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## Nutritional factors affecting levan production by *Bacillus* sp.V8 strain isolated from rhizosphere bean (*Vicia faba*) plant

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This investigation was designed to study the production of levan by fermentation; a number of 37 bacterial isolates were obtained from rhizosphere of different plants in Egypt and tested for levan production. Only one isolate (V8) was selected as a high efficiency levan producing bacterium and identified as *Bacillus lentus* V8 based on phenotypic identification by Bergey's Manual of Systematic Bacteriology and confirmed with 16S rRNA gene sequencing. The specific growth rate ( $\mu$ ) and doubling time ( $t_d$ ) were calculated at the log phase of growth curves being  $0.097\text{h}^{-1}$  and 7.15 h, respectively. Levan concentration was increased after 96 h (stationary phase). The effect of some nutritional factors on growth and levan production by *Bacillus* sp.V8 was investigated. 25% sucrose was the best carbon source for levan production followed by 25% black strap sugar cane molasses. Addition of fructose to the fermentation medium considerably reduced the levan production due to the inhibition of levan sucrose activity.  $1.5\text{ g l}^{-1}$  ammonium phosphates was most effective as nitrogen sources.  $7.2\text{ g l}^{-1}$   $\text{K}_2\text{HPO}_4$  found to be optimal for levan production, which gave 39.97 [or]  $36.60\text{ g l}^{-1}$  of levan dry weight on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses using shake flasks as a batch culture, respectively.

**Keywords:** *Bacillus* sp. V8, carbon sources, levan production, nitrogen sources, 16S rRNA gene sequencing

### Introduction

Levan is one of two main types of fructans, which are natural homopolymers of fructose (Arvidson *et al.*, 2006). It is a naturally occurring polymer of  $\beta$ -D-fructofuranose with  $\beta$  (2  $\rightarrow$  6) linkages between repeating five-member fructofuranosyl rings and branching at C-1 (Arvidson *et al.*, 2006; Barone and Medynets, 2007).

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It has great potential as a functional biopolymer in foods or a feed additive with prebiotic, and also used widely in food industries as it add sweetness to food, emulsifier, stabilizer and thickener, surface-finishing agent, encapsulating agent and carrier for flavour, fragrances, filler, bulking agent and substitute for gum Arabic (Abdel-Fattah *et al.*, 2005; Shih *et al.*, 2005); medical and pharmaceutical industries in different forms as hypocholesterolemic and anti-carcinogenic agent (Ben Ammar, 2002), coating material in a drug delivery formulation, blood plasma extender, modify tumor cell's membrane and a tablet binder (Abdel-Fattah *et al.*, 2005; Shih *et al.*, 2005) and levan derivatives such as sulfated, phosphated, or acetylated levans are asserted to be anti-AIDS agents (Clarke *et al.*, 1997). In addition, levan is used in chemical industries (Hae-Eun *et al.*, 2003; Kang *et al.*, 2009).

In commercial processes, sugars have been widely used as substrates for biological production of levan polymer. The biological production can use cheap raw materials, such as syrups and molasses have long been used as substrates for the fermentative production of commercial levan polysaccharides (Oliveira *et al.*, 2007; Küçükasik *et al.*, 2011) due to their many advantages like high sucrose and other nutrient contents, low cost and ready availability.

Levan is synthesized by bacteria from sucrose by levan sucrases. The production of levan polymer has been studied in bacterial genera such as *Pseudomonas* (Hettwer *et al.*, 1995), *Streptococcus* (Simms *et al.*, 1990), *Acetobacter* (Hernandez *et al.*, 1995), *Bacillus* (Ben Ammar *et al.*, 2002; Meng and Futterer, 2003; Abdel-Fattah *et al.*, 2005; Ghaly *et al.*, 2007), *Lactobacillus* (van Hijum *et al.*, 2004) and *Zymomonas* (Bekerset *et al.*, 2005; Kang *et al.*, 2009).

The objective of the present study was to isolate a bacterium, *Bacillus lentus* V8 strain from rhizosphere soil which could produce a large amount of levan polymer and study the influence of some nutritional factors on levan production by *Bacillus lentus* V8 strain such as carbon, nitrogen sources and  $K_2HPO_4$  concentrations.

## **Materials and methods**

### ***Isolation of levan producing bacteria***

Four soil samples were collected from the rhizosphere of four plants grown in Monufia Governorate, Egypt. These plants namely, bean (*Vicia faba*), pea (*Pisum sativum*), okra (*Abelmoschus esculentus*) and corn (*Zea mays*). Streak and serial dilution techniques were used for isolation of levan producing bacteria from rhizosphere soils on Abdel-Fattah *et al.* (2005) medium as a basal medium (Tauro *et al.*, 1986). The growing slimy colonies on plates (incubated

at 30°C for 2-5 days) were picked under aseptic conditions, purified and maintained on nutrient agar slants, grown at 30°C for 24 h, and stored at 4°C.

### ***Phenotypic and Genotypic Identification***

Identification of selected isolate was according to their morphological (Gram and endospore stained were observed under light microscope) and biochemical tests (catalase, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, indole production and Voges–Proskauer) based on Bergey's Manual of Systematic Bacteriology (Niall and Paul, 2009). It was then confirmed with 16S rRNA gene sequencing. DNA extraction was performed based on the use protocol of Gene Jet genomic DNA purification Kit (Thermo Scientific). The 16S rRNA gene was amplified with polymerize chain reaction (PCR) machine by using Maxima Hot Start PCR Master Mix (Thermo). The universal 16S primers were used for the amplification of the 16S rDNA gene fragment (F1 5' AGAGTTTGATCCTGGCTCAG 3' and R1 5' GGTTACCTTGTTACGACTT 3'). The DNA fragment was gel purified using the Gene JET™ PCR Purification Kit (ThermoScientific) (Marko *et al.*, 1982; Boom and Sol, 1990). The 16S rDNA amplified PCR product was used for the sequencing on the GATC Company by use ABI 3730xl DNA sequencer by using reverse primer based on the method of Sanger *et al.* (1977). The obtained sequence of 16S rDNA was compared with the database at the NCBI site (<http://www.ncbi.nlm.nih.gov>) by alignment with nucleotide collection database and *Bacilli* (taxid: 1386) for the organism. Then multiple sequence alignment was developed for these homologous sequences using the algorithm described in Clustal Omega. A phylogenetic tree was then drawn using the Neighbour joining method.

