CLONING AND EXPRESSION OF LEVANSUCRASE (*SacB*) GENE FROM *BACILLUS LICHENIFORMIS* MJ8 IN *ESCHERICHIA COLI* AND ENZYMATIC SYNTHESIS OF LEVAN

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ABSTRACT

In this study, a highly levansucrase-producing strain was isolated, identified as *Bacillus lichniformans* MJ8, and registered with the accession number OM672244.1 in the NCBI database. The *SacB* gene responsible for levansucrase production was transferred from this bacterium into *Escherichia coli*. It was found that the gene contains 1449 bp nucleotides, encoding 482 amino acids. The gene has been given the accession number ON811641.1 in the NCBI gene bank. The transformation was achieved by a cloning the *SacB* gene to plasmid pTG19-T, which was transferred to *Escherichia coli* DH5α. The *Escherichia coli* BL21 (DE3), with pet-28a (+) vector, was used to express the gene. One mM IPTG is induces the cloned gene to produce *SacB* protein. The levansucrase activity was 14.31 U/ml after transformation. The study also included the identification and characterization of levan produced by the bacteria using HPLC and FTIR techniques.

Keywords: 16S rRNA, PCR, plasmid, HPLC, BLAST, NCBI, FTIR
***Part of a Ph.D. dissertation of the first author**

أستنسال جين *SacB* المنتج لأنزيم Levansucrase من بكتريا *Bacillus licheniformis* MJ8 في بكتريا *Escherichia coli* لإنتاج Levan

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المستخلص

في هذه الدراسة تم الحصول على عزلة ذات قابلية عالية على إنتاج أنزيم Levansucrase شخصت على أنها تعود الى بكتريا *Bacillus lichniformans* MJ8 وسجلت في المركز الوطني لمعلومات التكنولوجيا الحيوية NCBI برقم وصول OM672244.1، ضخم جين *SacB* المسؤول عن إنتاج الأنزيم وتم أستنسالهُ في بكتريا *Escherichia coli*، 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. تضمنت الخطوة الأولى من عملية الأستنسال نقل الجين المضخم إلى البلازميد الناقل pTG19-T وأدخاله إلى *E. coli* DH5α، أما الخطوة الثانية اشتملت على نقل جين *SacB* إلى البلازميد التعبيري (+) pet-28a والتعبير عنه بوجود المحفز IPTG بتركيز 1mM في وسط تنمية الخلايا المؤهلة *E. coli* BL21 (DE3). بلغ فعالية الأنزيم بعد الأستنسال 14.3 وحدة/مل. كما تضمنت الدراسة إنتاج Levan وتشخيصه بتقنية كروماتوكرافيا السائل عالي الأداء (HPLC) وبوساطة طيف الأشعة تحت الحمراء (FTIR).

الكلمات المفتاحية: عزل، التشخيص الجيني، جهاز الفايتك2، البلازميدات، بنك الجينات
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INTRODUCTION

Levansucrase (E.C. 2.4.1.10.), which belongs to family 68, builds Levan from fructose residues of sucrose by releasing glucose and adding fructose molecules to an expanding levan fructooligosaccharide (L-FOS) chain (15). Levan is a fructooligosaccharide that is a typical homopolysaccharide composed primarily of units by β(2→6) glycosidic in the main chain and β(2→1) in branch points. Plants and microorganisms are two main producers of levan, such as numerous algal cells, yeasts, fungi, and bacteria (7, 29, 37). Many studies show that levan may help bacteria and plants deal with biological stresses by regulating cell osmotic pressure and making them more resistant to drought, salt, and low temperatures (23, 10, 42). Levan can also be used in many industries because it is both valuable and essential. Jakob et al. (16) discovered that levan, a hydrocolloid microgel, may bolster wheat bread and extend product life. Ragab et al. (34) said levan could be important for peptic ulcer problems. Levan's prebiotic properties have been confirmed by several additional studies (33, 1), as well as its antioxidant (8), anti-obesity (28), anti-fungal (39), antidiabetic (9), and anti-tumor properties (43). But the production of Levan by the traditional method using native bacteria is costly due to different molecular factors that switch off the gene expression of the levansucrase enzyme responsible for manufacturing this polymer. Therefore, this study aims to find a *Bacillus* sp. isolate that can produce a high level of levansucrase after cloning the gene into *Escherichia coli*, which increases levan production, and study its identification and characterization using HPLC and FTIR techniques.

MATERIALS AND METHODS

Source of bacterial strain : The bacterial strain used in this study was discovered in Baghdad, Iraq, in the rhizosphere of the Sativa plant. And identified as *Bacillus lichniformans* strain MJ8 using different techniques, including morphological and biological testing (20) and Vitek 2 compact system analysis (12). In addition to the molecular method using 16S rRNA gene identification (3, 5), the strain was then registered as a novel strain under Accession Number OM672244.1.

