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Application of response surface methodology for glucan production from *Leuconostoc dextranicum* and its structural characterization

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ABSTRACT

Sequential optimization strategy based on statistical experimental designs was employed to enhance glucan production by *Leuconostoc dextranicum* NRRL B-1146 in flask culture. A two-level Plackett–Burman design was employed first where 11 variables were studied for their influence on glucan production. Sucrose, peptone and yeast extract were the most significant variables improving glucan production. A three-level Box–Behnken factorial design was employed for maximizing the glucan production. A mathematical model was developed to show the effects of each medium component and their combinatorial interactions on glucan production. The optimal medium composition for maximum glucan production was sucrose 5.95%, peptone 0.52% and yeast extract 2.9%. This composition predicted 1063 mg/l glucan, the experimentally found glucan was 1015 ± 4.5 mg/l that showed a good agreement with the predicted value. The purified glucan was homogenous and its structural characteristics investigated by FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopic techniques showed that it contained α-(1 → 6) and α-(1 → 4) linkages.

1. Introduction

Polysaccharides from different sources are being screened and have found many potential applications recently. More and more polysaccharides have been reported to exhibit a variety of biological activities, while traditionally they have found numerous applications in the pharmaceutical, food, agriculture and fine chemical industries (Sutherland, 1998). It is generally accepted that polysaccharides enhance various immune responses *in vivo* and *in vitro*. Lactic acid bacteria produce a wide variety of exopolysaccharides. Homopolysaccharide synthesis in lactic acid bacteria has been mainly studied in oral *Streptococci*, *Leuconostoc* species and *Lactobacillus* species (Monsan et al., 2001). These homopolysaccharides share the feature of being synthesized by extracellular glucansucrases using sucrose as the glucosyl donor.

Leuconostoc species are commercially exploited for the production of glucans. They are potential therapeutic agents and are also used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries (Korakli & Vogel, 2006). Glucans differ in the type of glucosidic linkages, degree and type of branching, length of glucan chains, molecular mass, and confirmation of polymers (Monchois, Willemot, & Monsan, 1999). Depending on the main chain glucosidic linkages in glucan, three different types of α-glucans synthesized by *Leuconostoc* species are known *viz.* dextran with α-(1 → 6), mutan with α-(1 → 3) and alternan with α-(1 → 6) and α-(1 → 3) linkages. There are only few reports on the presence of α-(1 → 6) and α-(1 → 4) linkages from *Leuconostoc* species (Monsan et al., 2001).

Leuconostoc mesenteroides subsp. *dextranicum* is commonly found in starter cultures used in dairy industry, where they are involved in texture and flavor production (Cogan & Jordan, 1994). *Leuconostoc dextranicum* NRRL B-1146 is being explored for the production of glucan. This strain has been reported to produce a highly linear glucan with 97% α-(1 → 6) linkages (Jeanes et al., 1954). This data were based on a primitive structural characterization of polysaccharide using periodate oxidation. Our preliminary investigations has led to the finding that the polysaccharide from this strain is unique with linkages of α-(1 → 6) and α-(1 → 4) in its glucan. Polysaccharides with such linkages have recently been shown to be promising anti-cancer agents (Cao et al., 2006; Liu et al., 2007). The production of polysaccharides with such linkages has been reported from chinese herb *Angelica sinensis* (Oliv.) Diels (Cao et al., 2006), *Strongylocentrotus nudus* a sea urchin (Liu et al., 2007). The glucan with α-(1 → 6) and α-(1 → 4) linkages has been reported from *L. dextranicum* isolated from palm wine (Uzochukwu, Balogh, Loefler, & Ngoddy, 2002) and from *Lactobacillus reuteri* (Kralj et al., 2004).

We report a sequential optimization strategy for glucan production by *L. dextranicum* NRRL B-1146 through statistically designed experiments. First, Plackett–Burman screening design was applied to address the most significant variables affecting glucan production. Second, a Box–Behnken design was used to describe the

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nature of the response surface in the experimental region, to search optimal medium composition for maximizing glucan yield. The structure of the purified glucan was analyzed by FT-IR, ^{1}H and ^{13}C NMR spectroscopic techniques.

