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Production of levan by *Bacillus licheniformis* NS032 in sugar beet molasses-based medium

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KEYWORDS: levan; molasses; molecular weight

ABSTRACT

The production of levan by *Bacillus licheniformis* NS032 in a medium based on sugar beet molasses was studied. High polysaccharide yields were produced by using diluted molasses (100-140 g/L of total sugars) with the addition of commercial sucrose up to 200 g/L of total sugars, as well as K_2HPO_4. A levan yield of 53.2 g/L was obtained on a medium optimized by response surface methodology, containing 62.6% of sugar originating from molasses, and 4.66 g/L of phosphate, with initial pH value of 7.2. In comparison to the media with 200 and 400 g /L sucrose, in the molasses optimized medium, the observed bacterial growth was faster, while the maximum

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production of polysaccharide was achieved over a shorter time interval (48 h). The polysaccharide produced in molasses medium had a weight average molecular weight of 5.82 x 10^6 Da, degree of branching 12.68%, viscosity of 0.24 dL/g, and based on methylation analysis and NMR data, it did not significantly differ from levan obtained in the medium with 200 g/L sucrose.

1. Introduction

Levan is considered as one of the most promising microbial exopolysaccharides for various industrial sectors [1]. This β-(2→6) fructose polymer has received considerable research attention due to its antitumor, immunomodulating and prebiotic activities as well as many distinguishing properties such as low viscosity, strong adhesiveness, self-assembling ability, high water and chemical holding capacities, along with high water solubility, non-toxicity and good biocompatibility [2,3,4].

Levan is produced from sucrose by a variety of bacteria through the action of the enzyme levansucrase. Although the levans from the genera *Acetobacter*, *Bacillus, Erwinia, Halomonas*, *Paenibacillus, Pseudomonas, Streptococcus* and *Zymomonas* are the most studied, the production of this polysaccharide is widely distributed in nature. Recently, more than 100 bacterial genera were identified as levansucrase sources [4]. Physicochemical properties important for technological applications as well as biological activities of different levans depend on the length of the polymer chain and the degree of branching [5,6]. It is considered that the differences in molecular weight and size of levan molecule are mostly caused by the microbial source [3]. Therefore, to explore the full potential of this polysaccharide from novel sources and establish precise structure-activity relationships, more detailed examination of structure and functional properties is required.

To reduce the production cost of microbial polysaccharides, the use of sugar-based raw materials as nutritive substances can be advantageous [7-11]. Molasses is a food industry by-product of the final crystallization stage in the process of cane or beet sugar production. Since it is an agricultural product, its composition varies depending on the variety and the quality of sugar beet or cane,

climate and soil conditions, and the technology and processing parameters used in sugar beet/cane processing plants [12]. The non-sucrose fraction of raw materials such as molasses is of particular importance for microbial growth and metabolite production in fermentations. However, molasses can contain substances that are inhibitory for microorganisms, and which are necessarily removed (at least partly) by a pretreatment process [13].

Previously, our research group reported efficient, high levan production by *B. licheniformis* NS032 in media with 200 and 400 g/L sucrose using an inorganic nitrogen source [14]. However, considering its high sucrose content, molasses would seem to be a natural alternative to synthetic media for levan biosynthesis. Molasses has already been used for levan production [15,16,17] by *P. polymyxa*, *Z. mobilis* and *Halomonas sp.* AAD6. The *Z. mobilis* levan yield on sugar cane molasses, clarified by centrifugation (250 g/L of carbohydrate concentration), was significantly lower (2.53 g/L) than on respective sucrose medium (21.7 g/L) [16]. For levan from *Halomonas sp.* AAD6, sugar beet molasses (30 g/L) treated by TCPHAC (tricalcium phosphate, sulfuric acid, and activated carbon pretreatment) was used, and levan yield (12.4 g/L), and particularly levan theoretical yield (79%), was higher than in sucrose medium (1.84 g/L, 7%) [17]. For levan production with *P. polymyxa, a* high yield (38 g/L) comparable to the yield in sucrose medium (36 g/L) was achieved only on sugar beet molasses (150 g/L) subjected to expensive pretreatments such as gel filtration columns [15]. Recently, high levan production (49.86 g/L) by *Bacillus sp.* V8 on untreated cane sugar molasses was reported, comparable with production in sucrose medium (57.95 g/L, 250 g/L sucrose) [18]. Most studies on optimization of levan production using molasses suggest that the high yields of levan are associated with some form of molasses pretreatment, which is the reason to consider this strategy in the following research.

The aims of this study were to investigate the appropriate pretreatment of molasses to make it suitable for levan production by *B. licheniformis* NS032 and to study the effect on levan production when sucrose in the fermentation medium was replaced with sugar beet molasses. Growth kinetics in media with two sucrose levels and molasses were compared, as well as the fine structural characteristics of the obtained levans.

2. Materials and methods

2.1. *Microorganism and sucrose media*

Bacillus licheniformis NS032 was used to biosynthesize levan during fermentation. For low (S200) and high (S400) sucrose systems, fermentations were carried out in basal medium, (BM) (g/L: K_2HPO_4 3, KH_2PO_4 3, NaCl 1, $MgSO_4$ x $7H_2O$ 0.2, $FeSO_4$ x $7H_2O$ 0.001) with 200 and 400 g/L sucrose, 2.4 and 4.6 g/L NH_4Cl and initial pH 7.0 and 7.4, respectively, according to our previously reported procedure [14].

2.2. *Molasses: Origin, chemical analysis and pretreatment for fermentation medium*

The molasses used in this study was obtained from the Senta Sugar Factory, Serbia. Dry weight was determined by drying a molasses sample to a constant weight at 105 °C. Ash content was determined by igniting the oven-dried sample in a muffle furnace at 550 °C. Contents of C, N and S were determined using a Vario EL II CHNS/O Elemental Analyser. Minerals were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using iCAP 6500 Duo after HNO_3 digestion. For chloride determination, the ion selective electrode, Orion 9617BNWP, was used. Betaine determination was performed using the method reported by Focht [19]. Sucrose was determined using the Sucrose/D-Fructose/D-Glucose Assay Kit (Megazyme).

A pretreatment procedure was sought that would allow the direct use of molasses, diluted to a total sucrose content of 200 g/L, as the fermentation medium. Molasses (400 g) was mixed with water (500 mL), and afterwards, the solution was acidified to pH 3.5 with H_2SO_4 (4 mol/L) and left overnight. The formed sediment was separated by filtration, and the resulting solution was neutralized with KOH to pH 7. The obtained solution was treated with activated carbon (3%, w/V), applying heating at 60 °C for 1 h while stirring. The mixture was filtered through a Whatman No1 filter and diluted to a total sucrose content of 200 g/L in order to obtain the pretreated molasses.

To find the optimal concentration of pretreated molasses for levan production, a dilution series was prepared with total sugar concentrations in the 100-200 g/L range, with and without added commercial sucrose, K_2HPO_4 (3 g/L) and yeast extract (1.6 g/L).

2.3 *Fermentations*

Fermentations were conducted in 500 mL flasks with 200 mL of medium in an incubator at 37 °C without agitation for 5 days. Flasks were inoculated with 2% suspension prepared from 24 h nutrient agar slant cultures of *B. licheniformis* NS032 in physiological saline. Aliquots of approximately 10 mL were withdrawn in sterile conditions from the fermenting cultures every 12 h.

2.4. *Optimization of molasses medium*

Evaluation of the effect of molasses supplementation in the medium on levan production was made using response surface methodology (RSM) on the basis of Box-Behnen design with 17 runs and 5 replicates. The full experimental design is shown in Supplementary data, Table S1. The influence of three independent variables: molasses percentage (percentage of sucrose orginating from molasses in a total sugar concentration of 200 g/L, %MS200) (50-75%), pH (6-7.5) and K_2HPO_4 (3-9 g/L) concentration were investigated. The formulations and regression analysis were performed using Design Expert software, version 7.0. (Stat-Easy Inc., Minneapolis, USA).

