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Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production

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Genetically engineered *Escherichia coli* KO11 is capable of efficiently producing ethanol from all sugar constituents of lignocellulose but lacks the high ethanol tolerance of yeasts currently used for commercial starch-based ethanol processes. Using an enrichment method which selects alternatively for ethanol tolerance during growth in broth and for ethanol production on solid medium, mutants of KO11 with increased ethanol tolerance were isolated which can produce more than 60 g ethanol L^{-1} from xylose in 72 h. Ethanol concentrations and yields achieved by the LY01 mutant with xylose exceed those reported for recombinant strains of *Saccharomyces* and *Zymomonas mobilis*, both of which have a high native ethanol tolerance.

Keywords: ethanol; ethanol tolerance; fermentation; xylose; biomass; lignocellulose

Introduction

The conversion of renewable biomass into fuel ethanol offers the potential to reduce imported petroleum while providing many environmental benefits [10,25,27]. Continuing research is directed at reducing the cost of proposed processes through improvement in the biocatalyst. Over one billion gallons of fuel ethanol are produced annually in the US from starch-derived glucose using *Saccharomyces* yeasts, approximately 1% of the total automotive fuel [32]. Commercial conversion of glucose to ethanol by yeast benefits from the ethanol tolerance of this biocatalyst with product concentrations exceeding 90 g L^{-1} [36]. This high product concentration is an important factor in minimizing the cost of purification, nutrients, and equipment.

New biocatalysts are being developed for the fermentation of biomass-derived pentose sugars such as xylose, the most abundant component of hemicellulose [11,19,23,28,37,39]. Considerable progress has been made using four different recombinant approaches with patents issued for two [18,20,33] and others pending: (1) the transfer of genes encoding the ethanol pathway from *Zymomonas mobilis* into enteric bacteria which have the native ability to metabolize all pentose (xylose and arabinose) and hexose sugar constituents [4,23,38]; (2) the transfer of xylose utilization genes from *Escherichia coli* into *Z. mobilis*, a native ethanol producer [39]; (3) the transfer of xylose utilization genes from pentose metabolizing yeast into *Saccharomyces* [28]; and (4) the transfer of xylose utilization genes from thermophilic bacteria into *Saccharomyces* [37]. The microbial platforms used in the latter three approaches have higher native resistance to ethanol during glucose fermentation than enteric bacteria [7,9,14,26], a potential advantage.

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Many studies [9,21,35] have identified the permeability barrier provided by the cell membrane as the primary site of ethanol damage. Exposure to ethanol also induces stress proteins (chaperonins) which may aid protein folding and contribute to survival [3,30]. However, over-expression of native *E. coli* or *Z. mobilis* stress genes did not increase the final concentrations of ethanol produced by strain KO11 (ethanologenic derivative of *E. coli* B; [31]) under conditions in which sugar was not a limiting factor (Ingram, unpublished).

In this study, we describe a novel selection method using both solid and liquid medium which allowed the isolation of KO11 mutants that have the ability to produce over 60 g ethanol L^{-1} (7.5% w/v; 1.3 molar) from 140 g xylose L^{-1}.

Materials and methods

Bacterial strains and media

E. coli KO11 and three ethanol-resistant mutants of this strain (LY01-LY03) were investigated. Strain KO11 is an ethanol-producing recombinant in which the *Z. mobilis* genes for ethanol production (*pdc*, *adhB*) and the *cat* gene (immediately downstream) have been integrated into the *E. coli* B chromosome [31]. In this strain, resistance to chloramphenicol (600 mg L^{-1}) was used to select for increased expression of *pdc*, *adhB* and *cat*. Cultures were grown in modified Luria-Bertani (LB) broth [2] containing per liter: 5 g NaCl, 5 g yeast extract, 10 g tryptone, chloramphenicol (0, 40 or 600 mg), and sugar (20–140 g). Stock cultures of alcohol-resistant mutants were maintained on solid medium containing: glucose (20 g L^{-1}), chloramphenicol (600 mg L^{-1}), isopropanol (10 g L^{-1}), and agar (15 g L^{-1}). Isopropanol was used in solid medium instead of ethanol due to its lower volatility. Ethanol was added to LB broth on a weight basis to prepare a stock solution (100 g kg^{-1}) which was diluted as necessary for growth and survival experiments. LB broth containing ethanol was filter sterilized using 0.45-μm filters.