### ***Inoculum preparation and batch fermentation***

Inoculum was prepared by inoculating one loopful of culture into a conical flask (250 ml in volume) containing 50 ml of nutrient broth at 30°C for 24 h on a rotary shaker at 100 rpm. The content of these flask was used as standard inoculum (1ml contained  $7 \times 10^6$  viable cells) for shake flasks. This was used as inoculum for the levan production medium. Fermentation was carried out in 250 ml plugged Erlenmeyer flasks, each containing 100 ml sterile productive medium (Abdel-Fattah *et al.*, 2005) It has the following composition ( $\text{gl}^{-1}$ ): Yeast extract, 2.5; Sucrose, 200;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2;  $\text{K}_2\text{HPO}_4$ , 5.5 and pH

adjusted to 7.8, and inoculated with 5% of standard inoculum then incubated for 96 h at 30°C on a rotary shaker at 100 rpm. At the end of fermentation, Samples of 10 ml were taken to determine the cell dry weight and levan concentration.

### ***Sources of carbon, nitrogen and K<sub>2</sub>HPO<sub>4</sub> concentrations***

This experiment was conducted to study the effect of different carbon sources on levan polymer production by the most efficient strain. The appropriate carbon source was selected by replacing the original carbon source of the used medium with equivalent carbon amount of each of the tested carbon sources (fructose, black strap sugar cane molasses or sugar cane juice). Different sucrose or black strap sugar cane molasses concentrations were used ranging between 5% and 30% for levan production by tested strain on productive medium. The prescribed nitrogen source of the fermentation medium was replaced by equivalent nitrogen amount of each of the tested organic [beef extract, peptone, malt extract, casein, corn steep liquor (CSL), soy bean husk and wheat bran] and inorganic [ammonium phosphate, ammonium chloride, ammonium oxalate & ammonium sulphate]. Different nitrogen (ammonium phosphate) concentrations were used ranging between 0.5 and 2.5 g l<sup>-1</sup> for levan production by tested isolate. Five trials of K<sub>2</sub>HPO<sub>4</sub> concentrations, ranged from 1.8 to 9.0 g l<sup>-1</sup> were added to the productive medium to test their effect on levan production.

### ***Analytical methods***

At the end of fermentation period, cultures were centrifuged at 10000 rpm for 10 min. The pellets were used as source of cell dry weight, washing twice with distilled water and drying at 80°C to a constant weight. The supernatant was used for precipitation of levan polymer, precipitated by adding 1.5 volumes of ice-cold absolute ethanol to supernatant and left for an hour. The precipitated pellets were washed twice by distilled water, and then the pellets were collected by centrifugation at 10000 rpm/10 min described by Reiss and Hartmeier (1990). The precipitate was determined as polymer dry weight by dried at 110°C for 24 h and weighted according to the method of Reiss and Hartmeier (1990) and determined levan as fructose using glucose oxidase kits (the precipitate was hydrolyzed with HCL 0.5% (v/v) for 60 min at 100°C and estimated as fructose units by glucose oxidase kits from (BIO-ADWIC) EL NASR PHARMACEUTICAL CHEMICALS Co. (Egypt). Specific growth rate ( $\mu$ ) (h<sup>-1</sup>) and doubling time ( $t_d$ ) (h), multiplication rate (MR) and number of

generations (N) were calculated using the following equations according to Doelle (1975). The following formulas were used to calculate these parameters: Specific growth rate ( $\mu$ ) ( $\text{h}^{-1}$ ) =  $(\ln X - \ln X_0) / (t - t_0)^{-1}$ , Doubling time ( $t_d$ ) (h) =  $\ln_2 (\mu)^{-1}$ , Multiplication rate (MR) =  $1/(t_d)^{-1}$  and Number of generation (N) =  $(\log X - \log X_0) / (\log 2)^{-1}$ .

### Statistical analysis

The collected data were statistically analyzed using IBM® SPSS® Statistics software (2011) and the correlation coefficient and regression were analyzed with Microsoft Office Excel (2010).

## Results and discussions

### Isolation of levan producing bacteria

In this study, thirty seven isolates of bacteria were isolated from rhizosphere soils of different plants on productive medium. 4, 5, 9 and 19 isolates of bacteria were obtained from okra (*Abelmoschus esculentus*), pea (*Pisum sativum*), corn (*Zea mays*) and bean (*Vicia faba*), respectively (Table 1). The highest number and percentage of these isolates were obtained from bean (*Vicia faba*) followed by corn (*Zea mays*) being 19 (48.7%) and 9 (23.1%) isolates, respectively. These isolates were classified in to three categories namely high, moderate and weak levan producing isolates which gave levan concentrations ranged from 8 to  $>18 \text{ gl}^{-1}$ , 2 to  $> 8 \text{ gL}^{-1}$  and 0.56 to  $> 2 \text{ gl}^{-1}$ , respectively. The highest number of these isolates were presented in the second category (19 bacterial isolates) followed by third and first categories being 11 and 7 bacterial isolates, respectively.