2.5. *Growth, levan and glucose determination*

The growth of *B. licheniformis* NS032 was determined by measuring dry cell weight (DCW). Fermentation broth (8 mL) was centrifuged at 5000 g for 10 minutes. The pellet obtained was washed with 4 mL of distilled water to remove adsorbed polysaccharide and dried to a constant weigh at 105 °C. Supernatant obtained after biomass removal was used for determination of levan and glucose produced during fermentation. Two volumes of ice cold ethanol were added to the supernatant (2 mL), and the mixture was allowed to stand at 4 °C overnight to precipitate polysaccharide. The levan content was determined using the Fructan Megazyme Kit after hydrolysis of crude polysaccharide with 0.1% oxalic acid. Supernatant (0.25 mL) was clarified by Carrez reagent and used for glucose determination using the D-Glucose Assay Kit (GOPOD Format, Megazyme).

2.6. Levansucrase activity measurements and cellular localization

Soluble levansucrase (LS) in the fermentation medium and cell associated LS were separated by centrifugation at 5000 g for 10 minutes. Cells were washed twice with 50 mM potassium phosphate buffer, pH 6.0, and resuspended in the initial volume of the same buffer.

LS activity was assayed in a reaction mixture containing 0.8 M sucrose in 50 mM potassium phosphate buffer, pH 6.0, followed by incubation at 40 °C for 2 hours. The content of reducing sugars (glucose and fructose) released was determined by DNS assay. One unit of enzyme activity is expressed as the µmol of reducing sugars liberated from sucrose per min of reaction.

2.7. *Levan isolation and purification*

After removal of biomass by centrifugation, levan was obtained from supernatant by two successive precipitations using two volumes of ice cold ethanol. Precipitate was dissolved and dialyzed (molecular weight cut-off 14000, Sigma D9402) against running water for at least three days to completely remove low molecular weight impurities.

2.8. *Optical activity*

Optical rotation values were determined using a Rudolph Research Analytical IV polarimeter at 25 °C (C=0.1 g/100 mL, H_2O).

2.9. *Nuclear magnetic resonance (NMR) spectroscopy*

All NMR spectra were measured on a Bruker AVANCE III 500 spectrometer (500.26 MHz for ^{1}H and 125.80 for ^{13}C nuclei), using a 5 mm broadband probehead. Spectra were obtained at 298 K in D_2O (15 mg/mL), with DSS as an internal reference standard (0 ppm) for both the ^{1}H and ^{13}C spectra. Homo- and heteronuclear two dimensional (2D) spectra H-H COSY (correlation spectroscopy), H-C HSQC (Heteronuclear Single-Quantum Coherence) and H-C HMBC (Heteronuclear Multiple Bond Correlation) applying standard Brucker's pulse sequences were used for full assignment of the signals. Levan from *Erwinia herbicola* (Sigma L8647) was used for comparison.

2.10. *Methylation analysis*

The degree of branching of levans produced was determined by per-O-methylation and subsequent reductive cleavage and acetylation. O-per-methylation was performed by the Ciucano method [20]. The reaction was conducted at room temperature in 20 mL vials with silicone septa-lined screw caps in an inert gas atmosphere. Levan (60 mg), dried overnight at 60 °C, was dissolved in 6 mL dimethyl sulfoxide by mixing with a magnetic stirrer for 1 h, and afterwards,

powdered dry NaOH (0.6 g) and CH_3I (0.8 mL) were added to the mixture, which was stirred overnight. The next day, addition of NaOH (0.4 g) and CH_3I (0.6 mL) was repeated and the reaction was continued overnight. The methylation reaction was quenched with water, the sample was dialyzed against distilled water and extracted twice with $CHCl_3$. Extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness. IR spectroscopy was used to check the completeness of methylation.

Methylated levan was subjected to reductive cleavage as described previously [21,22]. Briefly, methylated levan (1 mg) was added to reducing agent (550 µL comprising 170.5 µL BF_3 etherate, 220 µL triethylsilane, 35.2 µL CF_3COOH and 124.3 µL CH_2Cl_2) and allowed to react for 24 h at 0 °C. Acetic anhydride (50 µL) was added and allowed to react for 1 h at room temperature. The solutions were extracted with CH_2Cl_2 three times, combined extracts were dried with anhydrous Na_2SO_4, evaporated to dryness and analyzed by gas chromatography/mass spectrometry. GC/MS analysis was conducted on a GCxGC/MS (Shimadzu, Japan) with the thermal modulator turned off. Separation was performed on an Rtx® column (Restek, 30 m x 0.25 mm i.d., 0.25 µm) with helium as the carrier gas. The column temperature was programmed linearly in a range 40–310 °C at a rate of 10 °C/min with an initial 1 min and final 7 min hold. Peak identification was performed by interpretation of fragmentation patterns of the EI mass spectra according to literature data, and quantitative evaluation was carried out by integration of peak areas [23,24].

2.11. *Viscosity*

Levan was dissolved in distilled water to concentrations of 0.6-1.0 g/dL. The intrinsic viscosities, [η], of levan samples were calculated based on flow time, t, at 25 ± 0.1 °C, using an Ubbelohde viscometer (l = 86.05 mm, R = 1 mm). The mean value of three measurements of t was taken to calculate the relative viscosity, $\eta_r = t/ t_o$, where t_o is the time flow of the solvent. Based on the relative viscosity, the specific viscosity (η_{sp}) was calculated as: $\eta_{sp} = \eta_r - 1$. Reduced viscosity is calculated by the formula (1):

$$\eta_{red} = \eta_{sp}/c \qquad (1)$$

where c is the concentration, in g/dL.

Extrapolation of the straight-line of η_{red} in function of solution concentrations, to zero concentration, gave [η].

2.12. *Determination of molecular weight*

The SEC system setup (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) with a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz) and MALLS (SLD 7000 PSS, Mainz) was used as described previously in detail [25]. The samples (2 mg/mL) were injected with a flow rate of 0.5 mL/min into a Suprema precolumn and three Suprema SEC columns 100, 3000 and 3000 (PSS, Mainz). The columns and the detectors were held at 50 °C. The mobile phase was 0.05 M $NaNO_3$ containing 0.02% NaN_3. Calibration was performed using a standard pullulan kit (PSS, Mainz, Germany) with molecular weights from 342 to 805 000 Da.

3. Results and discussion

3.1. Sugar beet molasses as a substrate for levan production by B. licheniformis NS032

3.1.1. Composition and pretreatment

The following composition was determined for the molasses used in this study: 77.8% dry weight, 38.64% C, 2.02% N, 5.93% H, 0.56% S, 6.3 % ash, 49.4 % sucrose, 5.9 % betaine, raffinose 0.55%, 1.25% chloride, 1.98% potassium, 1.12% sodium, 0.44% calcium, 70 ppm iron, 51 ppm magnesium, 4.2 ppm zinc, 2.5 ppm manganese, 1.9 ppm nickel, 0.71 ppm copper and < 0.05% phosphate.

To remove undesired compounds from molasses medium with 200 g/L total sucrose concentration, in the current study, a pretreatment procedure with H_2SO_4 and activated carbon was initially utilized. However, this procedure alone did not efficiently reduce the inhibition of levan synthesis (Fig. 1, S200, MS200). Further dilution of molasses solution to a concentration of 100 g/L sucrose plus the commercial sucrose (Fig. 1, MS100, S100) resulted in increased levan synthesis, which nevertheless, was still below levan production in the control. Addition of phosphate resulted in enhanced levan synthesis with a productivity (0.25-0.27 g levan/ g sucrose) similar to that obtained in S200 medium (Fig. 1, MS100, S100, P). Addition of yeast extract did not have significant impact

on levan productivity. Increasing the proportion of molasses in the medium to a sucrose in molasses:commercial sucrose ratio of 160:40 or greater inhibited levan synthesis, independently of the addition of phosphate. Our previous study has shown that for *B. licheniformis* NS032, the maximum productivity (0.26-0.29 g levan/g available sucrose) was achieved in BM with 100-200 g/L sucrose [14].

