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Levan production by *Zymomonas mobilis* in batch and continuous fermentation systems

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ABSTRACT

Levan production in batch and continuous fermentation systems by *Zymomonas mobilis* B-14023 was investigated. The culture medium used in both of the fermentation systems contained sucrose and various organic nitrogen sources. Maximum concentration of levan was produced with yeast extract among the nitrogen sources tested. Response surface methodology was used to investigate the effects of three factors on the concentration of levan in batch cultures of *Z. mobilis*. Maximum levan concentration was 40.2 g/L and this concentration was reached at the optimum levels of process variables, which were 299.1 g/L initial substrate concentration, 42.3 h incubation time, and initial pH 6.0. Continuous fermentation experiments were done in packed bed bioreactor using Ca-alginate immobilized *Z. mobilis* cells. The highest levan concentration (31.8 ± 0.21 g/L) was obtained at a dilution rate of 0.14 h^{-1} while maximum volumetric productivity (6.556 g/(L h)) was obtained at a dilution rate of 0.22 h^{-1}. Increasing the dilution rate resulted in decreased levan and increased residual sugar concentrations.

1. Introduction

Levan is one of two main types of fructans, which are naturally occurring homopolymers of fructose. Its main chain is composed of repeating fructofuranosyl rings connected by β-(2,6) links. Branching of the main chain results when fructofuranosyl rings connect through β-(2,1) linkages (Arvidson, Rinehart, & Gadala-Maria, 2006). Viscosity, solubility in water and oil, suspending and rheological properties, compatibility with salts and surfactants, stability to heat, acid and alkali, film formation, holding capacity for water and chemicals, and biological properties make levan a unique polymer for use in many fields (Bekers et al., 2005). Levan can be used not only in the food industry as a fructose source, an emulsifying and encapsulating agent and a texture forming compound, but also in medicine as an immunomodulator, a blood plasma substitute, a prolongator of the effect of drugs and as a sorbent of cholesterol in the intestinal tract (Bekers et al., 2001; de Oliveira, da Silva, Buzato, & Celligoi, 2007). Antitumor activity of levan produced by *Zymomonas mobilis* was also reported (Calazans, Lopes, Lima, & de França, 1997; Calazans, Lima, de França, & Lopes, 2000).

Z. mobilis is a Gram negative, ethanol-producing bacterium that can also produce various byproducts such as levan, sorbitol, gluconic acid and fructooligosaccharides in sucrose media. Levan production by *Z. mobilis* is catalyzed by the levansucrase enzyme that hydrolyzes sucrose and polymerizes the fructose into levan polymer (transfructosylation reaction). Sucrose concentration and temperature are the most important factors that regulate the activity of the levansucrase enzyme in *Z. mobilis*. High sucrose concentrations and high temperatures stimulate the fructooligosaccharide synthesis while low values of these parameters increase ethanol production (Bekers et al., 2000). Besides *Zymomonas*, other microorganisms belonging to the genera *Bacillus*, *Streptococcus*, *Pseudomonas*, *Xhanthomonas* and *Aerobacter* are also known to produce levan but their productivities are low (Yoshida, Suzuki, & Yagi, 1990).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors and searching optimum conditions for desirable responses. The conventional practice of RSM is time consuming because the methodology allows variation of one variable at a time. This restriction can be overcome by using RSM in such a way that can identify and quantify the various interactions among different parameters. RSM has been extensively used to optimize the cultural medium conditions and other parameters in bioprocesses (Göksungur, Uzunoğulları, & Dağbağlı, 2011; Li, Bai, Cai, & Ouyang, 2002; Ürküt, Dağbağlı, & Göksungur, 2007).

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In the previous researches of our group, pullulan production by *Aureobasidium pullulans* was optimized by using RSM in a stirred tank bioreactor (Göksungur, Dağbağlı, Uçan, & Güvenç, 2005) and in batch and repeated batch fermentation systems using Ca-alginate immobilized *A. pullulans* cells (Ürküt, Dağbağlı, & Göksungur, 2007). However, only few reports are available on optimization of levan production using statistical techniques. Borsari, Celligoi, Buzato, and Silva (2006) investigated substrate type (sugar cane juice and sucrose) and fermentation systems (batch and fed batch) and their effect on levan production using a complete factorial design. Melo, Lopes, and Catazans (2007) studied levan production using fractional factorial design and they found the optimum conditions for levan production as 100 rpm agitation, 20 °C and 250 g/L of initial sucrose concentration resulting in 14.67 g/L concentration of levan. de Oliveira, da Silva, Buzato, and Celligoi (2007) studied the use of alternative regional low-cost substrates (sucrose, molasses and sugar cane syrups), sugar concentration, fermentation time and fermentation medium constituents using statistical tools.

Immobilization of whole cells has many advantages over free cells, such as relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control and reduced susceptibility of cells to contamination. The entrapment of cells in Ca-alginate gel beads is the most widely used method for the immobilization of viable cells due to its simplicity, non-toxicity, mild gelation conditions and ease of use (Ürküt, Dağbağlı, & Göksungur, 2007). This simple and mild immobilization technique involves the drop-wise addition of cells suspended in sodium alginate into a solution of calcium chloride whereon the cells are immobilized in precipitated calcium alginate gel in the form of beads (Rosevear, 1984). Bekers et al. (2001) immobilized *Z. mobilis* cells by attachment to stainless steel wire spheres (WS) and Al_2O_3 granules and compared fermentation efficiency of attached cells with cells entrapped in Ca-alginate. No previous work used Ca-alginate immobilized *Z. mobilis* cells for continuous levan production in a packed bed bioreactor.