**Table 1.** Production of cell dry weight and levan polymer by bacteria isolated from rhizosphere of different plants

Isolate No.	Cell dry weight $\text{gl}^{-1}$	Levan as		Isolate No.	Cell dry weight $\text{gl}^{-1}$	Levan as	
		dry weight $\text{gl}^{-1}$	fructos e $\text{mgl}^{-1}$			dry weight $\text{gl}^{-1}$	fructos e $\text{mgl}^{-1}$
V1	0.63 <sup>H</sup>	1.68 <sup>I</sup>	8.0 <sup>x</sup>	P1	1.58 <sup>g</sup>	4.46 <sup>w</sup>	7.4 <sup>z</sup>
V2	1.08 <sup>y</sup>	1.44 <sup>K</sup>	6.5 <sup>C</sup>	P2	1.60 <sup>f</sup>	6.48 <sup>r</sup>	9.7 <sup>o</sup>
V3	1.01 <sup>B</sup>	1.02 <sup>M</sup>	3.8 <sup>J</sup>	P3	0.84 <sup>D</sup>	2.08 <sup>F</sup>	2.3 <sup>M</sup>
V4	0.57 <sup>I</sup>	1.08 <sup>L</sup>	8.0 <sup>y</sup>	P4	1.04 <sup>A</sup>	6.30 <sup>t</sup>	9.8 <sup>n</sup>
V5	0.75 <sup>F</sup>	1.44 <sup>K</sup>	6.8 <sup>B</sup>	P5	1.49 <sup>j</sup>	13.69 <sup>b</sup>	16.9 <sup>b</sup>
V6	0.15 <sup>P</sup>	3.20 <sup>B</sup>	8.7 <sup>t</sup>	O1	1.04 <sup>A</sup>	6.94 <sup>o</sup>	6.2 <sup>E</sup>
V7	0.22 <sup>O</sup>	10.88 <sup>d</sup>	13.1 <sup>d</sup>	O2	1.58 <sup>g</sup>	10.78 <sup>e</sup>	13.0 <sup>e</sup>

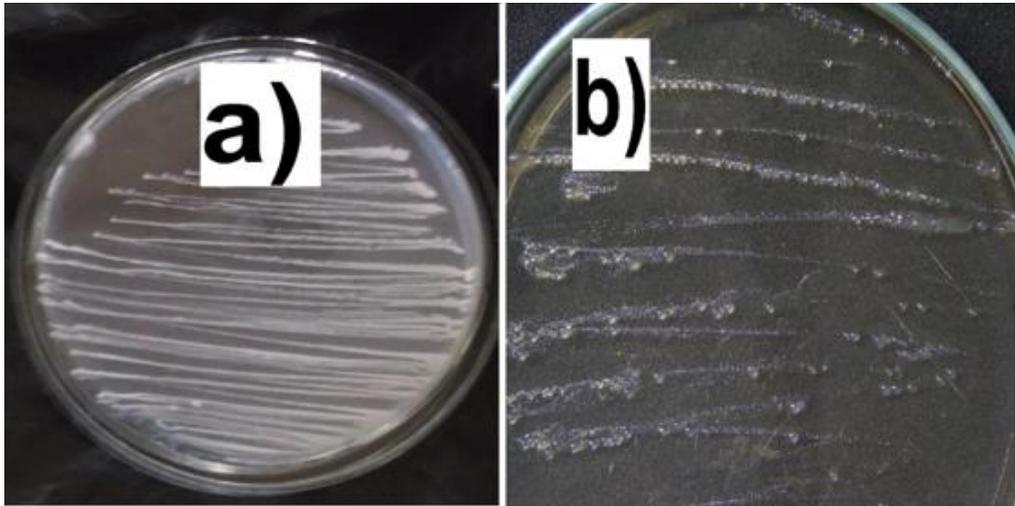
V8	1.65 <sup>d</sup>	18.82 <sup>a</sup>	22.4 <sup>a</sup>	O3	1.23 <sup>s</sup>	8.04 <sup>i</sup>	12.5 <sup>h</sup>
V9	1.13 <sup>v</sup>	1.92 <sup>H</sup>	1.5 <sup>N</sup>	O4	1.12 <sup>w</sup>	0.56 <sup>P</sup>	0.4 <sup>O</sup>
V10	1.92 <sup>a</sup>	4.52 <sup>v</sup>	8.9 <sup>s</sup>	C1	0.55 <sup>J</sup>	2.62 <sup>D</sup>	2.3 <sup>M</sup>
V11	1.05 <sup>z</sup>	1.96 <sup>G</sup>	2.9 <sup>L</sup>	C2	0.82 <sup>E</sup>	3.32 <sup>A</sup>	4.5 <sup>H</sup>
V12	0.23 <sup>N</sup>	0.66 <sup>O</sup>	0.4 <sup>O</sup>	C3	1.53 <sup>h</sup>	12.5 <sup>c</sup>	13.7 <sup>c</sup>
V13	0.9 <sup>C</sup>	3.82 <sup>y</sup>	8.1 <sup>w</sup>	C4	1.53 <sup>h</sup>	6.55 <sup>q</sup>	10.7 <sup>m</sup>
V14	0.45 <sup>L</sup>	3.54 <sup>z</sup>	12.0 <sup>j</sup>	C5	1.31 <sup>q</sup>	2.94 <sup>C</sup>	9.4 <sup>q</sup>
V15	1.42 <sup>m</sup>	10.68 <sup>f</sup>	11.9 <sup>k</sup>	C6	1.63 <sup>e</sup>	7.14 <sup>m</sup>	12.1 <sup>i</sup>
V16	1.87 <sup>b</sup>	3.20 <sup>B</sup>	4.8 <sup>G</sup>	C7	0.67 <sup>G</sup>	0.78 <sup>N</sup>	4.1 <sup>I</sup>
V17	0.48 <sup>K</sup>	1.66 <sup>J</sup>	8.9 <sup>s</sup>	C8	1.16 <sup>u</sup>	5.42 <sup>u</sup>	3.2 <sup>K</sup>
V18	1.66 <sup>c</sup>	4.36 <sup>x</sup>	9.2 <sup>r</sup>	C9	1.32 <sup>p</sup>	7.54 <sup>k</sup>	6.4 <sup>D</sup>
V19	0.42 <sup>M</sup>	2.14 <sup>E</sup>	6.9 <sup>A</sup>				

(V, P, O & C) - Bacterial isolates were obtained from bean (*Vicia faba*), pea (*Pisum sativum*), okra (*Abelmoschus esculentus*) and corn (*Zea mays*).

