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ORIGINAL PAPER

Adaptive evolution of nontransgenic *Escherichia coli* KC01 for improved ethanol tolerance and homoethanol fermentation from xylose

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Abstract Due to its excellent capability to ferment five-carbon sugars, *Escherichia coli* has been considered one of the platform organisms to be engineered for production of cellulosic ethanol. Nevertheless, genetically engineered ethanologenic *E. coli* lacks the essential trait of alcohol tolerance. Development of ethanol tolerance is required for cost-effective ethanol fermentation. In this study, we improved alcohol tolerance of a nontransgenic *E. coli* KC01 (*ldhA pflB ackA frdBC pdhR*::*pflB*p6-*aceEF-lpd*) through adaptive evolution. During ~350 generations of adaptive evolution, a gradually increased concentration of ethanol was used as a selection pressure to enrich ethanol-tolerant mutants. The evolved mutant, *E. coli* SZ470, was able to grow anaerobically at 40 g l^{-1} ethanol, a twofold improvement over parent KC01. When compared with KC01 for small-scale (500 ml) xylose (50 g l^{-1}) fermentation, SZ470 achieved 67% higher cell mass, 48% faster volumetric ethanol productivity, and 50% shorter time to complete fermentation with ethanol titer of 23.5 g l^{-1} and yield of 94%. These results demonstrate that an industry-oriented nontransgenic *E. coli* strain could be developed through incremental improvements of desired traits by a combination of molecular biology and traditional microbiology techniques.

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Introduction

Ethanol produced from renewable cellulosic biomass has the potential to provide an environmentally friendly transportation fuel, decrease our dependence on foreign oil, and create jobs. However, cellulosic ethanol faces many challenges and has yet to become an economically viable transportation fuel [1]. One of these challenges is the lack of natural microorganisms that ferment all biomass-derived sugars (C6 and C5 sugar mixtures) into ethanol with high yield (homoethanol fermentation). To develop such an organism, either a homoethanol pathway has been cloned into C5-fermenting hosts [4, 24] or C5 sugar catabolic pathways have been engineered into homoethanol-producing hosts [6, 7, 12–14, 25]. These transgenic strains are currently the leading candidates for industry-oriented cellulosic ethanol fermentation. Nevertheless, considering the potential public perception of and/or attitudes towards genetically modified organisms (GMOs) and regulations when applied on a large scale, nontransgenic strains may be preferred to transgenic ones, provided that they can achieve similar fermentation efficiencies using cellulosic-derived C6/C5 sugars.

Previously, we engineered a native homoethanol pathway (pyruvate ⟹ acetyl-CoA ⟹ acetaldehyde ⟹ ethanol) in *Escherichia coli* B which has an efficient ability to utilize all biomass-derived C6/C5 sugars [26, 27]. The resulting nontransgenic strain, *E. coli* SZ420 (*ldhA pflB frdBC ackA pdhR*::*pflB*p6-*aceEF-lpd*), fermented glucose and xylose to ethanol as a sole product. However, unlike native ethanol-producing strains such as *Saccharomyces cerevisiae* and

Zymomonas mobilis, the engineered homoethanol-producing *E. coli* SZ420 lacks the trait of alcohol tolerance. This strain was unable to grow anaerobically in LB medium containing 5 g l^{-1} ethanol and was unable to complete 50 g l^{-1} xylose fermentation. Subsequently, we selected a fast-growing mutant from SZ420 through a 3-month metabolic evolution procedure [3]. This mutant, KC01, was able to grow anaerobically in LB medium containing 15–20 g l^{-1} ethanol and completed 50 g l^{-1} xylose fermentation with longer than 120 h fermentation time.

In this study, we further enhanced alcohol tolerance of KC01 through a 2-month adaptive evolution procedure by selection of ethanol-tolerant mutants in screw-cap tubes containing gradually increased concentrations of ethanol (10–40 g l^{-1}). The adaptively evolved mutant, SZ470, was able to grow anaerobically in LB medium containing 40 g l^{-1} ethanol. When compared with KC01 for small-scale (500 ml) xylose (50 g l^{-1}) fermentation, SZ470 achieved 67% higher cell mass, 48% faster volumetric ethanol productivity, and 50% shorter time to complete fermentation with ethanol titer of 23.5 g l^{-1} and yield of 94%.