Isolation of KO11 mutants with increased ethanol tolerance

Mutants of KO11 were selected by diluting broth cultures (1:20 to 1:2000) into 10 ml of fresh LB broth containing ethanol and glucose (50 g L^{-1}). Subcultures (10 ml in 18 × 150-mm culture tubes) were incubated for 24 h or longer at 35°C without agitation to permit growth. As cell density increased in successive transfers, ethanol concentrations were increased. After every 3–4 liquid transfers, cultures were diluted and spread on solid medium (LB containing 20 g glucose L^{-1} and 600 mg chloramphenicol L^{-1}) and large, raised colonies selected to enrich for the subset of ethanol-resistant mutants which grew rapidly and retained efficient ethanol production. Ethanol-resistant mutants were stored on plates containing 1% isopropanol (30 °C) and in 40% glycerol at −75°C.

Effects of ethanol and sugars on growth

Culture tubes were prepared with 10 ml of LB broth containing 50 g glucose L^{-1} and various concentrations of ethanol, or various concentrations of sugar without ethanol. These were inoculated to an initial $OD_{550\,nm}$ of 0.05 and incubated at 35°C without agitation. Cell mass as $OD_{550\,nm}$ was monitored during incubation and reported either as a growth curve or as growth after 24 h.

Survival in LB containing 10% (w/v) ethanol

Survival of mutant strains was compared to KO11 after dilution into LB broth (50 g glucose L^{-1}) containing 100 g ethanol L^{-1}, a concentration at which growth is blocked. Cell suspensions (approximately 0.05 OD at 550 nm) were prepared in LB broth using cells from overnight plates. These were preheated to 35°C and mixed at time zero with an equal volume of preheated broth containing 200 g ethanol L^{-1}. Broth lacking ethanol served as a control. Serial dilutions were spread on solid medium at zero time (no ethanol only), 0.5 min, and 5 min. Colonies were counted after overnight incubation at 30°C to determine survival as colony forming units (CFU). Results are expressed as a percentage of the control which lacked ethanol.

Fermentation

Inocula were grown for 16 h (30°C) without agitation in LB broth containing glucose or xylose (50 g L^{-1}). Cells were harvested by centrifugation (6000 × *g*, 5 min, ambient temperature). Fermentation was initiated by adding sufficient cells to provide 1.0 OD at 550 nm (approximately 330 mg L^{-1}, dry cell weight). Batch fermentations were conducted at 35°C (100 rpm) in modified 500-ml Fleaker™ beakers containing 350 ml of LB broth supplemented with glucose or xylose (90 or 140 g L^{-1}) [5]. Sugar solutions were sterilized by autoclaving separately. Automatic addition of 2 N KOH was used to prevent acidification below pH 6. Increases in pH which occur at the end of fermentation were not regulated. Samples were removed to measure cell mass and ethanol concentration. Base consumption and pH were also recorded.

Analytical procedures

Cell density was measured at 550 nm using a Bausch & Lomb Spectronic 70 spectrophotometer (Union, NJ, USA) and converted to dry cell weight based on a standard curve for KO11. Ethanol was measured by gas chromatography with n-propanol as an internal standard [38] using a Varian Star 3400 CX gas chromatograph (Santa Clarita, CA, USA).

Results

Isolation of ethanol-tolerant mutants of E. coli KO11

A combination liquid and solid medium was used to successfully enrich for strains with mutations which increase ethanol tolerance and maintain the genetically engineered trait of efficient ethanol production (Figure 1). KO11 was initially transferred into LB broth containing 35 g ethanol L^{-1} and grew poorly. After five sequential transfers, the ethanol concentration in the broth was increased to 40 g L^{-1}; after 13 transfers, the ethanol concentration was increased to 45 g L^{-1}; after 14 transfers, the ethanol concentration was increased to 50 g L^{-1}. Dilution and plating on solid media was interspersed between liquid transfers after every 3–4 transfers to enrich for strains which retained ethanol production (large, raised colony phenotype for high ethanol production). Cultures were maintained in 50 g ethanol L^{-1} for 3 months before clones were selected. Dilution into higher concentrations of ethanol did not yield mutants with further increases in ethanol resistance which also retained rapid growth and efficient ethanol production, although attempts to select even more resistant organisms are being continued. It should be noted that additional ethanol (10–20 g L^{-1}) is produced during fermentative growth and provides further selective pressure.

