

Metabolic and evolutionary engineering of diploid yeast for the production of first-and second-generation ethanol

Yang Sun

Jilin Agricultural University

Qian Xue

Capital Normal University

Junyan Hou

Capital Normal University

Meilin Kong

Capital Normal University

Xiaowei Li

Capital Normal University

Boyang He

Huazhong Agriculture University

Mingjie Jin

Nanjing University of Science and Technology

Zhipeng Lei

Jilin Agricultural University

Shuobo Shi

Beijing University of Chemical Technology

Limin Cao (✉ caolimin@cnu.edu.cn)

Capital Normal University <https://orcid.org/0000-0001-6435-7170>

Research

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Abstract

Background: *Saccharomyces cerevisiae* has been widely used in the fermentation of plant-derived sugars to produce ethanol, called first-generation (1G) bioethanol, but made an impact on global food markets. Significant efforts have been therefore to employ non-food lignocellulosic feedstocks for bioethanol production, known as second-generation (2G) bioethanol. However, *S. cerevisiae* cannot naturally utilize xylose, a major component in lignocellulosic hydrolysates, and it has low tolerance to common carboxylic acid inhibitors present in lignocellulosic hydrolyzates. Metabolic engineering and evolutionary engineering have shown great power in strain improvement, which were also adopted here to solve these limiting factors in developing 2G bioethanol.

Results: An efficient expression of a six-gene cluster, including *XYL1/XYL2/XKS1/TAL1/PYK1/MGT05196*, was achieved in the evolved *S. cerevisiae* diploid strain A21Z, showing the ability to use mixed glucose and xylose. The engineered strain A21Z expressing the six-gene cluster displayed a high xylose consumption after 96 h, reaching 90.7% of the theoretical yield in ethanol production. To investigate its industrial characteristics, A31Z was obtained by direct evolution of A21Z under the treatment of industrial hydrolysate from wheat straw. Under different fermentation conditions with 1G and 2G feedstock candidates, A31Z showed a markedly improved xylose fermentation performance. A31Z could produce more ethanol and less glycerol compared to the control Angel from corn starch during 120 h, with a final ethanol production at 122.32 g/L. The ability to produce higher ethanol production was also found under the fermentation using carbon source from hydrolysis of Dried Distillers Grains with Solubles (DDGS) or whole corn.

Conclusions: Here, we report an effective strategy to improve xylose fermentation with an evolutionary engineering in the industrial *S. cerevisiae* diploid strain A31Z. This study demonstrated that a constructed A31Z has the higher xylose consumption and efficient ethanol production in mixed glucose and xylose with acetate. A31Z also gave a good ethanol production in 1G and 2G industrial feedstocks, indicating its significant contribution in the transition stage from the 1st generation to the 2nd generation bioethanol.

Background

Due to the concerns of increased global energy demand and climate change caused by increasing carbon dioxide emissions, alternative energy sources, such as 2G bioethanol, are rapidly developed in recent decades as a renewable and environmental friendly biofuel [1, 2]. To enhance the economic viability of environmental sustainability of biofuels, it is necessary to produce more ethanol production from abundant, inexpensive, and renewable lignocellulosic biomass as feedstocks [3].

The highly complex and recalcitrant lignocellulosic biomass can be hydrolyzed into fermentable sugars: mostly glucose from primary component of cellulose and pentoses from hemicellulose [4]. One of them, xylose is as the second most abundant sugar after glucose. Industrial yeast *Saccharomyces cerevisiae* is a well-studied and robust cellular factory, but it cannot natively assimilate xylose. A complete and

efficient utilization of both glucose and xylose by metabolic engineering yeast strains have been the limiting factor to produce economically viable ethanol from xylose using lignocellulosic feedstocks [5]. Two engineering strategies have been taken to engineer xylose assimilation in yeast the natural xylose metabolizing enzymes, xylose isomerase (XI), or xylose reductase and xylitol dehydrogenase, called the oxidoreductase pathway by sequential redox reactions with an intermediate xylitol. In either strategy, the xylulose produced is phosphorylated to xylulose-5-phosphate by xylulokinase (XK), which feeds into the pentose phosphate pathway (PPP). Strategies have also been done for altered xylose transporters and modified expression of genes encoding non-oxidative PPP and key gene PYK1 in EMP in *S. cerevisiae*. Additionally, directed evolution with xylose in the growth medium has led to improved ethanol production [6, 7].

In order to facilitate the enzymatic hydrolysis of lignocellulosic material in ethanol yield, based on its complex structure, it must be subjected to pretreatment under harsh conditions [8, 9]. The purpose of pretreatment is highly needed to break down the dense structure of the cell wall in the raw material of lignocellulosic biomass to release the cellulose and hemicellulose fibers, which ensure that the enzyme and the substrate can have a full and effective contact [10]. Two typical chemical pretreatment methods such as dilute acid and dilute alkali pretreatment have gained interest in recent years because of the effective biomass hydrolysis results [11, 12]. Related parameters contain pretreatment time, concentration or the solid loading among many others. However, more efficient pretreatment technologies should focus on an in-depth understanding of its biosynthesis and regulation for engineering plant cell walls with lower recalcitrance [1]. Dr. Peng group in Wuhan studied four pairs of *Miscanthus* samples with distinct cell wall composition with varied biomass saccharification, determined three main factors to affect lignocellulose recalcitrance, and established a novel standard to judge any types of biomass process technology for high ethanol production [13, 14]. The produced inhibitors that decreased the yeast fermentation performance [15], including furans, phenolic compounds, weak acids, etc., can be an even greater problem when higher substrate concentrations are used in the fermentation process [16]. The limited tolerance of *S. cerevisiae* to the acetate inhibitor present in corn stover is also a major challenge in bioethanol production. Moreover, the pre-treatment strategies for every biomass types need further to be understood in terms of the degree of hydrolysis to determine the optimum conditions for maximum hydrolysis and minimum production of microbial growth inhibition by-products such as acetate [17]. Short or long-term adaptation of the yeast to its hydrolysates during cell propagation has been shown to improve its tolerance, and thus its fermentation performance. Therefore, there is a need to optimize the producing strains in higher tolerance to degradation products.

After pretreatment, the polysaccharides are hydrolyzed into monosaccharides to improve ethanol fermentation [18]. In fact, it is very necessary to develop the reduced the costs of pretreatment, saccharification, and subsequent distillation to increase the development of bioethanol commercialization. The attractive enzymatic hydrolysis has its inherent advantages due to low energy requirements for distillation, mild or small reaction conditions (tanks and distillation columns), and lack of corrosion issues in spite of its higher cost and high specificity [18]. At present, many types of biomass have been used to evaluate the effects of high solid loading (> 15% solids, w/w) on different unit

operations with the use of process stream to improve the operational costs for sugar conversion [19, 20]. After high solid loading, especially in conjunction with hydrolysis following high solid pretreatment, the high the concentration of reducing sugars, the high the ethanol concentration and, thus, it led to the low the bioethanol purification cost, reduced the economic cost of the fermentation or facilities, and the simplified process[21]. Dr. Jin group in Nanjing confirmed that high solids loadings (> 18, wt%) process in enzymatic hydrolysis and fermentation result in high bioethanol production at a low cost [22]. Jin group has integrated ethanol production and fermentation process including dilute alkali or acid pretreatment, substrate mixture ratios and solids loadings from mixtures of corn and corn stover, which are as very rich resources as lignocellulosic feedstocks in China [23, 24].

