

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

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* 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 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 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. 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 30°C for 60 h at 150 rpm. 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, 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. 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 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, 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 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, characterization the levan polymer by ^{13}C 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^{-1}): ammonium sulphate, 1.5; sucrose [or] black strap sugar cane molasses*, 250; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 (53.48 %) and other metals necessary for the fermentation process. 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 after oven-drying 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 of 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 determine the optimum incubation temperature for levan production, fermentation was carried out at various temperatures in the range of 25, 30, 35, 40 and 50°C. 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 was 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) (h^{-1}) = $(\ln X - \ln X_0) / (t - t_0)^{-1}$, 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 (g L^{-1}) / initial sugar (g L^{-1}) x 100, effective yield (%) = cell dry weight (g L^{-1}) / Initial substrate concentration x 100, productivity ($\text{g L}^{-1} \text{h}^{-1}$) = levan concentration (g L^{-1}) / fermentation time (h) and yield coefficient relative to biomass (gg L^{-1}) = polymer conc. (g L^{-1}) / gram biomass dry weight (g L^{-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 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]. 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 238.8609 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]. Ash content was determined according to the method of Serra-Bonvehi and Escola-Jorda [17].

2.5. Statistical analysis

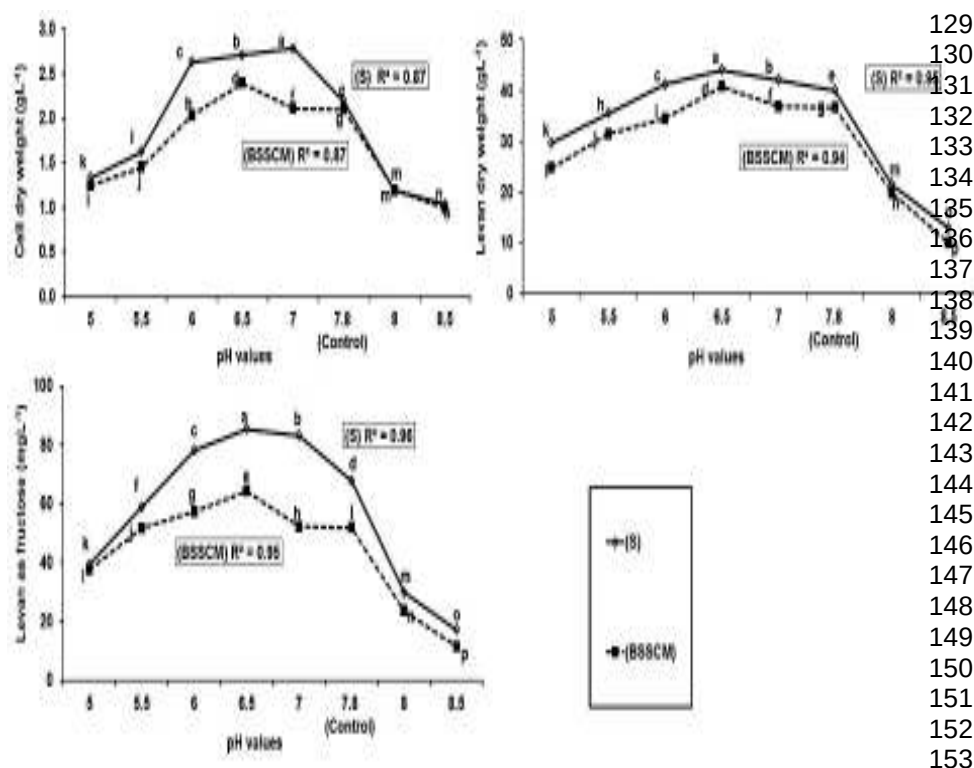
The collected data were statistically analysed using IBM® SPSS® Statistics software [18] and the correlation coefficient and regression were analyzed with Microsoft Office Excel 2010.

