African Journal of Microbiology Research Vol. 4 (11) pp. 1105-1109, 4 June, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 © 2010 Academic Journals

*Full Length Research Paper*

# Comparison of cell growth and ethanol productivity on different pretreatment of rice straw hemicellulose hydrolysate by using *Candida shehatae* CICC 1766

**Wan-Li Sun[1,2] and Wei-Yi Tao[1*]**

[1]Key Lab of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China.
[2]College of Chemistry and Bioengineering, Yichun University, Yichun, China.

Accepted 10 May, 2010

**In this work, the cell growth and ethanol productivity was tested for lignocellulosic biomass sugars of overliming-detoxified and NaOH-neutralized rice straw acid hydrolysate by using *Candida shehatae* CICC 1766. When the acetic acid reached 3 g/L in defined xylose medium, the final ethanol concentration was 33% of that in the medium without acetic acid addition. *C. shehatae* CICC 1766 could bear lower pH (ethanol yield 13.5 g/L at pH 4.0) in overliming-detoxified hydrolysate. At the optimal pH 5.0 the ethanol yield attended to 16.1 g/L. Ethanol yield in NaOH-neutralized hydrolysate was 13.7 g/L which is better than in overliming-detoxified hydrolysate (7.0 g/L). It suggested that NaOH-neutralized hydrolysate could be directly fermented by using *C shehatae* CICC 1766.**

**Key words:** Rice straw, *Candida shehatae*, hemicellulose hydrolysate, ethanol fermentation.

## INTRODUCTION

Lignocellulose has been considered to be an attractive raw material for fuel ethanol production (Nigam, 2002). In order to release free sugar monomers from the lingocellulose matrix and produce fuel ethanol, the hydrolysis of lignocellulose requires one or more pretreatment steps. Dilute sulfuric acid hydrolysis is thought to be one of the promising pretreatment methods and was extensively employed in industry (Nichols et al., 2005; Mosier et al., 2005). However, during the pretreatment of lignocellulosic biomass, a broad range of inhibitory compounds are formed (Palmqvist et al., 2000).

Acid hydrolysate comprises a complex mixture of components, in which more than 35 potential microbial inhibitors have been identified (Luo et al., 2002). The inhibiting compounds are divided in three main groups based on origin: weak acids, furan derivatives, and phenolic compounds. Dilute acid pretreatment in particular also may cause formation of furfural and 5-hydroxymethylfurfural (HMF) from the dehydration of released sugars. These compounds have been characterized as inhibitors of microbial growth and negatively affect fermentation of the sugars (Almeida et al., 2007). Therefore, for successful hydrolysate fermentation process of the lignocellulosic hydrolysate should be detoxified or an inhibitor-tolerant microorganism should be used (Almeida et al., 2007; Hahn-Hägerdal et al 2007).

Various physical and chemical methods can be used to detoxify the lignocellulosic hydrolysates. Physical detoxification methods are based on the principle that inhibitors can be removed by phase equilibrium separations, such as liquid–liquid extraction or evaporation (Palmqvist and Hähn-Hägerdal, 2000). Chemical detoxifications such as overliming, are based on chemical modifications of the inhibitors to a less toxic or non-toxic products (Palmqvist and Hähn-Hägerdal, 2000).

Overliming with a combination of high pH and temperature (Martinez et al., 2001) has for a long time been considered as a promising detoxification method for dilute sulfuric acid-pretreated hydrolysate of lingocellulosic biomass. This process has been demonstrated to help with the removal of volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) from the hydrolysate (Martinez et al., 2000; Ranatunga et al.,

*Corresponding author. E-mail: wytao1946@163.com. Tel: +86-510-85918205.

2000). Dilute acid pretreatment technology was able to produce acceptable xylose yields of 75 - 90% by conversion of hemicellulose (Mosier et al., 2005; Eggeman and Elander, 2005; Sun and Cheng, 2002), which account for nearly one-third carbohydrate of the lingocellulose. Xylose utilization is one of the prerequisites to make lignocellulosic ethanol processes to be more economically competitive (Galbe and Zacchi, 2002).

