Biofuel Research Journal 48 (2025) 2569-2581

Biofuel Research Journal

Journal homepage: www.biofueljournal.com

Original Research Paper

Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production

Pan Zhu[1,*], Yufei Li[1], Zihan Zhao[2], Xinyi Sun[1]

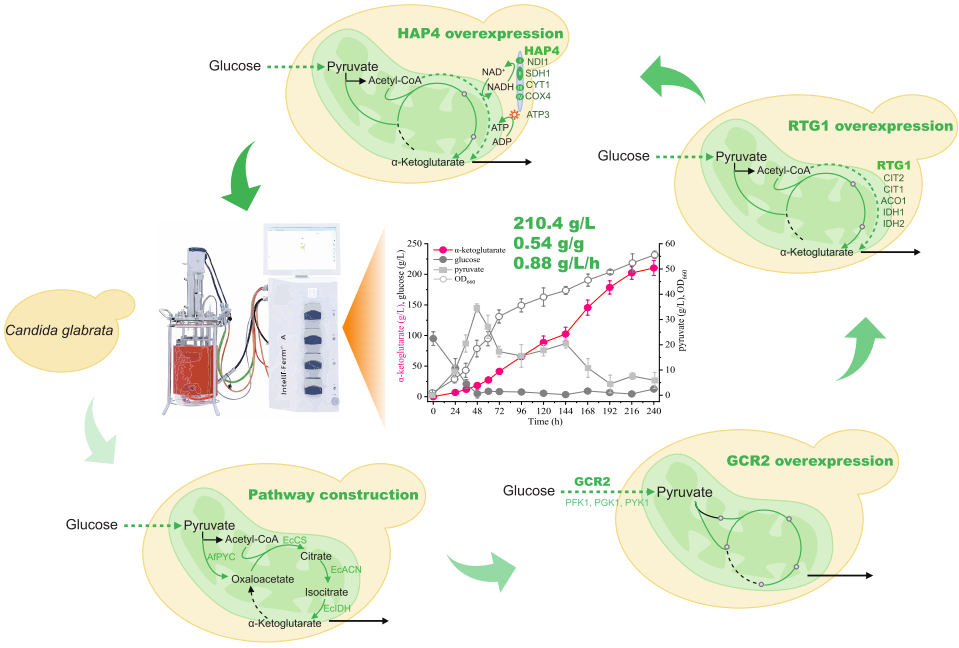
[1]*School of Life Sciences and Health Engineering, Jiangnan University, Wuxi 214122, China.*

[2]*School of Biotechnology and Key Laboratory of Industrial Biotechnology of Ministry of Education, Jiangnan University, Wuxi 214122, China.*

HIGHLIGHTS

- *Candida glabrata* was used as cell factories for efficient production of α-ketoglutarate (AKG).
- Transcription factor engineering was conducted to reconstruct metabolic network for AKG production.
- Transcription factors GCR2 and RTG1 were reinstalled and optimized to redistribute carbon flux.
- Transcription factor HAP4 was utilized to rewire electron transport chain for improving redox balance.
- The engineered strain produced 210.4 g/L AKG with a yield of 0.54 g/g and productivity of 0.88 g/L/h.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 July 2025
Received in revised form 15 November 2025
Accepted 15 November 2025
Published 1 December 2025

Keywords:
Transcription factor engineering
Candida glabrata
α-ketoglutarate
Pyruvate carrier
Pathway optimization
Bioeconomy

ABSTRACT

α-Ketoglutarate, a key intermediate in the TCA cycle, is crucial for amino acid synthesis and nitrogen transport. However, microbial engineering for α-ketoglutarate production is hindered by the intrinsic inefficiency of the metabolic network. In this study, transcription factor engineering was performed for the reconstruction of the metabolic network to boost α-ketoglutarate biosynthesis in *Candida glabrata*. Transcription factors GCR2 and RTG1 were first reinstalled to kick-start the glycolytic pathway and the TCA cycle, respectively, and then optimized to redistribute carbon flux between the two pathways. In addition, pyruvate carriers, MPC1 and MPC2, were introduced to facilitate the transport of cytoplasmic pyruvate to mitochondria, thereby feeding it into the TCA cycle for α-ketoglutarate biosynthesis. Next, transcription factor HAP4 was utilized to rewire the electron transport chain for improving redox balance and reducing overflow metabolism, thereby channeling more carbon flux to α-ketoglutarate production. Finally, the engineered strain *C. glabrata* KGA17 was capable of producing 210.4 g/L α-ketoglutarate in a 5-L bioreactor. This approach showed significant promise for developing efficient microbial cell factories for high-value chemical production.

©2025 Alpha Creation Enterprise CC BY 4.0

* Corresponding author at:
E-mail address: zhupan@jiangnan.edu.cn

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. 10.18331/BRJ2025.12.4.5.

Contents

1. Introduction

α-Ketoglutarate, a crucial intermediate in the tricarboxylic acid (TCA) cycle and a precursor to glutamate or glutamine, plays a pivotal role in balancing carbon and nitrogen metabolism in microorganisms. It has been widely used in the food, pharmaceutical, fine chemistry, and animal feed industries (Finogenova et al., 2005; Otto et al., 2011). Currently, α-ketoglutarate is produced through three different methods: chemical synthesis, enzymatic conversion from glutamate, and microbial fermentation. The predominant industrial method, chemical synthesis, suffers from low yields and the use of toxic chemicals, thereby rendering it economically unviable and limiting its wide application (Stottmeister et al., 2005; Zhou et al., 2012). Enzymatic production, reliant on glutamate, involves costly enzymes and complex processes. These limitations have spurred significant interest in microbial fermentation for α-ketoglutarate production.

Numerous microorganisms have been utilized for α-ketoglutarate production (**Table 1**). Naturally occurring producers such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Bacillus megaterium* have been isolated and used as biocatalysts in microbial fermentation for α-ketoglutarate production. However, their industrial application is significantly limited by low yields or long fermentation times (Zhou et al., 2010). Consequently, the engineered strains, such as *Yarrowia lipolytica* (Zhou et al., 2010) and *Candida glabrata* (Zhang et al., 2009), are employed for α-ketoglutarate production. α-Ketoglutarate production has been enhanced by overexpressing key enzymes in the TCA cycle to increase precursor supply. With glycerol as a carbon source, α-ketoglutarate production with *Y. lipolytica* mutant was increased to 132.7 g/L by

Table 1.
Microbial engineering for α-ketoglutarate production.

Strains	Titer (g/L)	Yield (g/g substrate)	Productivity (g/L/h)	Ref.
Escherichia coli W3110△4-$P_{(H)}$CA$I_{(H)}$A	32.2	0.32 g/g glucose	0.54	Chen et al. (2020)
E. coli THRD△rhtA△ilvIH/pBV220-ilvA	40.8	0.19 g/g glucose	1.46	Zhang et al. (2016)
Corynebacterium glutamicum JH107	51.1	-	0.43	Lee et al. (2013)
Yarrowia lipolytica T1	47.6	0.48 g/g glycerol	0.33	Guo et al. (2015
Y. lipolytica M6	50	0.5 g/g glycerol	0.35	Guo et al. (2016)
Y. lipolytica-ACL	56.5	0.57 g/g glycerol	0.39	Zhou et al. (2012)
Y. lipolytica-RoPYC2	62.5	0.63 g/g glycerol	0.43	Yin et al. (2012)
Y. lipolytica WSH-Z06	66.2	0.66 g/g glycerol	0.35	Yu et al. (2012)
Y. lipolytica H222-MH1	72	0.6 g/g glycerol	0.7	Holz et al. (2011)
Y. lipolytica H355A(FUM1-PYC1)	138	0.51 g/g glycerol	1.44	Otto et al. (2012)
Y. lipolytica VKM Y-2412	172	0.70 g/g ethanol	0.53	Kamzolova et al. (2012)
Y. lipolytica H355A(PYC1-IDP1) T5	186	0.36 g/g glycerol	1.59	Yovkova et al. (2014)
Torulopsis glabrata CCTCC M202019	30	0.3 g/g glucose	0.47	Huang et al. (2006)
T. glabrata PDC1-1	37.7	0.38 g/g glucose	0.52	Zhang et al. (2009)
T. glabrata CCTCC M202019	43.7	0.44 g/g glucose	0.68	Liu et al. (2007)
C. glabrata KGA17	210.4	0.54 g/g glucose	0.88	**This Study**

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

overexpressing fumarase (FUM1) and pyruvate carboxylase (PYC1) (Otto et al., 2012). Metabolic engineering strategies for regulating cofactor supply and regeneration are investigated to improve α-ketoglutarate production. For example, intracellular acetyl-CoA was increased by overexpressing acetyl-CoA synthetase and ATP-citrate lyase, thereby shifting metabolic flux from pyruvate to α-ketoglutarate in *Y. lipolytica*. In a 3-L fermenter, α-ketoglutarate production with *Y. lipolytica*-ACL reached 56.5 g/L with a productivity of 0.39 g/L/h (Zhou et al., 2012). Additionally, α-ketoglutarate production was also increased by engineering pyruvate dehydrogenase E1, E2, and E3 components to supply and regenerate acetyl-CoA in the TCA cycle. Specifically, the elevated expression level of the α-subunit of pyruvate dehydrogenase E1 component resulted in α-ketoglutarate production up to 43.3 g/L with a productivity of 0.30 g/L/h (Guo et al., 2014). With glucose as a carbon source, carbon flux in *C. glabrata* is redirected from pyruvate to α-ketoglutarate by manipulating the activity of the pyruvate dehydrogenase complex, pyruvate carboxylase, pyruvate decarboxylase, and α-ketoglutarate dehydrogenase complex. This strategy achieved an α-ketoglutarate concentration of 37.7 g/L with a productivity of 0.52 g/L/h (Zhang et al., 2009), surpassing the productivity of *Y. lipolytica* and suggesting *C. glabrata* as a potential alternative for metabolic engineering to enhance α-ketoglutarate production. To sum up, these strategies mainly focus on rewiring metabolic pathways to enhance α-ketoglutarate production, but metabolic network is not systematically optimized, thereby limiting the efficiency of α-ketoglutarate production.

Transcription factor engineering is a valuable alternative to control multiple steps in a particular metabolic network. At present, numerous transcription factors have been shown to stimulate the production of a particular metabolite by overexpressing them to regulate the relevant metabolic pathways. Various transcription factors involved in lipid synthesis in animals, plants, and microorganisms have been identified (Courchesne et al., 2009). Zinc-finger protein transcription factors can enhance the production of pharmaceutical proteins by binding to DNA sequences within the promoter regions of therapeutic proteins in mammalian cell lines (Reik et al., 2007). MYB and bHLH transcription factors can significantly improve flavonoid biosynthesis, specifically anthocyanin and seed coat tannin, when overexpressed in *Arabidopsis thaliana* (Vom Endt et al., 2002). ORCA2 protein can boost alkaloid production in plants by upregulating multiple genes in the biosynthesis pathway of terpenoid indole alkaloids (TIA), thereby increasing TIA formation (Vom Endt et al., 2002). However, these strategies have not been widely applied in engineering yeasts for the biosynthesis of chemicals.

