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Research Article

# Production of Levansucrase from Local Isolate *Bacillus Lichniformans* MJ8 and Enzymic Synthesis and Characterization of Levan

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## ABSTRACT

From fifty purified *Bacillus* spp. Isolates, twenty-six isolates of Levansucrase producing were found. They were isolated from different sources in Baghdad. These isolates were screened for their abilities to produce Levansucrase, it was found that the isolates designated S7, S8, S9, S10, S11, S12, S13, S14, F3, F4, and F5 (as named in this study) the higher producers of this enzyme by Levan formation in the sticky mucous membrane visible around the colonies, which was considered a primary indicator of the production Levansucrase. All isolates were culturally and morphologically identified to confirm they belong to the genus *Bacillus* sp. The secondary screening was performed on these isolates by estimation Levansucrase assay on the medium of Mineral Salts Broth modified by adding 20% sucrose, as the bacterial isolate *Bacillus* sp. S8 was obtained from the soil near the rhizosphere of the stevia plant and was superior in the amount of Levansucrase produced, reaching 529.87 U/ml. The identification tests of this isolate were carried out by studying the biochemical tests using the Vitek2 compact system. The test results were confirmed by 16S rRNA gene identification using Polymerase Chain Reaction (PCR) and its nitrogen base sequencing. The results revealed that this isolate belongs to this local isolate *Bacillus licheniformis* and identified *Bacillus licheniformis* strain MJ8, and it was registered under the Accession Number in Gene Bank: OM672244.1. The Levan was identified and character after extracting from the efficient local isolate using a High-Performance Liquid Chromatography (HPLC) technique, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), and Atomic Force Micrograph (AFM) analysis.

**Keywords:** Levansucrase; HPLC; 16S rRNA; *Bacillus licheniformis*; Vitek2; PCR

## INTRODUCTION

Levansucrase E.C (2.4.1.10) uses fructose residues to build up Levan, which belongs to family 68 of glycoside hydrolases. Levansucrase hydrolyzes sucrose, liberates glucose, and transfers fructose molecules to a growing Levan Fructooligosaccharide (L-FOS) chain [1]. Levan, a common homopolysaccharide, is composed of fructose units predominantly by β-(2→6) glycosidic linkage in the main chain with β-(2→1) linkage at branch points, making the fructooligosaccharide a unique carbohydrate polymer. Levan is produced by plants and microorganisms, including many algal cells, yeasts, fungi, and bacteria [2-4]. Previous studies have suggested that Levan can be involved in biological stress resistance in bacteria and plants, such as modulating the osmotic pressure of cells and improving drought resistance, salinity resistance, and low-temperature protection [5-7]. Besides, Levan has broad applications in many fields due to its functional and vital properties, found that Levan forms a hydrocolloid microgel, which can be used as an ingredient in wheat bread to extend the shelf life of products [8]. Levan could play a fundamental role in solving peptic ulcer problems [9]. Several other studies have confirmed that Levan has prebiotic characteristics [10,11] and antioxidative [12], anti-obesity [13], antifungal [14], antidiabetic [15], and antitumor [16] effects. Because of Levan's excellent properties and application prospects, researchers must improve its yield and quality.

Given the lack of studies that dealt with the production of this polymer in Iraq, this study aimed to isolate bacteria that can produce Levansucrase enzyme obtained from different sources in Baghdad and identify the best producer by Vitek 2 compact system and 16S rRNA gene nitrogen base sequencing. Purification and Identification of Levan by High-Performance Liquid Chromatography (HPLC) and Fourier-transform Infrared Spectroscopy (FTIR).

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## MATERIALS AND METHODS

### Sources of isolation

The sources of insulation included samples of rotten fruit, natural juice residues, sweets ready for consumption from vendors' carts, rancid jam, and different soils taken from other areas in Baghdad. The Distilled water pH 7.0 was used to suspend 10 grams of each sample and treated at 80°C for 10 minutes to kill the vegetative cell of the bacteria. 1 ml suspension was added to 20 ml of nutrient agar and incubated at 37°C overnight. The single colonies with the morphological characteristics of *Bacillus* sp. were selected and subjected to primary screening.

### Identification of isolates

The taxonomic keys are mentioned in Bergey's manual of systemic bacteriology [17,18]. The process of identifying isolates *Bacillus* sp. was adopted depending on the cultural and morphological characteristics.

**Primary screening:** The isolates were then screened for the ability to produce Levansucrase by streaking on Mineral Salts Agar modified medium was adopted according to [19] which contains 20% sucrose, 0.3% of $KH_2PO_4$, 0.3% of $K_2HPO_4$, 0.3% of $MgSO_4.7H_2O$, and 1.5% of Agar, the pH adjusting to 0.7 and incubated at 37°C for 48 hours. The ability of the isolates was revealed by the high formation of a sticky mucous membrane around bacterial growing colonies on the surface as an indicator of their ability to produce the enzyme and Levan formation. This isolate was subjected to secondary screening.

**Secondary screening (Levansucrase Production):** A loopful of the bacteria purified isolates was activated in Nutrient Broth (NB) for 24 hours at 37°C, ($2 \times 10^8$ cells/ml) of activated bacterial was transferred to Levansucrase production Mineral Salts Broth modified medium containing 20% sucrose was adopted according to Shih, et al. The pH of the medium was adapted to pH 7.0, an incubator shaker with a speed of 150 rpm at 37°C for 48 hours [19]. The growth cultures were filtered by centrifuged at 10000 × g for 20 min at 4°C, and the clear supernatant was the crude enzyme.

### Levansucrase activity assay

The amount of reduced sugar was determined by a spectrophotometer at A540 Straight Line Equation (y=0.7171 × -0.004). Standard glucose was used as a calibration curve [20]. Levansucrase assay in crude enzyme was determined according to [21]. The assay reaction Incubation at 37°C for 30 minutes contained mixed 0.5 ml crude enzyme and 0.5 ml substrate (5% sucrose in 100 ml sodium phosphate buffer pH 7.0). The reaction stopped by adding 1 ml of 3, 5 Dinitro Salicylic acid reagents (DNS), and the tubes were heated for 5 minutes. The absorbance was consistently at 540 nm. One enzyme (unit/ml) is defined as the amount of enzyme that catalyzes the liberation of 1 µml glucose per minute under the assay conditions.

### Identification of the most efficient local isolates *Bacillus* sp. (S8)

**Vitek2 of compact system identification:** Identifying the isolate (S8) was achieved by a special diagnostic kit for the Bacillaceae family (BCL), containing 46 biochemical tests. The procedure was followed according to the instructions of the manufacturer BioMerieux.

