Renewable Energy 34 (2009) 421–424

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

# Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production

Rajeev K. Sukumaran, Reeta Rani Singhania, Gincy Marina Mathew, Ashok Pandey*

*Biotechnology Division, National Institute for Interdisciplinary Science and Technology (formerly Regional Research Laboratory), CSIR, Trivandrum-695 019, India*

ARTICLE INFO

*Article history:*
Available online 15 July 2008

*Keywords:*
Bio-ethanol
Cellulase
Beta glucosidase
*Trichoderma*
*Aspergillus*
Solid state fermentation

ABSTRACT

A major constraint in the enzymatic saccharification of biomass for ethanol production is the cost of cellulase enzymes. Production cost of cellulases may be brought down by multifaceted approaches which include the use of cheap lignocellulosic substrates for fermentation production of the enzyme, and the use of cost efficient fermentation strategies like solid state fermentation (SSF). In the present study, cellulolytic enzymes for biomass hydrolysis were produced using solid state fermentation on wheat bran as substrate. Crude cellulase and a relatively glucose tolerant BGL were produced using fungi *Trichoderma reesei* RUT C30 and *Aspergillus niger* MTCC 7956, respectively. Saccharification of three different feed stock, i.e. sugar cane bagasse, rice straw and water hyacinth biomass was studied using the enzymes. Saccharification was performed with 50 FPU of cellulase and 10 U of β-glucosidase per gram of pretreated biomass. Highest yield of reducing sugars (26.3 g/L) was obtained from rice straw followed by sugar cane bagasse (17.79 g/L). The enzymatic hydrolysate of rice straw was used as substrate for ethanol production by *Saccharomyces cerevisiae*. The yield of ethanol was 0.093 g per gram of pretreated rice straw.



## 1. Introduction

Fuel ethanol production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable production of renewable transportation fuels. Ethanol has a higher octane rating than gasoline and produces fewer emissions, therefore being widely recognized as a substitute and/or additive to gasoline [15]. Due to these apparent advantages and also being a renewable alternative to existing transport fuels, there is now an increased interest in commercializing technologies for ethanol production from inexpensive biomass [10]. Most of the fuel ethanol produced in the world is currently sourced from starchy biomass or sucrose (molasses or cane juice), but the technology for ethanol production from non-food plant sources is being developed rapidly so that large-scale production will be a reality in the coming years [4]. The production of ethanol from lignocellulosic biomass involves the different steps of pretreatment, hydrolysis (saccharification) and ethanol recovery [14]. Hydrolysis of biomass is essential for generation of fermentable sugars which are then converted to ethanol by microbial action. Two methods, i.e. acid hydrolysis and enzymatic hydrolysis are primarily employed for biomass hydrolysis with varying efficiencies depending on treatment conditions, type of biomass and the properties of the hydrolytic agents. The former is a mature technology but with the disadvantages of generation of hazardous acidic waste and the technical difficulties in recovering sugar from the acid. The enzymatic method, however, is more efficient and proceeds under ambient conditions without generation of any toxic waste. The latter method which is under rapid development has immense potentials for improvement in cost and efficiency [7]. Commercialization of ethanol production from lignocellulosic biomass is hindered mainly by the prohibitive cost of the currently available cellulase preparations – the enzymes used for saccharification. Reduction in the cost of cellulases can be achieved only by concerted efforts which address several aspects of enzyme production from the raw material used for production to microbial strain improvement. Use of cheaper raw materials and cost effective fermentation strategies like solid state fermentation can improve the economics of cellulase production.

Reduction in cost of "bio-ethanol" may also be achieved by efficient technologies for saccharification which includes the use of better "enzyme cocktails" and conditions for hydrolysis. Cellulases are a group of enzymes which include endoglucanases which hydrolyze the cellulose polymer exposing reducing and non-reducing ends of the linear polymer of glucose units: "exoglucanases and cellobiohydrolases" which act on these ends to release "cellobiose" and "cellooligosaccharides"; and β-glucosidases (BGL) which cleaves the cellobiose units to liberate glucose – the end product [11]. The organisms currently employed for commercial cellulase production produce very less quantities of BGL compared to the

* Corresponding author.Tel.: +91 471 2515279; fax: + 91 471 2491712
*E-mail address:* ashokpandey56@yahoo.co.in (A. Pandey).

