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Overexpression of *SFA1* in engineered *Saccharomyces cerevisiae* to increase xylose utilization and ethanol production from different lignocellulose hydrolysates

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ABSTRACT

Here, an engineered *Saccharomyces cerevisiae* strain SFA1^{OE} was constructed by overexpressing *SFA1* in a reported WXY70 with effective six-gene clusters. Under simulated maize hydrolysate, SFA1^{OE} produced an ethanol yield of 0.492 g/g total sugars within 48 h. The productivity of SFA1^{OE} was comprehensively evaluated in typical hydrolysates from stalks of maize, sweet sorghum, wheat and *Miscanthus*. Within 48 h, SFA1^{OE} achieved an ethanol yield of 0.489 g/g total sugars in the optimized hydrolysate of alkaline-distilled sweet sorghum bagasse derived from Advanced Solid-State Fermentation process. By crossing SFA1^{OE} with a DQ1-derived haploid strain, we obtained an evolved diploid strain SQ-2, exhibiting improved ethanol production and thermotolerance. This study demonstrates that overexpressing *SFA1* enables efficient fermentation performance in different lignocellulosic hydrolysates, especially in the hydrolysate of alkaline-distilled sweet sorghum bagasse. The increased cellulosic bioethanol production of SFA1^{OE} provides a promising platform for efficient biorefineries.

1. Introduction

Efficient development of cellulosic ethanol can stabilize energy supply and improve the ecological environment caused by fossil fuel combustion, and therefore has become the focus of current research (Zhang et al., 2019). Considering that cellulosic hydrolysate usually contains inhibitors that affect the metabolic efficiency of yeast strains, especially acetate inhibitors in maize hydrolysates (Zhang et al., 2019), constructing efficient engineering strains and finding suitable hydrolytic system are the key technical bottlenecks of cellulosic ethanol production.

Three xylose metabolic pathways have been adopted to metabolically engineer *S. cerevisiae* for efficient utilization of xylose and glucose in cellulose hydrolysates: the Dahms or Weimberg pathway; the X-1-P or R-1-P pathway and the XR-XDH-XK or XI-XK pathway (Cao et al., 2014; Li et al., 2019). All *ADH* genes such as *SFA1* have the potential to influence the production of ethanol in *S. cerevisiae* is suited to optimizing yeast metabolic pathways (Brown et al., 2018). Due to the significance of *SFA1* to ethanol biosynthesis, we overexpressed *SFA1* in a reported strain WXY70 (named as SFA1^{OE}), which was derived from an evolved strain CE7 by expressing two copies of six-gene clusters *XYL1*(*K270R*)-*XYL2*- *XKS1*-*TAL1*-*PYK1*-*MGT05196* (Zhang et al., 2019), to see whether it can further influence fermentation capability. Additionally, we conducted the evolutionary engineering of SFA1^{OE} and further acquired a diploid strain SQ.

Comparative fermentation performances of the constructed strain SFA1^{OE} with its control or relative strains were evaluated in fermentation of hydrolysates from typical industrial lignocellulosic substrates

