Enhanced synthesis of S-adenosyl-L-methionine through Combinatorial metabolic engineering and Bayesian optimization in Saccharomyces cerevisiae

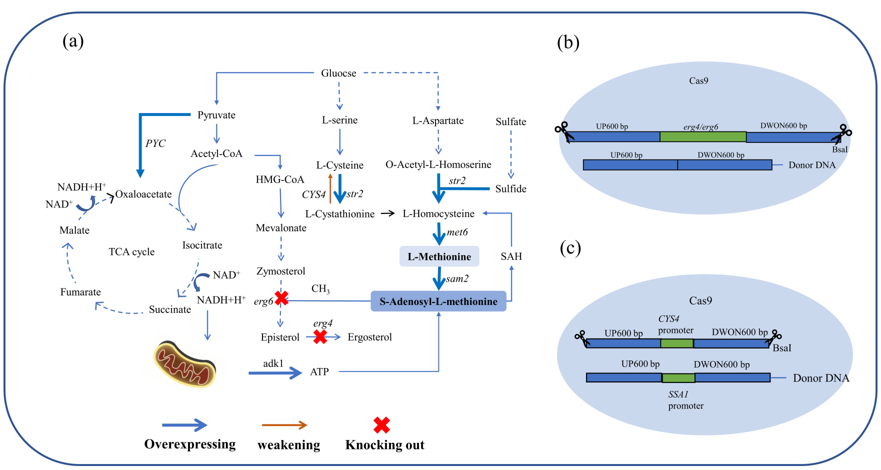
Guoqiang Xu[1], Wenhan Xiao[1], Xiangliu Shi[1], Haowei Huang[1], Xiaogang Wang[2], Wenshu Liang[1], Jianguo Xu[2], Fei Liu[2], Xiaomei Zhang[2], Jinsong Shi[2], and Zhenghong Xu[1]

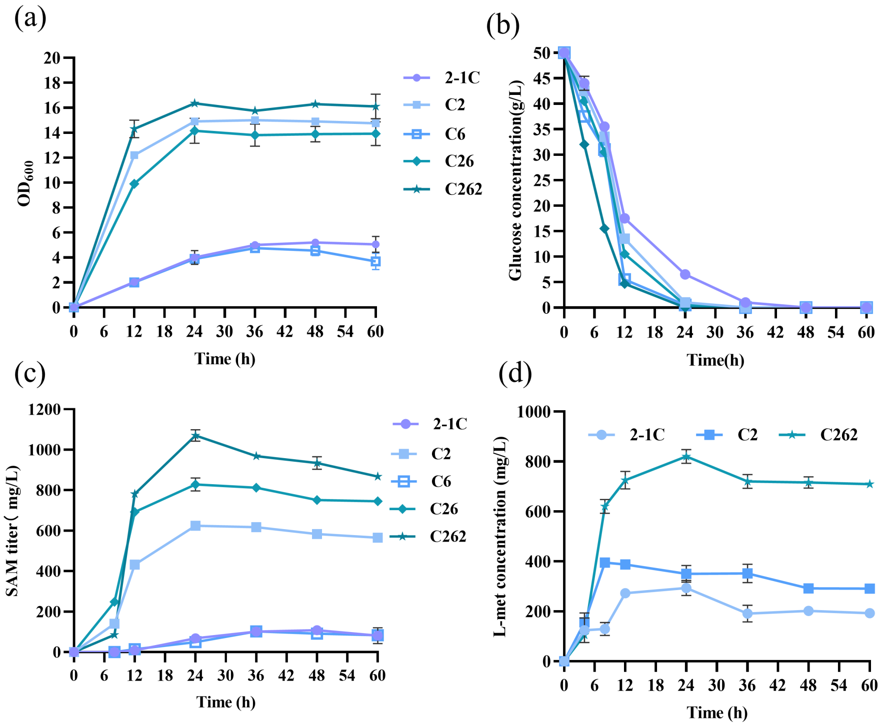
[1]Jiangnan University Key Laboratory of Industrial Biotechnology Ministry of Education
[2]Jiangnan University Wuxi School of Medicine

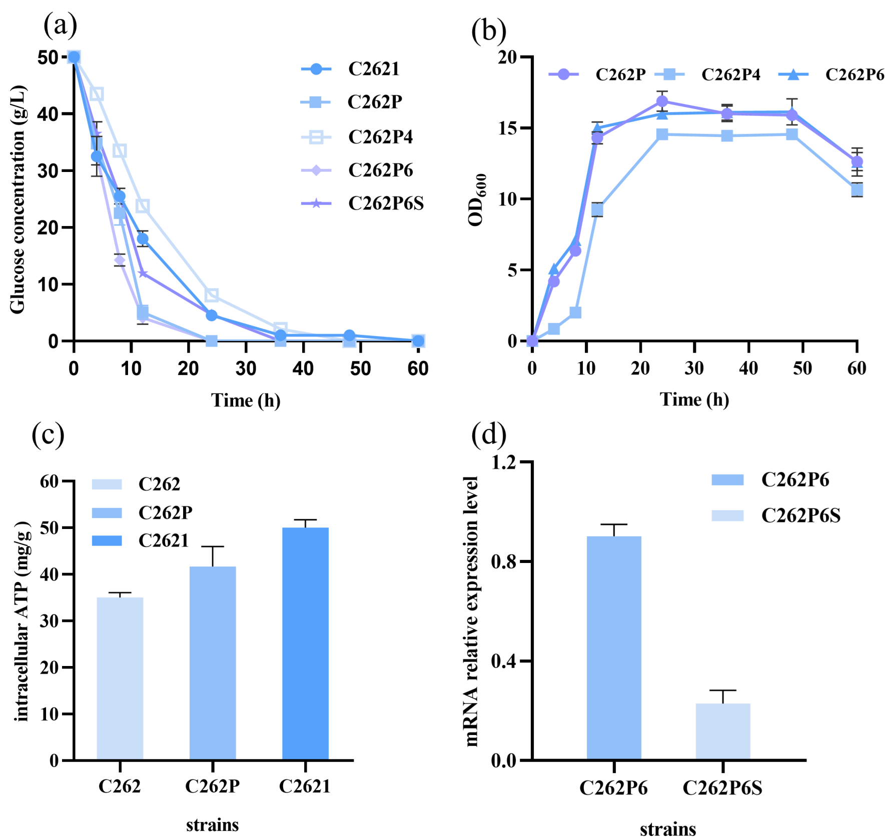
June 15, 2023

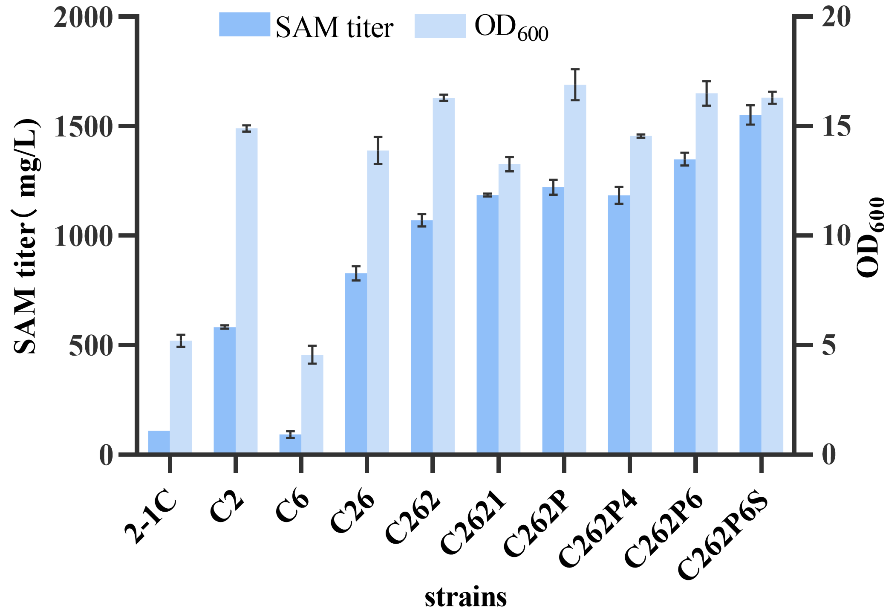
Abstract

S-adenosyl-L-methionine (SAM) is a substrate for many enzyme-catalyzed reactions and provides methyl groups in numerous biological methylations, and thus has vast applications in the medical field. *Saccharomyces cerevisiae* has been engineered as a platform with significant potential for producing SAM, although the current production has room for improvement. To surpass the restriction, a series of metabolic engineering strategies were employed to enhance the synthesis of SAM in this study. These strategies included enhancing SAM synthesis by overexpression of *SAM2, met6*, and *str2,* increasing ATP supply by integration of *adkI* and *PYC*, and down-regulating SAM metabolism by disrupting *erg4* and *erg6* and replacing the original promoter of *CYS4* with a weaker promoter. After combinatorial metabolic engineering, Bayesian optimization was conducted on the obtained strain C262P6 to optimize the fermentation medium. A final yield of 2972.8 mg/L at 36 h with 29.7% of the L-Met conversion rate in the shake flask was achieved, which was 26.3 times higher than that of its parent strain and the highest reported production in the shake flask to date. This paper establishes a feasible foundation for the construction of SAM-produced strains using metabolic engineering strategies and demonstrates the effectiveness of Bayesian optimization in optimizing fermentation medium to enhance the generation of SAM.

