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Research Article

Optimization of Process Parameters for Xylanase Production by *Bacillus atropheaus* [KJ 590121] SD9 Isolated from Sludge using Response Surface Methodology

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Abstract

In recent year, xylanase has become an essential option for eco-friendly industrial biotechnological applications and there is a rising demand for large scale production. In this study, *Bacillus atropheaus* SD9 isolated from sludge was tested for the xylanase production under submerged cultivation conditions. Maximum xylanase activities were achieved by optimizing process conditions i.e. temperature, pH, inoculums size and substrate concentration that significantly affect the xylanase production. Central Composite Design (CCD) quadratic response surface was applied to explicate these four factors. Statistical analysis of the results showed that optimized parameters had a significant effect on xylanase production. These optimization conditions resulted in a 3-fold increased level of the xylanase (85.16 U/g) production after 120 h of fermentation; whereas the value predicted by the quadratic model was 80.07 U/g/ml. High xylanase activity may broaden the prospectus of industrial scale. This statistical approach has been proved a powerful tool for the development of cultural conditions and to gather sufficient information with minimum number of trials for enhanced production of xylanase from this strain.

Keywords: Xylanase; *Bacillus atropheaus*; Response surface methodology; Central composite design

Introduction

Xylanases has become an essential option for eco-friendly industrial applications and there is rising demand for large scale production. Xylanase is a hemicellulolytic polysaccharide consisting of 1,4 linked β- D- xylopyranose residues, most commonly used for beer and juice clarification, pre-bleaching of kraft pulp, improving digestibility of animal feed, bread making and degumming of vegetable fibres such as jute ramie and hemp [1].

The choice of appropriate substrate is of great importance for successful production of xylanases. The substrate not only serve as source of carbon and energy source, but also provides the necessary inducing compounds for the organisms, preferentially for an extended period of time [2]. Lignocellulosic biomass represents an inexpensive and unexploited resource can emerge as suitable substrate for cost effective production of xylanase. Among lignocellulosic materials, forest residue is a major carbon sink in the forest ecosystems and accounts for roughly 20% of the terrestrial feed stock carbon storage, offering an enormous, renewable source of feedstock for biofuel production and so far there is no self sufficient process or technology available to convert this lignocellulosic biomass for bioenergy generation [3].

Cost-effective production of xylanases from low-cost agro-industrial residues is vital for their extensive and economic industrial application. To obtain the best outcome of a biotechnological process, it is imperative to consider the individual and combined influence of all involved parameters. Culture medium optimization by the traditional "one factor-at-a-time" technique requires a considerable amount of time and work. This limitation can be eliminated by factorial design optimization and response surface analysis. Plackett–Burman design is a powerful and efficient mathematical approach to screen and evaluate the important factors that influence the response without describing interaction among them. RSM comprises statistically designed experimental techniques for estimating the coefficients in a mathematical model and predicting the response and checking the applicability of the model [4,5].

Most of the literature concerning xylanases dealt with their purification and characterization, and relatively fewer studies have been done regarding production, optimization of xylanase [6]. Statistical optimization allows rapid screening of a number of factors and factor interactions and the role of each component. Response surface methodology is gaining recognition as a powerful approach for production of industrial important products such as chemicals and enzymes [7].

The objective of present study is to statistically optimize the process parameters such as temperature, pH, and inoculums size and substrate concentration for production of xylanase from *Bacillus atropheaus* SD9 under Submerged Fermentation (SmF) by central composite design in response surface methodology.

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Materials and Methods

Microorganism

Bacillus atropheaus SD9 was isolated from a sludge sample obtained from the sludge effluent in Parwanoo region of Himachal Pradesh showed true potential in extracellular xylanase production. This strain was propagated on nutrient agar medium at 35°C and maintained at 4°C and sub-cultured every month.

Identification

The isolate was identified on basis of various morphological characteristics (colony, size, shape, margin elevation, color, Gram's nature) and biochemical tests (catalase, H2S, urease, citrate utilization, MR- VP, hydrolysis of gelation casein, indole test). All the tests were performed according to Bergey's manual of systematic bacteriology [8].