Cultures which grew in 50 g ethanol L^{-1} were diluted

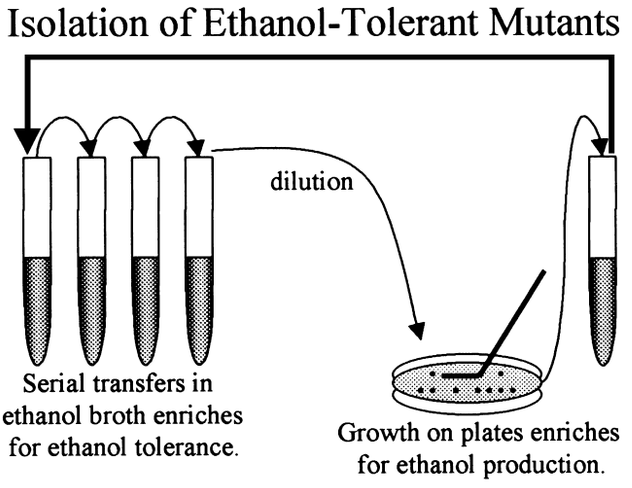

Figure 1 Procedure used for the isolation of ethanol-tolerant mutants. Strain KO11 was inoculated into broth and transferred serially into increasing concentrations of ethanol, as growth permitted, to select for the ability to grow in the presence of ethanol and spread on LB plates containing chloramphenicol (600 mg L^{-1}) to enrich for large raised colonies which produce neutral rather than acidic fermentation products and express high levels of chloramphenicol acyl transferase. Large raised colonies (high ethanol production) were harvested and used to inoculate ethanol broth for continuing selection. After many cycles over a 3-month period, individual clones were selected and tested for their ability to grow in the presence of ethanol. The best clones were designated LY01, LY02, and LY03.

and spread on solid medium containing high chloramphenicol to allow isolation of individual clones. Large raised colonies were further purified on plates. To test for ethanol resistance, single colonies were screened for growth in comparison to the parent. Of the 138 colonies tested, 128 grew in the presence of 45 g ethanol L^{-1}, well above the maximum concentration permitting growth of the parent KO11 (35 g L^{-1}). Twenty clones were tested for ethanol production in pH-controlled fermentors. Three clones were selected for further study: LY01, LY02, and LY03.

Ethanol tolerance

Mutants LY01, LY02, and LY03 were more resistant to growth inhibition by ethanol than the parent KO11 (Figure 2a) although all grew more slowly than the parent in broth lacking ethanol (Figure 2b). The initial growth of these mutants in ethanol broth was identical. Data are shown for one mutant (LY01) and the parent (Figure 2c and d). Ethanol resistance was equivalent with either glucose or xylose as the fermentable sugar. LY01 grew slowly in the presence of 50 g ethanol L^{-1} with a 4–6 h doubling time while KO11 failed to increase in optical density during incubation in LB broth containing 35 g ethanol L^{-1} during 6 h of incubation. With 35 g ethanol L^{-1}, LY01 grew with a doubling time of approximately 2.5–3.0 h. Growth with glucose was consistently faster than with xylose, with or without added ethanol. Similar ethanol tolerance was retained with all fermentable sugars examined, including lactose, arabinose, mannose, galactose, sucrose and raffinose (data not shown). These results indicate the absence of mutations which are detrimental to sugar uptake or metabolism.

Survival during brief exposure to a high concentration of ethanol (100 g ethanol L^{-1}) was investigated in the absence of measurable growth (Figure 3). The three mutants exhibited over 50% survival (CFU) during a 0.5-min exposure as compared to less than 10% survival for the parent, KO11. Differences were less dramatic after 5 min of exposure although the mutants were clearly more resistant than KO11.

