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Levan Production using *Pseudomonas Brassicacearum* Isolated from Rhizosphere Soil of *Cowpea* Farm in Iraq

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Abstract: The present investigation, was designed in order to isolate bacteria from rhizosphere soil of *Cowpea* farm in Iraq which is capable of levan production. The selected levan producing bacterium was identified as *Pseudomonas brassicacearum* based on the phenotypic identification by Bergey's Manual of Systematic Bacteriology and confirmed with the 16S rRNA gene sequencing. Acid hydrolyzed levan sample was subjected to TLC technique which revealed that, levan is composed of only one sugar which is fructose. R_f values of levan produced by *P. brassicacearum* was 0.42 which is identical or close to R_f values of fructose of 0.42. The effect of some factors on levan production by *P. brassicacearum* was investigated. The results showed that the best carbon source for levan production was sucrose 7.77 g/l, and casein was the best nitrogen source for levan production which gave 8.56 g/l, followed by ammonium sulfate and corn steep liquor which they gave 8.52, 8.09 g/l respectively. The highest levan yield was at the sucrose concentration of 300 g/l which gave 7.95 g/l. At initial pH of 7.8, *P. brassicacearum* gave their highest levan production that was 7.77 g/l. Levan production by *P. brassicacearum* continued increasing until it reached its maximum production at 40ºC which was 8.24 g/l. The optimal incubation period for levan production, was estimated at 8.70 g/l after 72 h of incubation.

Key words: rhizosphere soil, *P. brassicacearum*, levan production, carbon sources, 16S rRNA gene sequencing.

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Introduction

Levan is a homo-polysaccharides (Poly-fructan, biopolymer) with β- 2,6- linked branches that consist of β- 2,1- linked side branched, The enzyme responsible for levan synthesis generally is called as levansucrase, from sucrose- based substrate that catalases biosynthesis of levan by converting fructose on the sucrose donor to an acceptor molecule (1). In nature levan is derived from several sources such as Bacteria, Yeasts, molds and plants (2, 3, 4, 5). The unique properties of levan like odorless, nontoxic, biologically active, tasteless, non-mutagenic, made it suitable to use in more than one site of potential applications in food, pharmaceutical, Immunostimulant, cosmetics, antimicrobial and antitumor. There are many researchers work in the

field of medicine and pharmaceutical applications, such as antipathogenic bacteria and antiviral (7, 8). Levan also used as anticancer and antitumor agent (9). Furthermore, levan is used as Immune modulator (10), anti-inflammatory (11) and as immuno-stimulant by the activation of non-specific defense mechanisms to protect fish against pathogens (12). The earlier levan production from bacteria has been carried out by using of different bacterial species such as *P. fluorescencs* (13). *P. syringae* (14), *Leuconostoc kimchi* (15). (16) Reported that there are several bacterial species producing extracellular levan, like *Zymomonas mobilis*, *Bacillus subtilis*, *Bacillus polymyxa* and *Acetobacter xylinum*. However, Bacterial production of levan and other exopolysaccharides in rhizosphere soil play an important role in the regulation of carbon source diffusion in addition to its role in the increasing of water retention in the microbial and plants root environment. One of the rhizosphere bacterial species that capable to produce levan in the soil is *Pseudomonas spp*. which their production to levan is increased during desiccation (17), the increase in production leads to increase the aggregation of soil, and thus improve growth conditions of rhizosphere microbes and plants (18). *Pseudomonas brassicacearum* is a gram negative, motile bacterium. It's aerobic, oxidase and DNase positive and levan producing bacterium that have a rod shape with about 1.0-1.5 µm in length and 0.5 µm in width. These bacteria form a mucoid colonies with entire edges when grown on TSA. At the late stationary phase, these bacteria produce a diffusible, brown-orange colored pigment on TSA and TSB media. In addition to that, these bacteria produce a fluorescent pigment when grown on CAA media (19). Bacteria in young cultures form small, mucous colonies and this represents phase I. In the present work, the aim was the isolation and the identification of new *P. brassicacearum* which are able to produce levan. It also aimed to optimize levan production by *P. brassicacearum* isolate.

Materials and Methods

Samples Collection

A levan producing *P. brassicacearum* used in this study was isolated from rhizosphere soil of cowpea (*Vinga unguiculata* L.), sample of soil were collected carefully from different fields of cowpea in Iraq by uprooting the root system, and placed them in clean sterile nylon bags for transport to a laboratory. The samples were stored at 4 °C for bacterial isolation.

