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ENHANCED PRODUCTION OF DEXTRANSUCRASE BY *L. MESENTEROIDES* MTCC 867 USING RESPONSE SURFACE METHODOLOGY

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Abstract
In the present study, basal MRS medium was developed by screening of different media components for dextransucrase production for *L. mesenteroides* MTCC 867. Important MRS media components were screened by Placket Burman method. Further response surface methodology was applied for important media components using central composite design for enhanced enzyme production. The optimum values for the tested variables were; sucrose 34.01%, bacteriological peptone 1.22%, yeast extract 0.88% and K_2HPO_4 1.62%. A quadratic polynomial equation was suggested and then validated experientially. Validation was carried out in shake flasks under conditions predicted by the model. The predicted yield was 782.68 DSU/mL. On experimentation 760.58 DSU/mL dextransucrase was obtained. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. This activity is very high as compared to most of the reports. The dextransucrase production was increased by 8.63 fold over the basal medium.

Key Words: Dextransucrase; Fermentation; Leuconostoc species; Placket-Burman; Response surface methodology.

Introduction

Dextransucrase (EC 2.4.5.1) are extracellular enzymes mainly produced by many lactic acid bacteria such as *Leuconostoc* and *Streptococcus* species [1]. Dextransucrase from *L. mesenteroides* are used in industry for the production of oligosaccharides and dextran. When efficient acceptors like maltose or isolmaltose, are added to the reaction medium dextransucrase synthesizes low molecular weight oligosaccharides instead of high molecular weight polymers. These oligosaccharides are useful in food as food additives because of their desirable physiochemical properties in food and their prebiotics effect on intestinal bacteria, as well as in feed, pharmaceutical, or cosmetic industries and human nutritional applications, as they are specifically metabolized by beneficial saprophyte flora [2]. Dextransucrase is a sucrose inducible enzyme and has gained importance because it produces dextran having 95% linear α-(1→6) glucopyronosyl linkages units and 5% α-(1→2), α-(1→3) and α-(1→4) branched linkage [3]. Dextran has significant commercial values in blood plasma substitute formulations [4]. Considering its wide clinical and commercial applications, efforts are ongoing to enhance dextransucrase and dextran production. The effect of nutrients and other culture conditions such as effect of temperature, pH, minerals, carbon and nitrogen source on the production has been reported by several researchers [5,6,7,8,9,10].

Optimization of fermentation media to have a balanced proportion of various nutrients is very important to get optimum microbial growth and enzyme yield. However many efforts are not carried out to enhance enzyme activity by optimizing the nutritional requirements and the environmental conditions for submerged fermentation. Medium optimization by one factor at a time involves changing the independent variable while fixing the others at certain levels. However, such single dimensional search is laborious and time consuming, especially for a large number of variables [11,12,13]. Hence statistical methods are preferred, as they reduce the total number of experiments and understand the interactions among the nutrients at varying concentration.

In the present work, we report the media optimization for dextransucrase production for *L. mesenteroides* MTCC 867 strain using sequential

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statistical method. It was imperative to design suitable production media as MRS media is specifically used for lactobacilli fermentation. [14]. Most influential media components were analyzed by Plackett-Burman method. RSM was used for further optimization of important media components to enhance the yield. Hence, in the present study sequential optimization strategy was successfully applied to optimize media component for enhanced dextransucrase production.

Materials and methods

Microorganism

Leuconostoc mesenteroides MTCC 867 from Microbial Type Culture Collection and Gene Bank, (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India was used in present study. The organism was maintained through periodic transfer at 25°C on MRS agar slants. These slants were kept at 4°C for further experiments.

Culture medium

The basal modified sMRS media (glucose replaced by sucrose) was used for the experiments with following composition: (g/L): proteose peptone, 10; yeast extract, 5; beef extract, 10; sucrose, 20; triammonium citrate, 2; sodium acetate, 5; tween 80, 1.0; MgSO4, 0.1; $MnSO_4$, 0.05. Medium pH was adjusted to 7.0 before steam sterilization (121ºC, 15 lbs, 20 min). Inoculum was prepared in 250mL Erlenmeyer flasks containing 100mL sterile sMRS medium (180rpm for 12 h at 25ºC). The optical density of the inoculum was adjusted to 1.0 before inoculation and 2% inoculum was used for all experiments unless otherwise mentioned.

