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Purification and Characterization of Recombinant Levansucrase From *Bacillus lichniformans* MJ8

Mustafa Mohammed Omar[1]
mustafa.mohamed@uokirkuk.edu.iq

Jasim Mohammed Awada[2]
jasim.awda@coagri.uob

[1] Department of forestry, college of Agriculture, Kirkuk University, Kirkuk, Iraq.

[2] Department of food science, College of Agricultural Engineering Sciences, Baghdad University, Baghdad, Iraq

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Abstract

The ability to produce levansucrase was estimated in fifty *Bacillus* spp. isolates obtained from various sources. One isolate was found to be the higher producer of this enzyme through primary and secondary screening, which was performed by estimating levansucrase activity. It was found to be 6.05 U/ml. The results show, after identification by the Vitek 2 compact system with 92% probability and 16S ribosomal RNA gene sequencing, that this isolate is *Bacillus licheniformis*, designated in this study as MJ8, and registered in the Gene Bank under the accession number OM672244.1. Then, the gene of (*SacB*) was studied in detail. It contained 1449 nucleotides (accession number ON811641.1), expressed for 482 amino acids and 29 amino-acid as signal peptides. A recombinant plasmid, pET28a(+), with the levansucrase (*SacB*) gene, was over expressed in high efficiency *E. coli* BL21 (DE3). Then the enzyme was purified in three steps; firstly, it was precipitated with ammonium sulfate 40-80%. Secondly, DEAE cellulose ion exchange chromatography was applied then the gel filtration chromatography. The activity of levansucrase at the final purification steps reached 48.65 U/ml with a purification fold of 18.0 and a recovery of 48.9%. The levansucrase characterization was also studied. It was found that the molecular weight by SDS-PAGE and gel filtration methods was 43 kDa and 47 kDa, respectively, and the optimum temperature activity and stability were 45 ◦C, 30-35 ◦C, respectively while the pH activity and stability were 6.0, and 7.0, The levan was produced from cloned (*SacB*) levansucrase, separated, purified, and identified by NMR, AFM, and SEM analyses showed that its structure mostly consists of fructose residues.

Key words: NCBI, purification, SDS-PAGE, AFM, NMR, gel filtration.

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Correspondence Author: Mustafa M. Omar - Email mustafa.mohamed@uokirkuk.edu.iq

Introduction

The enzyme levansucrase (E.C. 2.4.1.10) catalyzes the transfer of fructose using a non-Leloir glycosyltransferase mechanism, with sucrose serving as the main non-activated donor substrate. The fact that these non-Leloir enzymes don't need expensively activated donors, like the nucleotide phosphate sugars required for Leloir enzymes, gives them great biotechnological potential. The hydrolysis of sucrose releases gibbs binding energy that can be used to form saccharides [1]. Their construction of levan, a β-(2→6) fructose polymer with a small amount of β-(2→1)-linked branch chains, is of particular interest, depending on the enzyme sources, the level of polymerization and the kind of branching differ [2, 3]. They can be widely utilized as viscosities, stabilizers, emulsifiers, gelling agents, or water-binding agents in food and non-food industries. Due to various bioactivities, including antiviral, antitumor, and immunostimulant activities, they may also have potential pharmaceutical applications [4]. Some levansucrase oligomerizes sucrose to produce fructooligosaccharides (FOS) and levan. These oligomers are referred to as beneficial prebiotics because they have low caloric values, lower lipid and cholesterol levels, aid in ion absorption from the gut, and promote bifidobacterial growth in the human colon [5]. Levansucrase can catalyze fructose transfer to various acceptors, such as alcohols and mono- or oligosaccharides, by using sucrose as a glycosyl donor. Galactose and xylose, two different sugar residues that cap fructooligosaccharides, may change their prebiotic and biochemical properties [6]. Some hetero-oligosaccharides can be used as prebiotics and sweeteners [7]. The reported levansucrase primarily concentrates on forming levan and FOS, despite the promising possibility of developing fructose compounds with novel extended functions. However, few have had their acceptor specificity for trans glycosylation thoroughly characterized [8]. The study aimed to isolate *Bacillus* spp. highest levansucrase producer was obtained from various sources, from the Baghdad governorate, soils were taken from the fields of the College of Agricultural Engineering Science and home garden at Amiriya area, and samples of damaged fruit, remnants of natural juices, and spoiled jam. Furthermore, rotten sweets are packed and ready for consumption (street vendors' carts). And identify the species using Vitik2 and 16S ribosomal RNA analysis, then cloned the (*SacB*) levansucrase gene and overexpressed it in high-efficiency *E. coli*. Purified levansucrase and characterization were then synthesized of the levan polymer for identification structure analysis.

