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# Stastical Optimization Of Process Variables For Corncob Hemicellulose Hydrolysate To Xylitol By Debaryomyces hansenii var hanseii

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**Abstract:** This study deals with the optimization of the process variables in Xylitol production by *Debrayomyces hansenii var hansenii* (MTTC 3034) using corncob hemicellulosic hydrolysate obtained by acid hydrolysis. The influence of various process variables namely temperature, pH, substrate concentration, agitation speed, inoculum size on the xylitol production was evaluated. The optimal levels of these variables were quantified by the response surface methodology, which permitted the establishment of a significant mathematical model with a co-efficient determination of $R^2$= 0.89. The validation experimental was consistent with the prediction model. The interactive effects of temperature and substrate concentration, temperature and agitation speed were determined to be significant. The optimum levels of process variables are: temperature- 31.8 $^oC$, pH- 7.25, substrate concentration- 3.5 g/l, inoculum size- 3.6 ml, agitation speed- 201.6 rpm. These conditions were validated experimentally which revealed an enhanced xylitol yield of 0.77 $gg^{-1}$.
**Keywords:** Xylitol, Corncob, *Debaryomyces hansenii var hanseni*, Optimization, RSM.

## INTRODUCTION

Lignocellulosic materials from vegetable residues and agro-industrial by-product could be used as potential high energetic or nutritious sources. Moreover, it accumulation in the solid form in the nature causes serious problems of environmental contamination (1-3). It is necessary to find new technologies to use this renewable biomass in different processes to produce economically valuable products. The biotechnological approach is one way to use this biomass as a micro-organism substrate for production of several useful feed stocks.

Among various agricultural waste, 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. Corncob contains approximately 35% hemicelluloses fraction, which can be easily hydrolyzed to constituent carbohydrates. These carbohydrates mainly consist of the xylose and other minor pentose (4-6). Corncob is an ideal raw material to produce xylitol by bioconversion.

Xylitol is a five-carbon sugar alcohol obtained from xylose reduction. The annual xylitol market is estimated to be $340 million, priced at $4-5 $kg^{-1}$ (7). It is a natural functional sweetener, has a sweetness equal to that of sucrose and can replace sucrose on a weight to weight basis (8). It promotes oral health and caries prevention (9). Xylitol has found increasing use in the food industry due to these properties. Xylitol is also an sugar substitute for diabetics (10) because insulin is not needed to regulate its metabolism.

Currently in industrial scale, xylitol is produced by the catalytic hydrogenation of D-xylose in hemicellulosic hydrolysate (11,12). With this method, however it is difficult to obtain a high yield (50-60% based on xylan converted) because considerable amounts of by-product are formed that make downstream processing for the purification of xylitol expensive (11-14). Xylitol production through bioconversion has been proposed as an alternative process utilizing microorganism such as

bacteria (15,16), filamentous fungi (17) and yeasts (18-21). Among those, yeast have been shown to possess some desirable properties has a potential xylitol producer (22-23). Therefore in the present study, yeast strain of species *Dabaryomyces hansenii var hansenii* was selected for xylitol production.

In microbial xylitol production from corncobs, the cobs are first hydrolysed to produce from hemicelluloses by acid hydrolysis and the corncob hydrolysate is then used as the medium for xylitol production. This study investigates the effect of process variables such as pH, temperature, substrate concentration, inoculums size and agitation speed on xylitol yield. Response surface methodology (RSM) is a mathematical and statistical analysis, which is useful for the modeling and analysis problems that the response of interest is influenced by several variables (24). RSM was utilized extensively for optimizing different biotechnological process (25,26).

In the present study, the optimization of process variables for xylitol production by *debaryomyces hansenii var hansenii* using RSM was reported. The central composite design [CCD] was applied to determine the optimum level of each of the significant variables.

## MATERIALS AND METHODS

**Microorganisms and maintenance:**

The yeast strain *Debaryomyces hansenii var hansenii* (MTCC 3034) was collected from Microbial Type Culture Collection & Gene bank, Chandigarh. The lyophilized stock cultures were maintained at 4 $^{o}$C on 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 the pH - 7. It is sub-cultured every thirty days to maintain viability.

**Size reduction:**

Corncob was collected from agricultural farms at perambalur, Tamilnadu, India. The collected raw material was dried in sunlight for 2 days, crushed and sieved for different mesh size ranging from 0.45 mm to 0.9 mm (20–40 mesh) and used for further studies. The composition of the corncob is given in Table 1 which used for xylitol production.

