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Effects of nutrients and process variables on xylitol production by *Debaryomyces hansenii* var *hansenii* using wheat straw

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ABSTRACT

The effects of nutrients and process variables on xylitol production by *Debaryomyces hansenii var hansenii* in wheat straw hemicellulose hydrolysate was studied. Screening and optimization were done using statistical methodology based on experimental designs. Various nutrients were designated and screened by Plackett-Burman design. Selected nutrients are optimized to maximise the xylitol production by Box-Behnken design of Response surface methodology (RSM). The optimum level (g/L) : $(NH_4)_2SO_4$ – 4.34, $MgSO_4.7H_2O$- 1.3, peptone- 5.1 and KH_2PO_4- 3.63 and influence of various process variables on the xylitol production was evaluated. The optimum levels were quantified by central composite design using RSM. The optimal process variables levels were temperature (31.3° C), substrate concentration (3.5 g/L), pH (7.35), agitation speed (194.4 rpm) and inoculum size (3.5 mL). Under the conditions the xylitol production attained the maximum yield of (0.75 g g^{-1}). Under appropriate level of nutrients and process variables, the experimental analysis reveals the maximum yield of xylitol for wheat straw using *Debaryomyces hansenii* var *hansenii*.

Introduction

Xylitol can be defined as a rare sugar, as it exists only in low amount in nature. It has beneficial health properties and represents an alternative to current conventional sweeteners (Granstrom *et al.,* 2007). Currently, xylitol is produced by catalytic reduction of a xylose solution with a high degree of purity using the catalyst Raney nickel. This process is expensive, because it requires the use of high pressure and several steps of xylose purification. In order to produce this chemical in a more environmental-friendly manner, research has been conducted on alternative strategies that utilises microorganisms for conversion of xylose to xylitol from hemicellulosic hydrolysate (Prakasham *et al.,* 2009; Granstrom *et al.,* 2007). Among those, yeast has some desirable properties and is proven to be a potential xylitol producer (Domýnguez *et al.,* 1997; Gýrio *et al.,* 1994).

In recent years, considerable research in converting agricultural waste, which is a renewable and abundantly available material, into value-added products has been carried out. Lignocellulosic materials are mainly composed cellulose, hemicellulose and lignin. The hemicellulose is the second most heterogeneous polymer of pentoses (arabinose, xylose), hexoses (glucose, galactose and mannose) and sugar acids. Unlike cellulose, hemicellulose has not been found to be chemically homogeneous. Xylans (glucomannans, mannans, galactans and arabogalactans) are the most abundant hemicelluloses (He *et al.,* 1993). Hemicellulose is composed of linear and branched heteropolymers of L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose for instance (Sun, 1999). The hemicellulose fraction can be hydrolyzed and used for fermentation of xylose with a view of producing xylitol (Canettieri *et al.,* 2001).

Microbial xylitol production from agricultural wastes containing hemicelluloses could be a possible candidate because it has the potential to realise cheaper production of xylitol with low environmental impact by the effective utilisation of renewable resources (Roberto *et al.,* 1995). Wheat straw, an agricultural residue, is annually generated in abundance around the world. The average yield of straw is 1.3–1.4 kg per kg of grain. Wheat straw is a lignocellulosic material containing

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35–40% cellulose, 30–35% hemicellulose, 10–15% lignin, 5–10% mineral and small amount of other components. Cellulose is a crystalline glucose polymer and hemicellulose is a complex amorphous polymer. The most abundant building block of hemicelluloses is xylan, a xylose polymer.

In the present study, yeast strain of species *Dabaryomyces hansenii var hansenii* was selected for xylitol production. To find out the possibilities and optimal process conditions for maximum production of xylitol, the investigations include the bioconversion of xylitol influenced by the factors of various ingredients concentration in culture medium. So, their screening and optimization study is very important. The Plackett-Burman screening design is applied for knowing the most significant nutrients enhancing xylitol production. Then, Box-Behnken design and central composite design (CCD) was applied to determine the optimum level of each of the significant nutrients and process variables respectively.

