FERMENTATION OF L-ARABINOSE, D-XYLOSE AND D-GLUCOSE BY ETHANOLOGENIC RECOMBINANT KLEBSIELLA OXYTOCA STRAIN P2

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SUMMARY
Recombinant Klebsiella oxytoca strain P2 carrying genes for pyruvate decarboxylase and alcohol dehydrogenase from Zymomonas mobilis was evaluated for its ability to ferment arabinose, xylose and glucose alone and in mixtures in pH-controlled batch fermentations. This organism produced 0.34-0.43 g ethanol/g sugar at pH 6.0 and 30°C on 8% sugar substrate and demonstrated a preference for glucose. Sugar utilization was glucose > arabinose > xylose and ethanol production was xylose > glucose > arabinose.

INTRODUCTION
Currently, over one billion gallons of ethanol are produced annually in the United States, with approximately 95% derived from corn. Now with increased attention to clean air and oxygenates for fuels, an opportunity exists for an expanded alcohol fuel industry. Technologies for lowering costs associated with ethanol production can improve the competitiveness of ethanol as a fuel or fuel additive. Lignocellulosic biomass, particularly corn fiber, represents a renewable resource which is available in sufficient quantities from corn wet milling industry to serve as a low cost feedstock. It is essential that both cellulose and hemicellulose components of corn fiber be fermented for efficient and economical conversion of corn to fuel ethanol. A typical hydrolyzate of corn fiber obtained from wet milling contains a variety of sugars such as xylose, arabinose and glucose. Most of the xylose- and glucose-fermenting yeasts such as Pichia stipitis, Candida shehatae and Pachysolen tannophilus do not have the ability to produce ethanol from L-arabinose (Slininger et al., 1985; Neirnack et al., 1982; Schneider et al., 1983; Du Preez et al., 1986; Delgenes et al., 1988; Maleszka et al., 1982; Deverell, 1983). Because corn fiber

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contains ~ 12% arabinose, there is an increasing demand to find an efficient arabinose-fermenting organism which can also ferment other sugar components of hemicellulose. *Klebsiella oxytoca* strain P2 is a recombinant organism in which *Zymomonas mobilis* alcohol dehydrogenase (*adh B*) and pyruvate decarboxylase (*pdc*) genes have been integrated into the chromosome and expressed at high levels to divert metabolism of pyruvate to ethanol (Ohta et al., 1991) and a lesser quantity of acetic acid. The purpose of the present study was to compare the growth and fermentation performance of this ethanologenic recombinant *K. oxytoca* strain P2 on arabinose, xylose and glucose.

**MATERIALS AND METHODS**

**Bacterial strain.** *Klebsiella oxytoca* strain P2 described previously (Wood and Ingram, 1992) was used. In this recombinant, the *Zymomonas mobilis* genes encoding alcohol dehydrogenase (*adh B*) and pyruvate decarboxylase (*pdc*) have been integrated into the *pfl* gene within the chromosome of *K. oxytoca* M5A1 (Ohta et al., 1991). Strain P2 was maintained in glycerol vials at -20°C for use as a working stock.

**Preparation of inocula for fermentations.** Strain P2 was plated on amended Luria broth (containing per liter: 20 g xylose, 10 g tryptone, 5 g yeast extract, 5 g NaCl and 40 mg chloroamphenicol) solidified with 1.5% agar. Plates were incubated at 30°C. Cells from a single well-isolated colony were inoculated into a 500 ml flask containing 200 ml of Luria broth with 50 g/l xylose. Cultures were incubated for 24 h at 30°C without agitation. Cells were harvested by centrifugation (18,000 g, 15 min) and used to inoculate culture medium at an initial cell density of approximately 320 mg dry weight per liter (Wood and Ingram, 1992).

**Fermentation experiments.** Batch culture experiments were carried out in pH-controlled 500 ml flakers with a working volume of 350 ml essentially as described by Beall et al. (1991). Luria broth containing 8% sugar was used as substrate. Sugars were sterilized separately. A 2 M KOH solution was used for pH control. All fermentations were performed at pH 6.0 and 30°C for 114 h. Samples were withdrawn periodically to determine cell mass, ethanol content and residual sugar. Base consumption and pH were also recorded.

**Analytical procedures.** Optical density was measured at 550 nm. For ethanol and sugar analysis, samples were clarified by centrifugation (12,000 x g, 5 min) to remove cells. Supernatant solutions were stored at -20°C prior to analysis. Ethanol concentration was determined by gas chromatography with a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector and a Porapak Q column (Millipore corporation, Waters Chromatography Division, Milford, MA). The injector, column and detector temperatures were 200, 150, and 250°C, respectively. Sugar analysis was performed by high pressure liquid chromatography (HPLC, Spectra-Physics, San Jose, CA). An Aminex ion exchange HPX-87H column (Bio-Rad Laboratories, Hercules, CA) was used. The elution of the sugars was performed at room temperature with 0.005 M H₂SO₄ at a flow rate of 0.6 ml/min. Peaks were detected by refractive index, and identified and quantified by comparison to retention times of saccharide standards (arabinose, glucose and xylose).
RESULTS AND DISCUSSION