**16S rRNA gene Identification:** The DNA of bacteria was extracted by using a DNA extraction kit [22], the PCR amplified the gene using primers, forward (5`AGAGTTTGATCCTGGCTCAG3) and reverse (5`TACGGYTACCTTGTTACGACTT3) [23]. Amplification by (PCR) with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and a final extension at 72°C for 7 min.

### Electrophoresis for amplification products

The product of PCR has relocated in 1% Agarose gel loaded a 2 µl DNA ladder 1500 bp for 45 min at 90 V. After that, to analyze the nucleotide sequence, the product was sent to the Macrogen Company.

### Levan isolation and purification

The method described by Srikanth, et al. was adopted in extracting Levan from the Mineral Salts Broth medium containing 20% sucrose and after the incubation period ends, transferred to a water bath at boiling to 100°C for 30 min and left to cool. Centrifugation was carried out at 10000 × g for 30 minutes to obtain two layers of the supernatant and sediment [24]. To eliminate the effectiveness of the extracellular enzyme's remnants in the supernatant, it was returned to the water bath for 5 min and left at 25°C for cooling and then increasing the pH of the supernatant within a range of 9.0-10.0 using potassium hydroxide 1 M KOH. Afterward, chilled ethanol was added at -20°C and a concentration of 80% at 1:2 (volume of the solution: volume of the extraction solvent). Then 1 ml of $CaCl_2$ was added to it at a concentration of 1% with continuous stirring for 20 min to enhance the precipitation process of the Levan and left to the next day preserved at a temperature of 4°C to form an easy to separate aqueous layer. The precipitated granules were collected by centrifugation at 10000 × g for 15 min and washed again with chilled ethanol of the same concentration of 80% at a rate of 1:4 (weight: volume of extraction solvent), and placed in a pre-prepared sterile glass petri dish and left at 45°C to dry. The purification stage was then carried out according to the method described by Dahech, et al. in purifying the Levan obtained by re-dissolving it again after being dried with De-Ionized Water (DIW) [25]. Then a dialysis process was performed with dialysis bags prepared for this with a pore diameter ranging from 12-14 kDa, which was placed in a container containing deionized water and left at 4°C for 72 hours, with replacing water two to three times a day. This diameter was mentioned by Gonzalez, at al. which is the elimination of low molecular weights from proteins, nuclear acids, or other organic compounds that can be found in the middle of fermentation [26]. Subsequently, a lyophilized operation at -55°C was performed using the Christ Alpha 1-2 LD lyophilized device of German origin to obtain a dry powder and store it in the refrigerator.

### Identification of levan

**High-Performance Liquid Chromatography (HPLC) analysis:** This analysis was carried out using the USA HPLC system; The aqueous extract was separated on Fast Liquid Chromatographic (FLC) $NH_2$ column, 3 µm particle size (50 × 4.6 mm) refractive index detector, Shimadzu RID-10A Mobile phase: deionized water: acetonitrile (50:50, V/V), flow rate 0.1 mL/min, temperature 30°C, injection volume 20 µl. A sample (1 mg) of a standard Levan produced from Erwinia herbicola bacterial isolation, which is

supplied by the German company Sigma-Aldrich. Moreover, under experimental conditions, each of the standard solutions of sugars (fructose, glucose, and sucrose), were dissolved in 10 ml of the mobile phase to the obtained standard of 100 µg/ml and the same for Levan and diluted to 25 µg/ml for each standard, then eluted on HPLC under the optimum separation conditions. The resulting solution was filtered again through a 0.45 um pore size membrane (Millipore) and then chromatographed using isocratic elution with an acetonitrile-water mixture (50:50, v/v) at a flow rate of 1 mL/min and the column temperature of 40°C.

**Fourier Transform Infrared Spectroscopy (FTIR) analysis:** This analysis has been carried out using (Bruker–Tensor 27 with an ATR unit). Compared with the standard sample Levan from Erwinia herbicola (Sigma-Aldrich) and local isolate *B. lichniformans* MJ8. The instrument operates in the wave number range of (600-4000) which measures the amount of IR radiation reflected or transmitted through a sample. The result is obtained in a graphical chart, in which the X–axis represents the wave number while the Y-axis represents the transmittance %.

**Scanning Electron Microscope (SEM) analysis:** Scanning Electron Microscope (SEM, Axia ChemiSEM) technology was used to determine the surface morphology of the Levan. The SEM stubs were attached to the freeze-dried pure Levan using double-faced adhesive tape before having the gold coating applied in an ion sputtering device. At a 10 kV acceleration voltage, the microstructures of samples at various magnifications were examined. SEM pictures of Levan samples at magnifications of 2500, 5000, 7000, and 13000x were collected for this investigation.

**Atomic Force Micrograph (AFM) analysis:** Atomic Force Micrograph (AFM, FlexAFM) analysis was used to determine the surface morphology and roughness of Levan. In an airtight container, the 1 mg/ml Levan solution was created and constantly swirled for 1 hour at 40°C. 5 L of the Levan solution was absorbed into the mica sheet after it had reached room temperature. Levan's AFM pictures were captured in tapping mode after a drying period at room temperature.