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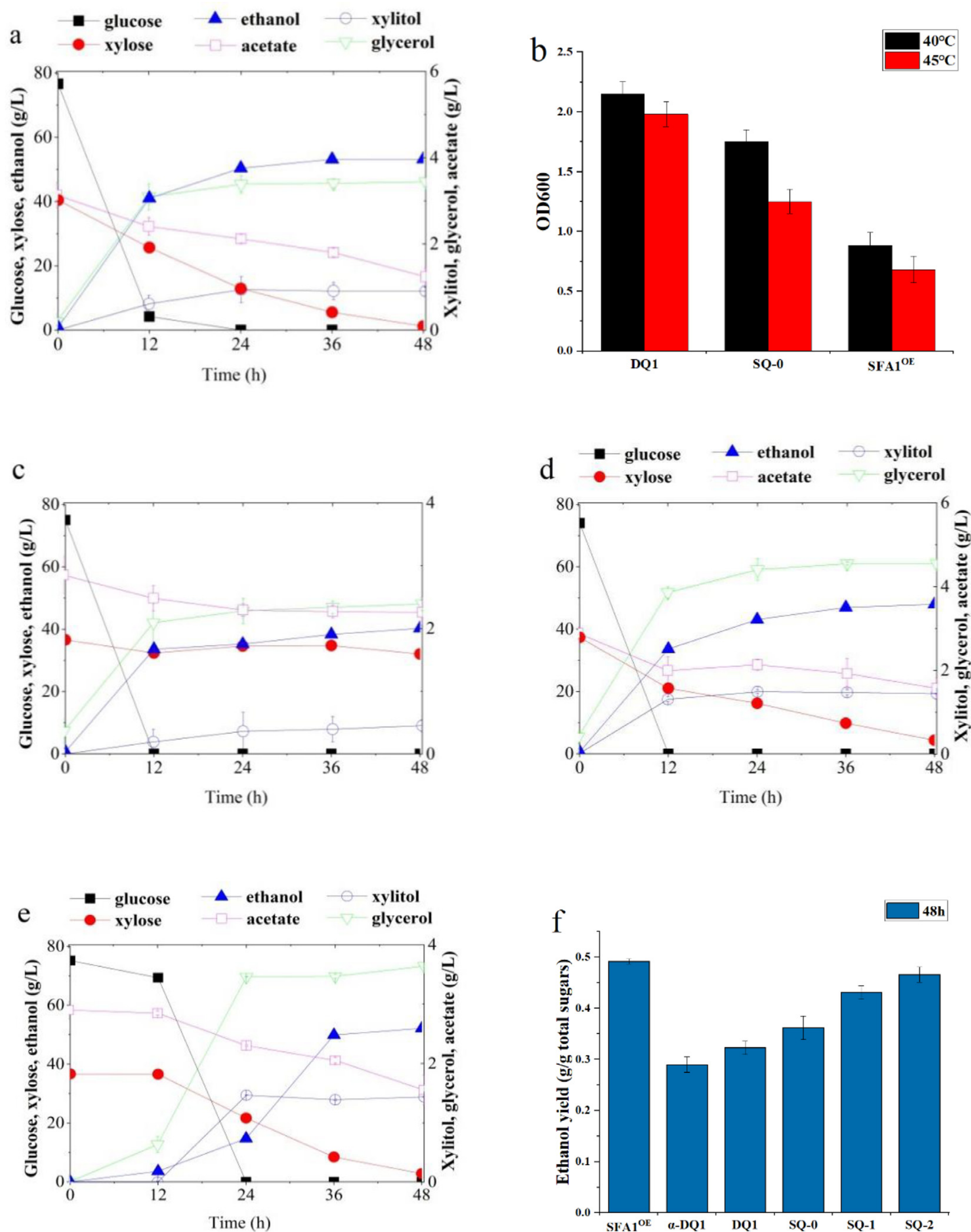

Fig. 1. a–f. Fermentation and growth profiles of strain SFA1^{OE} and its relative diploid strain SQ in simulated industrial fermentation conditions. (a) The fermentation profile of SFA1^{OE}. (b) The growth of diploid strain DQ1, haploid strains SQ-0 and SFA1^{OE} at 40 °C and 45 °C as measured by OD_{600}. (c–e) The fermentation profile of three sequentially evolved diploid strains SQ-0 (c), SQ-1 (d) and SQ-2 (e). (f) The ethanol yields of yeast strains SFA1^{OE}, α-DQ1, DQ1, SQ-0, SQ-1 and SQ-2 at 48 h.

in major Chinese cities including Shanghai, Beijing, Wuhan and Nanjing (Chen et al., 2018; Yu et al., 2014; Zahoor et al., 2017; Zhang et al., 2019). Consequently, the confirmed strain SFA1^{OE} could provide a useful platform for efficient biorefineries in the future.