Materials and methods

The strains of bacteria and culture media

Bacillus lichniformans MJ8 novel strain isolated from the soil of the Sativa plant was obtained from fields of the College of Agricultural Engineering Sciences/University of Baghdad-Iraq. The strain is registered in the National Center for Biotechnology Information (USA) under OM672244.1. *Escherichia coli* DH5α competent cells obtained from (Izmir Institute of Technology-Turkey) used to construct clones in pTG19-T vectors were cultured in Luria-Bertani medium (HiMedia Co., India) with Agar (HiMedia Co., India), and 50 µg/ml Amp, 50µg X-Gal, 0.1 M IPTG (Promega Co., USA) incubated at 37 °C. For testing Blue-White Screening for inducible expression, the levansucrase *SacB* gene was ligated with the pET28a(+)vector and transferred for over-expression into high-efficiency *E. coli* BL21(DE3) (Promega Co., USA). The transformant white colony cells were grown in L.B. Broth with 30 mg/L kanamycin incubated at 220 rpm, 37 °C [9].

Cloned (*SacB*) levansucrase and overexpression

The total DNA from *Bacillus lichniformans* strain MJ8 was extracted using Presto Mini DNA Bacteria Kit (Geneaid Co., Taiwan) [10]. The specific primers for the (*SacB*) amplification were designed based on (*B. licheniformis* DSM 13, GenBank No. AE017333), using the Primer-BLAST tool from NCBI. Forward 5′-**ATTGATGAACATCAAAAACATYGCT**-3′ and reverse 5′-**CGTTTTATTWGTTTACCGTTARTTG**-3′

and the product length expected was 1449 bp. The primers were designed by (Alpha DNA Co., USA) and the amplification by (PCR) Bioneer Co. to amplify the gene. As shown in Table (1), the PCR was programmed and was performed using a Master Mix (Promega Co., USA). The subcloning procedure was performed using (TA cloning kit Sinacolon Co., IRAN). The full-size gene was mixed with the pTG19-T vector and transformed directly into *E. coli* DH5α competent cells for the construction of clones. The obtained colonies grew on (LB) Agar containing 50µg/ml Amp, 50µg X-Gal, and 0.1 M IPTG for the Blue-White Screening test. The white colonies were isolated and purified using Plasmid Mini Extraction Kit (Bioneer Co., USA) and sequentially digested by *Bam*HI. The resulting product that contains the levansucrase (*SacB*) gene separated from the pTG19-T plasmid was purified from agarose gel with a Gel Extraction Kit (Bioneer Co., USA). The positive clones in the pET28a(+) vectors with levansucrase *SacB* were transformed into *E. coli* BL21(DE3) cells for overexpression.

Table 1. Cycling conditions of levansucrase gene amplification

Steps	cycles	Time	Temperature °C
Denaturation	1	5:00	95
Denaturation	35	00:45	95
Annealing	35	00:45	50
Extension	35	00:45	72
Final extension	1	10	72
Cooling	1	∞	4

DNA sequences analysis

The DNA sequence analysis was carried out by (Macrogen Co., Korea). GenBank has assigned accession numbers to the levansucrase (*SacB*) gene from strain MJ8 (ON81164.1). The DNA sequence was analyzed by BLAST from the (NCBI), with MEGA11 software 11.0.1 [11].

Production (*SacB*) Levansucrase from high-efficiency *E. coli*

The *E. coli* recombinant cell containing the pET28a(+) with (*SacB*) gene was grown in (LB) broth, with 50 mg/mL kanamycin, on a shaker incubator (220 rpm) at 37°C. When the optical density reached 0.6-0.8 on 600 nm, the (IPTG, 1.0 mmol/L) was added as an inducer. Then the (*SacB*) overexpression overnight at 37°C. The cells have collected at a speed of 8500xg for 15 min at 4 °C. The harvested cells were suspended in lysis buffer; then, the cells were sonicated by using 6-8 cycles of 10 s strokes with 30s rest and centrifuged at 12,000×g for 30 min. The supernatant was a crude enzyme [12, 13, 14].

The steps of purification levansucrase

All purification steps were performed at 7 ◦C. The resultant supernatant was precipitated with ammonium sulfate at 40-80% saturation and then desalted using dialysis bags, 12-14 kDa, against deionized water for 72 h. The sample was applied to a column (17×2.5cm) DEAE-cellulose (Whatman Co., England) with a flow rate of 40ml/h; they were collected in 3 ml tubes manically, which had been pre-equilibrated with 0.05M potassium phosphate buffer (pH 7.0). Then it was eluted by NaCl gradients from 0.1 to 0.5M solution (pH 7.0). The fractions that showed enzyme activity, which was concentrated from the ion exchange step, on a Sephadex G-100 (Pharmacia Co., Sweden) gel filtration column (60×1.5cm), which had been pre-equilibrated with the same buffer with a flow rate of 45 ml/h, they were collected in 3 ml tubes manically. The active fractions were collected and concentrated using polyethylene glycol 6000. The volume of the enzyme extract was estimated, and the activity (U/mL) and protein concentration (mg/mL) were calculated. The received data were processed using (Microsoft Office, Excel) [14].

