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Identification and application of novel low pH-inducible promoters for lactic acid production in the tolerant yeast *Candida glycerinogenes*

Qianqian Hou,[1,2] Qiang He,[3] Gengliang Liu,[1,2] Xinyao Lu,[1,2] Hong Zong,[1,2] Wenqiang Chen,[3] and Bin Zhuge[1,2,*]

The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China,[1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China,[2] and Shaanxi Engineering Research Center of Edible and Medicated Fungi, School of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong 723000, China[3]

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Bioproduction of organic acids under low pH condition without adding inducer and neutralizer is an economical craft to decrease downstream cost. *Candida glycerinogenes* had higher tolerances to low pH and lactic acid than *Saccharomyces cerevisiae*. QRT-PCR analysis showed that four of fifteen candidates functioned as potentially acid-inducible promoters in *C. glycerinogenes*. In particular, P*Cggmt1* showed the strongest induction ability at pH 2.5. Fluorescence analysis indicated that the induction ability of P*Cggmt1* gradually increased as the pH decreased. In addition, P*Cggmt1* had the binding sites of Msn2p/4p, Azf1p, and Nrg1p. P*Cggmt1* was further used to control the expression of lactate dehydrogenase gene from *Rhizopus oryzae* in *C. glycerinogenes*. Compared with pH 5.5, the specific activity of lactate dehydrogenase and lactic acid titer at pH 2.5 were increased by 229% and 218%, reaching 13.8 mU/mg and 12.3 g/L, respectively. These results presented here showed a potential to produce organic acid economically at low pH by the stress tolerant *C. glycerinogenes* and the novel low-pH inducible promoter P*Cggmt1*.

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[**Key words:** *Candida glycerinogenes*; Low pH-inducible promoter; Tolerant yeast; Lactate dehydrogenase; Lactic acid]

Conversion of renewable feedstock to valuable chemicals such as organic acids through microbial fermentation has attracted great attention. In this process, the acidic stress induced by the accumulated organic acids presents a key challenge to cell growth and product synthesis (1). Adding alkaline agents such as NaOH, Na_2CO_3, or $CaCO_3$ is a common strategy to maintain a suitable pH level. However, this also produces organic salts, resulting in osmotic stress inhibiting cell growth and organic acid production and additional downstream separation costs (2,3). Biosynthesis of organic acids by robust strains is an effective choice to overcome these problems.

Candida glycerinogenes is an industrial strain with excellent tolerance to hyperosmotic stress, organic acid, and heat (4). It has been commercially applied for the bioproduction of glycerol under high osmotic pressure. It has been also engineered to produce xylonate (5–7), exhibiting a potential as a platform strain for producing organic acid at low pH. However, in the process of organic acid fermentation, low pH not only affected cell growth but also decreased the transcription and the expression level of key enzymes, which in turn affected the accumulation of organic acids. Therefore, it is necessary to screen acid-inducible promoters to regulate the expression of organic acid synthesis-related genes, without additionally adding inducers and neutralizing agents, thereby improving the fermentation performance in an acidic environment (8).

Traditional promoters, such as P*gpd* (9), P*gap*, and P*aox1* (10), only transcribe and express genes at moderate pH, but have low activity under low pH conditions. Thus, screening and application of low pH-inducible promoters are important for organic acid production under acidic conditions. In *Lactococcus lactis*, low pH-inducible promoters have been investigated, which could be induced at pH 5.5 rather than at pH 7.0 (11). In *Saccharomyces cerevisiae*, a series of strong synthetic promoters has been designed, characterized, and applied in the production of lactic acid under acidic conditions (pH $\leq$ 3) (12). Similarly, a low pH-inducible promoter of *Aspergillus niger*, P*gas*, has been identified and showed an ability to effectively promote gene expression and produce itaconic acid at pH 2.0 (13). Therefore, the screening of low pH-inducible promoters will be beneficial to the production of organic acids by *C. glycerinogenes* at low pH.

