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Effect of Corn Steep Liquor on Fermentation of Mixed Sugars by *Candida shehatae* FPL-702

HASSAN K. SREENATH[1,2] AND THOMAS W. JEFFRIES*[,1,3]

[1] *Institute of Microbial Biochemical Technology, Forest Products Laboratory, 1 Gifford Pinchot Drive, Madison, WI 53705;* [2] *DFRL, India; and* [3] *Department of Bacteriology, University of Wisconsin-Madison, Madison, WI*

ABSTRACT

Candida shehatae FPL-702, is a mutant strain obtained from *C. shehatae* ATCC 22984 by selection for rapid growth on L-xylose and xylitol in the presence of respiratory inhibitors. This strain produced more ethanol from xylose than any other pentose-fermenting yeast strain tested. These included the parental *C. shehatae* strain ATCC 22984 and other wild-type or mutant strains of *Pichia stipitis* and *Pachysolen tannophilus*. *C. shehatae* FPL-702 showed initial rapid growth on xylose. During fed-batch shake-flask fermentation of glucose, FPL-702 produced 3.5% (w/v) ethanol. Ethanol production from glucose increased to 5.0% (w/v) when corn steep liquor (CSL) was added in the fermentation medium. In contrast, CSL reduced consumption of xylose and ethanol production in shake-flask fermentations of xylose and mixtures of xylose and glucose. However, CSL also reduced xylitol formation. Maximum ethanol production from a mixed-sugar fermentation was 3.25% (w/v). *C. shehatae* FPL-702 did not use L-arabinose during mixed-sugar fermentation in the presence or absence of CSL, but it did grow on arabinose and convert it into arabitol. In the fed-batch reactor, this strain exhibited improved ethanol production rates from mixed sugar in the presence of CSL.

Index Entries: *Candida shehatae; Pichia stipitis; Pachysolen tannophilus;* mixed sugar; fermentation; ethanol; corn steep liquor.

INTRODUCTION

The microbial conversion of agricultural and forestry residues into ethanol continues to be of worldwide interest. The hydrolysates of cellulose and hemicelluloses are commercially attractive sources of fermentable sugars, such as glucose, xylose, and arabinose. The efficient fermentation of these sugar mixtures is essential for the development of an economical process to make ethanol from biomass *(1–5)*. Various investigators have studied yeasts and other organisms for the conversion of glucose and xylose to ethanol *(5–9)*. Xylose is abundant in hardwood and corn-processing residues. L-Arabinose is usually present only in small amounts, but it is significant in some agricultural residues. Yeast species exhibiting significant etha-

*Author to whom all correspondence and reprint requests should be addressed.

nol production from xylose include *Candida tenuis, Candida shehatae, Pichia segobiensis, Pichia stipitis, Pachysolen tannophilus,* and *Bretannomyces naardenensis (5).* These strains generally exhibit lower ethanol production from D-xylose than from D-glucose. The oxidoreductive pathways from xylose to xylulose could be the limiting step in xylose metabolism *(10,11).* Strategies for obtaining various yeast mutants with enhanced fermentation of xylose and glucose have been developed in this laboratory and elsewhere *(12–15).* The results suggest that changes in the levels of enzymes involved particularly in enzymes of xylose metabolism may enhance the rate of growth and xylose fermentation *(16). C. shehatae* FPL-702 is a mutant strain that was obtained through selective enrichment and plating on L-xylose and xylitol in the presence of respiratory inhibitors *(17).* In the present work, we have studied *C. shehatae* FPL-702 for xylose and mixed-sugar fermentations.

MATERIALS AND METHODS

Yeast Strains

The wild-type strains of three xylose-fermenting yeasts, *P. tannophilus* NRRL-Y-2460, *P. stipitis* CBS 6054, and *C. shehatae* ATCC 22984, were compared with derived mutants that we had identified through previous screening. *P. tannophilus* FPL-NO_3NO_3-4, *P. stipitis* FPL-061, and *C. shehatae* FPL-702 were selected for comparison. Each strain was grown and maintained on fresh plates of yeast extract peptone xylose (YEPX) agar for 48 h at 31–32°C.

