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Fermentation of xylose and rice straw hydrolysate to ethanol by *Candida shehatae* NCL-3501

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Candida shehatae **NCL-3501 utilized glucose and xylose efficiently in batch cultures. The specific rate of ethanol production was higher with mixtures of glucose and xylose (0.64–0.83 g g^{-1} cells d^{-1}) compared to that with individual sugars (0.38–0.58 g g^{-1} cells d^{-1}). Although the optimum temperature for growth was 30°C, this strain grew and produced appreciable levels of ethanol at 45°C. A stable ethanol yield (0.40–0.43 g g^{-1} substrate utilized) was obtained between 10 g L^{-1} and 80 g L^{-1} of initial xylose concentration. Conversion efficiency was further improved by immobilization of the cells in calcium alginate beads. Free or immobilized cells of *C. shehatae* NCL-3501 efficiently utilized sugars present in rice straw hemicellulose hydrolysate, prepared by two different methods, within 48 h. Ethanol yields of 0.45 g g^{-1} and 0.5 g g^{-1} from autohydrolysate, and 0.37 g g^{-1} from acid hydrolysate were produced by free and immobilized cells, respectively.**

Keywords: *Candida shehatae*; xylose; rice straw hydrolysate; ethanol; immobilized cells

Introduction

As reserves of crude oil become increasingly scarce, the production of chemical feedstocks and liquid fuels from renewable resources becomes an intriguing possibility [4,12]. Agricultural and wood residues are the two largest sources of lignocellulosic materials. Recent advances in the hydrolysis of hemicellulose and cellulose may eventually create an abundant supply of inexpensive carbohydrates [7,10]. For complete utilization of these sugars to ethanol, an organism that can convert pentoses, as well as hexoses, would be favourable [19]. *Saccharomyces cerevisiae*, the yeast most widely used for the production of ethanol, cannot ferment pentoses.

In recent years, some yeast species have been found that efficiently carry out the direct conversion of D-xylose to ethanol under aerobic or anaerobic conditions [8,13,15,18]. Among these yeasts, *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* have shown the greatest potential. While screening yeast strains for pentose fermentation and identifying them we found a strain of *C. shehatae* NCL-3501 which efficiently utilizes a wide range of sugars, as well as hydrolysates of natural substrates, under varying environmental conditions [1]. The potential of *C. shehatae* NCL-3501 for fermentation of xylose and rice straw hemicellulose hydrolysate by free and immobilized cells is explored in this paper.

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Materials and methods

Microorganism

Candida shehatae NCL-3501 was obtained from National Chemical Laboratory, Pune, India. The culture was maintained on agar slants containing (g L^{-1}): glucose 50, yeast extract 4, peptone 5, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5 and agar 20; pH 5.5 stocks were subcultured every month and stored at 4°C.

Culture conditions

The culture medium contained (g L^{-1}): NH_4Cl_2 0.5, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 1.5, $CaCl_2 \cdot 2H_2O$ 0.1, $FeCl_3 \cdot 2H_2O$ 1.008 and $ZnSO_4 \cdot 7H_2O$ 0.001. Each carbon source was supplied at the level mentioned in the text. The pH of the medium was adjusted to 5.5 before autoclaving it. An inoculum was prepared by harvesting the yeast cells, grown in cotton-plugged 250-ml flasks containing 50 ml medium (xylose, 10 g L^{-1}) with shaking at 150 rpm on a rotary shaker for 24 h at 30°C. A cell suspension was prepared in sterile water. The cell number was maintained by adjusting the o.d. at 600 nm to 0.6–0.8 (corresponding to a dry weight of 2.0–2.5 g L^{-1} of cells), which resulted in a cell suspension containing approximately 1.6×10^8 cells ml^{-1}. The fermentation media were inoculated with 0.5 ml of this suspension.

Batch fermentation with free or immobilized cells was carried out in 250-ml Erlenmeyer flasks containing 150 ml of the culture medium, supplemented with xylose or rice straw hydrolysate, at 30°C and an agitation of 150 rpm on a rotary shaker. Fermentation flasks received 0.5 ml of the free cell inoculum or 50 beads of immobilized cells (approximately 1.6×10^6 cells $bead^{-1}$) providing similar cell densities in both types of cultures.