In this study, transcription factor engineering was used for the reconstruction of the metabolic network to enhance α-ketoglutarate biosynthesis in *C. glabrata* (**Fig. 1**). Transcription factors GCR2, RTG1,

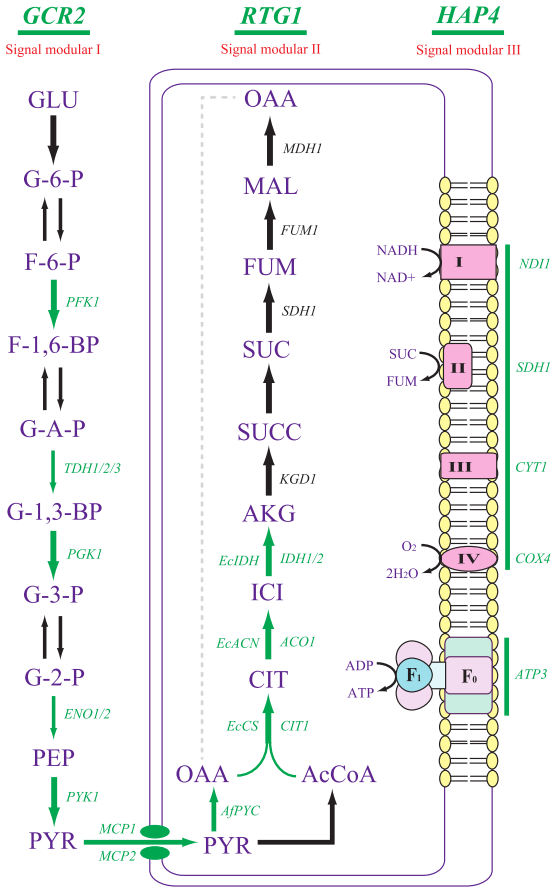


Fig. 1. Transcription factor engineering of *Candida glabrata* for α-ketoglutarate production.
Abbreviations: GLU: glucose; G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F-1,6-BP: fructose-1,6-bisphosphate; G-A-P: glyceraldehyde-3-phosphate; G-1,3-BP: glycerate-1,3-bisphosphate; G-3-P: glycerate-3-phosphate; G-2-P: glycerate-2-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; OAA: oxaloacetate; AcCoA: acetyl coenzyme A; CIT: citrate; ICT: isocitrate; AKG: α-ketoglutarate; SUCC: succinyl coenzyme A; SUC: succinate; FUM: fumarate; MAL: malate; PFK1: phosphofructokinase α subunit; TDH1/2/3: glyceraldehyde-3-phosphate dehydrogenase isozyme1/2/3; PGK1: phosphoglycerate kinase; ENO1/2: enolase 1/2; PYK1: pyruvate kinase 1; MPC1/2: pyruvate carrier subunits from *Saccharomyces cerevisiae*; AfPYC: pyruvate carboxylase from *Aspergillus flavus*; EcCS: citrate synthase from *E.coli*; EcACN: aconitase from *E.coli*; EcIDH: isocitrate dehydrogenase from *E.coli*; CIT1: mitochondrial citrate synthase; ACO1: mitochondrial aconitase; IDH1/2: isocitrate dehydrogenase subunit 1/2; KGD1: α-ketoglutarate dehydrogenase E1; SDH1: succinate dehydrogenase flavoprotein subunit; FUM1: mitochondrial fumarate hydratase; MDH1: mitochondrial malate dehydrogenase; NDI1: NADH:ubiquinone oxidoreductase; CYT1: cytochrome c1: COX4: cytochrome c oxidase subunit 4; ATP3: F_1F_0-ATP synthase γ subunit.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

and HAP4 were simultaneously reinstalled to activate the glycolytic pathway, the TCA cycle, and the electron transport chain, respectively. To increase mitochondrial pyruvate availability, pyruvate carriers, MPC1 and MPC2, were introduced to transport cytoplasmic pyruvate to mitochondria for α-ketoglutarate production using the TCA cycle. Further, GCR2 and RTG1 expression was optimized to redistribute carbon flux between the glycolytic pathway and the TCA cycle. These strategies systematically upgraded the metabolic network, enabling the final engineered strain *C. glabrata* KGA17 to produce 210.4 g/L α-ketoglutarate in a 5-L bioreactor.

2. Materials and Methods

2.1 Strains and plasmids

C. glabrata ATCC 55 was used as a host strain and modified to produce α-ketoglutarate. *E. coli* JM109 and shuttle plasmids pY13 and pY26 were used for plasmid construction. All strains and plasmids used in this study are listed in **Table S1** in **Supplementary Information**.

2.2 DNA manipulation

Plasmid construction was performed using Gibson assembly according to the protocol of 2X MultiF Seamless Assembly Mix (ABclonal). Transcription factors *GCR2* (CAGL0G05467g), *RTG1* (CAGL0C05335g), and *HAP4* (CAGL0K08624g) were amplified by PCR using the chromosomal DNA of C. *glabrata* ATCC 55 as a template. Pyruvate carrier subunits, *MPC1* (YGL080W) and *MPC2* (YHR162W), were amplified from the genomic DNA of *Saccharomyces cerevisiae*. The pyruvate carboxylase (AfPYC, AFLA_112120) gene was amplified by PCR from the complementary DNA (cDNA) of *Aspergillus flavus* ATCC13697. Citrate synthase (EcCS, b0720), aconitase (EcACN, b0118), and isocitrate dehydrogenase (EcIDH, b1136) genes were amplified by PCR from the chromosomal DNA of *E. coli* W3110. AfPYC, EcCS, EcACN, and EcIDH were targeted into mitochondria using the N-terminal mitochondrial localization signal (CoxIV) (Li et al., 2015). These genes were inserted into plasmids pY13 or pY26 to construct gene expression cassettes, which were then PCR-amplified for chromosomal integration. To construct recombinant C. *glabrata* strains, chromosomal integration was conducted using the CRISPR-Cas9 system (Enkler et al., 2016). The upstream and downstream homologous arms, gene expression cassettes, and Cas9-sgRNA plasmid were co-transformed into C. *glabrata* with electroporation (Zhou et al., 2009), and plated on SD/-Ura solid medium. Transformants were verified by colony PCR and gene sequencing.

2.3 Medium

LB medium was used to cultivate *E. coli* strains: 5 g/L yeast extract, 10 g/L peptone, and 5 g/L NaCl. Ampicillin (100 mg/mL) was added as required. SD/-Ura medium (synthetic dextrose medium without uracil) was used for yeast selection, which was purchased from WEIDI (WEIDI, China). YPD medium was used to cultivate yeast seed: 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. KAF medium (α-ketoglutarate fermentation medium in shake flasks) was used for yeast fermentation in shake flasks: 80 g/L glucose, 20 g/L high-fructose corn syrup, 10 g/L peptone, 5 g/L KH_2PO_4, 0.8 g/L $MgSO_4·7H_2O$, 6.6 g/L K_2SO_4, 3 g/L sodium acetate, 12 mg/L thiamine-HCl, 30 mg/L biotin, 0.4 mg/L pyridoxine-HCl, and 8 mg/L nicotinic acid. High-fructose corn syrup (F42) was purchased from B.L.B. (Baolingbao, China). $CaCO_3$ was sterilized by dry-heating sterilization and then used as a pH buffer.

KAFF medium (α-ketoglutarate fermentation medium in bioreactors) was used for yeast fermentation in a 5-L bioreactor: 80 g/L glucose, 20 g/L high-fructose corn syrup, 10 g/L peptone, 5 g/L KH_2PO_4, 0.8 g/L $MgSO_4·7H_2O$, 6.6 g/L K_2SO_4, 3 g/L sodium acetate, 12 mg/L thiamine-HCl, 30 mg/L biotin, 0.4 mg/L pyridoxine-HCl, and 8 mg/L nicotinic acid. When glucose in the fermentation medium KAFF was exhausted, 800 g/L glucose was fed to maintain glucose concentration at 0-10 g/L.

2.4 Culture conditions

For shake flask fermentation, a yeast colony was inoculated into 250 mL shake flasks containing 25 mL YPD medium and incubated at 30°C and 200 rpm for 24 h. The broth was centrifuged, the supernatant liquid was removed, and the pellet was resuspended in KAF medium. The cell suspension was divided equally among 500 mL shake flasks containing 50 mL fresh KAF medium with an initial biomass OD_{660} of 1.0. Fermentation for α-ketoglutarate production was then performed at 200 rpm and 30°C for 72 h.

For fed-batch fermentation, seed cultures were prepared by cultivating a single colony in 250 mL shake flasks with 25 mL YPD medium at 30ºC and 200 rpm for 24 h. Then, 1.0 mL seed cultures were transferred into 500 mL shake flasks containing 50 mL fresh KAFF medium and incubated under the same conditions for 24 h. Finally, seed cultures were incubated in a 5-L bioreactor (Intelli-Ferm A, China) with 1.5 L KAFF medium with an initial biomass OD_{660} of 1.0. Fermentation was conducted at 30°C for 10 d with agitation at 400 rpm and an aeration rate of 2.0 vvm. The culture pH was maintained at 5.5 using 4 M NaOH.

2.5 ARTP mutagenesis

Seed cultures were incubated in YPD medium at 30°C for 12 h, and then washed with PBS buffer (100 mM, pH 7.2) three times. After that, the cell suspension was diluted to an OD_{660} of 3.0 with sterile saline. Cell suspension (10 μL) was spread onto the sterilized metal slides for Atmospheric and Room Temperature Plasma (ARTP) mutagenesis (TMAXTREE, China) (Zhu et al., 2024).

For the primary screen, 200 α-ketoglutarate high-producing candidate strains were selected from the mutant library and incubated in tubes with fresh KAF medium. These cultures were fermented at 30ºC and 200 rpm for 72 h, and their α-ketoglutarate titer was quantified. Candidate strains with α-ketoglutarate production surpassing that of the original strain were selected for secondary screening. For the secondary screen, seed cultures of the selected strains were incubated in 500 mL shake flasks with 50 mL fresh KAF medium, and fermented at 30ºC and 200 rpm for 72 h. After that, α-ketoglutarate production of each strain was measured, and the best-performing strain was ultimately selected and obtained.

2.6 Analytical methods

The optical density at 660 nm (OD_{660}) was assayed by a spectrophotometer. The concentrations of glucose, ethanol, glycerol, pyruvate, and α-ketoglutarate were determined by high-performance liquid chromatography (HPLC) (Xu et al., 2012). Pyruvate transportation was analyzed with the isolated mitochondria using the inhibitor-stop method (Herzig et al., 2012; Li et al., 2015), and the uptake rate of pyruvate was calculated during the first 20 min of the reaction.

2.7 Intracellular NADH and NAD^+ determination

Intracellular NADH and NAD^+ were extracted by freezing-thawing in methanol (Qin et al., 2011). NADH and NAD^+ concentrations were determined by the Enhanced NAD^+/NADH Assay Kit with WST-8 (Beyotime, China).