Culture Medium

All fermentation media contained 1.7 g/L filter-sterilized yeast nitrogen base without ammonium sulfate or amino acids (Difco, Detroit, MI). Urea, 2.27 g/L, and peptone, 6.56 g/L, were used as nitrogen sources. Individual sugars, such as xylose, glucose, and arabinose, and sugar mixtures were autoclaved separately and added to the medium after they had cooled to room temperature. The final concentration of various sugars ranged from 60–180 g/L. The final pH was 4.0–4.5.

Inoculum Preparation

One loopful of cells from a 48-h-old YEPX plate was inoculated into 50 mL of culture medium containing 4% xylose in replicate 125-mL Erlenmeyer flasks and cultivated with shaking at 84 rpm for 24 h at 27–28°C. The cultures were centrifuged at $3.4 \times 10^3 g$ for 10 min, suspended in 10.0 mL distilled water, and used as the inoculum. The optical density of each cell suspension was measured and adjusted to the constant value by dilution with water.

Shake-Flask Fermentation

Two conditions were used for shake-flask studies. The first used the same conditions as employed for inoculum preparation. The second started with an initial low level of sugar (40 g/L) in 25 mL of medium in a 125-mL Erlenmeyer flask shaken at 84 rpm, and incubated at 27–28°C. After 18 h, we added aqueous sugar solution to make the final sugar concentration 125 g/L in 50 mL of medium. These fermentations were carried out in triplicate flasks either with individual sugars (glucose, xylose, or arabinose) or with a 40:40:20 mixtures of these same sugars.

This experiment was replicated with and without corn steep liquor (CSL) (28 g/L). The CSL was added in two batches of 14 g/L each at the outset of the experiment and after 18 h. The inoculum (5–6 g dry wt cells/L) was prepared as described earlier. The fermentation was monitored for 3–10 d by removing 1.3-mL samples for sugar, ethanol, and cell analyses. In the fermentation without CSL, an extra 28 g/L of yeast nitrogen base supplemented with urea and peptone was added to the medium for comparison.

Fed-Batch Fermentation

The batch fermentor (Multigen 2-L, New Brunswick Scientific, New Brunswick, NJ) was sterilized by autoclaving, and sterile medium containing yeast nitrogen base urea peptone was aseptically added. The fermenter run used two conditions. The first employed the sugar mixture alone (containing 40:40:20 glucose:xylose:arabinose). The second employed the sugar mixture plus 28 g/L CSL. Both vessels started off with approx 690 mL of medium containing 24 g sugar along with the cell inoculum of *C. shehatae* FPL-702 (4 g/L, 24-h-old) and 1.5 mL antifoam (FG-10, Dow Corning Corporation, Midland, MI), agitated at 300 rpm and sparged with approx 2.3 L air/L reactor volume/min. The air flow was continuously monitored by flow meter. After 15 h, each reactor received 84 g of additional sugar mixture in a volume of 400 mL, and at 36 h, each reactor received 54 g of sugar mixture in a volume of 200 mL. One final addition of sugar (105 g in 210 mL) was made at 63 h. Hence, the total sugar mixture used in this batch fermentation was 178 g/L, and the final working volume was 1.5 L. The temperature was maintained at 27–28°C, and the pH was maintained at 4.0–4.5. Five-milliliter samples were taken periodically for various analyses. In a separate experiment, a 20% sugar mixture was fermented with 28 g/L CSL at one step by *C. shehatae* FPL-702 under identical conditions.

Analytical Methods

Cell densities were measured at 525 nm. Dry weights were correlated with optical densities (OD) of cell suspensions between 0.05 and 0.5 OD units at 525 nm. An OD of 1.0 was equivalent to 0.21 mg dry wt of cells/mL. Ethanol was determined by gas chromatography *(18)*. Glucose, xylose, arabinose, xylitol, and other fermentation byproducts were determined by high-performance liquid chromatography (Hewlett Packard series 1050, Wilmington, DE) with a refractive index (RI) detector using an Aminex Carbohydrate HPX 87C, column (300 x 7.8 cm) maintained at 85°C *(19)*. The mobile phase was degassed distilled water at a flow rate of 0.5 mL/min at a pressure of 50–55 bar. The filtered clear sample (980 µL) was mixed with 20 µL of sucrose (500 g/L) as internal standard before injection.

