Lactobacillus spp.	+	LA6
3	Lactobacillus spp.	-	LA8
4	Lactobacillus spp.	-	LA12
5	Lactobacillus spp.	-	LA13
6	Lactobacillus spp.	-	LA14
7	Lactobacillus spp.	-	LA15
8	Lactobacillus spp.	-	LA17
9	Lactobacillus spp.	++	LA19
10	Lactobacillus spp.	+	LA20
11	Lactobacillus spp.	+	LA21
12	Lactobacillus spp.	-	LA22
13	Lactobacillus spp.	-	LA26
14	Lactobacillus spp.	-	LA27
15	Lactobacillus spp.	-	LA29
16	Lactobacillus spp.	+++	LA30
17	Lactobacillus spp.	+++	LA31
18	Lactobacillus spp.	-	LA32
19	Lactobacillus spp.	+	LA33
20	Lactobacillus spp.	+	LA34
21	Lactobacillus spp.	++	LA35
22	Lactobacillus spp.	-	LA36
23	Lactobacillus spp.	+	LA37
24	Lactobacillus spp.	++	LA38
25	Lactobacillus spp.	+++	LA39
26	Lactobacillus spp.	+	|LA40
27	Lactobacillus spp.	++	LA41

+++: high levan production, ++: moderate levan production, +: weak levan production, - : no levan production

3.3. Secondary screening on liquid media

Based on the results of primary screening, levan concentration was estimated. The results presented in Table 2 showed that the isolate *Lactobacillus* spp. (LA31) had the highest productivity of levan, with an average concentration of approximately 23 g/L (dry weight). The remaining isolates had concentrations ranging from 8.85 g/L to 17.6 g/L. Therefore, the isolate (LA31) was selected for the remaining experiments in the current study.

Table 2: Secondary screening for levan production by *Lactobacillus spp*. isolates in liquid medium

Bacterial isolates	Levan dry weight Average g/L
Lactobacillus spp. (LA3)	8.85
Lactobacillus spp. (LA30)	11.5
Lactobacillus spp. (LA31)	23
Lactobacillus spp. (LA35)	17.6
Lactobacillus spp. (LA38)	10.4
Lactobacillus spp. (LA39)	13.25

3.4. Molecular identification of Lactobacillus spp. (LA31) by 16s rRNA:

According to the findings of *16S rRNA* gene sequence analysis, the isolate LA31was identified as *L. fermentum* SHN1 (new scientific name, *Limosilactobacillus fermentum*). The nucleotide sequence was submitted and deposited at GenBank under the accession number OQ588768. The neighbor-joining method was employed to conduct a phylogenetic study of *L. fermentum* SHN1 (accession number OQ588768). The results revealed a substantial level of similarity and close clustering with other *L. fermentum* strains retrieved from the NCBI GenBank database (Figure 2).

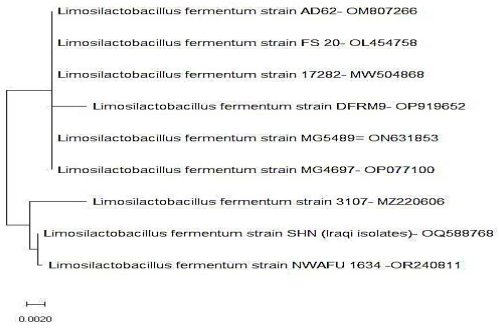


Figure 2: Phylogenetic tree represents the relationship between the sequence of the 16S rRNA gene of *L. fermentum* SHN1 and the related isolates. The phylogenetic tree was created with MEGA11 using neighbor-joining method.

3.5. Determination of the optimal conditions for levan production

3.5.1. The effect of pH on levan production

The experiment involved testing several pH values (4, 5, 6, 6.5, 7, and 8) to determine the optimal pH for levan synthesis. The production of Levan by *L. fermentum* SHN1 demonstrated a gradual and consistent rise with an increase in pH values from 6 to 6.5 (Figure 3). *L. fermentum* SHN1 exhibited its maximum levan production of 23.89 g/L at a pH of 6.5. Belghith *et al*. (2012) deduced that the enzyme levansucrase exhibited its optimal activity at a pH of 6.5; this finding could potentially account for the observed increase in levan production. Abou-taleb *et al*. (2015) also found that the maximum quantity of levan produced by *Bacillus lentus* V8 strain on a sucrose supplemented medium occurred at a pH of 6.5. The findings obtained from our work are in consistence with those of Khassaf *et al*. (2019) who reported that the optimum pH for levan production by *Bacillus subtilis* was 6.5.

