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Optimization of levan production from *Bacillus licheniformis* using response surface methodology

Imen Dahech*, Rania Bredai, Karima Srih
Biochemistry Laboratory, Faculty of Sciences of Sfax, PB 802, 3018 Sfax, (TUNISIA)
E-mail : imenbiologie@yahoo.fr

ABSTRACT

Optimized conditions for the levan production are necessary to increase its industrial application. This study aimed to optimize the production of levan synthesized by *Bacillus licheniformis* by factorial design and response surface methodology. The variables involved in this study were sucrose concentration (X1), temperature (X2), agitation (X3) and yeast extract concentration (X4). In view of the independent variables studied, the experiment showed the best results at: sucrose concentration (300 g/l), temperature 40°C, agitation 150 rpm and yeast extract concentration 3 g/l when an average production of 30.66 g/l was obtained.

KEYWORDS

Bacillus licheniformis;
Levan;
Factorial design;
Optimization.

INTRODUCTION

Levan, a β-(2→6) fructan biopolymer with occasional β-(2→1) branching, was found in many plants and microbial products[1]. It presents low viscosity, high solubility in water and biocompatibility[2] as well as other properties that can have industrial applications in the fields of cosmetics and pharmaceuticals as an antitumoral, immune modulator and hypocholesterolemic agent[3,4,5]. In the food industry, it is used as a fructose source and for the production of fructooligosaccharides[6], thickener, stabilizer, encapsulating agents and carrier for flavor and fragrances[7].

This high molecular weight polysaccharide is produced from sucrose-based substrate by transfructosylation reaction of levansucrase (EC 2.4.1.10) by a variety of microorganisms. The levan produced can be of high or low molecular weight, according to the fermentation conditions and this defines its application[8-10].

Although many investigations on the levan production have been reported, all suffer the disadvantages of low yield and contaminating of impure products. The application of statistical designs for experiments and its modeling is important in a scientific study because it defines the effect of various factors and its interaction that leads to the optimization of the process. This instrument has been used in biotechnology by various authors[11-13].

The response surface methodology (RSM) is an optimization technique based on factorial design that has been used to model several industrial processes, minimizing the empiricism that is involved in the technique of trial and error[14]. This method has been used successfully by some authors in levan production[15,16].

The objective of the present study was to assess,

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by statistical methods, the production of levan by *B. licheniformis*, varying the sucrose concentration, temperature, agitation and yeast extract concentration.

MATERIALS AND METHODS

Microorganism and maintenance culture medium

The *B. licheniformis* strain was used. The bacteria were maintained at 4°C in a culture medium containing in g/l: Na_2HPO_4 3.5, NaH_2PO_4 0.8, $MgSO_4$ 0.2, $NaNO_3$ 3.5, yeast extract 5.0 and sucrose 5.0. The cultures were kept at 4°C and renewed every 4 weeks.

Inoculum preparation

Inoculum was prepared from activated culture using 125 ml Erlenmeyer flasks with 25 ml of inoculum medium, containing in g/l: sucrose 5, trypton 10, NaCl 5.0 and yeast extract 5.0, pH 7.4. After incubation for 18 h at 40°C, the inoculum was centrifuged for 20 min at 9000 rpm. The cells were decanted and resuspended in sterile distilled water. The cell concentration was determined by turbidimetry at 600 nm.

Fermentation conditions

The fermentation medium consisted in g/l: Na_2HPO_4 3.5, NaH_2PO_4 0.8, $MgSO_4$ 0.2, yeast extract 5.0 and sucrose 200. Following heat sterilization (121°C) for 20 min, each flask was inoculated with 5% (v/v) of the subculture and incubated for 24 h at 40°C on a rotary shaker (200 rpm). Experiments were carried out in triplicate. The culture time (h), pH, agitation (rpm) and sugar concentration (g/l) of fermentations were varied according to the factorial planning (TABLE 1).

Enzyme source and assay

The fermentations were harvested by centrifugation at 9000 rpm, 4°C for 20 min. The supernatant was used as enzyme source. Levansucrase activity was estimated according to the method of Yanase et al,[17] with some modification. 0.5 ml of culture filtrate was incubated with 1 ml of 20% sucrose and 1ml of 0.1 M acetate buffer at pH 5.2 and incubated at 30°C for 15 min. The reducing sugars produced were measured by glucose oxidase kits. One unit of enzyme activity was defined as the amount of enzyme that produces reducing sugars equivalent to 1µmol of glucose per min.