Molecular characterization using 16s rRNA PCR technique

The isolate SD9 were identified at genomic level using 16S rRNA techniques.

Isolation of genomic DNA

The genomic DNA of the strain was isolated according to Genei DNA isolation kit.

PCR amplification of 16s rRNA region

PCR amplification was done to confirm the identity of the bacterial strains, the small sub-unit 16s rRNA genes were amplified from the genomic DNA with 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'TACCTTGTTACGACTT3') primers to get an amplicon size of 1500 bp. Amplifications were carried out in 20 µl reaction mixture consisting of 10 x buffer, 2.0 µl; 2.0 µl; 2.0 mMdNTPs, 2.0 µl; 3.0 U/µl Taq DNA polymerase, 0.2 µl; 100 ng/µl of each primer, 1.0 µl; template DNA, 1.0 µl and sterilized distilled water 12.8 µl in a Biorad (USA) thermal cycler using the PCR conditions 95°C for 2 min (denaturation), 52.3°C (StrainSD9)) for 1 min (annealing) and 72°C for 3 min (extension). The total number of cycles was 40, with the final extension of 72°C for 10 min. The amplified products (50 µl) were size separated on 1% agarose gel prepared in 1% TAE buffer containing 0.5 µg/ml ethidiumbromide and photographed with the gel documentation system (Biorad, USA). A 100 bp DNA ladder (Genei) was used as molecular weight size markers.

Purification of the PCR product

The PCR product (1500 bp) was purified from contaminating products by electro elution of the gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µl of nuclease free water.

Nucleotide sequencing

Sequencing pattern – The PCR amplicons obtained by amplifying PCR products was diluted in Tris buffer (10 mM, pH 8.5). The dilution used was 1:1000 in order to obtain the DNA concentration required for sequencing (30 ng/µl), the sequencing reaction required 8 µl DNA. The primer used in all sequencing reactions was 16 SF (5'AGAGTTTGATCCTGGCTCAG3') at a concentration of 3 µM. Sequencing was then performed using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA).

BLASTN analysis

Translated nucleotide sequence was then analyzed for similarities by using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/). Genotypically strain was identified by 16SrRNA technique.

Further genotypic identification was carried out using 16SrRNA technique. Genomic DNA of isolate was isolated by using DNA purification kit. The DNA was quantified by using standard protocol [9]. The isolated genomic DNA was used in PCR to amplify small subunit of 16S rRNA using universal primer having product size of approximately, 1500 bp. The PCR product so obtained after amplification was visualized using ethidium bromide on 2% agarose gel. Amplified PCR products were purified and got sequenced by the services provided by Xcelris Labs Ltd, Ahmdabad- India to confirm the results.

Production of xylanase

The synthetic medium composed of KH_2PO_4: 2.0g, $CaCl^{-2}$: 0.3g, $MgSO_4$. $7H_2O$- 0.3g, peptone: 5 g, yeast extract: 3 g, maltose: 3 g, Xylose: 5g, xylan: 5g, D.W: 1000ml was optimized as the best medium for xylanase production. 10% of inoculum was added to the medium and the flasks were incubated at 35°C for 120 h on an orbital shaker. The extract was centrifuged at 10,000g and at 4°C for 10 min, and the clear supernatant was assayed for xylanase activity. For optimization studies, different parameters were varied according to the experimental data.

Xylanase assay

Xylanase activity in the cultural filtrate was determined according to the method of Miller [10]. One unit of xylanase activity was defined as the µmol of xylose liberating per minute. One unit (1 IU) of xylanase activity is defined as the amount of enzyme required to release 1 µmol xylose per min under the assay conditions.

Response surface methodology

A factorial CCD for four factors with replicates at the center point and star point was used in this investigation. The variables used were temperature, pH, inoculums size and substrate concentration (pine wood biomass), each at four coded levels as shown in Table 1.

Statistical analysis

The statistical software package Design-Expert (Stat-Ease, Minneapolis, MN) was used for a regression analysis of experimental data and to plot the response surface. Variance Analysis (ANOVA) was used to estimate the statistical parameters.

Results and Discussion

The present isolate used has been isolated from sludge sample and

Table 1: Variables and their levels for the central composite experimental design.