2. Materials and methods

2.1. Microorganism and inoculum preparation

The strain *L. dextranicum* NRRL B-1146 was procured from Agricultural Research Service Culture Collection, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose) (Goyal & Katiyar, 1996), as stab at 4 °C and sub-cultured every 2 weeks. Two loops of culture were taken from stab and grown in 5 ml Tsuchiya medium (Tsuchiya et al., 1952) in a test tube at 28 °C for 12 h. One percentage of this broth was transferred to 250 ml Erlenmeyer flasks containing 100 ml of Tsuchiya medium (pH 6.9) and were grown for 12 h at 28 °C. This culture was used to inoculate 100 ml of media in 250 ml Erlenmeyer flasks at a level of 5% (v/v).

2.2. Production and purification of glucan

The production of glucan was carried out in 250 ml Erlenmeyer flasks containing 100 ml media as per experimental design (Tables 1 and 3). The inoculated flasks were incubated under static conditions at 28 °C for 48 h. All the runs were replicated and the glucan content was estimated. The culture supernatant was obtained by centrifuging the broth at 10,000*g* for 10 min at 4 °C. The crude glucan was precipitated by the addition of 3 volumes of 95% (v/v) prechilled ethanol at 4 °C and centrifuged at 13,000*g* for 30 min. The process was repeated and the glucan was determined by phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) in a micro titer plate (Fox & Robyt, 1991). The ethanol precipitate of the glucan obtained from the optimized media was dissolved in 50 mM Sodium phosphate buffer to a concentration of 10 mg/ml, and loaded onto a Sephadex G-100 column (1.5 cm × 30 cm, Sigma Chemical Co., St. Louis, USA). The column was eluted with the same buffer at a flow rate of 0.1 ml/min. The total sugar content of glucan was determined by phenol–sulfuric acid method (Fox & Robyt, 1991) using glucose as the standard. The fractions containing glucan were pooled and lyophilized for further analysis. The glucan could be associated with the protein so the protein concentration of all the fractions was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with bovine serum albumin as the standard.

2.3. Plackett–Burman design

Response surface methodology (RSM) is a collection of statistically based experimental designs, which have been established as a convenient method for optimizing various processes. Some of the popular choices include the Plackett–Burman design, the Box–Behnken design and the central composite design (Karthikeyan, Rakshit, & Baradarajan, 1996). The purpose of the first optimization step was to identify which ingredient(s) of the medium has a significant effect on glucan production. Based on Plackett–Burman factorial design, each variable was examined in two levels: −1 for low level and +1 for high level (Plackett & Burman, 1946). Table 1 shows the variables under investigation as well as levels of each variable used in the experimental design, whereas Table 2 represents the design matrix. Plackett–Burman experimental design is based on the first order polynomial model:

$$Y = \beta_o + \sum \beta_i x_i \quad (1)$$

Table 1
Assigned concentrations of variables at different levels and the regression analysis of the Plackett–Burman design

Variable	Levels (% w/v)		*t* Stat	*P*-value	Confidence level (%)
	−1	+1			
Sucrose (X_1)	1.0	6.0	6.83	0.0001	99.99
Peptone (X_2)	0.5	3.0	2.51	0.029	97.1
Yeast extract (X_3)	0.5	3.0	10.08	0.0001	99.99
Beef extract (X_4)	0.5	3.0	1.39	0.193	80.7
K_2HPO_4 (X_5)	1.0	4.0	−0.93	0.37	63
Tween 80 (X_6)	0.1	1.0	1.09	0.299	70.1
$MgSO_4$ (X_7)	0.01	0.1	−0.07	0.945	5.5
$MnSO_4$ (X_8)	0.001	0.01	0.66	0.522	47.8
$FeSO_4$ (X_9)	0.001	0.01	0.34	0.739	26.1
$CaCl_2$ (X_{10})	0.001	0.01	−1.83	0.094	90.6
NaCl (X_{11})	0.001	0.01	−0.39	0.707	29.3

where Y is the response (glucan yield mg/ml), β_o is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. This model does not describe interaction among variables and it is used to screen and evaluate the important variables that influence the response. In the present work, 11 assigned variables were screened in 12 experimental designs. All experiments were carried out in duplicate and the averages of the glucan content were taken as response (Table 2). From the regression analysis the variables, which were significant at 90% level ($P \leqslant 0.1$) were considered to have greater impact on glucan production. The experimental design and statistical analysis of the data were done by using Minitab statistical software package (Release 15).

