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Saccharomyces cerevisiae Strain Improvement Using Selection, Mutation, and Adaptation for the Resistance to Lignocellulose-Derived Fermentation Inhibitor for Ethanol Production

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Twenty-five *Saccharomyces cerevisiae* strains were screened for the highest sugar tolerance, ethanol-tolerance, ethanol production, and inhibitor resistance, and *S. cerevisiae* KL5 was selected as the best strain. Inhibitor cocktail (100%) was composed of 75 mM formic acid, 75 mM acetic acid, 30 mM furfural, 30 mM hydroxymethyl furfural (HMF), and 2.7 mM vanillin. The cells of strain KL5 were treated with γ-irradiation, and among the survivals, KL5-G2 with improved inhibitor resistance and the highest ethanol yield in the presence of inhibitor cocktail was selected. The KL5-G2 strain was adapted to inhibitor cocktail by sequential transfer of cultures to a minimal YNB medium containing increasing concentrations of inhibitor cocktail. After 10 times of adaptation, most of the isolated colonies could grow in YNB with 80% inhibitor cocktail, whereas the parental KL5 strain could not grow at all. Among the various adapted strains, the best strain (KL5-G2-A9) producing the highest ethanol yield in the presence of inhibitor cocktail was selected. In a complex YP medium containing 60% inhibitor cocktail and 5% glucose, the theoretical yield and productivity (at 48 h) of KL5-G2-A9 were 81.3% and 0.304 g/l/h, respectively, whereas those of KL5 were 20.8% and 0.072 g/l/h, respectively. KL5-G2-A9 reduced the concentrations of HMF, furfural, and vanillin in the medium in much faster rates than KL5.

Keywords: Lignocellulose-derived fermentation inhibitor, resistance, adaptation, *Saccharomyces cerevisiae*, ethanol production

Introduction

Lignocellulosic plant biomass is the earth's most abundant low-cost source for the production of biofuels such as bioethanol that does not compete with food provision [21]. Despite of this advantage, one of the main problems to be overcome in the industrial production of lignocellulosic bioethanol is that during the pretreatment of the lignocellolosic materials to obtain sugar-rich hydrolysates, several compounds that are toxic for microbial growth and fermentation are released to hydrolysates, due to the use of high temperatures and chemicals [22]. As a consequence, the fermentation of sugar-rich hydrolysates containing these inhibitory compounds is characterized by a low ethanol yield and productivity [10]. To tackle the problem of toxicity, a detoxification step such as overliming and ion exchange resin separation of the hydrolysate prior to fermentation has been proposed [3, 33]. However, these additional steps may require large investments, and a significant quantity of sugars could be lost [22]. Adaptation of yeast to inhibitory compounds could be an alternative to overcome this inhibitor problem [11, 25].

Yeast *Saccharomyces cerevisiae* is known to convert several inhibitors to less-toxic derivatives at the cost of an extended lag phase and reduced ethanol productivity [13, 23]. Cell adaptation to the inhibitors increased the inhibitor conversion rate, and thus improving ethanol production [17]. Lignocellulose-derived inhibitors are composed of several compounds that trigger a complex stress-related response in *S. cerevisiae*, involving the interaction of several

pathways and expression of genes with multiple functions [15]. However, genetic engineering approaches for obtaining an inhibitor-tolerant yeast strain can have uncertain results in an industrial setting with wide-ranging inhibitor concentrations. Moreover, it is not possible to identify all the genes for inhibitor bioconversion and tolerance and their complicated network [15].

Directed evolution or adaptive strategies is a way to mimic the natural selection conducted by nature. Directed evolution is based on the experimental improvement of cellular properties through iterative genetic diversification (spontaneous or induced mutation) and selection pressure [28]. A truly mechanistic understanding of the action of inhibitors and responsible genes is not needed [2]. Therefore, the directed evolution technique is a faster and more convenient way to obtain inhibitor-tolerant strains than the genetic engineering approach [2]. So far, several papers were published on the improvement of inhibitor tolerance by adaptive strategies, but all of them used only an adaptation step [7, 9, 11, 18, 20].

In this investigation, a yeast strain highly efficient in producing ethanol from lignocellulosic hydrolysate containing various inhibitors was developed by a combination of selection, mutation, and adaptation. Initially, efficient ethanol producers were selected based on sugar tolerance, ethanol tolerance, and fermentation speed. Among the selected strains, the strains showing higher resistance to inhibitors were selected and subjected to mutation by gamma-ray. Finally, the resistance of the selected strain was much improved by sequential adaptations to inhibitors.