Although according to reports, H_2SO_4 treatment was expected to efficiently remove heavy metals from molasses [17,26], analysis showed that contents of the metals tested (Fe, Ni, Cu and Zn) were not reduced in our pretreated molasses when compared to non-treated molasses (data not shown). However, the calcium concentration was reduced by 35%, contributing, along with the reduced colour caused by the activated carbon pretreatment, to easier isolation of levan. A similar finding was reported by Lee [10].

Molasses contains nitrogenous compounds and is expected to satisfy bacterial nitrogen demand. However, comparison of the chemical composition of our S200 medium and pretreated molasses showed the C/N ratio of molasses is too low (19 in molasses and 134 in S200, i.e. 7 times lower). At the same time, concentrations of Fe, Na and chlorides in our molasses medium diluted to a concentration of 200 g/L sucrose concentration were too high. It is evident from results presented in Fig. 1 that the highest levan yields could be achieved by a combination of two approaches. Addition of commercial sucrose would contribute to increasing the C/N ratio, and dilution of molasses would reduce the inhibitory effect of high concentrations of mineral compounds. The stimulation of polysaccharide production under nitrogen-limiting conditions is well established [11,27].

The phosphate concentration in molasses is low and presumably insufficient to support bacterial growth, so therefore, as shown in Fig. 1, the addition of phosphate had a positive effect on levan production. The stimulative effect of phosphate in molasses-containing medium on polysaccharide production was previously reported [7,9]. For instance, Kalogiannis [7] found that phosphate has two different roles in xanthan production: it acted as a nutrient and as a buffering agent to reduce pH fluctuations.

3.1.2. *Maximizing levan yield using RSM*

In the present study, RSM was applied to estimate the best molasses:sucrose ratio to maximize levan production and find possible interactions with phosphate concentration and initial medium pH. Molasses percentage, %MS200 (50-75%) was selected on the basis of results presented in Fig. 1. Control of pH value has proved to be one of the most important factors in optimizing the fermentation medium, not only for *B. licheniformis* NS032, but also for other levan producers [14,27,28,29].

Measured levan concentrations were in the range 6.53 to 52.85 g/L (Supplementary data, Table S1).

On the basis of the obtained data, a quadratic model was constructed. The model was significant (F value 26.98, with $p < 0.0001$), with only a 0.01% chance that the effects of the studied variables could not be explained by this model. Analysis of variance (ANOVA) for this model and the second order polynomial equation explaining the model are presented in Supplementary data, Table S2. Although according to ANOVA the terms X_2, X_1X_3 and X_2^2 were found to be non-significant, we included them in the main equation in order to present the entire picture of the system.

Contour plots showing interactions between the two variables when the third is fixed are presented in Fig. 2. The highest predicted levan content was obtained when molasses contributed from 53 to 59 % of sucrose in the medium and phosphate content was in the 6-8 g/L range (Fig. 2a). The initial pH of the medium exhibited a more powerful effect than molasses percentage. Levan concentrations higher than 45 g/L were anticipated with pH values higher than 6.5 and %MS200 lower than 68.75 % (Fig. 2b). Irrespective of phosphate concentration, the maximum levan yield was predicted when the initial pH of the medium was higher than 6.6 (Fig. 2c).

The numerical optimization function using the Design expert program predicts *B. licheniformis* NS032 could produce levan concentrations of 53.6 g/L in the following conditions: 62.6 % molasses in total sucrose of 200 g/L; 4.66 g/L phosphate; initial pH 7.2. This optimized medium, termed M200, was used for further work to study the relationships between *B. licheniformis* growth and levan production.

3.1.3. *Growth curves and change of pH during levan production*

Growth curves of *B. licheniformis* and changes in concentration of levan and glucose in media with different initial sucrose and molasses concentrations are shown in Fig. 3. In medium with an initial

200 g/L sucrose, maximum biomass was achieved at 48 h (1.18 g/L) with maximum biomass growth rate of 0.029 g DCW/Lh. After that time, a slow decline of biomass was measured. The maximum levan concentration was reached at 72 h (51.62 g/L), and this levan concentration remained relatively constant until the end of the experiment (120 h). The highest rate of glucose production was 1.98 g glucose/Lh at 48 h, and the glucose concentration in the fermentation broth decreased after 96 h. The yield of levan in terms of biomass, $Y_{P/X}$, was 48.7 g/g.

In medium with an initial 400 g/L of sucrose (Fig. 3b), the highest biomass growth rate was achieved at 48 h (0.043 g DCW/Lh), and the biomass continued to increase slowly until 96 h (2.78 g/L). Thereafter, an abrupt decrease occurred. Maximum production of levan was reached at 96 h (101.2 g/L, $Y_{P/X}$=36.4 g/g), while the highest rate of levan production was 1.7 g/Lh at 84 h. The highest glucose production rate was measured at 36 h (2.2 g glucose/Lh). The glucose production rate was exponential until 48 h and retained the values of approximately 70 g/L until 96 h, when an increase to values of about 110 g/L occurred. This was probably linked with the decrease in bacterial growth.

In M200 optimized medium with molasses (Fig. 3c), the fastest biomass growth rate was 0.095 g DCW/Lh at 48 h, maximum biomass of 3.7 g/L occurred at 72 h, and maximum levan production (53.2 g/L, $Y_{P/X}$=17.55 g/g) was measured at 48 h. After 24 h fermentation, the rate of levan production, 2.07 g levan/Lh was parallel with the highest glucose production, 2.74 g glucose/Lh. The concentration of glucose rapidly decreased after 48 h, and biomass slowly decreased after 72 h, while the levan concentration was maintained at approximately 50 g/L after 48 h.

Consumption of sucrose in S200 medium after 72 and 120 h was 82.4% and 94.6% respectively, while in S400 it was approximately 85% and 88.1% after 96 h or 120 h, i.e., the end of the observed time. In M200 molasses medium, only 18% of the sucrose was left after 48 h, and after 72 h the entire amount of sucrose had been consumed. Ratios of $levan_{max}/sucrose_{consumed}$ were 0.31, 0.29 and 0.33 for S200, S400 and M200, respectively.

The changes in pH during fermentation in M200 and sucrose media were also compared (Fig. 3d). In both sucrose media, a rapid drop of pH occurred during the first 24 h, and afterwards, the pH remained in the range of 5.2-5.4 (S200) or 4.5-4.8 (S400) until the end of fermentation. On the contrary, in M200, the pH was slightly reduced and was maintained from 6.1 to 6.9 throughout the

fermentation. The overall appearance of the pH curves is in accordance with reported data [27,30,31].

From Fig. 3, it is evident that, under the conditions described, levan was synthesized during all growth phases of *B. licheniformis* NS032, and the highest rate of levan production coincided with the highest bacterial growth rate. Of particular importance is that the level of levan did not decrease significantly by the end of the observed period, 120 h of fermentation. However, in M200 optimized medium, a slight decrease of levan content was measured after 96 h. Since the pH of the medium was still greater than 6 at this time (Fig. 3d), this levan reduction is probably connected with bacterial cell lysis.

Comparing Figs 3a and b, the higher sucrose concentration led to increased bacterial growth rate, and consequently, increased biomass. Although levan production was enhanced, the efficiency of sucrose conversion in S400 medium was lower than in S200 medium. Similar findings were previously reported [28]. However, Wu *et al*. [28] found the maximum levan production rate in medium with 250 g/L sucrose and in 24 h fermentation time. In the present study, the time required to reach the maximum concentration of levan was extended in S400 (96 h) compared with S200 (72 h). In addition, in our M200 optimized molasses medium, the excess of nitrogen stimulated rapid growth of *B. licheniformis* NS032 biomass and rapid secretion of levan to maximum levels, which indicates the possibility of shortening the length of fermentation (Fig 3c). The stimulative effect of molasses on microbial cell growth as a result of high nitrogen content has already been reported [8].

In order to further clarify the effect of sucrose on levan production, the activity of LS and its cellular localization during fermentation was determined. The levan producing activity was detected both in cells and in fermentation broth. LS activity for soluble and cell associated fractions were 0.29 and 0.34 U/mL for S200 medium, 0.31 and 0.33 U/ml for M200 medium and 0.51 and 0.21 U/ml for S400 medium. It is evident that LS activity of the soluble fraction in the S400 medium was almost twice as high as in the media with 200 g/L sucrose. In addition, the distribution between the soluble and the cell associated fractions was different, since in M200 and S200 media, the cell associated activities were 52-54% of total LS activity, while in S400 medium, this fraction accounted for only 29% of total LS activity.

