Asian Journal of Biological and Life Sciences | Original Research

Performance of response surface model for increase of dextransucrase production by *leuconostoc mesenteroides FT 045B* under different experimental conditions

Mary Helen Palmuti Braga Vettori[1], Cristian Jaques Boner de Lima[1], Kate Cristina Blanco[1], Mariana Cortezi[1], Jonas Contiero[1]*

1) Unesp - Univ Estadual Paulista, Biological Sciences Institute, Department of Biochemistry and Microbiology, Laboratory of Industrial Microbiology, 24A Ave., Bela Vista, IBRC, Rio Claro, SP, CEP 13506-900, Brazil

Submitted : 04.03.2012 Accepted :25.3.2012 Published : 10.5.2012

Abstract

Optimization of the medium for dextransucrase production by *Leuconostoc mesenteroides FT 045B* was carried out using response surface methodology. A central composite design was employed to determine maximal dextransucrase activity at optimal values of sugarcane molasses, corn steep liquor and potassium phosphate. The combined effects of these nutrients on dextransucrase activity were used to evaluate agitation speed and aeration rate through a full factorial design. A model with satisfactory fit was achieved in both cases. Dextransucrase activity was significantly affected by the independent variables corn steep liquor and K_2HPO_4 using the central composite design and by the independent variables agitation speed and aeration rate using the full factorial design. Maximal dextransucrase activity of 4.03 U/mL was obtained with sugarcane molasses 40 g/L, K_2HPO_4 20 g/L, corn steep liquor 20 g/L and 3.99 U/mL with agitation speed 100 rpm and aeration rate 1 vvm.

INTRODUCTION

Dextransucrase (glucansucrase or glucosyltranferase) EC 2.4.1.5 is an extracellular enzyme that synthesizes dextran from sucrose. Dextran is a D-glucose polymer composed of α(1-6) linkages in the linear chain and α(1,2), α(1,3) and/or α(1,4) branch linkages [1]. Various species of the genera Streptococcus, Leuconostoc and Lactobacillus are able to synthesize dextran from sucrose [2]. Dextran is an important polysaccharide that is currently the subject of many studies due to its successful industrial applications [3-9].

A number of authors have described the effects of sucrose concentration, aeration rate, agitation speed, medium pH, incubation temperature, nature of the yeast extract and other nutritional requirements on the production of dextransucrase, dextran and fructose from sucrose using different strains of Leuconostoc mesenteroides [10-12]. For the FT 045B strain, however, only the effect of temperature and a culture optimization condition have been studied and no experimental design has been applied to evaluate dextransucrase production by this strain [13].

Statistical experimental methods have been employed in recent years to optimize media for industrial purposes. Response surface methodology (RSM) is a collection of statistical methods for designing experiments in which several factors are varied simultaneously, models are built and optimal conditions are determined [14]. RSM has been successfully used in the optimization of bioprocesses, as it reduces the number of experiments, improves the statistical interpretation possibilities and indicates whether parameters interact.

The main objective of the present paper was to apply response surface methodology based on a central composite design (CCD) and full factorial design (FFD) for the optimization of culture medium components and culture conditions in order to enhance dextransucrase production by Leuconostoc mesenteroides FT 045B.

MATERIALS AND METHOD

Organism

Leuconostoc mesenteroides FT 045B isolated from an alcohol and sugar mill plant was provided by the Microbiology Industrial Process Control Division of Fermentec (Brazil), and the nucleotide sequence was submitted to GenBank sequence database provided by the National Center for Biotechnology Information (NCBI), GenBank ID: JF812153-1.

Central composite design

An experimental CCD was carried out in order to identify and optimize the nutrients in the production medium [sugarcane molasses (SCM), corn steep liquor (CSL) and potassium phosphate (K_2HPO_4)] that have a significant effect on dextransucrase production. For two factors, this design was made up of a full 2^3 factorial design, with eight cube points, augmented with three replications of the center points and six-star points, that is, points having an axial distance to the center of ±α (1.682) for one factor (totaling 17 experiments), while the other factor is at level 0. All experimental variables, factors and interaction effects on the response were investigated. The independent variables, experimental range and levels investigated for the CCD are given in Table 1.

