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Production Optimization and Partial Characterization of Xylanase from *Brevibacillus borstelensis* – MTCC 9874 Isolated from Soil Sample of Eastern Nepal and its Medium Optimization

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***Brevibacillus borstelensis* – MTCC 9874 was screened from 202 microorganisms isolated from seven different places of Kavre and Morang districts of Nepal by primary and secondary screening methods for xylanolytic activity. The microorganism was grown for 48 hours in five different mediums and minimal salt-yeast extract nutrient medium with xylan (1%w/v) was selected as a medium for further study based on xylanolytic activity measured using DNS method. Study of effect of temperature on xylanolytic activity showed that maximum xylanolytic activity (6.58±1.1 IU/ml) was found at 60°C. Optimum pH was found to be 7.6 (Xylanolytic activity = 6.81±2.32 IU/ml). Thermal stability study showed that the enzyme has a good stability at 40°C (91.12%).**

Plackett Burman design (Minitab 15.1) with seven variables viz. yeast extract, ammonium sulphate, sodium chloride, magnesium sulphate, calcium carbonate, trace element solution (pH 8) and result showed that yeast extract and xylan were significant factors for xylanase production (> 95% confidence levels). Full factorial Centre composite design (CCD) was used to optimize the two significant factors. Response surface and contour plot were used to locate the optimal value of the two factors.

Key words: Xylanase, Plackett-Burman design, Xylanolytic activity, response surface methodology, DNS method.

Xylan is the main hemicellulosic polysaccharide found in plant cell walls and is composed of a backbone chain of β-1,4-linked xylosyl residues and short side chains of arabinosyl, glucoronosyl and acetyl residues[1]. Its enzymatic hydrolysis requires endo- β-1,4-xylanase (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8) that cleaves glycosidic bonds to produce xylooligosaccharides and β -1,4-D-xylosidase (β-1, 4 - xyloside xylohydrolase, EC 3.2.1.37), responsible for the final breakdown of small xylooligosaccharides into xylose[2].

Xylan-degrading enzymes have attracted much attention because of their many important practical applications in various industrial processes, including the modification of cereal-

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based foodstuffs, improving the digestibility of animal feedstocks and delignification of paper pulp. Eco-friendly applications in paper/paper product manufacture and recycling, in textile manufacture, in baking, in the release of aroma and anti-oxidant molecules, and in the production of biopharmaceuticals, which are targeted at both selective and extensive modification of xylans, have provided an increased impetus to identify and obtain new xylanases with different specificities and properties[3].

The objective of this study was to characterize xylanase and optimize medium for production of xylanase. In order to improve characterized enzyme production, conventional method based on the 'change–one-factor-at-a-time" in which one independent variable is studied while fixing all others at a specific level, may lead to unreliable results and inaccurate conclusion. In addition to this, it is also expensive and time-consuming for large number of variables[4]. To overcome these limitations, Plackett-Burman design (PBD) followed by Response surface methodology (RSM) can be employed to optimize enzyme activity, performing a minimum number of experiments.

MATERIAL AND METHODS

Microorganism

Brevibacillus borstelensis – MTCC 9874 was isolated from Eastern Sugar Mills, Amadeva, Morang district, Eastern Nepal. It has been selected due to xylan-degrading properties noticed by primary screening method using xylan agar plate and congo red[5]. Its characterization was done using 16S rRNA analysis at Institute of Microbial Technology (IMTECH), Chandigadh, India. Isolates were maintained at 4-8°C on half nutrient agar slants.

Substrates, medium and submerged fermentation

Xylan from birchwood (Sigma, USA) was used as a substrate. 50 ml of growth medium (Birch wood xylan = 1%, Yeast extract = 0.2%, Peptone = 0.5%, MgSO4 = 0.05%, NaCl = 0.05% and $CaCl_2$ = 0.015%) maintained to pH 7.5 was used in submerged fermentation[5]. The flasks were incubated in a rotary shaker incubator (Sonar, India) at 55°C; 200 rpm for 4 days. The contents of the flask of different time intervals were centrifuged in a cooling centrifuge (Remi C-24, India) at -10°C: 10000 rpm. The supernatants were used as a source of enzyme and were stored separately in refrigeration at -20°C until they were analyzed. Each experiment was done in triplicate.

Xylanase activity assay

Xylanase activity was assayed using hydrolyzing xylan from birchwood (Sigma, USA). Liberated reducing sugars were quantified by dinitrosalicylic acid (DNS) method[6]. 1 IU/ml is expressed as 1 microgram of xylose formed per ml per minute. Each experiment was done in triplicate.