Levansucrase Activity Assay

Levansucrase activity was assayed according to (32) with some modifications. The reaction mixture reaction was prepared in a test tube containing 0.5 ml substrate (5% (w/v) sucrose dissolved in phosphate buffer (0.05 M, pH 7.0) and 0.5 ml of crude enzyme. After 10 minutes of incubation at 37 °C, the reaction was stopped by adding 1 ml of DNS and heating the mixture for 5 minutes, and adding 5 ml of water. The absorbance reading at 540 nm for a series of diluted standard glucose was used as a calibration curve according to (26). One unit of the enzyme was defined as the amount of enzyme needed to liberate 1 µml of glucose/min under reaction conditions.

Levansucrase gene amplification

Determine the *B. licheniformis* levansucrase gene :The National Center for Biotechnology Information-BLAST used to choose several *B. licheniformis* strains (Table 1) to compare sequences between the levansucrase gene and the SnapGene 6.0.5 software (Fig. 1) was used to create primers for amplifying the levansucrase (*SacB*) gene.

Table 1. Strains of *B. licheniformis* that were used to find the location of the *SacB* gene

The strains in the NCBI database	Accession number
***Bacillus licheniformis* strain DSM 13**	**NC- 006322.1**
***Bacillus licheniformis* strain BL1202**	**NZ- CP017247.1**
***Bacillus licheniformis* strain BK2 levansucrase**	**MF774878.1**
***Bacillus licheniformis* strain SK-1 levansucrase**	**JN712303.1**
***Bacillus licheniformis* strain TH4-2 levansucrase**	**JN712304.1**

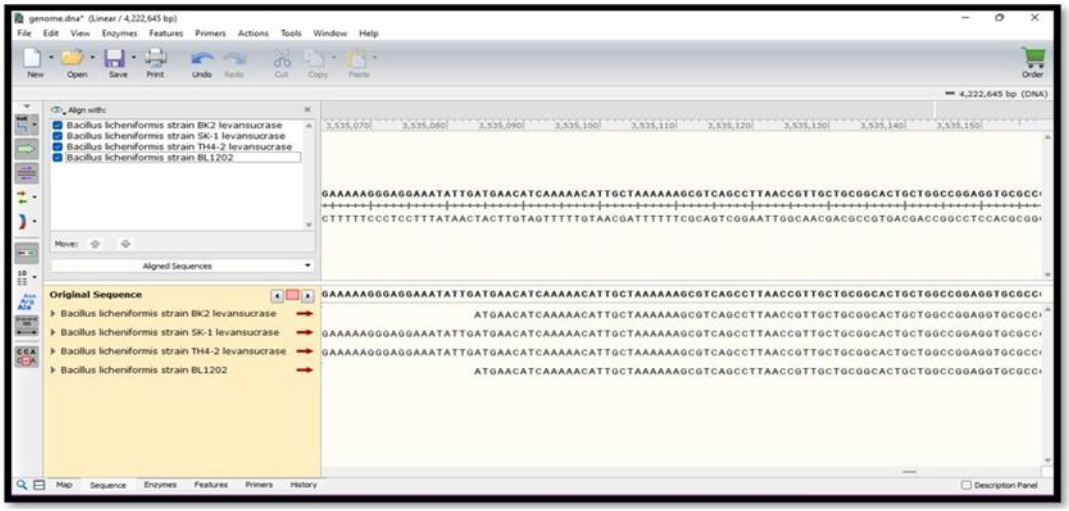

Fig. 1. Find and compare the levansucrase gene using Software SnapGene 6.0.5

Design specific primers for Levansucrase (*SacB*) gene amplification : The Primer-BLAST tool from NCBI was used to create the specific primers (Fig. 2). The primers were prepared by Alpha DNA Company, forward 5′-TTGATGAACATCAAAAACATYGCT-3′) and reverse (5′-GTTTTATTWGTTTAC CGTTARTTG-3′), and the product length expected was 1449 bp.

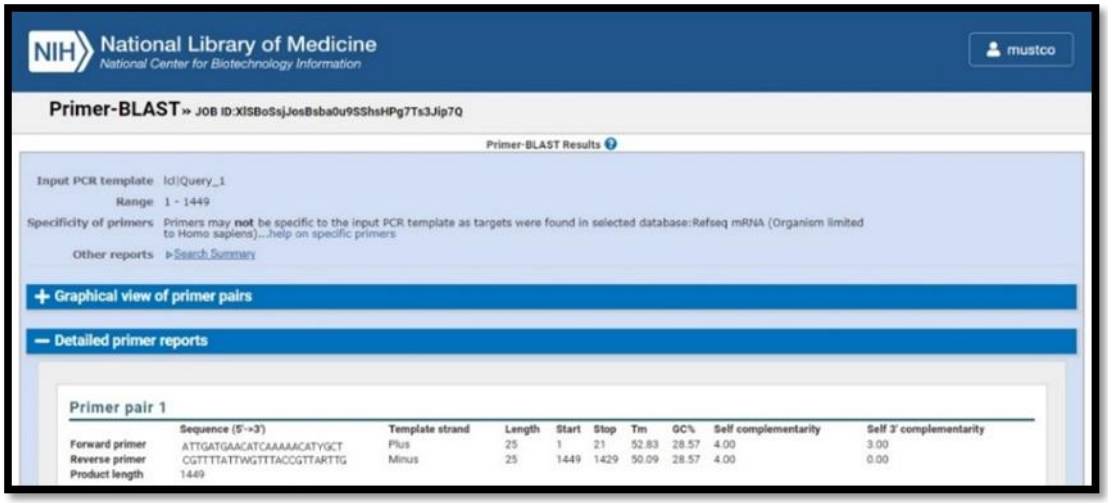

Fig. 2. Primer-BLAST tool applying specific Primers designed to amplify the levansucrase gene