2.4. Box–Behnken design

In order to describe the nature of the response surface in the experimental region, a Box–Behnken (Box & Behnken, 1960) design was applied. The advantage of Box–Behnken design is that it has only three levels, coded −1, 0, and +1 for low, middle and high concentrations, respectively. This needs few number of experiments, is more efficient and easier to arrange and to interpret in comparison to others (Bosque-Sendra, Pescarolo, Cuadros-Rodriguez, Garcia-Campana, & Almansa-Lopez, 2001). Therefore, this statistical technique was used in this study. The variables of highest confidence levels were prescribed into the three levels, coded −1, 0, and +1. A total of 15 runs, performed in duplicate, were used to optimize the range and levels of chosen variables, *viz*., sucrose, peptone and yeast extract. The range and levels of the variables investigated in this study are given in Table 3. The relationships and interrelationships of the variables were determined by fitting the second order polynomial equation to data obtained.

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response variable; β_o, β_i, β_{ii} and β_{ij} are constant regression coefficients of the model; and X_i and X_j ($i = 1, 3$; $j = 1, 3$, $i \neq j$) represent the independent variables (medium components in the form of coded values). The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Minitab statistical software package (Release 15) was used for the performing the regression and graphical analysis of data obtained.

2.5. Optical rotation and Fourier-transform infrared spectrometric (FT-IR) analysis

The polysaccharide was characterized using a Fourier Transform Infra-Red Spectrophotometer (Perkin-Elmer Instruments, Spectrum One FT-IR Spectrometer). The dried polymer was grinded

Table 2
Plackett–Burman design for 11 variables with coded values along with the observed results

Run order	Sucrose	Peptone	Yeast extract	Beef extract	K_2HPO_4	Tween80	$MgSO_4$	$MnSO_4$	$FeSO_4$	$CaCl_2$	NaCl	Glucan (mg/ml)
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	0.668 ± 0.12
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	0.416 ± 0.03
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	0.413 ± 0.07
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	0.659 ± 0.09
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	0.398 ± 0.05
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	0.761 ± 0.11
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	0.598 ± 0.00
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	0.396 ± 0.17
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	0.159 ± 0.00
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	0.268 ± 0.01
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	0.153 ± 0.04
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.087 ± 0.07

with Potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}, with 20 scans. Optical rotation was measured at 27 °C using a (Perkin Elmer Instruments, Model 343) polarimeter.

2.6. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectral analysis of glucan

^1H and ^{13}C NMR spectra for glucan were recorded at 30 °C with a NMR spectrometer (Varian, Model AS400) 400 MHz equipped with VnmrX for Sun Microsystems Ver. 6.1 software (operating frequencies 400 MHz for ^1H and 100 MHz for ^{13}C NMR). The samples were dissolved in D_2O (99.96%) at concentrations of 5 mg/ml (for ^1H NMR) and 20 mg/ml (for ^{13}C NMR). ^1H chemical shifts were referenced to internal D_2O (4.8 ppm at 30 °C).

3. Results and discussion

3.1. Evaluation of variables affecting glucan production

Glucans are synthesized by extracellular glucansucrases using sucrose as the glucosyl (glucose) donor with the release of free fructose. The conventional fermentation for production of glucan involves three phases: cell growth, enzyme production phase and glucan synthesis. The fermentation was allowed to proceed for 48 h and the amount of glucan produced was estimated. Plackett–Burman design was used as the first approach to screen the relatively important factors affecting glucan production from *L. dextranicum* NRRL B-1146. The averages of glucan production (mg/ml) for 12 different trials of the 11 medium components are presented in Table 2. The *t*-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance (Karthikeyan et al., 1996). Sucrose, peptone and yeast extract were found to be the most significant variables enhancing glucan production (Table 2). $CaCl_2$ was found to be significant although with a negative coefficient. The addition of $CaCl_2$ was avoided in subsequent experiments as it did not aid in enhancing glucan production and the minor requirements of the microorganisms were fulfilled by the complex components of yeast extract and beef extract.