The stability of the ethanol-tolerance trait in LY01, LY02, and LY03 was examined after 30 daily transfers on solid medium lacking alcohol. Cultures were tested in LB broth containing 35–50 g ethanol L^{-1} (50 g glucose L^{-1}). Results were identical to those described previously in Figure 2b and c, indicating that ethanol-selection was not

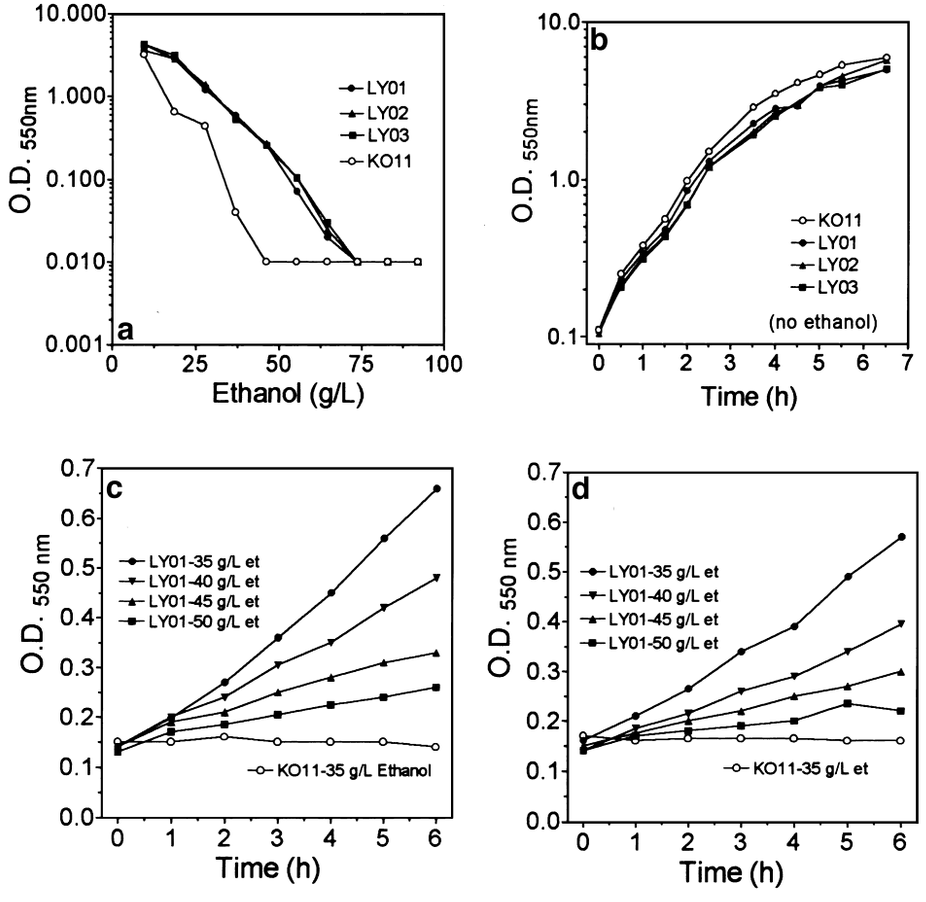

Figure 2 Effect of ethanol on growth. (a) Growth after 24 h (glucose); (b) Initial growth in the absence of ethanol (glucose); (c) Initial growth with glucose in the presence of ethanol; (d) Initial growth with xylose in the presence of ethanol.

