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Development of an Efficient Fermentation Process by Response Surface Methodology for Enhanced Dextransucrase Production from *Leuconostoc lactis* KU665298

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Abstract: Dextransucrase is an important extracellular glucosyltransferase which catalyzes the synthesis of dextran from sucrose and valuable oligosachharides. Dextran is a high molecular weight glucose polymer, which has diverse applications in the area of medicine, food and fine chemical industries. A CCD based statistical approach was employed for the enhanced dextransucrase production by *Leuconostoc lactis* KU665298, isolated from sugarcane mill bagasse sample. The most determining factors (i.e. incubation time, inoculum size, sucrose and K_2HPO_4) for dextransucrase production by *L. lactis* were screened by Plackett Burman design. Maximum dextransucrase activity of 3.6 U/mL was obtained with the experimental conditions of incubation time 18h with inoculum volume 6.5 (%, v/v) in a medium (pH 8), containing (%, w/v) sucrose 2, K_2HPO_4 2.5, which corresponded well with the predicted value of 3.05 U/mL by the model.

Key words: Dextransucrase, dextran, *Leuconostoc lactis*, RSM.

Introduction

Dextransucrase (EC 2.4.1.5) is a bacterial enzyme produced extracellularly by various lactic acid bacteria from genera *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella* [4,7,8,17]. It is an inducible glucosyltranferase, which belongs to the glycoside hydrolase family [11,14] (GH 70). Primary or substrate reaction catalyzed by dextransucrase is the synthesis of dextran by transferring D-glucosyl units from sucrose to dextran polymer chain and releasing fructosyl unit free [10,1527,28]. Dextran is polymeric chain of glucosyl units largely linked with α (1-6) linkage and side chains having α (1-2), α (1-3), and α (1-4) linkages depending upon the producing strain [2,16,20,21,34]. It is considered as the first microbial exopolysaccharide asserted for commercial utility and received approval for sustenance use by the Food and Drug Administration [7,12]. Dextran has been extensively utilized in biomedical, pharmaceutical, chemical and food industries for uses such as drug delivery, blood plasma volume expansion, cell encapsulation, emulsification, stabilization, gelling, thickening and tissue engineering, depending on its solubility and molecular mass [3,9,13,18,21,22]. Cross linked dextran is extensively applied for purification of proteins in research [23]. Biomedical application of dextran also includes its usage in coating of magnetic nanoparticles for magnetic resonance imaging and gene therapy [31,32].

Dextransucrase also catalyses a secondary or acceptor reaction, in which the presence of appropriate acceptor (glucose, maltose[2 5], isomaltose, etc.) besides sucrose, shifts the synthesis from dextran formation to prebiotic oligosaccharides production [5]. Recently, dextransucrase has also been utilized for enzymatic synthesis of important polyphenolic compounds such as chlorogenic acid glucoside [29] and caffeic acid glucoside [30].

Keeping in view the importance of dextran-su-

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crase, it became necessary to develop an efficient fermentation process for the enhanced production of enzyme. The 'one-variable at a time' approach has previously been utilized to study the effect of nutrients and culture conditions for dextransucrase production by Leuconostoc strains. However, this approach is time consuming and does not account for the interactive effect of various components. In the present study, optimal conditions for maximum dextransucrase production by isolated *L. lactis* KU665298 has been determined by applying response surface methodology based on central composite design (CCD), in which several parameters were varied simultaneously, models were built and interactive effects of parameters were studied.

Materials and methods

Microorganism and cultivation conditions

A dextransucrase producing culture was isolated from sugarcane baggase sample of sugarcane mill, karnal, India. It was identified as *Leuconostoc lactis* KU665298 by 16s rRNA sequencing and nucleotide sequence was submitted to GenBank sequence database. Chemicals and media ingradients used in experiment, were of analytical grade and from Hi-Media Pvt. Ltd., India. *L. lactis* KU665298 was maintained in modified MRS medium [6] (pH 8) plates (with the composition (%, w/v): Sucrose 2, Peptone 1, Yeast extract 0.5, $MnSO_4$ 0.005, $MgSO_4$ 0.01, Sodium acetate 0.5, agar 2) at 4°C and subcultured every three weeks.

