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# Cellulosic Bioethanol Production by Sequential Fermentation using Agricultural Waste

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**Abstract :** Cellulase catalyzes the conversion of cellulose into monomeric units which has many biologically important applications. Cost of production of cellulase which is great hindrance in the current era can be greatly reduced by using lignocellulosic wastes as substrate for the enzyme production. The current study mainly focuses on the production and optimization of cellulose using jackfruit waste as substrate and Aspergillus fumigatus JCF as microorganism. Substrate was pretreated with different chemicals and 0.5N NaOH was selected as the best pretreatment method. The enzyme with activity of 3.3 IU/ml was further used for the production of bioethanol through simultaneous saccharification and fermentation using agricultural wastes as substrate. The presence of yeast along with cellulase enzyme greatly reduces the accumulation of sugar in the fermentation media. Bioethanol production was tried using both treated and untreated substrates. Out of all the substrates tried pretreated sugarcane leaves liberated maximum bioethanol of about 18g/l.
**Keywords:** Cellulase; *Aspergillus fumigatus* JCF; Jack fruit perianths; Pretreatment; Response Surface Methodology; Simultaneous saccharification and fermentation.

## Introduction

Cellulose is one of the important constituent of plants. It is referred as "biological currency" because it is an abundantly available biopolymer which can be used for the production of many useful products[1,2]. Lignocelluloses form a major portion of agricultural wastes and forest wastes. The key step in the exploitation of cellulose is its hydrolysis into monomeric sugars and their eventual conversion into valuable compounds for the release of energy[3]. Cellulases are group of enzymes which include endoglucanases (EC 3.2.1.4), cellobiohydrolases ( EC3.2.1.91) and β–glucosidases (EC3.2.1.21)[4,5]. Cellulase plays an important role in the conversion of cellulose into monomeric units glucose. Thus cellulases have many important applications like formulation of washing powder,animal feed production[6], textile industry, pulp and paper industry[7], starch processing, lactic acid production[8], alcohol fermentation, extraction of fruits and vegetable juices[9].

Many microbes help in the cellulase synthesis which mainly includes bacterial and fungal species. Fungal species secrete more cellulase than bacterial species[10]. Filamentous fungi especially *Aspergillus*, *Penicillium* and *Trichoderma* species are efficient producers of cellulase enzyme[11,12]. High cost of raw materials is the major problem in the enzyme production can be overcome by microbial fermentation using low cost substrates. Research is going on extensively in this area. Some have tried the cellulase production with substrates like wheat straw[13], rice straw etc and fruit processing waste such as apple pomace and grape pomace, pineapple waste[14-16]. But studies on the use of jackfruit waste as raw material are very few in the literature.

The hydrolysis of cellulose will be greatly affected by the porosity of lignocellulosic biomass, by cellulose crystallinity, and by lignin and hemicellulose content[17]. Pretreatment procedures will remove lignin and hemicelluloses, thereby reducing cellulose crystallinity and increase the porosity of the materials. Pretreatment can be done by physical, chemical and biological methods. The operating conditions of fermentation need to be optimized for maximum production in industrial technology. Optimization using statistical optimization through central composite design considering all interactions effects of the factors reduces the time consumption which is a major drawback of optimization through one factor at a time study[18-20].

In the present scenario entire world is looking forward for an alternate energy source due to increased concern regarding environmental pollution, energy security etc[21,22] . Bioethanol produced through saccharification and fermentation is a good solution to the situation[23]. Enzymatic saccharification of cellulosic biomass has been considered as an environmentally friendly method. The present study mainly focuses on the optimized production and application of cellulase for increased bioethanol production.

## Materials and methods

### Raw Material

Jack fruit perianths procured locally from Kerala was used for the production of cellulase enzyme. Saw dust and sugar cane leaves were collected from the market in Chennai. Water hyacinth was collected from Kerala. Saw dust, sugar cane leaves and water hyacinth were used for bioethanol production. The jackfruit waste was brought to the lab and dried overnight at 60ºC and mill-ground. The particles taken from 240µm mesh were used as the carbon source for cellulase production. Feedstocks for bioethanol production like saw dust, water hyacinth and sugar cane were boiled, mashed and then autoclaved. All the samples were pretreated by using chemicals as per the procedure. The samples were then neutralized and dried properly at 60ºC until constant weight. This was then stored under dried conditions for further studies.