Table 1 Ethanol production from different substrates by *Candida shehatae* NCL-3501

Substrate	Biomass ($g\ L^{-1}$)	Ethanol yield ($g\ g^{-1}$)	Volumetric productivity ($g\ L^{-1}\ h^{-1}$)	Specific rate of ethanol production ($g\ g^{-1}$ cells d^{-1})
Glucose	11.0	0.44	0.17	0.38
Xylose	6.2	0.41	0.15	0.58
Galactose	3.1	0.28	0.06	0.48
Xylose/glucose (2 : 1)	6.6	0.44	0.22	0.83
Xylose/glucose (1 : 2)	7.0	0.38	0.18	0.64

Initial substrate concentration 10 $g\ L^{-1}$, fermentation time 24 h.

Immobilization of cells

Yeast cells were immobilized in calcium alginate using the method of Kierstan and Bucke [11]. Cells were harvested after 24 h of incubation in a culture medium containing 10 $g\ L^{-1}$ xylose. The cell suspension prepared in 0.85% saline was mixed with 3.2% (w/v) sodium alginate solution. The beads were formed by pumping the mixture through a peristaltic pump (ATTO, Tokyo, Japan) at a flow rate of 50 ml min^{-1} and gently dropping the mixture into cold 0.2 M $CaCl_2 \cdot 2H_2O$ solution under sterile conditions. The beads obtained were about 2 mm in diameter as measured by a vernier calliper and contained approximately 1.6×10^6 cells $bead^{-1}$. The beads were stored in saline at 4°C for further use.

Pre-treatment of rice straw

For preparation of the acid hydrolysate, finely chopped rice straw (5–6 mm) with 4.4% H_2SO_4 (1 : 10, solid : liquid) was placed in an Erlenmeyer flask and heated in a boiling water bath for 1 h [5]. The contents were filtered through a muslin cloth and the pH of the filtrate was adjusted to 5.5 by adding 1 M $Ca(OH)_2$.

Autohydrolysate was prepared by heating finely chopped (5–6 mm) rice straw, soaked in water, at 170°C (7.6 kg cm^{-2}) for 30 min in a high pressure reactor. After the reactor had cooled to room temperature, the water extract (containing mainly pentose sugars) was filtered and the clear filtrate was used as autohydrolysate after adjusting its pH to 5.5.

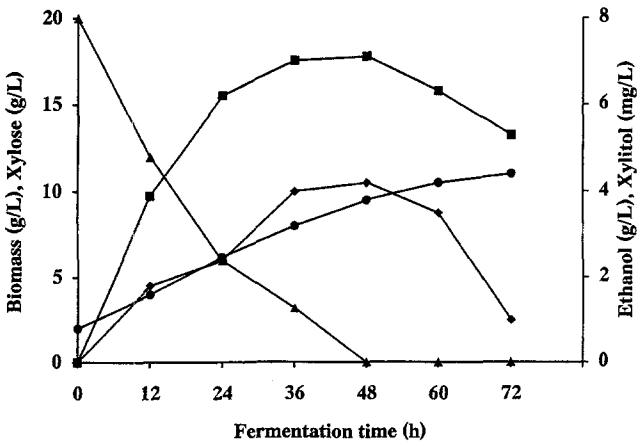

Figure 1 Time course of D-xylose utilization and ethanol production by *Candida shehatae* NCL-3501. Fermentation was carried out in 250-ml Erlenmeyer flasks containing 150 ml medium (xylose, 20 $g\ L^{-1}$) at 30°C on a rotary shaker (150 rpm). —▲—, Xylose; —●—, biomass; —■—, ethanol; —◆—, xylitol.