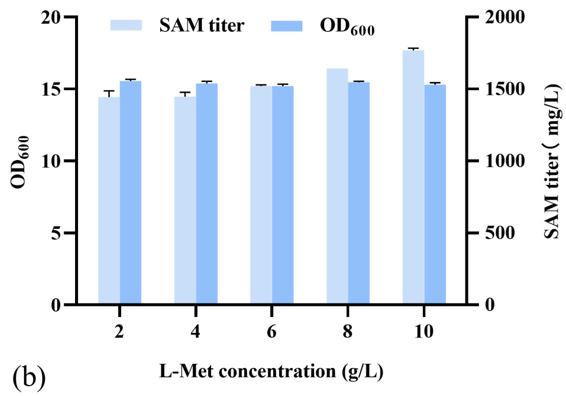



(a)
OD600
Time (h)
2-1C
C2
C6
C26
C262
(b)
Glucose concentration(g/L)
Time(h)
(c)
SAM titer (mg/L)
Time (h)
2-1C
C2
C6
C26
C262
(d)
L-met concentration (mg/L)
Time (h)
2-1C
C2
C262

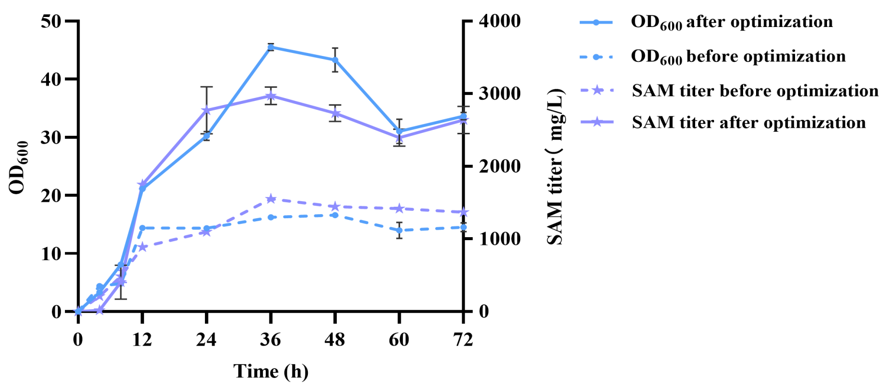

(a)
Glucose concentration (g/L)
Time (h)
C2621
C262P
C262P4
C262P6
C262P6S
(b)
OD600
Time (h)
C262P
C262P4
C262P6
(c)
intracellular ATP (mg/g)
strains
C262
C262P
C2621
(d)
mRNA relative expression level
strains
C262P6
C262P6S


SAM titer
OD600
SAM titer (mg/L)
OD600
2000
1500
1000
500
0
20
15
10
5
0
2-1C
C2
C6
C26
C262
C2621
C262P
C262P4
C262P6
C262P6S
strains

(a)


SAM titer
OD600
OD600
SAM titer (mg/L)
20
15
10
5
0
2000
1500
1000
500
0
2
4
6
8
10
L-Met concentration (g/L)

(b)


OD600 after optimization
OD600 before optimization
SAM titer before optimization
SAM titer after optimization
OD600
SAM titer (mg/L)
50
40
30
20
10
0
4000
3000
2000
1000
0
0
12
24
36
48
60
72
Time (h)

Enhanced synthesis of S-adenosyl-L-methionine through Combinatorial metabolic engineering and Bayesian optimization in *Saccharomyces cerevisiae*

Wenhan Xiao[1,2,4,7], Xiangliu Shi[1,2,4], Haowei Huang[1,2,4,7], Xiaogang Wang[5], Wenshu Liang[1,2,4], Jianguo Xu[3,6], Fei Liu[5], Xiaomei Zhang[3,7], Guoqiang Xu[1,2,4,6,7]*, Jinsong Shi[3,7], Zhenghong Xu[1,2,4,7]

[1]The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China;

[2]National Engineering Research Center for Cereal Fermentation and Food Biomanufacturing, Jiangnan University, Wuxi 214122, China;

[3]Laboratory of Pharmaceutical Engineering, School of Life Science and Health Engineering, Jiangnan University, Wuxi 214122, China;

[4]Jiangsu Provincial Engineering Research Center for Bioactive Product Processing Technology, Jiangnan University, Wuxi 214122, China;

[5]Key Laboratory of Advanced Control for Light Industry Processes, Ministry of Education, Jiangnan University, Wuxi, Jiangsu 214122, China;

[6]Wuxi Fuqi Pharmaceutical Co., Ltd, Wuxi 214100, China;

[7]Yixing Institute of Food and Biotechnology Co., Ltd, Yixing 214200, China.

*Corresponding author at: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, National Engineering Research Center for Cereal Fermentation and Food Biomanufacturing, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China;

E-mail address: xuguoqiang@jiangnan.edu.cn

Abstract

S-adenosyl-L-methionine (SAM) is a substrate for many enzyme-catalyzed reactions and provides methyl groups in numerous biological methylations, and thus has vast applications in the medical field. *Saccharomyces cerevisiae* has been engineered as a platform with significant potential for producing SAM, although the current production has room for improvement. To surpass the restriction, a series of metabolic engineering strategies were employed to enhance the synthesis of SAM in this study. These strategies included enhancing SAM synthesis by overexpression of *SAM2, met6*, and *str2,* increasing ATP supply by integration of *adkI* and *PYC*, and down-regulating SAM metabolism by disrupting *erg4* and *erg6* and replacing the original promoter of *CYS4* with a weaker promoter. After combinatorial metabolic engineering, Bayesian optimization was conducted on the obtained strain C262P6 to optimize the fermentation medium. A final yield of 2972.8 mg/L at 36 h with 29.7% of the L-Met conversion rate in the shake flask was achieved, which was 26.3 times higher than that of its parent strain and the highest reported production in the shake flask to date. This paper establishes a feasible foundation for the construction of SAM-produced strains using metabolic engineering strategies and demonstrates the effectiveness of Bayesian optimization in optimizing fermentation medium to enhance the generation of SAM.

KEYWORDS

S-adenosyl-L-methionine; combinatorial metabolic engineering; *Saccharomyces cerevisiae;* ATP; L-Met; CRISPR; Bayesian optimization

1. Introduction

S-adenosyl-L-methionine (SAM) is a physiologically active molecule in every living body. It is a substrate for many enzyme-catalyzed reactions and provides methyl groups in many biological methylations (Li et al., 2021). In the medical field, SAM can be used to treat arthritis, heavy depression, liver diseases and low sperm activity in infertile patience (Roje, 2006). SAM is formed by L-methionine (L-Met) and ATP as direct precursors catalyzed by ademetionine synthase in organisms. At present, the synthesis methods mainly include chemical synthesis, enzymatic conversion and microbial fermentation. The chemical synthesis method requires multi-step reactions, and the product is not easy to separate (Matos et al., 1987), so it is difficult to adapt to the conditions of industrial production; the enzymatic method synthesizes SAM by directly throwing the precursor, the product has high purity and is easy to extract, but its availability is limited by the harsh requirements for enzyme purity and high production cost (Park et al., 1996); the fermentation method on the other hand has the advantages of low production cost and simple processes. Therefore, industrial mass production of SAM is mainly by microbial fermentation.

The construction strategies of SAM high-yield strains mainly include: (1) Increasing L-Met supply. L-Met is the direct precursor for SAM synthesis, and its supply is of great importance for SAM synthesis. Ruan modified the SAM synthesis pathway in *Bacillus amyloliquefaciens*, and then analyzed the changes of intermediate metabolites (Ruan et al., 2019). A decrease of the content of both aspartic acid and L-Met was found. They speculated that due to the overexpression of the *SAM2* gene, there was a greater substrate consumption of L-Met. It was reported that the overexpression of cystathionine-γ-synthase gene can significantly increase the production of L-Met in *E. coli* (H. Li et al., 2017b) and that heterologous expression of *S. cerevisiae*-derived YML082W (a parallel homolog of *str2*) in *B. amyloliquefaciens* increased the SAM production of recombinant strain HZ-12 in the initial fermentation medium (Ruan et al., 2019). Zhao optimized the yield of engineered bacteria by adding L-Met, and finally obtained a yield of 8.81 g / L in a 10 L fermenter (Zhao, Shi, et al., 2016). This reveals that using the gene manipulation to drive metabolic flux can effectively improve SAM synthesis, and it also proves that L-Met plays an important role in improving microbial metabolism and synthesis of SAM. (2) Increasing ATP supply. The biosynthesis of SAM requires the participation of ATP, of which the intracellular supply level is an important factor that determines whether SAM can be excessively synthesized (D. Li et al., 2017). Because ATP not only effects the cell growth on a general level, but also it provides an adenosine for SAM synthesis. ATP supply in microbial cells can be improved by a variety of approaches, such as addition of energy substrates, metabolic engineering to modulate pH, ATP production or ATP consumption pathways, and control of respiratory chain reactions (Jin et al., 1997). It was reported that a higher level of SAM production was achieved by enhancing the ATP supply produced by the respiratory chain, which was stimulated by an increase in TCA circulating flux (Hayakawa et al., 2015). In addition to these strategies, controlling dissolved oxygen levels to generate sufficient ATP during batch cultures or high cell density cultures can also be effective in increasing the production of targeted metabolites (Wang et al., 2016). Chen established a dynamic ATP regulation strategy in *Escherichia coli* and the intracellular ATP level was maintained at 0.60 g / mg DCW, which increased SAM by 82.18% (Chen et al., 2020). Hu knocked out the *sod1* gene in *S.cerevisiae* to increase the supply of ATP, and SAM production increased by 22.3% (Hu et al., 2023). Yawei Chen improved the oxygen carrying capacity of cells by introducing Vitreoscilla hemoglobin and phosphite dehydrogenase to ensure the supply capacity of ATP when cell growth reaching to a certain level and resulted in 37% and 24% SAM increase, respectively et al. (Chen & Tan, 2018). (3) Downregulating SAM further metabolism. SAM can provide methyl for the ergosterol synthesis pathway. Thus, downregulating its further metabolism can presumably reduce SAM from further consuming, hence SAM accumulation increase, Shobayashi successfully screened a strain that lacked the ergosterol pathway, and its SAM production was 3.5 times that of its parents (Shobayashi et al., 2006); Mizunuma identified a *sah1* mutant that suppressed the Ca^{2+}-sensitive phenotypes of the *zds1*Δ strain and its SAM accumulation was 37.2-fold higher than the wildtype (Mizunuma et al., 2004). (4) Downregulating the competitive pathway of SAM synthesis. Cong Jing knocked out the *thrB* gene in *B. amyloliquefaciens* cutting off the threonine synthesis branch path and enhanced the metabolic flow of the SAM pathway increasing SAM by 42% (Jiang et al., 2020). He knocked out *CYS4* gene in *Pichia pastoris* disrupting the reflux from L- cystathionine to cysteine and the recombinant produced as twice as SAM compared to its parent strain (He et al., 2006).