2.8 Transcriptional analysis

The total RNA was isolated using the Yeast Total RNA Isolation Kit (Sangon Biotech, China). Reverse transcription was conducted according to the protocol accompanying the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara). Real-time quantitative PCR was performed as described in previous reports (Chen et al., 2013). The β-ACTIN gene was used as the internal control.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

2.9 Enzyme activity assay

Cell extracts were prepared for the determination of enzyme activity (Liu et al., 2004). Enzyme activities in glycolysis, such as phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerate kinase (PGK), enolase (ENO), and pyruvate kinase (PYK), were assayed as described in previous reports (Hauf et al., 2000). Enzyme activities in the TCA cycle, such as citrate synthase (CIT) and isocitrate dehydrogenase (IDH), were measured as described previously (Sugden and Newsholme, 1975). In addition, α-ketoglutarate dehydrogenase complex (KGD) activity was determined by measurement of the absorbance at 340 nm (A_{340}) as described in previous reports (Zhang et al., 2009). Malate dehydrogenase (MDH) activity was determined by measuring A_{340} to monitor NADH oxidation as described previously (Xu et al., 2012).

2.10 Protein content assay

To quantify protein contents, 1 mL of cell culture underwent two washes with PBS buffer. Then, the samples underwent centrifugation at 5000 rpm for 2 min to remove culture medium. Next, 10 mg of yeast cells were weighed, suspended in 1 mL of PBS, and disrupted using an ultrasonic cell disintegrator for 15 min. Finally, the resulting supernatant was obtained by centrifuging at 12,000 rpm for 5 min, after which protein contents were assessed by a BCA Protein Assay Kit.

2.11 Metabolic activity assay

Metabolic activity was analyzed by taking 1 mL of cell culture, washing it twice with PBS buffer, and then resuspending it to an OD_{660} of 10 in 1 mL of PBS. Subsequently, a 10% (v/v) resazurin solution (1 g/L) was added to the suspension, followed by an incubation period of 2 h at 37°C in the absence of light with agitation. Following the incubation, the fluorescence intensity was determined using a SpectraMax M3 spectrofluorometer with excitation at 560 nm and emission at 590 nm.

3. Results and Discussion

3.1 Pathway construction for α-ketoglutarate production

The TCA cycle is the primary pathway for α-ketoglutarate biosynthesis in *C. glabrata*, which was catalyzed by pyruvate carboxylase (PYC), citrate synthase (CIT), aconitase (ACO), and isocitrate dehydrogenase (IDH). To enhance this pathway, PYC from *A. flavus* (AfPYC), along with CIT (EcCS), ACO (EcACN), and IDH (EcIDH) from *E. coli*, were introduced into *C. glabrata* (**Fig. 2a**). Overexpressing these four enzymes increased the specific activities of AfPYC, EcCS, EcACN, and EcIDH by 191.7%, 105.9%, 117.8% and 32.6%, respectively, compared to *C. glabrata* KGA00 (**Fig. 2b**). Consequently, the engineered strain *C. glabrata* KGA04 produced 6.6 g/L α-ketoglutarate, which was 106.3% higher than *C. glabrata* KGA00 (**Fig. 2c**, **Table S2**). Additionally, pyruvate and OD were

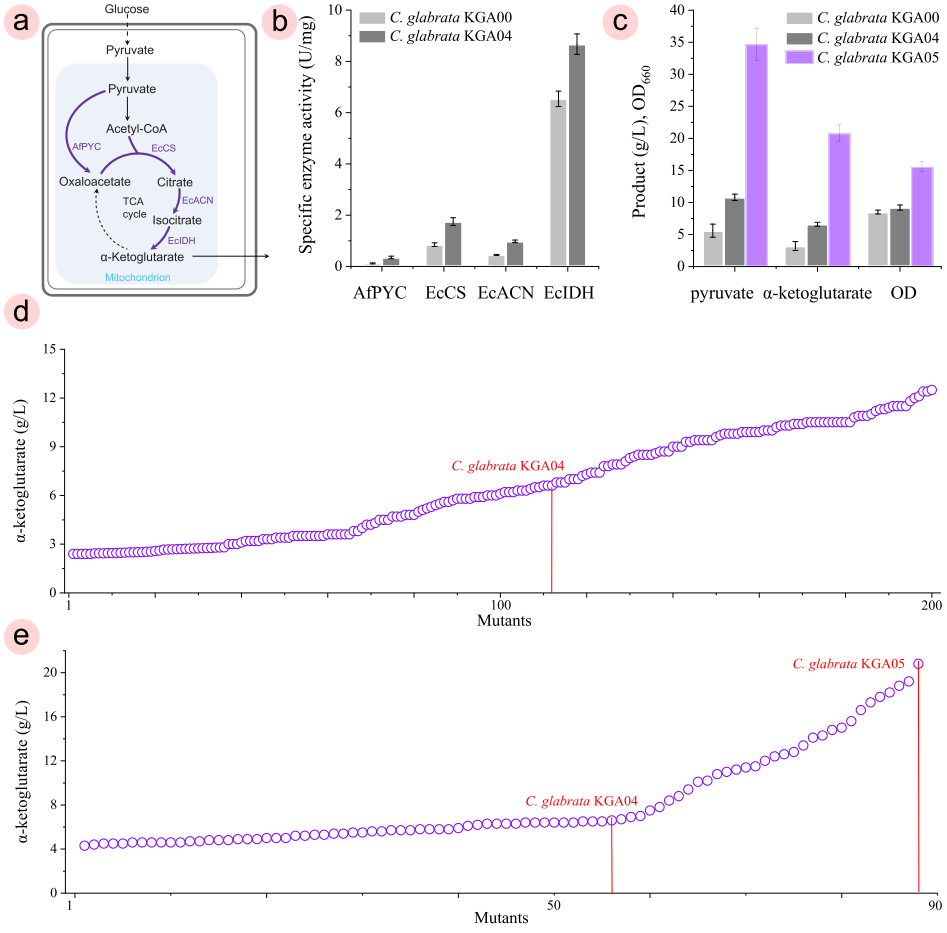


Fig. 2. Pathway construction and ARTP mutagenesis for α-ketoglutarate production. **a:** Schematic representation of α-ketoglutarate biosynthesis through the TCA pathway. **b:** The specific activities of AfPYC, EcCS, EcACN, and EcIDH. **c:** Effect of gene expression and ARTP mutagenesis on the production of pyruvate, α-ketoglutarate, and OD. **d:** Effect of ARTP mutagenesis on α-ketoglutarate production through primary screen. **e:** Effect of ARTP mutagenesis on α-ketoglutarate production through secondary screen.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

increased by 92.9% and 8.2%, respectively (Fig. 2c). These results confirmed the successful enhancement of the TCA cycle for α-ketoglutarate production.

To further enhance α-ketoglutarate production, *C. glabrata* KGA04 underwent ARTP mutagenesis to generate high-producing mutants. From the primary screen, 88 *C. glabrata* mutants were identified with superior α-ketoglutarate production compared to *C. glabrata* KGA04 (Fig. 2d). Secondary screen of these 88 positive mutants revealed 32 *C. glabrata* mutants with α-ketoglutarate production surpassing *C. glabrata* KGA04 (Fig. 2e). The best performing strain, *C. glabrata* KGA05, showed α-ketoglutarate production up to 20.8 g/L, which was 215.2% higher than *C. glabrata* KGA04 (Fig. 2c, Table S2). In addition, pyruvate and OD were increased by 221.3% and 69.6%, respectively (Fig. 2c). These findings showed that ARTP mutagenesis was efficient for enhancing α-ketoglutarate production.

3.2 GCR2 overexpression improves carbon flux in glycolysis

To search for the transcriptional activator of glycolysis, the genome-scale transcriptional regulatory network (TRN) of *C. glabrata* was used, which was reconstructed based on genomic and transcriptomic data (Xu and Liu, 2019). The results showed that glycolysis was regulated by GCR2, GCR1, AZF1, TYE7, and CAT8 (Xu and Liu, 2019) (Fig. 3a). GCR1 and GCR2 are positive regulatory transcription factors required for the optimal expression of many glycolytic genes in yeasts (Clifton and Fraenkel, 1981). GCR1 has specific DNA-binding activity for an essential element in glycolytic gene promoters, and GCR1 and GCR2 function as a transcriptional activation complex. GCR1 can provide the specific DNA-binding function, and GCR2 can offer the activation function (Uemura and Jigami, 1992; Nishi et al., 1995). Thus, GCR2 was selected for activating glycolysis.

To investigate the relation between the *GCR2* gene and gene expression in glycolysis, we analyzed transcriptional levels and enzyme activity of eight genes in glycolysis: *PFK1*, *TDH1*, *TDH2*, *TDH3*, *PGK1*, *ENO1*, *ENO2*, and *PYK1*. By overexpressing GCR2, the transcriptional levels of *PFK1*, *TDH1*, *TDH2*, *TDH3*, *PGK1*, *ENO1*, *ENO2*, and *PYK1* genes in strain *C. glabrata* KGA06 were all upregulated compared with *C. glabrata* KGA05 (Fig. 3b). Among them, *PFK1*, *PGK1* and *PYK1* genes showed a 3.33-, 5.88-, and 9.2-fold increase in its transcriptional levels, respectively (Fig. 3b). In addition, GCR2 overexpression resulted in a 54.5%, 83.0%, and 121.8% increase in the specific activity of PFK, PGK, and PYK compared to *C. glabrata* KGA05, respectively (Fig. 3c). These results indicated that gene expression in glycolysis showed a dramatic dependence

