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Microbial Levan from *Arthrobacter globiformis* Strain KX 146411.1: Characterization and Enhancement of Production

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Abstract: Levan producing bacteria was isolated from rhizosphere soil. The molecular identification of this isolate was conducted using 16S rRNA, which resulted in a sequenced region of 1295 base pairs. The sequence alignment in the gene bank indicated that this isolate has a high percentage of similarity (98%) to the retrieved consensus sequence of *Arthrobacter globiformis* strain JCM 1332. The produced levan was characterized using TLC. The effects of nutritional and physical factors on this isolate's levan production were investigated. The results demonstrated that the optimal sources for carbon and nitrogen during levan production were sucrose and casein, yielding 7.8 g/l and 8.24 g/l of levan, respectively. The highest levan yield 8.6, 7.8, 8.6, 8.53 and 8.27 g/l were obtained at 300 g/l sucrose, pH of 7.8, 33°C, 72 h period of incubation and 150 rpm respectively.

Keywords: *Arthrobacter globiformis* , strain KX 146411.1 , levan , 16S rRNA .

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Introduction:

Levan is a homopolyfructose that is produced naturally by plants as well as microorganisms (1). It is a branched exopolysaccharide that is composed of D-fructo-furanosyl groups linked to each other by β-(2,6) links in the main chain and by β-(2,1) links at the branching points (2). Levan has several advantages for bacterial strains which have the ability to produce it. These advantages include bacterial survival in soil, phytopathogenesis and mutualism (3). The extracellular microbial enzyme levansucrase (EC 2.4.1.10) is responsible for levan formation using sucrose as a substrate. This enzyme cleaves sucrose molecule, using fructose residues to build up levan polymer chain (4). Various bacterial species produce levan by sucrose fermentation and this process is accomplished by levansucrase (5). There are many bacterial species which have the ability to produce levan by sucrose fermentation, and most of them are found in rhizosphere soil like, *Zymomonas, Pseudomonas, Mycobacterium, Corynebacterium, Erwinia, Bacillus, Azotobacter* and some other species (4,6). *Arthrobacter globiformis* strain JCM 1332 is a soil inhabitant bacterium that shows uncommon morphological changes in its life cycle represented by shifting from gram negative rods that are 0.6-0.8 µm in width and 1.0 to 1.5 µm in length in young cultures to gram positive cocci that are 0.6-0.8 µm in diameter in the older ones. In addition to that, they form large spherical bodies in liquid media that have 1-2 µm. These bacteria have a good growth on agar medium that contains ammonium salts, nitrate or urea as the only source for nitrogen (7).

Its non-motile bacterium and its colonies do not secret a distinctive pigment on yeast extract-peptone media (8). However, the production of levan by all bacterial strains is affected by several factors like, carbon source, nitrogen source, inoculum size, fermentation period and initial pH as well as phosphate sources, oxygen content, temperature, agitation rate and nutrient content of the fermentation media (1,9). There are many techniques that had been used to characterize levan polymer, such as chemical hydrolysis by thin layer chromatography (TLC) and also by different physiochemical techniques like Fourier Transform Infrared (FTIR), Thermal Gravimetric Analysis (TGA), High Performance Liquid Chromatography (HPLC) and ^{1}Hydrogen-Nuclear Magnetic Resonance and ^{13}Carbon-Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR spectroscopy) (1,10). Levan properties give its importance in various fields depending on its molecular weight like food, pharmaceuticals, cosmetics, blood plasma extender, and also as prebiotics, anti-inflammatory, anti-oxidant, sweetener, as industrial gums, as industrial rubber, flavors and fragrances carrier emulsifier, thickener and stabilizer (1,11,12,13,14). In the present work, *Arthrobacter globiformis* strain KX 146411.1 was isolated and identified and the effect of nutritional and physical factors on levan production by were studied.

Materials and Methods:

Materials:

Fifteen rhizosphere soil samples were collected from three legumes farms in Kana'an village, Diyala Governorate, during the period from October 2014 to January 2015. The rhizosphere samples were from two plants, cowpea (*Vinga unguiculata* L.) and broad bean (*Vicia faba*). The collection had been conducted by uprooting the whole root system and stored in sterilized containers and numbered. These samples were transferred to the laboratory in a cool box and then, kept at 4 ºC (15). Commercial sucrose and Commercial date syrup used for levan production were obtained from Baghdad markets. Sugarcane molasses was obtained from a sugar factory in Al-Hawamdiya City, Cairo, Egypt.

