

Original Research Article

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

ABSTRACT

Aims: to optimize a fermentation conditions for microbial production of levan polymer on a low-price productive medium. Also to purified levan from *Bacillus* sp. V8 strain, characterized the levan polymer by ^{13}C NMR spectroscopy and physicochemical properties.

Study design: Optimization of environmental factors for levan production. Purification and identification of polymer.

Place and Duration of Study: Agric. Microbiology Dept., Fac. of Agriculture, Ain Shams Univ., Cairo, Egypt, between January 2012 and December 2013.

Methodology: *Bacillus* sp. V8 strain was used as procedure of levan. This strain was grown on productive medium and the end of fermentation take sample to determined cell dry weight and levan polymer. Study the optimal condition for levan production 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* V8 strain on medium supplemented with sucrose or black strap cane sugar cane molasses was investigated. Results indicated that the productive medium with initial pH 6.5, 10% inoculum size, incubated at 30 °C, 150 rpm shaking speed gave 51.40 or 45.34 gL⁻¹ of levan dry weight on medium supplemented with sucrose or black strap cane 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 2.89 gL⁻¹ was obtained after 48 h or 66 h, respectively. The highest levan dry weight (57.95 or 49.86 gL⁻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, levan as fructose, polymer dry weight yield, effective yield and $Y_{D/X}$, 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. Physical and chemical properties levan produced were white color, soluble in water and precipitated in Alcohol, it contains 40.86% carbon, 0.074% nitrogen and 0.98% ash.

Conclusion: The maximum levan production from *Bacillus lentus* V8 strain on productive medium supplemented with sucrose or black strap cane sugar cane molasses was obtained with the process conditions of pH 6.5, 10% inoculum size and incubated at 30°C for 60 h at 150 rpm. Characterized the levan polymer by ^{13}C NMR spectroscopy and physicochemical properties.

Keywords: Levan polymer; batch fermentation; bacteria; production; raw materials; polymer identification

1. INTRODUCTION

Levan is a β -2,6-linked fructose homopolymer with some β -2,1-linked branched point, 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 sugarbeet molasses. The organism 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 sp. Levansucrase was produced from various microorganisms [4, 5]. levan is amorphous or microcrystalline, white powder, soluble in cold water, very soluble in hot water, insoluble in 75% alcohol, anon-toxic, biologically active, extracellular polysaccharide, non-viscous, nontransparent suspension and deflect visible light [6, 7, 8]. The physiological effects of levan are dependent on its size and linkage type, and the fermentability of levan is therefore an important issue. The aim of this study was to optimize a fermentation conditions for microbial production of levan polymer on a low-price productive medium using shake flasks as a batch culture. Therefore, we purified levan from *Bacillus lentus* V8 strain, characterized the levan polymer by ¹³C NMR spectroscopy and physicochemical properties.

2. MATERIAL AND METHODS

2.1. Microorganism and fermentation condition

Microorganism used in this study was *Bacillus lentus* V8 strain. The strain was described previously by Abou-Taleb et al. [9] and grown in productive medium modified by Abou-Taleb et al. [9]. This medium contained (g/L): Ammonium sulphate, 1.5; Sucrose [or] Black strap sugar cane molasses, 250; MgSO₄.7H₂O, 0.2; K₂HPO₄, 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). The fermentation was carried out in 250 ml plugged Erlenmeyer flasks containing 100 ml sterile productive medium at 30°C 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 10000 rpm for 10 min at 4°C. 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 [10]. 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 [10] or 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).