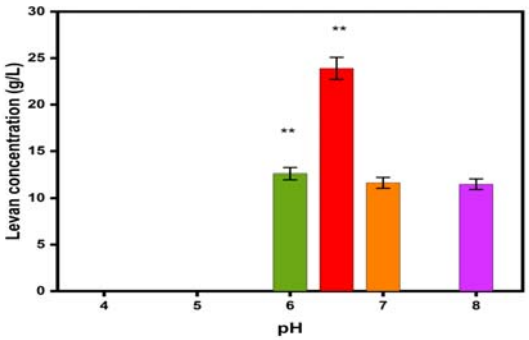


Figure 3: The effect of initial pH values on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P \leq 0.05$), (**$P \leq 0.01$) and (***$P \leq 0.001$).

The effect of carbon sources on levan production

To find out the optimal carbon source for levan yield enhancement, five different carbon sources (fructose, maltose, lactose, mannose, glucose, and sucrose) were employed. Based on the results, sucrose was shown to be the most effective carbon source for levan production by *L. fermentum* SHN1 resulting in a yield of 24.4 g/L. In comparison, mannose, fructose, maltose, glucose, and lactose yielded (2.1, 3.9, 5.1, 9, 11) g/L, respectively (Figure 4). Using fructose as the exclusive carbon source leads to a reduction in levan synthesis. According to reports, the production of levan declined when fructose was utilized instead of sucrose in the productive medium. These findings suggest that sucrose is the most effective stimulant and material for the enzyme levansucrase (Van Hijum *et al*., 2006; Al-qaysi *et al*., 2016; Moussa *et al*., 2017). Other bacterial species that demonstrate similar results regarding sucrose as the most favorable carbon source include, *B. licheniformis* (Dahech *et al*., 2012), *B. lentus* (Abou-Taleb *et al*., 2014) and *Z. mobilis* (Senthilkumar and Gunasekaran, 2005).

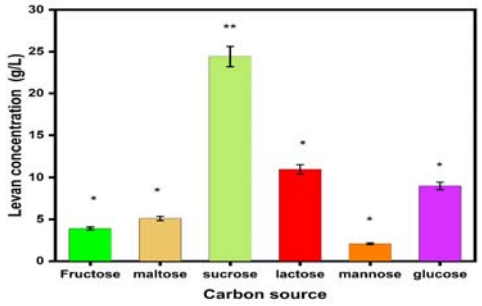


Figure 4: The effect of different types of carbon sources on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P \leq 0.05$), (**$P \leq 0.01$) and (***$P \leq 0.001$).

The effect of sucrose concentration

The amount of levan produced by *L. fermentum* SHN1 was affected by using different concentrations of sucrose. The findings indicated that the maximum concentration of levan (25.95 g/L) was identified at 100 g/L. Further increase in sucrose concentration led to an extreme decrease in the yield of the produced levan (Figure 5). It has been determined that the concentration of sucrose is the most significant factor influencing levan molecular weight (Wu *et al*., 2013). *Leuconostoc mesensteroides* produced a similar result, with the maximum levan yield at 10% sucrose content (Khudair *et al*., 2018). Also, Chidambaram *et al*. (2019) reported that levan production by *B. subtilis* was optimized at a sucrose concentration of 100 g/ L. On the other hand, Aramsangtienchai *et al*. (2020) reported that levan production by *Tanticharoenia sakaeratensis* increased steadily from 2.1 g/ L to 24.7 g g/ L as the sucrose concentration increased from 50 g/ L to 200 g g/ L.

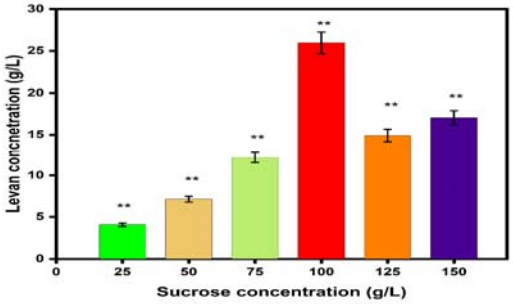


Figure 5: The effect of different concentrations of sucrose on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P \leq 0.05$), (**$P \leq 0.01$) and (***$P \leq 0.001$).

