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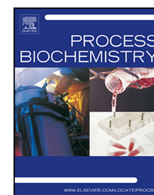

Microbial levan from *Brachybacterium phenoliresistens*: Characterization and enhancement of production

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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 1298 base pairs. The sequence alignment in the gene bank indicated that this isolate has a high percentage of similarity (99%) to the retrieved consensus sequence of *Brachybacterium phenoliresistens* strain phenol-A. The produced levan was characterized using TLC, FTIR, 1H NMR and 13C NMR spectroscopy techniques. 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 casein during levan production were sucrose and casein, yielding 7.88 g/land 8.12 g/l of levan, respectively. The highest levan yield (7.97 g/l) was obtained at a sucrose concentration of 300 g/l. At an initial pH of 7.8, this bacterium yielded their highest levan production of 7.88 g/l. The optimal incubation period was 72 h with a yield of 8.58 g/l, the optimal temperature was 30 °C and resulted in 7.87 g/l, and the highest levan production yield was obtained at 150 rpm and yielded 8.12 g/l.

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1. Introduction

Levan is a homopolyfructose that is produced naturally by plants, as well as microorganisms [1]. Levan 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 branches [2]. Levan lends several advantages for bacterial strains which produce it, including 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 molecules, and using fructose residues, builds the levan polymer chain [4]. Levan has many proprieties that make it a unique polymer including its biological properties, chemical and water holding capacity, viscosity, ability for film formation, ability to dissolve in oil and water, in addition to its acid, alkali and heat stability [5].

There are many bacterial species which have the ability to produce levan, and most of them are found in rhizosphere soil, for example, *Zymomonas, Pseudomonas, Mycobacterium, Corynebacterium, Erwinia, Bacillus, Azotobacter* among others [4,6]. *B. phenoliresistens* strain phenol-A is a small gram-positive bacterium that has a coccal to ovoid shape. These bacteria are facultative anaerobic but they grow better in aerobic conditions, are non-spore forming and non-motile bacteria. This bacterium was named based on its resistance to phenol [7]. However, the production of levan by all bacterial strains is affected by several factors like, carbon source, nitrogen source, inoculum size, fermentation period, initial pH [8], as well as phosphate sources, oxygen content, temperature, agitation rate and nutrient content of the fermentation media [9].

Many techniques have been used to characterize the levan polymer, such as chemical hydrolysis by thin layer chromatography (TLC) and using physiochemical techniques, such as Fourier Transform Infrared (FT-IR), ^{1}Hydrogen-Nuclear Magnetic Resonance and ^{13}Carbon-Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR spectroscopy) [10]. In a previous study we isolated *B. phenoliresistens* from rhizospheric soil, which can produce a large amount of the levan polymer. Levan was characterized using chemical analysis, (FT-IR) spectroscopy and (NMR) spectroscopy. The influences of nutritional and physical factors on levan production from *Brachybacterium phenoliresistens* were studied.

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2. Materials and methods

2.1. Materials

Fifteen rhizosphere soil samples were collected from three legume farms in Kana'an village, Diyala Governorate. The rhizosphere samples were from two plants, cowpea (*Vigna unguiculata* L.) and broad bean (*Vicia faba*). Sugarcane molasses was obtained from a sugar factory in Al-Hawamdiya City, Cairo, Egypt. Commercial date syrup, which used for levan production was obtained from Baghdad markets.

2.2. Strain isolation and identification

Ten grams of rhizosphere soil were suspended in 90 ml of sterilized peptone water (prepared by dissolving 10 g of peptone in 1 l of distilled water). Soil suspension was serially diluted, 10^{-1}, 10^{-2} and 10^{-3} and streaked on nutrient agar plates (HIMEDIA-INDIA) as duplicates [11]. These plates were incubated aerobically at 37 °C for 24–48 h. Growing colonies were picked up in aseptic conditions and streaked on sucrose nutrient agar (200 g of sucrose in 1 l of nutrient agar sterilized by the autoclaving at 121 °C for 15 min) and incubated at 37 °C for 24–48 h. Mucoid growing colonies were selected as levan producers and subjected to further identification steps [12]. Selected isolates were identified morphologically and biochemically according to the Bergey's Manual of Systematic Bacteriology (results are not shown) [13]. 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 (Revere primer 5′-GGTTACCTTGTTACGACTT-3′). The PCR product was purified using the Gene JET™ 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) [14].

