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Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11

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Hemicellulose hydrolysates of the agricultural residues bagasse, corn stover, and corn hulls plus fibers were readily fermented to ethanol by recombinant *Escherichia coli* strain KO11. Corn steep liquor and crude yeast autolysate served as excellent nutrients. Fermentations were substantially complete within 48 h, often achieving over 40 g ethanol L^{-1}. Ethanol yields ranged from 86% to over 100% of the maximum theoretical yield (0.51 g ethanol g $sugar^{-1}$).

Keywords: ethanol; *E. coli*; biomass; lignocellulose; pentose; hemicellulose

Introduction

The increasing use of oxygenates as fuel additives provides an opportunity for the large-scale expansion of fuel ethanol production [33]. However, the commercial viability of ethanol production from corn in the United States is dependent on the sale of fiber residues and stillage as an animal feed or co-product [15]. Since the animal feed market is relatively inelastic, any large expansion of the ethanol industry will require development of alternative uses for corn hull and fiber residues or the use of less expensive feedstocks.

Corn hulls and fibers represent 10% of the dry weight of the processed grain from wet milling of which 70% is carbohydrate. Approximately 85% of this carbohydrate is starch and hemicellulose [9,20], potentially useful feedstocks for additional ethanol production [6]. In 1984, Osborn and Chen [30] demonstrated that this material can be readily hydrolyzed by dilute acids and enzymes to produce a mixture of glucose, xylose, arabinose and a small amount of galactose. However, no organisms were available at that time which could rapidly and efficiently convert mixtures of pentose and hexose sugars to ethanol. Subsequently, *Escherichia coli* was genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) from the bacterium *Zymomonas mobilis* [17]. Two ethanologenic strains have been used in subsequent investigations, *E. coli* ATCC 11303 containing the plasmid pLOI297 [1] and strain KO11 [29], a related strain in which the ethanol pathway genes have been integrated into the chromosome. Other bacteria have also been engineered in a similar manner [3,16]. In these organisms, the *Z. mobilis* genes are expressed at high levels, redirecting central metabolism to produce ethanol and carbon dioxide as the primary products of fermentation. A recent study by Zhang *et al* [36] has reported a complementary approach, the genetic engineering of an *E. coli* xylose pathway into *Z. mobilis* strain CP4. However, the sugar range of this organism remains quite limited. No studies have been reported concerning the stability or hardiness of this genetically engineered *Z. mobilis* for bioconversions of lignocellulose hydrolysates.

Several articles have been published which summarize the literature concerning the toxicity of hemicellulose hydrolysates and bioconversion by yeasts and bacteria [7,13,18,27,28,32]. The reader is referred to these for further background information. A recent comparison of yeasts and bacteria using dilute acid hydrolysates of corn cob hemicellulose as a substrate concluded that the recombinant *E. coli* strain KO11 was superior to other pentose-fermenting organisms in ethanol productivity, ethanol yield, and resistance to inhibitors generated during hydrolysis [14]. Additional studies with ethanologenic *E. coli* have reported the successful fermentation of hemicellulose hydrolysates from hardwoods [22,23,35], softwoods [2], newsprint [25], corn cobs [4,24], corn hulls and fibers [4], and orange peels [10]. *E. coli* strain KO11 has the native ability to metabolize all sugars which are constituents of hemicellulose, starch, and pectin. However, expensive laboratory grade complex nutrients were utilized in most studies and attempts to substitute inorganic salts resulted in slower rates and lower yields [12,21]. Three of these feedstocks, corn hulls and fibers, corn stover, and bagasse, could be readily used to expand the capacity of existing ethanol plants with modest capital investment [19].

This report summarizes some of the fermentation results using *E. coli* strain KO11 as the biocatalyst. Hemicellulose hydrolysates were prepared from these three feedstocks.