3.2. *Structural characterization of levan samples*

The polysaccharide produced by *B. licheniformis* NS032 in M200 molasses medium under optimized conditions, as shown in Fig. 3c, was free of protein and nucleic acids according to elemental analysis. The high negative optical rotation and absence of sugars other than fructose upon hydrolysis indicated the similarity of this polysaccharide (labeled as LM) to levan samples LS1 and LS2 produced from S200 and S400 media, respectively. The levans were further characterized by methylation analysis, molecular weight determination, NMR spectroscopy, viscosity and optical activity measurement. The results are summarized in Table 1, and details are discussed below.

Two levans (LM and LS1) had similar molecular weights and viscosities, while levan LS2 differed in these characteristics (Table 1). As for the other characteristics examined, there were no easily distinguishable differences between all three levans.

3.2.1. *Molecular weight*

Both levans LM and LS1, produced in media with 200 g/L total sucrose, had relatively high molecular weights (range > 10^6 Da), while levan LS2, from medium with 400 g sucrose/L, had a lower molecular weight (in the range of 10^5 Da) (Table 1). The average degree of polymerization calculated using M_w and the molecular weight of β-(2→6)-linked fructose (162.16) was in the 3.5-4.5 x 10^4 range for levans LM and LS1 and 3.2 x 10^3 for levan LS2.

Levans from different microorganisms have molecular weights in the range 10^4 to 10^{10} Da, while levan from *Kozakia baliensis* stands out due to its exceptionally high molecular weight of 2.466 x 10^9 Da [5]. Levans from the most-studied Gram positive bacterial genera, *Bacillus* and *Paenibacillus,* have molecular weights in the 10^5 to 10^7 Da range (32,33], while for levan from *B. licheniformis* 8-37-0-1, a molecular weight of 2.826 x10^4 Da was reported [34].

It is well known that fermentation conditions can affect the molecular weight of levan. For example, high molecular weight levan was produced in the presence of 40% ethanol, 20% polyethylene glycol, acetonitrile or 0.08 M phosphate, while low molecular weight levan was produced in medium containing 0.8 M phosphate [35]. Recently, it was reported that pH of fermentation medium affected the molecular weight of *Halomonas* levan [29].

The difference in molecular weights between our levans shown in Table 1 indicates the possibility of controlling the molecular weight by the concentration of sucrose in medium. Although the presence of two enzymes with different substrate affinities cannot be excluded, a possible explanation for the molecular weight differences would be the presence of a bimodular molecular weight distribution in levan produced by *Bacillus licheniformis* NS032. The phenomenon of simultaneous production of levans of high and low molecular weight was observed for some microorganisms such as *B. subtilis* natto (2.3×10^6 and 7.2×10^3 Da [28,30] and *B. licheniformis* (6.12×10^5 and 1.1×10^4 Da) [36]. Two elongation mechanisms for levan synthesis, processive and non-processive, were proposed to explain such dual molecular weight distribution. It was shown that the modulation of levan molecular weight distribution in *B. subtilis* can be achieved by change in enzyme concentration independently of sucrose concentration [37].

The assumption of dual molecular weight distribution in *B. licheniformis* NS032 can be supported by some observations. Firstly, as mentioned in section 3.1.3, the soluble LS activity was found to be higher in medium with 400 g/L of sucrose than in S200 and M200 media. Secondly, the presence of a small amount of high molecular weight levan in the LS2 levan could have contributed to its higher PDI in relation to LS1 and LM levans. Finally, the absence of low molecular weight fractions in levans LS1 and LM could be explained by the isolation procedure that involved a dialysis step, resulting in removal of compounds with molecular weight lower than 1.4×10^4.

3.2.2. *Viscosity*

The intrinsic viscosities for the levans produced in the current study ranged from 0.14 to 0.25 dL/g. The low intrinsic viscosity of this polysaccharide in comparison with other high molecular weight microbial polysaccharides indicates its compact and spherical shape in solution, making it of significant importance for potential use in food and pharmaceutical industries [3,5]. The following data for levans originating from different sources are reported: 0.14 dL/g *Bacillus sp.,* 0.22-0.33 dL/g *Z. mobilis*, 0.17 dL/g *Pseudomonas*, 0.13-0.16 dL/g *S. salivarius* and 0.38 dL/g *Microbacterium laevaniformans* [38].

3.2.3. *Branching*

In order to determine the extent of branching, per-O-methylation followed by reductive cleavage, acetylation and GC/MS analysis were performed. Fig. 4 shows GC/MS chromatograms of the products obtained. The details of peaks identifications are presented in Supplementary data, Table S3.

Levan contains three types of D-fructofuranosyl residues each giving rise to two products (anhydroalditols). (2→6) linked residues yielded peaks with the largest area: 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-mannitol and 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol. Peaks with the lowest retention times were 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol and 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol, corresponding to terminal (nonreducing) groups, while 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol and 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol are products of branched residues.

Apart from that, in levan LS2, 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol was detected, indicating the presence of terminal α–D-glucose [39]. According to levan's biosynthesis mechanism, each levan molecule contains a sucrose residue with D-glucose as the non-reducing end-group. The molecular weight of levan LS2, estimated on the basis of its 0.87 % glucose content, was 2 x10^4 Da. It can be assumed that glucose could not be detected in levans LM and LS1 due to their high molecular weights [40].

There was no significant difference in the extent of branching between levans LM, LS1 and LS2. The degree of branching presented in Table 1 was estimated on the basis of the percentage of 3,4-di-*O*-methyl derivatives. In general, for high molecular weight polysaccharides, the percentage of branch points should be equal to that of the fructose non-reducing end-groups [41,42].

The extents of branching measured for our levans are in agreement with those reported for microbial levans by various authors: 14.28 % for *B. licheniformis* 8-37-0-1 [34], 12% for *P. polymyxa* NRRL B-18475 [32] and 10.5 % for *B. subtilis* [42]. Although a wide range of branching degree of different levans (5-22%) was reported by Lindberg [41], most data suggest narrower ranges of 9-15%, indicating some similarity in branching of bacterial levan molecules [42]. However, some microbial levans are listed as linear with low degrees of branching of < 5%, according to NMR, e.g. levan from *A. xylinum* [43], acetic acid bacteria [5] and *Lactobacillus reuteri* 121 [44].

3.2.4. NMR spectroscopy

NMR spectroscopy was also used to confirm the linkages observed by GC/MS and to try to detect any structural difference between the levans. In ^{13}C spectra of all levans (Supplementary data, Fig. S1), six main peaks are visible (106.91, 83.00, 79.00, 77.91, 66.09, 62.62 ppm) with positions identical to those reported for levans from *B. licheniformis* 8-37-0-1 [34], *A. xylinum* NCI 1005 [43] and acetic acid bacteria [5]. As reported previously, the chemical shift of anomeric carbons (106.91 ppm) indicates the presence of β-D-fructofuranosyl residues, and the presence of a downfield-shifted signal at 66.09 ppm (C6) is indicative of levan-type fructan in contrast to inulin-type fructan [45].

In the ^{1}H NMR spectra of levans LM and LS1, the signals occurred only in the 3.5-4.25 ppm region, while in levan LS2, an additional signal (5.41 ppm) in the anomeric proton region was found (Supplementary data, Fig. S2). As previously mentioned, due to the biosynthesis mode, each levan molecule contains a glucose residue, and therefore, the absence of H1 signal from α–D-Glc indicates the high molecular weight of the polymer.

Full assignment of chemical shifts in NMR spectra shown in Table 2 was completed by 2D spectroscopy (H-H COSY, H-C HSQC, H-C HMBC, Supplementary data, Figs 3-4). NMR spectra of the levans showed the presence of at least three sets of signals corresponding to β-fructofuranosyl units: A - main chain (-2→6-); B - Fru*f* from main chain with branching in position 1 (-1, 2→6-); C - terminal non-reducing Fru*f* from main/branching chain (2-).