Isolation of Bacteria

Ten grams of soil had been mixed in 90 ml of sterilized peptone water and then the soil suspension serially diluted. The 10^{-1}, 10^{-2} and 10^{-3} dilutions were streaked on nutrient agar plates (HIMEDIA-INDIA), the pH adjusted to 7.5, and was sterilized by autoclaving at 121 °C for 20 min, after that, all the plates were incubated anaerobically at 37 °C for 24-48 h. Morphologically different growing colonies were picked up and subcultured by streaking method on sucrose nutrient agar media (which composed of 200g/l sucrose and 28g/l nutrient agar, this media was sterilized by Autoclaving at 121 °C for 20 min.) and then incubated anaerobically for 24-48 h at 37 °C. Mucoid colonies were

chosen for identification and characterization (20).

Phenotypic and Genotypic Identification

Biochemical and physiological characterization of selected bacteria were performed according to Berge's Manual of Systematic Bacteriology (21), bacterial isolates were characterized according to both their phenotypic traits: such as shape, size, margin, surface, elevation, color, pigmentation and staining by Gram stain, and also Biochemical test. The later was performed using standard biochemical and physiological testes that included, catalase test (3% H_2O_2), oxidase reaction (Kovacs method) and diffusible pigment production, In addation carbohydrate fermentation was accomplished by using medium containing specific carbohydrate source (sucrose, glucose, lactose, mannitol, maltose, rhaminose) and starch hydrolysis was achieved by culture isolation on Starch nitrate agar medium, After incubation for 24 h, at 37 °C, the plate was covered with iodine, and a clear zone around the growth of colonies was detected on the medium plate. Gelatin hydrolysis was achieved by stab tubes of nutrient agar containing gelatin, after inoculation with tested bacteria, liquefaction of gelatin was observed (22). The Identification was then confirmed by 16S rRNA gene sequence. Genomic DNA from the isolates was extracted using of Gene JET^{TM} genomic DNA purification Kit (Thermo). After DNA extraction, PCR amplification reaction was performed by using PCR master mix (Thermo) in 50 µl final volume, consist of 25 µl maximum hot start PCR master mix (2X), using universal bacterial primer sets: 1µl (20 µM 16S RNA Forward primer 5`-AGAGTTTGATCCTGGCTCAG-3`) 1 µl (20 µM 16S RNA Revere primer 5`-GGTTACCTTGTTACGACTT-3`), 18 µl deionized water, nuclease-free and 5 µl DNA template. The PCR reaction was incubated in a thermocycler under following conditions, the initial denaturation at 95°C for 5 min. followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30sec extension at 70 °C for 2 min and final extension at 72°C for 10 min. Finally, the reaction was hold at 4°C hold. The PCR products were verified by agarose gel electrophoresis and purified with the genomic DNA purification Kit (Qiagen Science, MD), the product sequences were aligned with specific public databases that are available in the Genbank resources using NCBI site (http://www.ncbi.nlm.nih.gov/blast / Blast. cgi). In addition, Phylogenetic tree was generated by alignment of the obtained sequences of 16s rDNA of the tested bacteria with the data base sequences in the gene bank.

Levan Production

Inoculum Preparation and Fermentation Conduction

The first step for bacterial biopolymer (levan) production was done by preparation of standard inoculum (1ml contained 7 x 10^6 viable cell according to McFarland tube) for fermentation, which prepared by inoculating a loopful of pure bacterial culture into Erlenmeyer conical flask (250 ml volume) that contains 50 ml nutrient broth medium (HIMEDIA-India), and incubated at 36 °C for 24 h. The productive medium that modified by

(23) was used as a fermentation medium to produce levan, and is composed of (g/l): commercial sucrose, 200; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 5.5. The pH was adjusted to 7.8. The fermentation process was carried out by dispering of this medium into 250 ml Erlenmeyer conical flasks which then sterilized by autoclaving at 121 °C for 20 min. The flasks were inoculated with 2.5 ml (5%) of standard inoculum to each flask, and incubated (fermented) statically at 37°C for 24 h.