2.3. Production, isolation and estimation of levan

Bacterial inoculum was prepared using 50 ml of sterilized nutrient broth in a 250 ml Erlenmeyer conical flask. This medium was inoculated by one loopful of bacterial culture, shaken well and incubated for 24 h at 30 °C and 37 °C. The content of these flasks were used as standard inoculum (1 ml contained 7×10^6 viable cells) for shake flasks [15]. After the incubation period, fermentation was conducted by the inoculation of 100 ml of levan production medium. The levan production medium was prepared by dissolving: yeast extract, 2.5 g; sucrose, 200 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; K_2HPO_4, 5.5 g in 1 l of distilled water and the pH was adjusted to 7.8. The medium was distributed between 250 ml Erlenmeyer conical flasks, which were sterilized by autoclaving at 121 °C for 15 min [16]. The flasks were inoculated with 5% of the inoculum and incubated at 30 °C in a shaker incubator at 100 rpm for 24 h. At the end of the fermentation period, 10 ml of the culture from each flask was taken in plain tubes and centrifuged at 10,000 rpm for 10 min, to get cell free supernatants, which were then used to precipitate levan. Levan precipitation was accomplished by the addition of 1.5 vols of absolute ethanol to the supernatant and incubated for 60 min at 37 °C. The precipitated levan was centrifuged at 10,000 rpm for 10 min to obtain levan pellets. Levan pellets produced during the previous step was hydrolysed using 0.5% (v/v) HCl for an hour in a water bath at 100 °C. Levan concentration was measured as fructose units using a glucose oxidase kit [15]. The measurement was conducted according to the Glucose – TR manufacturer procedure (SPINREACT- Spain). Blank was prepared by adding 10 µl of distilled water to 1 ml of working reagent (WR), and the standards were prepared by the addition of 10 µl of standard to 1 ml of WR. 10 µl of each hydrolysed levan sample was added to 1 ml of WR. These solutions were mixed well and incubated for 10 min at 37 °C. Spectrophotometer was adjusted to zero using the blank sample, and then the standard and sample absorbances (A) were read at 505 nm. Fructose units were calculated as the following.

$$\frac{(A)\,sample}{(A)\,standard} x100 \ (standard\ conc.) = \frac{mg/dl}{100} = g/l$$

2.4. Characterization of levan

2.4.1. Fourier transform-infrared spectroscopy

The IR spectrum was recorded on the FTIR-8201PC Spectrophotometer in the (4000–400 cm^{-1}) region at mid temperature (26 °C ± 1 °C) [17].

2.4.2. ^1H and ^{13}C NMR spectra

NMR spectroscopy was used for levan analysis. 0.5 mg of levan (purified from insoluble and paramagnetics materials) was dissolved in dimethylsulfoxide (DMSO) for each of ^1H NMR and ^{13}C NMR analysis. Spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^1H NMR spectra were run at 300 MHz and ^{13}C NMR spectra were run at 75.46 MHz. Chemical shifts quoted in and related to that of the solvents [17].

2.5. Optimization of levan production

The strategy for optimization of fermentation parameters was accomplished by evaluating the effects of an individual parameter and incorporating it at the optimum level before optimizing the next parameter.

2.5.1. Effect of carbon sources

This experiment was conducted by replacing sucrose in the production medium with equivalent amount of alternatives. Fructose and agricultural materials (date syrup and sugarcane molasses) were used. Flasks were autoclaved, inoculated and incubated.

2.5.2. 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. Flasks were autoclaved, inoculated and incubated.