**Acid hydrolysis:**

The pretreatment was carried out in 500 ml glass flasks. 2 g corncobs at a solid loading of 10% (w/w) was mixed with dilute sulfuric acid (1% (w/w)) and pretreated in an autoclave at 120 $^{\circ}$C with residence time of 1 hour. The liquid fraction was separated by filtration and the unhydrolysed solid residue was washed with warm water (60 $^{\circ}$C). The filtrate and wash liquid were pooled together.

**Table 1. Composition of the corncob hemicellulose hydrolysate**

| Components | Amount (g/l) |
|---|---|
| Xylose | 28.7 |
| Glucose | 5.4 |
| Arabinose | 3.7 |
| Cellobiose | 0.5 |
| Galactose | 0.7 |
| Mannose | 0.4 |
| acetic acid | 2 |
| Furfural | 0.8 |
| Hydroxymethyl furfural | 0.2 |

**Detoxification:**

Hemicellulose acid hydrolysate was heated to 100 $^{\circ}$C, and maintained for 15 min to reduce the volatile components. The hydrolysate was then overlimed with solid $Ca(OH)_2$ up to pH 10, in combination with 0.1% sodium sulfite and filtered to remove the insoluble material. The filtrate was adjusted to pH 7 with $H_2SO_4$. The water phase was used for xylitol production.

**Activated charcoal treatment:**

Activated charcoal treatment is an efficient and economic method of reduction in the amount of phenolic compounds, acetic acid, aromatic compounds, furfural and hydroxymethylfurfural normally found in hemicellulosic hydrolysates. After centrifugation, the solutions were mixed with powdered charcoal at 5% (w/v) for 30 and stirred (100 rpm) at 30 $^{\circ}$C. The liquor was recovered by filtration, chemically characterized and used for culture media.

**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 selected nutrient, was used as the fermentation medium. The fermentation medium was compressed of the following (g/l); $KH_2PO_4$-2.74; yeast extract- 3.45; $MgSO_4.7H_2O$- 1.02; $(NH_4)_2SO_4$-3.94. It was sterilized at 120 $^{o}$C for 20 mins. After cooling the flasks to room temperature, the flasks were inoculated with grown culture broth. The fermentation was carried out with different parameter levels given in Table 2 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:**

Sugar and sugar alcohols in the culture broth were measured by high performance liquid chromatography (HPLC), model LC-10-AD (Shimadzu, Tokyo, Japan) equipped with a refractive index (RI) detector. The chromatography column used was a Aminex HPX-87H (300 x 7.8mm) column at 80 $^{o}$C with 5mM $H_2SO_4$ as mobile phase at a flow rate of 0.4 ml/min, and the injected sample volume was 20 µL.

## OPTIMIZATION OF XYLITOL PRODUCTION

**Design of Experiment (DOE):**

The RSM has several classes of designs, with its own properties and characteristics. Central composite design (CCD), Box–Behnken design and three-level factorial design are the most popular designs applied by the researchers. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach.

**Central composite design:**

The CCD is used to study the effects of the variables towards their responses and subsequently in the optimization studies. This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments, as well as to analyze the interaction between the parameters. In order to determine the existence of a relationship between the factors and the response variables, the data collected are analyzed in a statistical manner, using regression. A regression design is normally employed to model a response as a mathematical function (either known or empirical) of a few continuous factors and good model parameter estimates are desired (24).

The coded values of the process parameters are determined by the following equation

$$x_i = \frac{X_i - X_o}{\Delta x}, \quad \ldots. (1)$$

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 estimate the response function as a second order polynomial

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

where $Y$ is the predicted response, $_0$ constant, $_i$, $_j$, $_{ij}$ are coefficients estimated from regression. They represent the linear, quadratic and cross products of $X_i$ and $X_j$ on response.

**Model Fitting and Statistical Analysis:**

The regression and graphical analysis with statistical significance are carried out using Design-Expert software (version 7.1.5, Stat-Ease, Inc., Minneapolis, USA). In order to visualize the relationship between the experimental variables and responses, the response surface and contour plots are generated from the models. The optimum values of the process variables are obtained from the regression equation.

The adequacy of the models is further justified through analysis of variance (ANOVA). Lack-of-fit is a special diagnostic test for adequacy of a model that compares the pure error, based on the replicate measurements to the other lack of fit, based on the model performance (27). *F*-value, calculated as the ratio between the lack-of-fit mean square and the pure error mean square, is the statistic parameter used to determine whether the lack-of-fit is significant or not, at a significance level.