Selection of medium

The microorganism was incubated for 48 hours in five different media selected from literature[7, 8, 2, 9 & 10]. Submerged fermentation was carried out in the media separately at 55°C; 200 rpm for 48 hours and the best among the five was selected based on xylanase activity assay measured using DNS method.

Effect of temperature and pH on xylanase activity

The optimum temperature of crude xylanase for hydrolysis of xylan from birchwood was determined by assaying the enzyme activity using DNS method at 40, 45, 50, 55, 60, 65, 70, 75 and 80ºC for 10 minutes in phosphate buffer pH 7.6. The optimum pH was determined by measuring the activity at 60ºC kept for 1 hour over the pH range of 4 to 9. Phosphate buffers pH 4, 6, 6.6, 7, 7.6, 8 and 9 and Acetate buffer pH 5 were used during the study[11].

Thermal stability

Supernatant (1ml) was incubated at different temperature (30, 40, 50, 60 and 70ºC) for 10 minutes. Then xylan (2% w/v) was added in the supernatant and kept at 60 ºC for 10 minutes. Residual xylanase activity of each sample was quantified using DNS method[12 & 13].

EXPERIMENTAL

Plackett-Burman design

Plackett Burman experimental design with seven variables viz. yeast extract, ammonium sulphate, sodium chloride, magnesium sulphate, calcium carbonate, trace element solution (pH 7.6) and xylan in submerged fermentation (SmF) was

performed using Minitab 15.1 to screen the nutrients that were significantly affecting xylanase production[14]. The seven factors were screened in twelve experimental run in addition with three runs at their centre point. The enzyme activity assay was carried out in triplicate and average of it is reported as response/enzyme activity (Table 1).

Each factor was examined at two levels: -1 for low level and +1 for high level and centre point were run to evaluate the linear and curvature effects of the variables based on Plackett-Burman factorial design. The design is based on the first-order polynomial model:

$$Y = \beta_0 + \Sigma \beta_i x_i \quad ...(1)$$

Where Y is the response (enzyme activity), β_0 is the model intercept, β_i is the linear coefficient and x_i is the level of the independent variables. This model does not describe interaction among variables and is used to screen the significant factors that influence response (enzyme activity)[15].

The factors significant at 95% level ($p<0.05$) were considered to have significant effect on xylanase production from the regression analysis of variables.

Central composite design, response surface methodology and statistical analysis

Significant factors that contribute in xylanase production were further optimized by response surface methodology using Minitab 15.1[15 & 16]. Two level full factorial central composite design with 4 star points (a=1.41421) and a centre point with one replication resulting in a total of 13 experiments were used to optimize the chosen key variables for xylanase productivity in SmF. All the experiments were done in triplicate and average of enzyme activity is reported in Table 2.

Second-order polynomial equation was fitted to correlate the relationship between variables and response (xylanase activity) in order to predict optimal point[17]. The equation is:

$$Y = \beta_0 + \Sigma_{i=1}^{k} \beta_i X_i + \Sigma_{i=1}^{k} \beta_i X_i + \Sigma_1 \Sigma_3 \beta_i X_i X_2 \quad ...(2)$$

Where Y is the predicted response, k is the number of factor variables, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient.

Minitab 15.1 was used to evaluate analysis of variance (ANOVA) to determine significance of each term in the equation fitted and to estimate goodness of fit in each case[15]. Based on experimental results, response surface was drawn to show individual and cumulative effects of variables and the mutual interaction between them.

RESULTS AND DISCUSSION

Identification of organism

16S rRNA gene analysis (1393 bp) of the isolate carried out in IMTECH, Chandigadh,

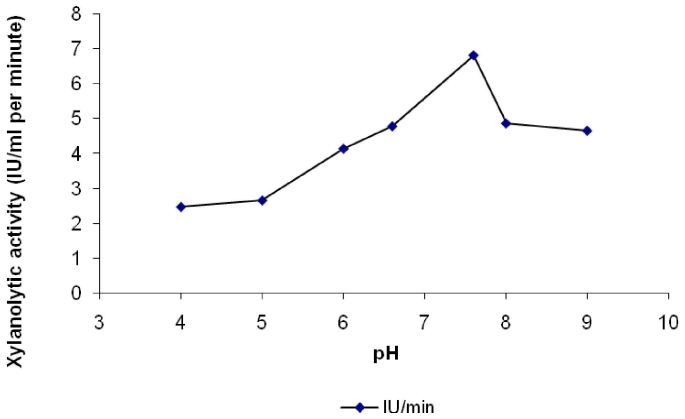

Fig. 1. pH vs. xylanolytic activity for determination of optimum pH for maximum xylanolytic activity