2.2. Study the optimal condition for levan production

This experiment was carried out to detect the optimum pH value for levan production. So, eight values of pH ranged between 5 and 8.5 were investigated. To find out the effect of different degrees of incubation temperatures (25, 30, 35, 40 & 50°C) on levan production. To select the best shaking speed leading to highest levan production, 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 were grown in Erlenmeyer flasks (500 ml in volume) containing 350 ml productive medium and incubated at 30°C for 96h on rotary shaker (150 rpm). Samples (15 ml) were taken from the growing culture periodically every 2 hrs under aseptic conditions and centrifuged at 10.000 rpm/ 10 min, the pellet (bacterial cells) was washed twice with distilled water, then dried at 70°C 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), doubling time (t_d), multiplication rate (MR) and number of generation (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 fermentation being yield, effective yield, productivity and yield coefficient relative to biomass 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 4°C during 16 h followed by dialysis overnight against demineralized water. Subsequently, the polymer was precipitated with 2 volumes of 96% ethanol, centrifuged for 10 min at 10.000 rpm, freeze dried and stored at -20°C for further analysis [15] (Van Hijum et al. 2001). The purified polymer was characterized using nuclear magnetic resonance (^{13}C -NMR; NMR Lab., Microanalytical unite (MAU), Fac. of pharmacy, Cairo Univ., Egypt) ~~were run~~ using BRUKER-400 spectrometer. Sample of levan was dissolved in D_2O solution (Sigma Aldrich), on cold and poured into 5 mm tubes. After the spectra with the spectral width of 238.8609 ppm with an acquisition time of 1.363sec 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]. Total nitrogen was determined polymer using Kjeldahel method as described by Jackson [16]. Ash content was determined according to the method of Serra-Bonvehí and Escola-Jorda [17].

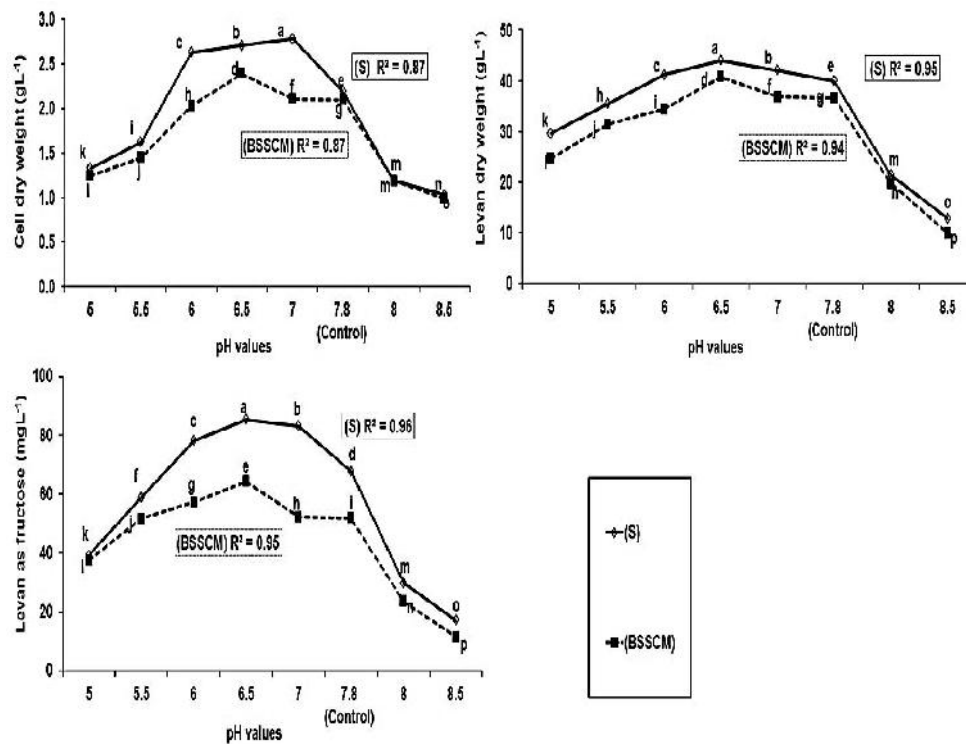
2.5. Statistical analysis

The collected 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 studied 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 g L^{-1} of cell dry weight, 44.00 [or] 40.80 g L^{-1} of levan dry weight and 85.40 [or] 64.30 mg L^{-1} of levan as fructose for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap cane 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] 10.00 g L^{-1} of levan dry weight and 17.20 [or] 11.50 mg L^{-1} of levan as fructose 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, levan dry weight and levan as fructose on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses for *Bacillus lentus* V8 strain ranged between 0.87 & 0.96. These results coincide with Wendt [19]; Ernandes and Garcia-Cruz [20] who noticed that levan production by *Z. mobilis* was decreased at pH value ranged 3.8 to 4.5, due to acid formation. Shih et al. [21] 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 [22] who observed that the production of levan by *Z.*



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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 30°C using shake flasks as a batch culture.

