



Efficient Production of Glucaric Acid by Engineered *Saccharomyces cerevisiae*

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ABSTRACT Glucaric acid is a valuable chemical with applications in the detergent, polymer, pharmaceutical and food industries. In this study, two key enzymes for glucaric acid biosynthesis, MIOX4 (*myo*-inositol oxygenase) and Udh (uronate dehydrogenase), were fused and expressed with different peptide linkers. It was found that a strain harboring the fusion protein MIOX4-Udh linked by the peptide (EA₃K)₃ produced the highest glucaric acid titer and thereby resulted in glucaric acid production that was 5.7-fold higher than that of the free enzymes. Next, the fusion protein MIOX4-Udh linked by (EA₃K)₃ was integrated into delta sequence sites of the *Saccharomyces cerevisiae* *opi1* mutant, and a strain, GA16, that produced a glucaric acid titer of 4.9 g/L in a shake flask fermentation was identified by a high-throughput screening method using an *Escherichia coli* glucaric acid biosensor. Strain improvement by further engineering was performed to regulate the metabolic flux of *myo*-inositol to increase the supply of glucaric acid precursors. The downregulation of *ZWF1* and the overexpression of *INM1* and *ITR1* increased glucaric acid production significantly, and glucaric acid production was increased to 8.49 g/L in the final strain GA-ZII in a shake flask fermentation. Finally, in a 5-L bioreactor, GA-ZII produced a glucaric acid titer of 15.6 g/L through fed-batch fermentation.

IMPORTANCE Glucaric acid is a value-added dicarboxylic acid that was synthesized mainly through the oxidation of glucose chemically. Due to the problems of the low selectivity, by-products, and highly polluting waste of this process, producing glucaric acid biologically has attracted great attention. The activity of key enzymes and the intracellular *myo*-inositol level were both rate-limiting factors for glucaric acid biosynthesis. To increase glucaric acid production, this work improved the activity of the key enzymes in the glucaric acid biosynthetic pathway through the expression of a fusion of *Arabidopsis thaliana* MIOX4 and *Pseudomonas syringae* Udh as well as a delta sequence-based integration. Furthermore, intracellular *myo*-inositol flux was optimized by a series of metabolic strategies to increase the *myo*-inositol supply, which improved glucaric acid production to a higher level. This study provided a way for constructing a glucaric acid-producing strain with good synthetic performance, making glucaric acid production biologically in yeast cells much more competitive.

KEYWORDS D-glucaric acid, *Saccharomyces cerevisiae*, metabolic engineering, *myo*-inositol, biosensor

Glucaric acid (GA) was classified as “one of the top 12 value-added chemicals from biomass” in 2004 (1). As a naturally produced aldardic acid, glucaric acid can be found in many types of fruits and vegetables as well as in mammalian tissues (2). Glucaric acid has diverse applications in the detergent, polymer, pharmaceutical and food industries (3–5). The dicarboxylic acid structure makes glucaric acid a useful bio-derived precursor for adipic acid (6), hydroxylated nylons, and other ester/amide

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TABLE 1 Titters of glucaric acid in different host cells

Host	Pathways or genes	Level of production (g/L)	Carbon source(s)	Reference
<i>E. coli</i> BL21 Star(DE3)	<i>INO1, mMIOX, udh</i>	1.13	Glucose	9
<i>E. coli</i> BL21 Star (DE3)	<i>INO1, mMIOX, udh</i>	1.42	Sucrose	14
<i>E. coli</i> MG1655(DE3)	<i>INO1, mMIOX, udh</i>	1.56	Glucose	10
<i>S. cerevisiae</i> CEN.PK2-1	<i>mMIOX, udh</i>	1.6	Glucose, myo-inositol	15
<i>S. cerevisiae</i> CEN.PK2-1	<i>tmMIOX, udh</i>	1.76	Glucose, myo-inositol	36
<i>E. coli</i> BL21 Star(DE3)	<i>INO1, mMIOX, udh</i>	2.5	Glucose	11
<i>E. coli</i> cell-free lysate	<i>mMIOX, udh</i>	3.0	Glucose-1-phosphate	26
<i>E. coli</i> MG1655(DE3)	<i>INO1, SUMO-mMIOX, udh</i>	4.85	myo-Inositol	12
<i>E. coli</i> BL21 Star(DE3)	<i>AtMIOX, udh</i>	5.35	Glucose, glycerol	6
<i>S. cerevisiae</i> BY4471	<i>AtMIOX, udh</i>	6.0	Glucose, myo-inositol	20
<i>P. pastoris</i> GS115	<i>mMIOX, udh</i>	6.61	Glucose, myo-inositol	21
<i>E. coli</i> cell-free lysate	<i>mMIOX, udh</i>	7.3	Sucrose	23
<i>S. cerevisiae</i> BY4471	<i>AtMIOX, udh</i>	10.6	Glucose, myo-inositol	22
<i>S. cerevisiae</i> INVSc1	<i>AtMIOX, udh</i>	11.21	Glucose, myo-inositol	19
<i>S. cerevisiae</i> BY4471	<i>AtMIOX, udh</i>	15.6	Glucose, myo-inositol	This study

polymers (1, 7). Glucaric acid is synthesized mainly through the oxidation of glucose by using nitric acid as the oxidizing agent, which is a nonselective process and produces many by-products and highly polluting waste (8). Additionally, the extraction of glucaric acid from plants is uneconomical due to its low concentration. Therefore, it is highly desirable to produce glucaric acid by a biological method such as microbial fermentation.

There are two main ways to synthesize glucaric acid biosynthetically: one is to introduce heterologous glucaric acid synthesis pathways into *Escherichia coli* or yeast cells, and the other is to use multiple-enzyme biocatalytic methods *in vitro* (Table 1). The glucaric acid biosynthetic pathway was first constructed successfully in *E. coli* by introducing three heterologous enzymes, i.e., Ino1 (*myo*-inositol-1-phosphate synthase) from *Saccharomyces cerevisiae* (converts glucose to *myo*-inositol), MIOX (*myo*-inositol oxygenase) from mouse (oxidizes *myo*-inositol to glucuronic acid), and Udh (uronate dehydrogenase) from *Pseudomonas syringae* (oxidizes glucuronic to glucaric acid), producing 1.13 g/L glucaric acid from glucose (9). The glucaric acid titer was increased by 42% after the knockdown of the phosphofructokinase (Pfk) activity (10). By the colocalization of the three heterologous enzymes using modular and synthetic scaffolds, the glucaric acid titer was increased to 2.5 g/L, from glucose and *myo*-inositol (11). To further improve glucaric acid production, four genes, *zwf*, *pgi*, *uxaC*, and *gudD*, which encode glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, uronate isomerase, and glucarate dehydratase, respectively, were deleted, and the glucaric acid titer reached 4.85 g/L, from glucose and *myo*-inositol (12, 13). Another glucaric acid synthesis pathway, starting from sucrose, was constructed by expressing three enzymes, i.e., sucrose permease (CscB), invertase (CscA), and D-fructokinase (CscK), in *E. coli* BL21(DE3), which produced 1.42 g/L glucaric acid from sucrose (14). The highest glucaric acid titer reached 5.35 g/L, from glucose and glycerol, by combining the strategies of glucaric acid synthetic pathway fine-tuning and cofactor regeneration (6).