The objective of the second experiment was to obtain a more precise estimate of the optimal operating conditions for the factors involved. Thus, a full factorial design (FFD) circumscribed 3^2 experimental design was used to optimize the levels of agitation speed (100, 150 and 200 rpm) and aeration rate (0.0, 0.5 and 1.0 vvm), resulting in a total of nine experiments. Samples were taken every 2 h throughout 10 h of fermentation.

In performing the regression equation for both the CCD and FFD, the test variables were coded based on Equation. 1:

$$x_i = \frac{(X_i - X_{cp})}{\Delta X_i}$$

Table 1 : Experimental range and levels of independent variables used in central composite design

Independent Variables		Range and levels				
		-α	-1	0	+1	+α
Production medium optimization (g/L)						
Sugar cane molasses (SCM)	X_1	23.2	30	40	50	56.8
Corn steep liquor (CSL)	X_2	0	3.11	10.11	17.11	21.77
Potassium phosphate (K_2HPO_4)	X_3	11.59	15	20	25	28.41

Table 2: Central composite experimental design and response values of dextransucrase activity at 24 h of fermentation of *L. mesenteroides FT045B*

Experiment	X_1[a]	X_2[b]	X_3[c]	Dextransucrase activity (IU/mL)
1	-1.000	-1.000	-1.000	1.23
2	-1.000	-1.000	1.000	1.52
3	-1.000	1.000	-1.000	2.62
4	-1.000	1.000	1.000	3.25
5	1.000	-1.000	-1.000	1.43
6	1.000	-1.000	1.000	1.81
7	1.000	1.000	-1.000	2.29
8	1.000	1.000	1.000	2.50
9	-1.682	0.000	0.000	0.41
10	1.682	0.000	0.000	0.23
11	0.000	-1.682	0.000	1.79
12	0.000	1.682	0.000	4.03
13	0.000	0.000	-1.682	3.25
14	0.000	0.000	1.682	3.38
15	0.000	0.000	0.000	2.96
16	0.000	0.000	0.000	2.96
17	0.000	0.000	0.000	2.96

[a] SCM concentration; [b] CSL concentration; [c] K2HPO4 concentration.

in which x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_{cp} is the real value of an independent variable at the center point and ΔX_i is the step change value.

Statistical analysis

The Statistic 7.0 software package (Stat Soft, Tulsa, OK, USA) was used for the experimental designs and regression analysis of the experimental data. RSM was used to understand the interaction of different variables and applied to identify optimal levels [14]. Analysis of variance (ANOVA) of the model was performed. The quality of the polynomial model equation was statistically judged by the coefficient of determination (R^2) and its statistical significance was determined by an F-test. The significance of the regression coefficients was tested by a t-test.

Inoculum and flask fermentation

The following was the growth medium for *L. mesenteroides FT 045B* (g/L): SCM 40, **K_2HPO_4** 20, $CaCl_2$ 0.02, $MgSO_4$ 0.2, NaCl 0.01, $FeSO_4$ 0.01 and $MnSO_4$ 0.01. The initial pH was adjusted to 7.4 and the medium was then sterilized at 121 ºC for 15 min. The strain was stored at -20 ºC in cryogenic culture solution and then transferred (7.5 mL) to the growth medium in Erlenmeyer flaks containing a working volume of 142.5 mL. The growth medium was incubated for 36 h at 30 ºC and 150 rev min^{-1} in a thermo-shaker incubator. A total of 5% (v/v) of the inoculum was transferred to 300-mL Erlenmeyer flasks containing 142.5 mL of production medium agitated at 150 rmp in a incubator at 24 ± 1 ºC for 48 h. The production medium consisted of the same salts as in the growth medium, with the addition of CSL ranging from 3.109 and 15 g/L, SCM ranging from 30 and 50 g/L and

K_2HPO_4 ranging from 15 and 25 g/L.