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

121 sugar cane molasses for *Bacillus lentus* V8 strain ranged between 0.87 & 0.96. The
 122 maximum levan production at initial pH 6 value might be due to induction of levansucrase to
 123 synthesize levan. These results coincide with Wendt [19]; Ernandes and Garcia-Cruz [20]
 124 who noticed that levan production by *Z. mobilis* was decreased at pH value ranged 3.8 to
 125 4.5, due to acid formation. Shih et al. [21] who showed that the optimum pH for cell growth
 126 and levan production *Bacillus subtilis* (natto) takahashi was pH 6. This result was
 127 disagreement with Ananthalakshmy and Gunasekaran [22] who observed that the production
 128 of levan by *Z. mobilis* B-4286 at an initial pH of 5.0.



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

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

159 Values in the same line (followed by letters within an alphabetic series) sharing the same letter do
 160 not differ significantly, according to Duncan's at 5 % level. Values followed by letters in different
 161 alphabetic series are significant.

163 3.2. Incubation temperature

164 Results in Fig. 2 recorded that the degrees of incubation temperatures from 25 to 35°C
 165 achieved high cell dry weight, levan production and reached maximum production at
 166 incubation temperature of 30°C being 2.71 [or] 2.39 gL⁻¹ of cell dry weight, 44.00 [or] 40.80
 167 gL⁻¹ of levan dry weight and 85.40 [or] 64.30 mgL⁻¹ of levan as fructose for *Bacillus lentus* V8
 168 strain on modified productive medium supplemented with sucrose [or] black strap sugar
 169 cane molasses, respectively. The increase of levan production at 30°C could be due to
 170 activation of levansucrase for converted sucrose into levan. It could be noticed from Fig. 2
 171 that the values of correlation coefficient between incubation temperature and each of cell dry
 172 weight, levan dry weight and levan as fructose on medium supplemented with sucrose [or]
 173 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.

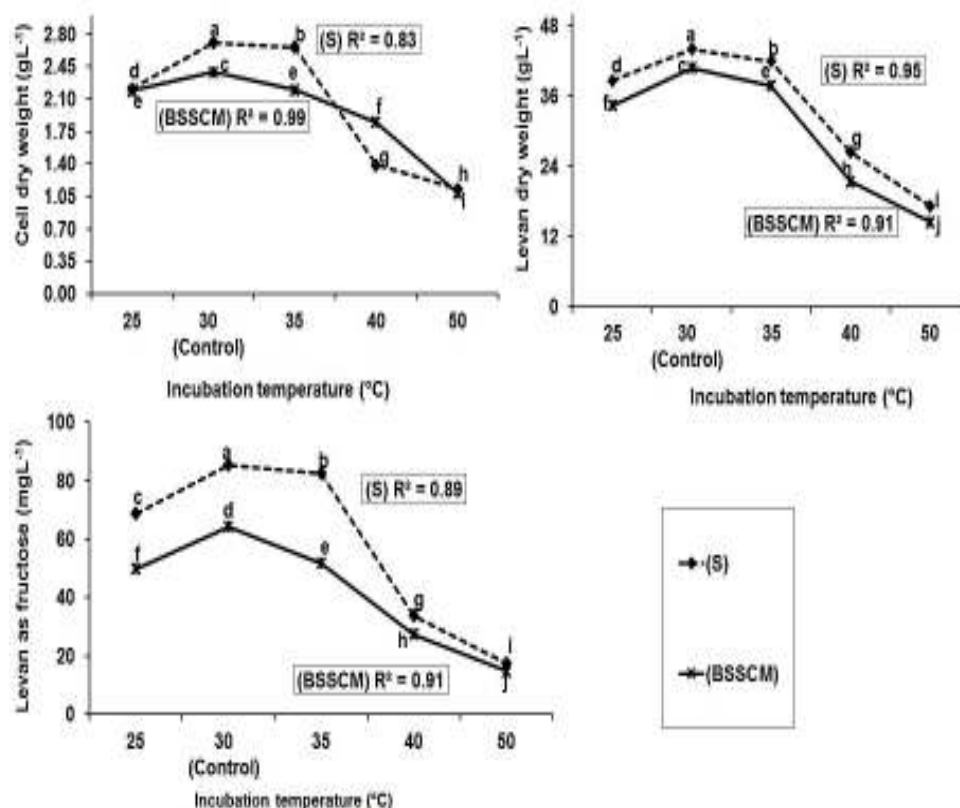


Fig. 2. Effect of incubation temperature on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture.