This study examined levan production from sucrose by free and immobilized *Z. mobilis* NRRL B-14023 cells in batch and continuous fermentation systems. RSM was used to optimize fermentation parameters to obtain maximum levan concentration in the batch fermentation system. Three factors (initial substrate concentration, incubation time and initial pH) considered to have significant effect on levan production were selected for optimization studies. *Z. mobilis* cells were immobilized in Ca-alginate gel beads and levan was produced in a packed bed bioreactor in the continuous fermentation system. This work is the first article on continuous levan production in a packed-bed bioreactor by Ca-alginate immobilized *Z. mobilis* cells.

2. Materials and methods

2.1. Microorganism and culture conditions

Z. mobilis NRRL B-14023 used throughout this study was kindly supplied by the US Department of Agriculture, Agricultural Research Service. The strain is maintained on culture medium at 4 °C and transferred monthly to fresh medium incubated without aeration or mixing at 28 °C for 1 day. The composition of the culture medium used (Bekers et al., 2001) was as follows (in g/L): sucrose, 50; yeast extract, 7.0; K_2HPO_4, 2.5; $(NH_4)_2SO_4$, 1.6; $MgSO_4{\cdot}7H_2O$, 1.0 (pH 5.0 ± 0.2). All chemicals used in this study were of analytical grade; Na-alginate (Sigma, A-2033, St. Louis, USA) used for immobilization of microbial cells was obtained from brown algae.

2.2. Batch fermentation experiments

The production medium used had the following composition (g/L): sucrose, 150–300; yeast extract, 2.5; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5 (pH 5.0 ± 0.2). After adjusting the pH to 5.0 with 1 N HCl, the substrate was sterilized at 121 °C for 20 min. Fermentations were carried out batchwise in 250 mL flasks with 100 mL working volume in an incubator at 28 °C without aeration or mixing. Alternative nitrogen sources (corn steep liquor, peptone, tryptone, urea, and malt sprouts) were substituted with yeast extract (2.5 g/L) on equal nitrogen bases to determine the effect of different sources of nitrogen on levan production. The optimization studies were done by RSM and the levels of incubation time, initial substrate concentration and initial pH are given in Table 1.

2.3. Cell immobilization

Z. mobilis cells grown in 25 mL culture medium were added and mixed with an equal volume (1:1, v/v) of 4% Na-alginate (Sigma, A-2033) solution till a homogenous solution was formed. A 50 mL aliquot of alginate-cell suspension containing 2% Na-alginate was added dropwise with a peristaltic pump to a hardening solution of 500 mL, 2% (w/v) $CaCl_2$. Alginate drops solidified upon contact with $CaCl_2$, forming beads (2.0–2.4 mm in diameter) and thus entrapping the bacterial cells. The beads were allowed to solidify for 30 min; they were then washed with sterile physiological solution to remove excess calcium ions and cells. To increase the entrapped cell population, the beads were incubated overnight in the culture medium at 28 °C and stored at 4 °C until use.

2.4. Continuous fermentation experiments

Continuous levan production was carried out using a jacketed Pyrex column packed with 2.0–2.4 mm diameter Ca-alginate beads containing entrapped bacterial cells. The packed-bed bioreactor used had the following characteristics: inner diameter 1.57 cm, column height 49 cm, total reactor volume 95 mL, bed volume 85 mL, void volume 22 mL, and packing weight 67 g. Prior to use, the packed-bed bioreactor was filled with Ca-alginate beads and the production medium fed from the bottom of the column continuously by means of a peristaltic pump (Chemap AG) through silicon tubing. Effluent liquid overflowed from the outlet port at the top of the column, keeping the level of liquid constant inside the column. The beads were trapped inside the bioreactor by a metal mesh filter covered with a plastic barrier. The temperature of the bioreactor was maintained at 28 °C by circulating water at a constant temperature from a circulator bath through the jacket of the column. The dilution rates were 0.14, 0.22, 0.29 and 0.4 h^{-1} throughout the packed-bed bioreactor experiments and the system was considered to be at steady state after at least five residence times. Levan production was monitored by measuring the concentration of the polymer in the effluent liquid overflowing from the column. The optimized initial substrate concentration and pH values were used in the packed-bed bioreactor experiments.

2.5. Analytical techniques

After each fermentation, the culture was centrifuged (5000 × *g* for 10 min) and cell growth determined by measuring the optical density at 590 nm relating it to biomass with a dry matter calibration curve. Levan was determined as suggested by Viikari (1984). The fermentation medium was centrifuged at 5000 × *g* for 10 min to remove biomass and the supernatant precipitated by 75% ethanol. pH was adjusted to 10 with 0.1 M KOH to facilitate precipitation. The precipitate was centrifuged at 5000 × *g* for 5 min and hydrolyzed with 0.1 M HCl for 1 h at 100 °C. The content of levan

Table 1
Experimental design.