Lactic acid is the building block of the important biopolymer poly-lactic acid (14) and has important applications in food, pharmaceutical, chemical, and environmental industries. Heterologous expression of lactate dehydrogenase (LDH) is a common strategy to improve lactic acid production. In this study, based on the acid-tolerant properties of *C. glycerinogenes*, a novel acid-inducible promoter was screened and used to control the expression of *ldh* from *Rhizopus oryzae* in *C. glycerinogenes* to promote lactic acid production at pH 2.5.

* Corresponding author at: The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China. Tel./fax: +86 510 85918150.
E-mail address: bzhuge@126.com (B. Zhuge).

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MATERIALS AND METHODS

Strains, media, and growth conditions All the strains used are listed in Table S1. *Escherichia coli* JM109 used for plasmid construction was cultured in Luria–Bertani medium supplemented with 100 μg/mL ampicillin. *S. cerevisiae* ZWA46, *C. glycerinogenes* UA5 (15) and derived strains were pre-cultured in YEPD medium (10 g/L yeast extract, 20 g/L tryptone and 20 g/L glucose) or SD medium (6.7 g/L yeast nitrogen base without amino acids and 20 g/L glucose). To assess the toxicity of pH or lactic acid, *C. glycerinogenes* UA5 and *S. cerevisiae* ZWA46 were cultured for 60 h at 37°C and 100 rpm in 50 mL YEPD medium with different pHs (2.25, 2.5, 2.75, 3.0, 4.0, and 5.0) or different concentrations of lactic acid (0, 30, 40, 50, and 60 g/L) at pH 5.5, respectively. *R. oryzae* was cultured in PDA medium (200 g/L potato infusion and 20 g/L glucose). For lactic acid production, recombinant strain was cultured in 250 mL flasks containing 50 mL fermentation medium (100 g/L glucose, 2 g/L urea and 5 g/L corn syrup) at pH 2.5 or 5.5, 37°C and 100 rpm for 60 h. Dilute hydrochloric acid was used to adjust the pH of the liquid medium.

RNA preparation and qRT-PCR assays The cells cultured for 16 h were harvested after treatment and used for total RNA extraction by a Trizol reagent (BBI, Toronto, Canada). One microgram RNA from each sample was used to synthesize the cDNA by using Hiscript II Q RT SuperMix (Vazyme, Nanjing, China). Quantitative PCR was performed using AceQ qPCR SYBR Green Master Mix (Vazyme) and the housekeeper gene 18S rDNA was used as the internal reference. All the experiments were performed in triplicate. Primers are listed in Table S2.

Plasmids construction Green fluorescent protein gene (*GFP*) was amplified from the commercial plasmid pCAMBIA1302 and then inserted into *Not* I and *Kpn* I sites of expression vector pURURA (6,15) to generate pUGA. The acid-inducible promoters (2.0 kb upstream of target genes) were amplified from the genomic DNA of *C. glycerinogenes* and then inserted into vectors pUGA and pURURA to generate different recombinant plasmids (Table S1). The lactate dehydrogenase gene (*ldh*) was amplified from the cDNA of *R. oryzae* and inserted into vector pURGMTA to generate pUGLA. Recombinant plasmids were linearized and integrated into *C. glycerinogenes* chromosome to obtain the different recombinant strains as previous paper described (6). All primers are listed in Table S2.

Fluorescence microscopy The recombinant strains harboring *GFP* were cultured to log phase in YEPD medium. Cells were collected, washed twice with PBS buffer, and then incubated in YEPD medium with different pHs (2.0, 3.0, 4.0, 5.0) for 6 h. The samples were collected, washed by PBS buffer, and then visualized by fluorescence microscopy (Olympus X53, excitation wavelength 488 nm, emission wavelength 520 nm, Olympus, Tokyo, Japan). Optical density (IOD) analysis was performed using Image-Pro Plus 6.0.

Enzyme activity assay The activity of LDH was measured by converting lactic acid to pyruvate and reducing NAD^+ to NADH. The amount of 1 μmol NADH produced per minute of protein at 30°C was defined as one unit of activity (16). Cells were harvested by centrifugation and disrupted by liquid nitrogen grinding, and then dissolved in PBS buffer. Crude enzyme extracts were obtained by centrifugation. Protein concentrations of cell extracts were measured by Non-Interference Protein Assay Kit (Sangon Biotech, Shanghai, China).