### Pretreatment

The delignification of the jackfruit perianths was achieved by hydrolyzing the sample with 0.5 M sulfuric acid, hydrochloric acid, citric acid, sodium hydroxide and potassium hydroxide. The mixture was kept at 50ºC for 2hr. Hydrolysate was neutralized, washed and centrifuged to remove precipitate. The precipitate and filtrate was used for the estimation of ash, lignin, cellulose and hemicelluloses. The pretreated substrate was weighed to measure the weight loss and the filtrate was used to determine the soluble lignin by modified Klason lignin method[24]. Cellulose and hemicelluloses amount was determined according to the procedure by Ververis et al[25]. Precipitate was dried overnight in oven and stored under sterilized conditions for further use.

### Microorganisms

A previously isolated cellulase producing fungi, *Aspergillus fumigatus* JCF from spoiled jackfruit was used in this study. It was grown on czapex dox agar medium at 30ºC. After complete sporulation it was stored at 4ºC in czapex dox agar medium. Fungal strain was dislodged from the agar medium for further study, by adding 5ml sterilized water containing 0.1% Tween 80[26]. The spores were dislodged from the agar slant and inoculated into presterilized media.

### Experimental design

Central composite design of response surface methodology was used for the cellulase production by *Aspergillus fumigatus* JCFconsisting of four factors at three level patterns (Table 1). Ammonium sulphate ($X_1$), $KH_2PO_4$($X_2$), Tween 80 ($X_3$) and time of incubation ($X_4$) are chosen as the independent variables and cellulase activity as the dependent output variable for central composite design. The polynomial quadratic equation fitted to evaluate the effect of each independent variable to the response is given in eq.(1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_1^2 + \beta_6 \beta X_2^2 + \beta_7 X_3^2 + \beta_8 X_4^2 + \beta_9 X_1 X_2 + \beta_{10} X_1 X_3 + \beta_{11} X_1 X_4 + \beta_{12} X_2 X_3 + \beta_{13} X_2 X_4 + \beta_{14} X_3 X_4 \quad \text{.....(1)}$$

where Y is the predicted response, $X_1$,$X_2$,$X_3$,$X_4$ are the coded independent input variables, $\beta_0$ is the intercept term, $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$ are the linear coefficients showing linear effects, $\beta_5$, $\beta_6$, $\beta_7$, $\beta_8$ are the quadratic coefficients showing squared effects and $\beta_9$, $\beta_{10}$, $\beta_{11}$, $\beta_{12}$ $\beta_{13}$, $\beta_{14}$ are the cross product coefficients showing interaction effects.

Optimum values of variables were obtained by using regression analysis. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) [27, 28].

**Simultaneous saccharification and fermentation for bioethanol production**

One gram of saw dust, sugar cane leaves and water hyacinth was mixed with 100 ml citrate buffer in a 250 ml flask. Media was prepared by mixing yeast extract (0.1%, w/v) and peptone (0.1%, w/v) to the above solution. Media prepared was sterilized and inoculated with baker's yeast and 4ml of crude cellulase enzyme (3.34 IU/ml). The fermentation was carried out at 120rpm and 30ºC. The sample was collected at regular intervals of 12 hours. Residual sugar and ethanol concentration was determined for each sample. The residual sugar concentration was measured by DNS assay[29].This procedure was repeated with samples treated with sodium hydroxide. NaOH treatment was conducted for the delignification of saw dust, sugar cane leaves and water hyacinth.

**Analytical Methods**

**Assay of cellulase enzyme activity**

Endoglucanase activity (CMCase) was measured using a reaction mixture containing1.5 ml of 1% carboxymethyl cellulose (CMC) in 50 mM sodium citrateacetate buffer (pH 4.8) and 1.5 ml of filtrate. The reaction mixture was incubated at 50 ± 2ºC for 10 min, and the reducing sugar produced was determined by DNS method[29]. One unit (IU) of cellulase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min.

***Determination of Bioethanol***

The amount of ethanol produced in the fermentation media was estimated by using dichromate method. The 1 ml of cell free extract was diluted four times and 1 ml of potassium dichromate was added. After keeping all tubes containing the above mixture in ice water 5 ml of concentrated sulfuric acid was added gently through the walls. Then the optical density was measured on spectrophotometer at 660 nm [30].