Factor	X_1 initial sugar concentration (g/L)			X_2 incubation time (h)			X_3 initial pH			Levan (g/L)
	Level			Level			Level			
	−1	0	+1	−1	0	+1	−1	0	+1	
	250	300	350	24	36	48	4.5	5.0	5.5	
Run	Experimental level for X_1			Experimental level for X_2			Experimental level for X_3			
1	250			24			4.5			18.63
2	350			48			5.5			33.00
3	350			36			5.0			30.02
4	300			36			5.5			40.17
5	250			36			5.0			29.85
6	300			36			5.0			36.20
7	300			24			5.0			27.33
8	300			36			5.0			36.26
9	300			36			5.0			36.60
10	250			48			5.5			28.18
11	300			48			5.0			30.12
12	300			36			5.0			36.20
13	350			24			4.5			14.46
14	300			36			4.5			29.07
15	250			48			4.5			6.89
16	300			36			5.0			36.50
17	300			36			5.0			36.10
18	350			48			4.5			16.40
19	350			24			5.5			17.11
20	250			24			5.5			25.38

was analyzed as fructose by the DNS method (Miller, 1959). Total sugar was determined according to the phenol sulfuric acid method using sucrose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The data reported are the average values ±SD of three replicate experiments.

The dilution rate (D, h^{-1}) was calculated by dividing the flow rate of the medium by the bed volume of the bioreactor. Levan productivity was calculated using the equation $R = D \times P$ where D is the dilution rate (h^{-1}) and P is the levan concentration (g/L). Effective (g levan produced g^{-1} initial sugar) and conversion (g levan produced g^{-1} sugar consumed) yields are expressed as the percentage of levan produced per initial quantity of sugar in the medium and percentage of levan produced per quantity of sugar consumed, respectively.

2.6. Experimental design and statistical analysis

The statistical analysis of the data was performed using Minitab Statistical Software (Release 13.20). Details of response surface methodology can be found elsewhere (Myers & Montogomery, 1995). The levels of factors used in the experimental design are listed in Table 1. The data of the factors were chosen after a series of preliminary experiments. Twenty experiments were conducted using a face central composite statistical design ($\alpha = 1$) for the study of three factors each at three levels (Table 1). The levels were −1, 0, +1 where 0 corresponded to central point. The actual level of the central point of each factor was calculated using the following equation (Myers & Montogomery, 1995).

$$\text{Coded value} = \frac{\text{Actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2}$$

Response surface model was fitted to the response variable, namely levan concentration (g/L). The second order response function for three quantitative factors is given by Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where X_1, X_2 and X_3 represent the levels of the factors according to Table 2 and β_0, β_1, …, β_{23} represent coefficient estimates with β_0 having the role of a scaling constant.

3. Results and discussion

3.1. Kinetics of levan production by Z. mobilis in batch culture

Z. mobilis cells were used for levan production in synthetic medium containing 150 g/L initial sugar. Fermentations were carried out batchwise in 250 mL flasks with 100 mL working volume in an incubator at 28 °C without aeration or mixing. *Z. mobilis* B-14023 produced a large quantity of extracellular levan when grown on 15% sucrose solution. Levan formation resulted in a cloudy solution immediately after inoculation was quantified by precipitation. As seen in Fig. 1, levan concentration increased up to 48 h of fermentation and then decreased. The highest concentration of levan obtained at 48 h of incubation was 15.52 ± 0.43 g/L. Bekers et al. (2005) reported that the degradation of levan in the culture liquid was a result of acid hydrolysis. They stated that organic acids produced by *Z. mobilis* hydrolyzed levan to reducing sugars and at low pH and high medium temperature levan might be utilized by *Z. mobilis* as a carbon source.

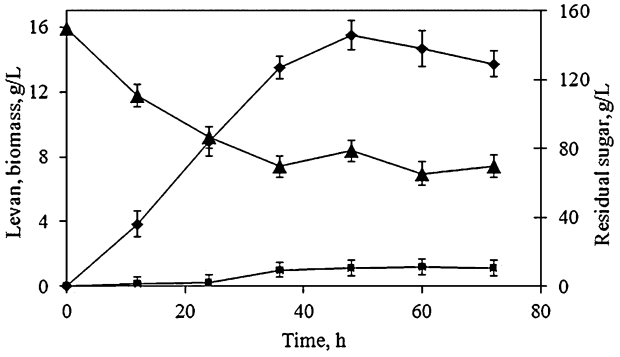

Fig. 1. Kinetics of levan production and sugar consumption for fermentation media containing 150 g/L of initial sugars (♦: levan, ■: biomass and ▲: residual sugar) (28 °C, pH 5.0, average of three replicates ± standard deviation).

Table 2
ANOVA and estimated regression coefficients for levan concentration (R^2 = 0.993).