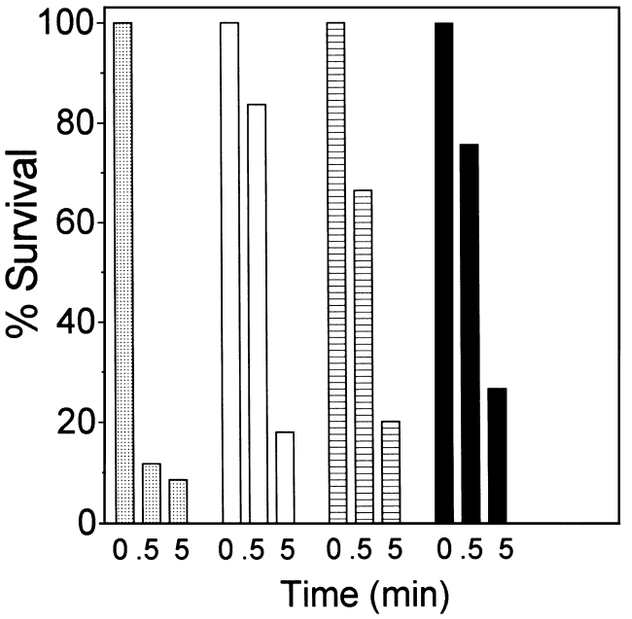

Figure 3 Survival in broth during exposure to a high concentration of ethanol (100 g L^{-1}). ▨, KO11; □, LY01; ▤, LY02; ■, LY03.

essential to maintain resistance. However, as a precaution, ethanol-resistant mutants were maintained on solid medium supplemented with 10 g isopropanol L^{-1}.

Osmotic tolerance and thermal tolerance

The retention of osmotic tolerance to sugars is essential for the fermentative production of high levels of ethanol and the utility of ethanol-tolerant mutants. As illustrated in Figure 4, tolerance to xylose and glucose were similar on a molar basis. With both sugars, KO11 appeared slightly more resistant to osmotic stress than the ethanol-resistant mutants. Thermal tolerance was also investigated and no differences were observed during growth at 48°C. Both KO11 and the mutants grew slowly at this temperature (data not shown). These studies indicate that neither osmotic tolerance nor thermal tolerance were greatly impaired during selection for increased tolerance to ethanol.

Fermentation of sugars to ethanol

Twenty of the most promising mutants were initially screened for their ability to produce ethanol from 140 g glucose L^{-1} and 140 g xylose L^{-1} in pH-controlled fermentors (data not shown). All but three of these were superior to KO11. Results for the three best mutants are shown in Figure 5. LY01 produced ethanol more rapidly during the fermentation of 140 g sugar L^{-1}, reached higher final ethanol concentrations, and achieved higher yields than the parent with xylose or glucose (Table 1). Cell mass produced during the fermentation was consistently higher with these mutants than with KO11. Base consumed for the neutralization of organic acids and dissolved CO_2 was higher for glucose than xylose, presumably due to a higher rate of CO_2 generation during glucose fermentation. Roughly equivalent amounts of KOH were required to maintain pH 6.0 for KO11 and the mutants.

The addition of base resulted in a small dilution of product. To facilitate the calculation of yield, total ethanol produced ($Ethanol_P$) per initial liter of fermentation broth was corrected for dilution (Table 1) by multiplying the maximum measured concentration of ethanol ($Ethanol_M$) by the dilution factor (D_f) resulting from the addition of base according to the following equation:

$$Ethanol_P = Ethanol_M \times D_f.$$

Since pH was maintained with 2 N KOH, added base in milliliters is equal to half the mmoles required for pH adjustment (Table 1):

$$D_f = (1000 + \tfrac{1}{2}\ \text{mmoles KOH}) \div 1000.$$

Thus after 96 h, the ethanol yield for KO11 with 140 g sugar L^{-1} was 74% and 80% of the theoretical maximum (0.51 g ethanol per g hexose or pentose) for glucose and xylose, respectively. With xylose, LY01 achieved 85% of the maximum theoretical yield of ethanol after 72 h and reached a final ethanol concentration of over 60 g L^{-1} (7.5% ethanol by volume). With glucose, 96 h was required to achieve a similar yield.