## Results and Discussion

**Pretreatment of substrate**

The effect of alkali and acid pretreatment on chemical composition of rice straw such as cellulose, hemicellulose and lignin was analyzed. Cellulose, hemicellulose and lignin contents of samples with and without pretreatment are shown in Table 1 where maximum cellulose content (32.18%) was obtained from 0.5 M NaOH pretreated substrate. Effect of pretreatment on cellulase production was studied by fermentation of various pretreated substrates. The results are shown in Fig 1. It shows that substrate treated with sodium hydroxide gave maximum yield.

**Table 1. Biochemical composition of Jackfruit waste under various pretreatment chemicals**

| Treatment method | Cellulose(%) | Hemicellulose(%) | Lignin(%) |
|---|---|---|---|
| Before Treatment | *29.17* | 15.78 | 4.28 |
| After Treatment | | | |
| 0.5 M NaOH | 35.18 | 14.2 | 1.30 |
| 0.5 M KOH | 33.9 | 12.7 | 2.44 |
| 0.5 M $H_2SO_4$ | 31.23 | 10.89 | 1.35 |
| 0.5 M HCl | 30.83 | 9.28 | 1.32 |

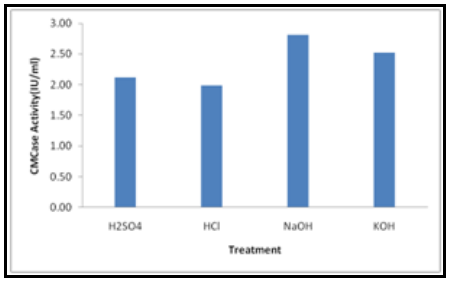


**Fig 1. Effect of pretreatment on CMCase activity**

**Optimization of parameters by Response surface methodology**

For maximizing the cellulase production four important parameters were optimized by response surface method through central composite design. The range and levels of the variables used in the experimental design are shown in Table 2. The parameters selected for the study included Ammonium sulphate ($(NH_4)_2SO_4$ (1-5%)), Potassium dihydrogen orthophosphate ($KH_2PO_4$ (0.5-2.5%), Tween 80(35-95) and Time of incubation (3.5-5.5days). By applying multiple regression analysis on the experimental data, a second-order polynomial equation was obtained as given in eq.(2)

$$Y = 3.34 + 0.14X_1 + 0.079X_2 - 0.25X_3 + 0.08X_4 - 0.09X_1X_2 - 0.18X_1X_3 + 0.001X_1X_4 + 0.099X_2X_3 + 0.065X_2X_4 + 0.24X_3X_4 - 0.52X_1^2 - 0.53X_2^2 - 0.15X_3^2 \quad \text{……..(2)}$$

**Table 2 . Response surface design along with experimental and predicted values for optimization of selected variables for CMCase production**

| Run | X1(%) | X2(%) | X3(µl) | X4(hrs) | Observed CMCase Activity (IU/ml) |
|---|---|---|---|---|---|
| 1 | 3.00 | 1.50 | 65.00 | 4.50 | 3.37 |
| 2 | 5.00 | 1.50 | 65.00 | 4.50 | 1.63 |
| 3 | 4.00 | 1.00 | 80.00 | 5.00 | 1.74 |
| 4 | 3.00 | 0.50 | 65.00 | 4.50 | 1.09 |
| 5 | 3.00 | 1.50 | 65.00 | 4.50 | 3.30 |
| 6 | 2.00 | 2.00 | 50.00 | 5.00 | 1.81 |
| 7 | 4.00 | 2.00 | 50.00 | 5.00 | 2.44 |
| 8 | 2.00 | 1.00 | 50.00 | 5.00 | 1.49 |
| 9 | 4.00 | 1.00 | 50.00 | 4.00 | 2.97 |
| 10 | 3.00 | 1.50 | 65.00 | 4.50 | 3.33 |
| 11 | 3.00 | 2.50 | 65.00 | 4.50 | 1.51 |
| 12 | 4.00 | 2.00 | 50.00 | 4.00 | 2.15 |
| 13 | 2.00 | 1.00 | 80.00 | 4.00 | 1.12 |
| 14 | 2.00 | 2.00 | 80.00 | 5.00 | 2.27 |
| 15 | 3.00 | 1.50 | 35.00 | 4.50 | 3.34 |
| 16 | 4.00 | 2.00 | 80.00 | 4.00 | 1.47 |
| 17 | 2.00 | 1.00 | 80.00 | 5.00 | 1.91 |
| 18 | 4.00 | 1.00 | 50.00 | 5.00 | 2.36 |
| 19 | 2.00 | 2.00 | 80.00 | 4.00 | 1.50 |
| 20 | 3.00 | 1.50 | 65.00 | 4.50 | 3.29 |
| 21 | 4.00 | 2.00 | 80.00 | 5.00 | 2.04 |
| 22 | 2.00 | 2.00 | 50.00 | 4.00 | 2.12 |
| 23 | 2.00 | 1.00 | 50.00 | 4.00 | 1.95 |
| 24 | 4.00 | 1.00 | 80.00 | 4.00 | 1.18 |
| 25 | 3.00 | 1.50 | 65.00 | 4.50 | 3.34 |
| 26 | 1.00 | 1.50 | 65.00 | 4.50 | 1.06 |
| 27 | 3.00 | 1.50 | 95.00 | 4.50 | 2.35 |
| 28 | 3.00 | 1.50 | 65.00 | 5.50 | 2.77 |
| 29 | 3.00 | 1.50 | 65.00 | 3.50 | 2.65 |
| 30 | 3.00 | 1.50 | 65.00 | 4.50 | 3.37 |