Levansucrase (*SacB*) gene Amplification

The reaction mixture (DNA template 4 µl, primers forward and reverse 2 µl, PCR premix 2 µl, free nuclease water up to 20 µl) Promega Co. used PCR Bioneer Co. to amplify the gene. As shown in Table (2), the PCR was programmed, and 2 µl PCR product with 2 µl of 1Kb DNA ladder (Promega Co.) was transported in 1% agarose gel for 45 minutes at 90 volts; the outputs of the amplified gene were sent to the Korean Macrogen Co. to determine the sequences. The sequence of nitrogenous bases of the *SacB* gene registries at accession number ON81164.1 in the NCBI gene bank.

Table 2. 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**

Cloning and Expression of *SacB* gene

The first step of cloning was the levansucrase gene extracted and purified from agarose using Gel/PCR DNA extraction kit from Bioneer Co. (Table 3).

Table 3. The components of agarose Gel/PCR DNA extraction kit

Component	Volume
MF Buffer	115ml
Wash Buffer	Ethanol 95%
Elution Buffer	5 ml
MF Column	200 PCS
Collection Tube	200 PCS

The gene was ligated with a pTG19-T vector using T4 DNA ligase the ligated mix was prepared according to the manufacturer's instructions as shown in (Table.4).

Table 4. The ligation mix

Component	Volume (microliter)
pTG19-T vector 25 ng/ul	2
SacB gene	2
T4 DNA ligase 200u/ul	2
10X Buffer ligase	1
Free nuclease water	Up to 10

The cloned pTG19-T vectors were inserted into *E.coli* DH5α competent cells using a TA cloning kit. The transformed cells were spread on LB (Luria Bertani) plates containing 50µg/ml of ampicillin and 80µg of each X-Gal 20mg/ml and 100mM IPTG and spread on the surface, and then incubated overnight at 37 °C. The pTG19-T-*SacB* gene was extracted from (white colonies) of *E.coli DH5α* positive cells by Plasmid Extraction kit (Bioneer Co). *SacB* gene was restricted from the pTG19-T vector using *BamHI* restriction endonucleases and purified by migrating in 1% Agarose and extracting. The extracted DNA fragments were ligated with the *BamHI* restriction site in the pET-28a(+) expression vector using T4 DNA ligase. This recombinant plasmid was put into *E. coli* BL21 (DE3) competent cells were cultured in an L.B (Luria Bertani) broth medium containing kanamycin 50 ug/ml in a shaking incubator at 37 °C with 220 rpm until the turbidity at 600 nm reached 0.6–0.8. The induction was done by adding one mM IPTG and 0.2 mM CuSO4. The time of installation was four h at 37°C. After being collected, the cells were suspended in a lysis buffer; the cells were sonicated by using 6–8 cycles of 10-30 seconds, then centrifuged for 30 minutes at (12,000 xg). The crude enzymes were in the supernatant (14).

Levan production, extraction, and purification: The polymer were synthesized according to (41), with some modifications. Recombinant levansucrase (14.31 U/ml) produced from *E. coli* incubated with potassium phosphate buffer 0.05 mM (pH 7.0) containing 100 g/L of sucrose and 0.05 g/L of $CaCl_2$ at 37 ◦C for 48 h. After the end of synthesis, the pH was adjusted to 9.0–10.0 by potassium hydroxide (1 M). Then chilled ethanol 80% (-20 °C) was added at a ratio of 1:2 and 1 ml of 1%; $CaCl_2$ was stirred for 20 min, then left for 24 h in a refrigerator (4 °C), and centrifugation at 10000 xg for 15 min. The process was repeated with a 1:4 ratio of chilled ethanol; then the precipitate was placed in a petri-dish at 45 °C. According to Bajpail et al. (4), the semi-dried levan was suspended in a small amount of de-ionized water and dialyzed (12 to 14 kDa) for 72 hours at 4 °C. González et al. (11) mentioned this diameter as a way to get rid of low molecular weight 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.

Determination of Levan Identity and Characteristics: HPLC analysis: The USA HPLC system FLC (Fast Liquid Chromatographic) NH2 column (50×4.6 mm) 3 µm was used to conduct this analysis. A reflective index detector separated the aqueous extract for I.D. detection (Shimadzu RID-10A). The rate flow for the mobile phase is 0.1 mL/min containing deionized water in 50 volumes with 50 volumes of acetonitrile at 30 °C. The injection sample 20µl included, in addition to the levan produced in this study, two standard levan, one produced from the bacteria *Erwinia herbicola* (SKU number L8647-1G) and another from chicory (SKU number F8052-50G), both supplied by Sigma-Aldrich and also included in the standard (fructose, glucose, and sucrose) from HiMEDIA Co. The standards were prepared by dissolving a sufficient amount of the sugars in (10 ml of the mobile phase 100 µg/ml and then diluted to 25 µg/ml) and filtered through Millipore with a 0.45-µm pore size membrane; The separation process was carried out

sequentially under the optimal conditions of the experiment.

