Research Article

FERMENTATIVE PRODUCTION AND STATISTICAL OPTIMIZATION OF ACTIVE PHARMACEUTICAL INGREDIENT, XYLITOL USING NOVEL ISOLATES OF *CANDIDA PARAPSILOSIS* BKR1

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ABSTRACT

Xylitol is a natural polyol and most widely known for its sugar substitute properties in diabetic patients. It is also used against the oral bacterial population. Most fascinating approach for commercial production of xylitol involves the suitable yeast fermentation. In this present investigation, factorials Optimization of these medium and process conditions are studied. Xylitol production by *Candida parapsilosis* strain BKR1 using Plackett-Burman and RSM are reported in modified minimal medium. The Plackett-Burman screening design reports the significant medium components are Xylose, yeast extract, Pottasium Dihydrogen phosphate and magnesium sulphate. Further factorial optimization using face centred central composite design reveals the optimum levels of the significant medium components as Xylose – 104.69 g/l, Yeast Extract – 4.12 g/l; KH_2PO_4 – 2.84 g/l and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 2.09 g/l. Also the process parameters such as agitation, pH, Temperature and Inoculum level were optimized and validated as Agitation: 107 rpm, pH – 5, Temperature – 29.9°C, Inoculum level – 1 ml.

KEYWORDS: Xylitol, *Candida parapsilosis* BKR1, medium optimization, Response surface methodology, pentose sugar pathway

INTRODUCTION

Xylitol is one of the expensive natural sweeteners and gains considerable attention in the food processing and healthcare industries. They have numerous applications such as tooth decay, ear infection for children and sugar substitute for diabetic patients in the medical/ pharmaceutical sector^{1, 2}. Xylitol does not undergo Maillard reaction, and so it does not darken or reduce the nutritional value of the proteins. Hence in the food industry, xylitol is used in the ingredients and formulations of food to improve storage properties, colour and taste of food products. It is used solely or in combination with other sugar substitutes in the manufacture of sugarless chocolates. Chewing gums, hard caramels, liquorice sweets, wafer fillings, chocolate, pastilles, and other confectioneries for diabetics³. Xylitol can be metabolized in the absence of insulin and can replace sugar on a weight for weight basis⁴ making it a suitable sweetener for diabetic patients⁵. Production of xylitol from lignocellulosic hydrolyzates without detoxification, in which the xylose fermentation strain *Candida* sp. is showed high furfural tolerance and is used to convert xylose into xylitol from various source of non-detoxified lignocellulosic hydrolyzates. The overall utilization of xylose in hydrolyzate reaches over 95 percent⁶. Bioconversion of xylitol is invariably affected by medium composition and process conditions which emphasize the importance of optimization study. Response surface methodology (RSM) is a significant statistical analysis tool useful for optimization of medium requirements and process conditions⁷. Using factorial design, model equations were developed for optimizing different biotechnological intervention of xylitol production⁸. In chemical method of xylitol production, several energy expensive steps involved in the purification of xylose from wood hemicellulose⁹. The microbial conversion of

xylose to xylitol is beneficial especially due to relatively lenient process and evasion of toxic catalysts such as nickel¹⁰. Xylitol production through biotechnological approaches has been recommended due to the involvement of microbes such as bacteria, fungi and yeast¹¹. Among other microorganisms, yeast has some appropriate properties and was recognized to be a prospective candidate for xylitol production^{12, 13}. In the present study, the fermentative production, optimization of medium composition using Plackett-Burman Design, Factorial Design of process variables using Response Surface Methodology (RSM) and its corresponding analysis of variance by novel isolate *Candida parapsilosis* BKR1 were reported. The Plackett-Burman screening design is applied for identifying the most significant nutrient stimulating the xylitol production. Face Centred central composite design (FC-CCD) and Box-Behnken design (BBD) were applied to determine the optimum levels of each significant nutrient and process variable, respectively.