3.2.4.1. *2D spectra of levans LM and LS1*

The NMR spectra of levans LM and LS1 were identical, and therefore, detailed explanations are given only for levan LM.

The COSY spectrum of levan LM showed cross peaks between H3/H4 (4.18/4.08), H4/H5 (4.08/3.94), H5/H6 (3.94/3.88), H5/H6' (3.94/3.54), H6/H6' (3.88/3.54) and H1/H1' (3.76/3.66) (Supplementary data, Fig. S3a). The difference in chemical shifts between 6 and 6' protons in fructan-type polysaccharides such as levan and inulin is very large, which might be explained by the shielding effect of the electron cloud of oxygen [40]. The shielding effect also causes an overlapping of H5 and H6 signals [5,43].

Providing direct C-H correlation the HSQC spectrum enables assignment of protonated carbon atoms and some of the severely overlapped H-signals which could not be recognized otherwise (Fig. 5a; Supplementary data, Fig. S3b), especially for fragments B and C (Table 2). The absence of cross peaks between C2 and any other H confirmed its quaternary anomeric carbon character [40]. The presence of two cross peaks H5/C5 (3.94/83.00 and 3.85/83.73), indicating the two types of chains associated with β-(2→6) and β-(1→2) linkages, is specific for levan and could be used for monitoring the hydrolysis of this polysaccharide (Fig. 5b) [46].

Since it shows cross peaks between H and C at two or three bonds distance, the HMBC spectrum is of special significance for polysaccharides, enabling identification of the atoms having glycosidic bonds. In the anomeric region of the HMBC spectrum of levan LM, the C2/H6 cross peak at 106.91/3.88 ppm confirmed the β-(2→6) linkage between two D-fructofuranosyl units supporting the main chain structure. Despite the strong H1, H1'/C2 cross peaks within the same D-Fru*f*-units (over two bonds) and the large overlapping, the cross peak at 3.74, 3.66/106.33 ppm (fragment B) could be evidence of (2→1) linkages between neighboring D-Fru*f* residues at the branching sites (-1,2-, 2-). Correlations C2/H3 and C2/H5 within the same D-Fru*f* units in particular fragments were also observed. (Fig. 5c). The cross peak corresponding to β-(2→6) linkages *i.e*. between C2 of one D-Fru*f* unit and H6 of the next one in the main chain (A) may not be present in the HMBC spectrum of some levans [47], although the presence of this cross peak was reported for levan from *P. bovis* sp nov BD3526 [48].

3.2.4.2. *NMR spectra of LS2 levan*

Full 2D NMR spectra of LS2 levan are shown in Supplementary data Fig. S4 and partial spectra in Fig. 6. a-c. Only the differences from levans LM and LS1 are discussed.

The presence of α-D-Glc was indicated by the anomeric signals (5.41 and 95.23 ppm, for H and C, respectively; fragment D, Table 2) in 1D spectra of LS2 (Supplementary data, Figs S1c, S2c). The 2D cross peaks provided the further evidence (Fig. 6a-c; Supplementary data, Fig. S4). In the COSY spectrum, the cross peak at 5.41/3.54 ppm corresponds to H1 and H2, while the cross peak at 5.41/95.23, in the HSQC spectrum, connects H1 with C1 of glucose. The cross peak at 5.41/106.0, corresponding to H1 of glucose and C2 of the neighboring fructose, was present in the HMBC spectrum. The positions of these cross peaks are in accordance with previously reported

data for sucrose [40], 6-kestose [49] and low molecular weight fructans [50,51]. The molecular weight of LS2 levan, calculated on the basis of the peak areas corresponding to anomeric H from glucose and H6' from fructose, was 8.89 x 10^3 Da.

3.2.4.3. *Evidence of branching based on NMR spectra*

^{13}C NMR has often been used to distinguish linear and branched levans from plants or bacteria [45,52]. Intense and sharp signals in linear levan and weak C3 signals downfield from the major C3 signal, or the presence of minor peaks upfield of C6 in branched molecule are referred to as the key features of the spectra [53,54]. Furthermore, no branching was observed in the ^{13}C NMR spectra of levans from acetic acid bacteria or levan from *P. bovis* BD352 [5,48].

Fig. 7 shows positions of the minor peaks upfield of the anomeric C2 atom in ^{13}C spectra of the levans in the current study, along with levan from *E. herbicola*. It is evident that in all levans, three anomeric signals appeared: the main peak at 106.91 and two minor peaks at 106.33 and 106.21 ppm. Additional peaks of lower intensity were present: 106.81, 106.5 in all levans, while some peaks were visible only in certain levans (105.97 only in LS2 levan, 106.66 ppm in LM and *E. herbicola* levan, 106.77 and 106.17 ppm in levans LM and LS2). Such distribution of minor peaks indicates there was no significant difference in branching extent and pattern between the investigated levan polysaccharides. These data are in accordance with the similarity in branching degree we obtained by methylation analysis.

4. Conclusion

The composition of the fermentation medium based on sugar beet molasses was optimized for the production of levan by *B. licheniformis* NS032. A levan yield of 53.2 g /L was obtained on a medium with 62.6% of sucrose originating from molasses in a total sugar concentration of 200 g/L, 4.66% of phosphate and with an initial pH 7.2. For pretreatment of molasses, the sulfuric acid and activated carbon procedures were used, which, by lowering the calcium concentration in the fermentation medium, contributed to easier isolation of polysaccharide by ethanol precipitation. To the best of our knowledge, this is the highest yield of levan on molasses-based medium reported so far. The bacterial growth and levan production in molasses optimized medium and media with two levels of sucrose (200 and 400 g/L) were compared. Higher growth rate was achieved in the

molasses medium, while the maximum production of levan occurred as early as within 48 hours, allowing a shorter fermentation time. Apart from that, levan obtained from molasses medium was structurally compared to levans produced in media with 200 and 400 g/L of sucrose. The polysaccharide obtained in the molasses medium had an weight average molecular weight (M_w) of 5.82 x 10^6 Da, degree of branching 12.68% and intrinsic viscosity of 0.24 dL/g. Based on the molecular weight, viscosity and NMR data, no significant difference was found between levans produced in media with total sugar content of 200 g/L independently of sucrose origin. Considering the high levan yields obtained in media with different concentrations and origins of sucrose, *B. licheniformis* NS032 could be a promising candidate for scaling up the levan production process. Future work should aim at using levans of different molecular weights and their modified derivatives with extended functional properties in high value applications. Apart from that, further studies are needed to characterize LS from *B. licheniformis* NS032 and to compare its molecular, biochemical and kinetic characteristics with other LS.

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Figure captions

Fig. 1. The effect of the ratio of molasses to total sucrose, phosphate and yeast extract on levan production by *B. licheniformis* NS032.
(S200 – control, basal medium containing 200 g/L sucrose; see 3.1. for details;
MS200, MS100, MS120, MS140, MS160, MS180 – molasses diluted to the final sugar concentration indicated as numbers and expressed in g/L;
S100, S80, S60, S40, S20 – commercial sucrose added to the medium in concentration indicated as numbers and expressed in g/L;
P, added phosphate (0.3 %); YE, added yeast extract (0.16 %);
Values are means from 3 independent replicates.)

Fig. 2. Contour plots of levan yields (g/L) predicted by RSM for cultivation of *B. licheniformis* NS032 on molasses medium. Interaction of a) the molasses percentage and KH_2PO_4 concentration at a constant pH of 6.75, b) the molasses percentage and initial pH at a constant KH_2PO_4 concentration (6 g/L), c) initial pH and KH_2PO_4 concentration at a constant molasses percentage (62.5 %).

Fig. 3. The time course of *Bacillus licheniformis* NS032 fermentation in different media. Growth, levan and glucose production in media with 200 g sucrose/L (S200) (a), 400 g/L sucrose (S400) (b), molasses (M200) (c); Change of pH during levan production (d).

Fig. 4. TIC chromatograms of partially methylated anhydroalditolacetates for levans LM (a), LS1 (b) and LS2 (c). Peaks are labelled as: 1 – terminal glucose, (2+3) – terminal fructose monomers, (4+5) – linked monomers in main chain, (6+7) – branching points.

