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gTME for Improved Adaptation of *Saccharomyces cerevisiae* to Corn Cob Acid Hydrolysate

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Abstract Global transcription machinery engineering was employed to engineer xylose metabolism, tolerance and adaptation to lignocellulosic hydrolysates. Mutation of the transcription factor *spt15* was introduced by error-prone PCR, then transformed the recombinant plasmid pYX212-spt15 into *Saccharomyces cerevisiae* YPH499 which was not able to grow on xylose, and screened on media using lignocellulosic hydrolysates as the sole carbon source. The maximum sugar yield was obtained by the hydrolysis form with 3% HCl (*m*/*v*) using autoclaving at 120°C, for 2 h with a solid to liquid ratio of 1:10. The corn cob hydrolysate contained 68.41 g/L xylose and 7.67 g/L glucose. The recombinant strain showed modest growth rate when cultured in the cellulosic hyrolysates with different pretreatment and pH conditions. The results showed that spt15-29 reached the maximum xylose and glucose utilization of 65.7% and 87.0%, respectively, the maximum ethanol concentration was 11.9 g/L, after 71 h, using the acid hydrolysate with the initial pH of 5.

Keywords gTME · Corn cob acid hydrolysate · *Saccharomyces cerevisiae* · Cofermenting xylose and glucose

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Introduction

Bioconversion of forestry and agricultural residues into ethanol as an alternative fuel is receiving increased attention owing to concerns over the rising cost of petrol, global warming, food shortages and the decreasing supply of non-renewable fuels [1]. Plant wastes, including agricultural residues, waste paper, and forestry residues, need to be disposed. They are the most abundant carbohydrate on earth [2]; they are renewable [3] and are not in competition with food sources. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and ligin, such as corn cob which has higher content of hemicellulose than other plant. China is a major corn-producing country, and effective use has not been made of the corn cobs in China. However, the corn cobs have not been effectively exploited in China, a major corn-producing country. It makes sense if we can economically convert the corn cob into ethanol and other products [4].

Engineering *Saccharomyces cerevisiae*, the preferred microorganism for ethanol production, to ferment cellulosic hydrolysates to ethanol is an important technology under development Unfortunately, the natural *S. cerevisiae* cannot utilize xylose and it has a low tolerance to cellulosic hydrolysates [5, 6], which leads to a decrease of the ethanol yield and utilization of sugar. A great deal of research has been done over the last two decades to improve the ability of *S. cerevisiae* to simultaneously coferment xylose and glucose and also to enhance the tolerance of the strain to lignocellulosic hydrolysates. These limitations preclude the simultaneous exploration of multiple gene modifications and confine gene modification searches to restricted, sequential approaches that often have difficulties reaching a global phenotype optimum due to the complexity of metabolic landscapes [7, 8].

Global transcription machinery engineering (gTME) is an approach for reprogramming gene transcription to elicit cellular phenotypes, which is important for technological applications [9, 10]. Transcription factor spt15 in transcription initiation complex is a TATA-binding protein, which can transcript most of the mRNA gene in the genome when binding to RNA Pol II and more than ten kinds of general transcription factors. Mutations in the spt15 lead to over expression of related genes and improve the tolerance of *S. cerevisiae* to ethanol [9] and xylose [11].

In our study, gTME was employed to engineer xylose metabolism, tolerance and adaptation to lignocellulosic hydrolysates. Mutation of the transcription factor *spt15* was introduced by error-prone polymerase chain reaction (PCR), and the recombinant plasmid pYX212-spt15 was then transformed into *S. cerevisiae* YPH499 and screened on media using corn cob acid hydrolysate as the sole carbon source. One recombinant strain spt15-29 which grew well on such media was chosen for further research.

These results demonstrated the applicability of gTME to genetical modification and the adaptation of *S. cerevisiae* to metabolize xylose, while improving tolerance and adaptation to lignocellulosic hydrolysates. The method gTME will have potential for the optimization of industrial strains in the future (Fig. 1).