Levansucrase activity and protein assays

The enzyme's activity was determined according to [15], with some modifications. 0.5 ml of the substrate (5% sucrose (W/V) sodium phosphate buffer 0.05M, pH 7.0) and 0.5 ml of crude enzyme and incubated at 37°C for 10 minutes. Then 1 ml of (DNS) was added to stop the reaction and heat for 5 min. The absorbance was measured at 540 nm. Standard glucose was achieved as a calibration curve, according to [16, 17]. One unit of the enzyme was defined that produced 1 µml of glucose/min under stander conditions. The protein concentrations were determined according to Bradford [18].

The levansucrase molecular weight estimation

Two methods were used to determine the MW of the enzyme: one of them was polyacrylamide gel electrophoresis (SDS-PAGE), according to [19], and the second was gel filtration chromatography. In the first method, coomassie brilliant blue R-250 was used for staining the protein bands on a gel. The ladder proteins ranged from 10 to 250 kDa (Promega Co., USA). In the second method, Gel filtration chromatography via a Sephadex G-100 column (60×1.5 cm) was used with the same conditions mentioned for the purification enzyme. Blue dextran was used to estimate the void volume (Vo) on (OD 600). The elution volume (Ve) was calculated using standard proteins on (OD 280), which included ovalbumin (45 kDa), trypsin (23 kDa), bovine serum albumin (67 kDa), and alkaline phosphatase (140 kDa), with concentration (2 mg/ml).

Characterization of levansucrase, temperature, and pH

The influence of temperature on enzyme activity during the standard reaction mixture described above, and the levansucrase effect of stability for 30 min at 20–90 ◦C. In addition, the effect of pH on activity and stability between (3.0 and 9.0) was studied by dissolving the substrate in buffers with different pH and then determining the subsequent enzyme activity and incubating the levansucrase with buffers for 24 h at 7 ◦C. All the buffer concentrations in 0.05M: were sodium acetate (pH 3.0–4.0–5.5), potassium phosphate (pH 6.0–7.5), and Tris-HCl (pH 8.0–9.0); And it's done received data were processed using (Microsoft Office, Excel).

Polymers Biosynthesis

The polymers were synthesized according to [14] by incubating levansucrase produced from recombinant *E. coli* with an activity 14.93 U/ml with 100 ml of buffer potassium phosphate pH 7.0, 0.05 M, containing 100 g sucrose and 0.05 g $CaCl_2$ at 37 ◦C for 48 hours.

The Polymers Separation

The polymers were separated after synthesis by adjusting the pH of the production medium to 9.0–10.0 by potassium hydroxide (1 M). Then chilled ethanol 80% at -20 °C was added at a rate of 1:2 and 1 ml of 1%, $CaCl_2$, then stirred for 20 min and left for 24 h at refrigerator temperature; after that, centrifuged at speed 10000 xg for 15 min. The precipitated was washed with chilled ethanol at the rate of 1:4 and placed at 45 °C. According to Bajpail *et al*. [20], the dried levan was again dissolved in de-ionized water for purification. Next, it was dialyzed for 72 hours at 4 °C using dialysis bags with pore diameters ranging from 12 to 14 kDa against deionized water with changing the water as needed. Mentioned this diameter as a way to get rid of low MW substances like proteins, nuclear acids, and other organic substances that are present in levan. After that, the levan obtains a dry powder using a lyophilization operation at -55 °C and stores it in a refrigerator [21].

The polymer identification structure analysis

Identification of polymer by NMR

The test 1H-NMR and 13C-NMR resonance spectrum, used (Bruker Co., 400 MHz, Switzerland), for analysis of the polymer sample produced in this study and compared with the standard levan obtained from Sigma Co., Germany, produced from *Erwinia herbicola* (SKU number L8647-1G). The samples were dissolved in a 60 mg/ml concentration in heavy water D_2O at a temperature of 25°C. The chemical shifts were expressed in ppm [22].

Identification of polymer by AFM

The Surface morphology and roughness were obtained using Atomic Force Micrograph (AFM) analysis. First, the levan solution with a 1 mg/ml concentration was prepared and stirred continuously for 1 hour at 40 °C in an airtight bottle. Once cooled to 25 °C, 5µL of levan solution was absorbed into the mica sheet. After levan dried at room temperature, the AFM images were taken by tapping on them.

Identification of polymer by SEM

The levan surface shape was looked at using the scanning electron microscope (SEM Axia Co., Germany) method. Pure freeze-dried levan was stuck to the SEM stubs with double-sided tape, and conductive gold was put on top

of it in an ion-sputtering machine, at a speed of 10 kV, the microstructures of samples with different magnifications were looked at. This study obtained SEM images of Levan samples at magnifications of 2500, 5000, 7000, and 13000×.

