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Metabolic Engineering of Nonmodel Yeast *Issatchenkia orientalis* SD108 for 5-Aminolevulinic Acid Production

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ABSTRACT

Biological production of 5-aminolevulinic acid (5-ALA) has received growing attention over the years. However, there is the tradeoff between 5-ALA biosynthesis and cell growth because the fermentation broth will become acidic due to the production of 5-ALA. To address this limitation, we engineered an acid-tolerant yeast, *Issatchenkia orientalis* SD108, for 5-ALA production. We first discovered that the cell growth rate of *I. orientalis* SD108 was boosted by 5-ALA and its endogenous ALA synthetase (ALAS) showed higher activity than those homologs from other yeasts. The titer of 5-ALA was improved from 28 mg/L to 120-, 150-, and 300 mg/L, by optimizing plasmid design, overexpressing a transporter, and increasing gene copy number, respectively. After redirecting the metabolic flux using the pyruvate decarboxylase (*PDC*) knockout strain (SD108*ΔPDC*) and culturing with urea, we increased the titer of 5-ALA to 510 mg/L, a 13-fold enhancement, proving the importance of the newly identified IoALAS with higher activity and the strategic selection of nitrogen sources for knockout strains. This study demonstrates the acid-tolerant *I. orientalis* SD108*ΔPDC* has a high potential for 5-ALA production at a large scale in the future.

1 | Introduction

5-Aminolevulinic acid (5-ALA) is a nonproteinogenic amino acid with many applications in the pharmaceutical and agricultural industries (Yi et al. 2021). For example, 5-ALA is an important precursor for heam, which is responsible for oxygen transportation in human cells (Liu et al. 2014). Besides, chlorophyl synthesis for oxygen production in plants also requires 5-ALA as a precursor (Hotta et al. 1997). Notably, 5-ALA has been approved as a drug for cancer therapy and cancer localization (Kennedy, Pottier, and Pross 1990; Lee et al. 2022; Liu et al. 2024). Its production can be achieved by either chemical routes or biological routes. The chemical production route is not environmentally friendly and expensive (Sasaki, Watanabe, and Tanaka, 2002), while the biological route is regarded as an advantageous approach for 5-ALA production.

Two distinct biosynthetic pathways have evolved for the production of 5-ALA. The C5 pathway, predominantly observed in higher plants, microalgae, and bacteria (Beale 1970). This pathway involves the conversion of L-glutamate into 5-ALA through a sequence of three reactions mediated by glutamate-tRNA ligase, glutamyl-tRNA reductase, and glutamate-1-semialdehyde aminotransferase, making it more challenging to work in nonmodel strains. On the other hand, the C4 pathway, discovered by Shemin and Russell (Shemin and Russell 1953), is primarily found in animals, yeast, and certain photosynthetic bacteria. In this pathway, succinyl-CoA and glycine undergo a condensation reaction catalyzed by the enzyme 5-ALA synthase (ALAS) and the cofactor pyridoxal 5′-phosphate, producing 5-ALA, CO_2, and coenzyme A.

On the other hand, engineering an acid-tolerant host for acid production is an attractive approach, as it can reduce downstream processing costs. Although *Escherichia coli* was engineered to produce 5-ALA with a titer of 63.4 g/L (Zhou et al. 2024), maintaining neutral pH during *E. coli* fermentation is still required, which significantly increases the cost of 5-ALA production (Elfsson et al. 1999; Uehlinger et al. 2000). Using yeast as a host can generally address the low-pH requirement since yeast is more tolerant of acidic conditions. However, previous studies only showed that *Saccharomyces cerevisiae* (Zhang et al. 2018) and *Yarrowia lipolytica* (Cui et al. 2021) produced 5-ALA of 0.52 and 2.2 g/L, respectively. This indicates the importance of both yeast host selection, and the gene screening in optimizing 5-ALA production.

In this study, we adopted another acid-tolerant yeast strain which is *Issatchenkia orientalis* (also named *Pichia kudriavzevii* or *Candida krusei* formerly) (Douglass et al. 2018). It is renowned for its high tolerance to extremely acidic stress and was used as a robust organism for production of organic acids such as succinic acid (Tran et al. 2023) and D-lactic acid (Park et al. 2018). Recently, a comprehensive set of genetic tools was developed for engineering *I. orientalis* SD108 strain, including episomal plasmids and genetic elements (Cao et al. 2020), the CRISPR/Cas9 system (Tran et al. 2019), and the landing pad system for efficient multicopy gene integration (Fatma et al. 2023).

With all the available genetic tools, we sought to explore *I. orientalis* SD108 as a platform organism for 5-ALA production. We first justified the utilization of this yeast strain for 5-ALA production and demonstrated its potential as a host for 5-ALA production. We then optimized the gene selection, plasmid backbone, transporter, and gene copy number to enhance the 5-ALA production. Furthermore, we used a block strategy to redirect the carbon flux to 5-ALA production. Finally, we tested different nitrogen sources to increase the production in minimal medium.

2 | Material and Methods

2.1 | Strains, Media, and Plasmids

All the strains were listed in Table 1, while Supporting Information S1: Tables S1 and S2 included all plasmids and primers in this study. For *E. coli* culture, LB medium was used at 37°C.

TABLE 1 | Strains used in this study.