Despite the fact that a considerable titer of SAM has been achieved through microbial fermentation, the production cycle still remains long while the production intensity remains low. Thus, in order to address these problems, a comprehensive method that concludes the four strategies mentioned above and an algorithm called Bayesian optimization were utilized in this study to produce SAM. Among all the microbes that has been used to produce SAM, *S. cerevisiae* has been proved to be an ideal industrial chassis cell. *S. cerevisiae* is harm-free for researchers when conducting an experiment for it's considered as "GRAS"-generally regarded as safe by the FDA (Dong-Min et al., 2011). And because it has vacuoles filled with negatively charged polyphosphates, it can enrich positively charged SAM (Chan & Appling, 2003), and the gene manipulation technology in *S. cerevisiae* is more sophisticated than other chassis. Thus, *S. cerevisiae* is ideal for SAM synthesis by fermentation. In this study, we firstly strengthened SAM synthesis pathway by overexpressing key genes including *SAM2* encoding methionine adenosyltransferase, *met6* encoding 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase and *str2* encoding cystathionine gamma-synthase aiming to enhance L-Met supply. Then *adk1* encoding adenylate kinase and an exogenous gene *PYC* encoding pyruvate carboxylase originated from *Rhizopus oryzae* was respectively expressed to ensure ATP supply. Several genes modulating ergosterol synthesis pathway, where SAM acts as a methyl radical donor, namely *erg4* encoding delta(24(24(1)))-sterol reductase and *erg6* encoding sterol 24-C-methyltransferase was knocked out respectively by using gene editing technology and the recombinant with the best performance was chosen for further investigation. Furthermore, by using gene editing technology and promoter engineering, we downregulated the competing pathway of SAM, which was controlled by *CYS4* gene coding cystathionine beta-synthase that converts L-cystathionine to cysteine, and its transcription level was compared.

The optimization of the culture medium is a necessary step in enhancing the synthesis of SAM. To achieve this goal, a strategy based on Bayesian optimization was adopted due to the high complexity of the biochemical system and economic constraints. Bayesian optimization derives from the field of machine learning and has superiority in handling the problems related to a black-box system (Gel et al., 2018; Shahriari et al., 2015). The relationship between the conditions of the fermentation, such as the initial composition of the culture medium, the concentrations of the precursors, and time at which precursor is added to the culture, and the yield of bio-product is difficult to determine. However, the Bayesian optimization method predicts the yield and its uncertainty corresponding to a condition by merely utilizing the data from previous experiments. After the prediction, the method determines the next conditions to be tested by optimizing an acquisition function. The result of the test is then integrated with data from previous tests. The prediction and determination steps are executed iteratively until the desired performance of the test is obtained. This method guarantees economic and data efficiency because this method does not require systematic design of experiments and data from experiments performed on the similar strains are of value to be utilized. Moreover, in the sequential testing, the procedure could be terminated with desired results achieved, demonstrating its flexibility in the application. The strategy based on the systematical method and data used to be processed are presented in the next section.

This study engineered four strategies from metabolic engineering and acquired a strain that produced as 26.3 times as SAM than its parent strain, reaching 2972.8 mg/L at 36 h with 29.7% of the L-Met conversion rate after medium optimization by Bayesian optimization, and the production intensity reached 145.7 mg/L/h at 12 h, which is higher than most of the recombinants ever reported. This paper establishes a feasible foundation for the construction of SAM produced strains using metabolic engineering strategies and demonstrates the effectiveness of Bayesian optimization in optimizing fermentation medium to enhance the generation of SAM.

2. Materials and methods

2.1 Strains, plasmids, and culture media

All strains and plasmids used in this study are listed in Table 1.

E. coli JM109 was used for plasmid amplification. *E. coli* was cultured in LB medium (1% peptone, 0.5% yeast powder, and 1% NaCl) supplemented with *Amp* during screening, at 37 °C under shaking at 220 rpm. *S. cerevisiae* was cultured in SD medium (2% glucose, 1.34% YNB, and amino acid mixed solution) removed the corresponding amino acid during screening, at 30 °C under shaking at 220 rpm. Engineered strains was cultured in O-medium (5% glucose, 1% peptone, 0.5% yeast powder, 0.05% $MgSO_4 \cdot 7H_2O$, 0.4% KH_2PO_4, 0.2% K_2HPO_4, and 0.15% L-Met), at 30 °C under shaking at 220 rpm.

2.2 Primers used in this study

All primers used in this study are listed in Table 2.

2.3 Genetic manipulation

2.3.1 Overexpression of targeted genes

The target fragment *SAM2*, *met6*, *str2* and *adk1* from *S. cerevisiae* BY4741 and *PYC* from *Rhizopus oryzae* reported in the NCBI database as a template was obtained by PCR amplification using *SAM2*F, *SAM2*R *met6F*, *met6*r *str2*F, *str2*R, *adk1*F, *adk1*R, *PYC*F and *PYC*R as primers (Table1). The plasmid pRS306 and the target fragment *SAM2* were double digested with *BamH* I and *Hind* III, and then ligated with T4 ligase. The ligated product was transformed into *E.coli*, and the recombinant plasmid pRS306-*SAM2* , pRS305-*met6str2* , pRS303-*adk1* and pRS303-*PYC*,was obtained by screening and verification. The *E.coli* with different recombinant plasmids was cultured in a LB medium for 14-16 hours and 2-4 ml of bacteria was obtained for plasmid extraction using plasmid extraction kits. Chemical transformation requires the linearization of the plasmid at the defective marker. LeuF and LeuR were used as primers for leucine labeling reverse PCR linearization. UraF and UraR were used as primers for uracil labeling reverse PCR linearization. HisF and HisR were used as primers for histidine labeling reverse PCR linearization. The linearized plasmid was transformed into according strains by lithium acetate transformation method, and coated on the corresponding SD defective medium, and cultured at 30 °C for 2-3 d.

2.3.2 Disrupting targeted genes

The 600 bp before and after *erg4* and *erg6* were amplified by primers *erg4*UF, *erg4*UR, *erg4*DF, *erg4*DR, *erg6*UF, *erg6*UR, *erg6*DF and *erg6*DR with homologous arms. And the amplified products were connected by fusion PCR resulting in two 1200 bp DNA sequences named donor DNA-*erg4* and donor DNA-*erg6* respectively. The specific sRNAs of *erg4* and *erg6* were designed by https://www.atum.bio. The designed sRNA and the knockout plasmid PCRCT-LBH containing Cas9 protein were digested and ligated by BsaI. The ligation product was transferred into the E. coli JM109, and the colonies were selected for colony PCR and sent to the enterprise for sequencing. The donor DNA and sequencing verified knockout plasmid were transformed into corresponding strains, and the product was coated into the defective SD medium. After 2-3 days of culture at 30 °C, single colonies were picked for colony PCR.

2.3.3 Replacement of promoters

Based on the 'www.fruitfly.org', a promoter predicting website, the location and length of promoters of *SSA1* and *CYS4* gene were predicted, and the results that rated the highest were chosen. The 800 bp before and after *CYS4* and *SSA1* promoters were amplified by primers *CYS4*UF, *CYS4*UR, *CYS4*DF, *CYS4*DF, *SSA1*F, and *SSA1*R with homologous arms respectively. And the amplified products were connected by fusion PCR resulting in a 1600 bp DNA sequence named donor DNA-*SSA1*.

2.4 Analytical methods

2.4.1 Determination of biomass

The fermentation broth was diluted with deionized water and mixed evenly, so that the OD_{600} value was between 0.2-0.8, the absorbance value at the wavelength of 600 nm was detected, and the absorbance value was multiplied by the dilution factor to obtain the biomass (OD_{600}). Take 1 mL of fermentation broth and centrifuge at 12,000 rpm for 2 min, pour off the supernatant to collect the bacterial cells, place the bacterial slurry in a 105 °C oven to dry to constant weight, and accurately weigh its weight on a balance, which is DCW.

2.4.3 Determination of glucose content

Take 1 mL of fermentation broth and centrifuge at 12,000 rpm for 2 mins, take out the supernatant and dilute it so that the final concentration of glucose is within the detection range of 0-1 $g \cdot L^{-1}$ of the equipment, and the concentration is measured with a Sillman biosensor. Multiplied by the dilution factor is the unconsumed glucose content in the fermentation broth.

2.4.4 Determination of SAM content

Take 1 mL of fermentation broth, centrifuge at 12,000 rpm for 2 min, discard the supernatant, add 2 mL of 1.5 M perchloric acid solution, shake at 30 °C for 2 h, and then centrifuge at 8,000 rpm for 10 min, and filter the supernatant through a 0.22 μm membrane After HPLC detection. The chromatographic column is Hypercil GOLDTM aQ C18 (4.6 mm×250 mm), maintained to 80% acetonitrile solution, mobile phase: 0.01 $mol \cdot L^{-1}$ ammonium formate, containing 3% (v/v) acetonitrile, adjusted with formic acid to the pH was 3.0, the flow rate was 1.0 $mL \cdot min^{-1}$, the detection wavelength was 254 nm, and the injection volume was 20 μL. The content of SAM was quantified by the external standard method.

2.4.5 Determination of L-Met content

The mobile phase was: 10% methanol, the flow rate was 1.0 $mL \cdot min^{-1}$, the detection wavelength was 210 nm, and other conditions were the same as the detection of SAM content.

2.4.6 Determination of ATP content

The mobile phase was 95% (v/v) 0.05 $mol \cdot L^{-1}$ sodium phosphate buffer (pH=6.0) and 5% (v/v) methanol, and other conditions were the same as the detection of SAM content.

2.4.7 Determination of mRNA expression level

Real-time fluorescence quantitative PCR. *S. cerevisiae* bacteria were sampled at 60 h and total

RNA was extracted using a UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech). The titer and purity of RNA were determined, and RNA was stored at -80°C until use. Reverse transcription to obtain cDNA was performed according to the instructions of the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Biomedical Technology (Beijing) Co., Ltd.). A ChamQ Universal SYBR qPCR Master Mix Kit (Nanjing Vazyme Biotech Co. Ltd) was used to accomplish quantitative real-time PCR with specially designed primers (Table1). Experimental data were analyzed by GraphPad 8.0.