0960-1481/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.renene.2008.05.008

other cellulase proteins and they are inhibited by their own product glucose. Due to this control on saccharification imposed by product inhibition of BGL, the efficiency of enzymatic hydrolysis cannot be improved much by increasing enzyme loading and much of the enzyme added for saccharification remains unutilized. BGL active at higher concentrations of glucose can therefore improve the efficiency of hydrolysis several fold.

The present study evaluated the application of *Trichoderma reesei* cellulase and *Aspergillus niger* BGL for biomass hydrolysis and ethanol fermentation. Crude cellulase and BGL were produced by solid state fermentation on wheat bran by *T. reesei* RUT C30 and *A. niger* MTCC 7956, respectively, and were used for hydrolysis of biomass. Comparative evaluation of biomass saccharification was performed with different feed stock. Potential for ethanol production using saccharified biomass was also evaluated by fermenting rice straw hydrolysate with *Saccharomyces cerevisiae*.

## 2. Materials and methods

### 2.1. Biomass feed stock and pretreatment

Rice straw (RS) and sugar cane bagasse (SCB) were procured locally and dried water hyacinth leaves (WH) were a gift. The biomass feed stock was brought to the lab and further dried overnight at 70 °C in a hot air oven to remove residual moisture. Each feed stock was milled to reduce the size prior to pretreatment. One batch of each sample was pretreated with dilute acid and the other with dilute alkali. Briefly, the samples were reacted with 0.1 N HCl or 0.1 N NaOH for 1 h at 120 °C in an autoclave. After cooling, the samples were washed several times in tap water to neutralize the pH followed by a final rinse in distilled water, after which they were air dried by spreading on paper. The pretreated feed stock was either used immediately for hydrolysis experiments or stored in airtight containers at 4 °C until used.

### 2.2. Microorganisms and inoculum preparation

The fungus *T. reesei* RUT C30 was a kind gift from Prof. George Scakacs, Technical University of Budapest. *A. niger* MTCC 7956 was isolated from decaying wood in our lab and was identified at the Microbial Type Culture Collection (MTCC), Chandigarh, India. *S. cerevisiae* NCIM 3288 was purchased from the National Collection of Industrial Microorganisms, India. Both the fungi were maintained on potato agar slants and sub-cultured fortnightly. The yeast culture was maintained on YEPD agar slants and sub-cultured weekly. For preparation of fungal inocula, about 2 ml of sterile distilled water containing 0.1% Tween 80 was introduced into the sporulated slants of each fungus and the spores were dislodged into the liquid by gentle pipetting. The spore suspensions of *T. reesei* and *A. niger* containing $\sim 10^7$ spores/ml was used as inoculum for production of cellulase and BGL, respectively. In the case of yeast, the culture was grown in YEP broth for 12 h with 180 rpm agitation on rotary shaker and the culture was used at 10% v/v as inoculum for alcohol fermentation.

### 2.3. Enzyme production

The mineral salt medium used for both cellulase and BGL productions had a composition in g/L: urea, 0.3; $KH_2PO_4$, 2; $(NH_4)_2SO_4$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; peptone, 0.75; yeast extract, 0.25; $CaCl_2 \cdot 2H_2O$, 0.4; and trace elements: $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot 7H_2O$, 0.0016, $ZnSO4 \cdot 7H_2O$, 0.0014; and $CoCl_2$, 0.002. Wheat bran was used as substrate for production of the enzyme under solid state fermentation. Erlenmeyer flasks containing 5 g of substrate was moistened with the mineral salt medium so as to achieve either 40% or 57% of initial moisture for cellulase and BGL productions, respectively. The flasks were inoculated with 1 ml of *T. reesei* spore suspension for cellulase production and with *A. niger* spore suspension for BGL production. The contents were mixed thoroughly and were incubated at 30 °C and 95% relative humidity. Incubation period was 96 h for cellulase production and 72 h for BGL production. The enzymes were recovered by extraction with 0.1 N citrate buffer (pH 4.8) after the incubation period. The extract was centrifuged to remove debris at 10,000 rpm for 10 min at 4 °C and was used as the crude enzyme sample. Concentration of the enzymes was done by precipitation with 4 volumes of ice cold acetone. The precipitates obtained were recovered by centrifugation at 10,000 rpm for 10 min at 4 °C and were resuspended in 50 mM citrate buffer (pH 4.8). These preparations were used for hydrolysis of biomass.