India, revealed that the organism is *Brevibacillus borstelensis*. The sequence of 1393 base pairs was as follows:

CGAGCGAGTCCCTTCGGGGGCTAGCGGCGGACGGGTGAGTAACACGTAGGC
AACCTGCCCGTAAGCTCGGGATAACATGGGGAAACTCATGCTAATACCGGAT
AGGGTCTTCTCTCGCATGAGAGGAGACGGAAAGGTGGCGCAAGCTACCACTT
ACGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAG
GCGACGATGCGTAGCCGACCTGAGAGGGTGACCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCCACAATGGA
CGAAAGTCTGATGGAGCAACGCCGCGTGAACGATGAAGGTCTTCGGATTGTA
AAGTTCTGTTGTCAGAGACGAACAAGTACCGTTCGAACAGGGCGGTACCTTG
ACGGTACCTGACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAG
GCGGCTATGTAAGTCTGGTGTTAAAGCCCGGGGCTCAACCCCGGTTCGCATC
GGAAACTGTGTAGCTTGAGTGCAGAAGAGGAAAGCGGTATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTCTG
GTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGTTTCA
ATACCCTCAGTGCCGCAGCTAACGCAATAAGCACTCCGCCTGGGGGAGTACG
CTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCCGCTGACCGTCCTAGAGATAGGGCTTCCCTTCGGGGCAGCGGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTATCTTTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGA
GACTGCCGTCGACAAGACGGAGGAAGGCGGGGATGACGTCAAATCATCATG
CCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAACGGGAAG
CTAGCTCGCGAGAGTATGCCAATCTCTTAAAACCAGTCTCAGTTCGGATTGCA
GGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACG
GGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCGCAAGGAGCC

Enzymatic Production

Maximum xylanolytic activity among five medium after SmF for 48 hours was seen in minimal salt-yeast extract nutrient medium {Yeast extract = 6g/L, Ammonium sulphate = 0.1g/L, Sodium chloride = 0.3g/L, Magnesium sulphate = 0.1g/L, Calcium carbonate = 0.02g/L and Trace element solution (pH 8) = 1 ml) with xylan (1%w/v) in which the composition of Trace element solution contained (g/L): Ferrous sulphate = 1, Zinc sulphate = 0.9 and Manganese sulphate = 0.2[8]. Thus, this medium was chosen for medium and condition optimizations study.

Effect of pH and temperature

Maximum xylanolytic activity was measured in between pH 6.6 and 8 (Fig. 1). Xylanolytic activity measured at pH 7.6 was 6.81±2.32 IU/ml. The enzyme activity at pH 6.6 and 8 were 70.12 and 71.33% of that at pH 7.6 respectively. Commercial xylanase preparations are available to the pulp and paper industry, however, the optimal characteristics of the constituent enzymes in these preparations are poorly suited to the extreme conditions of temperature (60-80°C) and pH (> pH l0.0) encountered during kraft pulp bleaching. Consequently there is a need for xylanases which are both thermostable and alkalitolerant[18]. Maximum xylanolytic activity was found in between 50 and 70ºC (Fig. 2). Xylanolytic activity measured at 60ºC was 6.58±1.1 IU/ml. The enzyme activity at 50 and 70ºC were 90.96 and

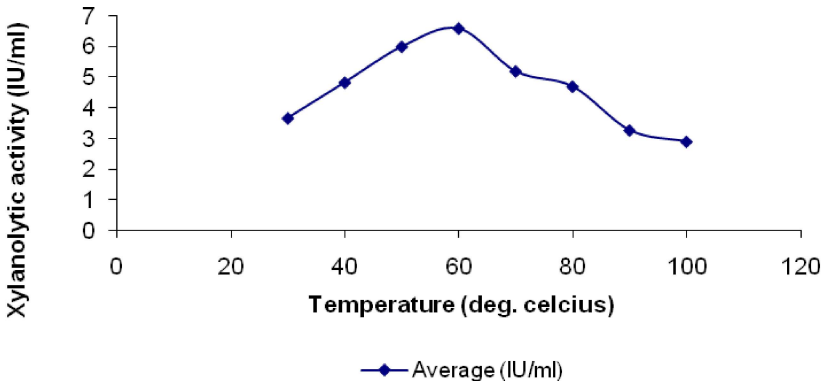

Fig. 2. Temperature vs. xylanolytic activity for determination of optimum temperature for maximum xylanolytic activity