Results and Discussion

Cloning, Expression, and production of Levansucrase from recombinant *E. coli*

Levansucrase (*SacB*) gene from *B. lichniformans* MJ8 strains was successfully cloned and showed that it contained 1449 nucleotides (accession number ON811641.1) translated to 482 amino-acid and 29 amino-acid signal peptides. The *SacB* from *B. subtilis* and *B. megaterium* were also found to bear a 29 amino-acid signal peptide in the reported literature [23, 24]. This means that the theoretical molecular mass of the enzyme is approximately 53 kDa, given that the average molecular weight of amino acids is 110 kDa. The sequence alignment showed that the MJ8 strain sequence showed 100% identity with nine strains of 16S rRNA ribosomal RNA gene partial sequence registered in NCBI [25] (Figure 1). The levansucrase activity in wild-type strain MJ8 was 6.05 U/ml, while for the (*SacB*) expressed in *E. coli* BL21 (DE3) was 14.93 U/ml.

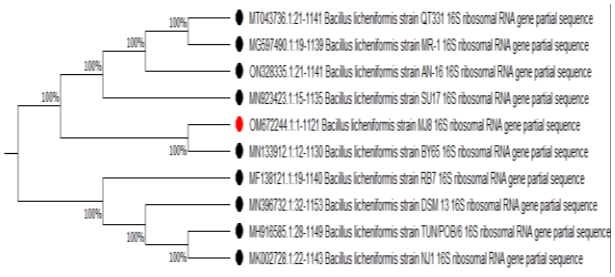

Figure 1. Phylogenetic analysis of the sequencing identity % of 16S ribosomal RNA gene of NCBI strains with *local Bacillus licheniformis* MJ8 strain.

The levansucrase purification

The purification of the cloned enzyme was performed through different steps. Firstly, 100 ml of crude protein was applied to precipitate by ammonium sulfate. The recovered fraction was used for additional purification after being tested for enzyme activity. Then, the DEAE-cellulose ion exchange chromatography column was applied for further purification. Finally, the proteins were eluted with NaCl. The chromatogram in Figure (2) shows that there were 4 peaks of protein and just one peak of levansucrase activity in the fraction numbers 67-82, which were collected and concentrated. Sephadex G-100 column was used as the last step of purification. The result indicated that only a single peak contains levansucrase activity, as shown in Figure (3). The separation of the enzyme in the recovery phase and its absence from the washing phase is evidence that the protein is bound to the negative ion exchanger and that the net charges carried on the enzyme under separation conditions are negative [26].

The Fold of levansucrase at the last step of purification was 18.0, with a 48.9% recovery and an enzyme activity of 48.65 U/ml. The summary of the purification of the levansucrase enzyme is shown in (Table 2). These results indicate that the enzyme was purified at a higher level than in some literature [27].

Molecular mass determination

The SDS-PAGE method

The levansucrase after the purification steps was analyzed by SDS-PAGE; the result showed a single band on a polyacrylamide gel, as shown in Figure (4), which indicates that the levansucrase was successfully purified. The molecular weight was estimated based on the enzyme protein band that appeared in the polyacrylamide gel after electrophoresis. The standard curve in Figure: 5 shows the relationship between the log of the MW of standard proteins and relative mobility (Rm) for them, indicating that the log of the MW of the levansucrase is 1.64. Thus, its MW is approximately 43 kDa, noting that the loading dye's relative mobility (Rm) was 9.8 cm. It is mentioned that the purpose of using SDS in electrophoresis is to cancel the effect of charges. Thus, the difference in size or MW is the only factor for separating the protein of enzyme and other proteins on a polyacrylamide gel [27].

The Gel filtration chromatography method

The gel filtration method was used on a Sephadex G100 column to estimate the molecular weight of the levansucrase using the standard curve that represents the relationship

between the logarithm of the MW of the standard proteins and the Ve/Vo. The results show that the logarithm of the MW of the enzyme was 1.68, so the MW is calculated at approximately 47 kDa (Figure 6).

Table 2. The purification steps of levansucrase produced from recombinant *E. coli*

Purification1 steps	Volume 1(ml)	Enzyme1 activity1 (U/ml)	Protein concentration1 (mg/ml)	Specific1 activity1 (U/mg)	Total1 activity1 (U)	Total1 protein (mg)	Purification1 fold	Recovery (%)
Crude enzyme	100	14.93	0.565	26.42	1493.00	56.500	1	100
Precipitation 40-80%	20	55.61	1.420	39.16	1112.20	28.400	1.5	74.5
Dialysis	21	52.56	1.121	46.89	1103.76	23.541	1.8	73.9
DEAE-cellulose	21	43.76	0.198	221.01	918.96	4.158	8.4	61.6
Sephadex G-100	15	48.65	0.102	476.96	729.75	1.530	18.0	48.9