Dextransucrase assay

L. lactis was grown in modied MRS medium broth at 25°C temperature and 150 rpm agitation rate in a temperature controlled orbital shaker for 24 h. Culture broth was then centrifuged and dextransucrase activity was estimated in cell free supernatant, as it is produced extracellularly by *L. lactis*. Dextransucrase activity was estimated by measuring the reducing sugar released from sucrose using the oxide-reduction technique of the dinitosalacylic acid reagent [19]. In the presence of reducing sugars, the 3, 5-dinitrosalicylic acid reduces to 3-amino-5-nitrosalicylic acid, resulting in color change (yellow to Red colouration) which is quantified by measuring absorbance at 540 nm.

Definition of dextransucrase activity

The enzyme activity was expressed in terms of units. One unit (U/ml) of dextransucrase activity was defined as the amount of enzyme that liberates 1 ìmol of reducing sugar (fructose) per min per mL at 25°C in 25 mM sodium acetate buffer (pH 5.0) in a 2.0 % (w/v) sucrose solution.

Screening of most influential physicochemical conditions on the basis of Plackett-Burman design

Plackett-Burman design was applied for the screening of most influential medium ingredients and fermentation conditions for dextransucrase production. A total of nine components *viz.* sucrose, pH, Temperature, agitation rate, inoculum size, peptone, beef extract, K_2HPO_4 and incubation time were chosen to establish the fundamental factors significantly affecting the dextransucrase production. A set of 12 combinations was reproduced by the software, each being illustrated at two levels, high (+1) and low (-1), in which the dextransucrase activity was estimated distinctly. Components showing the positive effect on dextransucrase activity were represented by a Pareto graph. Software Design Expert (Version 10.0) was employed for Plackett–Burman design and regression analysis. The effect of each variable was determined by following equation:

$$E = (\Sigma M^{+} - \Sigma M^{-})/N \ldots\ldots\ldots\ldots\ldots \text{Eq.1}$$

where 'E' is the effect of the tested variable and M^{+} and M^{-} are responses (dextransucrase activity) of experiments at which the parameter was at its higher and lower level, respectively and N is the number of experiment carried out.

Central composite design and statistical analysis

CCD of RSM was applied to optimize the production conditions of most influential factors namely, incubation time, inoculum size, sucrose and K_2HPO_4, keeping rest of the conditions and ingredients same. A total of 30 experimental runs were conducted in CCD and enzyme activity (U/mL) was taken as the response. Design Expert 10 was employed to perform statistical analysis of the data and to evaluate the analysis of variance (ANOVA) for the estimation of significance of

each term and goodness of fit in each case. 3-D response surface plots were generated to determine the individual and interactive effects of these variables.

Results and discussion

Screening of most influential physicochemical conditions on the basis of Plackett-Burman design

The nine independent variables (sucrose, pH, Temperature, agitation rate, inoculum size, peptone, beef extract, K_2HPO_4 and incubation time) were chosen and their effect on dextransucrase production was observed. 12 different combinations of these variables were accomplished by Plackett-Burman design. Experiments were conducted with these different combinations of fermentation conditions and dextransucrase activity was recorded in each case (Table 1). Further, a Pareto chart was constructed to find out the variables, showing positive effect on dextransucrase production. Among these nine independent variables, incubation time, inoculum size, sucrose and K_2HPO_4 were found to have a positive effect on dextransucrase production by *L. lactis* (Figure 1). Recently, dextran production by *Weissella cibaria* NITCSK4 has also been optimized by applying Plackett-Burman design and RSM-Genetic Algorithm based technology, and a 51 % higher dextran production was obtained [12].

Central composite design and statistical analysis

Four parameters i.e. incubation time, inoculum size, sucrose and K_2HPO_4 had positive effect on dextransucrase production by *L. lactis*. Different experimental runs (30) were designed by employing CCD on these parameters and keeping rest of the physicochemical conditions same (Table 2). Maximum dextransucrase activity of 3.6 U/mL was obtained with the experimental conditions of incubation time 18h with inoculum volume 6.5 (%, v/v) in a medium containing (%, w/v) sucrose 2, K_2HPO_4 2.5, and rest of the conditions remained same. The optimized conditions obtained by employing RSM for dextransucrase production by *Leuconostoc mesenteroids* NRRL B-640 were 30 g/l sucrose, 18.9 g/l yeast extract, 19.4 g/l K_2HPO_4 and 15 g/l beef extract [24]. Statistical evaluation was also done for dextran and dextransucrase production by *Lactobacillus acidophilus* ST76480.01, in which dextran yield was significantly effected was increasing sucrose concentration and 15 % sucrose concentration was