Materials and methods

Preparation of hemicellulose hydrolysates for fermentation

Hydrolysis was carried out in a continuously rotating reactor with direct steam injection. A typical charge for this

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Table 1 Sugar composition of hemicellulose hydrolysates from agricultural residues

Agricultural residue	Sugar composition (% of total)				Hydrolysate	
	Glucose	Xylose	Galactose	Arabinose	Total sugars (g L^{-1})	Sugar yield (g g substrate^{-1})
Corn hulls + fibers	27	39	11	23	100–140	0.50–0.70
Corn stover	19	61	7	12	80–130	0.22–0.27
Bagasse	6	89	0	14	70–110	0.20–0.25

reactor was 7 kg total weight and included dilute sulfuric acid as the catalyst. Hydrolysis conditions were essentially as reported [4] with minor modifications for each feedstock. These conditions are based on the extensive studies by Grohmann *et al* [11]. Hemicellulose hydrolysate containing dissolved sugars was recovered using a Damon/IEC EXD basket centrifuge (Needham, MA, USA) at $5000 \times g$ and stored at 4°C. Prior to use, hydrolysates were overlimed to pH 9 with calcium hydroxide [8, 26, 31, 34] and adjusted to pH 6.0–6.5 with HCl.

Bacterial strain

E. coli strain KO11 has been previously described [29] and contains genes encoding the ethanol pathway from *Z. mobilis*. This organism was grown in xylose broth (composition per liter: 5 g Difco Yeast Extract (Difco, Detroit, MI, USA), 10 g Difco Tryptone, 5 g NaCl, 50 g xylose, and 40 mg chloramphenicol) and on solid medium (1.5% agar) containing a lower concentration of xylose (20 g L^{-1}). Stock cultures were prepared by diluting cultures with an equal volume of 80% (w/v) glycerol and stored at –80°C.

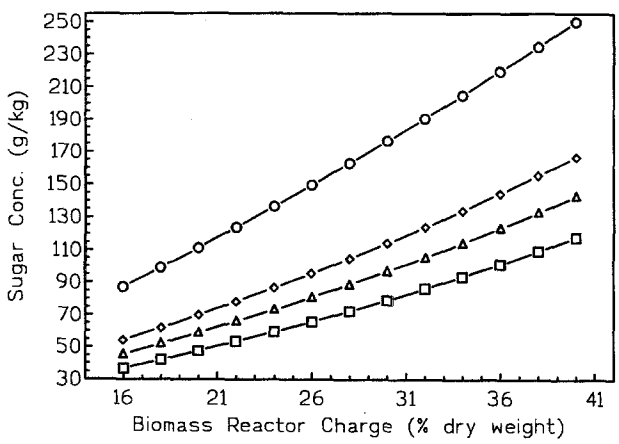

Figure 1 Predicted effect of moisture content in the biomass reactor on the resulting sugar concentrations in hemicellulose hydrolysates. Curves are shown for four different sugar yields which span the range of most lignocellulosic materials. Assumed sugar yield in g sugar g biomass^{-1}: ○, 0.50; ◇, 0.30; △, 0.25; □, 0.20. Approximate sugar in g kg^{-1} = (1000 * sugar yield * %dry weight/100)/[1 – (% dry weight/100)(1 – sugar yield)]. Note that sugar concentration is expressed on a weight/weight basis. Since densities of these sugar solutions are greater than 1.0 g ml^{-1}, sugar concentrations on a volume basis are higher

Preparation of inocula

Fresh colonies were transferred to 250-ml Erlenmeyer flasks containing 50 ml xylose broth and incubated for 12 h at 35°C on a rotary shaker. Resulting cultures were diluted 10-fold into 500-ml fermentors [5] containing xylose broth (lacking chloramphenicol) and incubated for 12 h at 35°C. KOH (2 N) was added automatically to maintain pH 6.0–6.5. When needed, further scale up of inoculum was carried out in a similar manner using large fermentors. In some cases, inocula were also grown in hemicellulose hydrolysate supplemented with Difco nutrients (10 g Tryptone and 5 g Yeast Extract L^{-1}), corn steep liquor (1–5% by volume), or crude yeast autolysate (0.5–2% by volume). Samples of