In our previous work, based on same haploid yeast strain, we showed that overexpression of three xylose utilization genes *XYL1*, *XYL2* and *XKS1* for an engineered *WXY12* strain improved the xylose consumption [25]. In the meanwhile, overexpressing four PPP genes increased ethanol production from xylose by previous report [26]. In addition, we further overexpressing six gene cluster in yeast, the resulting strain *WXY74*, to improve xylose utilization in the present of acetate in our lab [27]. Our constructed haploid engineered strains including *WXY12* and *WXY74* produced higher ethanol production among several yeast strains in different labs. Here, we demonstrated that metabolic/evolutionary engineering yeast diploid strain enhanced the activity of xylose consumption for new obtained *A21Z* strain, which increases ethanol production and significantly affects the metabolic efficiency. We then compared five representative pretreatments and enzymatic hydrolysis differences lies in Shanghai, Nanjing, Wuhan and Beijing focused on corn stover (CS) between the target *A21Z* strain and commercial strain Angel yeast and examined the effects of target genes on xylose metabolism under different fermentation conditions. These contain green SSCF without waste water, mixtures of corn and CS for first and second ethanol production, three specials biomass like bioenergy crop *Miscanthus* with distinct cell wall composition, four kinds of special biomass with solid acid pretreatments. We thusly confirmed this robust engineered strain *A21Z* that improved xylose utilization and obtained stable high ethanol production in the different fermentation strategies. In turn, the effects of various fermentation strategies at different condition by robust ethanol production of *A21Z* strain were also investigated. Rational metabolic and evolutionary engineering strategies for the efficient co-fermentation of mixed sugars with acetate inhibitor from CS that have been performed for strain development might be modified for robust ethanol production. Therefore, balanced integration and system optimization are needed to maximize economic and environmental benefits for cellulosic ethanol production.

Results

One xylose baseline industrial diploid yeast strain constructing and its followed evolutionary engineering

In this study, the combined metabolic and evolutionary engineering strategies were used to improve the metabolic utilization efficiency of glucose and xylose, acetate tolerance of *S. cerevisiae* from lignocellulosic hydrolysate. Due to the better fermentation performance of diploid yeast strain in vitality and endurance compared to that of haploid strain, two diploid yeasts, *S. cerevisiae* Angel and *S.*

cerevisiae Henderson, were selected as the starting strains to be modified. As expected, neither the natural Angel yeast nor the Henderson yeast can use xylose. Their comparative fermentation was conducted in the same condition containing 50 g/L glucose and 50 g/L xylose with 6 g/L acetate, which was displayed as below (Fig. 1 a and b). As can be seen from the Fig. 1, two diploid strains both made good use of glucose while only used 3 g/L xylose at the end of fermentation. Ethanol production had been on an upward trend before 12 h, and after that it had been on a downward trend, indicating that the yeast preferred glucose which was consumed before it began to metabolize little xylose [28]. The preferred glucose consumption of *S. cerevisiae* is the direct result of the sugar specificities of the hexose transporters. Xylitol formation of Henderson yeast was slightly higher than that of Angel yeast, and there was no significant difference in glycerol accumulation. Angel and Henderson yeast strain both consumed most of initial acetate, resulting in an end concentration at 2.3 and 1.2 g/L acetate, respectively.

To enable the ability for xylose utilization, one xylose metabolic pathway carried by two plasmids *fps1-nat^r* (*XYL1, XYL2, XKS1*) and *pUC-TTRR* (*TAL1, TKL1, RKI1, RPE1*) was introduced into Angel and Henderson strain, producing two engineered strains ABN and BBN, respectively [26, 29]. The fermentation performance of each engineered strain was evaluated in mixed sugar medium with acetate. ABN and BBN both consumed all glucose within 12 h, while little differences were observed in xylose consumption and ethanol production after glucose depletion. Between two engineered diploid strains, ABN showed a higher efficiency in xylose consumption and ethanol production with 27.4 g/L ethanol produced from 50 g/L glucose and 50 g/L xylose in 120 h. The amount of residual xylose was dramatically decreased to 28.8 g/L in 120 h. BBN utilized approximately 10.9 g/L xylose and produced 25.0 g/L ethanol (Fig. 1 c and d). Therefore, xylose metabolic efficiency of ABN is superior to BBN accompanied with similar glycerol accumulation and lower xylitol accumulation. Therefore, ABN with better performance was selected to conduct directional evolution experiment under the pressure of acetate. Unexpectedly, the capability of two engineered strains ABN and BBN to use acetate was much lower than that of their own original strain, indicating that metabolism of acetate was inhibited when the overexpressed xylose metabolism was enhanced.

Directed evolution and genetic modification of diploid strains

The presence of acetate can seriously hinder the yeast growth in the process of mixed sugar fermentation and results in lower xylose utilization and ethanol formation. When a large amount of acetate exists in the medium, it leads to intracellular acidification and hinders the utilization of xylose. The improvement of acetate metabolic pathway can effectively accelerate the utilization of acetate. Directed evolution was adopted here to effectively increase the metabolic efficiency of mixed sugar with acetate. Compared with haploid yeast, diploid yeast with larger in volume and stronger in vitality is often used in industrial ethanol fermentation. In this study, the originally diploid strain ABN was evolved to obtain the strain A1 [27]. On this basis, A2 was obtained by evolving A1 in the YP medium with xylose acclimation containing 8 g/L acetate. The xylose consumption and ethanol production of A1 and A2 strains were compared in fermentation medium with 79.1 g/L glucose, 39 g/L xylose and 3.1 g/L acetate (Fig. 1 e and f). Samples were taken every 12 h during 96 h fermentation process. Although the two tested strains

consumed glucose within 12 h, there also existed some differences about xylose consumption and ethanol production after glucose depletion. A1 produced 43.6 g/L ethanol with consumption of approximately 17.9 g/L xylose relative to 45.2 g/L ethanol production with 21.4 g/L xylose consumption of A2, reaching 88.1% and 88.2% of the theoretical yield in ethanol production, respectively. In contrast to A1, A2 consumed 3.5 g/L more xylose and produced 1.6 g/L more ethanol, resulting in its better obvious fermentation advantage. It can be likely that the original strain had less genetic modification, which was not conducive to producing more favorable mutations (Fig. 1 e and f).