Levan Extraction

At the end of fermentation period, samples 10 ml were centrifuged at 10000 rpm for 10 min to remove bacterial cell free supernatant was used to precipitate levan by the addition 1.5 volumes of absolute ethanol, and incubated for one hour at 37°C. Then the levan were collected by centrifugation at 10000 rpm for 10 min. The precipitated pellets were hydrolyzed by HCl 5% (v/v) for one hour at 100 °C. Levan was estimated corresponding to fructose units by using Glucose oxidase kit (SPINREACT) and the absorbance was read at wavelength 505 nm. (38).

Characterization of Levan by Thin-Layer Chromatography

Equal weights (1 mg) of glucose, sucrose and fructose were dissolved in 1 ml of 1% ethanol. Then, a 10 µL of hydrolyzed levan and other sugar suspensions were spotted by capillary tubes at equal distances and about 2 cm of the lower edge of the plate. Then, TLC plate was placed in a closed jar that contains the mobile phase which is composed of chloroform /acetic acid /water [v/v] (6:7:1). Levan and other sugars were diffused through a silica gel plate. After drying, the plate was sprayed by a mixture of H_2SO_4 and ethanol at [v/v] (9:1) and placed in oven for 5-10 min, at 90 ºC. Levan components were appeared as dark colored spots (24).

Optimization of Levan Production

Effect of Carbon Sources

This experiment was conducted to study the effect of various carbon sources on levan production and the possibility of using cheap and available carbon source alternatives in its production. Fructose and agricultural materials like Date syrup and sugarcane molasses were used. Dates syrup was obtained from Baghdad markets and sugarcane molasses was obtained from a sugar factory in Al-Hawamdiya city, Cairo, Egypt. These materials were used by replacing sucrose in the productive medium with the equivalent amounts of alternatives (38). All flasks were incubated at 37 °C for 24 h. Levan amount was estimated as described above.

Effect of Nitrogen Sources

This experiment was conducted in order to investigate the effect of various nitrogen sources and to determine the most suitable nitrogen source on levan production. Organic (Corn Steep Liquor and Casein) and inorganic (Ammonium sulfate and Ammonium phosphate) nitrogen sources were used as alternatives for the original nitrogen source in the productive medium. These materials were used by replacing yeast extract in the productive medium with equivalent amounts of alternatives. Flasks were incubated and levan

amount was estimated as described above (38).

Effect of Sucrose Concentrations

This experiment was conducted to identify the effect of various sucrose concentrations on levan production. Different sucrose concentrations (50g, 100g, 150g and 300g) were used in productive medium preparation. Flasks were incubated, and levan amount was estimated as described above (38).

Effect of Initial pH

This experiment were performed to study the effect of various pH values on levan production. Four groups of productive medium flasks were prepared, each group was adjusted to a particular pH value (7, 7.5, 8, and 8.5) by adding 1 N HCL and 1 N NaOH. Flasks were incubated and levan amount was estimated as described above (38).

Effect of Temperature

To study the effect of temperature on levan production, different incubation temperatures were used (30, 33, 40 °C) for 24 hr. At the end of fermentation period, levan amount was estimated as described above (38).

Effect of the Incubation Period

To study the effect of the various incubation times, production medium was incubated for (24, 48, 72 and 96) hr. at 37 °C. This experiment was conducted to determine the effect of incubation time on bacterial growth and levan production. At the end of fermentation period, levan amount was estimated as described above (38).

Statistical Analysis

The effect of different factors on levan production were tested statistically by using Duncan's test multiple range test at the level of $P < 0.05$. Least significant difference –LSD test was used to significantly compared between means in this study (25).

Results and Discussion

1. Bacterial Identification

The isolate was identified through molecular characterization by 16S rRNA sequencing and phenotypic characterization by using standard physiological and biochemical tests. The isolate showed small and mucus, yellow pigmented colonies which released a fluorescence pigment in the growth medium. Microscopic examinations revealed that the isolate was Gram negative rods, single or paired. The isolate showed different biochemical results shown in the table (1).

Table (1) Biochemical and Physiological tests of the isolate

No. of Test	Test	Result
1	Colony texture	Mucoid
2	Gram stain	Negative (-ve)
3	Growth on MacConkey medium	Positive (+ve)
4	Catalase	Positive (+ve)
5	Oxidase	Positive (+ve)
6	Blood Hemolysis	Negative (-ve)
7	Oxidation test	A/K*
8	Urea hydrolysis	Negative (-ve)
9	Starch hydrolysis	Positive (+ve)
10	Gelatin hydrolysis	Positive (+ve)
11	Production of byoverdine (Green fluorescent pigment)	Positive (+ve)
Fermentation of Carbohydrates		
1	Sucrose	Positive (+ve)
2	glucose	Positive (+ve)
3	lactose	Positive (+ve)
4	mannitol	Positive (+ve)
5	maltose	Positive (+ve)
6	rhaminose	Positive (+ve)