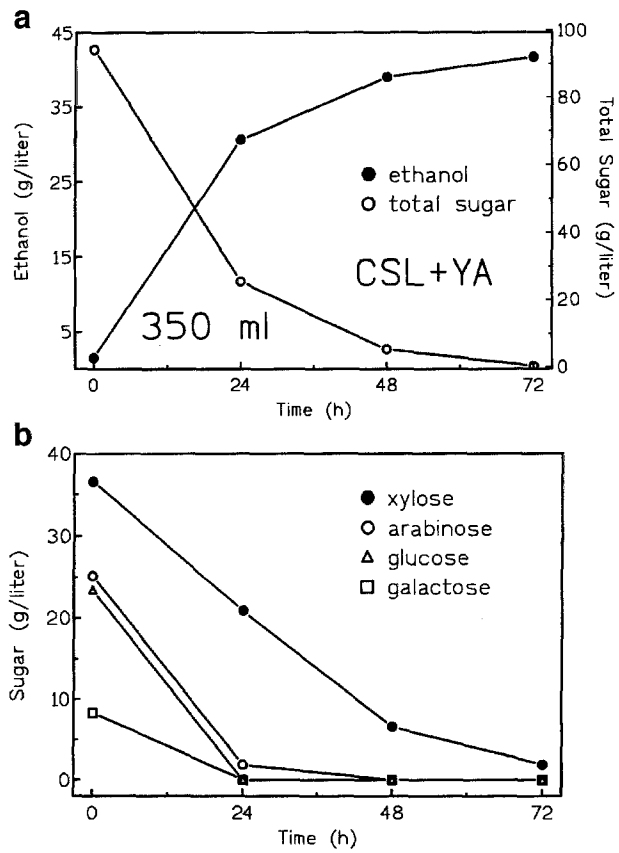

Figure 2 Fermentation of mixed laboratory sugars simulating the composition of hydrolysates from corn hulls and fibers. Corn steep liquor and crude yeast autolysate were used as nutrients with fermentation volumes of 350 ml. *E. coli* strain KO11 was the biocatalyst. (a) Ethanol production; (b) individual sugars

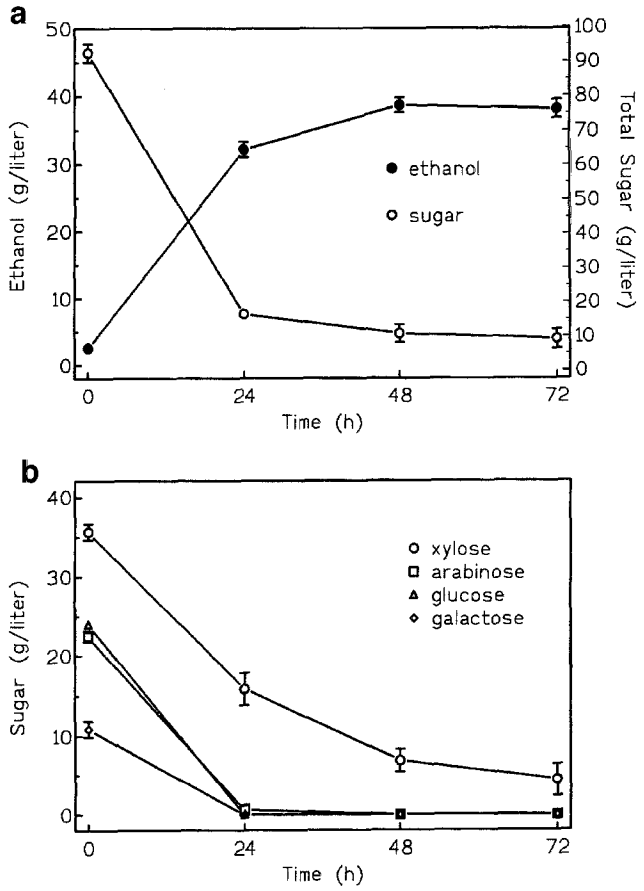

Figure 3 Fermentation of hemicellulose hydrolysates from corn hulls and fibers. Corn steep liquor and crude yeast autolysate served as nutrients with fermentation volumes of 350 ml. Results represent an average of three fermentations and include standard deviations. *E. coli* strain KO11 was the biocatalyst. (a) Ethanol production; (b) individual sugars

corn steep liquor were obtained from four different corn wet millers.

