Overexpression of \textit{SFA1} in engineered \textit{Saccharomyces cerevisiae} to increase xylose utilization and ethanol production from different lignocellulose hydrolysates

Lang Zhu\textsuperscript{a}, Pengsong Li\textsuperscript{b}, Tongming Sun\textsuperscript{a}, Meilin Kong\textsuperscript{a}, Xiaowei Li\textsuperscript{a}, Sajid Ali\textsuperscript{a}, Wenbo Liu\textsuperscript{a}, Sichun Fan\textsuperscript{a}, Jingchun Qiao\textsuperscript{a}, Shizhong Li\textsuperscript{c}, Liangcai Peng\textsuperscript{d,e}, Boyang He\textsuperscript{d,e}, Mingjie Jin\textsuperscript{f}, Wei Xiao\textsuperscript{b,g}, Limin Cao\textsuperscript{a},\textsuperscript{⁎}

\textsuperscript{a} College of Life Sciences, Capital Normal University, Beijing 100048, China
\textsuperscript{b} Beijing Key Laboratory for Source Control Technology of Water Pollution, Engineering Research Center for Water Pollution Source Control and Eco-remediation, College of Environmental Science and Engineering, Beijing Forestry University, Beijing 100083, China
\textsuperscript{c} MOST-USDA Joint Research Center for Biofuels, Beijing Engineering Research Center for Biofuels, Institute of New Energy Technology, Tsinghua University, Beijing 100084 China
\textsuperscript{d} Biomass and Bioenergy Research Centre, Huazhong Agricultural University, Wuhan 430070, China
\textsuperscript{e} College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China
\textsuperscript{f} School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing 210094, China
\textsuperscript{g} Department of Biochemistry, Microbiology and Immunology, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada

\textbf{ARTICLE INFO}

\textbf{Keywords:}
Lignocellulosic
Ethanol
Xylose
\textit{SFA1}
\textit{Saccharomyces cerevisiae}

\textbf{ABSTRACT}

Here, an engineered \textit{Saccharomyces cerevisiae} strain SFA1\textsubscript{OE} was constructed by overexpressing SFA1 in a reported strain WXY70 with effective six-gene clusters. Under simulated maize hydrolysate, SFA1\textsubscript{OE} produced an ethanol yield of 0.492 g/g total sugars within 48 h. The productivity of SFA1\textsubscript{OE} was comprehensively evaluated in typical hydrolysates from stalks of maize, sweet sorghum, wheat and \textit{Miscanthus}. Within 48 h, SFA1\textsubscript{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\textsubscript{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\textsubscript{OE} provides a promising platform for efficient biorefineries.

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

Comparative fermentation performances of the constructed strain SFA1\textsubscript{OE} with its control or relative strains were evaluated in fermentation of hydrolysates from typical industrial lignocellulosic substrates or R-1-P pathway and the XR-XDH-XK or XI-XK pathway (Cao et al., 2014; Li et al., 2019). All \textit{ADH} genes such as \textit{SFA1} have the potential to influence the production of ethanol in \textit{S. cerevisiae} is suited to optimizing yeast metabolic pathways (Brown et al., 2018). Due to the significance of \textit{SFA1} to ethanol biosynthesis, we overexpressed \textit{SFA1} in a reported strain WXY70 (named as SFA1\textsubscript{OE}), which was derived from an evolved strain CE7 by expressing two copies of six-gene clusters \textit{XYL1}(K270R)-\textit{XYL2}-\textit{XKS1}-\textit{TAL1}-\textit{PYK1}-\textit{MGT05196} (Zhang et al., 2019), to see whether it can further influence fermentation capability. Additionally, we conducted the evolutionary engineering of SFA1\textsubscript{OE} and further acquired a diploid strain SQ.

Comparative fermentation performances of the constructed strain SFA1\textsubscript{OE} with its control or relative strains were evaluated in fermentation of hydrolysates from typical industrial lignocellulosic substrates.
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 SFA1OE 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 SFA1OE 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 SFA1OE 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.