Inoculum and bioreactor fermentation

The fermentation inoculum was prepared up to 5% (v/v) of the total volume of the production medium. The inoculum medium (pH 6.7) and reactor with production medium were sterilized at 121 ºC for 15 min. The microorganism was transferred from the stock culture to the growth medium, incubated for 36 h at 30 ºC in stirred flasks (150 rpm) and then transferred to the reactor. The working volume for fermentation was 1.2 liters, with pH controlled at 6.7 by the addition of NaOH solution 5M and the temperature maintained at 26 ± 1 ºC.

Analytical methods

Dextransucrase activity was assayed by measuring the amount of dextran produced [15]. The enzyme digest was prepared by the addition of 1880 µL of buffer (pyridinium/acetate, pH 5.2) to 2000 µL of 400 mM sucrose, which was pre-warmed to the desired temperature (30 °C in the experiments for this study). The reaction was initiated by the addition of 120 µL of sufficiently diluted dextransucrase. Aliquots of 500 µL of the reaction digest were taken at various times and added directly to 1000 µL of cold (0-10 °C) anhydrous ethanol, mixed and centrifuged for 15 min at 13,000 × g using tarred centrifuge tubes. The supernatants were removed and the precipitates were dissolved in 500 µL of water, mixed and added to 1000 µL of anhydrous ethanol, mixed, centrifuged, dissolved in 500 µL of water and precipitated a third time. The precipitates were treated four times with 1000 µL of acetone and once with 1000 µL of anhydrous ethanol, dried under an infrared heat lamp and weighed. The number of µmoles of D-glucose incorporated into dextran was obtained by (µg of weighed dextran) divided by 162 (the anhydrous weight of D-glucose incorporated into dextran), from which the International Unit (IU) was computed (µmoles of D-glucose incorporated into dextran per min per mL of dextransucrase).

RESULTS AND DISCUSSION

Optimization of fermentative production of dextransucrase medium

RSM was employed to optimize maximal dextransucrase activity. The experiments were carried out based on a CCD, which is an optimal design that allows the calculation of the effects and interactions of each of the four factors chosen with the best precision possible and a minimal number of experiments. Table 2 displays the results of dextransucrase activity for each of 17 experiments at 24 h of fermentation.

The highest dextransucrase activity was acquired with the highest CSL concentration (Experiment 12). Considering SCM as the carbon source and CSL as the nitrogen source, the experiments conducted with constant K_2HPO_4 concentration revealed that an increase in nitrogen/carbon ratio leads to an increase in dextransucrase activity, as demonstrated in Experiments 1 and 3 (1.23 and 3.25 IU/mL, respectively), 5 and 8 (1.43 and 2.50 IU/mL, respectively), 13 and 12 (3.25 and 4.03 IU/mL, respectively) and 11 and 14 (1.79 and 3.38 IU/mL, respectively). Lopretti and Martinez, in 1999 [16], report the same association between this ratio and dextransucrase production.

The response surface quadratic model was performed in the form of analysis of variance (ANOVA) and the results are summarized in Table 3.

Table 3 displays three linear terms, three quadratic terms and three interaction terms. The significance of each coefficient was determined using the F test and *p*-values. The corresponding variables were more significant when the absolute F-value was larger and the *p*-value was smaller. The results ($p > 0.05$) revealed that the linear term of X_1 (SCM), quadratic term of X_2 (CSL) and interaction terms of X_2X_3 (CSL and K_2HPO_4) and X_1X_2 (SCM and K_2HPO_4) could be discarded, thereby allowing the model to be

Table 3: Analysis of variance for quadratic model determined from central composite design (CCD)