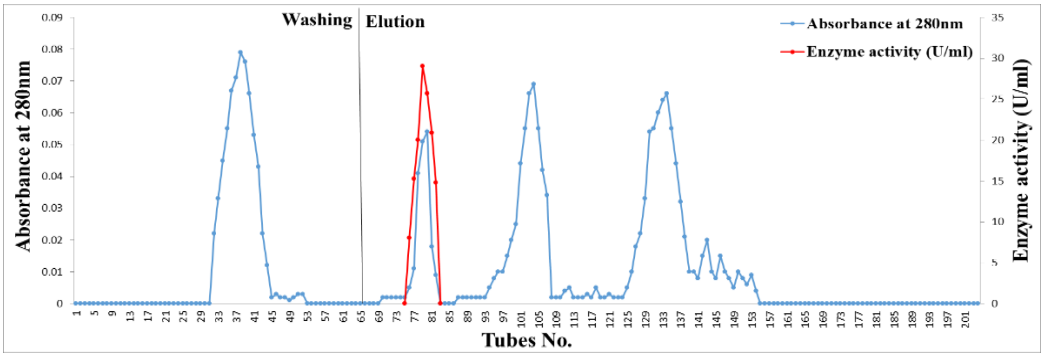

Figure 2. The third step for purification of levansucrase using DEAE-cellulose chromatography

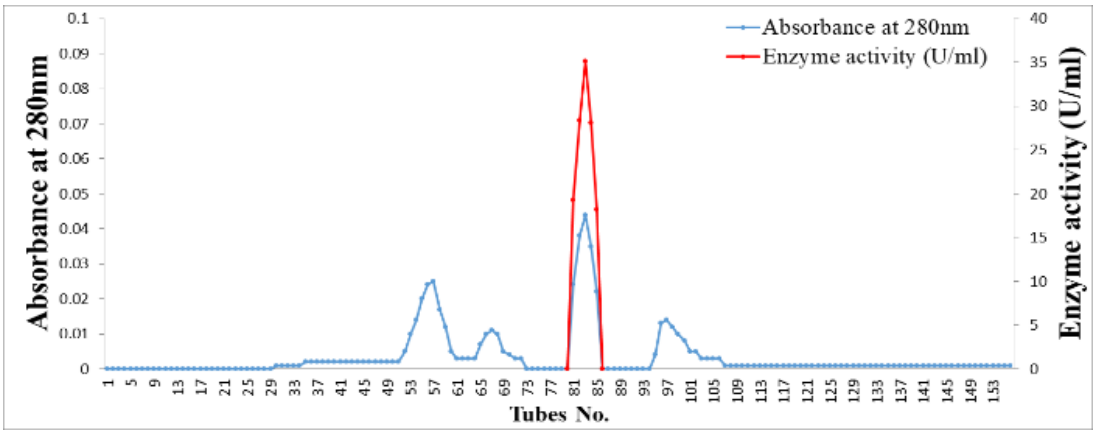

Figure 3. The fourth step for purification of levansucrase using Sephadex G-100 chromatography

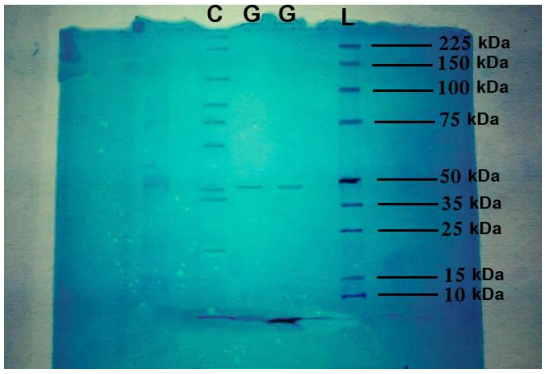

Figure 4. Electrophoresis of levansucrase produced in this study on SDS-PAGE, where C: crude enzyme G: after the gel filtration L: standard proteins

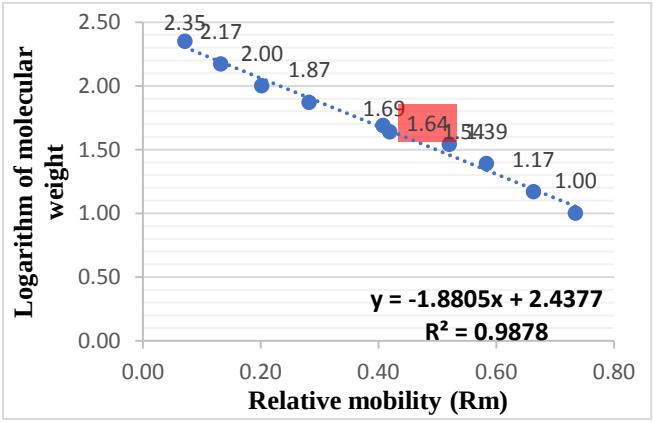

Figure 5. Standard curve connects the logarithm of the molecular weight of standard proteins with relative mobility (Rm) by electrophoreses method