Materials and Methods

Yeast Strains and Culture Condition

Twenty-five strains (KL1-KL25) of *S. cerevisiae*, relatively high ethanol producers selected from more than 300 stock cultures in our laboratory, were used in this study. The stock cultures were collected from domestic and foreign culture collections (KTCC, ATCC, NRRL) and university laboratories. Some yeast strains were isolated from soils sampled at local distilleries. Among them, KL18 and KL20 are ATCC 26603 and ATCC 26602, respectively. *S. cerevisiae* KL5 was selected among the 25 strains as the highest sugar-tolerant, ethanol-tolerant, and ethanol-producing strain. *S. cerevisiae* KL5-G2-A9 is an inhibitor resistance strain derived from KL5 after mutation and adaptation. For activation of yeast cells, cells were grown on a YPD agar plate that consisted of 1% yeast extract (Y), 2% peptone (P), and 2% dextrose (D) solidified with 2% agar, at 33°C for 2 days. YP agar plates containing 20%, 30%, or 40% (w/v) glucose and YPD agar plates containing 5%, 10%, or 15% ethanol were 1st selection media for sugar- and ethanol-tolerance. YP broth containing 23% (w/v) dextrose and a Durham fermentation tube was a 2nd selection medium for ethanol production. For liquid culture, cells were inoculated into a 250 ml Erlenmeyer flask containing 50 ml of YPD and the flask was incubated in a rotary shaking incubator operated at 33°C and 200 rpm.

Inhibitor Cocktail

The inhibitor cocktail (100%) consisted of 75 mM (3.5 g/l) formic acid (Sigma-Aldrich), 75 mM (4.5 g/l) acetic acid (Sigma-Aldrich), 30 mM (2.9 g/l) furfural (Sigma-Aldrich), 30 mM (3.8 g/l) HMF (Sigma-Aldrich) [19], and 2.7 mM (0.41 g/l) vanillin (Sigma-Aldrich) [7]. A five times concentrated inhibitor cocktail was prepared by dissolving HMF and vanillin in redistilled water. Formic acid, acetic acid, and freshly redistilled furfural were added and the inhibitor cocktail was then filter sterilized.

γ-Ray Mutagenesis and Selection of Inhibitor-Resistant Strains for Ethanol Production

One colony of KL5 was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of YPD and the flask was incubated at 33°C and 200 rpm for 24 h. The cells in the culture were washed twice with 0.85% NaCl and suspended in the same solution, and the cells were irradiated with γ-ray (^{60}Co) under dosage of 5 kGy. The survived cells were spread onto a YNB agar plate containing 40% inhibitor cocktail and incubated at 33°C for 2 days, and fast growing colonies with round morphology were selected. The cells of each colony were inoculated into a cap tube with 10 ml of YNB containing 5% glucose and 40% inhibitor cocktail, and the cap tube was incubated for 4 days at 33°C. Relatively higher ethanol-producing strains in the cap tubes were selected. One loopful of cells of each selected culture, grown at 33°C for 24 h on YNB with 5% glucose and 40% inhibitor cocktail, was inoculated into a 250 ml Erlenmeyer flask with 50 ml of YNB with 5% glucose and 40% inhibitor cocktail and the flasks were incubated at 33°C for 4 days. Mutant strains producing more ethanol than parental strains at the end of fermentation were selected.

Adaptation

Sequential cultivation with a stepwise increase in inhibitor cocktail concentration was performed as follows. The cultivation of KL5 mutant strain was carried out in YNB medium containing inhibitor cocktail, and the culture was transferred to medium containing a higher inhibitor cocktail concentration, followed by sequential cultivations. The initial inhibitor cocktail concentration was set at 40% (v/v) and it was changed to 42%, 44%, 46%, 48%, 50%, 52%, 54%, 56%, 58%, and 60% stepwise.

Growth Assessment in Fermentation-Derived Inhibitor

Mutants showing better tolerance than the wild-type strain were further assessed for growth in YNB containing inhibitor or inhibitor cocktail. One milliliter of an inoculum was transferred to 50 ml of the YNB containing various concentrations (20–100%) of

inhibitor or inhibitor cocktail in a 250 ml Erlenmeyer flask. The flask was incubated with shaking (200 rpm) at 33°C. Growth was evaluated periodically by withdrawing samples and measuring the optical density of the culture at 600 nm.

