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Production, Purification and Characterization of Levan Polymer from *Bacillus lentus* V8 Strain

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To optimize fermentation conditions for microbial production of levan polymer on a low-price productive medium. Also to purified levan from *Bacillus* sp. V8 strain, characterization the levan polymer by ^{13}C NMR spectroscopy and physicochemical properties.
Study Design: Optimization of environmental factors for levan production. Polymer purification and identification.
Place and Duration of Study: Agric. Microbiology Dept., Fac. of Agriculture, Ain Shams Univ., Cairo, Egypt, between January 2012 and December 2013.
Methodology: *Bacillus lentus* V8 strain was used as producer of levan. This strain was grown on productive medium. Cell dry weight and levan polymer were determined in all the samples at the end of the fermentation period. Study on optimal levan production conditions such as initial pH, incubation temperatures, shaking speed, inoculum size. The parameters of growth and levan were calculated from the exponential phase. Isolation and identification of levan polymer.
Results: Effect of some environmental factors on growth and levan production by *Bacillus lentus*

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V8 strain on medium supplemented with sucrose or black strap sugar cane molasses was investigated. Results indicated that the productive medium with initial pH 6.5, 10% inoculum size, incubated at 30ºC, 150rpm shaking speed gave 51.40 or 45.34gL^{-1} of levan dry weight on medium supplemented with sucrose or black strap sugar cane molasses, respectively. With respect to biological activity of *Bacillus lentus* V8 strain on modified medium supplemented with sucrose or black strap sugar cane molasses using shake flasks as a batch culture, results reveal that the fermentation period for highest cell dry weight 2.99 or 2.89gL^{-1} was obtained after 48 h or 66 h, respectively. The highest levan dry weight (57.95 or 49.86gL^{-1}) and biopolymer yield (23.18 or 36.93%) on modified medium supplemented with sucrose or black strap sugar cane molasses were achieved after 60 h, respectively. A highly positive correlation coefficient between incubation period and cell dry weight, levan dry weight, polymer dry weight yield, effective yield and $Y_{p/x}$, R^2 values ranged from (0.91 to 0.97) and (0.89 to 0.96) on medium supplemented with sucrose or black strap sugar cane molasses, respectively. The levan produced by tested strain was identified by ^{13}C NMR and characterized by being white color, soluble in water and precipitated with alcohol, it contains 40.86% carbon and 0.98% ash.

Conclusion: The highest amount of levan was produced by *Bacillus lentus* V8 strain on productive medium supplemented with sucrose [or] black strap sugar cane molasses with pH 6.5, 10% inoculum size and incubated at 30ºC for 60h at 150rpm. Characterization the levan polymer by ^{13}C NMR spectroscopy and physicochemical properties.

Keywords: Levan polymer; microbial production; batch fermentation; raw materials; identification; purification.

1. INTRODUCTION

Levan is a β -2,6-linked fructose homopolymer, and it has been found in many plants and microbial products. Bacterial levans are much larger than those produced by plants, with multiple branches and molecular weights (2-100 million Da) [1,2]. *Bacillus polymyxa* (NRRL-18475) produced a levan-type fructan (β- 2, 6 fructofuranoside) when grown on sucrose, sugarcane juice, and sugar beet molasses. This species converted about 46% of the fructose moiety of sucrose to levan when grown on sucrose medium by levansucrase [3]. Levansucrase is responsible for levan formation during sucrose fermentation of different bacterial species [4,5]. Levan is amorphous or microcrystalline, white powder, soluble in cold water, very soluble in hot water, insoluble in 75% alcohol, non-toxic, biologically active, extracellular polysaccharide, non-viscous, nontransparent suspension and reflect visible light [6-8]. The physiological effects of levan are dependent on its size and linkage type, and the fermentability of levan is therefore an important issue. Here in this study, we optimized the fermentation conditions for producing levan polymer using microbial production on a low-price productive medium. Therefore, we purified levan from *Bacillus lentus* V8 strain, characterized the levan polymer by ^{13}C NMR spectroscopy and investigated their physicochemical properties.

2. MATERIALS AND METHODS

2.1 Microorganism and Fermentation Condition

Bacillus lentus V8 strain was used to produce levan. This strain was characterized previously by Abou-Taleb et al. [9] and grown in productive medium modified by Abou-Taleb et al. [9]. This medium contained (gL^{-1}): ammonium sulphate, 1.5; sucrose [or] black strap sugar cane molasses*, 250; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 7.2 and pH adjusted to 7.8 before sterilization. Inoculum of the bacteria was prepared in nutrient broth medium and used at 5% (v/v). *Black strap sugar cane molasses, a by-product of sugar industry, contains high sugar concentration (54%) and other metals necessary for the fermentation process. The fermentation was carried out in 250ml plugged Elenmeyer flasks containing 100 ml sterile productive medium at 30ºC on rotary shaker at 100 rpm for 96 h. At the end of fermentation, samples of 10 ml were withdrawn to determine the cell dry weight and levan concentration from triplicates shaken flasks.