2.5.3. Effect of sucrose concentrations

This experiment was carried out to identify the effects of various sucrose concentrations on levan production. Different sucrose concentrations (50, 100, 150 and 300 g/l) were used in the production medium preparation. Flasks were autoclaved, inoculated and incubated.

2.5.4. Effect of initial pH

This experiment was performed to study the effects of various pH values on levan production. Production medium were adjusted to pH values of 7, 7.5, 8, and 8.5. Flasks were autoclaved, inoculated and incubated.

2.5.5. 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, flasks were autoclaved, inoculated and incubated.

2.5.6. Effect of incubation period

To study the effect of various incubation periods, production medium was incubated for 24, 48, 72 and 96 h. Flasks were autoclaved, inoculated and incubated.

2.5.7. Effect of agitation rate

This experiment was conducted at two agitation rates (150 and 200 rpm). Flasks were autoclaved, inoculated and incubated.

2.6. Statistical analysis

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

3. Results and discussion

3.1. Screening of levan producing bacteria

Screening for levan production was performed by culturing isolates on nutrient agar and sucrose nutrient agar media. After incubation, the isolates that were grown on sucrose nutrient agar showed mucoid and slimy colonies, which are considered indicators for levan production, especially when sucrose was the sole carbon source in the medium [15,16,18].

3.2. Identification of the bacterial isolate

The isolate was identified as *B. phenoliresistens* 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 (Fig. 1) confirmed their high similarity (99%) to *B. phenoliresistens* strain phenol-A. The nucleotide sequence for the isolate was deposited in the GenBank under the accession number (KX139300).

Table 1
Comparison of ^{13}C NMR values of *B. phenoliresistens* levan with ^{13}C NMR values of *Z. mobilis* levan and *Bacillus licheniformis* levan.

Carbon atom	Standard Levan of *Z. mobilis*	*B.licheniformis* Levan	*B. phenoliresistens* Levan
C-1	60.761	59.784	59.073
C-2	104.641	104.176	103.834
C-3	77.683	76.179	77.021
C-4	75.754	75.120	74.183
C-5	80.783	80.257	82.253
C-6	63.957	63.358	62.007

3.3. Characterization of levan

3.3.1. (FTIR) spectroscopy analysis of levan

The FTIR spectrum of *B. phenoliresistens* KX139300 levan showed several characteristic peaks (Fig. 2). The characteristic O—H broad stretching peak was found approximately 3394.1/cm. A weak band of C—H was noticed at approximately 2932.23/cm. The stretching of C═O was observed at 1647.88/cm. Some sharp bands were present approximately 1000/cm, which are specific for carbohydrates. These results confirmed that the characterized product was levan and is consistent with levan produced by *Pseudomonas fluorescens* [17], *Acetobacter xylinum* [8] and comparable with reference levan [19].

3.3.2. NMR analysis of levan

The ^{1}H NMR spectrum resonance of *B. phenoliresistens* levan, (Fig. 3), indicated that the identified compound of this isolate contains 6 unexchangeable protons signals (3.393, 3.474, 3.511, 3.582, 3.625 and 4.650) corresponding to the resonance of the standard levan of *Zymomonas, Mobilis* [20] and of *Bacillus licheniformis* levan [10]. This result was also consistent with the levan obtained from *B. amyloliquefaciens*, which produces levan that showed six proton shifts between (3.4 and 4.2) ppm and corresponded to the levan standard [21].

^{13}C NMR spectra of levan produced by *B. phenoliresistens* showed six resonance signals as shown in Fig. 4. C-1: 59.073, C-2: 103.834, C-3: 77.021, C-4: 74.183, C-5: 82.253, C-6: 62.007 were present and corresponded to the resonance signals of the standard levan of *Z. mobilis* [20] and to the resonance signals of *B. licheniformis* [10], which produces levan with six resonance shifts between 60 and 115 ppm, and are close to the signal shifts of the standard levan (Table 1).