2.5 Fermentation optimization design

2.5.1 Modeling the biomass yield

Given the fact that the yield of SAM positively correlates with biomass concentrations, which are easier to be measured than SAM, in this study, a procedure was conducted to find the medium that maximize the biomass concentrations. The medium to be optimized in this work included the type of carbon source and its concentration, the concentrations of other components mentioned in the O-medium. To determine the relationship between the yield of biomass and the condition of the fermentation, a Gaussian process regression with a prior zero-mean assumption was adopted using the data from fermentation of the C262P6 strain. The prediction of the mean of the yield $\mu(x) \in R$ corresponding to a condition $x \in R^{m\times 1}$, where m is the number of the components, and the uncertainty of the prediction $\sigma^2(x) \in R$ are calculated as

$$\mu(x) = K(x,X)[K(X,X) + \sigma_n^2 I]^{-1}Y, \tag{1}$$

$$\sigma^2(x) = K(x,x) - K(x,X)[K(X,X) + \sigma_n^2 I]^{-1}K(X,x), \tag{2}$$

where $X \in R^{n\times m}$ denotes the components studied in the previous experiments, $Y \in R^{n\times 1}$ is the mean of the corresponding yield, $\sigma_n^2 \in R^{1\times n}$ denotes sample variance, $I \in R^{n\times n}$ is a unit matrix, and n is the number of the samples. $K(X_1, X_2) \in R^{p\times p}$ is a radial basis function matrix (p is the sum of the columns of the input X_1 and X_2) whose element of ith row and jth column is defined as

$$k(x_i, x_j) = \sigma_f^2 \exp\left[-\frac{(x_i - x_j)^2}{2l^2}\right], \tag{3}$$

where x_i, x_j are the ith and jth column of the augmented matrix $[X_1\, X_2]$ respectively, σ_f and l are hyperparameters which is the estimated by maximizing the likelihood $p(Y|X, \sigma_f, l)$.

2.5.2 Condition to be tested

The condition to be tested is determined by seeking a solution that maximizes acquisition function. There are several types of acquisition functions, and in this study adopted was probability of improvement:

$$P(f(x) \geq f(x^*) + \xi\ ) = \Phi\left[\frac{\mu(x) - f(x^*) - \xi}{\sigma(x)}\right], \tag{4}$$

where x is the condition to be tested, $\mu(x)$ and $\sigma(x)$ are the corresponding prediction calculated by Eq.

(1) and Eq. (2), x^* is the condition that corresponds to the highest yield in the previous experiments, f is the function mapping the condition and yield, ξ is a trade-off coefficient which is adjusted by the willing to exploit or explore, and Φ is the cumulative distribution function of standard normal distribution. The search for a such x was conducted with a genetic algorithm. After the test of searched condition, result would be integrated into the data for prediction to determine the next condition to be tested. Details about this algorithm can be found in the reference (Gel et al., 2018; Shahriari et al., 2015).

3 Results

3.1 Enhancing L-Met supply by overexpressing *SAM2, met6* and *str2*

As the direct precursor of SAM, enhancement of not only endogenous but also exogenous L-Met supply can effectively boost SAM production by bacterial fermentation (Chu et al., 2013). Therefore, we intended to enhance the expression of genes coding key enzymes in the SAM synthesis pathway. Firstly, we overexpressed *SAM2* in the chassis strain *S. cerevisiae* CEN.PK 2-1C, and resulted in a recombinant named C2. And the corresponding parameters were measured and analyzed (Fig2). The results showed that the cell growth of C2 was greatly strengthened (Fig2a) and the SAM titer also exhibited a substantial increase reaching 616.5 mg/L, which is 4.7 times higher than its parent strain (Fig2c) proving that overexpressing *SAM2* can drastically improve cell growth as well as SAM production.

Afterward, *met6* encoding 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase which is the enzyme controlling the last step of L-Met synthesis was overexpressed in *S. cerevisiae* CEN.PK 2-1C and resulted in a recombinant named C6. And the corresponding biomass and SAM titer were measured and analyzed (Fig2). The biomass of the engineered strain C6 was not much different from its parent strain, indicating that the overexpression of the *met6* gene did not cause a burden on the growth of the strain. (Fig2a) The SAM titer of C6 was 102.9 mg / L after 24 h fermentation, which was not significantly different from that of 2-1C. (Fig2c) Thus, *met6* gene was subsequently overexpressed in C2 and the resulting strain C26 was fermented in a flask shake. The corresponding biomass and SAM titer were measured and analyzed (Fig 2). C26 entered the stationary phase at 36 h, and the glucose was also depleted as it entered the stationary phase. (Fig 2b) The co-expression of *SAM2* and *met6* genes did not cause metabolic pressure on the strain, and the growth status of the strain was not significantly affected (Fig 2a). The SAM yield and production intensity of engineered strain C26 were 837.2 mg/L and 34.8 mg/ L /h after 24 h fermentation, which was 34.1% and 34.3% higher than that of *SAM2* alone respectively. (Fig2c)

In order to further enhance the L-Met supply of the SAM synthesis pathway, a gene that was involved in several reactions (Fig 1a) in the SAM synthesis pathway *str2* gene encoding cystathionine gamma-synthase was overexpressed in C26 and resulted in a recombinant named C262. A 60-hour fermentation was performed on C262. The cell growth, glucose concentration, and SAM title of the three strains were compared (Fig 2). C262 entered the stable phase at 24 h, and at the same time, glucose was also consumed as it entered the stable phase (Fig 2ab). This revealed that the co-expression of *str2* gene did not cause metabolic stress on the strain and that the growth status of the strain had no obvious effect. The SAM titer and production intensity of engineering strain C262 was 1,070.8 mg·L-1 and 44.61 mg/L/h after 24 h of fermentation, which was 71.60% and 72.72% respectively higher than that of C2 and 27% and 28.1% respectively higher than that of C26 (Fig 2c). The above phenomenon indicated that co-expression of *str2* and *met6* gene had a significant effect on SAM production. At the same time, the intracellular L-Met accumulation of C262 and C2 was compared (Figure 2d). From the perspective of fermentation time, the highest production of L-Met appeared before the highest production of SAM, and the intracellular concentration of L-Met decreased with the increase of SAM production and then maintained at a certain level; the accumulation of intracellular L-Met in C262 was significantly increased by 55.0%, confirming the important role of *met6* and *str2* in L-Met synthesis, which can greatly increase the supply of intracellular L-Met. Thus, we chose the engineered strain C262 for further research.

3.2 Enhancing ATP supply by overexpressing *adk1* and *PYC* gene

Undoubtedly, ATP plays an important role in cell growth as well as SAM synthesis, for it provides the energy that biochemical reactions needed in bacteria and it serves as an adenosine donor in SAM synthesis. Pyruvate carboxylase (*PYC*) is the metabolic step limiting the production of target carboxylic acids (Malubhoy et al., 2022). Adenylate kinase encoded by *adk1* gene of *S. cerevisiae* catalyzes AMP to ATP (Cheng et al., 2010), which partly distributes to SAM synthesis. Therefore, overexpression of *adk1* and *PYC* were conducted in engineered strain C262 resulting in two recombinants named C2621 and C262P respectively. Later, a 60-h fermentation of C262, C2621, and C262P was performed and their cell growth, glucose concentration, and SAM title were compared (Fig 3,4). It was shown that SAM titer reached 1185.8 mg/L and 1222.0 mg/L after 24 h fermentation when overexpressing *PYC* and *adk1* respectively (Fig 4). Compared with C262, the SAM titer of C2621 and C262P increased by 10.7% and 14.2% respectively.

At the same time, the intracellular ATP supply of engineered strains C262, C2621, and C262P were compared. (Figure 3c) Compared with C262, the intracellular ATP supply of C2621 and C262P increased by 42.88% and 19.19% respectively. Despite the increase of ATP supply caused by overexpression of *adk1*, it also showed a burden on cell growth, however, this phenomenon did not appear in C262P with less ATP supply increase. (Figure 4) Therefore, C262P was chosen for further investigation.

3.3 Enhancing SAM synthesis by downreglutating SAM further metabolism pathway

SAM acts as a methyl radical donor (Roje, 2006) via the reaction where Zymosterol is converted into Ergosterol which is an important constituent of cytomembrane (Qu et al., 2019). This process is controlled by a series of genes including *ergX* genes（X represents different numbers 1,2,3,4…）, which can be divided into two categories regarding cell growth: essential genes and nonessential genes. Among these genes, *erg4* and *erg6* are nonessential genes which means disrupting them will not affect cell growth generally while reducing further consumption of SAM. Downregulating *erg4* and *erg6* may be able to weaken the further metabolism of SAM, hence the increase of SAM accumulation. Thus, Crispr-Cas9 technology was utilized to disrupt *erg4* and *erg6* in C262P and resulted in two recombinants named C262P4 and C262P6. Then C262P, C262P4, and C262P6 were cultured in a shake flask and the corresponding SAM titer, cell growth, and glucose concentration were compared (Fig 3,4).

Disrupting *erg6* has brought a 10.39% SAM increase compared with C262P, reaching 1349.7 mg/L, while disrupting *erg4* not only did not show SAM increase but the cell growth of C262P4 throughout the whole fermentation process was significantly inhibited compared with C262P and C262P6. (Fig 4) And the glucose consuming rate of C262P4 was also greatly lower than that of C262P and C262P6. (Fig 3a) Therefore, the recombinant C262P6 that showed SAM increase yet no cell growth burden was chosen for further investigation.