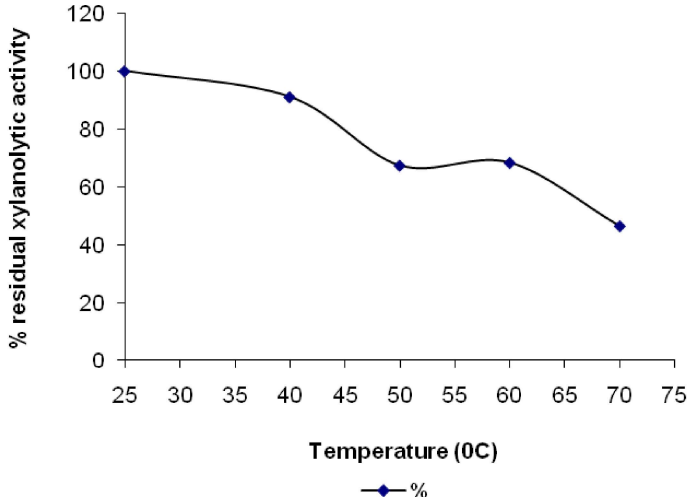

Fig. 3. Thermostability study of xylanase

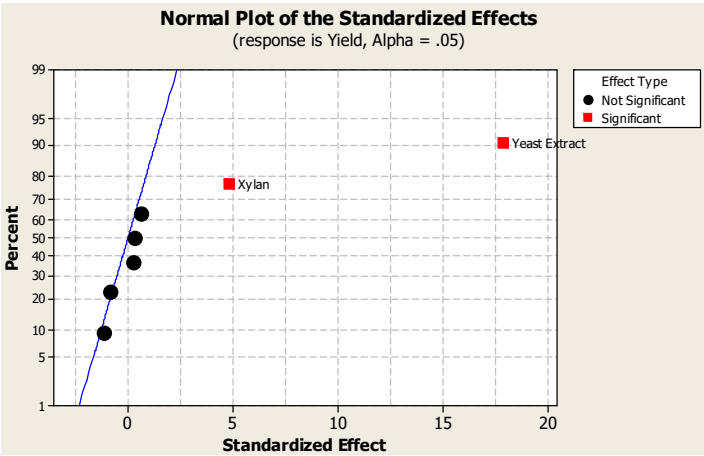

Fig. 4. Factors (Xylan and Yeast extract) that are having significant effect in xylanase production

78.85% of that at 60ºC respectively. The enzyme was found alkalophilic but maximum xylanolytic activity was found below pH 10. The enzyme could fulfill the temperature requirement for pulp and paper industries as it had shown maximum xylanolytic activity in between 50 and 70°C.

Thermal stability of xylanase

The residual activities of crude xylanase incubated at 40, 50, 60 and 70ºC for a period of 10 minutes were estimated at optimum temperature. The enzyme was relatively more stable for 10 minutes in between 25 and 40ºC (Fig. III). 8.89, 32.45, 31.51 and 53.31% of the original activities were lost at 40, 50, 60 and 70ºC.

Table 1. Plackett-Burman design showing seven variables with coded values along with the observed results for xylanase production