Strains	Genotype
E. coli DH5α	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d lacZΔM15 Δ(lacZYA-argF) U169, hsdR17(rK- mK+), λ–*
SD108	*I. orientalis* SD108 *ΔURA3*
SD108-*IoALAS*	*I. orientalis* SD108 *ΔURA3::IoURA3p-IoURA3-IoPDCt-TDH3p-IoALAS-IoURA3t*
SD108-*ScALAS*	*I. orientalis* SD108 *ΔURA3::IoURA3p-IoURA3-IoPDCt-TDH3p-ScALAS-IoURA3t*
SD108-*YlALAS*	*I. orientalis* SD108 *ΔURA3::IoURA3p-IoURA3-IoPDCt-TDH3p-YlALAS-IoURA3t*
IH	*I. orientalis* SD108 *ΔURA3* with integration of *GPMp-IoHEM25* at LP36 site
SH	*I. orientalis* SD108 *ΔURA3* with integration of *GPMp-ScHEM25* at LP36 site
IR	*I. orientalis* SD108 *ΔURA3* with integration of *GPMp-IoAQR1* at LP36 site
SR	*I. orientalis* SD108 *ΔURA3* with integration of *GPMp-ScAQR1* at LP36 site
AI	*I. orientalis* SD108 *ΔURA3* harboring p101a-*IoALAS*
AS	*I. orientalis* SD108 *ΔURA3* harboring p101a-*ScALAS*
EI	*I. orientalis* SD108 *ΔURA3* harboring p101e-*IoALAS*
ES	*I. orientalis* SD108 *ΔURA3* harboring p101e-*ScALAS*
SD108 *ΔLSC1*	*ΔURA3 ΔLSC1*
SD108 *ΔLSC2*	*ΔURA3 ΔLSC2*
SD108 *ΔPDC*	*ΔURA3 ΔPDC*
SD108 *ΔGPD*	*ΔURA3 ΔGPD*
SD108 *ΔPDC ΔLSC2*	*ΔURA3 ΔPDC ΔLSC2*
LP	Integration of repeated sequence in *I. orientalis* SD108 *ΔURA3*
3A	*I. orientalis ΔURA3* LP integrated with 3 copies of *GPMp-IoALAS*
5A	*I. orientalis ΔURA3* LP integrated with 5 copies of *GPMp-IoALAS*
A3A	3A strain harboring p101e-*IoALAS*
A5A	5A strain harboring p101e-*IoALAS*

For culturing the *I. orientalis*, both YPAD (10-g/L yeast extract, 20-g/L peptone, 20-g/L glucose, and 100-mg/L semiadenine sulfate) and SC-URA (1.7-g/L yeast nitrogen base, 5-g/L ammonium sulfate, 1.92-g/L SC-URA, and 20-g/L glucose) were used at 30°C. LB and YPAD are both rich medium to maintain faster cell growth of bacteria and yeast, respectively. For 5-ALA production, SC-URA was supplemented with 3-g/L glycine, 1-g/L succinate, and 50-mM pyridoxal phosphate (PLP). The genetic constructions were completed using Hi-Fi assembly and Q5 polymerase.

2.2 | Plasmid Transformation, Gene Integration and Knockout in *I. orientalis*

I. orientalis SD108*ΔURA3* was cultured in YPAD medium overnight and inoculated to a fresh medium to an OD of 0.2. After the OD reached 0.8 to 1 (i.e., around 4 h), cells were collected and washed with sterilized water twice. Next, the DNA transformation mixture consisting of 240-μL PEG2000 at 50%, 50-μL ssDNA at 2 mg/mL, 36 μL of 1-M lithium acetate, 30 μL concentrated cell, and 1000-ng plasmid was placed at 42°C for 1 h and spread on the selection plate afterwards. The same protocol was used for gene integration by using CRISPR/Cas9 plasmid and the additional linear DNA containing the homology arm (Tran et al. 2019). To confirm the integration, colony PCR was conducted using a site-specific primer and a gene-specific primer.

2.3 | Culture Conditions

For the 5-ALA tolerance test, the colonies of wildtype *I. orientalis* SD108 were picked in YPAD and cultured at 30°C with shaking at 250-rpm overnight. The culture was then 1% inoculated to 2-mL SC-URA medium with 50-g/L glucose and different concentrations of 5-ALA at 30°C with shaking at 250 rpm for 4 days. Biomass can be calculated by a factor of 0.18 g/L/OD. For the SC-URA medium supplemented with 5-ALA, the medium was adjusted to pH 5.6 before inoculation. For 5-ALA production, the SD108 harboring the plasmid with the *URA3* marker were picked directly into 2-mL SC-URA (containing 5-g/L ammonium sulfate) or SC-URA-urea (replacing 5-g/L ammonium sulfate with 2.5-g/L urea) with 50-g/L glucose and then cultured at 30°C with shaking at 250 rpm for 4 days or 8 days.

2.4 | 5-ALA Quantification

For quantification of 5-ALA, a colorimetric method was used. Briefly, 200 μL supernatant was mixed 200-μL 1-M sodium acetate buffer (pH 4.6) and 40-μL acetylacetone. The mixture was boiled for 10 min for 5-ALA derivation. For color formation (i.e., pink color), Ehrlich's reagent containing 100 g/L *para*-dimethylaminobenzaldehyde (DMAB), 160-mL/L hypochlorous acid in glacial acetic acid was mixed with the mixture and then reacted in the dark for 10 min. The absorbance at 553 nm was measured and quantified using the authentic 5-ALA (Sigma, USA).