Fig. 5. Expanded regions of 2D NMR spectra of LM levan produced by *B. licheniformis* NS032. a) C6-region of the HSQC spectrum, b) C5-region of the HSQC spectrum, c) C2-region of the HMBC spectrum.

Fig. 6. Expanded anomeric regions of 2D NMR spectra of LS2 levan produced by *B. licheniformis* NS032. a) COSY b) HSQC c) HMBC.

Fig. 7. Expanded anomeric region in ^{13}C NMR spectra of levans LM (a), LS1(b) and LS2(c) from *B. licheniformis* NS032, and levan from *E. herbicola* (d).

Table 1

Structural and physicochemical characteristics of levans produced by *B. licheniformis* NS032 during fermentation in three different media.

	Levan/Medium		
	LM/M200	LS1/S200	LS2/S400
M_n^*	3.75 x 10^6	6.79 x 10^6	8.25 x 10^4
M_w	5.82 x 10^6	7.24 x 10^6	5.13 x 10^5
M_z	7.42 x 10^6	7.96 x 10^6	3.45 x 10^6
PDI	1.55	1.07	6.22
Branching, %	12.68±0.71	11.04±0.57	11.99±0.63
Viscosity, dL/g	0.24	0.25	0.14
Optical activity	-40.4	-41.8	-40.9

*M_n-Number average molecular weight, M_w-Weight average molecular weight, M_z- Z average molecular weight, PDI (Polydispersity index) = M_w/M_n

Table 2

Chemical shifts (ppm) in ^{1}H and ^{13}C NMR spectra of levans produced by *B. licheniformis* NS032.

		1,1’	2	3	4	5	6, 6’
A, β-(2→6)-Fru*f*	H	3.76, 3.66		4.18	4.08	3.94	3.88, 3.54
	C	62.62	106.91	79.00	77.91	83.00	66.09
B, β-(1,2→6)-Fru*f*	H	3.74, 3.66		4.18	4.08	3.94	3.88, 3.54
	C	63.11	106.33*	79.41*	77.83*	83.00	65.91*
C, ter-Fru*f*	H	3.87, 3.68		4.22	4.09	3.85	3.84, 3.72
	C	62.76	106.21*	79.56*	76.96*	83.73	64.88*
D, ter-Glc*p*	H	5.41	3.54	-**	-	-	-
	C	95.23	-	-	-	-	-

* Interchangeable among B/C fragments; **Overlapping signals.

HIGHLIGHTS

- For levan production by *Bacillus licheniformis* NS032 a new cost-effective medium based on sugar beet molasses was optimized.
- High levan yield (53.2 g/L) was obtained on molasses-based medium in 48 h fermentation time.
- The kinetics of bacterial growth and levan production in molasses medium and media with two levels (200 and 400 g/L) of sucrose were compared.
- Levans produced in molasses-based medium and sucrose media were compared on the basis of molecular weight, degree of branching, NMR data and viscosity.

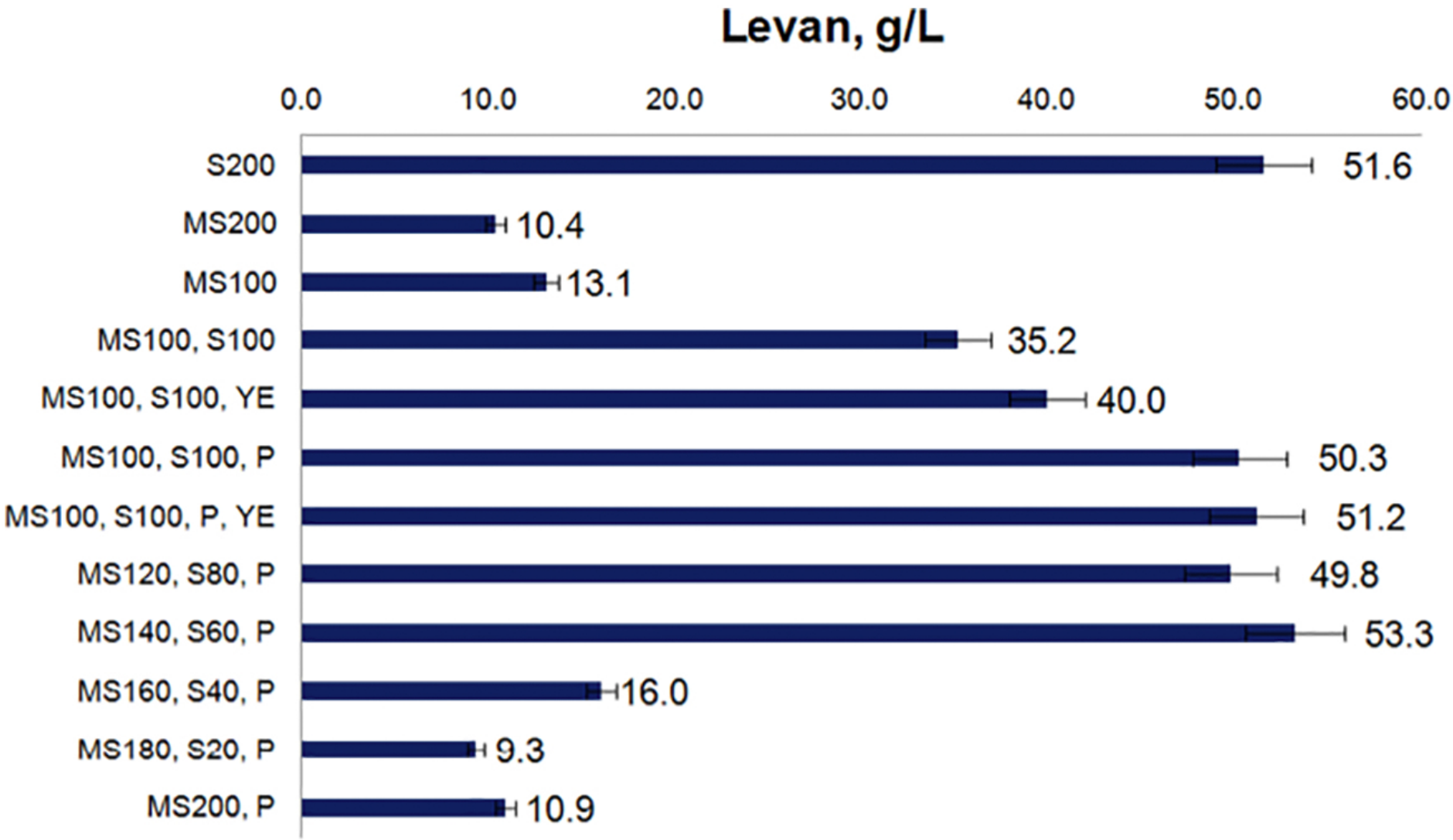
Levan, g/L
0.0
10.0
20.0
30.0
40.0
50.0
60.0
S200
51.6
MS200
10.4
MS100
13.1
MS100, S100
35.2
MS100, S100, YE
40.0
MS100, S100, P
50.3
MS100, S100, P, YE
51.2
MS120, S80, P
49.8
MS140, S60, P
53.3
MS160, S40, P
16.0
MS180, S20, P
9.3
MS200, P
10.9