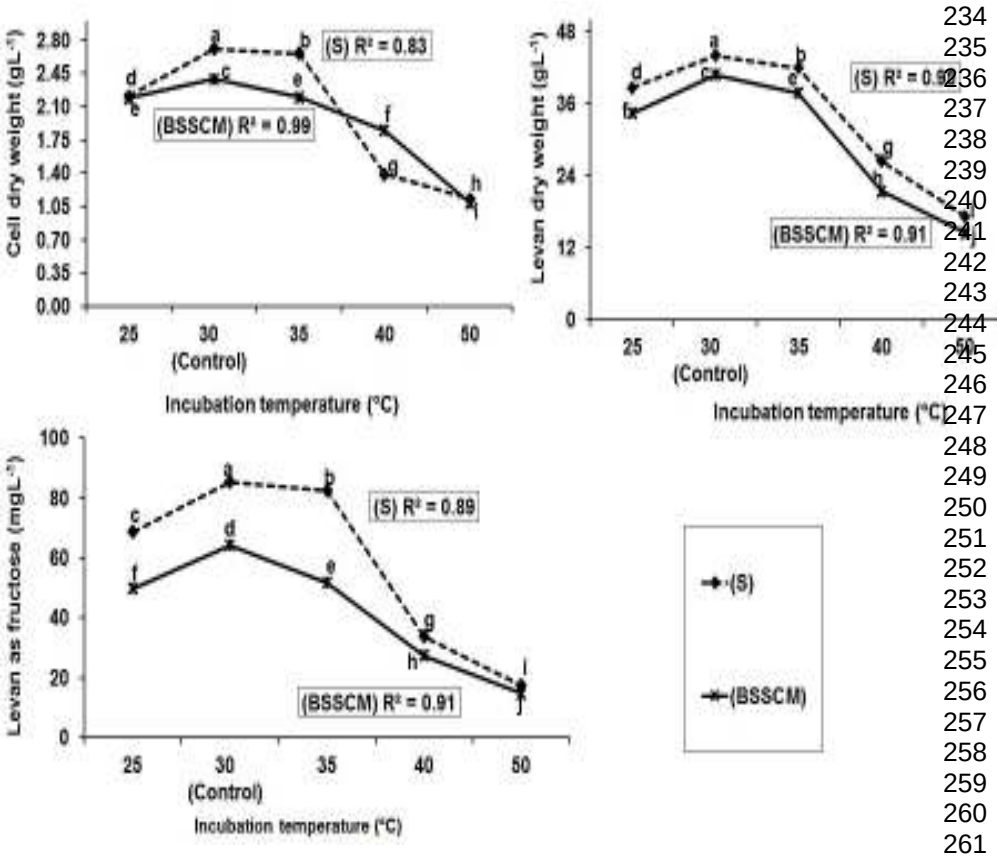
S= Medium supplemented with sucrose; BSSCM= Medium supplemented with black strap sugar cane molasses.

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.

3.3. Shaking speed

Results illustrated in Fig. 3 clearly show that the shaking speed from 100 - 200 rpm achieved high levan production by *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

228 levan to half of the highest yield. Whereas, Vinhas et al. [23]; Alegre et al. [24] who found
 229 that the highest yield of levan production by *Z. mobilis* using Erlenmeyer flasks without
 230 agitation. Also, Han and Clarke [6] who reported that the polysaccharide production
 231 increased when the culture was slightly agitated during fermentation.
 232
 233



262
 263 **Fig. 3. Effect of shaking speed on the production of levan by *Bacillus lentus* V8 strain**
 264 **on modified productive medium supplemented with S [or] BSSCM during 96 h using**
 265 **shake flasks as a batch culture.**

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

268 Values in the same line (followed by letters within an alphabetic series) sharing the same letter do
 269 not differ significantly, according to Duncan's at 5 % level. Values followed by letters in different
 270 alphabetic series are significant.