Materials and Methods

Preparation and Hydrolysis of the Pyrolysate

The pyrolysate was prepared by pyrolysis of corn cob by the following method: added 2% (*m*/*v*), 3% (*m*/*v*) sulfuric acid, hydrochloric acid and phosphoric acid were added to a

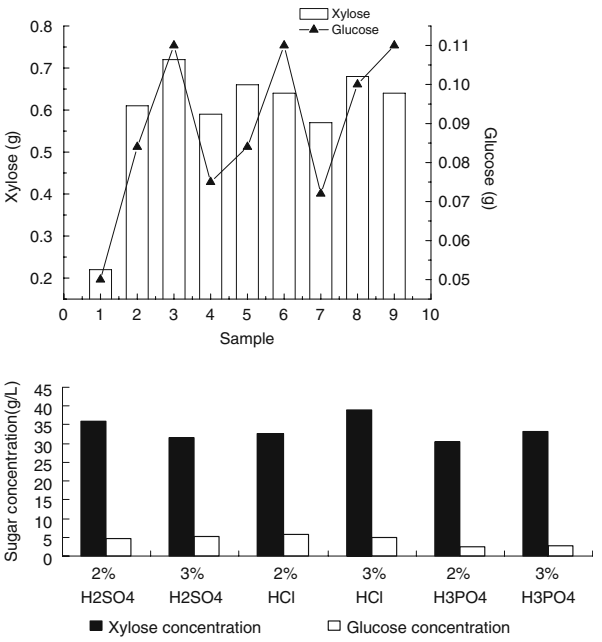

Fig. 1 Xylose and glucose yield of corn cob with different acid treatment

100-mL Erlenmeyer flask containing 5 g dried corn cob with a solid (corn cob) to liquid (acid) ratio of 1:10, and the mixture was hydrolyzed by autoclaving at 120°C for 2 h. The resulting hydrolysate was adjusted to pH 6.0 with solid $Ca(OH)_2$ and the resulting precipitate was then removed by filtration through a 0.45-μm membrane. The sugar concentrations obtained are given in the Fig. 1.

Microorganism and Growth Conditions

S. cerevisiae YPH499 (Stratagene, USA) was the source microorganism. It was able to grow on glucose but not on xylose.

The expression vector used for the *spt15* was pYX212 (Jiangnan University, Wuxi, China).

Add T at blunt end [12] of pYX212, subsequently ligated to *spt15* then transformed into YPH499 by lithium acetate transformation [13]. Plasmid-contained yeast strains were grown on yeast extract peptone dextrose (YPAD) media supplemented with an appropriate carbon source. The yeast extract-peptone medium consisted of 10 g/L yeast extract and 20 g/L peptone and 0.075 g/L adenine sulphate. The carbon source used for YPH499-derived yeast spt15-29 was corn cob hydrolysate.

Precultures for liquid culturing were prepared as follows: a few colonies from pure cultures on plates were inoculated into 5 mL of YPAD medium supplemented with 20 g/L xylose and grown over one to two nights at 30°C, 200 rpm, 30 mL precultures were inoculated from the previous preculture using the same medium and grown similarly. The 30-mL precultures were centrifuged, washed with distilled water, and used as inocula. The initial OD of the actual cultures (optical density at 600 nm (OD600)) was usually 0.5.

Culture conditions for the fermentation experiments were as follows: the actual cultures were inoculated without washing of the precultured cells to OD600 of 0.2–0.5 from the above-mentioned 30-mL precultures inoculated to flasks of 100 mL. The fermentation

medium contained corn cob hydrolysate with 3% HCl autoclaved at 120°C with a solid to liquid ration of 1:10 for 2 h, $(NH_4)_2SO_4$ 5 (g/L), KH_2PO_4 1 (g/L), NaCl 0.1 (g/L), $MgSO_4.7H_2O$ 0.5 (g/L), $CaCl_2$ 0.1 (g/L), and yeast extract 0.2 (g/L). The pH of the medium was adjusted with HCl and NaOH. The medium was then sterilized for 15 min under 0.1 MPa. Culture condition was 30°C, 200 rpm for 72 h.