### 2.4. Biomass saccharification and alcohol fermentation

Enzymatic saccharification of biomass was done by incubating 1 g of pretreated biomass (sugar cane bagasse, rice straw or water hyacinth) with both the crude cellulase and BGL preparations at 45 °C, in stoppered 250 ml flasks in a total volume of 50 ml made up with 50 mM citrate buffer (pH 4.8). The flasks were agitated at 100 rpm in a water bath shaker. Two different combinations of enzyme loading and incubation time were tried for hydrolysis of pretreated biomass (Table 1).

Ethanol production was studied using the enzymatic hydrolysate of rice straw. Alkali pretreated rice straw was hydrolyzed under the conditions in method B which gave higher yield on sugars with bagasse. The hydrolysate was concentrated by evaporation to reducing sugar content of either 6% or 12%. The hydrolysate was sterilized by filtration and was inoculated with 10% v/v of a 12 h old seed culture of *S. cerevisiae*. Incubation was carried out in stoppered flasks at room temperature (28 ± 2 °C) without agitation. Samples (1 ml) were withdrawn at regular intervals and centrifuged for 10 min at 4 °C at 13,000 rpm. The supernatant was filtered using 0.45 m filters and the ethanol content was analyzed by gas chromatography.

### 2.5. Analytical methods

Filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation according to Ghose [2] and expressed as filter paper units (FPU). Endoglucanase activity was determined with carboxy methyl cellulose as substrate as outlined in Ref. [2] and one unit of CMCAse was defined as the amount of enzyme required for liberating 1 mM of reducing sugar per ml per minute and was expressed as U/gds (units per gram dry substrate) or U/ml. Total cellulase activity was also expressed similarly when the activity was less than that could be expressed as FPU. β-glucosidase activity was determined using *p*-nitrophenyl β-D-glucopyranoside as substrate according to the IUPAC method [1]. Estimation of total reducing sugar in the enzymatic hydrolysate of biomass was done by DNS method [6] and the estimation of ethanol was done by gas chromatography as outlined in NREL Laboratory Analytical protocol # 011 [13].

**Table 1**
Testing of saccharification conditions for hydrolysis of sugar cane bagasse

| Method | Cellulase loading (FPU/g) | BGL loading (U/g) | Incubation time (h) | Reducing sugar yield g/L |
|---|---|---|---|---|
| A | 80 | 10 | 24 | 3.79 |
| B | 50 | 5 | 48 | 17.64 |

**Table 2**
Comparison of the crude enzyme preparations from *T. reesei* RUT C30 and *A. niger* MTCC 7956

| Fungus | Type of enzyme preparation | Total cellulase activity (Filter paper assay) | | Endoglucanase activity (CMCase assay) | | BGL activity (pNPG assay) | |
|---|---|---|---|---|---|---|---|
| | | FPU/gDS | FPU/ml | U/gDS | U/ml | U/gDS | U/ml |
| *Trichoderma reesei* RUT C30 | Crude cellulase (SSF extract) | 22.8 | 1.14 | 299.55 | 14.98 | 4.5 | 0.22 |
| | Concentrated Cellulase (10×)[a] | NA | 13.65 | NA | 45.22 | NA | 1.72 |
| *Aspergillus niger* MTCC 7956 | Crude BGL (SSF extract) | 4.55[b] (U/gDS) | 0.22[b](U/ml) | 135.44 | 6.77 | 21.39 | 2.84 |
| | Concentrated BGL (30×)[a] | NA | 0.24 | NA | 196.15 | NA | 83.62 |

NA : not applicable.
[a] The crude extract from solid state fermentation was precipitated with 4 volumes of ice cold acetone and the precipitate was resuspended in appropriate volume of 50 mM citrate buffer (pH 4.8).
[b] Too less to be expressed as FPU. One unit in this case is the amount of enzyme liberating 1 μM of glucose /ml/min.

