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Application of factorial design to the study of xylitol production from corncob hemicellulose hydrolysate by *Candida guilliermondii*

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Abstract

Optimization of the culture medium and process variables for xylitol production from corncob hemicelluloses hydrolysate was carried out using *Candida guillermondii* (NCIM 3124). The optimization was performed with statistical methodology based on experimental designs. The screening of nine nutrients for their influence on xylitol production was achieved using a Plackett-Burman design. The four selected components were optimized with Box-Behnken design using response surface methodology (RSM). The optimum level (g/l) is: $MgSO_4.7H_2O$- 1.34, yeast extract- 4.34, KH_2PO_4- 2.94 and xylose- 9.49 and influence of various process variables on the xylitol production was evaluated. The optimal levels were quantified by the central composite design using RSM. The optimum level of process variables are: temperature (29.88 ^oC), substrate concentration (3.26 g/l), pH (7.25), agitation speed (170.42 rpm), inoculum size (3.41 ml). These conditions were validated experimentally which revealed an enhanced xylitol yield of 0.73 g/g.

Keywords: Xylitol, Corncob, *Candida guillermondii*, Optimization, RSM.

Introduction

Xylitol ($C_5H_{12}O_5$), a five-carbon polyol with sweetness comparable with that of sucrose, has found increasing use in food and pharmaceutical industries due to several advantages. It has outstanding organoleptic and anticariogenic properties (Shen et al. 2001). Besides, it prevents osteoporosis (Mattila et al. 2002), can be consumed by diabeties (Parajo et al. 1998), and can replace antibiotics in the treatment of acute otilis media (Uhari et al. 2000; Tapiainen et al. 2002). Xylitol is also used in chewing gums, candy, soft drinks, ice creams and hygiene products.

In fruits naturally contain xylitol in lower amount and makes it extraction difficult so it is not economically feasible (Makinen and Soderling 1980). In industries xylitol production involves the chemical reduction of xylose obtained by acid hydrolysis of xylion present in the hemicelluloses of plant structural tissues (Bar 1991). Owing to the use of high pressure and temperature, as well as the need for expansive separation and purification steps in the chemical reduction process (Winkelhausen et al. 1998; Cao et al. 1994; Parajo et al. 1998; Nidetzky et al. 1996) , xylitol production through bioconversion has been proposed as an alternative process utilizing microorganism such as bacteria (Izumovi et al. 1988), filamentous fungi (Dahiya 1991) and yeast (Meyrial et al. 1991; Nishio et al. 1989; Gong et al. 1983; Vandeska et al. 1996). Among the microorganisms yeast have been shown to possess some desirable properties has a potential xylitol producer (Dominguez et al. 1997; Girio et al. 1994). Therefore in the present study, yeast strain of species *Candida guillermondii* were selected for xylitol production.

Among various agricultural wastes, corncob is regarded as promising agricultural resources for microbial xylitol production because corn is widely cultivated, and corncobs are rich in hemicellulose but are not effectively utilized. In microbial xylitol production from corncobs, the cobs are first hydrolysed and to produce from hemicelluloses by acid hydrolysis and hydrolysate for used as the medium of xylitol production. Bioconversion of xylitol is influenced by the factors of various ingredients in culture medium. So their optimization study is very important. Response surface methodology (RSM) is a mathematical and statistical analysis, which is useful for the modeling and analysis problems are response of interest is influenced by several variables (Montogomery 2001). RSM were utilized extensively for optimizing different biotechnological process (Li et al. 2007; Naveena et al. 2005).

In the present study, the screening and optimization of medium composition, process variables for xylitol production by Candida guillermondii using Plackett-Burman and RSM are reported. The Plackett-Burman screening design is applied for knowing the most significant nutrients enhancing xylitol production. In Box-Behnken design and central composite design (CCD) were applied to determine the optimum level of each of the significant nutrients and process variables respectively.