It appears that both high MIOX activity and *myo*-inositol flux are essential for high-level glucaric acid production (15). Unlike *E. coli*, yeasts can biosynthesize *myo*-inositol from glucose and absorb extracellular *myo*-inositol using specific transporters (16–18), so yeasts should be more suitable for glucaric acid production than *E. coli*. Recently, glucaric acid biosynthetic pathways have been successfully constructed in *S. cerevisiae* (15, 19, 20) and *Pichia pastoris* (21), which produced 1.6 and 6.61 g/L glucaric acid, respectively. Previously, we constructed the Bga-3 strain of *S. cerevisiae* by integrating the *Arabidopsis thaliana miox4* gene and the *P. syringae udh* gene into the delta sequence site of an *opi1* mutant, which produced 6 g/L glucaric acid (20). The addition of MgCl₂ to the fermentation medium increased

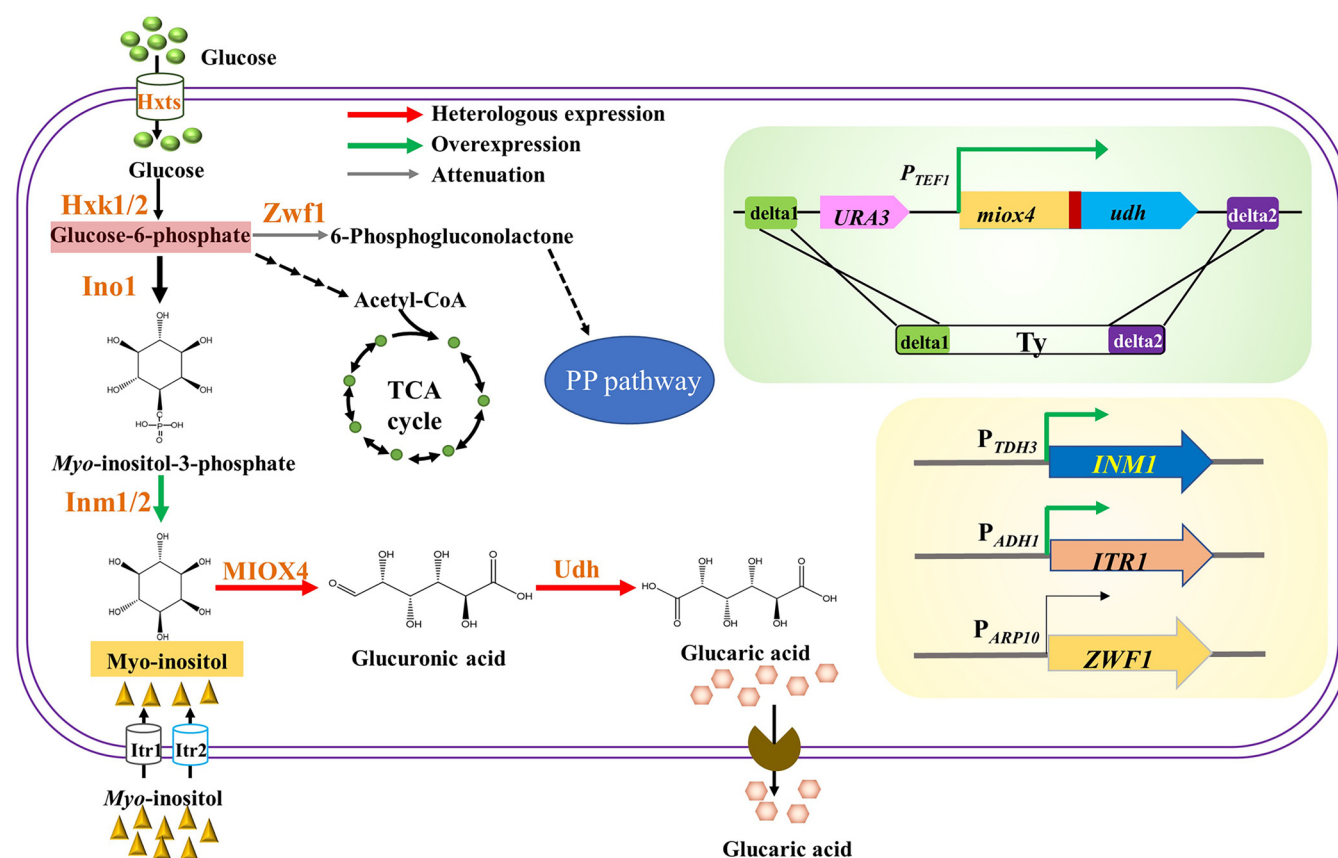


FIG 1 The glucaric acid pathway in *S. cerevisiae* and metabolic strategies for improving its production. TCA, tricarboxylic acid; PP, pentose phosphate; Hxt, hexose transporter; Hxk, hexokinase; Ino1, *myo*-inositol-3-phosphate synthase; Inm, *myo*-inositol monophosphatase; MIOX4, *myo*-inositol oxygenase from *Arabidopsis thaliana*; Udh, urinate dehydrogenase from *P. syringae*; ITR, *myo*-inositol transporter; Ty, transposon yeast.

the glucaric acid titer to 10.6 g/L (22). These findings indicate that yeast cells are much better producers of glucaric acid than *E. coli* cells.

An alternative to *in vivo* glucaric acid biosynthesis is the *in vitro* application of isolated enzymes. The first cell-free multienzyme system for the production of glucaric acid combined 7 enzymes, sucrose phosphorylase (SP), phosphoglucomutase (PGM), *myo*-inositol-1-phosphate synthase (MIPS), *myo*-inositol monophosphatase (IMPase), MIOX, Udh, and NADH oxidase (NOX), which formed a multienzyme cascade system to convert 17 g/L sucrose to 7.3 g/L glucaric acid (23). In other studies, hemicellulose and hardwood xylan were used as the substrates to produce glucaric acid by an *in vitro* multienzyme system (24, 25), and an immobilized multienzyme system was constructed, achieving 1.7 g/L glucaric acid from glucose-1-phosphate (26).

In this study, *Arabidopsis thaliana* MIOX4 (AtMIOX4) and *P. syringae* Udh were fused using 13 different peptide linkers and expressed in *S. cerevisiae* to determine the best MIOX4-Udh fusion protein for high-level glucaric acid production. The efficiency of the pathway was further increased by integrating the MIOX4-Udh fusion protein into the delta sites of the *S. cerevisiae* genome. Engineered strains with the highest levels of glucaric acid production were obtained using the *E. coli* glucaric acid-responsive biosensor. The *myo*-inositol supply was also improved by a series of metabolic strategies to increase glucaric acid production (Fig. 1), and strain GA-ZII, capable of producing 15.8 g/L glucaric acid, was developed.

RESULTS

Fusional expression of AtMIOX and Udh to increase glucaric acid production.