Materials and Methods

Collection and maintenance of organism

The yeast strain *Dabaryomyces hansenii var hansenii* was collected from National collection of industrial microorganisms, Pune, India. The lyophilized stock cultures were maintained at 4° 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 subcultured 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 was supplemented with different nutrients concentration for tests according to the selected factorial design and sterilized at 120° C for 20 mins. After cooling, the flasks were kept at room temperature and the flasks were inoculated with 1 ml of grown culture broth. The flasks were maintained at 30°C under agitated at 200 rpm for 48 hours. After the optimization of medium composition, the fermentation was 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 33 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 optimise the fermentation (Ramesh *et al.,* 2013; Naveena *et al.,* 2005; Li *et al.,* 2007; Montgomery, 2001).

Results and Discussion

To determine which variables significantly affect xylitol production by Plackett–Burman design, nine variables were 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 Figure-1 the variables, viz., KH_2PO_4, Yeast extract, $MgSO_4.7H_2O$ and Peptone were selected for further optimization to attain a maximum response.

The levels of factors and the effect of their interactions on xylitol production wre 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

Table-1. *Plackett–Burman Experimental Design for nine variables on xylitol production*

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	-1	0.13
2	1	-1	-1	-1	1	1	1	-1	1	0.42
3	1	1	-1	1	1	-1	1	-1	-1	0.36
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.40
5	-1	-1	-1	1	1	1	-1	1	1	0.18
6	-1	-1	1	1	1	-1	1	1	-1	0.35
7	1	1	1	-1	1	1	-1	1	-1	0.53
8	-1	1	1	-1	1	-1	-1	-1	1	0.41
9	1	-1	1	-1	-1	-1	1	1	1	0.50
10	1	1	-1	1	-1	-1	-1	1	1	0.60
11	-1	1	1	1	-1	1	1	-1	1	0.42
12	1	-1	1	1	-1	1	-1	-1	-1	0.57

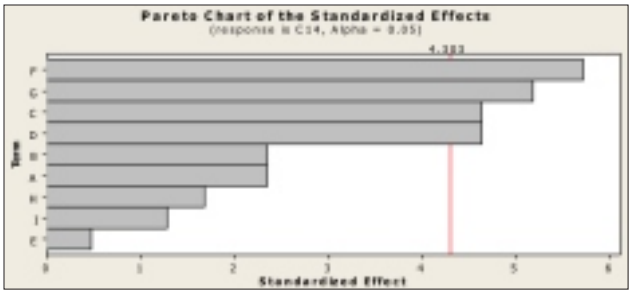

Fig. 1. *Pareto chart showing the effect of media components on xylitol production*

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 and the results are analyzed by ANOVA.

Table-2. *Ranges of variables used in Box-Behnken design for xylitol production*

S.No	Variables	Code	Levels (g/L)		
		-1	0	1	
1	$(NH_4)_2SO_4$	A	2	4	6
2	$MgSO_4.7H_2O$	B	0.6	1.2	1.8
3	Peptone	C	3	5	7
4	KH_2PO_4	D	1	3	5

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

Runs	A	B	C	D	Xylitol Yield (g g^{-1})	
					Experimental	Predicted
1	0	-1	1	0	0.50	0.49
2	0	1	0	-1	0.27	0.29
3	0	0	1	1	0.59	0.57
4	1	0	1	0	0.48	0.52
5	0	0	0	0	0.70	0.70
6	0	1	0	1	0.59	0.59
7	-1	0	0	1	0.55	0.57
8	-1	1	0	0	0.35	0.32
9	0	0	1	-1	0.51	0.46
10	0	-1	-1	0	0.40	0.39
11	-1	0	0	-1	0.34	0.36
12	0	0	0	0	0.70	0.70
13	0	0	-1	-1	0.44	0.40
14	-1	-1	0	0	0.55	0.53
15	-1	0	1	0	0.57	0.56
16	-1	0	-1	0	0.43	0.42
17	0	0	-1	1	0.55	0.54
18	1	0	0	1	0.56	0.54
19	1	0	0	-1	0.51	0.50
20	1	1	0	0	0.65	0.60
21	0	0	0	0	0.70	0.70
22	1	-1	0	0	0.38	0.35
23	0	1	1	0	0.44	0.46
24	1	0	-1	0	0.52	0.56
25	0	-1	0	1	0.39	0.40
26	0	1	-1	0	0.45	0.46
27	0	-1	0	-1	0.41	0.44
28	0	0	0	0	0.70	0.70
29	0	0	0	0	0.70	0.70