3.4 Enhancing SAM synthesis by downregulating competing pathways

There is a cystathionine-β-synthase (CBS) in the SAM synthesis pathway of *S. cerevisiae*, which is encoded by the *CYS4* gene. It can catalyze the synthesis of cystathionine from homocysteine, leading to the reflux of cystathionine and reducing its flow to the SAM precursor L-Met. It was reported that disrupting *CYS4* in *Pichia pastoris* has been shown to significantly increase SAM production (He et al., 2006), but it also results in cysteine deficiency, requiring the addition of cysteine to the fermentation medium, which increases production costs. Whereas Qin (Qin et al., 2020) engineered *CYS4* by replacing its promoter with a weaker promoter called P_{G12} in *Pichia pastoris* and successfully lower the enzyme activity of CBS, which led to a 39.8% SAM increase comparing to its parent strain. *SSA1* promoter is also a weak promoter (Peng et al., 2015), and by using gene editing technology to replace the original *CYS4* promoter with the *SSA1* promoter, the transcription level of *CYS4* can be greatly reduced, thereby reducing the activity of CBS and the reflux of cystathionine, and promoting SAM synthesis. In this study, in order to downregulate the expression of *CYS4* and to reduce production cost simultaneously, the original promoter was replaced by a weaker promoter *SSA1*, and resulted in a recombinant named C262P6S. Subsequently, C262P6 and C262P6S were cultured in a shake flask and the corresponding SAM titer, cell growth, and glucose concentration were compared (Fig 3,4). In the first 18 hours, the cell growth of C262P6S was slightly lower than that of C262P6 and remained similar to C262P6 in the rest of the fermentation process (Fig 3b). And the SAM titer of C262P6S reached 1551.9 mg/L, which was 15.0% higher than that of C262P6 (Fig 4).

Furthermore, the transcriptional level of *CYS4* with its original promoter and with the *SSA1* promoter was compared (Fig 3d). The results showed that the transcriptional level of *CYS4* with the *SSA1* promoter was 28.3% than that of *CYS4* with its original promoter, which proved that replacing the origin promoter of *CYS4* with *SSA1* effectively diminished the expression level of *CYS4* and led to the degradation of L-cystathionine refluxing to cysteine ultimately causing SAM accumulation increase.

3.5 Optimization of medium

As L-Met showed no significant impact on cell growth but does on SAM, it was individually optimized using a set of concentration gradients (Fig 5a). The concentration gradient of L-Met was set from 2 to 10 g/L and their influences on the SAM titer and cell growth of the engineered strain were compared. Different concentrations of L-Met showed no burden on the cell growth of the recombinant, while the SAM titer showed a positive correlation with L-Met concentration. When the L-Met concentration was below 6 g/L, it showed no insignificant impact on SAM titer, however, SAM titer started to increase as the L-Met concentration grew to 6 g/L and above. Considering the production cost, further increase of L-Met concentration was no longer conducted and 10 g/L L-Met was chosen to perform further fermentation.

Then, a fermentation verification of the engineered strain was performed on the medium that was acquired by Bayesian optimization and precursor optimization. The carbon source was determined to be sucrose with a concentration of 90.7 g/L and the optimal concentrations of peptone and yeast powder were found to be 28.0 g/L and 23.8 g/L, respectively. The concentrations of inorganic salt remain the same as those of the O-medium based on the result that MgSO4 showed a negative correlation with biomass accumulation (not present) and that KH_2PO_4/K_2HPO_4 primarily serves to maintain pH at which the strain exhibits the highest growth rate. The OD600 value reached 46.5 at 36 h, which is 2.73 times higher than that of the recombinant's OD600 value under unoptimized conditions. (Fig 5b) What's more, the SAM titer reached 2972.8 mg/L at 36 h, increasing 91.6% compared to the engineered strain in the unoptimized medium, demonstrating the effectiveness of medium optimization in enhancing the synthesis of SAM. Notably, the SAM titer reached 1748.1 mg/L at 12 h and the production intensity reached 145.7 mg/L/h, which is the highest level ever reported of using *S. cerevisiae* as the chassis cell to produce SAM on the shake flask level.

4 DISCUSSION

In this study, a combinatorial method that included four metabolic strategies was engineered in *S. cerevisiae* CEN.PK 2-1C. Firstly three key genes (*SAM2*, *met6*, and *str2*) in the SAM synthesis pathway were overexpressed in order to enhance the L-Met supply. Then the supply of ATP was augmented by overexpressing the *adk1* gene and introducing an exogenous gene *PYC*. By utilizing CRISPR-Ca9, the further metabolism of SAM and its competing pathway was successfully downregulated. Notably, overexpression of the *str2* gene and *PYC* gene was discovered for the first time to be effective in SAM synthesis due to their great significance in sulfur metabolism and energy supply respectively. What's more, Bayesian optimization was firstly introduced in SAM production.

There are two ademetionine synthases in *S. cerevisiae*, which are encoded by *SAM1* and *SAM2*, respectively. *SAM1* is inhibited by the feedback of excessive L-Met, while *SAM2* does not, so the *SAM2* gene from *S. cerevisiae* was selected to overexpress to obtain a high-yield SAM strain (Kodaki et al., 2003). Firstly, a recombinant of which the SAM synthesis pathway was enhanced by overexpressing *SAM2*, *met6*, and *str2* was obtained. However, SAM titer did not show an obvious increase when overexpressing the *met6* gene alone, it was speculated that the L-Met cannot be transformed into SAM in time due to the lack of simultaneous overexpression of *SAM2* gene. Kanai constructed XΔ*ado1* from the X2180-1A strain, and the SAM accumulation of the former was 30 times that of the latter (Kanai et al., 2013). Microarray analysis showed that the expression of the L-Met synthesis pathway was enhanced in the XΔado1 strain, and it was speculated that overexpression of *met6* would leads to the decrease of homocysteine and the accumulation of L-Met; Heterologous expression of S.cerevisiae-derived YML082W (a parallel homologous gene of *str2*) in *B.amyloliquefaciens* significantly increased the SAM production of recombinant strain HZ-12 in the initial fermentation medium (Ruan et al., 2019). Overexpression of cystathionine-γ-synthase gene can also significantly increase the production of L-Met in E.coli (H. Li et al., 2017a). *Str2* gene enables cystathionine gamma-synthase activity which is involved in transsulfuration enhancing sulfur metabolism regarding in SAM synthesis pathway. Thus, the SAM increase brought by overexpression of *str2* may be contributed to its ability to accumulate L-Met and to provide sulfur for SAM synthesis.

In this study, *PYC* was connected for the first time with SAM production and showed a positive effect. *PYC* can catalyze the synthesis of oxaloacetic acid from pyruvic acid and strengthen the citric acid cycle. Besides, it links the high-capacity glycolytic pathway in *S. cerevisiae* to the synthetic pathway of the desired product (Xu et al., 2017). Xu conducted a heterologous expression of pyruvate carboxylase (*PYC*) encoding gene from Rhizopus oryzae resulted in an increase in fumaric acid titer to 226.0 mg/L from 194.0 mg/L in *S. cerevisiae* (Xu et al., 2022). When overexpressing *PYC*, excess oxaloacetic acid will be transported to mitochondria for glucose synthesis, providing a substrate supply for SAM synthesis. As an agonist of *PYC*, AcCoA will increase the supply of AcCoA when *PYC* is overexpressed, so that more reducing NADH and $FADH_2$ will be produced in the tricarboxylic acid cycle. Excessive

NADH and $FADH_2$ can produce ATP molecules under the catalysis of the *adk1* gene through the electron transport chain in mitochondria, and some of the ATP will act as adenosine donors along with L-Met to form SAM. Overexpression of *adk1* can enhance the process of AMP transforming to ATP, hence the significant intracellular ATP supply increase. It was proven that the cell growth was inhibited while intracellular ATP level increased to a certain level (Hayakawa et al., 2016), which is identical to the case of overexpressing of *adk1*.

As a methyl donor, SAM participates in the pathway of ergosterol synthesis in *S. cerevisiae*. Zhao measured the content of ergosterol after disrupting *erg4* in *S. cerevisiae* BY4741, and the results showed that the content of ergosterol was only 33.8% of that of its parent strain (Zhao, Hang, et al., 2016). It was speculated that the non-prosperous ergosterol synthesis could be responsible for no improvement of SAM production and greatly inhibited cell growth after the disruption of *erg4*. Shobayashi detected the content of ergosterol after deleting *erg4* gene in *S. cerevisiae*, and no ergosterol was not detected in the extracts of *erg4* disruptants whereas the intermediate compound of ergosterol that originated from *erg4* mutation was (Shobayashi et al., 2006). Thus, it was speculated that the deprivation of ergosterol caused by disrupting *erg4* incapacitates the usual synthesis of cytomembrane, hence the lower cell growth. Notably, in the recombinant C262P4, as the cells struggle to grow, other sterols functioning as ergosterol like its intermediate compound replace its place in the cytomembrane enabling cells to grow consecutively. However, substances that supported the formation of cytomembrane in C262P4 in the later period of its fermentation were yet to be confirmed. Thus, researches focus on ergosterol synthesis could investigate the dynamic metabolic process in recombinants where *erg4* is disrupted. When knocking out *erg6*, the process of zymosterol transforming to ergosterol was entirely disrupted which incapacitates SAM to provide methyl for ergosterol synthesis whereas only the last step of ergosterol synthesis was disrupted when knocking out *erg4*. This may be the reason why the SAM titer showed a considerable increase in C262P6 while not in C262P4.

Finally, while many mechanisms of SAM synthesis have been discussed above, those related to cell growth remain complex and intricate to explore. To overcome this limitation, Bayesian optimization was employed due to its superiority in addressing black-box problems, i.e., biological systems, in this study. The choice of sucrose instead of glucose could be rationalized by considering the dynamics of diauxic growth (Narang & Pilyugin, 2006) and nitrogen source at optimal concentrations provided sufficient material for cell construction and metabolism. The optimization step utilized potential metabolic flux and aided the cell in achieving its maximum SAM production.

ACKNOWLEDGMENTS

We thank Prof. Mattheos Koffas, Rensselaer Polytechnic Institute, United States of America, Prof. Hui Li and Prof. Xiaojuan Zhang, Jiangnan university, China, for their suggestions and critical comments. This work was partially supported by the National Key Research and Development Program of China (2020YFA0908300), the Jiangsu Provincial Natural Science Foundation (No. BK20191333), Industry prospect and key technology R & D(G20211001), the national first-class discipline program of Light Industry Technology and Engineering (LITE2018-11), the Program of Introducing Talents of Discipline to Universities (No. 111-2-06).