S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane molasses.

Letters on top of bars in the same column or line are different letters indicate to significant differences and the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

3.2. Incubation temperature

Results in Fig. 2 recorded that the degrees of incubation temperatures from 25 to 35°C achieved high cell dry weight, levan production and reached maximum production at incubation temperature of 30°C being 2.71 [or] 2.39 gL⁻¹ of cell dry weight, 44.00 [or] 40.80 gL⁻¹ of levan dry weight and 85.40 [or] 64.30 mgL⁻¹ of levan as fructose for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. It could be noticed from Fig. 2 that the values of correlation coefficient between incubation temperature and each of cell dry weight, levan dry weight and levan as fructose 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 30°C (84%) and decreased significantly to 60% and 20.8% by conducting the reaction at 35°C and 40°C, respectively. Also Shih et al. [21] who found the suitable temperature range for growth and levan production was from 25 to 40 °C.

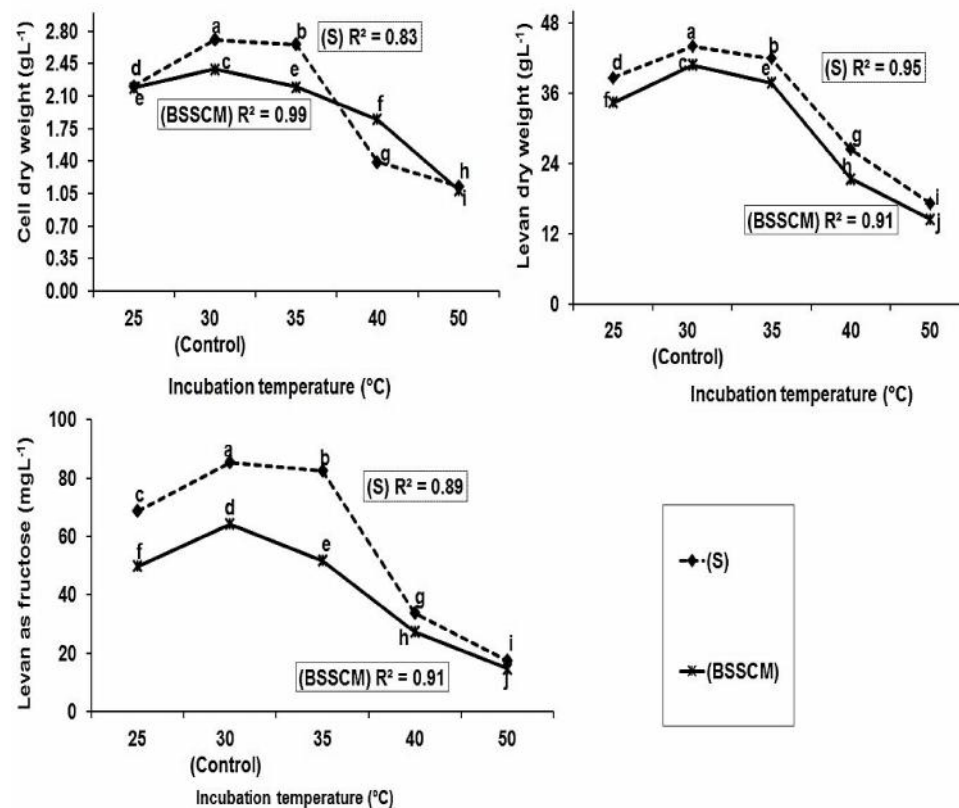


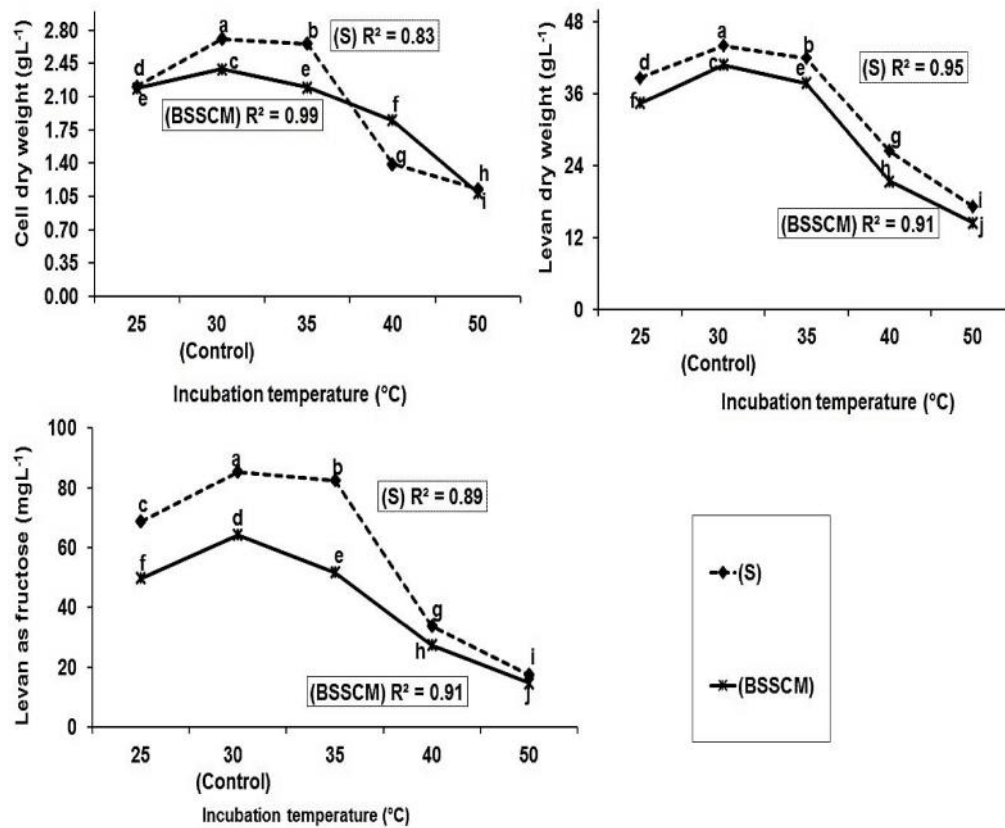
Fig. 2. Effect of incubation temperature on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture.

S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane molasses.

Letters on top of bars in the same column or line are different letters indicate to significant differences and the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

3.3. Shaking speed