Fig. 1. a–f. Fermentation and growth profiles of strain SFA1OE and its relative diploid strain SQ in simulated industrial fermentation conditions. (a) The fermentation profile of SFA1OE. (b) The growth of diploid strain DQ1, haploid strains SQ-0 and SFA1OE at 40 °C and 45 °C as measured by OD600. (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 SFA1OE, α-DQ1, DQ1, SQ-0, SQ-1 and SQ-2 at 48 h.
2.2. Yeast fermentation and metabolite analysis and evolutionary engineered strains

The yeast cell culture, evolutionary engineering, fermentation analysis and metabolite measurement was conducted using strain SFA1OE 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 KH2PO4, 2 g/L (NH4)2SO4, 1 g/L MgSO4 and 10 g/L yeast extract for fermentation. The initial cell density for fermentation was OD600 = 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 pre-treated by rational enzymatic hydrolysis as described (Chen et al., 2018). Briefly, 12% maize flour (stalks) hydrolysed and 0.1% amylase were co-fermented in a shaking bottle at 30 °C for 72 h, with an initial OD600 of 1.0.

3. Results and discussion

3.1. Construction of haploid and diploid SFA1OΔE 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, SFA1OΔE and sfα1a 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 SFA1OΔE and WXY70 were 1.20 g/L and 4.03 g/L, respectively, demonstrating that SFA1OΔE has improved xylose metabolism capacity. WXY70 and SFA1OΔE 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, SFA1OΔE exhibited improved acetate metabolism capacity. On the other hand, sfα1a 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 MATa haploid strain α-DQ1, and crossed it with the MATa haploid strain SFA1OΔE to obtain a diploid strain SQ-0. We tested the high-temperature resistance of DQ1, SQ-0 and SFA1OΔE. 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 SFA1OΔE (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, where 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 (SFA1OΔE, α-DQ1, DQ1, SQ-0, SQ-1 and SQ-2) still reveals that SFA1OΔE has the best fermentation performance (Fig. 1f).

To further evaluate the industrialization profile of SFA1OΔE, we conducted a fermentation test in a common acidic blasting maize stalk and performed microscopic examination (Unpublished results). SFA1OΔE 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 SFA1OΔE was higher than the reported industrial strain (Unpublished results). It is likely that SFA1OΔE 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 SFA1OΔE in different hydrolysates

The above experimental data encouraged us to test the fermentation capacity of SFA1OΔE 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 SFA1OΔE, glucose and xylene release, and the increase in ethanol and glycerol, production remained within a stable range. An evolved SFA1OΔE 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 SFA1OΔE 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
xylan has been converted into xylose and oligo-xylan 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, SFA1OE 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 SFA1OE 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, SFA1OE 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 SFA1OE strains under different culture conditions. The comparative fermentations were done

Fig. 2. a–f. Fermentation and growth profiles of strain SFA1OE and its relative strains in different hydrolysates. (a) Fermentation and growth profile of SFA1OE. (b) Ethanol yield (% dry matter) of Miscanthus, maize and wheat straw with control Angel yeast, SFA1OE and CE7. (c) SFA1OE in maize medium. (d) SFA1OE in maize distiller’s grains. Fermentation profiles of (e) SFA1OE and (f) Zymomonas mobilis TSH-01 in the hydrolysate of alkaline-distilled sweet sorghum bagasse.
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, SFA1OE and Angel consumed xylose about 63.5% and 11.3%, respectively. We performed and compared the fermentation capacities of SFA1OE and control industrial Angel yeast in a typical maize hydrolysate and maize distiller's grain in the Jin lab. These results showed that SFA1OE 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 SFA1OE 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). SFA1OE had much better fermentation performance than TSH-01. Within 16 h, SFA1OE 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), SFA1OE 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 SFA1OE 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 SFA1OE strain can ensure efficient and full use of sweet sorghum stalks for bioethanol production.

4. Conclusions

We obtained a target strain SFA1OE 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. SFA1OE 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


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.

Acknowledgements

The authors sincerely thank Prof. Jie Bao for comparative fermentation experiments to evaluate SFA1OE in Shanghai. This study was funded by the National Natural Science Foundation of China [Grant No. 31570044] to Limin Cao.

References