The xylose consumption and ethanol production of strains expressing different promoters were compared under glucose and xylose co-fermentation conditions in previous research [27]. The remained xylose after fermentation in strain WXY46 (six gene cluster: *XYL1(K270R)-XYL2-TAL1-PYK1-MGT05196-PYK1-MGT05196*) expressing xylose pathway genes by constitutive promoters was significantly lower than that of strain WXY48 expressing only xylose pathway genes by HSP promoters and strain WXY63 expressing xylose pathway genes by only TCA promoters. In consistent with that the ethanol yield of WXY46 was also higher than that of strain WXY48 or WXY63. Therefore, we expressed the six gene cluster of higher ethanol production on A2 to obtain a better A21Z strain. We wanted to investigate whether A2 can further promote the ethanol production and general applicability of the six gene cluster. The A22Z was obtained by integrating the 2z-e7 cluster (*XYL1 (K270R)-XYL2-TAL1-kIPYK1-MGT05196-kIPYK1-MGT05196*) into A2 as same above. The final xylose consumption and the ethanol yield of A21Z were 37.5 g/L and 53.5 g/L, respectively, reaching 90.7% of the theoretical yield in ethanol production. The A22Z produced ethanol of 51.1 g/L with consuming xylose of 33.2 g/L, reaching 90.1% of the theoretical yield in ethanol production. The amount of residual xylose of A21Z was dramatically decreased to 1.1 g/L in 96 h relative to the control strain A2, whose produced ethanol was also 8.3 g/L less than A21Z. Therefore, compared with A2, A21Z and A22Z showed significant improvement in xylose consumption rate, ethanol production rate and sugar alcohol conversion rate. However, the fermentation effect of A22Z was slightly worse than that of A21Z (Fig. 2 a and b).

Adaptive acclimation of the target strain *S. cerevisiae* A21Z in hydrolysate at the solids loading of 15% wheat straw stover

The lignocellulose is usually used to produce bio-based chemicals such as ethanol by anaerobic fermentation at present. Because of the complicated components of the wheat straw hydrolysate, inhibitors are accumulated in the pretreatment step. Although some inhibitors are removed after detoxification, the remaining inhibitors still negatively affects the cell growth of the fermentation. The adaptive domestication strategy is the commonly used method so that the target strains can be better adapted to the fermentation environment of industrial hydrolysate. In this study, Fig.3 a and b indicated that the glucose concentration of the mixture of the yeast seed A21Z and treated wheat straw hydrolysate at 10.0% (v/v) was 45.85 g/L; the initial concentration of xylose, ethanol and glycerol were 17.07 g/L, 1.84 g/L, 1.01 g/L, respectively. Stability of commonly used measures of target strain in the 15% solids loading wheat straw hydrolysate mainly referred to the utilization of glucose, xylose, and increasing ethanol and glycerol generation. 15% wheat straw stover hydrolysate was utilized by A21Z to produce

ethanol directly (Fig. 3 a, b). In the initial transfer process, target strain A21Z could not utilize 15% wheat straw stover hydrolysate stably to enrich ethanol. While with the adaptive evolution, the releasing glucose and xylose with the increasing ethanol and the glycerol generation kept in a stable range. This means that the strain had adapted to the environment of 15% solid content of wheat straw detoxified hydrolysate, and the simultaneous saccharification and co-fermentation (SSCF) could be carried out to further evaluate the target strain.

Simultaneous saccharification and co-fermentation (SSCF) of *S. cerevisiae* A21Z and its acclimated A31Z

Wheat straw was dry acid pretreated and bio detoxified before the simultaneous saccharification and co-fermentation (SSCF). The co-fermentation strains using glucose, xylose and other kinds of saccharides were one of the key factors for simultaneous saccharification. SSCF was the better choice for the use of xylose rich lignocellulosic materials to produce ethanol and A21Z was the better target strain for mixed sugar fermentation at laboratory fermentation stage. With the adaptive acclimatization, the further adapted strain A31Z derived from A21Z in wheat straw stover hydrolysate was evaluated by SSCF. In the pre-hydrolysis stage, as shown in Fig.3 c, glucose increased with the saccharification time with a certain cellulase dosage of 9.71% (W/W) in fermentation medium. Xylose was constant in the saccharification time because the majority of xylan was already converted to xylose and oligo-xylan in the pretreatment step. Glucose and xylose were about 77.08 g/L and 35.95 g/L with the saccharification time of 12h. In the consequent SSCF stage, with the function of adapted *S. cerevisiae* A31Z, the initial glucose was quickly converted into ethanol within 24 h and then started to utilize glucose from cellulose hydrolysis. Xylose conversion decreased with the fermentation time step by step. Finally, at the cellulase dosage of 15 mg total protein /g, adapted *S. cerevisiae* A31Z produced 56.68 g/L of ethanol, leading to sugar alcohol conversion rate of 63.13%; the xylose conversion rate of A31Z achieved 84.90% (Fig.3 c). Approximately, half ethanol came from the initial glucose released from the pre-hydrolysis, the other half was from xylose and the glucose released during the SSCF [30].

Fermentation performance of different yeasts in enzymatic hydrolysis of *Miscanthus*, maize and wheat straw

Lignocellulosic biomasses such as *Miscanthus*, maize and wheat straw are important feed stocks for 2G bioethanol. Here, sugars from enzymatic hydrolysis of *Miscanthus*, maize and wheat straw were used as substrates for the yeasts to produce ethanol, respectively. Sugar yields (% dry matter) released could be found in Table S1, and relative cell wall composition from these biomasses could be found in Table S2. As shown in Fig. 4, the control strain Angel used in a company for ethanol production, the evolved strain A31Z and our previous reported strain CE7 were chosen to ferment these hydrolyses at 37°C. Ethanol yield and concentration produced could also found in Table 2. It showed that strain A31Z could give a higher production of ethanol in the hydrolysis of *Miscanthus*. Under the condition of other two hydrolyses, there is no significant difference in the ethanol production among these three yeasts. However, as shown

in Table 3, there is a significant improvement of pentose utilization rate of A31Z in all three kinds of hydrolyses.

Fermentation performance of A31Z using corn as feedstocks

The engineered strain A31Z was given a better performance in pentose utilization, and showed a good potential to be used as a cell factory for 2G ethanol. Here we also evaluated the performance of A31Z and a commercial strain Angel as control strain in 1st generation yeast-based production process of bioethanol from corn (Fig. 5a and 5b). Corn contained approximately two-thirds starch, and the corn starch was converted to glucose with the process described in the method part. After 120 h fermentation with extracted glucose from corn starch, the control strain Angel could produce 119.40 g/L ethanol production, while A31Z could produce 122.32 g/L ethanol, as shown in Fig. 5a and 5b. However, the byproduct glycerol was reduced about 20% in A31Z. It was believed that glycerol and ethanol levels are inversely related [31], and the slightly increased ethanol in A31Z could be due to the decreased glycerol.