Source	Sum of Squares	Degrees of freedom	Mean square	F-Value	Value P > F
(1) SCM (g/L) (L)[d]	0.038	1	0.038	1.776	0.224
SCM (g/L) (Q)[e]	9.574	1	9.574	451.763	0.000
(2) CSL (g/L) (L)	5.157	1	5.157	243.347	0.000
CSL (g/L) (Q)	0.000	1	0.000	0.000	0.994
(3) K_2HPO_4 (g/L) (L)	0.215	1	0.215	10.129	0.015
K_2HPO_4 (g/L) (Q)	0.215	1	0.215	10.162	0.015
(1L) For (2L) (interaction)	0.301	1	0.301	14.194	0.007
(1L) For (3L) (interaction)	0.014	1	0.014	0.642	0.449
(2L) For (3L) (interaction)	0.001	1	0.001	0.048	0.832
Error	0.148	7	0.021		
Total SS	18.268	16			

[d] (L)= linear variable; [e] (Q)= squared variables

reduced.

The adjusted second-order polynomial empirical equation that represents enzyme yield is expressed in Equation 2 (Ya):

$Ya= 2.926 + 0.618X_2 + 0.127X_3 - 0.196X_1X_2 \quad 0.947X_1X_1 - 0.029X_2X_2 + 0.114X_3X_3/2/$

The goodness of fit of the model was checked by the coefficient of determination (R^2) and coefficient of multiple correlation (R). In this case, the R^2 value (0.988) for Equation 2 indicates that 98.8% of the sample variation in dextransucrase activity was attributed to the independent variables and 1.2% of the total variation cannot be explained by the model.

The effects of the independent variables and their interactions on the formation of the product are illustrated in the analysis of the response surfaces (Figure 1) constructed from Equation 2. An important interaction was found between the variables studied.

Figure 1 demonstrates that an increase in CSL concentration led to increased dextransucrase activity. Maximal dextransucrase activity (4.03 IU/mL) was obtained with 40 g/L of SCM. The optimal range for dextransucrase activity was 16 to 21.77 g/L of CSL and 32 to 44 g/L of SCM. The surface plot (Figure. 2) revealed an increasing trend for dextransucrase activity with the increase in K_2HPO_4 (range: 26 to 28.41 g/L) and when the SCM concentration was close to the central point (32 to 44 g/L). Higher or lower concentrations of SCM did not favor dextransucrase activity. Figure 2(C) confirms the tendency observed in Figures 1(A) and (B), in which dextransucrase activity was improved when the K_2HPO_4 and CSL concentrations were above the central point and when the SCM concentration was close to central point.

The same behavior regarding dextransucrase activity in function of SCM concentration is reported by Jeanes, *et al*, in 1957 [17], who found that a sucrose concentration above 60 g/L inhibited growth cell and dextransucrase production, and by Lopretti and Martinez, in 1999 [16], who found that dextransucrase demonstrated a Michaelis-Menten kinetic model, as a high concentration of SCM (above 50 g/L) inhibited dextransucrase activity.

The increase in enzyme activity with the increase in K_2HPO_4 concentration can be explained in light of findings described by Yusef *et al*, in 1997 [18], who report that an increase in K_2HPO_4 maintains the pH and inhibits enzyme denaturation, thereby favoring dextransucrase production. It has been reported that enzyme production can be enhanced by increasing the amounts of yeast extract (nitrogen source) and K_2HPO_4. Barker and Ajongwen in 1991 [9], Tsuchiya *et al*. in 1952 [10], Stacey in 1942 [20] and Hehre in 1946 [21] studied the effects of concentrations of sucrose, CSL and phosphate on dextran production in whole cultures using high sucrose concentrations. The authors optimized the composition of ingredients and recommend an optimal concentration of 2% each of sucrose, CSL and phosphate for dextransucrase production.

Optimization of dextransucrase production by combination of aeration rate (AR) and agitation speed (AS)

The effect of AR (vvm) and AS (rpm) on enzyme yield by *Leuconostoc mesenteroides FT 045B* was studied using the FFD, totaling 9 experiments. Table 4 displays the results regarding dextransucrase activity in each experiment at 8 h of fermentation.