2.5 | Metabolite Analysis Using HPLC

Extracellular metabolites were analyzed and quantified using high-performance liquid chromatography (HPLC, Agilent 1200 Series; Agilent Technologies, Wilmington, USA) equipped with a refractive index detector and Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA, USA) (Kang et al. 2022). Metabolites and sugars were analyzed at 50°C with 5-mM H_2SO_4 as the mobile phase, with a flow rate at 0.6 mL/min. The culture broth was centrifuged at 15,000 x g for 10 min, and supernatants were obtained for analysis of extracellular metabolites and sugars in the medium.

3 | Results and Discussion

3.1 | Exploration of *I. Orientalis* as a Robust Host for 5-ALA Production

To enable industrial 5-ALA production, we first examined the robustness of acid-tolerant *I. orientalis* SD108 for 5-ALA production. At first, we found that there was a potential 5-ALA transporter encoded by *UGA4* (Garcia et al. 1997) and 5-ALA was converted to heme inside the cell (Figure 1A). Due to the toxicity effect of 5-ALA, which has been reported to inhibit bacterial cell growth, thus activated the plant cell growth (Yi et al. 2021), we cultured *I. orientalis* in SC-URA medium with different concentrations of exogenous 5-ALA (up to 50 g/L) for 4 days. As shown in Figure 1B, the residual 5-ALA was 100% at low concentrations (i.e., 2–25 g/L), while it decreased to 95.8% and 94.3% at high concentrations (25 and 50 g/L), indicating that 5-ALA was not consumed at low concentrations by *I. orientalis* but would be consumed only around 5% at high concentrations. Additionally, the cell growth rate, as indicated by the final OD value, was accelerated by 5-ALA and the OD increased from 40 to 68 when 50-g/L 5-ALA was exogenously added, suggesting that 5-ALA did not inhibit cell growth and instead facilitated cell growth (Figure 1C). Therefore, *I. orientalis* SD108 is a viable host for 5-ALA production.

3.2 | Evaluation of Three *ALAS* Genes From Different Yeast Strains for 5-ALA Production

Next, we selected three *ALAS* genes from yeast strains including *I. orientalis* (i.e., the host strain itself), *S. cerevisiae*, and *Y. lipolytica* for evaluation. Since we did not do codon optimization for all three genes, we calculated the CAI (codon usage index) first and they are 0.79, 0.75, and 0.65 for *IoALAS*, *ScALAS,* and *YlALAS*, respectively, suggesting that the expression of *IoALAS* and *ScALAS* should be similar, while the expression of *YlALAS* may possibly be lower due to lower CAI value (Figure 2A). Overexpression of *IoALAS* resulted in a higher 5-ALA production (27.2 mg/L) compared to that of *ScALAS* (21.2 mg/L) and *YlALAS* (15.6 mg/L) (Figure 2B). This finding aligns with previous reports, indicating lower enzyme activity of *Yl*ALAS compared to *Sc*ALAS (Cui et al. 2021). Although previous studies predominantly utilized *Sc*ALAS for 5-ALA production (Cui et al. 2021;

