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A N I N T E R N A T I O N A L J O U R N A L

Scale Up of Dextran Production from a Mutant of *Pediococcus pentosaceus* (SPAm) Using Optimized Medium in a Bioreactor

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ABSTRACT

The mutant of Pediococcus pentosaceus *(SPAm) produced earlier by UV-mutagenesis exhibiting higher dextransucrase activity as compared to wild-type was used. The generated mutant SPAm gave 12.2 mg/ml, a 20% higher dextran than wild-type. Response surface methodology was carried out for further enhancement of dextran production. To enhance dextran production by the mutant SPAm, Plackett-Burman Design and a 2^2 full factorial Central Composite Design was employed. After response optimization, the optimum concentration of sucrose and yeast extract was 5.115% (w/v) and 0.635% (w/v), respectively. The experimental values of dextran 36.0 mg/ml at flask level and 35.0 mg/ml at bioreactor level were in good agreement with the predicted value of 40.8 mg/ml. The increase in dextran production by the mutant SPAm using the optimized medium was 3 fold higher as compared to unoptimized medium.*

Key words: *Pediococcus pentosaceus*, dextran, Plackett-Burman Design, Central Composite Design, Response Surface Methodology

INTRODUCTION

Dextrans $(C_6H_{10}O_5)n$ are a class of homopolysaccharides produced by the lactic acid bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella* (Katina et al., 2009; Patel et al., 2011). Dextrans consist of D-glucose units polymerized predominantly in α-(1→6) linkage (Padmanabhan et al., 2003) and α-(1→2), α-(1→3), α-(1→4) glycosidic linked branches (Kim et al., 2003). Dextrans have enormous industrial applications due to their non-ionic, inert, stable, porous, gelling and pseudoplastic attributes (Patel et al., 2011). These are used as food syrup stabilizers, matrix of chromatography columns, blood plasma substitutes, antithrombogenic agents, treatment for iron deficiency anaemia, drug carriers (Purama and Goyal, 2005; Patel et al., 2011). Dextrans have also been reported to arrest the replication of human immunodeficiency virus (HIV-1), the causative agent of the dreaded AIDS (Baba et al., 1990; Ko et al., 1991). Dextran hydrogels are used in various pharmaceutical and biomedical applications such as contact lenses, cell encapsulation for drug delivery, burn wound dressing and in spinal cord regeneration (Aumelas et al., 2007). Dextrans act as protective coating against oxidation for metal nanoparticles (Bautista et al., 2005) and shield against biofouling in

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biomaterials (Sengupta et al., 2006).) Use of dextrans have ramified into paper, metal-plating processes and enhanced oil recovery (Patel et al., 2011). Dextrans from various microbial sources have been studied and characterized to evaluate their industrial potentials (Purama et al., 2009; Majumder and Goyal, 2009; Patel et al., 2010).

The concentrations of medium components are crucial parameters regulating the microbial metabolite production. The direct and traditional measurement techniques as 'one factor at a time' used for optimization of multivariable system is time consuming, labour intensive and prone to erroneous data. Statistical approaches such as Plackett-Burman Design and Central Composite Design by Response surface methodology (RSM) are commonly used methods to optimize culture medium, enzyme synthesis, aqueous two phase separation of proteins and glucan production (Liu and Wang, 2007; Majumder et al., 2009a, Majumder et al., 2009b). To enhance the production of dextran concentration, combinatorial interactions of medium components is a good strategy. Considering the multifarious utility of dextran, a natural isolate of lactic acid bacterium, *Pediococcus pentosaceus* (SPA) producing significantly high dextran concentration was screened from the soil of Assam. The dextran production ability of *Pediococcus* genus was reported for the first time (Patel and Goyal, 2010b). Further this was the first report on *Pediococcus pentosaceus* showing dextran production which is more or comparable to other commercial Leuconostoc strains (Majumder et al., 2009a; Majumder et al., 2009b). In the present work, a high dextran yielding mutant (SPAm) developed from wild-type *Pediococcus pentosaceus* by UV-mutagenesis was used. The dextran concentration from the mutant SPAm was enhanced by statistical optimization of the medium. Plackett-Burman Design was used to screen the significant factors and Central Composite Design was used to investigate their interactive effects.