Materials and methods

Strains, media, and growth conditions

Bacterial strains *E. coli* B (ATCC11033), *E. coli* KC01 (NRRL B-59543) and its derivative alcohol-tolerance mutant *E. coli* SZ470 (NRRL B-59542) were used in this study. KC01 is a fast-growing mutant selected from non-transgenic ethanologenic *E. coli* SZ420 (*E. coli* B *ldhA pflB ackA frdBC pdhR*::*pflB*p6-*aceEF-lpd*) [3, 26, 27]. Bacterial cultures were grown in Luria–Bertani (LB) broth (g l^{-1}: tryptone 10, yeast extract 5, NaCl 5) supplemented with 50 g l^{-1} xylose, or on LB plates (agar 15 g l^{-1}) containing 50 g l^{-1} xylose. For the fermentations, the seed cultures were inoculated into the media which, essentially, became anaerobic as the growing cells consumed the small amount of oxygen present in the media, rather than by inoculating cells into anaerobic media under strictly anaerobic conditions.

Adaptive evolution

Escherichia coli KC01 was streaked on LB xylose plates and incubated at 35°C for 16 h. One colony was used to inoculate a 10-ml screw-cap tube containing 10 ml LB xylose medium, and incubated at 35°C for 24 h. One hundred microliters of this 24 h culture was inoculated into a 10-ml screw-cap tube filled with 9.9 ml LB xylose medium containing 10 g l^{-1} ethanol and was incubated at 35°C for 24 h. Continuously repeating this transfer procedure seven times at 10 g l^{-1} ethanol, the culture was then sequentially transferred to ethanol concentrations of 15, 20, 25, 30, 35, and 40 g l^{-1}. This daily transfer procedure was repeated for 1 week at each ethanol concentration.

Ethanol challenge

One colony of SZ470 or KC01 was inoculated in a 10-ml test-tube containing 5 ml LB xylose medium and incubated at 35°C for 3 h in a rotator. The culture (0.5 ml) was added to 4.5 ml LB xylose medium supplemented with 0.75 g ethanol (final ethanol concentration 150 g l^{-1}). The culture was then mixed and held for 2 min. Serial dilutions of this ethanol-challenged culture were then made immediately using fresh LB xylose medium. The dilution (0.1 ml) was spread onto LB xylose plates and incubated at 35°C overnight to count the surviving cells (by colony number). A similar dilution procedure and plate count were applied to cultures that did not undergo the ethanol challenge, used as the control. Cell survival rate was calculated as follows: number of colonies of ethanol-challenged culture/number of colonies of control culture.

Alcohol tolerance

Screw-cap tubes (10 ml) were prepared with 9.9 ml LB xylose medium and various concentrations of ethanol (0–50 g l^{-1}) or butanol (0–10 g l^{-1}). Overnight cultures (100 μl) were inoculated into these tubes and incubated at 35°C without shaking. Cell growth was measured after 24 h incubation. The tolerance was measured by efficient cell growth (3–4 doublings) at the highest alcohol concentration and by the ethanol concentration that inhibited 50% of cells (ID_{50}, ethanol concentration that allows 50% cell growth compared with control).

Fermentations

Seed cultures were prepared by inoculating colonies from fresh LB xylose plates into a 250-ml flask containing 20 ml LB broth with 50 g l^{-1} xylose. After incubating for 11 h (35°C, 155 rpm), 2 ml of the culture was used to inoculate a 500-ml fermentation vessel (Fleaker™, Corning) containing 350 ml LB broth with 50 g l^{-1} xylose (inoculum 16.5 mg l^{-1} cell dry weight). Fermentations were maintained at 35°C, 100 rpm mixing, and pH 6.0 by automatic addition of 2 N KOH. All fermentations were carried out in three or more replicates.

