Auxotrophs compromise bio-production in yeast Saccharomyces cerevisiae

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Abstract

Auxotrophic marker genes have widely used for genetic engineering in yeast. However, the effects of auxotrophic strains that are deficient in synthesis of amino acids or nucleotides on the growth and production are rarely reported. In this study, a total of eight auxotrophic strains with single knockout of selective markers were obtained to evaluate cell growth and free fatty acid (FFA) production in Saccharomyces cerevisiae with supplementing different concentrations of amino acids or nucleotides. Generally, except for gene ADE2, most auxotrophic strains possessed decreased cell growth and FFA production, which could be remedied by the higher concentrations of supplements. leu2Δ damaged both growth and production even with supplementation of 1000 mg/L leucine. Therefore, this study shows that auxotrophs compromise the metabolic engineering endeavor and provides a guidance in supplementing amino acids or nucleotides during fermentations for maximizing bio-productions.

1 Introduction

Bio-manufacturing via microbial cell factories represents for an alternative production route for compounds previously obtained from natural resources or chemical synthesis [1,2], considering advantages in climate insensitivity, low land occupancy and environmental friendliness [3]. At present, the production of bulk chemicals, organic acids [4], fatty alcohols[5], and alkanes [6] have been realized in microbes. In addition, with the help of developed technologies of systems biology, protein engineering, cofactor engineering and computer-aided modeling [7,8], complex biosynthetic pathways have been designed to enable the productions of compounds like cannabidiol [9] and scopolamine [10]. In particular, the industrial-scale productions of artemisinin [11], farnesene [12] and squalene[13] demonstrate the greatest potential of microbial cell factories.

The auxotrophic marker gene is wildly used for convenient genetic manipulation during cell factory construction. However, the auxotrophic genotype might compromise the bio-production due to the insufficient supply of nutrients. For example, the auxotroph is often constructed by disrupting some essential genes for amino acids synthesis, such as leucine (*LEU2*), histidine (*HIS3*) and uracil (*URA3*) for convenient genetic manipulation in yeast*Saccharomyces cerevisiae* [14]. Traditional genetic manipulation relies on the insertion of heterologous metabolic pathways into the locus of auxotrophic markers [15]. Despite the fast development of molecular biology, auxotrophic selective markers are still used to maintain plasmid stability, and even sgRNA expression in the CRISPR/Cas9 system [16]. However, amino acids, as the precursors of protein synthesis, participate in various metabolic pathways and are crucial for microbial growth and production[17]. Therefore, the exogenous addition of amino acids during fermentations [15], may be not enough to support efficient cell growth to decrease product yields, and even misjudge the feasibility of metabolic engineering strategy[18], which, however, has not been paid much attentions by researchers.

Here, we evaluated the effects of eight commonly used auxotrophic markers, with grouping them into synthesis of acetyl-CoA, α-ketoglutarate, and succinyl-CoA derived modules (Figure 1), in cell growth and bio-productions of free fatty acid (FFA) in *S. cerevisiae* [19]. By comparing the prototrophic and auxotrophic strains with exogenous supplements of different concentrations of amino acids or nucleotides, we concluded that except for gene *ADE2* , most auxotrophic strains possessed decreased cell growth and FFA production, which could be remedied by the higher concentrations of supplements. *leu2* Δ damaged both growth and production even in 1000 mg/L of supplemented leucine, which is not suitable as a screening marker. This study reminded us to be cautious to use auxotrophic cell factory in bio-productions, and the recovery of these genes in fermentations is supposed to be essential for enhancing product yields.

2 MATERIALS AND METHODS

2.1 Strains and media

All strains and plasmids used in this study were listed in supplementary tables S1 and S2, respectively. *S. cerevisiae* were cultivated in YPD medium containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose. *Εσϛηερισηια ϛολι ΔH5α* was grown in LB medium (5 g/L yeast extract, 10 g/L NaCl and 10 g/L tryptone), and 100 mg/mL ampicillin was added. Synthetic Dropout (SD) medium was composed of 20 g/L glucose and 6.7 g/L amino acid-free yeast nitrogen source (YNB). Minimal (Delft) medium was adopted to cultivate *S. cerevisiae*strains [20] containing 20 g/L glucose, 2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4$*$7H_2O$, 2 ml/L trace metals, 1 ml/L vitamin solution, and amino acids were supplemented if necessary. Solid plates contain 20 g/L agar.