2. Materials and methods

2.1. Construction of yeast engineered strains and plasmids

Ligation of a linearized fragment T1 from plasmid pT1-0 (L1-pPGK1-tPGI-L2) and the *SFA1* gene amplified from WXY70 genomic DNA resulted in plasmid pT1-1 via a method of Golden gate assembly (Zhang et al., 2019). pT1-1 was linearized by PCR after *DpnI* enzyme digestion to yield a L1-pPGK1-SFA1-tPGI-L2 fragment. Other two linearized fragments CAT8up-AbA-L1 and L2-CAT8down were PCR amplified from plasmids pT5-0 and pT3-0, respectively. These three fragments were used to co-transform WXY70 at the *CAT8* locus to form an engineered strain SFA1^{OE} derived by a strong *PGK1* promoter. The relevant plasmids and strains WXY70, CE7 and WXY74 have been previously reported (Zhang et al., 2019). The separated spores derived from a diploid DQ1 and haploid SFA1^{OE} were crossed to obtain a new diploid strain SFA1-DQ1, which was confirmed by genomic PCR (Qureshi et al., 2015). Its derived strains SQ-0, SQ-1 and SQ-2 were obtained by evolutionary engineering.

2.2. Yeast fermentation and metabolite analysis and evolutionarily engineered strains

The yeast cell culture, evolutionary engineering, fermentation analysis and metabolite measurement was conducted using strain $SFA1^{OE}$ as previously described (Zhang et al., 2019). The experiments were performed in biological triplicate.

2.3. Fermentation of hydrolysate of alkaline-distilled sweet sorghum bagasse

The sweet sorghum bagasse, with 1–2 mm in diameter and 3–50 mm in length, was generated during solid-state fermentation and then alkaline-distilled and hydrolyzed using the method as described(Yu et al. 2014). After hydrolysis with a solid: liquid ratio of 1:5, the hydrolysate was centrifuged to remove the insoluble solids, and supplemented with 2 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4$ and 10 g/L yeast extract for fermentation. The initial cell density for fermentation was OD_{600} = 2.0. The fermentation experiments were conducted at 30 °C in triplicate.

2.4. Enzymatic hydrolysis of lignocellulosic biomasses

The dried *Miscanthus*, maize and wheat straws were pretreated under steam explosion and ground into powders through 40 mesh screening as biomass samples. 0.3 g sample was incubated with 0.012 g mixed-cellulases co-supplied with 0.8% Tween-80 at 5% solid loading under 150 rpm shaking at 50 °C for 48 h as previously described (Zahoor et al., 2017).

2.5. Fermentation from hydrolysate of maize starch and whole maize

Starch samples were mixed with lysozyme and treated for 4 h to obtain hydrolysates. Which were hydrolyzed and fermented. The products were then concentrated at 85 °C and 0.09 MPa for 30 min to remove residual ethanol, followed by adding cellulose and xylanase to the sample and incubation with 250 rpm shaking for 24 h at 50 °C. Finally, the hydrolysis pH was adjusted to 4.6 to adapt to DDGS (Dried Distillers Grains with Soluble). Maize stalks and maize flour were pretreated by rational enzymatic hydrolysis as described (Chen et al., 2018). Briefly, 12% maize flour (stalks) hydrolysate and 0.1% amylase were co-fermented in a shaking bottle at 30 °C for 72 h, with an initial OD_{600} of 1.0.