Assay Methods

Sugars and xylitol in culture supernatants were analyzed with a Dionex high performance liquid chromatography (HPLC) system consisting of an UltiMate 3000 pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment, a Lichrospher Nh2 column (4.6×250 nm; Hanbang Technology Ltd. Jiangsu, China), and a Shodex 101 refractive index (RI) detector. The column temperature was maintained at 30°C, and the elution solvent was with a mixture of acetonitrile and purified water with the volume ratio of 80:20, with a flow rate of 1.0 mL/min. Ethanol concentrations were determined by the SBA 40 C biosensor (Biology Institute of Shandong Academy of Sciences, China). The pH during fermentation was measured using an Orion 410 A+pH meter (Thermo Electron Corporation).

Results and Discussion

Construction and Selection of the Recombinant Yeast Expression Vector pYX212-spt15

The error-prone PCR was carried out under the template of *spt15* using a GenemorphII Random mutagenesis kit. The error-prone PCR products were identical to the PCR products determined by electrophoresis, PYX212-spt15 was then transformed into *S. cerevisiae* YPH499, which could not utilize the xylose. See the article "gTME for improved xylose fermentation of *S. cerevisiae*" [11].

The strain was subsequently plated on the SD medium of corn cob hydrolysate (Fig. 2). The combinant yeast spt15-29 was isolated from the plate containing corn cob acid hyrolysate as the sole carbon source.

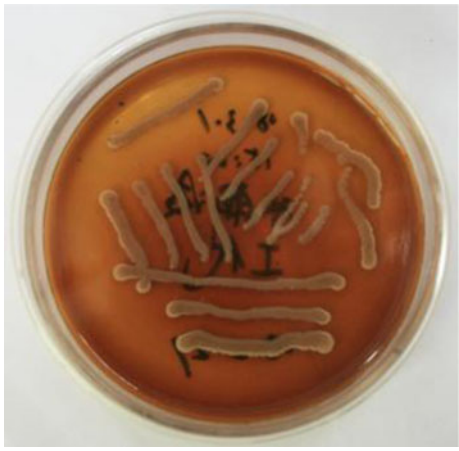

Fig. 2 Colonies growing on the corn cob hydrolysate plate

Analysis of the Fermentation Products by spt15-29

Acid Hydrolysis of Corn Cob

In our preliminary study, the influence of sugar yield by different acid hydrolysis methods has been studied using corn cob as the feedstock. Corn cob has a high amount of hemicellulose, which is composed mainly of xylose, arabinose and mannose. Acidolysis is suitable for hemicellulose hydrolysis, and the content of xylose in corn cob hydrolysis is much higher than glucose. The maximum sugar yield (43.8%) was obtained by the hydrolysis with 3% HCl (*m*/*v*) using autoclaving at 120°C, for 2 h, the solid and liquid ratio of 1:10. The results are shown in Fig. 1. It has practical significance to obtain a *S. cerevisiae* strain which can coferment xylose and glucose to produce ethanol. The study was performed using corn cob hydrolysis under the optimal conditions described herein.

Growth Curve of YPH499 and spt15-29 in the Acid Hydrolysate with Different Initial pH

Compared with the parent strain of YPH499, spt15-29 had higher growth rate in the medium with varying initial pH in the acid hydrolysate. The growth rates of the recombinant spt15-29 with varying initial pH are shown as Fig. 3. From the value of OD600, the recombinant showed modest growth rates in the alkaline condition. Both of the two strains could not grow when the pH of the culture medium was one.

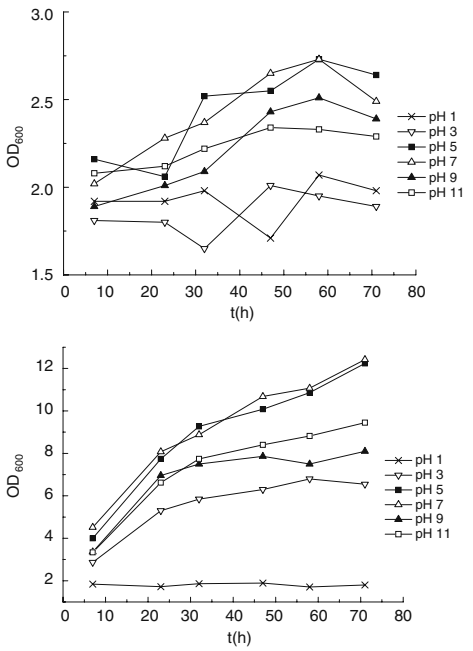

Fig. 3 The growth curve of YPH499 and spt15-29 in the acid hydrolysate with different initial pH