Statistical significance of the model was checked by ANOVA. The Model F-value of 46.81 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. The regression results from the data of central composite design experiments are shown in Table 3. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case $X_1$, $X_2$, $X_3$, $X_4$, $X_1X_2$, $X_1X_3$, $X_2X_3$, $X_3X_4$, $X_1^2$, $X_2^2$, $X_3^2$ and $X_4^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 29.60 implies the Lack of Fit is significant. There is only a 0.08% chance that a "Lack of Fit F-value" this large could occur due to noise."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 20.489 in the current study indicates an adequate signal. This model can be used to navigate the design space.

**Table 3. Analysis of Variance Table for Response Surface Quadratic Model**

| Source | Sum of squares | df | Mean square | F value | P value Prob > F | Significance |
|---|---|---|---|---|---|---|
| Model | 17.43 | 14 | 1.24 | 46.81 | <0.0001 | Significant |
| X1 | 0.45 | 1 | 0.45 | 17.04 | 0.0009 | Significant |
| X2 | 0.15 | 1 | 0.15 | 5.78 | 0.0296 | Significant |
| X3 | 1.51 | 1 | 1.51 | 56.92 | <0.0001 | Significant |
| X4 | 0.14 | 1 | 0.14 | 5.34 | 0.0354 | Significant |
| X1X2 | 0.12 | 1 | 0.12 | 4.55 | 0.0499 | Significant |
| X1X3 | 0.55 | 1 | 0.55 | 20.54 | 0.0004 | Significant |
| X1X4 | 1.900E-0050 | 1 | 1.900E-0050 | 7.143E-004 | 0.9790 | |
| X2X3 | 0.16 | 1 | 0.16 | 5.87 | 0.0285 | Significant |
| X2X4 | 0.067 | 1 | 0.067 | 2.51 | 0.1343 | |
| X3X4 | 0.90 | 1 | 0.90 | 33.69 | <0.0001 | Significant |
| $X1^2$ | 7.47 | 1 | 7.47 | 280.97 | <0.0001 | Significant |
| $X2^2$ | 7.74 | 1 | 7.74 | 290.92 | <0.0001 | Significant |
| $X3^2$ | 0.59 | 1 | 0.59 | 22.22 | 0.0003 | Significant |
| $X4^2$ | 0.90 | 1 | 0.90 | 33.82 | <0.0001 | Significant |
| Residual | 0.40 | 15 | 0.027 | | | |
| Lack of Fit | 0.39 | 10 | 0.039 | 29.60 | 0.0008 | Significant |
| Pure error | 6.626E-003 | 5 | 1.325E-003 | | | |
| Corr.Total | 17.83 | 29 | | | | |

In the current study determination coefficient (R-squared) was found to be 0.9659.When the R-squared value is closer to 1, the model will be stronger and will predict the result in a better way[31].The "Pred R-Squared" of 0.8727 is in reasonable agreement with the "Adj R-Squared" of 0.9567, that is the difference is less than 0.2."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio in the current study was 20.489 which indicate an adequate signal. This model can be used to navigate the design space.

Under the optimum conditions predicted enzymatic activity was found to be 3.34 IU/ml. To validate the predicted response, experiments were conducted in triplicates under optimized conditions. The cellulase activity was found to be 3.32 IU/ ml which was close to the predicted value.

