A synthetic hybrid promoter for D-xylonate production at low pH in the tolerant yeast Candida glycerinogenes

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ABSTRACT
The tolerant yeast Candida glycerinogenes, with high D-xylonate and low-pH tolerances, was used as the host for D-xylonate production at low pH in this study. A low-pH inducible promoter, pGUKd, was engineered using the core promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (pGAP) combined with the upstream activating sequence of the promoter of the guanylate kinase gene (pGUK1) that had substituted pH-responsive TF binding sites. The recombinant cells that expressed GFP from the hybrid promoter pGUKd displayed dramatically increased fluorescence intensity at pH 2.5, thus verifying that pGUKd is a low-pH inducible promoter. The promoter pGUKd was then used to express the D-xylene dehydrogenase gene xylB, resulting in increased expression levels of xylB at low pH. The recombinant protein exhibited higher specific activities under lower pH conditions and produced 38 g/l D-xylonate at pH 2.5. This rate is much higher than that produced by fermentation at pH 5.5. These results suggest that the novel hybrid promoter pGUKd functions to direct the production of D-xylonate at low pH, and we provide a candidate genetic tool for the stress tolerant yeast C. glycerinogenes.

Introduction
D-xylonate derived from the hemicellulose sugar D-xylose is an important platform chemical with versatile applications as a complex agent, chelator, dispersant or the precursor of 1,2,4-butanetriol. It could also serve as a non-food-derived replacement for D-gluconic acid, which is widely used in the food, chemical, dye and pharmaceutical industries. Microbial production of D-xylonate with high yields and production rates was described when bacteria such as Gluconobacter oxydans and Pseudomonas putida were used. However, the commercial production of D-xylonate has not been developed until now. The bacteria produce a wide range of oxidases with low specificity, resulting in a mixture of sugar acids when using complex substrates for fermentation. In addition, the bacteria could not tolerate inhibitors found in the lignocellulosic hydrolysates. Recently, yeasts that are highly tolerant to these inhibitors, such as Saccharomyces cerevisiae, Kluyveromyces lactis and Pichia kudriavzevii, were found to be suitable for producing D-xylonate when they were genetically modified to produce a specific D-xylose dehydrogenase gene.

The inherent characteristics of this yeast’s tolerance to low pH is of great value for producing organic acids in acidic environments because fermentation at low pH reduces the need for a neutralizing base, minimizes contamination risks, and is desirable for efficient recovery of organic acids in downstream processes. The use of the nonconventional yeast P. kudriavzevii to produce D-xylonate at low pH resulted in lower yields of D-xylonate and lower productivity. In the present study, a hybrid low-pH inducible promoter pGUKd was engineered and then introduced into the stress tolerant yeast Candida glycerinogenes to improve the production of D-xylonate at low pH. Our study provides a candidate tool for the genetic
engineering of the potential cellular factory *C. glycerinogenes*.

**Materials and methods**

**Strains, media and growth conditions**

All the strains used in this study are listed in Supplementary Table 1. *C. glycerinogenes* UA5 and its derived strains were pre-cultured in YPD medium (20 g/l glucose; 20 g/l peptone; 10 g/l yeast extract) or synthetic dextrose (SD) medium (0.67 g/l yeast nitrogen base without amino acids; 20 g/l glucose) supplemented with the nutrients required for auxotrophic mutants. To assess the toxicity of D-xylonate, *C. glycerinogenes* was cultured in medium containing different concentrations of D-xylonate at pH 2.5 or 5.5. For D-xylonate production, yeasts were cultured in a 5 l jar fermenter containing 3 l of defined media (10 g/l glucose; 50 g/l xylose; 20 g/l peptone; 10 g/l yeast extract) at pH 2.5 or 5.5, 37°C, 1.5 vvm and 500 rpm agitation. *Escherichia coli* JM109 was cultured in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) for plasmid propagation.

**Construction of hybrid low-pH induced promoter**

The hybrid low-pH induced promoter was designed as described previously. The upstream activating sequence (UAS) of a native pH response promoter of the guanylate kinase gene GUK1 was used as the foundation and then combined with the 146 bp core promoter of pGAP that was characterized in our previous study.

**Construction of expression vectors**

The *GFP* coding sequence was amplified from the commercial plasmid pCAMBIA1302 using primers GFP-F and GFP-R and cloned into the integrative expression vector pURGAP under the promoter pGAP to generate pURGAP-GFP. The pGAP promoter was replaced by the hybrid promoter pGUkI after digestion with the restriction enzymes *Sac* II and *BamH* I. The integrative expression vector pURGAP, using *URA5* as a selection marker, was described in our previous study. To construct D-xylose dehydrogenase (*xylB*) expression cassettes, the *xylB* gene from *Caulobacter crescentus* was synthesized with optimal *S. cerevisiae* codons by the online service of GeneArt and then inserted into the pURGAP after digestion with *BamH* I and *Kpn* I to generate pURGAP-xylB. The recombinant vector was linearized by using the restriction enzyme *Hind* III and then used for transformation. All of the primers used in this study are listed in Supplementary Table 2.