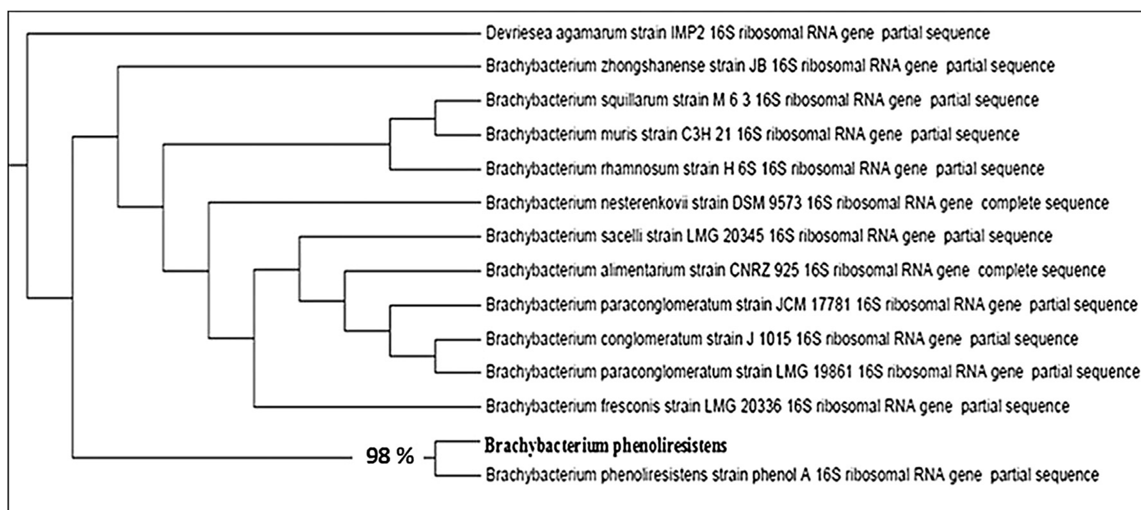

Fig. 1. Phylogenetic consensus tree based on the alignment of 1298 bases of *B. phenoliresistens* 16S rRNA gene sequences showing the relationship of *B. phenoliresistens* to closet bacteria on Gene Bank.

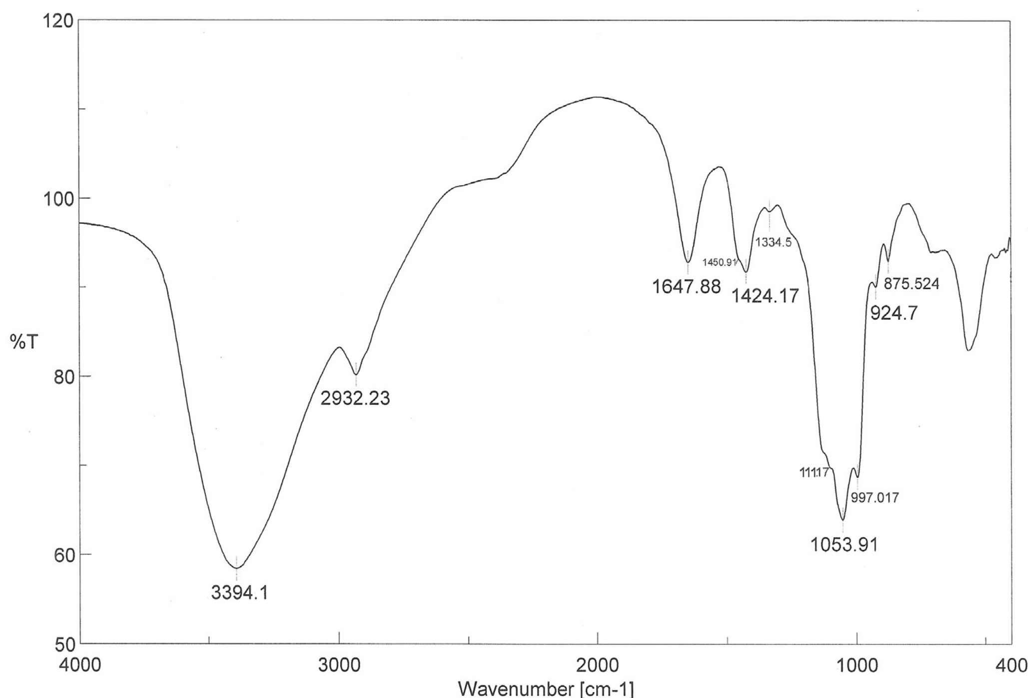

Fig. 2. The FTIR spectrum of levan from *B. phenoliresistens*.