The second order regression equation provided the levels of xylitol production as a function of $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, peptone and KH_2PO_4 which can be presented in terms of coded factors as in the following equation.

$$Y = 0.70 + 0.026A + 1.000E\text{-}002B + 0.025C + 0.062D + 0.12AB - 0.045AC - 0.040AD - 0.028BC + 0.085BD - 7.500E\text{-}003CD - 0.090A^2 - 0.15B^2 - 0.091C^2 - 0.11D^2 \quad .....(1)$$

Where Y is the xylitol yield (g g^{-1}) and A, B, C and D is $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, peptone and KH_2PO_4 respectively. ANOVA for the response surface is shown in Table-4. The model *F*-value of 25.18 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 A, C, D and all the square effects of A, B, C, D and the combination of AB, AC, AD and BD are significant for xylitol production. The co-efficient of determination (R^2) for xylitol production is calculated as 0.9618, which is very close to 1 and can explain up to 96.00% variability of the response. The predicted R^2 value of 0.7800 is in reasonable agreement with the adjusted R^2 value of 0.9236. An adequate precision value greater than 4 is desirable. The adequate precision value of 16.795 indicates an adequate signal and suggests that the model can be to navigate the design space.

Table-4. *Analyses of variance (ANOVA) for response surface quadratic model for the production of xylitol*

Source	Sum of square	df	Mean square value	F-value	P-value
Model	0.39	14	0.028	25.18	< 0.0001
A-$(NH_4)_2SO_4$	8.01E-03	1	8.01E-03	7.17	0.018
B-$MgSO_4.7H_2O$	1.20E-03	1	1.20E-03	1.07	0.3176
C-Peptone	7.50E-03	1	7.50E-03	6.71	0.0214
D-KH_2PO_4	0.047	1	0.047	41.96	< 0.0001
AB	0.055	1	0.055	49.43	< 0.0001
AC	8.10E-03	1	8.10E-03	7.25	0.0175
AD	6.40E-03	1	6.40E-03	5.73	0.0313
BC	3.03E-03	1	3.03E-03	2.71	0.1221
BD	0.029	1	0.029	25.87	0.0002
CD	2.25E-04	1	2.25E-04	0.2	0.6605
A^2	0.053	1	0.053	47.03	< 0.0001
B^2	0.15	1	0.15	137.24	< 0.0001
C^2	0.054	1	0.054	48.34	< 0.0001
D^2	0.082	1	0.082	73.48	< 0.0001
Residual	0.016	14	1.12E-03		
Lack of Fit	0.016	10	1.56E-03		
Pure Error	0	4	0		
Cor Total	0.41	28			

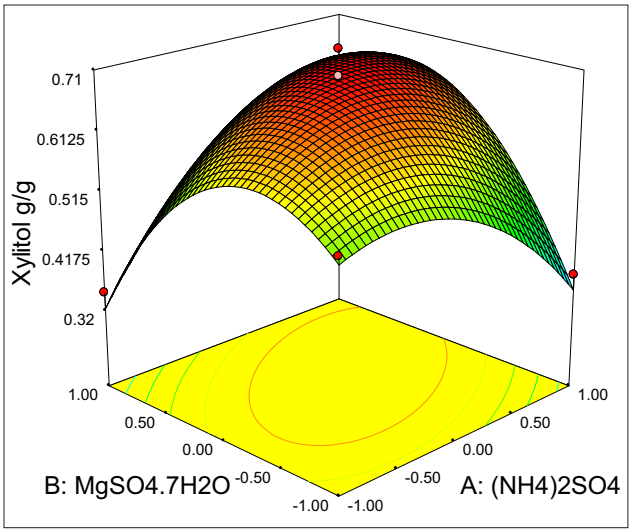

Fig. 2. *3D Plot showing the effect of $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ on xylitol yield*