To enhance the conversion efficiency in the ethanol production process, the organism used should be able to ferment all monosaccharides in the medium. A number of naturally occurring xylose-fermenting yeast species, including *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus,* have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production (Agbogbo et al., 2006). To date, there have been mostly reported for *P. stipitis* (Nigam, 2002; Agbogbo and Coward-Kelly, 2008; Huang et al., 2009). However, very few studies were done by *C. shehatae* and *P. tannophilus.* This work studied of ethanol production from the defined media and fermentation of overliming-detoxified and NaOH-neutralized hydrolysates using *C. shehatae* CICC 1766.

## MATERIALS AND METHODS

### Microorganism and growth media

*C. shehatae* CICC 1766 was obtained from the China Center of Industrial Culture Collection. The stock culture was grown at 28˚C for 3 days on agar plate containing glucose 10 g/L, yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, agar 20 g/L. A colony from the plate was then transferred by loop to a 250 mL Erlenmeyer flask containing 50 ml of YPX growth medium and incubated at 30˚C in a rotary shaker at 150 rpm for 36 h.

### Preparation of rice straw hydrolysate

Rice straw was collected from fields near Wuxi, Jiangsu province, China. Prior to dilute acid hydrolysis, the raw straw material was sliced to a suitable size (between 0.4 and 0.9 mm) and dried at 65˚C overnight to ensure a low content of moisture. Dried rice straw was pretreated with 1.5% (w/v) sulfuric acid at 10% (w/v) solids loading in an autoclave at 121˚C with residence times of 60 min. The liquid fraction was separated by filtration and stored in ice refrigerator at 4˚C.

### Preparation of the overliming-detoxified and NaOH-neutralized hydrolysates

The overliming-detoxified hydrolysate: The concentrated acid hydrolysate (100 mL) was heated to 50˚C, and held at that temperature for 15 min to remove or reduce the concentration of volatile components. This was followed by the addition 34 g/L of calcium hydroxide (lime), which raised the pH of the hydrolysate to 10.0. Agitation was then carried out for 30 min. The calcium sulfate ($CaSO_4$) sludge and the liquid were separated by filtration and finally the pH of the filtered overliming-detoxified hydrolysate was adjusted with sulfuric acid ($H_2SO_4$) to 5.0. NaOH-neutralized hydrolysate: The concentrated acid hydrolysate was directly neutralized with 10 M NaOH to pH 5.0.

### The defined medium and rice straw hydrolysate medium

The defined medium consisted of xylose 50 g/L or xylose 40 g/L, glucose 10 g/L, yeast extract 3 g/L, urea 0.25 g/L, $CaCl_2$ 0.25 g/L, $MgSO_4$ 0.25 g/L, $KH_2PO_4$ 2.5 g/L and pH 5.0. The overliming-detoxified hydrolysate medium (A): The overliming-detoxified hydrolysate contained xylose 50.4 g/L, glucose 3 g/L, acetic acid 2.51 g/L, furfural 0.01 g/L. The overliming-detoxified hydrolysate medium (B): The overliming-detoxified hydrolysate contained xylose 50.4 g/L, glucose 3 g/L, acetic acid 2.51 g/L, furfural 0.01 g/L, yeast extract 3 g/L, urea 0.25 g/L, $CaCl_2$ 0.25 g/L, $MgSO_4$ 0.25 g/L, and $KH_2PO_4$ 2.5 g/L.

The NaOH-neutralized hydrolysate medium (A): The NaOH-neutralized hydrolysate contained xylose 53.3 g/L, glucose 2.5 g/L, acetic acid 1.58 g/L, furfural 0.016 g/L. The NaOH-neutralized hydrolysate medium (B): The NaOH-neutralized hydrolysate contained xylose 53.3 g/L, glucose 2.5 g/L, acetic acid 1.58 g/L, furfural 0.016 g/L, yeast extract 3 g/L, urea 0.25 g/L, $CaCl_2$ 0.25 g/L, $MgSO_4$ 0.25 g/L, and $KH_2PO_4$ 2.5 g/L.

### Shaking flask experiments

The defined medium and all treated hydrolysates were fermented by *C. shehatae* at 30˚C, in 150-ml shake-flasks containing 50 ml medium at 150 rpm. Fermentation medium was inoculated with 20% (v/v) seed cultures to give an initial cell concentration 1.6 g/L. Fermentation time was 96 h.