**DNA transformation of *C. glycerinogenes***

*C. glycerinogenes* UA5 was pre-cultured in YPD for 16 h and then inoculated into fresh medium at 30°C for 4 h. Cells were collected by centrifugation at 5000 g for one minute, washed twice with sterile water and then resuspended in 360 μl transformation buffer containing 240 μl of 50% PEG3350 (w/v), 36 μl of 1 M lithium acetate, 10 μl of 5 g/l ssDNA, deionized water and approximately 5 μg of linearized plasmid DNA. The suspension was mixed thoroughly and then subjected to heat shock for 60 min at 42°C. Cells were then collected by centrifugation at 5000 g for 5 min, washed twice with sterile water and then spread on selective plates.

**Real-time PCR**

The relative expression levels of *xylB* were determined using real-time PCR (RT-PCR). The total RNA extraction, cDNA synthesis and RT-PCR were performed as described previously. The primers were designed using Beacon Designer 7 and are listed in Supplementary Table 2.

**Fluorescence microscopy**

The recombinant strains expressing *GFP* were cultured to log phase in YPD medium at pH 5.5. Samples were collected, washed twice with distilled water, and then incubated in YPD medium at pH 2.5 or 5.5 for 6 h. The samples were then washed and visualized by fluorescence microscopy (Olympus X53, excitation wavelength 488 nm, emission wavelength 520 nm).

**Analysis methods**

Biomass was measured with the optical density at 600 nm after the appropriate dilutions. The value of OD_{600} was calibrated to the dried cell weight (DCW) by using the equation 1 OD_{600} = 0.33 g DCW l^{-1}. D-xylonate, glycerol, D-xylose and glucose concentrations were analyzed by high-performance liquid chromatography (HPLC; DIONEX, USA) using an Aminex HPX–87H column (Bio-Rad; USA) and an
RI-101 refractive index detector (Shodex; Japan). The mobile phase was 5 mM H$_2$SO$_4$, with a flow rate of 0.6 ml/min at 60°C.

**Measurement of Enzyme activity**

The cells were harvested by centrifugation and disrupted by agitation with glass beads (Sigama, 425 × 600 mm) by vortexing 15 times, alternating 30 s of vortexing with 1 min on ice. The xylose dehydrogenase activities of crude cell extracts were measured as described previously. The protein content of the extracts was determined using the bicinchoninic acid method (CW Biotech, BAC protein assay kit).

**Results and discussion**

**Effects of D-xylonate and low pH on the growth of C. glycerinogenes UAS**

*C. glycerinogenes* that previously showed multi-tolerance toward hyperosmotic stress, high-temperature, ethanol and acetic acid, was tested for its D-xylonate tolerance at pH 5.5 and 2.5 in this study. As shown in Figure 1, in the presence of 50 or 100 g/l D-xylonate, the biomass did not differ significantly at either pH 5.5 or pH 2.5. However, an increase in the concentration of D-xylonate to 200 g/l resulted in a dramatic reduction especially under the condition of pH 2.5. In this case, only 0.8 g l$^{-1}$ biomass was produced. In addition, D-xylonate was not consumed by the culture, suggesting that *C. glycerinogenes* lacks a degradation pathway for D-xylonate. D-xylonate was reported to impose considerable stress on the cell wall of *S. cerevisiae* in a transcriptome study; however, no similar research has been performed in a nonconventional yeast. The High-Osmolarity-Glycerol pathway, of which we have characterized the key component Hog1 kinase, might also contribute to the weak acid and low-pH tolerance.

In conclusion, *C. glycerinogenes* displayed greater tolerance to D-xylonate than *S. cerevisiae*, suggesting that *C. glycerinogenes* is a potential host for D-xylonate production at low pH.

**Construction of the hybrid low-pH induced promoter pGUKd**

During D-xylonate production by yeast, the pH of the medium decreased gradually as acidic products accumulated. A low-pH inducible promoter may have potential application for D-xylonate production in low-pH fermentation. In this study, we engineered a synthetic promoter for the nonconventional yeast *C. glycerinogenes* that is inducible under low-pH conditions.