**Validation of the experimental model:**

The statistical model was validated with respect to xylitol production under the conditions predicted by the model in shake-flasks level. Samples were drawn at the desired intervals and xylitol production was determined as described above.

**Table 2. Ranges of variables used in RSM**

| S. N. | Variables | Code | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | | | -2.37 | -1 | 0 | 1 | 2.37 |
| 1 | Temperature ($^{o}$C) | A | 20 | 25 | 30 | 35 | 40 |
| 2 | Substrate concentration (g/l) | B | 1 | 2 | 3 | 4 | 5 |
| 3 | pH | C | 6 | 6.5 | 7 | 7.5 | 8 |
| 4 | Agitation speed (rpm) | D | 50 | 100 | 150 | 200 | 250 |
| 5 | Inoculum size (ml) | E | 1 | 2 | 3 | 4 | 5 |

**Table 3. Central Composite Design (CCD) in coded levels with Xylitol yield as response**

| Runs | A | B | C | D | E | Xylitol yield (g $g^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Experiment | Predicted |
| 1 | -2.37 | 0 | 0 | 0 | 0 | 0.54 | 0.48 |
| 2 | -1 | 1 | 1 | 1 | 1 | 0.70 | 0.73 |
| 3 | -1 | -1 | 1 | 1 | -1 | 0.45 | 0.50 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0.79 | 0.76 |
| 5 | 1 | 1 | 1 | 1 | -1 | 0.60 | 0.66 |
| 6 | -1 | 1 | 1 | -1 | -1 | 0.58 | 0.60 |
| 7 | 0 | 0 | 0 | 0 | 0 | 0.79 | 0.76 |
| 8 | 1 | 1 | -1 | -1 | -1 | 0.41 | 0.49 |
| 9 | 0 | 0 | 0 | -2.37 | 0 | 0.69 | 0.61 |
| 10 | -1 | 1 | -1 | 1 | -1 | 0.56 | 0.57 |
| 11 | 1 | -1 | 1 | 1 | 1 | 0.72 | 0.72 |
| 12 | 1 | 1 | 1 | 1 | 1 | 0.78 | 0.76 |
| 13 | 0 | 0 | -2.37 | 0 | 0 | 0.42 | 0.39 |
| 14 | 0 | -2.37 | 0 | 0 | 0 | 0.45 | 0.43 |
| 15 | -1 | -1 | -1 | -1 | 1 | 0.43 | 0.46 |
| 16 | 0 | 0 | 0 | 0 | -2.37 | 0.57 | 0.48 |
| 17 | -1 | 1 | -1 | 1 | 1 | 0.62 | 0.63 |
| 18 | 2.37 | 0 | 0 | 0 | 0 | 0.63 | 0.60 |
| 19 | -1 | -1 | 1 | 1 | 1 | 0.63 | 0.58 |
| 20 | 1 | 1 | -1 | 1 | 1 | 0.66 | 0.68 |
| 21 | -1 | 1 | 1 | -1 | 1 | 0.63 | 0.69 |
| 22 | -1 | -1 | -1 | 1 | 1 | 0.41 | 0.44 |
| 23 | -1 | 1 | -1 | -1 | -1 | 0.48 | 0.53 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0.79 | 0.76 |
| 25 | 0 | 2.37 | 0 | 0 | 0 | 0.70 | 0.63 |
| 26 | 0 | 0 | 2.37 | 0 | 0 | 0.67 | 0.62 |
| 27 | 0 | 0 | 0 | 0 | 0 | 0.79 | 0.76 |
| 28 | 1 | -1 | 1 | -1 | -1 | 0.55 | 0.58 |
| 29 | -1 | 1 | -1 | -1 | 1 | 0.65 | 0.61 |
| 30 | -1 | -1 | -1 | 1 | -1 | 0.40 | 0.39 |
| 31 | 0 | 0 | 0 | 0 | 0 | 0.76 | 0.76 |
| 32 | 1 | 1 | 1 | -1 | 1 | 0.65 | 0.65 |
| 33 | 0 | 0 | 0 | 0 | 0 | 0.71 | 0.76 |
| 34 | 0 | 0 | 0 | 0 | 2.37 | 0.66 | 0.66 |
| 35 | -1 | 1 | 1 | 1 | -1 | 0.65 | 0.65 |
| 36 | 0 | 0 | 0 | 2.37 | 0 | 0.72 | 0.72 |
| 37 | -1 | -1 | 1 | -1 | -1 | 0.51 | 0.52 |
| 38 | 1 | -1 | -1 | 1 | -1 | 0.51 | 0.54 |
| 39 | -1 | -1 | -1 | -1 | -1 | 0.38 | 0.41 |
| 40 | 1 | -1 | 1 | 1 | -1 | 0.64 | 0.64 |
| 41 | 0 | 0 | 0 | 0 | 0 | 0.79 | 0.76 |
| 42 | 1 | 1 | -1 | 1 | -1 | 0.63 | 0.60 |
| 43 | 1 | 1 | 1 | -1 | -1 | 0.56 | 0.55 |
| 44 | 1 | -1 | -1 | 1 | 1 | 0.61 | 0.60 |
| 45 | 1 | -1 | -1 | -1 | 1 | 0.55 | 0.54 |
| 46 | 1 | -1 | 1 | -1 | 1 | 0.61 | 0.66 |
| 47 | 1 | 1 | -1 | -1 | 1 | 0.55 | 0.57 |
| 48 | -1 | -1 | 1 | -1 | 1 | 0.59 | 0.59 |
| 49 | 0 | 0 | 0 | 0 | 0 | 0.70 | 0.76 |
| 50 | 1 | -1 | -1 | -1 | -1 | 0.49 | 0.48 |