To optimize the glucaric acid-producing pathway by increasing the catalytic efficiency

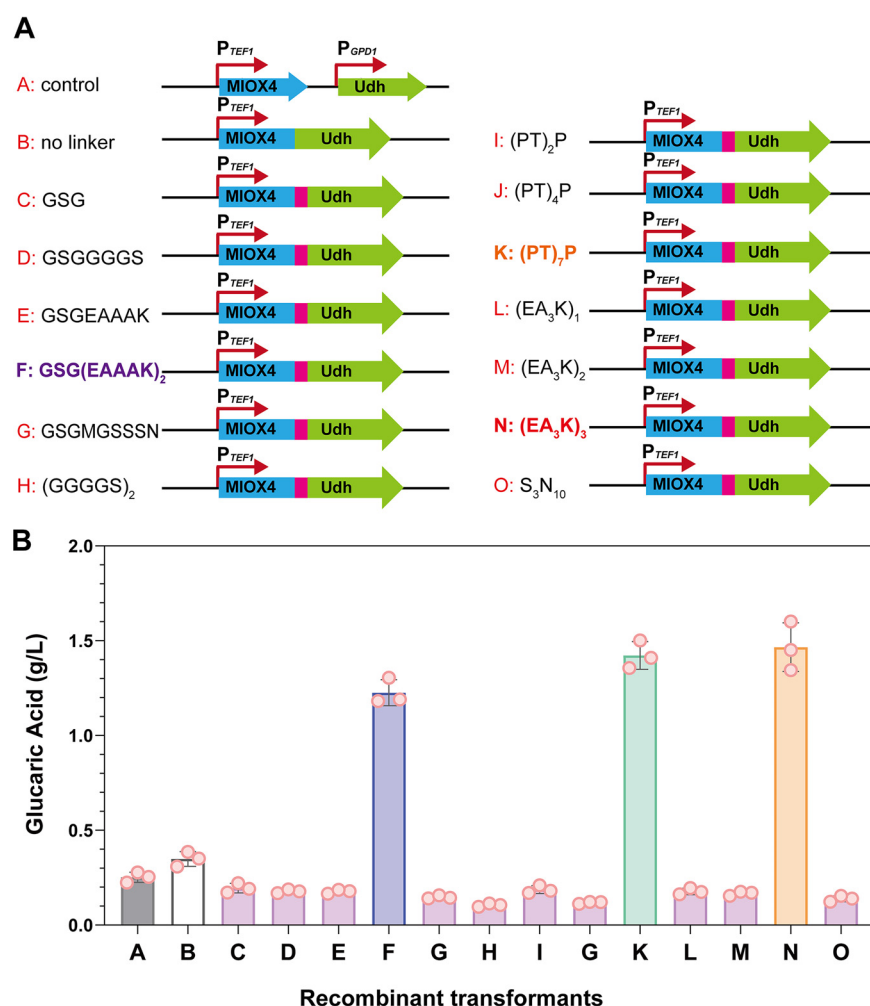


FIG 2 Effects of the expression of the MIOX4-Udh fusion protein linked by different peptides on glucaric acid production. (A) Structure of the MIOX4-Udh fusion protein with 13 different linkers. (B) Shaken-flask fermentation results for strains harboring the MIOX4-Udh fusion protein listed in panel A. All experiments were performed in triplicate, and the error bars represent the means $\pm$ standard deviations.

of AtMIOX and Udh, AtMIOX and Udh were fused with 13 different peptide linkers by overlap extension PCR (Fig. 2A; see also Fig. S1 in the supplemental material), and they were then integrated into the *OPI1* promoter of the *S. cerevisiae opi1* mutant. Both the free enzymes and directly fused MIOX4 and Udh, without a linker, were used as negative controls. The shake flask fermentation results indicated that strains expressing the fusion protein MIOX4-Udh linked by the peptides GSG(EAAAK)₂, (PT)₇P, and (EA₃K)₃ (linkers F, K, and N, respectively) significantly increased the accumulation of glucuronic acid compared with the two control strains (Fig. 2B). Specifically, the level of glucuronic acid production by the strain expressing MIOX4-Udh linked by (EA₃K)₃ reached 1.46 g/L, 5.7 times higher than that of the strain expressing the unlinked enzymes and 4.2 times higher than that of the strain expressing MIOX4-Udh fused without a linker. This indicates that the fusion of AtMIOX and Udh with an appropriate linker peptide can increase glucuronic acid production and that the catalytic activities of MIOX and Udh are rate-limiting factors for the production of glucaric acid.

Increasing glucaric acid production by the delta sequence-based integration of the fusion protein MIOX4-(EA₃K)₃-Udh. To increase the expression level of the fusion protein MIOX4-(EA₃K)₃-Udh, the proteins were integrated into the delta sites of the *S. cerevisiae* genome (Fig. 3A) since there are more than 400 delta sequences in the yeast genome (27). After integration, about 1,000 transformant strains were screened for