The samples were centrifuged at 10000rpm for 10 min at 4ºC. The pellets were used as source of cell dry weight, washing twice with distilled water and drying at 80ºC 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, then the pellets were collected by centrifugation at 10000rpm/10 min described by Reiss and Hartmeier [10]. The precipitate was determined as polymer dry weight after oven-drying at 110ºC for 24h and weighted according to the method of Reiss and Hartmeier [10].

2.2 Optimal Conditions of Levan Production

To optimize the pH for levan production, eight values of pH ranged between 5 and 8.5 were investigated. To determine the best incubation temperature for levan production, fermentation experiment was carried out at various temperatures in the range of 25, 30, 35, 40 and 50ºC. Different speeds of rotary shaker being 0 (static), 50, 100, 150, 200 and 250 rpm/ min were used during the fermentation process. The inoculum size was optimized for maximal levan production; the productive medium was inoculated with 2.5, 5, 10 and 15% of the standard inoculum.

2.3 Biological Activity of Levan Producing Bacteria

In this experiment, the tested strain was grown in Erlenmeyer flasks (500 ml in volume) containing 350ml productive medium and incubated at 30ºC for 96h on rotary shaker (150 rpm). Samples (15ml) were taken from the growing culture periodically every 2 hrs under aseptic conditions and centrifuged at 10.000rpm/10min, the pellet (bacterial cells) was washed twice with distilled water, then dried at 70ºC to a constant weight. The relation between time and cell dry weight (growth curve) was plotted. The parameters of growth, namely specific growth rate (μ) (h^{-1}) = (ln X –ln X0) $(t - t0)^{-1}$, doubling time (t_d) (h) = ln_2 $(\mu)^{-1}$, multiplication rate (MR) and generation number (N) were calculated from the exponential phase according to Doelle [11]. Levan was determined as levan dry weight and levan as fructose described by Reiss and Hartmeier [10]. Parameters of levan production being yield (%) = levan concentration (gL^{-1})/initial sugar (gL^{-1}) x 100, effective yield (%) = cell dry weight (gL^{-1})/Initial substrate concentration x 100, productivity ($gL^{-1}h^{-1}$)= levan concentration (gL^{-1})/fermentation time (h) and yield coefficient relative to biomass (ggL^{-1})= polymer conc. (gL^{-1})/gram biomass dry weight (gL^{-1}) were calculated according to Ramadan et al. [12]; Lee [13]; Grothe et al. [14], respectively. Correlation coefficient and regression equations analysis between incubation time and each of cell dry weight, polymer as (dry weight & fructose), yield, effective yield, productivity and yield coefficient relative to biomass were calculated.

2.4 Purification and Characterization of Levan Polymer

The precipitated polymer (pellet) was resuspended in demineralized water at 4ºC during 16h followed by dialysis overnight against demineralized water. Subsequently, the polymer was precipitated with 2 volumes of 96% ethanol, centrifuged for 10min at 10.000 rpm, freeze dried and stored at -20ºC for further analysis [15]. The purified polymer was characterized using nuclear magnetic resonance (^{13}C-NMR; NMR Lab., Microanalytical unite (MAU), Fac. of pharmacy, Cairo Univ., Egypt) using BRUKER-400 spectrometer. Sample of levan was dissolved in D_2O solution (Sigma Aldrich), cold and poured into 5 mm tubes. After the spectra with the spectral width of 238.8609ppm with an acquisition time of 1.363 sec were run, about 100 transients were acquired in the presence of a sealed external capillary of neat TMS (tetra methyl silan, Sigma Aldrich) to allow referencing of the chemical shifts. Organic carbon of levan was determined according to the method suggested by Jackson [16]. Ash content was determined according to the method of Serra-Bonvehi and Escola-Jorda [17].

2.5 Statistical Analysis

Data were statistically analysed using IBM® SPSS® Statistics software [18] and the correlation coefficient and regression were analyzed with Microsoft Office Excel 2010.