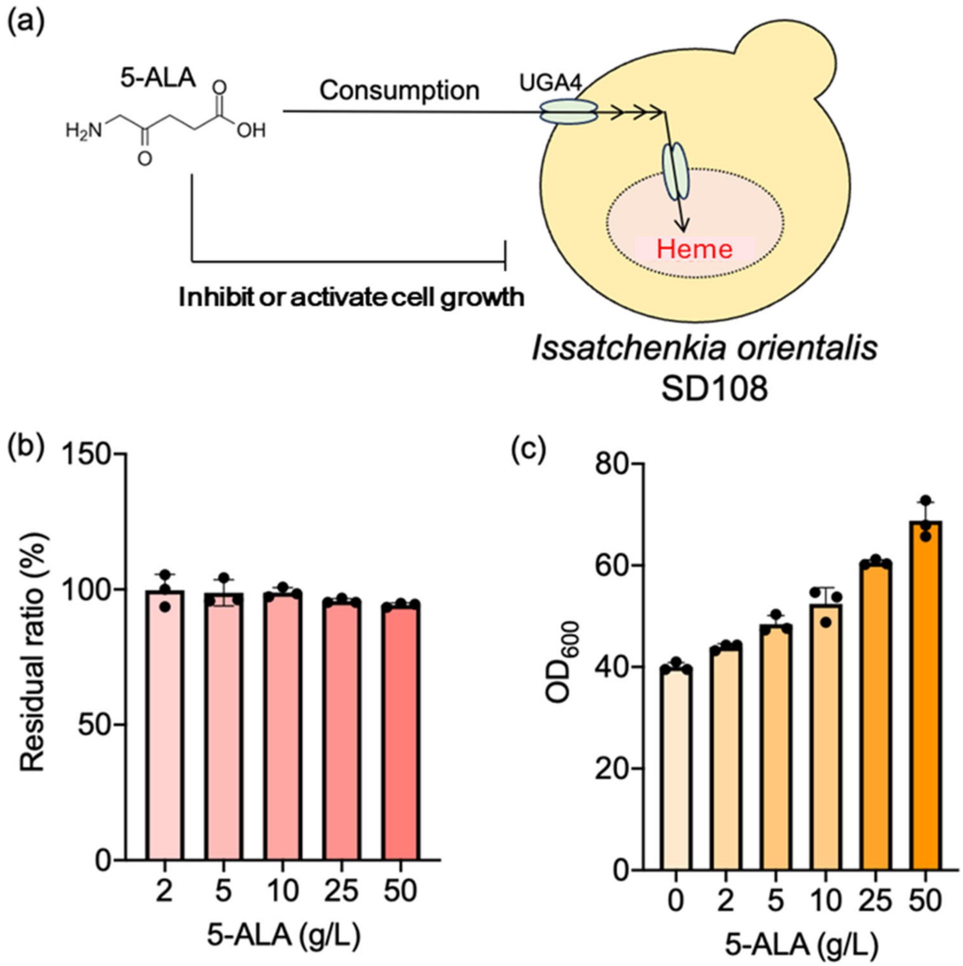

FIGURE 1 | Evaluation of *Issatchenkia orientalis* SD108 as a potential host for 5-ALA production. (a) Presence of a potential 5-ALA transporter in the genome of *I. orientalis* SD108, which facilitated 5-ALA uptake. On the other hand, 5-ALA may also inhibit or activate the cell growth. (b) Assessment of 5-ALA consumption by *I. orientalis* SD108 cultured in SC-URA medium for 4 days. (c) Final optical density (OD) measurements at day 4. All experiments were conducted in three independent replicates.

Hara et al. 2019; Mao et al. 2020), we identified a potentially more active ALAS enzyme from *I. orientalis*.

To make sure that *Io*ALAS has better performance than *Sc*ALAS, we compared these two ALASs in the following experiments. *Io*ALAS and *Sc*ALAS were subcloned into plasmids p101a and p101e for 5-ALA production in SC-URA medium (Figure 2C). Cells with episomal expression of *IoALAS* gene (i.e., AI and EI strain) outperformed those with episomal expression of the *ScALAS* gene (i.e., AS and ES strain) in 5-ALA production, reaching a peak concentration of 120 mg/L using EI strain. The difference between p101e and p101a is only on the terminator and the spacing between ARS and CEN. To further confirm the plasmid effect, we tested all the four strains in YPAD medium, a non-selection medium. Notably, strains harboring p101a produced higher titers in YPAD medium, suggesting possible chromosomal integration of the plasmid by unknown reasons. Conversely, strains with p101e exhibited 5-ALA dropping from 120 mg/L (Figure 2C) to 18.6 mg/L (Supporting Information S1: Figure S1). Here, by using an episomal plasmid and changing the plasmid backbone, we increased the 5-ALA titer from 28 to 120 mg/L, a 4.3-fold enhancement. As a result, *Io*ALAS performed better than *Sc*ALAS.

3.3 | Optimization of Carbon Source Concentration and Culture Conditions

Next, we sought to optimize the medium composition and culture conditions to improve 5-ALA production. We examined the effect of glucose as a main carbon source in different inoculations (Figure 3A) because of potential inhibition of the heme production pathway from glucose (Lee et al. 2003). Increased initial glucose concentration enhanced 5-ALA production, reaching up to 180 mg/L in both 2/14 (2-mL media in 14-mL culture tube) and 2/28 condition (2-mL media in 28-mL culture tube). Besides, the effect of exogenous addition of glycine was examined because glycine is an important precursor for 5-ALA production. Increased addition of glycine did not enhance the 5-ALA production (Figure 3B), while the biomass in terms of OD at the fourth day reduced from 40 to 30 when the glycine concentration was increased from 3 to 9 g/L (Figure 3C). The growth inhibition by glycine was also observed in *S. cerevisiae* with 80% growth inhibition from 0.075 to 0.75 g/L of glycine (Hara et al. 2019), while only 25% inhibition occurred in *I. orientalis* from 3 to 9 g/L of glycine. Overall, medium composition optimization indicated either succinyl-CoA availability is limiting, or glycine transport may be a bottleneck for 5-ALA production.