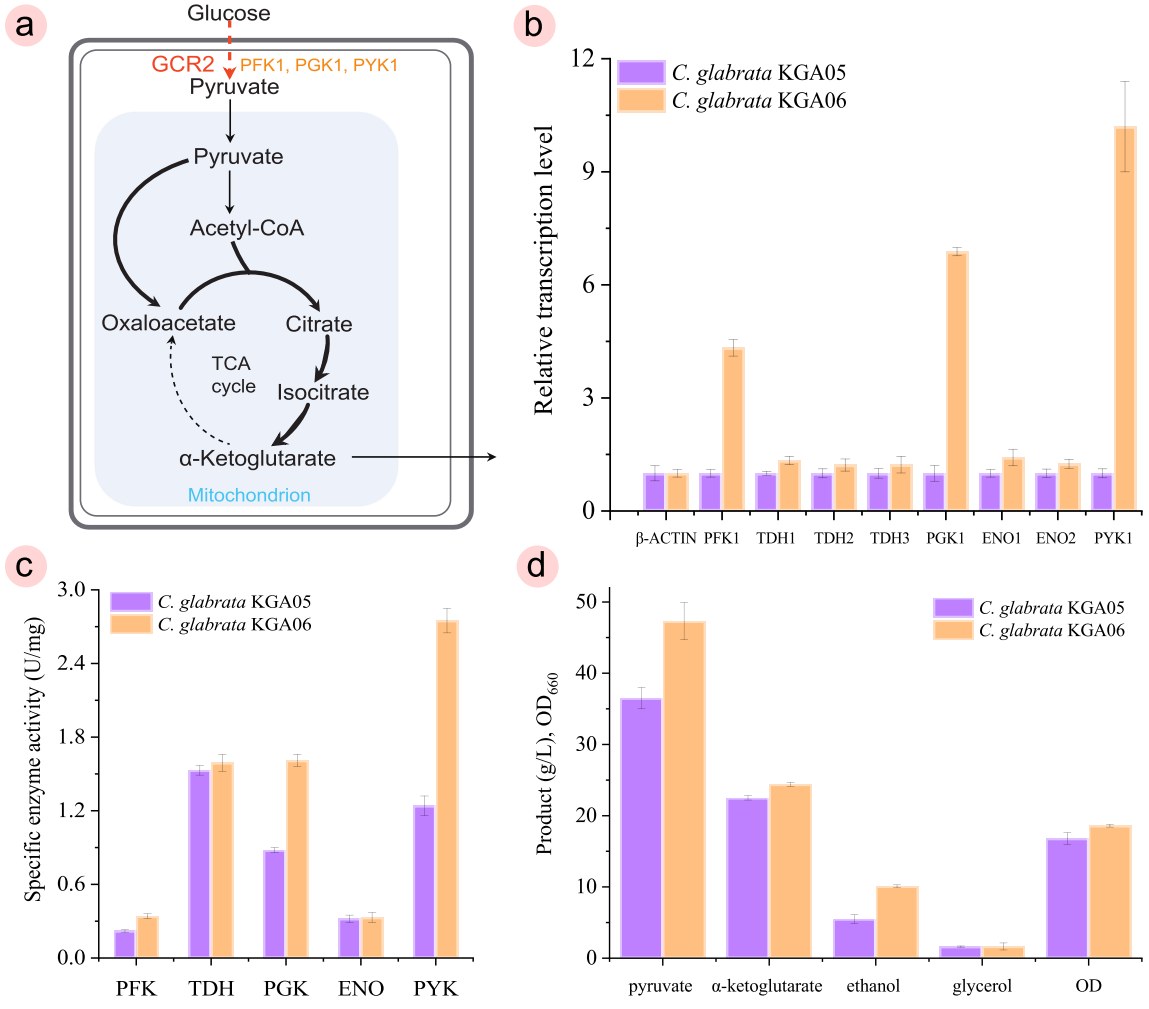


Fig. 3. GCR2 overexpression for improving carbon flux in glycolysis. **a:** Schematic representation of α-ketoglutarate biosynthesis through improving carbon flux in glycolysis. **b:** The relative transcription level of genes *PFK1*, *TDH1*, *TDH2*, *TDH3*, *PGK1*, *ENO1*, *ENO2*, and *PYK1*. **c:** The specific activities of PFK, TDH, PGK, ENO, and PYK. **d:** Effect of GCR2 overexpression on the production of pyruvate, α-ketoglutarate, ethanol, glycerol, and OD.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

on GCR2 gene, suggesting that this modification in GCR2 was beneficial to increase the glycolytic flux in *C. glabrata*.

GCR2 overexpression caused an increase in gene transcriptional level and enzyme activity in glycolysis, thereby increasing the production of pyruvate and α-ketoglutarate. The final concentrations of pyruvate (47.3 g/L) and α-ketoglutarate (24.4 g/L) with *C. glabrata* KGA06 were increased by 29.6% and 8.4% compared to *C. glabrata* KGA05, respectively (**Fig. 3d**, **Table S2**). In addition, GCR2 overexpression improved cell growth, and the OD of *C. glabrata* KGA06 was increased by 10.7% compared to *C. glabrata* KGA05 (**Fig. 3d**). Overexpression of *GCR2* also resulted in an 83.6% increase in ethanol production, while the concentration of glycerol in *C. glabrata* KGA06 was similar to *C. glabrata* KGA05 (**Fig. 3d**). These results suggested that the efficiency of transporting pyruvate into mitochondria should be improved to reduce ethanol and increase α-ketoglutarate production.

3.3 MCP1/2 overexpression driving pyruvate transportation

In *S.* cerevisiae, pyruvate carriers, MPC1 and MPC2, are crucial for transporting pyruvate across mitochondrial membranes (Bricker et al., 2012; Herzig et al., 2012). To explore this function, the *MPC1* and *MPC2* genes from *S. cerevisiae* were simultaneously overexpressed in *C. glabrata* KGA06, resulting in *C. glabrata* KGA07 (**Fig. 4a**). This modification increased the uptake rate of pyruvate to 9.5 pmol/min in *C. glabrata* KGA07, which was 69.6% higher than in *C. glabrata* KGA06 (**Fig. 4b**). Correspondingly, the OD of *C. glabrata* KGA07 was slightly increased from 18.6 to 20.1 g/L, and the production of α-ketoglutarate was increased to 28.1 g/L, which was 15.2% higher than *C. glabrata* KGA06 (**Fig. 4c**, **Table S2**). Additionally, ethanol and glycerol production were reduced to 7.5 and 1.55 g/L, exhibiting a 25.7% and 6.1% decrease, respectively (**Fig. 4c**). These results indicated that MPC1 and MPC2 overexpression increased mitochondrial pyruvate availability, which was conducive to enhancing α-ketoglutarate production through the TCA cycle.

3.4 RTG1 overexpression enhances carbon flux in the TCA cycle

To search for the transcriptional activator of the TCA cycle, the genome-scale TRN of *C. glabrata* was used; the findings revealed that the TCA cycle was regulated by RTG1, RTG3, AZF1, STB5, and SIP4 (Xu and Liu, 2019) (**Fig. 5a**). The expression of *RTG* genes (*RTG1*, *RTG2*, and *RTG3*) can influence in the expression of a subset of nuclear genes, such as *CIT2* gene in the glyoxylate cycle, in response to changes in the functional state of mitochondria. As basic helix-loop-helix/leucine zipper (bHLH/Zip) transcription factors, RTG1 and RTG3 can bind as a heterodimer to activate transcription at the novel DNA target site GTCAC (Jia et al., 1997). RTG2 can encode a cytoplasmic protein with an amino-terminal ATP-binding domain similar to the hsp70/actin/sugar kinase superfamily of ATP-binding proteins (Bork et al., 1992). Thus, RTG1 was selected for activating the TCA cycle.

To investigate the relation between the *RTG1* gene and gene expression in the TCA cycle, we analyzed transcriptional levels and enzyme activity of nine genes in the TCA cycle: *CIT2*, *CIT1*, *ACO1*, *IDH1*, *IDH2*, *KGD1*, *SDH1*, *FUM1*, and *MDH1*. By overexpressing *RTG1*, the transcriptional levels of *CIT2*, *CIT1*, *ACO1*, *IDH1*, and *IDH2* genes in *C. glabrata* KGA08 showed a 4.56-, 2.42-, 1.35, 6.88- and 7.92-fold increase compared with *C. glabrata* KGA07, respectively (**Fig. 5b**). However, the transcription of the remaining TCA cycle genes *KGD1*, *SDH1*, *FUM1*, and *MDH1* was essentially unaffected by *RTG1* overexpression (**Fig. 5b**). In addition, *RTG1* overexpression resulted in a 151.8% and 42.5% increase in the specific activity of CIT and IDH compared with *C. glabrata* KGA07, respectively (**Fig. 5c**). KGD and MDH activity in *C. glabrata* KGA08 were almost concordant with *C. glabrata* KGA07 (**Fig. 5c**). These results indicated that gene expression in the TCA cycle showed a dramatic dependence on RTG1 gene, suggesting that this modification in RTG1 could probably improve carbon flux in the TCA cycle.

RTG1 overexpression channeled more carbon flux from pyruvate to α-ketoglutarate production. The increase in expression levels of *CIT2*, *CIT1*, *ACO1*, *IDH1*, and *IDH2* genes in *C. glabrata* KGA08 was associated with a large decrease in pyruvate accumulation and a significant increase in α-ketoglutarate production. Pyruvate concentration was decreased from 40.4 g/L to 6.3 g/L, but α-ketoglutarate concentration was increased from 28.1 g/L to 38.4 g/L (**Fig. 5d**, **Table S2**). These results indicated that, by overexpression of *RTG1*, pyruvate flux traveled through the TCA cycle and was then effectively redirected to α-ketoglutarate. In addition, this modification in *RTG1* improved cell growth, and the OD of *C. glabrata* KGA08 was increased by 32.3% compared with *C. glabrata* KGA07 (**Fig. 5d**). Overexpression of *RTG1* also resulted in a 120.0% increase in ethanol titer, while the concentration of glycerol was similar to *C. glabrata* KGA07 (**Fig. 5d**). These results suggested that further stepwise improvement should be made to channel more carbon flux to α-ketoglutarate.

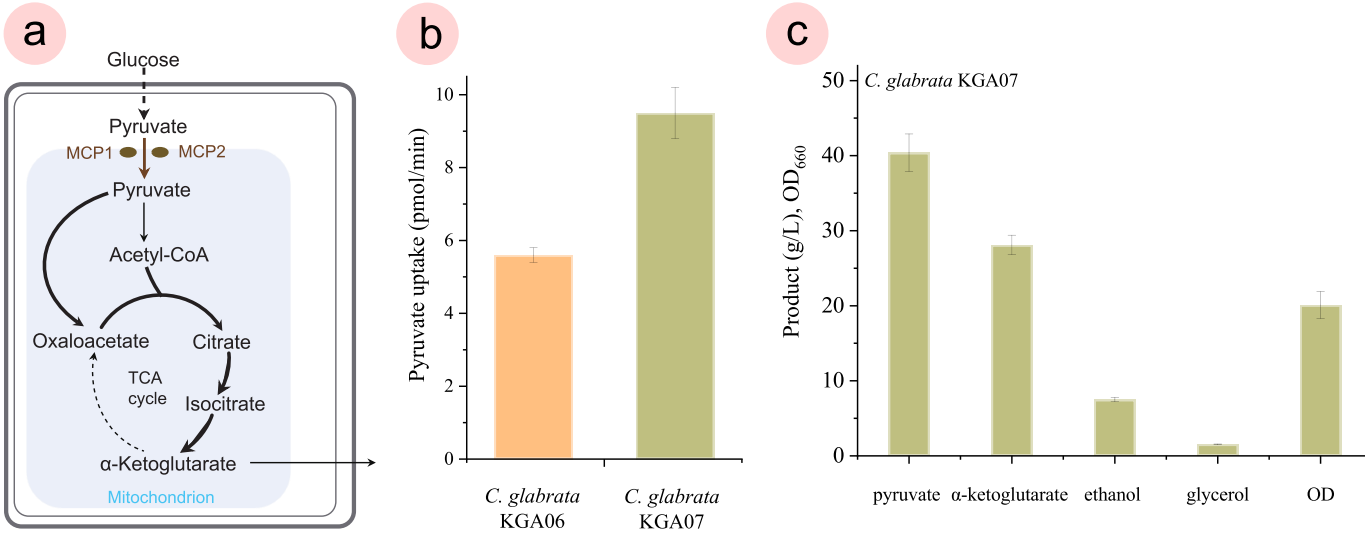


Fig. 4. MCP1/2 overexpression for driving pyruvate transportation. **a:** Schematic representation of pyruvate transportation from the cytosol to the mitochondria. **b:** Effect of MCP1/2 overexpression on pyruvate uptake. **c:** Effect of MCP1/2 overexpression on the production of pyruvate, α-ketoglutarate, ethanol, glycerol, and OD.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