Fermentation

The fermentation was conducted in either a flask or a fermenter. For flask fermentation, a 250 ml Erlenmeyer flask sealed with silicon stoppers and with a working volume of 50 ml of YNB or YP containing different concentrations of inhibitor cocktail was used. One loopful of cells of activated yeast was inoculated into a 250 ml Erlenmeyer flask, and the flask was incubated at 33°C for 4 or 5 days. For fermentation using a fermenter, a 5 L jar fermenter (KF-5 L; Kobiotech Co. Ltd., Korea) equipped with sensors to measure temperature, dissolved oxygen, and pH, and working volume of 3 L of YNB containing different concentrations of inhibitor cocktail was used. The pH of the fermenter medium was maintained at 5.0 using a pH controller with 4 N NaOH and 4 N HCl. Activated yeast culture broth (5%) was inoculated to the fermenter and the fermentation was conducted at 33°C for 4 days.

Analysis

Fermentation samples were centrifuged at 8,000 ×*g* for 2 min in an Eppendorf centrifuge to obtain the supernatants before analysis. All samples were filtered through 0.20 μm membrane filters and diluted prior to HPLC analysis. The analysis was performed using a Shiseido HPLC system (Shiseido, Tokyo, Japan) and the S-MC data system (Shiseido). Glucose and ethanol were separated on an SUGAR SH1011 column (Showa Denko K.K., Tokyo, Japan) operating at 50°C for 30 min, and inhibitor cocktail was separated on the same column at 50°C for 60 min. As the mobile phase, 5 mM H_2SO_4 was supplied at a flow rate of 0.6 ml/min. Detection was performed using a refractive index detector (RI 101; Showa Denko K.K.). The ethanol produced in the medium by yeast fermentation was expressed in % (v/v).

Results and Discussion

Selection of Sugar- and Ethanol-Tolerant Strains with High Fermentation Speed

The tolerance of 25 yeast strains was examined using YP agar plate containing high concentration of glucose (20 ~ 40% (w/v)) or ethanol (5 ~ 10% (v/v)), and their fermentation speeds were examined by CO_2 gas formation using Durham fermentation tube. The results showed that, in a medium containing 20% glucose, all 25 strains grew well. In a medium containing 30% glucose, seven strains (KL4, KL5, KL6, KL16, KL17, KL20, and KL24) showed growth, and KL4, KL5, and KL24 strains showed significantly better growth than the others. In a medium containing 40% glucose, KL5 and KL24 showed poor growth, but the rest of the strains did not grow at all.

In the medium containing 5% ethanol, all the strains grew well. In the medium containing 10% ethanol, KL5, KL6, KL9, KL18, and KL24 showed slight growth, but in the medium containing 15% ethanol, all the strains did not grow.

In the test with Durham fermentation tubes, KL4, KL5, and KL24 showed distinctively higher fermentation speed; judged by the higher speed of CO_2 gas formation, than the other strains.

From the above results, it was concluded that KL4, KL5, and KL24 showed the highest ethanol and sugar tolerance, and fermentation speed among the 25 strains .

Selection of Strains Producing Higher Concentration of Ethanol

Ethanol fermentation in YP containing 25% glucose was performed in a 250 ml flask, and among the 25 strains, KL4, KL5, and KL24 produced the highest concentration of ethanol, at 10.9%, 11.1%, and 10.9%, respectively. The ethanol fermentation of the three strains was tested again in a 5 L fermentor and KL4, KL5, and KL24 strains were confirmed as the highest ethanol producers (10.35 ± 0.03%, 10.70 ± 0.01%, and 10.53 ± 0.02%, respectively).

The above results indicated that KL4, KL5, and KL24 were the strains producing the highest ethanol concentration among the 25 strains as well as being strains with the highest ethanol and sugar tolerance and fermentation speed. Therefore, these three strains were selected for further study. In fermentative ethanol production, sugar tolerance [6], ethanol tolerance, and high fermentation speed [31] are important characteristics for selection of efficient yeast strains for ethanol fermentation. Favaro *et al*. [6] also used high concentration up to 20% glucose to select yeast strains before testing their tolerance to various inhibitors or inhibitor cocktails.