## RESULTS AND DISCUSSIONS

The levels of process variables temperature, substrate concentration, pH, agitation speed, and inoculum size and the effect of their interactions on xylitol production were determined by central composite design of RSM. The design matrix of experimental results by tests planned according to the 52 full factorial designs.

Fifty experiments were preferred at different combinations of the factors shown in Table 2 and the central point was repeated eight times (4, 7, 26, 29, 33, 35, 43, 51). The predicted and observed responses along with design matrix are presented in Table 3 and the results were 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 inoculum size, which can be presented in terms of coded factors as in the following equation:

$$Y = 0.76+0.025A+0.041B+0.049C+0.024D+0.037E-0.028AB-(3.437E-003)AC+0.019AD+(2.813E-003)AE-0.010BC+0.013BD+(4.687E-003)BE+(9.375E-004)CD+(4.687E-003)CE-(3.125E-004)DE-0.038A^2-0.043B^2-0.046C^2-0.017D^2-0.033E^2 \quad \ldots(3)$$

Where Y is the Xylitol yield (g $g^{-1}$), A, B, C, D and E are temperature, substrate concentration, pH, agitation speed and inoculum size respectively. ANOVA for the response surface is shown in (Table 4). The model *F*-value of 11.54 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, B, C, D, E and all the square effects of A, B, C, D, E and the combination of A*B and A*D were significant for xylitol production. The co-efficient of determination ($R^2$) for Xylitol production was calculated as 0.8900, which is very close to 1 and can explain up to 89.00% variability of the response. The predicted $R^2$ value of 0.6956 was in reasonable agreement with the adjusted $R^2$ value of 0.8114. An adequate precision value greater than 4 is desirable. The adequate precision value of 11.347 indicates an adequate signal and suggests that the model can be to navigate the design space.

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.

The interaction effects of variables on xylitol production were 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 production) and contour plots from the interactions between the variables are shown in Figures 1-10. Figure 1 shows the dependency of xylitol production on temperature and pH. The xylitol production increased with increase in temperature to about 31 $^{o}$C and thereafter xylitol production decreased with further increase in temperature. The same trend was observed in Figures 2-10. Increase in pH resulted increase in xylitol production up to 7. This is evident from above Figures shows the dependency of substrate concentration, agitation speed, inoculum size on xylitol production. 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 results are shown in Table 3. The predicted values from the regression equation closely agreed with that obtained from experimental values.