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

### ***Selection of the obtained bacterial isolates***

Screening of bacterial isolates for levan was conducted by using the plate agar with and without sucrose, as a preliminary study for selecting the levan producers, all 37 of bacterial isolates showed signs of growth on basal medium agar and demonstrated positive results in plate agar with sucrose. Since the sole carbon source in basal medium agar was sucrose, therefore the result of the test was a strong evident that levan sucrose was produced in order converted the soluble sucrose into the polysaccharide  $\beta$ -D fructoside (levan) and glucose. The most efficient levan producing bacteria V8 isolate was grown on plate agar with and without sucrose. When the culture was grown on agar with sucrose, the colonies had a mucoid slimy appearance which indicated the production of the levan from sucrose (Figure.1- a, b). These results are in agreement with those of Ghaly *et al.* (2007) who stated that *Bacillus licheniformis* was grown on plate agar with sucrose, the colonies had a mucoid slimy appearance which indicated the production of the polysaccharide. Data presented in Table 1 clearly show that only one out of thirty seven levan producing isolates gave the highest value of levan dry weight (18.82  $\text{g l}^{-1}$ ) and levan as fructose (22.4 $\text{mg l}^{-1}$ ) after 4 days incubation period on productive medium. Whereas the other isolates (36 isolates) recorded the values of levan dry weight ranged from 0.56 to 12.50  $\text{g L}^{-1}$  and levan as fructose ranged from 0.4 to 13.7 $\text{mg L}^{-1}$ . These results are in agreement with those of Kang *et al.* (2002); Meng and Futterer, (2003); Abdel-Fattah *et al.* (2005); Bekers *et al.* (2005); Ghaly *et al.* (2007); Kang *et al.* (2009) who indicated that microorganisms known with ability of levan production include some species of the genera *Acetobacter*, *Pseudomonas*, *Bacillus*, *Lactobacillus*, *Pediococcus* and *Zymomonas*.



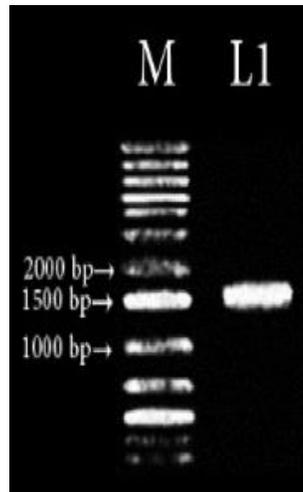
**Fig. 1.** Growth of the most efficient levan producing bacteria V8 isolate on basal medium Abdel-Fattah *et al.* (2005): a) Growth without sucrose b) Growth with sucrose (the mucoid slimy appearance indicates the presence of levan).

### ***Phenotypic and Genotypic Identification***

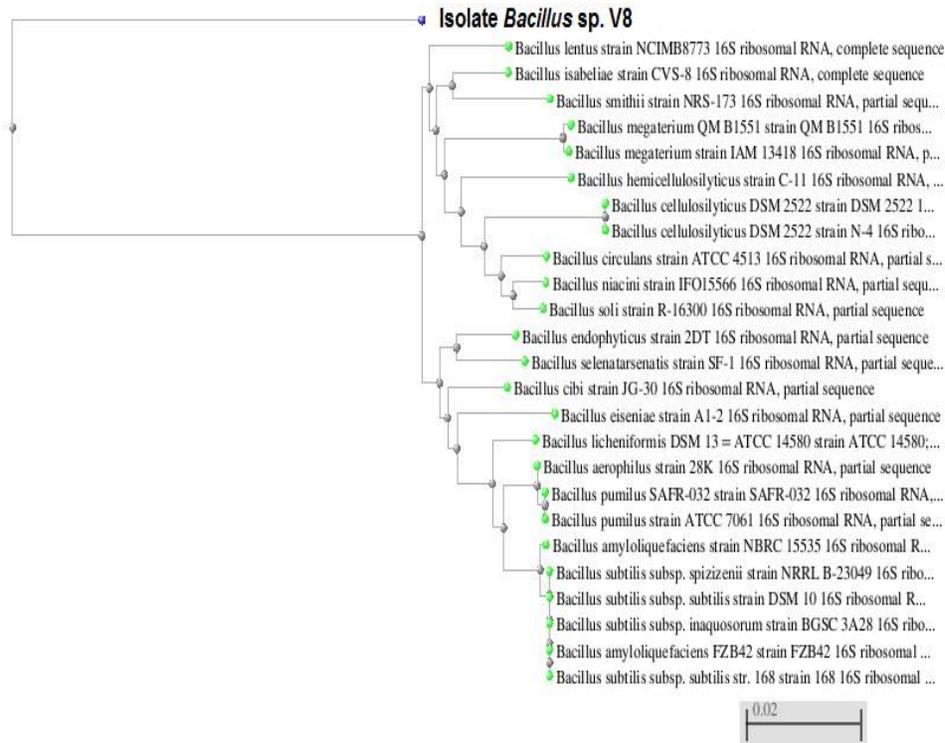
The selected isolate was identified of morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology according to (Niall and Paul, 2009). The results obtained showed that the isolate V8, preliminary classified to be *Bacillus* genus. This isolate was Gram positive, rod in shape, motile, an endospore-forming bacterium. The isolate showed positive results for catalase and starch hydrolysis, while gave negative results for gelatin hydrolysis, casein hydrolysis, indole production and Voges–Proskauer. PCR amplification of its 16S rDNA gene showed a 1.5kb band on electrophoresis for the isolate *Bacillus* sp. V8 (Figure 2). The analysis of 16S rRNA gene of isolate *Bacillus* sp. V8 sequenced with R1 primer at the reverse directions produced 1226 bp was compared by using BLAST similarity searches, and the closely related sequences were obtained from the database at the NCBI site (GenBank).