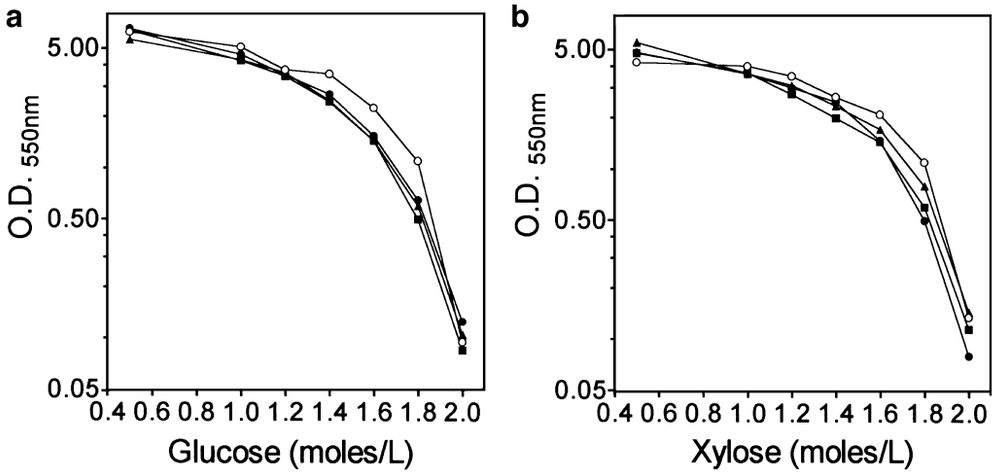

Figure 4 Osmotic tolerance of growth after 24 h. (a) Glucose; (b) Xylose. —○—, KO11; —●—, LY01; —▲—, LY02; —■—, LY03.

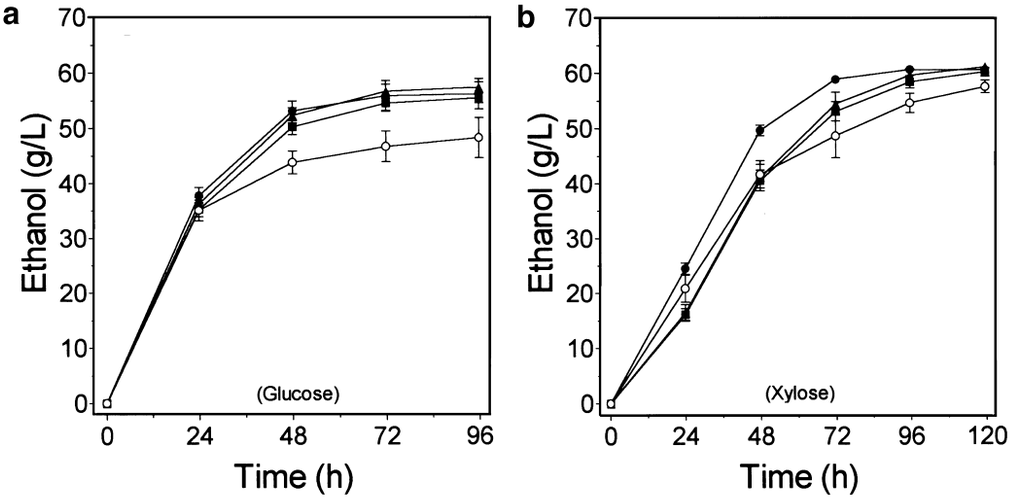

Figure 5 Ethanol production during fermentation of LB broth containing 140 g sugar L^{-1}. Bars represent standard deviations. (a) Glucose. (b) Xylose. —○—, KO11; —●—, LY01; —▲—, LY02; —■—, LY03.

Table 1 Comparison of glucose and xylose fermentation by engineered strains of bacteria and yeasts