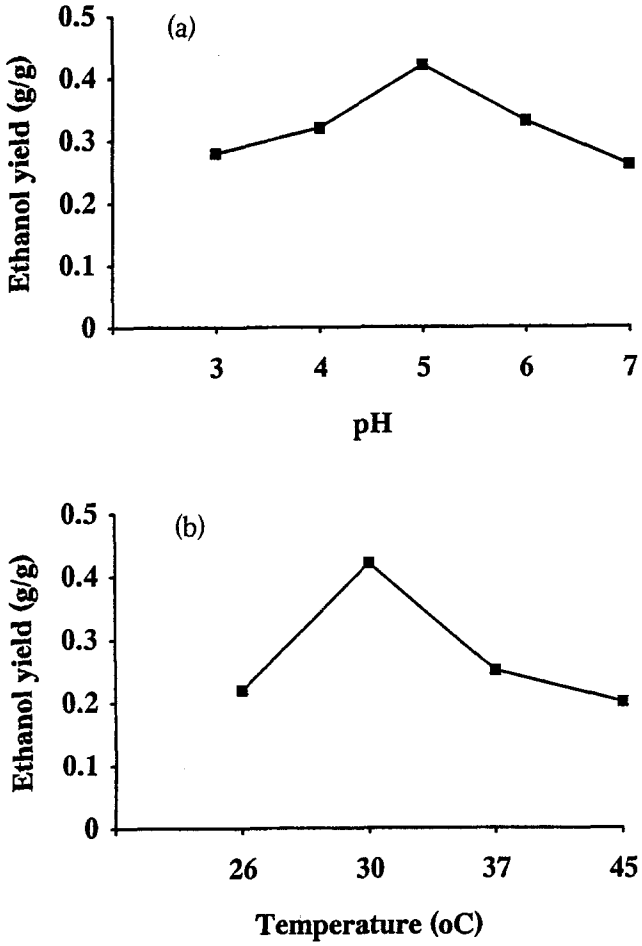

Figure 2 Effect of pH (a) and temperature (b) on ethanol production by *Candida shehatae* NCL-3501.

Acid hydrolysate and autohydrolysate contained 20 $g\ L^{-1}$ and 23.1 $g\ L^{-1}$ total sugars, respectively, and were used without further treatment.

Analytical methods

To determine the dry weight of the yeast cells, cultures were centrifuged ($8250 \times g$, 10 min) at 4°C, washed twice with distilled water and a sample was dried to a constant weight at 60°C. Ethanol content was determined by gas chromatography (AIMIL-5700, Nucon Engineers Ltd,

India) using a Nucon chromatographic column packed with Chromosorb. N_2 was supplied as the carrier gas. Flame (carrier) ionization detector and injector temperatures were maintained at 200°C and 180°C, respectively. The method of Morsel and Heyer [16] was followed by xylitol determination. Xylose and total sugar contents were determined by the orcinol [20] and phenol-sulfuric acid method [2], respectively.

Results and discussion

Glucose and xylose were fermented rapidly by *C. shehatae* NCL-3501. The fermentation of 10 g L^{-1} of sugar was essentially complete within 24 h, with the maximum yields of ethanol from glucose and xylose being 0.44 and 0.41 g g^{-1} substrate utilized, respectively (Table 1). Galactose utilization and conversion to ethanol was slow compared to glucose and xylose. However, the maximum specific rate of ethanol production from galactose as substrate (0.48 g ethanol g^{-1} cells d^{-1}) was higher than that from glucose (0.38 g g^{-1} d^{-1}) but lower than that from xylose (0.58 g g^{-1} d^{-1}). These values were found to be higher than those reported earlier for *P. tannophilus* (0.22 g g^{-1} d^{-1}) and *C. tropicalis* (0.20 g g^{-1} d^{-1}) [9]. *C. shehatae* NCL-3501 also utilized mixtures of xylose and glucose efficiently. Interestingly, the specific rate of ethanol production with mixtures of sugars was much greater than with individual sugars. It has been reported that xylose-fermenting yeasts preferentially utilize glucose in mixtures of xylose and glucose because of severe repression of D-xylose catabolism [3,8,13]. Once glucose has been consumed, enzymes for D-xylose catabolism are synthesized.

A typical profile of xylose (20 g L^{-1}) fermentation by *C. shehatae* NCL-3501 is shown in Figure 1. The level of ethanol reached a maximum after 36 h of fermentation, and remained more or less constant until 48 h and decreased thereafter. About 84% of the available xylose was utilized within 36 h, giving an ethanol yield of 0.43 g g^{-1} sugar utilized. Xylitol was produced during the initial period of fermentation, and its level decreased after 48 h. Biomass content continued to increase up to 72 h, though at a slower rate after 36 h. An increase in biomass concomitant with the decrease in ethanol and xylitol indicates that this strain efficiently utilizes ethanol and xylitol as carbon source in the absence of sugars. *C. shehatae* CSIR-Y492 and *Pichia stipitis* CSRI-Y633 utilize xylitol for growth but not for ethanol production [3]. Concurrent production and consumption of ethanol by *Pachysolen tannophilus* has also been demonstrated [14].