Preparation of crude yeast autolysate

Yeast autolysate (YA) was prepared by incubating a 20% (w/v) suspension of yeast (Fermipan, King of Prussia, PA, USA) in distilled water for 24 h at 48°C. This preparation was stored frozen at −20°C.

Fermentation experiments

Fermentations were conducted at pH 6.0–6.5 and 35°C in a variety of vessels ranging from 500-ml [5] to 200-L. Agitation was provided in the smallest units by a magnetic stirring bar; impellers provided mixing in the larger fermentors. Difco Yeast Extract, Difco Tryptone, corn steep liquor and crude YA were used as nutrients. Except for seed preparation, no antibiotics were added to the fermentations.

Analyses

Cell mass was estimated by measuring optical density at 550 nm. Ethanol was measured using a Varian Star 3400 gas chromatograph (Sunnyvale, CA, USA) equipped with a J&W Scientific DB640 capillary column (Folsom, CA, USA) (80°C, isothermal), autosampler, electronic integrator and FID detector (N_2 carrier gas). Sugar concentrations were measured using a Biorad HPLC (Richmond, CA, USA) equipped with an Aminex HPX-87P column and electronic integrator. Distilled water was used as the mobile phase.

Results and discussion

Sugar composition of hemicellulose hydrolysate derived from agricultural residues

Many excellent studies [11,27,28] have reported the acid concentrations, pHs, temperatures, pressures and times needed to hydrolyze hemicellulose for lignocellulosic materials. With corn hulls plus fibers, corn stover, and bagasse, we were able to produce hemicellulose syrups containing relatively high sugar concentrations (Table 1). As expected, sugar yield from corn hulls and fibers was the highest due to the unusual abundance of starch and hemicellulose [30]. Sugar yields obtained with bagasse and corn stover are representative of plant stems and leaves. Xylose, glucose and arabinose were the most abundant sugars in hemicellulose hydrolysate from all three materials tested.

Sugar yield has a profound effect on the design of reactors for the production of hemicellulose hydrolysates. A beer alcohol concentration of around 40 g L^{-1} is frequently used as a target for biomass fermentations [15,18]. To achieve this, syrups must be produced which contain at least 80 g sugar L^{-1}. Figure 1 shows the predicted relationship between moisture content in the reactor charge and the sugar concentration in the resulting liquid phase after hydrolysis. Note that this is expressed on a weight/weight basis. Sugar concentration per liter is slightly higher (correcting for densities of approximately 1.05 g ml^{-1} for 100 g sugar L^{-1}). Thus a very high solids content is required to produce hemicellulose syrups of over 80 g L^{-1} from substrates such as bagasse and corn stover. For materials such as stems and leaves, at least 1/3 of the sugar will remain trapped after an initial solids separation. Thus some form of washing will also be required and will further dilute the sugars. In contrast, the high sugar yields from corn hulls and fibers make these residues a near ideal starting material.

Fermentation of hemicellulose hydrolysate from corn hulls and fibers

The fermentation of hydrolysates from corn hulls and fibers represents the most challenging of the three substrates examined with respect to sugar composition. No single sugar dominates this hydrolysate and the biocatalyst must be able to switch between different pathways for sugar metabolism at various stages of fermentation. Figure 2a,b shows the fermentation of a sugar mixture simulating this hydrolysate using industrial nutrients. Glucose, galactose, and arabinose were preferentially metabolized. These three sugars were essentially absent after 24 h of fermentation. Xylose was metabolized more slowly with approximately 2 g L^{-1} remaining after 72 h. These results demonstrate that *E. coli* strain KO11 can metabolize a complex mixture of sugars using corn steep liquor as the primary nutrient.