**Simultaneous saccharification and bioethanol production**

The simultaneous Saccharification and fermentation process, firstly described by Takagi et al[32], combines enzymatic hydrolysis of cellulose with simultaneous fermentation of its main derived sugar (glucose) to ethanol. In this process lignocellulosic materials with complex cellulose will be broken down into simple sugars by cellulolytic action, which will be followed by fermentation for the production of bioethanol. Current work reveals a comparative SSF study for bioethanol production by using substrates with and without pretreatment with sodium hydroxide. The yeast *Saccharomyces* sp. is the choice of organisms due to its high bioethanol production efficiency than others[33] .Bioethanol is a form of renewable energy produced from agricultural feedstocks. Cellulosic bioethanol offers promise because cellulose fibers, a major and universal component in plant cells walls, can be used to produce ethanol[34]. So here cheaply available agricultural wastes like saw dust, sugar cane leaves and water hyacinth were used for the production of reducing sugar and thereby bioethanol.

According to Schell and Walter in 1991[35], simultaneous Saccharification and fermentation (SSF) is thought to be the best process for enzymatic conversion of cellulose to ethanol. Samples were treated with crude cellulase enzyme which released reducing sugars which then was acted upon by yeast for the production of bioethanol. Highest amount of reducing sugar was released from sugar leaves which was about 16.5 g/l. Water hyacinth and saw dust released about 15.5g/l and 15.22 g/l respectively. Fermentation by yeast on these Saccharified samples released bioethanol. Maximum bioethanol released by sugar cane leaves was about 15.41g/l and water hyacinth and saw dust released about 15.08 and 14.78 g/l respectively. When the pretreated substrates were used for simultaneous saccharification and fermentation more release of reducing sugar was observed. Pretreatment makes the lignocellulosic substrate amenable to hydrolysis and fermentation by breaking down complex molecules. Action of cellulase enzyme on NaOH treated sugar cane leaves, water hyacinth and saw dust released maximum of 20g/l, 19.19g/l and 18.43g/l reducing sugar respectively. These Saccharified samples released maximum bioethanol concentration of about 18g/l ,17.88g/l and 16.37g/l respectively on fermentation with baker's yeast. The results of these studies are shown in Fig 2 and Fig 3.Bioethanol production by microbial extracellular enzymatic hydrolysis and fermentation on cellulosic substrates yielded of 8.9g/l [36].