## RESULTS AND DISCUSSION

### Isolation and primary screening

The isolation and primary screening process were carried out simultaneously by applying selective pressure on the isolation sources. The main aim was to obtain the bacterial isolates from *Bacillus* spp. The selective isolates, which colonies have high viscosity, Therefore, these isolates were characterized by their wavy edges, rounded, slightly lobed, irregular shape, creamy, brownish in color, and sticky at times [27,28]. The results of the morphological tests also showed that they possess microscopic characteristics similar to the genus *Bacillus*, in that they possess bacillary or semi-bacillary forms that are grouped in the form of short chains and often in the form of pairs. It also can move, using the flagella that enable it to move in liquid and semi-liquid mediums, gram-positive, endospore-forming, with the difference in the spore location according to the isolate species. These characteristics agree with what was mentioned by the taxonomic sources approved and are unique in identifying *Bacillus* bacteria [18]. The isolates that show high viscosity around their colonies in the form of slimy mucoid spots on the surface as an apparent indication of their ability to produce the enzyme Levansucrase as a result of fermentation of sucrose added to isolation media using the Mineral Salts Agar (MSA) modified by adding sucrose at a rate of 20%. And it is worth noting that the mucoid name method was launched on this method as one of the indications for the positive examination in the two media. Thus, 26 isolates were obtained from different isolation sources, distinguished by their ability to produce sticky material around their colonies, eleven of which are S7, S8, S9, S10, S11, S12, S13, S14, F3, F4, and F5 isolated from the soil of the potato crop, soil of sativa plant, persimmon fruit. A rate of 42.30% was distinguished by their high viscosity similar to the glue around their colonies (colloidal white colonies), in addition to the amount of sticky material produced, which are among the critical indicators of the ability of these isolates to produce the Levansucrase (Levan) as shown. However, eight isolates from different isolation sources, at a rate of 30.76% showed a lower viscosity than their predecessor's medium around their colonies. During the same 48-hour incubation period, besides, three isolates in the same incubation period gave a very weak viscosity, at a rate of 26.92%, as shown. It is worth noting that the treatment carried out on the isolation sources led to the elimination of the vegetative cells of the bacteria. In contrast, the spores resisted the heat, which grew after providing the appropriate conditions. The development of the bacterial isolates obtained in aerial conditions prevented the growth of the Clostridium bacteria, which form the spores naturally grown in anaerobic conditions (Table 1) (Figure 1).

**Table 1:** The efficiency of 26 isolates producing Levansucrase enzyme in Mineral Salts Agar modified with 20% sucrose is estimated based on Levan formation represented in the sticky mucous membrane around the colonies after 48h at 37°C.

| Isolates | Isolation source | Sample | Levan forming |
|---|---|---|---|
| 1S | Amiriya area | Soil of the house garden | ++ |
| 2S | Amiriya area | Soil of the house garden | ++ |
| S3 | Amiriya area | Soil of the house garden | ++ |
| S4 | Amiriya area | Soil of the house garden | ++ |
| S5 | Amiriya area | Soil of the house garden | + |
| S6 | Amiriya area | Soil of the house garden | + |
| S7 | Fields of the Field Crops Department | Soil of the Potato crop | +++ |
| S8 | Fields of the Field Crops Department | Soil of the Potato crop | +++ |
| S9 | Fields of the horticultural department | Soil of Sativa plant | +++ |
| S10 | Fields of the horticultural department | Soil of Sativa plant | +++ |
| S11 | Fields of the horticultural department | Soil of Sativa plant | +++ |
| S12 | Fields of the horticultural department | Soil of Sativa plant | +++ |
| S13 | Fields of the horticultural department | Soil of Sativa plant | +++ |

| | | | |
|---|---|---|---|
| S14 | Fields of the horticultural department | Soil of Sativa plant | +++ |
| C1 | Sweets from vendors' carts | Rotten sweets | + |
| C2 | Sweets from vendors' carts | Rotten sweets | ++ |
| C3 | Sweets from vendors' carts | Rotten sweets | + |
| C4 | Sweets from vendors' carts | Rotten sweets | + |
| F1 | Rotten fruit | Persimmon fruit | + |
| F2 | Rotten fruit | Persimmon fruit | ++ |
| F3 | Rotten fruit | Persimmon fruit | +++ |
| F4 | Rotten fruit | Persimmon fruit | +++ |
| F5 | Rotten fruit | Persimmon fruit | +++ |
| 1J | Natural juice residues | Fruit peels | + |
| 2J | Natural juice residues | Fruit peels | ++ |
| A1 | Rotten jam | Carrot jam | ++ |

**Note:** +++: High viscosity; ++: Medium viscosity; + :Low viscosity

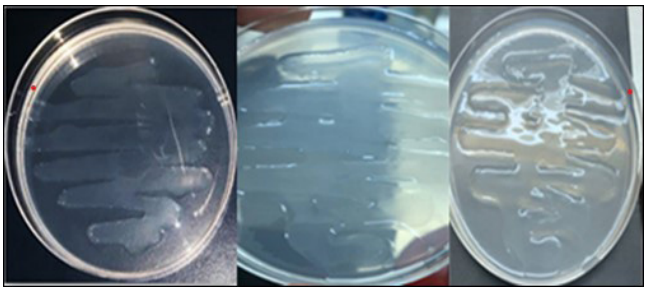

**Figure 1:** Sticky colonies of isolates were obtained by the mucoid method on the medium of Mineral Salts Agar (MSA) modified with 20% sucrose after incubation for 48 hours.

## Secondary screening

Submerged fermentation was accomplished for the production of Levansucrase as reported earlier among 26 tested bacterial isolates, 11 Levan bacterial strains (top Levansucrase producer), namely (S7, S8, S9, S10, S11, S12, S13, S14, F3, F4, F5) were selected for further investigations for subjected to secondary screening by assay enzyme activity (U/ml). The isolate S8 in secondary screening showed maximum activity of 529.87 U/ml, followed by S9 (396.4598 U/ml) and F4 (379.9935 U/ml). The minimum activity was found in S11 (195.4855 U/ml) (Figure 2).

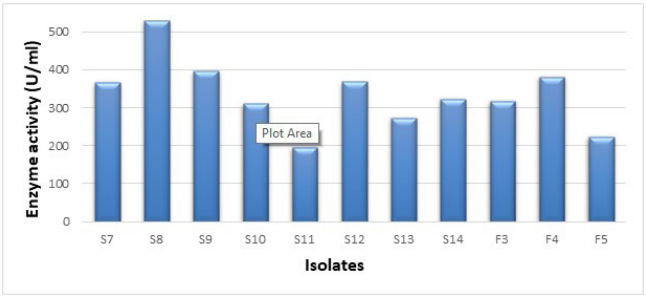


**Figure 2:** Quantitative secondary screening to test the efficiency of *Bacillus* spp.

Isolates for the production of Levansucrase activity (U/ml) in Mineral Salts Broth medium modified with 20% sucrose at pH 7.0, a temperature of 37°C, inoculum volume of 2 × $10^8$ cells/ml, an incubation period of 48 hours, and a stirring speed of 150 rpm.