TABLE 1 : Full factorial design for investigation of the factors: Sucrose concentration, temperature, agitation and yeast extract concentration on the levan production by *B. licheniformis*

Runs	Variables				Levan (g/l)
	X_1	X_2	X_3	X_4	
1	-1	-1	-1	-1	
2	1	-1	-1	-1	
3	-1	1	-1	-1	
4	1	1	-1	-1	
5	-1	-1	1	-1	
6	1	-1	1	-1	
7	-1	1	1	-1	8
8	1	1	1	-1	30.66
9	-1	-1	-1	1	
10	1	-1	-1	1	
11	-1	1	-1	1	
12	1	1	-1	1	
13	-1	-1	1	1	
14	1	-1	1	1	
15	-1	1	1	1	10.66
16	1	1	1	1	28.25
17	0	0	0	0	11.33
18	0	0	0	0	10.66
19	0	0	0	0	9.21

		Real levels		
		-1	0	1
X_1	Sucrose concentration (g/l)	10	20	30
X_2	Temperature (°C)	20	30	40
X_3	Agitation (rpm)	50	100	150
X_4	Yeast extract concentration (g/l)	3	5.5	8

Levan determination

The levan concentration was determined for all the fermentations after centrifugation at 9000 rpm and 4°C for 20 min. The levan formed was estimated by reducing sugar using the dinitrosalicylic acid method[18] after precipitating the supernatant with ice-cold absolute ethanol and acid hydrolysis.

Cellular biomass

The biomass was determined in a spectrophotometer based on 600 nm a calibration curve. The fermented broth was centrifuged at 4000g for 20 minutes. The sediment was suspended in distilled water and centrifuged again twice at the same conditions and the cell sediment was diluted in appropriate volumes for spectro-

photometric determinations.

Factorial design

Once the variables having the greatest influence on the responses were identified, a CCD[19] was used to optimize the levels of these variables. A factorial design was performed in order to determine the optimal conditions for the production of levan by *B. licheniformis*. In this study, the independent variables chosen were sucrose concentration, temperature, agitation and yeast extract concentration.

The studied dependent variable (Y) was levan production, expressed in g/l, and the results were analyzed by the STATISTICA applicative 7.0 (multiple regression) including parameters estimation and analyses of variance (ANOVA).

RESULTS AND DISCUSSION

Based on earlier studies, sucrose concentration, temperature, agitation and yeast extract concentration medium were identified as the major factors affecting levan production by *B. licheniformis*. In the present work, these variables were statistically optimized with the help of a factorial design using RSM. The experimental design and the results are shown in TABLE 1. The best results obtained in the levan production occurred with the highest sugar concentration (300 g/l), observed in the trials 8 and 16 with production of 30.66 and 28.25 g/l levan, respectively. Different authors had noticed the importance of carbohydrate concentration on levansucrase activity. At high sucrose concentrations, oligosaccharides and polysaccharide polymerization was the reaction most catalyzed by levansucrase[20]. Sucrose was considered a good inducer of the synthesis of levansucrase. Shih *et al.* (2005)[21] observed that 200 g/l of sucrose produced 40-50 g/l of levan from *B. subtilis* isolated from Natto. TABLE 1 showed that the sucrose concentration had a positive linear effect on the production of levan. These results indicated that in order to maximize the production of levan, the concentration of sucrose must be increased. According to results, the best levan production (30.66 g/l) was obtained using 300 g/l of sucrose, at temperature 40ºC, orbital agitation at 150 rpm and yeast extract concentration.

The second degree polynomial, which includes the quadratic terms, presents only those coefficients that were considered significant by analysis of variance (TABLE 2):

$$Y = 3.11 + 1.68\,X_1 + 4.18\,X_2 + 3.28\,X_3 + 0.01\,X_4 + 2.52\,X_1X_2 + 2.52\,X_1X_3 - 0.32\,X_1X_4 + 4.85\,X_2X_3 + 0.016\,X_2X_4 + 0.016\,X_3X_4$$

The value of the adjusted coefficient of determination (R^2), observed for the levan production response (90%) and non-significant lack of fit (TABLE 3) suggested a good fit of the model to the experimental data and that it could be used for prediction purposes.