(FTIR) analysis: The Bruker–Tensor 27 with an ATR unit was used to conduct this analysis. The levan was compared to the levan from *Erwinia herbicola* (Sigma-Aldrich) as a standard. The device calculates the amount of infrared energy that passes through a sample in the 600–4000 cm–1 wavenumber range. The result is shown on a graph, where X is the wave number, and Y is the percentage of light that gets through.

RESULTS and DISCUSSION

The Novel Strain Isolation and Identification: This study tested 26 Bacillus sp. isolates from various foods and soils from different locations to produce levansucrase. One isolate with the highest activity of 6.05 U/ml was selected for further research (Fig. 3). This isolate was initially diagnosed through morphological and biological tests (20) using a Vitek 2 compact system analysis. This analysis has shown that the isolate under study belongs to *B. licheniformis*, with a 94% probability. The identification was finally confirmed by a molecular method using 16S rRNA gene amplification (2, 5, 13, 35). which also approved that the isolate belongs to *Bacillus licheniformis* with 98.93-100.00 % similarity using BLAST from NCBI (Table 5). According to these results, it was registered in the database with accession number OM672244.1 and designated as *Bacillus licheniformis* MJ8. It should be mentioned that Santhana et al. (36) also isolated a strain of *Bacillus licheniformis* from the hot springs area in Ranony Province, Thailand, at 50 °C that showed high enzyme productivity registered under accession number FJ171619.1, which was used then for cloning the gene responsible for the production of levan into *Escherichia coli*. And Pongsakorn et al. (32) The *Bacillus amyloliquefaciens* KK9, isolated from soil in Thailand and identified by 16S rRNA, the sequence of recombinant plasmid was verified by nucleotide sequencing and assigned to GenBank in Accession number KC477262. Permatasari et al. (31, 44) isolated *Bacillus* sp. from the soil with high levansucrase enzyme production and identified the 16S rRNA gene. It is identified as *B. licheniformis* strain BK2, accession number MF774878.1 in the NCBI. The *SacB* gene from this strain was cloned in a high-efficiency component cell of *Escherichia coli*.

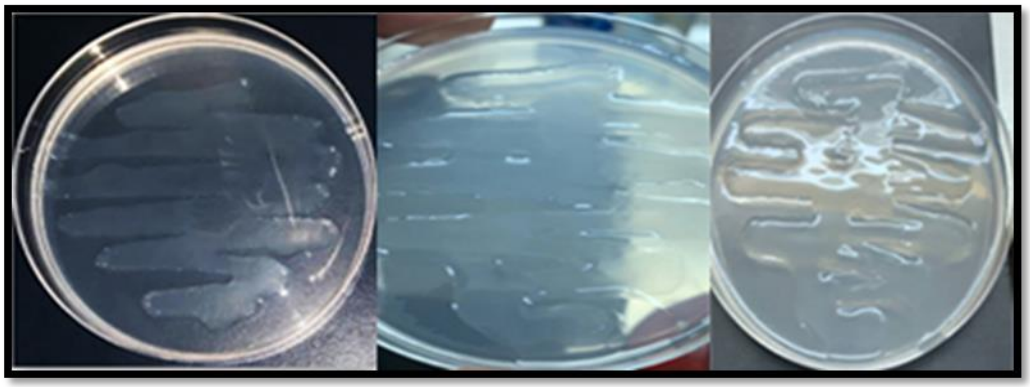

Fig. 3. Levan produced from Bacillus licheniformis MJ8 on M.S.A. media containing 20% Sucrose after incubation at 37°C for 48h

Isolation and Identification of *SacB* gene

The *Bacillus licheniformis* MJ8 *SacB* gene was found to consist of approximately a single band of ≈1500 bp after electrophoresis (Fig. 4); it contains 1449 bp of nucleotides, encoding 482 amino acids. The gene sequence has an identity between 94.06% and 100.00% BLAST results (Table 6). Previous studies have reached the same conclusions. The *SacB* gene of *B. amyloliquefaciens* KK9, amplified and cloned in *Escherichia coli*, has a molecular size of 1422 bp, according to (32). Santhana et al. (36) found that the levansucrase-producing gene in *B. licheniformis* RN-01, amplified for cloning in *E. coli*, had a size of 1446 bp. While trying to clone the *SacB* gene into *Escherichia coli* bacteria, Lili et al. (19) discovered that the gene's molecular size reached 1449 bp after being isolated from *B. licheniformis*. Min et al. (25) found that the *SacB* gene was encoded by 1428 bp nucleotides from *B. velezensis* BM-2 and can be cloned and expressed in competitive *Escherichia coli* BL21 (DE3).