MATERIALS AND METHODS

Media and Microorganism

The yeast strain *Candida parapsilosis* strain BKR1 (NCBI Accession No: KC462059) was isolated as described earlier¹⁴ and the strains were maintained at 4°C in culture medium supplemented with 20 g agar. The medium composition (g/l) is given as: malt extract - 3.0; yeast extract - 3.0; peptone - 5.0; glucose - 10.0, with pH 7. It was stored at 4°C as agar slants and sub-cultured every thirty days to maintain viability. Composition of the modified minimal medium: Ammonium sulphate - 3 g/l; KH_2PO_4 – 5 g/l; Yeast extract – 3 g/l; MgSO_4 – 0.2 g/l; ZnSO_4 – 6 mg/l; CuSO_4 – 0.4 mg/l; FeCl_3 – 15 mg/l; CoCl_2 – 0.45 mg/l; MnSO_3 – 5 mg/l; CaCl_2 – 200 mg/l; Xylose

– 100 g/l. Optimization studies were carried out in the minimal medium.

Fermentation conditions

Xylitol Fermentation was carried out in Erlenmeyer flasks of 250 ml with 100 ml of modified minimal medium (MMM). Each flasks are supplemented with different nutrient concentration for tests according to the selected factorial design and maintained aseptically. After cooling the flasks at room temperature, all the flasks were inoculated with 1 ml of grown culture broth, individually. All the inoculated flasks were maintained at 30°C under agitation at 100 rpm for 48 h based on the results obtained from preliminary screening carried out for five days.

Analytical methods

The quantification of xylose and xylitol were characterized by high performance liquid chromatography using ion moderated partition chromatography column SHODEX SC 1011 sugar column of 300 X 7.8 mm dimension. Samples are eluted with deionized HPLC grade water at a flow rate of 0.5 ml/min at 80°C and detected with a differential refractometer detector (WATERS 410)¹⁵.

Optimization of xylitol production

Plackett–Burman experimental factorial design is a fractional approach and the main outputs of such a design may be estimated as the difference between the average of measurements made at the high level (+1) and low level (-1) of the factor. For the screening of significant medium component in xylitol production, Plackett-Burman experimental design assumes there is only linear correlation and no interaction among the variables. To identify the most significant variables affecting the xylitol production, Plackett-Burman design was employed. The low level (-1) and high level (+1) of Eleven variables are tabulated (Table 1) and were screened in 13 experimental runs as shown in Table 2. The insignificant ones were eliminated in order to obtain a reasonable set of factors convenient to derive a conclusive result. The statistical software package Design Expert 7.0.0 (Stat-ease Inc., USA) was employed to interpret and analyse the experimental data. The significant factors were enlisted based on PBD approach which leads to the usage of central composite design (CCD) and Box-Behnken Design (BBD) to obtain a quadratic model for medium components and process parameters, respectively.

Response Surface Methodology

The face centred central composite design and Box-Behnken design were used to study the effects of variables on their responses and subsequently in the response surface optimization studies. Both the methods are suitable for fitting the model to a quadratic surface and also aid to optimize the actual parameters with least number of experiments, as well as to study the interaction among the process/ medium parameters. In order to determine the existence of a relationship between the factors and response variables, the unprocessed data were analysed through statistical means, using correlation and regression methods. A regression design is typically employed to model a response as a mathematical equations of a few continuous factors and good model parameter assessments are desired⁶.

The coded values of the process parameters are estimated using the following equation:

$$X_i = (X_i - X_0) / \Delta x_i \quad (\text{Eqn. 1})$$

where x_i – coded value of the i^{th} variable, X_i – uncoded value of the i^{th} test variable and X_0 – uncoded value of the i^{th} test variable at center point. The regression analysis is performed to evaluate the response function as a second order polynomial expression,

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i X_i + \sum_{i=1, i < j}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j \quad (\text{Eqn. 2})$$

where Y is the predicted response, β_0 constant, β_i, β_j and β_{ij} are coefficients estimated from regression. They represent the linear, quadratic and cross products of X_i and X_j on output functions (responses).