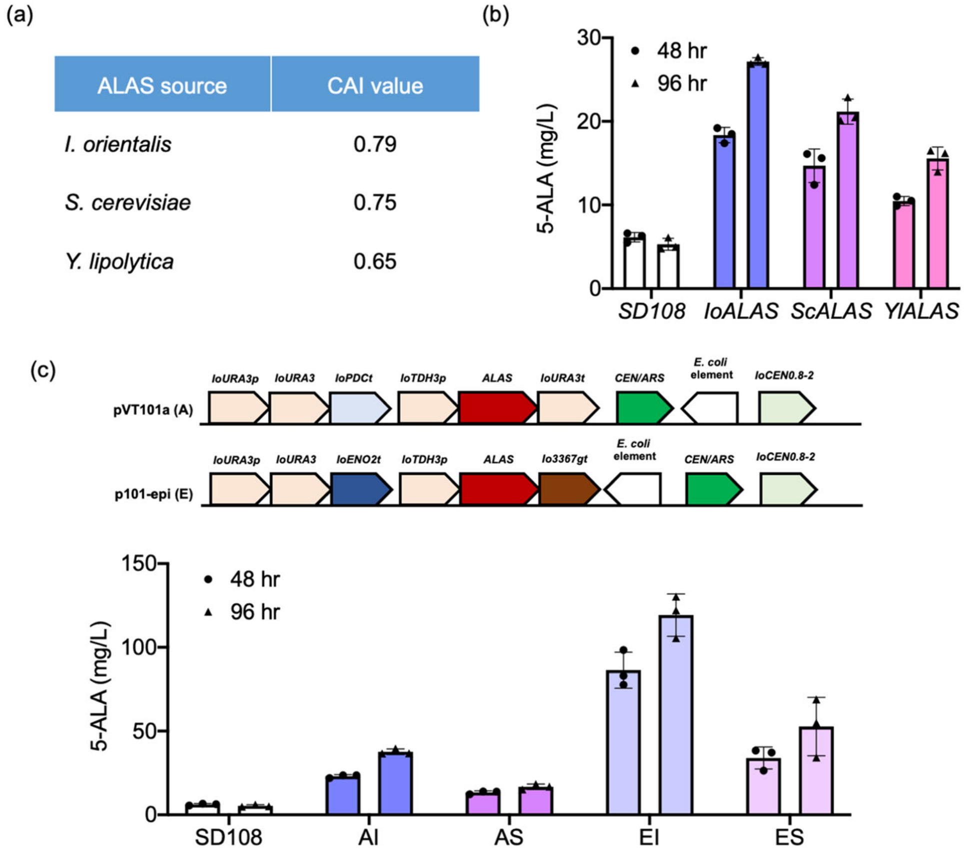

FIGURE 2 | Comparison of *ALAS* gene candidates from three yeast species. (a) Codon Adaptation Index (CAI) values for the *ALAS* genes *from I. orientalis, S. cerevisiae*, and *Y. lipolytica*. (b) 5-ALA production using different *ALAS* genes integrated into the genome, tested in SC-URA medium over 4 days. (c) Production of 5-ALA using episomal plasmids (p101a and p101e) in SC-URA medium over 4 days. AI: SD108 harboring p101a-*IoALAS*; AS: SD108 harboring p101a-*ScALAS*; EI: SD108 harboring p101e-*IoALAS*; ES: SD108 harboring p101e-*ScALAS*. All experiments were performed in three independent replicates.

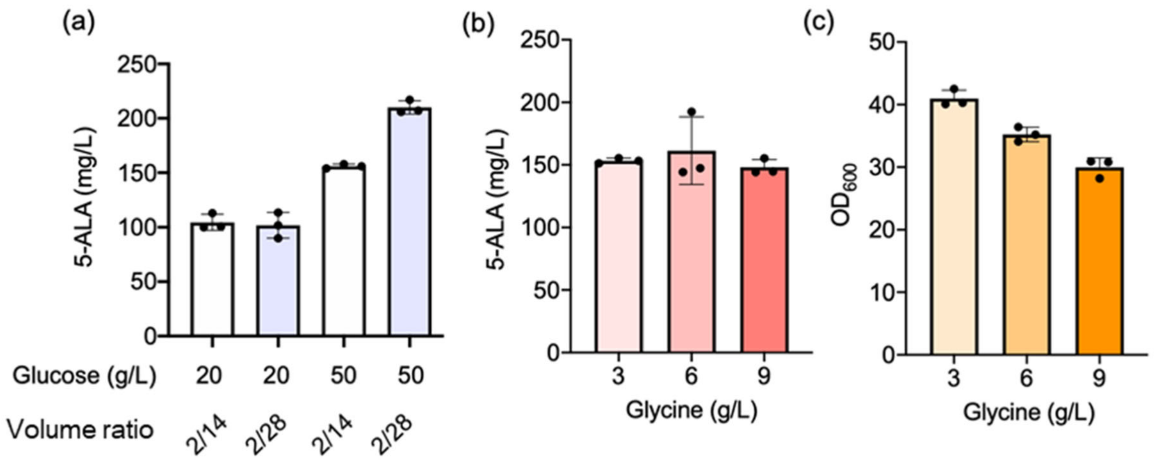

FIGURE 3 | Optimization of 5-ALA production through medium and culture conditions. (a) Effect of glucose concentration and volume ratios at 2/14 or 2/28 (i.e., 2-mL media in 14- or 28-mL culture tube) on 5-ALA production. Effect of glycine concentration on (b) 5-ALA production, and (c) cell growth. All experiments were performed in three independent replicates.

3.4 | Evaluation of the Effect of Transporter Overexpression and Gene Copy Number

Transporter genes are critical to metabolic compounds formation in genetic cell, we further examined the effects of two transporter genes, including *HEM25* (a mitochondrial glycine transporter) and *AQR1* (a plasma membrane transporter for amino acid secretion) on 5-ALA production (Figure 4A). *AQR1* encoded a gene responsible for excreting the threonine/homoserine and it has been reported that overexpression of a threonine/homoserine exporter could enhance 5-ALA production in both *E. coli* and *C. glutamicum* (Kang et al. 2011;