Analysis methods Biomass was represented by the dried cell weight (DCW). Lactic acid, glycerol, ethanol, and glucose concentrations were analyzed by high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and an RI-101 refractive index detector (Shodex, Tokyo, Japan). The mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min at 60°C. All experiments were repeated in triplicate.

Statistical analysis The data are expressed as the mean ± SD of three independent experiments. To investigate statistical differences, ANOVA with multiple comparisons were conducted on the Statistical Package for Social Scientists (SPSS) version 20.0 (IBM, Armonk, NY, USA). Samples with P values of <0.05 were considered statistically different.

The accession numbers of nucleotide sequences The acid-inducible promoter sequences from *C. glycerinogenes* were sequenced by Sangon Biotech and deposited in the National Center for Biotechnology Information (NCBI). The GenBank accession numbers of the genes and corresponding promoters are MK027743 to MK027746.

RESULTS

Growth characteristics of *C. glycerinogenes* and *S. cerevisiae* under low pH and lactic acid condition We compared the tolerance of strains to different environmental stresses through changes in final biomass. In the initial stage of fermentation, the growth rates of the strain decreased as the pH decreased or the concentration of lactic acid increased. The maximum specific growth rates of *Candida glycerinones* were much higher than those of *S. cerevisiae* under the same conditions. Besides, the delay phase of *C. glycerinones* was shorter (Fig. 1A, B, D, E). The final biomass of *C. glycerinones* was not significantly impacted by the changes in pH level. The biomass of *S. cerevisiae* was decreased by 20.6% at pH 2.5 and its cell growth was almost completely repressed at pH 2.25 (Fig. 1C). pH value was not changed significantly during the cultivation.

During the cultivation with lactic acid at pH 5.5, the pH level of the media kept stable and the lactic acid concentration was not changed, indicating that *C. glycerinogenes* and *S. cerevisiae* cannot decompose lactic acid. Low lactic acid concentrations showed similar slightly negative effects on cell growths of *C. glycerinogenes* and *S. cerevisiae*. At the lactic acid concentration of 60 g/L, the biomass of *C. glycerinogenes* was 2.8 g/L, decreased by 50.7%, but the cell growth of *S. cerevisiae* was completely repressed (Fig. 1F). These

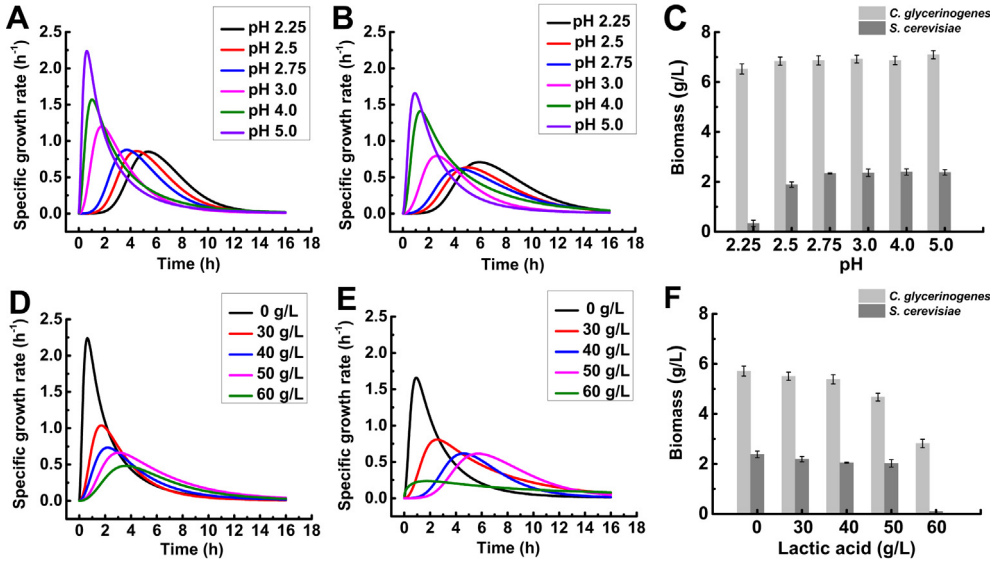

FIG. 1. Cell growth of *C. glycerinogenes* and *S. cerevisiae* at different pHs (2.25, 2.5, 2.75, 3.0, 4.0, and 5.0) and different concentrations of lactic acid at pH 5.5 (0, 30, 40, 50, and 60 g/L). Specific growth rates under different pHs and different concentrations of lactic acid in *C. glycerinogenes* (A, D) and *S. cerevisiae* (B, E). Biomasses of *C. glycerinogenes* and *S. cerevisiae* under different pHs (C) and different concentrations of lactic acid (F). The data are expressed as the mean ± SD of three independent experiments.