Tolerance of Yeast Strains Towards Inhibitor Cocktail in Ethanol Fermentation

The effect of different concentrations of inhibitor cocktail on the ethanol fermentation by the three selected strains was examined and the results are shown in Fig. 1. With increased concentrations of inhibitor cocktail, the ethanol production of all the three strains decreased accordingly. The KL4 and KL24 strains stopped ethanol production at 50% cocktail, whereas KL5 still produced ethanol up to 70% cocktail. Therefore, the KL5 had the highest tolerance in ethanol fermentation towards the inhibitor cocktail.

Since significant differences in inhibitor resistance exist among *S. cerevisiae* strains, as shown in this study as well as

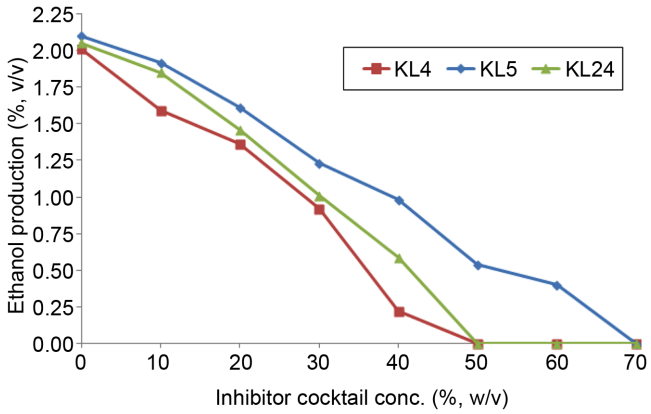

Fig. 1. Ethanol fermentation of KL4, KL5, and KL24 in YNB containing different concentrations of inhibitor cocktail.
The fermentation was carried out for 4 days using a 250 ml Erlenmeyer flask.

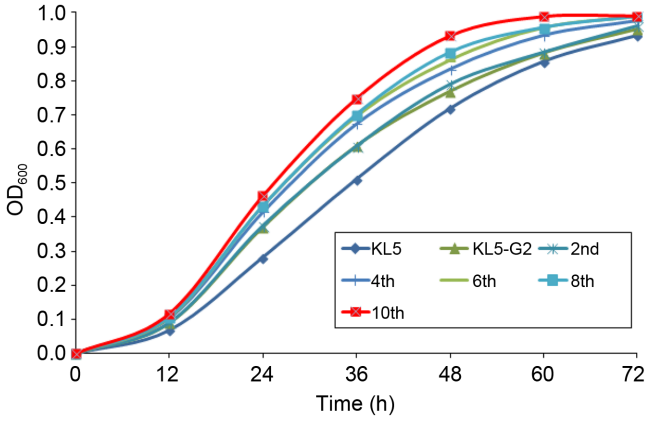

Fig. 2. Time courses of cell growth of KL5 and KL5-G2 mutants at different adaptation steps in YNB containing 50% inhibitor cocktail and 5% glucose.

in other reports (1, 4, 19), it is important to screen yeast strains for ethanol production from lignocelluloses hydrolysate containing inhibitors.

The inhibitory effects of weak acids such as acetic and formic acids have been ascribed to uncoupling and intracellular anion accumulation [26], causing a decrease of cytosolic pH [24], which in turn results in ATP depletion [32]. Increased toxicity of formic acid seems to be associated with its smaller molecular size, which may facilitate its diffusion through the plasma membrane and probably result in higher anion toxicity [2]. The inhibitory effects of furans such as HMF and furfural can be explained by a redirection of yeast energy to fixing the damage caused by furans and by reduced intracellular ATP and NAD(P)H levels, either by enzymatic inhibition or consumption/regeneration of cofactors [2]. The inhibition mechanism of phenolic compounds such as vanillin has not been completely elucidated. Phenolic compounds may act on biological membranes [8].

Improvement of Inhibitor Tolerance by γ-Ray Mutation

To improve the tolerance of the yeast strains to the inhibitors, cells of KL4, KL5, and KL24 were irradiated with γ-ray and improved cells were selected according to their growth and fermentation speed in YNB media containing inhibitor cocktail. In the 1st selection, cells with relatively higher growth rate in YNB media containing 40% inhibitor cocktail were selected. In the 2nd selection, cells with higher fermentation speed were selected in YNB media containing 40% inhibitor cocktail.