RESULTS AND DISCUSSION

Fermentation of Xylose and Arabinose by Wild and Mutant Yeast Strains

Ethanol production was observed only on D-xylose. Each of the mutant strains outperformed its parent (Fig. 1). In the case of *C. shehatae* FPL-702 and *P. tannophilus* FPL-NO_3NO_3-4, volumetric productivities were clearly higher, whereas in *P. stipitis* FPL-061, volumetric productivities were similar to the parent, but the specific fermentation rate was about 30–40% higher as was previously reported

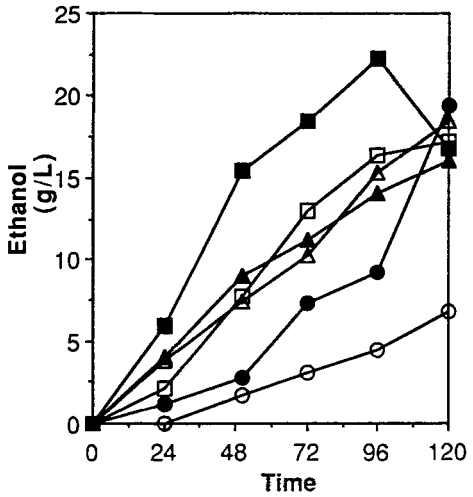

Fig. 1. Ethanol production from xylose fermentation in some wild type yeasts and their derived mutants. □, *C. shehatae* ATCC 22984; ■, *C. shehatae* FPL-702; △, *P. stipitis* CBS 6054; ▲, *P. stipitis* FPL-061; ○, *P. tannophilus* NRRL Y-2460; ●, *P. tannophilus* FPL-NO_3-NO_3-4 (=ATCC 60393).

(20). Cells grew on both D-xylose and L-arabinose, but growth on arabinose was very slow. Two of the yeast mutants, *C. shehatae* FPL-702 and *P. tannophilus* FPL-NO_3NO_3-4, clearly outgrew the parental strains on arabinose.

In order to evaluate our best mutant *C. shehatae* FPL-702, we performed a fed-batch fermentation in shake flasks and 2-L batch reactor in the presence and absence of CSL. CSL is an inexpensive major byproduct of the corn starch processing. The composition depends on the method of preparation, but it is generally a rich source of nitrogen, water-soluble vitamins, amino acids, minerals, and other growth stimulants *(21)*. Such components of fermentation medium can support growth and fermentation activity and influence ethanol production *(22–24)*. Hence, CSL was added to the fermentation medium mainly to increase nitrogen sources in addition to amount of nitrogen (urea) available in the fermentation medium *(25)*.

Effect of CSL on Fermentation of Glucose and Xylose

During fed-batch fermentation of glucose, *C. shehatae* FPL-702 produced 3.5% (w/v) ethanol in the absence of CSL. In the presence of CSL, ethanol production from glucose increased from 3.5–5.0% (w/v) (Fig. 2). The rate of glucose utilization nearly doubled in the presence of CSL in first 24 h of fermentation (Fig. 3). In contrast, CSL reduced xylose utilization by 32% in the first 72 h of fermentation (Fig. 4). This could possibly be owing to oxygen limitations and other inhibitory components of CSL involving membrane transport of pentoses in the absence of glucose *(6,25,26)*. CSL addition also reduced the rate of ethanol formation and xylitol production. CSL has high lactic and phytic acid contents that could affect sugar metabolism, but we have not examined these factors.