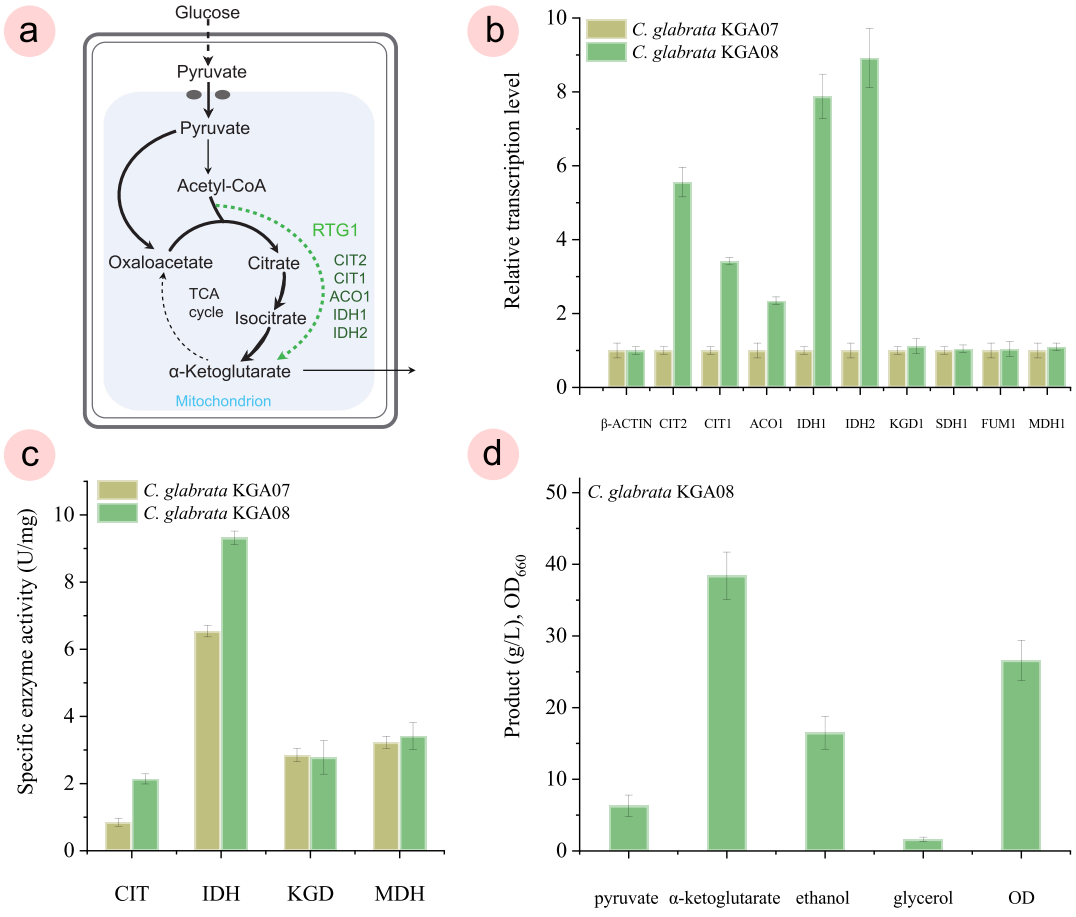


Fig. 5. RTG1 overexpression for enhancing carbon flux in the TCA cycle. **a:** Schematic representation of α-ketoglutarate biosynthesis through enhancing carbon flux in the TCA cycle. **b:** The relative transcription level of genes *CIT2*, *CIT1*, *ACO1*, *IDH1*, *IDH2*, *KGD1*, *SDH1*, *FUM1*, and *MDH1*. **c:** The specific activities of CIT, IDH, KGD, and MDH. **d:** Effect of RTG1 overexpression on the production of pyruvate, α-ketoglutarate, ethanol, glycerol, and OD.

3.5 GCR2 and RTG1 optimization increasing α-ketoglutarate production

To optimize the α-ketoglutarate synthesis pathway, *GCR2* and *RTG1* were overexpressed in *C. glabrata* at different expression levels. Gene expression strengths were categorized into three levels by adjusting gene copy numbers (**Fig. 6a**): high level (three copies, H), medium level (two copies, M), and low level (one copy, L). Accordingly, GCR2 and RTG1 expression cassettes with different strengths were designed and assembled in *C. glabrata* to obtain the best metabolic distribution for enhancing α-ketoglutarate production. When the strengths of *GCR2* and *RTG1* were respectively maintained at medium and high levels in *C. glabrata* KGA13, the concentration of α-ketoglutarate (48.5 g/L) was increased by 26.3% compared to *C. glabrata* KGA08 (**Fig. 6b**, **Table S2**). In addition, the OD of *C. glabrata* KGA13 corresponded to a 14.7% increase compared to *C. glabrata* KGA08 (**Fig. 6c**). However, the concentrations of pyruvate, ethanol, and glycerol were similar to *C. glabrata* KGA08 (**Fig. 6c**). These results were possibly due to the enhanced activity of glycolysis and the TCA cycle for balancing NADH surplus through ethanol and glycerol production.

3.6 HAP4 overexpression promotes the electron transport chain

To search for the transcriptional activator of the electron transport chain, the genome-scale TRN of *C. glabrata* was used; the findings revealed that the electron transport chain was regulated by HAP2/3/4 (Xu and Liu, 2019) (**Fig. 7a**). As a transcriptional activator complex, HAP2/3/4 complex can facilitate the expression of electron transport chain genes through interacting with a CCAAT sequence within the UAS2UP1 element (Olesen and Guarente, 1990). HAP2 and HAP3 are primarily responsible for site-specific DNA binding, while HAP4 provides the main transcriptional activation domain (Olesen and Guarente, 1990). Thus, HAP4 was selected for activating the electron transport chain.

Increasing NADH oxidation is an effective approach to reducing ethanol and glycerol production by enhancing the electron transport chain *via* a transcription factor. To put this approach into practice, intracellular NADH, NAD^+, and NADH/NAD^+ ratio were first determined for strains *C. glabrata* KGA05 and *C. glabrata* KGA13. The oxidoreduction level in strain *C. glabrata* KGA13 revealed a higher level of NADH/NAD^+ ratio (**Fig. 7c**), indicating that the excess NADH could not be oxidized during overflow metabolism. Thus, regulation of NADH level may be an effective approach to further improve α-ketoglutarate production. Then, to investigate the relation between the *HAP4* gene and gene expression in the electron transport chain, we analyzed the transcriptional level of five genes in the electron transport chain: *NDI1*, *SDH1*, *CYT1*, *COX4*, and *ATP3*. By overexpressing *HAP4*, the transcriptional levels of *NDI1*, *SDH1*, *CYT1*, *COX4*, and *ATP3* genes in *C. glabrata* KGA17 were all significantly upregulated compared with *C. glabrata* KGA13 (**Fig. 7b**). These results indicated that gene expression in the electron transport chain showed a dramatic dependence on the *HAP4* gene, suggesting that this modification in *HAP4* could probably improve NADH oxidation.

HAP4 overexpression could facilitate NADH oxidation through the electron transport chain to enhance α-ketoglutarate production. The increase

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

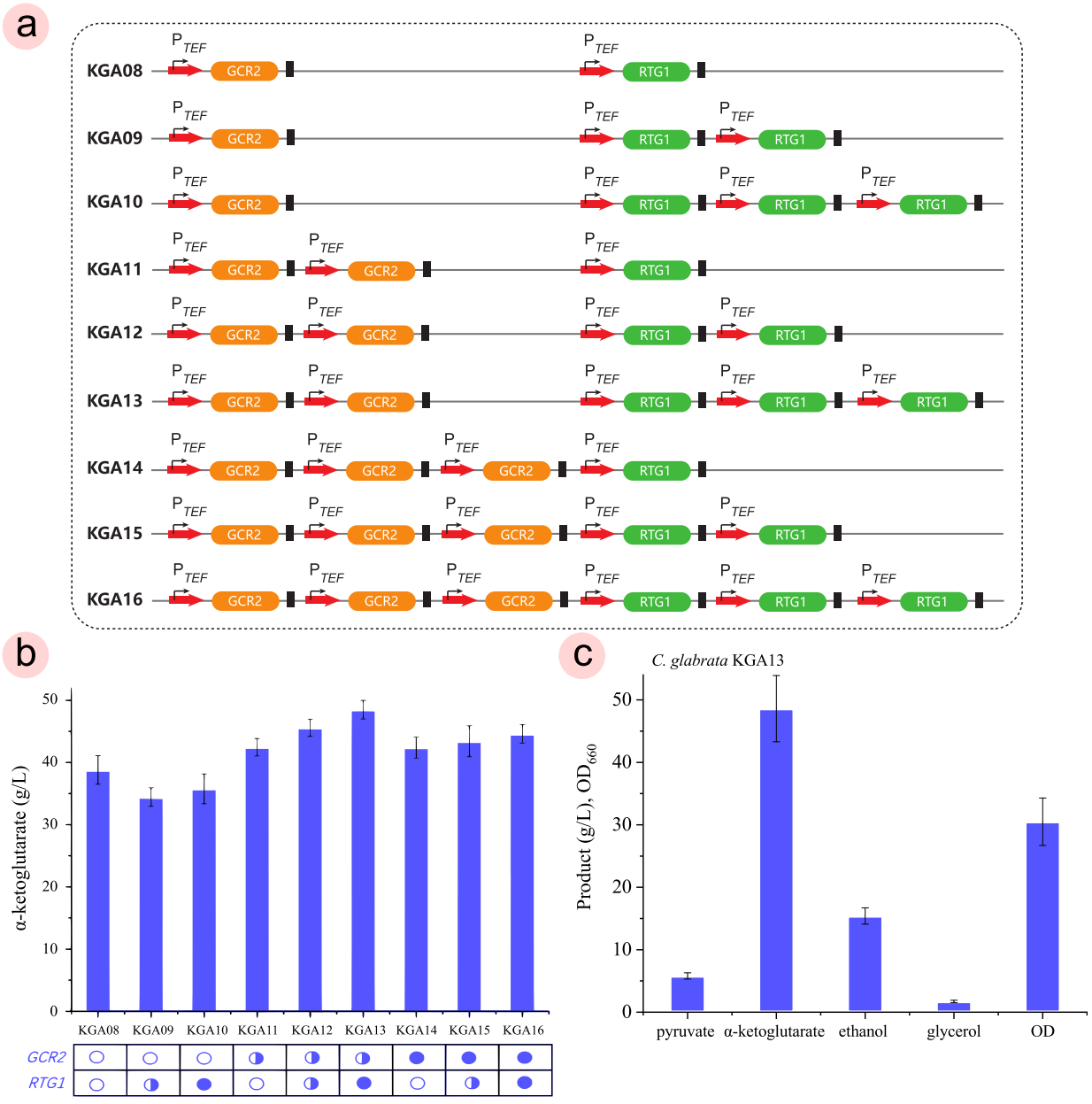


Fig. 6. GCR2 and RTG1 optimization for increasing α-ketoglutarate production. **a:** A series of GCR2 and RTG1 expression cassettes designed at different expression levels. **b:** The concentrations of α-ketoglutarate achieved by different GCR2 and RTG1 expression cassettes. **c:** *C. glabrata* KGA13 (two copies of *GCR2* and three copies of *RTG1*) for the production of pyruvate, α-ketoglutarate, ethanol, glycerol, and OD.