Established on secondary screening, the S8 isolate was selected as the best producer of Levansucrase and subjected to biochemical tests and molecular identification. Some studies have indicated the successful use of the reagent method now primary and secondary screening to isolate the microorganism species producing Levansucrase, [29] were able to obtain 42 isolates of soil samples collected from Jinan city in China from bacteria belonging to the genus *Bacillus* that produce the enzyme Levansucrase through the formation of fibrin, as isolates numbered 8-37-0-1 showed the highest productivity, The selected isolate was diagnosed through the 16S rRNA gene and using what is available in the gene bank, it was found that it belongs to the type *Bacillus licheniformis* and registered with the number KF647836.1 [30] isolated *Bacillus licheniformis* strain RN-01, diagnosed it on the molecular plane, and recorded it in GenBank No. FJ171619.1 and isolated at 50°C from soil models in the Rinong Province, Thailand, hot springs area showed high enzyme productivity. Levansucrase was used to transfer the gene responsible for the production of this enzyme and its coloration in *E. coli* bacteria [20]. While found the possibility of isolating Levansucrase-producing bacteria from soil samples collected from Maitreya Station in Antarctica, and after conducting the primary and secondary screening process for the isolates, it was possible to obtain a high-productivity isolate, and after performing the diagnostic process for a gene 16S rRNA and after receiving the mentioned gene sequences and using the available information in the NCBI Gene Bank, it was found that the isolate belongs to *Bacillus licheniformis*, this strain was registered as ANT 179 with access number JQ000031.1. isolated *B. licheniformis* strain BK2 from the soil, diagnosed it through the 16S rRNA gene, and registered it in GenBank No. MF774878.1, as this isolate was characterized by its high ability to produce Levansucrase enzyme and transfer the gene responsible for producing this enzyme to *E. coli* BL21 bacteria with increased ability to express [31].

## Identification of the most efficient local isolates *Bacillus* sp. (S8)

**Vitek2 compact system identification:** The isolate (S8) was identified using the Vitek2 compact system. The result showed similarity of this isolate to *Bacillus licheniformis* by a probability of 92%, as shown in Figure, thus confirming its belonging to the target species specifically for the study Soliman, et al. pointed out that the adoption of traditional methods in biochemical tests is somewhat complicated due to the need for large quantities of materials, some of which may be expensive, as well as the long time that some tests may take compared to the technology of the Vitek2 system [32]. The Bacillaceae family (BCL) card is highly accurate in identifying several species of aerobic bacteria that form endospores, valenza, et al. reported a significant difference between the traditional methods of identification results and the results of the Vitek2 system [33,34] (Figure 3).

## Confirmatory molecular identification of *Bacillus licheniformis* S8

The gel electrophoresis Agarose results were shown using a U.V light detector of DNA extracted from isolate S8 by PCR amplification of gene 16S rRNA assay (Figure 4).

| Identification Information | Card: | BCL | Lot Number: | 2391888103 | Expires: | Jan 27, 2023 12:00 CST |
|---|---|---|---|---|---|---|
| | Completed: | Jan 20, 2022 02:40 CST | Status: | Final | Analysis Time: | 13.80 hours |
| Organism Origin | VITEK 2 | | | | | |
| Selected Organism | 92% Probability | | Bacillus licheniformis | | | |
| | Bionumber: | 1332361755476270 | | | Confidence: | Good identification |

| Biochemical Details | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | BXYL | + | 3 | LysA | - | 4 | AspA | - | 5 | LeuA | (+) | 7 | PheA | + | 8 | ProA | - |
| 9 | BGAL | + | 10 | PyrA | + | 11 | AGAL | - | 12 | AlaA | - | 13 | TyrA | + | 14 | BNAG | - |
| 15 | APPA | + | 18 | CDEX | + | 19 | dGAL | - | 21 | GLYG | - | 22 | INO | + | 24 | MdG | + |
| 25 | ELLM | + | 26 | MdX | - | 27 | AMAN | - | 29 | MTE | + | 30 | GlyA | + | 31 | dMAN | + |
| 32 | dMNE | + | 34 | dMLZ | - | 36 | NAG | + | 37 | PLE | + | 39 | IRHA | - | 41 | BGLU | + |
| 43 | BMAN | - | 44 | PHC | - | 45 | PVATE | (+) | 46 | AGLU | + | 47 | dTAG | + | 48 | dTRE | + |
| 50 | INU | - | 53 | dGLU | + | 54 | dRIB | + | 56 | PSCNa | - | 58 | NaCl 6.5% | + | 59 | KAN | - |
| 60 | OLD | + | 61 | ESC | + | 62 | TTZ | + | 63 | POLYB_R | - | | | | | | |

**Figure 3:** Results of biochemical tests contained in the Bacillaceae family card (BCL) in the Vitek 2 compact system for the most efficient local isolate *Bacillus licheniformis* S8

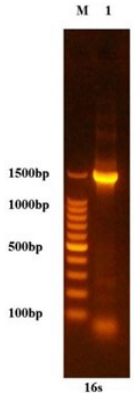


**Figure 4:** Electrophoresis of the PCR product on an agarose gel of *Bacillus licheniformis* S8 efficient local isolate using the 16S rRNA gene.
**Note:** Marker Ladder: 100-1500 bp; M: negative control; Numbers 1: local isolate

**Sequence analysis:** PCR products were sent to Microgen Company/ Korean to determine the sequences of the nitrogen bases. The result showed the molecular size of the gene amplification band was 1121 bp compared with ladder size under the same conditions. These sequences were compared with the available information on those found in the NCBI through the PLAST Nucleotide program. Identified isolation shows a 100% match between this isolate and the strains of *Bacillus licheniformis* bacteria registered in NCBI.

In the study of nitrogenous bases sequence of DNA to identify *B. lichniformans* stated that the 16S rRNA gene had been successfully used to study nitrogenous bases sequence of DNA to distinguish between different types of bacteria, including *Bacillus* spp. specifically, thus, it gives conclusive results in the identification [35]. The nitrogen base sequences from *Bacillus licheniformis* S8 registered on the NCBI belong to *Bacillus licheniformis* strain MJ8 Accession number: OM672244.1 and to draw the phylogenetic tree for the local isolate through the Blast Tree View (BTV) program, depending on the 16S rRNA sequences, this program is adopted when using the maximum probability of finding the phylogenetic tree with the selection of the most accurate form of the possibility of the degree of evolutionary kinship between isolates. The below figure showed an apparent convergence between the local isolate understudy, *Bacillus licheniformis* strain MJ8, and the recorded standard strains in the NCBI GenBank. It is reported that many researchers have used identification at the molecular level to identify *Bacillus licheniformis* (Figure 5) [36-39].