Effect of CSL on Fermentation of Mixed Sugars

During mixed-sugar fermentation, glucose utilization was rapid in the presence of CSL and was complete in 48 h. Xylose consumption, however, was initially

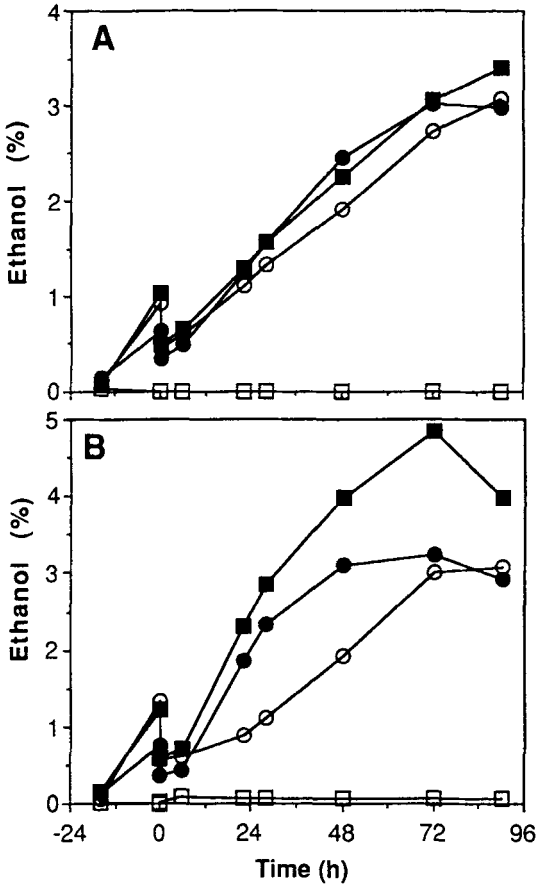

Fig. 2. Ethanol production in *C. shehatae* FPL-702 from fed-batch fermentation of various sugars and sugar mixture in the presence **(A)** and absence **(B)** of CSL. □, arabinose; ■, glucose; ○, xylose; ●, sugar mixture.

faster (Fig. 5). In the absence of CSL, glucose was slowly consumed in 72 h, and xylose consumption did not stop. The maximum ethanol production from mixed sugars in the presence of CSL was 3.25% (w/v) (Fig. 5), and the rate of ethanol production was enhanced compared to fermentation of mixed sugars in the absence of CSL.

Fermentation of Arabinose

Small amounts of arabinose were utilized from the sugar mixture in the presence or absence of CSL up to 100 h of fermentation. This was followed by a steep increase in the rate of arabinose utilization. Although arabinose was not fermented, arabitol and xylitol were formed as end products (Fig. 6). Small amounts of ethanol were detected in the flasks receiving arabinose plus CSL. This may be owing to the presence of some hexose sugars (e.g., glucose, maltose) or lactic acid in CSL (*25*). When *C. shehatae* FPL-702 was continuously adapted on arabinose by serial transfer, the strain converted nearly all of the arabinose into arabitol in a period of 20 d. However, fermentation of arabinose was not apparent.

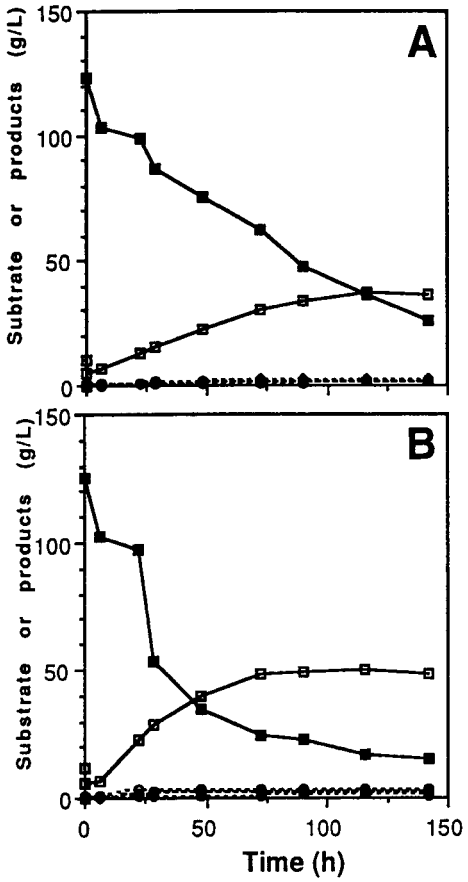

Fig. 3. Effect of CSL on shake-flask fermentation of glucose by *C. shehatae* FPL-702: **(A)** without CSL and **(B)** with CSL. ■, glucose; ◆, ribitol; □, glycerol; ◇, arabitol; ●, xylitol; □, ethanol.