Results illustrated in Fig. 3 clearly show that the shaking speed from 100 - 200 rpm achieved high levan production in *Bacillus lentus* V8 strain. The maximum cell dry weight and levan production achieved at 150 rpm were 2.83 [or] 2.59 gL⁻¹ of cell dry weight , 47.80 [or] 41.60 gL⁻¹ of levan dry weight and 93.50 [or] 66.80 mgL⁻¹ of levan as fructose on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses, respectively. A high positive correlation coefficient between shaking speed and each of cell dry weight was (0.96 or 0.96), levan dry weight was (0.98 or 0.97) and levan as fructose was (0.93 or 0.88) for *Bacillus lentus* V8 strain on modified productive medium supplemented with sucrose or black strap sugar cane molasses, respectively (Fig. 3). Data are in agreement with those obtained by Shih et al. [21] 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. [23]; Alegre et al. [24] 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.



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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.

Letters on top of bars in the same column or line are different letters indicate to significant differences and the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

3.4. Inoculum size

This experiment was performed to study the effect of inoculum size on growth and levan production. The fermentation medium was inoculated with 2.5 to 15 %. Results illustrated by Fig. 4 show that the inoculum size of 10% achieved the highest growth and levan production being 2.87 [or] 2.72 gL⁻¹ of cell dry weight, 51.40 [or] 45.34 gL⁻¹ of levan dry weight and 95.20 [or] 71.10 mgL⁻¹ of levan as fructose 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 (R²) between inoculum size and each of cell dry weight, levan dry weight and levan as fructose 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 [25] who used 10% (v/v) of 12 h old seed culture of *Z. mobilis* for levan production. Whereas, Shih et al. [21] who used 5% (v/v) inoculum of *B. subtilis* (natto) Takahashi for levan production.