## Identification of levan

**HPLC analysis:** HPLC technology is adopted to identify polysaccharides obtained by extraction method and product from *B. lichniformans* is the most efficient locally produced and isolated to confirm it belongs to Levan. From beloew Figure 6 shows the retention time for the standard solutions of fructose, glucose, and sucrose and the standard sample of Levan produced from the *E. herbicola* and for the sample under study and respectively. Since it is noticed that one peak appears for the Levan under study and its retention time matches the retention time for the standard of Levan, as it was (2.540 and 2.523) minutes, respectively. However, the retention time for fructose, glucose, and sucrose was 2.546, 4.228, and 5.148 minutes, respectively. These data confirm that the polysaccharide produced is pure and consists of fructose units and is a type of homopolysaccharides [40]. In addition to being a type of fructan belonging to the group of Fructooligosaccharide (FOS) [41]. Furthermore, Benigar, et al. succeeded in extracting Levan from isolates *Zymomonas mobilis* and *E. herbicola* and measuring their structures by several techniques; it was found that Levan is composed of fructose, while Pei, et al. demonstrated that the backbone of Levan consisted of 2,6-substituted β-fructose using the HPLC technique [42,43] (Figure 6).

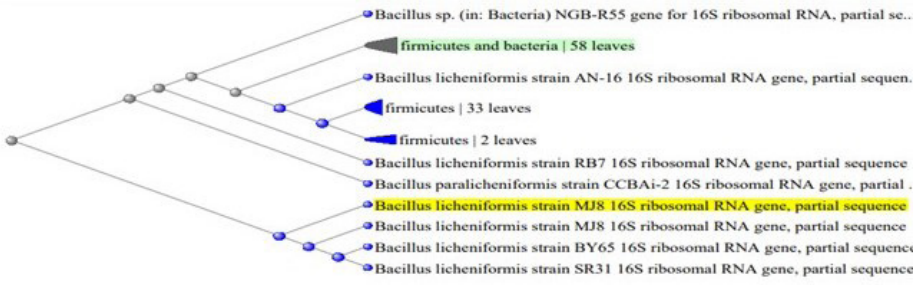


**Figure 5:** Genetic tree of a local isolate with several strains of the same bacteria in the NCBI GenBank based on the Blast Tree View (BTV) program.

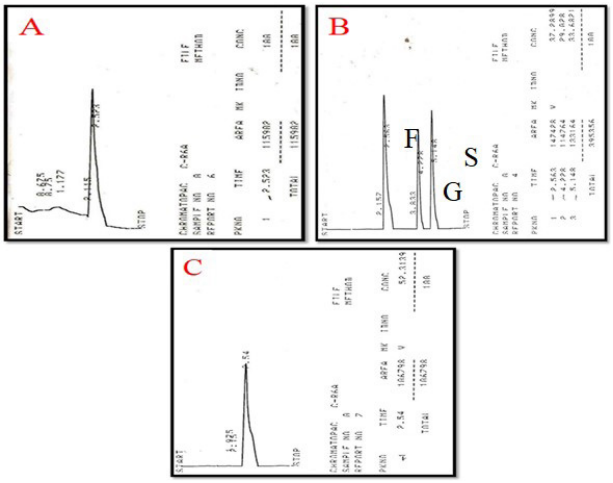


**Figure 6:** HPLC analysis of Levan production.
**Note:** A: Standard Levan (Sigma-Aldrich) from Erwinia herbicola; B: Standard Sugar (F: Fructose; G: Glucose; S: Sucrose); Levan produce from *B. lichniformans* MJ8

**FTIR analysis:** The results of this technique showed the presence of the adjustable frequency group C-O for both samples standard from *E. herbicola* (Sigma-Aldrich) and Levan from *B. lichniformans* at the wavelength of 1123.44 $cm^{-1}$ and 1122.07 $cm^{-1}$, respectively. In contrast, the bending group starts at CH, OH at 1325.66 $cm^{-1}$ and 1326.78 $cm^{-1}$ corresponding to the corresponding frequency with this group at wavelength 1424.50 $cm^{-1}$ and 1425.42 $cm^{-1}$, respectively. In contrast, the stretchable frequency of ketone groups appeared at 1645.44 $cm^{-1}$ and 1660.14 $cm^{-1}$, respectively, whereas at the 8.58 $cm^{-1}$ and 2933.13 $cm^{-1}$ for the model, the adjustable frequency of the C-H group appeared for the two samples, respectively, studied the characterization of Levan produced from Pseudomonas fluorescens and, based on the FTIR analysis, found that the structure of Levan was homologous to the standard Levan sample which the broad stretching peak of O-H stretching around 3319.26 $cm^{-1}$, C-H vibration was observed at about 2935.48 $cm^{-1}$, carbonyl C=O stretching noted at 1722.31 $cm^{-1}$ [44]. The FTIR spectrum analysis of Levan produced from *L. mesenteroides* showed the presence of hydroxyl stretching and vibrations in polysaccharides [45]. Based on FTIR spectra analysis of Levan produced from *B. licheniformis* showed that the stretching of O-H vibrations appeared at the wavelength around 3300 $cm^{-1}$, while the band at around 2900 represents C-H stretching, the peak at the wavenumber of 1660 $cm^{-1}$ is typical for C=O stretching, also analysis the Levan secreted from Halomonas and Chromohlobacter by FTIR and found that the O-H stretching at the wavenumber range from 3600-3200 $cm^{-1}$, the C-H stretching at the wavenumber range from 3000-2800 $cm^{-1}$, while the vibration of C=O at the wavenumber of 1641.16 $cm^{-1}$, and the region of typical carbohydrate at the fingerprints wavenumber range of 800-1000 $cm^{-1}$ (Figure 7) [46,47].

**Scanning Electron Microscope (SEM) analysis:** In order to examine the Levan's microstructure and surface morphology, which may aid in comprehending its physical characteristics, a scanning electron microscopy investigation was carried out. Below figure displays the surface morphology micrographs of Levan at 2500, 5000, 7000, and 13000. Levan in this research had a highly branched and porous structure, as shown in the SEM photos. Levan was thought to be most likely to be used in the food and cosmetics sectors as a texturing, thickening, stabilizing, and water-binding agent due to its highly branched and porous structure, which was thought to be favorable to the development of hydrated polymer [48-50]. Additionally, SEM scans showed that Levan had a glossy, sheet-like surface that might be used to create a plasticized film [51]. The microstructure of the Levan generated by *Bacillus mojavensis* and *Brenneria* sp. in this work was comparable to that of glucan produced by *Leuconostoc pseudomesenteroides* XG5, which had a smooth and sparkling surface and a highly branching structure [50]. A consistent porosity network was seen in EniD312 (Figure 8) [52,53].