Selection of the important media components for process modeling

The Plackett-Burman factorial design was used to select significant medium components affecting the dextransucrase production. Sucrose, bacteriological peptone, yeast extract, proteose peptone, ammonium dihydrogen orthophosphate, K_2HPO_4, $MgSO_4$, $MnSO_4$, sodium acetate and pH were analyzed as possible factors affecting production. Each variable was represented at two levels, upper ("high (+)") and lower ("low (-)") levels of the range covered by each variable and the response [15]. A 12-run Plackett-Burman experimental design was used to screen factors (Table 1), and experimental responses were analyzed by first order model by the following equation

$Y = \beta_o + \Sigma \beta_i x_i$

Where Y is the response for dextransucrase production, β_o is the model intercept and β_i is the linear coefficient, and xi is the level of the independent variable. This model does not describe the interaction among factors and it is used to screen and evaluate important factors that influence the response. From the regression analysis of the variables, the factors having significant effect on dextransucrase production were further optimized by RSM.

Optimization using response surface methodology

The four components viz; sucrose, bacteriological peptone, ammonium dihydrogen orthophosphate and sodium acetate were identified by Plackett-Burman method which affect dextransucrase production. Hence to find the interactive effects of the four variables, these were further optimized to enhance the yield by RSM using Design-Expert Version 6.0.10, Stat-Ease Inc. Minneapolis, USA software. The central composite design (CCD) was employed. The coded terms and actual values are presented in Table 2. Regression analysis was performed on the data obtained. A second-order polynomial equation was used to fit the data by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a four factor system the model equation is

$$Y = \beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

Where Y (dextransucrase) is the predicted response; β_o is the intercept; β_1, β_2, β_3 and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD are independent variables. The proportion of variance explained by the polynomial models obtained was given by multiple coefficient of determination, R^2. The fitted polynomial equation was expressed as three dimensional response surface plots to find the concentration of each factor for maximum dextransucrase production. These diagram shows relationship between the responses and the experimental levels of each factor used in the design. To optimize level of each factor for maximum response 'Numerical optimization' process was employed. The combination of different optimized parameters, which gave maximum dextransucrase yield, was tested experimentally to validate the model.

Analysis of Dextransucrase

The fermented broth was centrifuged (10000 rpm, 4ºC, and 30 min) to obtain clear supernatant. The supernatant was used for dextransucrase estimation.

Dextransucrase activity was determined by measuring the total amount of reducing sugars produced (calculated as fructose) using 3, 5-di-nitrosalicylic acid (DNSA) method [16]. Sucrose (10%) in acetate buffer (0.05 M, pH 5.2) was used as a substrate. For estimation of the enzyme activity, 0.9 mL of substrate solution was mixed with 0.1 mL of enzyme broth and incubated at room temperature for 15 min. The reaction was stopped by addition of 1 mL of DNSA reagent. The reaction mixture was heated in a boiling water bath for 15 min. After cooling, 10 mL of distilled water was added to each tube and mixed on a vortex mixer. The reducing sugars produced were determined spectrophotometrically at 540 nm using Hitachi UV-VIS spectrophotometer.

One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates one µmol of reducing sugar from sucrose in one min at pH 5.2 and 30°C. The relation between these two units was found to be 1U mL = 20.52 DSU mL [17].

Results and Discussion

Simple basal sMRS culture medium gave dextransucrase activity of 88.12 DSU/mL. Hence to enhance this activity placket-burman and response surface methodology was applied sequentially. The conventional optimization technique, e.g. one-factor-at-a-time method, is not only tedious and time-consuming, but also misleading of result interpretation, especially for the interactions among different factors which they are unable to detect. The sequential strategy of using Plackett-Burman and RSM together is an efficient statistical technique for the optimization of multiple variables in order to predict the best conditions with a minimum number of experiments.