MATERIAL AND METHODS

Culturing of mutant (SPAm) of *Pediococcus pentosaceus*

The mutant SPAm (Patel and Goyal, 2010a) was obtained from the wild-type *Pediococcus pentosaceus* (Patel and Goyal, 2010b). The cultures were maintained as stab in modified MRS agar (Goyal and Katiyar, 1996) (containing 2% (w/v) sucrose) at 4°C and sub-cultured every 2 weeks. From the cultures maintained as MRS agar stab at 4°C, 1 loopful was inoculated in the enzyme production medium described by Tsuchiya et al. (Tsuchiya et al., 1952) and grown at 25°C at 180 rpm for 12h. This medium consisted of (%, w/v) sucrose, 2; yeast extract, 2; K_2HPO_4, 2; $MgSO_4$. $7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.001; $FeSO4.7H_2O$, 0.001; $CaCl_2$, 0.001; NaCl, 0.001 and the pH of medium was adjusted to 6.9. 1% of this broth was transferred to 250 ml Erlenmeyer flask containing 50 ml of statistically designed medium with variable composition and incubated at the above mentioned culture conditions.

Estimation of dextran concentration

The carbohydrate content in the cell free supernatant of the lactic acid bacterium mutant SPAm grown in statistically designed enzyme production medium with variable ingredient composition was determined by phenol-sulphuric acid method (Dubois et al., 1956) in a micro-titre plate (Fox and Robyt, 1991). To 25 µl of sample containing dextran in a microtitre plate, 25 µl of 5% (v/v) phenol was added. The mixture was mixed by shaking the plate on a vortex mixer for 30s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture. The plate was again shaken for 30s to ensure proper mixing of the contents of the wells. Then the plate was wrapped in cling film and incubated in water bath at 80°C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on a multimode microplate reader (Tecan, model Infinite™ 200). A Standard graph was plotted using dextran (40 kDa) in the concentration range 0.1-1 mg/ml.

Optimization procedure and experimental designs

Statistical designs were applied in two steps. The first step was to identify the significant nutrients for dextran production using Plackett-Burman Design (Plackett and Burman, 1946) and the second step was to optimize these significant nutrients using Central Composite Design (CCD) in response surface methodology (RSM). The experimental design and statistical analysis of the

data were done by Minitab statistical software (Majumder et al., 2009a).

Plackett-Burman Design

Five medium components were selected for Plackett-Burman Design *viz.* sucrose, yeast extract, K_2HPO_4, Tween 80 and $CaCl_2$. Among the nutrients, sucrose was selected as the carbon source as it is the substrate and inducer of dextran production (Tsuchiya et al., 1952) full stop needed. Yeast extract powder was chosen as the nitrogen source for its significant effect on dextran production (Majumder et al., 2009a). K_2HPO_4 was chosen as it acts as a buffering agent in the fermentation medium to maintain its pH for a longer duration (Goyal and Katiyar, 1997). The surfactant Tween 80 was selected as it alters the membrane permeability and enhances the release of the extracellular dextransucrase, which is expected to increase dextran biosynthesis (Goyal and Katiyar, 1997). $CaCl_2$ was considered for its stimulatory effect on dextransucrase production (Qader et al., 2008). Each of the 5 factors was examined in two levels: low level (-1) and high level (+1) (Plackett and Burman, 1946). The factors considered, their levels and the design matrix were presented in Table 1. Plackett-Burman experimental design is based on the first order polynomial model:

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

Where, Y is the response (dextran concentration), 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 is useful for screening and evaluation of the key factors that influence the response. In this work, 5 variables were screened in 16 run orders. The experiments were carried out in duplicate and the averages of the dextran concentrations were taken as response (Table 1). From the regression analysis the variables, which were significant at 95% level ($P < 0.05$) were considered to have greater impact on dextran production and were further optimized by a central composite design. The experimental design and statistical analysis of the data were done by Minitab statistical software package (Version15) (Majumder et al., 2009a).