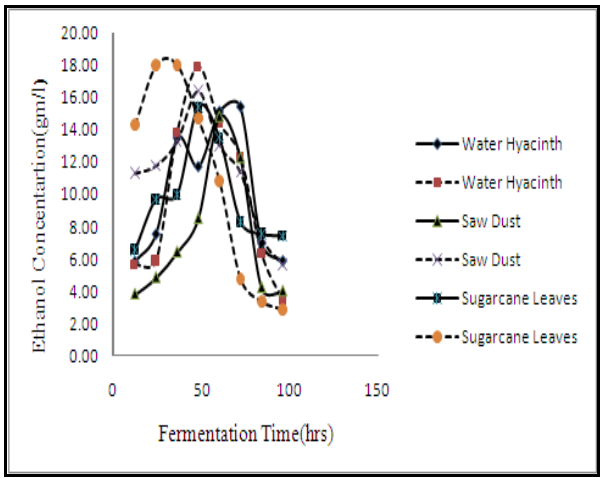


**Fig 2 Bioethanol production in SSF**

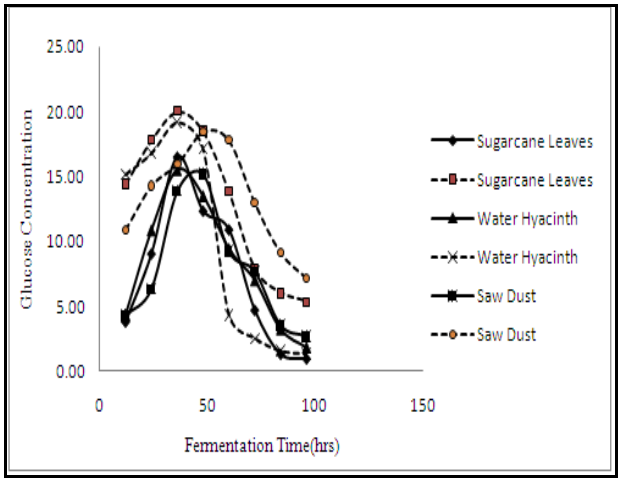


**Fig 3 Reducing sugar released in saccharification**

## Conclusion

The current study mainly focused on the effect of different parameters on cellulase production. Pretreatment greatly increases the porosity of substrates so that activity of microbes will easier for the enzyme production. Out of the different chemicals tried for pretreating the substrate, 0.5N NaOH released maximum amount cellulose. Using response surface methodology, factors like ammonium sulphate $KH_2PO_4$, Tween 80 and time of incubation were optimized for enhanced cellulase production. The maximum activity predicted by the model was in agreement with that of the experimental values. Consequently, the cellulase enzyme produced by *Aspergillus fumigatus JCF*, was employed for bioethanol production using simultaneous saccharification and fermentation. Out of the six substrates tried for bioethanol production pretreated sugarcane leaves produced maximum bioethanol at shake flask level. Thus the present study reveals that, by using the agricultural wastes cellulase production can be economically increased thereby bioethanol production can be enhanced.

**References:**

1. Himmel M.E., Ruth M.F. and Wyman C.E., Cellulase for commodity products from cellulosic biomass, Curr. Opin. Biotechnol,1999,10,358–64.
2. Wei Wang., Tongqi Yuan., Kun Wang., Baokai Cui. and Yucheng Dai., Statistical optimization of cellulase production by the brown rot fungi, *Fomitopsis palustris*, and its application in the enzymatic hydrolysis of LHW-pretreated woody biomass, Process. Biochem,. 2012,47,2552-2556.
3. Olofsson K., Wiman M. and Liden G., Controlled feeding of cellulases improves conversion of xylose in simultaneous saccharification and cofermentation for bioethanol production, J. Biotechnol. 2010, 145,168–75.
4. Jagtap S. and Rao M., Purification and properties of a low molecular weight1,4-beta-d-glucangluco hydrolase having one active site for carboxymethylcellulose and xylan from an alkalothermophilic *Thermomonospora* sp, Biochem.Biophys.Res.Commun., 2005, 329(1),111–6.