The NCBI site showing the highest percentage of similarity of 83% with *Bacillus lentus* strain NCIMB8773 16S ribosomal RNA, complete sequence (accession number:040792.1), *Bacillus isabelliae* strain CVS-8 16S ribosomal

RNA, complete sequence (accession number:042619.1) and *Bacillus aerophilus* strain 28K 16S ribosomal RNA, partial sequence (accession number:042339.1). Multiple sequence alignment was developed using sequences of the isolate *Bacillus* sp. V8 \_rev 1226bp with the sequences of highest 25 previous homologous sequences, then phylogenetic tree was drawn using Clustal Omega as shown in Figure3. Concerning the phylogenetic tree constructed in this study it was interesting to note that the isolate *Bacillus* sp. V8 is so close to *Bacillus lentus* (accession number: 040792.1).



**Fig. 2.** PCR products for 16S gene M: 1kbp DNA ladder, Lane 1; isolate *Bacillus* sp. V8.

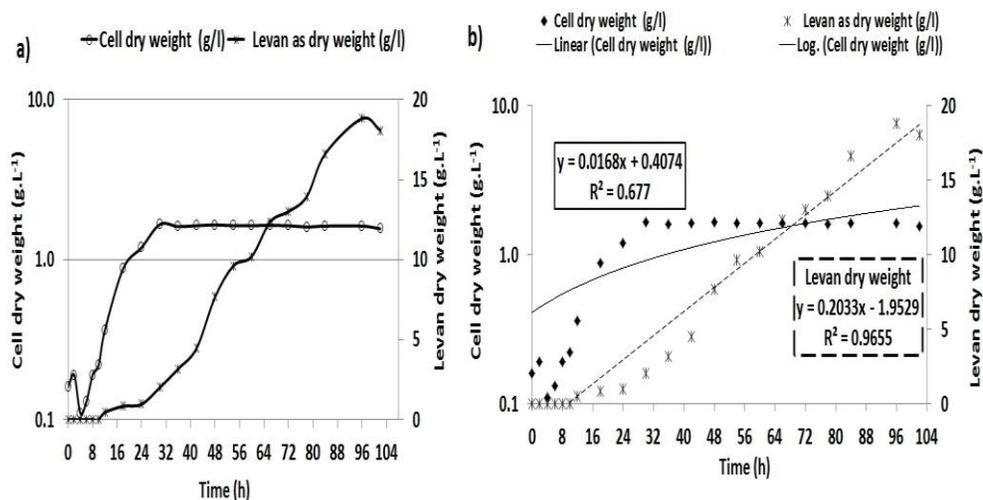


**Fig. 3.** Neighbour-joining tree based on 16S rRNA gene sequences of the genus *Bacillus* obtained from BLAST search showing the position of isolate *Bacillus* sp. V8 and related strains.

### ***Study of growth and levan production Profile***

The samples were taken every two hours during the log phase and then every six hours. The *Bacillus* sp. V8 strain, was grown on productive medium (Abdel-Fattah *et al.*, 2005) and incubated at 30°C for 96 h. Results recorded that *Bacillus* sp. V8 strain was grown exponentially during the first 10-30 h (Figure 4- a) of the incubation period. The growth was found to be more constant (stationary phase) during the second, third and fourth days, then slightly decreased after 96 h of the incubation period (decline phase). The growth parameters for tested strain were calculated at the log phase of growth curve. Results recorded that the specific growth rate ( $\mu$ ) being ( $0.097\text{h}^{-1}$ ), the doubling time ( $t_d$ ) being (7.15h), multiplication rate (MR) being (0.140 h) and the number of generation (N) being (2.80) for *Bacillus* sp. V8 strain. Results also show that the strain *Bacillus* sp. V8 delayed to produce levan after 12 h. Sharp increase in levan concentration was recorded during the first 48 h, and then a gradual increase to reach the maximum value occurred after 96 h. Thereafter, levan concentration was decreased. Data from Figure 4- b also

show a positive correlation coefficient ( $R^2$ ) between the time of incubation and each of cell dry weight and levan dry weight being 0.68 and 0.97, respectively. Obtained results are generally in agreement with those obtained by Shih *et al.* (2005) who found that the levan production was noted after a few hours of cell growth and reached the maximum after cell growth reached the stationary phase by *B. subtilis* (natto) Takahashi. No polysaccharide was produced during the death phase.