3. RESULTS AND DISCUSSION

3.1 Effect of Initial pH

The effect of pH value on the production of levan was investigated using best conditions of incubation. Data illustrated in Fig. 1 show that cell dry weight and levan production gradually increased as the pH values increased from 5.5 to 6.5 and reach their maximum at initial pH of 6.5 being 2.71 [or] 2.39 gL^{-1} of cell dry weight and

44.00 [or] 40.80gL^{-1} of levan dry weight for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. The levan production was gradually decreased as the pH values increased from 7.0 to 8.5 and reduced to reach the lowest value at pH 8.5 being 12.85 [or] 10.00gL^{-1} of levan dry weight on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively for *Bacillus lentus* V8 strain. Also results from Fig. 1 clearly show a high positive correlation coefficient between initial pH values and each of cell dry weight and levan dry weight on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses for *Bacillus lentus* V8 strain ranged between 0.87 and 0.96. The maximum levan production at initial pH 6.5 value might be due to induction of levansucrase at pH values ranging between 5.0 and 6.5 to synthesize levan, as reported by Vega- Paulino and Zúniga [19]. These results coincide with Wendt [20]; Ernandes and Garcia-Cruz [21] who notified that levan production by *Z. mobilis* was decreased at pH value ranged 3.8 to 4.5, due to acid formation**.** Shih et al. [22] who showed that the optimum pH for cell growth and levan production *Bacillus subtilis* (natto) takahashi was pH 6. This result was disagreement with Ananthalakshmy and Gunasekaran [23] who observed that the production of levan by *Z. mobilis* B-4286 at an initial pH of 5.0.

3.2 Incubation Temperature

Different temperatures ranged from 25 to 35ºC achieved high cell dry weight, levan production and reached maximum production at incubation temperature of 30ºC being 2.71 [or] 2.39 gL^{-1} of cell dry weight and 44.00 [or] 40.80gL^{-1} of levan dry weight for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively (Fig. 2). The increase of levan production at 30ºC could be due to activation of levansucrease to convert sucrose into levan [24]. The values of correlation coefficient between incubation temperatures and each of cell dry weight and levan dry weight on medium supplemented with sucrose [or] black strap sugar cane molasses for *Bacillus lentus* V8 strain ranged from (0.83-0.99) with high positive correlation coefficient. Results are in line with those of Abdel-Fattah et al. [5] who reported that the conversion of fructose to levan by *B. subtilis* NRC33a was highest at 30ºC (84%) and decreased significantly to 60% and 20.8% by conducting the reaction at 35ºC and 40ºC, respectively. Also Shih et al. [22] who found the suitable temperature range for growth and levan production was from 25 to 40ºC.

3.3 Shaking Speed

The maximum cell dry weight and levan production reached at 150 rpm were 2.83 [or] 2.59 gL^{-1} of cell dry weight and 47.80 [or] 41.60gL^{-1} of levan dry weight on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively (Fig. 3). A high positive correlation coefficient between shaking speed and each of cell dry weight was 0.96 [or] 0.96gL^{-1} and levan dry weight was 0.98 [or] 0.97 gL^{-1} for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. Data are in agreement with those obtained by Shih et al. [22] who found that the suitable shaking speed was from 150 to 200 ppm; however, the still culture significantly reduced the productivity of levan to half of the highest yield. Whereas, Vinhas et al. [25]; Alegre et al. [26] who found that the highest yield of levan production by *Z. mobilis* using Erlenmeyer flasks without agitation. Also, Han and Clarke [6] who reported that the polysaccharide production increased when the culture was slightly agitated during fermentation.

3.4 Inoculum Size

The effect of inoculum size on cell growth and levan production was also investigated. The fermentation medium was inoculated with 2.5 to 15%. The inoculum size of 10% had the highest growth and levan production being 2.87 [or] 2.72gL^{-1} of cell dry weight and 51.40 [or] 45.34gL^{-1} of levan dry weight by *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. It could be noticed from Fig. 4 that a high positive correlation coefficient (R^2) between inoculum size and each of cell dry weight and levan dry weight on modified productive medium supplemented with sucrose [or] black strap cane molasses by *Bacillus lentus* V8 strain ranged from 0.99-1.0. These results are in agreement with those of Senthilkumar and Gunasekaran [27] who used 10% (v/v) of 12h old seed culture of *Z. mobilis* for levan production. Whereas, Shih et al. [22] who

used 5% (v/v) inoculum of *B. subtilis* (natto) Takahashi for levan production.

3.5 Biological Activity of Levan Produced by *Bacillus lentus* V8 Strain on Modified Productive Medium Supplemented with Sucrose [or] Black Strap Sugar Cane Molasses