Fed-batch Fermentation of Mixed Sugars

The aeration rate in the shake-flask experiment was low, and pH was not controlled. In xylose-fermenting yeasts, the fermentative performance greatly depends on the presence of oxygen *(1,6,7)*. At high sugar concentrations, polyol production and osmotic inhibition become problems. These effects can be mitigated by periodically adding sugar in small amounts to the active fermentation. We therefore scaled up the mixed-sugar fermentation in the presence and absence of CSL to 1.5 L in stirred-tank reactors (Table 1). In the fed-batch reactor with CSL, 23 g/L of ethanol were produced in 26 h after fermenting a 10% sugar mixture. When the sugar concentration was increased to 12.5% at 36 h, a total of 28 g/L ethanol was obtained at 48 h. At 63 h, mixed sugar was added to 18% (final), which resulted in production of a maximum of 40 g/L ethanol in 84 h in the reactor. Ethanol production was rapid as long as glucose was present, but tended to stall between additions of mixed sugars. Glucose was consumed rapidly followed by a slow xylose assimilation. Such preferential utilization of glucose compared to xylose could result from diffrential transport as proposed by Slininger et al. *(26)*. Arabinose was also consumed, but at a lower rate, and its principal products were

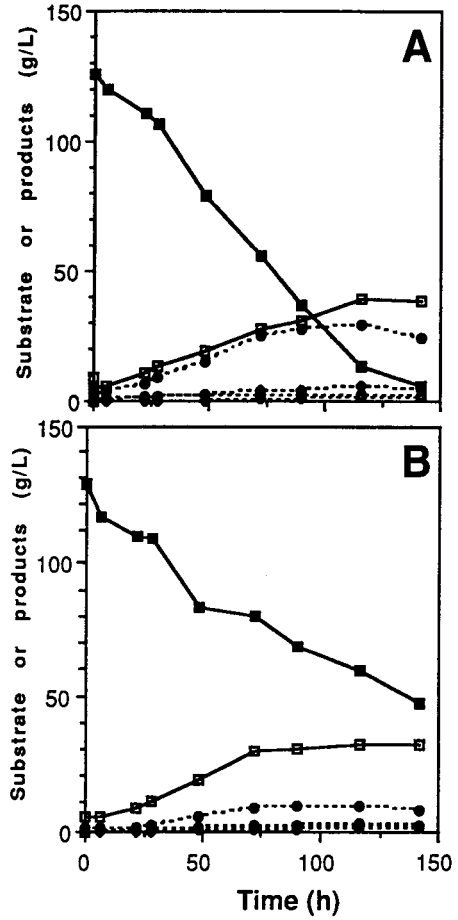

Fig. 4. Effect of CSL on shake-flask fermentation of xylose by *C. shehatae* FPL-702: **(A)** without CSL and **(B)** with CSL. ■, xylose; ◆, ribitol; □, glycerol; ◇, arabitol; ●, xylitol; □, ethanol.

xylitol and arabitol. In the absence of CSL, the maximum ethanol obtained after mixed-sugar fermentation was 25% less, and both glucose and xylose were consumed at lower rates than that observed in the fermentation with CSL. CSL increased cell growth (as determined by dry wt) and decreased polyol production. CSL appeared to increase the consumption of xylose in the mixed-sugar fed-batch fermentation (Table 1). The basis for this increase is not fully understood—especially given the apparent repressive effect observed in the batchwise utilization of xylose alone. However, in the mixed-sugar fed-batch fermentation, CSL was only added at the beginning of the experiment and was not added with the successive batches of sugar. It is possible that the stimulatory effects of CSL on growth compensated for inhibition of xylose utilization. With the successive additions of mixed sugars, glucose appeared to suppress ethanol production from xylose, and with the high aeration rate employed, some ethanol was oxidized. The batch fermentation of 20% mixed sugars with CSL by *C. shehatae* FPL-702 produced a maximum ethanol concentration of 34 g/L within 36 h, giving an ethanol yield of 0.32 (Fig. 7). Further, the ethanol concentration remained constant for another