Run Order	Yeast Extract (g/L)	NH_4SO_4 (g/L)	NaCl (g/L)	$MgSO_4$ (g/L)	$CaCO_3$ (g/L)	Trace element solution in ml (pH 7.6)	Xylan (g/L)	Xylanase Activity (IU/ml)
1	30(+1)	1.4(+1)	3(+1)	0.1(-1)	2(+1)	2.7(+1)	5(-1)	9.27
2	6(-1)	1.4(+1)	3(+1)	0.75(+1)	0.02(-1)	2.7(+1)	10(+1)	3.10
3	6(-1)	0.1(-1)	0.3(-1)	0.1(-1)	0.02(-1)	1(-1)	5(-1)	1.69
4	18(0)	0.75(0)	1.65(0)	0.425(0)	1.01(0)	1.85 (0)	7.5(0)	6.75
5	6(-1)	1.4(+1)	3(+1)	0.1(-1)	2(+1)	1(-1)	5(-1)	1.61
6	30(+1)	0.1(-1)	0.3(-1)	0.1(-1)	2(+1)	2.7(+1)	10(+1)	9.65
7	18(0)	0.75(0)	1.65(0)	0.425(0)	1.01(0)	1.85 (0)	7.5(0)	6.89
8	6(-1)	0.1(-1)	3(+1)	0.75(+1)	2(+1)	1(-1)	10(+1)	3.74
9	30(+1)	0.1(-1)	3(+1)	0.1(-1)	0.02(-1)	1(-1)	10(+1)	11.06
10	18(0)	0.75(0)	1.65(0)	0.425(0)	1.01(0)	1.85 (0)	7.5(0)	7.49
11	6(-1)	1.4(+1)	0.3(-1)	0.1(-1)	0.02(-1)	2.7(+1)	10(+1)	4.72
12	30(+1)	0.1(-1)	3(+1)	0.75(+1)	0.02(-1)	2.7(+1)	5(-1)	8.62
13	30(+1)	1.4(+1)	0.3(-1)	0.75(+1)	2(+1)	1(-1)	10(+1)	9.89
14	30(+1)	1.4(+1)	0.3(-1)	0.75(+1)	0.02(-1)	1(-1)	5(-1)	8.43
15	6(-1)	0.1(-1)	0.3(-1)	0.75(+1)	2(+1)	2.7(+1)	5(-1)	1.58

Table 2. Full factorial central composite design matrix for two variables in five settings in real and coded units (parenthesis) and response of xylanase activity

Run Order	Xylan (g/L)	Yeast Extract (g/L)	Xylanase Activity (IU/ml)	
			Observed	Predicted
1	10 (0)	30 (0)	9.81	9.5840
2	5 (-1)	20 (-1)	6.63	7.2141
3	10 (0)	30 (0)	10.07	9.5840
4	15 (+1)	20 (-1)	5.23	5.5517
5	5 (-1)	40 (+1)	9.57	10.0658
6	2.93 (-2)	30 (0)	8.99	8.3957
7	10 (0)	44.14 (+2)	11.61	11.2637
8	10 (0)	30 (0)	9.82	9.5840
9	10 (0)	30 (0)	9.35	9.5840
10	15 (+1)	40 (+1)	9.87	10.1034
11	17.07 (+2)	30 (0)	7.47	7.2468
12	10 (0)	15.86 (-2)	6.50	6.0288
13	10 (0)	30 (0)	8.87	9.5840

Thermal stability study showed that the enzyme has a good stability at 40ºC (91.11%) in comparison with initial xylanolytic activity. Xylanolytic activity was found to be decreased when temperature was increased. The enzyme showed xylanolytic activity equal to 68.5% at 60°C though it was 91.11% at 40°C. It showed that this xylanase is less thermostable in comparison with other xylanases as well which are stable in between 50 and 65°C and at more than 75°C[19].

Plackett-Burman Design

The result of Plackett-Burman design showed that yeast extract and xylan are significant factors for xylanase production (Fig. 4). Magnesium sulphate and calcium carbonate were found insignificant with negative coefficients for the enzyme activities. Sodium chloride, ammonium sulphate and trace element solution (pH 8) were also found insignificant (Fig. 4).

Therefore, amount of all the ingredients of medium which were insignificant in production of xylanase were kept constant in subsequent experiments. The model equation for xylanase production neglecting insignificant variables is as follows:

$$Y_{activity} = - 1.36 + 0.366 X + 0.289 Y \quad ...(3)$$

Where, $Y_{activity}$ = Enzyme activity, X = Xylan and Y = Yeast extract

Central Composite Design and statistical analysis

The result of enzyme activity when *Brevibacillus borstelensis* was incubated for 6 days as per 13 experiment of CCD showed that maximum enzyme activity (11.61 IU/ml) was found in run 7 in which 10 and 44.14 g/L of xylan and yeast extract were used respectively (Table 2).

Table 3. Results of regression analysis of the full factorial Central Composite Design (CCD)

Term	Coefficient	SE Coefficient	T- statistics	p - value
Constant	9.58400	0.2617	-0.257	0.804
Xylan (X)	-0.40620	0.2069	3.458	0.009
Yeast extract (Y)	1.85083	0.2069	4.617	0.002
Xylan×Xylan (X^2)	-0.88138	0.2219	-3.508	0.008
Yeast extract × Yeast extract (Y^2)	-0.46888	0.2219	-2.233	0.056
Xylan × Yeast extract	0.4250	0.2926	1.452	0.190