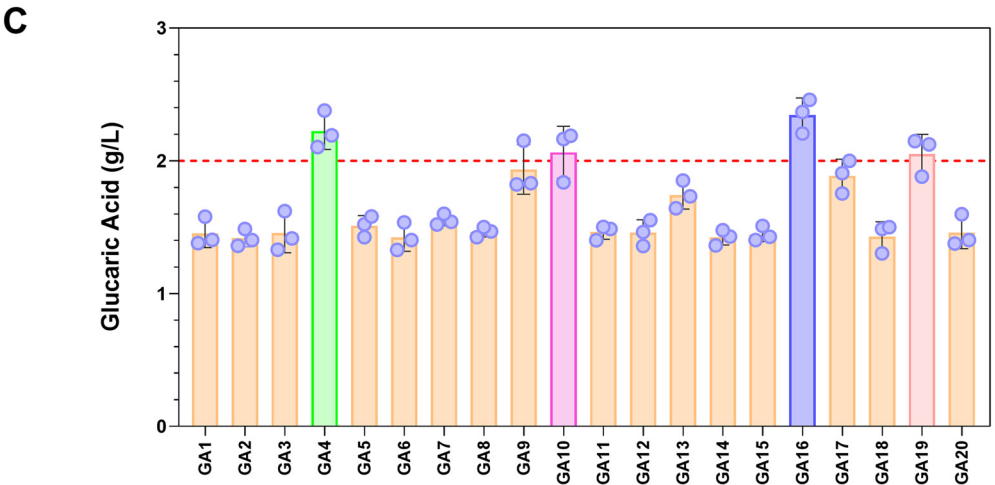
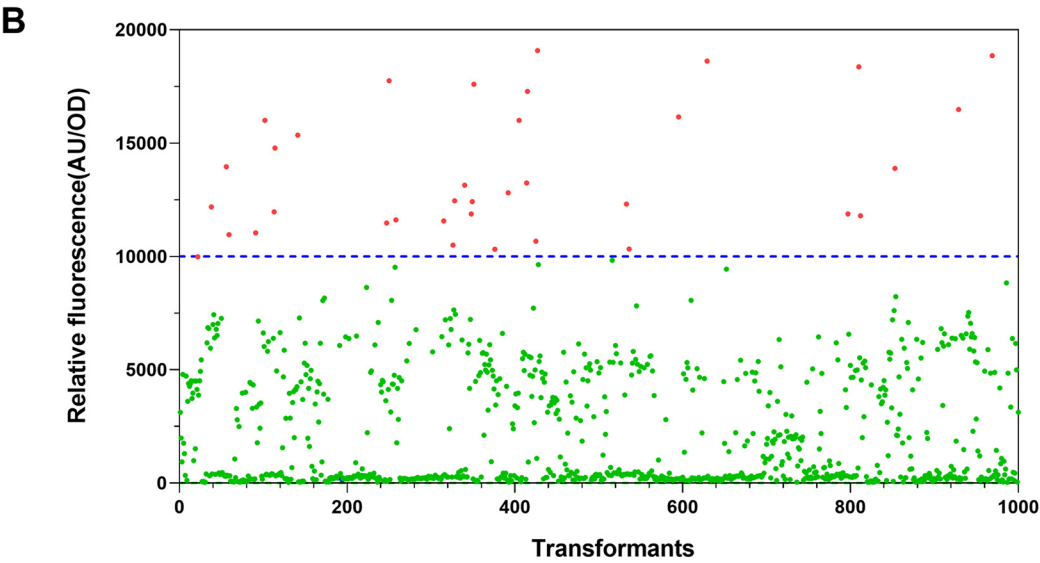
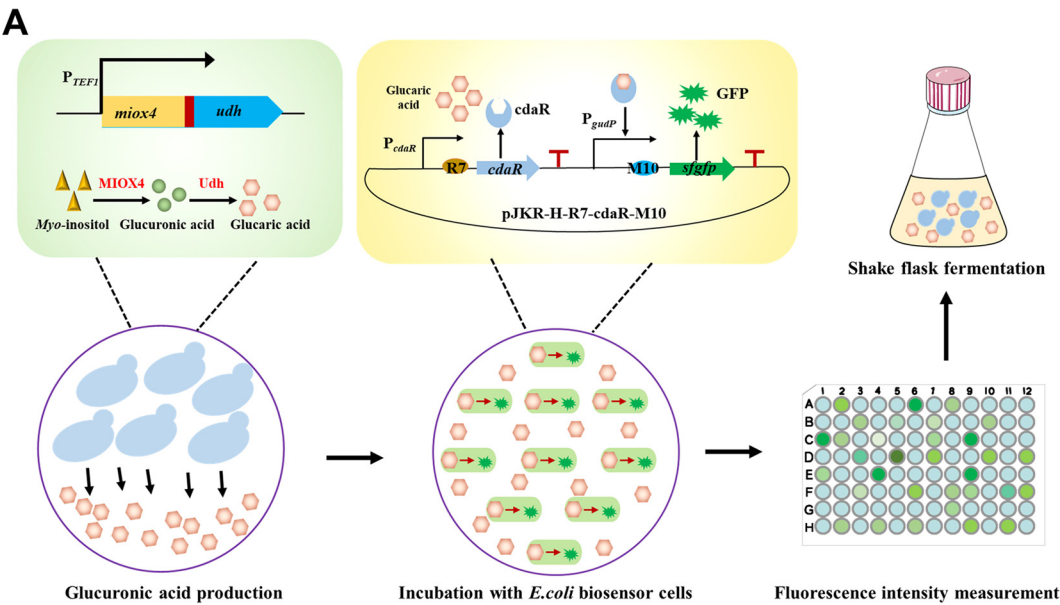


FIG 3 Screening for strains producing high titers of glucaric acid using the *E. coli* glucaric acid biosensor. (A) Schematic diagram of the strategy for screening for strains producing high titers of glucaric acid. The glucaric acid-producing strains (Continued on next page)

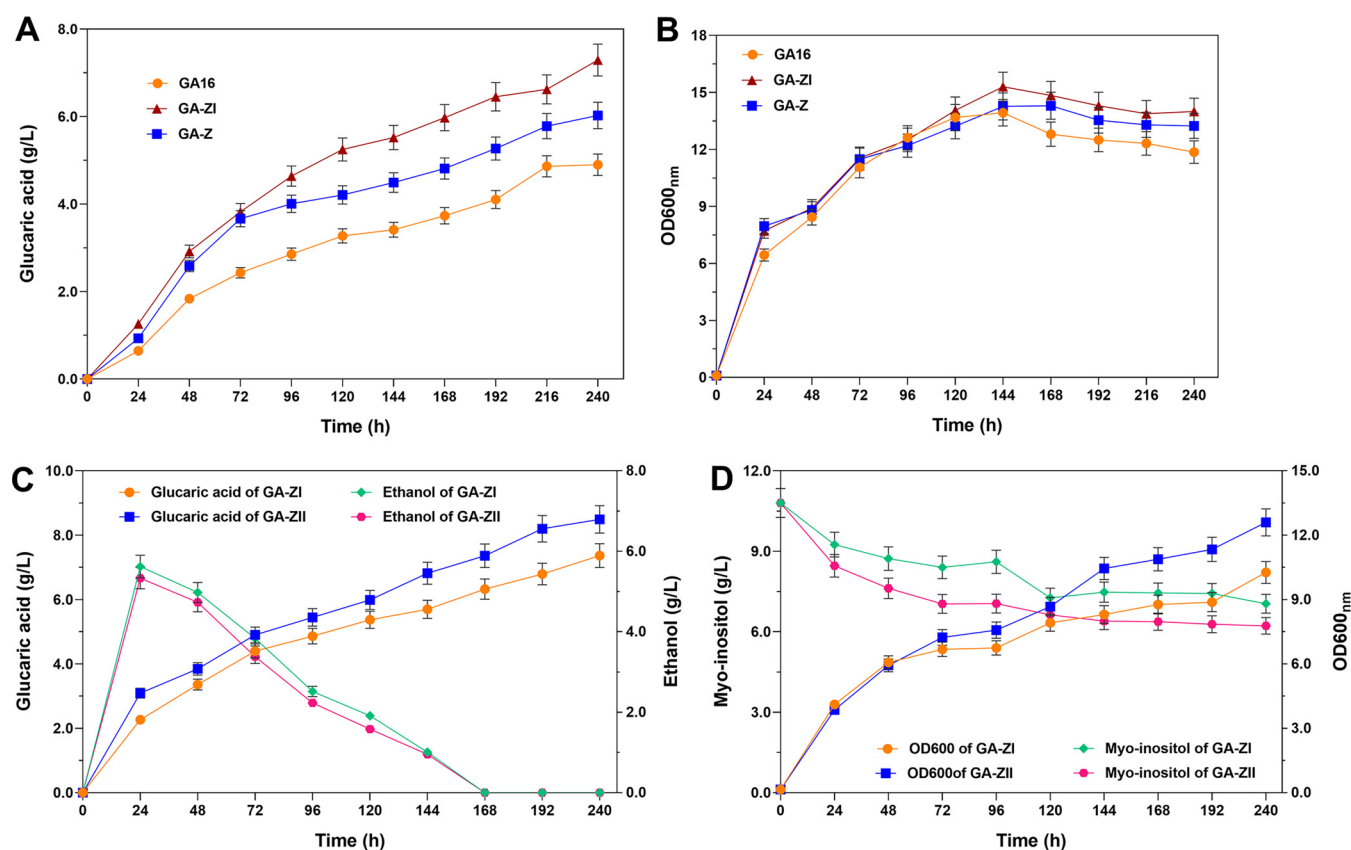


FIG 4 Shaken-flask fermentation results for glucaric acid by improving the supply of the *myo*-inositol precursor. (A and B) Glucaric acid production (A) and growth (B) of strains with downregulated *ZWF1* and overexpressed *INM1*. (C) Glucaric acid and ethanol titers of strains with overexpressed *ITR1*. (D) Growth and the rest of the *myo*-inositol in the culture medium of strains with overexpressed *ITR1*. All experiments were performed in triplicate, and the error bars represent the means $\pm$ standard deviations.