### Analytical methods

The amount of monosaccharides in the reaction mixture was quantified by HPLC using Agilent technology 1100 series, equipped with RID-HP1047A and kromasil $NH_2$ (4.6 mm × 250 mm, 5 µm), with an elution system of V (acetonitrile): V (water) = 80:20 at a flow rate of 1 ml/min, and injection volume was 10 µl. Samples were filtered through 0.25 µm filter and injected into the chromatograph. The ethanol concentration was determinated by GC using Shimadzu GC-2010 with FID/HS and PEG-20M column (30 m × 0.32 mm), and with nitrogen as carrier gas at flow rate of 1.5 mL/min. The temperature for injector and detector was 200 and 250˚C, respectively, and for the column oven was maintained at 40˚C for 5 min, ramped to 180˚C at a rate of 10˚C /min, and subsequently maintained at 180˚C for 5 min.

## RESULTS AND DISCUSSION

### Effect of acetic acid on ethanol production

When the acid hydrolysate was overlimed with solid $Ca(OH)_2$, volatile compounds, such as furfural and 5-hydroxymethylfurfural, were removed only a portion, acetic acid was slightly affected by overliming, meanwhile, detoxification also resulted in the loss of sugar. This observation was reported during the neutralization of water-hyacinth hemicellulose acid hydrolysate (Nigam, 2002). In order to investigate the effect of acetic acid on the formation of ethanol by *C. shehata* CICC 1766, cells were grown in defined medium containing acetic acid at different levels. The obtained results signified that the

**Table 1.** Ethanol production in batch cultures by *C. shehatae* CICC 1766 from defined xylose containing acetic acid at different levels.

| Acetic acid | xylose | | Ethanol production | | |
|---|---|---|---|---|---|
| (g $L^{-1}$) | Initial (g $L^{-1}$) | Residual (g $L^{-1}$) | Concentration (g $L^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | Yield (g $g^{-1}$ xylose) |
| 0 | 50 | 0 | 16.4 | 0.342 | 0.33 |
| 0.25 | 49.5 | 2.0 | 13.5 | 0.281 | 0.28 |
| 0.5 | 50.5 | 4.8 | 10.6 | 0.221 | 0.23 |
| 1.0 | 49.8 | 5.0 | 11.9 | 0.248 | 0.26 |
| 2.0 | 49.3 | 14.5 | 9.0 | 0.188 | 0.26 |
| 3.0 | 49.7 | 15.1 | 5.5 | 0.115 | 0.16 |

**Table 2.** Ethanol production by *C. shehatae* CICC 1766 from rice straw overliming-detoxified hydrolysate.

| pH | xylose | | Ethanol production | | |
|---|---|---|---|---|---|
| | Initial (g $L^{-1}$) | Residual (g $L^{-1}$) | Concentration (g $L^{-1}$) | Productivity (g $L^{-1}$ $h^{-1}$) | Yield (g $g^{-1}$ xylose) |
| 4 | 48.8 | 4.6 | 13.5 | 0.161 | 0.30 |
| 4.5 | 49.7 | 3.8 | 15.4 | 0.183 | 0.33 |
| 5.0 | 50.4 | 0 | 16.1 | 0.192 | 0.32 |
| 5.5 | 49.1 | 1.7 | 14.7 | 0.175 | 0.31 |
| 6.0 | 50.9 | 3.7 | 13.2 | 0.157 | 0.28 |

xylose consumption strongly depended on the amount of acetic acid. When the acetic acid reached 3 g/L, the residual xylose was 15.1 g/L, and the final ethanol concentration was decreased to 33% of that in the medium without acetic acid addition (Table 1). The presence of acetic acid in the medium inhibited the sugar utilization and fermentation activity of the yeast. This result is in agreement with that reported by (Diaz et al., 2009). Thus, *C. shehata* CICC 1766 was sensitive to acetic acid. The addition of acetic acid to the defined xylose medium resulted in the intracellular pH decrease of the cell. To maintain intracellular pH, protons must be transported out of the cell by an ATP-consuming transport system (Neidhardt et al., 1987). Consequently, the maintenance energy requirement will be higher in the defined xylose medium containing acetic acid, which, together with the lower energy yield from xylose, negatively influences the fermentation rate. A possible explanation might be that ethanol production from xylose yields less adenosine triphosphate (ATP) on a molar basis (Lawford and Rousseau, 1991).

To compare with acid hemicelluose hydrolysate containing xylose and glucose, fermentation was carried out in a defined xylose and xylose/glucose medium. The results showed that, the glucose was consumed prior to xylose for cell growth during early stages of the defined glucose/xylose mixture fermentation and the cell concentrations were higher in 0 - 30 h. However, the cell concentrations were high in the xylose medium 30 h later, and the final ethanol concentration, yield and productivity was high in the xylose medium. This phenomenon can be explained by higher biomass in defined xylose medium.