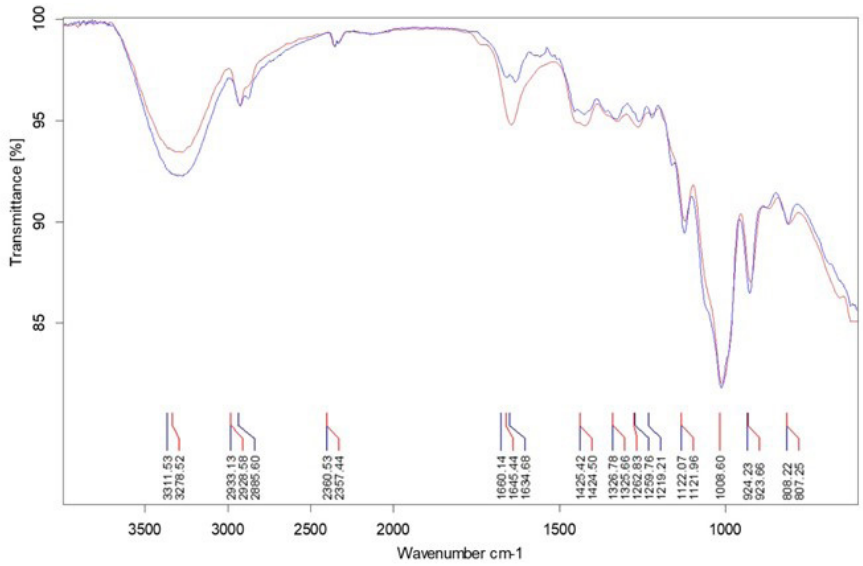


**Figure 7:** Infrared spectroscopy (FT-IR) of a Levan produced from the most efficient local isolate *B. lichniformans* MJ8 (Blueline) and the comparison sample for Levan produced by *E. herbicola* (Redline)
Note: (▬) Standard; (▬) Sample

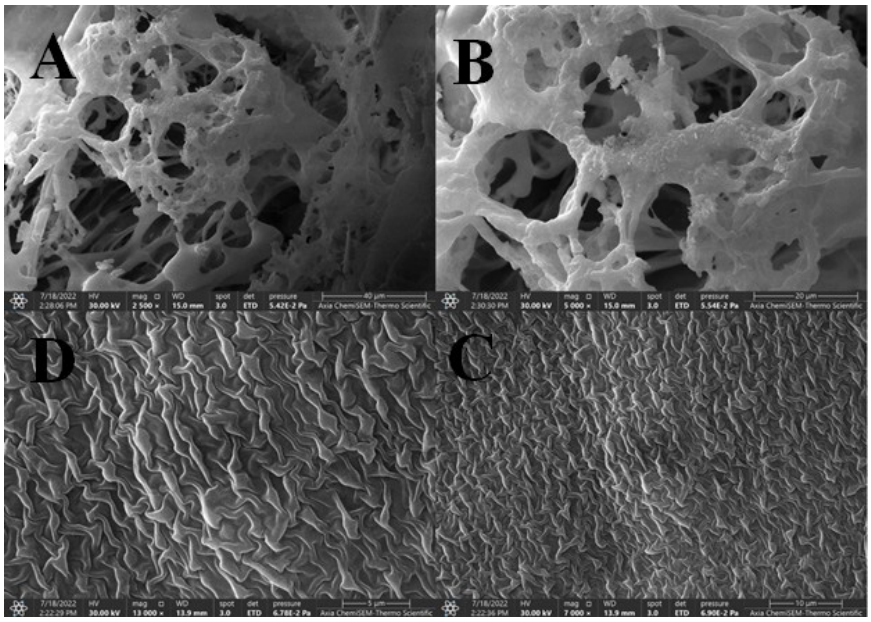


**Figure 8:** SEM images of purified Levan at 2500 × (A), 5000 × (B), 7000 × (C), 13000 × (D) magnification.

**Atomic Force Micrograph (AFM) analysis:** AFM, which was created on the basis of SEM, is a helpful technique for evaluating polymer morphology with high resolution and ease of use. Levan's topography AFM pictures showed many ellipsoidal or spheroidal particles and lumps that resembled spikes, demonstrating that polysaccharides had a significant attraction for the water molecules [23,54]. The mean roughness was 1.48 nm, the average roughness was 3.41 nm, and the highest peak height of the rounded lumps was 166 nm. Levan's maximal height was much greater than the height of a single polysaccharide chain (0.1–1 nm), indicating that Levan's intermolecular and intramolecular aggregation may have contributed to the densely packed molecular structure shown in AFM pictures [48]. In contrast to *Lactobacillus reuteri* E81 glucan, which had tangled networks [55], and Mesona blumes gum EPS polymer, which had an uneven structure like the worm, a comparable result was observed for the EPS polymer from *Lactobacillus sakei* L3 (Figure 9) [56].

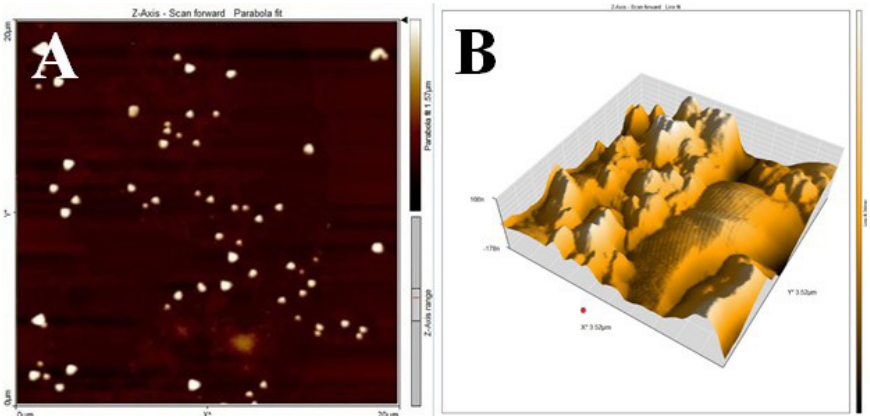


**Figure 9:** AFM images of purified Levan (A) planar and (B) cubic

## CONCLUSION

A novel strain of *Bacillus* sp. that can produce Levansucrase was isolated from the soil rhizosphere of the Stevia plant. The local isolate, *Bacillus* sp. (S8), showed it has a high ability to produce enzymes using the medium of Mineral Salt Broth. The identification tests with the Vitek2 system and at the molecular level confirmed that this local isolate belongs to *B. lichniformans* and were called *B. lichniformans* MJ8. The identification results of Levan using HPLC technology showed an indication of the presence of fructose when acidic decomposition. The possibility depends on one of the characterization techniques to identify Levan. The best technique for Levan characterization was shown to be the FTIR technique. Moreover, the composition and structure of the synthesized Levan were highly similar to that of natural Levan. In the future, we will further study the biological function of the Levan synthesized by the enzymatic method, and then explore the application way of the Levan.