in the expression levels of *NDI1*, *SDH1*, *CYT1*, *COX4*, and *ATP3* in *C. glabrata* KGA17 was associated with a large decrease in ethanol and glycerol accumulation and a significant increase in α-ketoglutarate production. The final concentrations of ethanol and glycerol with *C. glabrata* KGA17 were decreased by 68.2% and 60.2% compared with *C. glabrata* KGA13, respectively (Fig. 7d). At the same time, the NADH/NAD^+ ratio in *C. glabrata* KGA17 was reduced by 44.1% (Fig. 7c). In addition, the final concentration of α-ketoglutarate was increased to 53.4 g/L, showing 10.1% higher than *C. glabrata* KGA13 (Fig. 7d, Table S2). These results indicated that *HAP4* overexpression reduced overflow metabolism and channeled more carbon flux to α-ketoglutarate production. To assess the potential metabolic burden induced by multi-copy transcription factor expression, we determined metabolic activity and protein contents between *C. glabrata* KGA05 and *C. glabrata* KGA17 (Fig. S1). Compared with *C. glabrata* KGA05, *C. glabrata* KGA17 harboring multi-copy transcription factor expression showed an 8.4% decrease in metabolic activity and a 13.8% increase in total protein contents. These results suggested the existence of a measurable and manageable metabolic burden. Notably, no significant delays in cell growth or reductions in biomass were observed under fermentation conditions. Thus, although *C. glabrata* KGA17 suffered from a slight metabolic burden due to multi-copy transcription factor expression, it did not significantly compromise the performance of engineered *C. glabrata* strains in α-ketoglutarate production.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

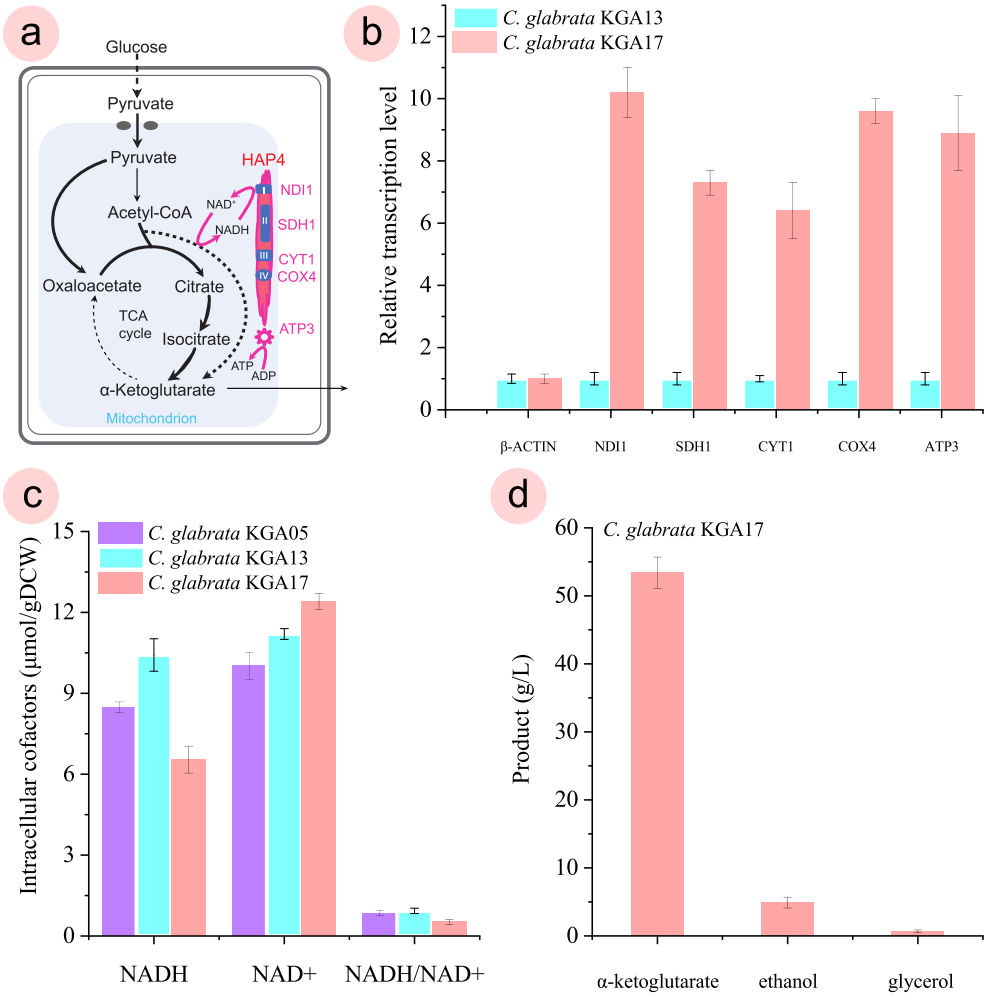


Fig. 7. HAP4 overexpression for promoting the electron transport chain. **a:** Schematic representation of α-ketoglutarate biosynthesis through promoting the electron transport chain. **b:** The relative transcription level of genes *NDI1*, *SDH1*, *CYT1*, *COX4*, and *ATP3*. **c:** The levels of intracellular NADH, NAD^+, and the $NADH/NAD^+$ ratio in different strains. **d:** *C. glabrata* KGA17 with HAP4 overexpression for the production of α-ketoglutarate, ethanol, and glycerol.

3.7 Production of α-ketoglutarate in a 5-L bioreactor

Following shake flask fermentations, we performed fed-batch fermentations to increase α-ketoglutarate production with the engineered strain *C. glabrata* KGA17 in a 5-L bioreactor (**Fig. 8**). During the fermentation process, glucose was rapidly consumed during cell growth and nearly depleted at 48 h. After that, a fed-batch strategy was adopted to maintain glucose concentration at 0-10 g/L. In addition, *C. glabrata* KGA17 continued to grow and obtained a maximal OD of 55.6. α-Ketoglutarate accumulated gradually, achieving the final concentration of 210.4 g/L. At the same time, α-ketoglutarate yield and productivity were increased to 0.54 g/g and 0.88 g/L/h, respectively. These results demonstrated the exceptional potential of *C. glabrata* KGA17 for efficient α-ketoglutarate production.

Transcription factors can regulate specific gene sets through binding to DNA sequences and interacting with other proteins (Broun, 2004). For one thing, transcription factors can activate the transcription machinery to enhance the rate of transcription of a particular group of genes (Grotewold, 2008). For another thing, transcription factors can act as repressors to subtly decrease metabolite production without complete repression (Grotewold, 2008). Thus, transcription factor engineering is a developing biotechnology that aims to boost metabolite production by regulating metabolic pathways for the accumulation of target metabolites (Courchesne et al., 2009). Transcription factor-based strategies for constructing microbial cell factories mainly focus on improving DNA binding capacity of transcription factors, modifying transcription factors involving an associated tolerance regulatory network, regulating the specificity and location of transcription factor DNA binding sites, modulating the operational and dynamic range of transcription factors, designing high-performance quorum sensing tools, and coupling transcription factors with other regulatory elements (Deng et al., 2022). These strategies have been widely used in the biosynthesis of high-value products: biofuels such as ethanol, isobutanol, farnesene and fatty alcohol; pharmaceuticals such as *N*-acetylglucosamine, erythritol, 2′-fucosyllactose, and lysine; organic acids such as 3-hydroxypropionic acid, pyruvic acid, shikimic acid, glucaric acid, and fatty acid (Li et al., 2020; Deng et al., 2022; He et al., 2023).

In *S.* cerevisiae, the Rpb7 subunit of RNA polymerase II was engineered using error-prone PCR for screening ethanol-tolerant and high-producing mutant strains. The mutant strain was able to produce 122 g/L ethanol, which was 40% higher than that of the parental strain (Qiu and Jiang, 2017). Based on the fatty acyl-CoA-responsive transcription factor FadR from *E. coli*, metabolite biosensors were developed for high-throughput screening of fatty alcohol high-producing *S. cerevisiae*. By coupling these biosensors with RTC3, GGA2, and LPP1 overexpression, fatty alcohol production showed an approximately 80% increase compared with that of the parental strain (Dabirian et al., 2019). Similarly, an erythritol-responsive biosensor based on transcription factor EryD was used for maximizing erythritol production in *Y. lipolytica*, and erythritol titer reached 148 g/L in a 3-L fed-

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

batch fermenter (Qiu et al., 2020). In *C. glabrata*, hypoxia-inducible factor 1 (H1F-1) was engineered to enhance the transcription of key enzymes involved in pyruvic acid biosynthesis under low dissolved oxygen (DO) levels, resulting in pyruvic acid production up to 53.1 g/L in a 5-L bioreactor under 10% DO (Luo et al., 2020). In our study, transcription factors GCR2, RTG1, and HAP4 impacted numerous genes involving glycolysis, TCA cycle, and electron transport chain, resulting in a coordinated up-regulation of these pathways. This up-regulation in *C. glabrata* KGA17 boosted α-ketoglutarate production, reaching 210.4 g/L in a 5-L bioreactor, which was the highest α-ketoglutarate production reported so far through microbial fermentation.

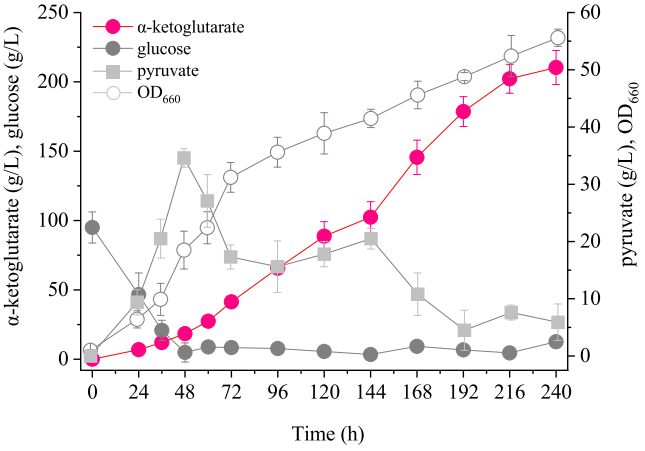


Fig. 8. α-Ketoglutarate production by *C. glabrata* KGA17 in a 5-L bioreactor.

Although transcription factor engineering has made great progress in developing efficient microbial cell factories, it is still in its early stages. The remaining challenge is to enable transcription factor engineering to become a highly efficient toolkit for constructing microbial cell factories to achieve the industrial application for the biosynthesis of high-value products. First, the conventional method for identifying transcription factors is time-consuming and labor-intensive, because it often relies on the individual or subjective assessment of predicted transcription factors from databases. This method may overlook additional relevant transcription factors. To facilitate rapid, efficient, and specific screening of transcription factors associated with target genes, future studies can develop transcription factor microarray kits through high-throughput microarray technology to detect transcription factors with modified activity. Second, despite the discovery of numerous functions and mechanisms associated with transcription factors, a comprehensive understanding of the specific processes and regulatory mechanisms is essential for revealing many unidentified challenges in the basic cellular processes of microorganisms. This in-depth analysis and comprehension can better help researchers to develop highly effective methods to systematically regulate cellular metabolism. In this regard, future research can focus on the coordinated regulation of metabolic pathways and their related transcription factors to simultaneously achieve the improvement of environmental stress resistance and the efficient production of various chemicals. Third, while significant advancements have been made in artificially designed transcription factors, traditional design methods face many limitations, such as limited data, low versatility, and inadequate rational design. Consequently, it is still challenging to develop highly orthogonal and high-quality synthetic transcription factors.