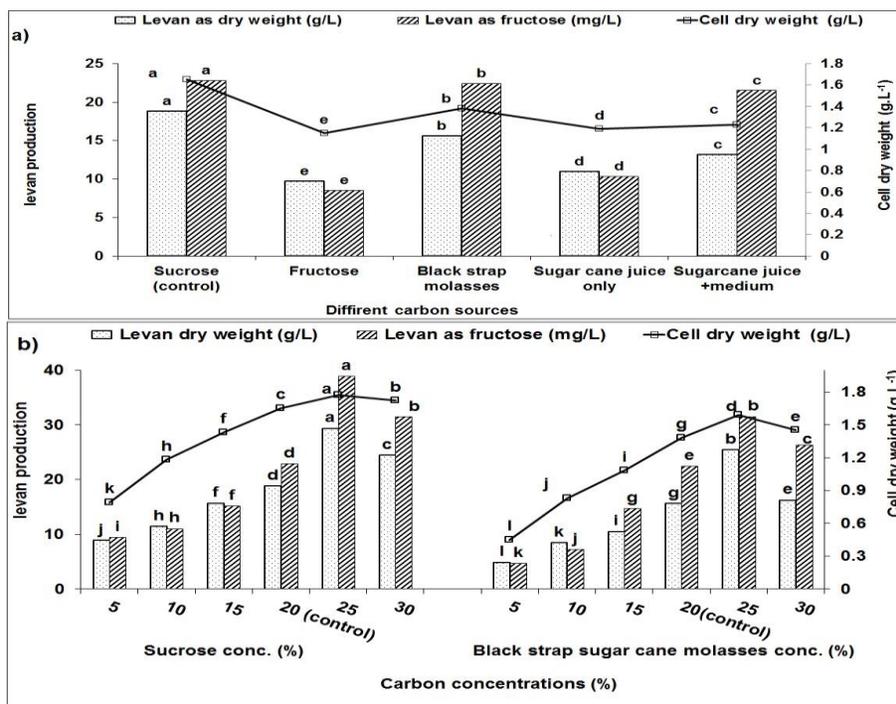
**Fig. 4.** levan production and growth curve of *Bacillus sp.* V8 strain. a) Cell dry weight ( $g\ L^{-1}$ ) and levan dry weight ( $g\ L^{-1}$ ) of *Bacillus sp.* V8 strain. b) Correlation coefficient between the time of incubation and each of cell dry weight and levan dry weight.

### ***Effect of carbon sources***

Four carbon sources were used as illustrated by Figure 5- a. It was found that sucrose was the best one as a sole carbon source for cell dry weight ( $1.65\ g\ L^{-1}$ ), levan dry weight ( $18.82\ g\ L^{-1}$ ) and levan as fructose ( $22.8\ mg\ L^{-1}$ ) followed by black strap sugar cane molasses giving  $1.38\ g\ L^{-1}$  of cell dry weight,  $15.62\ g\ L^{-1}$  of levan dry weight and  $22.4\ mg\ L^{-1}$  of levan as fructose for *Bacillus sp.* V8strain. Small amount of levan production being  $9.68\ g\ L^{-1}$  of levan dry weight and  $8.50\ mg\ L^{-1}$  of levan as fructose were obtained by the tested strain in media supplemented with fructose. Media supplemented with sucrose (or) black strap sugar cane molasses gave the highest cell dry weight and yield of levan by tested strain. In a study for selecting a cheap agricultural or manufacturing by-product for higher levan production, it was found that black strap sugar cane molasses contains high sugar concentration and other metals necessary for the fermentation process and is inexpensive. Molasses has been successfully used for fermentative production of polysaccharides such as curdlan (Lee *et al.*, 2003) and xanthan (Kalogiannis *et al.*, 2003). These results are in agreement

with Senthilkumar and Gunasekaran (2005); Shih *et al.* (2005); Oliveira *et al.* (2007); Ghaly *et al.* (2007) who indicated that the best carbon source was sucrose for levan production by different bacterial genera. In addition, data are in agreement with those obtained by Küçükasık *et al.* (2011) who stated that sugar beet molasses pretreated with TCP followed by acidification with sulfuric acid gave the highest yields of levan. Also, Han and Watson (1992) reported sugarcane juice and beet molasses can be made a good substrate for levan production by various modifications.

Data illustrated in Figure 5- b clearly show that 25% sucrose [or] black strap sugar cane molasses gave the highest figures of cell dry weight (1.77 [or] 1.59  $\text{gl}^{-1}$ ) and levan production (29.32 [or] 25.40  $\text{gl}^{-1}$  of levan dry weight & 38.8 [or] 31.5  $\text{mgL}^{-1}$  of levan as fructose) for *Bacillus* sp. V8strain. Statically analysis revealed a high positive correlation coefficient ( $R^2$ ) between sucrose concentrations and each of cell dry weight (0.87 ( $y = 0.1897x + 0.7593$ )), levan dry weight (0.86 ( $Y=3.8429x + 4.6333$ )) and levan as fructose (0.83 ( $y = 5.7583x + 1.2827$ )) produced by *Bacillus* sp. V8strain.



**Fig. 5.** Cell dry weight and levan production by *Bacillus* sp. V8 strain. a) Different carbon sources b) Different sucrose concentrations (%) and black strap cane molasses concentrations (%).

Whereas the highest correlation coefficient  $R^2$  was recorded at black strap sugar cane molasses concentrations and each of cell dry weight and levan as fructose being 0.88 ( $Y=0.2166x+0.372$ ) and 0.88 ( $y = 5.3886x - 1.0600$ ), respectively and moderate correlation coefficient ( $R^2$ ) of levan dry weight being 0.69. These results are in line with Oliveira *et al.* (2007) who clearly that 250  $g\ l^{-1}$  sucrose gave the highest yield of levan by *Z. mobilis* ATCC 31821.

However, the sucrose concentration at its upper (350  $g\ l^{-1}$ ) and lower (150  $g\ l^{-1}$ ) limits reduced levan production. Senthilkumar and Gunasekaran (2005) obtained similar results showed that increase in sucrose concentration from 50 to 200  $g\ l^{-1}$  enhanced levan production from 15.5 to 22.8  $g\ l^{-1}$  with concomitant increase in biomass from 1.1 to 2.4  $g\ l^{-1}$  at 30°C.