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Materials and methods

Microorganisms and maintenance

The yeast strain *Candida guillermondii* (NCIM 3124) was collected from National collection of industrial microorganisms, Pune, India. The lyophilized stock cultures were maintained at 4 ^{o}C on culture medium supplemented with 20 g agar. The medium composition (g/l) was compressed of the following: Malt extract - 3.0; Yeast extract - 3.0; Peptone - 5.0; Glucose -10.0 and pH - 7. It is sub-cultured every thirty days to maintain viability.

Preparation of hemicelluloses hydrolysate

Corncob hemicelluloses hydrolysate was prepared by sequence of process namely size reduction, acid hydrolysis, detoxification, activated charcoal treatment as previously described by Ramesh et al. 2013.

Fermentation Conditions

Fermentation was carried out in 250 ml Erlenmeyer flasks with 100 ml of pretreated corncob hemicelluloses hydrolysate at pH 7. This is supplemented with different nutrients concentration for tests according to the selected factorial design and sterilized at 120 ^{o}C for 20 mins. After cooling the flasks are kept at room temperature, the flasks were inoculated with 1 ml of grown culture broth. The flasks were maintained at 30 ^{o}C under agitated at 200 rpm for 48 hours.

After the optimization of medium composition, the fermentation were carried out with different parameter levels and optimized media for tests according to the selected factorial design. During the preliminary screening process, the experiments are carried out for 5 days and it was found that the maximum production was obtained in 48 hours. Hence experiments were carried out for 48 hours.

Analytical Methods

Xylitol concentrations were determined by high performance liquid chromatography (Ramesh et al. 2013).

Optimization of Xylitol production

Design of Experiment (DOE)

The RSM combined with a 3^3 full factorial experimental design was used to point out the relationship existing between the response functions and the process variables as well as to determine the conditions of these variables able to optimize the fermentation (Ramesh et al. 2013).

Results and discussion

To determine which variables significantly affect xylitol production by Plackett–Burman design. Nine variables are screened in 12 experimental runs (Table 1) and insignificant ones are eliminated in order to obtain a smaller, manageable set of factors. The low level (-1) and high level (+1) of each factor (-1, +1) were listed (g/l): K_2HPO_4 (6.6, 7), yeast extract (1.5, 5), peptone (2, 5), KH_2PO_4 (1.2, 3.6), xylose (9.8, 10.2), $(NH_4)_2SO_4$ (1, 4), $MgSO_4.7H_2O$ (0.7, 1.3), malt (2.8, 3.2) and glucose (9.8, 10.2) and they are coded with A, B, C, D, E, F, G, H, I respectively.

Plackett-Burman experiments (Table 1) showed a wide variation in xylitol production. This variation reflected the importance of optimization to attain higher productivity. From the pareto chart shown in Fig. 1 the variables, viz., $MgSO_4.7H_2O$, yeast extract, KH_2PO_4 and xylose were selected for further optimization to attain a maximum response.

Table 1. Plackett – Burman Experimental Design for nine variables

Run Order	A	B	C	D	E	F	G	H	I	Xylitol yield (g/g)
1	-1	1	-1	-1	-1	1	1	1	-1	0.28
2	1	-1	-1	-1	1	1	1	-1	1	0.20
3	1	1	-1	1	1	-1	1	-1	-1	0.45
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.43
5	-1	-1	-1	1	1	1	-1	1	1	0.23
6	-1	-1	1	1	1	-1	1	1	-1	0.38
7	1	1	1	-1	1	1	-1	1	-1	0.53
8	-1	1	1	-1	1	-1	-1	-1	1	0.52
9	1	-1	1	-1	-1	-1	1	1	1	0.60
10	1	1	-1	1	-1	-1	-1	1	1	0.62
11	-1	1	1	1	-1	1	1	-1	1	0.43
12	1	-1	1	1	-1	1	-1	-1	-1	0.59