3. Results and discussion

3.1. Construction of haploid and diploid $SFA1^{OE}$ strains and fermentation analysis

Both overexpression and deletion of *SFA1* with bifunctional activities of alcohol and formaldehyde dehydrogenases are reported to have positive effects on ethanol production (Brown et al., 2018), which is suitable to the high ethanol environment. In this study, $SFA1^{OE}$ and *sfa*1Δ strains were constructed in the starting strain WXY70, and fermented in a controlled medium mimicking maize stalk hydrolysate (Fig. 1a). At 48 h, the remaining xylose contents of $SFA1^{OE}$ and WXY70 were 1.20 g/L and 4.03 g/L, respectively, demonstrating that $SFA1^{OE}$ has improved xylose metabolism capacity. WXY70 and $SFA1^{OE}$ produced 51.36 g/L and 53.20 g/L ethanol, or ethanol yields of 0.470 and 0.492 g/g total sugars, reaching 92.0% and 96.5% of the theoretical value, respectively. Additionally, compared with WXY70, $SFA1^{OE}$ exhibited improved acetate metabolism capacity. On the other hand, *sfa*1Δ achieved an ethanol yield of 0.468 g/g total sugars, which was lower than that of WXY70 and in contrast with the previous report (Brown et al., 2018). It is speculated that the discrepancy is due to different industrial strain backgrounds and potential interactions formed by the six-gene clusters in WXY70. Thus, the introduction of xylose metabolism-related gene *SFA1* with enhanced alcohol dehydrogenase activity into yeast strain could increase the ethanol yield. The transcript level of *SFA1* showed a gradual increase up to 2.3-fold over time, indicating that the metabolic activity of *SFA1* was continuously strengthened to improve ethanol production (Unpublished results). It is speculated that *SFA1* performs similar *ADH5* activity to catalyze the aldehyde to alcohol (Brown et al., 2018).

Industrial fermentation often uses diploid strains due to its increased tolerance to ethanol and other inhibitors in hydrolysates. An adapted yeast diploid strain DQ1 had an ethanol concentration of 71.4 g/L in SSCF with a yield of 80.3% and had a with high-temperature resistance (Qureshi et al., 2015). However, DQ1 cannot utilize xylose during the fermentation process. Therefore, we induced DQ1 to undergo meiosis and sporulation, isolated an *MATα* haploid strain α-DQ1, and crossed it with the *MATa* haploid strain $SFA1^{OE}$ to obtain a diploid strain SQ-0. We tested the high-temperature resistance of DQ1, SQ-0 and $SFA1^{OE}$. At 40 and 45 °C, DQ1 displayed a characteristic high-temperature resistance, and the high temperature resistance phenotype is significantly improved in SQ-0 compared to $SFA1^{OE}$ (Fig. 1b); however, the ethanol production and xylose consumption of SQ-0 failed to achieve our expectation (Fig. 1c). Therefore, we conducted a two-month domestication process in the presence of acetate. Under the condition of 20 g/L xylose and 5 g/L acetate, two evolved strains SQ-1 and SQ-2 were obtained for one and two months, respectively. At 48 h, SQ-0 and SQ-1 produced 40.3 and 48.0 g/L ethanol with an ethanol yield of 0.361 and 0.431 g/g total sugars, respectively, while SQ-2 produced 52.2 g/L ethanol with an ethanol yield of 0.466 g/g total sugars (Fig. 1d-e). The consumption of xylose and acetate from SQ-0 to SQ-2 also increased from 4.5 g/L to 33.9 g/L, and from 0.59 g/L to 1.35 g/L, respectively. Hence, compared with the original SQ-0, the resulting SQ-2 after two-month evolutionary engineering displays significantly improved cellulosic ethanol production, as well as increased tolerance to high temperature and acetate, the major hydrolysate inhibitor. Nevertheless, fermentation evaluation on six strains ($SFA1^{OE}$, α-DQ1, DQ1, SQ-0, SQ-1 and SQ-2) still reveals that $SFA1^{OE}$ has the best fermentation performance (Fig. 1f).