AUTHOR CONTRIBUTIONS

Wenhan Xiao and Xiangliu Shi carried out experiments and data analysis. Haowei Huang, Xiaogang Wang and Wenshu Liang helped to carry out experiments. Jianguo Xu, Hui Li, Xiaojuan Zhang, Xiaomei Zhang, Jinsong Shi, Fei Liu and Zhenghong Xu conceived, planned, and supervised the study. Guoqiang Xu coordinated the research team, interpreted the results, and helped draft the manuscript. All authors read and approved the final manuscript.

Uncategorized References

Chan, S. Y., & Appling, D. R. (2003). Regulation of S-adenosylmethionine levels in *Saccharomyces cerevisiae*. *journal of biological chemistry*, *278*(44), 43051-43059. https://doi.org/10.1074/jbc.M308696200

Chen, Y., & Tan, T. (2018). Enhanced S-Adenosylmethionine Production by Increasing ATP Levels in Baker's Yeast (*Saccharomyces cerevisiae*). *Journal of Agricultural and* Food *Chemistry*, *66*(20), 5200-5209. https://doi.org/10.1021/acs.jafc.8b00819

Chen, Y. W., Liao, Y., Kong, W. Z., & Wang, S. H. (2020). ATP dynamic regeneration strategy for enhancing co-production of glutathione and S-adenosylmethionine in *Escherichia coli*. *Biotechnology Letters*, *42*(12), 2581-2587. https://doi.org/10.1007/s10529-020-02989-9

Cheng, X., Xu, Z., Wang, J., Zhai, Y., Lu, Y., & Liang, C. (2010). ATP-dependent pre-replicative complex assembly is facilitated by *Adk1*p in budding yeast. *journal of biological chemistry*, *285*(39), 29974-29980. https://doi.org/10.1074/jbc.M110.161455

Chu, J., Qian, J., Zhuang, Y., Zhang, S., & Li, Y. (2013). Progress in the research of S-adenosyl-L-methionine production. *Applied Microbiology & Biotechnology*, *97*(1), 41-49.

Dong-Min, Chung, Yung-Chul, Chung, Pil, Je, MaengHyo-Kon, & Chun. (2011). Regioselective deglycosylation of onion quercetin glucosides by *Saccharomyces cerevisiae*. *Biotechnology Letters*.

Gel, E., Ntaimo, L., Shier, D., & Greenberg, H. J. (2018). Recent Advances in Optimization and Modeling of Contemporary Problems || Bayesian Optimization. *10.1287/educ.2018*, 255-278.

Hayakawa, K., Kajihata, S., Matsuda, F., & Shimizu, H. (2015). (13)C-metabolic flux analysis in S-adenosyl-L-methionine production by *Saccharomyces cerevisiae*. *Journal of Bioscience and Bioengineering*, *120*(5), 532-538. https://doi.org/10.1016/j.jbiosc.2015.03.010

Hayakawa, K., Matsuda, F., & Shimizu, H. (2016). Metabolome analysis of *Saccharomyces cerevisiae* and optimization of culture medium for S-adenosyl-L-methionine production. *AMB Express*, *6*(1), 38. https://doi.org/10.1186/s13568-016-0210-3

He, J., Deng, J., Zheng, Y., & Gu, J. (2006). A synergistic effect on the production of S-adenosyl-L-methionine in *Pichia pastoris* by knocking in of S-adenosyl-L-methionine synthase and knocking out of cystathionine-beta synthase. *Journal of Biotechnology*, *126*(4), 519-527. https://doi.org/10.1016/j.jbiotec.2006.05.009

Hu, Z. C., Zheng, C. M., Tao, Y. C., Wang, S. N., Wang, Y. S., Liu, Z. Q., & Zheng, Y. G. (2023). Improving ATP availability by *sod1* deletion with a strategy of precursor feeding enhanced S-adenosyl-L-methionine accumulation in *Saccharomyces cerevisiae*. Enzyme and Microbial Technology, *164*, 110189. https://doi.org/10.1016/j.enzmictec.2022.110189

Jiang, C., Ruan, L., Wei, X., & Guo, A. (2020). Enhancement of S-adenosylmethionine production by deleting *thrB* gene and overexpressing *SAM2* gene in *Bacillus amyloliquefaciens*. *Biotechnology Letters*, *42*(11), 2293-2298. https://doi.org/10.1007/s10529-020-02945-7

Jin, S., Ye, K., & Shimizu, K. (1997). Metabolic flux distributions in recombinant *Saccharomyces cerevisiae* during foreign protein production. *Journal of Biotechnology*, *54*(3), 161-174.

Kanai, M., Masuda, M., Takaoka, Y., Ikeda, H., Masaki, K., Fujii, T., & Iefuji, H. (2013). Adenosine kinase-deficient mutant of Saccharomyces cerevisiae accumulates S-adenosylmethionine because of an enhanced methionine biosynthesis pathway. *Applied microbiology and biotechnology*, *97*(3), 1183-1190. https://doi.org/10.1007/s00253-012-4261-3

Kodaki, T., Tsuji, S., Otani, N., Yamamoto, D., Rao, K. S., Watanabe, S., Tsukatsune, M., & Makino, K. (2003). Differential transcriptional regulation of two distinct S-adenosylmethionine synthetase genes (*SAM1* and *SAM2*) of Saccharomyces cerevisiae. *Nucleid Acids Research* (3), 303-304. https://doi.org/10.1093/nass/3.1.303

Li, D., Wang, D., & Wei, G. (2017). Efficient co-production of S-adenosylmethionine and glutathione by *Candida utilis*: effect of dissolved oxygen on enzyme activity and energy supply. *Journal of Chemical Technology and Biotechnology*, *92*(8), 2150-2158. https://doi.org/10.1002/jctb.5226

Li, H., Wang, B. S., Li, Y. R., Zhang, L., Ding, Z. Y., Gu, Z. H., & Shi, G. Y. (2017a). Metabolic engineering of *Escherichia coli* W3110 for the production of L-methionine. *Journal of industrial microbiology & biotechnology*, *44*(1), 75-88. https://doi.org/10.1007/s10295-016-1870-3

Li, J., Sun, C., Cai, W., Li, J., Rosen, B. P., & Chen, J. (2021). Insights into S-adenosyl-l-methionine (SAM)-dependent methyltransferase related diseases and genetic polymorphisms. *Mutation Research-reviews In Mutation Research*, *788*, 108396. https://doi.org/10.1016/j.mrrev.2021.108396

Malubhoy, Z., Bahia, F. M., de Valk, S. C., de Hulster, E., Rendulić, T., Ortiz, J. P. R., Xiberras, J., Klein, M., Mans, R., & Nevoigt, E. (2022). Carbon dioxide fixation via production of succinic acid from glycerol in engineered *Saccharomyces cerevisiae*. *Microbial Cell Factories*, *21*(1), 102. https://doi.org/10.1186/s12934-022-01817-1

Matos, J. R., Raushel, F. M., & Wong, C. H. (1987). S-adenosylmethionine: studies on chemical and enzymatic synthesis. biotechnology and applied biochemistry, *9*(1), 39-52.

Mizunuma, M., Miyamura, K., Hirata, D., Yokoyama, H., & Miyakawa, T. (2004). Involvement of S-

adenosylmethionine in G1 cell-cycle regulation in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(16), 6086-6091. https://doi.org/10.1073/pnas.0308314101
Narang, A., & Pilyugin, S. S. (2006). Bacterial gene regulation in diauxic and nondiauxic growth.
Park, J., Tai, J., Roessner, C. A., & Scott, A. I. (1996). Enzymatic synthesis of S-adenosyl-L-methionine on the preparative scale. *Bioorganic & Medicinal Chemistry Letters*, *4*(12), 2179-2185. https://doi.org/10.1016/s0968-0896(96)00228-3
Peng, B., Williams, T. C., Henry, M., Nielsen, L. K., & Vickers, C. E. (2015). Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities. *Microbial Cell Factories*, *14*, 91. https://doi.org/10.1186/s12934-015-0278-5
Qin, X., Lu, J., Zhang, Y., Wu, X., Qiao, X., Wang, Z., Chu, J., & Qian, J. (2020). Engineering *Pichia pastoris* to improve S-adenosyl- l-methionine production using systems metabolic strategies. *Biotechnology and Bioengineering*, *117*(5), 1436-1445. https://doi.org/10.1002/bit.27300
Qu, S., Yang, K., Chen, L., Liu, M., Geng, Q., He, X., Li, Y., Liu, Y., & Tian, J. (2019). Cinnamaldehyde, a Promising Natural Preservative Against Aspergillus flavus. *Frontiers in Microbiology*, *10*, 2895. https://doi.org/10.3389/fmicb.2019.02895
Roje, S. (2006). S-Adenosyl-L-methionine: beyond the universal methyl group donor. *Phytochemistry*, *67*(15), 1686-1698. https://doi.org/10.1016/j.phytochem.2006.04.019
Ruan, L., Li, L., Zou, D., Jiang, C., Wen, Z., Chen, S., Deng, Y., & Wei, X. (2019). Metabolic engineering of *Bacillus amyloliquefaciens* for enhanced production of S-adenosylmethionine by coupling of an engineered S-adenosylmethionine pathway and the tricarboxylic acid cycle. *Biotechnology for Biofuels*, *12*, 211. https://doi.org/10.1186/s13068-019-1554-0
Shahriari, B., Swersky, K., Wang, Z., Adams, R. P., & Freitas, N. D. (2015). Taking the Human Out of the Loop: A Review of Bayesian Optimization. *Proceedings of the IEEE*, *104*(1), 148-175.
Shobayashi, M., Mukai, N., Iwashita, K., Hiraga, Y., & Iefuji, H. (2006). A new method for isolation of S-adenosylmethionine (SAM)-accumulating yeast. *Appl Microbiol Biotechnol*, *69*(6), 704-710. https://doi.org/10.1007/s00253-005-0009-7
Wang, D., Wang, C., Wu, H., Li, Z., & Ye, Q. (2016). Glutathione production by recombinant *Escherichia coli* expressing bifunctional glutathione synthetase. *Journal of Industrial Microbiology and Biotechnology*, *43*(1), 45-53. https://doi.org/10.1007/s10295-015-1707-5
Xu, G., Shi, X., Gao, Y., Wang, J., Cheng, H., Liu, Y., Chen, Y., Li, J., Xu, X., & Zha, J. (2022). Semi-rational evolution of pyruvate carboxylase from *Rhizopus oryzae* for elevated fumaric acid synthesis in Saccharomyces cerevisiae. *Biochemical Engineering Journal*(177-), 177.
Xu, G., Wu, M., & Jiang, L. (2017). Site-saturation engineering of proline 474 in pyruvate carboxylase from *Rhizopus oryzae* to elevate fumaric acid production in engineered *Saccharomyces cerevisiae* cells. *Biochemical Engineering Journal*, *117*(part_PB), 36-42.
Zhao, W., Hang, B., Zhu, X., Wang, R., Shen, M., Huang, L., & Xu, Z. (2016). Improving the productivity of S-adenosyl-l-methionine by metabolic engineering in an industrial *Saccharomyces cerevisiae* strain. *Journal of Biotechnology*, *236*, 64-70. https://doi.org/10.1016/j.jbiotec.2016.08.003
Zhao, W., Shi, F., Hang, B., Huang, L., Cai, J., & Xu, Z. (2016). The Improvement of SAM Accumulation by Integrating the Endogenous Methionine Adenosyltransferase Gene *SAM2* in Genome of the Industrial *Saccharomyces cerevisiae* Strain. Applied *Biochemistry and Biotechnology*, *178*(6), 1263-1272. https://doi.org/10.1007/s12010-015-1943-1