Synthetic hybrid promoters are composed of 2 modular components, the core promoter element and the enhancer elements. The 146 bp-core sequence of a native promoter of glyceraldehyde-3-phosphate dehydrogenase gene (pGAP) containing the transcription start site and TATA box was used as the core promoter element to ensure gene constitutive expression. The upstream activating sequence (UAS) of the promoter of the guanylate kinase gene (pGUK1), which is a low-pH inducible promoter with a low basal promoter strength, was used as the core promoter element to ensure gene constitutive expression in *C. glycerinogenes*. The upstream activating sequence (UAS) of the promoter of the guanylate kinase gene (pGUK1), which is a low-pH inducible promoter with a low basal promoter strength, was used as the foundation for transcription factor binding site modification. The binding sites of various pH-response transcription factors, Msn2/4 (AGGGG), Swi4 (TTCGCGT), and Rlm1 (CTATAAATAG), and output-improved transcription factors, Azf1 (TTTCTTTT) and Swi5 (TGCTGGT), were substituted into the UAS of pGUK1, and the resultant hybrid promoter pGUKd is shown in Figure 2.

The expression strength of the hybrid promoter pGUKd was compared with that of pGAP by expressing the reporting gene GFP. As shown in Figure 3, the pGUKd-GFP transformant displayed a dramatic increase in fluorescence intensity at pH 2.5, suggesting that the hybrid promoter acted as a low-pH inducible promoter.
Expression of xylose dehydrogenase gene xylB

Since no episomal vectors are available for C. glycerinogenes, the xylB gene was integrated into the 5.8S rDNA site by expression cassettes that harboured pGAP promoters as the control and the low-pH inducible promoter pGUKd to generate recombinants pGAP-xylB and pGUKd-xylB. The recombinants were cultured in YPD medium at different pH conditions for 20 h to assess the relative expression levels of xylB. As shown in Figure 4A, the expression levels of xylB driven by pGUKd increased as the pH decreased within the indicated range, while it was constitutively expressed under the control of the pGAP promoter. The relative expression level of xylB at pH 2.0 increased 18.2-fold compared with that at pH 7.0, suggesting that the hybrid promoter pGUKd functioned during low-pH fermentation. The specific activities of the D-xylose dehydrogenase of pGUKd-xylB at pH 2.5 increased by 3-fold compared with that at pH 5.5 (Fig. 4B). The recombinant pGAP-xylB exhibited lower specific activities at pH 2.5, possibly be due to the fact that the optimum pH for D-xylose dehydrogenase was determined under alkaline conditions.

Figure 2. The sequence of hybrid low-pH inducible promoter pGUKd. The core-promoter of pGAP is double-underlined containing a TATA box. The binding sites of various transcription factors of Msn2/4 (italics), Azf1 (bold), Swi4 (wavy underline), Rlm1 (bold underline) and Swi5 (underline) were substituted into the upstream activating sequence of pGUK1, to generate pGUKd.

Figure 3. GFP expression under the control of pGUKd and pGAP promoters at different pH conditions.

Figure 4. The inducing capacity of pGUKd by expressing xylB. A, the relative expression levels of xylB at different pH conditions. B, the specific activities of D-xylose dehydrogenase of the recombinant using different promoters of pGUKd and pGAP. All data from three independent replicates were used to calculate the mean and standard deviation.
Production of D-xylionate by *C. glycerinogenes* pGUKd-xylB

D-xylionate production by *C. glycerinogenes* pGUKd-xylB was performed in media containing 50 g/l D-xylose in a 5 l jar fermentor. Since *C. glycerinogenes* cannot utilize D-xylose as a carbon source, 10 g/l D-glucose was provided for cell growth as a co-substrate. The final biomass was not significantly affected by differing the pH value, although the strain exhibited a significant growth delay at pH 2.5. Like *S. cerevisiae*, *C. glycerinogenes* does not have a specific D-xylose uptake protein, and D-xylose transport is performed by the hexose transporters when glucose is absent. We engineered *C. glycerinogenes* to produce xylitol with a relatively high rate of D-xylose consumption, implying that *C. glycerinogenes* may possess an efficient uptake mechanism for D-xylose. The D-xylose was almost completely consumed at pH 2.5 within 48 h, while 14.4 g/l D-xylose remained in the culture at pH 5.5. *C. glycerinogenes* pGUKd-xylB produced 38.0 g/l D-xylonate at pH 2.5, with a rate of 0.79 g/l/h, which was much higher than that cultured at pH 5.5 (Fig. 5). Xylitol was produced as a by-product under both pH conditions. This could be eliminated by deleting the D-xylose reductase gene in future studies. It is clear that *P. kudriavzevii* produced much higher levels of D-xylonate than *C. glycerinogenes* by using a pulsed D-xylose feeding strategy. However, lower pH values significantly affected the production of D-xylonate, resulting in a lower yield and rate of production. The use of the hybrid promoter in our study provided a potential solution for efficiently producing D-xylonate at low pH. Since *C. glycerinogenes* is an acid tolerant yeast, this promoter may also have potential applications in the production of other organic acids at low pH.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.
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