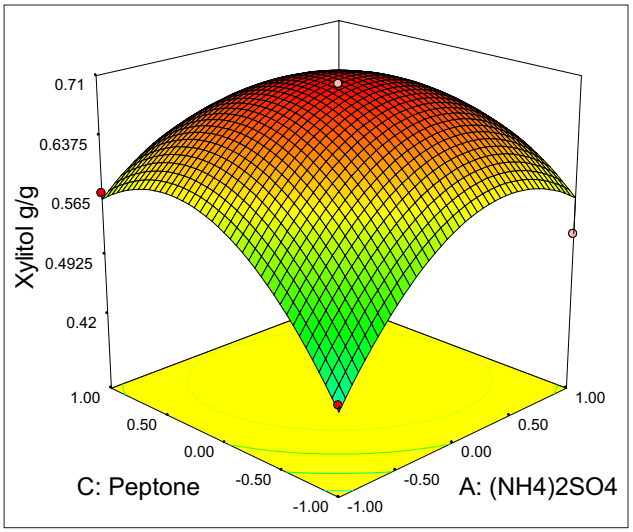

Fig. 3. *3D Plot showing the effect of $(NH_4)_2SO_4$ and peptone on on xylitol yield*

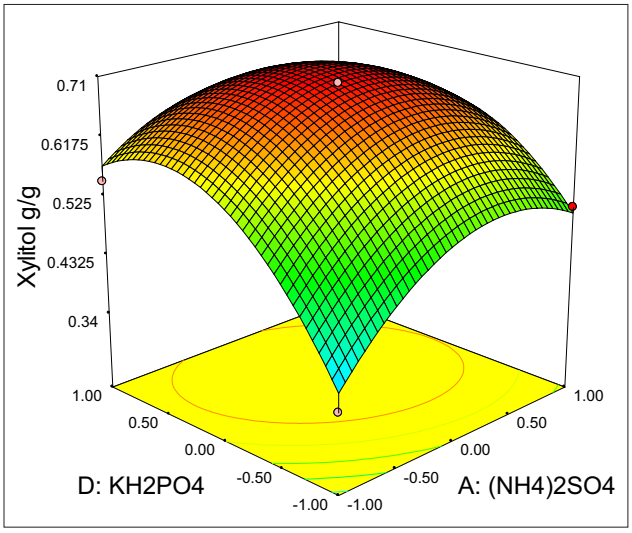

Fig. 4. *3D Plot showing the effect of $(NH_4)_2SO_4$ and KH_2PO_4 on xylitol yield*

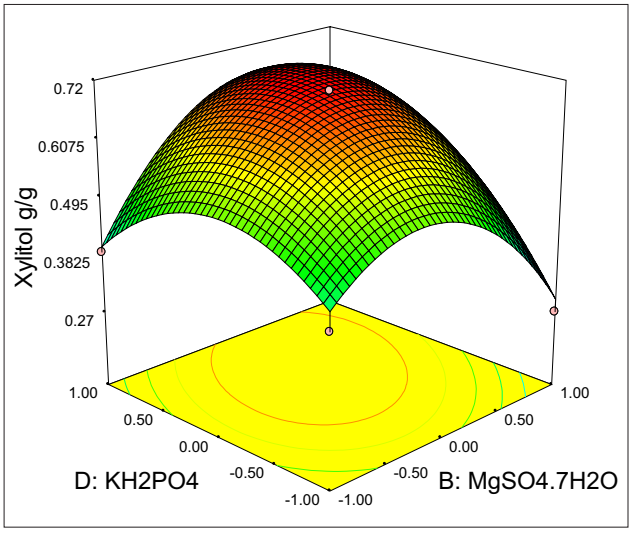

Fig. 5. *Plot showing the effect of $MgSO_4.7H_2O$ and KH_2PO_4 on xylitol yield*

Central composite design (CCD) was used for optimization of the process variables namely temperature (°C), 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). The design matrix of experimental results by tests planned according to the 50 full factorial designs and the central point is repeated eight times. The predicted and observed responses along with design matrix are presented in Table-5 and the results are analyzed by ANOVA.