To further evaluate the industrialization profile of $SFA1^{OE}$, we conducted a fermentation test in a common acidic blasting maize stalk and performed microscopic examination (Unpublished results). $SFA1^{OE}$ produced an ethanol yield of about 0.873 g/g total sugars from the maize hydrolysate, which was close to the ethanol yield of control industrial characteristics. The mortality of $SFA1^{OE}$ was higher than the reported industrial strain (Unpublished results). It is likely that $SFA1^{OE}$ has reached the industrial ethanol yield using the blasting of maize stalks and has more cellular activity.

3.2. Determination of the fermentation capacity of $SFA1^{OE}$ in different hydrolysates

The above experimental data encouraged us to test the fermentation capacity of $SFA1^{OE}$ in a wide range of hydrolysates, including those from different biomass materials treated with different methods. Even after pretreatment and detoxification, the wheat straw hydrolysate still has remaining inhibitors that interfere with cell growth during fermentation. After adaptive acclimation in the wheat straw hydrolysate during 84 days about 1000 generations to yield an evolved strain $SFA1^{OE}$, glucose and xylose release, and the increase in ethanol and glycerol, production remained within a stable range. An evolved $SFA1^{OE}$ had adapted to the 15%-solid-wheat-straw detoxified hydrolysate environment and produced a stable ethanol production, suggesting that overexpressed *SFA1* could keep stable genetics phenotype (Unpublished results). This $SFA1^{OE}$ was further evaluated using the Simultaneous saccharification and co-fermentation (SSCF) method (Fig. 2a). In the pre-hydrolysis stage, glucose increased with increase in the amount of cellulose and saccharification time; while most of the

