www.sphinxsai.com

ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.7, pp 3883-3888, Sept-Oct 2014

Response surface optimization for co- production of cellulase and xylanase enzymes by *Trichoderma reesei* NRRL–3652

A. Manivannan and R.T.Narendhirakannan*

**Department of Biotechnology, School of Biotechnology and Health Sciences
Karunya University (Karunay Institute of Technology and Sciences)
Coimbatore – 641 114, India**

***Corres.author: bionaren_phd@yahoo.co.in
Phone : 0422 – 2614300, Fax : +91- 422- 2615615**

Abstract: Response Surface Methodology (RSM) was employed to optimize carbon (water hyacinth and xylose), nitrogen mixture of (yeast extract) and peptone sources, a Box- Behnken design based on a three level, three variable design was used to calculate the interactive effects. Under optimized condition, enzymes activity of cellulase and xylanase were predicted to be 24.56 IU/ml and 20.33 IU/ml respectively, which well compared to the actual experimental yield of 23.19 IU/ml and 21.47 IU/ml. Along with cellulase, xylanase was produced simultaneous during the course of the process. The effective xylanase production was found as a function to xylose. The optimum enzyme activity of *Trichoderma reesei* was appeared to be one among few fungal strains having both cellulase and xylanase productivity.
Key words: Response surface methodology, Box-Behnken design, cellulase, xylanase, *Trichoderma reesei.*

Introduction

The growing demand for energy for transportation, heatingand industrial processes, and to provide raw material for the industry is one of the greatest challenges for society in the 21st century[1].Bioethanol made biologically from lignocellulosic biomass, including agricultural and forestry residues, municipal wastes and woody crops being widely accepted as a unique feedstock with powerful economic, environmental and tactical aspects[2]. Lignocellulosic materials could be naturally degraded to monomeric sugars by enzymatic hydrolysis done by cellulase and xylanase, which are widely spread among bacterial and fungal species[3].A cellulolyticenzyme system is a complex system of enzymescomposed of endoglucanase (endo-1, 4 β-D-glucanase,EC 3.2.1.4), exo-glucanase (1,4–β-Dglucan-cellobiohydrolase,EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucanohydrolase, cellobiase, EC 3.2.1.21) that acts synergistically todegrade cellulosic substrate[4,5]. As well the xylanses(EC 3.2.1.18) are a complex system, it includes:xylanases (1, 4-β-D-xylan xylanohydrolase, EC 3.2.1.8)and xylosidases (1,4 β-D-xylan xylohydrolase, EC3.2.1.37)[6,7].High cost of theseenzymes, however presents a significant barrier tocommercialization of ethanol and chemicals. Due to theheterogeneity and complexity of lignocelluolyticbiomass conversion requires multiple enzymeactivities. An efficient and cost effective enzymesystem should contain balanced activities of cellulases(both endo and exo-glucanase), β-glucosidase andxylanase, and such a system should also have hightiter of these activities to offset the cost of ethanol production[8,9].

The use of cheap biomass resources as substrate can help to reduce substrates costs account for enzyme production. Water hyacinth biomass is a low- cost and abundant biomass material containing about cellulose, 34.19% hemicelluloses, 17.66% and lignin, 12.22%[10]. Which can serve as aneffective substrates for enzyme production. Statistical optimization for enhancing the co-production of cellulase and xylanase enzymes has

advantage of screening of most significant variables to produce enzyme up to maximum amount. Many microorganisms have been reported to produce cellulase and xylanase enzymes, though the majority of the work has focused on the use of fungal enzymes to hydrolyze the lignocellulytic materials in to monomeric sugars[8,9].*Trichoderma reesei*, shows a relatively higher enzyme production of both cellulase and xylanase could improve the yield by optimizing the fermentation conditions[11]. In the present study, production and optimization of cellulase and xylanase with high productivity using water hyacinth biomass has been studied using Response Surface Methodology.

Materials and Methods

Substrate preparation

The aquatic plant Water hyacinth was collected from the natural pond, Periya kullam (Big Lake), in Coimbatore city, Tamil Nadu, India. Water hyacinth *Eicchornia crassipes (Mart.) Solms* has been authenticated from Botanical Survey of India (BSI) BSI/SRC/5/23/2012-13/Tech. 464- TNAU Coimbatore, Tamil Nadu, India. Water hyacinth plant was washed thoroughly several times with tap water to remove adhering dirt, chopped into small pieces (~1-2 cm), blended to small particles (~3-5 mm), and finally dried in a hot air oven at 105℃ for 6 h.