### ***Effect of nitrogen sources***

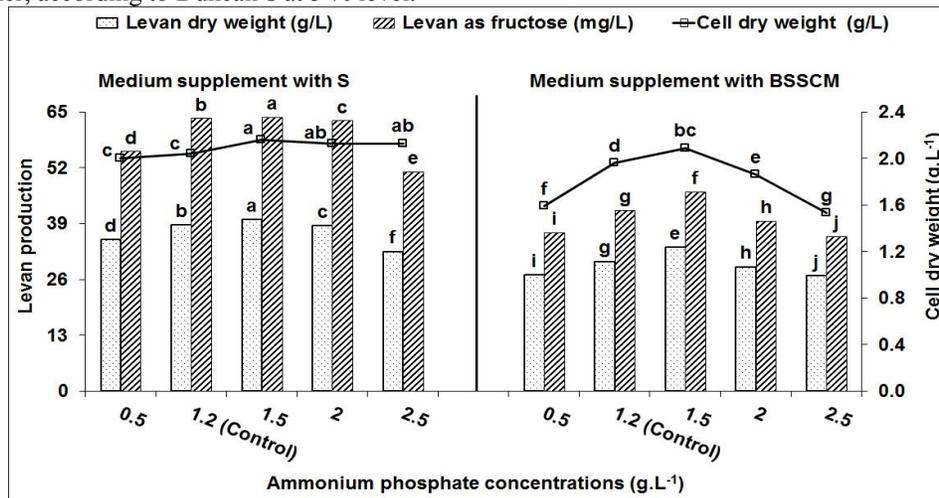
Data presented in Table 2 clearly show that the sources of nitrogen greatly affected the production of levan. Ammonium phosphate was the best nitrogen source for *Bacillus* sp. V8 strain giving 2.04  $g\ l^{-1}$  cell dry weight, 38.71  $g\ l^{-1}$  levan dry weight and 63.4  $mg\ l^{-1}$  levan as fructose in productive medium containing 25% sucrose, followed by casein, ammonium oxalate, beef extract, malt extract & yeast extract, and giving 1.96  $g\ l^{-1}$  cell dry weight, 30.12  $g\ l^{-1}$  levan dry weight and 42.0  $mg\ l^{-1}$  levan as fructose in productive medium supplemented with 25% black strap sugar cane molasses, followed by ammonium sulphate, casein, corn steep liquor (CSL), malt extract and yeast extract. These results are not compatible with Garcia-Cruz (1997); Ernandes and Garcia-Cruz (2011) who stated that, the ammonium salts may influence negatively in the production of levan. Wendt (2001); Abdel-Fattah *et al.* (2005); Oliveira *et al.* (2007) also mention that yeast extract was the best nitrogen source for the production of levan by *Z. mobilis* or *Bacillus subtilis* NRC33.

Data illustrated by Figure 6 show that 1.5  $g\ l^{-1}$  ammonium phosphate gave the highest figure of cell dry weight (2.16 [or] 2.09  $g\ l^{-1}$ ), levan dry weight (39.92 [or] 33.40  $g\ l^{-1}$ ) and levan as fructose (63.70 [or] 46.30  $mg\ l^{-1}$ ) on medium supplemented with sucrose [or] black strap sugar cane molasses as carbon source, respectively for *Bacillus* sp. V8 strain. Statistically analysis revealed a moderate correlation coefficient ( $R^2$ ) between nitrogen concentrations and cell dry weight (0.66 with regression equation ( $Y=0.035x + 1.987$ )) on a medium supplemented with sucrose. Whereas, the lowest correlation coefficient between nitrogen concentrations and each of cell dry weight (on medium supplemented with black strap sugar cane molasses), levan dry weight and levan as fructose (on medium supplemented with sucrose [or] black strap sugar cane molasses).  $R^2$  values ranged from 0.01 to 0.47.

**Table 2.** Effect of different nitrogen sources on levan production by *Bacillus* sp. V8 strain on productive medium containing sucrose and black strap sugar cane molasses

Different nitrogen sources	Sucrose			Black strap sugar cane molasses		
	Cell dry weight $g\ L^{-1}$	Levan as dry weight $g\ L^{-1}$	fructose $mg\ L^{-1}$	Cell dry weight $g\ L^{-1}$	Levan as dry weight $g\ L^{-1}$	fructose $mg\ L^{-1}$
Yeast extract (Control)	1.77 <sup>g</sup>	29.32 <sup>g</sup>	38.9 <sup>g</sup>	1.59 <sup>k</sup>	25.40 <sup>k</sup>	31.5 <sup>k</sup>
Beef extract	1.98 <sup>b</sup>	30.00 <sup>e</sup>	48.9 <sup>c</sup>	1.11 <sup>p</sup>	20.00 <sup>p</sup>	22.0 <sup>f</sup>
Peptone	1.27 <sup>n</sup>	20.00 <sup>p</sup>	21.8 <sup>s</sup>	1.00 <sup>q</sup>	15.80 <sup>q</sup>	17.8 <sup>t</sup>
Malt extract	1.90 <sup>e</sup>	30.00 <sup>e</sup>	37.6 <sup>j</sup>	1.70 <sup>i</sup>	26.12 <sup>j</sup>	30.0 <sup>i</sup>
Casein	1.96 <sup>c</sup>	30.26 <sup>b</sup>	49.8 <sup>b</sup>	1.73 <sup>h</sup>	27.05 <sup>h</sup>	38.8 <sup>h</sup>
Corn steep Liquor (CSL)	1.29 <sup>m</sup>	20.00 <sup>p</sup>	22.4 <sup>q</sup>	1.73 <sup>h</sup>	27.00 <sup>j</sup>	38.6 <sup>j</sup>
Soybean husk	1.13 <sup>o</sup>	10.96 <sup>s</sup>	11.3 <sup>x</sup>	0.97 <sup>r</sup>	13.29 <sup>r</sup>	12.4 <sup>w</sup>
Wheat bran	1.13 <sup>o</sup>	10.94 <sup>t</sup>	13.1 <sup>v</sup>	0.92 <sup>s</sup>	10.00 <sup>u</sup>	13.2 <sup>u</sup>
Ammonium phosphate	2.04 <sup>a</sup>	38.71 <sup>a</sup>	63.4 <sup>a</sup>	1.96 <sup>c</sup>	30.12 <sup>c</sup>	42.0 <sup>e</sup>
Ammonium chloride	1.67 <sup>j</sup>	20.16 <sup>o</sup>	23.4 <sup>o</sup>	1.13 <sup>o</sup>	20.68 <sup>m</sup>	23.6 <sup>n</sup>
Ammonium oxalate	1.92 <sup>d</sup>	30.08 <sup>d</sup>	47.0 <sup>d</sup>	1.33 <sup>i</sup>	23.34 <sup>l</sup>	28.3 <sup>m</sup>
Ammonium sulphate	1.33 <sup>i</sup>	20.22 <sup>n</sup>	22.5 <sup>p</sup>	1.85 <sup>f</sup>	29.66 <sup>f</sup>	41.3 <sup>f</sup>