Analyses

Cell mass was estimated by optical density (1.0 ml cells at 1.0 OD_{550} equals approximately 0.33 mg cell dry weight)

using a Unico1100 spectrophotometer with a round culture tube (diameter 1.0 cm) as cuvette [3]. Ethanol concentrations were measured by gas chromatography (GC; Varian CP3800 equipped with a flame ionization detector and a capillary column). 1-Propanol was used as an internal standard for ethanol measurements. The concentrations of sugars and organic acids were determined by high-performance liquid chromatograph (HPLC; Waters) equipped with a refractive index detector. Products were separated using a BioRad HPX 87H column with 4 mM H_2SO_4 as mobile phase (10 µl sample injection volume, 0.4 ml min^{-1} mobile phase running speed, 45°C column temperature).

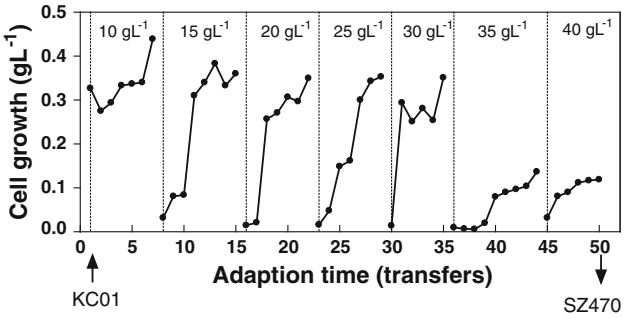

Fig. 1 Adaptive evolution of *E. coli* KC01 for ethanol tolerance. Bacterial cells (100 µl) were transferred into a 10-ml screw-cap tube containing 9.9 ml LB xylose medium supplemented with different amounts of ethanol (10–40 g l^{-1}) and incubated at 35°C for 24 h. After seven transfers at one ethanol concentration, the transfer was repeated at a higher level of ethanol

Results

Selection of an alcohol-tolerant mutant by adaptive evolution

The cell growth and ethanol production rates of nontransgenic *E. coli* SZ420 were very low in glucose fermentation, and even lower in xylose fermentation [26]. A fast-growing mutant KC01, selected from SZ420, had better cell growth and ethanol production rates [3], but was still unable to grow anaerobically with 15–20 g l^{-1} ethanol. To further improve its ethanol tolerance, KC01 was subjected to an adaptive evolution procedure to select ethanol-tolerant mutants. Specifically, the culture was sequentially transferred to LB xylose medium containing increased concentrations of ethanol at 10, 15, 20, 25, 30, 35, and 40 g l^{-1}. The culture was transferred daily for 1 week for each ethanol concentration. The cell growth during this adaptive evolution is presented in Fig. 1. The cells grew well with 6–7 doublings in 24 h at ethanol concentration of 10 g l^{-1} (Fig. 1a). However, at ethanol concentrations greater than 10 g l^{-1}, the culture grew poorly initially with 3–4 doublings, but eventually grew well with 6–7 doublings after multiple transfers. After the adaptive evolution at 40 g l^{-1} ethanol, the culture was streaked on LB xylose plates for isolated colonies. One of the colonies was selected and named *E. coli* SZ470 after comparing 10 colonies for their anaerobic growth in LB xylose containing 40 g l^{-1} ethanol. It should be noted that evolution with higher than 40 g l^{-1} ethanol was attempted and did not yield a further improved mutant.

Growth characteristics of SZ470

The selected mutant SZ470 had a smaller colony size than parent KC01 on LB xylose plates. This different phenotype may indicate that genetic mutation(s) occurred in SZ470 during adaptive evolution. In screw-cap tube grown cultures with LB xylose medium, SZ470 grew faster and achieved 15–20% higher cell mass than KC01 in 24 h (Fig. 2a, b), although there was no growth difference in the initial 10 h. The improved cell growth of SZ470 might be related to its adaptively evolved ethanol tolerance, because significant amounts of ethanol (6.8 g l^{-1}) were produced during the 10–24 h period.