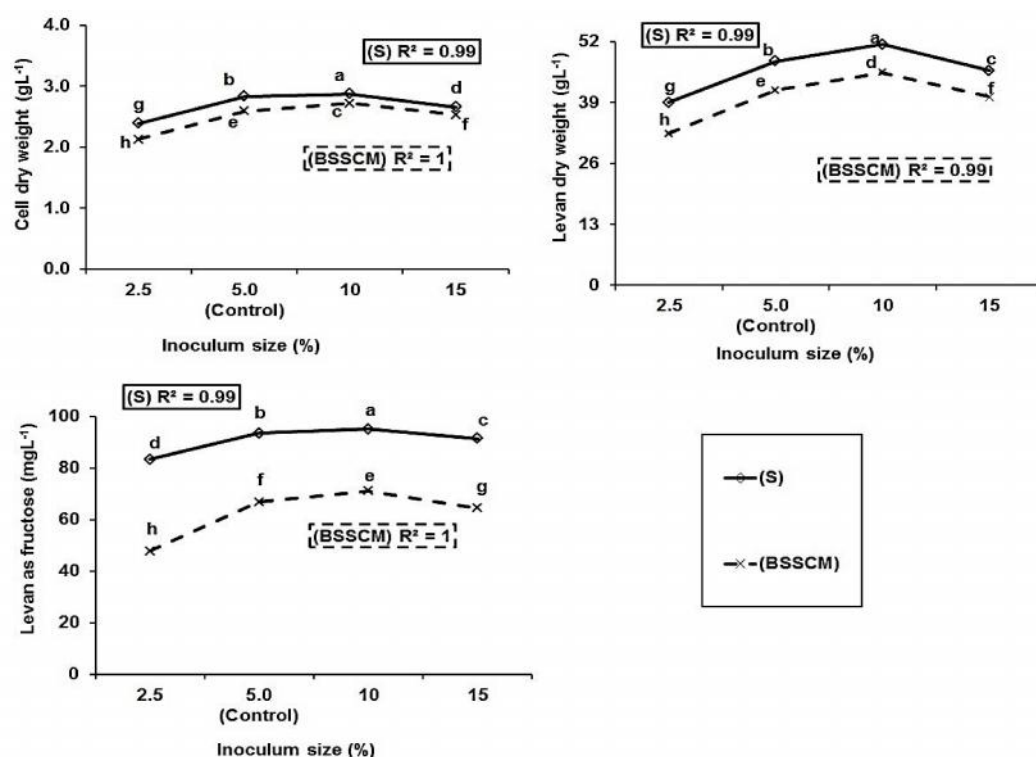


Fig. 4. Effect of inoculum size on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture.

S= Medium supplemented with sucrose; BSCM= Medium supplemented with black strap sugar cane molasses.

Letters on top of bars in the same column or line are different letters indicate to significant differences and the same letter do not significantly differ from each other, according to Duncan's at 5 % level.

3.5. Biological activity of levan producing *Bacillus* sp. V8 strain on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses

From the previous experiment results, it could be concluded that the highest yield of levan was obtained by *Bacillus lentus* V8 strain grown on 250 gL⁻¹ sucrose [or] black strap sugar cane molasses as a carbon source supplemented with 1.5 gL⁻¹ (NH₄)₃PO₄ as a nitrogen source, nutritional elements and pH adjusted to 6.5 using shake flasks as a batch culture for 96 h at 30°C and 150 rpm. Data show that tested strain grew exponentially with a specific growth rate (μ) of 0.041 h⁻¹ [or] 0.033 h⁻¹, were noticed during the first period of 18 - 48 h [or] 18 - 54 h 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 and revealed the growth parameters for this strain which were calculated from the growth curve. Results in Figs. 5, 6 show the cell dry weight, levan production, polymer yield (%), effective yield (%), $Y_{p/x}$ (ggL⁻¹) and productivity (gL⁻¹h⁻¹) for *Bacillus lentus* V8 strain during propagation in modified productive medium supplemented with 250 gL⁻¹ sucrose [or] black strap sugar cane molasses (54% sugar) for 96 h at 30°C. Results showed that increasing the period led to gradual increase in cell dry weight to recording the maximum biomass production being 2.99 [or] 2.89 gL⁻¹ on modified productive medium supplemented with sucrose after 48 h [or] black strap sugar cane molasses after 66 h, respectively. The highest levan production being 57.95 [or] 49.86 gL⁻¹ of levan dry weight, 103.1 [or] 82.7 mgL⁻¹ of levan as fructose and 23.18 [or] 36.93% of polymer yield on modified productive medium supplemented with sucrose [or] black strap sugar cane molasses after 60 h, 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 gL⁻¹h⁻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 gL⁻¹h⁻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, levan as fructose, polymer dry weight yield, effective yield, $Y_{p/x}$ and polymer productivity, R² values ranged from 0.91 to 0.97 on modified productive medium supplemented with sucrose.