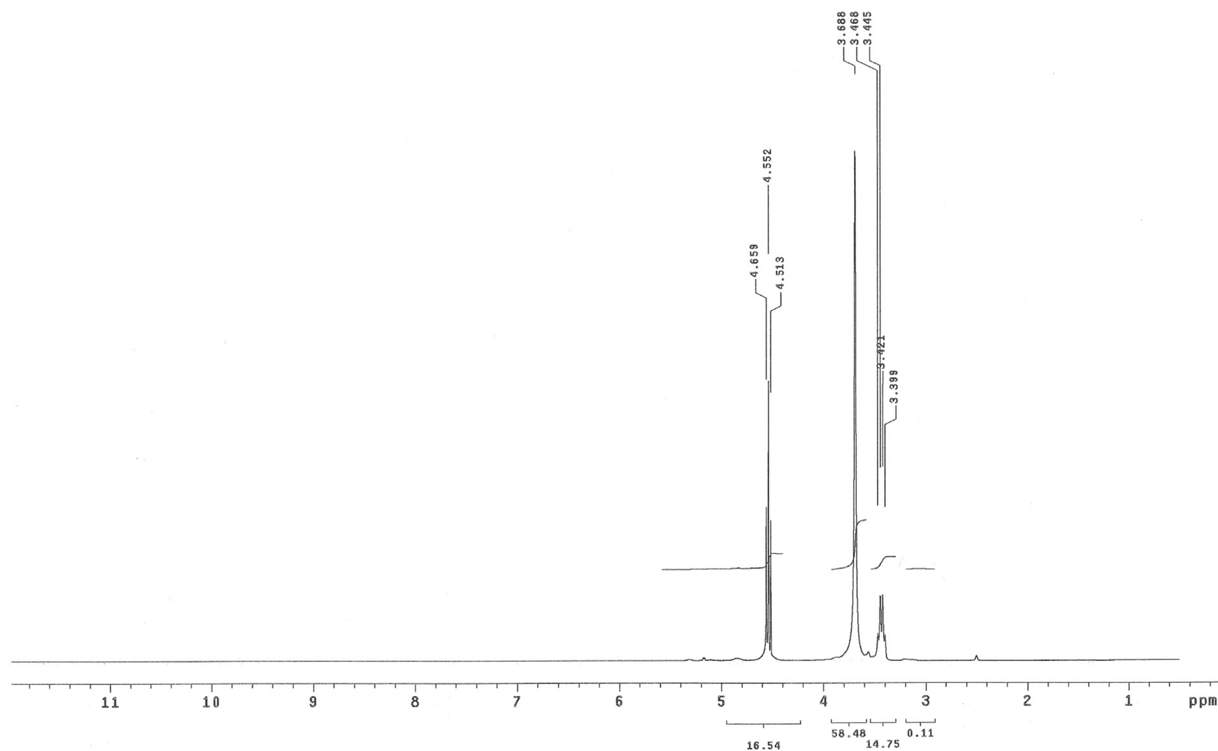

Fig. 3. The ^{1}H NMR spectrum of levan from *B. phenoliresistens*. The spectrum was recorded at 300 MHz for sample in DMSO solution.