Table 1 - Plackett-Burman Design for two levels of 5 variables in uncoded values along with the observed dextran concentrations.

Run Order	Sucrose (A)	Yeast extract (B)	K_2HPO_4(C)	Tween 80 (D)	$CaCl_2$ (E)	Dextran (mg/ml)
1	6	1	1	1	0.002	15.6
2	1	1	3	0.1	0.0002	5.2
3	6	1	1	0.1	0.0002	16.5
4	1	3	1	0.1	0.0002	2.9
5	1	3	1	1	0.002	3.0
6	1	3	3	0.1	0.002	4.2
7	6	3	1	1	0.0002	11.5
8	1	3	3	1	0.0002	3.0
9	6	1	3	1	0.0002	16.8
10	6	3	3	0.1	0.0002	13.7
11	1	1	3	1	0.002	5.4
12	1	1	1	0.1	0.002	5.6
13	6	1	3	0.1	0.002	21.7
14	6	3	3	1	0.002	12.2
15	6	3	1	0.1	0.002	11.8
16	1	1	1	1	0.0002	7.2

Central Composite Design

The 2^2 full-factorial central composite design (CCD) with two medium constituents, i.e. sucrose and yeast extract was generated by Minitab statistical software. In this study, the experimental plan consisted of 13 run orders. The experiments were carried out in duplicate and the averages of the dextran concentration were taken as the response. The relationships among the variables were determined by fitting the following second-order polynomial equation to the data obtained from 13 experiments.

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (2)$$

Where, Y is the predicted response, k is the number of factor variables, β_o is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, βij is the interaction coefficient. X_i is the factor variable in its coded form. The data were analyzed statistically by ANOVA method. P-values below 0.1 were regarded as statistically significant. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and the significance of the regression coefficients were checked by F-test and p-value (Zhong and Wang, 2010).

Validation of the model at shake flask and bioreactor level

The confirmation and validation of data under shake flask and bioreactor level was done by using the RSM optimized medium composition. In one set, the experiment was performed in 500 ml flask with 200 ml optimized culture medium and grown at 25°C at 180 rpm. The parameters like dextran concentration, enzyme activity, pH, sucrose concentration and cell optical density were analyzed at every 2h interval as described above. In another set of experiment the fermentation was carried out in 1L volume of culture medium in a 3L bioreactor (Applikon, model Bio Console ADI 1025). 1% inoculum from 12h grown culture was inoculated in the bioreactor. The pH 7.0 and temperature 25°C were kept constant throughout the fermentation process. The Dissolved Oxygen (DO) was adjusted to 100% before inoculation. The agitation was set to 180 rpm at the beginning of the run but changed accordingly to keep the DO above 40%. The parameters like dextran concentration, enzyme activity, sucrose concentration and cell optical density were analyzed at every 2h interval. The cell optical density was taken at 600 nm. The dextran concentration was measured as described in earlier section. The enzyme activity was analysed by estimating the reducing sugars by the Nelson-Somogyi protocol (Nelson, 1944; Somogyi, 1945). The sucrose concentration was determined by estimating the reducing sugars by the method of Sumner and Sisler (1944).

RESULTS AND DISCUSSION

Estimation of dextran of wild-type *Pedio-coccus pentosaceus* and its mutant SPAm

The mutant SPAm showed 12.2 mg/ml dextran, whereas the wild-type *Pediococcus pentosaceus* exhibited 10.2 mg/ml. The mutant showed higher dextran production by 20% as compared to the wild-type. The dextran structure from the mutant as analysed by FTIR, NMR spectroscopy was identical to that of wild-type, however, surface morphology by SEM analysis showed larger pore size of dextran from the mutant (data not shown).