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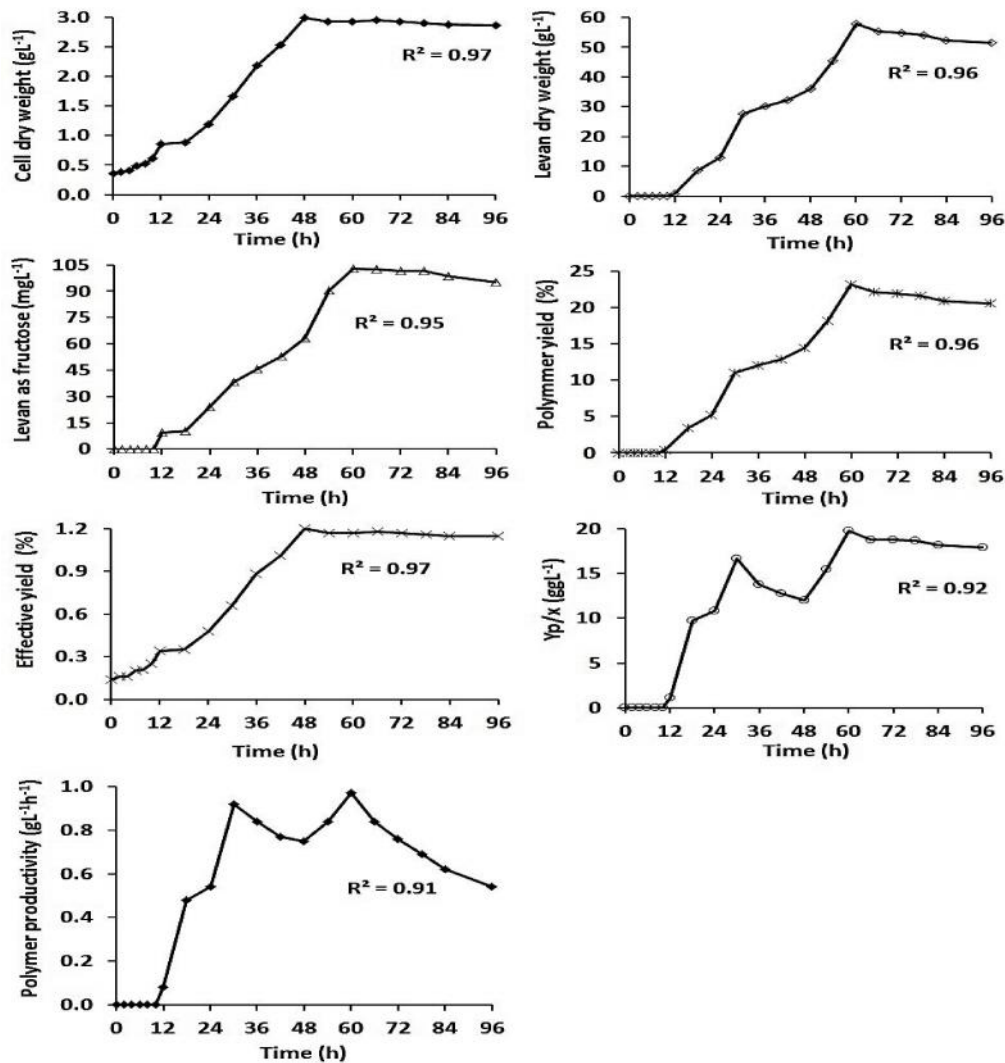
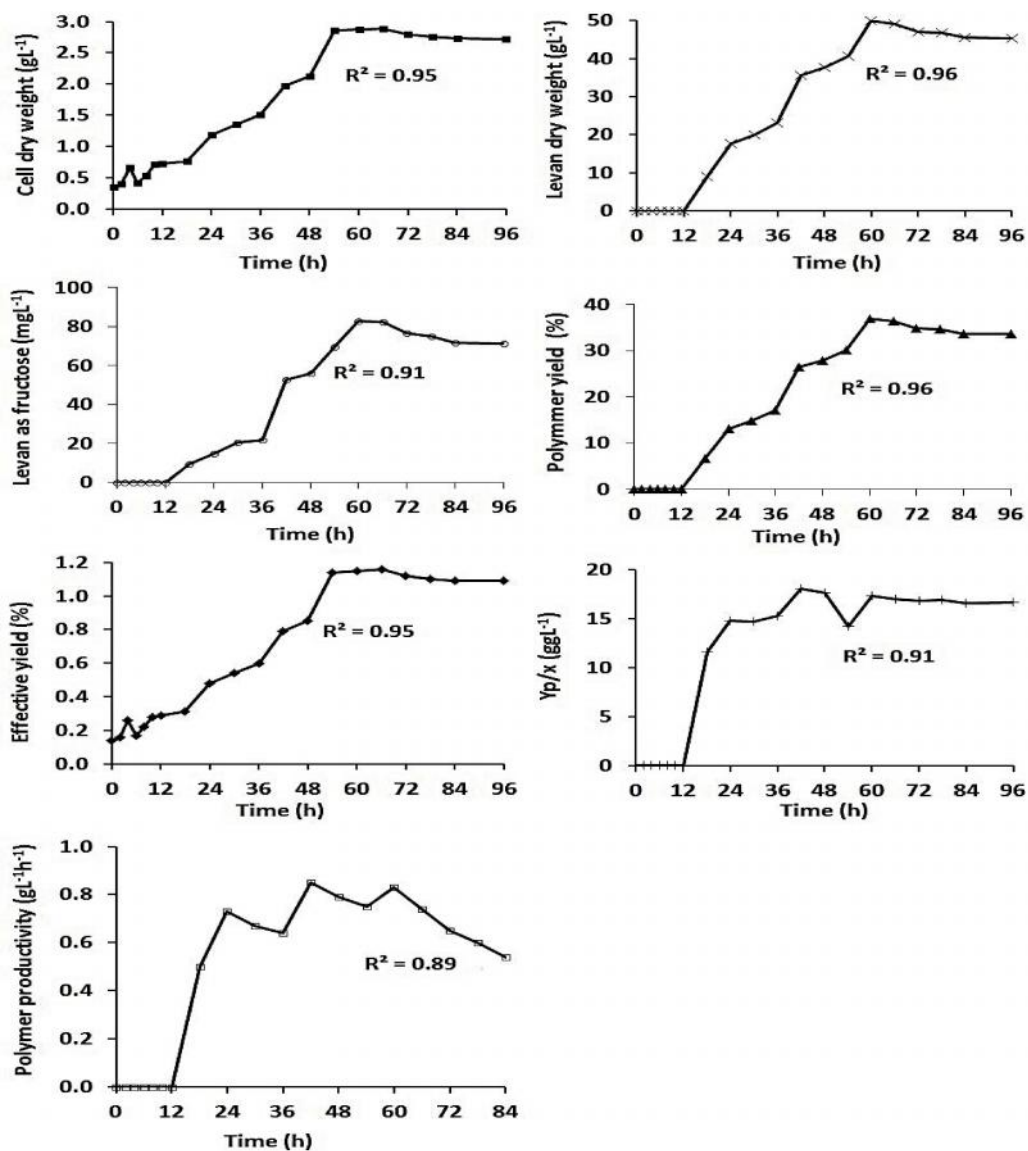