Microorganism

The fungal strain *Trichoderma reesei* NRRL - 3652 was acquired from Agricultural Research Service - New York. Stock of the fungal were maintained on Potato Dextrose Agar (PDA) slants at 4℃.

Pretreatment

Water hyacinth was treated with varying concentrations of H_2SO_4 (0.1, 0.5, 1, 1.5 or 2%) at a ratio of 1: 8 and the reactive mixture was kept at 121℃ for 1h. We selected the 1:8 solid- liquid ratio based on the sugar yield obtained from the different ratios applied in our preliminary trials.

Enzyme production

The unoptimized fermentation medium composed of (g/l): Xylose 10 g, KNO_3 4.5g, Yeast extract 5 g, Peptone 5 g and a trace element solution 2.7 ml/l that comprised (g/l): $ZnSO_4.7H_2O$ 0.14 g, $MnSO_4.H_2O$ 0.16 g, $FeSO_4$. $7H_2O$ 0.5 g, $CoCl_2$. $2H_2O$ 0.2g in distilled water. The pH of the medium was adjusted to 7.2 after sterilization using sterile 1 N NaOH. Erlenmeyer flasks (250 ml) containing 50 ml sterile culture medium were inoculated with 2.5 ml inoculum. The flasks were incubated at 28 ℃ for 120 h in 120 rpm on an orbital shaker. The extract was centrifuged at 10,000 rpm at 4℃ for 10 min, and the clear supernatant was assayed for cellulase and xylanase activity. For optimization studies, the composition of the culture medium was varied according to the experimental data, while the pH, temperature and time of fermentation were not varied.

Enzyme activity

Cellulase and xylanase enzymes were analyzed using CM-Cellulose (2% w/v), birch wood xylan (1% w/v) respectively following the method given by Ghose (1987)[12]. The amounts of released glucose and xylose during estimation was quantified using respective standards[13]. One IU of activity was expressed as the amount of enzyme required to release 1µmol of product/min under assay conditions.

Response surface methodology

The three most important variables, viz. Water hyacinth (A), yeast extract + peptone (B) and Xylose (C) were selected with three coded levels (1,0,+1), as shown in Table 1. RSM using Box and Behnken factorial design[14]with quadratic model was employed to study the combined effect of three independent variables using Design-expert 8.0.7.1 (Stat-Ease, USA) trial version software.

The relation between the coded forms of the input value and the actual value of the water hyacinth, yeast extract + peptone and xylose are described in equation:

$$X_i = \frac{(Z_i - Z_0)}{\Delta Z}$$

Where X_i is a coded value and Z_i the actual value of the factor, Z_0 the actual value of the same variable at the center point, ΔZ the step change of the variable.

The average Cellulase activity (IU/ml) and xylanase activity (IU/ml) were taken as dependent variables or responses Y_1 and Y_2. Regression analysis was performed on the data obtained. The regression model between dependent variables (Y) and independent variable was:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ij} X\, i^2 + \sum_{i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j$$

Where Y is predicted response, and i, j are linear, quadratic coefficients, respectively b and k are regression coefficient and number of factors studied in the experiment, respectively.

Results and discussion

To optimize carbon (water hyacinth), nitrogen (mixture of yeast extract and peptone) and xylose, BBD, consisting of a set of 17 experiments with five replicates at central point was conducted. The results obtained by BBD were analyzed by standard analysis of variance (ANOVA), and design matrix of the independent variables in coded units along with predicted and experimental values of response is given in Table 2.All the experiments were performed in 250 ml Erlenmeyer flask containing 50ml of media. The quadratic model expressed by equation represents cellulase (Y_1) and xylanse (Y_2) activityas a function of water hyacinth (A), mixture of peptone and yeast extract (B) and Xylose (C).

$$Y_1 \text{ (IU/ml)} = 13.03 + 1.06A + 0.80B - 0.14C - 1.05AB + 8.67AC - 3.47BC - 0.04A^2 - 0.03B^2 + 0.01C^2$$

$$Y_2 \text{ (IU/ml)} = 12.07 + 1.20A + 0.90B - 0.05C - 2.42AB + 8.71AC - 2.26BC - 0.05A^2 - 0.04B^2 + 5.48C^2$$