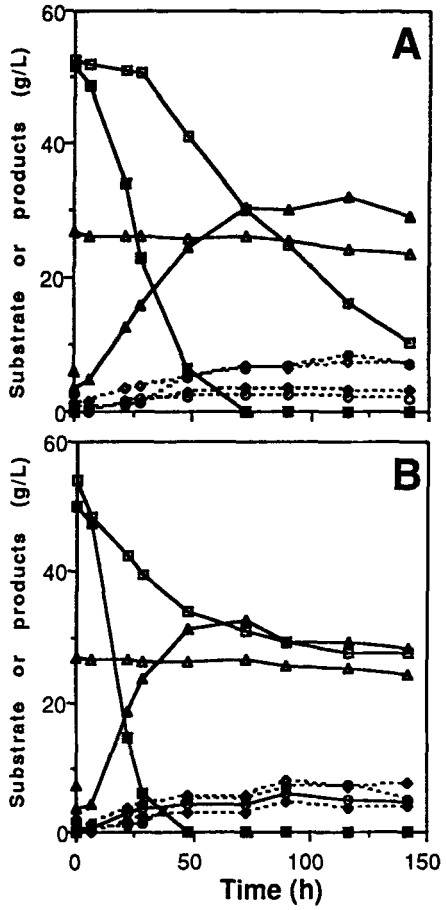

Fig. 5. Effect of CSL on shake-flask fermentation of sugar mixture by *C. shehatae* FPL-702: **(A)** without CSL and **(B)** with CSL. ■, glucose; □, xylose; △, arabinose; ◆, ribitol; ○, glycerol; ◇, arabitol; ●, xylitol; ▲, ethanol.

24 h before decreasing. The decrease in ethanol concentration could be owing to reassimilation of produced ethanol by this yeast with increasing oxygenation in the fermentation medium as reported by other workers *(27)*. In the presence of glucose, xylose utilization was greatly reduced, suggesting that catabolite repression blocked simultaneous utilization of glucose, xylose, and arabinose. In previous studies with *C. shehatae* ATCC 22984, a rapid fed-batch fermentation of pure xylose attained 56 g/L of ethanol concentration within 48 h *(28)*. The present trials with *C. shehatae* FPL-702 showed a lower product concentration (but better yield) than previously reported. This difference could be attributed to inoculum preparation, cell density, the media used, and how the sugars were added. In the previous trial with the parent ATCC 22984, the inoculum was grown under fully aerobic conditions in a continuous culture mode, the initial cell density was 20 g/L, the media contained higher concentration of essential nutrients, and a single sugar (xylose) was added continuously. In the present trial, the inoculum was grown under oxygen-limited conditions in a batch mode, the initial inoculum was 4 g/L, the medium was not optimal, and mixed sugars were added batchwise. Given

