

# Assessment of Levan Production from Sucrose by *Zymomonas mobilis* CCT 4494

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## Abstract:

Levan is a polymer composed of fructose units linked by  $\beta(2\rightarrow6)$  glycosidic bonds. It can be found in many plants and microbial products, and it has many applications in different industrial fields, such as foods and pharmaceuticals. The aim of this study was to analyze levan production from sucrose by *Zymomonas mobilis* CCT 4494. A  $2^{4-1}$  fractional design was carried out to evaluate the effects of pH, temperature, sucrose concentration and time. The statistical analysis ( $p < 0.10$ ) showed that the effect of the sucrose concentration was the most important influence on the levan concentration followed by the effect of the temperature. The best levan biosynthesis condition was at 25 °C, with initial pH value of 4.5, 250 g L<sup>-1</sup> of sucrose concentration during 72 h of fermentation. Furthermore, highest levan production was 7.6 g L<sup>-1</sup>, at 25 °C with sucrose concentration of 250 g L<sup>-1</sup>.

**Keywords:** batch fermentation; biopolymer; exopolysaccharide; statistical methodology

## 1. Introduction

Levan is a natural exopolysaccharide composed of fructose polymer (fructan). Its main chain is composed of repeating fructofuranosyl rings connected by  $\beta$ -(2,6) links. Branching of the main chain results when fructofuranosyl rings connect through  $\beta$ -(2,1) linkages [1].

Microbial levans are produced from sucrose-based substrate by transfructosylation reaction of levansucrase ( $\beta$ -2,6-fructan:D-glucose-fructosyl transferase, EC 2.4.1.10) [2]. Different microorganisms in the fermentation process can produce levan by the action of the levansucrase enzyme [3, 4], such as bacteria *Zymomonas mobilis* [3-8] and *Bacillus subtilis* [9, 10].

Levan is an exopolysaccharide that has a wide variety of applications. Viscosity, solubility in water and oil, suspending and rheological

properties, compatibility with salts and surfactants, heat stability, acid and alkali, film formation, holding capacity for water and chemicals, and biological properties make levan a unique polymer for use in many different fields [11].

Levan can be used in medicine as a blood plasma extender [12], a hypoglycemic and antitumor activity [13]. It has high potential applications in food industry as a gum, sweetener, emulsifier, formulation aid, stabilizing thickener, surface-finishing agent, encapsulating agent and a carrier for color or flavors [3, 14].

*Z. mobilis* produce ethanol as a main product in sucrose medium and as a byproduct can produce levan, fructooligosaccharides, sorbitol and gluconic acid. Levan production by *Z. mobilis* is catalyzed by the levansucrase enzyme that hydrolyzes sucrose and polymerizes the

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fructose into levan polymer (transfructosylation reaction) [5].

Applying statistical methodology to study the different parameters that affect the production of biotechnological compounds is important because it defines the effects of several factors and their interactions that may lead to the optimization of the process [15].

The present study assessed, using statistical methodology, the effects of the pH of the medium, temperature, sucrose concentration and fermentation time on levan production by *Zymomonas mobilis* CCT 4494.

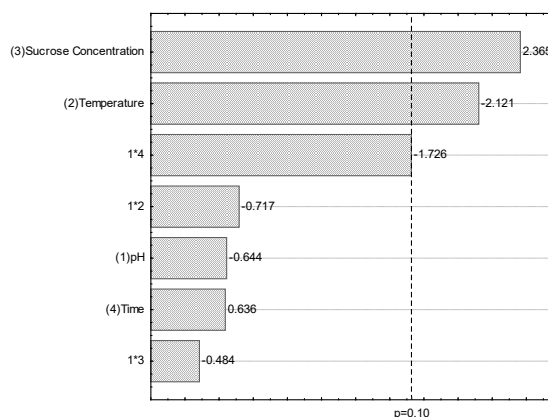
## 2. Results and Discussion

Table 1 shows the average results of the final pH, production of biomass and levan obtained in the  $2^{4-1}$  fractional design to evaluate the main effects of the pH, temperature, sucrose concentration and time on the levan production. A total of 9 experimental runs were carried out and a variation in mass was obtained, which ranged from 2.6 to 7.6 g L<sup>-1</sup> in the runs 5 and 6, respectively.

The statistical analysis showed that the effect of the sucrose concentration was the most important influence on the levan concentration followed by the effect of the temperature (Fig. 1). No statistical significance ( $p < 0.10$ ) was observed for the factor pH or fermentation time. The positive effect of sucrose concentration indicates that high mass of polysaccharide was obtained when high sucrose concentration was used. And the negative effect of temperature indicates that fermentation temperature at 25 °C had a higher levan production than 35 °C.