The specific amounts of exogenous amino acids or nucleotides added in media are as follows. In preliminary experiments, low (20-60 mg/L, Figure S2) and high (100 mg/L, Figure S3) concentrations of amino acids or nucleotides were added in the culture medium, respectively, to determine the growth and production of the auxotrophic strains. Furthermore, we set 100 mg/L as the baseline to test the essential amounts of amino acids and nucleotides for each auxotroph to restore cell growth and bio-productions. For example, the concentrations of leucine, tryptophan, lysine (Figure 2) and methionine (Figure 3) were increased from 100 mg/L to 500 and 1,000 mg/L, respectively. The concentrations of histidine, arginine (Figure 4), uracil and adenine (Figure 5) were decreased from 100 mg/L to 20 and 60 mg/L.

2.2 Construction of engineered strain

All *S. cerevisiae* strains used in this study were derived from CEN.PK113-11C (*MATα ΣϒΨ2 MAΛ2-8ϛ ηισ3Δ1 υρα3-52*). The primers used in this study were listed in Table S3. Firstly, strain YHM01 was obtained via integrating *Cas9* gene expression box at neutral site XI of FFA producing strain YJZ06 [19]. ScYan01 (*ura3* Δ) and ScYan08 (*his3* Δ) were obtained by *in situ* restoration of gene *HIS3* and *URA3* in strain YHM01, respectively. Furthermore, other auxotrophic strains were constructed based on ScYan01, adopting the CRISPR/Cas9 system with different sgRNA and donor DNA (approximately 500 bp upstream and downstream homologous arms) [16], obtaining engineered strains with double deficient genes of *URA3* and other screening marker. Finally, the single auxotrophic strains were obtained by *in situ*repair of *URA3* gene. Gene *HIS3* was *in situ*supplemented in strain ScYan08, generating strain ScYan09 as the positive control, without any deletions of auxotrophic genes. The obtained engineering strains were tested on SD + / - amino acid or nucleotide (20 mg/L) culturing at 30 for 2 - 3 days, as shown in Figure S1.

2.3 Growth curve measurement

Strains were inoculated with initial OD_{600} of 0.2 in Delft minimal medium, supplementing different concentrations of amino acids or nucleotide. Samples were taken at 0 h, 6 h, 12 h, 24 h, 36 h and 48 h, respectively, and the optical density was measured at 600 nm (OD_{600}) by visible light spectrophotometer. Samples were diluted to OD_{600} = 0.2 - 0.8 for measurement, and OD_{600} values were calculated to demonstrate cell growth.

2.4 Quantification of free fatty acids

All strains were precultured in YPD medium for 24 h and then transferred to Delft minimal medium con-

taining 20 g/L glucose and specific amino acids or nucleotides at 30, 220 rpm. After cultivation for 48 h, FFA were determined according to the previous method[19]. In brief, 100 μL sample was taken for FFA extraction. Then, the 100 μL ddH_2O, 10 μL 40% tetrabutylammonium hydroxide and 200 μL dichloromethane (200 mM methyl iodide as methyl donor and 100 mg/L pentadecanoic acid as an internal standard) were successively added to the sample. The mixtures were shaken for 30 minutes at 1,600 rpm in whirlpool mixer, and centrifugation was performed at 3,000 g for 10 minutes to promote phase separation. The 200 μL dichloromethane was transferred to GC bottles containing glass insert. The samples were dried at room temperature for 4 h, and re-suspended with 200 μL N-hexane. Finally, the samples were analyzed by gas chromatography. Specific procedures is shown by predecessors [19].

3 RESULTS

3.1 Auxotrophs of *S. cerevisiae* decreased both cell growth and FFA production

Eight auxotrophs that are deficient in synthesis of some specific amino acids and nucleotides, including *ura3* Δ, *his3* Δ,*met15* Δ, *leu2* Δ, *trp1* Δ, *ade2* Δ, *arg4* Δ, and *lys2* Δ, were constructed in an FFA producing strain YJZ06[19]. These strains grew normally in minimal plates supplementing the corresponding nutrition (Figure S1). While cultivating in minimal media containing commonly used concentrations of 20 mg/L of uracil and histidine, 60 mg/L of leucine[14], 20 mg/L of methionine and arginine[21], 40 mg/L of tryptophan[22], 30 mg/L of lysine [23]and 50 mg/L of adenine [24], a serious decreased growth and FFA titers were observed. In particular, *ura3* Δ,*met15* Δ, *leu2* Δ, *arg4* Δ, and *lys2* Δ strains had 13.5-35.0% of maximum biomass and 23.7-44.2% of FFA in compared with the prototrophic strain (Figure S2). In this case, we suspected that the supplemented amino acids and nucleotides were not enough to support cell growth and FFA production. We increased the concentrations of amino acids and nucleotides to 100 mg/L to cultivate the strains (Figure S3). Growth behaviors of strains like *ura3* Δ, *his3* Δ,*arg4* Δ, and *ade2* Δ had been greatly improved with a slight influence on FFA titers, but strains of *leu2* Δ, *met15* Δ,*trp1* Δ, and *lys2* Δ still grew poorly with an extremely low FFA titers, which demonstrated that effects of auxotrophic strains were distinguished, and different concentrations of amino acids and nucleotides were required. Therefore, we set 100 mg/L as the baseline to test the essential amounts of amino acids and nucleotides to restore cell growth and bio-productions.