Table 2 Effect of xylose concentration on ethanol production of *Candida shehatae* NCL-3501

Xylose (g L^{-1})	Fermentation time (h)	Ethanol (g L^{-1})	Yield (g g^{-1})	Productivity (g L^{-1} h^{-1})
10	24	3.6	0.41	0.15
20	36	7.0	0.43	0.19
30	48	10.8	0.41	0.22
40	48	14.0	0.41	0.29
50	64	18.0	0.43	0.28
60	72	20.0	0.40	0.27
70	96	25.0	0.43	0.26
80	120	30.0	0.41	0.25
90	120	30.0	0.38	0.25

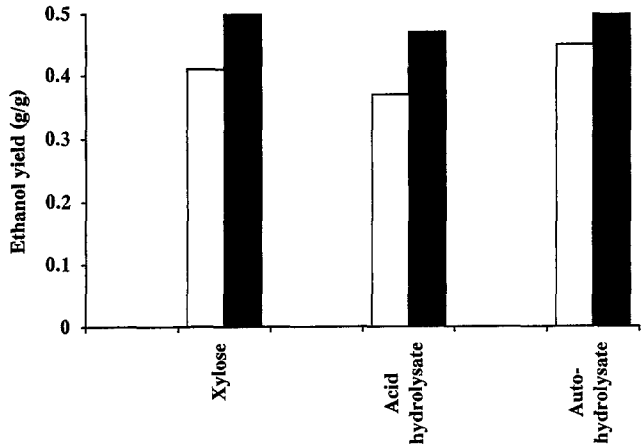

Figure 3 Fermentation of xylose and rice straw hydrolysates by free and immobilized cells of *Candida shehatae* NCL-3501. Acid hydrolysate and autohydrolysate contained 20 g L^{-1} and 23.1 L^{-1} total sugars, respectively; whereas xylose was used at 20 g L^{-1}. □, Free cells; ■, immobilized cells.

The effect of the initial culture pH on ethanol production was examined within the pH range 3–7. *C. shehatae* NCL-3501 performed best around pH 5 (Figure 2a). The temperature for maximum ethanol yield by *C. shehatae* NCL-3501 was 30°C (Figure 2b). However, this strain grew and produced appreciable levels of ethanol at 45°C. This ability indicates a potential for use of this strain in simultaneous saccharification and fermentation of lignocellulosic biomass where the process temperature is kept around 40°C [7].

C. shehatae NCL-3501 grew and utilized xylose efficiently at xylose levels ranging from 10 to 90 g L^{-1} (Table 2). Maximum ethanol productivity occurred at 40–50 g L^{-1} of xylose. A higher product concentration was achieved at 80–90 g L^{-1} of xylose, but the reactor productivity was lower due to slower metabolic rate. When the initial xylose concentration exceeded 80 g L^{-1}, the ethanol concentration was maximum at 120 h; then the concentration of residual sugar declined only slightly, while ethanol levels began to fall. This observation also suggested the concurrent production and utilization of ethanol by *C. shehatae* NCL-3501. The ethanol yield was stable between 10 g L^{-1} and 80 g L^{-1} of initial xylose concentration. These results suggest that the fermentation of very high sugar concentrations are limited mainly by the ethanol tolerance of the yeast.

Figure 3 shows the batch fermentation of xylose and rice straw hydrolysate by free and immobilized cells of *C. shehatae* NCL-3501. Rice straw hydrolysate prepared by two different methods was evaluated since it would be a practical substrate for conversion to liquid fuels. Acid hydrolysate and autohydrolysate contained 20 g L^{-1} and 23.1 g L^{-1} total sugars, respectively, and both substrates supported good growth and ethanol yields. While ethanol yields of 0.45 g g^{-1} and 0.5 g g^{-1} sugar utilized were produced from autohydrolysate in 48 h by free and immobilized cells, respectively, corresponding ethanol yields from

acid hydrolysate were 0.37 g g^{-1} and 0.47 g g^{-1}, respectively. The lower ethanol yields with acid hydrolysate may be due to the presence of inhibitory compounds (furfural, phenolics etc) which should be almost absent in autohydrolysate [17,19]. Immobilized cells exhibited better performance than free cells on all the substrates tested, probably due to the protection of the cells from inhibitors by the support [18,19].