Figure 1

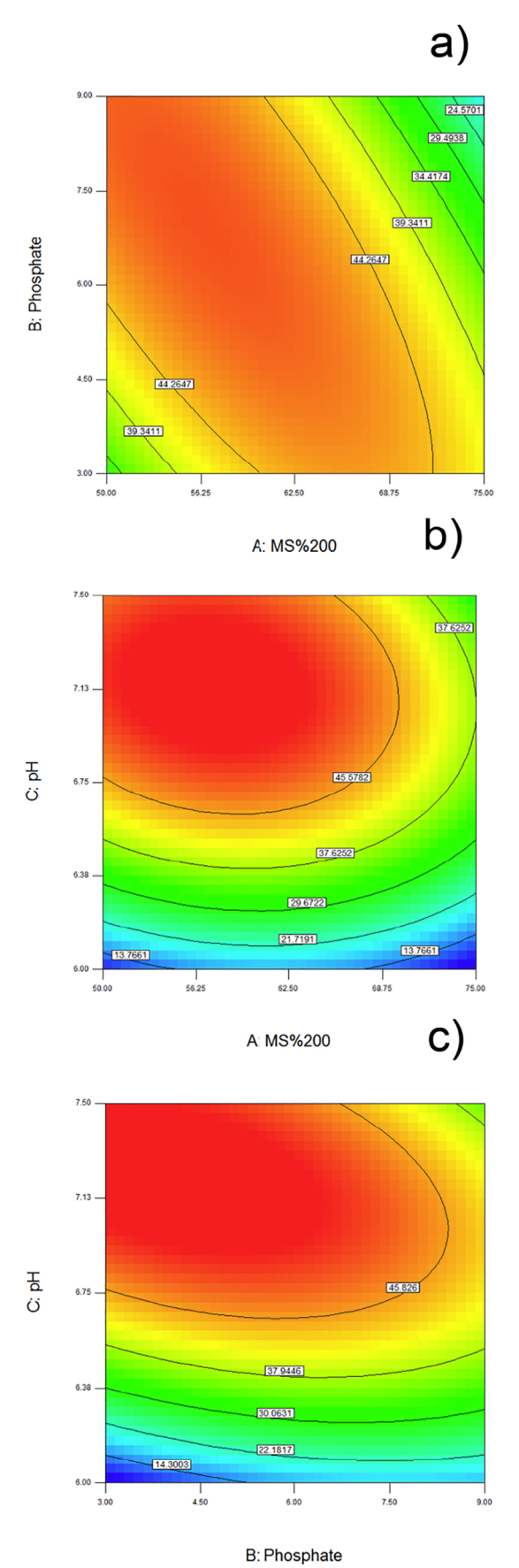
a)
B: Phosphate
9.00
7.50
6.00
4.50
3.00
24.5701
29.4938
34.4174
39.3411
44.2647
44.2647
39.3411
50.00
56.25
62.50
68.75
75.00
A: MS%200
b)
C: pH
7.60
7.13
6.75
6.38
6.00
37.6252
45.5782
37.6252
29.6722
21.7191
13.7661
13.7661
50.00
56.25
62.50
68.75
75.00
A: MS%200
c)
C: pH
7.50
7.13
6.75
6.38
6.00
45.625
37.9446
30.0631
22.1817
14.3003
3.00
4.50
6.00
7.50
9.00
B: Phosphate

Figure 2

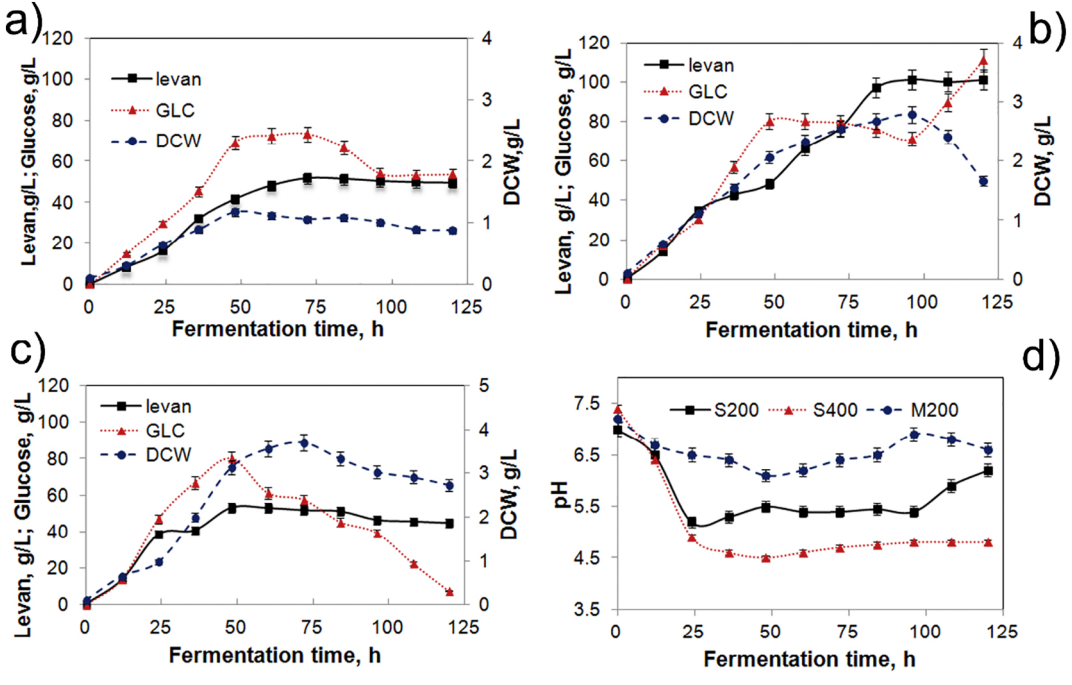
a)
Levan,g/L; Glucose, g/L
DCW,g/L
levan
GLC
DCW
Fermentation time, h
b)
Levan, g/L; Glucose, g/L
DCW, g/L
levan
GLC
DCW
Fermentation time, h
c)
Levan, g/L; Glucose, g/L
DCW, g/L
levan
GLC
DCW
Fermentation time, h
d)
pH
S200
S400
M200
Fermentation time, h

Figure 3

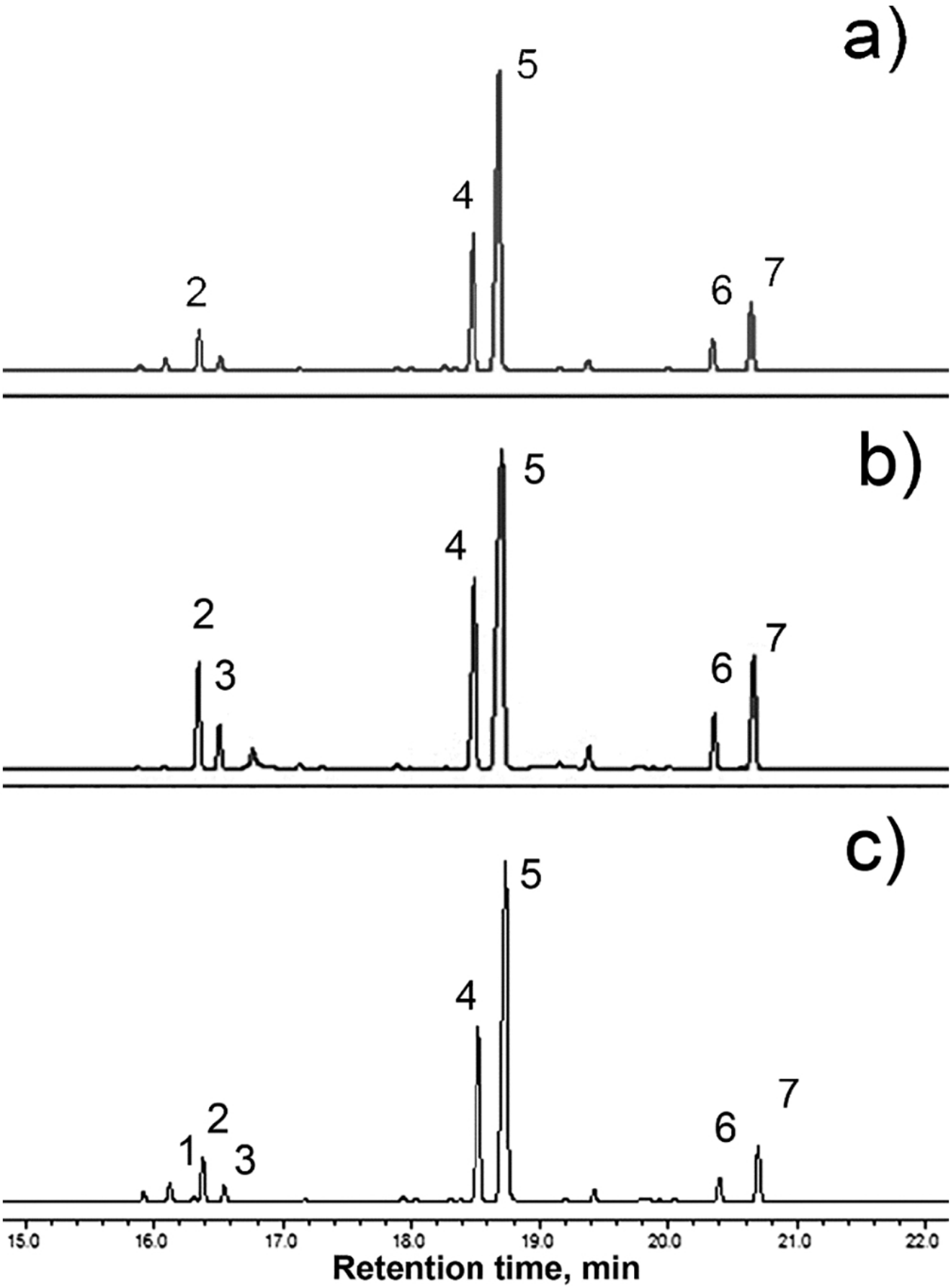
a)
5
4
2
6
7
b)
5
4
2
3
6
7
c)
5
4
2
1
3
6
7
15.0
16.0
17.0
18.0
19.0
20.0
21.0
22.0
Retention time, min