Standard deviation of error term in the model, S = 0.585229; Sum of squares of the prediction errors, PRESS = 12.0131; R^2 = 93.71%; R^2 (predicted) = 68.50% & R^2 (adjusted) = 89.22%

Table 4. Analysis of variance (ANOVA) for the model regression representing xylanase activity

Source	DF	Sequencial SS	Adjusted SS	Adjusted MS	F-ratio	p-value
Regression	5	35.7374	35.7374	7.1475	20.87	0.000
Linear	2	28.7245	28.7245	14.3623	41.93	0.000
Square	2	6.2904	6.2904	3.1452	9.18	0.011
Interaction	1	0.7225	0.7225	0.7225	2.11	0.190
Residual error	7	2.3974	2.3974	0.3425		
Lack of fit	3	1.4899	1.4899	0.4966	2.19	0.232
Pure error	4	0.9075	0.9075	0.2269		
Total	12	38.1349				

DF = Degree of freedom; SS = Sum of square; MS = Mean square

Minitab 15.1 was used to find out quadratic mathematical model [Equation 4]:

$$Y_{activity} = 9.484 - 0.406\ X + 1.851Y - 0.881X^2 - 0.469Y^2 + 0.425XY \quad ...(4)$$

Where, $Y_{activity}$ = Enzyme activity, X = Xylan and Y = Yeast extract

The model (equation 4) did not show lack of fit (p = 0.232) as shown in Table 3. The model can explain 93.71% (R^2 = 93.71%) of the variation in xylanase activity and can predict 68.5% (R^2 predicted = 68.5%) of the xylanase activity (Table 4).

Response surface to estimate dependent variable, xylanase activity, over independent variables, xylan and yeast extract concentrations as per equation (4) is shown in Fig. V. The surface was curved in structure with the change in xylan concentration showing that '0' level of xylan (10g/L) is showing peak xylanase activity where as '+2' level of yeast extract concentration (44.14 g/L) has shown maximum xylanase activity. Contour plots obtained from Response surface methodology suggested optimized concentrations of xylan (10g/L) when yeast extract concentration was 44.14g/L (Fig. 6).

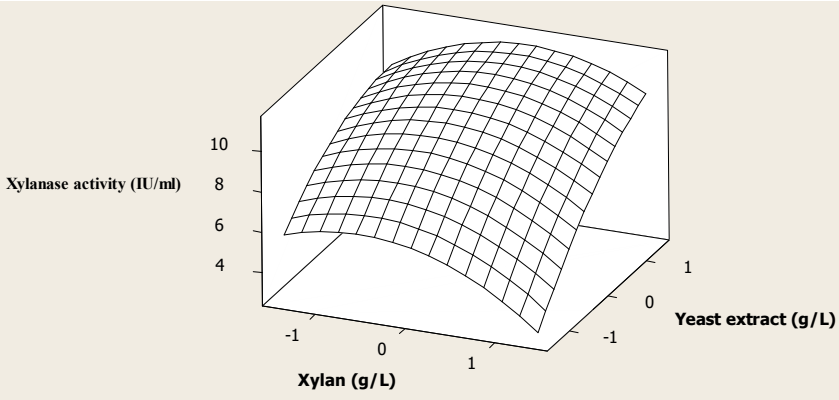

Fig. 5. Response surface described by equation 4 which represents xylanase activity (IU/ml) as a function of xylan and yeast extract concentration

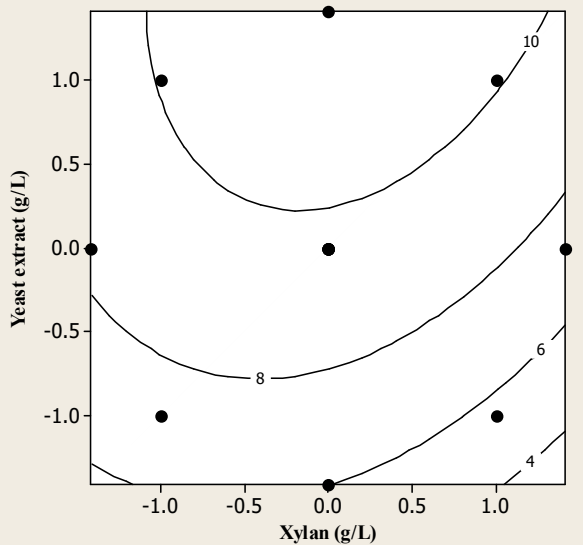

Fig. 6. Contour plot of xylanolytic activity (IU/ml) vs. yeast extract (g/L) and xylan (g/L)

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