Source	DF[a]	Coefficient	Seq SS[b]	Adj SS[c]	Adj MS[d]	*F*	*P*
Regression	9		1576.09	1576.09	175.121	2E+03	<0.001
Constant		−426.757					<0.001
Linear	3		355.02	355.02	118.341	1E+03	<0.001
Sugar conc.		1.61030					0.044
Time		−0.631952					<0.001
pH		80.5715					<0.001
Square	3		1020.38	1020.38	340.128	4E+03	<0.001
Sugar conc. × sugar conc.		−0.00264503					<0.001
Time × time		−0.0543235					<0.001
pH × pH		−7.70634					<0.001
Interaction	3		200.69	200.69	66.895	838.12	<0.001
Sugar conc. × time		0.00557660					<0.001
Sugar conc. × pH		−0.0439856					<0.001
Time × pH		0.593507					<0.001
Residual error	10		0.80	0.80	0.080		
Lack of fit	5		0.61	0.61	0.121	3.18	0.115
Pure error	5		0.19	0.19	0.038		
Total	19		1576.89				

[a] Degrees of freedom.
[b] Sequential sum of squares.
[c] Adjusted sum of squares.
[d] Adjusted mean square.

The maximum biomass concentration obtained was 1.42 ± 0.04 g/L and levan production was noted immediately after the lag phase of the microorganism and the concentration was maximum after the cells entered the stationary phase (data not shown). Low biomass production is normally observed in *Z. mobilis* because *Z. mobilis* uses the Entner–Doudoroff pathway for carbon catabolism and produces only 1 mol ATP per mol of glucose consumed and that result for *Z. mobilis* is one of the lowest molar growths reported for bacteria (de Oliveira et al., 2007).

As expected, the concentration of residual sugars decreased during the fermentation, coinciding with an increase in the concentration of levan during the fermentation, however, sugar was not utilized completely. This might be due to high initial substrate concentration resulting in incomplete utilization of sugar in the fermentation medium. Maximum productivity (0.375 g/(L h)) was obtained at the 36th hour of fermentation. Effective and conversion yield values obtained were 10.3% and 21.7%, respectively. It can be concluded from these yields that almost 10% of initial sucrose and 20% of consumed sucrose were utilized by the microorganism to produce levan. Viikari (1984) also found that the amount of levan produced by *Z. mobilis* was equivalent to almost 10% of the original sucrose.

3.2. Effect of nitrogen sources on levan production

Yeast extract is a source of vitamins (pantothenic acid, nicotinic acid, biotin, thiamine and pyridoxine) essential for cell growth and a stimulating factor for levan synthesis (de Oliveira et al., 2007). However, the high cost of yeast extract has a negative impact on the economics of its use in industrial scale processes. To improve the economic parameters of levan fermentation, comparable nitrogen sources were substituted with yeast extract (2.5 g/L) on equivalent nitrogen bases using synthetic medium containing 150 g/L initial sucrose concentration. The quantity of each nitrogen source used was equivalent to 2.5 g/L yeast extract nitrogen. The nitrogen sources and the amounts used (g/L) were: corn steep liquor, 3.8; malt sprouts, 4.6; peptone, 1.75; urea, 0.56; tryptone, 1.93. The results showed that levan synthesis by *Z. mobilis* was markedly influenced by the nitrogen source used. As seen in Fig. 2, yeast extract yielded the highest concentration of levan when compared with the other nitrogen sources (15.52 ± 0.23 g/L). Corn steep liquor and malt sprouts yielded 13.91 ± 0.27 g/L and 10.30 ± 0.28 g/L of levan, respectively. The other nitrogen sources were utilized by the microorganism, however, they yielded lower levan levels than yeast extract (Fig. 2). Our results showed that corn steep liquor can be used as an alternative nitrogen source in levan production.

Parallel to our findings, de Oliveira et al. (2007) stated that yeast extract was significant for levan production. They used a factorial planning and found that yeast extract + KH_2PO_4 and yeast extract + $MgSO_4$ were significant for levan production in the commercial sucrose medium and sugar cane syrup, respectively. They obtained the highest levan production when KH_2PO_4 was used with yeast extract. Melo et al. (2007) used a fractional factorial design to study levan production by *Z. mobilis* and they found that the initial concentration of yeast extract did not influence levan production. Bekers et al. (2002) used yeast extract, rye stillage and sugar beet molasses stillage as nitrogen source in levan and ethanol production by *Z. mobilis*. They found that molasses stillage stimulated the ethanol synthesis, while rye stillage was more preferable for levan production. They also found that 0.5% of yeast extract could be substituted by 10% of stillage.

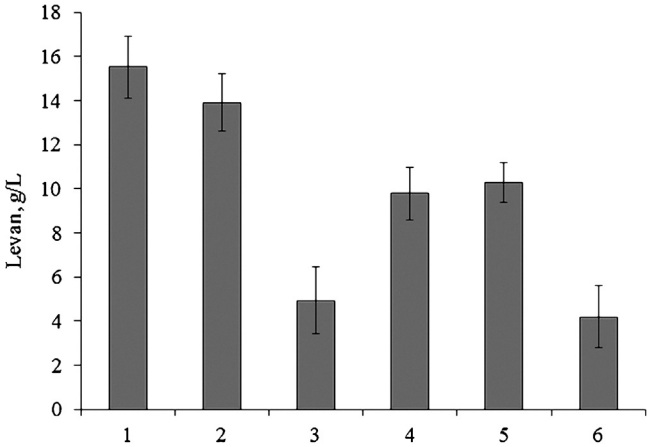

Fig. 2. The effect of different nitrogen sources on levan production (28 °C, pH 5.0, initial substrate concentration = 150 g/L, average of three replicates ± standard deviation) (1: yeast extract, 2: corn steep liquor, 3: peptone, 4: tryptone, 5: malt sprouts and 6: urea).