Hydrolysates prepared from corn hulls and fibers were also investigated using industrial nutrients. Similar levels of ethanol were produced with these hydrolysates (Figure 3a,b; Table 2). However, 8–10 g xylose L^{-1} remained after

Table 2 Ethanol production from hemicellulose hydrolysates by *E. coli* strain KO11

Nutrients	Fermentation volume (L)	Sugar (g L^{-1})	Base[a] (ml L^{-1})	Max EtOH (g L^{-1})	Time (h)	Total EtOH[b] (g L^{-1})	Q_p (gE L^{-1} h^{-1})	Yield (gTE gS^{-1})	Conver.[c] (%)
Mixed sugars simulating corn hull hemicellulose hydrolysate									
CSL, YA	0.35	96	67	41.7	72	44.5	0.62	0.46	90
Corn hulls plus fibers									
CSL, YA	0.35	94	27	44.0	72	45.0	0.63	0.48	94
CSL, YA	0.35	94	27	42.1	72	43.2	0.60	0.46	90
Corn stover									
Difco	0.35	77	10	37.9	36	39.0	1.08	0.51	100
CSL, YA	0.35	75	8	38.1	48	39.0	0.81	0.52	103
CSL, YA	11.0	81	nm	37.5	60	37.5	0.63	0.46	90
CSL, YA	1.6	80	0	42.4	48	42.4	0.88	0.53	104
CSL, YA	25.0	69	nm	35.0	48	35.0	0.73	0.51	100
Difco	150.0	90	nm	40.8	48	40.8	0.85	0.45	88
Bagasse									
Difco	0.35	80	nm	35.0	72	35.0	0.49	0.44	86
YA	0.35	81	nm	35.4	72	35.4	0.49	0.44	86
CLS, YA	0.35	80	nm	36.9	60	36.9	0.62	0.46	90
CSL, YA	25.0	71	nm	36.5	48	36.5	0.61	0.51	100
Difco	1.6	75	nm	36.2	48	36.2	0.75	0.48	94

Abbreviations: Difco, 5 g Difco Yeast Extract and 10 g Difco Tryptone L^{-1}; CSL, corn steep liquor; YA, crude yeast autolysate; Max EtOH, highest concentration of ethanol achieved during fermentation; nm, not measured; Q_p, average volumetric productivity calculated by dividing total ethanol by fermentation time; gTE gS^{-1}, g total ethanol divided by the grams of sugar
[a]2 N KOH consumed to maintain pH 6.0–6.5 during fermentation
[b]Corrected for dilution by base
[c]Conver (%), ethanol yield (g g^{-1}) divided by 0.51 × 100

72 h despite the high yields per gram of sugar. Based on subsequent studies with other substrates, it is likely that this problem of incomplete xylose utilization with hydrolysate can be solved by reducing the initial sugar concentration below 85 g L^{-1}.

Fermentation of hemicellulose hydrolysate from corn stover

Corn stover hemicellulose hydrolysate was an excellent substrate for fermentation (Figure 4; Table 2). Similar results were obtained with fermentation volumes from 0.35 L to 150 L. Corn steep liquor and crude yeast autolysate were equivalent to Difco products as nutrients. The fermentation of all sugars was essentially complete within 48 h, reaching final ethanol concentrations of around 40 g L^{-1}. Although considerable study has been devoted to inhibitors generated during the production of acidic hemicellulose hydrolysates [27,28], a simple liming procedure was sufficient to remove inhibitors and allow bioconversion by *E. coli* strain KO11. Yields in these fermentations approached or exceeded the theoretical maximum, 0.51 g ethanol g $sugar^{-1}$.