Table 1: Plackett-Burman design experiments for media components [a]

Run	A: Sucrose	B: Bacteriological Peptone	C: Yeast Extract	D: Proteose Peptone	E:Amm Dihydrogen Orthophosphate	F: K_2HPO_4	G: $MgSO_4$	H: $MnSO_4$	J: Sodium acetate	K: pH	L: D1	Dextransucrase (DSU/ml)
1	30(+1)	2(+1)	0.1(-1)	2(+1)	1(+1)	0.1(-1)	0.05(+1)	0.001(-1)	0.1(-1)	6(-1)	1	155.95
2	10(-1)	2(+1)	0.1(-1)	0.1(-1)	0.1(-1)	1(+1)	0.05(+1)	0.01(+1)	0.1(-1)	8(+1)	1	355.97
3	10(-1)	0.1(-1)	0.1(-1)	2(+1)	1(+1)	1(+1)	0.005(-1)	0.01(+1)	1(+1)	6(-1)	1	200.64
4	30(+1)	2(+1)	2(+1)	0.1(-1)	1(+1)	1(+1)	0.005(-1)	0.01(+1)	0.1(-1)	6(-1)	-1	151.89
5	10(-1)	2(+1)	2(+1)	0.1(-1)	1(+1)	0.1(-1)	0.005(-1)	0.001(-1)	1(+1)	8(+1)	1	168.76
6	30(+1)	0.1(-1)	0.1(-1)	0.1(-1)	1(+1)	1(+1)	0.05(+1)	0.001(-1)	1(+1)	8(+1)	-1	38.44
7	30(+1)	2(+1)	0.1(-1)	2(+1)	0.1(-1)	0.1(-1)	0.005(-1)	0.01(+1)	1(+1)	8(+1)	-1	61.69
8	10(-1)	0.1(-1)	2(+1)	2(+1)	1(+1)	0.1(-1)	0.05(+1)	0.01(+1)	0.1(-1)	8(+1)	-1	295.65
9	10(-1)	0.1(-1)	0.1(-1)	0.1(-1)	0.1(-1)	0.1(-1)	0.005(-1)	0.001(-1)	0.1(-1)	6(-1)	-1	80.94
10	10(-1)	2(+1)	2(+1)	2(+1)	0.1(-1)	1(+1)	0.05(+1)	0.001(-1)	1(+1)	6(-1)	-1	195.64
11	30(+1)	0.1(-1)	2(+1)	0.1(-1)	0.1(-1)	0.1(-1)	0.05(+1)	0.01(+1)	1(+1)	6(-1)	1	175.51
12	30(+1)	0.1(-1)	2(+1)	2(+1)	0.1(-1)	1(+1)	0.005(-1)	0.001(-1)	0.1(-1)	8(+1)	1	55.94

[a] Values in parentheses are coded variables, actual values in (%w/v)
[b] Readings are average of two determinations

Selection of important factors by Plackett-Burman design

Plackett-Burman design was adopted to select most significant medium components. Table 1 shows the design along with response of different experimental trials. The standard analysis of variance (ANOVA) results calculated from experimental runs shown in Table 3. Contrast coefficients allow determination of the effect of each constituent. A large coefficient either positive or negative indicates that a factor has a large impact on response. A coefficient close to zero means that a factor has little or no effect. The P-value is the probability that the magnitude of a contrast coefficient is due to random process variability and it serves as a tool for checking the significance of each of the coefficients. A low P-value indicates a 'real' or significant effect. Analysis of P-value showed that among the variables tested sucrose, beef extract, yeast extract, triammonium citrate, pH and sodium acetate had significant effect on dextransucrase production ($P<0.05$). ANOVA for dextransucrase production (Y, DSU/mL) indicated the 'F-value' to be 940.59, which implied the model to be significant. ANOVA indicated the R^2 value of 0.9997 for response Y. This again ensured a satisfactory adjustment of the model to the experimental data, and indicated that the model could explain 99% variability in the response. The adequate precision which measures the signal to noise ratio was 100.40 for response, which indicates an adequate signal. A ratio of > 4 is desirable. This model can be used to navigate the design space for response Y. The 'Pred R-Squared' of 0.9914 is in reasonable agreement with 'Adjusted R-Squared' of 0.9987 for Y. The model equation can be shows as

$$\text{Dextransucrase, } Y\ (\text{DSU/mL}) = +139.94 - 5.48 \times \text{sucrose} + 21.29 \times \text{bacteriological peptone} + 13.13 \times \text{yeast extract} + 15.85 \times \text{Amm Dihydrogen Orthophosphate} + 11.11 \times K_2HPO_4 + 1841.85 \times MgSO_4 + 10105.19 \times MnSO_4 - 47.34 \times \text{sodium acetate} + 24.04 \times D1$$

Sucrose was selected as an essential carbon source as it acts as an inducer for dextransucrase synthesis.