5. Guo R., Ding M., Zhang S.L., Xu G.L. and Zhao F.K., Molecular cloning and characterization oftwonovel cellulase genes from the mollusc Ampullaria crossean, J.Comp. Physiol[B], 2008,178 (2),209–15.
6. Han W. and He M., The application of exogenous cellulase to improve soil fertility and plant growth due to acceleration of straw composition, Bioresour. Technol., 2010,101, 3724–3731.
7. De Vries R.P., Regulation of *Aspergillus*genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production, Appl. Microbiol. Biotechnol., 2003, 61, 10-20.
8. EI-Hawary F.I., Mostafa Y.S. and Laszlo E., Cellulase production and conversion of rice straw to lactic acid by simultaneous saccharification and fermentation, Acta Aliment. Hang, 2003,30, 281–295.
9. Bhat M.K., Cellulase and related enzymes in biotechnology, Biotechnol. Adv., 2000,18, 355–383.
10. Amouri B. and Gargouri A., Characterization of a novel β-glucosidase from a *Stachybotrys* strain. Biochem.Eng., 2006, 32, 191-197.
11. Archer D.B. and Peberdy J.F., The molecular biology of secreted enzyme production by fungi, Crit. Rev. Biotechnol., 1997,17,273–306.
12. Schulein M., Cellulases of *Trichoderma reesei*. In: Wood WA, Abelson JN, editors. Methods in enzymology. 1988,160, 234-42.
13. Gomathi D., Muthulakshmi C., Guru Kumar D., Ravikumar G., Kalaiselvi M. and Uma C, Submerged fermentation of wheat bran by *Aspergillus flavus*for production and characterization of carboxy methyl cellulase, Asian Pacific Journal of Tropical Biomedicine, 2012, S67-S73
14. Hang Y.D. and Woodams E.E., Apple Pomace: Potential substrate for production of b-Glycosidase by *Aspergillus foetidus*, Lebensm-Wiss. U-Technol., 1994, 27,587-589.
15. Omojasola P., Folakemi Jilani., Priscilla O. and Ldiyemi S.A, Cellulase production by some fungi cultured on pineapple waste, Nat. Sci., 2008, 6, 64-79.
16. Sun H., Ge X., Hao Z. and Peng M, Cellulase production by *Trichoderma* sp. on apple pomace under solid state fermentation, Afr. J. Biotechnol., 2001, 9,163-166 .
17. Zhang Q. and Cai W., Enzymatic hydrolysis of alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3, Biomass. Bioenerg.,2008, 32,1130–1135.
18. Kang S.M., Ko E.H., Lee J.S. and Kim S.W., Over production of β- glucosidase by *Aspergillus niger*mutant from lignocellulsic biomass, Biotechnol. Lett., 1999, 21,647-650.
19. Kumar P. and Satyanarayana T., Optimization of culture variables for improving glucoamylase production by alginate-entrapped *Thermomucor indicaeseudaticae* using statistical methods, Bioresour. Technol., 2007,98,1252–1259.
20. Khambhatya Y., Modya K., Jha B. and Gohel V., Statistical optimization of medium components for j-carrageenase production by *Pseudomonas elongate*, Enzyme Microb. Technol., 2007, 40,813–822.
21. Mei X., Liu R., Shen F. and Wu H., Optimization of fermentation conditions for the production of ethanol from stalk juice of sweet sorghum by immobilized yeast using response surface methodology, Energ. Fuels., 2009, 23,487–91.
22. Han M., Kim Y., Kim Y., Chung B. and Choi G.W., Bioethanol production from opti-mized pretreatment of cassava stem, Korean. J. Chem .Eng., 2011, 28(1),119–25.
23. Khattak W.A., Ul-Islam M. and Park J.K.m Prospects of reusable endogenous hydrolyzing enzymes in bioethanol production by simultaneous saccharification and fermentation, Korean .J. Chem. Eng., 2012, 29(11),1467–82.
24. KCL., Massan ja puun kokanaisligniinipitoisuus (Total lignin content of wood and pulp-in Finish) KCL Espoo: Finland.,1982, 115b, 3.
25. Ververis C., Georghiou K., Danieldis D., Hatzinikolaou D.G., Santas P., Santas R. and Corleti V., Cellulose,hemisellulose,lignin and ash content of some organic materials and their solubility for use as paper pulp supplements, Bioresour. Technol. , 2007, 98,296-301.
26. Smith P.J., Rinzema A., Tramper J., Schlosser E.E. and Knolw W., Accurate determination of process variables in a solid state fermentation system, Process Biochenz.,1996, 31, 669-678.
27. Jin Zhou., Yong-Hong Wang., Ju Chu., Ling-Zhi Luo., Ying-Ping Zhuang. and Si-Liang Zhang., Optimization of cellulase mixture for efficient hydrolysis of steam-explodedcorn stover by statistically designed experiments, Bioresour. Technol., 2009, 100,819-825
28. Khuri A.I. and Cornell J.A., Response Surface: Design and Analysis. Marcell Decker Inc, New York. 1987.
29. Miller G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., 1959,31, 426–427.
30. Balasubramanian K., Ambikapathy V. and Panneerselvam A., Journal of Microbiology and Biotechnology Research., 2011, 1(4),158-163.

31. 31.Md. Zahangir Alam, Suleyman A. and Muyibi Rosmaziah Wahid., Statistical optimization of process conditions for cellulase production by liquid state bioconversion of domestic wastewater sludge, Bioresour. Technol., 2008, 99,4709–4716.
32. Takagi M., Abe S., Suzuki S., Emert GH. and Yata N., In Proceedings of Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Protein., 1977, 551-571.
33. Inderwildi O.R. and King D.A., "Quo Vadis Biofuels", Energy.Environ. Sci., 2009, (4), 343. doi:10.1039/b822951c.
34. Jones R., Pamment P.N. and Greenfied P.F., Alcoholic fermentation by yeasts.The effect of environment and other variables, Process. Biochem.,1981, 16,42-49.
35. Schell D. and Walter E., Simultaneous Saccharification and Fermenation of Corn Stover Hydrolysate to Ethanol, 1991, Paper 72. Biotech. Symp. for Fuels and Chemicals. Colorado Springs.
36. Arthe R., Rajesh R., Rajesh E.M., Rajendran R. and Jeyachandran S., Production of bioethanol from cellulosic cotton waste through microbial extracellular enzymatic hydrolysis and fermentation, Electronic Journal of Environmental, Agricultural and Food Chemistry, 2008,7 (6),2984-2992.

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