Table 1. Plackett-Burman experimental design for evaluating the influence of various independent variables on dextransucrase production

Run	Sucrose (%, w/v)	pH	Temp. (°C)	RPM	I.V. (%, w/v)	Peptone (%, w/v)	Beef extract (%, w/v)	K_2HPO_4 (%, w/v)	I.T. (h)	Response (U/mL)
1	4	6	20	100	6	0.5	4	4	6	1.4
2	1	6	20	100	2	0.5	0.5	1	6	1.09
3	1	10	20	250	6	0.5	4	4	30	1.6
4	1	6	40	100	6	3	0.5	4	30	0.58
5	4	10	20	250	6	3	0.5	1	6	0.6
6	4	10	20	100	2	3	0.5	4	30	1.92
7	4	6	40	250	6	0.5	0.5	1	30	0.79
8	4	6	40	250	2	3	4	4	6	0.17
9	1	10	40	100	6	3	4	1	6	0.12
10	1	6	20	250	2	3	4	1	30	0.96
11	4	10	40	100	2	0.5	4	1	30	0.456
12	1	10	40	250	2	0.5	0.5	4	6	0.1

Temp: incubation temperature; I.V: inoculum volume; I.T: incubation time

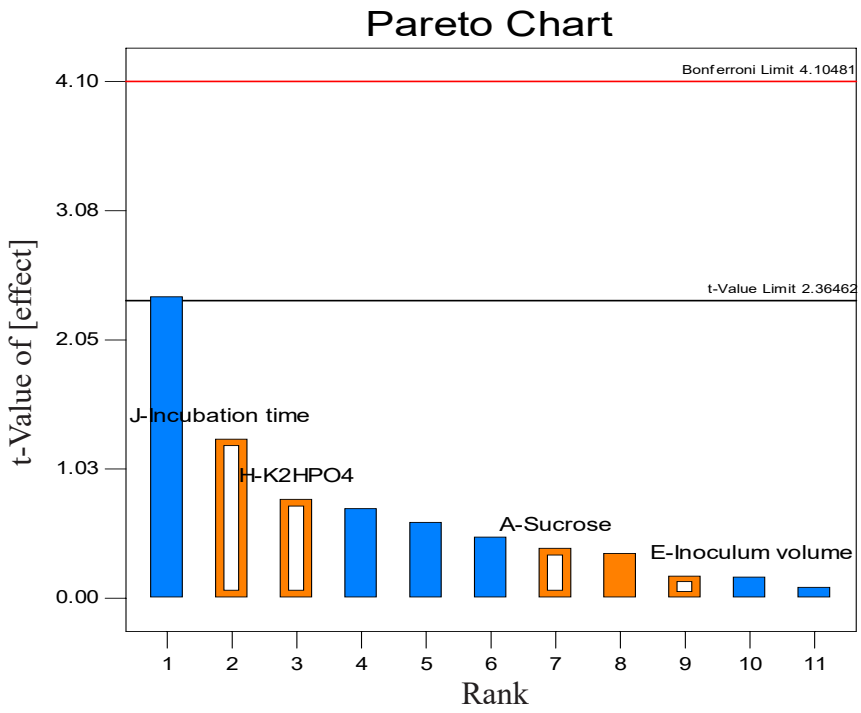

Fig. 1. A pareto chart showing the positive and negative effects of selected variables on dextransucrase production

Table 2. Central composite experimental design by using factors having positive effect on dextransucrase activity

Run	Incubation time (h)	K_2HPO_4 (%, w/v)	Sucrose (%, w/v)	Inoculum volume (%, v/v)	Response (U/mL)
1	18	0.5	2	4.5	2.3
2	6	1.5	1	3.5	0.66
3	18	2.5	2	6.5	3.6
4	30	1.5	1	5.5	2.6
5	30	3.5	3	5.5	2.1
6	30	1.5	3	3.5	2.13
7	18	2.5	2	2.5	2.42
8	6	3.5	3	5.5	0.87
9	18	2.5	2	4.5	2.5
10	18	2.5	0	4.5	0.01
11	30	3.5	1	3.5	1.71
12	18	2.5	4	4.5	2.6
13	6	1.5	3	3.5	0.81
14	18	2.5	2	4.5	2.9
15	6	3.5	1	3.5	0.77
16	-6	2.5	2	4.5	0.00
17	30	1.5	1	3.5	1.76
18	18	2.5	2	4.5	2.78
19	30	3.5	1	5.5	1.93

table 2. (continued).