## 3. Results

### 3.1. Production of enzymes for biomass hydrolysis

Crude cellulase and BGL preparations used in this study were produced by solid state fermentation on wheat bran in an effort to reduce the cost of ethanol production. Solid sate fermentation needs lesser infrastructure and relatively less skilled manpower besides being able to use cheaper raw materials for enzyme production [8,9]. All these add to the economic advantage of this mode of fermentation for enzyme/metabolite production. SSF also produces a more concentrated product, which in this case is very much advantageous. Both the enzymes were produced on the same substrate and same mineral salt medium, but using different organisms and fermentation conditions. Crude cellulase preparation was produced employing *T. reesei* RUT C30 on wheat bran as substrate as described previously [12]. BGL was produced using *A. niger* MTCC 7956 using conditions optimized in our laboratory (unpublished results). Crude cellulase preparation had appreciable levels of filter paper and CMCase activity, but lesser BGL activity (Table 2). On the contrary, the crude BGL preparation had a lesser filter paper and CMCase activity while the BGL activity was several fold higher. In another study, it was found that the *A. niger* BGL retained approximately 20% of activity in the presence of 0.5 M glucose, while the *T. reesei* enzyme was not active at that concentration of glucose (unpublished results).

### 3.2. Biomass saccharification

Two different combinations of cellulase and BGL loading and incubation time was tried for biomass saccharification using alkali pretreated sugar cane bagasse as a model substrate. From the results presented in Table 1, it can be seen that method B which used the cellulase and BGL loading of 50 FPU and 10 U, respectively per gram of biomass; and an incubation time of 48 h performed several fold better than a higher enzyme loading and lesser incubation time. Cellulases need to penetrate the polymer to access and hydrolyze it, unlike many common enzymes which take in their substrates to the active site pockets. Cellulases have specific domains for binding their substrate so that the enzyme sits on the polymer and effects a slow degradation (reviewed in Ref. [5]). Above a given threshold concentration of cellulase defined by the biomass type and degree of polymerization, the concentration of enzyme might be having lesser effects on cellulose hydrolysis compared to the effect of reaction time. This is probably the reason for more efficient hydrolysis at an increased incubation time, even when used along with lesser enzyme loading. Since method B gave better reducing sugar yield, this was used for studying the saccharification of other feed stock.

Sugar cane bagasse, rice straw and water hyacinth biomass were tried as feed stock for production of fermentable sugars. The feed stocks were pretreated either with 0.1 N HCl or with 0.1 N NaOH as out lined in Section 2, and were used as raw materials for the saccharification experiments. Table 3 shows the yield of reducing sugars from the feed stock resources after 48 h of incubation.

The method of pretreatment had a pronounced effect on the yield of reducing sugars in the case of both water hyacinth biomass (WH) and rice straw (RS). However, with sugar cane bagasse, the effects of the methods of pretreatment were not much different in improving digestibility. Alkali treatment of WH and RS could result in a better yield of reducing sugars compared to acid treatment. Highest yield of reducing sugar ($26.3 \pm 0.97$ g/L) was obtained from alkali treated rice straw followed by alkali treated bagasse ($17.79 \pm 0.21$). While both acid and alkali treatment can remove the lignin in the biomass to retrieve comparatively purer cellulose, the acid treatment results in hydrolysis of hemicellulose sugars and their removal. Alkali treatment on the other hand tends to preserve the hemicellulose polymer [3]. Since the enzymes used for hydrolysis are both crude preparations, the possibility of hemicellulases like xylanases and arabino-furanosidases being found in them cannot be ruled out and this would have resulted in the observed higher yield of reducing sugars; due to the hydrolysis of hemicelluloses along with cellulose. Among the feed stock tried, the highest yield of reducing sugars was obtained from rice straw followed by sugar cane bagasse and the lowest yield was obtained from water hyacinth biomass. It can be observed that both the rice straw and sugar cane bagasse contained higher cellulose content than water hyacinth and the superior yield is due to the increased concentration of cellulose which can be acted upon by the enzymes. The total amount of sugars released from 1 dry gram of each feed stock is given in Table 3. It may be noted that yield/g biomass have rather higher values and with rice straw it is even higher than 1 g. These errors might have been resulted due the differences in the method of estimation of the weights. While the biomass weights were estimated using an analytical balance, the value for reducing sugars was calculated from a spectrophotometric determination of sugars and these values may not be directly comparable.