high-level glucaric acid production by 24-well microplate fermentation, followed by a comparison of the relative fluorescence values using the *E. coli* glucaric acid biosensor (Fig. 3B). The results showed that there were 36 single colonies with a relative green fluorescent protein (GFP) fluorescence value (ratio of the GFP fluorescence to the optical density at 600 nm [OD₆₀₀]) of $>10,000$ (Fig. 3B), the best 20 of which were screened by 10-mL shake tube fermentations and determinations of the glucaric acid titer (Fig. 3C). Three colonies, GA4, GA10, and GA16, had glucaric acid titers of >2 g/L, the highest of which was produced by GA16, so it was selected for further studies. It was shown that strain GA16 produced 4.9 g/L of glucaric acid in a 50-mL shake flask fermentation (Fig. 4A), which was 28.9% higher than the level produced by the previously constructed Bga-3 strain (20).

Improving the supply of *myo*-inositol precursors to increase glucaric acid production. *myo*-Inositol is the key precursor for glucaric acid and can be synthesized from glucose-6-phosphate by *S. cerevisiae*. However, in addition to being a *myo*-inositol precursor, glucose-6-phosphate also plays roles in many other metabolic pathways such as the pentose phosphate pathway and the glycolytic pathway. To increase the accumulation of glucose-6-phosphate, the *ZWF1* gene, encoding glucose-6-phosphate

FIG 3 Legend (Continued)

were obtained by integrating the MIOX4-Udh fusion protein into delta sequence sites of the *opi1* mutant and cultured in a 24-well plate. After fermentation, the liquid supernatant was assayed by an *E. coli* glucaric acid biosensor in a 96-well plate. The strains were selected according to the fluorescence intensity and then cultured in a 10-mL shake tube. Strains with increased glucaric acid titers were selected using an HPLC method and then transferred to a 50-mL shake flask for further fermentation analysis. AU, arbitrary units. (B) Relative fluorescence of $\sim 1,000$ transformants selected using the *E. coli* glucaric acid biosensor. (C) Fermentation results for 20 screened colonies in 10-mL shake tube fermentations. All experiments were performed in triplicate, and the error bars represent the means $\pm$ standard deviations.

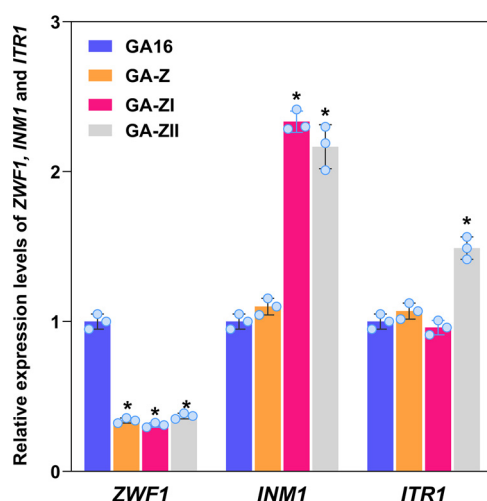


FIG 5 Relative gene expression levels of *ZWF1*, *INM1*, and *ITR1* in strains GA16, GA-Z, GA-ZI, and GA-ZII. The asterisks show a statistically significant difference at a *P* value of <0.01. All experiments were performed in triplicate, and the error bars represent the means $\pm$ standard deviations.

dehydrogenase (28–30), was downregulated by replacing its promoter with P_{ARP10r} according to the RNA sequencing (RNA-seq) results from our previous study (22), in strain GA16 to produce strain GA-Z (Fig. S2). The shake flask fermentation results showed that the glucaric acid titer of GA-Z reached 6.0 g/L, 22.4% higher than that of GA16 (Fig. 4A), indicating that reduced glucose-6-phosphate flux through the pentose phosphate pathway can increase glucaric acid production.

In our previous study, the overexpression of *INM1* (encoding inositol monophosphate 1-phosphatase) significantly increased glucaric acid production (22), so *INM1* was next overexpressed by replacing its promoter with the constitutive promoter P_{TDH3} in strain GA-Z to construct strain GA-ZI (Fig. S3). It was shown that the glucaric acid titer was increased to 7.3 g/L in strain GA-ZI by shake flask fermentation, which was 21.7% higher than that of GA-Z (Fig. 4A). In addition, there was no significant difference among the three strains GA16, GA-Z, and GA-ZI in their cell growth rates (Fig. 4B), suggesting that the downregulation of *ZWF1* and the overexpression of *INM1* did not influence cell growth.

To improve the ability of the engineering strain to take up extracellular inositol and thereby increase the cytosolic *myo*-inositol precursor concentration, the *myo*-inositol transporter *Itr1* was constitutively expressed by replacing its natural promoter with the constitutive promoter P_{ADH1} in strain GA-ZI to construct GA-ZII from GA-ZI (Fig. S4). After shake flask fermentation, the glucaric acid titer reached 8.5 g/L, 16.4% higher than that of GA-ZI (Fig. 4C). In addition, the level of residual *myo*-inositol of GA-ZII in the culture medium was significantly lower than that of GA-ZI, indicating that the overexpression of *Itr1* increases the uptake of extracellular *myo*-inositol from the extracellular medium (Fig. 4D). Interestingly, the cell growth rate of GA-ZII was also increased by 27.6% compared with that of GA-ZI, indicating that high cytosolic *myo*-inositol levels promote yeast cell growth (Fig. 4D).

To confirm the above-described gene regulatory changes, the expression levels of *ZWF1*, *INM1*, and *ITR1* were compared among GA16, GA-Z, GA-ZI, and GA-ZII by quantitative real-time PCR (qRT-PCR). It was shown that the expression levels of *ZWF1* in GA-Z, GA-ZI, and GA-ZII were significantly lower than that in GA16, whereas the expression levels of *INM1* in GA-ZI and GA-ZII and *ITR1* in GA-ZII were all significantly higher (Fig. 5). These results indicated that the expression levels of *ZWF1*, *INM1*, and *ITR1* were all crucial for the *myo*-inositol supply.