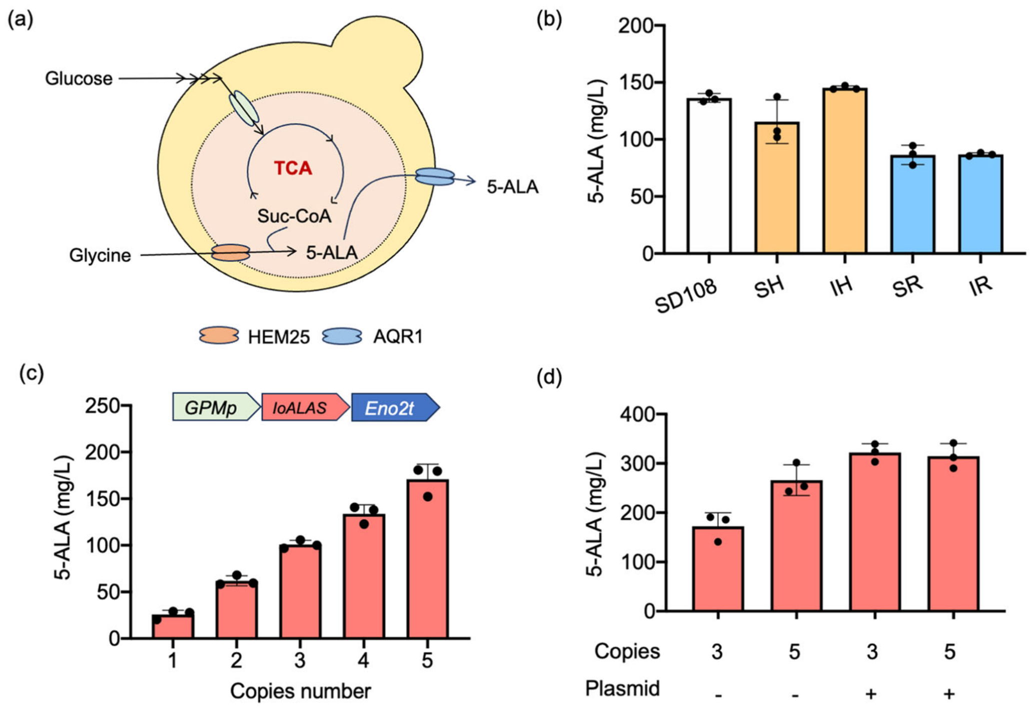

FIGURE 4 | Effect of transporter overexpression and gene copy number on 5-ALA production. (a) Overview of transporter functions in yeast. (b) 5-ALA production using strain with individual chromosomal expression of *IoHEM25* (IH), *ScHEM25* (SH), *IoAQR1* (IR), and *ScAQR1* (SR) and expression of *IoALAS* from p101e-*IoALAS*. (c) Impact of gene copy numbers on 5-ALA production. (d) Effect of p101e-*IoALAS* plasmid in three or five copies strains (i.e., 3A, 5A, A3A, and A5A) on 5-ALA production. All the experiments were performed in three independent replicates.

Yang et al. 2016). Therefore, we integrated the transporter expression cassettes into the chromosome. Next, we transformed p101e-*IoALAS* into each strain and evaluated the transporter effect on 5-ALA production. The results showed that overexpression of *AQR1* did not enhance 5-ALA production, but overexpression of *IoHEM25* (IH strain) can slightly increase the 5-ALA production (Figure 4B). Therefore, the transportation of glycine and availability of succinyl-CoA should be improved simultaneously. In addition, we attempted to increase gene copy numbers, but the plasmid construction failed and the multiple copies of *TDH3p-IoALAS* integration using a landing pad strategy failed as well. As an alternative strategy, we integrated multiple copies of the *GPMp-IoALAS*. We changed the promoter from *TDH3p* to *GPMp* and found only a slight difference of 5-ALA production (i.e., 41 mg/L for *GPMp* and 50 mg/L for *TDH3p* in Supporting Information S1: Figure S2) in YPAD medium using one copy of *GPMp-IoALAS* or *TDH3p-IoALAS*. Thus, we integrated 1 to 5 copies of *GPMp-IoALAS* into the chromosome and the 5-ALA titer increased linearly as the gene copy number increased (Figure 4C), which led to a titer of 150 mg/L with five copies. However, all the strains harbored the CRISPR/Cas9 plasmid, which reduced the cell growth and 5-ALA production. Therefore, we selected the strains with three copies and five copies of *GPMp-IoALAS* (i.e., the 3-A strain and the 5-A strain). PCR confirmation is shown in Supporting Information S1: Figure S3, which demonstrates that the CRISPR/Cas9 plasmid has been removed, followed by the transformation of either the empty plasmid to recover the uracil auxotroph or the p101e-*IoALAS* plasmid (Figure 4D). After plasmid removal, the 3- and 5-A strains produced 172 and 266 mg/L, respectively. With another copy of *ALAS* gene from p101e-*IoALAS*, the production was even enhanced to 320 mg/L and no difference was observed in the A3A and A5A strains, indicating the activity of ALAS is not limiting at this stage.

3.5 | Improvement of 5-ALA Production by Gene Deletion

A time-course analysis of 5-ALA production and metabolite profiling were shown in Figure 5A,B, respectively. The 5-ALA titer increased to 150 mg/L at day 5 while metabolite analysis showed glucose depletion within 3 days and accumulation of ethanol and glycerol up to 4 g/L. To reduce the fermentative products, we first tested two single knockout strains, i.e., SD108 *ΔPDC* and SD108 *ΔGPD*, for 5-ALA production. The *PDC* gene encodes the pyruvate decarboxylase catalyzing the first step for ethanol production through the acetyl-CoA pathway and the *GPD* gene encodes glycerol-3-phosphate dehydrogenase catalyzing the glycerol production (Figure 5C). The *PDC* knockout strain enhanced 5-ALA production from 160 to 199 mg/L, while the *GPD* knockout strain significantly reduced the 5-ALA production to 66 mg/L (Figure 5D), suggesting glycerol could contribute more to 5-ALA production.