Effect of the Growth of spt15-29 by Different Initial pH in the Acid Hydrolysate

Low inoculum cultures were started using an overnight culture of yeast at an OD600 of 0.1 in 50 mL of medium containing 20 g/L of glucose. High inoculum cultures were created by growing 250 mL of yeast in a 1,000 mL flask for 2 days, and then collected by centrifugation at 5,000 g for 25 min. The cell pellet was then resuspended in 3 mL of YPD without glucose. This solution was then inoculated into 100 mL of the medium in a 500 mL flask to obtain a starting OD600 of around 0.5. The carbon source of the culture was corn cob hydrolysate. Fermentations were performed at 30°C, 200 r/min. Samples were taken and supernatant analysis was conducted to measure sugar concentrations.

Xylose is the main component of corn cob hydrolysis, the strain YPH499 cannot grow well in the hydrolysis conditions, and the utilization ratio of glucose was lower than that in the culture medium in which glucose is the sole carbon source. The results showed that spt15-29 grew more rapidly and had higher sugar utilization in comparison to YPH499. Due to toxicity factors in corn cob hydrolysis, the utilization ratio of xylose in recombinant strain spt15-29 was lower than that of spt15-25 [11] grown in pure xylose or a sugar mixture. Spt15-25 was screened in a culture medium containing xylose as the sole carbon source. However, similar to spt15-25, the strain spt15-29 can ferment xylose and glucose at the same time, with a higher utilization ratio of glucose than the original strain YPH499. In addition, the utilization ratio of sugar is higher under alkaline conditions than inder acidic conditions. YPH499 reached the maximum glucose utilization and ethanol yield of 67.4%; spt15-29 reached the maximum glucose and xylose utilization of 87.0% and 65.7%, respectively, after 71 h in the acid hydrolysate with the initial pH 5 (see Figs. 4, 5, 6, and 7).

The xylitol concentration in the supernatant was detected by HPLC as described in the Assay methods section.