Evaluation of ethanol tolerance

To better understand the anaerobic cell growth of SZ470 and KC01, the ethanol tolerance of these cultures was evaluated by two different approaches. The first approach was to determine the survival rate of the cells challenged by 150 g l^{-1} ethanol for 2 min. The results are shown in Table 1. This ethanol challenge caused minimal damage to SZ470 cells, whose survival rate was as high as 100%, while over 60% of KC01 cells were damaged and killed during the challenge. The second approach was to determine the anaerobic cell growth in LB xylose medium containing different amounts of ethanol (0–50 g l^{-1}). The results are presented in Fig. 2 and Table 2. At ethanol concentrations of 5–40 g l^{-1}, SZ470 grew efficiently (greater than 3–4 doublings, 0.11–0.49 g l^{-1} cell dry weight), with 50% cell inhibition at 30–35 g l^{-1} ethanol (Table 2; Fig. 2b). In contrast, KC01 could achieve cell growth of greater than 3–4 doublings (0.13-0.38 g l^{-1} cell dry weight) at much lower ethanol concentrations (5–20 g l^{-1}), with 50% cell inhibition at 10–15 g l^{-1} ethanol (Table 2; Fig. 2a). These results indicate that SZ470 gained at least a twofold enhanced ethanol tolerance over that of KC01.

It is interesting to note that SZ470 also showed better tolerance to other alcohols (such as butanol) than KC01, even though the cells had not been adaptively evolved in other alcohol conditions. As shown in Fig. 2c and d, SZ470

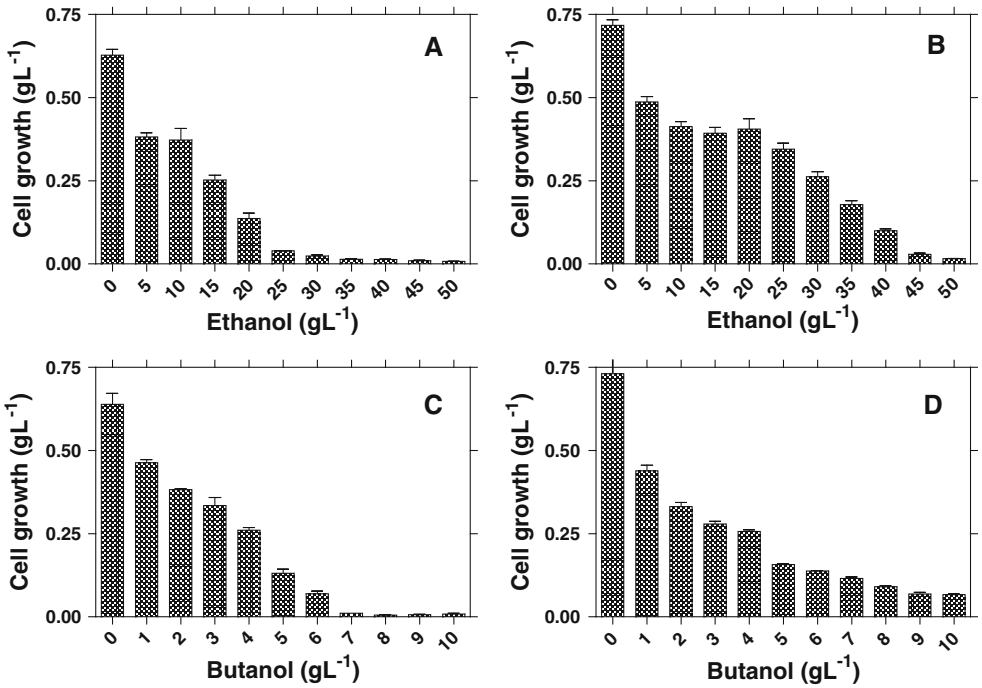

Fig. 2 Evaluation of alcohol tolerance. Screw-cap tubes (10 ml) were prepared with 9.9 ml LB xylose broth containing 50 g l^{-1} xylose and various concentrations of ethanol (0–50 g l^{-1}) or butanol (0–10 g l^{-1}). Overnight cultures (100 μl) were inoculated into these tubes (initial cell density 3.3 mg l^{-1} cell dry weight) and incubated at 35°C without shaking. Cell growth was measured after 24 h of incubation. **a** *E. coli* KC01, ethanol; **b** SZ470, ethanol; **c** KC01, butanol; **d** SZ470, butanol