Strain	Sugar ($g\ L^{-1}$)	Replicates	Cell mass[a] ($g\ L^{-1}$)	Base ($mmol\ L^{-1}$)	Ethanol produced[b]		Yield[c] (% theor.)
					Time (h)	Amount ($g\ L^{-1}$)	
KO11	glu (140)	9	2.8 ± 0.9	186 ± 32	96	52.7 ± 3.6	73.8 ± 5.0
LY01	glu (140)	3	4.0 ± 0.8	171 ± 30	96	61.0 ± 3.3	85.4 ± 4.7
LY02	glu (140)	7	3.1 ± 0.7	135 ± 64	96	61.1 ± 1.9	85.6 ± 2.6
LY03	glu (140)	7	3.6 ± 0.7	153 ± 65	96	59.6 ± 1.9	83.4 ± 2.6
KO11	xyl (140)	8	3.1 ± 0.5	105 ± 12	120	59.5 ± 2.3	83.3 ± 3.2
LY01	xyl (140)	6	3.6 ± 0.4	80 ± 4	96	63.2 ± 1.0	88.5 ± 1.6
LY02	xyl (140)	6	3.3 ± 0.4	89 ± 11	120	63.8 ± 1.6	89.4 ± 2.3
LY03	xyl (140)	6	3.5 ± 0.3	95 ± 10	120	62.9 ± 1.3	88.1 ± 1.9
KO11	xyl (90)	6	3.2 ± 0.2	64 ± 10	48	41.0 ± 0.5	89.3 ± 1.2
LY01	xyl (90)	5	3.8 ± 0.3	54.9 ± 9	48	42.4 ± 0.7	91.9 ± 1.5
Zm CP4[d] (pZB5)	xyl (52)				15	25	94
Sac 1400[e] (pLNH33)	xyl (52)		9.4		36	20.5	78
Sac 1400[e] (pLNH33)	xyl (~193) fed batch				193	50.3	51
Sac 1400[e] (pLNH33)	glu excess fed batch		~13		67	133	
*Sc*H158[f] pBXI	xyl (29)				10	~2	~14

[a]Cell dry weight calculated from maximum $OD_{550\ nm}$ (0.33 g dry weight per liter at 1.0 OD).
[b]Ethanol produced per liter of fermentation broth, corrected for dilution by base (2N KOH).
[c]Theoretical yield of ethanol from both xylose and glucose is 0.51 g ethanol g^{-1} sugar.
[d]Values reported for genetically engineered *Z. mobilis* harboring plasmid pZB5 containing pentose utilization genes from *E. coli* [39].
[e]Values reported or estimated (~) for genetically engineered *Saccharomyces* strain 1400 harboring plasmid pLNH33 containing xylose utilization genes from *Pichia stipidis*, a pentose-fermenting yeast [28].
[f]Values reported or estimated (~) for genetically engineered *S. cerevisiae* strain H158 harboring plasmid pBXI containing a xylose isomerase gene from the thermophilic bacterium, *Thermus theromophilus* [37].

Discussion

Ethanol-resistant mutants of *E. coli* have been previously isolated after nitrosoguanidine mutagenesis by three successive serial transfers in broth containing 38 g ethanol L^{-1} [9]. Although these mutants were clearly more ethanol-resistant than the K12 parent, higher resistance to ethanol is needed in biocatalysts used for commercial ethanol production. Previous investigations into phosphonomycin-resistant mutants of *E. coli* KO11 (ethanologenic derivative of *E. coli* B) resulted in the fortuitous recovery of mutants such as strain SL40 which produced ethanol more rapidly than the parent and achieved a maximum ethanol concentration of 56 g L^{-1} [29]. Previous attempts with liquid enrichment alone successfully isolated ethanol-tolerant mutants of KO11 but these mutants failed to fully retain ethanol production capability (Ingram, unpublished data). Although the reason for this is unclear, the respiratory system is functional and we suspect that the dissolved oxygen in fresh medium during serial transfers and mixing may provide an initial growth advantage for cells with reduced fermentative capacity (reduced levels of *pdc* or *AdhB*). The new approach used in this study alternated between selection for ethanol resistance in broth and selection for rapid growth on solid medium containing a high level of chloramphenicol, and yielded strains which are better suited for ethanol production. Inclusion of the solid medium step took advantage of the apparent linkage between expression of the *pdc* and *adhB* genes to that of the *cat* gene immediately downstream [31], and the tendency of acid production to limit colony size (and cells per colony) in clones with reduced ethanol production [22,23]. The colony size of native *E. coli* on solid medium containing a fermentable sugar is limited by acidification of the environment with lactic and acetic acids. Highly ethanologenic clones produce little acid to limit growth and thus form larger, more elevated colonies than clones in which ethanol production has been partially replaced by organic acids as products of fermentation [22,23]. The high level of chloramphenicol (600 mg L^{-1}) in these plates provides a second basis for selection. Clones in which expression of ethanologenic genes is reduced often also have reduced expression of the downstream *cat* gene. Thus pooling large colonies from plates, harvested by scraping into fresh LB broth, served to enrich for clones in which the traits of rapid growth, efficient ethanol production, and high level expression of *cat* (also *pdc* and *adhB* which are upstream) have been retained.