The second order regression equation provided the levels of xylitol production as a function of temperature, substrate concentration, pH, agitation speed and inoculums size, which can be presented in terms of coded factors as in the following equation:

$$Y = 0.74 + 0.011A + 0.036B + 0.050C + 0.050D + 0.050E - 0.044AB + 0.014AC + 0.043AD - 2.500E\text{-}003AE - 6.250E\text{-}004BC + 0.023BD - 8.125E\text{-}003BE - 3.750E\text{-}003CD + 5.000E\text{-}003CE - 2.500E\text{-}003DE - 0.066A^2 - 0.041B^2 - 0.038C^2 - 0.038D^2 - 0.046E^2 \quad \text{.....(2)}$$

Where Y is the xylitol yield (g g^{-1}), A, B, C, D and E are temperature, substrate concentration, pH, agitation speed and inoculums size respectively.

ANOVA for the response surface is shown in Table-6. The model *F*-value of 21.92 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

Table-5. *CCD in coded levels with xylitol yield as response on xylitol production*

Runs	A	B	C	D	E	Xylitol Yield (g g^{-1})	
						Experimental	Predicted
1	-2.37	0	0	0	0	0.35	0.34
2	-1	1	1	1	1	0.67	0.69
3	-1	-1	1	1	-1	0.32	0.38
4	0	0	0	0	0	0.75	0.74
5	1	1	1	1	-1	0.65	0.64
6	-1	1	1	-1	-1	0.59	0.54
7	0	0	0	0	0	0.75	0.74
8	1	1	-1	-1	-1	0.21	0.29
9	0	0	0	-2.37	0	0.46	0.40
10	-1	1	-1	1	-1	0.54	0.54
11	1	-1	1	1	1	0.70	0.73
12	1	1	1	1	1	0.72	0.72
13	0	0	-2.37	0	0	0.46	0.40
14	0	-2.37	0	0	0	0.48	0.42
15	-1	-1	-1	-1	1	0.40	0.45
16	0	0	0	0	-2.37	0.41	0.36
17	-1	1	-1	1	1	0.60	0.61
18	2.37	0	0	0	0	0.45	0.39
19	0	0	0	0	0	0.75	0.74
20	-1	-1	1	1	1	0.60	0.49
21	1	1	-1	1	1	0.63	0.61
22	-1	1	1	-1	1	0.59	0.64
23	-1	-1	-1	1	1	0.40	0.42
24	-1	1	-1	-1	-1	0.47	0.47
25	0	0	0	0	0	0.75	0.74
26	0	2.37	0	0	0	0.60	0.60
27	0	0	2.37	0	0	0.65	0.64
28	0	0	0	0	0	0.75	0.74
29	1	-1	1	-1	-1	0.46	0.46
30	-1	1	-1	-1	1	0.60	0.55
31	-1	-1	-1	1	-1	0.34	0.32
32	0	0	0	0	0	0.74	0.74
33	1	1	1	-1	1	0.58	0.52
34	0	0	0	0	0	0.75	0.74
35	0	0	0	0	2.37	0.61	0.60
36	-1	1	1	1	-1	0.60	0.60
37	0	0	0	2.37	0	0.65	0.64
38	-1	-1	1	-1	-1	0.40	0.41
39	1	-1	-1	1	-1	0.45	0.48
40	-1	-1	-1	-1	-1	0.32	0.35
41	1	-1	1	1	-1	0.60	0.59
42	1	1	-1	1	-1	0.56	0.53
43	1	1	1	-1	-1	0.38	0.41
44	1	-1	-1	1	1	0.55	0.59
45	1	-1	-1	-1	1	0.49	0.45
46	1	-1	1	-1	1	0.54	0.60
47	1	1	-1	-1	1	0.33	0.37
48	-1	-1	1	-1	1	0.53	0.54
49	0	0	0	0	0	0.75	0.74
50	1	-1	-1	-1	-1	0.34	0.33

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 all the squares effects of A, B, C, D, E and the combination of AB, AD and BD are significant for xylitol production. The co-efficient of determination (R^2) for xylitol production is calculated as 0.9380, which is very close to

Table-6. *Analyses of variance (ANOVA) for response surface quadratic model for the production of xylitol*