Adequacy of the polynominal equation was tested by *F* test and analysis of variance (ANOVA) for response surface quadratic model is given in Table 3 the ANOVA values for the two responses viz. cellulase and xylanse activity from the RSM experiments. The *p* value serves as a tool for checking the significance of each of the coefficient. The model terms having *p* value<0.05were considered significant whereas less than 0.0001 treated as highly significant. ANOVA for Celulase production Y_1 (IU/ml) indicated the *F* value to be 4.19, which implied model to be significant model terms having values of '*P>F* less than 0.05 were considered significant, whereas those greater than 0.10 are insignificant. Correspondingly ANOVA for xylanase production Y_2 (IU/ml) indicated the *F* value to be 29.20, which implied the model was significant. It could be concluded from the Table 3 that coefficient of linear and quadratic effect of each model term water hyacinth (A), mixture of peptone and yeast extract (B) and Xylose (C) are significant. It is indicating that both carbon and nitrogen source can act as restrainingsubstrates and little change in their concentration will affect enzyme production[15].

ANOVA indicated the R^2value of 0.8434 and 0.9741 respectively, for responses $Y_1$and Y_2. This again confirmed an acceptable quadratic model to the experimental data, and showed that the model could explain 90-95% of the variability in the response. The adequate precision which measures the signal to noise ratio was 6.231 and 15.637 for responses $Y_1$and Y_2 respectively, which indicates a suitable signal.

The optimal parameter resulting combination in cellulase and xylanase yield were obtained by solving the system of partial derivatives for the different independent variables. The model predicted the optimum concentrations of A, B and C were 10 g/l, 2 g/l and 18 g/l with 24.56 IU/ml cellulase activity respectively, correspondingly 10 g/l, 2 g/l and 2 g/l was obtained as optimum concentrations with 20.33 IU/ml xylanase activity. To determine the optimum level of each variable for maximum response and to understand the interaction of variables the response surface curves were plotted (Fig 1).

Experimental validation of the model

Experiment was conducted with optimized conditions predicted by RSM analysis to verify the accuracy of the model. The cellulase and xylanse activity was found to be 23.19 IU/ml and 21.47 IU/ml respectively. The optimized yield obtained is in accord with predicted ones (Table 2). This study shows rationally good production on natural substrate like water hyacinth biomass and further enhancement in production with the satisfactory amendment of other parameters.

Conclusion

The present study reconnoitered the co- production of cellulase and xylanase enzymes by *Trichoderma reesei* NRRL – 3652 as this strain was found to be one among few fungal strain to produce both cellulase and xylanse enzyme. Considering this facets, the present organism is appropriate for commercial utilization as it

uses naturally available water hyacinth as a substrate. Which will ultimately help to develop a cost-effective process for bioethanol production.

The experimental models derived from response surface optimization of the independent variables lead to the 4 fold increase in the cellulase and xylanase enzyme activity through primary screening experiments and confirmed that optimum conditions for enzymes production can be effectivelyprophesied by RSM.

Table 1 Experimental range, level and code of independent variables

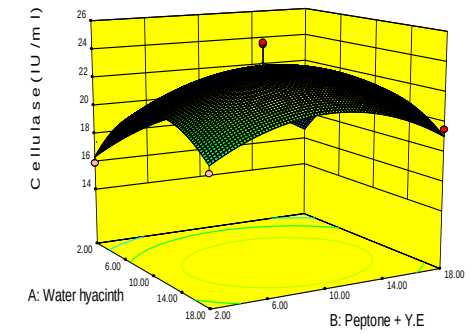
Independent variables	Symbol coded	Range and levels		
		-1	0	+1
Water hyacinth (g/l)	A	2	10	18
Peptone and Yeast Extract (g/l)	B	2	10	18
Xylose (g/l)	C	2	10	18

Table 2 Box- Behnken design matrix along with the experimental and predicted value of cellulase and xylanase activity