The polynomial model describing the correlation between the 11 variables and the glucan yield could be presented as

$$Y_{mg/ml} = 0.4145 + 0.1136X_1 + 0.4169X_2 + 0.1678X_3 + 0.0231X_4 - 0.0156X_5 + 0.0181X_6 - 0.0017X_7 + 0.011X_8 + 0.0057X_9 - 0.0305X_{10} - 0.0064X_{11} \quad (3)$$

The production of glucan is linked to the amount of glucansucrase secreted into the medium. The different nutrients used in the Plackett–Burman design, for the production of glucan were chosen based on the nutrients that affected glucansucrase production (Majumder & Goyal, 2007). Sucrose was chosen as the carbon source as it was the inducer for the production of glucansucrase and also the substrate for glucan production (Tsuchiya et al., 1952). The +1 level of sucrose was chosen at 6% (w/v) as higher concentrations than 6% lead to increased viscosity making the separation of cells difficult. Different nitrogen sources were chosen as they were found to significantly affect the production of glucan from *Leuconostoc* species (Barker & Ajongwen, 1991; Karthikeyan et al., 1996).

3.2. Interaction between medium components and their optimization for glucan production

At the end of screening experiment, sucrose, peptone and yeast extract were believed to play a significant role in glucan production. The Box–Behnken design for the three variables and the corresponding experimental data are shown in Table 3A. The results of the second-order response surface model fitting in the form of ANOVA are given in Table 3B. The goodness of the model was checked by the determination coefficient R^2 (0.9865) explaining 98.65% of the variability of the response. The value of adjusted R^2 (0.9692) was also very high, indicating high significance of the model. The coefficients of the regression analysis are shown in Table 4 and the polynomial model for glucan yield, *Y* can be expressed by Eq. (4).

Table 3A
Box–Behnken design matrix of three variables and the experimentally observed response

Run no.	Coded factor values			*Y* glucan (mg/ml)
	Sucrose X_1 (%,w/v)	Peptone X_2 (%,w/v)	Yeast extract X_3 (%,w/v)	
1	−1 (1.0)	−1 (0.5)	0 (1.75)	0.570 ± 0.0136
2	1 (6.0)	−1 (0.5)	0 (1.75)	0.796 ± 0.0277
3	−1 (1.0)	1 (3.0)	0 (1.75)	0.507 ± 0.0563
4	1 (6.0)	1 (3.0)	0 (1.75)	0.621 ± 0.0181
5	−1 (1.0)	0 (1.75)	−1 (0.5)	0.178 ± 0.0006
6	1 (6.0)	0 (1.75)	−1 (0.5)	0.327 ± 0.0085
7	−1 (1.0)	0 (1.75)	1 (3.0)	0.656 ± 0.0381
8	1 (6.0)	0 (1.75)	1 (3.0)	0.912 ± 0.0136
9	0 (3.5)	−1 (0.5)	−1 (0.5)	0.338 ± 0.0201
10	0 (3.5)	1 (3.0)	−1 (0.5)	0.345 ± 0.0228
11	0 (3.5)	−1 (0.5)	1 (3.0)	0.830 ± 0.0185
12	0 (3.5)	1 (3.0)	1 (3.0)	0.707 ± 0.0523
13	0 (3.5)	0 (1.75)	0 (1.75)	0.441 ± 0.0035
14	0 (3.5)	0 (1.75)	0 (1.75)	0.469 ± 0.0266
15	0 (3.5)	0 (1.75)	0 (1.75)	0.440 ± 0.0071

Table 3B
ANOVA for quadratic model

Source	SS[a]	DF[b]	MS[c]	*F*-value	Prob. (*P*) > *F*
Model	0.6246	9	0.0696	57.037	<0.0001
Residual (error)	0.00854	7	0.0012	–	–
Lack of fit	0.00785	3	0.0026	15.268	0.0118
Pure error	0.00686	4	0.00017		
Total	0.6350	16			

R^2 = 0.9865; CV = 6.58; Adj. R^2 = 0.9692.
[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean square.