Run	Incubation time (h)	K_2HPO_4 (%, w/v)	Sucrose (%, w/v)	Inoculum volume (%, v/v)	Response (U/mL)
20	42	2.5	2	4.5	1.56
21	18	2.5	2	4.5	2.89
22	6	3.5	1	5.5	0.68
23	18	2.5	2	4.5	2.89
24	30	1.5	3	5.5	2.45
25	6	3.5	3	3.5	0.67
26	6	1.5	1	5.5	0.8
27	30	3.5	3	3.5	1.93
28	6	1.5	3	5.5	0.96
29	18	4.5	2	4.5	2.7
30	18	2.5	2	4.5	2.2

optimum for highest dextransucrase activity (4.64 DSU/mL/h) [1].

The outcomes of CCD were implemented into second order polynomial equation for the prediction of response on the bases of coded value:

$$Responses=R1=+2.69+0.56A-0.030B+0.26C+0.18D-0.064AB+0.013AC+0.072AD-3.125E-003BC-0.059BD-0.017CD-0.56A^2-0.13B^2-0.43C^2+5.208E-0.004D^2 \quad \text{Eq.2}$$

Among four process order suggested through ANOVA by Design expert 10.0, cubic and quardratic process order were proved to be the best due to low standard deviation (0.53) and high R-squared value (0.8411) respectively (Table 3). It was also suggested by a study[24] that the high determination coefficient (R^2= 0.959) of model is responsible for high degree of variability of response (96 %).

Further, the 3D response surface plots (Fig. 2a to 2f) described the independent and combined effects of each variable upon the response i.e., dextransucrase activity. Figure (2a) represents the interaction between incubation time (A) and K_2HPO_4 (B) where the shape of the response surface indicates the effect of these two variables. Higher values of variable A favor the production of dextransucrase achieving maxima at 18 h, whereas values of variable B (K_2HPO_4) do not have a significant effect on response i.e., dextransucrase activity. In Fig. (2f), both the lower and higher values of variable C (sucrose concentration) lead to decrease in the dextransucrase production whereas values of variable D (inoculum volume) do not have a much significant effect on dextransucrase activity. Thus, the response surface plots suggested that independent variables incubation time (A) and sucrose (C) have significant effect on dextransucrase production. Vettori *et al*.,[34] has applied CCD based RSM for increase of dextransucrase production by *L. mesenteroids* FT 045B and highest dextransucrase activity was recorded with sugarcane molasses 40g/L, K_2HPO_4 20 g/L and corn steep liquar 20 g/L.

Table 3. Quadratic fitted model analysis for dextransucrase production by *L. lactis*

Standard deviation	0.53
Mean	1.81
C.V. %	29.48
Press	22.74
R^2	0.8411
Adjusted- R^2	0.6928
Predicted-R^2	0.1507
Adequate Precision	9.866