Production of glucaric acid by fed-batch fermentation in a 5-L bioreactor. To optimize glucaric acid production, fed-batch fermentation was performed in a 5-L bioreactor with GA-ZII in yeast extract-peptone-dextrose (YPD) medium. Ten grams per

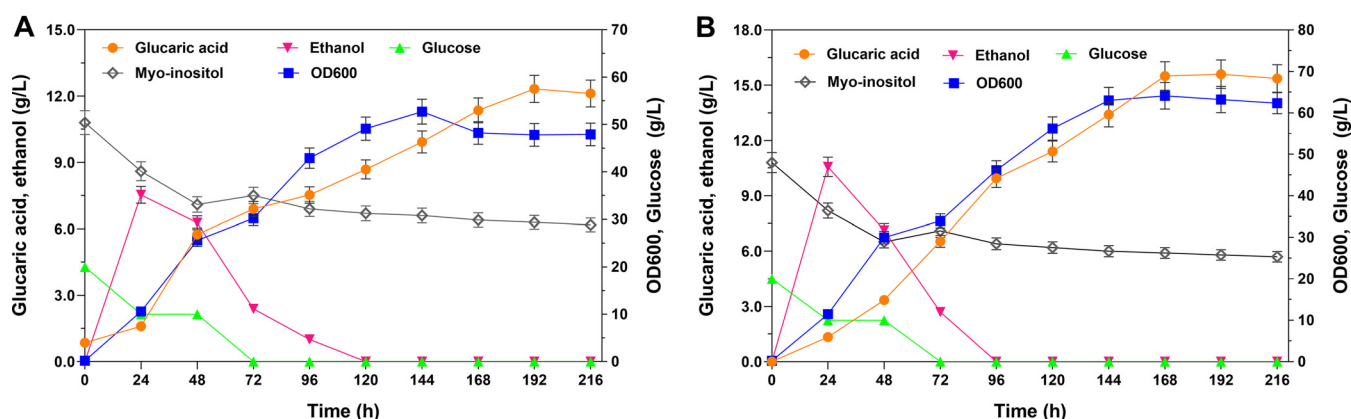


FIG 6 Fed-batch fermentation of strain GA-ZII in a 5-L bioreactor. (A) Glucaric acid production, ethanol production, glucose consumption, and growth of strain GA-ZII in YPD medium. Ten grams per liter of glucose was fed at 24 and 48 h. (B) Glucaric acid production, ethanol production, glucose consumption, and growth of strain GA-ZII in YPD medium with 100 mM MgCl_2 . Ten grams per liter of glucose was fed at 24 and 48 h. The error bars represent the standard deviations from three independent assays.

liter of glucose was added to the fermentation medium after 24 and 48 h. It was shown that strain GA-ZII produced a maximum glucaric acid titer of 12.3 g/L at 192 h, which was 44.7% higher than that in the shake flask fermentation (Fig. 6A). The by-product ethanol accumulated only in the initial 24 h of fermentation, but it was all metabolized by GA-ZII after 120 h (Fig. 4A). The biomass of GA-ZII peaked at 52.7 (OD_{600}) after 144 h.

In our previous study, we found that the addition of MgCl_2 could increase both the biomass and the glucaric acid titer (22), so 100 mM MgCl_2 was added to the fed-batch fermentation medium. The results showed that the glucaric acid titer was increased to 15.6 g/L at 168 h, which was 26.8% higher than that in the absence of MgCl_2 (12.3 g/L), and the fermentation time for the highest glucaric acid yield was reduced by 24 h. The biomass of GA-ZII strain reached above 60 with the addition of 100 mM MgCl_2 (Fig. 6B), which was 22% higher than that without MgCl_2 ; the ethanol utilization rate increased; and the ethanol was consumed after 96 h, 24 h earlier than without MgCl_2 . Overall, the addition of MgCl_2 increased the cell growth rate, the rate of reutilization of ethanol, and glucaric acid production.

DISCUSSION

This study aimed to construct an engineered yeast strain that could produce glucaric acid with high efficiency. The two key steps in the glucaric acid pathway are the accumulation of the precursor *myo*-inositol and the conversion (via glucuronic acid) of *myo*-inositol to glucaric acid. Here, to increase the efficiency of the conversion of *myo*-inositol to glucaric acid, the enzymes MIOX4 and Udh, which catalyze this conversion, were fused using 13 different peptide linkers, and the most efficient MIOX4-Udh fusion protein was then selected on the basis of the glucaric acid titer. The most efficient fusion protein was then integrated into multiple delta sites in the genome of the *S. cerevisiae opi1* mutant to increase its expression level. In addition, to increase the supply of the precursor *myo*-inositol, a series of strategies for the metabolic regulation of the glucaric acid pathway was implemented. After the optimization of the fermentation conditions, the engineered strain GA-ZII produced 15.6 g/L glucaric acid after fed-batch fermentation in a 5-L bioreactor.

The activity of MIOX4 is the rate-limiting step of the glucaric acid pathway because of its low stability (15). To enhance the solubility and stability of MIOX, an artificial SUMO (small ubiquitin-related modifier) tag was linked to the N terminus of MIOX in the recombinant *E. coli* GA-producing strain, increasing glucaric acid production by 1.25-fold compared with wild-type MIOX (12). Recently, it was reported that directed evolution had also been used to investigate highly active mutants of MIOX by high-throughput screening (31, 32). Using a glucaric acid-responsive biosensor, two MIOX mutants, D82Y and S173N, were found to have activities that were 3.8- and 2.7-fold

higher, respectively, than that of wild-type MIOX (31). In this study, MIOX4 and Udh were fused using different peptide linkers and coexpressed to increase the efficiency of the conversion of *myo*-inositol to glucaric acid. The fusion protein MIOX4-Udh, linked by the $(EA_3K)_3$ peptide, produced the highest glucaric acid titer, which was 5.7 times higher than those of the mixed separate enzymes. We also found that the expression of the fusion of MIOX4 and Udh with linker F $[GSG(EA_3K)_2]$ and linker K $[(PT)_7P]$ benefited glucaric acid production. It has been reported that poly(G) and S_3N_{10} linkers both had high cleavage rates (33), and the GGGGS linker could improve the specific activity of MIOX when Udh was fused to the N terminus of MIOX (21). However, we found that both the $(GGGGS)_2$ and S_3N_{10} linkers reduced the enzyme activities of MIOX4 and Udh in this study. It has been demonstrated that the degree of stabilization of the $(PT)_xP$ linker is positively related to its length (33). Therefore, $(PT)_7P$ is the most stable among three the linkers $(PT)_2P$, $(PT)_4P$, and $(PT)_7P$ used in this study, which is consistent with our results. The EA_3K linker is a rigid unit, and more rigid units in $(EA_3K)_n$ could form a more helical conformation, which is usually considered a very stable structure due to the many H bonds (34). Here, we found that the fusion protein MIOX4-Udh linked by $(EA_3K)_3$ significantly increased the level of glucaric acid production, which was consistent with previous results showing that the linker $(EA_3K)_3$ could improve Udh-MIOX activity when Udh was fused to the N terminus of MIOX (21). Our results indicated that a stable peptide linker can elevate the activity of MIOX4 and thus promote glucaric acid production. To further increase the catalytic efficiency, the $(EA_3K)_3$ -linked MIOX4-Udh fusion protein was integrated into multiple delta sequence sites of the *S. cerevisiae opi1* mutant genome. To identify strains producing the highest levels of glucaric acid efficiently, we also developed a high-throughput screening method using the *E. coli* glucaric acid-responsive biosensor constructed in our previous study (35). In addition, further factors that influence MIOX activity should be addressed in future research; i.e., the catalytic activity of MIOX is source dependent (36) and influenced by the dissolved oxygen (DO) concentration in the fermentation medium (37, 38). Other sources of MIOX and the control of DO may further increase MIOX catalytic activity.