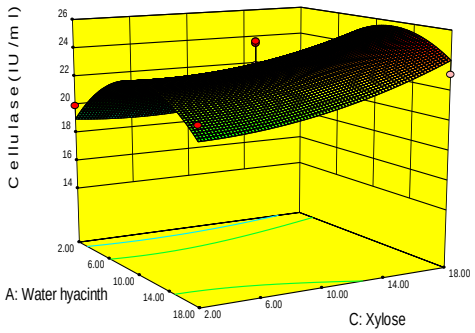
Run no.	A:Water hyacinth (g/l)	B:YeastExtract + peptone (g/l)	C: Xylose (g/l)	Cellulase activity (IU/ml)		Xylanase activity (IU/ml)	
				Predicted	Observed	Predicted	Observed
1	0	0	0	16.35	15.89	16.13	16.08
2	-1	0	-1	18.97	18.47	19.13	19.10
3	-1	0	+1	16.72	17.22	16.88	16.91
4	+1	-1	0	19.07	19.53	19.26	19.31
5	-1	+1	0	19.00	19.94	18.89	18.92
6	+1	+1	0	20.36	21.34	20.47	20.47
7	0	-1	+1	20.44	19.47	20.44	20.44
8	0	+1	-1	24.03	23.09	24.25	24.22
9	0	+1	+1	20.12	19.64	20.31	20.33
10	0	0	0	20.80	19.36	20.46	20.41
11	0	-1	-1	23.12	24.56	22.68	22.73
12	+1	0	-1	22.91	23.39	23.41	23.39
13	-1	0	0	23.32	21.55	24.18	23.35
14	0	0	0	23.32	23.27	24.18	25.17
15	0	0	0	23.32	24.73	24.18	23.86
16	+1	0	+1	23.32	22.16	24.18	23.41
17	0	0	0	23.32	24.87	24.18	25.09

Table 3 Analysis of variance (ANOVA) for the fitted quadratic model of cellulase and xylanase activity.

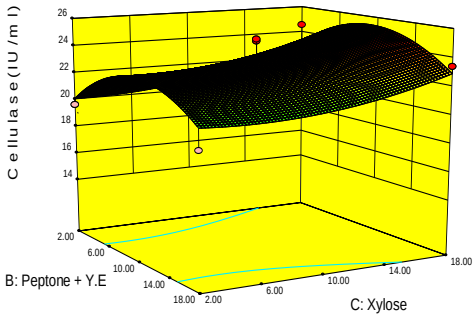
Cellulase activity (IU/ml)						**Xylanase activity (IU/ml)**				
Source	SS	DF	MS	*F* Value	*P>F*	SS	DF	MS	*F* Value	*P>F*
Model	97.33	9	10.81	4.19	0.0361	120.36	9	13.37	29.20	<0.0001
A	12.28	1	12.28	4.76	0.0656	14.45	1	14.45	31.54	0.0008
B	0.11	1	0.11	0.043	0.8420	0.40	1	0.40	0.86	0.3834
C	13.08	1	13.08	5.07	0.0591	14.18	1	14.18	30.96	0.0008
AB	0.018	1	0.018	7.060E-003	0.9354	0.096	1	0.096	0.21	0.6608
AC	1.23	1	1.23	0.48	0.5119	1.24	1	1.24	2.71	0.1434
BC	0.20	1	0.20	0.077	0.7898	0.084	1	0.084	0.18	0.6812
A^2	41.99	1	41.99	16.27	0.0050	52.00	1	52.00	113.53	<0.0001
B^2	23.86	1	23.86	9.24	0.0188	33.29	1	33.29	72.68	<0.0001
C^2	2.71	1	2.71	1.05	0.3398	0.52	1	0.52	1.13	0.3229
Residual	18.07	7	2.58			3.21	7	0.46		
Lack of fit	9.20	3	3.07	1.38	0.3693	0.014	3	4.92E-003	5.753E-003	0.9993
Pure error	8.87	4	2.22			3.19	4	0.80		
R^2 : 0.8434, adj R^2: 0.6421, Pre R^2: 0.6356						R^2 : 0.9741, adj R^2: 0.9407, Pre R^2: 0.9578				
C.V: 7.62%, adequate precision: 6.231						C.V: 3.17 %, adequate precision: 15.637				
SS Sum of squares of model parameters, DF degree of freedom, MS mean square of model parameters										