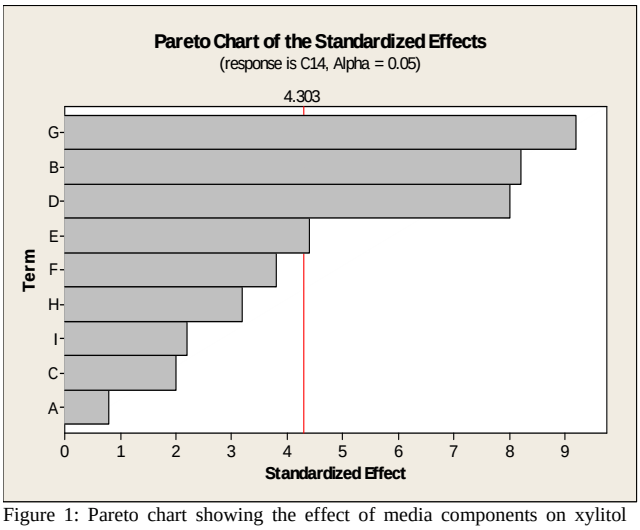

Figure 1: Pareto chart showing the effect of media components on xylitol production.

Table 2. Ranges of variables used in Box-Behnken design

S.No	Variables	Code	Levels (g/l)		
			-1	0	1
1	$MgSO_4.7H_2O$	A	0.6	1.2	1.8
2	Yeast Extract	B	2	4	6
3	KH_2PO_4	C	1	3	5
4	Xylose	D	8	10	12

The selected nutrients levels and effect of their interactions on xylitol production were determined by Box-Behnken design of RSM. The design matrix of experimental results by tests planned according to the 29 full factorial designs. Twenty nine experiments were preferred at different combinations of the factors shown in Table 2 and the central point was repeated five times. The predicted and observed responses along with design matrix are presented in Table 3.

The quadratic models in terms of coded variables are shown in the following equation (1), where (Y) represents xylitol yield (g/g), as a function of $MgSO_4.7H_2O$ (A), yeast extract (B), KH_2PO_4 (C), xylose (D).

Table 3. Box-Behnken design in coded levels with xylitol yield as response

Runs	A	B	C	D	Xylitol Yield (g/g)	
					Experimental	predicted
1	0	-1	1	0	0.39	0.42
2	0	1	0	-1	0.26	0.27
3	0	0	1	1	0.50	0.47
4	1	0	1	0	0.43	0.46
5	0	0	0	0	0.66	0.65
6	0	1	0	1	0.52	0.52
7	-1	0	0	1	0.44	0.48
8	-1	1	0	0	0.29	0.26
9	0	0	1	-1	0.48	0.45
10	0	-1	-1	0	0.34	0.35
11	-1	0	0	-1	0.32	0.36
12	0	0	0	0	0.66	0.65
13	0	0	-1	-1	0.37	0.35
14	-1	-1	0	0	0.47	0.46
15	-1	0	1	0	0.51	0.48
16	-1	0	-1	0	0.40	0.37
17	0	0	-1	1	0.53	0.51
18	1	0	0	1	0.50	0.50
19	1	0	0	-1	0.45	0.45
20	1	1	0	0	0.59	0.55
21	0	0	0	0	0.65	0.65
22	1	-1	0	0	0.33	0.30
23	0	1	1	0	0.38	0.40
24	1	0	-1	0	0.48	0.51
25	0	-1	0	1	0.35	0.34
26	0	1	-1	0	0.38	0.40
27	0	-1	0	-1	0.41	0.41
28	0	0	0	0	0.66	0.65
29	0	0	0	0	0.66	0.65

$$Y = 0.66 + 0.029A + 0.11B + 0.016C + 0.046D + 0.11AB - 0.040AC - 0.018AD - 0.012BC + 0.080BD - 0.035CD - 0.099A^2 - 0.16B^2 - 0.10C^2 - 0.11D^2 \quad \ldots.(1)$$