Factor Coded	Name	Low Actual	High Actual
A	Temperature	30	45
B	pH	4	7
C	Inoculum size (%)	7	12
D	Substrate concentration (%)	0.5	2.5

Overall second order polynomial equation for xylanase production is given in equation in form of coded factors $Y = 80.07 + 3.71*A + 4.47*B + 1.69*C + 5.89*D - 8.39*A^2 - 2.72*B^2 - 5.12*C^2 - 6.77*D^{2+} 0.41*A*B - 1.99*A*C - 0.29*A*D - 1.45*B*C - 1.02*B*D + 1.91*C*D$.

Where A: temperature; B: pH; C: Inoculum size; D: Substrate concentration.

Table 2: Morphological and biochemical characteristics of SD9.

CHARACTERISTICS			
A. Morphological tests		**B. Biochemical tests**	
Color	Cream	Catalase	+ve
Form	Circular	H_2S test	-ve
Elevation	Pulvinate	Urease	-ve
Margin	Entire	Citrate utilization	+ve
Gram's nature	Gram positive (+ve)	Methyl red test	-ve
Shape	Rod	Voges Prokaurer test	+ve
		Hydrolysis of gelatin	-ve

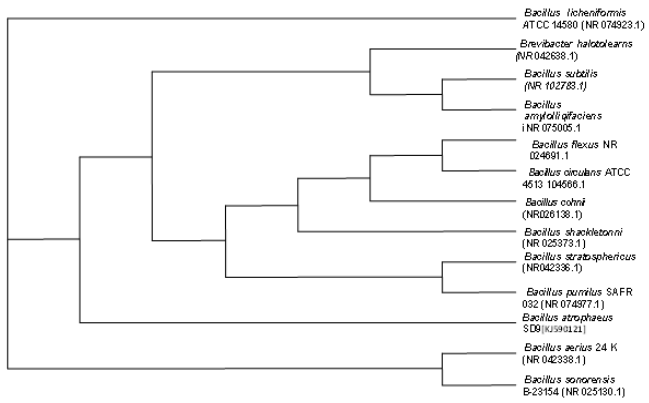

Figure 1: Neighbour joining tree constructed from clustal X showing Phylogenetic relationship of *Bacillus atropheaus* SD9 [KJ 590121] based on distance matrix analysis of 16S rRNA sequences.

showed potential xylanase production. Morphological, physiological and biochemical tests were carried out for this isolate according to Bergey's manual of systematic bacteriology as shown in Table 2. It was catalase positive, rod shaped and tentatively identified as Bacillus on basis of morphological and biochemical tests.

Sequence of the isolate so obtained was submitted to NCBI database and matched with already existing sequences. Sequence similarity search (mBLAST, NCBI) for SD9 showed 99% species specific alignment with *Bacillus atropheaus* SD9.

The 16SrRNA sequences of the isolate have been registered under gene bank databases with accession numbers *Bacillus atropheaus* SD9 [KJ 590121]. Phylogenetic tree of *Bacillus atropheaus* SD9 with respect to other Bacillus sp. bacteria as inferred by neighbor joining method have been presented in Figure 1.

Response Surface Methodology (RSM) had not only been used for optimization of medium components in the fermentation process [11] but also for studying the combined effects of culture parameters. Preliminary experiments on xylanase production from the *Bacillus atropheaus* SD9 that the most important environmental factors were pH, temperature, and inoculums size. Hence these factors were considered as independent variables and their effect on xylanase was studied using CCD of RSM as described by Montgomery [12]. Results were analyzed by Analysis Of Variance (ANOVA) [1]. This assisted in elucidating the main, square as well as the interaction effects among various variables.

The results of CCD experiment for studying the effects of four independent variables on xylanase production are presented in Table 3 along with predicted and observed responses. The differences between few runs of experimental and predicted activities were due to determination coefficients where few percentages of total variations are not explained by the model.

