Metabolic Engineering of *Saccharomyces cerevisiae* Suitable for the Second-generation Bioethanol Production

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ABSTRACT

Multiple utilization of renewable resources including lignocellulose feedstock is a potential strategy in response to shortage of fossil energy in the future. As an attractive microbial cell factory, *Saccharomyces cerevisiae* has been explored for production of biofuels, biochemicals and high-value compounds due to its robustness and high tolerance in industrial harsh conditions. To realize co-fermentation of xylose and glucose and enhance tolerance to lignocellulosic inhibitors, two novel heterologous genes, the *Meyerozyma guilliermondii* MGT05196N360F mutant encoded a specific xylose transporter and the *Ru-xylA* (where *Ru* represents the rumen) gene encoded xylose isomerase (XI) with higher activity in *S. cerevisiae*, were integrated into genomic DNA of the diploid robustness strain BSIF. Meanwhile, further modifications including overproduction of the xylulokinase and the non-oxidative pentose phosphate pathway (PPP), and inactivation of the aldose reductase and alkaline phosphatase, were also performed. By combining above-mentioned rationally designed genetic modifications and alternant evolution in xylose and leach liquor of steam-exploding corn stover, the final strain LF1 exhibited excellent xylose fermentation, the higher sugars synchronization utilization and the enhanced inhibitor resistant capacity. Further, mutant LF1-6, with enhanced tolerance against Huarun (a factory name) leachate of straw pretreatment feedstock with higher toxicity was selected from the mutagenesis library generated via a multiplex atmospheric and room temperature plasma (ARTP) method. The growth and sugar metabolism of mutants were significantly increased in medium containing 50% Huarun leachate, but LF1 was completely inhibited. However, the metabolic rate of xylose of LF1-6 was lower than LF1 in medium containing pure xylose compared to leachate. In fact, this phenomenon of antagonism between high metabolic rate of xylose and high tolerance to inhibitors was found in our previous research, but the related mechanism remains unclear. Then, LF1-6M was obtained after second-round ARTP mutagenesis, which relieved this phenomenon of antagonism. We have performed multi-level omics analysis to further study this antagonism mechanism.

1. Evaluation of *S. cerevisiae* strains as the chassis cell for second-generation bioethanol production.

A diploid wild-type *S. cerevisiae* strain BSIF, isolated from tropical fruit in Thailand, was selected out from several robust *S. cerevisiae* strain because of its excellent characteristics. The maximal specific growth rate of BSIF was as high as 0.65 h^{-1} in yeast extract peptone dextrose medium, the ethanol yield was 0.45 g g^{-1} consumed glucose (Table 1). This strain exhibited superior tolerance to high temperature, hyperosmotic and oxidative stress (Fig.1), and better growth performance in lignocellulosic hydrolysate compared with other strains(Fig.2). Furthermore, the BSIF has better xylose utilization capacity when the initial xylose metabolic pathway was introduced (Fig.3). All above-mentioned results indicate that BSIF is an excellent chassis strain for lignocellulosic ethanol production.

Table 1. Metabolic characteristics of *S. cerevisiae* strains in glucose

	NAN27	BSIF	RC212	CICC31034	6508
μ_{max} [a]	0.590±0.005	0.652±0.004	0.609±0.006	0.632±0.005	0.556±0.022
Y_{Eth} [b]	0.421±0.003	0.451±0.016	0.441±0.001	0.423±0.007	0.425±0.001
V_{Glu} [c]	1.183±0.106	1.966±0.055	1.939±0.021	1.653±0.071	1.238±0.048