3.4. Optimization of levan production

3.4.1. Effect of carbon sources on levan production

To identify the best carbon source that can yield the highest amount of levan, three carbon sources (fructose, date syrup and molasses) were used instead of sucrose, which was the sole carbon source in the original production medium. Sucrose was the best carbon source for levan production by *B. phenoliresistens* which yielded 7.88 g/l, followed by fructose which yielded 6.90 g/l (Fig. 5). These results indicate that sucrose is the best inducer and substrate for levansucrase [22]. Other bacterial species that showed similar results with sucrose as the best carbon source include *Z.*

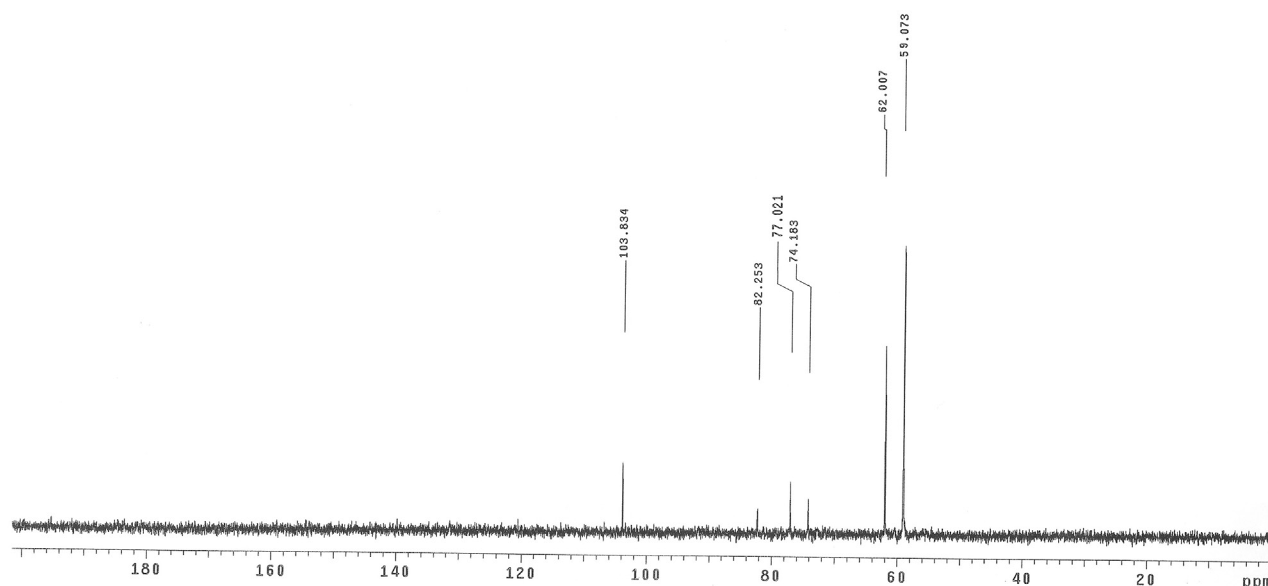

Fig. 4. The ^{13}C NMR spectrum of levan from *B. phenoliresistens*. The spectrum was recorded at 75.46 MHz for sample in DMSO solution.

mobilis [23], *B. subtilis* (Natto) [24], *B. licheniformis* [10] and *B. lentus* [15].

The results show significant differences between sucrose and fructose as carbon sources. Furthermore, using date syrup and molasses individually as carbon sources resulted in a similar amount of levan production with no significant differences in their production levels (Fig. 5). *B. phenoliresistens* levan production yields were 5.38 and 5.49 g/l when date syrup and molasses were used as carbon sources, respectively. These results show that we can choose an inexpensive agricultural or manufactured by-product for levan production. Levan is also produced by *Halomonas* sp. [25] and *B. lentus* [15] using molasses as a carbon source. Molasses is considered a good carbon source for levan production, especially when subjected to several modifications [20].

3.4.2. Effect of nitrogen sources on levan production

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 levan production and yielded 8.12 g/l (Fig. 5), although there were no significant differences in this result when compared to those obtained by using yeast extract in the original production medium.