Fig. 5.

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.

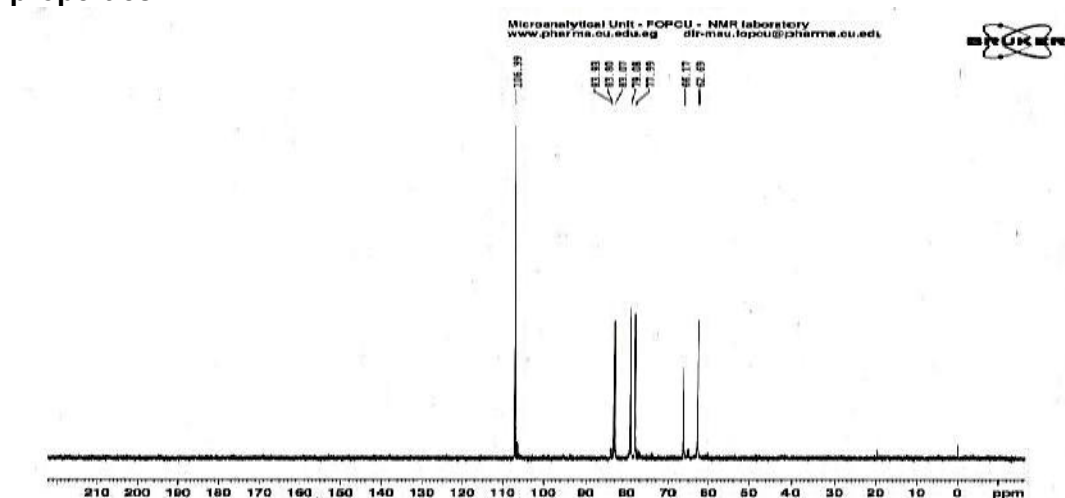
It was also noticed that the highest positive correlation coefficient between fermentation period and cell dry weight ($R^2 = 0.95$), levam dry weight ($R^2 = 0.96$), levam as fructose ($R^2 = 0.91$), polymer dry weight yield ($R^2 = 0.96$), effective yield ($R^2 = 0.95$) and $Y_{p/x}$ ($R^2 = 0.91$)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



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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 30°C using shake flasks as a batch culture.

3.6. Levan characterization by ^{13}C NMR spectroscopy and physicochemical properties



C NMR spectrum of the purified 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. ^{13}C NMR indicated the presence of beta 2, 6 linkages which is 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* [27]. However, carbon content was recorded in produced levan being 40.86%. The amount of total nitrogen and ash were 0.074% and 0.98%, respectively. 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 [27] 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, total nitrogen and ash in levan produced by *Bacillus polymyxa* being 44.03%, 0.09% and 0.86%, respectively.

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

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

Carbon atom (^{13}C NMR)	Chemical shift (ppm)		
	<i>Bacillus polymyxa</i> *	<i>Lactobacillus reuteri</i> strain 121 **	<i>Bacillus lentus</i> 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 [26].

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

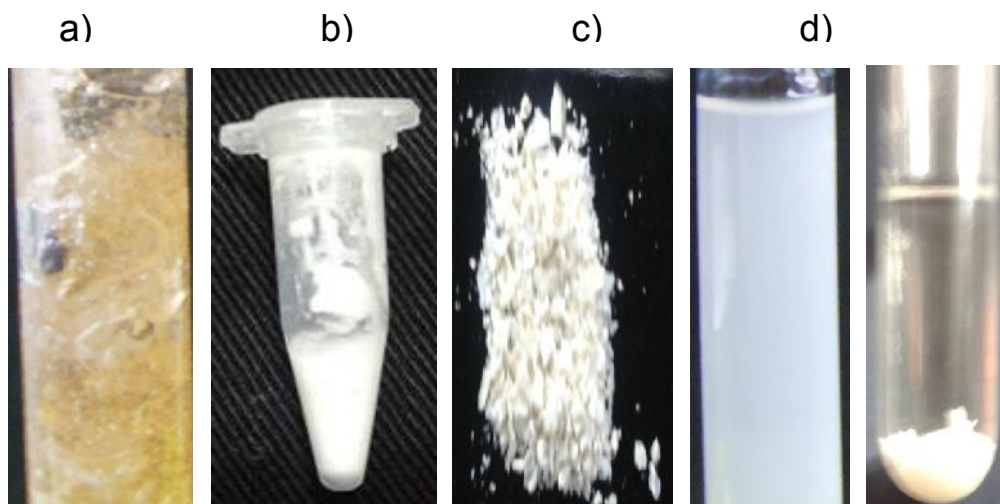


Fig. 8. Levan polymer gum produced by *Bacillus lentus* V8 strain

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 cane sugar cane molasses was obtained with the process conditions of pH 6.5, 10% inoculum size, incubated at 30 and 150 rpm shaking speed for 60 h. Physical and chemical properties levan produced were white color, soluble in water and precipitated in Alcohol, it contains 40.86% carbon, 0.074% nitrogen and 0.98% ash.

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