Source	Sum of square	Df	Mean square value	F-value	P-value
Model	0.95	20	0.048	21.92	< 0.0001
A-Temperature °C	4.83E-03	1	4.83E-03	2.23	0.1464
B-Substrate Concentration g/L	0.057	1	0.057	26.1	< 0.0001
C-pH	0.11	1	0.11	49.3	< 0.0001
D-Agitation speed rpm	0.11	1	0.11	49.3	< 0.0001
E-Inoculum size mL	0.11	1	0.11	50.39	< 0.0001
AB	0.063	1	0.063	29.05	< 0.0001
AC	6.05E-03	1	6.05E-03	2.79	0.1057
AD	0.058	1	0.058	26.65	< 0.0001
AE	2.00E-04	1	2.00E-04	0.092	0.7636
BC	1.25E-05	1	1.25E-05	5.76E-03	0.94
BD	0.017	1	0.017	7.89	0.0088
BE	2.11E-03	1	2.11E-03	0.97	0.3319
CD	4.50E-04	1	4.50E-04	0.21	0.6522
CE	8.00E-04	1	8.00E-04	0.37	0.5484
DE	2.00E-04	1	2.00E-04	0.092	0.7636
A^2	0.24	1	0.24	110.43	< 0.0001
B^2	0.093	1	0.093	42.8	< 0.0001
C^2	0.081	1	0.081	37.42	< 0.0001
D^2	0.081	1	0.081	37.42	< 0.0001
E^2	0.12	1	0.12	54.64	< 0.0001
Residual	0.063	29	2.17E-03		
Lack of Fit	0.063	22	2.86E-03	228.44	< 0.0001
Pure Error	8.75E-05	7	1.25E-05		
Cor Total	1.01	49			

1 and can explain up to 94.00% variability of the response. The predicted R^2 value of 0.7726 is in reasonable agreement with the adjusted R^2 value of 0.8952. An adequate precision value greater than 4 is desirable. The adequate precision value of 14.983 indicates an adequate signal and suggests that the model can be to navigate the design space.

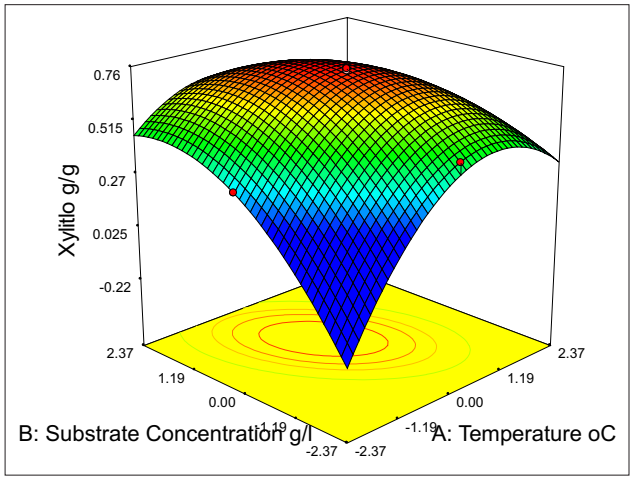

Fig. 6. *3D Plot showing the effect of temperature and substrate concentration on xylitol yield*

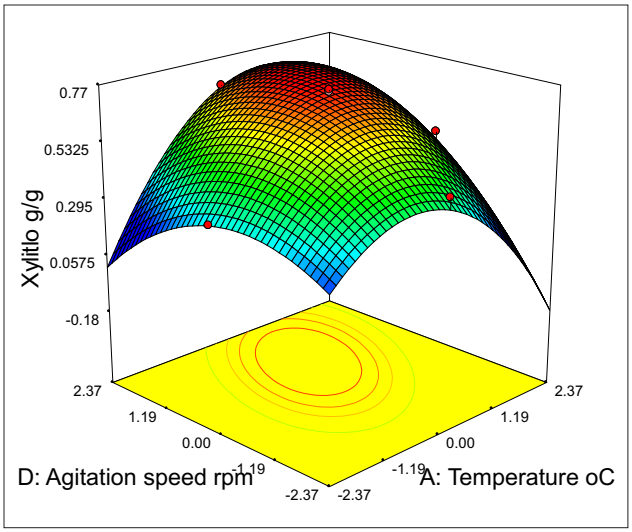

Fig. 7. *3D Plot showing the effect of temperature and agitation speed on xylitol yield*