To fit the response function and experimental data, regression analysis was performed and the second order model for the response was evaluated by ANOVA. The regression for the response was statistically significant. For the response (Y), the model did not show any lack of fit and determination coefficient (R^2) for xylitol production obtained was 0.9639, which explained 96% of the variability in response. The predicted R^2 value of 0.7931 was in reasonable agreement with the adjusted R^2 value of 0.9279. An adequate precision value greater than 4 is desirable. The adequate precision value of 17.024 indicates an adequate signal and suggests that the model can be to navigate the design space. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. The smaller the magnitude of the P, more significant is the corresponding coefficient. P value less than 0.05 indicate the model terms are significant. From the P value it was found that, the variables, A, D, A^2, B^2, C^2, D^2, A*B, A*C, B*D and C*D were significant for xylitol production. The above model can be used to predict the xylitol production within the limits of the experimental factors that the actual response values agree well with the predicted response values.

Central composite design

Central composite design was used for optimization of the process variables namely temperature (^oC), substrate concentration (g/l), pH, agitation speed (rpm) and inoculum size (ml) for xylitol yield. Five process variables and assessed at 5 coded levels as previously done by Ramesh et al. 2013. Table 4 lists the predicted and observed response along with design matrix of experimental results by tests planned according to the full factorial designs. The xylitol yield was selected as the response due to the different cycles of the runs. Fifty experiments were performed in triplicate and the central point was repeated eight times.

Table 4. Central Composite design (CCD) in coded levels with xylitol yield as response

Runs	A	B	C	D	E	Xylitol yield(g/g)	
						Exp	Pred
1	-2.37	0	0	0	0	0.56	0.55
2	-1	1	1	1	1	0.70	0.65
3	-1	-1	1	1	-1	0.35	0.39
4	0	0	0	0	0	0.72	0.72
5	1	1	1	1	-1	0.68	0.64
6	-1	1	1	-1	-1	0.53	0.56
7	0	0	0	0	0	0.72	0.72
8	1	1	-1	-1	-1	0.32	0.25
9	0	0	0	-2.37	0	0.45	0.40
10	-1	1	-1	1	-1	0.52	0.45
11	1	-1	1	1	1	0.65	0.66
12	1	1	1	1	1	0.60	0.64
13	0	0	-2.37	0	0	0.27	0.29
14	0	-2.37	0	0	0	0.47	0.45
15	-1	-1	-1	-1	1	0.51	0.54
16	0	0	0	0	-2.37	0.21	0.27
17	-1	1	-1	1	1	0.59	0.63
18	2.37	0	0	0	0	0.45	0.48
19	0	0	0	0	0	0.72	0.72
20	-1	-1	1	1	1	0.55	0.56
21	1	1	-1	1	1	0.59	0.52
22	-1	1	1	-1	1	0.58	0.64
23	-1	-1	-1	1	1	0.58	0.57
24	-1	1	-1	-1	-1	0.40	0.41
25	0	0	0	0	0	0.73	0.72
26	0	2.37	0	0	0	0.50	0.54
27	0	0	2.37	0	0	0.60	0.59
28	0	0	0	0	0	0.72	0.72
29	1	-1	1	-1	-1	0.49	0.49
30	-1	1	-1	-1	1	0.59	0.56
31	-1	-1	-1	1	-1	0.34	0.32
32	0	0	0	0	0	0.72	0.72
33	1	1	1	-1	1	0.56	0.50
34	0	0	0	0	0	0.72	0.72
35	0	0	0	0	2.37	0.59	0.55
36	-1	1	1	1	-1	0.61	0.55
37	0	0	0	2.37	0	0.50	0.57
38	-1	-1	1	-1	-1	0.45	0.44
39	1	-1	-1	1	-1	0.43	0.40
40	-1	-1	-1	-1	-1	0.32	0.31
41	1	-1	1	1	-1	0.65	0.58
42	1	1	-1	1	-1	0.39	0.43
43	1	1	1	-1	-1	0.51	0.51
44	1	-1	-1	1	1	0.60	0.56
45	1	-1	-1	-1	1	0.39	0.40
46	1	-1	1	-1	1	0.50	0.55
47	1	1	-1	-1	1	0.28	0.32
48	-1	-1	1	-1	1	0.61	0.58
49	0	0	0	0	0	0.72	0.72
50	1	-1	-1	-1	-1	0.25	0.26