results indicate that *C. glycerinogenes* has better tolerances to low pH and high lactic acid concentration than *S. cerevisiae*.

Screening and identification of low pH-inducible promoters Based on previous literature (17) about *S. cerevisiae* and the transcriptome data of *C. glycerinogenes* under acidic conditions, fifteen potential acid stress-responsive genes were selected (Table S3). Most of the tested genes were upregulated and only *Cgady2* was downregulated at pH 2.5 (Fig. 2A). Among the upregulated genes, *Cggmt1*, *Cgguk1*, *Cgadh3*, and *Cgtpo2* were upregulated by more than 5-fold (Fig. 2A). The relative transcriptional level of *Cggmt1* increased significantly at low pH and reached a maximum at pH 2.0 which was 20.8-fold that at pH 5.0 (Fig. 2B). The relative transcriptional level of *Cgguk1* peaked at pH 3.0. In contrast, the relative transcriptional levels of *Cgadh3* and *Cgtpo2* only showed weak upregulations at pH 2.0 and 3.0 (Fig. 2B). The results indicate that the promoters of *Cggmt1*, *Cgguk1*, *Cgadh3*, and *Cgtpo2* are potentially pH-inducible, and the promoter of *Cggmt1* is more strongly induced by low pH.

Expression of green fluorescent protein gene controlled by the selected low pH-inducible promoters To compare the pH-inducible strength of the promoters, fluorescence intensities of the recombinant strains *C. glycerinogenes*-P*Cggmt1-GFP*, *C. glycerinogenes*-P*Cgguk1-GFP*, *C. glycerinogenes*-P*Cgadh3-GFP*, and *C. glycerinogenes*-P*Cgtpo2-GFP* were investigated at different pHs. All the recombinants showed similar fluorescence intensity at pH 5.0 (Fig. 3A, B). As the pH decreased, the strengths of P*Cggmt1* and P*Cgguk1* were increased significantly, reaching a maximum at pH 2.0. The fluorescence intensity of *C. glycerinogenes*-P*Cggmt1-GFP* at pH 2.0 was 1.4-fold and 1.6-fold that of *C. glycerinogenes*-P*Cgguk1-GFP* and *C. glycerinogenes*-P*Cgadh3-GFP*, respectively (Fig. 3B). In contrast, the strength of P*Cgtpo2* almost kept stable at different pHs (Fig. 3B). These results indicate that P*Cggmt1* is strongly induced by low pH.

Expression of *ldh* controlled by P*Cggmt1* to promote lactic acid production in *C. glycerinogenes* At pH 5.5, the fermentation broth of *C. glycerinogenes* UA5 only contained ethanol and glycerin, and no lactic acid was produced, indicating that *C. glycerinogenes* UA5 cannot produce lactic acid naturally (Fig. S1). The low pH-inducible promoter P*Cggmt1* was used to control the expression of *ldh* from *R. oryzae*. The *ldh* was successfully expressed in *C. glycerinogenes*. At pH 5.5, the specific activity of LDH was 4.2 mU/mg. The specific activity of LDH reached 13.8 mU/mg at pH 2.5, which was 3.3 times that at pH 5.5 (Fig. 4A). The lactic acid production of *C. glycerinogenes*-P*Cggmt1-ldh* was further performed under pH 5.5 and pH 2.5 conditions. At the end of cultivation, the pH was slightly decreased to 5.4 and 2.4, respectively, which was almost kept stable. Low pH improved glucose consumption rate, and glucose was completely consumed at 36 h in both conditions (Fig. 4B, C). The biomass and ethanol accumulation of *C. glycerinogenes*-P*Cggmt1-ldh* was decreased by 34% and 25% at pH 2.5, respectively. However, the titer of lactic acid was improved from 3.9 g/L to 12.3 g/L, which was increased by 218% at pH 2.5. Lactic acid can be stably accumulated in fermentation. In addition, there was no significant difference in the production of glycerol under both pH conditions (Fig. 4B, C).