Table 4
Model coefficient estimated by multiple linear regression

Model term	Parameter estimate	Standard error	Computed *t*-value	*P*-value
Intercept	0.4516	0.02366	19.024	0.000
X_1	0.0933	0.01449	6.441	0.001
X_2	−0.0440	0.01449	−3.041	0.029
X_3	0.2395	0.01449	16.533	0.000
X_1^2	0.0682	0.02133	3.201	0.024
X_2^2	0.1050	0.02133	4.926	0.004
X_3^2	−0.0003	0.02133	−0.017	0.987
X_1X_2	−0.0278	0.02049	−1.358	0.232
X_1X_3	0.0266	0.02049	1.301	0.250
X_2X_3	−0.0324	0.02049	−1.584	0.174

$$Y = 0.4516 + 0.0933X_1 - 0.0440X_2 + 0.2395X_3 + 0.0682X_1^2 + 0.1050X_2^2 - 0.0003X_3^2 - 0.0278X_1X_2 - 0.0266X_1X_3 - 0.0324X_2X_3 \quad (4)$$

The ANOVA of quadratic regression model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test with a very low probability value [($P_{model} > F$) = 0.0001] (Table 3B). The relatively lower value of coefficient of variation (CV = 6.58%) indicates a better precision and reliability of the experiments carried out.

The significance of each coefficient can be seen from the *t* and *P* values listed in Table 4. Yeast extract (X_3) had a significant effect ($P < 0.0001$) on glucan yield, *Y* as it had the largest coefficient followed by sucrose (X_1). Positive coefficient was observed for the quadratic term of sucrose (X_1^2) and peptone (X_2^2) and interaction term X_1X_3. However, peptone (X_2), quadratic term of yeast extract (X_3^2) and interaction terms (X_1X_2 and X_2X_3) had a negative effect on *Y*. The probability value of the coefficient of quadratic effect of yeast extract (X_3^2) was very high (0.987) indicating that only 1.3% of the model was affected by this variable. Linear effect of yeast extract and sucrose are highly significant and explains that they can act as limiting nutrients and subtle variation in their concentration will alter the product formation.

2-Dimensional contour plots are the graphical representations of the regression equation and help in the identification of the type of interactions between test variables. The circular contour plots indicate that the interaction between the corresponding variables is negligible. An elliptical or saddle nature of the contour plots indicates significance of the interactions between the corresponding variables. The interaction between sucrose and peptone was negligible (Fig. 1A) and there was an increase in glucan production when high concentrations of sucrose (4–6%) and lower concentrations of peptone (0.5–2%) were used.

The interactions between yeast extract and peptone are not perfectly elliptical (Fig. 1B) and optimum glucan production can be obtained at concentrations of yeast extract (2–3%) and peptone (1.5–2.5%). The plot for sucrose and yeast extract depicts interaction between them (Fig. 1C) and maximum glucan production can be obtained at relatively high concentrations of sucrose (4–6%) and yeast extract (2–3%). The concentration of medium components selected for maximum glucan production using the point prediction tool of Minitab software were as follows: sucrose 5.95%, peptone 0.52% and yeast extract 2.9%. These values predicted 1063 mg/l glucan production. These optimized values of nutrient parameters were validated by flask culture study in triplicate sets of experiments and the maximum glucan production obtained was 1015 ± 4.5 mg/l which was in good agreement with the predicted value.

3.3. Structural analysis of glucan by FT-IR spectroscopy

The purity of the glucan produced from the optimized medium is an important factor for the potential use of the biopolymer in pharmaceutical and food-related applications. The crude glucan obtained by alcohol precipitation was purified by a Sephadex G-100 column. The glucan was eluted by buffer, as a single peak as detected by the phenol–sulfuric acid assay (Fig. 2). The protein content of the eluted fractions was determined by the method of Lowry (Lowry et al., 1951). Less than 1% protein was found and this was supposed to be protein associated with the glucan. Glucansucrase is associated with the polymer glucan and minor fraction of the alcohol precipitate is contaminated with glucansucrase associated glucan. This was eluted as a minor peak during chromatography on Sephadex G-100 (Fig. 2). The polysaccharide containing fraction was pooled, freeze-dried and used for subsequent analysis. The polysaccharide appeared as a white powder, and had a optical rotation ($[\alpha]_D^{20}$ + 52 (*c* 0.2, H_2O)) indicating the D-configuration of the glucosyl residues (Liu et al., 2007).