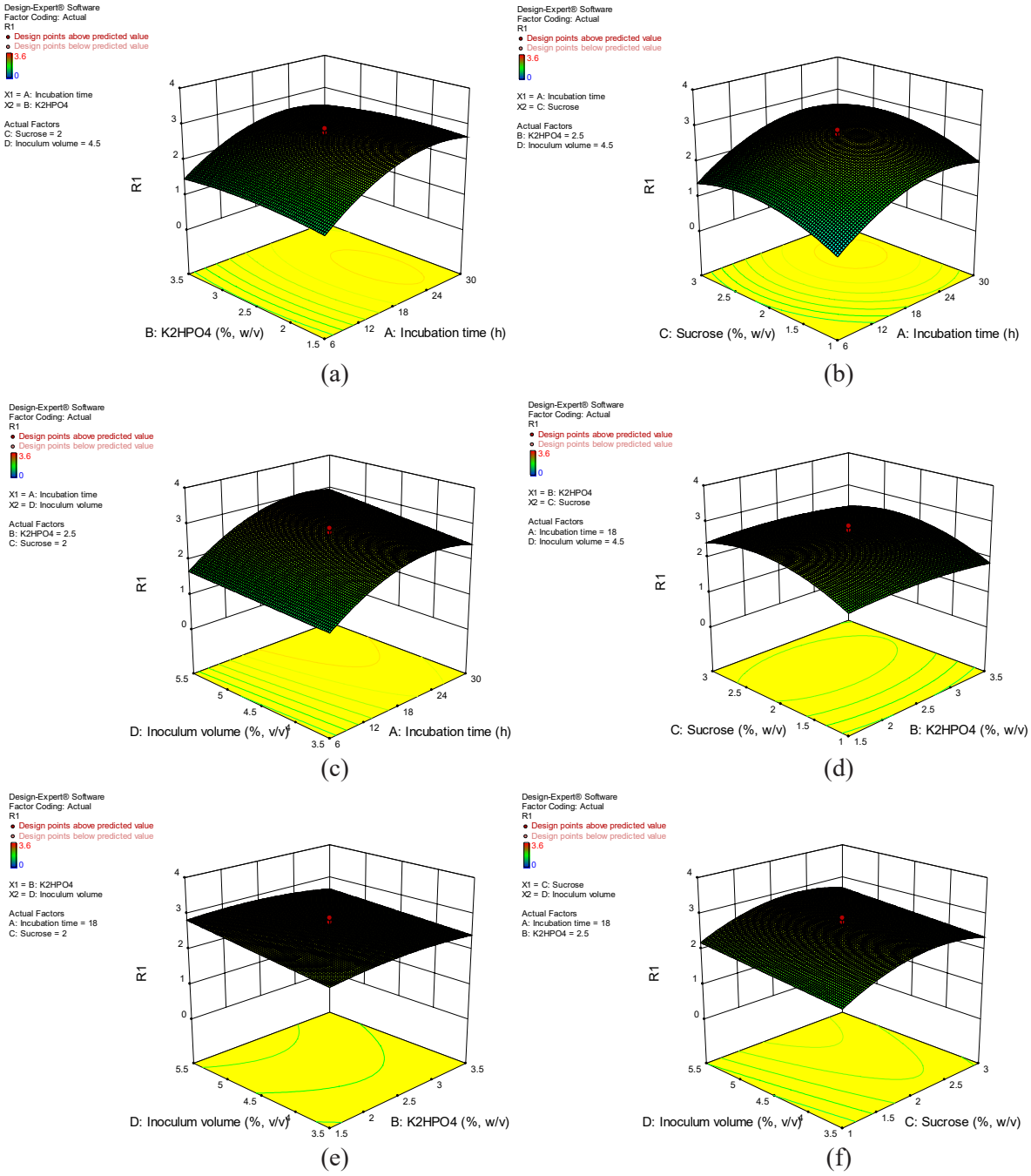

Fig. 2. Response surface plots for the yield of dextransucrase from *L. lactis*, variable components were (a) K_2HPO_4 and incubation time, (b) sucrose and incubation time, (c) inoculum volume and incubation time, (d) sucrose and K_2HPO_4, (e) inoculum volume and K_2HPO_4, (f) inoculum volume and sucrose

Validation of model

The maximum dextransucrase activity (3.60 U/mL), obtained experimentally, was more than the predicted value of 3.05 U/mL, computed by the empirical model (Eq. 2). Usefulness of RSM to actuate the most befitting parameters for dextransucrase production was reflected by a high degree of similarity between the predicted (3.05 U/mL) and experimental data (3.60 U/mL) of the response (Fig. 3). In a study conducted by Purama

and Goyal [24], the experimental value (10.7 U/mL) for maximum dextransucrase activity corresponded well with the predicted value (10.9 U/mL) by the model.

The figure 4 illustrates the deviation of different variables for the production of dextransucrase by *L. lactis* from the reference point. Depending on the conditions of experiments, the dextransucrase activity ranged from 0 to 3.60 U/mL. Model was tested statistically by Fisher's F ANOVA. The P value (P<0.10) of statistical parameters were used to confirm the significance of