Fermentations of arabinose, xylose and glucose separately and in two different combinations by the recombinant *K. oxytoca* strain P2 were investigated in pH-controlled batch fermentations at pH 6.0 and 30°C. The two combinations - mixture A contained arabinose, xylose and glucose (1:1:1) and mixture B contained arabinose, xylose and glucose (1:2:1). The results are summarized in Table 1. The organism grew well on each individual sugar and their mixtures, and produced ethanol. Growth was best on glucose (cell mass, 3.5 g/l) but ethanol yield was best on xylose (33.3 g/l). These fermentations produced 0.34, 0.37, and 0.42 g ethanol per g of arabinose, glucose and xylose, respectively. Ethanol production from mixtures A and B were

Table 1. Fermentation of L-arabinose, D-xylose and D-glucose by recombinant *Klebsiella oxytoca* strain P2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cell mass\textsuperscript{b} (g/l)</th>
<th>Base consumed\textsuperscript{c} (mmol/l)</th>
<th>Maximum ethanol\textsuperscript{d} (g/l)</th>
<th>Ethanol yield\textsuperscript{e} (g/g S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>3.2 (0.4)</td>
<td>98 (1)</td>
<td>27.2 (0.0)</td>
<td>0.34 (0.0)</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.3 (0.2)</td>
<td>101 (12)</td>
<td>33.3 (2.8)</td>
<td>0.42 (0.03)</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.5 (0.1)</td>
<td>64 (1)</td>
<td>29.8 (0.1)</td>
<td>0.37 (0.0)</td>
</tr>
<tr>
<td>Mixture A (Ara, xyl, glu; 1:1:1)</td>
<td>3.3 (0.7)</td>
<td>94 (10)</td>
<td>29.7 (0.8)</td>
<td>0.37 (0.01)</td>
</tr>
<tr>
<td>Mixture B (Ara, xyl, glu; 1:2:1)</td>
<td>2.8 (0.1)</td>
<td>101 (17)</td>
<td>34.2 (5.9)</td>
<td>0.43 (0.07)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values reported are from duplicate experiments for a single sugar and triplicate experiments for mixture of sugars. The standard deviations are reported in the parenthesis.

\textsuperscript{b} Cell mass was calculated from the maximal OD\textsubscript{560} (approximately 0.32 g dry weight/liter/OD unit).

\textsuperscript{c} Base (KOH) refers to that added automatically to maintain pH at 6.0 during fermentation and is related to the amount of acid produced as a coproduct of fermentation.

\textsuperscript{d} Ethanol values were corrected for dilution by base addition during fermentation.

\textsuperscript{e} Ethanol yield in g per g of substrate available for fermentation.
Fig. 1 (A, B). Ethanol production by *Klebsiella oxytoca* strain P2 from arabinose, xylose and glucose at pH 6.0 and 30°C. Substrate used, 80 g/l. Symbols: o, arabinose; ●, xylose; ▼, glucose; △, mixture A of arabinose, xylose and glucose (1:1:1); □, mixture B of arabinose, xylose and glucose (1:2:1).

0.37 and 0.43 g per g substrate, respectively. The theoretical yield of ethanol from both pentose and hexose sugars is assumed to be 0.51 g ethanol per g sugar. Considerable difference was observed in the amount of base required to maintain pH during fermentation of glucose and other sugars. Base consumption was 64, 98 and 101 mmol/l for fermentation of glucose, arabinose and xylose, respectively (Table 1). Ethanol production from sugar substrates is shown in Fig. 1 (A,B). Growth of the organism and time of maximum alcohol production were influenced by the substrate. Rates of sugar utilization by recombinant *K. oxytoca* strain P2 at pH 6.0 and 30°C are shown in Fig. 2. It is evident that the recombinant organism utilized each individual sugar (glucose, arabinose and xylose) very well (Fig. 2A). With the mixture of sugars, the organism showed a preference for glucose (Fig. 2B,C). In addition, xylose utilization was slow. Approximately 47 and 29% of supplied xylose were left unutilized in mixtures A and B, respectively even after 114 h fermentation. Maximum ethanol yields were 0.44 and 0.50 g per g of sugar utilized from mixtures A and B, respectively. This indicates that ethanol production was better from sugar mixtures than individual sugars alone. The reason of this interactive
Fig. 2 (A, B, C). Time course of substrate utilization by recombinant Klebsiella oxytoca strain P2 at pH 6.0 and 30°C. Substrate used, 80 g/l (total). (A), arabinose, xylose and glucose separately; (B), mixture A of arabinose, xylose and glucose (1:1:1); (C), mixture B of arabinose, xylose and glucose (1:2:1). Symbols: o, arabinose; ●, xylose and ▲, glucose.

relationship is not clear. Also, it is not clear why arabinose was utilized more rapidly than xylose. According to Doran and Ingram (1993), the upper limit for ethanol tolerance in the recombinant organism may be 45-47 g/l. While we have not tried to optimize the conditions for efficient production of ethanol using the recombinant organism, the results illustrate that the organism has the capacity to utilize a variety of sugars including arabinose. Attempts are under
way to determine how the organism performs on various pretreated corn fiber substrates.

REFERENCES


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