The effect of nitrogen sources on levan production

A total of six nitrogen sources, namely peptone, yeast extract, casein, KNO3, NH4Cl, and a combination of peptone and yeast extract, were used in order to determine the optimal nitrogen source for levan synthesis. As illustrated in Figure (6), a combination of peptone + yeast extract at a ratio (2:0.5) was the optimal nitrogen source for levan synthesis, resulting in a yield of 24.21g/L. The data presented in Figure 6 clearly demonstrate that the nitrogen sources have a significant impact on the synthesis of levan by *L. fermentum* SHN1 strain. The highest levan dry weight was achieved when (peptone+ yeast extract) was used as a nitrogen source, while the lowest levan dry weight (6.1 g/L) was obtained when yeast extract was used alone. Lactic acid bacteria (LAB) are highly specialized microbes that grow on complicated organic substances. These bacteria need carbohydrates, nucleotides, amino acids, peptides, vitamins and minerals for their growth because of the absence of specific metabolic pathways.

For example, in the absence of exogenous amino acids, LAB cannot grow at the expense of mineral nitrogen (Morishita *et al*., 1981). To meet their nutritional requirements in complex growth media, expensive and undefined compounds such as peptone, meat extract, and yeast extract are typically added (De Man *et al*., 1960). In line with our discoveries, de Oliveira *et al*. (2007) confirmed that yeast extract played a crucial role in the formation of levan. The combination of yeast extract with KH_2PO_4 and yeast extract with $MgSO_4$ was found to have a substantial impact on levan production. Additionally, Silbir *et al*. (2014) discovered that yeast extract produced the greatest amount of levan in comparison to other nitrogen sources. The greatest EPS production by two strains of *Lactobacillus plantarum* isolated from cow milk was obtained when yeast extract used as a N_2 source. This effect is explained by the composition of yeast extract which includes amino acids, peptides, carbohydrates, and salts (Imran *et al*., 2016).

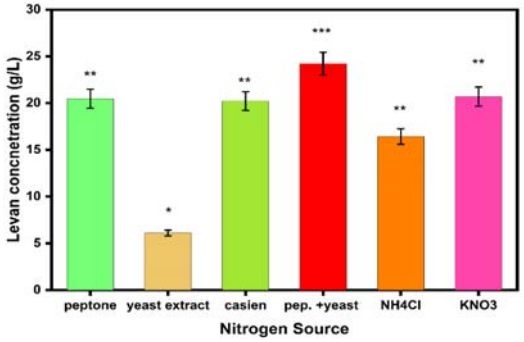


Figure 6: The effect of different types of nitrogen sources on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P \leq 0.05$), (**$P \leq 0.01$) and (***$P \leq 0.001$).

The effect of inoculum size

The impact of various inoculum volumes on the production of levan was examined and illustrated in Figure (7). *L. fermentum* SHN1 was incubated with various inoculum sizes (0.5, 1, 2, 4, 8, 10, 15, 20) % of inoculum (1.5x10 ^{8} CFU/ml, OD600 = 0.5). The findings indicated that an inoculum volume of 1% was the optimal for levan production, resulting in a yield of 25 g/L. On the other hand, using of 2% inoculum size resulted in a reduced levan dry weight to 17.52 g/L.

The reduction in the production of levan could be associated with the elevation in cell growth in the production media, which resulted in the consumption of nutrients in a short period of time that leading to a quick consumption of carbohydrates and decreased levan production. The findings were consistent with those of Khassaf *et al*. (2019) who reported that the greatest quantity of levan was generated when an inoculum volume of 1% was employed. In addition, Küçükaşik *et al*. (2011) employed a 1% inoculum size to achieve the maximum levan synthesis from molasses by *Halomonas* spp. However, the current study differs from what some other researchers found when using of 5% inoculum size for levan production (Dahech *et al*. 2014). This could be attributed to variations in the growth rate among various isolates. Additionally, the isolate employed in this study is regionally isolated, which means that its development and production may differ from that of strains and isolates that are universal.