Fermentation of hemicellulose hydrolysate from bagasse

Hemicellulose hydrolysate syrups from bagasse were readily fermented to ethanol by *E. coli* strain KO11 (Table 2; Figure 5). The small amounts of glucose and arabinose were metabolized within the first 24 h. Complete utilization of xylose required 48–72 h. The larger scale fermentations (1.6-L and 25-L) performed as well or better than the early studies with 0.35-L fermentors. Again, fermentations containing industrial nutrients were equivalent to those with Difco products. Ethanol yields were from 86% to 100% of the theoretical maximum.

Conclusions

1) *E. coli* strain KO11 can efficiently metabolize complex mixtures of sugars derived from the acid hydrolysis of lignocellulosic biomass. 2) Inexpensive materials such as crude yeast autolysate and corn steep liquor can be used effectively as nutrients for this organism. Performance of fermentations with these industrial nutrients was equivalent to Difco nutrients for hemicellulose hydrolysates with *E. coli* strain KO11 as the biocatalyst. 3) For hemicellulose hydrolysates from most agricultural residues, beer ethanol concentrations will be limited by sugar concentration rather than the ethanol tolerance of *E. coli* strain KO11. Beer ethanol levels of 45 g L^{-1} were achieved in hemicellulose hydrolysates. 4) Corn hulls and fibers contain an unusually high level of carbohydrates which can be converted to monomers by dilute acid. This material represents an excellent feedstock for the expansion of grain-based ethanol production.

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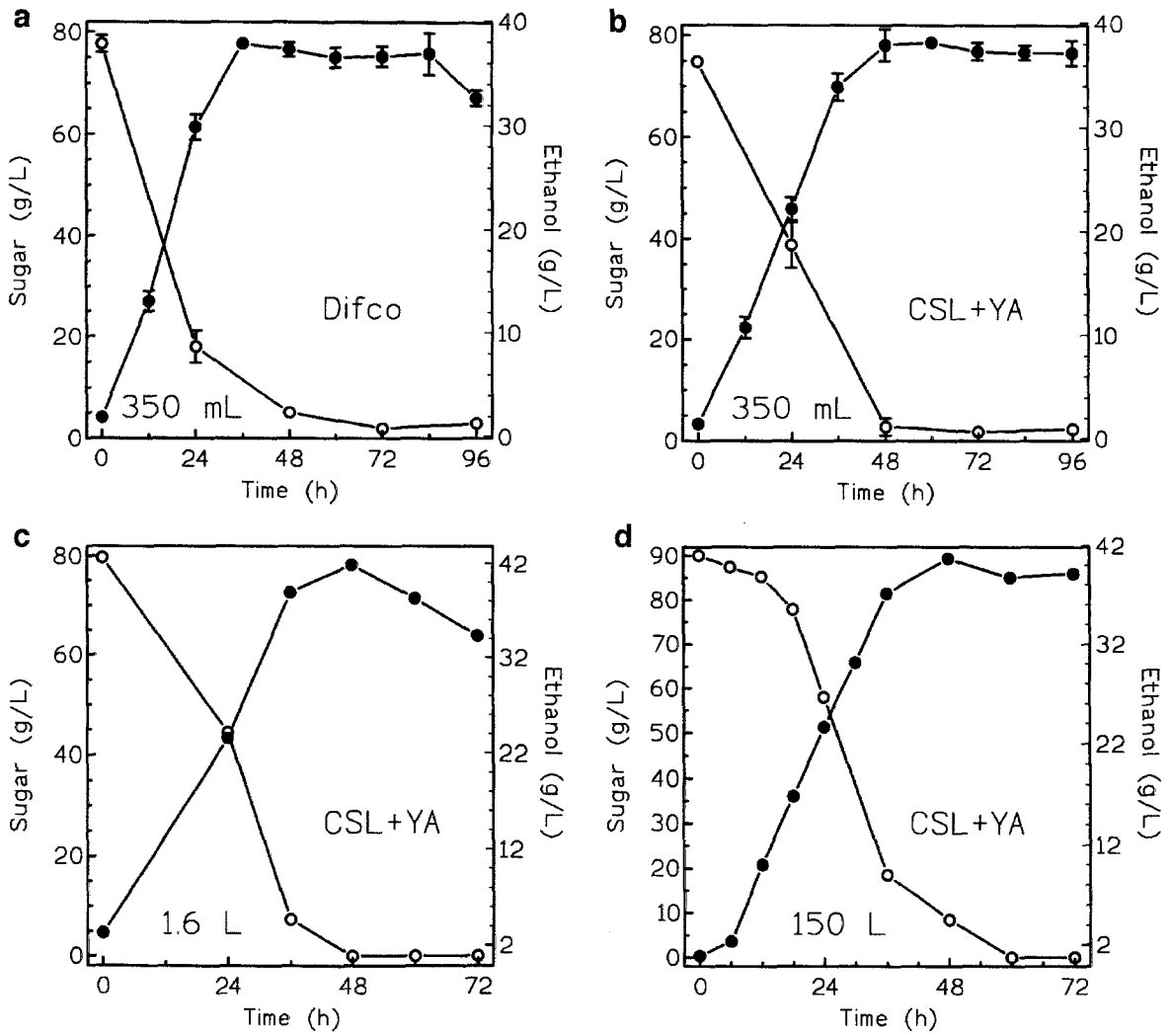