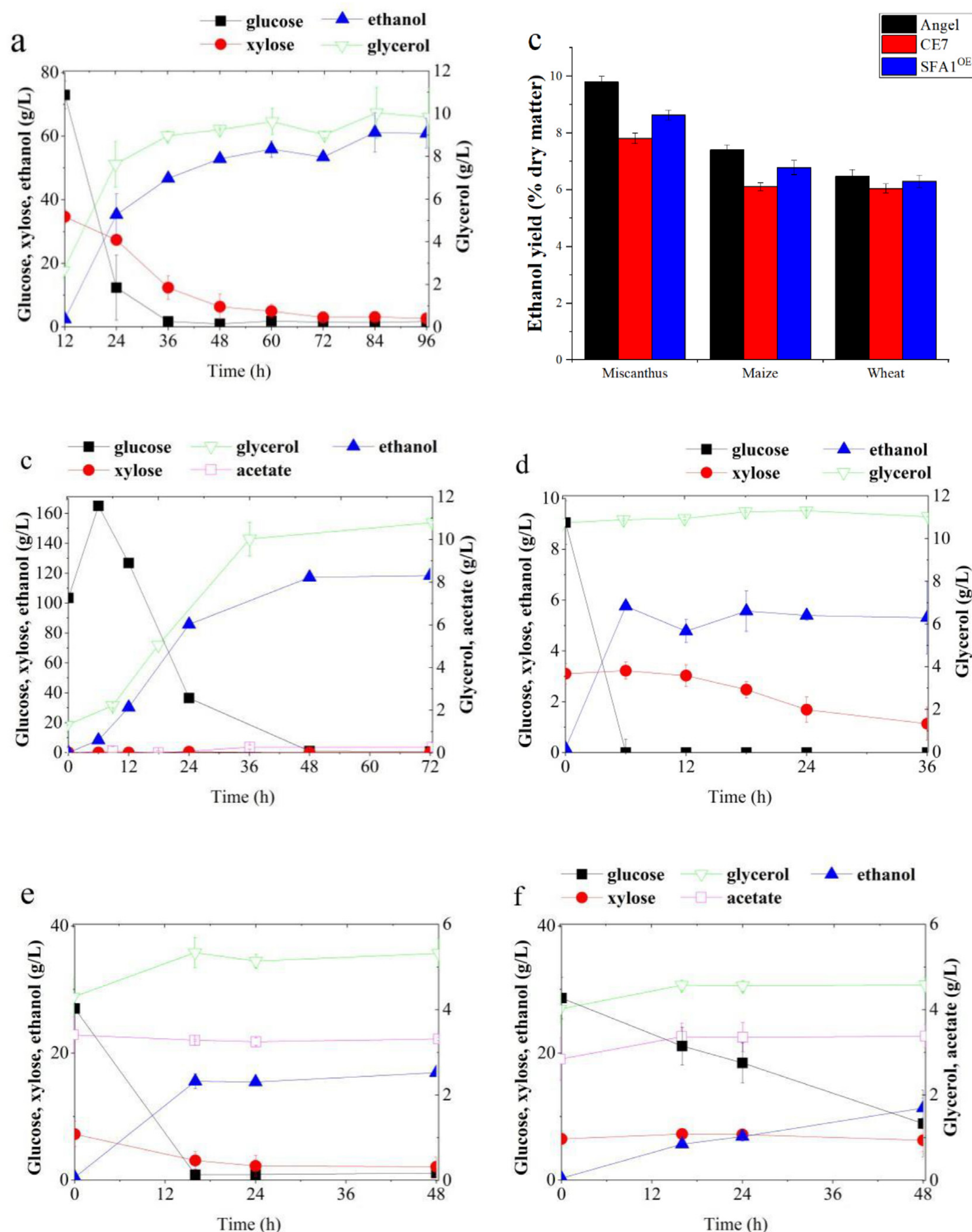

Fig. 2. a–f. Fermentation and growth profiles of strain SFA1^{OE} and its relative strains in different hydrolysates. (a) Fermentation and growth profile of SFA1^{OE}. (b) Ethanol yield (% dry matter) of *Miscanthus*, maize and wheat straw with control Angel yeast, SFA1^{OE} and CE7. (c) SFA1^{OE} in maize medium. (d) SFA1^{OE} in maize distiller's grains. Fermentation profiles of (e) SFA1^{OE} and (f) *Zymomonas mobilis* TSH-01 in the hydrolysate of alkaline-distilled sweet sorghum bagasse.

xylan has been converted into xylose and oligoxylan to result in the maintained xylose. In the subsequent SSCF stage, the initial glucose was quickly converted to ethanol within 24 h, and then the cellulose-hydrolyzed glucose began to be utilized. As time went on, the conversion rate of xylose gradually decreased. Finally, when the cellulose dosage reached 15 mg/g, SFA1^{OE} produced 62.0 g/L of ethanol and had the xylose utilization by 92.7%, outperforming previously reported haploid XR-XDH strains (Zhang et al., 2019). These results demonstrate that the *SFA1* gene effectively regulates the utilization of mixed sugar.

Lignocellulosic biomass such as *Miscanthus*, maize and wheat straw are important raw materials for bioethanol. Peng and colleagues used distinct cell wall polymer deconstruction to improve the biomass digestibility of *Miscanthus* (Li et al., 2018). The raw materials from *Miscanthus*, maize and wheat straw were enzymatically digested into mixed sugars as substrates to produce ethanol. At 48 h, the xylose utilization by SFA1^{OE} in wheat and maize were 27.1% and 44.3%, in comparison to 22.5% and 39.7% by an industrial standard Angel yeast, respectively (Fig. 2b). There is no significant difference in the utilization of xylose in *Miscanthus* for three strains. Among them, SFA1^{OE} had the higher ethanol production in *Miscanthus* hydrolysate and the higher xylose utilization in wheat hydrolysate.

Jin and colleagues focused on the feasibility of utilizing *S. cerevisiae* in the combined fermentation of glucose and xylose (Wang et al., 2014). Prof. Jin used the first- and second-generation maize combined the fermentation technologies to evaluate Angel and SFA1^{OE} strains under different culture conditions. The comparative fermentations were done

at 30 °C, shaker at 150 rpm, for 72 h (Fig. 2c). The fermentation on maize distiller's grains was performed (Fig. 2d). At 48 h, SFA1^{OE} and Angel consumed xylose about 63.5% and 11.3%, respectively. We performed and compared the fermentation capacities of SFA1^{OE} and control industrial Angel yeast in a typical maize hydrolysate and maize distiller's grain in the Jin lab. These results showed that SFA1^{OE} had better xylose utilization efficiency than Angel.