## Ethanol production from overliming-detoxified hydrolysate under different pH

One type of inhibitory effect commonly detected in hydrolysate fermentations is caused by weak acids such as acetic acid, and this adverse effect is due to the undissociated molecular form, which is pH dependent (Palmqvist and Hähn-Hägerdal, 2000; Almeida et al., 2007). The inhibitory effect increases with a decrease in pH as the number of undissociated molecules is higher at low pH. Minimization of acetic acid inhibition in hydrolysate has been successfully performed by controlling the pH of the fermentation at pH 6.0 or above pH 6.0 (Palmqvist and Hähn-Hägerdal, 2000; Agbogbo and Wenger, 2007; Nigam, 2001a). With a rise in the pH of the hydrolysate, however, the risk of microbial contamination rises during the fermentation. Ethanol fermentation from rice straw overliming-detoxified hydrolysate (A) was conducted under various pHs (Table 2). When pH increased from 4.0 - 5.0, ethanol productivity and yield also rose, which indicated that acetate inhibition could be alleviated to some extent by pH adjustment. The optimum pH for *C. shehatae* CICC 1766 to ferment hydrolysate was 5.0. It was different from the reported optimum pH of 5.5 for *C. shehatae* to ferment hydrolysate, such as *C. shehatae* NCIM 3501 by(Chandel et al., 2007), *C. shehatae* NCL 3501 by (Abbi et al., 1996a) and *C. shehatae* ATCC 22984 by (Yu et al.,

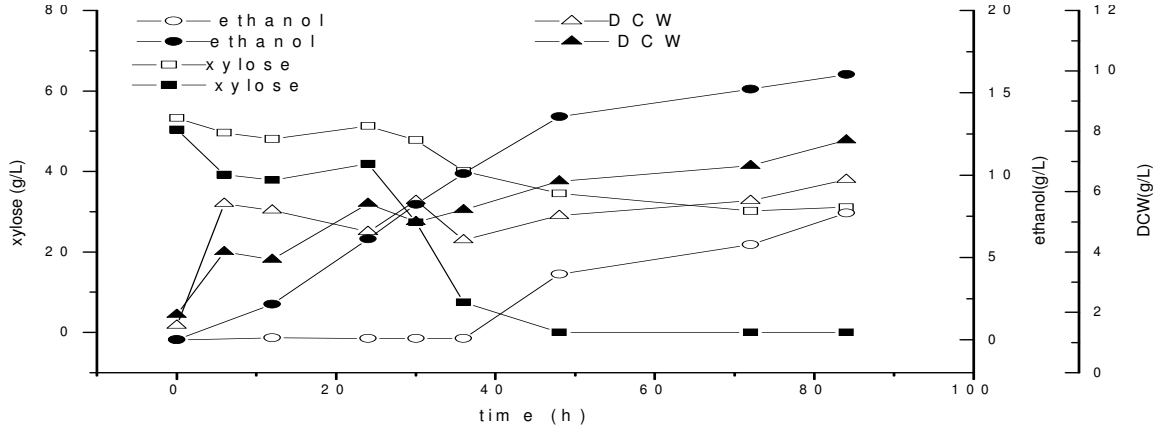


**Figure 1.** Time course of ethanol fermentation by *C. shehatae* CICC 1766 with overliming-detoxified hydrolysate (A) (filled symbols) and NaOH-neutralized hydrolysate (A) (open symbols).