The results were analyzed by using ANOVA (Analysis of Variance) appropriate for the experimental design shown in Table 3. The ANOVA of the quadratic model indicates the model to be significant. The Model F-value of 6.29 implied the model to be significant. There is only a 0.07% chance that a "Model F-Value" this large could occur due to noise. Model P value (Prob>F) is very low [<0.0001]. The P values are used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The F value and the corresponding P values, along with the coefficient estimates are given in Table 4. The smaller the magnitude of the P, the more significant is the corresponding coefficient.

The optimum temperature for enzyme production by an organism varies since it is likely to affect growth of the organism. The present findings are in accordance with those of [13] who observed highest xylanase production by Bacillus circulans at 35°C using xylan. Other Bacillus sp. have been reported to exhibit maximum xylanase titreat 30°C [14], 500C and 55°C [15].

The Pred R-Squared of 0.7145 is in reasonable agreement with the Adj R-Squared of 0.7257. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable.

A ratio of 7.964 indicates an adequate signal. This model can be used to navigate the design space. The fit of the model was also expressed by the coefficient of regression R2, which was found to be 0.8268 indicating that 82.68% the variability in the response could be explained by the model. The closer the value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values. Here the value of R2 (0.8268) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. This implies that the prediction of experimental data is quite satisfactory.

To study the interactive effect of two factors on the xylanase production, the response surface methodology was used and 3D surface t was drawn. 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 interaction effects of these two factors. The interaction effects of the variables and optimal levels of the each variable were determined by plotting the response surface graphs. The 3D response surface graphs and contour plots are shown in Figure 2. The elliptical shape of the contour indicates good interaction of the two variables. There was a significant interaction between every two variables.

So, compared with the traditional 'one- variable at- a-time' approach which is unable to detect the frequent interactions occurring between two or more factors although they often do occur, RSM has immeasurable effects and tremendous advantages. The response surfaces of mutual interactions between the variables were found to positive in all cases. Optimum condition is the one

Table 3: Parameters, experimental runs and responses of Placket-Burman design used for selection of significant parameters.

Run	Factor 1 A: Temperature	Factor 2 B: pH	Factor 3 C: Inoculum Size	Factor 4 D: Substrate Concentration	Response Enzyme activity	Predicted Enzyme Activity
1	37.5	5.5	9.5	1.5	84.39	80
2	37.5	5.5	9.5	1.5	83.28	80.07
3	37.5	5.5	9.5	1.5	59.16	60.23
4	37.5	5.5	9.5	1.5	84.57	80.07
5	30	7	12	2.5	65.56	66.69
6	45	7	7	0.5	66.73	64.69
7	30	4	7	2.5	47.84	49.43
8	30	4	12	0.5	44.24	45.27
9	22.5	5.5	9.5	1.5	39.21	39.08
10	37.5	8.5	9.5	1.5	88.2	78.1
11	45	4	7	2.5	58.82	59.43
12	37.5	5.5	14.5	1.5	62.84	62.84
13	30	4	12	2.5	63.2	63.51
14	52.5	5.5	9.5	1.5	54.8	53.9
15	45	7	12	2.5	68.2	70.35
16	37.5	5.5	9.5	1.5	85.9	80.07
17	45	7	12	0.5	56.2	57.37
18	45	4	12	0.5	48.32	48.46
19	30	7	7	0.5	46.59	51.9
20	37.5	5.5	9.5	1.5	57.09	80.07
21	45	4	7	0.5	48.38	50
22	30	4	7	0.5	42.71	38.84
23	37.5	5.5	4.5	1.5	57.1	56.68
24	30	7	12	0.5	54.89	52.55
25	37.5	5.5	9.5	1.5	85.16	80.27
26	45	7	7	2.5	68.3	70.02
27	37.5	5.5	9.5	3.5	65.8	64.78
28	37.5	5.5	9.5	-0.5	-	-
29	45	4	12	2.5	68.1	63.54
30	30	7	7	2.5	60.26	58.39

at which the maximum xylanase production is attained. Such an optimum condition for xylanase production can be obtained by solving the second order polynomial equation using RSM. The central point is the point at which the slope of the contour is zero in all directions. The coordinates of the central point within the highest contour levels in each of these figures will correspond to the optimum values of the respective constituents. The optimum values drawn from these figures are in close agreement with those obtained by optimizing the regression model Eq. (3). The optimum values for maximum xylanase production were: substrate concentration -1.50%, temperature – 35.5°C, pH: 5.50, inoculum size: 9.50% and substrate concentration1.50% the optimal values for the variables as predicted were found to be within the design region. This shows that the model correctly explains the influence of the chosen variables on the xylanase production. Similarly Central composite techniques have been used to optimize maximum xylanase yield by Schizophyllum commune and Thermomyces with activity of 5.74U/ml and 2.74 respectively under submerged fermentation.