Fitting the model

The interpretation of data in Table 1 revealed that there was an extreme variation in the dextran concentration in the 16 trials ranging from 2.9 mg/ml to 21.7 mg/ml. This variation can be attributed to the variable medium composition. Regression coefficients of the 5 ingredients were analysed (Table 2).

Table 2 - Statistical analysis of Plackett-Burman Design showing coefficient, T and P values for each variable

Variable	Coefficient	T-value	P-value
Intercept	9.762	22.81	0.000
Sucrose (A)	5.212	12.16	0.000
Yeast extract(B)	-1.987	-4.63	0.001
K_2HPO_4 (C)	0.500	1.18	0.264
Tween 80 (D)	-0.438	-1.01	0.338
$CaCl_2$ (E)	0.175	0.39	0.702

Lower the P-value higher the significance of the variable (Majumder et al., 2009a). The variable sucrose and yeast extract had confidence levels greater than 95% as revealed by Pareto chart, so were considered significant. Sucrose having coefficient 5.212 was positively significant and its presence in the medium in higher concentrations enhanced dextran production. Yeast extract having coefficient -1.987 was found negatively significant and its presence in the medium in lower concentrations stimulated dextran production. Excluding insignificant variables the model equation for dextran concentration after screening by Plackett-Burman Design can be written as

$Y = 9.762 + 5.212A – 1.987B$ (3)

Where, Y= Dextran concentration, A = Sucrose and B = Yeast extract

On the basis of the significant coefficients (Table 2), sucrose and yeast extract were selected for further medium optimization to maximize dextran production. All other variables used in all the trials were kept to their median levels. At the end of the screening experiments for different nutritional factors, the conditions were optimized by Central Composite Design (CCD). Taking five levels of both the significant factors, thirteen experiments were carried out from the design (Table 3). The results of the second order response surface model fitting in the form of ANOVA are given in Table 4. To test the fit of the model, the regression equation and determination coefficient R^2 were evaluated. The model presented a high R^2 value of 94.35% and adjusted R^2 value of 90.32% for dextran concentration. The coefficients of regression for the dextran concentration were calculated and the following regression equation was obtained.

$$Y= 27.2171 + 24.5989X_1 - 25.3429X_2-12.5250 X_1X_2 +7.5709X_1^2 – 0.9282X_2^2 \quad (4)$$

Where, Y = Response (Dextran concentration), X_1 = Sucrose and X_2 =Yeast extract.

The ANOVA of quadratic regression models for dextran concentration demonstrated that the model is highly significant, and is evident from the Fisher's F-test with a very low probability value [(Pmodel > F = 0.0000)]. The significance of each coefficient was determined by t-values and P-values which are listed in Table 5. Larger magnitude of t-test and smaller P-value indicates the high significance of the corresponding coefficient (Tanyildizi et al., 2005).

The result showed that the interaction, X_1X_2 (sucrose. yeast extract) is highly significant with a P value of 0.066 and a negative coefficient of −12.5250.

Table 3 - 2^2 full factorial Central Composite Design matrix of two variables in uncoded units and experimental response.

Run order	Sucrose (%, w/v) X_1	Yeast extract (%, w/v) X_2	Dextran concentration (mg/ml)
1	3.0	2.75	15.9
2	3.0	2.75	15.8
3	1.5	1.25	8.6
4	4.5	4.25	23.8
5	3.0	4.865	11.3
6	0.885	2.75	6.8
7	5.115	2.75	28.3
8	3.0	2.75	15.8
9	3.0	0.635	16.2
10	4.5	1.25	35.7
11	1.5	4.25	7.9
12	3.0	2.75	15.9
13	3.0	2.75	15.8

Table 4 - ANOVA of Central Composite Design for dextran concentration.

Source	DF	Sum of squares	Mean Square	F-value	Prob. P > F
Model	5	773.81	65.743	23.4	0.00
Residual (error)	7	46.30	1.138	-	-
Lack of fit	3	46.29	2.277	5143.5	0.00
Pure error	4	0.012	0.000	4.74	-
Total	12	-	-	-	-

Table 5 - Model coefficient of dextran estimated by multiple linear regression.