Strain isolation and identification:

Ten grams of rhizosphere soil were suspended in 90 ml of sterilized peptone water and mixed well. Then, soil suspension was serially diluted. 10^{1}, 10^{2} and 10^{3} dilutions had been streaked on nutrient agar plates as duplicates (16). These plates were incubated aerobically at 37ºC for 24-48 h. Bacterial colonies were picked up in aseptic conditions and streaked on sucrose nutrient agar 200g of sucrose to 1000 ml of nutrient agar, and mixed well by stirring and heating until boiling and sterilized by the autoclave at 121°C (15 Ib/In^{2}) for 15 min and then incubated at 37ºC for 24-48h. Mucoid growing colonies had been selected as levan producer and then subjected to further identification steps (17). Selected isolates were identified morphologically and biochemically according to the Bergey's Manual of Systematic Bacteriology (18). Molecular identification was done using 16S rRNA gene sequencing. DNA extraction was performed based on the manufacturer's instructions for Gene Jet genomic DNA purification Kit (Thermo Scientific). DNA samples were

amplified through polymerize chain reaction (PCR) with Thermocycler, Labnet (USA) using Maxima Hot Start PCR Master Mix (Thermo) and universal bacterial primer sets: (Forward primer 5`-AGAGTTTGATCCTGGCTCAG-3`) and (Reverse primer 5`-GGTTACCTTGTTACGACTT-3`). The PCR product was purified using the Gene JETTM PCR Purification Kit (Thermo Scientific) and was sequenced commercially by Sigma-Egypt using forward and reverse primers. Sequencing data were aligned with the publicly available database (GenBank) using National Center for Biotechnology Information (NCBI-BLAST) (19).

Production, isolation and estimation of levan:

Bacterial inoculum was prepared by the preparation of 50 ml of sterilized nutrient broth that contained in 250 ml in volume Erlenmeyer conical flask. This medium was inoculated by one loopful of bacterial culture, shake well and incubated for 24h at 37ºC. The contents of these flasks were used as standard inoculum (1ml contained 7×10^6 viable cells) (15). Fermentation was conducted by the inoculation of 100 ml of levan production medium with 5% of the inoculum. Levan Production Medium was prepared by dissolving yeast extract, 2.5g; sucrose, 200g; $MgSO_4.7H_2O$, 0.2g; K_2HPO_4, 5.5g in 1 liter of distilled water by stirring and heating until boiling. The pH had been adjusted to 7.8. The medium was distributed in Erlenmeyer conical flasks (250 ml in volume) and then, these flasks were sterilized by the autoclave at 121°C (15 Ib/In^2) for 15 min (5). The flasks were incubated at 30ºC in shaker incubator at 100 rpm for 24h (15). Levan production was carried out according to Abou-Taleb *et al.* (15) with some modifications. At the end of fermentation period, 10 ml of the culture from each flask was taken in plain tubes and centrifuged at 10000 rpm for 10 min, to get cell free supernatant, which was then used to precipitate levan. Levan precipitation was accomplished by the addition of 1.5 volumes of absolute ethanol to the supernatant and incubated for 60 min at 37ºC. The precipitated levan had been centrifuged at 10000 rpm for 10 min, to obtain levan pellets. Levan determination was carried out by hydrolyzing levan pellets that were produced by the previous step using 0.5% (v/v) HCl for an hour in a water bath at 100ºC. Levan was estimated as fructose units by using glucose oxidase kit (15). The estimation was conducted according to the manufacturer's procedure. Briefly, the blank was prepared by adding 10µL of distilled water to 1ml of working reagent (WR), and the standard was prepared by the addition of 10µL of standard to 1ml of WR. 10µL of each hydrolyzed levan sample was added to 1ml of WR. These solutions were mixed well and incubated for 10 min at 37ºC. Spectrophotometer was adjusted to zero by blank, and then standard and samples absorbance (A) were read at 505 nm. Fructose units were calculated as the following:

$$\frac{\text{(A) Sample}}{\text{(A) Standard}} \times 100\ (\text{Standard conc.}) = \text{mg/dl} \div 100 = \text{g/l}$$