***A/K; A= Acid production; K**

This bacteria was shown to be positive for the catalase, oxidase test, Green fluorescent pigment and growth on MacConkey agar medium. Hydrolytic reaction of the tested strain showed that it is positive for starch and gelatin, whereas negative hydrolysis for urea was detected. The isolate was positive fermentation for all sugars (Sucrose, glucose, lactose, mannitol, maltose, and rhaminose) that used in the present study, while it was negative for Gram stain and blood hemolysis. The 16S rRNA gene was amplified in the isolate of representative strain using universal primers and sequence analysis was accomplished, and compared with the isolates that retrieved by NCBI-Blast and aligned with clustal2. Phylogenetic tree showed that new strain is identified as a strain of *P. brassicacearum.* The results of 16S rDNA sequencing showed high homology score, which the value of similarity was 99 % with isolates of *P. brassicacearum* especially *P. brassicacearum* subsp. *brassicacearum* NFM421 and *Pseudomonas brassicacearum* subsp. *neoaurantiaca* strain CIP 109457, that presents on the NCBI (Figure 1) using of reverse primers as a complement strand, produced complete DNA genome that is about 1154 bp, According to morphological, biochemical, physiological and genotyping characteristics, this bacteria has a high similarities with strains of *P. brassicacearum* (26, 27).

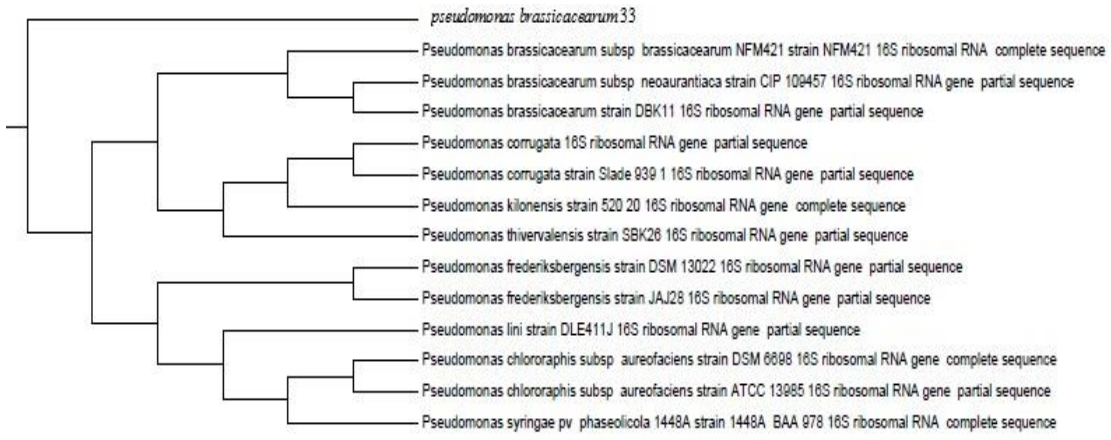

Figure (1) phylogenetic tree represents the relationship among the sequence of 16S rRNA gene of *P. brassicacearum* and the related isolates

2. Analysis of Levan by Thin-Layer Chromatography Technique

Acid hydrolyzed levan sample was subjected to TLC technique which revealed that levan is composed of only one sugar that is fructose. R_f values of levan produced by *P. brassicacearum* was 0.42, which is closer to R_f values of fructose 0.42. These results are in agreement with (28) and (25) who demonstrated that, levan analysis by TLC technique will liberate fructose only as a final hydrolysis product. Fructose and other sugars appeared as dark spots on TLC plates (Figure 2). Monosaccharaides (fructose and glucose) took the highest position on the chromatography, and this due to their low molecular masses, and also to the sucrose that is composed of glucose and fructose.