Model Term	Coefficient	Standard error of Coefficient	T-value	P-value
Inter-cept	27.2171	15.936	1.708	0.131
X_1	24.5989	14.587	1.686	0.136
X_2	-25.3429	14.105	-1.797	0.115
X_1^2	7.5709	4.362	1.736	0.126
X_2^2	-0.9282	4.362	-0.213	0.838
X_1X_2	-12.5250	5.752	-2.177	0.066

Analysis of response surface

The 3D response surface and 2D contour plots are the graphical representations of regression equations (Zhong and Wang, 2010). They conveniently illustrate the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The shapes of the contour plots indicate the significance of mutual interactions between the variables. Circular contour plot symbolises negligible interactions between the corresponding variables, whereas elliptical contour plot indicates the significant interactions between the corresponding variables (Zhong and Wang, 2010). The contour and surface plot representing the regression equation for yield of dextran from the mutant SPAm was presented in Figure 1.

The plot illustrated that the interaction between the independent variable sucrose and yeast extract (X_1X_2) is negative but very strong. The dextran production was increased at high sucrose and low yeast extract concentration (Fig. 1).

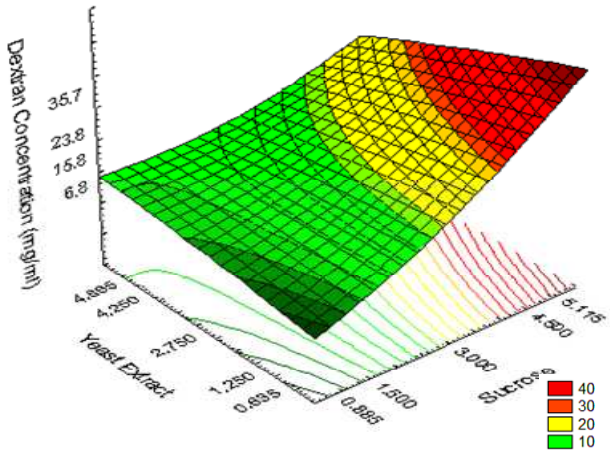

Figure 1 - Contour and surface plot of the combined effects of sucrose and yeast extract on dextran concentration of mutant (SPAm).

Optimization of medium composition and experimental validation of the model

Response optimization predicted the maximum dextran production of 40.8 mg/ml with desirability 1, at sucrose concentration 51.15 g/L and yeast extract concentration 6.35 g/L. Response surface methodology (RSM) showed that a medium containing (g/L) sucrose, 51.15; yeast extract, 6.35; K_2HPO_4, 20; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; NaCl 0.01 and Tween 80, 5 (ml/L) was optimum for the production of dextran. The dextran production by the mutant of *Pediococcus pentosaceus* (SPAm) using optimized medium was compared at shake flask level and batch fermentation in a bioreactor. The fermentation profile of dextran production from the mutant in flask and bioreactor is shown in Figure 2 and Figure 3, respectively. The maximum dextran concentration obtained experimentally using the above medium composition was 36.0 mg/ml at shake flask level which showed good agreement with the predicted value 40.8 mg/ml (Fig.2).

The increase in dextran concentration of the mutant after medium optimization (36.0 mg/ml) was 3 fold higher as compared to unoptimized medium (12.2 mg/ml). For the bioreactor the

online data such as dissolved oxygen, pH, temperature and agitation were monitored and the offline data like dextran concentration, enzyme activity, sucrose concentration and cell optical density were plotted with time (Fig.3).