The vibrational spectra have found important applications in the identification and analysis of sugars. The carbohydrates show high absorbance in the region of 1200–950 cm^{-1}, that is within the so-called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide allowing its possible identification (Cerna et al., 2003). The FT-IR spectrum of the polysaccharide showed a strong band at 3422 cm^{-1} assigned to the hydroxyl stretching vibration of the polysaccharide (Fig. 3). The band in the region of 2928 cm^{-1} was due to C—H stretching vibration and the band in the region of 1645 cm^{-1} corresponded to associated water. A characteristic absorption at 850 and 930 cm^{-1} were also observed indicating the α-configuration of the sugar units. There was no absorption at 890 cm^{-1}indicated the absence of β-configuration (Barker, Bourne, Stacey, & Whiffen, 1954). The absorption at 1155, 1080 and 1020 cm^{-1} indicated α-pyranose of the glucose residue. The band at about 1150 cm^{-1} was assigned to the valent vibrations of the C—O—C bond and glycosidic bridge. The bands at 1020 cm^{-1} and 1080 cm^{-1} are present in polysaccharide with α-(1 → 6) and α-(1 → 4) linkages and can be considered as a characteristic for the type of interunit link (Shingel, 2002).

3.4. Structural analysis of glucan by ^1H NMR and ^{13}C NMR spectroscopy

^1H NMR and ^{13}C NMR spectra further confirmed the homogeneity of the purified polysaccharide. The spectral features were typical of polysaccharide with α-(1 → 6) and α-(1 → 4) linkages based on the peak assignments from the literature (Uzochukwu et al., 2002; Cao et al., 2006; Liu et al., 2007). The ^{13}C NMR spectrum of polysaccharide is shown in Fig. 4A. Based on data available in literature, it was possible to identify that the resonances in the region of 102.2 and 97.7 ppm corresponded to C-1 of the 1,4-D-Glc*p* and 1,6-D-Glc*p* residues, respectively (Seymour, Knapp, & Bishop, 1976; Uzochukwu et al., 2002; Cao et al., 2006). The major resonance in the anomeric regions occur at 97.7 ppm rather than at about 90 ppm indicating that the C-1 is linked. The signal at