Exp- Experimental; Pre-Predicted

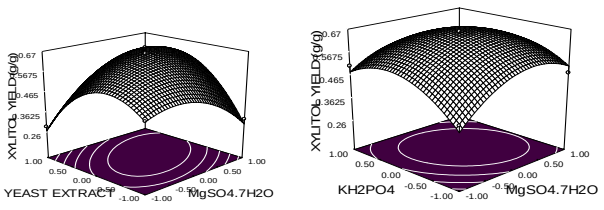

Figure 2: 3D Plots showing the interactive effect of $MgSO_4.7H_2O$ and yeast extract, $MgSO_4.7H_2O$ and KH_2PO_4 on xylitol yield.

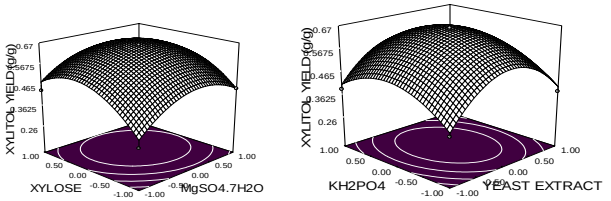

Figure 3: 3D Plot showing the interactive effect of $MgSO_4.7H_2O$ and xylose, yeast extract and KH_2PO_4 on xylitol yield.

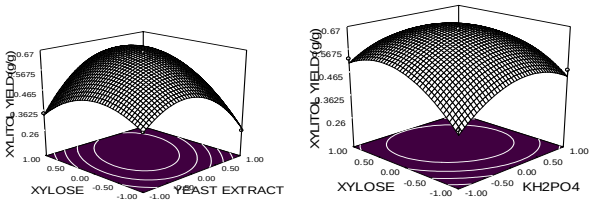

Figure 4: 3D Plot showing the interactive effect of yeast extract and xylose, KH_2PO_4 and xylose on xylitol yield.

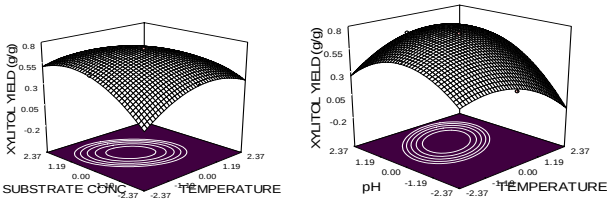

Figure 5: 3D Plot showing the interactive effect of temperature and substrate concentration, temperature and pH on xylitol yield.

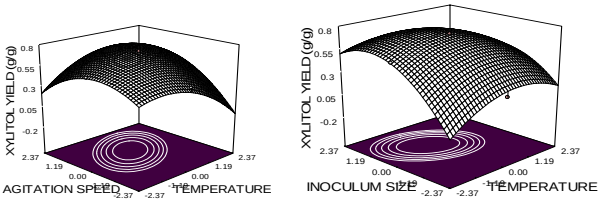

Figure 6: 3D Plot showing the interactive effect of temperature and agitation speed, temperature and inoculum size on xylitol yield.

The quadratic models in terms of coded variables are shown in the following equation (2), where (Y) represents xylitol yield (g/g), as a function of temperature (A), substrate concentration (B), pH (C), agitation speed (D) and inoculums size (E).