3.2 Large amounts of required amino acids that derived acetyl-CoA and succinyl-CoA

The six amino acids are divided into three groups according to their specific intermediates in the tricarboxylic acid (TCA) cycle, as acetyl-CoA module (leucine, tryptophan, lysine), succinyl-CoA module (methionine), and α-ketoglutarate module (histidine, arginine) (Figure 1). As shown in Figure S2, 100 mg/L of leucine, tryptophan, and lysine could not support efficient cell growth, and hence the concentrations were further increased to 500 mg/L and 1,000 mg/L. As expected, to restore cell growth and FFA production, the required amounts of amino acids in acetyl-CoA module are extremely high, up to 1,000 mg/L (Figure 2). For strains deficient in synthesis of tryptophan and lysine, the cell growth and production capacity were basically similar to those of control strain with 500 mg/L of supplements. However, compared with control strain, only 70% of maximum biomass and FFA titers was obtained even in 1,000 mg/L of leucine (Figure 2A, D), demonstrating leucine metabolism may be closely related to acetyl-CoA for cell growth and product formation.

Similar to those in acetyl-CoA module, methionine in succinyl-CoA module also needs higher concentration to restore cell growth. While supplementing 500 mg/L and 1,000 mg/L of methionine to cultivate the auxotrophic strain, the growth and FFA were similar to the control strain, which was obviously improved than those in 100 mg/L (Figure 3). The degradation of methionine generates succinyl-CoA through a multi-step reaction involved in intermediates of methylmalonyl-coA and methylmalonyl-SCOA (Figure 1), whose absence may disturb central metabolism for cell growth and production.

3.3 Σμαλλ αμουντς οφ ρεχυιρεδ αμινο αχιδς ιν α-κετογλυταρατε μοδυλε

The metabolism of arginine and histidine is involved in the synthesis of the intermediate α-ketoglutarate of TCA cycle (Figure 1), which also showed relatively small amounts of required supplements to restore cell

growth (Figure S3). Therefore, the lower concentrations of arginine and histidine (20, 60, and 100 mg/L) were adopted to cultivated the auxotrophic strains to evaluate their roles in cell growth and FFA production (Figure 4).

his3 Δ had little impact on cell growth and FFA production, and the auxotrophic strain reached the optimal behavior of control strain in 60 mg/L of histidine. The performance of strain did not continue to improve when the concentration was further increased to 100 mg/L. Different from histidine, while supplementing 60 mg/L of arginine, the OD_{600} and FFA titer were only 60% and 30% of those in control strain within 48 h, respectively (Figure 4B, D). The minimal required amount of arginine was approximately 100 mg/L.

3.4 Effects of nucleotides on growth and production

Except for amino acids, the deficient synthesis of nucleotides (uracil and adenine) is also commonly used screening markers. The absence or insufficiency of nucleotides will inevitably affect cell growth, considering their essential roles in translation process as three nucleotide codons in the central rule. We investigated the effects of exogenous addition of nucleotides with different concentrations on the growth and production in two nucleotide deficient strains (*ura3* Δ and *ade2* Δ). As 100 mg/L of supplements are sufficient (Figure S3), lower concentrations of 20, 60, and 100 mg/L were utilized to determine the growth and production capacity of the strains (Figure 5).

These two deficient strains possessed a similar characteristic in required amounts of nucleotides. The performances of the strains were similar to the control strain when only 60 mg/L uracil or adenine was added, and the increased concentrations did not further improved cell growth (Figure 5A, B). FFA titers of the strain *ura3* Δ supplemented with 60 mg/L and 100 mg/L uracil were about 90% of that in control strain (Figure 5C). In contrast, FFA titers of strain*ade2* Δ were 60% and 90% of that of control strain, respectively (Figure 5D).

4. DISCUSSION

Auxotrophic genes are commonly applied as screening markers in genetic manipulation of *S. cerevisiae* , but their negative roles in bio-productions in metabolic engineering are often overlooked. The deficient synthesis of amino acids or nucleotides may interfere with the homeostasis of microbial cell and hinder cell growth[18]. In this study, eight commonly used auxotrophic makers were selected to investigate their effects on cell growth and FFA production in *S. cerevisiae* .