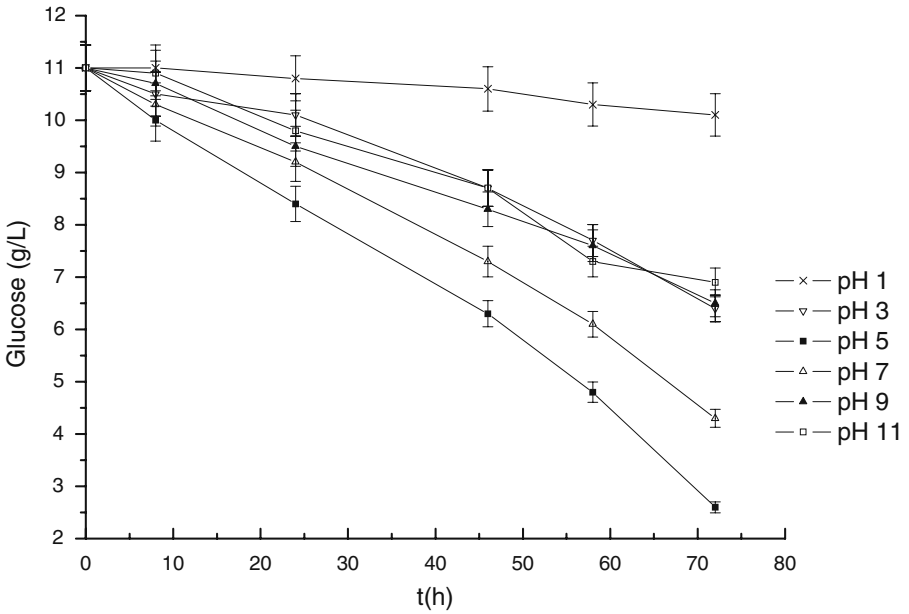

Fig. 4 Glucose utilization curve of YPH499

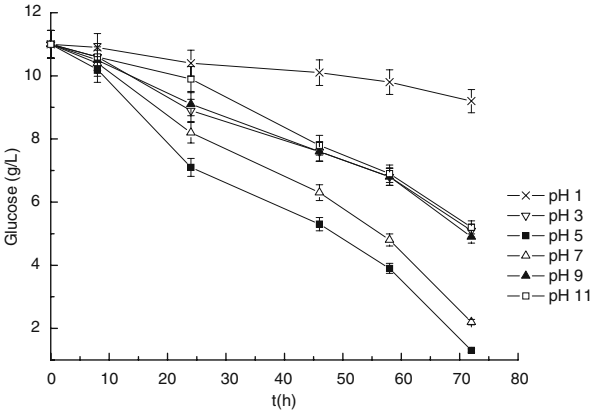

Fig. 5 Glucose utilization curve of spt15-29

The xylitol concentration was lower than the RI detector minimum of 0.7 g/L, which was the result obtained for the recombinant strain spt15-25 [11].

The Concentration of Ethanol by spt15-29 Anaerobic Fermentation

The parent strain YPH499 and the recombinant strainspt15-29 were grown in the corn cob hydrolysate at 30°C, 200 rpm for 71 h. YPH499 reached the maximum ethanol yield of 2.38 g/L, spt15-29 reached the maximum ethanol concentration was 11.9 g/L, after 71 h in the acid hydrolysate with the initial pH of 5 (Table 1).

gTME allows rapid effective and simple engineering of desired multiple phenotypes of microorganisms. Gene expression regulation plays a part in all levels in the course of the