Table 5. The sequencing Identity % of 16S rRNA gene of NCBI strains with *Bacillus licheniformis* MJ8

	The strains	Identity (%)	Accession
1	*Bacillus licheniformis* strain BY65	100.00	MN133912.1
2	*Bacillus licheniformis* strain SR31	100.00	MH010387.1
3	*Bacillus licheniformis* strain RB7	99.02	MF138121.1
4	*Bacillus licheniformis* strain QT331	98.93	MT043736.1
5	*Bacillus licheniformis* strain SU17	98.93	MN923423.1

Table 6. The sequencing Identity % of levansucrase gene of NCBI strains with *Bacillus licheniformis* MJ8

	The strains	Identity (%)	Accession
1	*Bacillus licheniformis* strain 8-37-0-1 levansucrase gene	100.00	KF647836.1
2	*Bacillus licheniformis* strain SK-1 levansucrase gene	100.00	JN712303.1
3	*Bacillus licheniformis* strain TH4-2 levansucrase gene	99.93	JN712304.1
4	*Bacillus licheniformis* strain RN-01 levansucrase gene	99.86	FJ171619.1
5	*Bacillus licheniformis* strain LN-05 levansucrase gene	94.06	MW972060.1

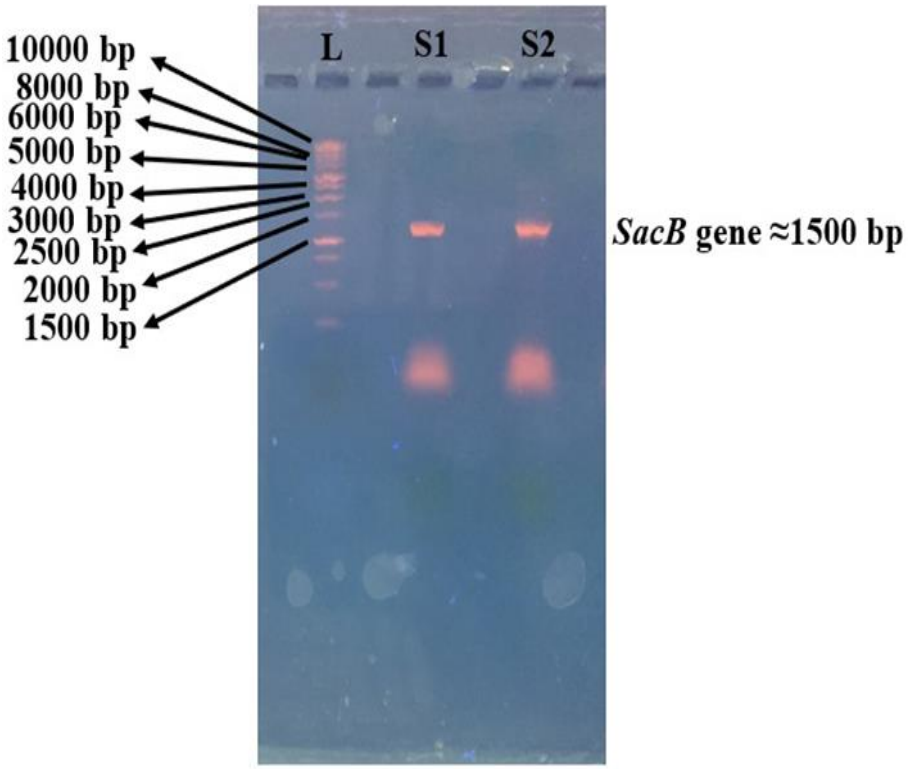

Fig. 4. Product of amplified levansucrase gene migration on Agarose (1%), S1; S2: sample, L: 1Kbp ladder

Cloning Levansucrase (*SacB*) gene

The *SacB* gene from the local isolate was extracted and inserted into the pTG19-T vector, transforming it into a competent cell. The transformed positives of *E. coli* DH5α (white colonies) were discovered by blue-white screening containing pTG19-T with the *SacB* gene. In contrast, blue colonies were found without recombinant plasmid (Fig. 5). The (pTG19-T-*SacB*) plasmid was extracted and digested with *BamHI* restriction endonuclease. The result showed two bands on the agarose pTG19-T with size ≈2900 bp and size ≈1500 bp *SacB*, correlated with a 1 Kb ladder (Fig. 6). These results confirmed the insertion of the levansucrase gene into *E. coli* DH5α.

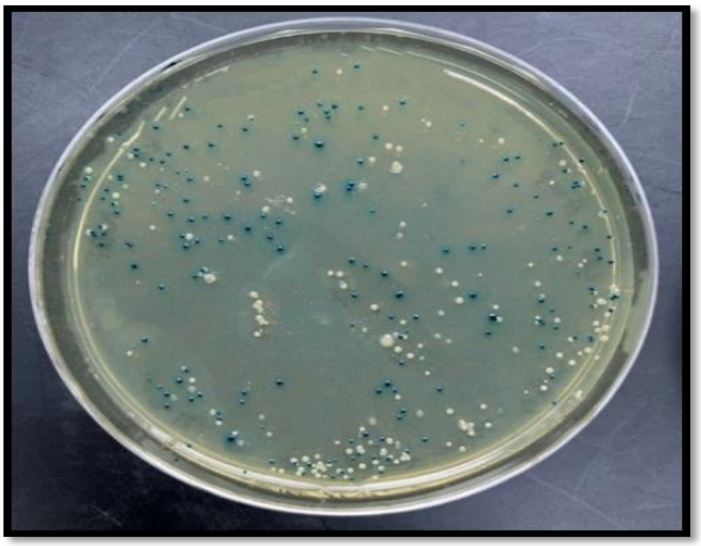

Fig. 5. The results of the Blue-White test for competent cell, the white colony have the recombinant plasmid pTG19-T-*SacB*