Figure legends

Figure 1. a. Schematic illustration of the metabolic engineering strategies involved in S-adenosylmethionine biosynthesis in this study. *SAM2*, methionine adenosyltransferase, *met6*, 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, *str2*, cystathionine gamma-synthase, *PYC*, pyruvate carboxylase, *erg6*, sterol 24-C-methyltransferase, *erg4*, delta(24(24(1)))-sterol reductase, *CYS4*, cystathionine beta-synthase, *SAH1*, adenosylhomocysteinase, TCA cycle tricarboxylic acid cycle, SAH S-adenosylhomocysteine; b. Schematic illustration of knocking out *erg4* and *erg6* using gene editing technology; c. Schematic illustration of replacing original promoter of *CYS4* with *SSA1* promoter using gene editing technology.

Figure 2. a. OD_{600} value of the corresponding recombinants constructed in the study; b. Glucose consumption of the corresponding recombinants constructed in the study; c. SAM titer the corresponding recombinants constructed in the study; d. L-Met content of 2-1C, C2 and C262

Figure 3. a. Glucose consumption of the corresponding recombinants constructed in the study; b. OD600 value of C262P, C262P4 and C262P6; c. Intracellular ATP supply level of C262, C262P and C2621; d. mRNA expression level of *CYS4* gene in C262P6 and C262P6S

Figure 4. Comparison chart of OD_{600} and SAM titer of the recombinant constructed in this study

Figure 5. a. Comparison chart of OD_{600} and SAM titer of the recombinant with different exogenous L-Met supply b. Comparison chart of OD_{600} and SAM titer of the recombinant in optimized and unoptimized medium

Table1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and characteristics	Source or reference
E. coli JM109		Lab collection
S. cerevisiae CEN.PK 2-1C	*MATa*; *his3Δ1*; *leu2Δ0*; *trp1Δ0*; *ura3Δ0*	Lab collection
C2	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*	This work
C26	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6*	This work
C262	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*	This work
C2621	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*, pRS304-*adk1*	This work
C262P	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*, pRS304-*PYC*	This work

Strain or plasmid	Relevant genotype and characteristics	Source or reference
C262P*Δ4*	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*, pRS304-*PYC,* deleting *erg4*	This work
C262P*Δ6*	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*, pRS304-*PYC,* deleting *erg6*	This work
C262P*Δ6S*	*S. cerevisiae* CEN.PK 2-1C harboring plasmids pRS306-*SAM2*, pRS305-*met6str2*, pRS304-*PYC,* deleting *erg6,* replacing *CYS4* promoter by *SSA1*	This work
pRS306	*E. coli* (*Amp^R*), *S. cerevisiae* (Ura)	Lab collection
pRS305	*E. coli* (*Amp^R*), *S. cerevisiae* (Leu)	Lab collection
pRS303	*E. coli* (*Amp^R*), *S. cerevisiae* (His)	Lab collection
pRS306-*SAM2*	*E. coli* (*Amp^R*), *S. cerevisiae* (Ura), pRS306 harboring gene *SAM2*	This work
pRS305-*met6*	*E. coli* (*Amp^R*), *S. cerevisiae* (Leu), pRS305 harboring gene *smet6*	This work
pRS305-*met6str2*	*E. coli* (*Amp^R*), *S. cerevisiae* (Leu), pRS305 harboring genes *met6* and *str2*	This work
pRS304-*adk1*	*E. coli* (*Amp^R*), *S. cerevisiae* (His), pRS303 harboring gene *adk1*	This work
pRS303- *PYC*	*E. coli* (*Amp^R*), *S. cerevisiae* (His), pRS303 harboring gene *PYC*	This work
PCRCT-LHB-*erg4*	*E. coli* (*Amp^R*), *S. cerevisiae* (Trp), PCRCT-LHB-Trp integrating sgRNA-*erg4*	This work
PCRCT-LHB-*erg6*	*E. coli* (*Amp^R*), *S. cerevisiae* (Trp), PCRCT-LHB-Trp integrating sgRNA-*erg6*	This work
PCRCT-LHB-*SSA1*	*E. coli* (*Amp^R*), *S. cerevisiae* (Trp), PCRCT-LHB-Trp integrating sgRNA-*CYS4*	This work

Table 2 Primers used in this study

Primers	Sequence
*SAM2*F	CGCGGATCCATGTCCAAGAGCAAAACTTTC
*SAM2*R	GGGGGCCCAAGCTTTTAAAATTCCAATTTCTTTGG
UraF	AGGCCTTTTGATGTTAGCAGAATTG
UraR	CTAGGTTCCTTTGTTACTTCTTCTGC
*met6*F	CTGCAGGAATTCGATATCAAGCTTATGGTTCAATCTGCTGTCTTAGGGTTC
*met6*R	GAGGTCGACGGTATCGATAAGCTTTTAATTCTTGTATTGTTCACGGAAGTACTTGGCG
LeuF	TAGGGCCATGAAAGCGGCCATTCTTG
LeuR	CAACATGAGCCACCATTGCCTATTTGGTCC
*adk1*F	GTCCCTATTTCAATCAATTGAA

Primers	Sequence
*adk1*R	GCAAATGGCATTCTGACATCC
*PYC*F	ATCCCCCGGGCTGCAGGAATTCATGCCTGCTGCACCAGTACGTGAACAC
*PYC*R	CGATAAGCTTGATATCGAATTCTTAGGCTTCCTCTTTGACAACCTTGGCCA
*erg4*UF	ATATATCTCACAGCTTGCGCAG
*erg4*UR	AGCGATGCTAATCTATGTACACTAC
*erg4*DF	GTAGTGTACATAGATTAGCATCGCTTGATGTATTTACGCGACAAAAGGG
*erg4*DR	CCTGCAGGTTATTACGTTGGC
*erg6*UF	CACTCATGGTTTAAGAAACAACTTTTCC
*erg6*UR	AAGTAAAACAGATAAGGGAAACTTGAATG
*erg6*DF	CCCTTATCTGTTTTACTTCGATATATACCTATTTTCCTATATATGCAGATAAA
*erg6*DR	CAACTTTATTATGAGGGAGTTGATTATTG
*CYS4*UF	AACCTTGCAGAGTCATTGTTCACATC
*CYS4*UR	GTGAAGTGCCTTGCGTTTACTTTAAC
*CYS4*DF	AACACTTGAAGATTTCGTTGTAGGCC
*CYS4*DR	CTTGGAAATACCGCTAATAGTCCCAC
*SSA1*F	GTAAACGCAAGGCACTTCACCCTTGATCGTTGGCAATAATGTCCAC
*SSA1*R	CAACGAAATCTTCAAGTGTTCGTTTAGAAGCTGTCATTTGCGTT
q*CYS4*F	TCTTCCGGTTCTGCCTTCAC
q*CYS4*R	GAGTCAAAACGGGCCAACAC
qATC1F	TCAGAGCCCCAGAAGCTTTG
qATC1R	GAGCCAAAGCGGTGATTTCC