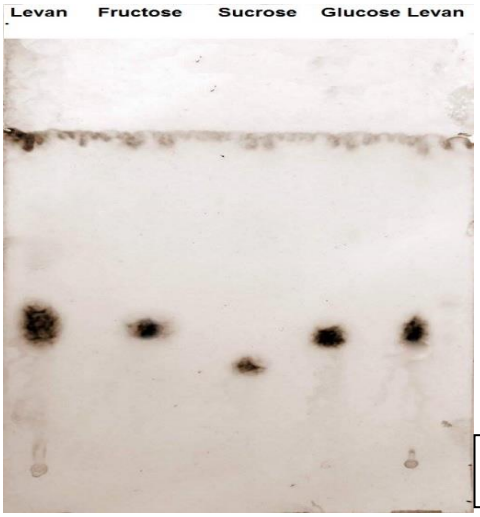

Figure (2) TLC analysis of levan of the isolate (3) treated with H_2SO_4 and ethanol at [v/v] (9:1) using silica gel plate with solvent system chloroform /acetic acid /water [v/v] (6:7:1)

3. **Optimization of Levan Production**

The main object of the present work was to investigate the selected strain of *P. brassicacearum* 33 to produce exopolysaccharide, that is called Levan, by using medium containing sucrose as carbon source and basal medium (Productive medium) supplemented with 20% of sucrose (29). The second aim was to optimize levan production under several factors affecting the growth of producing bacteria and amount of levan.

1. Effect of Carbon Sources

Levan production was optimized in order to identify the best carbon source that can give the highest levan yield. Three carbon sources (fructose, date syrup and molasses) were used instead of sucrose which was the sole carbon source in the original productive medium. As illustrated in table (2), sucrose was the best carbon source for levan production which gave 7.77 g/l, whereas fructose and date syrup did not show a significant difference in levan production 6.44 and 6.41g/l respectively. In addation, molasses yield low production when it was used as a carbon source 4.09 g/l. Sucrose is shown to be the best inducer and substrate for levansucrase (30). Using fructose as a sole carbon source decrease levan production. These results are in compatible with those of (31). It has been reported that the production of levan decreased when fructose was used as a carbon source instead of sucrose in the productive medium. The results of levan production using date syrup as a carbon source were in agreement with the results of (32) who revealed that the reduction in levan production is due to using the date syrup as a carbon source instead of sucrose. Levan production using molasses were also in agreement with the study of (33) and (24).

Table (2) Effect of Carbon Source on Levan Production (Mean ± SE)

Carbon source	Levan concentration(g/l)
Sucrose	7.77 ± 0.05 a
Fructose	6.44 ± 0.01 b
Date syrup	6.41 ± 0.05 b
Molasses	4.09 ± 0.17 c
LSD value	0.319 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is not significant difference from each other, according to Duncan's at 5% level.**

2. Effect of Nitrogen Sources

Four nitrogen sources (ammonium sulfate, ammonium phosphate, corn steep liquor and casein) were used to identify the best nitrogen source for levan production. As illustrated in table (3), casein was the best nitrogen source for levan production, it gave 8.56 g/l. This result was in

agreement with those of (24), study which indicated that, levan production rates is increased when casein was used instead of yeast extract in the original productive medium, increasing the biosynthesis production of Exo-polysaccharides can be effected by the combination of sugars unit and their concentration, some studies showed the concentration of EPS were higher with supplementation of medium by skim milk and whey protein that contain (Casein hydrolysates) (34). Ammonium sulfate $\{(NH_4)_2\ SO_4\}$ result was not significantly differs from the result that obtained from the use of casein 8.52 g/l. Increasing of levan production by using of ammonium salts was not agreed with (35) who showed that the possibility of negative influence of these salts upon levan production. Ammonium phosphate decreased levan production to 6.84 g/l and this result suggest that this source is less efficient for levan production by this isolate. Using of corn steep liquor gave a good production of about 8.09 g/l, that was not differs significantly from the result that obtained by ammonium sulfate.

Table (3) Effect of Nitrogen Source on Levan Production (Mean ± SE)

Nitrogen source	Levan concentration(g/l)
Yeast extract	7.77 ± 0.05 c
Ammonium sulfate	8.52 ± 0.01 ab
Ammonium phosphate	6.84 ± 0.23 d
Corn steep liquor	8.09 ± 0.15 b
Casein	8.56 ± 0.06 a
LSD value	0.437 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is no significant difference from each other, according to Duncan's at 5% level.**

3. Effect of Sucrose Concentrations

Different sucrose concentrations were used (50, 100, 150 and 300g) to identify the best concentration for levan production, as it showed in table (4). The highest levan yield was at the sucrose concentration of 300 g/l which gave 7.95 g/l. However, this result was not significantly differs from that those resulted from using of 200 g/l in the original productive medium which yield 7.77 g/l. These results are in agreement with (36) and (37) who stated that, the increase in levan production yield is proportional with sucrose concentrations in levan production medium.