DISCUSSION

Low pH and high osmotic stress are key challenges to microbial production of organic acids. *C. glycerinogenes* is a robust strain that has better acid tolerance and growth characteristics than *S. cerevisiae* under low pH and lactic acid conditions, and it is also a potential host strain for organic acids production. The high osmotic pressure-glycerol (HOG) and cell wall integrity (CWI) pathways are important to yeast in response to low pH stress (18). Our previous studies found that *C. glycerinogenes* had a stronger regulation on stress-response transcription factors than *S. cerevisiae*, leading to a more obvious up-regulation of stress-response genes (15,19). Besides, *C. glycerinogenes* showed a broader stress-response mechanism than *S. cerevisiae* under stress conditions (20). Therefore, these stronger and more complex responsive mechanisms may be responsible for the excellent acidic stress tolerance of *C. glycerinogenes*.

Four candidate genes, *Cggmt1*, *Cgguk1*, *Cgadh3*, and *Cgtpo2*, selected from 15 tested genes, were highly upregulated. In *S. cerevisiae*, *Sctpo2* encodes a polyamine transporter that is responsive to acid stress by transporting polyamines/protons (21). *Scadh3* encodes an alcohol dehydrogenase that is up-regulated under acidic conditions to generate energy for cell metabolism in *S. cerevisiae*. However, the roles of *Cggmt1* and *Cgguk1* under acidic stress are still unclear. *Cggmt1* encodes the GDP-mannose transporter importing GDP-mannose from the cytoplasm into the Golgi lumen (22). Therefore, the upregulation of *Cggmt1* may be beneficial to the function of Golgi lumen in the post-modification of protein, such as glycosylation, to generate more functional proteins against acidic stress. *Cgguk1* encodes the guanylate kinase, involving in ATP binding and purine nucleotide metabolic process. Up-regulation of *Cgguk1* may regulate the energy balance for cells to adapt to the acid stress environment. In addition, the obtained four pH-inducible promoters showed significant differences in the expression of *GFP* at different pHs. The inducible properties of the promoters may be affected by acid-related transcription factors. Further analysis may be helpful to elucidate the detailed mechanism of these genes and promoters in the response to acidic stress.

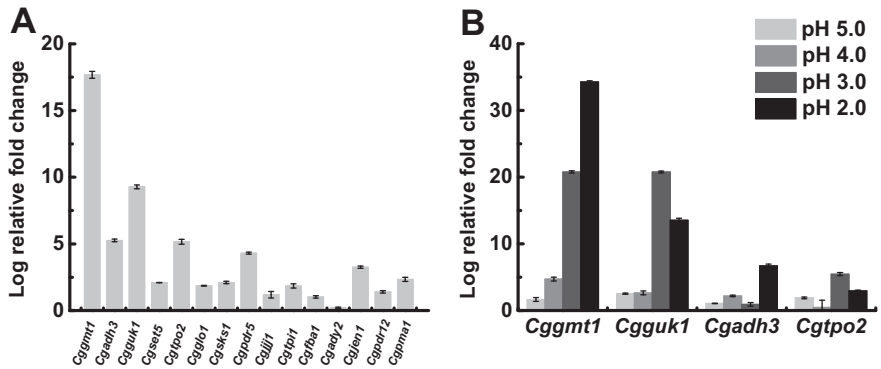

FIG. 2. RNA levels of different genes. (A) The relative transcriptional levels of selected genes at pH 2.5. (B) The relative transcriptional strengths of *Cggmt1*, *Cgguk1*, *Cgadh3*, and *Cgtpo2* at different pHs (2.0, 3.0, 4.0, and 5.0). The data are expressed as the mean ± SD of three independent experiments.