Further investigation was carried out for finding the optimum level of yeast extract and it was found that yeast extract concentrations higher than 2.5 g/L did not improve levan synthesis of the organism. Yeast extract concentration of 2.5 g/L was found to be optimum for levan production by *Z. mobilis* since levan concentration decreased at higher nitrogen concentrations (data not shown). This might be due to the fact that high concentration of nitrogen in the culture medium is repressing levan formation while encouraging the biomass production. Melo et al. (2007) used yeast extract concentrations of 2–5 g/L in levan production by *Z. mobilis* and found that yeast extract concentration did not affect levan synthesis. They also stated that yeast extract presented a higher effect on the biomass production. Similar findings were also reported for other microbial polysaccharides. Göksungur et al. (2011) found that 0.4 g/L yeast extract concentration was optimum for pullulan production by *Aureobasidium pullulans* and at a higher nitrogen concentration, pullulan concentration decreased. Schuster, Wenzig, and Mersmann (1993) found that the formation rate of pullulan increased up to ammonium sulphate concentrations of 1.2 g/L, however at higher nitrogen quantities, the product formation rate decreased since the microorganism was able to grow more intensively. Lazaridou, Biliaderis, Roukas, and Izydorczyk (2002) studied production of pullulan from molasses and found that pullulan formation occurred only after NH_4^+ was exhausted. They suggested that NH_4^+ may regulate the activities of key enzymes, causing a shift in the carbon flow to biomass production at the expense of polysaccharide synthesis when its concentration in the medium exceeds a certain level.

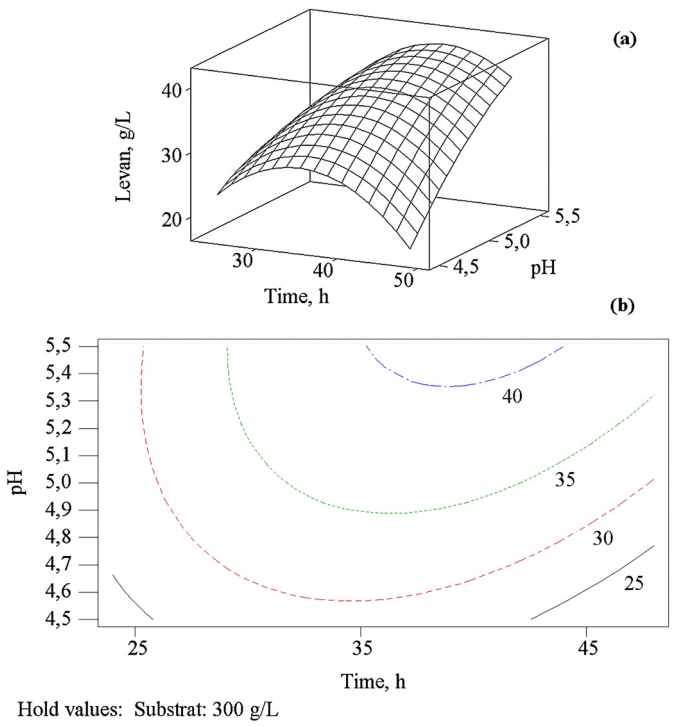

Fig. 3. Response surface plot (a) and contour plot of (b) the combined effects of incubation time and initial pH on levan production by *Z. mobilis* B-14023 with constant initial sugar concentration (300 g/L).

3.3. Optimization of levan production in batch culture

Our preliminary experiments showed that the initial substrate concentration, incubation time and initial pH influenced the production of levan by *Z. mobilis* B-14023. Thus response surface methodology was used to determine the optimum levels of these parameters leading to a maximum levan synthesis. The levels of these factors (Table 1) used in the optimization studies by RSM were determined by preliminary experiments. The effect of the three previously mentioned variables, each at three levels and their interactions on levan production has been determined by carrying out twenty experiments given by the face centered design (Table 1). The variables used for the analysis were initial substrate concentration (X_1), incubation time (X_2) and initial pH (X_3). The experimental responses for the 20 runs are presented in Table 1, which shows considerable variation in the amount of levan production depending on the three independent variables in the medium.

Analysis of variance (ANOVA) for the concentration of levan is presented in Table 2. The analysis gives the value of the model and determines the requirement of a more complex model with a better fit. As shown in Table 2, R^2 was 0.993 indicating that the model as fitted, explained 99.3% of the variability in levan concentration. *F*-Test for regression was significant at a level of 5% ($P < 0.05$), indicating that the model is fit and can adequately explain the variation observed in levan concentration with the designed levels of the factors. If the *F*-test for lack of fit is significant, then a more complicated model is needed to fit the data. As seen in Table 2, the lack of fit (0.115) was not significant at the 5% level ($P > 0.05$) indicating that the experimental data obtained fitted well with the model. These results show that the model chosen can satisfactorily explain the effects of initial substrate concentration, incubation time and initial pH on levan production by *Z. mobilis* B-14023 in shake flask cultures using synthetic medium containing sucrose as substrate.