271 272 **3.4. Inoculum size**

273 This experiment was performed to study the effect of inoculum size on growth and levan
 274 production. The fermentation medium was inoculated with 2.5 to 15 %. Results illustrated by
 275 Fig. 4 show that the inoculum size of 10% achieved the highest growth and levan production
 276 being 2.87 [or] 2.72 gL⁻¹ of cell dry weight, 51.40 [or] 45.34 gL⁻¹ of levan dry weight and
 277 95.20 [or] 71.10 mgL⁻¹ of levan as fructose by *Bacillus lentus* V8 strain on modified
 278 productive medium supplemented with sucrose [or] black strap sugar cane molasses,
 279 respectively. It could be noticed from Fig. 4 that a high positive correlation coefficient (R²)
 280 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.

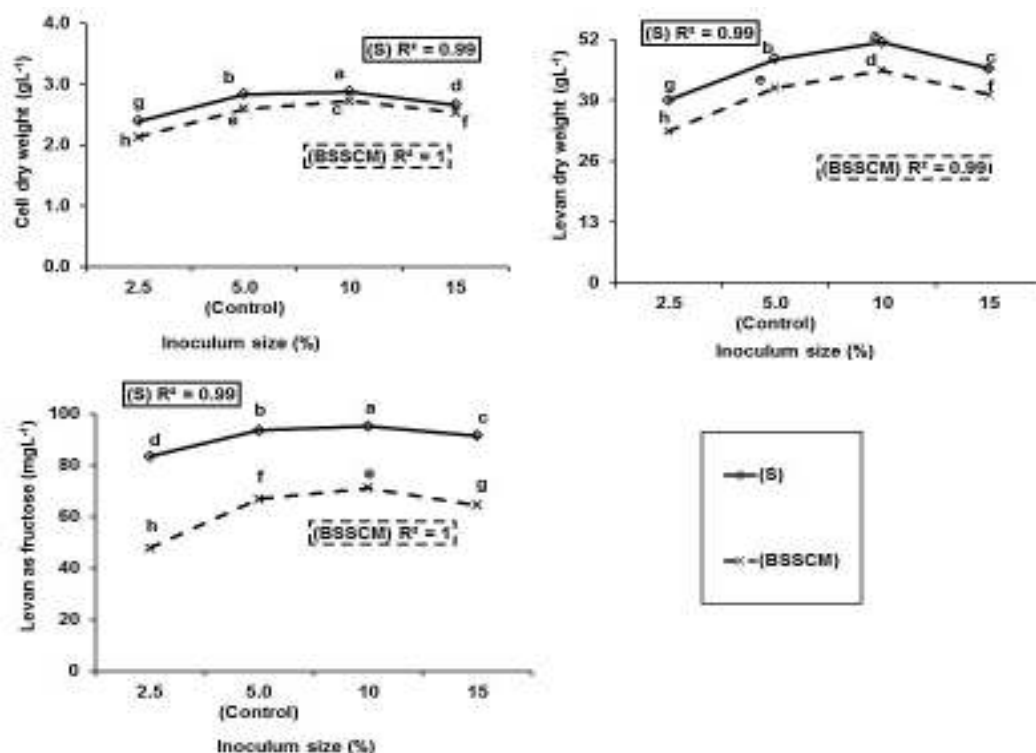


Fig. 4. Effect of inoculum size on the production of levan by *Bacillus lentus* V8 strain on modified productive medium supplemented with S [or] BSSCM during 96 h using shake flasks as a batch culture.