After the 1st and 2nd selections, KL4-G6 and KL4-G8 were selected from 232 γ-ray-mutated KL4 strains; KL5-G2 and KL5-G3 from 187 γ-ray-mutated KL5 strains; and KL24-G5 from 195 KL24-γ-ray-mutated strains, respectively. These five selected γ-ray mutants were evaluated again for their ethanol production in YNB media containing 40% inhibitor cocktail and 5% glucose. The results showed that KL4-G6, KL4-G8, KL5-G2, KL5-G3, and KL24-G5 produced 0.69%, 0.89%, 1.30%, 1.08%, and 0.87%, respectively. Among the five strains, KL5-G2 produced the highest amount of ethanol (1.30%), whereas the parental KL5 strain produced 1.01% ethanol. Among the three parental strains of KL4 (0.24%), KL5 (1.01%), and KL24 (0.55%), KL5 was the highest ethanol-producing strain in the presence of the inhibitor cocktail, and we also obtained the highest ethanol producer KL5-G2, which was derived from KL5. The KL5-G2 strain was selected for further study.

Improvement of Inhibitor Tolerance by Adaptation

KL5-G2 was adapted to inhibitor cocktail by 10 sequential cultivations of the cells with a stepwise increase in inhibitor cocktail concentration. As shown in Fig. 2, the cells showed a faster growth rate with increased number of adaptation step. The specific growth rate of KL5 and KL5-G2 was 0.0527 and 0.0613 h^{-1}, respectively. After the 10th adaptation step, the specific growth rate of KL5-G2 increased to 0.0825 h^{-1}.

After 10 adaptations, the culture broth was spread on YNB agar plate containing 50% inhibitor cocktail, and 12 colonies (A1–A12) showing faster growth than KL5-G2 were selected. The growth of these 12 strains in various concentrations of inhibitor cocktail was measured and the results are shown in Fig. 3. The growth of all the strains decreased with the increased concentrations of the inhibitor

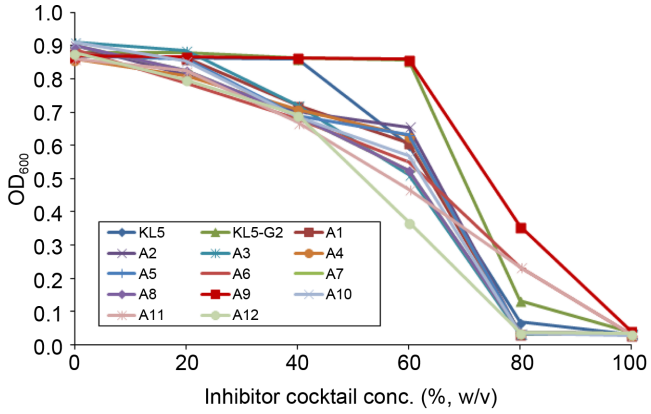

Fig. 3. The cell growth of KL5, and its mutant KL5-G2 and various adapted strains (KL5-G2-A1 to -A12), in YNB with different concentrations of inhibitor cocktail after 48 h.

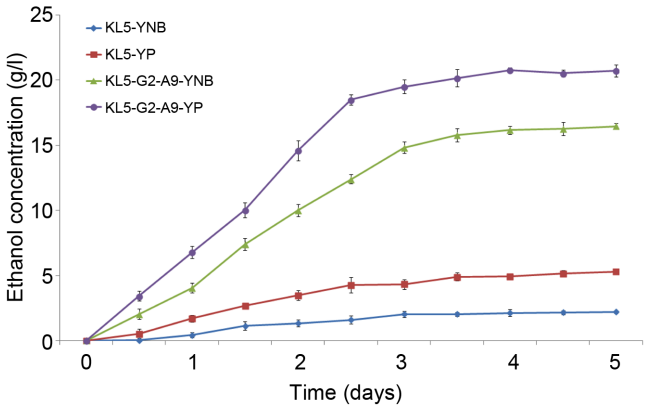

Fig. 5. Time courses of ethanol production by KL5 and KL5-G2-A9.

One loopful of activated cells of each strain was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of YNB or YP broth with 60% inhibitor cocktail and 5% glucose, and incubated for 5 days.

cocktail and completely stopped at 100% inhibitor cocktail. Among the 12 strains examined, KL5-G2-A9 exhibited the most improved tolerance to the inhibitor cocktail in growth.

The ethanol production of KL5-G2-A9 along with KL5 and KL5-G2 in various concentrations of inhibitor cocktail was examined, and the results are shown in Fig. 4. The KL5-G2-A9 strain showed much improved tolerance towards the inhibitor cocktail in ethanol fermentation. At 60% of inhibitor cocktail, KL5-G2-A9 produced 1.51% ethanol, whereas KL5 produced only 0.22% ethanol.