Characterization of levan:

This characterization had been accomplished by using Thin-Layer Chromatography (TLC). This analysis had been carried out by hydrolyzing levan by 5% HCl (v/v) and heating as described previously. Equal weights (1mg) of glucose, sucrose and fructose were dissolved in 1 ml of 1% ethanol. Then, 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, this plate was placed in a closed jar that contained the mobile phase which was composed of chloroform /acetic acid /water (6:7:1) by volumes. Levan and other sugars were diffused through silica gel plate, after a period of time and when the diffusion reached to about 15 cm, the plate was taken out of the jar and left to dry at room temperature. After drying, the plate was sprayed by a mixture of H_2SO_4 and ethanol at (9:1) by volumes and put in oven for 5-10 min at 90ºC. Levan components appeared as dark colored spots (10). R_f values of the ascending materials had been calculated as the following (20):

$$R_f = \frac{\text{Distance moved by substance}}{\text{Distance moved by the solvent front}}$$

Optimization of Levan Production:

Optimization of Levan Production was carried out according to the method described by Abou-Taleb *et al.* (15) and Abdel-Fattah *et al.* (5).

Effect of Carbon Sources:

This experiment was conducted by replacing sucrose in the production medium with equivalent amount of alternatives. Fructose and agricultural materials like Date syrup and sugarcane molasses had been used. All flasks were incubated at 37°C for 24 h. Levan amount was estimated as described above.

Effect of Nitrogen Sources:

This experiment was performed by replacing the original nitrogen source in the production medium which is yeast extract with equivalent amount of alternatives. Organic (Corn Steep Liquor and Casein) and inorganic (Ammonium sulfate and Ammonium phosphate) nitrogen sources were used as alternatives. All flasks were incubated at 37°C for 24 h. Levan amount was estimated as described previously.

Effect of Sucrose Concentrations:

This experiment was carried out to identify the effect of various sucrose concentrations on levan production. Different sucrose concentrations (50, 100, 150 and 300) g/liter were used in the production medium preparation. All flasks were incubated at 37°C for 24 h. Levan amount was estimated as described previously.

Effect of Initial pH:

This experiment was performed to study the effect of various pH values on levan production. Four groups of production 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. All flasks were incubated at 37°C for 24 h.

Levan amount was estimated as described previously.

Effect of Temperature:

This experiment was conducted by changing the incubation temperature used in the fermentation process. Different incubation temperatures (30, 33, 37 and 40°C) were used for 24 h. All flasks were incubated for 24 h. Levan amount was estimated as described previously.

Effect of Incubation Period:

To study the effect of various incubation periods, production medium was incubated for (24, 48, 72 and 96 h) at 37°C. This experiment was conducted to determine the effect of incubation time on levan production. At the end of each fermentation period, levan was obtained and estimated as mentioned previously.

Effect of Agitation Rate:

This experiment was conducted at two agitation rates (150 and 200 rpm). All flasks were incubated at 37°C for 24 h. Levan amount was estimated as described previously.

Statistical Analysis:

The effect of different factors on levan production was tested statistically by using Analysis System- SAS (2012). Least significant difference-LSD test was used to significant compare between means in this study.

Results and discussion:

Screening for levan production was performed by culturing of the obtained isolates on nutrient agar and sucrose nutrient agar media. After incubation, 36 isolates from the total number of the isolates that grown on sucrose nutrient agar showed mucoid and slimy colonies, which are considered as an indicator for levan production. The isolate was identified as *Arthrobacter globiformis* according to their morphological and biochemical characteristics, and through the 16S rRNA gene sequencing. The homology of the partial 16S rRNA gene sequences of the isolate was determined using the BLAST algorithm in NCBI. Phylogenetic comparison of the 16S rRNA sequence with those of the other bacterial isolates confirmed their high similarity (98%) to *Arthrobacter globiformis* strain JCM 1332. The nucleotide sequence for the isolate was deposited in the GenBank under the accession number (KX146411.1) (Figure. 1).