When the AS values were kept constant, the increase in AR values did not have a significant effect on enzyme activity, which only increased about 0.25 to 0.5 IU/mL in each case (Experiment A to C, D to F and G to I). AS had a significant influence over enzyme activity, as the increase in AS led to a decrease in dextransucrase activity (Experiments A, D and G; B, E and H; C, F and I). An optimal region was found when AR was increased (0.8 to 1.0 vvm) and AS was decreased (80-100 rpm), thereby demonstrating a variation of ± 10 rpm from 90 rpm and ± 0.2 vvm from 1.0 vvm for the maintenance of the optimized conditions.

The *Leuconostoc mesenteroides* NRRL B512 F strain is known to be micro-aerophilic and a number of authors state that oxygen positively affects the growth of this strain [22-27]. However, Goyal and Katiyar [28] found that a still-flask culture is an important condition for acquiring the best production of dextransucrase from *Leuconostoc mesenteroides* NRRL B 512 F.

Veljkovic et al. [11] obtained maximal extracellular dextransucrase production from *Leuconostoc mesenteroides* with the maximal oxygen uptake rate (about 1 mmol O_2/L.h). Alsop [3] found that air sparging provided higher dextransucrase yield in comparison to conditions without aeration or pure oxygen aeration. Champagne and Gardner [29] studied the effects of sugar aeration and fermentor size on viable counts of *Leuconostoc mesenteroides* BLAC grown in MRS broth or a carrot juice medium. In the carrot juice medium, aeration did not have a pronounced effect on the final population level, even though the quantity of viable cells was greater when the culture was aerated. Viable counts were not affected by scaling the volume of the fermentation from 2 to 15 L.

Regulation of the aeration of *Leuconostoc mesenteroides* NRRL B-1299 for dextransucrase production in sucrose fermentation has little effect on the culture profile and enzyme production when compared to that of *Leuconostoc mesenteroides* NRRL B 512 F [30]. Although many studies report that oxygen positively affects high enzyme production, there have been conflicting reports on the production of dextransucrase using shaken and static flask cultures [10,28,31]. Aeration and greater agitation rates of the culture media have been found to not favor the production of dextran or dextransucrase and mild aeration and agitation have been used with uncontrolled pH in these processes [19,32-34]. Goya et al. [35] studied this issue in greater detail, comparing static-flask culture with shaken-flask culture and found that enzyme activity in the static flask was 30% higher [10,19,31,33,36,37].

A possible explication for why AS can negatively affect dextransucrase production is that cells produce dextransucrase and lactic acid during fermentation. In the initial stage, these products are around each cell, dropping the pH at each specific site and forming the appropriate conditions for improving dextransucrase production (pH ~ 5.2). When the AS increases, a faster diffusion of pH occurs around the cell and the pH rises back to 6.7. Thus, the production of dextransucrase decreases and the previously produced dextransucrase is destabilized in such a high pH.

Table 5 displays the results of the second-order response surface models for dextransucrase activity in the form of analysis of variance (ANOVA). Statistical analysis of the data was performed using the Statistics 7.0 program to evaluate the variance and determine the significance of each term in the equations fitted to estimate the goodness of fit in each case.

The regression equation (Yb) demonstrated that dextransucrase activity was an empirical function of the test

variables in coded units, as shown in Equation 3:

$$Yb = 1.9422 - 1.0816Z_1 + 0.2150Z_2 + 0.5316Z_1^2 + 0.2816Z_2^2/3/$$

where Yb is the predicted response (dextransucrase activity) and Z_1 and Z_2 are the coded values of the test variables agitation speed and aeration rate, respectively.

The results reveal that the independent variables AS (1L) and AR (2L) had a strong positive linear effect on the response ($p > 0.05$) (Table 5). The same was observed with the squared variables AS (Q) and AR (Q). Among these, insignificant terms (on the basis of *p*-values greater than 0.05) were neglected. The R^2 value (0.996) indicates that 99.6% of the sample variation in dextransucrase was attributed to the independent variables and only 0.4% of the total variation cannot be explained by the model.