Comparison of Ethanol Production and Inhibitor Reduction Between KL5 and KL5-G2-A9

Ethanol fermentation was carried out by KL5 and KL5-G2-A9 using a minimal medium YNB and a complete medium YP, each containing 60% inhibitor cocktail. The results (Fig. 5) showed that, in both YNB and YP media, KL5-G2-A9 produced ethanol with a much higher theoretical yield and productivity. In YNB medium, the theoretical yield and productivity (at 48 h) of KL5-G2-A9 were 64.5% and 0.208 g/l/h, respectively, whereas those of KL5 were 8.6% and 0.027 g/l/h, respectively. In YP medium, the theoretical yield and productivity (at 48 h) of KL5-G2-A9 were 81.3% and 0.304 g/l/h, respectively, whereas those of KL5 were 20.8% and 0.072 g/l/h, respectively. Therefore, both yeast strains produced more ethanol in YP medium, having richer nutrients than YNB in the presence of inhibitor cocktail. It was also reported that amino acid enrichment of the culture medium enhanced the ability of cells to resist furfural and HMF exposure [29].

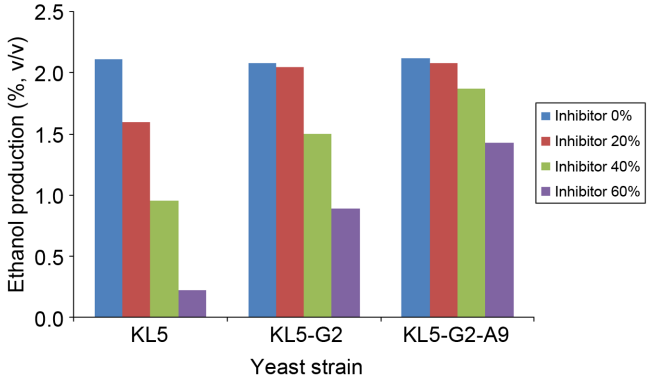

Fig. 4. The ethanol production of the KL5, KL5-G2, and KL5-G2-A9 strains in a 250 ml Erlenmeyer flask for 4 days.

The same tendency was observed in inhibitor reduction during this ethanol fermentation, especially in HMF, furfural, and vanillin (Fig. 6). In both YNB and YP media, KL5-G2-A9 decreased the concentrations of HMF, furfural, and vanillin in much faster rates than did KL5. In the YP medium, the inhibitors were also decreased at much faster rates by both yeast strains than in YNB. Among the various inhibitors, vanillin was diminished at the fastest rate and reached zero percent by KL5-G2-A9 in both YNB and YP media after 4 days. Both strains decreased the furfural faster than HMF, which is in line with previous work [6, 19]. However, the decrease of formic acid and acetic acid by both strains were not distinct compared with the other three inhibitors, which was also shown by Martin and Jonsson [19]. During the fermentation, the initial concentration (2.7 g/l) of acetic acid was slightly decreased to 2.3–2.5 g/l and a similar result was also observed in the reports of