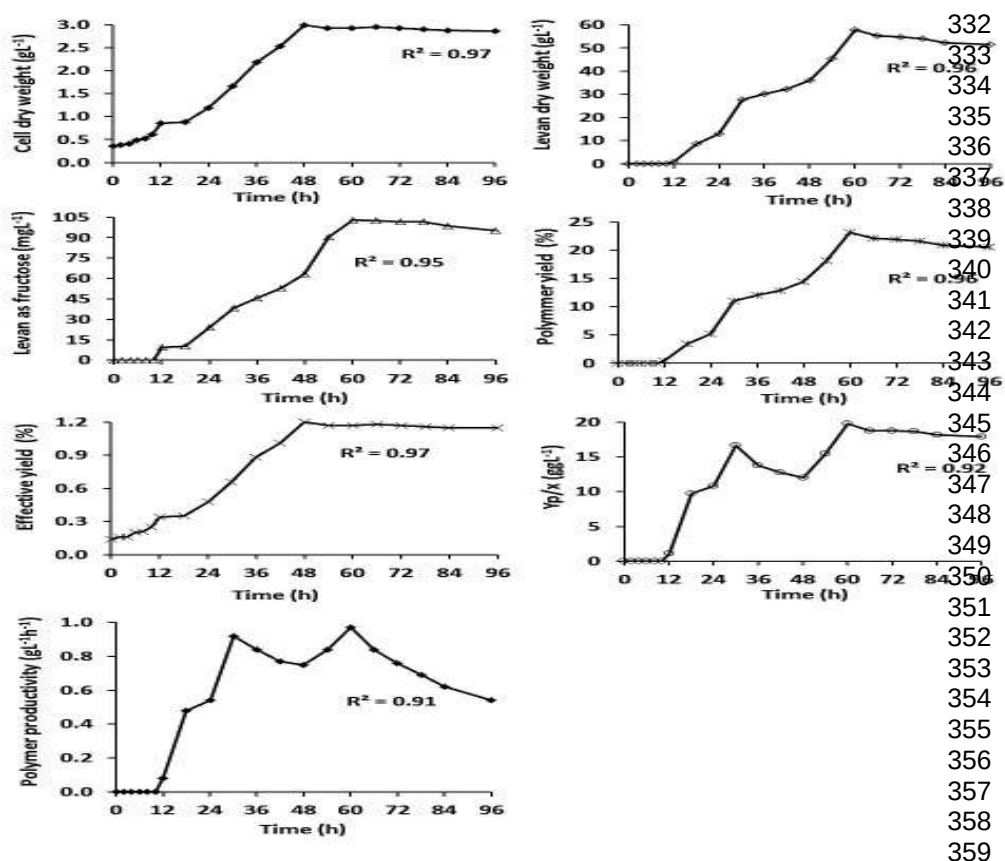
S= Medium supplemented with sucrose; BSCM= Medium supplemented with black strap sugar cane molasses.

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.

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 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 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 with a specific growth rate (μ) of 0.041 h⁻¹ [or] 0.033 h⁻¹. Doubling time (t_d), multiplication rate (MR) and generation number (N) were calculated from the growth curve being 16.91 h [or] 21.00 h, 0.06 [or] 0.005 and 1.77 [or] 1.71 on modified medium

310 supplemented with sucrose [or] black strap sugar cane molasses, respectively. Results in
 311 Figs. 5, 6 show the cell dry weight, levan production, polymer yield (%), effective yield (%),
 312 $Y_{p/x}$ (ggL⁻¹) and productivity (gL⁻¹h⁻¹) for *Bacillus lentus* V8 strain during propagation in
 313 modified productive medium supplemented with 250 gL⁻¹ sucrose [or] black strap sugar cane
 314 molasses (54% sugar) for 96 h at 30°C. Results showed that increasing the period led to
 315 gradual increase in cell dry weight to recording the maximum biomass production being 2.99
 316 [or] 2.89 gL⁻¹ on modified productive medium supplemented with sucrose after 48 h [or]
 317 black strap sugar cane molasses after 66 h, respectively. The highest levan production being
 318 57.95 [or] 49.86 gL⁻¹ of levan dry weight, 103.1 [or] 82.7 mgL⁻¹ of levan as fructose and
 319 23.18 [or] 36.93% of polymer yield on modified productive medium supplemented with
 320 sucrose [or] black strap sugar cane molasses after 60 h, respectively. The highest values of
 321 effective yield (1.18 [or] 1.16%) after 66 h on modified productive medium supplemented
 322 with sucrose [or] black strap sugar cane molasses, respectively. The highest amount of
 323 polymer dry weight productivity (0.97 gL⁻¹h⁻¹) and $Y_{p/x}$ (19.78 ggL⁻¹) were attained after 54 h
 324 of fermentation on modified productive medium supplemented with sucrose, but the highest
 325 values of polymer productivity (0.85 gL⁻¹h⁻¹) and $Y_{p/x}$ (18.07 ggL⁻¹) were achieved after 42 h
 326 of fermentation on modified productive medium supplemented with black strap sugar cane
 327 molasses. Data illustrated by Fig. 5 clearly show a highly positive correlation coefficient
 328 between incubation period and cell dry weight, levan dry weight, levan as fructose, polymer
 329 dry weight yield, effective yield, $Y_{p/x}$ and polymer productivity, R^2 values ranged from 0.91 to
 330 0.97 on modified productive medium supplemented with sucrose.
 331