In the near future, artificial intelligence technology can be used to develop more efficient, precise, and predictable fine-tuning of artificially designed transcription factors, which is no longer constrained to species or environmental conditions. Ultimately, the development of transcription factor engineering should not be limited to applications in model microorganisms such as *E. coli* and *S. cerevisiae*, but should be extended to other typical biotechnology workhorses such as *Pichia pastoris* and *Kluyveromyces marxianus* (Gu et al., 2025). Once these problems are solved, transcription factor engineering will provide new possibilities for developing efficient microbial cell factories to achieve the biomanufacturing of chemicals.

4. Conclusions

In this study, transcription factor engineering was used to develop efficient microbial cell factories for α-ketoglutarate production. Accordingly, transcription factors GCR2 and RTG1 were selected to activate the glycolytic pathway and the TCA cycle, respectively. In addition, mitochondrial pyruvate availability was increased by employing pyruvate carriers, MPC1 and MPC2, to transport cytoplasmic pyruvate into mitochondria, thus enhancing α-ketoglutarate production via the mitochondrial TCA cycle. To further improve α-ketoglutarate production, GCR2 and RTG1 expression levels were optimized, and the best-performing strain, *C. glabrata* KGA13, was able to produce 48.5 g/L α-ketoglutarate. To reduce overflow metabolism and enhance α-ketoglutarate production, transcription factor HAP4 was used to activate the electron transport chain and improve redox balance. In a 5-L bioreactor, the final titer, yield, and productivity of α-ketoglutarate with strain *C. glabrata* KGA17 were increased to 210.4 g/L, 0.54 g/g, and 0.88 g/L/h, respectively. These findings highlight the potential of *C. glabrata* as a promising platform for the large-scale production of various organic acids.

Acknowledgements

This work was financially supported by the Provincial Natural Science Foundation of Jiangsu Province (BK20241629) and the Fundamental Research Funds for the Central Universities (JUSRP124023).

Author Contributions

Pan Zhu: Writing-review & editing, Writing-original draft, Software, Investigation, Data curation, Funding acquisition, Conceptualization, Resources. **Yufei Li**: Resources, Formal analysis. **Zihan Zhao**: Investigation, Conceptualization, Resources, Methodology. **Xinyi Sun**: Software, Formal analysis.

Conflict of Interest

The authors declare that they have no conflicts of interest to disclose.

References

[1] Bork, P., Sander, C., Valencia, A., 1992. An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc. Natl. Acad. Sci. U.S.A. 89(16), 7290-7294.

[2] Bricker, D.K., Taylor, E.B., Schell, J.C., Orsak, T., Boutron, A., Chen, Y.C., Cox, J.E., Cardon, C.M., Van Vranken, J.G., Dephoure, N., Redin, C., Boudina, S., Gygi, S.P., Brivet, M., Thummel, C.S., Rutter, J., 2012. A mitochondrial pyruvate carrier required for pyruvate uptake in yeast, *Drosophila*, and humans. Science. 337(6090), 96-100.

[3] Broun, P., 2004. Transcription factors as tools for metabolic engineering in plants. Curr. Opin. Plant Biol. 7(2), 202-209.

[4] Chen, X., Dong, X., Liu, J., Luo, Q., Liu, L., 2020. Pathway engineering of *Escherichia coli* for α-ketoglutaric acid production. Biotechnol. Bioeng. 117(9), 2791-2801.

[5] Chen, X., Xu, G., Xu, N., Zou, W., Zhu, P., Liu, L., Chen, J., 2013. Metabolic engineering of *Torulopsis glabrata* for malate production. Metab. Eng. 19, 10-16.

[6] Clifton, D., Fraenkel, D.G., 1981. The gcr (glycolysis regulation) mutation of *Saccharomyces cerevisiae*. J. Biol. Chem. 256(24), 13074-13078.

[7] Courchesne, N.M.D., Parisien, A., Wang, B., Lan, C.Q., 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. J. Biotechnol. 141(1-2), 31-41.

[8] Dabirian, Y., Teixeira, P.G., Nielsen, J., Siewers, V., David, F., 2019. FadR-based biosensor-assisted screening for genes enhancing fatty acyl-CoA pools in *Saccharomyces cerevisiae*. ACS Synth. Biol. 8(8), 1788-1800.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

[9] Deng, C., Wu, Y.K., Lv, X.Q., Li, J.H., Liu, Y.F., Du, G.C., Chen, J., Liu, L., 2022. Refactoring transcription factors for metabolic engineering. Biotechnol. Adv. 57, 107935.
[10] Enkler, L., Richer, D., Marchand, A.L., Ferrandon, D., Jossinet, F., 2016. Genome engineering in the yeast pathogen *Candida glabrata* using the CRISPR-Cas9 system. Sci. Rep. 6, 35766.
[11] Finogenova, T.V., Morgunov, I.G., Kamzolova, S.V., Chernyavskaya, O.G., 2005. Organic acid production by the yeast *Yarrowia lipolytica*: a review of prospects. Appl. Biochem. Microbiol. 41(5), 418-425.
[12] Grotewold, E., 2008. Transcription factors for predictive plant metabolic engineering: are we there yet?. Curr. Opin. Biotechnol. 19(2), 138-144.
[13] Gu, Z.Y., Ding, D.Y., Shan, Z.P., Tang, Y.S., Chen, X.L., 2025. Metabolic engineering of the non-conventional yeast *Kluyveromyces marxianus* for enhancing the biosynthesis of succinic acid. Biofuel Res. J. 12(3), 2503-2516.
[14] Guo, H., Liu, P., Madzak, C., Du, G., Zhou, J., Chen, J., 2015. Identification and application of keto acids transporters in *Yarrowia lipolytica*. Sci. Rep. 5, 8138.
[15] Guo, H., Madzak, C., Du, G., Zhou, J., 2016. Mutagenesis of conserved active site residues of dihydrolipoamide succinyltransferase enhances the accumulation of alpha-ketoglutarate in *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 100(2), 649-659.
[16] Guo, H., Madzak, C., Du, G., Zhou, J., Chen, J., 2014. Effects of pyruvate dehydrogenase subunits overexpression on the alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06. Appl. Microbiol. Biotechnol. 98(16), 7003-7012.
[17] Hauf, J., Zimmermann, F.K., Müller, S., 2000. Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 26(9-10), 688-698.
[18] He, H.H., Yang, M.F., Li, S.Y., Zhang, G.Y., Ding, Z.Y., Zhang, L., Shi, G.Y., Li, Y.R., 2023. Mechanisms and biotechnological applications of transcription factors. Syn. Syst. Biotechnol. 8(4), 565-577.
[19] Herzig, S., Raemy, E., Montessuit, S., Veuthey, J.L., Zamboni, N., Westermann, B., Kunji, E.R., Martinou, J.C., 2012. Identification and functional expression of the mitochondrial pyruvate carrier. Science. 337(6090), 93-96.
[20] Holz, M., Otto, C., Kretzschmar, A., Yovkova, V., Aurich, A., Potter, M., Marx, A., Barth, G., 2011. Overexpression of alpha-ketoglutarate dehydrogenase in *Yarrowia lipolytica* and its effect on production of organic acids. Appl. Microbiol. Biotechnol. 89(5), 1519-1526.
[21] Huang, H.J., Liu, L.M., Li, Y., Du, G.C., Chen, J., 2006. Redirecting carbon flux in *Torulopsis glabrata* from pyruvate to α-ketoglutaric acid by changing metabolic co-factors. Biotechnol. Lett. 28(2), 95-98.
[22] Jia, Y., Rothermel, B., Thornton, J., Butow, R.A., 1997. A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. Mol. Cell. Biol. 17(3), 1110-1117.
[23] Kamzolova, S.V., Chiglintseva, M.N., Lunina, J.N., Morgunov, I.G., 2012. α-Ketoglutaric acid production by *Yarrowia lipolytica* and its regulation. Appl. Microbiol. Biotechnol. 96(3), 783-791.
[24] Lee, Y.B., Jo, J.H., Kim, M.H., Lee, H.H., Hyun, H.H., 2013. Enhanced production of alpha-ketoglutarate by fed-batch culture in the metabolically engineered strains of *Corynebacterium glutamicum*. Biotechnol. Bioprocess. Eng. 18(4), 770-777.
[25] Li, J.W., Zhang, X.Y., Wu, H., Bai, Y.P., 2020. Transcription factor engineering for high-throughput strain evolution and organic acid bioproduction: a review. Front. Bioeng. Biotechnol. 8, 98.
[26] Li, S., Liu, L., Chen, J., 2015. Compartmentalizing metabolic pathway in *Candida glabrata* for acetoin production. Metab. Eng. 28, 1-7.
[27] Liu, L., Li, Y., Zhu, Y., Du, G., Chen, J., 2007. Redistribution of carbon flux in *Torulopsis glabrata* by altering vitamin and calcium level. Metab. Eng. 9(1), 21-29.
[28] Liu, L.M., Li, Y., Li, H.Z., Chen, J., 2004. Manipulating the pyruvate dehydrogenase bypass of a multi-vitamin auxotrophic yeast *Torulopsis glabrata* enhanced pyruvate production. Lett. Appl. Microbiol. 39(2), 199-206.
[29] Luo, Z.S., Zeng, W.Z., Du, G.C., Chen, J., Zhou, J.W., 2020. Enhancement of pyruvic acid production in *Candida glabrata* by engineering hypoxia-inducible factor 1. Bioresour. Technol. 295, 122248.
[30] Nishi, K., Park, C.S., Pepper, A.E., Eichinger, G., Innis, M.A., Holland, M.J., 1995. The *GCR1* requirement for yeast glycolytic gene expression is suppressed by dominant mutations in the *SGC1* gene, which encodes a novel basic-helix-loop-helix protein. Mol. Cell. Biol. 15(5), 2646-2653.
[31] Olesen, J.T., Guarente, L., 1990. The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev. 4(10), 1714-1729.
[32] Otto, C., Yovkova, V., Aurich, A., Mauersberger, S., Barth, G., 2012. Variation of the by-product spectrum during alpha-ketoglutaric acid production from raw glycerol by overexpression of fumarase and pyruvate carboxylase genes in *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 95(4), 905-917.
[33] Otto, C., Yovkova, V., Barth, G., 2011. Overproduction and secretion of alpha-ketoglutaric acid by microorganisms. Appl. Microbiol. Biotechnol. 92(4), 689-695.
[34] Qin, Y., Johnson, C.H., Liu, L.M., Chen, J.A., 2011. Introduction of heterogeneous NADH reoxidation pathways into *Torulopsis glabrata* significantly increases pyruvate production efficiency. Korean J. Chem. Eng. 28(4), 1078-1084.
[35] Qiu, X.L., Xu, P., Zhao, X.R., Du, G.C., Zhang, J., Li, J.H., 2020. Combining genetically-encoded biosensors with high throughput strain screening to maximize erythritol production in *Yarrowia lipolytica*. Metab. Eng. 60, 66-76.
[36] Qiu, Z.L., Jiang, R.R., 2017. Improving *Saccharomyces cerevisiae* ethanol production and tolerance via RNA polymerase II subunit Rpb7. Biotechnol. Biofuels 10, 125.
[37] Reik, A., Zhou, Y., Collingwood, T.N., Warfe, L., Bartsevich, V., Kong, Y., Henning, K.A., Fallentine, B.K., Zhang, L., Zhong, X., Jouvenot, Y., Jamieson, A.C., Rebar, E.J., Case, C.C., Korman, A., Li, X.Y., Black, A., King, D.J., Gregory, P.D., 2007. Enhanced protein production by engineered zinc finger proteins. Biotechnol. Bioeng. 97(5), 1180-1189.
[38] Stottmeister, U., Aurich, A., Wilde, H., Andersch, J., Schmidt, S., Sicker, D., 2005. White biotechnology for green chemistry: fermentative 2-oxocarboxylic acids as novel building blocks for subsequent chemical syntheses. J. Ind. Microbiol. Biotechnol. 32(11-12), 651-664.
[39] Sugden, P.H., Newsholme, E.A., 1975. Activities of citrate synthase, NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenases, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in nervous tissues from vertebrates and invertebrates. Biochem. J. 150(1), 105-111.
[40] Uemura, H., Jigami, Y., 1992. Role of GCR2 in transcriptional activation of yeast glycolytic genes. Mol. Cell. Biol. 12(9), 3834-3842.
[41] Vom Endt, D., Kijne, J.W., Memelink, J., 2002. Transcription factors controlling plant secondary metabolism: what regulates the regulators?. Phytochemistry. 61(2), 107-114.
[42] Xu, G., Liu, L., Chen, J., 2012. Reconstruction of cytosolic fumaric acid biosynthetic pathways in *Saccharomyces cerevisiae*. Microb. Cell. Fact. 11, 24.
[43] Xu, N., Liu, L.M., 2019. Computational inference of the transcriptional regulatory network of *Candida glabrata*. FEMS Yeast Res. 19(4), foz036.
[44] Yin, X., Madzak, C., Du, G., Zhou, J., Chen, J., 2012. Enhanced alpha-ketoglutaric acid production in *Yarrowia lipolytica* WSH-Z06 by regulation of the pyruvate carboxylation pathway. Appl. Microbiol. Biotechnol. 96(6), 1527-1537.
[45] Yovkova, V., Otto, C., Aurich, A., Mauersberger, S., Barth, G., 2014. Engineering the alpha-ketoglutarate overproduction from raw glycerol by overexpression of the genes encoding NADP+-dependent isocitrate dehydrogenase and pyruvate carboxylase in *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 98(5), 2003-2013.
[46] Yu, Z., Du, G., Zhou, J., Chen, J., 2012. Enhanced alpha-ketoglutaric acid production in *Yarrowia lipolytica* WSH-Z06 by an improved integrated fed-batch strategy. Bioresour. Technol. 114, 597-602.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