Table (4) Effect of Sucrose Concentration on Levan Production (Mean ± SE)

Sucrose Concentrations (g/l)	Levan concentration(g/l)
50	4.22 ± 0.08 d
100	5.87 ± 0.05 c
150	6.62 ± 0.23 b
200 (Control)	7.77 ± 0.05 a
300	7.95 ± 0.07 a
LSD value	0.409 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is no significant difference from each other, according to Duncan's at 5% level.**

4. Effect of Initial pH

This experiment was conducted with different pH values 7, 7.5, 8 and 8.5 to identify the best pH value for levan production, that shown in table (5). Levan production yields were increased gradually when the pH values increased from 7 to 7.8, at initial pH of 7.8. *P. brassicacearum* gave their highest levan production that was 7.77 g/l. This result was not compatible to these declared by (38) who stated that, the optimal pH value for levan production by *Bacillus lentus* V8 was 6.5. Furthermore, the result was also disagreed with (39) who showed that, the optimal initial pH of *Z. mobilis* B-4286 was 5.0. When pH values were increased from 8 to 8.5, levan production was decreased gradually to reach to (6.18). At the pH value of 8.5, the result was inagreement with those of (38) who stated that levan production was decreased when pH values increased more than the optimal pH value of the production.

Table (5) Effect of Initial pH on Levan Production (Mean ± SE)

pH value	Levan concentration (g/l)
7	5.77 ± 0.01 d
7.5	6.68 ± 0.06 b
7.8	7.77 ± 0.05 a
8	6.34 ± 0.10 c
8.5	6.18 ± 0.16 c
LSD value	0.320 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is no significant difference from each other, according to Duncan's at 5% level.**

5. Effect of Temperature

The effect of temperature on levan production is illustrated in table (6). When this isolate was experimented for levan production at different incubation temperatures 30 ºC, 33 ºC, 37 ºC and 40 ºC.

Levan production by *P. brassicacearum* is continued to increase until reached its maximum production at 40ºC, with 8.24 g/l. The results were in compatible to those of (40) who declared the suitable temperature range for levan production is from 25ºC-40 ºC. However, each bacterial species has an optimal temperature for levan production which depends on levansucrase activation and fermentation conditions (41).

Table (6) Effect of Temperature on Levan Production (Mean ± SE)

Temperature	Levan concentration (g/l)
30º C	7.16 ± 0.06 d
33º C	7.43 ± 0.05 c
37º C	7.77 ± 0.01 b
40º C	8.24 ± 0.02 a
LSD value	0.135 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is no significant difference from each other, according to Duncan's at 5% level.**

6. Effect of Incubation Period

In order to identify the optimal incubation period for levan production, this experiment were conducted and levan yield was estimated at different incubation periods 24, 48, 72 and 97 hour and 40ºC, as it showed in table (6). The highest levan production by the isolate 8.70 g/l was brought after 72 h of incubation. Levan production was increased gradually to reach its maximum production at the optimal incubation period which represented by 72 h. The highest production was significantly differs from than that of the production obtained after 24 h of incubation. Subsequently, the production yields were decreased after 96 h of incubation. This result was disagreed with (24) who indicated that levan production by *Bacillus* sp. V8 is continued to increase after 72 h, and even after 96 h of incubation. (42, 37) showed that the highest levan production was obtained after 48 h of incubation. They suggest that the increase in levan production is due to levan degradation and acid hydrolysis.

Table (7) Effect of Incubation Period on Levan Production (Mean ± SE)

Incubation Period	Levan concentration (g/l)
24 h	7.77 ± 0.05 a
48 h	8.29 ± 0.15 b
72 h	8.70 ± 0.04 c
96 h	8.13 ± 0.14 b
LSD value	0.427 *
* (P<0.05).	

***represented values in the same column, that followed by the same letter, and there is no significant difference from each other, according to Duncan's at 5% level.**

Conclusions

The present research dealt with locally *P. brassicacearum* that are localized in rhizosphere soil and have the ability to produce levan in different quantities. The result showed the ability to improve levan production by locally isolated bacteria through the completion of fermentation process under optimized conditions that are specific to each isolate and the ability to produce levan by this isolate through the use of low-cost industrial by-products like molasses and corn steep liquor or cheap material like date syrup as carbon and nitrogen sources alternative.

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