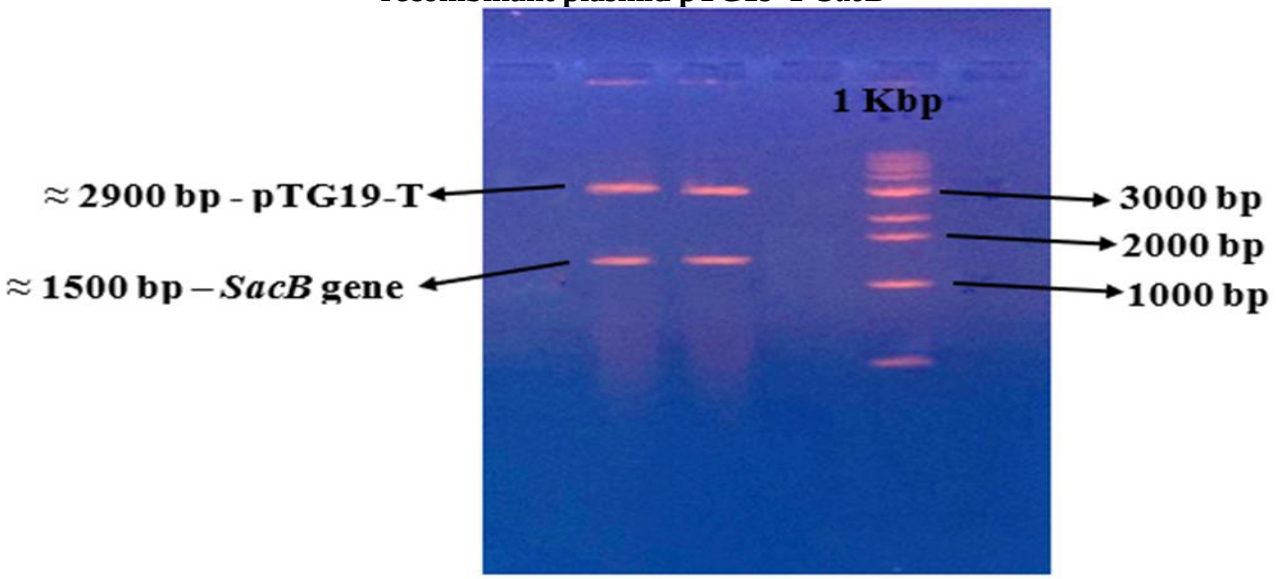

Fig. 6. The results of *BamHI* restriction enzyme of recombinant plasmid pTG19-T – *SacB*

Expressing of *SacB* gene from recombinant *Escherichia coli:* The *SacB* gene was purified and ligated with pET28a(+) expression vector (Fig. 7) using T4 DNA ligase and transformed into *E. coli* BL21 (DE3) cells. The transformed cells (Fig. 8) were picked on LB-agar medium, including kanamycin, according to the kanamycin resistance marker originating from the pET-28a(+) vector. IPTG inducted the levansucrase gene expression. The result showed that the levansucrase activity reached 14.31 U/ml. The high production of the enzyme by cloned cells is due to the nature of the plasmid used in the study, which was found in the cells in more than one copy (13).