Sweet sorghum not only supplies grain and soluble sugars, but also lignocellulosic resource, so it is regarded as a promising energy crop for bioethanol production. In an integrated process, the soluble sugars in sweet sorghum stalks were used to produce 1.5-generation bioethanol via an Advanced Solid-State Fermentation technology (Yu et al. 2014), and then ethanol distillation and alkaline pretreatment were performed simultaneously via the so-called alkaline-distillation process so that the lignocellulose in the sweet sorghum bagasse could be converted into ethanol (Li et al., 2013). In this study, we evaluated the feasibility of using SFA1^{OE} to produce cellulosic ethanol based on alkaline-distilled sweet sorghum bagasse, with the previously reported *Zymomonas mobilis* TSH-01 as the control strain (Li et al., 2013). SFA1^{OE} had much better fermentation performance than TSH-01. Within 16 h, SFA1^{OE} produced 16.28 g/L ethanol, with an ethanol yield of 0.449 g/g total sugars, or 88.0% of the theoretical maximum (Fig. 2e). In comparison, the control strain TSH-01 produced 5.65 g/L ethanol, with an ethanol yield of 0.161 g/g total sugars, or 31.6% of the theoretical maximum (Fig. 2f). At the end of the fermentation (48 h), SFA1^{OE} produced 17.77 g/L ethanol, with an ethanol yield of 0.489 g/g total sugars, or 96.0% of the theoretical maximum (Fig. 2e), whereas TSH-01 produced 11.35 g/L ethanol, with an ethanol yield of 0.326 g/g total sugars, or 64.0% of the theoretical maximum (Fig. 2f). These results indicate that SFA1^{OE} was more suitable for cellulosic ethanol production from the hydrolysate of alkaline-distilled sweet sorghum bagasse than the previously used *Z. mobilis* TSH-01. Considering that alkaline-distillation is a cost-effective process that combines 1.5-generation bioethanol production and pretreatment of lignocellulosic materials together, the promising SFA1^{OE} strain can ensure efficient and full use of sweet sorghum stalks for bioethanol production.

4. Conclusions

We obtained a target strain SFA1^{OE} and its further evolved diploid strain SQ-2 by metabolic and evolutionary engineering. The Systematic evaluation of *SFA1* contribution to cellulosic ethanol production was conducted in hydrolysates of maize straw, SSCF low-waste wheat straw, alkaline-distilled sweet sorghum bagasse, and various steam explosion biomass. SFA1^{OE} performed better than other strains in the hydrolysate of alkaline-distilled sweet sorghum bagasse derived from Advanced Solid-State Fermentation process, achieving an ethanol yield of 0.489 g/g total sugars. This study provides potential industrial strains for efficient cellulosic ethanol development.

CRediT authorship contribution statement

Lang Zhu: Investigation, Formal analysis, Writing - original draft. **Pengsong Li:** Investigation, Writing - review & editing. **Tongming Sun:** Writing - original draft. **Meilin Kong:** Writing - original draft. **Xiaowei Li:** Writing - original draft. **Sajid Ali:** Writing - original draft. **Wenbo Liu:** Investigation, Formal analysis. **Sichun Fan:** Investigation, Formal analysis. **Jingchun Qiao:** Investigation, Formal analysis. **Shizhong Li:** Investigation, Formal analysis. **Liangcai Peng:** Investigation, Formal analysis. **Boyang He:** Investigation, Formal analysis. **Mingjie Jin:** Supervision. **Wei Xiao:** Supervision, Writing - review & editing. **Limin Cao:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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