Upon a detailed examination of the data from Figure 5A,B, we found that 5-ALA reached 80 mg/L using 50-g/L glucose, while accumulating byproducts, including 4-g/L glycerol, 2-g/L ethanol, and 1 g/L acetate. From day 3 to day 5, cells began to consume byproducts for 5-ALA production. Therefore, 5-ALA production increased from 80 to 150 mg/L, which may possibly explain why the *GPD* knockout is negative to 5-ALA production. On the other hand, we also explored three single knockout strains, including SD108 *ΔLSC1*, SD108 *ΔLSC2,* and SD108

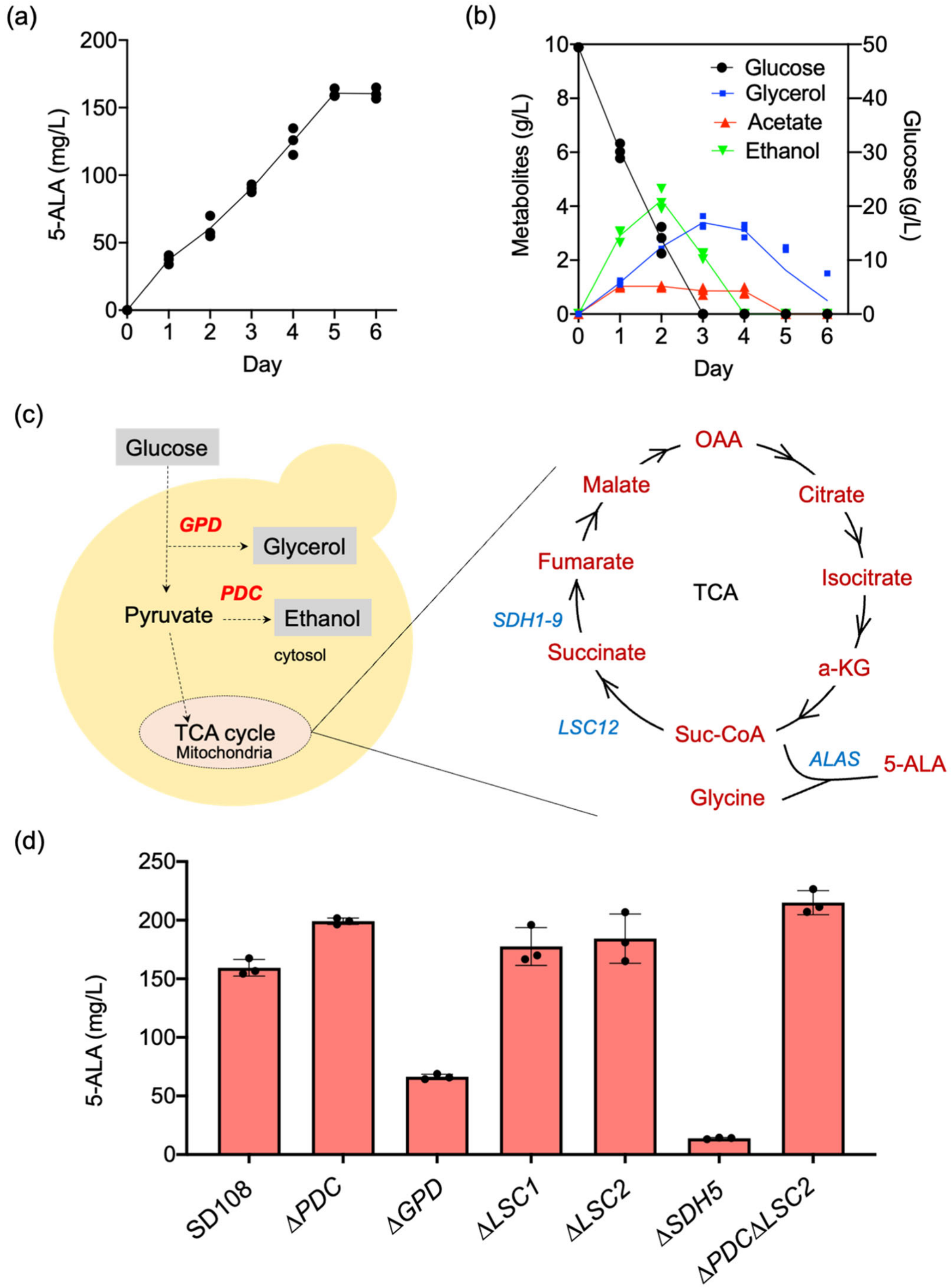

FIGURE 5 | Gene knockout for directing the carbon flux to TCA cycle for 5-ALA production. (a) Time-course of 5-ALA production using SD108 harboring p101e-*IoALAS*. (b) Glucose consumption and other metabolite accumulation along with culture time. (c) metabolic pathway for 5-ALA and byproduct production and (d) 5-ALA production using different knockout strains. All the experiments were performed in three independent replicates.