Based on previous results, it could be concluded that the highest yield of levan was obtained by *Bacillus lentus* V8 strain grown on 250gL^{-1} sucrose [or] black strap sugar cane molasses as a carbon source supplemented with 1.5gL^{-1} $(NH_4)_3PO_4$ as a nitrogen source, nutritional elements and pH adjusted to 6.5 using shake flasks as a batch culture for 96 h at 30ºC and 150 rpm. Data reflected the tested strain grown exponentially during the first period of 18 - 48h [or] 18 - 54h of fermentation period and also showed a highly positive correlation coefficient between cell dry weight of growth and fermentation period being 0.98 [or] 0.96 on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively with a specific growth rate (µ) of 0.041h^{-1} [or] 0.033h^{-1}. Doubling time (t_d), multiplication rate (MR) and generation number (N) were calculated from the growth curve being 16.91h [or] 21.00h, 0.06 [or] 0.005 and 1.77 [or] 1.71 on modified medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. Figs. 5, 6 reveal the cell dry weight, levan production, polymer yield (%), effective yield (%), $Y_{p/x}$ (ggL^{-1}) and productivity (g$L^{-1}h^{-1}$) for *Bacillus lentus* V8 strain during propagation in modified productive medium supplemented with sucrose [or] black strap sugar cane molasses for 96 h at 30ºC. Here we could conclude that increasing the period of incubation led to gradual increase in cell dry weight to recording the maximum biomass production being 2.99 [or] 2.89gL^{-1} on modified productive medium supplemented with sucrose after 48h [or] black strap sugar cane molasses after 66 h, respectively. The highest levan production being 57.95 [or] 49.86gL^{-1} of levan dry weight and 23.18 [or] 36.93% of polymer yield on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses after 60h, respectively. The highest values of effective yield (1.18 [or] 1.16%) after 66 h on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. The highest amount of polymer dry weight productivity (0.97 g$L^{-1}h^{-1}$) and $Y_{p/x}$ (19.78 ggL^{-1}) were attained after 54 h of fermentation on modified productive medium supplemented with sucrose, but the highest values of polymer productivity (0.85 g$L^{-1}h^{-1}$) and $Y_{p/x}$ (18.07 ggL^{-1}) were achieved after 42 h of fermentation on modified productive medium supplemented with black strap sugar cane molasses. Data illustrated by Fig. 5 clearly show a highly positive correlation coefficient between incubation period and cell dry weight, levan dry weight, polymer dry weight yield, effective yield, $Y_{p/x}$ and polymer productivity, R^2 values ranged from 0.91 to 0.97 on modified productive medium supplemented with sucrose.

The highest positive correlation coefficient was also observed between fermentation period and cell dry weight (R^2= 0.95), levan dry weight (R^2= 0.96), polymer dry weight yield (R^2= 0.96), effective yield (R^2 = 0.95) and $Y_{p/x}$ (R^2= 0.91) and polymer productivity being 0.89 on modified productive medium supplemented with black strap sugar cane molasses (Fig. 6).

3.6 The Characterization of Levan by ^{13}C NMR Spectroscopy and Physicochemical Properties

The ^{13}C NMR spectrum of the purified levan product showed six carbon signals at 106.99, 83.07, 79.08, 77.99, 66.17, and 62.69 ppm (Fig. 7). Results in Table 1 clearly show that the chemical shifts were representative to C-2, C-5, C-4, C-3, C-6 and C-1, respectively, of the levan produced by *Bacillus lentus* V8 strain. Also, the ^{13}C NMR indicated the presence of beta 2, 6 linkages which is main characteristic for levan polymer. These results are in agreement with those of van Hijum et al. [15]; Han [26] who used ^{13}C NMR to confirm that the C-2 resonance indicate the presence of β-fructofuranose. The physicochemical properties of levan produced from *Bacillus lentus* V8 strain during 66 h using shake flasks as a batch culture. Levan produced by *Bacillus* sp. V8 strain gave close physical properties of levan produced from *Bacillus polymyxa* [29]. However, carbon content was recorded in produced levan being 40.86%. The amount of ash was 0.98 %. Fig. 8-a, b, c and d) showed that levan produced by *Bacillus lentus* V8 strain was white color, very soluble in warm water and insoluble (precipitated) in 75% alcohol. Murphy [29] who found that levan was soluble in cold water and very soluble in hot water, and insoluble in 75% alcohol. Also found the carbon content and ash in levan produced by *Bacillus polymyxa* being 44.03% and 0.86%, respectively.

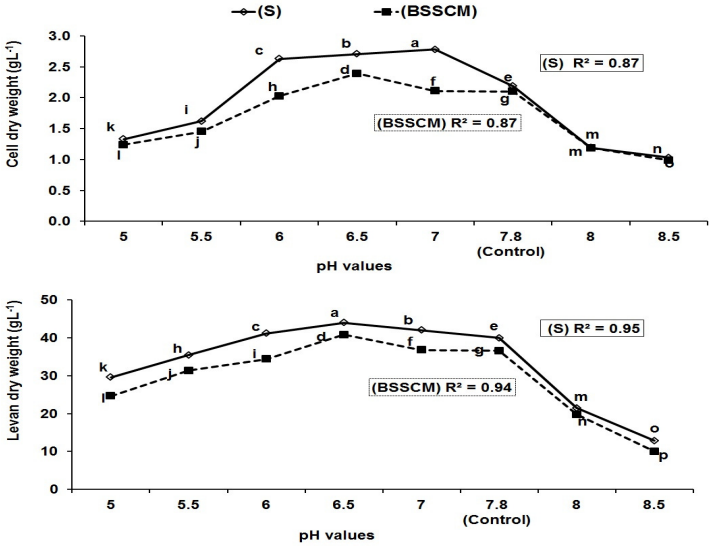

Fig. 1. Effect of different initial pH on biomass and levan production by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h at 30ºC using shake flasks as a batch culture