Corn contained approximately two-thirds starch. When corn is processed by yeast to produce ethanol, other component corn except starch could be recycled into a rich and nutritious feedstock, named DDGS (Dried Distillers Grains with Solubles). Glucose and xylose were the main sugars contained in DDGS. The A31Z was purposely engineered to use pentose, and was firstly evaluated its performance using a mixture of glucose/xylose as a mimic of DDGS hydrolysis (Fig. 5 c and d). As expected, there was a significant improvement when xylose was added in the medium, which is also consistent with our previous data [27]. A31Z made an ethanol production at 63.33 g/L, and Angel only produced 46.21 g/L ethanol. The difference was obviously due to the different xylose consuming ability of these two stains as shown in Fig. 5c and 5d. Then A31Z and Angel were fermented using DDGS hydrolysis directly (Fig. 5e and 5f). The fermentation of A31Z resulted in an ethanol production at 5.54 g/L from a total sugar at 11.17 g/L (contained 7.84 g/L glucose and 3.33 g/L xylose). In contrast, the fermentation of Angel only resulted in an ethanol production at 4.94 g/L from a total sugar at 9.56 g/L (contained 6.74 g/L glucose and 2.82 g/L xylose). It showed that there was a better performance in A31Z for ethanol production under the fermentation using DDGS.

The SSCF of whole corn in integrated ethanol production

It has been reported that the integrated fermentation of whole corn could result in a higher ethanol concentration and could facilitate the introduction of the 2G technology [32, 33]. In the above parts, the constructed strain A31Z had been shown a good fermentation ability from a mixture of glucose and xylose, 1G feedstock (such as corn) and 2G feedstock (such as *Miscanthus*). In this section, we ferment this strain in an integrated process to simultaneously ferment 1G and 2G feedstock. As shown in Fig. S1, the carbon source was obtained from corn flour (1G feedstock) and corn stover (2G feedstock). The SSCF experiments showed there was a significant improvement in the ability of ethanol production and xylose utilization in the strain A31Z (Table 4). It had been reported that the integrated 1G and 2G ethanol production process will result a better economic result when compared with the stand-alone 1G or 2G plant [34]. Here we showed the technical feasibility of A31Z in the integrated 1G + 2G ethanol production

process from whole corn with the current hydrolysis technology. In the future, the ethanol production ability can be further enhanced when advanced hydrolysis technologies were developed, which could result a better economic result and enabled its commercialization.

Discussion

This study provides a roadmap for how to progressively optimize a six-gene cluster of xylose metabolic pathway to improve ethanol production for a diploid yeast strain by using the effective metabolic and evolutionary engineering strategies. In our previous studies, the unbalanced or low expression level of xylose metabolism genes *XYL1* (K270R), *XYL2* and *XKS1* would lead to the imbalance of xylose metabolism pathway in yeast, resulting in the massive accumulation of xylitol, the intermediate product. Overexpressing the rate-limiting enzyme genes *PYK1* can promote the conversion of sugar into ethanol. High expression of key gene *TAL1* along with highly overexpressed above three genes simultaneously can promote xylose metabolism, otherwise a series of by-products such as glycerol and xylitol would be accumulated. Moreover, overexpression of xylose transporter *MGT05196* can improve the xylose uptake and its utilization efficiency. Some constitutive promoters as *PGK1*, *ADH1*, *TEF1*, *TDH3*, TCA cycle promoters as *MDH1*, *ACO1* and HSP type promoters, such as heat shock family *HSP26*, *HSP31* are used to drive the above xylose metabolic genes as a six gene cluster in a haploid evolved strain CE7 [27], resulting in improved the utilization rate of xylose and more ethanol production formation. This indicates that the proposed promoters of different fermentation stages (glucose and xylose stage) can effectively regulate the transcription levels of xylose metabolism in the presence of acetate. Based on the better ethanol producer haploid strain like *WXY46* with the efficient six gene cluster, it is expected to be more suitable for industrial hydrolysates for the common candidate diploid strain. Our results also showed that the overexpressed xylose metabolic pathway inhibited the acetate metabolism, and excessive acetate in turn inhibited xylose metabolism, which required us to combine metabolic and evolutionary engineering to optimize its fermentation capabilities simultaneously. By rational molecular modification and adaptive evolution, optimized overexpression of six gene cluster in haploid and diploid yeasts both can promote ethanol production effectively. Thus we obtained the improved ethanol productions of target engineered strains *ABN* and *A21Z* from 53.7–90.7% of the theoretical yield. The experimental results show that we have found a highly efficient and stable gene cluster of xylose metabolic pathway, which is at advanced stage and providing new experimental evidence and theoretical basis for applied study of bioethanol.

To optimize the SSCF potentials relative to cellulase activity and xylose with remaining inhibitors for cellulosic ethanol fermentation of *A21Z*, a successful dry biorefining approach was proposed starting from dry acid pretreatment, disk milling, and biodetoxification of lignocellulose feedstock such as wheat straw [30]. Such a well-recognized SSCF of low inhibitor and xylose conserved lignocellulose feedstock after dry biorefining achieved high ethanol production at moderate cellulase usage and minimum wastewater generation. In the meanwhile, the efficient co-expression of six gene cluster also contributed to the enhancement of the degradation of lignocellulose-derived inhibitors. For wheat straw, 56.7 g/L of ethanol for its evolved strain *A31Z* was produced with the overall yield of 63.1% from cellulose and xylose, in which the higher xylose conversion was 84.9%, at the moderate cellulase dosage of 15 mg/g.

The higher xylose conversion efficiency in SSCF fermentation accompanied by the minimum amount of wastewater generation is obtained in the current dry biorefining for cellulosic ethanol production, indicating that adapted diploid *S. cerevisiae* A31Z was a better SSCF fermentation strain evaluated by the advancing biorefining technology.

The target strain *S. cerevisiae* A31Z was evolved under the treatment of wheat straw stover hydrolysate. However, the accumulated mutations gave it a general phenotype in bioethanol production fermenting different hydrolysates, such as *Miscanthus* (Table 2), DDGS (Fig. 5e and 5f) and whole corn (Table 4). Further genome sequencing and evaluation of individual mutations could be a good strategy to reveal the potential mechanisms for resistant of the inhibitors in hydrolysate. Since there is a good performance in ethanol production of A31Z in different simulated industrial conditions, it means that A31Z would be a good workhorse to be evaluated in bigger scale. Our results indicate that a non-xylose utilization strain could be developed to significantly consume xylose and hydrolysis of 1G/2G feedstocks, which could further foster academic and commercial strain development for other large scale chemicals/fuels.

Conclusion

Currently, almost all commercial bioethanol is derived from corn or sugar cane, which is under criticisms of competition of land and water and elevation of food prices. Significant efforts have been made to the development of 2G bioethanol, also called the lignocellulosic bioethanol. Here we have made an integrated engineering of an ethanol producer A31Z by metabolic engineering and direct evolution. The strain has shown a good performance in ethanol production in mixed glucose and xylose. We further demonstrated that A31Z also gave a good ethanol production in 1G and 2G industrial feedstocks, indicating its significant contribution in the transition stage from the 1st generation to the 2nd generation bioethanol.