Twenty experiments were carried out from the design and by applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain levan production by *Z. mobilis*.

$$Y = -426.757 + 1.6103X_1 - 0.631952X_2 + 80.5715X_3 - 0.00264503X_1^2 - 0.0543235X_2^2 - 7.70634X_3^2 + 0.00557660X_1X_2 - 0.0439856X_1X_3 + 0.593507X_2X_3 \quad (2)$$

where X_1, X_2 and X_3 are the actual levels of factors shown in Table 1.

Regression analysis (Table 2) of the experimental data showed that the independent variables, X_1 (initial substrate concentration), X_2 (incubation time) and X_3 (initial pH) had a significant effect on levan production ($P < 0.05$). The positive coefficients for X_1 and X_3 indicate a linear effect to increase levan production, while negative coefficient for X_2 shows a linear effect to decrease levan production. Probability (P) values were used as a tool to check the significance of each of the coefficients. The smaller the magnitude of P value, the more significant was the correlation with the corresponding coefficient. Among the three factors tested, initial pH had the highest impact on levan concentration as given by the highest linear coefficient (80.5715), followed by initial substrate concentration (1.611030) and incubation time (−0.0631952). These factors also showed significant negative quadratic effects on levan production indicating that levan concentration increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Interaction between these parameters was also significant as shown by low P values ($P < 0.05$) for interactive terms.

Response surfaces were plotted by using Minitab Statistical Software to study the effects of parameters and their interactions on polysaccharide production. The results of levan production affected by initial substrate concentration, incubation time and initial pH are presented in Figs. 3–5. These types of plots show effect of two factors on the response (levan concentration) at a time and the other factor was kept at level zero. From the 3D response surface plots and the corresponding contour plots, the optimal values of the independent variables can be observed and the interaction

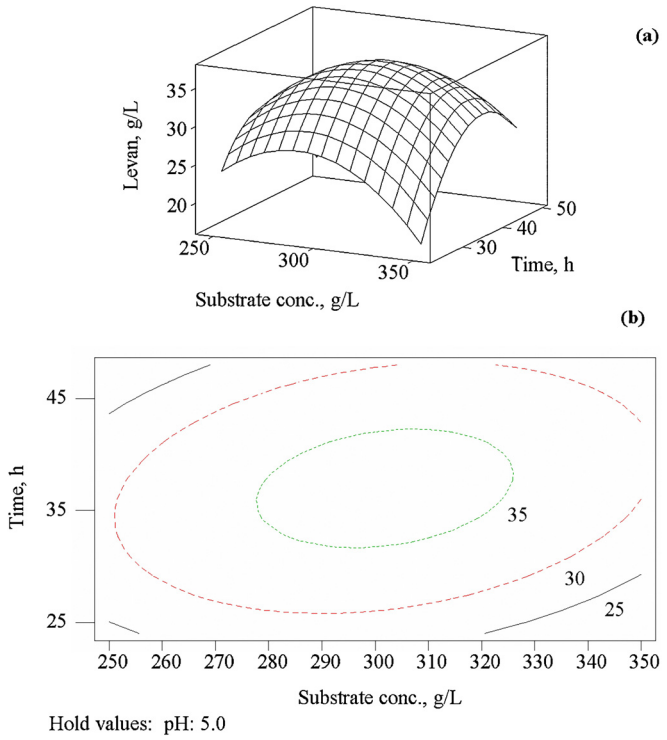

Fig. 4. Response surface plot (a) and contour plot of (b) the combined effects of initial substrate concentration and incubation time on levan production by *Z. mobilis* B-14023 with constant initial pH (5.0).

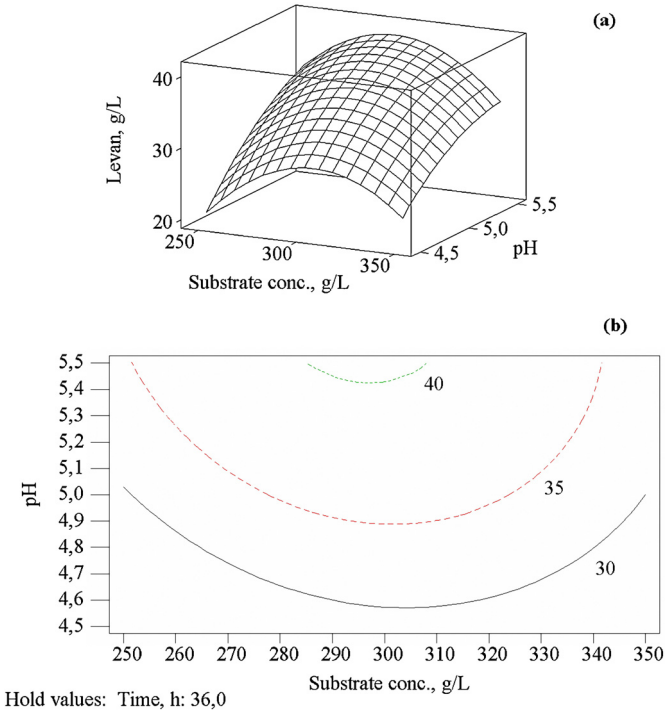

Fig. 5. Response surface plot (a) and contour plot of (b) the combined effects of initial substrate concentration and initial pH on levan production by *Z. mobilis* B-14023 with constant incubation time (36 h).

between each independent variable's pair can be easily understood. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. An elliptical nature of the contour plots indicates that the interactions between the independent variables are significant. The maximum value predicted by the surface was confined in the smallest ellipse in the contour diagram.