This increase in levan production could be due to the effect of a combination of sugar units and their concentration on exopolysaccharide biosynthesis. Some studies have shown that a higher concentration of exopolysaccharides when the medium was supplemented with skim milk and whey proteins [26]. Increase in levan production by *B. phenoliresistens* when casein was used as the nitrogen source was consistent with the increase in levan production by *B. lentus,* when casein was used instead of the yeast extract in the original production medium [15]. In addition to casein as the best nitrogen source, ammonium phosphate {$(NH_4)_3$ PO_4} yielded 7.88 g/l. This result did not differ significantly from that obtained using casein. A similar result was obtained by *B.lentus* which showed elevation in levan production when ammonium phosphate was used instead of yeast extract in the levan production medium [15]. Increase in levan production using ammonium salts was the opposite of the results obtained by *Z. mobilis* [23], which showed a decrease in levan production.

3.4.3. Effect of sucrose concentration on levan production

Various sucrose concentrations were used (50, 100, 150 and 300 g/l) to identify the best concentration for levan production by different isolates. The highest levan production by *B. phenoliresistens* was at the sucrose concentration of 300 g/l, which yields 7.97 g/l (Fig. 5). However, these results did not significantly differ from those when using 200 g/l of sucrose in the original production medium which yielded 7.88 g/l. Levan production increased notably from 3.48 to 7.97 g/l when sucrose concentration was increased from (50–300) g/l. *Z. mobilis* and *B. subtilis* NATTO revealed that the increase in levan production is proportional to the sucrose concentration in levan production medium [25,27,28].

3.4.4. Effect of initial pH on levan production

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 by *B. phenoliresistens* increased gradually when the pH values were increased from 7 to 7.8. At an initial pH of 7.8, *B. phenoliresistens* had its highest levan production of 7.88 g/l (Fig. 5). These results were significantly different from those obtained at pH values 7 and 7.5. These results were not consistent with the results obtained by *B. lentus* whose optimal pH value is 6.5 for levan production [29]. Additionally, these results were not consistent with the results obtained by *Z. mobilis* which showed its optimal initial pH to be 5.0 [30]. When pH values increased from 8 to 8.5, levan production gradually decreased, reaching 6.33 g/l. These results were significantly different from those obtained at pH value of 7.8. Similar results were observed in *B. lentus* which showed a decrease in levan production when pH values increased above the optimal pH because, the optimal pH value is the best pH value for the activation of levansucrase [29].

3.4.5. Effect of temperature on levan production

The optimal incubation temperature for levan production by *B. phenoliresistens* was 30 °C, at which the production was 7.87 g/l (Fig. 5). Levan production by this isolate decreased when the temperature was raised above the optimal temperature. These results were consistent with *B. subtilis* NRC 33a and *B. lentus*, which had an optimal temperature of 30 °C for levan production. These results indicate that this temperature might be the optimal temperature for levansucrase activation [16,29].