**Table 3**
Yield of reducing sugar from enzymatic hydrolysate of different biomass feed stock

| Pretreatment | Feed stock | Reducing sugar concentration (g/L) | Total sugar released per gram biomass (g/g) |
|---|---|---|---|
| Alkali | Water hyacinth biomass | $14.20 + 2.22$ | $0.71 + 0.11$ |
| | Rice straw | $26.30 + 0.97$ | $1.31 + 0.05$ |
| | Sugar cane bagasse | $17.79 + 0.21$ | $0.89 + 0.01$ |
| Acid | Water hyacinth biomass | $5.38 + 0.40$ | $0.27 + 0.02$ |
| | Rice straw | $10.98 + 0.50$ | $0.55 + 0.02$ |
| | Sugar cane bagasse | $15.82 + 0.14$ | $0.88 + 0.01$ |

**Table 4**
Ethanol production from rice straw hydrolysate by *Saccharomyces cerevisiae*

| Reducing sugar (RS) concentration (g/L) | Duration of incubation (h) | Ethanol concentration (g/L) | Ethanol yield (g/g DS) | Efficiency (RS to ethanol) % |
|---|---|---|---|---|
| 60 | 24 | 12.34 | 0.093 | 40.33 |
| 120 | 24 | 25.56 | 0.096 | 41.76 |

### 3.3. Ethanol production from rice straw hydrolysate

Enzymatic hydrolysate of rice straw with a reducing sugar concentration of 60 g/L or 120 g/L was used for the fermentation experiments. The results of the fermentation of alkali pretreated and enzymatically saccharified rice straw are given in Table 4. The maximum ethanol concentration (25.56 g/L) was obtained after 24 h using hydrolysate with 12% reducing sugar. With the hydrolysate having initial reducing sugar concentration of 6%, the maximum ethanol concentration obtained was 12.34 g/L in 24 h. The time course of ethanol production from rice straw hydrolysate containing 6% reducing sugar is shown in Fig. 1. The rate of ethanol production was 0.23 g/L/h for the initial 14 h and then increased to 1.01 g/L/h for 14–22 h. The yield of ethanol peaked at 24 h. There was no increase in the production after 24 h and the ethanol concentration remained at about 12 g/L. The ethanol yield obtained was 0.093 g and 0.096 g per gram of dry substrate (gDS) for initial sugar concentrations of 6% and 12%, respectively.

## 4. Discussion

Major bottleneck in biomass to ethanol conversion is the cost of cellulase enzymes and any strategy which can bring down the production cost of cellulases can significantly reduce the cost of bio-ethanol. Solid state fermentation is a well established technology for enzyme production and provides several advantages like lower cost of operation, lesser infrastructure requirements, ability to operate with less skilled manpower and above all the ability to use cheap agro-industrial residues and biomass as raw materials [8,9]. Here we have demonstrated that the crude cellulase and BGL preparations produced through SSF by fungi can be used for hydrolysis of biomass with considerable efficiency. The yield of reducing sugars from pretreated biomass is more than 85% in the best cases (Table 4) and the hydrolysate did not contain any inhibitory compounds as evidenced by the growth and ethanol production on this by *S. cerevisiae*. The method of pretreatment can influence the efficiency of hydrolysis and in this study it was found that alkali pretreatment resulted in a higher yield of reducing sugars in the enzymatic hydrolysis of rice straw, sugar cane bagasse and water hyacinth biomass. A trial conducted to evaluate the potential of ethanol production from the hydrolysate of rice straw produced using enzymes from SSF showed that ~100 mg (0.093 or 0.096 g) of ethanol may be obtained per gram of pretreated biomass. The efficiency of conversion of reducing sugars to ethanol was about 40%. The values obtained are for an un-optimized process and there are ample scopes to improve the efficiency of the process to obtain higher ethanol yields. This study forms the basic trials conducted to test the feasibility of using enzymes produced in our own laboratory for biomass hydrolysis and subsequent ethanol fermentation. We are now concentrating on the efforts to optimize the conditions of saccharification and fermentation and pretreatment. Attempts will be made to increase the sugar content of the hydrolysate so as to obtain a better yield of ethanol. Also more elaborate studies will be performed with compositional analyses of biomass. The study demonstrates the feasibility of using crude *T. reesei* RUT C30 cellulase and *A. niger* MTCC 7956 β-glucosidase in combination with hydrolysis of at least three biomass residues and the potential of using the hydrolysate generated from rice straw for ethanol production.