Methods

DNA manipulation

All DNA manipulations were carried out in *Escherichia coli* strain DH5 α as described [35].

Construction of yeast strains

cerevisiae haploid strains Angel and Henderson used in this study were obtained from *Angel Yeast Co., Ltd.* The reported a plasmid pUC-TTRR [26] and another reported plasmid pUC-fps1-nat [29] were transferred to Angel and Henderson for constructing strain ABN and BBN, respectively. Repeated batch cultivation of yeast strain ABN was performed for independent evolution using a previous reported method [27]. Strain A1 and A2 was obtained after three years evolution process. Strain A21Z was obtained by integrating the expression cassette 1z-e7 (*XYL1 (K270R)-XYL2-TAL1-PYK1-MGT05196-PYK1-MGT05196*) [27] into strain A2. A22Z was obtained by integration of another copy of the expression cassette 1z-e7 in A21Z. A31Z was obtained by adapted evolution with A21Z grown in 15%

wheat straw stover hydrolysate using a previous reported method [27]. *S. cerevisiae* strains used in this study are listed in Table S1. The method used for yeast transformation is the standard LiAc/SS carrier DNA/PEG method [36].

Medium

The YP medium contained 20 g/L tryptone and 10 g/L yeast extract.

Enzymatic hydrolysis of lignocellulosic biomasses

The biomass power (0.300 g), Miscanthus, maize and wheat straw, was respectively incubated with 0.012 g mixed cellulases (containing cellulases at 10.60 FPU g⁻¹ biomass and xylanase at 6.72 U g⁻¹ biomass from Imperial Jade Bio-technology Co., Ltd) containing 0.8% Tween-80 at 5% solid loading, and shaken under 150 rpm for 48 h at 50 °C. The samples were centrifuged at 3000g for 5 min, and the supernatants were collected for hexoses and pentose assay.

Fermentation from hydrolysis of corn starch and DDGS (Dried Distillers Grains with Solubles)

To obtain hydrolysis of corn starch, starch samples with 30% solids were mixed with the enzyme liquozyme with a loading at 0.064% under 85°C with a pH at 5.7 for 4 hours. Then the saccharification was performed with the addition of 0.1% starch hydrolyzing enzyme and 0.6% Novozymes Celluclast® with a pH at 4.8 for 0.5 hours. The corresponding fermentation was adopted under 30°C with a pH at 4.6 and 150 rpm for 72 hours. The initial OD₆₀₀ for the fermentation was 1.0. When the fermentation was finished, unconverted (residual) starch was recovered with the treatment of 1% H₂SO₄ under 95°C for 90 mins. Then the sample was evaporated to remove the remaining ethanol at 0.09mpa under 85°C for 30 mins. After the evaporation, water was added to the sample to recover the initial volume with a adjusted pH between 4.8 and 5.0. Subsequently, 0.6 % cellulases and 1.5% xylanase was added and under incubation with 50°C at 250 rpm for 24 hours. Finally, the hydrolysis was adjusted to pH 4.6 and adopted as the medium for fermentation from DDGS.

Simultaneous saccharification and co-fermentation (SSCF) of whole corn

The corn stover is pretreated using dilute sulfuric acid (1%) at a high temperature (160°C) for 10 minutes with a solid loading at 10%. Then the liberated hemicelluloses from corn stover were incubated under 50°C for 48 h at 250 rpm with mixed 40 mg cellulases per gram glucan (containing cellulases and xylanase with a ratio at 9:1). Meanwhile, the corn flour is treated under 85°C for 4 h for liquefaction using the enzyme liquozyme with a loading at 0.064%.

The schematic process of SSCF could be found in Fig. S1. The co-fermentation as performed in shake flaks under 30°C for 72 h containing 12% corn flour hydrolysis, 12% corn stover hydrolysis, and 0.1% diastase with a initial OD of 1.0.

Analysis methods

OD₆₀₀ and Cell dry weight (CDW) were measured described earlier [25]. Glucose, xylose, xylitol, ethanol and acetic acid were analyzed by HPLC using its related column and detector run with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.4 mL/min [25]. The pentose and hexose of soluble sugars were respectively detected as described previously [14].

Declarations

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Authors' Contributions

YS, QX, JYH, BYH and ZPL performed some of the experiments. MLK and XWL participated in some of the experiments and manuscript writing. MJJ participated in the design of the experiments and performed some of the experiments. SBS participated in the design of the experiments and contributed to manuscript writing. LMC supervised the research and participated in the design of the experiments and manuscript writing. All authors have contributed to scientific discussion. All authors read and approved the final manuscript

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Availability of data and materials

The data sets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Author details

1, Key Laboratory of Straw Biology and Utilization, the Ministry of Education, College of Life Science, Jilin Agricultural University, Changchun 130118, China; Yang Sun & Zhipeng Lei; 2, College of Life Sciences, Capital Normal University, Beijing 100048, China; Qian Xue, Junyan Hou, Meilin Kong, Xiaowei Li & Limin Cao; 3, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China; Boyang He; 4, School of Environmental and Biological Engineering, Nanjing University of Science and Technology, 200 Xiaolingwei Street, Nanjing 210094, China; Mingjie Jin; 5, Beijing Advanced Innovation Center for Soft Matter Science and Engineering, Beijing University of Chemical Technology, Beijing, China; Shuobo Shi

Corresponding authors

Correspondences to [Shuobo Shi](#) and [Limin Cao](#).

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Tables

Table 1. Strains used in this study

Name	Description	References
<i>S. cerevisiae</i> Angel	Angel yeast	Angel Yeast Co., Ltd China
<i>S. cerevisiae</i> Henderson	Henderson yeast	Angel Yeast Co., Ltd China
<i>S. cerevisiae</i> Angel	Angel yeast	One company in China
<i>S. cerevisiae</i> CE7	Evolution from E6	[27]
<i>S. cerevisiae</i> WXY74		[27]
<i>S. cerevisiae</i> ABN	Angel, <i>KanMX</i> :: PDC1p- <i>TKL1</i> - <i>TKL1t</i> /PGK1p- <i>TAL1</i> - <i>TAL1t</i> /TPI1p- <i>RKI1</i> - <i>RKI1t</i> /ADH1p- <i>RPE1</i> - <i>RPE1t</i>	[26]
<i>S. cerevisiae</i> BBN	Henderson, NAT:: <i>ADH1p</i> - <i>XYL1</i> (K270R)- <i>ADH1t</i> /PGK1p- <i>XYL2</i> -PGK1t/PGK1p- <i>XKS1</i> -PGK1t	[29]
A1	evolutionary engineering for ABN	This study
A2	evolutionary engineering for ABN	This study
A21Z	A2, <i>hygB</i> :: T1-Z1, T2-Z2, T3-Z3, T4-Z4 A2:: <i>2z-e7</i>	This study
A22Z	evolutionary engineering for A21Z	This study
A31Z		This study