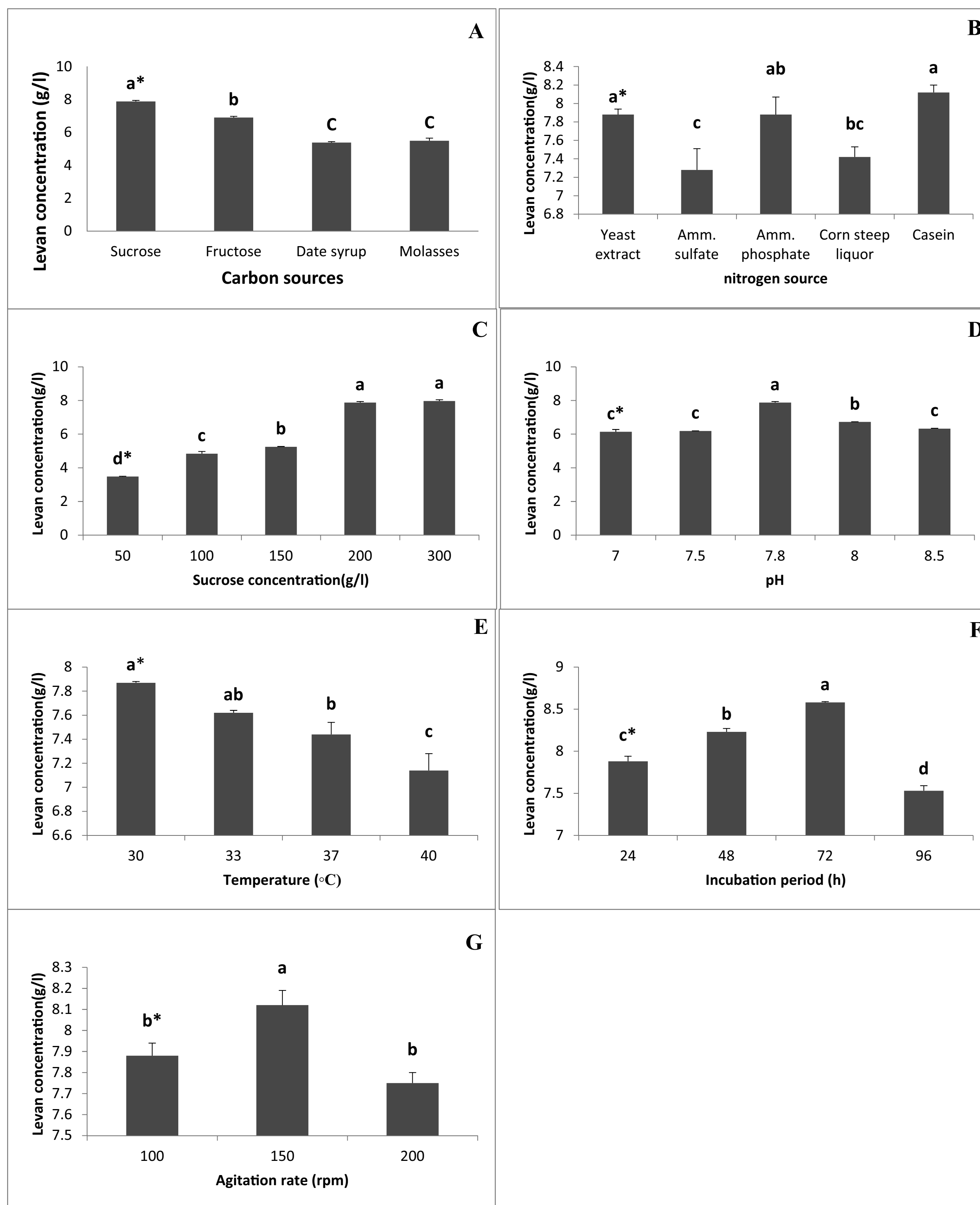

Fig. 5. Effect of biosynthesis conditions on the production of levan. (A) carbon sources, (B) nitrogen sources, (C) sucrose concentration, (D) initial pH, (E) temperature, (F) incubation period, (G) agitation rates.* the same letter on the column do not significantly differ from each other according to Duncan's at ($P < 0.05$). Data were expressed as (Mean ± SE).

3.4.6. Effect of the incubation period on levan production

To identify the optimal incubation period for levan production, levan yields were estimated at different incubation periods (24, 48, 72 and 96 h). The highest levan production by *B. phenoliresistens* was 8.58 g/l. after 72 h of incubation (Fig. 5). Levan production gradually increased after 24 h of incubation and reached its maximum production after 72 h of incubation.

These three isolates produced significantly higher levan at 72 h compared to that after 24 h of incubation. However, levan yields decreased after 96 h of incubation. Levan production by *B. lentus*

continued to increase after 72 h, and even after 96 h of incubation [15].

3.4.7. Effect of agitation rate on levan production

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 the *B. phenoliresistens* isolates yielding 8.12 g/l (Fig. 5). The result significantly differs from those obtained at 100 rpm and 200 rpm. Another levan producing bacteria *B. subtilis* and *B. lentus* produced maximum yields of levan at 150 rpm [29,31].

4. Conclusions

A novel strain of *B. phenoliresistens* that has the ability to produce levan was isolated and identified from soil rhizosphere. Levan's production by this strain was improved using optimized fermentation conditions. 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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