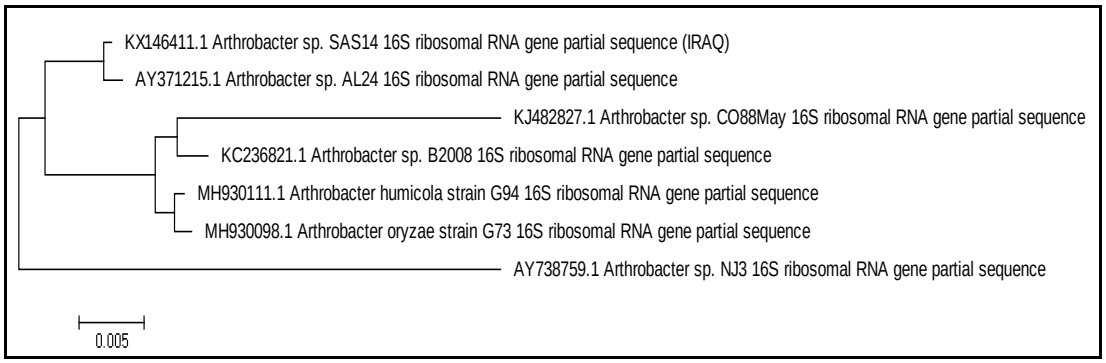

Figure (1): Phylogenetic consensus tree based on the alignment of 1295 bases of *Arthrobacter globiformis* 16S rRNA gene sequences on Gene Bank.

Identification of levan (Analysis of Levan by Thin-Layer Chromatography Technique):

Acid hydrolyzed levan samples of *A. globiformis* KX146411.1 were subjected to TLC technique.TLC had been revealed that levan is composed of only one sugar which is fructose. The Rf_f value of fructose standard was identical to Rf_f values of levan, which was 0.45 (Table 1). These results are in agreement with Muro *et al.* (21) and Dahech *et al.* (10) who reported 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). Monosaccharides (fructose and glucose) took the highest position on the chromatograph due to their low molecular mass. Sucrose took the lowest position because it is composed of glucose and fructose and this increases its molecular mass.

Table (1): Rf_f values of sugars and levan that are ascendant on TLC plate.

Saccharide	Rf_f value of *A. globiformis* KX146411.1
Levan	0.45
Glucose	0.39
Sucrose	0.31
Fructose	0.45

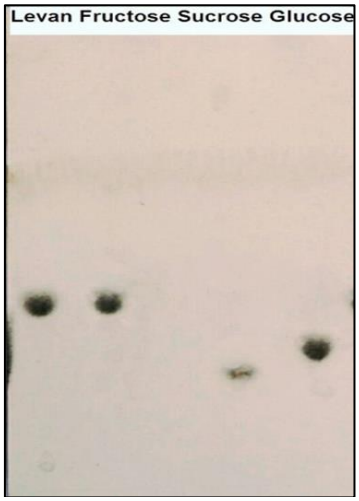

Figure (2): TLC analysis of levan produced by *A. globiformis* KX146411.1.

Optimization of Levan Production:

Effect of carbon sources on levan production:

In order to identify the best carbon source that can yield the highest amount of levan, three carbon sources (fructose, date syrup and molasses) were used compared with sucrose which was the sole carbon source in the original production medium. Sucrose was the best carbon source for levan production by *A. globiformis* KX146411.1 which gave 7.8 g/l. These results indicated that sucrose is the best inducer and substrate for levansucrase (22). These results were compatible with the results that was reported by others (12, 15, 23, 24, 25, 26). They showed that different bacterial species have the ability to produce levan using sucrose as the best