Table 2 Ethanol yield (% dry matter) and concentration (g/L) of different yeast using the hydrolysis of Miscanthus, maize and wheat straw

Strains	Hydrolysis								
	<i>Miscanthus</i>			maize			wheat straw		
Ethanol yield (% dry matter)									
Angel	9.80	±	0.22	7.41	±	0.19	6.47	±	0.24
CE7	7.82	±	0.19	6.11	±	0.15	6.05	±	0.16
A31Z	10.51	±	0.32	7.66	±	0.17	6.37	±	0.27
Ethanol concentration (g/L)									
Angel	4.73	±	0.11	3.57	±	0.09	3.12	±	0.12
CE7	3.77	±	0.09	2.95	±	0.07	2.92	±	0.08
A31Z	5.07	±	0.16	3.70	±	0.08	3.07	±	0.13

Table 3 Sugar-ethanol conversion rate (%) and pentose utilization rate (%) of different yeast using the hydrolysis of *Miscanthus*, maize and wheat straw

Strains	Hydrolysis								
	<i>Miscanthus</i>			maize			wheat straw		
Sugar-ethanol conversion rate (%)									
Angel	60.88	±	1.85	66.07	±	2.01	71.26	±	2.17
CE7	56.91	±	1.26	61.76	±	1.37	66.61	±	1.48
A31Z	59.94	±	2.07	65.05	±	2.25	70.16	±	2.42
Pentose utilization rate (%)									
Angel	38.38	±	0.95	22.45	±	1.46	39.65	±	1.19
CE7	28.01	±	1.21	21.15	±	1.43	37.67	±	0.86
A31Z	45.81	±	1.11	34.53	±	1.43	51.86	±	0.89

Table 4. Concentration (g/L) of Ethanol, sugars, and other main metabolites in SSCF experiments

Strain	Time (h)	Acetate (g/L)	Ethanol (g/L)	Glucose (g/L)	Xylose (g/L)	Glycerol (g/L)	Xylitol (g/L)	Maltose (g/L)
Angel	0	1.86	0.00	39.69	18.11	0.00	0.00	6.31
	24	1.87	51.95	1.48	11.16	4.30	1.69	0.85
	48	2.02	58.54	0.13	9.38	4.35	3.46	0.00
	72	2.25	60.73	0.08	8.69	4.41	4.44	0.00
A31Z	0	1.86	0.00	39.69	18.11	0.00	0.00	6.31
	24	1.65	54.60	2.33	3.62	5.21	1.11	1.87
	48	1.73	64.34	0.17	0.25	5.35	1.74	0.78
	72	2.05	67.18	0.05	0.18	5.28	2.21	0.30

Figures

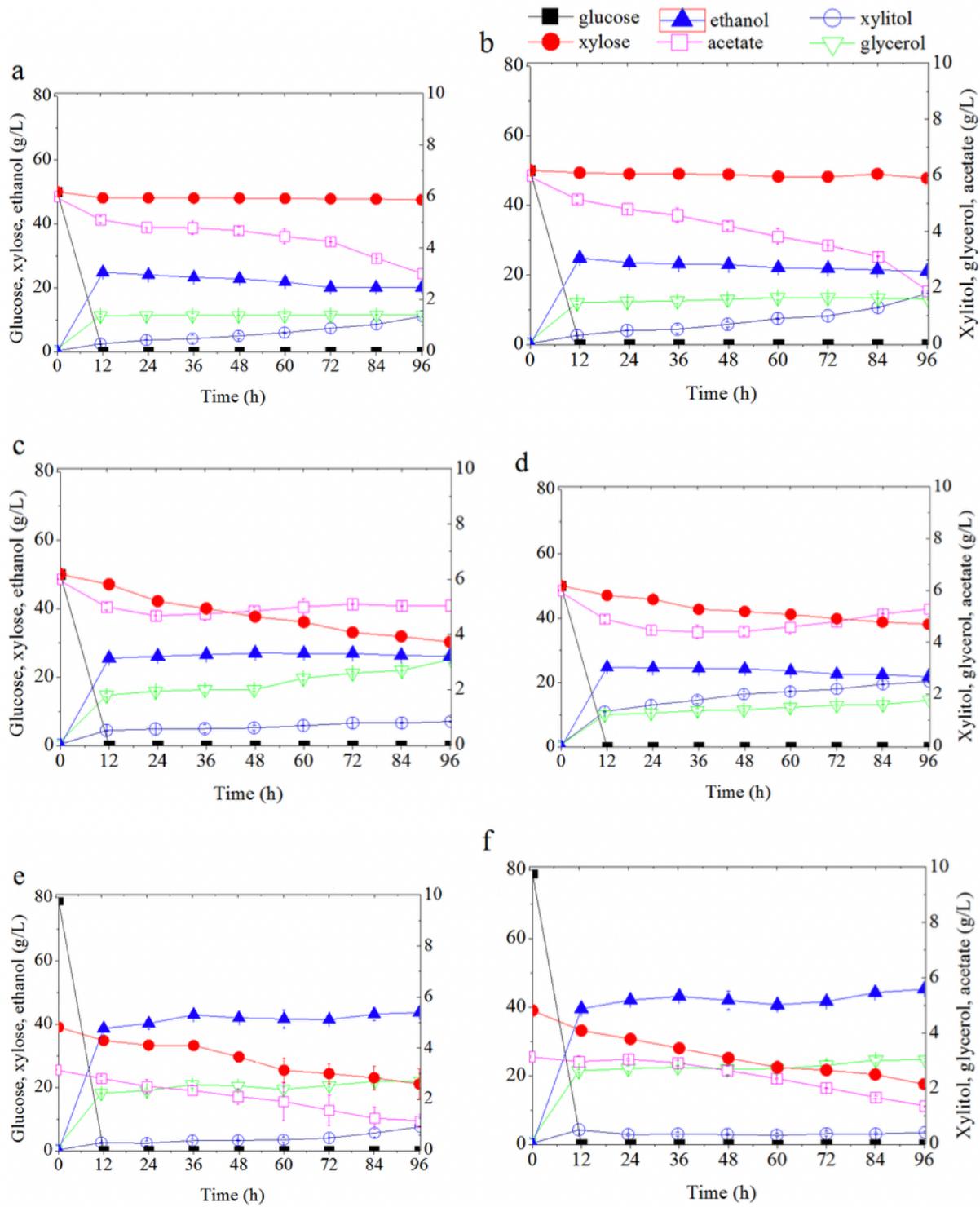


Figure 1

Time-dependent ethanol fermentation profiles of yeast strains. The fermentation profile of Angel yeast (a) and Henderson yeast (b) in mixed sugar medium of 50 g/L glucose and 50 g/L xylose containing 6 g/L acetate. The fermentation profile of ABN (c) and BBN (d) in mixed sugar medium of 50 g/L glucose and 50 g/L xylose containing 6 g/L acetate. The fermentation profile of A1 (e) and A2 (f) in YP medium with 3 g/L acetate, 80 g/L glucose and 40 g/L xylose. Symbol: glucose (black solid square), xylose (red solid triangle), ethanol (blue solid circle), xylitol (green hollow square), glycerin (yellow hollow circle), acetate (blue hollow inverted triangle). Each experiment was repeated three times, two parallel controls at a time.