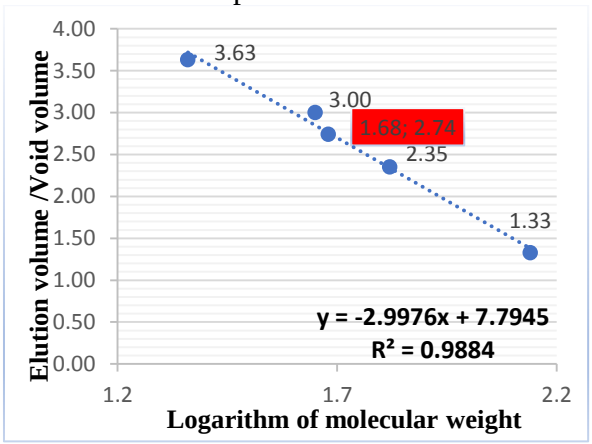

Figure 6. Standard curve connects the logarithm of the molecular weight of standard proteins with (Ve/Vo) by gel filtration method

The molecular weight estimated in this study by gel filtration is slightly different compared to that estimated by electrophoresis. That is expected due to differences in the condition of the methods used. Different studies have reached variable results in this field. Vigants *et al*. [28] produced the enzyme from *Zymomonas mobilis*, purified it in three steps, and estimated its molecular weight by electrophoresis. It was found to be approximately 50 kDa. At the same time, [29] found that the MW was 47 kDa of levansucrase produced by *Erwinia amylovora*. While Surawut *et al*. [30] estimated the molecular weight by electrophoresis (SDS-PAGE) of the cloned levansucrase, it was found to be 52 kDa. Mostafa *et al*. [31] found that the molecular weight is 44.5 kDa of the levansucrase produced by the *Aspergillus awamori* EM66. While [32] were able to estimate the MW of the levansucrase from *Klebsiella* strain L1 by electrophoresis, it was found that 43 kDa.

Characterization of levansucrase, temperature, and pH

As shown in Figure (7-8), the optimum pH for the activity was 6.0, which attained 17.37 U/ml. At the same time, the activity decreased at acidic pH numbers 3.0 and 4.0 and alkaline pH numbers 8.0 and 9.0. During the study of the pH effect on enzyme stability, it was found that levansucrase retained its total activity at pH 7.0. The condition of the pH value is critical for enzyme performance [33]. When the pH values alter from the optimal, the enzyme activity declines and decreases; these results agree with all other studies about this enzyme [34].

The optimum activity temperature was 17.81 U/ml at 45 °C, as shown in Figure (7-8). The reason for the increase in the activity of the enzyme with the increase in temperature is a result of the increase in the kinetic energy of the molecules and then the increase in collisions between the enzyme molecules and the molecules of the substrate [27].

During the study of the effect of temperature on levansucrase stability, it was found that the enzyme retains its total activity at 30-35 °C. After that, the activity begins to decrease gradually as the temperature rises. [35] reported that most levansucrase produced by *Bacillus sp*. are active at 30 °C, while Ammar *et al*. [36] reported that a levansucrase optimum activity at 60 °C, the activity was

measured at different temperatures for 10 min at pH 6.2, while the effect of temperature on the stability the relative activity was measured after heat treatment of the enzyme at different temperatures, the enzyme kept full activity at 40 °C, and the full pH activity at 6.0 and the full stability of the levansucrase was at 5.0, 6.0, 7.0. The levansucrase from *B. licheniformis* ANT 179 exhibited 50 % activity in a pH range of 4.0–12.0. Earlier reports indicated optimum levansucrase activity at pH 6.0 from *Bacillus subtilis* BB04 [37, 38].

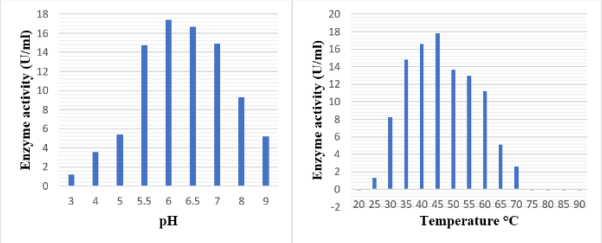

Figure 7. The optimum levansucrase activity pH and temperature

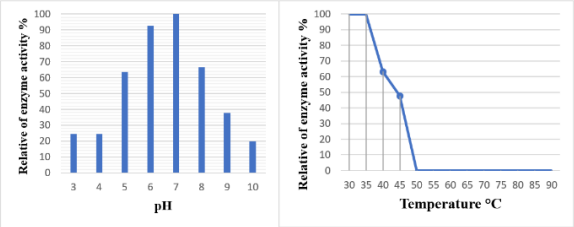

Figure 8. The optimum levansucrase stability pH and temperature

Polymer identification structure analysis

Identification of polymer by NMR

The FOS synthesized by levansucrase produced from recombinant *E. coli* was analyzed by both ^{13}C and ^{1}H NMR.^{13}C NMR analysis of the polymer produced from the cloned (*SacB*) levansucrase gene showed seven chemical shifts between 104 and 59 ppm, similar to chemical shifts of standard levan produced from *Erwinia herbicola*. It is also similar to the resonance signals of levan produced from *Bacillus licheniformis* obtained by [39] (Table 5). However, according to NMR analysis, only fructose units made up the levan polymer. [40] explained the reason for the difference in the positions of carbon atoms by a small amount is due to the type of microorganisms that produce levan. Other factors include molecular weight, production technique, number of side branches, degree of purity, extraction method, optimal conditions, and components of the medium production.