The best levan biosynthesis condition was at 25 °C, with initial pH value of 4.5, 250 g L<sup>-1</sup> of sucrose concentration during 72 h of fermentation. This production was greater than the one found by Ernandes and Garcia-Cruz [16], under the same conditions: sucrose concentration (250 g L<sup>-1</sup>) and agitation (200 rpm), the highest levan production was 4.5 g L<sup>-1</sup>. Higher values were found by Melo et al. [6], with maximum production of 14.6 g L<sup>-1</sup>, synthesized by *Z. mobilis* strain ZAG-12. However, the maximum levan concentration found by Silbir et al. [8] was 40.2 g L<sup>-1</sup>, concentration reached at

the optimum levels of process variables, which were 299.1 g L<sup>-1</sup> initial substrate concentration, 42.3 h incubation time, and initial pH 6.0.



**Figure 1.** Pareto Charts for variables pH, temperature, sucrose concentration and time for levan production.

Previous studies indicated that high sucrose concentrations have a positive effect on biopolymer formation during the fermentation with *Z. mobilis*. Ernandes and Garcia-Cruz [16] varied the initial sucrose concentration (50, 150 and 250 g L<sup>-1</sup>) and obtained maximum formation of levan with 250 g L<sup>-1</sup>.

Melo et al. [6] showed that the final levan concentration depends on the initial sucrose concentration, temperature and agitation velocity, and they concluded that the best conditions occurred at 100 rpm agitation, 20 °C and 250 g L<sup>-1</sup> of initial sucrose, resulting in 14.67 g L<sup>-1</sup> of levan. Also, they showed that increasing the initial concentration of sucrose from 150 g L<sup>-1</sup> to 250 g L<sup>-1</sup>, brings a 3.1 g L<sup>-1</sup> improvement in levan production. In the present study, with the same increase in the initial concentration of sucrose (150 to 250 g L<sup>-1</sup>), there was an increase of 3.6 g L<sup>-1</sup> of levan production, a similar value to the one encountered by Melo et al. [6].

The influence of temperature and sucrose concentration was also stated by Bekers et al. [5]. These authors reported that to produce levan, sucrose concentration and temperature were the most important factors in the fermentation medium to regulate levansucrase activity of *Z. mobilis*, and consequently the levan formation.

Ananthalakshmy and Gunasekaran [17] studied the effect of different fermentation conditions on levan production by *Z. mobilis* B-4286. They showed that levan production increased from 5.7 g L<sup>-1</sup> to 12.6 g L<sup>-1</sup> with an increase in initial sucrose concentration (50 to 150 g L<sup>-1</sup>).

From the obtained results, it can be observed that variables pH and fermentation time were not significant and pH had negative effect on the levan production, which means that when the pH enhance of 4.5 to 6.5, there was a decrease in the levan biosynthesis (Table 1, experiments 6 and 5) and time had a positive effect (Fig. 1), which means that the levan production was higher with more fermentation time.

The variables time and pH were not statistically influent. The fact that the initial pH did not influence the production of levan can be justified by the range adopted (4.5 to 6.5), which are among the recommended values of optimum pH for levan production. According Ernandes and Garcia-Cruz [18], the optimum pH for the activity of the enzyme levansucrase is 6.5. In cultures containing sucrose, lower pH values can be used for levan suitable production by the same enzyme. The inhibition of the enzyme occurs at extremely low values (pH<3.0) or, as found by Doelle et al. [19], very high values (above 8.0). This range of pH was also studied by Lyness and Doelle [20], these authors reported that the enzymatic activity for hydrolysis of sucrose was better at pH 6.5. Crittenden and Doelle [21] studied the levansucrase activity of *Z. mobilis* UQM 2716, and they concluded that the pH 5.5 was the best for the polysaccharide synthesis. Ananthalakshmy and Gunasekaran [17] had higher levan production with initial pH 5.0 than at pH 6.0 and 7.0. Later, Tano and Buzato [22] investigated the influence of initial pH (5.4; 5.9 and 6.3) of sugar cane juice in high concentration for levan production by *Z. mobilis*, and the levan concentration achieved was higher at pH 6.3.

Cell growth was low overall the experiments. The maximum biomass concentration obtained was 2.0 g L<sup>-1</sup>, associated to experiment 7, at the highest substrate concentration tested (250 g L<sup>-1</sup>) (Table 1). Other researchers have also found low biomass concentration. Silbir et al. [8] obtained 1.4 g L<sup>-1</sup> on a kinetics study of levan production

by *Z. mobilis* B-14023 in batch culture. These authors observed that levan production was produced immediately after the lag phase of the microorganism and its concentration was maximum after the cells entered the stationary phase. De Oliveira et al. [7] also found a low cell growth, they stated that low biomass production is normally observed with *Z. mobilis*, because this bacteria uses the Entner-Doudoroff pathway for carbon catabolism and produces only 1 mol ATP per mol of glucose consumed.

Since medium pH was not controlled with the fermentation progress, there was a reduction in the final pH. Analyzing the final pH of the fermentation broth obtained after fermentation process, observed that the final pH varied between 3.4 to 4.2 (Table 1). This reduction occurs due to acid formation and indicates the resistance of the bacteria at low pH [23].