TABLE 2 : Results of regression analysis of the factorial design

Term	Coefficient	T-statistic	P-value
Intercept	3.11	5.209	0.007
X_1	1.68	2.67	0.1172
X_2	4.18	16.555	0.0006*
X_3	3.23	9.915	0.0048*
X_4	0.01	0	0.9920
X_1X_2	2.52	4.004	0.0585
X_1X_3	2.52	4.004	0.0585
X_1X_4	-0.32	0.064	0.8034
X_2X_3	4.85	14.873	0.0009*
X_2X_4	0.016	0	0.9902
X_3X_4	0.016	0	0.9902

X_1: Sucrose concentration, X_2: Temperature, X_3: Agitation, X_4: Yeast extract concentration, *Significant (p<0.05)

TABLE 3 : Analysis of variance (ANOVA) for the model regression representing levansucrase activity

Source	SS	DF	MS	F-value	P-value
Model	1316.995	10	131.699	5.209	<0.001
Residual	530.987	21	25.285		
Lack of Fit	431.556	14	30.825	2.170	0.1531
Pure Error	99.431	7	14.204		
Total (corr.)	1847.982	31			

R^2 = 0.9; SS, sum of squares; DF, degrees of freedom; MS, mean square

Results show a positive effect of the independent variables X_2 (temperature) and X_3 (agitation) in levan production (Figure 1). Belghith et al.[22] studied the production of levan in *Bacillus sp.* and found optimal conditions at pH 6.5 and temperature of 50°C. Likewise, Dolle et al.[23] verified that 25ºC at pH 5.0 is the ideal condition for levan production. Moreover, Reiss & Hartmeier[24] demonstrated good productivity of this

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polymer at temperature varying between 27-30ºC at pH 5.0. Furthermore, low temperatures stimulate transfructolysation reactions and yet lengthen enzyme activity. On the other hand, higher temperatures stimulate sucrose hydrolysis[25-27].

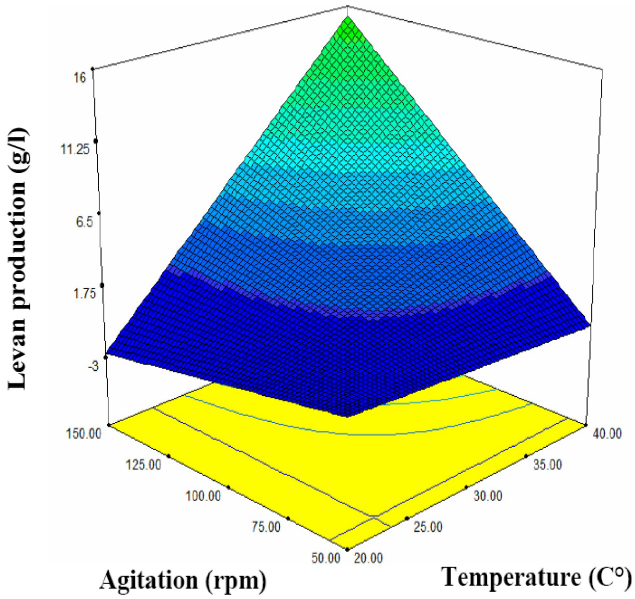

Figure 1 : Response surface/contour plot showing effect of independent variables: Sucrose concentration, temperature, agitation and yeast extract concentration on the levan production by *B. licheniformis*

The optimum conditions found here for the levan production from *B. licheniformis* were as follows: 300 g/l of sucrose, temperature 40°C, agitation 150 rpm and yeast extract concentration 3 g/l, when an average production of 30.66 g/l was obtained.

Magalhães[28] demonstrates that agitation facilitates medium homogeneity, allowing the access of the microorganism to the substrate. In fact, increasing agitation (≥ 100 rpm), improves levan yield and increase considerably medium oxygenation.

CONCLUSION

The experiments showed that yeast extract does not affect levan production by *B. licheniformis*. However, levan production is affected by initial concentration of sucrose, temperature and agitation in the experimental ranges studied. The best conditions for levan production (30.66 g/l) were 300 g/l of sucrose, at temperature 40ºC, orbital agitation at 150 rpm and yeast extract concentration 3 g/l.

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