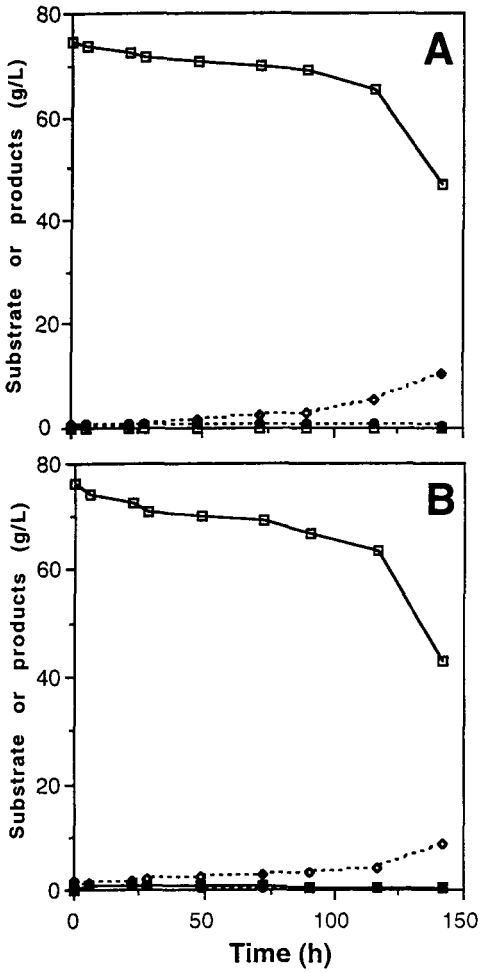

Fig. 6. Effect of CSL on shake-flask fermentation of arabinose by *C. shehatae* FPL-702: **(A)** without CSL and **(B)** with CSL. □, arabinose; ◇, arabitol; ●, xylitol; □, ethanol.)

these differences, it is apparent that here is further room for improvement with *C. shehatae* FPL-702.

CONCLUSIONS

In these comparative trials, the mutant strain *C. shehatae* FPL-702 fermented xylose better than its parent or any other of several yeasts tested. In the presence of CSL, FPL-702 efficiently consumed and fermented glucose. In contrast, a xylose/glucose sugar mixture was consumed at a lower rate, resulting in lower ethanol in shake-flask fermentations. In the fed-batch reactor, this strain exhibited improved ethanol rates from mixed sugar in the presence of CSL. Arabinose was not fermented by this strain in the presence or absence of CSL, but was converted to arabitol. The ethanol formation rate and yield from sugar mixture fermentation could be further accelerated by optimizing fermentation conditions, such as aeration, amount of inoculum, and media components, as well as from strain development techniques.

Table 1
Effect of CSL on Fermentation of Mixed Sugars in Fed-Batch Reactor in *C. shehatae* FPL-702: Summary of Various Fermentation Parameters

Fermentation parameters	Without CSL	With CSL
Glucose consumed (g/L)	107.0	107.0
Xylose consumed (g/L)	76.0	102.0
Arabinose consumed (g/L)	13.0	22.0
Ethanol concentration (g/L)	25.7	40.1
Arabitol produced (g/L)	12.2	14.8
Xylitol produced (g/L)	5.1	3.6
Glycerol produced (g/L)	4.8	2.5
Ribitol produced (g/L)	4.3	3.3
Cell dry weight (g/L)	15.6	20.2
Aeration (L/L/min)	1.2	1.2
Reactor volume (L)	1.5	1.5
Reactor pH	4.5	4.5
Reactor temperature (°C)	27.5	27.5

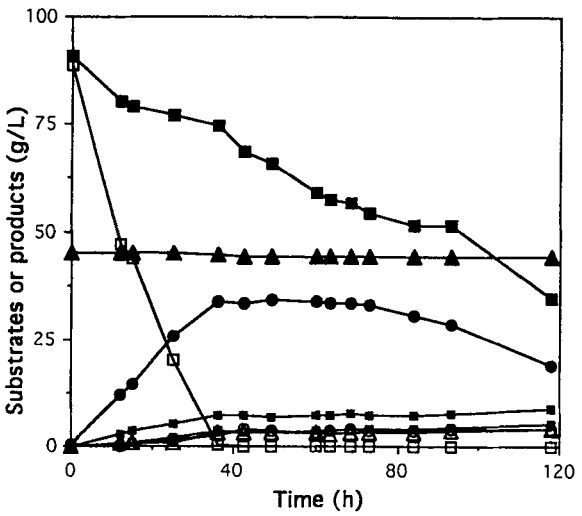

Fig. 7. Batch fermentation of 20% mixed sugars in fermentation reactor by *C. shehatae* FPL-702. □, glucose; ■, xylose; ▲, arabinose; ●, ethanol ; ○, ribitol; □, arabitol; ▪, glycerol; △, xylitol.

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