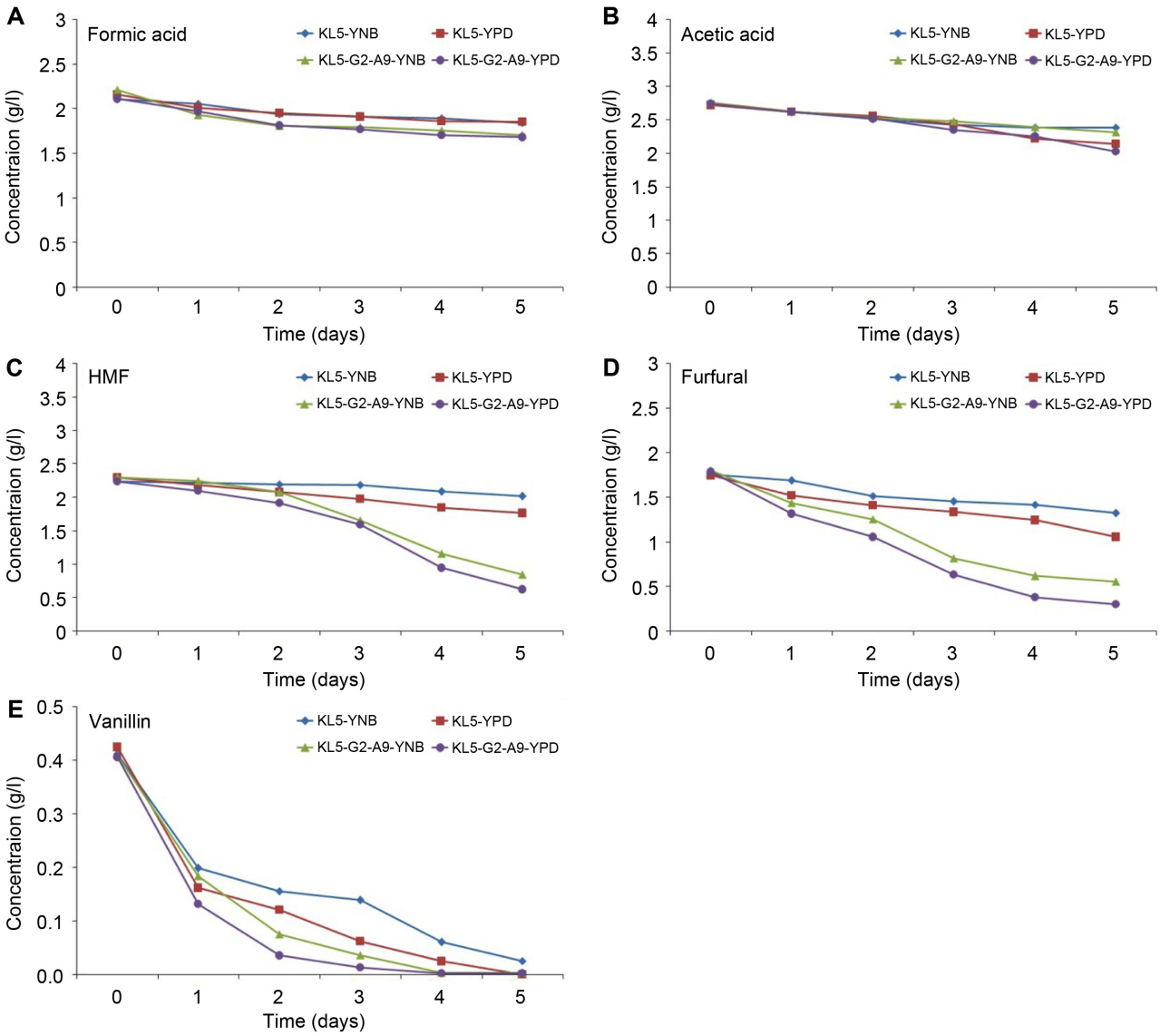

Fig. 6. Time courses of inhibitors reduction during ethanol fermentation by KL5 and KL5-G2-A9.
(A) Formic acid. (B) Acetic acid. (C) HMF. (D) Furfural. (E) Vanillin.
One loopful of activated cells of each strain was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of YNB or YP broth with 60% inhibitor cocktail and 5% glucose, and incubated for 5 days.

Landaeta *et al*. [11] and Lee *et al*. [14].

During fermentation, *S. cerevisiae* can convert furfural and HMF to their corresponding and less toxic alcohols, furfuryl alcohol [5, 27] and 2,5-*bis*-hydroxymethylfuran [30], respectively. A newly described aldehyde reductase enzyme encoded by *ARI1* of *S. cerevisiae* possessed reduction capabilities towards at least 14 aldehydes, including furfural, HMF, vanillin, and cinnamaldehyde [16]. In the case of aliphatic acids such as formic and acetic acids, inhibition of yeast was found to be apparent at concentrations exceeding 100 mM [12]. In our study, the concentration of formic and acetic acid was 45 mM, which was not the inhibitory concentration to ethanol fermentation, and no apparent reduction of the acids contents in the medium was observed (Fig. 6).

In conclusion, a *S. cerevisiae* strain highly resistant to inhibitors present in hydrolysate of lignocellulosics was developed using the sequential combination of selection, mutation, and adaptation. The developed strain showed a much improved theoretical ethanol yield and volumetric

productivity (7.5- and 7.7-fold, respectively, over the parent strain) in the medium containing 60% inhibitor cocktail. This result indicated that selection, mutation, and adaptation are efficient methods to improve the resistance of the yeast strains towards inhibitor cocktails for ethanol production.

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