The *myo*-inositol precursor supply was enhanced by improving the capacity of yeast cells to both synthesize *myo*-inositol and transport it into the cell from the medium to further increase glucaric acid production. *S. cerevisiae* cells obtain *myo*-inositol from two main sources: the *de novo* conversion of intracellular glucose to *myo*-inositol (16) and the import of extracellular *myo*-inositol by inositol transporters (17, 18). Glucose-6-phosphate is the major precursor for *myo*-inositol biosynthesis, which is first converted to inositol-1-phosphate by Ino1 (inositol phosphate synthase) and then dephosphorylated to *myo*-inositol by inositol monophosphatases (Inm1p and Inm2p) (39). Therefore, glucaric acid production is highly dependent on the availability of glucose-6-phosphate. However, as an intermediate of important primary metabolic pathways, much of the glucose-6-phosphate pool is diverted to the glycolysis, pentose phosphate, and gluconeogenesis pathways (40). Since the transcription of *INO1* is repressed by *myo*-inositol through the Opi1p repressor and the Ino2p and Ino4p activators (41), the *OPI1* gene was deleted in our previous study (20). In this study, to increase the glucose-6-phosphate supply, the *ZWF1* gene, encoding glucose-6-phosphate dehydrogenase, which catalyzes the first step of the pentose phosphate pathway (29, 30), was downregulated. The downregulation of *ZWF1* significantly increased the glucaric acid titer by redirecting the glucose-6-phosphate flux to *myo*-inositol production, increasing glucaric acid production by the GA-Z strain by 22.4% compared with the GA16 strain. To increase the glucose-6-phosphate supply and produce more *myo*-inositol, the *INM1* gene, encoding inositol monophosphatase, was overexpressed in yeast cells, and the glucaric acid titer was increased by 21.7% in the GA-ZI strain compared with the GA-Z strain, suggesting the importance of inositol monophosphatase for promoting *myo*-inositol synthesis and thereby increasing glucaric acid production.

In addition to *de novo myo*-inositol biosynthesis, yeast cells can also absorb *myo*-inositol from the fermentation medium via the inositol transporters Itr1 and Itr2 (17,

18). Since *Itr1* is the major *myo*-inositol transporter and its expression is repressed by high cytosolic *myo*-inositol levels, the *ITR1* gene was overexpressed in the GA-ZI strain. Here, 10.8 g/L *myo*-inositol was added to a shake flask fermentation and a fed-batch fermentation. During shake flask fermentation, about 3.5 g/L *myo*-inositol was consumed by the GA-ZI strain, which was increased to 4.6 g/L in the GA-ZII strain. At least 6.3 g/L *myo*-inositol was required to produce 7.3 g/L glucaric acid by the GA-ZI strain, and 7.3 g/L *myo*-inositol was required to produce 8.5 g/L glucaric acid by the GA-ZII strain in the shake flask fermentation. Here, we suppose that all of the *myo*-inositol taken from the extracellular culture medium was used to produce glucaric acid. The yields of glucaric acid were 1.167 g/g *myo*-inositol and 0.107 g/g glucose for the GA-ZI strain and 1.167 g/g *myo*-inositol and 0.104 g/g glucose for the GA-ZII strain. In fed-batch fermentation, 4.6 g/L *myo*-inositol was consumed by the GA-ZII strain, which was increased to 5.1 g/L by the GA-ZII strain after the addition of 100 mM MgCl_2 to the culture medium. The yields of glucaric acid for the GA-ZII strain were 1.167 g/g *myo*-inositol and 0.173 g/g glucose without 100 mM MgCl_2 and 1.167 g/g *myo*-inositol and 0.241 g/g glucose with 100 mM MgCl_2 .

In addition, *Itr1* and *Itr2* are also regulated at the posttranslational level and are then degraded in the vacuole in response to *myo*-inositol feedback inhibition (42). Future research could involve engineering *Itr1* and/or *Itr2* mutants that can transport *myo*-inositol constitutively, by rational design, or investigating heterologous high-activity *myo*-inositol transporters that could markedly increase glucaric acid production. In addition, *myo*-inositol is a sugar-like carbon source and is essential for both intracellular signal transduction and cellular structure (43, 44). It is important to carefully balance the metabolic flux of *myo*-inositol between primary cellular metabolism and glucaric acid production. Therefore, although a relatively much higher glucaric acid titer was achieved in this study, further research and development have the potential to achieve further increases.

Conclusions. Here, we constructed a glucaric acid-producing strain by the fusional expression of *MIOX4* and *Udh1* and integrated the two genes into multiple delta sequences of the yeast genome to increase their expression levels. To improve the supply of the *myo*-inositol precursor, the strain was metabolically engineered by the downregulation of *ZWF1* and the overexpression of *INM1* and *ITR1*. Finally, the inclusion of MgCl_2 in the fermentation medium and glucose supplementation during fermentation markedly increased glucaric acid production, and the glucaric acid titer was increased to 15.6 g/L from a fed-batch fermentation.

MATERIALS AND METHODS

Strains and growth conditions. Yeast cells were shake flask cultured in YPD medium (20 g/L glucose, 20 g/L tryptone, and 10 g/L yeast extract) and synthetic defined (SD) medium [20 g/L glucose; 1.7 g/L yeast nitrogen base; 5 g/L $(\text{NH}_4)_2\text{SO}_4$; and an amino acid mixture without uracil, leucine, histidine, or methionine added to 1/10 of the volume] at 30°C at 220 rpm. Agar (2%, wt/vol) was added to solid medium when needed. The *E. coli* glucaric acid biosensor was cultured in M9 medium (6.78 g/L Na_2HPO_4 , 3 g/L K_2HPO_4 , 0.5 g/L NaCl, 1 g/L NH_4Cl , 0.24 g/L MgSO_4 , 0.1 mmol/L CaCl_2 , 4 g/L glucose).

For shake flask fermentation, a yeast seed culture was first grown in YPD medium for 16 h at 220 rpm at 30°C, fresh YPD medium (50 mL) containing 10.8 g/L *myo*-inositol at 5% of the total volume was then added, and the mixture was fermented for 10 days at 220 rpm at 30°C. After 24 and 48 h of fermentation, 5 g/L glucose was added. The concentrations of glucose, glucaric acid, *myo*-inositol, and ethanol were measured by high-performance liquid chromatography (HPLC) as described previously (20).

Quantitative real-time PCR analysis of gene expression levels. Strains were first cultured overnight in YPD medium and then inoculated into fresh YPD medium and cultured to an OD_{600} of 1.2 to 1.5. The hot phenol method was used to extract the total RNA (45), and the first-strand cDNA was synthesized using a SuperRT cDNA synthesis kit (Cw Biotech, China) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using SYBR Premix Ex Taq (Cw Biotech) in a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) to determine the relative expression levels of *ZWF*, *ITR1*, and *INM1*, with *PGK1* as the reference gene. The results were calculated by the $2^{-\Delta\Delta\text{CT}}$ method (46) using the primers listed in Table S1 in the supplemental material.