Fig. 3 shows the 3D plot (a) and its corresponding contour plot (b) showing the effect of incubation time and initial pH on levan production, while initial substrate concentration was fixed at its middle level (300 g/L). As shown in Fig. 3, for the same levels of pH and incubation time, the concentration of levan decreased from the middle to high pH or incubation time levels. It is interesting to note that the maximum levan concentration was obtained at pH 6 which is higher than the middle level (pH 5).

Fig. 4 presents 3D plot (a) and its corresponding contour plot (b) showing the effects of initial substrate concentration and incubation time on levan production, while the initial pH was fixed at its middle level (pH 5). It is evident that levan production gradually decreased when incubation time exceeded optimal conditions (42 h). This indicated that an increase in incubation time would not further increase the production of levan. This fact is important in making the whole process economically more feasible and in making it possible to shorten the incubation time in the potential industrial application.

The 3D response surface plot (a) and the contour plot (b) in Fig. 5, which give levan production as a function of initial substrate concentration and initial pH at a fixed incubation time (36 h), indicated that levan production increased with the increase of substrate concentration from 250 to 300 g/L, but beyond 300 g/L, levan synthesis by the organism decreased gradually with the increase of carbon source. The optimum substrate concentration of 300 g/L is a high level of substrate concentration for many fermentation processes, however, it is well known that levan production by *Z. mobilis* occurs at high sucrose concentrations (de Oliveira et al., 2007). Contrary to our findings, Borsari et al. (2006) produced levan from sugar cane juice by *Z. mobilis* and they stated that levan production decreased by 46.2% with the increase of total sugar concentration from 200 g/L to 300 g/L. Melo et al. (2007) produced 14.67 g/L levan at an initial sucrose concentration of 250 g/L and they found 3.13 g/L increase in levan production by increasing the initial sucrose concentration from 150 g/L to 250 g/L.

Figs. 3–5 show in general that the maximum levan production was obtained at middle level of each pair of factors at a constant middle level of the other factor. Further increase in these factors above the middle level (initial substrate concentration: 300 g/L, incubation time: 42 h, initial pH: 6.0) showed decrease in levan concentration. In order to determine the maximum levan concentration corresponding to the optimum levels of initial substrate concentration, incubation time and initial pH, a second order polynomial model was used to calculate the values of these variables. The fitting of the experimental data to Eq. (2) allowed the determination of the levels of initial substrate concentration (X_1 = 299.1 g/L) incubation time (X_1 = 42.3 h), and initial pH (X_3 = 6.0) giving a maximum levan concentration of 42.6 g/L. The above data optimizes levan production from synthetic medium by *Z. mobilis* B-14023 in batch culture.

The final fermentation was performed using hydrolyzed starch medium with the optimized levels of initial substrate concentration (299.1 g/L) incubation time (42.3 h), and initial pH (6.0) given by the model. Maximum levan production (40.2 ± 0.57 g/L) which was slightly lower than the value given by the model (42.6 g/L), was obtained at 42.3 h of fermentation.

3.4. Production of levan by Ca-alginate immobilized *Z. mobilis* B-14023 using continuous culture fermentation

Although traditional batch fermentation appears to dominate the industry today, there is much interest in developing more rapid methods of producing bioproducts, such as continuous culture. In order to provide information on levan production and volumetric

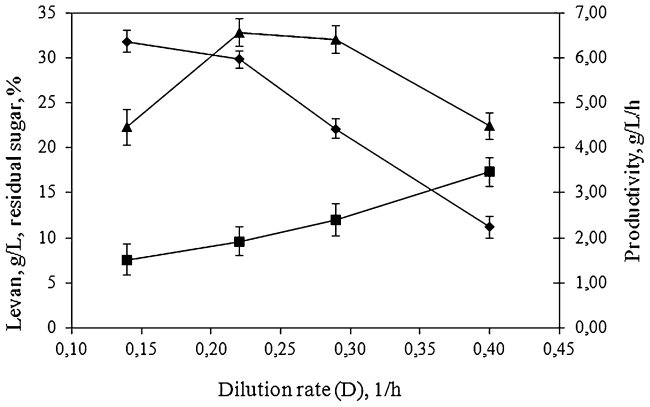

Fig. 6. The effect of dilution rate (D, h^{-1}) on the concentration of levan (g/L), residual sugar (%) and productivity (g/(L h)) in the effluent of continuous fermentation of Ca-alginate immobilized *Z. mobilis* B-14023 (♦: levan, ■: residual sugar and ▲: productivity) (28 °C, pH 6.0, initial substrate concentration = 299.1 g/L, average of three replicates ± standard deviation).