S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane mlasses; Values in the same line (followed by letters within an alphabetic series) sharing the same letter do not differ significantly, according to Duncan's at 5 % level. Values followed by letters in different alphabetic series are significant

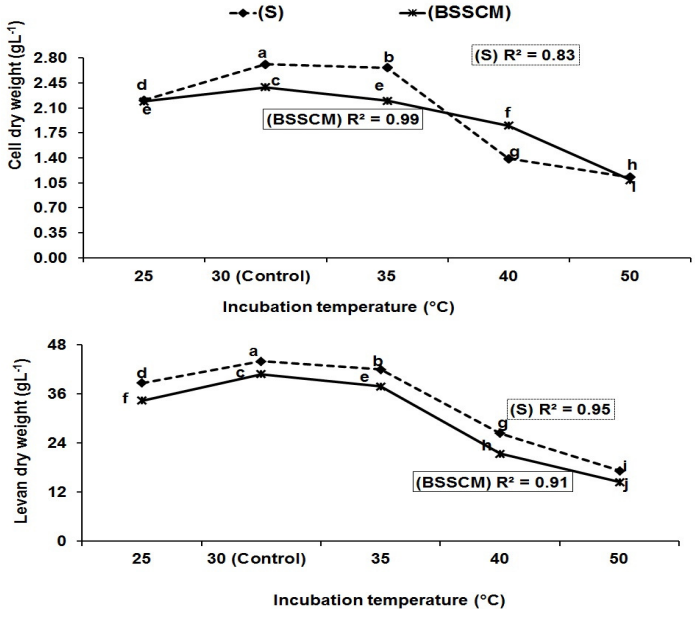

Fig. 2. Effect of incubation temperatures on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture

S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane mlasses; Values in the same line (followed by letters within an alphabetic series) sharing the same letter do not differ significantly, according to Duncan's at 5 % level. Values followed by letters in different alphabetic series are significant

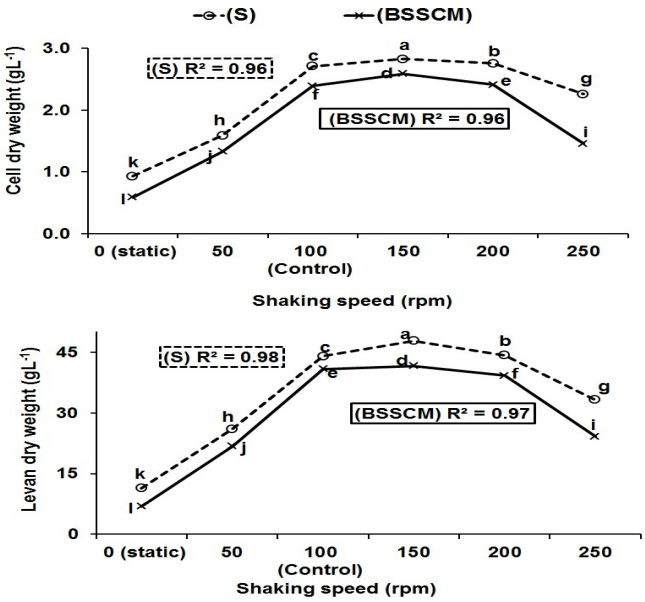

Fig. 3. Effect of shaking speed on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture

S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane molasses; Values in the same line (followed by letters within an alphabetic series) sharing the same letter do not differ significantly, according to Duncan's at 5 % level. Values followed by letters in different alphabetic series are significant

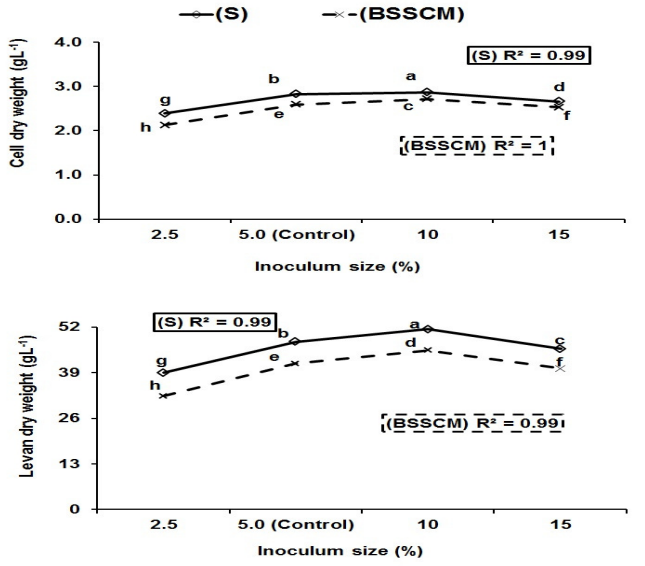

Fig. 4. Effect of inoculum size on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture

S= Medium supplemented with sucrose; BSCM= Medium supplemented with black strap sugar cane molasses; Values in the same line (followed by letters within an alphabetic series) sharing the same letter do not differ significantly, according to Duncan's at 5% level. Values followed by letters in different alphabetic series are significant

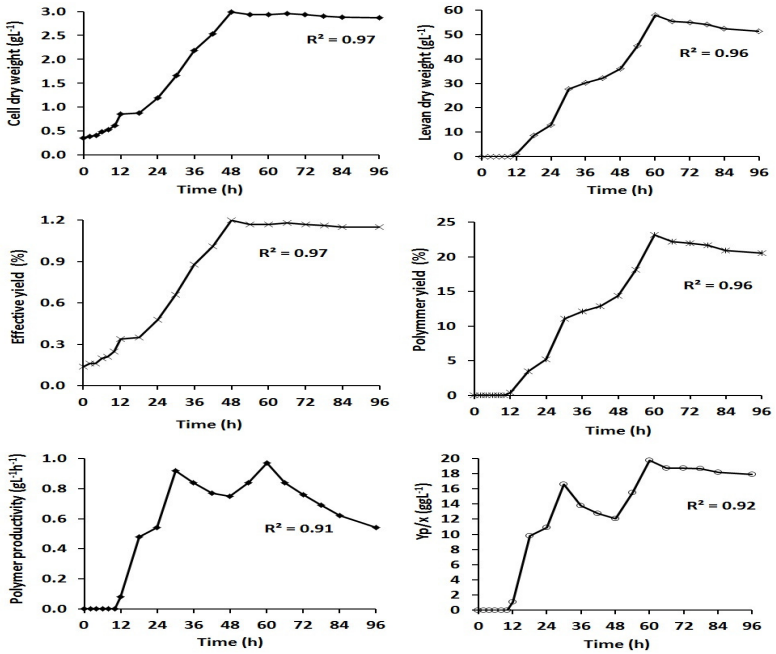

Fig. 5. Biological activity of *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose during 96 h incubation period at 30°C using shake flasks as a batch culture

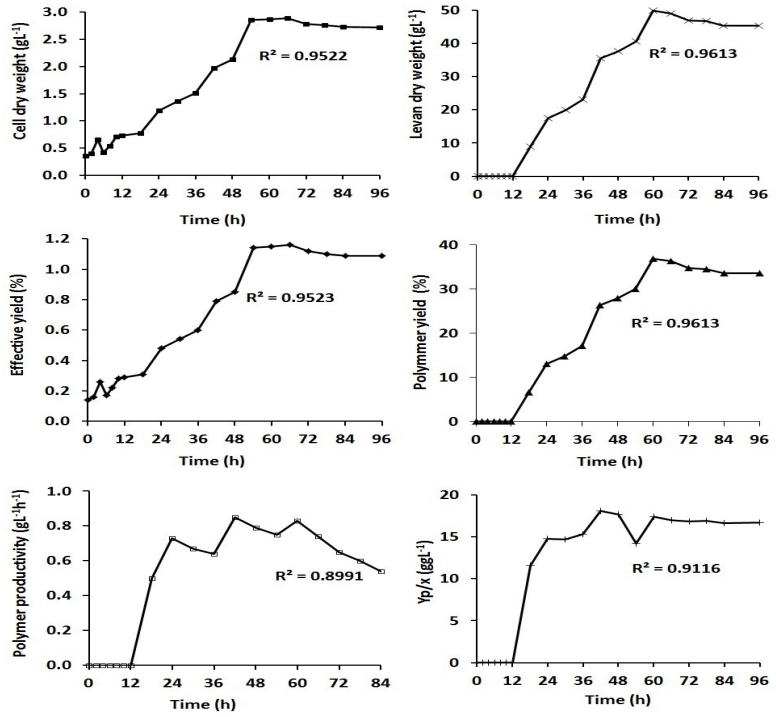

Fig. 6. Biological activity of *Bacillus lentus* V8 strain on modified productive medium supplemented with black strap sugar cane molasses during 96 h incubation period at 30ºC using shake flasks as a batch culture

Table 1. ^{13}C NMR chemical shifts of levan produced by *Bacillus lentus* V8 strain

Carbon atom (^{13}C NMR)	Chemical shift (ppm)		
	Bacillus polymyxa*	***Lactobacillus reuteri* strain 121****	***Bacillus lentus* V8 strain**
C-1	60.7	61.7	62.69
C-2	104.4	105.0	106.99
C-3	77.0	78.1	77.99
C-4	75.7	76.6	79.08
C-5	80.5	81.2	83.07
C-6	63.6	64.3	66.17