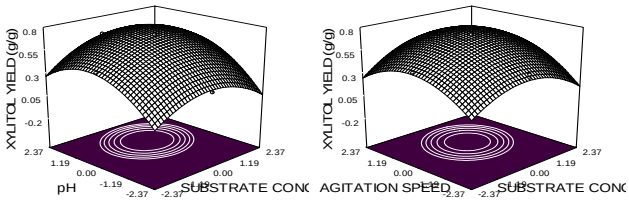

Figure 7: 3D Plot showing the interactive effect of substrate concentration and pH, substrate conc and agitation speed on xylitol yield.

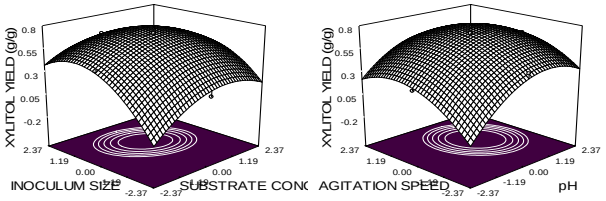

Figure 8: 3D Plot showing the interactive effect of substrate concentration and inoculum size, pH and agitation speed on xylitol yield.

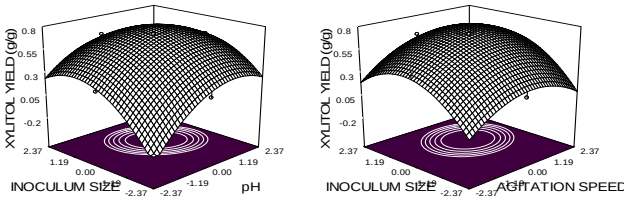

Figure 9: 3D Plot showing the interactive effect of pH and inoculum size, agitation speed and inoculum size on xylitol yield.

$$Y = 0.72 - 0.014A + 0.020B + 0.062C + 0.038D + 0.059E - 0.026AB + 0.027AC + 0.032AD - 0.023AE + 8.125E\text{-}003BC + 8.750E\text{-}003BD - 0.018BE - 0.013CD - 0.021CE - 4.375E\text{-}003DE - 0.037A^2 - 0.040B^2 - 0.049C^2 - 0.042D^2 - 0.055E^2 \quad \ldots\ldots\ldots\ldots (2)$$

To fit the response function and experimental data, regression analysis was performed and the second order model for the response was evaluated by ANOVA. The regression for the response was statistically significant. For the response (Y), the model did not show any lack of fit and determination coefficient (R^2) for xylitol production obtained was 0.9296, which explained 92% of the variability in response. The predicted R^2 value of 0.7319 was in reasonable agreement with the adjusted R^2 value of 0.8811. An adequate precision value greater than 4 is desirable. The adequate precision value of 14.799 indicates an adequate signal and suggests that the model can be to navigate the design space. The model F-value of 19.15 implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. Values of "prob > F" less than 0.05 indicate model terms are significant. Values greater than 0.1 indicates model terms are not significant. In the present work, linear terms of B, C, D, E and A^2, B^2, C^2, D^2, E^2 and the combination of A*B, A*C, A*D, A*E, B*E and C*E were significant for xylitol production.

Both design to investigate the interaction effects of variables on xylitol production were studied by 3D plots surface curves against any two independent variables for keeping another variable at its central (0) level. Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels are more helpful in understanding both the main and the interactive effects of two factors. The response surface curves for the xylitol yield were shown in Figs. 2-9. The nature of the response surface curves shows the interaction between the variables. The elliptical shape of the curve indicates good interaction between the two variables and circular shape indicates no interaction between the variables. From the figure it is observed that elliptical nature of the contour in graphs depicts the mutual interaction of all the variables. 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.