Table 5. ^{13}C-NMR values for levan produced in this study and the standard of *E. herbicola* with levan from *Bacillus licheniformis* [38]

Carbon atom ^{13}C	Chemical shift (ppm) Levan produced from cloned levansucrase	Standard Levan of *E. herbicola*	*Bacillus licheniformis* Levan [39]
C-1	104.15	104.16	104.17
C-2	103.56	103.56	-
C-3	80.23	80.24	80.25
C-4	76.18	76.18	76.17
C-5	75.11	75.12	75.12
C-6	63.32	63.32	63.35
C-7	59.77	59.79	59.78

The 1H NMR spectrum shows six protons between 3.5 and 4.0 ppm, similar to chemical shifts of standard levan produced from *Erwinia herbicola* and levan produced from *B. licheniformis* obtained by [40] (Table 6). Therefore, it could not be possible to detect ketoses molecules by ^{1}H NMR. In the case of high molecular weight levan, the number of fructose units is much larger than glucose units, making it impossible to identify the ketose molecules [41]. Consequently, it can be stated that this levansucrase is applicable to produce high molecular weight levan at high temperatures.

Table 6. ^{1}H-NMR values for levan produced in this study and the standard of *E. herbicola* with levan from *Bacillus licheniformis* [39]

Chemical shift (ppm)		
Levan produced from cloned levansucrase	**Standard Levan of *E. herbicola***	***Bacillus licheniformis* Levan** [39]
3.586	3.540	3.599
3.652	3.648	3.640
3.772	3.768	3.767
3.978	3.974	3.965
4.042	4.039	4.022
4.063	4.059	4.050

Identification of polymer by AFM

AFM, created based on SEM, is a valuable tool for characterizing polymer

morphology with high resolution and ease of use. Levan's topographical AFM images showed numerous ellipsoidal or spheroidal particles and lumps resembling spikes, demonstrating that oligosaccharides had a strong affinity for the water molecules [42, 43, 44]. The conclusion depicted in Figure 11 indicates that levan's intermolecular and intramolecular aggregation may cause the tightly packed molecular structure seen in AFM images [45]. The polymer from *Lactobacillus sake* 3 was reported to have produced similar results [43] but was distinct from the tangled networks of *Lactobacillus reuteri* E81 glucan [46], and Mesona blumes gum EPS polymer, which was shaped erratically like a worm [47].

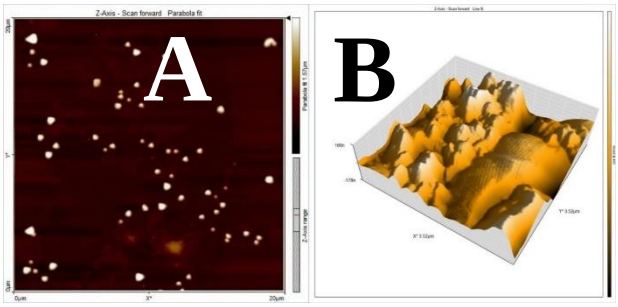

Figure (image) 11. Surface characteristics of levan produced in this study using atomic force microscopy (AFM), where here A: Two-dimensional (2D), B: Three-dimensional (3D)

Identification of polymer by SEM

The levan's surface morphology and microstructure were examined using scanning electron microscopy, which can be used to comprehend the levan's physical characteristics better. The surface morphology micrographs of levan at 2500, 5000, 7000, and 13000 (Figure 12).

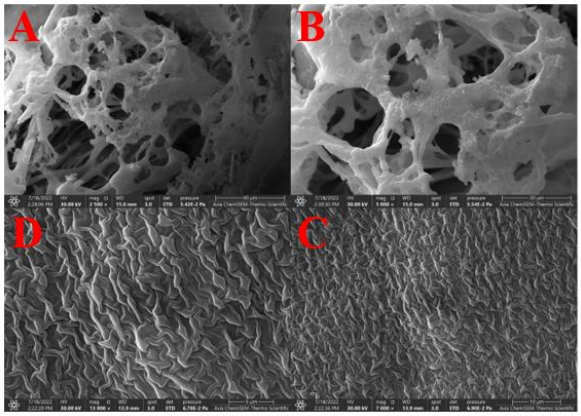

Fig. (image) 12. Scanning electron microscope image of levan produced in this study, where A: 2500X, B: 5000X, C: 7000X, D: 13000X

In this study, SEM images showed that Levan had a structure with many branches and many holes. Levan was thought to be most likely to be used as a thickening, stabilizing, and water-binding agent in the food and cosmetics industries because of its highly branched and porous structure, which produce it straightforward for hydrated polymers to form [45, 46, 49]. In addition, SEM images showed that levan had a glossy, sheet-like surface that could be used to create a plasticized film [50]. *Leuconostoc pseudomesenteroides* XG5 produced glucan with a smooth, glittering surface and a highly branched structure. The microstructure of the levan in this study was similar to that of this glucan [49], *Brenneria* sp. and levan from *Bacillus mojavensis* differed little from one another, however, and EniD312 showed a consistent porous network [51, 44].