Genetic DNA manipulations and strain construction. To construct the *MIOX*-*Udh* fusion protein, 14 *Udh-T_{CYC1}* fragments with different linkers (linkers C [GSG], D [GSGGGGS], E [GSGEAAAK], F [GSGEAAAKEAAAK], G [GSGSMGSSSN], H [GGGGSGGGGS], I [PTPTP], J [PTPTPTPTP], K [PTPTPTPTPTPTPTP], L [EAAAK], M [EAAAKEAAAK], N [EAAAKEAAKEAAAK], and O [SSSNNNNNNNNNN]) (33, 47–50) at the N terminus were fused to the C terminus of the *P_{TEF1}*-*MIOX4* fragment. First, the *P_{TEF1}*-*MIOX4* fragment was amplified from the

Bga-3 genome using the primer pair MIOX4-LF and MIOX4-LR, and the 14 Udh- T_{CYC1} fragments were amplified from the Bga-3 genome using the primers UDH-F0, UDH-F1, UDH-F2, UDH-F3, UDH-F4, UDH-F5, UDH-F6, UDH-F7, UDH-F8, UDH-F9, UDH-F10, UDH-F11, UDH-F12, UDH-F13, and UDH-R (Fig. S1A). The PCR products of the P_{TEF1} -MIOX4 fragment and the 14 Udh- T_{CYC1} fragments were fused by a sequential PCR method (51) to construct P_{TEF1} -MIOX4-Udh- T_{CYC1} fragments using the primers MIOX4-LF and T_{CYC1} -(opi1)R (Fig. S1B). The HIS3 fragment was then amplified with the primers HIS3-(opi1)F and HIS3-(opi1)R, using the genome of Bga-3 as a template. Finally, the 14 P_{TEF1} -MIOX4-Udh- T_{CYC1} fragments and the HIS3 fragment were integrated into the *OPI1* promoter region of the BY4741 *opi1* Δ mutant strain and screened on SD-His agar plates. To integrate the free enzymes of MIOX4 and Udh into the *OPI1* promoter region of the BY4741 *opi1* Δ mutant, the P_{TEF1} -MIOX4-HIS3-Udh- T_{CYC1} fragment was amplified using primer pair MIOX-TF and T_{CYC1} -(opi1)R (Fig. S1B) and then transformed into the BY4741 *opi1* Δ mutant strain. The positive integrations were confirmed by PCR using primers UDH-check-F and OPI1-check-R (Fig. S1C). All primers used here are listed in Table S1.

To integrate the optimal MIOX4-Udh fusion protein (fused by linker N) into the delta site of the BY4741 *opi1* Δ mutant, the delta1-URA3- P_{TEF1} -MIOX4-Udh- T_{CYC1} -delta2 fragment was fused by a sequential PCR method. First, the delta1 fragment was amplified from the Bga-3 genome using primers delta1-F and T_{ADH1} -R, and the URA3 fragment was amplified from the pRS316 plasmid (52) using primers URA3-F and URA3-R. The delta1 fragment, the URA3 fragment, and the fused MIOX4-Udh fragment were fused by the sequential PCR method using primers delta1-F and UDH-R3 to obtain the delta1-URA3- P_{TEF1} -MIOX4-Udh- T_{CYC1} -delta2 cassette, which was then transformed into the BY4741 *opi1* Δ mutant strain by the lithium acetate (LiAc) method and screened on SD-Ura plates. The positive transformants were identified by a high-throughput screening method using the *E. coli* glucaric acid biosensor constructed in our previous study (35).

To decrease the expression of *ZWF1*, its natural promoter was replaced by P_{ARP10} by integrating the MET- P_{ARP10} fragment into the promoter region of *ZWF1*. To obtain the MET- P_{ARP10} fragment, the MET15 and P_{ARP10} fragments were first amplified from the genomes of CEN.PK2-1C and BY4741 using primer pairs MET-F/MET-R and P_{ARP10} -F/ P_{ARP10} -R, respectively (Fig. S2A). Full-length MET15- P_{ARP10} was amplified by the sequential PCR method using primers MET-F and P_{ARP10} -R (Fig. S2B) and then transformed into the GA16 strain. The correct integrations were confirmed by PCR using primers P_{ARP10} -check-F and ZWF1-check-R (Fig. S2C).

To overexpress *INM1* and *ITR1*, their natural promoters were replaced by P_{TDH3} and P_{ADH1} by integrating the LEU2- P_{TDH3} and HIS3- P_{ADH1} fragments into the promoter regions of *INM1* and *ITR1*, respectively. To construct the LEU2- P_{TDH3} fragment and the HIS3- P_{ADH1} fragment, LEU2 and HIS3 were first amplified from plasmids pHAC181 and pRS313 (53) using primer pairs LEU2-F/R and HIS3-F/R, respectively. The *TDH3* and *ADH1* promoters were amplified from the BY4741 genome using primer pairs P_{TDH3} -F/R and P_{ADH1} -F/R, respectively (Fig. S3A and Fig. S4A). The LEU2- P_{TDH3} and HIS3- P_{ADH1} cassettes were then obtained by fusing LEU2 with P_{TDH3} and by fusing HIS3 with P_{ADH1} by the sequential PCR method using primer pairs LEU2-F/ P_{TDH3} -R and HIS3-F/ P_{ADH1} -R, respectively (Fig. S3B and Fig. S4B). The LEU2- P_{TDH3} cassette was transformed into the GA-Z strain, and the correct integration was confirmed by PCR using primers P_{TDH3} -check-F and INM1-check-R (Fig. S3C). The HIS3- P_{ADH1} cassette was then transformed into the GA-ZI strain to construct the GA-ZII strain. The correct transformation was confirmed by PCR using primers P_{ADH1} -check-F and ITR1-check-R (Fig. S4C).

Microplate screening using the *E. coli* glucaric acid biosensor. The selected transformants were inoculated into YPD medium (2 mL) in 48-well deep microplates with air-permeable lids. The plates were cultured for 12 h at 30°C at 250 rpm to generate the seed cultures, which were then transferred to 24-well deep microplates and incubated for 7 days. After centrifugation at 5,000 rpm for 5 min, the supernatants were retained. To determine GA production using the *E. coli* glucaric acid biosensor (35), *E. coli* BL21(DE3) cells containing plasmid R7M10 were cultured in M9 medium and then incubated with the culture supernatants in 96-well deep microtiter plates (Corning Inc., Corning, NY, USA) to an OD₆₀₀ of 0.6 to 0.8. The fluorescence assays were performed using a plate reader (BioTek, Winooski, VT, USA). The excitation and emission wavelengths were set at 530 nm and 488 nm, respectively. The green fluorescent protein (GFP) fluorescence/OD₆₀₀ ratio was used to calculate the relative glucaric acid concentration in each culture. The selected strains were then cultured in tubes containing YPD medium (10 mL) (plus 60 mmol/L inositol) for fermentation culture for 7 days. Glucose (5 g/L) was added at 24 and 48 h of fermentation.

Fed-batch fermentation and metabolite analysis. For seed preparation, the GA-ZII strain was first grown overnight in YPD medium at 30°C at 220 rpm and then transferred into 2.5 L YPD medium supplemented with 10.8 g/L myo-inositol in a 5-L bioreactor. In a previous study, we found that MgCl₂ could significantly increase glucaric acid production by improving the cell growth rate and the activity of MIOX4 (22). According to the concentration of MgCl₂ used in our previous study, 100 mM MgCl₂ was supplemented when fed-batch fermentation was performed. The fermentation conditions were as follows: a temperature of 30°C, a stirring speed of 600 to 800 rpm, an aeration rate of 2 Vessel Volume per Minute, L/min (vvm), and a dissolved oxygen (DO) level of 100%. During fermentation, 10 g/L glucose was fed into the fermentor at 24 and 48 h. At the end of the fermentation, the medium composition was analyzed as described previously (20).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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