Table 1 Cell survival rate of 150 g l^{-1} ethanol-challenged cultures

Strain	Control (colony)	Ethanol challenge (colony)	Survival rate (%)
KC01	248 ± 10	93 ± 8	37.5
SZ470	136 ± 12	142 ± 6	100

The cultures were exposed to 150 g l^{-1} ethanol for 2 min, and then were used to determine cell survival rate by plate colony count

Table 2 Incremental improvement of ethanol tolerance of non-transgenic *E. coli* strains

E. coli strains	ID^{a}_{50}	ID^{b}_{100}	Source
B (wild type)	16	23	[3]
SZ420	2–3	9	[3]
KC01	10–15	25	This study
SZ470	30–35	45–50	This study
LY01 (transgenic)	30–35	50	[23]
ET1 (transgenic)	30–35	50	[22]

[a] ID_{50} is the ethanol concentration that allows 50% cell growth compared with control. ID_{50} of LY01 and ET1 estimated from the cited paper

[b] ID_{100} is the ethanol concentration that allows minimal or no cell growth. ID_{50} of LY01 and ET1 estimated from the cited paper

cells achieved 3 doublings at butanol concentration of 10 g l^{-1}, while KC01 could grow to 3 doublings at butanol concentration 6 g l^{-1} and was unable to grow when the butanol concentration was greater than 7 g l^{-1}.

Homoethanol fermentation from xylose

To test whether the enhanced ethanol tolerance of SZ470 could be translated into efficient xylose fermentation, fermentations with 50 g l^{-1} xylose were evaluated in pH-controlled vessels (500 ml). As shown in Fig. 3 and Table 3, SZ470 maintained maximal cell growth for 36 h (0–36 h, Fig. 3a) and an efficient ethanol production rate for 60 h (12–72 h, Fig. 3b), achieved a maximum 4.66 g l^{-1} cell mass, and completed fermentation in 72 h (23.5 g l^{-1} ethanol titer, 94% yield) (Table 3). On the contrary, KC01 achieved maximum cell growth and efficient ethanol production rates for only 24 h (0–24 h), obtained a lower maximal cell mass (2.8 g l^{-1}) and ethanol titer (16 g l^{-1}), and was not able to complete the fermentation in 72 h (30% xylose remained) (Table 3; Fig. 3a, b). These results demonstrate that the twofold enhanced ethanol tolerance enabled SZ470 to achieve 67% higher cell mass and 48% higher average volumetric ethanol productivity with 50% shorter fermentation time compared with KC01. However, it is also interesting to note that SZ470 had 28% lower maximal cell growth rate (h^{-1}), 9% lower

maximal volumetric ethanol productivity, and 45% lower maximal specific ethanol productivity compared with KC01 (Table 3).

Stability of the adaptively evolved strain

The different colony size, cell growth rate, and ethanol tolerance between SZ470 and KC01 indicate that certain mutations had evolved in SZ470 during the adaptive evolution process. Understanding the mutations responsible for the phenotype of SZ470 was interesting but not the focus of this study. Instead, we were interested in the genetic stability of the ethanol tolerance gained during adaptive evolution. In this regard, SZ470 was transferred daily for an additional 2 months on LB xylose plates without added ethanol as a selection pressure and then tested for ethanol tolerance and xylose fermentation. The results showed that the acquired ethanol-tolerance trait was stably maintained in SZ470. This strain was still able to grow efficiently at ethanol concentration of 40 g l^{-1} and completely fermented 50 g l^{-1} xylose in 72 h (data not shown). In addition, SZ470 still maintained a smaller colony appearance than KC01 on LB xylose plates.