Yeast extract and bacteriological peptone were selected as the nitrogen source as they found to be significant for dextransucrase production from *Leuconostoc* species. Potassium dihydrogen orthophosphate acts as a buffering agent and helps to maintaining the pH in the fermentation process [5,6,10]. Sucrose, yeast extract, bacteriological peptone and K_2HPO_4 were selected as significant components for further optimization by RSM.

Response surface methodology

Optimum levels of the above mentioned factors and the effect of their interactions on enzyme production were determined by CCD. Table 2 shows the details of the actual and coded values employed in the RSM as well as the predicted and observed responses for enzyme production (Y). Second order regression equation provided the levels of dextransucrase production as a function of initial values of sucrose, beef extract, yeast extract and sodium acetate which can be predicted by the following equation.

$$\text{Dextransucrase (DSU/mL)} = 35.93 + 20.93 \times A + 15.76 \times B - 441.73 \times C + 271.01 \times D - 0.43 \times A^2 + 2.82 \times B^2 + 176.74 \times C^2 - 5.22 \times D^2 - 1.09 \times A \times B + 6.32 \times A \times C + 9.87 \times A \times D + 59.35 \times B \times C + 1.52 \times B \times D - 287.50 \times C \times D$$

Where A = sucrose, B = bacteriological peptone, C = yeast extract, D = K_2HPO_4

Table 2: Central composite design (CCD) matrix for RSM showing observed and predicted yield of dextransucrase

	Media Components (%)[a]				Dextransucrase (DSU/ml)	
Run	Sucrose (A)	Bacteriological Peptone (B)	Yeast Extract (C)	K_2HPO_4 (D)	Experimental	Predicted
1	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	550.56	560.29
2	20 (-1)	1.625 (+1)	0.875 (-1)	0.875 (-1)	471.74	408.60
3	30 (0)	1.25 (0)	1.25 (0)	2 (+2)	697.85	704.83
4	40 (+1)	1.625 (+1)	1.625 (+1)	0.875 (-1)	657.84	622.42
5	20 (-1)	1.625 (+1)	0.875 (-1)	1.625 (+1)	539.10	563.33
6	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	538.25	560.29
7	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	631.76	560.29
8	30 (0)	1.25 (0)	2 (+2)	1.25 (0)	570.82	588.28
9	20 (-1)	0.875 (-1)	0.875 (-1)	1.625 (+1)	498.60	521.79
10	30 (0)	2 (+2)	1.25 (0)	1.25 (0)	613.80	611.49
11	40 (+1)	0.875 (-1)	1.625 (+1)	1.625 (+1)	654.07	704.98
12	20 (-1)	0.875 (-1)	0.875 (-1)	0.875 (-1)	385.28	367.92
13	40 (+1)	1.625 (+1)	1.625 (+1)	1.625 (+1)	751.20	763.51
14	20 (-1)	0.875 (-1)	1.625 (+1)	0.875 (-1)	308.7	313.21
15	20 (-1)	1.625 (+1)	1.625 (+1)	0.875 (-1)	354.83	387.28
16	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	526.44	560.29
17	20 (-1)	0.875 (-1)	1.625 (+1)	1.625 (+1)	368.52	305.35
18	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	528.70	560.29
19	40 (+1)	0.875 (-1)	0.875 (-1)	1.625 (+1)	864.00	826.50
20	10 (-2)	1.25 (0)	1.25 (0)	1.25 (0)	81.61	114.88
21	40 (+1)	1.625 (+1)	0.875 (-1)	0.875 (-1)	490.72	548.84
22	40 (+1)	0.875 (-1)	0.875 (-1)	0.875 (-1)	512.30	524.55
23	30 (0)	0.5 (-2)	1.25 (0)	1.25 (0)	492.67	512.27
24	50 (+2)	1.25 (0)	1.25 (0)	1.25 (0)	670.72	654.74
25	30 (0)	1.25 (0)	0.5 (-2)	1.25 (0)	731.31	731.13
26	30 (0)	1.25 (0)	1.25 (0)	1.25 (0)	586.02	560.29
27	40 (+1)	0.875 (-1)	1.625 (+1)	0.875 (-1)	594.03	564.75
28	20 (-1)	1.625 (+1)	1.625 (+1)	1.625 (+1)	404.77	380.28
29	40 (+1)	1.625 (+1)	0.875 (-1)	1.625 (+1)	868.37	851.65
30	30 (0)	1.25 (0)	1.25 (0)	0.5 (-2)	399.56	409.87