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

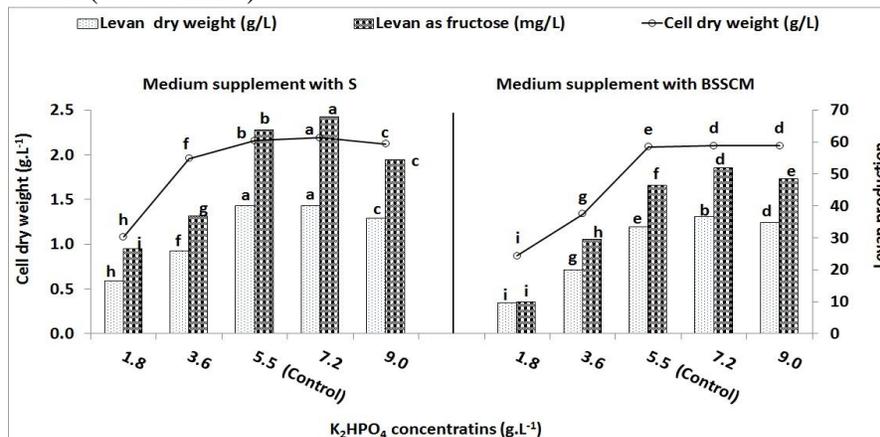


**Fig. 6.** Effect of ammonium phosphate concentrations as nitrogen source on levan production by *Bacillus* sp. V8 strain on medium supplement with sucrose or black strap sugar cane molasses. S- Sucrose; BSSCM- Black strap sugar cane molasses

**Effect of  $K_2HPO_4$  concentrations**

Results recorded in Figure 7 clearly show that the best concentration of  $K_2HPO_4$  was found to be 7.2  $g\ L^{-1}$ , which gave the highest cell dry weight (2.19 [or] 2.10 $g\ L^{-1}$ ), levan dry weight (39.97 [or] 36.60  $g\ L^{-1}$ ) and levan as fructose (67.8 [or] 51.8  $mg\ L^{-1}$ ) by *Bacillus* sp. V8strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. It

should be noticed that levan production, slightly increased as the  $K_2HPO_4$  concentrations increased from  $5.5\text{ gL}^{-1}$  as control to  $7.2\text{ gL}^{-1}$  on medium supplemented with sucrose [or] black strap sugar cane molasses for tested strain. Also it could be noticed that the values of correlation coefficient ( $R^2$ ) between  $K_2HPO_4$  concentrations and each of cell dry weight and levan production (levan dry weight and levan as fructose) on modified medium supplemented with black strap sugar cane molasses (0.80-0.82) were higher than recorded for medium supplemented with sucrose (0.60 - 0.68) during fermentation period for *Bacillus* sp. V8 strain. Obtained data confirmed the findings obtained by Vigants *et al.* (1998); Bekers *et al.* (2000) who found that sodium and potassium salts have a stimulating effect on levan synthesis. In addition, data are in agreement with those obtained by Jerez (1993) who found that the growth rate and the amount of levan formed were proportional to the increase of potassium concentration up to  $10\text{ gL}^{-1}$ . These results are in disagreement with those of Abdel-Fattah *et al.* (2005) who found that the addition of  $5.0\text{ gL}^{-1}$   $K_2HPO_4$  was most favorable for extracellular levan sucrose production ( $19.5\text{ U mL}^{-1}$ ).



**Fig. 7.** Influence of different  $K_2HPO_4$  concentrations on levan production by *Bacillus* sp. V8 strain on medium supplement with sucrose or black strap sugar cane molasses. S- Sucrose; BSSCM- Black strap sugar cane molasses

## Conclusion

*Bacillus* sp. V8 isolated from bean (*Vicia faba*) rhizosphere soil has the ability to produce a significant amount of extracellular levan polymer and identified using phenotypic characterizes and confirmed using 16SrRNA gene sequencing. Nutritional conditions optimization was done to optimize levan production by *Bacillus* sp. V8 strain. Investigated the effect of carbon, nitrogen sources and concentrations and  $K_2HPO_4$  concentrations in the productive

medium to be significant. Results of this study clearly showed that increased the production of levan by *Bacillus* sp. V8 strain from 18.82 to 39.97 [or] 36.60  $\text{g l}^{-1}$  on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses.

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