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

It was also noticed that the highest positive correlation coefficient between fermentation period and cell dry weight ($R^2 = 0.95$), levan dry weight ($R^2 = 0.96$), levan 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).

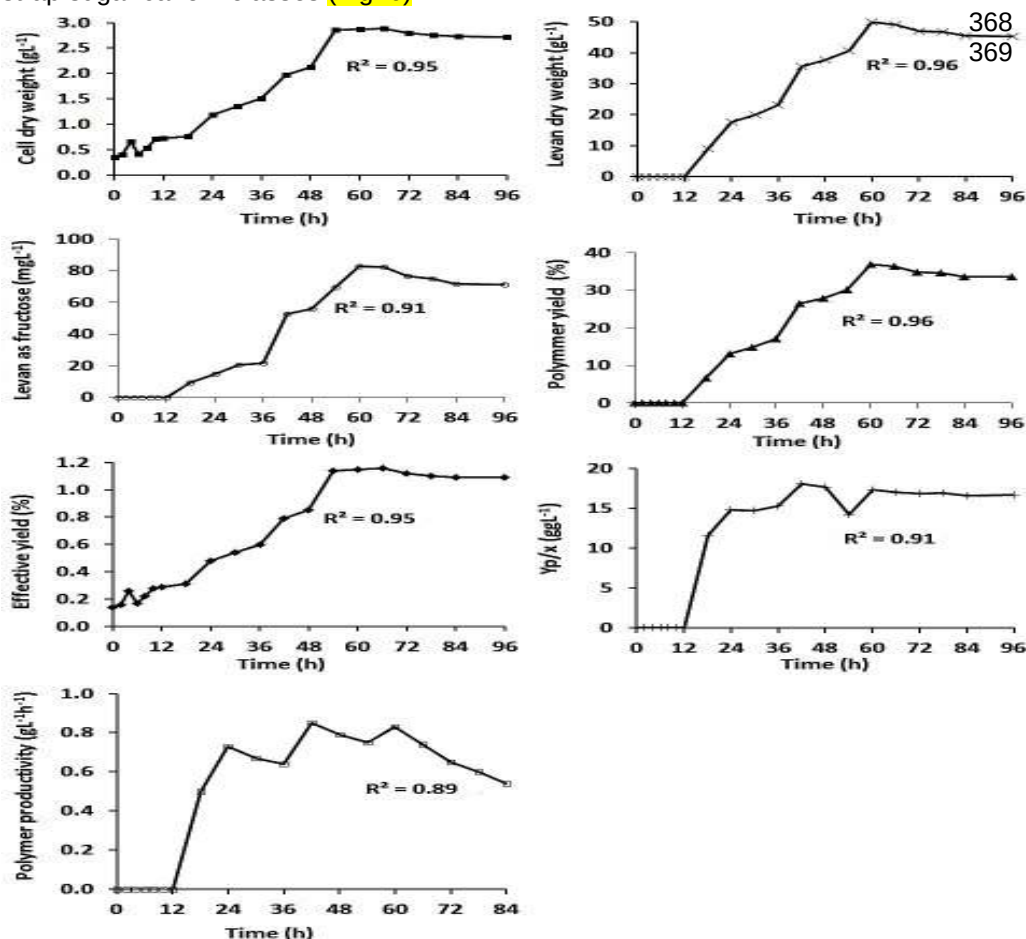


Fig. 6. Biological activity of *Bacillus lentus* V8 strain on modified productive medium supplemented with black strap sugar cane molasses during 96 h incubation period at 30°C using shake flasks as a batch culture.