Figure 4 Fermentation of hemicellulose hydrolysates from corn stover using *E. coli* strain KO11. Standard deviations are shown for (a) and (b) ($n = 3$). (a) Difco nutrients, 350-ml fermentation volume; (b) corn steep liquor plus yeast autolysate as nutrients, 350-ml fermentation volume; (c) corn steep liquor plus yeast autolysate as nutrients, 1.6-L fermentation volume; (d) corn steep liquor plus yeast autolysate as nutrients, 150-L fermentation volume. ●, Ethanol; ○, sugar

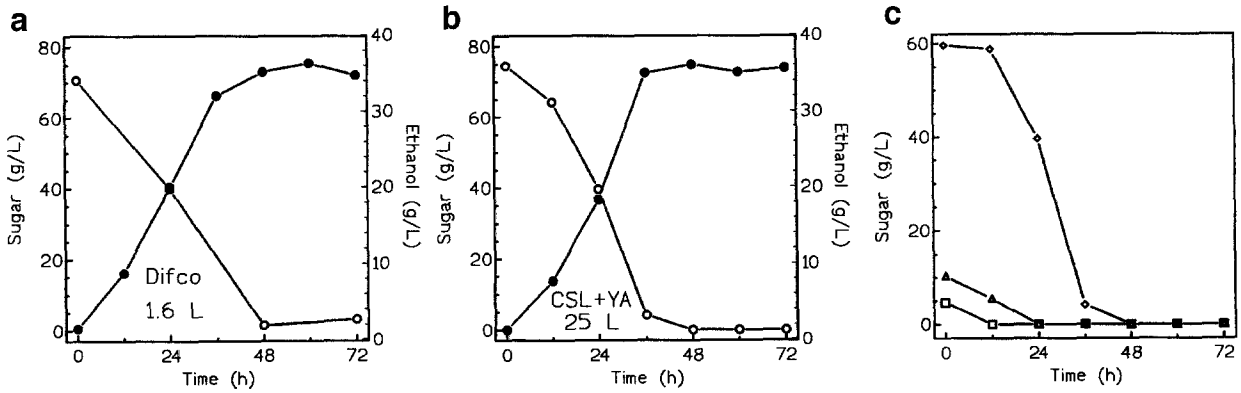

Figure 5 Fermentation of hemicellulose hydrolysates from sugar cane bagasse using *E. coli* strain KO11 as the biocatalyst. (a) Difco nutrients, 1.6-L fermentation volume; (b) corn steep liquor plus yeast autolysate as nutrients, 25-L fermentation volume; (c) sugars from (b). ●, Ethanol; ○, sugar; ◇, xylose; △, arabinose; □, glucose

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