**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 | Prob > F |
|---|---|---|---|---|---|
| Model | 0.59 | 20 | 0.030 | 11.54 | <0.0001 |
| A-Temperature($^o$ C) | 0.026 | 1 | 0.026 | 10.21 | 0.0034 |
| B-Substrate concentration(g/l) | 0.071 | 1 | 0.071 | 27.69 | <0.0001 |
| C-pH | 0.10 | 1 | 0.10 | 39.92 | <0.0001 |
| D-Agitation speed(rpm) | 0.024 | 1 | 0.024 | 9.42 | 0.0046 |
| E-Inoculum size(ml) | 0.059 | 1 | 0.059 | 23.21 | <0.0001 |
| AB | 0.026 | 1 | 0.026 | 10.10 | 0.0035 |
| AC | 3.781E-004 | 1 | 3.781E-004 | 0.15 | 0.7036 |
| AD | 0.012 | 1 | 0.012 | 4.54 | 0.0417 |
| AE | 2.531E-004 | 1 | 2.531E-004 | 0.099 | 0.7555 |
| BC | 3.403E-003 | 1 | 3.403E-003 | 1.33 | 0.2585 |
| BD | 5.778E-003 | 1 | 5.778E-003 | 2.26 | 0.1439 |
| BE | 7.031E-004 | 1 | 7.031E-004 | 0.27 | 0.6043 |
| CD | 2.813E-005 | 1 | 2.813E-005 | 0.011 | 0.9173 |
| CE | 7.031E-004 | 1 | 7.031E-004 | 0.27 | 0.6043 |
| DE | 3.125E-006 | 1 | 3.125E-006 | 1.220E-003 | 0.9724 |
| $A^2$ | 0.081 | 1 | 0.081 | 31.67 | <0.0001 |
| $B^2$ | 0.091 | 1 | 0.091 | 35.39 | <0.0001 |
| $C^2$ | 0.11 | 1 | 0.11 | 44.50 | <0.0001 |
| $D^2$ | 0.016 | 1 | 0.016 | 6.23 | 0.0185 |
| $E^2$ | 0.060 | 1 | 0.060 | 23.47 | <0.0001 |
| Residual | 0.074 | 29 | 2.56E-003 | | |
| Lack of fit | 0.064 | 22 | 2.904E-003 | 1.95 | 0.1841 |
| Pure Error | 0.010 | 7 | 1.486E-003 | | |
| Cor Total | 0.67 | 49 | | | |