Nucleotide Sequencing

Following sequence of isolate SD9 was obtained after sequence analysis.

Forward sequence

CTTTCGGGAGCCGGACAGATGGGAGCTTGCTCCCT
GATGTCAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTC
CGGGAAACCGGGGCTAATACCGGATGCTTGAT
TGAACCGCATGGTTCAATTATAAAAGGTGG
CTTCTGCTTACAATTTCCGAAGGACCCCGGGCCCAA
TTACTTATTTGGGAAGGTACCGCCCCACCAGGGCA
ACAATCCTTACCCAACTGGAAGGGTGGTC
CGCCCCCCTGGGAACGAAAACCCGGCCAAAACCCCACCG
GAAGGCACCATTAGGAATCCTCCCCAATGGAACAAAGTCC

Table 4: Analysis of Variance (ANOVA) for response surface quadratic model for the production of Xylanase.

Source	Coefficient	DF	Sum of squares	F	Prob>F
Model	80.07	14	4563.56	6.29	0.0007
A Temperature	3.71	1	329.6	6.36	0.0244
B pH	4.47	1	478.83	9.24	0.0888
C Inoculum size	1.69	1	68.55	1.32	0.2694
D Substrate concentration	5.89	1	595.09	11.48	0.0044
A^2	8.32	1	1878.5	36.25	0.0001
B^2	2.72	1	197.92	3.82	0.0709
C^2	5.15	1	707.77	13.66	0.0024
D^2	6.77	1	732.97	14.15	0.0021
AB	0.41	1	2.64	0.051	0.8247
AC	1.99	1	63.6	1.23	0.2866
AD	0.29	1	1.35	0.026	0.8743
BC	1.45	1	33.47	0.65	0.435
BD	1.02	1	16.81	0.32	0.578
CD	1.91	1	58.52	1.13	0.3059

Where A: Temperature; B: pH; C: Inoculum Size; D: Substrate Concentration; DF: Degree of freedom; F: F test

GAACGAACAACCCCCCGGGGATGAAGAAAGGTTTCCGA
ACCTAAAACTTTTTTGGTTAGGAAAAAACAATACCGT
TTCAAAAAGGGGGTACCTTGGCCGTTCCCAACC
CAAAAGCCAACGGCAAATACCTGGCAACCACCCCG
GTTATTCCTTAGTTGCAAGCGTTGGCCGGAATTAATG
GGCCTTAAGCGGCGGCAAGCCGTTTCCTTAGTCCT
AATGGAAGCCCCCGGTTCACCGGGAAGGCAATGGAAA
TGGGAACTTGAGTGAGGAGAGAGGTTGAATCC
AGGTGTGCGTGAATGCTAAGGTTGAAGACACCATGCCAAG
CAATCTTGTCGTACTGACCTAAGCCGAGCCTGGAAC
CACCGAATATTCCTGTTATTCAAGGCGTAACCAA
TAATGCTTAATGTAAGGTTCGCATTAATGCTGCAGCA
GGCATAAGCATTCGCTTGAAATACCGTCCTACCTGA
ATTACGGAATTAACGGTGCCAGCGTGGACTGATT
ATCAAGCAACCCGGACTAACAGTCTGAACTGCT
TGAACATAATAGCTCCTCGAGCAAGTAAATGTCATGT
GTGTCATGTGTGT G AGATGTG GGTATTCA TGAGCGCACA
GATCAGTG CAGCATC ATGCCATC TAGTACT