Model fitting and statistical analysis

The regression analysis with statistical significance and graphical interference were carried out using Design Experts 7.0 software. In order to visualize the association between the experimental and response variables, the surface and contour plots were observed from the models displayed through software options. The optimum values of the process variables were obtained from the regression equation generated based on the response values.

The competence of the models was further vindicated through analysis of variance (ANOVA). Lack-of-fit is exceptional diagnostic test for inspecting the proficiency of a model and compares the error, based on the replicate measurements to the other lack of fit, based on the model performance¹⁵. F-value, calculated ratio between the lack-of-fit mean square and the pure error mean square are statistic parameters used to determine whether the lack-of-fit is acceptable or not, at significant level. The statistical models were validated with respect to xylitol production under the conditions predicted by the model in laboratory level experiments. Samples were drawn at the desired intervals and xylitol production was determined as described elsewhere⁷.

RESULTS

Plackett-Burman experiments (Table 2) showed a conspicuous variation in xylitol production. The deviations reflected the importance of optimization to attain higher xylitol productivity from the industrial perspective. From the Pareto chart, (Figure 1) the variables, viz., Xylose, Potassium Dihydrogen Phosphate, Yeast extract and Magnesium sulphate were selected for further optimization to attain a maximum xylitol yield. The level of factors Xylose, Potassium Dihydrogen Phosphate, yeast extract & magnesium sulphate and the effect of their interactions on xylitol production were assessed through face centred central composite design of RSM.

Thirty experiments were performed at different combinations of the factors as shown in Table 3 and the central point was repeated six times (1, 2, 7, 9, 15 and 18) which is evident from the table itself. The predicted and experimental responses along with design matrix are presented in Table 3. ANOVA was most successful and prominent tool for analysis of statistical information which was employed in this study. The second order polynomial regression equation exhibits the different levels of xylitol production as a function of influencing variables, Xylose, Potassium Dihydrogen Phosphate, yeast

extract and magnesium sulphate, which can be represented in terms of coded factors as:

$$Y = 0.56 + 0.03*A + 0.01*B - 0.018*C + 0.023*D + 0.01*AB - 0.032*AC - 0.005*AD - 0.01*BC - 0.0025*BD + 0.0125*CD - 0.082*A^2 - 0.13*B^2 - 0.082*C^2 - 0.117*D^2 \text{ (Eqn. 3)}$$

where Y is the xylitol yield (g/g), A, B, C and D are Xylose, Potassium Dihydrogen Phosphate, yeast extract and magnesium sulphate, respectively.

Box-Behnken design was used for optimization of the process parameters such as agitation (rpm), temperature (°C), pH and inoculum level (ml/l) based on its influence to affect the xylitol yield during fermentation process. Range of the process parameters were assessed at 4 coded levels as listed in Table 4. The xylitol yield was selected as response due to different experimental runs. Twenty nine experiments were performed in triplicates to analyse the output response as tabulated in Table 5 which shows considerable reduction in standard error.

The quadratic models in terms of coded variables are shown in the following equation (4), where (Y) represents xylitol yield (g/g), as a function of Agitation (A), pH (B), Temperature (C) and Inoculum level (D).

$$Y = 0.534 + 0.04*A + 0.013*B - 0.02*C + 0.017*D + 0.0025*AC - 0.02*AD + 0.017*BC + 0.037*BD + 0.04*CD - 0.137*A^2 - 0.193*B^2 - 0.173*C^2 - 0.142*D^2 \text{ (Eqn. 4)}$$