Figure 1

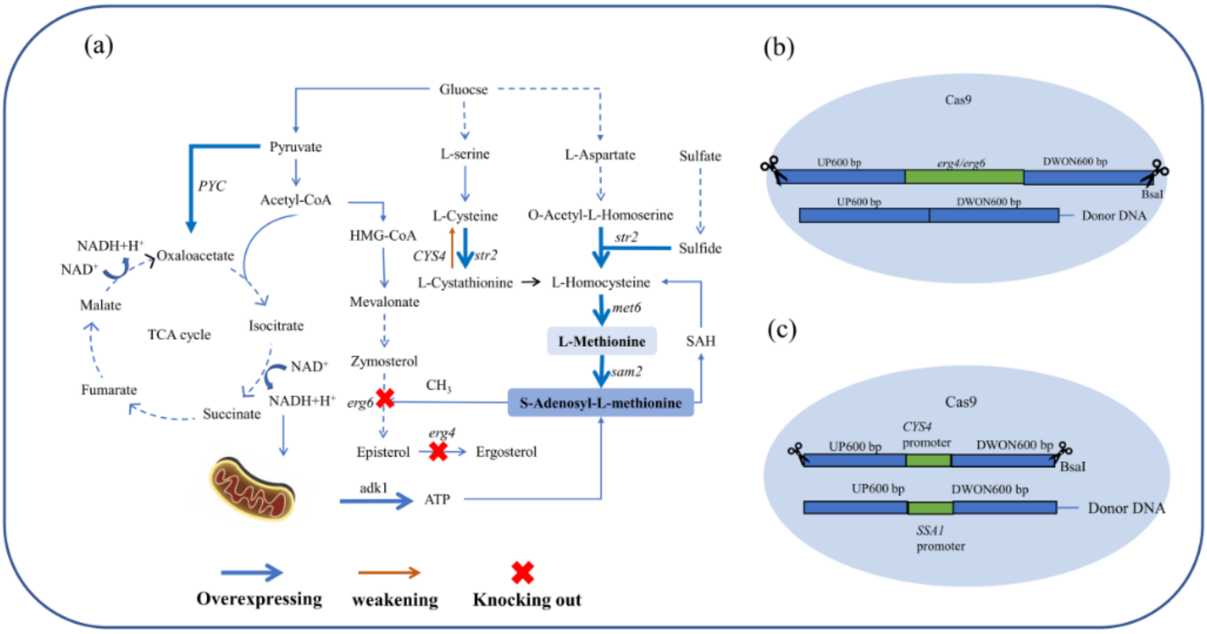


Figure 2

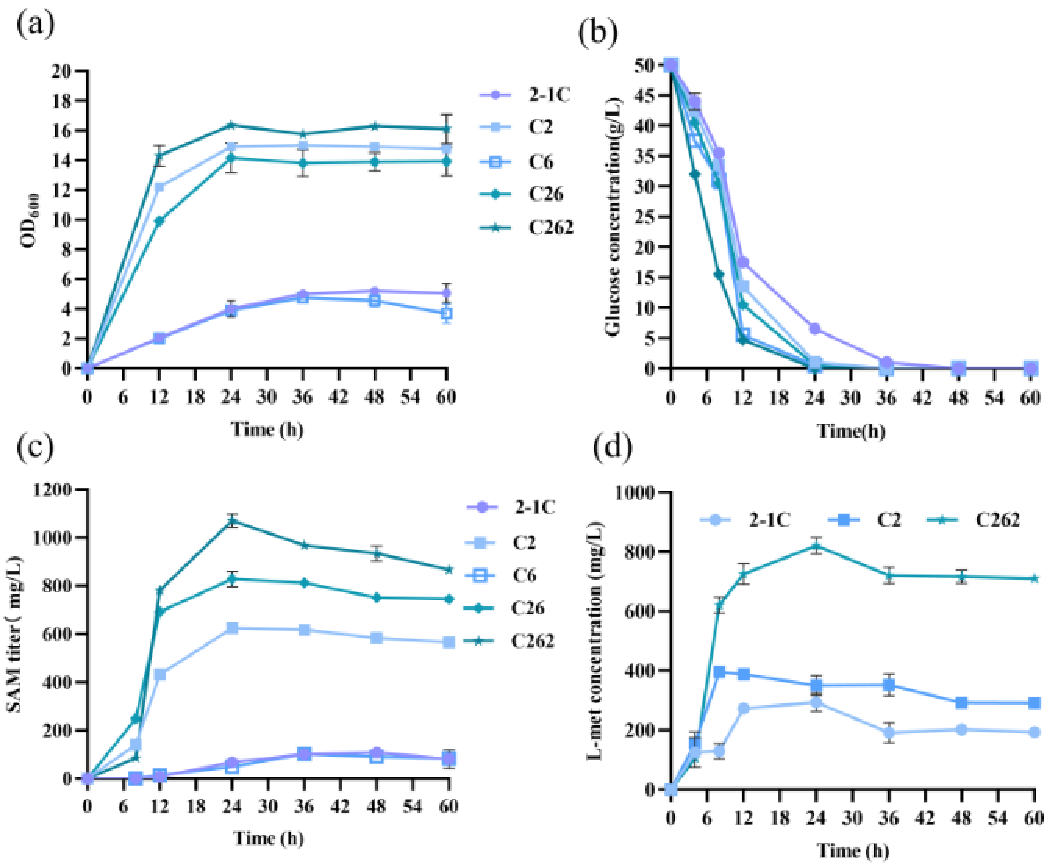


Figure 3

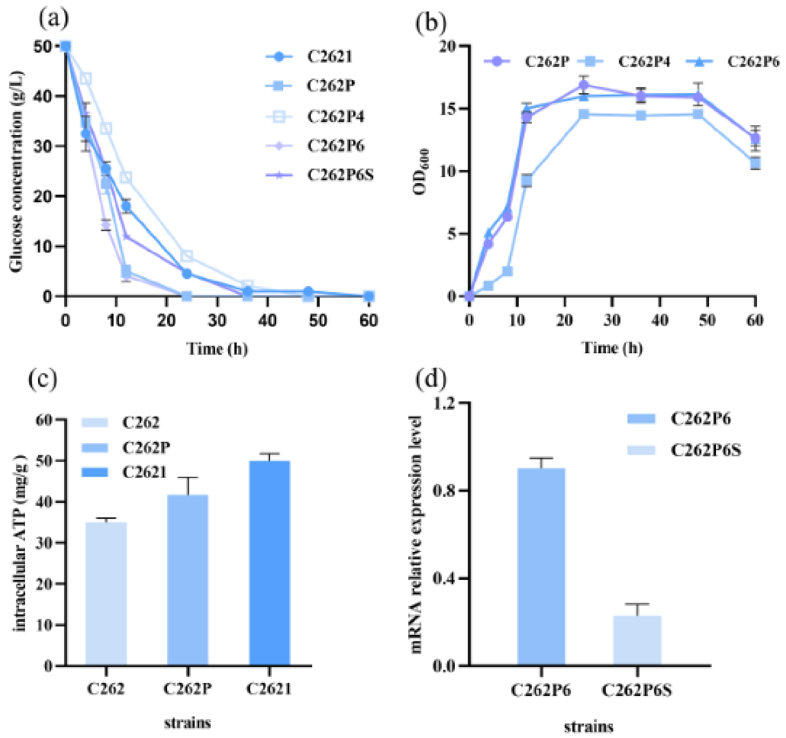


Figure 4

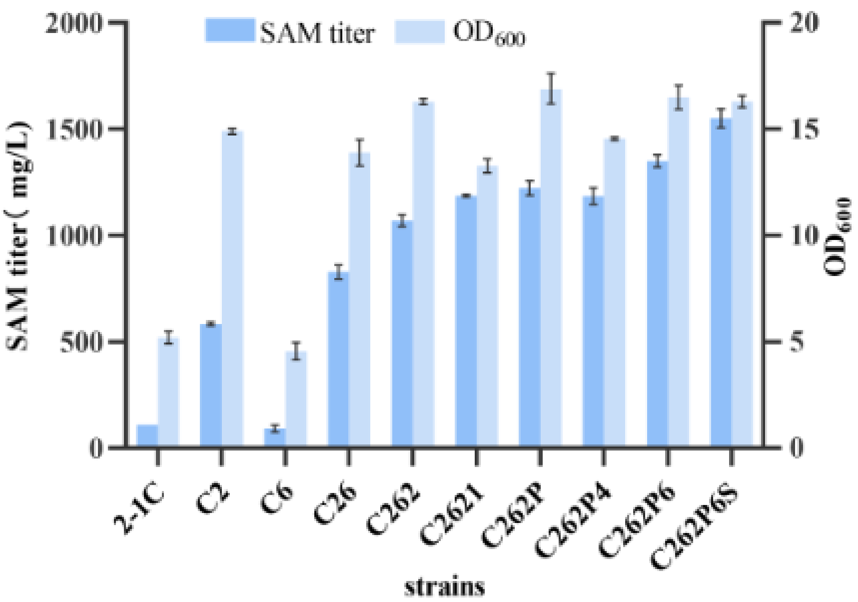
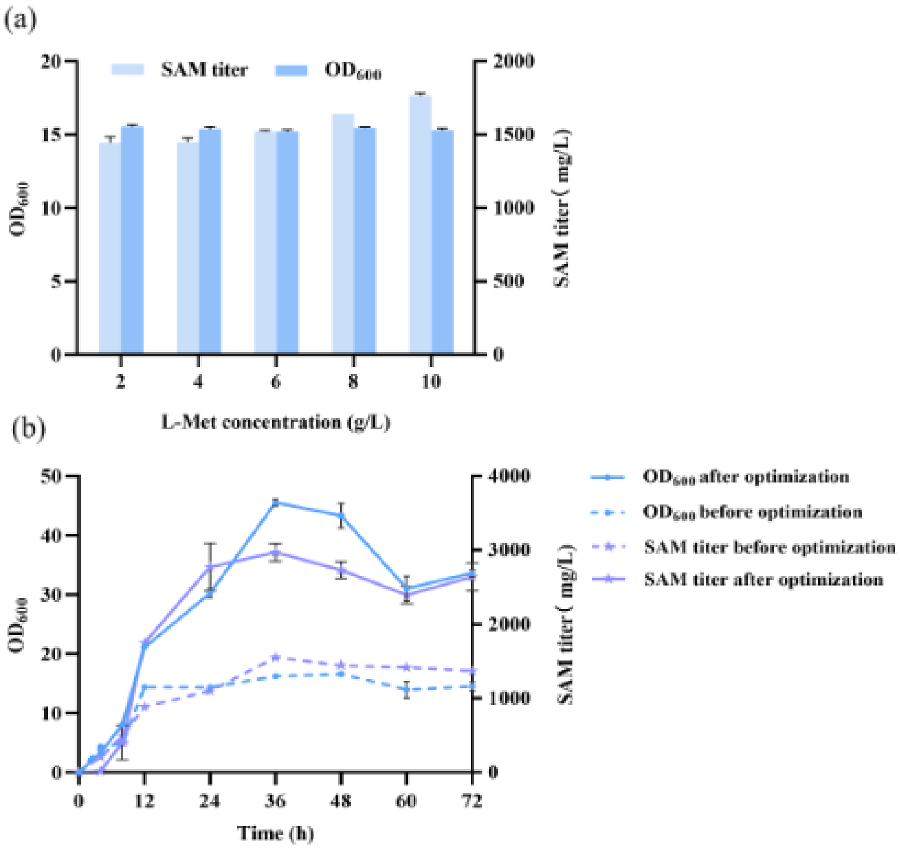


Figure 5



(a)
SAM titer
OD600
20
15
10
5
0
2000
1500
1000
500
0
OD600
SAM titer (mg/L)
2
4
6
8
10
L-Met concentration (g/L)
(b)
50
40
30
20
10
0
4000
3000
2000
1000
0
OD600
SAM titer (mg/L)
0
12
24
36
48
60
72
Time (h)
OD600 after optimization
OD600 before optimization
SAM titer before optimization
SAM titer after optimization