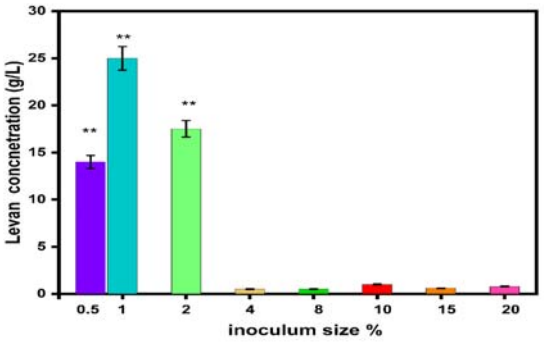


Figure 7: The effect of inoculum size on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P \leq 0.05$), (**$P \leq 0.01$) and (***$P \leq 0.001$).

3.5.2. The effect of the incubation period

Production of levan was estimated at various incubation periods (12, 24, 36, 48, 60, 72 and 96 hr.). The maximum levan level produced by *L. fermentum* SHN1 was 24.87 g/L and biomass of 8.25 g/L was achieved after 48hr of incubation (Figure 8).

The findings align with previous studies that have reported the highest yield of levan synthesis by *L. reuteri* FW2 was after incubation for 48 hours (Ahmed *et al.*, 2022). Based on another investigation, *Z. mobilis* achieved the maximum levan production after 42 hours of incubation (Silbir *et al.*, 2014). Sims *et al*. (2011) conducted a separate investigation and found that the highest amount of levan produced by the gut bacteria *L. reuteri* 100–23 was achieved after a period of 96 hr. of incubation. Under optimal conditions, the probiotic *Bacillus tequilensis*-GM produces 2.8 g/L of levan following 72 hr. of incubation (Abid *et al.*, 2019).

The isolate *L. fermentum* SHN1, isolated in this work, exhibits a significant capacity to produce abundant quantities of levan in a shorter incubation period compared to prior research. Consequently, it holds great potential for commercial applications.

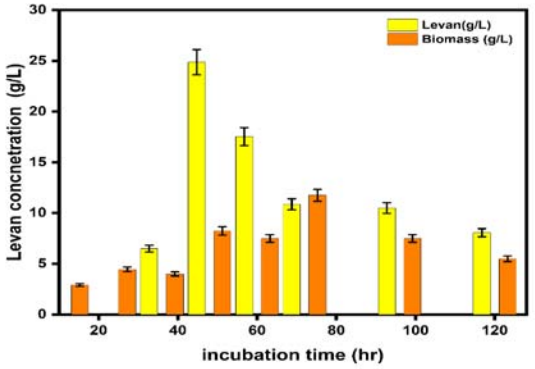


Figure 8: The effect of different incubation periods on levan production by *L. fermentum* SHN1 isolate. The experiment was repeated as triplicate and the values are represented as mean ± SD at (*$P < 0.05$), (**$P < 0.001$) and (***$P < 0.0001$).

3.6. Characterization of levan

3.6.1. Fourier Transform-Infrared spectroscopy (FTIR):

The Fourier Transform Infrared (FTIR) spectrum of *L. fermentum* SHN1 levan exhibited multiple distinct peaks that are indicative of its unique properties (Figure 9). The distinctive peak corresponding to the stretching of the O-H bond of polysaccharide was observed at about 3406.05 cm^{-1}. A medium C-H stretching vibration has been detected at 2935.46 cm^{-1}and C=O stretching was detected at 1650.95 cm^{-1}(Salman *et al.*, 2019). The peaks between 1126 and 900 cm^{-1} correspond to the characteristic features of polysaccharides (Kadhum and Haydar, 2020; Sánchez-León et *al*., 2023).

The observed three peaks at 1128.28 cm^{-1}, 1060.78 cm^{-1}, and 1014.49 cm^{-1} correspond to the ring vibration of C-OH groups and the glycosidic linkage C-O-C stretching vibration (Srikanth *et al.*, 2015b). The stretching vibration of the presence of C-H and the band at 1650.95 cm^{-1} were attributed to the bounding of water (Xu *et al.*, 2016). In addition, the presence of the furanoid ring and the bending vibration of D-type C-H in furanose were indicated by

peaks in the range of 927-810 cm-1, which are characteristic of carbohydrates (Ahuja *et al.*, 2013). As summarized in Table 3, the data revealed that the polymer synthesized by *L. fermentum* SHN1 was levan.