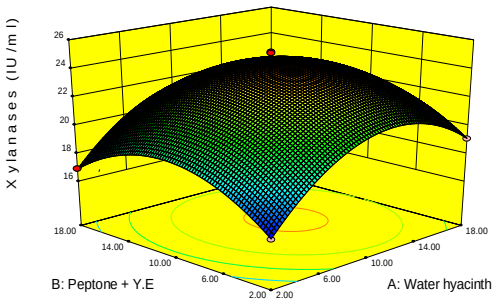
A



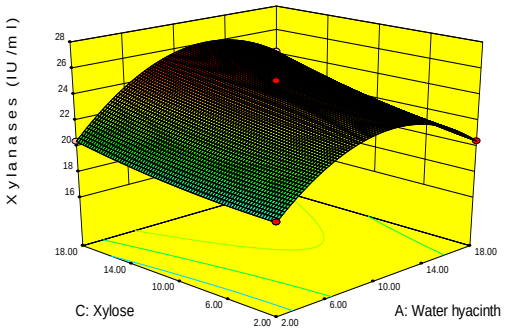
B



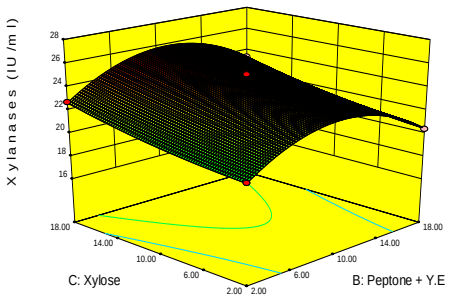
C



D



E



F

Figure 1 Response surface plot displaying relative effect of two variables of enzyme activity cellulase: A WH and Peptone + YE, B WH and xylose, C Peptone + YE and xylose; xylanse: D Peptone + YE and WH, E xylose and WH, F xylose and Peptone + YE

Acknowledgement: We would like to express our appreciation to Dr. Paul Dinakaran, Chancellor, Dr. S. Sundar ManoharanPh.D, Vice-Chancellor, Rev. (Dr.) Sarvjeet Herbert Ph.D and Dr. C. Joseph Kennady, Registrar, Dr. Patrick Gomez, Director, School of Biotechnology and Health Sciences, Karunya University, Coimbatore for providing necessary facilities to carry out the experiments.

References

1. Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G. and Zacchi, G., Bio-ethanol – the fuel of tomorrow from the residues of today, Trends in Biotechnology, 2006, 24, 549–556.
2. Wyman, C.E., What is (and is not) vital to advancing cellulosic ethanol, Trends in Biotechnology, 2007, 25, 153–157.
3. Xia, L.M. and Sheng, X.L., High-yield cellulaseproduction by *Trichoderma reesei* ZU-02 on corncobresidues, Bioresource Technology,2004,91(3), 259-62.
4. Esterhauer, H., Steiner, W., Labudova, I., Hermann, A. and Hayn, M., Production of *Trichoderma* cellulase in laboratory and pilotscale, Bioresource Technology,1991, 36, 51-65.
5. Holker, U., Hofer, M. and Lenz.J., Biotechnological advantages oflaboratory scale solid state fermentation with fungi, Applied Microbiology and Biotechnology, 2004, 64, 175-86.
6. Fang, H.Y., Changa, S.M., Hsieh, M.C.and Fang, T.J., Production,optimization growth conditions and properties of the xylanasefrom *Aspergillus carneus* M34, Journal of Molecular Catalysis B: Enzymatic, 2007,49, 36-42.
7. Lama, L., Calandrelli, V., Gambacorta, A. and Nicolaus, B., Purification and characterization of thermostable xylanaseand β-xylosidase by thermophilic bacterium *Bacillusthermantarcticus*, Research in Microbiology, 2004, 155, 283-89.
8. Viikari, L., Alapuranen, M., Puranen, T., Vehmaanpera, J. and Siika-Aho M., Thermostable enzymes in lignocellulose hydrolysis,Advances in Biochemical Engineering Biotechnology, 2007, 108, 121-45.
9. Safaa, M.A., Sana, H.O. and Nadia, A.S., Co-production of Cellulase and Xylanase Enzymes by Thermophilic *Bacillus subtilis* 276NS. International Journal of Biotechnology for wellness Industries, 2013, 2, 65-74.
10. Ahn, D.J., Kim, S.K. and Yun, H.S., Optimization of pretreatment and saccharification for the production of bioethanol from water hyacinth by *Saccharomyces cerevisiae*, Bioprocess and Biosystems Engineering, 2012, 35(1-2), 35-41.
11. Singhania, R.R., Sukumaran, R.K. and Pandey, A., Improved cellulase production by *T reesei* RUT C30 under SSF through process optimization. Applied Biochemistry and Biotechnology, 2007, 142(1), 60-70.
12. Ghose, T.K., Measurement of cellulase activities, Pure and Applied Chemistry, 1987, 59(2), 257–268.
13. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducingsugar, Analytical Chemistry, 1959, 31 (3), 426–428.
14. Box, G.E.P. and Behnken, D.W., Some three level designs for the study of quantitative variables, Technometrics, 1960, 2, 455-75.
15. Tanyildizi, M.S., Ozer, D. and Elibol, M., Optimization of α- amylase production by *Bacillus sp*. using response surface methodology, Process Biochemistry, 2005, 40, 2291-2296.

*****