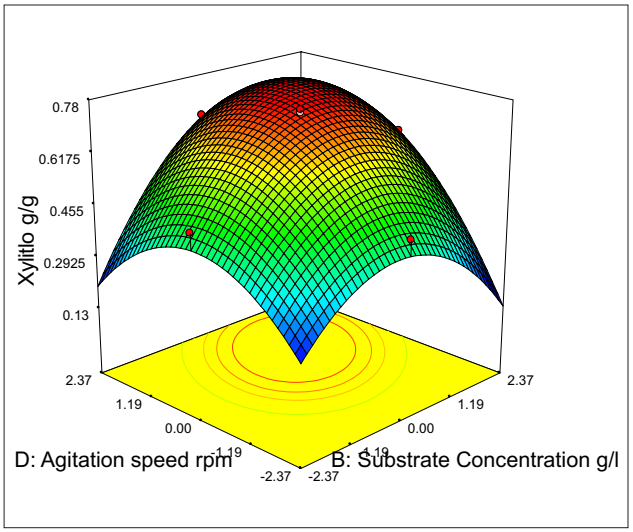

Fig. 8. *3D Plot showing the effect of substrate concentration and agitation speed on xylitol yield*

Both design the interaction effects of variables on xylitol production are studied by plotting 3D surface curves against any two independent variables, while keeping another variable at its central (0) level. The 3D curves of the calculated response (xylitol yield) and contour plots from the interactions between the variables are shown in Figures 2-5. Figure 2 shows the dependency of xylitol on $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$. The xylitol production increased with increase in $(NH_4)_2SO_4$ to about 4.34 g/L and thereafter xylitol production decreased with further increase in $(NH_4)_2SO_4$. The same trend is observed in Figures 3-5. This is evident from above figures that the dependency of $MgSO_4.7H_2O$, peptone and KH_2PO_4 on xylitol production. The optimal operation conditions of $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, peptone and KH_2PO_4 for maximum xylitol production are determined by response surface analysis and also estimated by regression equation. The predicted results are shown in Table-3. The predicted values from the regression equation closely agreed with that obtained from experimental values.

In CCD the Figure 6 shows the dependency of xylitol on temperature and substrate concentration. 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 Afschar, 1994). The xylitol production increased with increase in temperature to about 31.3° C and thereafter xylitol production decreased with further increase in temperature. The same trend is observed in Figures 7-8. This is evident from the figures that the dependency of pH, substrate concentration, agitation speed, inoculum size on xylitol production. The xylitol production is maximum at substrate concentration - 3.5 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 194 rpm. Xylitol production is high at 150 rpm is reported (Jeevan *et al.,* 2011). The optimal operation conditions of temperature, substrate concentration, pH, agitation speed and inoculum size for maximum xylitol production are determined by response surface analysis and also estimated by regression equation. The predicted results are shown in Table-5. The predicted values from the regression equation closely agreed with that obtained from experimental values.

Validation of the experimental model is tested by carrying out the batch experiment under optimal operation conditions (g/L): $(NH_4)_2SO_4$ – 4.34, $MgSO_4.7H_2O$- 1.3, peptone- 5.1 and KH_2PO_4- 3.63 established by the regression model. Under optimal process variables levels are temperature (31.3° C), substrate concentration (3.5 g/L), pH (7.35), agitation speed (194.4 rpm) and inoculum size (3.5 mL). Four repeated experiments are performed and the results are compared. The xylitol production (0.75 g g^{-1}) obtained from experiments as very close to the actual response (0.74 g g^{-1}) predicted by the regression model, which proved the validity of the model.

In this work, Plackett-Burman design was used to test the relative importance of medium components on xylitol production. The nutrients like $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, peptone and KH_2PO_4 are found to be significant for xylitol production. From further optimization studies the optimized values of the nutrients for xylitol production were as follows (g/L): $(NH_4)_2SO_4$– 4.34 , $MgSO_4.7H_2O$ - 1.3 , peptone- 5.1, KH_2PO_4- 3.63, Then the influence of various process variables,

namely, temperature, pH, substrate concentration, agitation speed, and inoculums size on the xylitol production was evaluated by CCD. The optimum levels of process variables are temperature- 31.3 ℃, substrate concentration- 3.5 g/L, pH- 7.35, agitation speed- 194.4 rpm and inoculums size- 3.5 mL. At this optimized medium concentrations and operation conditions the maximum xylitol production was found to be 0.75 g g-1. This study showed that the wheat straw is good tool to increase the xylitol production.

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