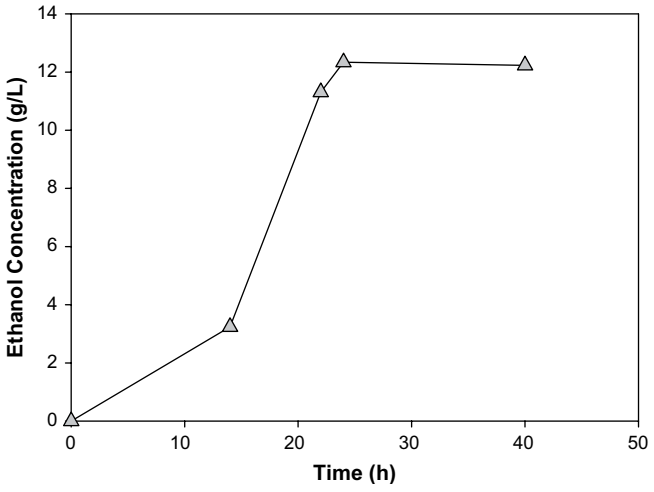


**Fig. 1.** Time course of ethanol production by *Saccharomyces cerevisiae* MTCC 7956 from alkali pretreated rice straw hydrolysate (6% initial reducing sugar concentration).

## Acknowledgement

We thank Mr Ajith Kumar M, Kerala Bureau of Industrial Promotions for providing us water hyacinth biomass. Authors acknowledge the financial support from CSIR under task force project CMM013, and in the form of research internships to RRS and GMM.

## References

[1] Ghose TK, Bisaria VS. Measurement of hemicellulase activities, part I – xylanases. Pure Appl Chem 1987;59(12):1739–52.
[2] Ghose TK. Measurement of cellulase activities. Pure Appl Chem 1987;59(2):257–68.
[3] Grey KA, Zhao L, Emptage M. Bioethanol. Curr Opin Chem Biol 2006;10:141–6.
[4] Lin Y, Tanaka S. Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 2006;69:627–42.
[5] Lynd LR, Weimer PJ, van Zyl WH, Pretorious IS. Microbial cellulase utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506–77.
[6] Miller GM. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.
[7] Mishima D, Tateda M, Ike M, Fujita M. Comparative study on chemical pretreatments to accelerate enzymatic hydrolysis of aquatic macrophyte biomass used in water purification processes. Bioresour Technol 2006;97(16):2166–72.
[8] Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation. I processes and products. Process Biochem 2000;35:1153–69.
[9] Raimbault M. General and microbiological aspects of solid substrate fermentation. Electronic J Biotechnol ISSN: 0717-3458. Available from: http://www.ejbiotechnology.info/content/vol1/issue3/full/9/index.html 15 December 1998 [accessed 15.03.07.].
[10] Schell DJ, Riley CJ, Dowe N, Farmer J, Ibsen KN, Ruth MF, et al. A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock. Bioresour Technol 2004;91:179–88.
[11] Schulein M. Cellulases of *Trichoderma reesei*. In: Wood WA, Abelson JN, editors. Methods in enzymology, vol. 160. New York: Academic Press; 1988. p. 234–42.
[12] Singhania RR, Sukumaran RK, Pandey A. Improved cellulase production by *Trichoderma reesei* RUT C- 30 under SSF through process optimization. Appl Biochem Biotechnol 2007;142(1):60–70.
[13] Templeton DW. Determination of ethanol concentration in biomass to ethanol fermentation supernatants by gas chromatography. National Renewable Energy Laboratory; 1994. Laboratory Analytical Procedure #011, Revision 05/05/1994.
[14] van Zessen E, Weismann M, Bakker RR, Elbersen HW, Reith JH, den Uil H. Lignocellulosic ethanol, a second opinion. Report 2GAVE-03.11. Netherlands Agency for Energy & Environment. Available from:, http://www.novem.nl/default.asp?menuId = 10&documentId = 34649; 2003 [accessed 04.06.05.].
[15] Wyman C. Biomass ethanol: technical progress, opportunities, and commercial challenges. In: Socolow R, Anderson D, Harte J, editors. Ann Rev Energy Environ, 24; 1999. p. 189–226. Annual Reviews, Palo Alto, CA.