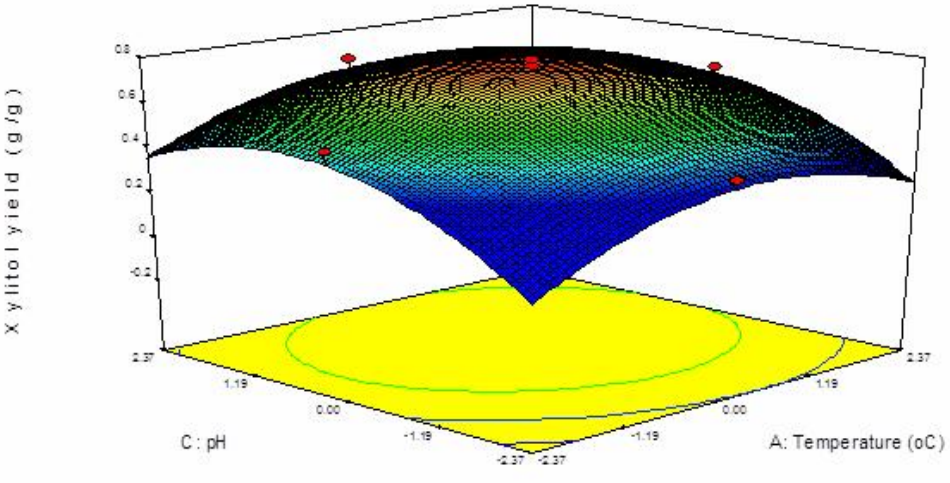


**Figure 1. 3D Plot showing the effect of temperature and pH on xylitol yield**

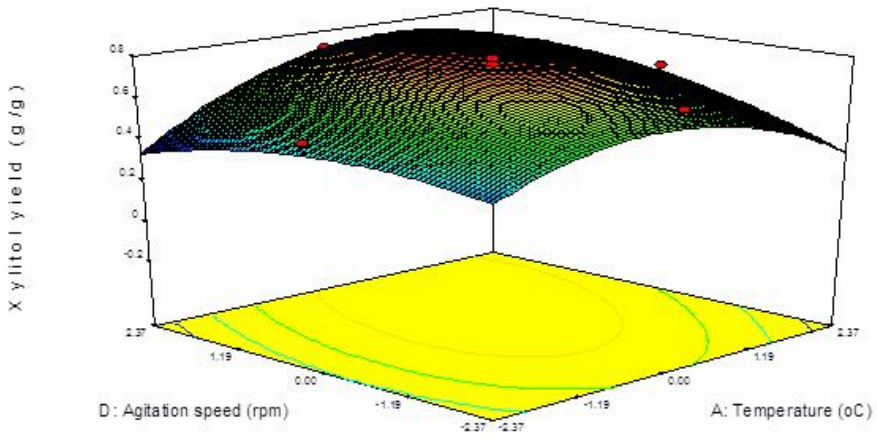


**Figure 2. 3D Plot showing the effect of temperature and agitation speed on xylitol yield**

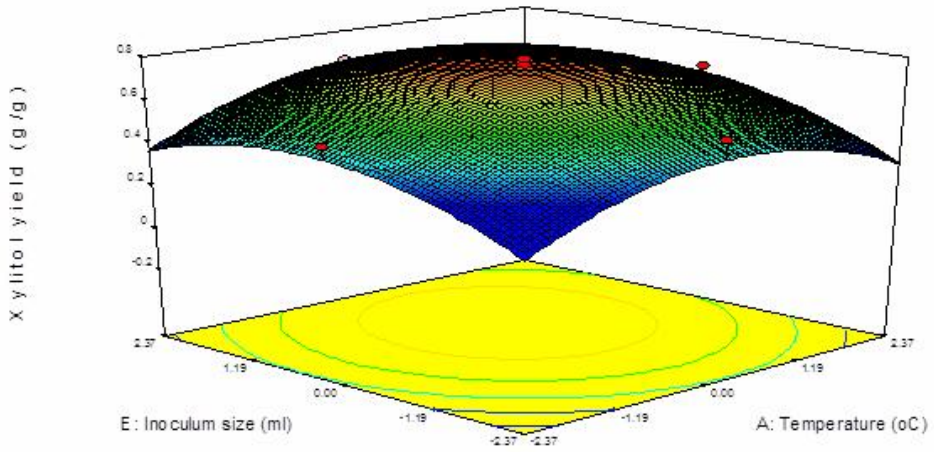


**Figure 3. 3D Plot showing the effect of temperature and inoculum size on xylitol yield**

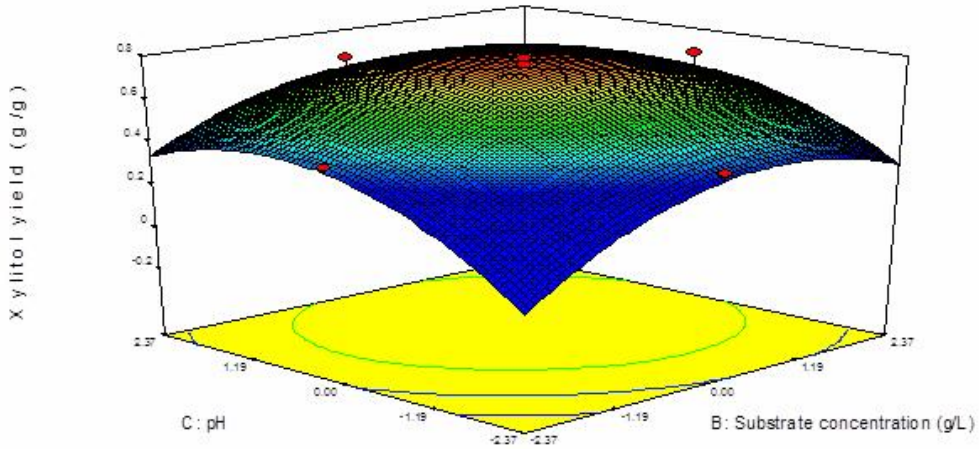


**Figure 4. 3D Plot showing the effect of substrate concentration and pH on xylitol yield**

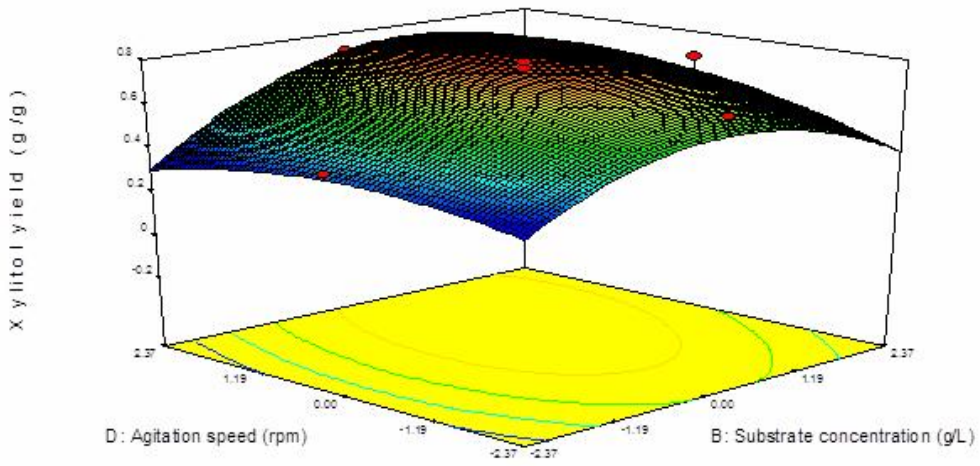


**Figure 5. 3D Plot showing the effect of substrate concentration and agitation speed on xylitol yield**

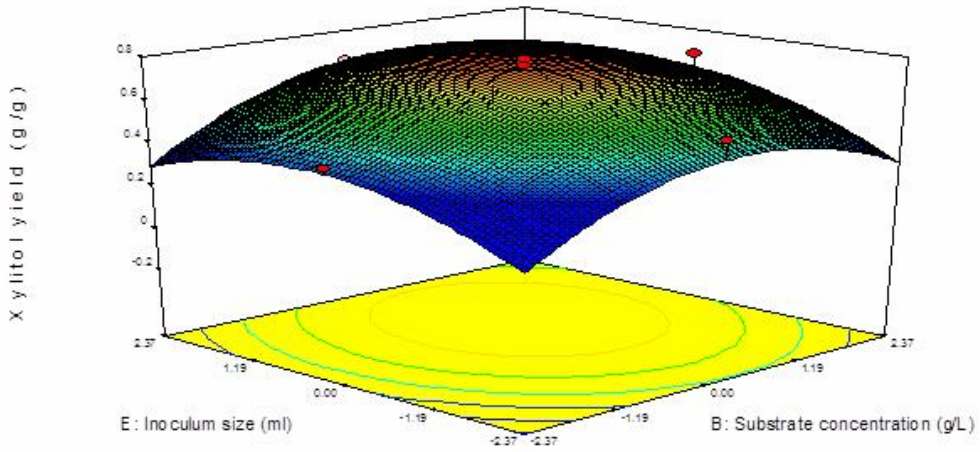


**Figure 6. 3D Plot showing the effect of Substrate concentration and inoculum size on xylitol yield**

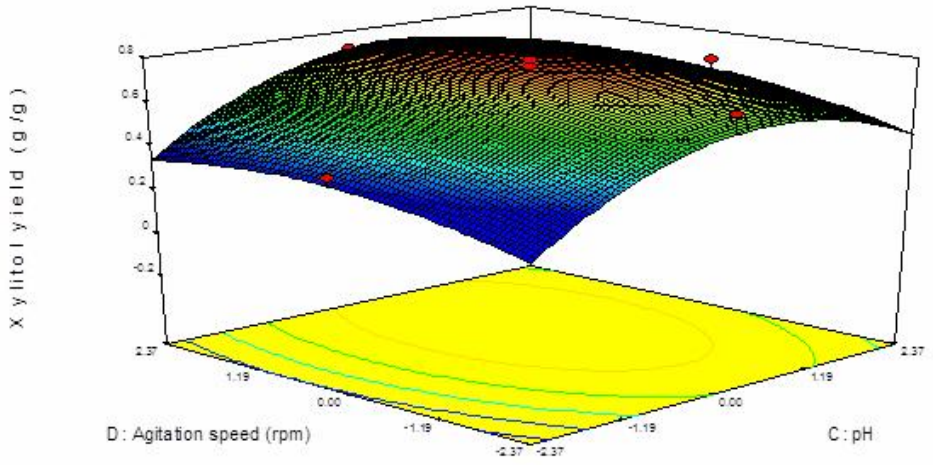


**Figure 7. 3D Plot showing the effect of pH and agitation speed on xylitol yield**

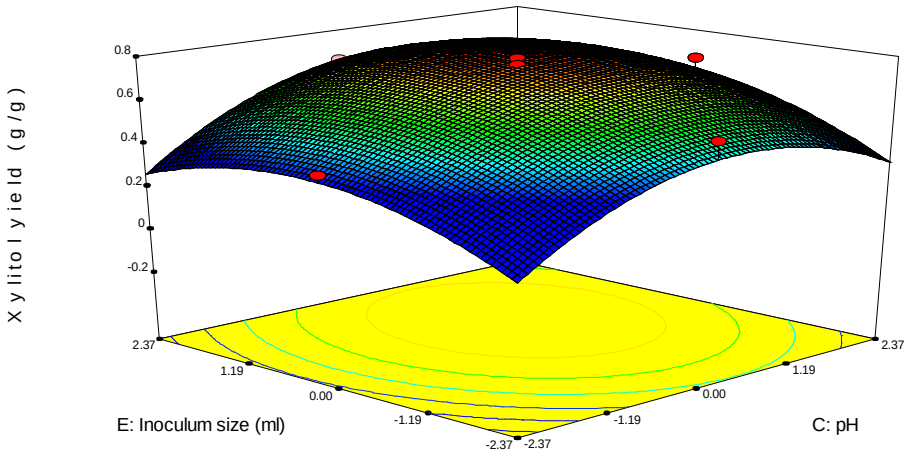