Discussion

Ethanol tolerance and efficient C5 sugar utilization are essential microbial traits for cellulosic ethanol fermentation. Molecular techniques have been used to establish a C5 sugar pathway in ethanol-producing hosts or to engineer a homo-ethanol pathway in hosts with native ability to ferment C5 sugars. Nevertheless, these molecular techniques have rarely been used to improve ethanol tolerance of potential microbial biocatalysts. Although previous reports show that ethanol damages the permeability barrier of bacterial cell membranes and therefore inhibits cell growth [8, 11, 18], it is believed that ethanol tolerance is a multiple-gene-controlled trait. Changes in membrane composition such as fatty-acid chain length, and/or *trans*-fatty acid, membrane protein, or phospholipid composition, were all reported to contribute to ethanol tolerance [2, 9, 10, 14–18]. Therefore, to improve the

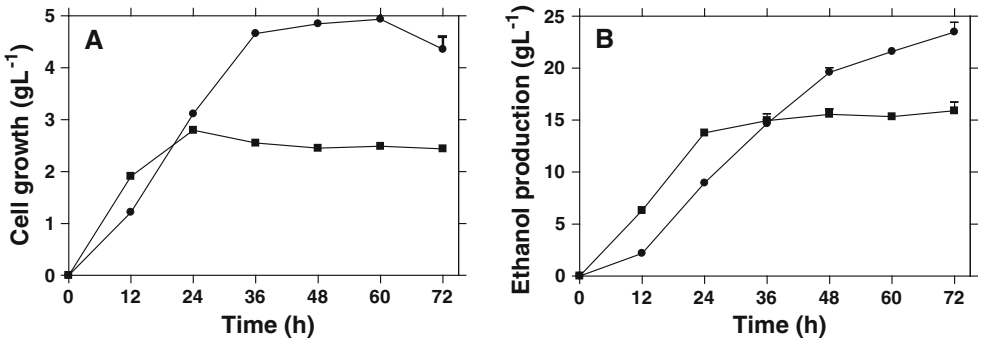

Fig. 3 Fermentations of 50 g l^{-1} xylose. The pH-controlled fermentation was carried out in a 500-ml vessel filled with 350 ml LB containing 50 g l^{-1} xylose (pH 6.0, 35°C, 100 rpm agitation). Samples were taken every 24 h to measure cell growth and ethanol production. **a** Cell growth; **b** ethanol production. *Symbols*: *filled squares*, KC01; *filled circles*, SZ470

Table 3 Summary of xylose fermentations

Strain	Cell growth[a]		Xylose used (g l^{-1})	Ethanol produced[b]		Volumetric productivity[c] (g l^{-1} h^{-1})		Specific productivity[c] (g g^{-1} h^{-1})	
	Mass (g l^{-1})	Rate (h^{-1})		Titer (g l^{-1})	Yield (%)	Maximum	Average	Maximum	Average
KC01	2.798	0.281	36.350	15.899	86	0.574	0.221	0.205	0.079
SZ470	4.660	0.202	49.125	23.498	94	0.520	0.326	0.112	0.070

Fermentations were carried out for 72 h at the following conditions: 500-ml vessel containing 350 ml Luria broth with xylose (50 g l^{-1}), 35°C, 100 rpm, and pH 6.0

[a] Cell growth rate was calculated from inoculation to early stationary phase (0–24 h for KC01, 0–36 h for SZ470)

[b] Yield was calculated as percent of theoretical maximum (0.51 g ethanol per g sugar used)

[c] Maximum volumetric and specific productivities were calculated from the most productive 24 h period (0–24 h for KC01, 12–36 h for SZ470). Average volumetric and specific productivities were calculated from the whole fermentation period (0–72 h)

ethanol tolerance of a microorganism, it is logical to target the whole genome through adaptive evolution and select a "guided mutant" using ethanol as a selection pressure instead of targeting a specific gene [5, 20, 22, 23].