Response surfaces were drawn for the experimental results obtained from the effect of different variables (AR and AS) on dextransucrase production to determine the individual and cumulative effects of these variables as well as their mutual interactions.

Figure 2 illustrates the response surface for the effect of AR and AS on dextransucrase activity.

The greatest dextransucrase activity was acquired with AS at 100 rpm and AR at 1.0 vvm (Figure. 2). An optimized region was found when AR was increased (between 0.8 to 1.0 vvm) and TA was decreased (between 80 to 100 rpm).

Dextransucrase activity obtained with the CCD was greater than that obtained with the FFD. Contiero, Cortezi, Marconato, Franchetti and Monti, in 2006 [38] studied the same *Leuconostoc mesenteroides* FT045B strain and found the optimal incubation temperature for enzyme production to be between 23 to 25 ºC, with 60% less enzyme production at temperatures from 27 to 29 ºC. In the present study, while the CCD was conducted at 24 ºC, the second experiment (FFD) was conducted at 26 ºC. This may explain why even when improving the culture conditions with AR and AS, it was not possible to achieve greater dextransucrase activity. The temperature of 26 ºC in the second experiment (FFD)

Table 4: Results for dextransucrase activity at 8 h of fermentation of *L. mesenteroides* FT 045B in function of different AS and AR ratios

Experiment	AS[f] (rpm)	AR[g] (vvm)	Dextransucrase activity (IU/mL)
A	100	0.0	3.74
B	100	0.5	3.50
C	100	1.0	3.99
D	150	0.0	1.98
E	150	0.5	1.96
F	150	1.0	2.45
G	200	0.0	1.37
H	200	0.5	1.43
I	200	1.0	1.94

[f]AS= agitation speed; [g]AR= aeration rate

Table 5: Statistical analysis of variance showing coefficient values and *p*-value for each variable for dextransucrase activity after 8 h

Source	Sum of Squares	Degrees of freedom	Mean square	F-Value	Value P > F
(1) AS[h] (rpm) (L)[j]	7.020	1	7.020	2493.953	0.000
AS (rpm) (Q)[k]	0.565	1	0.565	200.844	0.001
(2) AR[i] (vvm) (L)	0.277	1	0.277	98.532	0.002
AR (vvm) (Q)	0.159	1	0.159	56.370	0.005
(1L) for (2L) interation	0.026	1	0.0260	9.095	0.057
Erro	0.008	3	0.003		
Total SS	8.055	8			

[h]AS=agitation speed; [i]AR=aeration rate; [j](L)= linear variables; [k](Q)= squared variables

was used in order to simulate the best conditions that could be conducted in a real industry, thereby decreasing the cost related to maintaining fermentation at 24 ºC. Moreover, the pH in the first experiment was not controlled, which improved dextransucrase production, whereas the FFD was conducted under pH controlled at 6.7, thereby destabilizing dextransucrase activity and making its production more difficult. Likewise, Contiero, Cortezi, Marconato, Franchetti and Monti, in 2006 [38] and Lazic, Veljkovic, Vucetic and Vrvic, in 1992 [12] found that controlled pH in *Leuconostoc mesenteroides* fermentation caused a decrease in dextransucrase production.

CONCLUSION

The central composite design and response surface methodology enabled the determination of optimal operating conditions for obtaining greater dextransucrase production. The above results demonstrate a high potentiality of substituting sucrose and yeast extract with SCM and CSL, respectively. The optimization of the analyzed responses demonstrated that the best results for dextransucrase production by *Leuconostoc mesenteroides FT 045B* were obtained with an optimal range of 16 to 21.77 g/L of CSL, 32 to 44 g/L of SCM and 26 to 28.41 of K_2HPO_4 in 24 h of fermentation in a thermo shaker. Moreover, the greatest dextransucrase activity with the optimization of dextransucrase production was achieved with a combination of AR at 1.0 vvm and AS at 100 rpm.

ACKNOWLEDGEMENTS

The authors thank the FAPESP São Paulo Research Foundation for financial support, with grant number [2008/07410-1].

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