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

^{13}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 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 [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.

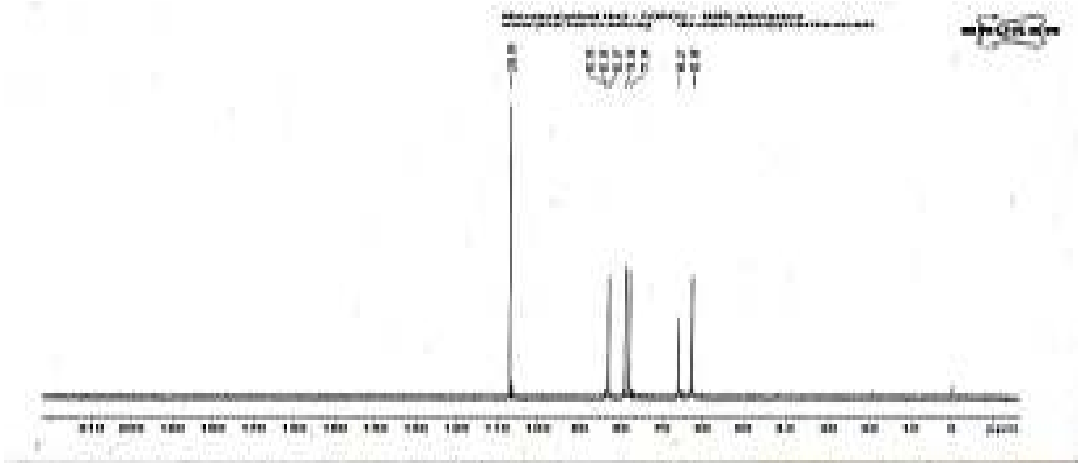


Fig. 7. ¹³C NMR of levan polymer purified produced by *Bacillus lentus* V8 strain.

Table 1. ¹³C 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].

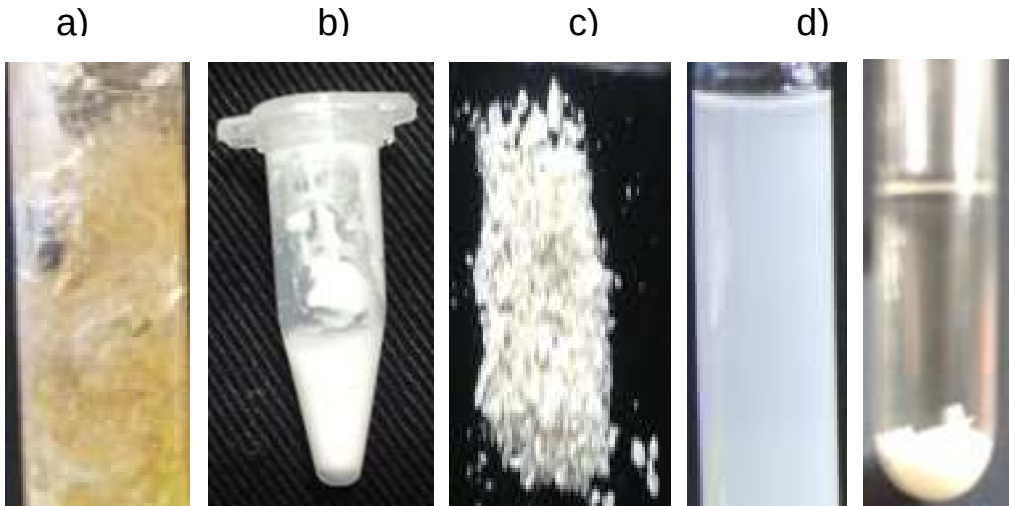


Fig. 8. Levan polymer gum produced by *Bacillus lentus* V8 strain

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 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 and 0.98% ash.

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