productivities over a range of dilution rates, *Z. mobilis* B-14023 cells immobilized in Ca-alginate beads were used in a packed bed bioreactor. The optimized initial sucrose concentration (299.1 g/L) and initial pH (6.0) values were used in continuous levan production experiments. The fermentation medium was loaded to a packed bed bioreactor containing Ca-alginate immobilized *Z. mobilis* B-14023 cells at 28 °C. Dilution rates (D, h^{-1}) analyzed were 0.14, 0.22, 0.29 and 0.4 h^{-1}. The system was considered to be at steady state after at least five residence times. The steady state concentrations of levan and residual sugar together with volumetric productivity for each dilution rate are shown in Fig. 6. Maximum levan concentration (31.8 ± 0.21 g/L) was attained at $D = 0.14\ h^{-1}$ with a residual sugar concentration of 75.4 g/L. Increasing the dilution rate decreased the levan and increased the residual sugar concentrations. Volumetric productivity values increased gradually with increasing dilution rates and gave a peak at 0.22 h^{-1}, where maximum volumetric productivity (6.556 g/(L h)) was obtained. High volumetric productivity resulted in incomplete utilization of substrate; hence the concentration of residual sugar was 96.2 g/L at this dilution rate. The maximum volumetric productivity obtained in this study was higher than that reported by Beker, Shvinka, Pankova, Laivenieks, and Mezhbarde (1990) for *Z. mobilis* grown continuously at $D = 0.05\ h^{-1}$ (3.20 g/(L h)).

Our results showed that there are two zones of dilution rate that are particular interest concerning levan production from sucrose: around $D = 0.22\ h^{-1}$ where the volumetric productivity of the system is at a peak, and below $D = 0.14\ h^{-1}$ where sugar utilization and levan production values are at maximum.

One of the main drawbacks of alginate gels is their susceptibility to cation chelating agents such as phosphate and lactate, which can cause instability of the beads (Göksungur, Gündüz, & Harsa, 2005). In this study, levan was produced with alginate beads for almost 2–3 days. Shrinkage, deformation and small cracks on the surface of the beads were observed at the end of this period. As the fermentation progressed further, the beads lost their hardness and were completely disrupted in the medium. It is possible that levan formation in the beads together with the increase in cell biomass and possibly CO_2 could lead to the disruption of beads. Bekers et al. (2001) compared levan production capacity of *Z. mobilis* cells entrapped in Ca-alginate beads with cells attached to stainless steel wire spheres and Al_2O_3 granules. They found that stainless steel wire spheres and Al_2O_3 granules with immobilized bacteria could be used as an inoculum generator for sucrose fermentation, however, the general producer of levan and ethanol was the free suspended cell biomass. They found that levan and CO_2 accumulated in the Ca-alginate beads, increasing their volume and resulting in degradation after 4–5 days.

Within the scope of the present work, packed bed bioreactors with their lower input and operation costs seem to be efficient and stable bioreactors for levan production. The important drawback of this type of bioreactor is the existence of pressure drop or resistance to flow across the bioreactor bed and deviation of the bioreactor from plug-flow behavior. This problem can be solved by using tapered columns or special column configurations (Göksungur & Zorlu, 2001). In this study, the major problem was the disruption of beads after a certain period of time. Although many attempts have been made to improve the stability of Ca-alginate beads, such as covering the beads with poly-L-lysine, treating the beads with polyethyleneimine, glutaraldehyde, hexamethylenediamine and coating the beads with chitosan (Göksungur, Gündüz, et al., 2005), further investigations will continue to focus on the improvement of the stability of Ca-alginate beads for levan production.

4. Conclusion

In this study levan was produced by *Z. mobilis* B-14023 cells using synthetic medium containing sucrose in batch and continuous culture. Initial substrate concentration, incubation time and initial pH were the three important process variables for levan production in batch culture. Linear, quadratic and interaction effects of these variables on levan production were determined using response surface methodology and the model chosen satisfactorily explained the effects of the above-mentioned process variables. By fitting the experimental data to a second order polynomial equation, the optimum levels of initial substrate concentration (X_1 = 299.1 g/L), incubation time (X_2 = 42.3 h) and initial pH (X_3 = 6.0) were determined. The maximum concentration of levan at the optimum process parameters was 40.2 g/L.

Cell immobilization in Ca-alginate gels offer a rapid, mild, simple, cheap and versatile technique which may be applied to a wide range of cells. However, there is little information in the literature about levan production using immobilized cells in continuous culture. In this study, continuous culture experiments were done in a packed bed bioreactor using Ca-alginate immobilized bacterial cells. The optimized conditions found in batch experiments were used in continuous culture experiments. In the continuous fermentation, two important zones of dilution rate were determined: $D = 0.22\ h^{-1}$ where the volumetric productivity of the system is at a peak (6.556 g/(L h)), and $D = 0.14\ h^{-1}$ where the highest levan concentration (31.8 g/L) was obtained.

This study shows that continuous fermentation in a packed bed bioreactor with immobilized *Z. mobilis* cells is an efficient and stable system for levan production with lower input and operation costs. However, the major drawbacks are the existence of pressure drop and disruption of Ca-alginate gel beads in the long term fermentation. Therefore, further research will focus on improvement of the stability of gel beads for continuous levan production in packed bed bioreactor.

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