## REFERENCES

1. Bergey DH. Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins. 1994.
2. Cerning J. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol Rev. 1990;7(1-2):113-130.
3. Öner ET. Microbial production of extracellular polysaccharides from biomass. Biofuel Biorefin. 2013:35-56.
4. Al-Turk A, Odat N, Massadeh MI. Isolation and Molecular characterization of antibiotic producing *Bacillus licheniformis* strains isolated from soil. J Pure Appl Microbiol. 2020;14(4):2363-2370.
5. Matsuhira H, Tamura KI, Tamagake H, Sato Y, Anzai H, Yoshida M. High production of plant type levan in sugar beet transformed with timothy (Phleum pratense) 6-SFT genes. J Biotechnol. 2014;192:215-222.
6. Dong CX, Zhang LJ, Xu R, Zhang G, Zhou YB, Han XQ, et al. Structural characterization and immunostimulating activity of a levan-type fructan from Curcuma kwangsiensis. Int J Biol Macromol. 2015;77:99-104.
7. Kirtel O, Menéndez C, Versluys M, van den Ende W, Hernández L, Toksoy Öner E. Levansucrase from Halomonas smyrnensis AAD6T: first halophilic GH-J clan enzyme recombinantly expressed, purified, and characterized. Appl Microbiol Biotechnol. 2018;102(21):9207-9220.
8. Jakob F, Pfaff A, Novoa-Carballal R, Rübsam H, Becker T, Vogel RF. Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function. Carbohydr Polym 2013;92(2):1234-1242.
9. Ragab TI, El Awdan SA, El-Bassyouni GT, Salama BM, Helmy WA, Esawy MA. Role of levan extracted from bacterial honey isolates in curing peptic ulcer: In vivo. Int J Biol Macromol. 2020;142:564-573.
10. Porras-Domínguez JR, Ávila-Fernández Á, Rodríguez-Alegría ME, Miranda-Molina A, Escalante A, González-Cervantes R, et al. Levan-type FOS production using a *Bacillus licheniformis* endolevanase. Process Biochem. 2014;49(5):783-790.
11. Adamberg K, Adamberg S, Ernits K, Larionova A, Voor T, Jaagura M, et al. Composition and metabolism of fecal microbiota from normal and overweight children are differentially affected by melibiose, raffinose and raffinose-derived fructans. Anaerobe. 2018;52:100-110.
12. Dahech I, Belghith KS, Hamden K, Feki A, Belghith H, Mejdoub H. Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats. Int J Biol Macromol. 2011;49(4):742-746.
13. Oh JS, Lee SR, Hwang KT, Ji GE. The anti-obesity effects of the dietary combination of fermented red ginseng with levan in high fat diet mouse model. Phytother Res. 2014;28(4):617-622.
14. Song B, Zhu W, Song R, Yan F, Wang Y. Exopolysaccharide from *Bacillus vallismortis* WF4 as an emulsifier for antifungal and antipruritic peppermint oil emulsion. Int J Biol Macromol. 2019;125:436-444.
15. Versluys M, Kirtel O, Toksoy Öner E, van den Ende W. The fructan syndrome: Evolutionary aspects and common themes among plants and microbes. Plant Cell Environ. 2018;41(1):16-38.
16. Yoo SH, Yoon EJ, Cha J, Lee HG. Antitumor activity of levan polysaccharides from selected microorganisms. Int J Biol Macromol. 2004;34:37-41.
17. Liu C, Lu J, Lu L, Liu Y, Wang F, Xiao M. Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1. Bioresour Technol. 2010;101(14):5528-5533.
18. Logan NA. Bacillus. Springer. 2009.
19. Shih IL, Yu YT, Shieh CJ, Hsieh CY. Selective production and characterization of levan by Bacillus subtilis (Natto) Takahashi. J Agric Food Chem. 2005;53(21):8211-8215.
20. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-428.
21. Collec GJ, Fraser GA, Marmion PB, Sinmons A. Edinburgh, Practical Medical Microbiology. Churchill Livingstone. 1996;11:163.
22. Dahech I, Harrabi B, Hamden K, Feki A, Mejdoub H, Belghith H, et al.Antioxidant effect of nondigestible levan and its impact on cardiovascular disease and atherosclerosis. Int J Biol. 2013;58:281-286.

23. Wang B, Song Q, Zhao F, Zhang L, Han Y, Zhou Z. Isolation and characterization of dextran produced by *Lactobacillus* sakei L3 from Hubei sausage. Carbohydr Polym. 2019.

24. Srikanth R, Siddartha G, Reddy CH, Harish BS, Ramaiah MJ, Uppuluri KB. Antioxidant and anti-inflammatory levan produced from Acetobacter xylinum NCIM2526 and its statistical optimization. Carbohydr Polym. 2015;123:8-16.

25. Dahech I, Bredai R, Srih K. Enzymatic synthesis of levan polysaccharide by *Bacillus licheniformis* levansucrase. Int J Biol Macromol. 2014;8(5):180-184.

26. González-Garcinuño Á, Masa R, Hernández M, Domínguez Á, Tabernero A, Del Valle EM. Levan-capped silver nanoparticles for bactericidal formulations: Release and activity modelling. Int J Mole Sci. 2019;20(6):1502.

27. Zhou X, Li Y. Techniques for oral microbiology. Mol Oral Microbiol. 2015:15-40.

28. Xavier JR, Ramana KV. Optimization of levan production by cold-active *Bacillus licheniformis* ANT 179 and fructooligosaccharide synthesis by its levansucrase. Appl Biochem Biotechnol. 2017;181(3):986-1006.

29. Liu J, Yang C, Chi Y, Wu D, Dai X, Zhang X, et al. Algicidal characterization and mechanism of *Bacillus licheniformis* Sp34 against Microcystis aeruginosa in Dianchi Lake. J Basic Microbiol. 2019;59(11):1112-1124.

30. Salama BM, Helmy WA, Ragab TI, Ali MM, Taie HA, Esawy MA. Characterization of a new efficient low molecular weight Bacillus subtilis NRC 16 levansucrase and its levan. J Basic Microbiol. 2019;59(10):1004-115.