Figure 4

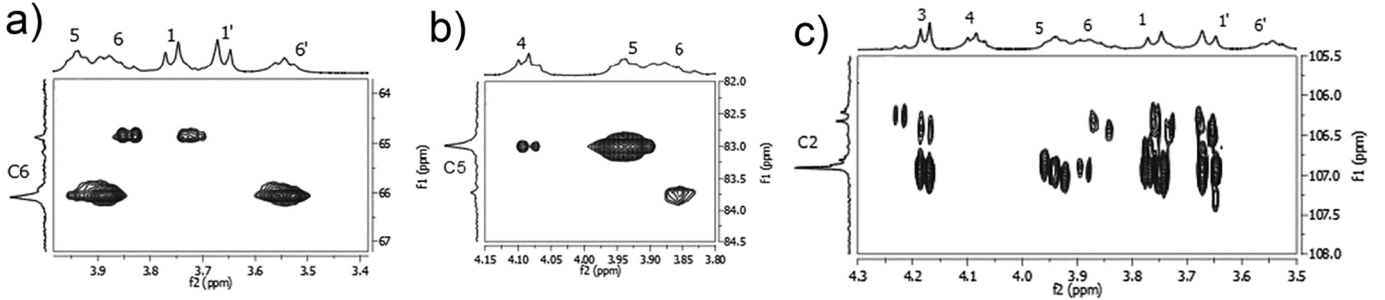
a)
5
6
1
1'
6'
C6
64
65
66
67
3.9
3.8
3.7
3.6
3.5
3.4
f2 (ppm)
b)
4
5
6
C5
f1 (ppm)
82.0
82.5
83.0
83.5
84.0
84.5
4.15
4.10
4.05
4.00
3.95
3.90
3.85
3.80
f2 (ppm)
c)
3
4
5
6
1
1'
6'
C2
105.5
106.0
106.5
107.0
107.5
108.0
f1 (ppm)
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
f2 (ppm)

Figure 5

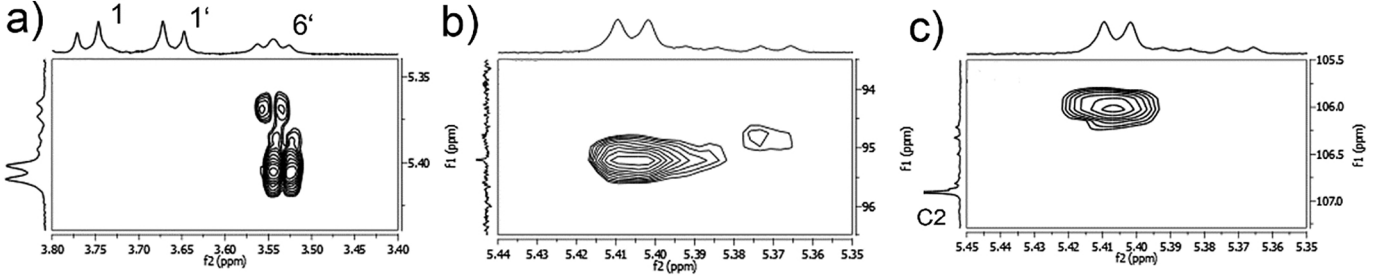
a)
1
1'
6'
5.35
5.40
3.80 3.75 3.70 3.65 3.60 3.55 3.50 3.45 3.40
f2 (ppm)
b)
f1 (ppm)
94
95
96
5.44 5.43 5.42 5.41 5.40 5.39 5.38 5.37 5.36 5.35
f2 (ppm)
c)
f1 (ppm)
105.5
106.0
106.5
107.0
C2
5.45 5.44 5.43 5.42 5.41 5.40 5.39 5.38 5.37 5.36 5.35
f2 (ppm)

Figure 6

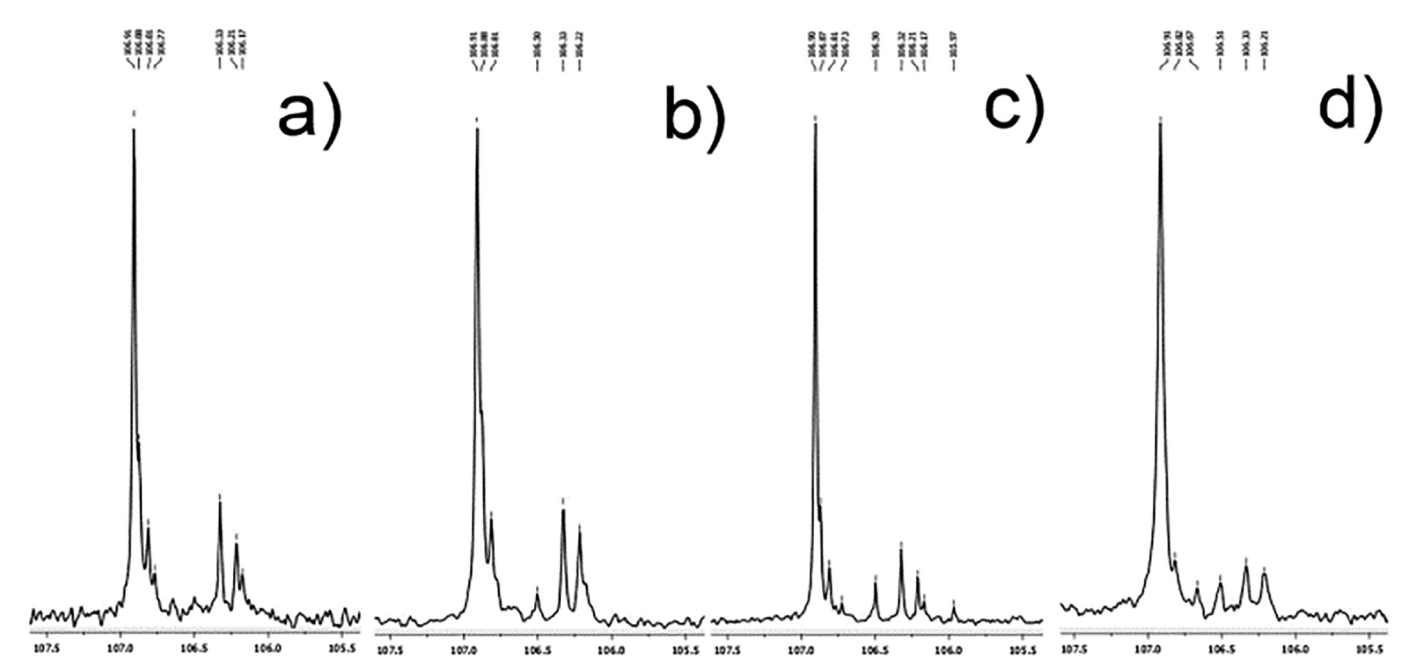

Figure 7