Conclusion

The bacteria present in the soil are an essential source for producing various compounds, including the levansucrase enzyme, as proved in this study. The gene responsible for producing this enzyme which is named *SacB*, was transferred by high efficiency to *Escherichia coli* and then purified using different steps. The final purification fold was 18.0, with a 48.9% recovery and an enzyme activity of 48.65 U/ml. The levansucrase produced by cloned bacteria had a strong appearance of homogeneity. The molecular weight of the cloned enzyme estimated by SDS-PAGE and the gel filtration

method was 43 kDa and 47 kDa, respectively. The optimum temperature and pH of enzyme activity were 45 °C was 6.0, respectively. Biosynthesis of Levan from cloned (*SacB*) levansucrase was performed and separated, and structure identification was studied. It displayed various acceptor specificities for oligosaccharides and excellent sucrose polymerization activity. The levansucrase may be helpful for the efficient synthesis of levan, which has important biological, pharmaceutical, hygienic, or industrial uses, as well as for the expansion of the repertoire of chemicals containing fructose that may either have inherent value on their own or serve as intermediates for further modification to become valuable products.

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تنقية وتوصيف أنزيم levansucrase المكلون من *Bacillus lichniformans* MJ8

مصطفى محمد عمر[1] **جاسم محمد عودة**[2]

mustafa.mohamed@uokirkuk.edu.iq Jasim.awda@coagri.uobaghdad.edu.iq

1 كلية الزراعة، جامعة كركوك، كركوك، العراق
2 كلية علوم الهندسة الزراعية، جامعة بغداد، بغداد، العراق

- تاريخ استلام البحث 04/2023 / 09 وتاريخ قبوله 07/ 05/2023
- البحث مستل من أطروحة دكتوراه للباحث الأول.

المستخلص

جرى أختبار قابلية 50 عزلة من بكتريا *Bacillus* spp. عزلت من مصادر مختلفة على إنتاج أنزيم levansucrase. أظهرت أحدى هذه العزلات القدرة العالية على أنتاج الأنزيم بفعالية 6.05 وحدة/مل بعد أن اجتازت مرحلة الغربلة الأولية والثانوية. شخصت على مستوى النوع بإستخدام تقنية Vitek2 وبنسبة أحتمالية بلغت 92% وكذلك دراسة تتابعات القواعد النتروجينية لجين 16S rRNA فتبين انها من نوع *Bacillus lichniformans* سلالة MJ8 برقم وصول OM672244.1. ضخم جين *SacB* المسؤول عن إنتاج الأنزيم، ومن خلال دراسة تتابعات القواعد النيتروجينية للجين المضخم والمسجل برقم وصول ON81164.1 في بنك الجينات، وجد أنه يتكون من 1449 زوج قاعدة تشفر 482 حامض أميني ويتكون من 29 ببتيدة مفردة. تم كلونة الجين *SacB* وأمكن التعبير عنه في الناقل التعبيري pET28a(+) من خلال خلايا عالية الكفاءة *E. coli* BL21 (DE3). أنتج الأنزيم من الخلايا المكلونة وتمت تنقيته بثلاث مراحل، الترسيب بكبريتات الأمونيوم، التبادل الآيوني DEAE cellulose ومن ثم الترشيح الهلامي Sephadex G-100، في نهاية مراحل التنقية أمكن الحصول على فعالية أنزيمية قدرها 48.65 وحدة/مل، نقيت 18.0 مرة، وبلغت نسبة أسترداد الأنزيم 48.9%. تم التأكد من نقاوة الأنزيم بالترحيل الكهربائي وبوزن جزيئي 43 كيلودالتون، 47 كيلودالتون بطريقة الترشيح الهلامي. بدراسة توصيف الأنزيم وجد أن درجة الحرارة والرقم الهيدروجيني الأمثل للفعالية هي 45 مْ و6.0 على التوالي. وأحتفظ الأنزيم بكامل فعاليته في الرقم الهيدروجيني 6.0 ودرجة حرارة 30-35 مْ. كما تضمنت الدراسة أنتاج الليفان من levansucrase المنتج من الخلايا المكلونة وتشخيصه بتقنية الرنين النووي المغناطيسي (NMR) و مجهر القوة الذرية (AFM) كذلك المجهر الألكتروني الماسح (SEM).

الكلمات المفتاحية: NCBI، تنقية، SDS-PAGE، AFM، NMR، الترشيح الهلامي