ΔSDH5, to increase the availability of succinyl-CoA (Figure 5C). *LSC1* and *LSC2* encode two subunits of succinyl-CoA ligase, which catalyzed the succinyl-CoA to succinate, thus we expected *LSC1* and *LSC2* knockouts could increase the availability of succinyl-CoA. *SDH5* encodes one of the essential enzymes in the succinate dehydrogenase complex, which converts succinate to fumarate. Cui and his coworkers demonstrated *SDH5* deletion is required for 5-ALA production in *Y. lipolityca* (Cui et al. 2021). For the gene target to increase the succinyl-CoA concentration, individual *LSC1* and *LSC2* gene knockout regarding to the *Δ*LSC1 and *Δ*LSC2 strains, could enhance 5-ALA production to 177 and 184 mg/L, respectively, while *SDH5* knockout significantly reduced the production to 14 mg/L. Next, we tested the double deletion of *PDC* and *LSC2* but did not observe any statistical difference in 5-ALA production between this double knockout strain and the *PDC* knockout strain ($p = 0.15$). As a result, the *PDC* knockout strain is robust for 5-ALA production.

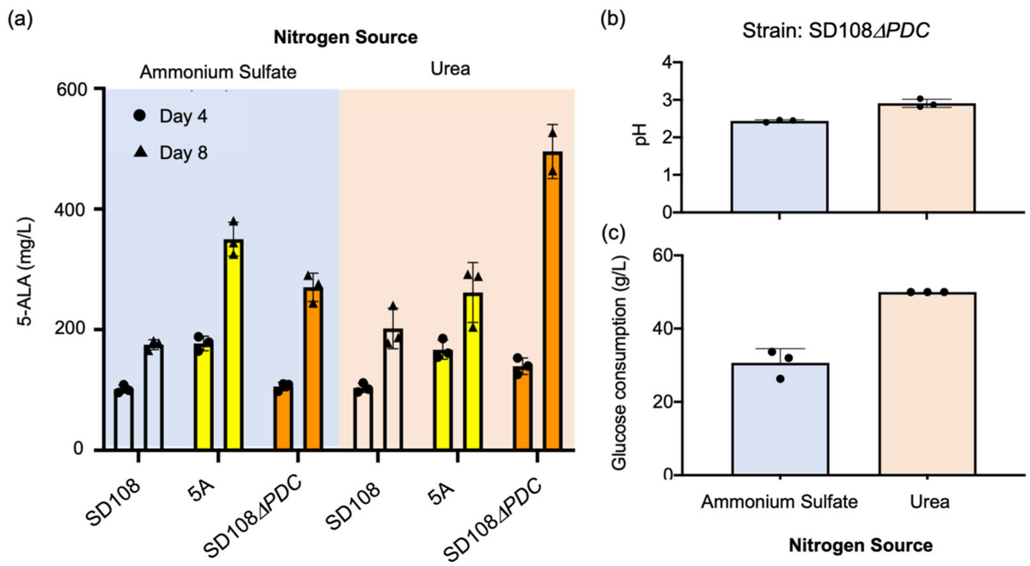

FIGURE 6 | Effect of nitrogen source on 5-ALA production in SD108, 5A, and SD108Δ*PDC* strains. (a) 5-ALA production in SD108, 5A, and SD108Δ*PDC* strains. (b) pH and (c) glucose consumption after 8-day culture of SD108Δ*PDC* strain. The nitrogen content of 5-g/L ammonium sulfate is equal to 2.5-g/L urea. All the experiments were performed in three independent replicates.

3.6 | Nitrogen Source for Further Enhancement of 5-ALA Production

When changing the nitrogen source to urea, the *PDC* knockout strain exhibited a substantial increase of 5-ALA from 260 to 510 mg/L (Figure 6A). Analysis of metabolite profiles in the *PDC* knockout strain revealed that the urea medium resulted in a higher pH at 2.9 compared to ammonium medium at pH 2.4 (Figure 6B). Similar behavior was observed in the SD108 strain (Supporting Information S1: Figure S4). The increased buffering capacity of the urea medium allowed for higher glucose consumption (i.e., 50 g/L) compared to ammonium medium (i.e., 31 g/L) (Figure 6C). This demonstrates that the choice of nitrogen source significantly impacts 5-ALA production by influencing the glucose consumption rate through improved buffering capacity for the *PDC* knockout strain.

4 | Conclusion

In this study, we explored *I. orientalis* SD108 as a robust host for 5-ALA production. It was found that the strain was tolerant to high concentrations of 5-ALA and its endogenous *ALAS* gene was more active than the homologs from *S. cerevisiae* and *Y. lipolytica*. Significant improvement of 5-ALA production was achieved by increasing the copy numbers of the *ALAS* gene, adjusting culture conditions, as well as redirecting the metabolic flux by gene knockout via CRISPR technology. Additionally, the study highlighted the impact of nitrogen source optimization on 5-ALA yield, particularly for the *PDC* knockout strain. Overall, we identified a new ALAS with higher activity and demonstrated *I. orientalis* as a promising platform host for industrial 5-ALA production in the future.

Author Contributions

Shih-I Tan: conceptualization, methodology, investigation, data curation, writing–original draft, writing–review and editing. **I-Son Ng:** supervision, writing–review and editing. **Huimin Zhao:** supervision, writing–review and editing, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.