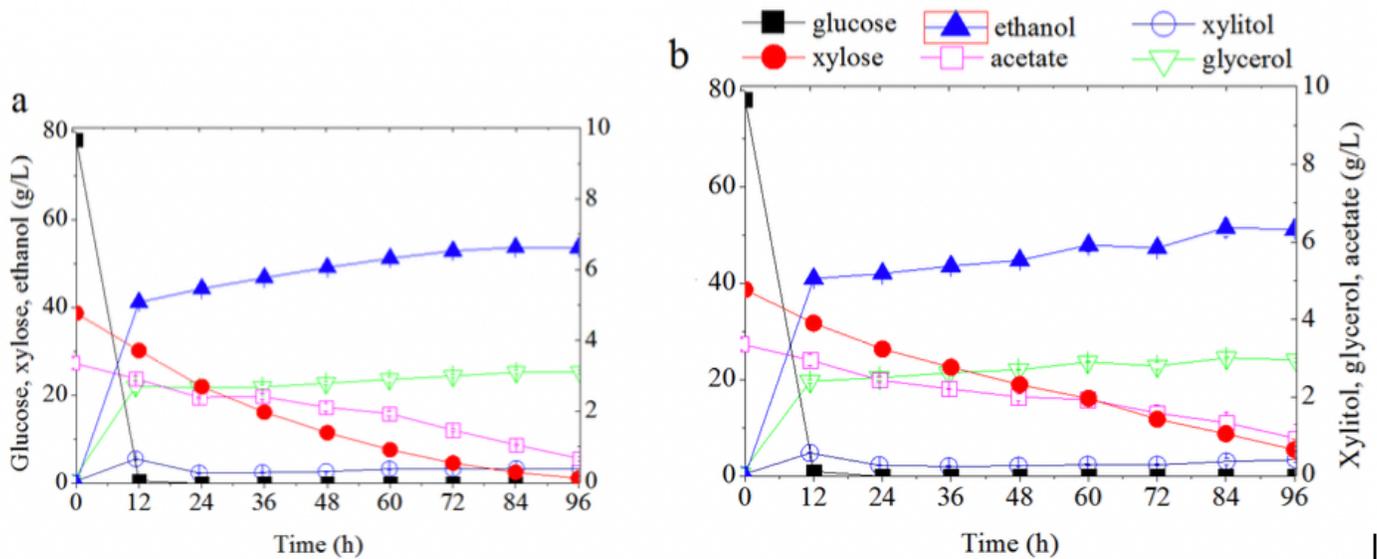


Figure 2

Time-dependent ethanol fermentation profiles of yeast strains. The fermentation profile of A21Z (a) and A22Z (b) in YP medium with 3 g/L acetate, 80 g/L glucose and 40 g/L xylose.

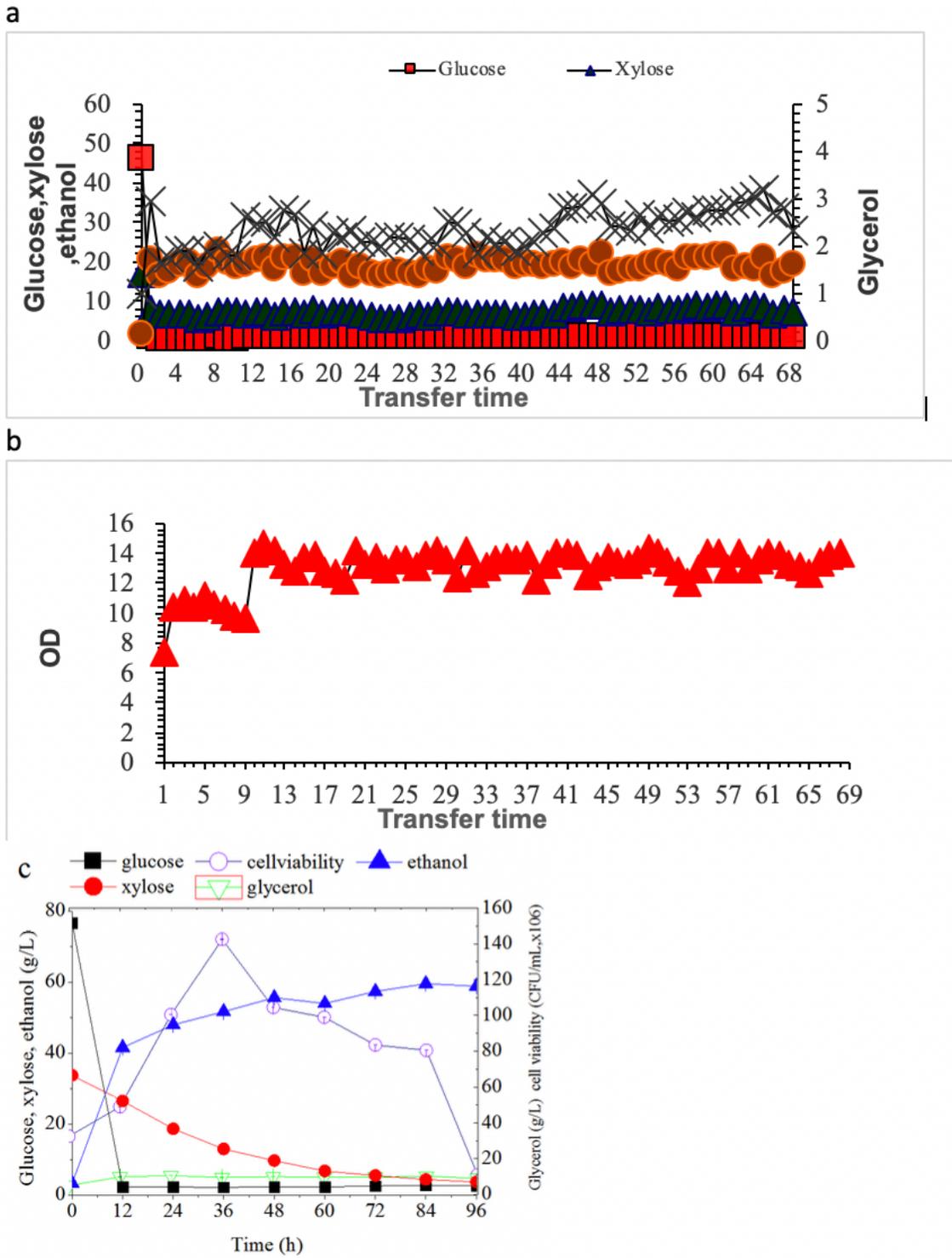


Figure 3

Time-dependent ethanol fermentation profiles of yeast strains. The fermentation profile of A21Z (a, b) in treated wheat straw hydrolysate with 45.85 g/L glucose, 17.07g/L xylose, 1.84 g/L ethanol and 1.01 g/L glycerol. The simultaneous saccharification and co-fermentation (SSCF) profile of A31Z(c).