**Figure 8. 3D Plot showing the effect of pH and inoculum size on xylitol yield**

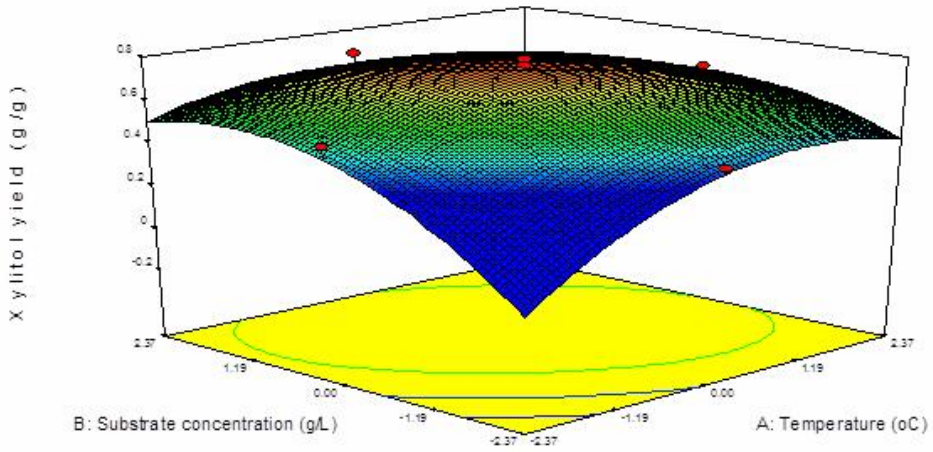


**Figure 9. 3D Plot showing the effect of substrate concentration and temperature on xylitol yield**

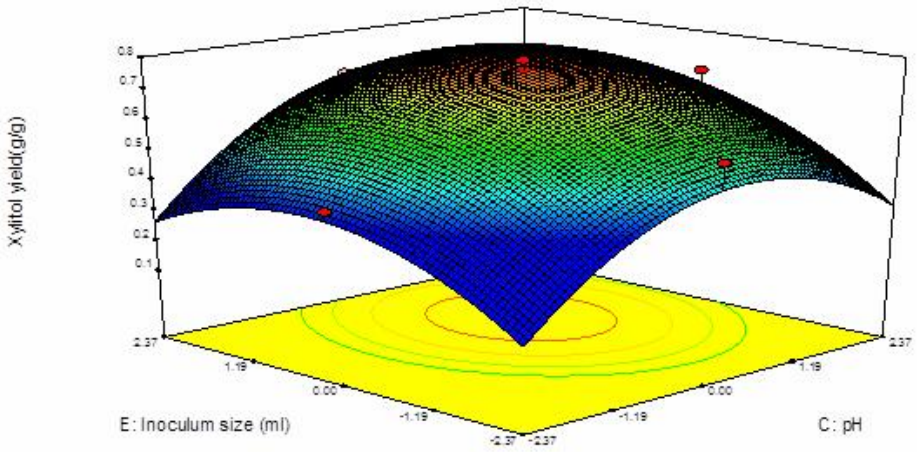


**Figure 10. 3D Plot showing the effect of pH and inoculum size on xylitol yield**

**Validation of the experimental model:**

Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions are temperature (31.8 $^{o}$C), substrate concentration (3.5 g/l), pH (7.25), agitation speed (201.6 rpm) and inoculum size (3.6 ml) established by the regression model. Four repeated experiments were performed and the results are compared. The xylitol yield of 0.77 g $g^{-1}$ obtained from experiments was very close to the actual response (0.76 g $g^{-1}$) predicted by the regression model, which proved the validity of the model.

## CONCLUSIONS

In this work, central composite design was proved to be powerful tool for the optimization of xylitol production by *Debaryomyces hensenii var hansenii* using corncob hemicelluloses hydrolysate. It was used to test the relative importance of process variables on xylitol production. From this optimization studies the optimized values of the process variables for xylitol production were as follows: temperature- 31.8 $^{o}$C, substrate concentration- 3.5 g/l, pH- 7.25, agitation speed- 201.6 rpm and inoculum size- 3.6 ml. This study showed that the corncob is a good source for the production of xylitol. Validation experiments verified the availability and the accuracy of the model. Using the optimized conditions, the xylitol production yield reaches 0.77 g $g^{-1}$. The results show a close concordance between the expected and obtained production level.

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