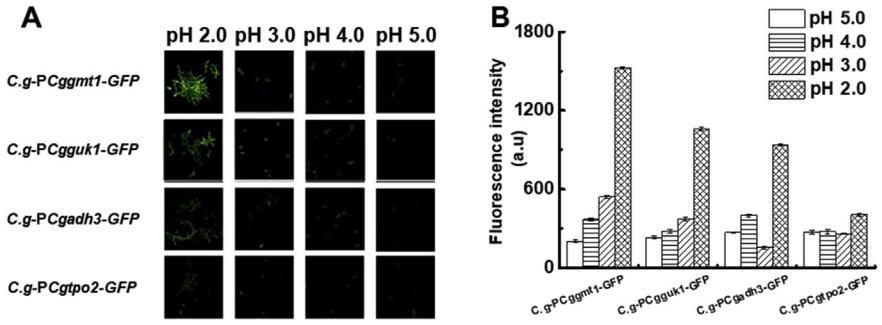

FIG. 3. Expression of *GFP* controlled by low pH-inducible promoters at different pHs (2.0, 3.0, 4.0, and 5.0). (A) Fluorescence images of *C. glycerinogenes*-P*Cggmt1-GFP*, *C. glycerinogenes*-P*Cgguk1-GFP*, *C. glycerinogenes*-P*Cgadh3-GFP*, and *C. glycerinogenes*-P*Cgtpo2-GFP* at different pHs. (B) Optical density analysis of the fluorescence intensities of the recombinants. The data are expressed as the mean ± SD of three independent experiments.

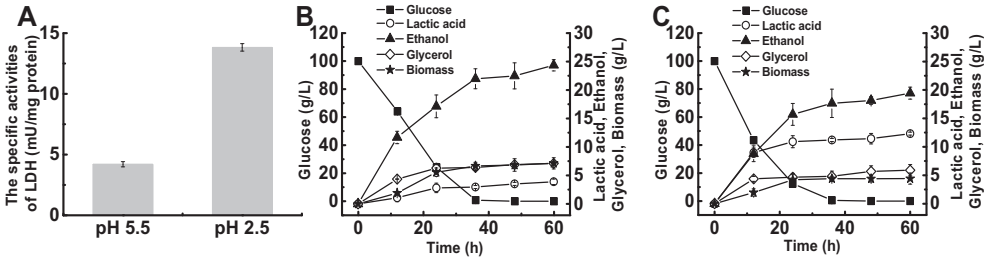

FIG. 4. Lactic acid fermentation of recombinant strain *C. glycerinogenes*-P*Cggmt1-ldh* under different pH conditions. (A) The specific activities of the lactic acid dehydrogenase of *C. glycerinogenes*-P*Cggmt1-ldh* at pH 5.5 and pH 2.5. (B) Lactic acid fermentation of recombinant strain at pH 5.5. (C) Lactic acid fermentation of recombinant strain at pH 2.5. Squares, glucose; circles, lactic acid; triangles, ethanol; diamonds, glycerol; pentagrams, biomass. The data are expressed as the mean ± SD of three independent experiments.

Low pH improved the expression level of *ldh* in *C. glycerinogenes*-P*Cggmt1-ldh*, leading to a higher LDH activity. The improvement of key enzyme activity directed metabolic flux to lactic acid production, resulting in higher lactic acid level and lower ethanol level. Notably, the glycerol production was not impacted at different pHs, suggesting the robustness of the glycerol synthesis pathway in *C. glycerinogenes*. As two main by-products, disruptions of the accumulations of glycerol and ethanol may be helpful for further improvement on lactic acid titer. Besides, increasing the expression level of LDH by multiple strategies, such as codon usage optimization, is also valuable for lactic acid accumulation.

In conclusion, *C. glycerinogenes* showed better tolerance to low pH and high lactic acid concentration. Some novel low pH-inducible promoters from *C. glycerinogenes* were screened and analyzed. The promoter of *Cggmt1*, P*Cggmt1*, showed the strongest low pH-inducible performance. Further, inducible expression of *ldh* by P*Cggmt1* improved the titer of lactic acid in *C. glycerinogenes* under acidic stress. The results presented here provided another strategy for organic acids production by a robust stress tolerance strain and a low-pH inducible promoter.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.01.005.

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