DISCUSSION

Statistical tools are prominent in analysing the experimental data with the response function usually evaluated through ANOVA significance test and found to express significant results for second order polynomial equation. Regression coefficient (R^2) was statistically calculated as 0.9954 for the xylitol yield (Y), which accounts for only 99% of the variability in response and purely found to show no lack of fit with the model developed. The predicted regression coefficient value of 0.9787 was in reasonable agreement with the adjusted R^2 value of 0.9912 which is evident from the analysis of variance and the contour plots shown in the figure 2 - 4. The adequate precision value of 39.116 indicates satisfactory signal and suggests that the model can be used to navigate in the given design space, since the threshold cut-off of undesired precision is mere 4. Based on the statistical report, there is only 0.01% chance that F-ratio might

increase very large due to noise signals/ standard deviations. The smaller the magnitude of P value, more significant is the corresponding coefficient which signifies the model can be technically adopted for the response chosen such as xylitol production⁷. From the P value, it was found that the interaction among the variables AB, AC, BC, CD was found to be in good agreement with the enhancement of xylitol production. Also the interaction among the above stated variables could decide the rate of xylitol production and it is rationale to understand that this is the rate limiting step for xylitol formation in the isolated novel *Candida parapsilosis* BKR1. The above derived model can be used to predict the xylitol production within the limits of the experimental conditions that the tangible response values agree well with the predicted response values¹¹.

The production of Xylitol with variable interactions were analyzed by plotting surface curves against any two independent variables, while keeping another variable at its central (0) level. The curves of calculating response (viz., Xylitol production) and contour plots from the interactions between the variables are depicted in Figures 2-4. Figures 2 show the dependency of xylitol on xylose substrate against yeast extract and magnesium sulphate as independent variable. The Xylitol production increased with increase of yeast extract concentration up to 4 g/l and then Xylitol production decreases with further growth in yeast extract. Similar effects were noted in Figures 3 and 4. Increase in KH_2PO_4 concentration resulted in the increase in Xylitol production up to 2.8 g/l. The optimal concentrations of $(NH_4)_2SO_4$, KH_2PO_4 , $MgSO_4 \cdot 7H_2O$ and yeast extract for enhancing xylitol yield were dogged by response surface plots and estimated by regression equation¹⁵.

Validation of the Experimental Model: Under the optimized operating concentration in modified minimal medium: Xylose – 104.69 g/l, Yeast Extract – 4.12 g/l; KH_2PO_4 – 2.84 g/l and $MgSO_4 \cdot 7H_2O$ – 2.09 g/l as suggested by the regression model, batch experiments were performed to validate the results¹⁴. Four repeated experiments were performed to check the consistency. The xylitol production (0.555g/g) obtained from experiments was in close agreement to the actual response (0.568 g/g) predicted by the regression model equations, which proved the cogency of the model. The response surface methodology was exhibiting similar analysis and interpretation as described elsewhere^{7, 8}.

Table 1: Plackett Burman Design for Eleven medium components

S No	Factor Code	Factor – Medium Composition	(-) level	(+) level
1	A	Ammonium Sulphate (g/l)	2	4
2	B	Potassium Dihydrogen Phosphate (g/l)	3	7
3	C	Yeast Extract (g/l)	2	4
4	D	Magnesium Sulphate (g/l)	0.1	0.3
5	E	Zinc Sulphate (mg/l)	4	8
6	F	Copper Sulphate (mg/l)	0.3	0.5
7	G	Ferric Chloride (mg/l)	10	20
8	H	Cobalt Chloride (mg/l)	0.3	0.6
9	J	Manganese Sulphite (mg/l)	3	7
10	K	Calcium Chloride (mg/l)	150	250
11	L	Xylose (g/l)	50	150

Table 2: Nutrient screening using Plackett Burman Experimental Design

Run	A (g/l)	B (g/l)	C (g/l)	D (g/l)	E (mg/l)	F (mg/l)	G (mg/l)	H (mg/l)	J (mg/l)	K (mg/l)	L (mg/l)	Xylitol yield (g/g)
1	0	0	0	0	0	0	0	0	0	0	0	0.59
2	+	-	+	+	+	-	-	-	+	-	+	0.34
3	-	-	-	+	-	+	+	-	+	+	+	0.31
4	-	-	-	-	-	-	-	-	-	-	-	0.16
5	+	+	-	+	+	+	-	-	-	+	-	0.25
6	+	-	-	-	+	-	+	+	-	+	+	0.21
7	-	+	-	+	+	-	+	+	+	-	-	0.24
8	-	+	+	-	+	+	+	-	-	-	+	0.34
9	-	+	+	+	-	-	-	+	-	+	+	0.45
10	+	-	+	+	-	+	+	+	-	-	-	0.21
11	+	+	+	-	-	-	+	-	+	+	-	0.20
12	-	-	+	-	+	+	-	+	+	+	-	0.15
13	+	+	-	-	-	+	-	+	+	-	+	0.23