Thus an important feature of *C. shehatae* NCL-3501 is efficient utilization of the major sugars found in complex lignocellulosic hydrolysate, which indicates the potential of this strain for practical processes. Also, pretreatment of hydrolysates before fermentation with this strain is not necessary, which is required in the case of other yeast strains [6,17].

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References

1 Abbi M. 1994. A study of fermentation of pentose sugars to ethanol by *Candida shehatae*. MSc Thesis, University of Delhi, New Delhi, India.
2 Dubois M, KA Gilles, IK Hamilton, PA Rebers and F Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.
3 du Preez JC, M Bosch and BA Prior. 1986. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis*. Appl Microbiol Biotechnol 23: 228–233.
4 Enari T-M and M-L Suihko. 1984. Ethanol production by fermentation of pentoses and hexoses from cellulosic materials. Crit Rev Biotechnol 1: 229–239.
5 Fan LT, MM Gharpuray and HY Lee. 1981. Evaluation of pretreatments for enzymatic conversion of agricultural residues. Biotechnol Bioeng Symp 11: 29–45.
6 Ferrari MD, E Neirotti, C Albornoz and E Saucedo. 1992. Ethanol production from eucalyptus wood hemicellulose hydrolysate by *Pichia stipitis*. Biotechnol Bioeng 40: 753–759.
7 Ghosh P and A Singh. 1993. Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass. Adv Appl Microbiol 39: 295–333.
8 Grootjen DRJ, LHHM Meijlink, RGJM van der Lans and KChAM Luyben. 1990. Cofermentation of glucose and xylose with immobilized *Pichia stipitis* and *Saccharomyces cerevisiae*. Enzyme Microb Technol 12: 860–864.
9 Jeffries TW. 1982. A comparison of *Candida tropicalis* and *Pachysolen tannophilus* for conversion of xylose to ethanol. Biotechnol Bioeng Symp 12: 103–114.
10 Jeffries TW. 1984. Emerging technology for fermenting D-xylose. Trends Biotechnol 3: 208–212.
11 Kiersten M and C Bucke. 1977. The immobilization of microbial cells, subcellular orgenelles and enzymes in calcium alginate gels. Biotechnol Bioeng 19: 387–397.
12 Kuhad RC and A Singh. 1993. Lignocellulose biotechnology: current and future prospects. Crit Rev Biotechnol 13: 151–172.
13 Laplace JM, JP Delegenes, R Moletta and J Navarro. 1993. Effect of culture conditions on the co-fermentation of glucose and xylose mixture to ethanol by a mutant *Saccaromyces diastaticus* associated with *Pichia stipitis*. Appl Microbiol Biotechnol 39: 760–763.
14 Maleszka R and H Schneider. 1984. Concurrent production and consumption of ethanol by cultures of *Pachysolen tannophilus* growing on D-xylose. Appl Environ Microbiol 44: 909–913.
15 Mishra P and A Singh. 1993. Microbial pentose utilization. Adv Appl Microbiol 39: 91–152.
16 Mörsel J-T and T Heyer. 1990. A colorimetric method for the determination of glycerides. Die Nahrung-food 34: 287–288.
17 Nishikawa N, R Sutcliffe and JN Saddler. 1988. The influence of lignin degradation products on xylose fermentation. Appl Microbiol Biotechnol 27: 549–552.
18 Schneider H. 1989. Conversion of pentoses to ethanol by yeasts and fungi. Crit Rev Biotechnol 9: 1–40.
19 Skoog K and B Hahn-Hägerdal. 1988. Xylose fermentation. Enzyme Microb Technol 10: 66–80.
20 Standing CN, AG Fredrickson and HM Tsuchia. 1972. Batch and continuous transient for two substrate system. Appl Microbiol 23: 354–359.