[a] Values in parentheses are coded variables
[b] Readings are average of two determinations

Table 4 shows ANOVA results for the RSM quadratic equation for response Y. According to the present model A, B, C, D, A^2, C^2, AD, and CD are significant model terms. ANOVA for dextransucrase production (Y, DSU/mL) indicated the 'F-value' to be 25.28, which implied the model to be significant.

Source	SM	DF	MS	F Value	Prob > F
Model	101599.5	9	11288.83	940.5954	0.0011
A	36101	1	36101	3007.967	0.0003
B	4911.524	1	4911.524	409.2324	0.0024
C	1869.097	1	1869.097	155.7348	0.0064
E	611.0836	1	611.0836	50.91602	0.0191
F	300.0641	1	300.0641	25.0016	0.0377
G	20608.94	1	20608.94	1717.155	0.0006
H	24813.92	1	24813.92	2067.517	0.0005
J	5446.483	1	5446.483	453.8057	0.0022
L	6937.385	1	6937.385	578.0289	0.0017
Residual	24.00359	2	12.0018		

SM, Sum of squares; DF, degree of freedom; MS, mean square, R^2: 0.9997; Adj R^2: 0.9987; Pred R^2: 0.9914; CV: 2.14

Table 3: Analysis of variance (ANOVA) results for the PB model for the response

Model terms having values of 'Prob>F' less than 0.05 are considered significant, whereas those greater than 0.10 are insignificant. The 'Lack of Fit P-value' of 0.3814 implies Lack of fit is not significant relative to pure error and that the model fits. ANOVA indicated the R^2 value of 0.9593 for response Y. This again ensured a satisfactory adjustment of the quadratic model to the experimental data, and indicated that this model could explain 95% response variability. The adequate precision which measures the signal to noise ratio was 22.61. A ratio of > 4 is desirable. This model can be used to navigate the design space for the response Y. The 'Pred R-Squared' of 0.8127 is in reasonable agreement with the 'Adjusted R-Squared' of 0.9214 for Y. A good correlation between observed and predicted results reflected the accuracy and applicability of central composite design for process optimization.

Table 4: Analysis of variance (ANOVA) results for the CCD quadratic model

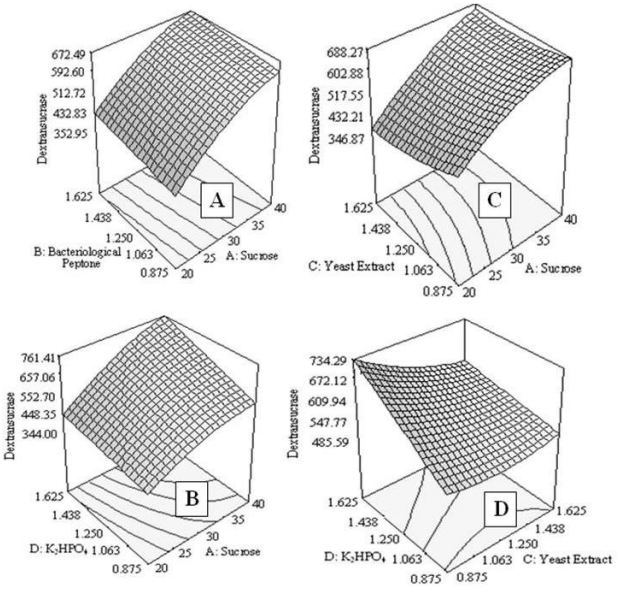
Source	SM	DF	MS	F Value	Prob > F
Model	751706.7	14	53693.33	25.28962	<0.0001
A	437175.5	1	437175.5	205.9102	<0.0001
B	14765.73	1	14765.73	6.954677	0.0187
C	30608.82	1	30608.82	14.41679	0.0018
D	130500.9	1	130500.9	61.46608	<0.0001
A^2	52789.61	1	52789.61	24.86396	0.0002
B^2	4.329342	1	4.329342	0.002039	0.9646
C^2	16944.51	1	16944.51	7.980882	0.0128
D^2	14.83244	1	14.83244	0.006986	0.9345
AB	268.8272	1	268.8272	0.126618	0.7269
AC	9008.079	1	9008.079	4.242815	0.0572
AD	21928.92	1	21928.92	10.32854	0.0058
BC	1114.821	1	1114.821	0.525082	0.4798
BD	0.738307	1	0.738307	0.000348	0.9854
CD	26154.06	1	26154.06	12.31859	0.0032
Residual	31847.06	15	2123.137		
Lack of Fit	23353.41	10	2335.341	1.374758	0.3814
Pure Error	8493.647	5	1698.729		