Table 3: Face Centred Central Composite design (FCCCD) in coded levels with xylitol yield as response

Run	A: Xylose	B: Yeastextract	C: KH ₂ PO ₄	D: MgSO ₄	Xylitol yield (g/g)	
					Experiment	Prediction
1	100	4	3	2	0.55	0.54
2	100	4	3	2	0.58	0.57
3	100	2	3	2	0.39	0.38
4	100	4	3	3	0.45	0.44
5	100	6	3	2	0.45	0.44
6	120	2	2	1	0.19	0.19
7	100	4	3	2	0.57	0.56
8	120	2	4	3	0.15	0.15
9	100	4	3	2	0.56	0.55
10	80	6	4	3	0.15	0.15
11	100	4	3	1	0.42	0.41
12	100	4	4	2	0.46	0.45
13	80	6	4	1	0.07	0.07
14	80	2	2	3	0.09	0.09
15	100	4	3	2	0.58	0.57
16	120	2	2	3	0.23	0.23
17	80	2	2	1	0.08	0.08
18	100	4	3	2	0.58	0.57
19	80	4	3	2	0.45	0.44
20	120	6	4	1	0.12	0.12
21	80	2	4	1	0.09	0.09
22	80	2	4	3	0.19	0.19
23	120	6	4	3	0.17	0.17
24	80	6	2	3	0.14	0.14
25	80	6	2	1	0.09	0.09
26	120	4	3	2	0.49	0.48
27	120	6	2	1	0.26	0.25
28	100	4	2	2	0.48	0.47
29	120	6	2	3	0.26	0.25
30	120	2	4	1	0.08	0.08

Table 4: Ranges of coded values in Box Benhem design

Process variable	Code	(-) Level	(+) Level
Agitation	A	50	150
pH	B	4	6
Temperature	C	28	32
Inoculum level	D	0.5	1.5

Table 5: Box Benhem Design in coded levels with xylitol yield as response

Run	A: Agitation (rpm)	B: pH	C: Temperature (°C)	D: Inoculum level (ml)	Xylitol yield (g/g)	
					Experiment	Predicted
1	100	4	30	0.5	0.21	0.20
2	150	5	30	1.5	0.3	0.28
3	50	4	30	1	0.16	0.15
4	150	6	30	1	0.25	0.24
5	100	6	30	1.5	0.25	0.24
6	100	6	30	0.5	0.16	0.15
7	100	5	30	1	0.52	0.49
8	100	5	28	1.5	0.24	0.23
9	50	5	30	0.5	0.18	0.17
10	150	5	32	1	0.23	0.22
11	100	6	32	1	0.19	0.18
12	100	6	28	1	0.19	0.18
13	100	5	32	1.5	0.28	0.26
14	100	5	30	1	0.53	0.50
15	100	5	30	1	0.54	0.51
16	50	5	32	1	0.16	0.15
17	100	4	32	1	0.12	0.11
18	50	5	30	1.5	0.23	0.22
19	100	4	30	1.5	0.15	0.14
20	50	6	30	1	0.18	0.17
21	100	5	30	1	0.55	0.52
22	100	5	30	1	0.53	0.50
23	150	5	30	0.5	0.33	0.31
24	100	5	32	0.5	0.12	0.11
25	100	4	28	1	0.19	0.18
26	150	5	28	1	0.27	0.25
27	150	4	30	1	0.23	0.22
28	50	5	28	1	0.21	0.20
29	100	5	28	0.5	0.24	0.23