carbon source. Furthermore, using date syrup and molasses individually as carbon sources instead of sucrose in the original production medium for *A. globiformis* KX146411.1 gave similar amount of production and there is no significant difference in their production (Table 2). Production was (4.95 and 5.06) g/l, when the same sources were used respectively. Levan production results using date syrup as a carbon source were in agreement with the results of Moosavi-Nasab *et al*. (26), who indicated a decrease in levan production when date syrup used as a carbon source, compared with that produced by sucrose. This is because of the presence of some sugars combination in the date syrup. These sugars are sucrose, glucose and fructose, which lead to the inhibition of the cell growth and to the production of the metabolites. Using fructose as a sole carbon source was shown to decrease levan production, and these results are consistent with those of Viikari and Gisler (27), who reported that the levan production was reduced when fructose is used as a carbon source, instead of sucrose in the production medium. Levan production using molasses were also reported by Küçükasık *et al*. (28) and Abou-Taleb *et al*. (15). In addition to these studies, Han and Watson (29) showed that molasses is considered as a good carbon source for levan production, especially when subjected to several modifications.

Table (2): Effect of Carbon Sources on Levan Production.

Carbon source	Levan Quantity g/l
	A. globiformis
Sucrose	7.80 ± 0.07 a
Fructose	6.70 ± 0.04 b
Date syrup	4.95 ± 0.03 c
Molasses	5.06 ± 0.13 c
LSD value	0.276 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE)**

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. Each source was used separately instead of the yeast extract in the original production medium. Casein was the best nitrogen source for the isolate that gave 8.24 g/l (Table 3). This elevation in the production is due to that, exopolysaccharide biosynthesis can be affected by the combination of sugars units and their concentration, some studies showed the concentration of exopolysaccharides were higher with the supplementation of medium by skim milk and whey proteins that contain (30). These results were in agreement with those of Abou-Taleb *et al.* (15). The study has indicated that levan production rates had been increased when casein used instead of the yeast extract in the original production medium. Ammonium sulfate $\{(NH_4)_2 SO_4\}$ is another nitrogen source that increased levan production for about 8.20 g/l by *A. globiformis* KX 146411.1. These results did not significantly differ from the results obtained from using of casein. These

two results were in agreement with Abou-Taleb *et al*. (15) who reported that levan production rates increased when ammonium sulphate was used instead of yeast extract in levan productive medium. Levan production by *A. globiformis* KX 146411.1 using ammonium phosphate and corn steep liquor were (7.90 and 7.64) g/l respectively. There were no significant differences in levan production by using these two sources on one hand, and the production obtained when using yeast extract on the other hand.

Table (3): Effect of Nitrogen Source on Levan Production

Nitrogen source	Levan Quantity g/l
	A. globiformis
Yeast extract	7.80 ± 0.07 b
Ammonium sulfate	8.20 ± 0.05 a
Ammonium phosphate	7.90 ± 0.09 b
Corn steep liquor	7.64 ± 0.12 b
Casein	8.24 ± 0.05 a
LSD value	0.263 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE)**

Effect of Sucrose Concentrations:

Different sucrose concentrations were used (50, 100, 150 and 300) g/l to identify the best concentration for levan production by different isolates. levan production of *A. globiformis* KX 146411.1 was at the concentration of 300 g/l which yields 8.06 g/l. However, these results did not significantly differ from those resulted when using 200 g/l in the original production medium (7.80 g/l).

Levan production increased notably from 4.16 g/l to 8.06 g/l when sucrose concentration increased from (50 to 300) g/l (Table 4). These results are in agreement with those of Senthilkumar and Gunasekaran (23), Santos *et al.* (31) and Silbir *et al.* (32). They revealed that the increase in levan production yield is proportional with sucrose concentration in levan production medium.

Table (4): Effect of Sucrose Concentration on Levan Production.

Sucrose Concentrations (g/l)	Levan Quantity g/l
	A. globiformis
50	4.16 ± 0.06 c
100	6.73 ± 0.21 b
150	6.78 ± 0.34 b
200	7.80 ± 0.07 a
300	8.06 ± 0.02 a
LSD value	0.633 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE)**

Effect of Initial pH:

This experiment was conducted with different pH values (7, 7.5, 7.8, 8 and 8.5) to identify the best pH value for levan production. Levan production yields were increased gradually when the pH values increased from 7 to 7.8.