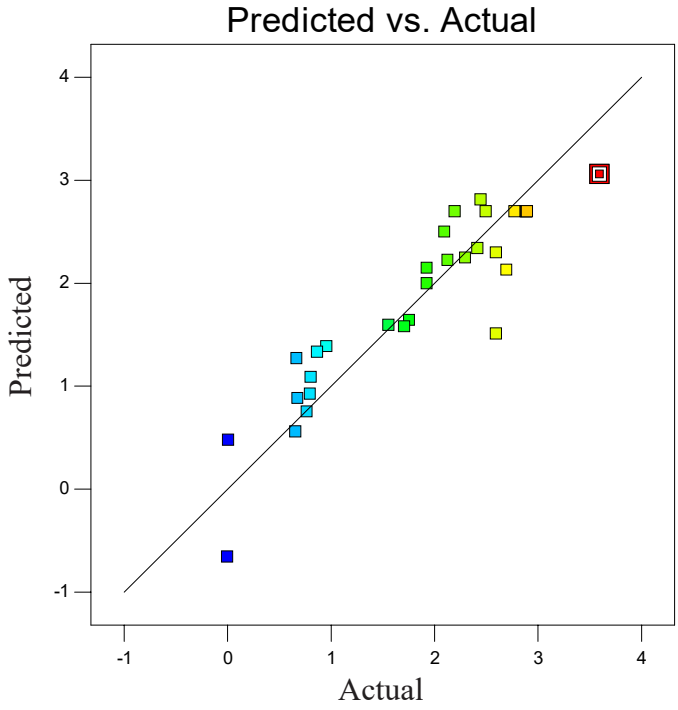

Fig. 3. Experimental verses predicted value of dextransucrase activity

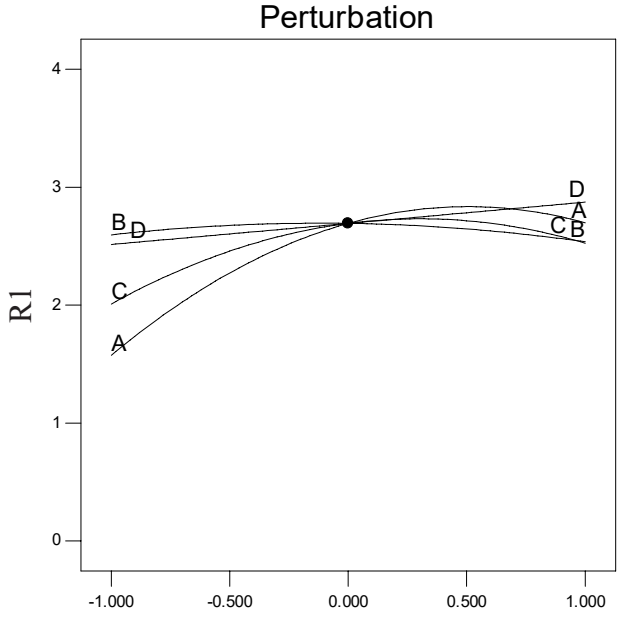

Fig. 4. Perturbation plot showed the optimum value for four variables

model. ANOVA of the quadratic regression model suggested that the model was significant as the value of p>F for the model is less than 0.001. The 'Lack of Fit F-value' of 4.71 implies that 'Lack of Fit' is not significant relative to pure error (Table 4). There is only a 5.06 % chance that this large F-value could occur due to noise. Non-significant lack of fit is good for experiment model as we want model to fit.

Conclusion

A Plackett–Burman design and CCD based RSM was employed to predict the optimal fermentation conditions for dextransucrase production by *L. lactis*. The RSM has proved as an efficient statistical tool for studying independent and cumulative effects of different variables on dextransucrase activity. A 2.4 fold increase in enzyme activity and reduction in production time (24 h to 18 h) were achieved by RSM, which are subsequently going to minimize the time and cost of dextransucrase production by *L. lactis* KU665298.

Table 4. Lack of fit test of CCD calculated value for deriving effective model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	22.52	14	1.61	5.67	0.0009	significant
A-Incubation time	7.61	1	7.61	26.82	0.0001	
B-K2HPO4	0.021	1	0.021	0.074	0.7892	
C-Sucrose	1.6	1	1.6	5.63	0.0314	
D-Inoculum volume	0.77	1	0.77	2.73	0.1193	
AB	0.066	1	0.066	0.23	0.6357	
AC	2.76E-003	1	2.76E-003	9.72E-003	0.9228	
AD	0.083	1	0.083	0.29	0.5972	
BC	1.56E-004	1	1.56E-004	5.51E-004	0.9816	
BD	0.056	1	0.056	0.2	0.662	
CD	4.56E-003	1	4.56E-003	0.016	0.9008	
A^2	8.51	1	8.51	30.01	< 0.0001	
B^2	0.44	1	0.44	1.56	0.2309	
C^2	4.97	1	4.97	17.53	0.0008	
D^2	7.44E-006	1	7.44E-006	2.62E-005	0.996	
Residual	4.25	15	0.28			
Lack of Fit	3.84	10	0.38	4.71	0.0506	not significant
Pure Error	0.41	5	0.082			
Cor Total	26.77	29				

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