Metabolic engineering has been previously used in the development of a nontransgenic *E. coli* B for homofermentative production of ethanol [26]. However, deletions of the competing fermentation pathways made the initially engineered strain SZ420 sensitive to ethanol. This strain was not able to grow in medium containing 9–10 g l^{-1} ethanol, and had a 50% cell inhibition ethanol concentration as low as 2.3 g l^{-1} (Table 2). A fast-growing mutant, KC01, selected from SZ420 had improved ethanol tolerance and achieved 50% cell inhibition at ethanol concentrations of 10–15 g l^{-1}, but was still unable to grow in 25 g l^{-1} ethanol [3]. To further improve its ethanol tolerance, KC01 was subjected to a 2-month adaptive evolution procedure by growing the cells in LB xylose medium with gradually increasing concentrations of ethanol. When ethanol was used as a selection pressure during the evolution process, only cells that had evolved sufficient ethanol tolerance would survive and replicate. These surviving cells would become the dominant population after multiple enrichment transfers in the same levels of ethanol. When this process was repeated with higher levels of ethanol, additional mutations would occur to allow the cells to tolerate the increased levels of ethanol. With 2 months of continuous evolution, a genetically stable ethanol-tolerant mutant, SZ470, was evolved and selected. The resulting mutant grew effectively in 40 g l^{-1}ethanol and had 50% cell inhibition at ethanol concentrations of 30–35 g l^{-1}. This ethanol tolerance level achieved by SZ470 was comparable to those achieved by the transgenic *E. coli* LY01 [23] and *E. coli* ET1 [22].

It should be noted that directly growing KC01 in LB xylose medium containing 40 g l^{-1} ethanol did not yield an ethanol-tolerant mutant. High concentrations of ethanol created too much pressure for cells to survive and evolve. This failed attempt might demonstrate the necessity of stepwise selection for genetic traits involving multiple genes. Stepwise increase in ethanol concentration as a selection pressure would allow related genes to mutate one at a time and eventually acquire the mutations needed for higher ethanol tolerance. Although we do not know how many and what type of mutations are needed to enable SZ470 to grow anaerobically at 40 g l^{-1} ethanol, an estimated 30 mutations occurred during this adaptive evolution process. This estimation was made based on the number of generations evolved ($\sim$350) and the mutation rate of 0.087 per generation for ethanologenic *E. coli* (Laura Jarboe, personal communication).

The enhanced ethanol tolerance (40 g l^{-1} ethanol) enabled SZ470 to complete 50 g l^{-1} xylose fermentation with ethanol as the sole product (94% ethanol yield). Surprisingly, SZ470 was not able to complete fermentations with 75 g l^{-1} xylose in multiple trials (data not shown). Since a maximal 38.25 g l^{-1} ethanol could be produced from 75 g l^{-1} xylose (theoretic yield: 0.51 g ethanol per g of sugar), ethanol tolerance should not be the limiting factor responsible for the incomplete fermentation. Determining the new limiting factor would be our next step for the incremental improvement of the nontransgenic *E. coli* strains for efficient ethanol fermentation from higher concentrations of sugars. Based on the engineered ethanol pathway in our strain: glucose (or 1.2 xylose) $\Longrightarrow$ (glycolysis) $\Longrightarrow$ 2 pyruvate + 2 NADH $\Longrightarrow$ (enhanced anaerobically expressed pyruvate dehydrogenase) $\Longrightarrow$ 2 acetyl-CoA + 4 NADH $\Longrightarrow$ (alcohol dehydrogenase) $\Longrightarrow$ 2 ethanol [26], we have speculated that alcohol dehydrogenase (AdhE) might be the new limiting factor, because pyruvate dehydrogenase had been highly expressed in a previous study [26, 27], and *E. coli* usually has an efficient glycolytic flux [21]. We will focus on improvements of *adhE* expression in SZ470 for efficient fermentation of xylose ($\geq$75 g l^{-1}) in a future report (manuscript in preparation).

In addition, the high concentration of ethanol produced during fermentation might have negative impacts on gene transcription and/or enzyme activity of xylose transporters [19], leading to incomplete fermentation of higher concentrations of xylose ($\geq$75 g l^{-1}). Improving xylose transportation may also enhance homoethanol fermentation from higher concentrations of xylose.

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