At initial pH of 7.8, isolates gave their highest levan production which was 7.80 g/l. These results significantly differ from those obtained at pH values 7 and 7.5. These results were not consistent with the results declared by Abou-taleb *et al.* (33) who showed that the optimal pH value for levan production by *Bacillus lentus* V8 was 6.5. Also, the results disagree with Ananthalakshmy and Gunasekaran (34) who showed that the optimal initial pH of *Z. mobilis* B-4286 is 5.0. When pH values increased from 8 to 8.5, levan production of *A. globiformis* KX 146411.1 were decreased gradually to reach 5.38 g/l. These results were significantly different from that obtained at pH value of 7.8 (Table 5). These results were in agreement with those of Abou-taleb *et al.* (33) who showed that levan production decreased when pH values increased above the optimal pH value of the production due to that, the optimal pH value is the best pH value for levansucrase activation.

Table (5) Effect of Initial pH on Levan Production.

pH value	Levan Quantity g/l
	A. globiformis
7	6.60 ± 0.04 b
7.5	6.68 ± 0.04 b
7.8	7.80 ± 0.07 a
8	5.95 ± 0.13 c
8.5	5.38 ± 0.26 d
LSD value	0.457 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE)**

Effect of Temperature:

The maximum levan production by *A. globiformis* KX 146411.1 was 8.60 g/l at 33ºC. Levan production continued to decrease when the temperature raised up over the optimal temperature, and the results differed significantly than that obtained by optimal temperature (Table 6). This result was in compatible to those of Shih *et al.* (12) who declared that the suitable temperature ranges of levan production has been found between (25ºC-40ºC). However, each bacterial species has an optimal temperature for levan production which depends on levansucrase activation (35).

Table (6): Effect of Temperature on Levan Production

Temperature	Levan Quantity g/l
	A. globiformis
30º C	7.80 ±0.09 b
33º C	8.60 ±0.02 a
37º C	7.59 ±0.05 bc
40º C	7.36 ±0.14 c
LSD value	0.239 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE).**

Effect of Incubation Period:

In order to identify the optimal incubation period for levan production, this experiment was conducted and levan yield was estimated at different incubation periods (24, 48, 72 and 96) h. The highest levan production by *A. globiformis* KX 146411.1 was 8.53 g/l after 72 h of incubation. Levan production increased gradually than that obtained after 24 h of incubation to reach their maximum production after 72 h of incubation. The highest production by this isolate was significantly different than the production obtained after 24 h of incubation (Table 7). Subsequently, levan yields were decreased after 96 h of incubation. This result was disagreeing with Abou-Taleb *et al.* (15) who indicated that, levan production by *Bacillus* sp. V8 is continued to increase after 72 h, and even after 96 h of incubation.

Table (7): Effect of Incubation Period on Levan Production

Incubation Period	Levan Quantity g/l
	A. globiformis
24 h	7.80 ± 0.07 c
48 h	8.17 ± 0.02 b
72 h	8.53 ± 0.03 a
96 h	7.90 ± 0.03 c
LSD value	0.108 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE).**

Effect of Agitation Rate:

Three agitation rates (100, 150 and 200) rpm was used to identify the best agitation rate for levan production. The highest levan production yield was obtained at 150 rpm by *A. globiformis* KX 146411.1 which gave 8.27 g/l. These results significantly differ from those obtained by 100 rpm and 200 rpm (Table 8). These results are incompatible with those obtained by Shih *et al.* (12) and Abou-Taleb *et al.* (15) who indicated that levan production reached their maximum yields at 150 rpm.

Table (8): Effect of Agitation Rate on Levan Production.

Agitation Rate(RPM)	Levan Quantity g/l
	A. globiformis
100	7.80 ± 0.07 b
150	8.27 ± 0.02 a
200	7.79 ± 0.02 b
LSD value	0.075 *
*** (P<0.05).**	

***Values in the same column followed by the same letter do not significantly differ from each other and this is according to Duncan's at 5% level. (Mean ± SE).**

Conclusions:

A novel strain of *A. globiformis* were found to inhabit the rhizosphere soil and have the ability to produce levan. Levan production by bacteria was improved through the completion of fermentation process under optimized

conditions that are different to each isolate. This strain has the ability to produce levan using low-cost, industrial by-products, such as molasses and corn steep liquor, or cheap materials, such as date syrup, as alternative carbon and nitrogen sources.

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