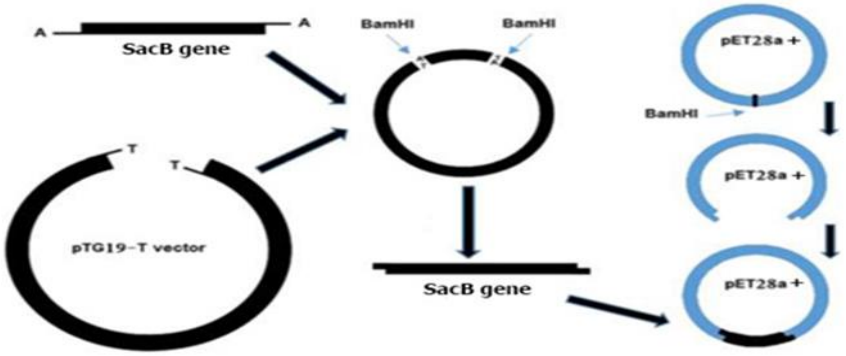

Fig. 7. Schematic representation of the *SacB* gene cloning

The cloning of the levansucrase *SacB* gene has been reported in several studies. Lorenzo et al. (21) found a possibility of cloning the *SacB* gene from *Erwinia amylovora* into *E. coli* BL21 (DE3) competent cells. The levansucrase gene was amplified from genomic DNA and ligated into a pETM-30 expression vector between the *NcoI* and *EcoRI* restriction sites. While Santhana et al. (36) *SacB* from *Bacillus licheniformis*, RN-01 strain was cloned and expressed in *Escherichia coli* with the highest level of the gene expression plasmid, pBluescript II SK. The *SacB* gene was found to be composed of a single 1446 bp. This was predicted to encode 482 amino acid residues. Lili et al. (19) were able to clone the same gene from *B. licheniformis* strain 8-37-0-1 to the high-efficiency *E. coli* BL21 (DE3) using the expression pET-21b(+) vector. Then, the gene encoding the enzyme was cloned, and it contained an ORF of 1449 nucleotides, encoding a 482 amino-acid protein with a predicted 29 amino-acid signal peptide. And in another study, Min et al. (25) found the possibility that the levansucrase gene of *B. velezensis* BM-2 was cloned to plasmid pET-32a-Acma-zz, and the recombinant plasmids were transferred to *Escherichia coli* BL21. A transformed clone was selected to efficiently express and secrete the fusion enzymes with an Acma-tag. The purification efficiency was 93.4%, with a specific activity of 16.589 U/mL protein.

Fig. 8. The transformed *E. coli* BL21 (DE3) contain pET28a(+)-*SacB* gene on L.B Kanamycin agar

Identification of Levan

HPLC analysis: To confirm that the polysaccharides from *Bacillus lichniformans* MJ8 in this study belonged to levan, HPLC technology was used to identify them. The retention times for the standard levan from *E. herbicola*, Chicory, and standard of fructose (F), glucose (G), and sucrose (S), in addition to the levan produced in this study, are shown in Fig. (9) A, B, C, and D, respectively. The R.T. for the levan under investigation, which was 2.523, 2.533, and 2.540 minutes, coincides with the standard of levan; one peak is observed for the levan under study. Fructose, glucose, and sucrose, on the other hand, had retention times of 2.546, 4.228, and 5.148 minutes, respectively (Table 7).

Table 7. The retention time for standard and levan produce from local isolate

Seq	Samples	Retention time (minute)
A	Standard levan of *E. herbicola*	2.523
B	Standard levan of Chicory	2.533
C	Fructose	2.563
	Glucose	4.228
	Sucrose	5.148
D	*B. lichniformans* MJ8 Levan	2.540

These results show that the levan produce in this study is pure, contains fructose units, and is a homopolysaccharide type (40), in addition to being a fructan type and a member of the fructooligosaccharide (FOS) group (24). Additionally, Benigar et al. (6) successfully produced and isolated levan from two isolates, Zymomonas mobilis and E. herbicola, and analyzed levan using HPLC technology. At the same time, Pei et al. (30) used the HPLC method to demonstrate that levan structural constituent was 2,6-substituted -fructose.