SM, Sum of squares; DF, degree of freedom; MS, mean square, R^2: 0.9593; Adj R^2: 0.9214; Pred R^2: 0.8127; CV: 8.45

Dextransucrase yield for different levels of significant variables was predicted from the respective surface response plots (Fig. 1. A-D). Each plot represents an infinite number of combinations of two test variables with the other two maintained at their respective central values. When all variables were kept at their central values dextransucrase yield was 526.44 DSU/mL. Fig. 1A shows the response for the interactive factors, bacteriological peptone and sucrose, where K_2HPO_4 and yeast extract were kept at central level. Dextransucrase

yield for this interaction was 672.49 DSU/mL; corresponding to the high amount of sucrose and bacteriological peptone. Fig. 1B shows the interaction between sucrose and K_2HPO_4, where yeast extract and bacteriological peptone were kept at central values. Increase in the sucrose and K_2HPO_4 concentration results in 761.41 DSU/mL. Fig. 1C shows interaction between yeast extract and sucrose where bacteriological peptone and K_2HPO_4 were kept at central values. Increase in concentration of yeast extract and sucrose results in dextransucrase yield of 688.27 DSU/mL. Fig. 1D shows the interaction between K_2HPO_4 and yeast extract which gives dextransucrase production of 734.29 DSU/mL, when sucrose and bacteriological peptone were kept at central values. It can be seen from three dimensional plots that at lower and higher concentration of sucrose, bacteriological peptone, yeast extract and results in lower dextransucrase yield. Higher sucrose concentration shows inhibitory action for dextransucrase yield due to increased viscosity during fermentation which results in mass transfer limitation of nutrients. Thus it is essential to balance these all nutrients to achieve maximum dextransucrase yield [6,7].

Fig. 1. Three dimentional response surface plots, interactive effect of (A) bacteriological peptone and sucrose, (B) K_2HPO_4 and sucrose, (C) yeast extract and sucrose, (D) K_2HPO_4 and yeast extract on dextransucrase (DSU/ml). The other two components are at their central values (central values (%w/v) are sucrose 30.00, bacteriological peptone 1.25, yeast extract 1.25 and K_2HPO_4 1.25).



The RSM model showed that a medium containing sucrose 34.01%, bacteriological peptone 1.22%, yeast extract 0.88% and K_2HPO_4 1.62% was optimum for the dextransucrase production. Validation was carried out in shake flasks under conditions predicted by the model. The predicted yield was 782.68 DSU/mL. On experimentation 760.58 DSU/mL dextransucrase was obtained. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The dextransucrase activity achieved in this work was compared with the reported literature [5,6,8,9,10]. Researchers showed that depending upon the strain used the enzyme activity vary from 3.2 DSU/mL to 450 DSU/mL. In the basal medium *L. mesenteroides* MTCC 867 produces 88.12 DSU/mL enzyme units which were further enhanced to 782.65 DSU/mL using statistical optimization technique.

Conclusion

In the present study sequential statistical strategies, Plackett-Burman design followed by RSM were used successfully to find the optimum values of the significant factors to achieve maximum dextransucrase production. The predicted yield was 782.68 DSU/mL. On experimentation 760.58 DSU/mL dextransucrase was obtained. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated. The dextransucrase production showed 5.97 fold increases over the central point and 8.63 fold increases over the basal medium. The statistical technique was used successfully to achieve maximum dextransucrase yield with respect to the literature reports.

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