**Assignment cited from Han [28].*
***Assignment cited from van Hijum et al. [15]*

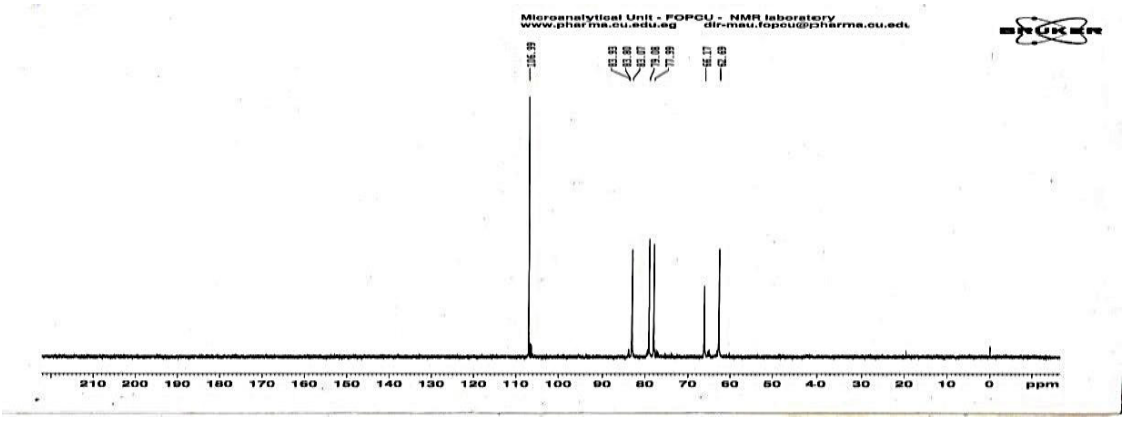

Fig. 7. ^{13}C NMR of levan polymer purified produced by *Bacillus lentus* V8 strain

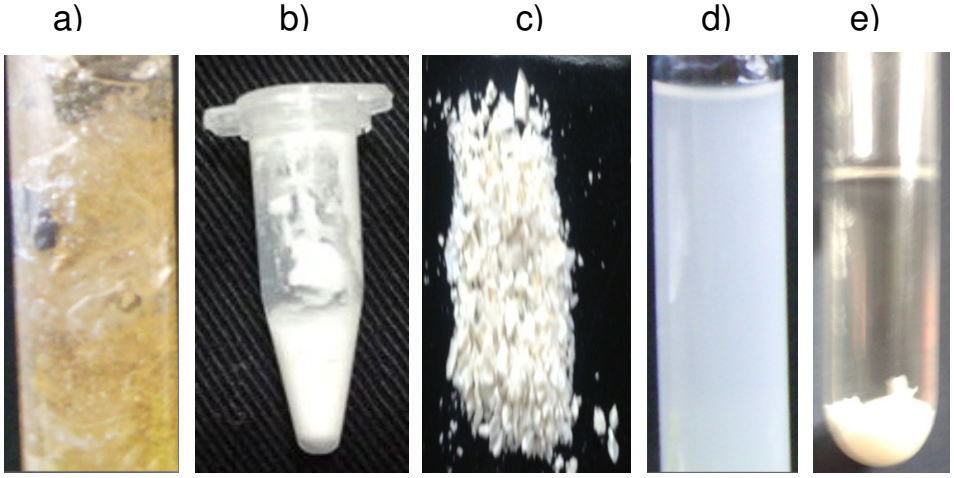

Fig. 8. Levan polymer gum produced by *Bacillus lentus* V8 strain
(a) Before purification b) After purification c) Levan white powder d) Soluble in water e) Insoluble (Precipitated) in 75% Alcohol

4. CONCLUSION

This study clearly show that the maximum levan production from *Bacillus lentus* V8 strain on productive medium supplemented with sucrose [or] black strap sugar cane molasses was obtained with the process conditions of pH 6.5, 10% inoculum size, incubated at 30ºC and 150