The FTIR spectrum obtained in this work from *L. fermentum* SHN1 is in agreement with the FTIR spectra of levan produced by *Pantoea agglomerans* ZMR7 (Al-Qaysi *et al.*, 2021), *Brachybacterium phenoliresistens* (Moussa *et al.*, 2017), and is similar to the standard levan from *E. herbicola* (Figure10).

Table 3. The comparison of FTIR values of levan produced by *L. fermentum SHN1* with other bacteria

Chemical groups	*E. herbicola* Standard levan (cm^{-1})	*B. phenoliresistens* Levan (cm^{-1})	*P. agglomerans* Levan (cm^{-1})	*L. fermentum* SHN1 Levan (cm^{-1}) this study
O-H	3429.20	3394.1	3417.68	3406.05
C-H	2927.74	2932.23	2935.66	2935.46
C=O	1637.45	1647.88	1639.49	1650.95
References		(Moussa et al., 2017)	(Al-Qaysi et al., 2021)	

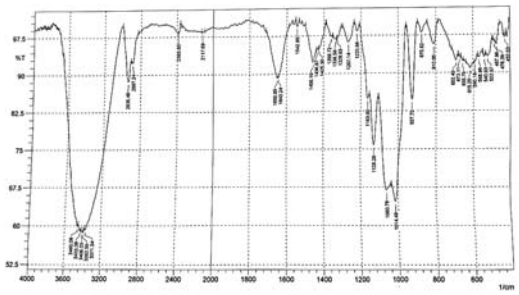


Figure 9: FT-IR spectrum of the levan obtained from *L. fermentum* SHN1 isolate.

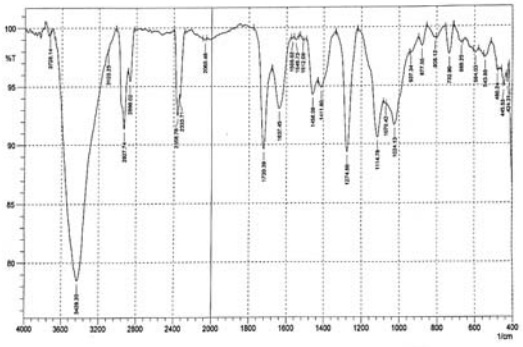


Figure 10: FT-IR spectrum of the Standard levan obtained from *E. herbicola.*

3.6.2. Nuclear Magnetic Resonance (NMR)

The ^{1}H-NMR spectra of levan exopolysaccharide produced by *L. fermentum* SHN1 displayed distinct signals that are indicative of levan. The proton signals were detected within the chemical shift range of 3.27 to 5.16 ppm (Figure11). The spectrum revealed that the isolated molecule exhibits proton signals that cannot be interchanged and correlated to the resonance of the standard levan from *E. herbicola* (Figure12). In addition, the composition of the produced levan by *L. fermentum* SHN1 was verified by using ^{13}C-NMR spectroscopy analysis as shown in Table 4. The ^{13}C NMR spectra exhibited six prominent resonances at 61.04, 104.68, 76.39, 75.75, 80.65, and 63.37, which were designated, in that order, for the C1 through C6 atoms comprising the structure of levan (Figure 13). Significantly, the ^{13}C NMR spectrum of EPS S81 displayed a signal at 63.2 ppm (C-6) that was displaced towards the lower end of the spectrum. This signal verified the existence of a β-(2 → 6) linkage, thereby establishing the structure as levan rather than inulin (Xu *et al.*, 2016; Taylan *et al.*, 2019). The signal alterations observed were consistent with those of other levans generated by different bacteria, including *Bacillus subtilis* MTCC441 (Veerapandian *et al.*, 2023), *P. agglomerans* ZMR7 levan (Al-Qaysi *et al.*, 2021), *B. phenoliresistens* (Moussa *et al.*, 2017) and *E.herbicola* (Figure 14).