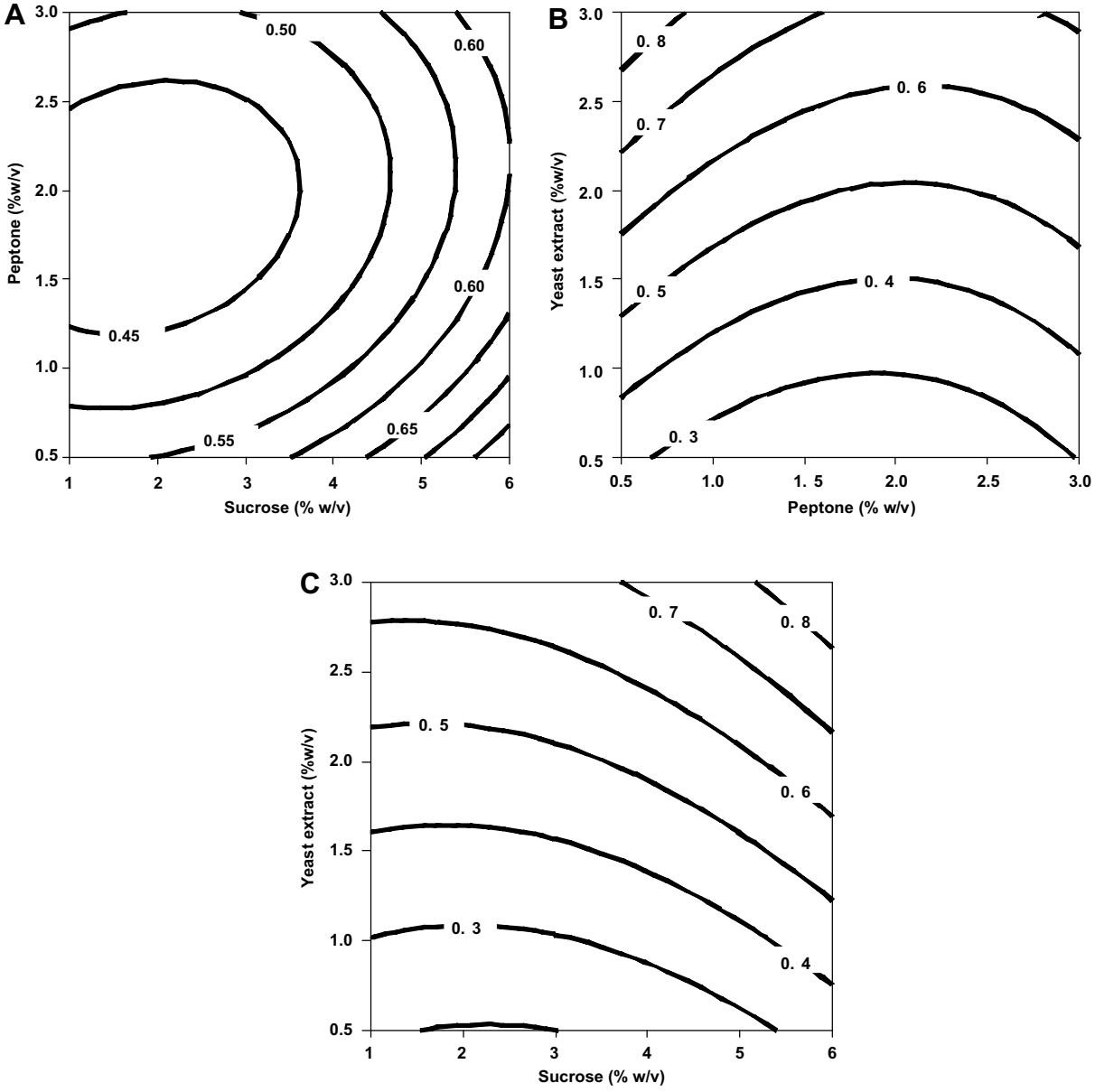

Fig. 1. Contour plots of the combined effects of sucrose, peptone and yeast extract on glucan production by *Leuconostoc dextranicum* NRRL B-1146. (A) Peptone and sucrose at fixed level of yeast extract; (B) yeast extract and peptone at fixed level of sucrose; (C) yeast extract and sucrose at fixed level of peptone.

66.9 ppm rather than at 60 ppm indicated that most of the C-6 is also linked (Seymour et al., 1976). Similarly the peaks at 102.2 and 78.5 ppm suggested the presence of α-(1 → 4) linkage. The linkages in the polysaccharide are α-glucosidic linkages, as indicated by the absence of chemical shifts downfield of 102.2 ppm.

The configurations for glucan were further confirmed by the ^{1}H NMR spectrum which displayed signals for anomeric protons at 5.38 and 4.91 which are assigned to α-(1 → 4) Glc*p* and α-(1 → 6) Glc*p*, respectively (Fig. 4B). The strong water (HOD) signal at 4.8 ppm obscures the α-anomeric signal at 4.9 ppm. The chemical shifts from 3.4 to 4.0 ppm are assigned to protons of carbons C-2–C-6 of glycosidic ring (Cheetam, Fiala-Beer, & Walker, 1991). Peak intensities of the ^{13}C and ^{1}H NMR have been previously used to determine the abundance of a particular linkage in the polysaccharide (Uzochukwu et al., 2002). The intensities of characteristic peaks of 97.7, 102.2 ppm and 5.38 and 4.9 ppm indicated the presence of equal abundance α-(1 → 4) linkages along with α-(1 → 6) linkages.

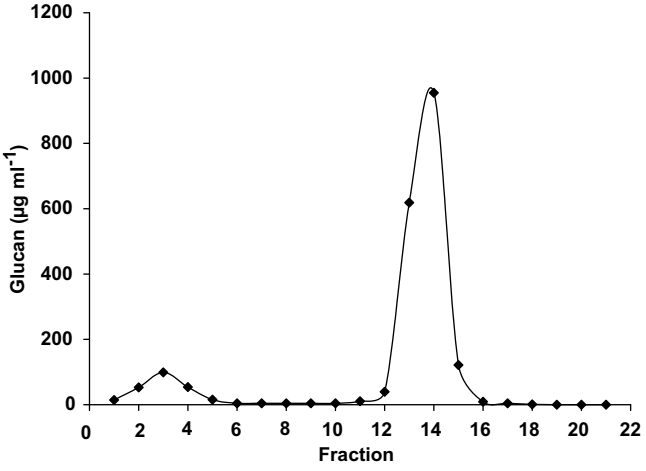

Fig. 2. Elution profile of glucan from Sephadex G-100 with 50 mM sodium phosphate buffer (pH 7.0) at 0.1 ml/min flow rate.