The six amino acids are divided into three groups based on their synthesis and degradation metabolism. Generally, except gene*ADE2* , all these markers decreased both cell growth and FFA production of the corresponding auxotrophs under the commonly used concentrations. Interestingly, these observed repressions could be remedied by the much higher concentrations of supplements than expected ([?]500 mg/L), which guided us to increase the supplemented concentrations while adopting auxotrophs. Among them, the minimal required amounts of amino acids from acetyl-CoA and succinyl-CoA modules are supposed to be much higher than those from α-ketoglutarate module and nucleotides (500 mg/L vs 60 mg/L), which may be attributed to the competition with cell growth. As reported, the strain *ade2* Δ will turn red under limited concentrations, which is suitable for high-throughput screening[25].

In particular, deficiency in leucine synthesis greatly decreased both cell growth and FFA production, even in medium supplementing 1,000 mg/L of leucine, as observed in other species [26-29]. There is a close relationship of gene *LEU2* with cell growth and lipogenesis, which has previously been reported that leucine metabolism, but not leucine itself played a key role as a signal to affect TOR activity. Here, leucine metabolism was inferred as an alternative way to offer cytoplasmic acetyl-CoA to regulate the cell growth and FFA production. Therefore, the auxotrophic strains may be not suitable for bio-productions, especially in the long-run fermentations.

5 CONCLUSION

In summary, it is not desirable to disrupt the synthesis of amino acids or nucleotides in order to construct

a heterologous metabolic pathway in metabolic engineering. Currently, the optimized CRISPR-Cas9 gene editing tool may provide an alternative solution to achieve the recovery and reuse of screening markers [16]. Meanwhile, numerous neutral loci in *S. cerevisiae* have been characterized for multiple gene integration [30]. The recovery of auxotrophic markers to generate the prototrophic strain is supposed to be essential to maximize bio-productions during the fermentation, and alleviate the interference of internal environment for the engineered strains.

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Conflict of interest: The authors declare no competing interests

Data availability

All related data and information are available from the corresponding author on reasonable request

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Figure 1 Synthesis and degradation of amino acids are closely related to cell growth and FFA production. Six amino acids are grouped as acetyl-CoA module (lysine, tryptophan, leucine), succinyl-CoA module (methionine), and α-ketoglutarate module (histidine, arginine). The investigated amino acids are colored in red, and compounds marked in orange are intermediates of amino acids involved in the tricarboxylic acid (TCA) cycle.

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Figure 2 Effects of auxotrophic genes from acetyl-CoA module on cell growth and FFA production. Control: prototrophic strain;*leu2* Δ: strain ScYan11 with single deletion of gene *LEU2* ;*trp1* Δ: strain ScYan12 with single deletion of gene *TRP1* ;*lys2* Δ: strain ScYan14 with single knocked out gene *LYS2* . Growth curve of *leu2* Δ, *trp1* Δ and *lys2* Δ were measured by adding exogenous leucine, tryptophan and lysine of 100, 500 and 1,000 mg/L, respectively (A, B and C). Meanwhile, fatty acid titers at fermentation endpoint (48 h) of *leu2* Δ, *trp1* Δ and*lys2* Δ were measured (D). All data was represented as the mean ± s.d. of three clone samples.

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Figure 3 Effects of auxotrophic genes from succinyl-CoA module on cell growth and FFA production. Control: prototrophic strain;*met15* Δ: strain ScYan10 with single deletion of gene *MET15* . Growth curve (A) and fatty acid titers (B) at fermentation endpoint (48 h) of *met15* Δ were measured by adding exogenous methionine of 100, 500 and 1,000 mg/L, respectively. All data was represented as the mean ± s.d. of three clone samples.

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Φιγυρε 4 Εφφεςτς οφ αυξοτροπηις γενες φρομ α-Κετογλυταρατε μοδυλε ον ςελλ γροωτη ανδ ΦΦΑ προδυςτιον. Control: prototrophic strain;*his3* Δ: strain ScYan8 with single damage of gene *HIS3* ;*arg4* Δ: strain ScYan13 with single removal of gene *ARG4* . Growth curve of *his3* Δ and *arg4* Δ were measured by adding exogenous histidine and arginine of 20, 60 and 100 mg/L, respectively (A and B). Fatty acid titers at fermentation endpoint (48 h) of*his3* Δ and *arg4* Δ were measured (C and D). All data was represented as the mean ± s.d. of three clone samples.

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Figure 5 Effects of auxotrophic genes from nucleotide module on cell growth and FFA production. Control: prototrophic strain;*ura3* Δ: strain ScYan1 with single damage of gene *URA3* ;*ade2* Δ: strain ScYan15 with single deletion of gene *ADE15* . Growth curve of strain *ura3* Δ and *ade2* Δ were measured by adding exogenous histidine of 20, 60 and 100 mg/L, respectively (A and B). Fatty acid titers at fermentation endpoint (48 h) of *ura3* Δ and *ade2* Δ were measured (C and D). All data was represented as the mean ± s.d. of three clone samples.