The individual mutants selected for evaluation (large, raised colonies) all retained the rapid growth and homoethanol traits of the parent. Strain LY01 appears to be the best KO11 mutant for xylose fermentation. In comparison to the parent, LY01 was more resistant to growth inhibition by ethanol, exhibited increased survival during exposure to high ethanol, and continued to metabolize sugars to produce higher product concentrations during fermentation.

The biochemical and genetic basis for increased ethanol tolerance in *E. coli* mutants remains unknown but is of considerable interest. The slow progressive improvement of ethanol tolerance during enrichment is consistent with a requirement for multiple mutations. Single step attempts failed to obtain clones capable of growth in 50 g ethanol L^{-1}. Previous studies have concluded that ethanol damages the permeability barrier provided by the cell membrane [9,12,34,35]. Increases in fatty acid chain length [15,24] and *trans*-fatty acids [12,24,34], changes in phospholipid composition [6,17,34], and an increase in the proportion of membrane protein [8,17] have been shown to correlate with increased ethanol tolerance in bacteria. Peptidoglycan synthesis also appears to be damaged by ethanol in some strains of *E. coli* [9,16]. Oxidative growth in the presence of high ethanol-concentrations or other chaotropic agents induced changes in morphology (filament formation) and cell lysis similar to effects caused by penicillin. Since these changes were partially antagonized by antichaotropic salts, ethanol-induced filamentation and lysis were attributed to a weakening of critical hydrophobic associations in protein complexes which assemble peptidoglycan [16,24].

Over 10 years ago, genes encoding the fermentation pathway from *Z. mobilis* were used to replace the two native acidogenic pathways (acetic and lactic acids) in *E. coli* and thus create a recombinant biocatalyst capable of efficiently fermenting all sugar constituents of hemicellulose to ethanol [23]. Since then, a variety of alternative approaches have been pursued to develop additional biocatalysts for the fermentation of xylose, the most abundant component of hemicellulose [28,37,39]. These new approaches have all focussed on the use of a homoethanol producer with much higher native alcohol tolerance than *E. coli* [13,14] and the addition of recombinant genes for xylose utilization. The best available published data for xylose fermentation by these alternative biocatalysts are included in Table 1 for comparison to KO11 and the ethanol-resistant mutant LY01. Ethanol-concentrations reported for engineered strains of *Z. mobilis* [39] and *Saccharomyces* [28] strains during the fermentation of xylose are far below those which can be achieved by these organisms with glucose. Thus far, ethanol concentrations achieved by KO11 with xylose remain higher levels than those reported for all other biocatalysts. The ethanol tolerances of KO11 and the ethanol-resistant mutants were essentially independent of the fermentable sugar being metabolized. Strain LY01 appears to be the best KO11 mutant for xylose fermentations. In simple batch fermentations with xylose alone, the maximum level of ethanol produced by LY01 was over 2.5-fold higher than that produced by *Z. mobilis* (pZB5), *Saccharomyces* 1400(pLNH33), and *S. cerevisiae* (pBXI). Ethanol yields per gram of xylose (0.45–0.47) for LY01 exceeded those of engineered yeasts (0.07–0.39) but were lower than reported for *Z. mobilis* (0.48) during the fermentation of a low xylose concentration (52 g L^{-1}). With near equal mixtures of xylose and glucose, however, *Saccharomyces* 1400(pLNH33) [28] can achieve higher levels than can currently be produced by LY01 with individual sugars or sugar mixtures.

The economical production of hemicellulose hydrolysates which contain high sugar concentrations remains an engineering challenge for biomass conversion [1,25]. With plant residues and hardwoods, pentose sugars (xylose and arabinose) dominate as constituents (>85% of total). As more concentrated syrups from biomass become available, LY01 should allow the commercial production of ethanol

concentrations equivalent to those currently produced from cane (100–140 g hexose L^{-1}) syrups by yeasts.

Acknowledgements

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