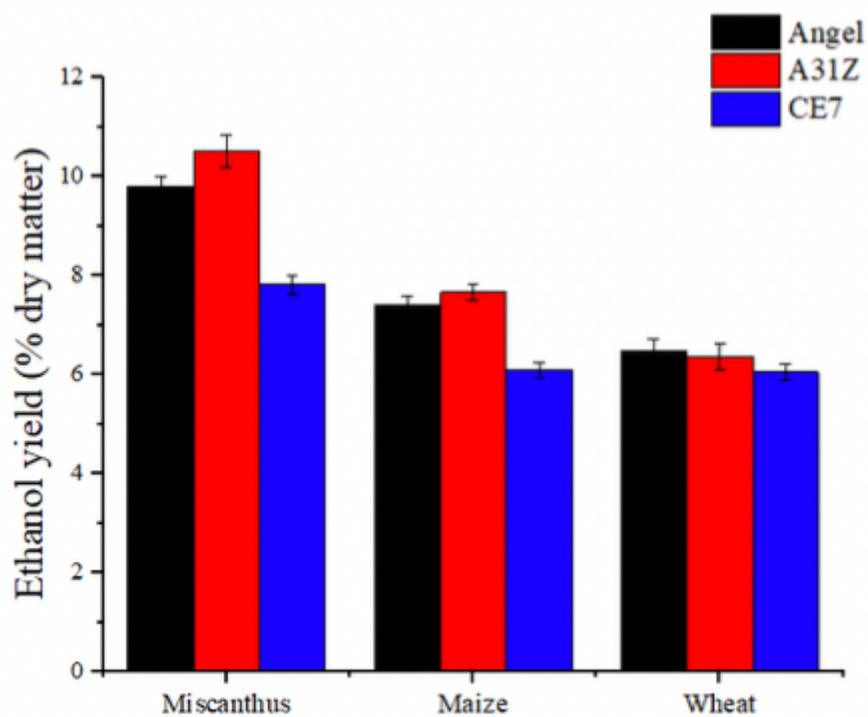


Figure 4

Time-dependent ethanol fermentation profiles of yeast strains. Ethanol yield (% dry matter) of Miscanthus, maize and wheat straw with Angel, A31Z and CE7 yeast strains.

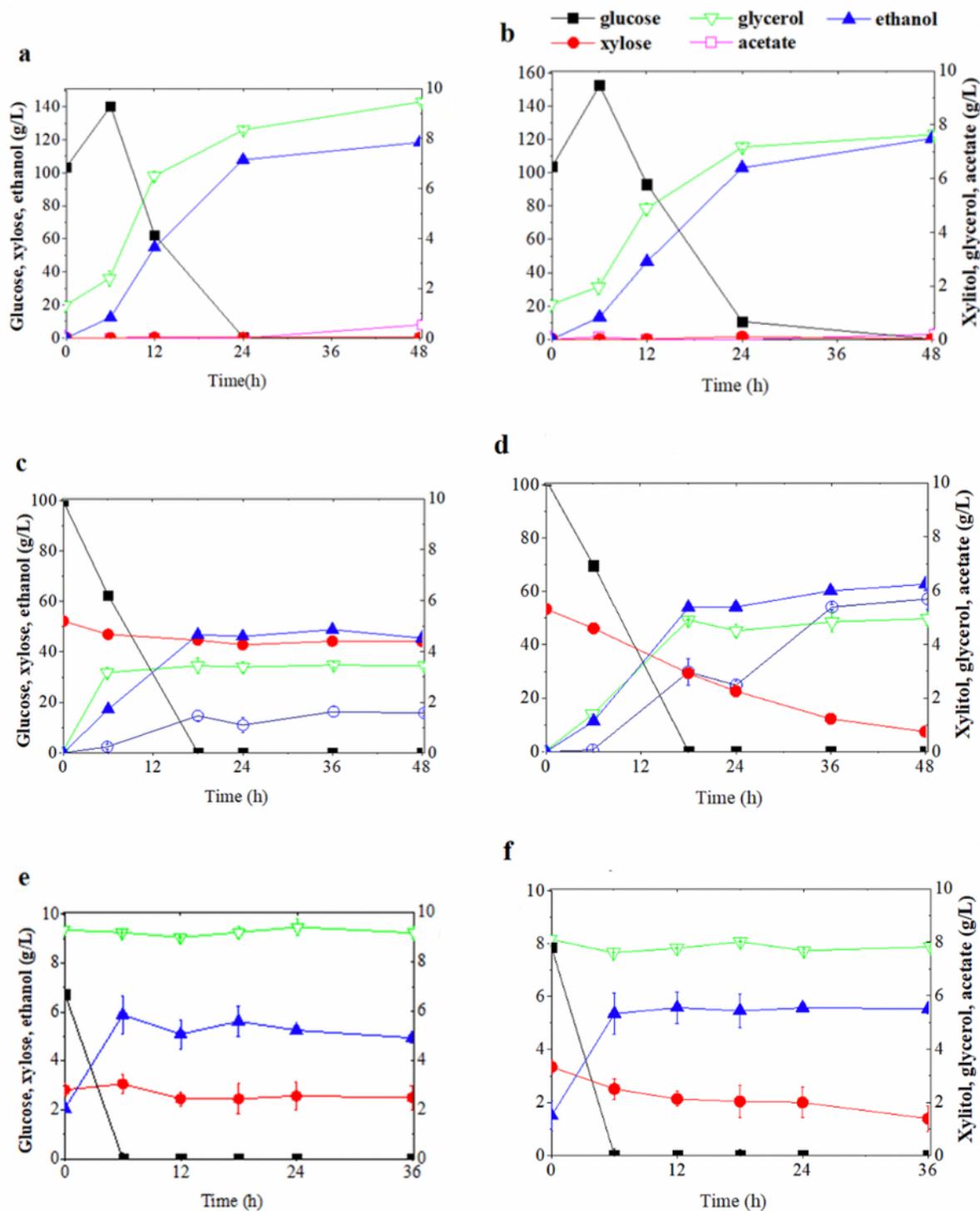


Figure 5

Time-dependent ethanol fermentation profiles of yeast strains The fermentation profile of Angel yeast (a) and A31Z yeast (b) in corn ethanol medium. The fermentation profile of Angel (c) and A31Z (d) in mixed sugar medium of 100 g/L glucose and 50 g/L xylose. The fermentation profile of Angel (e) and A31Z (f) from DDGS (Dried Distillers Grains with Solubles).

Supplementary Files

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