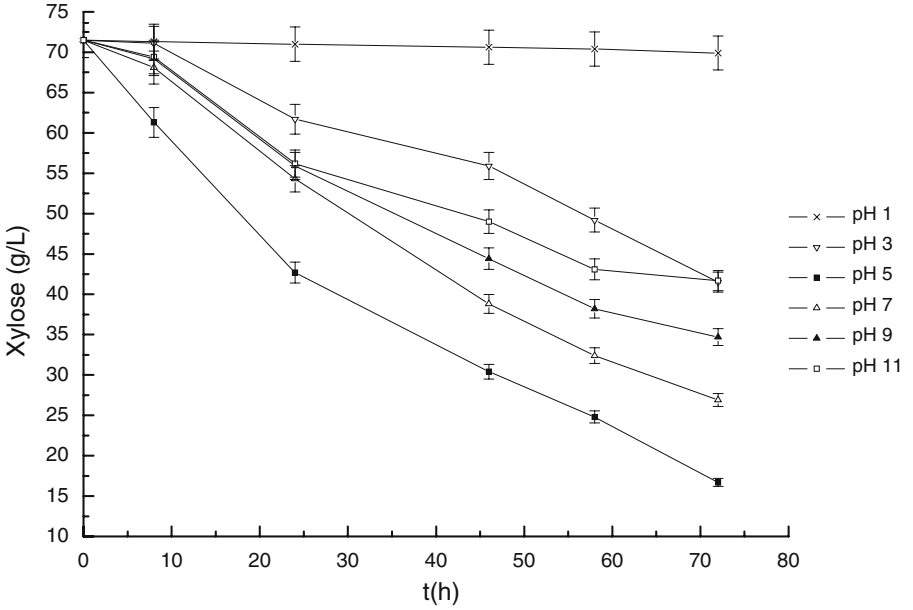

Fig. 6 Xylose utilization curve of spt15-29

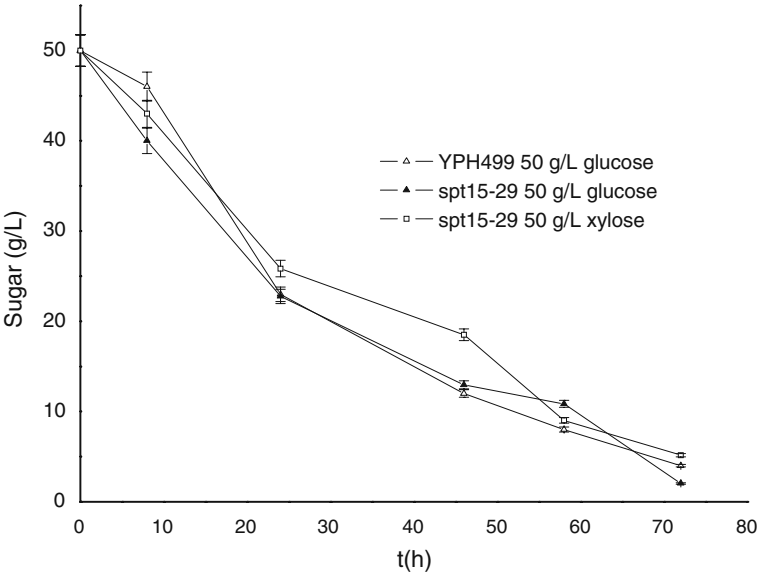

Fig. 7 The concentration of xylitol by spt15-29

genetic information transmission. As we know, transcription regulation is a highly efficient connection through networks of transcriptional regulators. Transcription factor *spt15* in a transcription initiation complex is a TATA-binding protein, which can transcript most of the mRNA genes in the genome when binding to RNA Pol II and more than ten kinds of general transcription factors. Mutations in the *spt15* lead to over expression of related genes and improve the tolerance of *S. cerevisiae* to ethanol [9] and xylose [11]. In this article, the tool of gTME outperformed traditional approaches, more quickly and effectively optimizing phenotypes. We demonstrate that the components of global cellular transcription machinery (*spt15*) can be engineered to allow global perturbations of the transcription, which can help unlock complex phenotypes.

gTME is an approach for reprogramming gene transcription to elicit cellular phenotypes, which is important for technological applications. In our study, gTME was employed to engineer xylose metabolism, tolerance and adaptation to lignocellulosic hydrolysates. The optimized corn cob hydrolysate contained 38.95 g/L xylose and 4.85 g/L glucose, under the condition as follows: 120°C, and a solid/liquid ratio of 1:10, 3% HCl for 2 h. The

Table 1 Ethanol yield of YPH499 and spt15-29

Initial pH	YPH499 Ethanol concentration (g/L)	spt15-29
1	0	0
3	0.13±0.01	2.7±0.01
5	2.37±0.01	11.9±0.01
7	1.28±0.01	6.9±0.01
9	0.20±0.01	3.7±0.01
11	0.17±0.01	3.2±0.01

recombinant strain had modest growth rate in the cellulosic hyrolysates with different pretreatment and pH conditions. After 71 h in the acid hydrolysate with the initial pH 5, YPH499 reached the maximum glucose utilization and ethanol yield of 67.4% and 2.38 g/L, respectively. spt15-29 reached the maximum xylose and glucose utilization of 65.7% and 87.0%, respectively, while the maximum ethanol concentration was 11.9 g/L.

Corn cob has a high amount of hemicellulose, which is composed mainly of xylose, arabinose and mannose. The results showed that spt15-29 grew more rapidly and had higher sugar utilization in comparison to YPH499. Due to toxicity factors in corn cob hydrolysis, the utilization ratio of xylose in recombinant strain spt15-29 was lower than that of spt15-25 grown in pure xylose or a sugar mixture. Spt15-25 was screened in a culture medium containing xylose as the sole carbon source. However, similar to spt15-25, the strain spt15-29 can ferment xylose and glucose at the same time, with a higher utilization ratio of glucose than the original strain YPH499. Meanwhile, the concentration of the by-product xylitol was very low.

Application of gTME to *S. cerevisiae* for improving xylose fermentation is theoretically feasible. In practice, tolerance and adaptation of the recombinant strain to lignocellulosic hydrolysates through cofermentation of glucose and xylose, under anaerobic conditions, has been improved in the present work, which also expands the possibilities for biotransformation of biomass to produce ethanol. The recombinant strain had modest growth rate in the cellulosic hyrolysates with different pretreatment and pH conditions [14, 15]. Through multiple transcript regulated genes, *S. cerivisiae* is able to be optimized for use in fermentation environments, which will facilitate ethanol production, pentose and hexose cofermentation, tolerance of ethanol and inhibitor in the acid hydrolysate. However, much more research needs to be done in order to realize the commercialization of the recombinant strain for ethanol produce, such as improving the tolerance of the depressor during fermentation, and reducing production cost, which will be meaningful for the biotransformation of vegetable fiber to ethanol.

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