Table 4: Comparison of ^{13}C NMR values of *L. fermentum* SHN1, and other bacteria

Carbon atom	Standard levan from *E. herbicola*	*B. phenoliresistens levan*	*P. agglomerans* levan	*L. fermentum* SHN1 *levan* this study
C-1	60.98	59.073	60.58	61.04
C-2	104.68	103.834	104.38	104.68
C-3	75.69	7.0217	75.83	76.39
C-4	76.30	74.183	76.74	75.75
C-5	80.62	82.253	80.35	80.65
C-6	63.37	62.007	63.15	63.37
Reference		(Moussa *et al.*, 2017)	(Al-Qaysi *et al.*, 2021)	

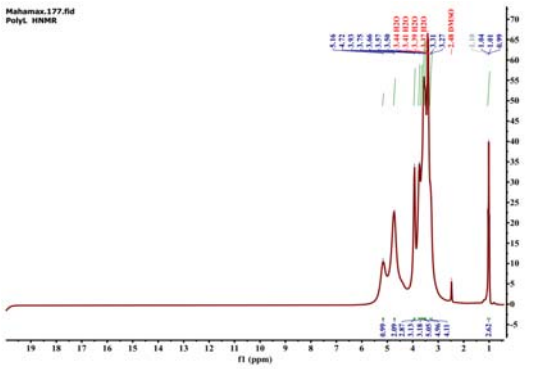


Figure 11: ^{1}HNMR spectrum for levan obtained from *L. fermentum* SHN1 isolate.

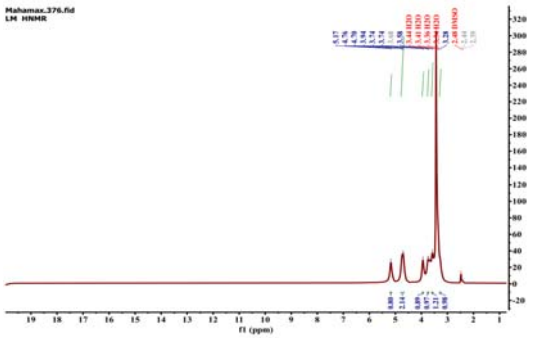


Figure 12: [1]HNMR spectrum for standard levan obtained from *Erwinia herbicola.*

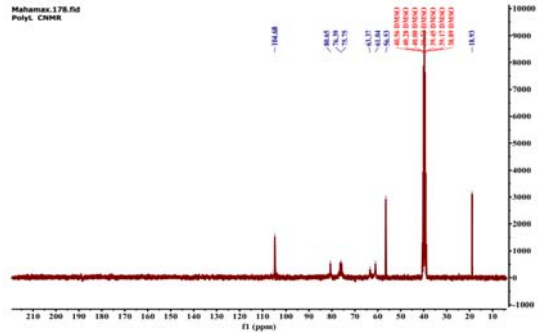


Figure 13:[13]C NMR spectrum for levan obtained from *L. fermentum* SHN1 isolate.

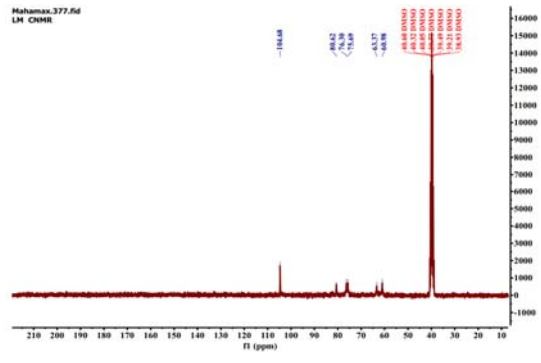


Figure 14: [13]C NMR spectrum for standard levan obtained from *Erwinia herbicola*

4. Conclusion

Based on previous findings, a novel levan (EPS) producing isolate of *L. fermentum* SHN1was isolated from milk and dairy products. This isolate was identified using phenotypic characteristic and *16S rRNA* gene sequencing. The production of levan was enhanced using optimized conditions (pH, carbon source, sucrose concentration, nitrogen source, inoculum size and incubation time). The partially purified biopolymer was characterized by FTIR and NMR. These analyses confirmed the presence of the functional groups within the levan structure and the similarity with the standard levan.

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