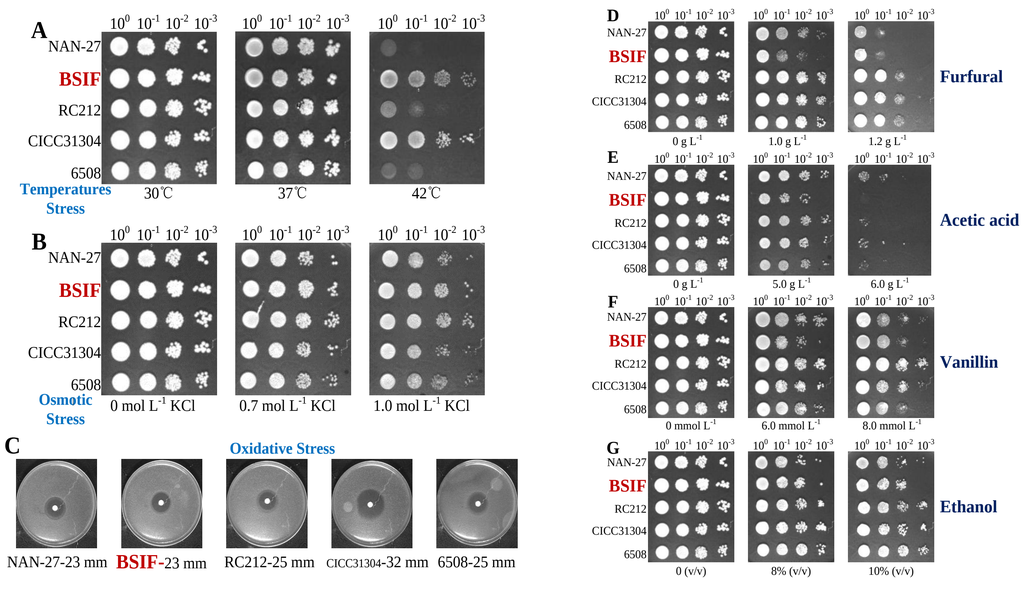

Fig.1 Growth of *S. cerevisiae* strains on the plate at different stress conditions.
(A), temperatures; (B), osmotic stress with high concentration of KCl; (C), oxidative stress with H_2O_2; (D) furfural; (E) Acetic acid; (F) Vanillin; (G) Ethanol.

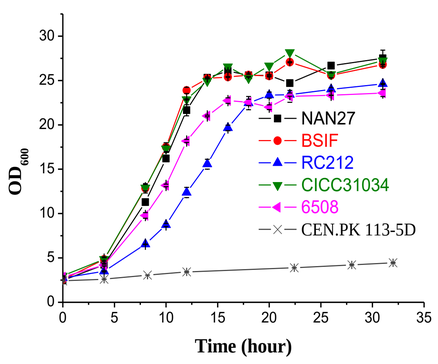

Fig.2 Oxygen-limited growth and fermentation in corn stover hydrolysate.

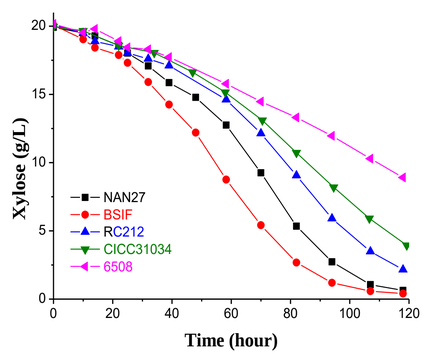

Fig.3 Xylose consumption of strains in shake flasks. The strains were endowed with the capacity of xylose assimilation by introduction the XR-XDH pathway from *Scheffersomyces stipitis*.

2. Construction of an efficient glucose and xylose co-fermentation *S. cerevisiae* strain through metabolic and evolution engineering.

BSIF Isolated from tropical fruit in Thailand

↓ Expressing Ru-*xylA*; overexpressing *XKS1* and genes of PPP; deleting the gene *GRE3* and *PHO13*.

BSN0 (*pho13::*XI, *gre3::*PPP, XK)

↓ Overexpressing Ru-*xylA* at *δ*-sequence by three rounds of integration.

BSN3 (*pho13::*XI, 3*δ::*XI, *gre3::*PPP, XK)

↓ Evolving in the medium using xylose as sole carbon source.

XH7 (*pho13::*XI, 3*δ::*XI, *gre3::*PPP, XK, AE)

↓ Evolving in the leach liquor of pretreated corn stover (PCS).

XHR11 (*pho13::*XI, 3*δ::*XI, *gre3::*PPP, XK, AE-PCS)

↓ Expressing the *MGT05196(N360S)* and further evolving in the medium using xylose as sole carbon source.

LF1
(*pho13::*XI, 3*δ::*XI, *gre3::*PPP, XK, AE-PCS, *N360S*, AE)

Fig.4 The detailed process of strain LF1 construction

Genetic modification and evolution was performed on BSIF. The detailed description was shown in the figure 4. The final strain (LF1) consumed 40 g L^{-1}Xylose or mixed sugars (80 g L^{-1} glucose and 40 g L^{-1} xylose) in 12 h and 16 h, respectively, with ethanol yield more than 0.473 g g^{-1}(Fig.5), reaching to 93 % of the theoretical yield. Then, the fermenting capacity of LF1 was investigated in different lignocellulosic hydrolysate from four companies, and all yields of conversion of glucose and xylose to ethanol exceeded 88%(data not shown).