In BBD the Fig. 2 shows the interactive effect of $MgSO_4.7H_2O$ and yeast extract, $MgSO_4.7H_2O$ and KH_2PO_4 on xylitol yield. The xylitol yield increased with increasing in $MgSO_4.7H_2O$ to about 1.3 g/l and after xylitol yield decreased with further increase in $MgSO_4.7H_2O$. The similar results were observed in Figs. 3-4. This is evident from above Figs shows the interactive effect of yeast extract, KH_2PO_4, xylose, on xylitol yield. The optimal operation conditions of $MgSO_4.7H_2O$, yeast extract, KH_2PO_4 and xylose for maximum xylitol yield were determined by response surface analysis and also estimated by regression equation. The predicted values from the regression equation closely agreed with that obtained from experimental values.

In the fermentation medium, temperature is a critical factor and has insightful influence on metabolic activities of microorganisms. The most appropriate temperature for xylitol production is 30 °C. However, the yield for xylitol production is temperature independent, if the yeast is cultured at a temperature of between 30 °C and 37 °C while temperature above 37 °C, the yield decreases dramatically (Silva and Afshar 1994). In CCD the Fig. 5 shows the interactive effect of temperature and substrate concentration, temperature and pH on xylitol yield. The xylitol yield is increased with increasing in temperature to about 30 ^oC and later xylitol yield is decreased with further increase in temperature. The same trend was observed in Figs. 6-9. This is evident from above Figs shows the dependency of pH, substrate concentration, agitation speed, inoculum size on xylitol yield. Increase in pH resulted increase in xylitol production up to 7. It has been observed by Cheng et al. 2009 that ascending in the pH from 4.5 to 6.0 leads to dramatic increase in xylitol and productivity. However, the highest yield of xylitol is found at pH 6.0. This is evident from above Figures shows the dependency of substrate concentration, agitation speed, inoculum size on xylitol production. The xylitol production is maximum at substrate concentration - 3.3 g/L. Initial xylose concentration of from 3.8 g/L showed a linear xylitol production rate in fermentations of *Candida sp. B-22* (Cao et al. 1994). The xylitol production is maximum at agitation speed 170 rpm. Xylitol production is high at 150 rpm is reported by Jeevan et al. 2011. The optimal operation conditions of temperature, substrate concentration, pH, agitation speed, inoculum size for maximum xylitol production were determined by response surface analysis and also estimated by regression equation. The predicted values from the regression equation closely agreed with that obtained from experimental values.

Validation of the experimental model

Validation of the experimental model was tested by the batch experiment with optimal operation conditions (g/l): $MgSO_4.7H_2O$- 1.339, yeast extract- 4.34, KH_2PO_4- 2.939, xylose- 9.49 established by the regression model. Under optimal process variables levels are temperature (29.88 ^oC), substrate concentration (3.26 g/l), pH (7.25), agitation speed (170.42 rpm), inoculum size (3.41 ml). Four repeated experiments were performed and the results are compared. The xylitol production (0.73 g/g) obtained from experiments were very close to the actual response (0.72 g/g) predicted by the regression model, which proved the validity of the model.

Conclusion

In this experiment, Plackett -Burman design were used to determine the relative importance of medium components on xylitol production. Among the variables, $MgSO_4.7H_2O$, yeast extract, KH_2PO_4 and xylose were found the most significant variables. From further optimization studies the optimized values of the nutrients for xylitol production were as follows (g/l): $MgSO_4.7H_2O$- 1.339, yeast extract- 4.34, KH_2PO_4- 2.939, xylose- 9.49. The influence of various process variables such as temperature, pH, substrate concentration, agitation speed and inoculum size on the xylitol production was evaluated by CCD, which permitted the establishment of a significant mathematical model with a co-efficient determination of R^2= 0.92.The interactive effects of temperature and all other variables, substrate concentration and inoculums size, pH and inoculums size were determined to be significant. The optimum levels of process variables are: temperature (29.88 ^oC), substrate concentration (3.26 g/l), pH (7.25), agitation speed (170.42 rpm), inoculum size (3.41 ml). This study showed that the corncob is a good source for the production of xylitol. Using the optimized conditions, the xylitol yield reaches 0.73 g/g. The results show a close concordance between the expected and obtained production level.

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