31. Permatasari NU, Ratnaningsih E, Hertadi R. The use of response surface method in optimization of levan production by heterologous expressed levansucrase from halophilic bacteria *Bacillus licheniformis* BK2. IOP Conf Ser Earth Environ Sci. 2018;209(1):012015.

32. Soliman NS, Aly SA. Occurrence and identification of yeast species isolated from Egyptian Karish cheese. J Yeast Fungal Res. 2011;2(4):59-64.

33. Funke G, Monnet D, de Bernardis C, von Graevenitz A, Freney J. Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-negative rods J Clin Microbiol. 1998;36(7):1948-1952.

34. Valenza G, Strasen J, Schäfer F, Frosch M, Kurzai O, Abele-Horn M. Evaluation of new colorimetric Vitek 2 yeast identification card by use of different source media. J Clin Microbiol. 2008;46(11):3784-3787.

35. Lakshmi GS, Bhargavi PL, Prakasham RS. Sustainable bioprocess evaluation for xylanase production by isolated Aspergillus terreus and Aspergillus fumigatus under solid-state fermentation using oil palm empty fruit bunch fiber. Curr Trends Biotechnol Pharm. 2011;5(4):1434-1444.

36. Anuraj NS, Sabnani MK, Yadav M, Jyotsana K, Deepika S, Rachna C, et al. Identification and characterization of proteases and amylases producing *bacillus licheniformis* strain EMBS026 by 16S rRNA gene sequencing. Int J Microbiol Res. 2012;4(5):231-235.

37. Omar MM, Jabber HA. Isolation, screening and identification of Bacillus sp. producing alkaline protease. Iraqi J Agric Sci. 2017;48(6):1493-1503.

38. Wahyuningrum D, Hertadi R. Isolation and characterization of levan from moderate halophilic bacteria *Bacillus licheniformis* BK AG21. Procedia Chem. 2015;16:292-298.

39. Phengnoi P, Charoenwongpaiboon T, Wangpaiboon K, Klaewkla M, Nakapong S, Visessanguan W, et al. Levansucrase from Bacillus amyloliquefaciens KK9 and its Y237S variant producing the high bioactive levan-type fructooligosaccharides. Biomolecules. 2020;10(5):692.

40. Sreenivasan S, Kandasamy R. Levan: A biocompatible homopolysaccharide excipient for stabilization of peptide drugs. Int J Pept Res Ther. 2017;23(3):305-311.

41. McCleary BV, Charmier LM, McKie VA, McLoughlin C, Rogowski A. Determination of fructan (inulin, FOS, levan, and branched fructan) in animal food (animal feed, pet food, and ingredients): single-laboratory validation, first action 2018.07. J AOAC Int. 2019;102(3):883-892.

42. Benigar E, Tomsic M, Sretenovic S, Stopar D, Jamnik A, Dogsa I. Evaluating SAXS Results on Aqueous Solutions of Various Bacterial Levan utilizing the String-of-Beads Model. Acta Chim Slov. 2015;62(3):509-517.

43. Pei F, Ma Y, Chen X, Liu H. Purification and structural characterization and antioxidant activity of levan from Bacillus megaterium PFY-147. Int J Biol Macromol. 2020;161:1181-1188.

44. Jathore NR, Bule MV, Tilay AV, Annapure US. Microbial levan from Pseudomonas fluorescens: Characterization and medium optimization for enhanced production. Food Sci Biotechnol. 2012;21(4):1045-1053.

45. Shukla R, Goyal A. Elucidation of structure and biocompatibility of levan from Leuconostoc mesenteroides NRRL B-1149. Curr Trends Biotechnol Pharm. 2013;7(2):635-643.

46. Nakapong S, Pichyangkura R, Ito K, Iizuka M, Pongsawasdi P. High expression level of levansucrase from *Bacillus licheniformis* RN-01 and synthesis of levan nanoparticles. Int J Biol Macromol. 2013;54:30-36.

47. Nasir DQ, Wahyuningrum D, Hertadi R. Screening and characterization of levan secreted by halophilic bacterium of Halomonas and Chromohalobacter genuses originated from Bledug Kuwu Mud Crater. Procedia Chem. 2015;16:272-278.

48. Sun N, Liu H, Liu S, Zhang X, Chen P, Li W, et al. Purification, preliminary structure and antitumor activity of exopolysaccharide produced by Streptococcus thermophilus CH9. mol. 2018;23(11):2898.

49. Yang Y, Feng F, Zhou Q, Zhao F, Du R, Zhou Z, et al. Isolation, purification and characterization of exopolysaccharide produced by Leuconostoc pseudomesenteroides YF32 from soybean paste. Int J Biol Macromol. 2018;114:529-535.

50. Zhou Q, Feng F, Yang Y, Zhao F, Du R, Zhou Z, et al. Characterization of a dextran produced by Leuconostoc pseudomesenteroides XG5 from homemade wine. Int J Biol Macromol. 2018 ;107:2234-2241.

51. Feng F, Zhou Q, Yang Y, Zhao F, Du R, Han Y, et al. Characterization of highly branched dextran produced by Leuconostoc citreum B-2 from pineapple fermented product. Int J Biol Macromol. 2018;113:45-50.

52. Haddar A, Hamed M, Bouallegue A, Bastos R, Coelho E, Coimbra MA. Structural elucidation and interfacial properties of a levan isolated from *Bacillus mojavensis*. Food Chem. 2021;343:128456.

53. Xu W, Liu Q, Bai Y, Yu S, Zhang T, Jiang B, et al. Physicochemical properties of a high molecular weight levan from Brenneria sp. EniD312. Int J Biol Macromol. 2018;109:810-818.

54. Ahmed Z, Wang Y, Anjum N, Ahmad A, Khan ST. Characterization of exopolysaccharide produced by *Lactobacillus* kefiranofaciens ZW3 isolated from Tibet kefir–Part II. Food Hydrocoll.2013;30(1):343-350.

55. İspirli H, Sagdic O, Yılmaz MT, Dertli E. Physicochemical characterisation of an α-glucan from *Lactobacillus* reuteri E81 as a potential exopolysaccharide suitable for food applications. Process Biochem. 2019;79:91-96.

56. Tao F, Biao GZ, Yu JZ, Ning ZH. Isolation and characterization of an acidic polysaccharide from Mesona Blumes gum. Carbohydr Polym. 2008;71(2):159-169.