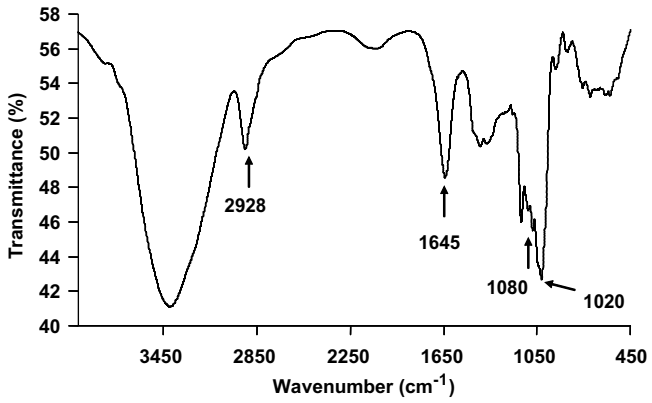

Fig. 3. FT-IR spectrum of glucan produced from *Leuconostoc dextranicum* NRRL B-1146.

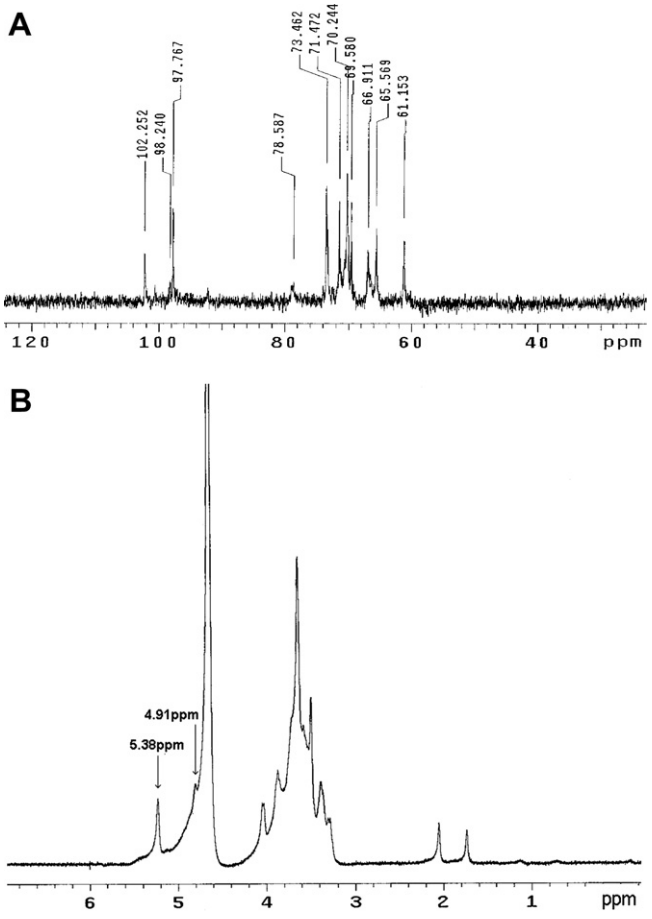

Fig. 4. NMR spectra of glucan from *Leuconostoc dextranicum* NRRL B-1146. (A) ^{13}C NMR spectrum of glucan and (B) ^{1}H NMR spectrum of glucan.

4. Conclusion

It is highly important to screen exopolysaccharide producing microorganisms with novel properties that could be of commercial value. In the present study, the effect of different nutrients on glucan production from *L. dextranicum* NRRL B-1146 was studied. Using Plackett–Burman design different ingredients were screened for their effect on glucan production. The results showed that sucrose, peptone and yeast extract significantly affect glucan production. Using a Box–Behnken design for the three selected ingredients, the interaction between the components and their optimum levels for maximum glucan production were determined. The experimentally found value of glucan produced in the flask culture using statistically designed medium was in perfect agreement with the predicted value. The glucan produced from *L. dextranicum* NRRL B-1146 with the statistically formulated medium was homogenous and free of contaminating polysaccharides as shown by size exclusion chromatography. The structural characterization of the glucan using FT-IR, ^{1}H NMR and ^{13}C NMR techniques indicated the presence of high abundance of α-(1 → 6) and α-(1 → 4) linkages. Polysaccharides in general and α-D-glucans in particular with these linkages have been shown to be of therapeutic potential and very promising anti-tumor agents.

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