[47] Zhang, C.L., Qi, J.S., Li, Y.J., Fan, X.G., Xu, Q.Y., Chen, N., Xie, X.X., 2016. Production of α-ketobutyrate using engineered *Escherichia coli* via temperature shift. Biotechnol. Bioeng. 113(9), 2054-2059.

[48] Zhang, D., Liang, N., Shi, Z., Liu, L., Chen, J., Du, G., 2009. Enhancement of α-ketoglutarate production in *Torulopsis glabrata*: redistribution of carbon flux from pyruvate to α-ketoglutarate. Biotechnol. Bioprocess. Eng. 14(2), 134-139.

[49] Zhou, J., Dong, Z., Liu, L., Du, G., Chen, J., 2009. A reusable method for construction of non-marker large fragment deletion yeast auxotroph strains: A practice in *Torulopsis glabrata*. J. Microbiol. Methods. 76(1), 70-74.

[50] Zhou, J., Yin, X., Madzak, C., Du, G., Chen, J., 2012. Enhanced alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06 by alteration of the acetyl-CoA metabolism. J. Biotechnol. 161(3), 257-264.

[51] Zhou, J., Zhou, H., Du, G., Liu, L., Chen, J., 2010. Screening of a thiamine-auxotrophic yeast for alpha-ketoglutaric acid overproduction. Lett. Appl. Microbiol. 51(3), 264-271.

[52] Zhu, P., Zhang, C., Chen, J.Y., Zeng, X., 2024. Multilevel systemic engineering of *Bacillus licheniformis* for efficient production of acetoin from lignocellulosic hydrolysates. Int. J. Biol. Macromol. 279(Part 1) ,135142.

Pan Zhu is an Assistant Professor at Jiangnan University. She received her Ph.D. degree in Biology from Tsinghua University in 2021. Currently, her research focuses on microbial metabolic engineering and synthetic biology. She has published peer-reviewed journal papers, including in Chem, Chinese Journal of Catalysis, International Journal of Biological Macromolecules, and Trends in Chemistry.

Yufei Li is an undergraduate student in the School of Life Science and Health Engineering at Jiangnan University. Her research focuses on yeast metabolic engineering.

Zihan Zhao is an undergraduate student in the School of Biotechnology at Jiangnan University. His research focuses on metabolic engineering of yeasts for organic acids biosynthesis.

Xinyi Sun is an undergraduate student in the School of Life Sciences and Health Engineering at Jiangnan University. Her research focuses on metabolic engineering of yeasts for organic acids biosynthesis.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

Supplementary Information

Table S1.
C. glabrata strains and plasmids used in this study.

Strains & Plasmids	Relevant characteristics	Ref.
Strains		
ATCC 55	*C. glabrata Δura3Δhis3Δtrp1*	Roetzer et al. (2008)
KGA00	ATCC 55	**This Study**
KGA01	ATCC 55; TPK3Δ::P_{TEF}-AfPYC	
KGA02	ATCC 55; TPK3Δ::P_{TEF}-AfPYC; HMS1Δ::P_{TEF}-EcCS	
KGA03	ATCC 55; TPK3Δ::P_{TEF}-AfPYC; HMS1Δ::P_{TEF}-EcCS; YAP3Δ::P_{TEF}-EcACN	
KGA04	ATCC 55; TPK3Δ::P_{TEF}-AfPYC; HMS1Δ::P_{TEF}-EcCS; YAP3Δ::P_{TEF}-EcACN; IME1Δ::P_{TEF}-EcIDH	
KGA05	KGA04; ARTP mutagenesis	
KGA06	KGA05; LIG4Δ::P_{TEF}-GCR2	
KGA07	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2	
KGA08	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1	
KGA09	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1	
KGA10	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1, P_{TEF}-RTG1	
KGA11	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2	
KGA12	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2	
KGA13	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1, P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2	
KGA14	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2, P_{TEF}-GCR2	
KGA15	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2, P_{TEF}-GCR2	
KGA16	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1, P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2, P_{TEF}-GCR2	
KGA17	KGA05; LIG4Δ::P_{TEF}-GCR2, P_{TEF}-MCP1/2; YAP5Δ::P_{TEF}-RTG1, P_{TEF}-RTG1, P_{TEF}-RTG1; PDE2Δ::P_{TEF}-GCR2, P_{TEF}-HAP4	
Plasmids		
pY26	2 μ, Amp, URA3, P_{GPD}, P_{TEF}	Chen et al. (2016)
pY13	CEN6/ARSH4, Amp, HIS3, P_{TEF}	
pY26-AfPYC	2 μ, Amp, URA3, P_{GPD}, P_{TEF}-AfPYC	**This Study**
pY13-EcCS	CEN6/ARSH4, Amp, HIS3, P_{TEF}-EcCS	
pY26-EcACN	2 μ, Amp, URA3, P_{GPD}, P_{TEF}-EcACN	
pY13-EcIDH	CEN6/ARSH4, Amp, HIS3, P_{TEF}-EcIDH	
pY26-GCR2	2 μ, Amp, URA3, P_{GPD}, P_{TEF}-GCR2	
pY13-MCP1/2	CEN6/ARSH4, Amp, HIS3, P_{TEF}-MCP1/2	
pY26-RTG1	2 μ, Amp, URA3, P_{GPD}, P_{TEF}-RTG1	
pY13-HAP4	CEN6/ARSH4, Amp, HIS3, P_{TEF}-HAP4	

Table S2.
C. glabrata strains for α-ketoglutarate production in shake flasks.

Strains	Titer (g/L)	Yield (g/g glucose)	Productivity (g/L/h)
C. glabrata KGA00	3.2	0.04	0.04
C. glabrata KGA04	6.6	0.08	0.09
C. glabrata KGA05	20.8	0.26	0.29
C. glabrata KGA06	24.4	0.31	0.34
C. glabrata KGA07	28.1	0.35	0.39
C. glabrata KGA08	38.4	0.48	0.53
C. glabrata KGA13	48.5	0.61	0.67
C. glabrata KGA17	53.4	0.67	0.74

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.

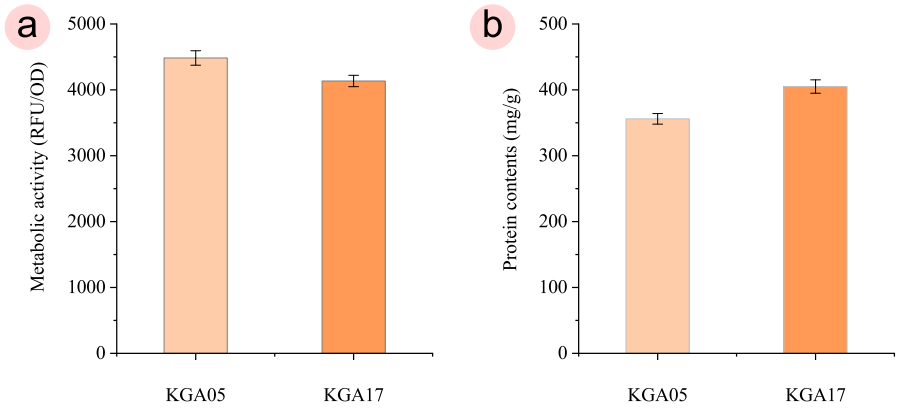


Fig. S1. Comparison of metabolic activity and protein contents between *C. glabrata* KGA05 and *C. glabrata* KGA17. **a:** Metabolic activity. **b:** Protein contents.

References

[1] Chen, X., Zhu, P., Liu, L., 2016. Modular optimization of multi-gene pathways for fumarate production. Metab. Eng. 33, 76-85.
[2] Roetzer, A., Gregori, C., Jennings, A.M., Quintin, J., Ferrandon, D., Butler, G., Kuchler, K., Ammerer, G., Schüller, C., 2008. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologous transcription factors. Mol. Microbiol. 69(3), 603-620.

Please cite this article as: Zhu P., Li Y., Zhao Z., Sun X. Cell factories and transcription factor engineering for bioproducts: the case of *Candida glabrata* for α-ketoglutarate production. Biofuel Research Journal 48 (2025) 2569-2581. DOI: 10.18331/BRJ2025.12.4.5.