### 3. Material and Methods

#### 3.1 Microorganism, Medium and Fermentations

The *Z. mobilis* CCT 4494 bacterium was obtained from Fundação Tropical de Pesquisas e Tecnologia André Tosello (Campinas, Brazil). The bacterial strain was cultivated and maintained in medium consisting of glucose (20 g L<sup>-1</sup>), peptone (10 g L<sup>-1</sup>) and yeast extract (10 g L<sup>-1</sup>). The pH value was adjusted to 6.5. The cultures were reactivated monthly, incubated at 30 °C for 24 h and stored at 4 °C. The cell concentration was determined by turbidimetry at 570 nm, using a spectrophotometer (Biochrom, Libra S22, Cambridge, UK).

The medium used for levan production was the synthetic medium, proposed by Rodriguez and Callieri [24], composed by sucrose (50-250 g L<sup>-1</sup>); yeast extract (5.0 g L<sup>-1</sup>); KH<sub>2</sub>PO<sub>4</sub> (1.0 g L<sup>-1</sup>); MgSO<sub>4</sub>·7H<sub>2</sub>O (1.0 g L<sup>-1</sup>) and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.0 g L<sup>-1</sup>). After adjusting the pH value to 4.5, 5.5 or 6.5 with 1 mol L<sup>-1</sup> HCl, the substrate was sterilized at 121 °C for 15 min using an autoclave (Phoenix Lufanco, AV, Araraquara, Brazil). The sucrose was sterilized separately from the salts, to avoid Maillard Reaction.

Fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium, placed on orbital shakers

(Marconi, MA830, Piracicaba, Brazil) under controlled temperature with a 200 rpm agitation.

### 3.2 Analytical Methods

After each fermentation, the final pH was determined directly in the fermented extract by potentiometry using pHmeter (Digimed, DM20, São Paulo, Brazil). The culture was centrifuged at 6,941 x g for 15 min using a centrifuge (EVLAB, EV:025, Londrina, Brazil) and biomass determined by turbidimetry at 570 nm relating it to a biomass with a dry matter calibration curve.

The levan was separated by ethanol precipitation, hydrolyzed with 0.5% HCl at 100 °C for 60 min. The content of levan was estimated by reducing sugar using the method of Somogyi [25] and Nelson [26].

### 3.3 Sequential Strategy of Experimental Designs

The sequential strategy of experimental design was adopted to evaluate the levan synthesis. The effects of the initial pH (variable  $X_1$ ), temperature (°C) (variable  $X_2$ ), sucrose concentration (variable  $X_3$ ) and incubation time (variable  $X_4$ ) were evaluated by means of a  $2^{4-1}$  fractional design (Table 1). All experiments were done in triplicate to obtain an estimative of experimental error, and another experiment was carried out in triplicate at the central point. A total of 27 experiments were carried out. The ranges of variation between the lowest and the highest limits of each independent variable were established from literature data and preliminary tests, using their real values, as shown in Table 2. The results were evaluated using the Statistica software (StatSoft, Inc., version 7.0, Tulsa, USA).

**Table 1.** Matrix of the  $2^{4-1}$  fractional design (coded values), used to study the influence of 4 factors on levan production, final pH and biomass by *Zymomonas mobilis* CCT 4494 from sucrose.

Run	Variables				$\text{pH}_{\text{final}}$	Biomass $\text{g L}^{-1}$	Levan
	$X_1$	$X_2$	$X_3$	$X_4$			
1	-1	+1	-1	+1	3.6±0.0	0.6±0.0	3.9 ±1.7
2	+1	-1	+1	-1	3.9±0.0	0.9±0.0	6.7 ±2.3
3	+1	-1	-1	+1	3.6±0.0	0.7±0.1	4.1 ±1.6
4	-1	-1	-1	-1	3.6±0.0	0.8±0.0	3.1 ±0.8
5	+1	+1	-1	-1	4.2±0.0	1.9±0.1	2.6 ±1.6
6	-1	-1	+1	+1	3.4±0.1	1.0±0.6	7.6 ±2.9
7	-1	+1	+1	-1	3.5±0.0	2.0±0.3	4.4 ±3.5
8	+1	+1	+1	+1	3.4±0.1	1.4±0.0	3.3 ±1.9
9	0	0	0	0	3.4±0.0	1.4±0.2	4.0 ±1.6

**Table 2.** Independents variables, levels and real values for fractional 24-1 factorial experiments.

		Real levels		
		-1	0	+1
$X_1$	Initial pH	4.5	5.5	6.5
$X_2$	Temperature (°C)	25	30	35
$X_3$	Sucrose concentration ( $\text{g L}^{-1}$ )	50	150	250
$X_4$	Incubation time (h)	24	48	72

## 4. Conclusions

Levan production was affected by initial concentration of sucrose and temperature in a fractional design studied. There was a direct relation between the higher production, low temperature and high sucrose concentration used. The levan production using *Zymomonas mobilis* CCT4494 was higher in fermentation with

250  $\text{g L}^{-1}$  sucrose concentration at 25 °C, with initial pH between 4.5 and 6.5 and fermentation time between 24 and 72 hours.

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