Reverse sequence

ATAACCAGAACTTCACCCCAATCATCTGTCC
CACCTTCGGCGGCTGGCTCCAAAAGGTT
ACCTCACCGACTTCGGGTGTTACAAACTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGA
ACGTATTCACCGCGGCATGCTGATCCGCGATTAC
TAGCGATTCCAGCTTCACGCAGTCGAG
TTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGG
GATTGGCTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGC
CCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGG
GCATGATGATTTGACGTCATCCCCACCTTCCTCCG
GTTTGTCACCGGCAGTCACCTTAGAGTGCCCAA
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TTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGT

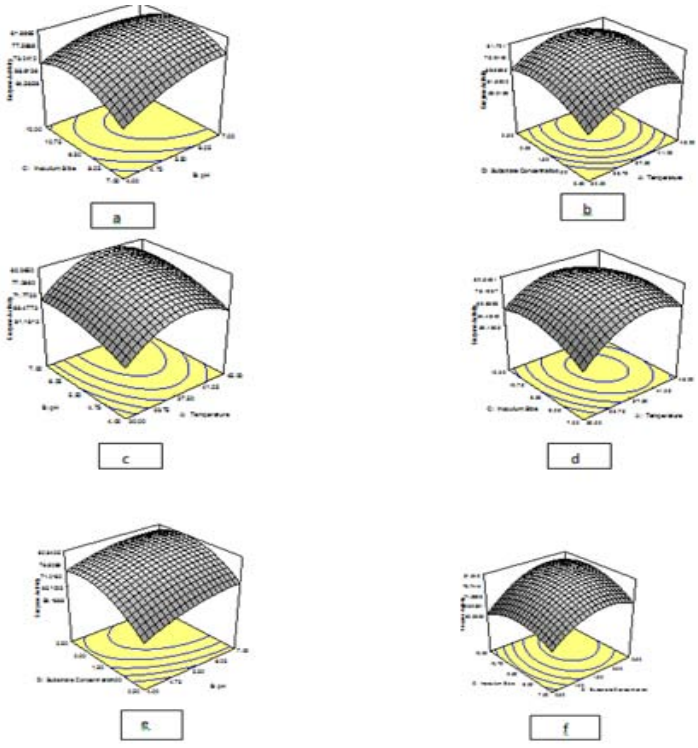

Figure 2: 3D Response surface plot showing interactive effect of a) pH and inoculums size b) temperature and substrate concentration c) temperature and pH d) temperature and inoculums size e) substrate concentration and pH f) substrate concentration and inoculums size on Xylanase activity.

CACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAG
GGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGC
GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGA
CCGTACTCCCCAGGCGGAGTGCTTAATGCGT
TTGCTGCAGCACTAAAGGGCGGAAACCCTCTA
ACACTTAGCACTCATCGTTTACGGCGTGTCTACCA
GGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGC
CTCAGC GTCAGTTACAGACCAGAGAGTCGCCTTCGCCACT
GGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACG
TGGAATTCCACTCTCCTCTTCTGCACTCAAAGTTCCCCA
GTTTCCAATGACCCTCCCCGGGTTGAGCCGGGGGCTTT
CACATCAACTTAAGAAACCGCCTGCGCGGCGCTT
TACGCCCAATAATTCCGGACAACGGCTTGCCACCT
ACGTATTACCGCGGCTGCTGGCCACGTATTAGCCTG
CTTTCTGGGTTAGGTACCGTCAGGTACCGGCCCTATC
CGAACGGGTACTTGGTTCTTCCCTAACAACAGAA
GTTTTACGGATCCGAAAAACCTTTCATCACCTCCA
GCGCGTTGCTCCGTTCGACTTTCGGTCCATTGGCCGA
GAATTCGTACGGCTGCCTCCGGTAGATCTGGACGTGG
TCTAGTCCAGTGT

Conclusion

This research work demonstrates that the response surface methodology can be a powerful and simple tool to effectively analyze the results and to determine optimal conditions for xylanase production. Using the optimized conditions the maximum xylanase production of 85.16U/g was obtained. Hydrolytic products of xylan, such as p-D-xylopyranosyl residues, can be converted into combustible liquids (ethanol), solvents and artificial low-calorie sweeteners. Xylanases have also been used for bio modification of fiber properties with the aim of improving drainage and beat ability in the paper mills before or after beating of pulp.

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