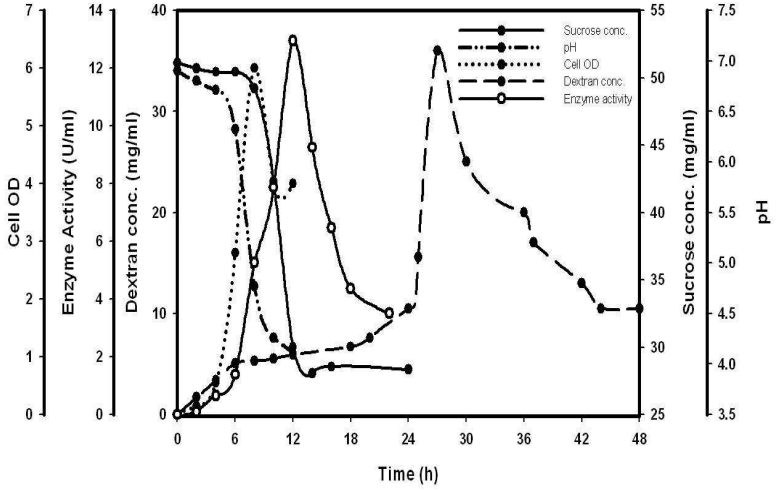

Figure 2 - Variation of dextran concentration, enzyme activity, sucrose concentration, cell optical density and pH during batch fermentation of the mutant of *Pediococcus pentosaceus* (SPAm) in flask.

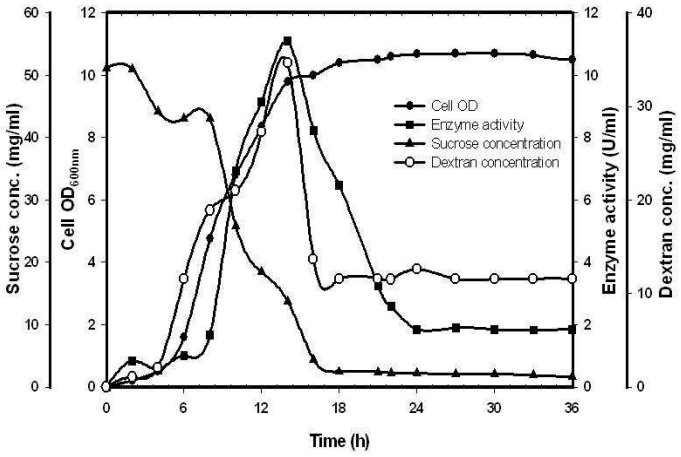

Figure 3 - Variation of dextran concentration, enzyme activity, sucrose concentration and cell optical density during batch fermentation of the mutant of *Pediococcus pentosaceus* (SPAm) in bioreactor.

The dextran concentration after 14-16h was 35 mg/ml which was also in good agreement with the predicted value. The %DO dropped to 40% after 6h of fermentation showing the micro-aerophillic nature of microorganism.

In both cases sucrose concentration profiles showed no consumption of sucrose during first 10-12h. On comparison of dextran and sucrose concentration profiles in the bioreactor, it was found that the dextran concentration peaked after the commencement of sucrose consumption. In bioreactor, the enzyme activity (10.9 U/ml) and the cell optical density both, reached maximum at 14h, which is in agreement with the report of growth associated biosynthesis of dextransucrase (Santos et al., 2000; Rodrigues et al, 2003, Michelena et al., 2003). Oxygen is known to have positive effects on the growth of certain strains of *L. mesenteroides* (Veljkovic et al. 1992). Thus the early production of dextran in bioreactor as

compared to flask culture, is possibly be due to the effect of oxygen mass transfer rates on biosynthesis of dextransucrase and hence dextran.

CONCLUSIONS

Dextran concentration of *Pediococcus pentosaceus* (SPAm) mutant was 12.2 mg/ml with unoptimized medium. Using statistical methods the medium composition was optimized. The response optimization gave 5.115% sucrose and 0.635% yeast extract resulting in enhanced dextran production. The predicted value of dextran (40.8 mg/ml) was in good agreement with the experimental values from flask culture (36.0 mg/ml) and from bioreactor run (35.0 mg/ml). The RSM optimized medium gave 3-fold higher dextran production from the mutant SPAm as compared to unoptimized medium. The results show that the optimized medium can be implemented for large scale dextran production from *Pediococcus pentosaceus* (SPAm).

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