Figure 1: Pareto chart showing the effect of media component on xylitol Production **Figure 2: Three dimensional plot showing interactive effect of Yeast extract & Xylose and Magnesium sulphate & Xylose on Xylitol**

yield

Figure 3: Three dimensional plot showing interactive effect of KH_2PO_4 & Xylose and KH_2PO_4 & Yeast extract on Xylitol yield

Figure 4: Three dimensional plot showing interactive effect of Magnesium sulphate & Yeast extract and KH_2PO_4 & Magnesium sulphate on xylitol yield

Figure 5: Three dimensional plot showing interactive effect of Agitation & pH and Agitation & Temperature

Figure 6: Three dimensional plot showing interactive effect of Agitation & Inoculum level and Temperature & pH

Figure 7: Three dimensional plot showing interactive effect of Inoculum level & pH and Inoculum level & Temperature

Box-Behnken Design

To accommodate the response function and experimental data, regression analysis were performed and the second order model for the reaction was valued by ANOVA which reveals the results are statistically significant. Lack of fit was not displayed by the chosen model during the statistical analysis and a regression coefficient of 0.9890 was obtained accounting for 98% variability in response for the xylitol yield (Y) as response function. The predicted R^2 value of 0.9411 was in sensible agreement with the adjusted R^2 value of 0.9780. The adequate precision value of 29.334 indicates an acceptable signal and suggests that the model can be used to navigate in the design space. F ratio of 90.09 implies the model is significant. From the P value it was found that, the interaction between the variables BD and CD was crucial and significant rate limiting regime for enhancing xylitol yield. The discussed model can be used to predict the xylitol yield within the limits of the experimental factors that the actual response values agree well with the predicted response values^{9, 13}.

The response surface curves for the xylitol yield and its interaction with other independent variables were shown in Fig. 5-7. From the diagrams, it was observed that increase in the agitation beyond 107 rpm reduces the xylitol yield which is in corroboration with many of the earlier research findings¹⁰. In general, the ovoid/ elliptical shape of the curve indicates good interaction between the two variables and circular shape indicates no interaction between the variables. From the figure 7, it is observed that elliptical nature of the contour in graphs depicts the mutual interaction of the variables BD and CD. There is a relative significant interaction between every two variables, and there is a maximum predicted yield as indicated by the surface confined in the smaller ellipse in the contour diagrams which is in corroboration with Walther et al., (2001)¹⁰.

Validation of the experimental model was examined by carrying out the batch experiment under optimal operation parameters: Agitation: 107 rpm, pH – 5, Temperature – 29.9°C, Inoculum level – 1 ml as referred by the regression model. Two repeated experiments were performed and the results are compared. The xylitol production (0.527 g/g) obtained from experiments was in significant agreement with that of the actual response (0.538 g/g) predicted by the regression model, which proved the validity of the model.

CONCLUSION

In the present investigation, Plackett-Burman design was adopted to understand the relative importance of different medium components involved with xylitol production. Among the medium constituents, Potassium Dihydrogen Phosphate, Magnesium sulphate, yeast extract and xylose were found to be predominant variables majorly regulating the fermentative production of xylitol in *Candida parapsilosis* BKR1 under optimized conditions. Recurrent factorial designs using Central Composite Design reveals the optimized value of significant variables for xylitol production are: Xylose – 104.69 g/l, Yeast Extract – 4.12 g/l; KH_2PO_4 – 2.84 g/l and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 2.09 g/l. The present study showed the significance of medium components during the commercial xylitol production. Based on the Box Behnken Method, the optimized conditions for the process parameters were found to be Agitation - 107 rpm, pH - 5, Temperature ~ 30°C, yeast Inoculum level - 1 ml. The xylitol yield was determined to be 0.55 g/g which supports the technical and commercial feasibility. In future, the strain improvement strategies could also help in the enhancement of the xylitol yield.

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