rpm shaking speed for 60h. Physical and chemical properties levan produced were white color, soluble in water and precipitated in Alcohol, it contains 40.86% carbon and 0.98% ash.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Keith J, Wiley B, Ball D, Arcidiacono S, Zorfass D, Mayer J, Kaplan D. Continuous culture system for production of biopolymer levan using *Erwinia herbicola*. Biotechnology and Bioengineering. 1991;38:557-560.
2. Arvidson SA, Rinehart BT, Gadala-Maria F. Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp. Carbohydrate Polymers. 2006;65:144-149.
3. Han YW, Watson MA. Production of microbial levan from sucrose, sugarcane juice and beet molasses. Journal of Industrial Microbiology.1992;9:257-260.
4. Ben Ammar Y, Matsubara T, Ito K. Characterization of a thermos table levansucrase from *Bacillus* sp. TH4-2 capable of producing high molecular weight levan at high temperature. Journal of Biotechnology. 2002;99:111-119.
5. Abdel-Fattah AF, Mahmoud DAR, Esawy MAT. Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymic synthesis of levan and fructo-oligosaccharides. Current Microbiology. 2005;51:402-407.
6. Han YW, Clarke MA. Production and characterization of microbial levan. Journal of Agricultural and Food Chemistry. 1990;38:393-396.
7. Ghaly AE, Arab F, Mahmoud NS, Higgins J. Production of levan by *Bacillus licheniformis* for use as a soil sealant in earthen manure storage structures. American Journal of Biochemistry and Biotechnology. 2007;3:47-54.
8. Melo IR, Pimental MF, Lopes CE, Calazans GM. Application of fractional factorial design to levan production by *Zymomonas mobilis*. Brazilian Journal of Microbiology. 2007;38:45-51.
9. Abou-Taleb KhA, Abdel-Monem MO, Yassin MH, Draz AA. Nutritional factors affecting levan production by *Bacillus* sp.V8 strain isolated from rhizosphere bean (*Vicea faba*) plant. The International Journal of Agricultural Technology. 2014;10:899-914.
10. Reiss M, Hartmeier W. Levan production with a flocculent strain of *Zymomonas mobilis*. Food Biotechology. 1990;4:69-75.
11. Doelle HW. Bacterial Metabolism. 2nd ed. Academic Press, New York.1975;738.
12. Ramadan EM, El-Sawy M, Gamal RF, Abdelhady HM. Growth parameters of yeast grown on agricultural residues using shake flasks as a batch culture. Annals of Agriculture Science, Fac. Agri., Ain Shams Univ., Cairo, Egypt. 1985;30:25-45.
13. Lee SY. Plastic bacteria. Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnology. 1996;14:431-438.
14. Grothe E, Moo-Young M, Chisti Y. Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme and Microbial Technology. 1999;25:132-141.
15. Van Hijum SA, Bonting K, Van Der Maarel MJ, Dijkhuizen L. Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiology Letters. 2001;205:323-328.
16. Jackson ML. Soil chemical analysis. Prentice Hall of India Pvt. Ltd., New Delhi. 1973;183-205.
17. Serra-Bonvehi J, Escola-Jorda R. Nutrient composition and microbiological quality of honeybee-collected pollen. J. Agric. Food Chem. 1997;5:725-732.
18. IBM® SPSS® Statistics Version 19.0, SPSS Inc., Chicago, Illinois; 2011.
19. Vega-Paulino RJ, Zúniga-Hansen ME. Potential application of commercial enzyme preparations for industrial production of short- chain fructooligosaccharides. Journal of Molecular Catalysis B: Enzymatic. 2012;76:44-51.
20. Wendt R. Studies levan production by *Zymomonas mobilis*. Dissertation, State University of Campinas, Campinas, Brazil. 2001;76f.
21. Ernandes FM, Garcia-Cruz CH. Nutritional requirements of *Zymomonas mobilis* CCT 4494 for levan production. Brazilian Archives of Biology and Technology. 2011;54:589-600.
22. Shih I, Shieh Y, Yu C, Hsieh C. Selective production and characterization of levan by

Bacillus subtilis (Natto) Takahashi. Journal of Agricultural and Food Chemistry. 2005;53:8211-8215.
23. Ananthalakshmy VK, Gunasekaran P. Optimization of levan production by *Zymomonas mobilis*. Brazilian Archives of Biology and Technology.1999;42:291-297.
24. Youssef GA, Youssef AS, Talha S, El-Aassar SA. Increased fructosyltranseferase (levansucrase) production by optimizing culture condition from *Pediococcus acidilactici* strain in shaking batch cultures. Life Science Journal. 2014;11:33-47.
25. Vinhas GM, Calazans GM, Lopes CE. Produc- of levanas by *Zymomonas mobilis* and ZAG-12 obten¸ c~ao the solubility curve of the levan-'agua-ethanol system; 2000.
26. Alegre RM, Wendt R, Rigo M. Levan production by isolated mutants of *Zymomonas mobilis*. Magazine Exatas and Natural Sciences. 2005;7:103-112.
27. Senthilkumar V, Gunasekaran P. Influence of fermentation conditions on levan production by *Zymomonas mobilis* CT2. Indian Journal of Biotechnology. 2005;4:491-496.
28. Han YW. Levan production by *Bacillus polymyxa*. Journal of Industrial Microbiology.1989;4:447-452.
29. Murphy D. Structure of a levan produced by *Bacillus polymyxa*. Canadian Journal of Chemistry. 1952;30:872-878.

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