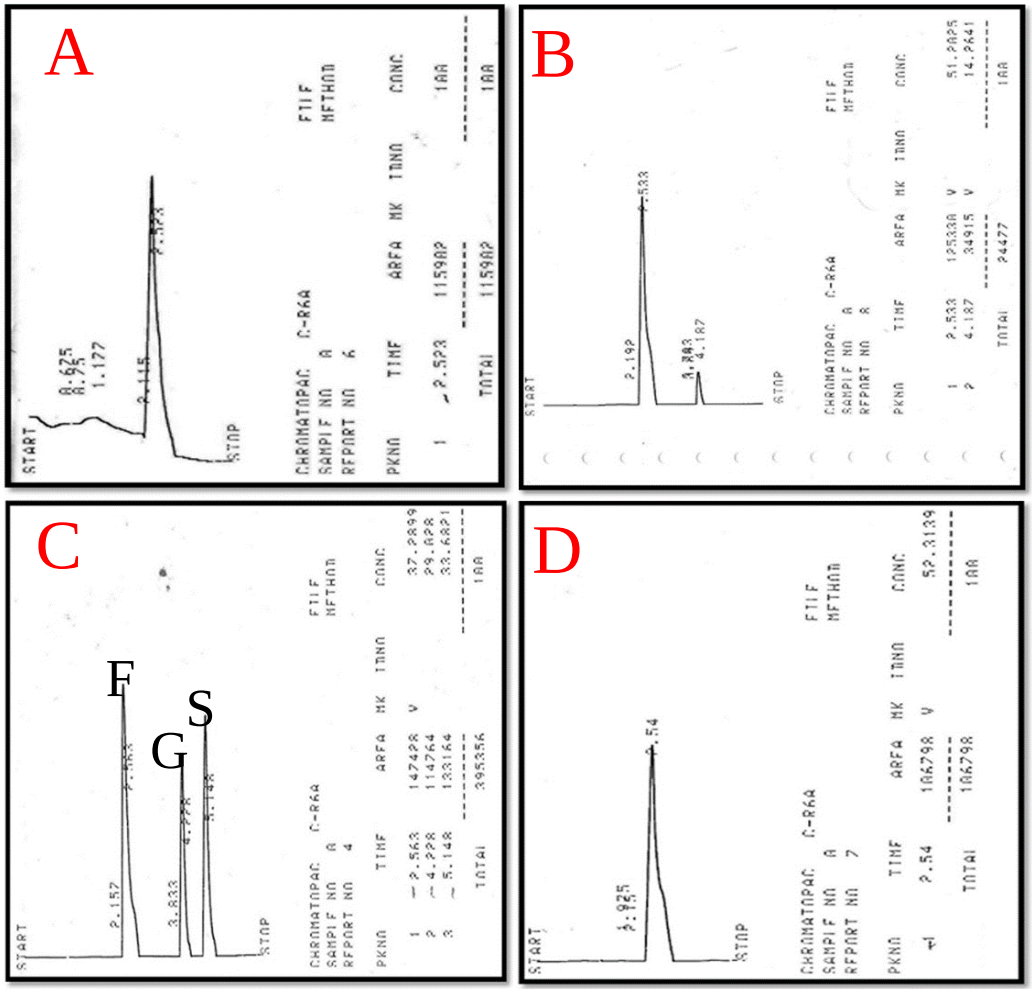

Fig. 9. HPLC analysis. A: standard.Levan from *Erwinia herbicola*, B: standard Levan from Chicory. C: sugar solution (F: Fructose, G: Glucose, S: Sucrose),.D: Levan produced in this study from *B. lichniformans* MJ8

FTIR analysis: According to the results of this method, both levan contained the adjustable frequency group C-O at wavelengths of 1123.44 and 1122.07 cm^{-1}, respectively (Fig. 10). The bending group, in contrast, begins at CH and OH at wavelengths of 1424.50 and 1425.42 cm^{-1}, respectively, which correspond to the respective frequencies of these groups at 1325.66 cm-1 and 1326.78 cm^{-1}. On the other hand, the ketone groups' stretchable frequencies were found to be 1645.44 cm^{-1} and 1660.14 cm^{-1}, while the C-H groups' adjustable frequencies were found to be 8.58 cm^{-1} and 2933.13 cm^{-1}, respectively, for the two samples. The structure of both levan was homologous with the broad stretching peak of O-H stretching at approximately 3319.26cm-1, C-H vibration noted at approximately 2935.48cm^{-1}, and carbonyl C=O spelling recorded at 1722.31cm^{-1}, according to research by Jathore et al. (18) produced levan from *Pseudomonas fluorescens*. Based on an analysis of the FTIR spectra of levan produced from *B. licheniformis*, According to Shukla and Goya's (38) analysis of the FTIR spectrum, polysaccharides in levan made from *L. mesenteroides* contain hydroxyl stretching and vibrations. Mamay et al. (22) reported that the extension of O-H vibrations first appeared at a wavelength of about 33300 cm-1. At the same time, the peak at wave number 1660cm^{-1} is typical for C=O stretching, while the band around 2900 represents C-H stretching. While Nasir et al. (27) used FTIR to analyze the levan secreted by Halo monas and Chromohalobacter, they discovered that the O-H stretching occurred between 3600 and 3200 cm^{-1}, the C-H stretching occurred between 3000 and 2800 cm^{-1}, the vibration of C=O occurred at 1641.16 cm^{-1}, and the region of typical carbohydrate occurred within the 800-1000 cm^{-1} wavenumber range.

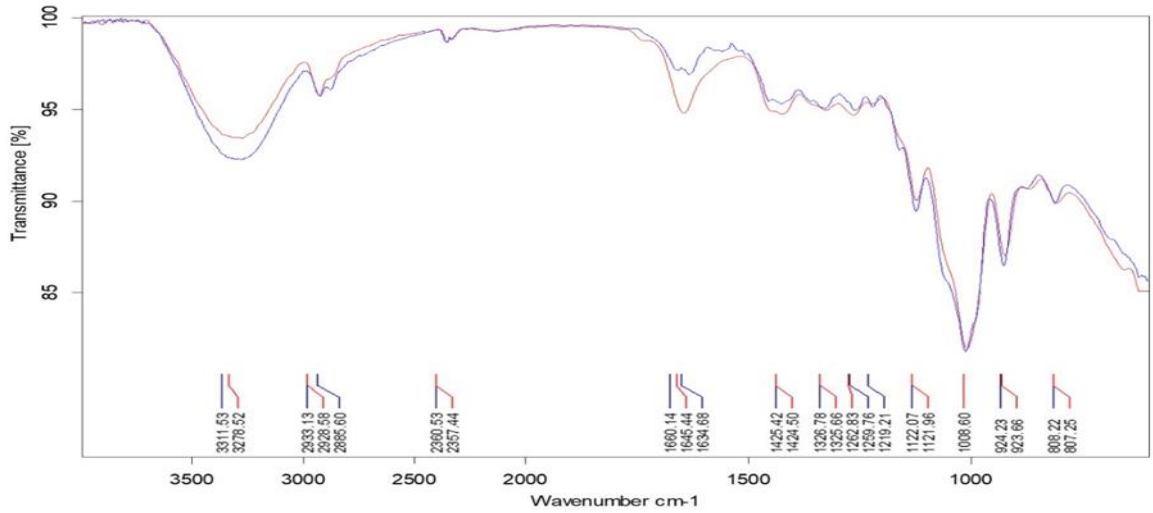

Fig. 10. Fourier-transform infrared spectroscopy used to compare standard levan from E. herbicola (Redline) and focal isolate B. lichniformans MJ8 (Blueline)

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