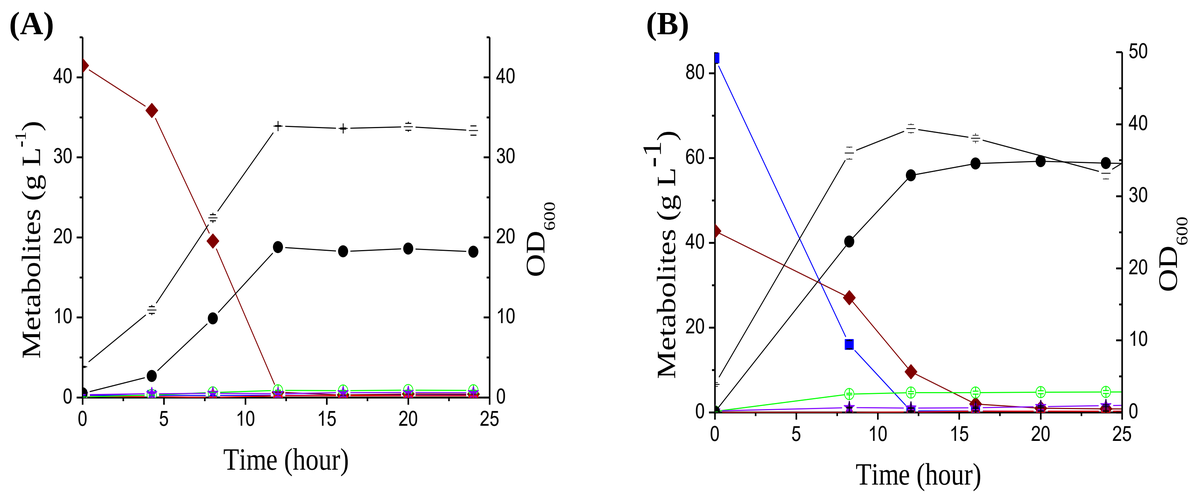

Fig. 5 The fermentation of LF1 in (A) 40 g L^{-1} xylose and (B) mixed sugars (80 g L^{-1} glucose, 40 g L^{-1} xylose). ■, glucose; ◆, xylose; ●,ethanol; —,OD_{600}.

3. Engineering strain LF1 to match lignocellulosic hydrolysate with higher toxicity(unpublished).

To further enhance tolerance of LF1 to inhibitors, we chose leachate of straw pretreatment feedstock (from Huarun company of China) with higher toxicity as screening stress. Our previous work also found a phenomenon of antagonism between high tolerance to inhibitors and high metabolic rate of xylose. The strain LF1-6M was obtained via two-round multiplex atmospheric and room temperature plasma (ARTP) mutagenesis, and its tolerance to inhibitors was enhanced and the above-mentioned phenomenon of antagonism was relieved(Fig.6).

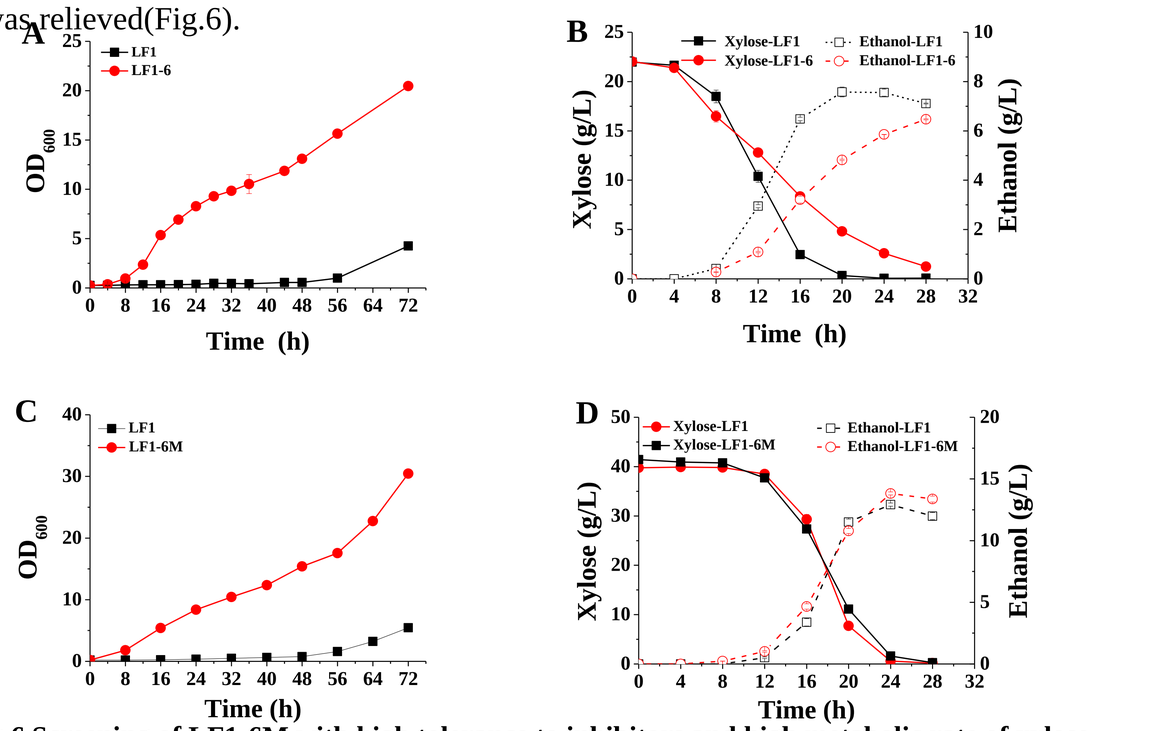

Fig.6 Screening of LF1-6M with high tolerance to inhibitors and high metabolic rate of xylose. (A) and (C) The growth of mutant strains in medium with 50% leachate of straw pretreatment feedstock;(B) and (D) The metabolic rate of xylose of mutant strains in yeast extract peptone medium with xylose;

Compared to LF1, LF1-6 with higher tolerance and lower metabolic rate of xylose was screened via one-round ARTP mutagenesis; LF1-6M with higher tolerance and equivalent metabolic rate of xylose was screened via two-round ARTP mutagenesis based on LF1-6.

Acknowledgement

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