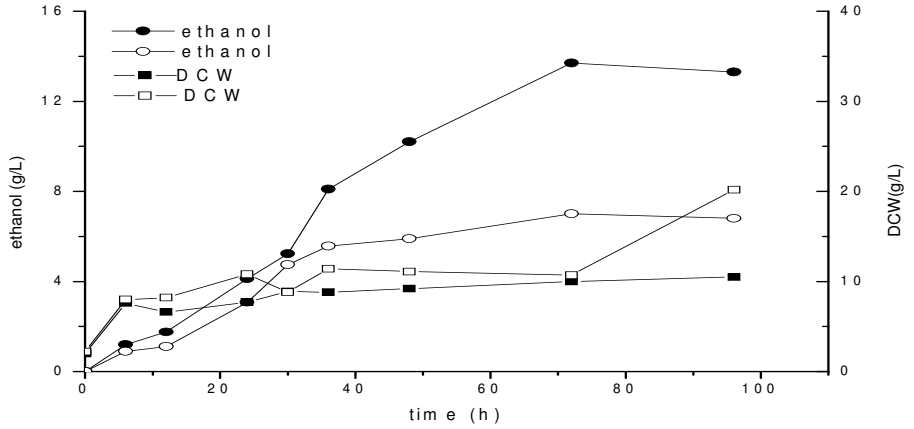


**Figure 2.** Time course of ethanol fermentation by *C. shehatae* 1766 with overliming-detoxified hydrolysate (B)
(open symbols) and NaOH-neutralized hydrolysate (B) (filled symbols).

1987). The strain still showed good fermentability even at pH 4.0.

## Fermentation of rice straw hemicellulose hydrolysate at pH 5.0

Figure 1 shows the cell growth rate was higher in overliming-detoxified hydrolysate (A) than that in defined xylose or xylose/glucose medium and NaOH-neutralized hydrolysate (A). An ethanol concentration (16 g/L) in defined xylose or xylose/glucose medium was similar to that in overliming-detoxified hydrolysate (A) during 72 h or 84 h. However, the ethanol concentration was low using the NaOH-neutralized hydrolysate (A), and xylose consumption was also low (residual xylose 31.1 g/L). The consumption rates of xylose were 0.89 and 0.27 g $L^{-1}$ $h^{-1}$ using overliming-detoxified hydrolysate and NaOH-neutralized hydrolysate for fermentation, respectively. The poor fermentability of NaOH-neutralized hydrolysates as compared to detoxified ones was primarily due to the presence of higher amount of phenolics, furans and acetic acid, which might have inhibited the fermentation efficiency of *C. shehatae* (Chandel et al., 2007). This result agrees with that reported by Diaz et al using *Pichia stipitis* (Diaz et al., 2009). However, the cell growth in NaOH-neutralized hydrolysate was faster than that overliming-detoxified hydrolysate during 0 - 6 h.

Interestingly, fermentation results (Figure 2) demonstrated that ethanol concentration was up to 13.7 g/L by NaOH-neutralized hydrolysate (B), while ethanol concentration was only 7 g/L by overliming-detoxified hydrolysate (B). This phenomenon was contrary to reports by Nigam (2002, 2001a) and Chandel et al. (2007), these reported ethanol concentration were within 12.9 - 18.0 g/L in overliming-detoxified hydrolysate, and within 1.8 - 2.5 g/L in NaOH-neutralized hydrolysate. It suggested that acid hemicellulose hydrolysate without

detoxification could be directly fermented by *C. shehatae* CICC 1766. At same time, the latter's biomass was always higher than the former during hydrolysate fermentation, and a biomass of 20.2 g DCW /L, twice as much as the former, was exhibited at 96 h. The most of the carbon sources were used for biomass might be one of the reasons. An increase in biomass concomitant with the decrease in ethanol indicates that this strain also utilizes ethanol as carbon source (Chandel et al., 2007; Abbi et al., 1996a; b). Meanwhile, this approach could achieve a good result in hemicellulose hydrolysate fermentation by *C. shehatae* CICC 1766.

## Conclusion

The final ethanol was higher in defined xylose medium than that in the defined xylose/glucose medium, and the glucose was consumed prior to xylose by *C. shehatae* CICC 1766 for cell growth in the defined xylose/glucose medium. The ethanol concentration was attended to 16.1 g/L in overliming-detoxified hydrolysate. We found *C. shehatae* CICC 1766 could directly ferment acid hemicellulose hydrolysate without detoxification, Ethanol concentration was 13.7 g/L, and the optimum pH was 5.0, which was different from the reported optimum pH of 5.5 for *C. shehatae* to ferment hydrolysate, *C. shehatae* CICC 1766 still showed good fermentability even at pH 4.0.

### REFERENCES


Abbi M, Kuhad RC, Singh A (1996a). Fermentation of xylose and rice straw hydrolysate to ethanol by Candida shehatae NCL-3501. J. Ind. Microbiol. Biot., 17: 20-23.

Abbi M, Kuhad RC, Singh A (1996b).Bioconversion of pentose sugars to ethanol by free and immobilized cells of Candida shehatae NCL-3501: fermentation behaviour. Process Biochem., 31: 555-560.

Agbogbo FA, Coward-Kelly G, Torry-Smith M, Wenger KS (2006). Fermentation of glucose/xylose mixtures using Pichia stipitis. Process. Biochem. 41:2333–2336. (Not cited in the main work)

Agbogbo FA, Wenger KS (2007).Production of ethanol from vorn stover hemicellulose hydrolyzate using Pichia stipitis. J. Ind. Microbiol. Biot., 34: 723-727.

Agbogbo FA, Coward-Kelly G (2008).Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast. Pichia. stipitis. Biotechnol. Lett., 30: 1515-1524.

Almeida JRM, Modig T, Petersson A, Hahn-Hägerdal B, Liden G, Gorwa-Grauslund MF (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J. Chem. Technol. Biotechnol., 82(4): 340-349.

Chandel AK, Kapoor RK, Singh A, Kuhad RC (2007). Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresource. Technol., 10: 1947-1950.

Diaz MJ, Ruiz E, Romero I, Cara C, Moya M, Castro E (2009). Inhibition of Pichia stipitis fermentation of hydrolysates from olive tree cuttings. World J Microbiol Biotechnol., 25:891-899.

Eggeman T, Elander RT (2005). Process and economic analysis of pretreatment technolodies. Bioresource. Technol., 96: 2019-2025.

Galbe M, Zacchi G (2002). A review of the production of ethanol from softwood. Appl. Microbiol. Biot., 59(6): 618-628.

Hahn-HB, Karhumaa K, Fonseca C, Spencer-M I, Gorwa-GMF (2007). Towards industrial pentose-fermenting yeast strains. Appl. Microbiol. Biot., 74(5): 937-953.

Huang CF, Lin TH, Guo GL, Hwang WS (2009). Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *Pichia stipitis*. Bioresource. Technol., 100(17): 3914-3920.

Lawford HG, Rousseau JD (1991). Ethanol production by recombinant *Escherichia coli* carrying genes from Zymomonas mobilis. Appl. Biochem. Biotechnol., 28/29: 221-236.

Luo C, Brink DL Blanch HW (2002).Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. Biomass. Bioenerg., 22:125-138

Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO (2000). Effects of $Ca(OH)_2$ treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnol. Bioeng., 69: 526-536.

Martinez A, Rodriguez ME, Wells ML, York SW, Preston JF, Ingram LO (2001). Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol. Progr., 17: 287-293.

Mosier N, Wyman CE, Dale BE, Elander R, Lee YY, Holtzapple M Ladisch M (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource. Technol., 96(6): 673-686.

Nichols NN, Dien BS, Guisado GM, Lopez MJ (2005). Bioabatement to remove inhibitors from biomass-derived sugar hydrolysates. Appl. Biochem. Biotechnol., 121-124: 379-390.

Neidhardt FC, Ingraham JL, Low BK, Magasanik B, Schaechter M, Umbarger HE (1987). Escherichia coli and Salmonella typhimurium. Cellular and molecular biology, 1. Library of Congress Cataloging-in-Publication Data. Washington, DC: American Society for Microbiology.

Nigam JN (2001a). Ethanol production from wheat straw hemicellulose hydrolysate by Pichia *stipitis*. J. Biotechnol., 87: 17-27

Nigam JN (2002). Bioconversion of water-hyacinth (Eichhornia crassipes) hemicellulose acid hydrolysate to motoe fuel ethanol by xylose-fermenting yeast. J. Biotechnol., 97: 107-116.

Palmqvist E, Hähn-Hägerdal B (2000). Fermentation of lignocellulosic hydrolysates.II: inhibitors and mechanisms of inhibition. Bioresource. Technol., 74: 25-33.

Palmqvist E, Hähn-Hägerdal A (2000). Fermentation of lignocellulosic hydrolysates.I: inhibitors and detoxification. Bioresource. Technol., 74: 17-24.

Ranatunga TD, Jervis J, Helm RF, McMillan JD, Wooley RJ (2000). The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics: role of inorganics, uronic acids and ether-soluble organics. Enzyme. Microbiol. Technol., 27: 240-247.

Sun Y, Cheng J (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource. Technol., 83: 1-11.

Yu S, Wayman M, Parekh SK (1987). Fermentation of ethanol to pentose containing spent sulfite liquor. Biotechnol.Bioeng., 24: 1144-1150.