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

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

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 agriculture and medical field. *Saccharomyces cerevisiae* has been engineered as a platform with significant potential for producing SAM, although the current production has room for improvement. Thus, a method that consists of a series of metabolic engineering strategies was established this study. These strategies included enhancing SAM synthesis, increasing ATP supply, and down-regulating SAM metabolism and downregulating competing pathway. 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-producing strains using metabolic engineering strategies and demonstrates the effectiveness of Bayesian optimization in optimizing fermentation medium to enhance the generation of SAM.

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. [1] In the medical field, SAM can be used to treat arthritis, heavy depression, liver diseases and low sperm activity in infertile patience. [2] 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, [3] so it is difficult to adapt to the conditions of industrial production; the enzymatic method synthesizes SAM by directly adding 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;[4] 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. [5] 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* [6] 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. [5] 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. [7] This reveals that using the gene manipulation to drive metabolic flux can effectively improve SAM synthesis. (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.[8] Because ATP not only effects the cell growth, 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. [9] 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. [10] In addition to these strategies, controlling dissolved oxygen levels to generate sufficient ATP can also be effective in increasing the production of targeted metabolites. [11] 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%. [12] Hu knocked out the *sod1* gene in *Saccharomyces cerevisiae* to increase the supply of ATP, and SAM production increased by 22.3%.[13] Yawei Chen improved the oxygen carrying capacity of cells by introducing Vitreoscilla hemoglobin and phosphite dehydrogenase to ensure the ATP supply when cell growth reaching to a certain level and resulted in 37% and 24% SAM increase, respectively. [14] (3) Downregulating the further metabolism of SAM. SAM can act as a methyl provider for the ergosterol synthesis pathway.

Thus, downregulating its further metabolism can presumably reduce SAM from further consuming, hence the 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; [15] Mizunuma identified a *sah1* mutant that suppressed the Ca^{2+}-sensitive phenotypes of the *zds1*Δ strain and its SAM accumulation was 37.2 times higher than that of the wildtype. [16](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 increasing SAM by 42%.[17] 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. [18]

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 have 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. [19] *S. cerevisiae* has vacuoles filled with negatively charged polyphosphates, it can enrich positively charged SAM, [20] and the gene manipulation technology in *S. cerevisiae* is more sophisticated than it is in other chassises. 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 to enhance L-Met supply. Then *adk1* encoding adenylate kinase and an exogenous gene *PYC* encoding pyruvate carboxylase originated from *Rhizopus oryzae* were respectively overexpressed 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 were knocked out respectively by using gene editing technology. 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.

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 .[21, 22] 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 employed 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-producing 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), at 30 °C under shaking at 220 rpm. Engineered strains were cultured in original 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 S1.

2.3 Overexpressing 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 were 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 (Table S1). 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. 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 medium, and cultured at 30 °C for 2-3 d.

2.4 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 (Figure 1B). 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 corresponding company 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.

2.5 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*(Figure 1C).

2.6 Determination of and Glucose Content and glucose

The fermentation broth was diluted with deionized water and mixed well, so that OD600 value is between 0.2 and 0.8, the absorbance value was detected at wavelength of 600nm. 1mL of fermentation broth was centrifuged at 12,000rpm for 2min, supernatant was diluted to make the final glucose concentration within the detection range of equipment 0- 1 g/L, the glucose concentration was measured with Silman biosensor.

2.7 Determination of SAM Content

Took 1 mL of fermentation broth, centrifuge at 12,000 rpm for 2 min, discard the supernatant, added 2 mL of 1.5 M perchloric acid solution, shook at 30 °C for 2 h, and then centrifuged at 8,000 rpm for 10 min, and filtered the supernatant through a 0.22 μm membrane after HPLC detection. The chromatographic column was Hypercil GOLDTM aQ C18 (4.6 mm×250 mm), mobile phase: 0.01 mol·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·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.

2.8 Determination of L-Met Content

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

2.9 Determination of ATP Content

The mobile phase was 95% (v/v) 0.05 mol·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.

2.10 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 UNlQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech). The titer and purity of RNA were determined, and RNA was stored at -80℃ 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 (Table S1). Experimental data were analyzed by GraphPad 8.0.

2.11 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 maximized 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

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

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

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

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

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 $p(Y|X, \sigma_f, l)$.

2.12 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:

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

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 such an x was conducted with a genetic algorithm. After the test of searched condition, the results 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 [21, 22].

3. RESULTS

3.1 Enhancing L-Met Supply by Overexpressing *SAM2, met6* and *str2*

As the direct precursor of SAM, enhancement of L-Met supply can effectively boost SAM production by bacterial fermentation. [23] Therefore, we intended to enhance the expression of genes coding key enzymes in the SAM synthesis pathway. Firstly, *SAM2* and *met6* controlling

the last step of L-Met synthesis were respectively overexpressed in *S. cerevisiae* CEN.PK 2-1C resulting two recombinants named C2 and C6. The corresponding parameters were measured and analyzed (Figure 2). The results showed that the cell growth of C2 was greatly strengthened and the SAM titer also exhibited a substantial increase reaching 616.5 mg/L, which is 4.7 times higher than its parent strain (Figure 2A). The OD_{600} and SAM titer (102.9 mg/L after 24 h fermentation) of the engineered strain C6 both showed no difference compare to its parent strain, indicating that the overexpression of the *met6* gene did not cause a burden on the growth of the strain (Figure 2AE).

Thus, *met6* gene was subsequently overexpressed in C2 and a series of fermentation of the resulting strain C26 was performed in a shake flask. C26 entered the stationary phase at 36 h, and the glucose was also depleted as it entered the stationary phase (Figure 2C). 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 (Figure 2B). 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 C2 (Figure 2C).

In order to further enhance the L-Met supply of the SAM synthesis pathway, the gene that was involved in several reactions (Figure 1A) in the SAM synthesis pathway *str2* gene was overexpressed in C26 and resulted in a recombinant named C262. A 60-hour fermentation was performed on C262. The SAM titer and production intensity of engineering strain C262 was 1,070.8 $mg \cdot L^{-1}$ 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 (Figure 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 2E). Notably, 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*.

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 needed in multiple biochemical reactions and it serves as an adenosine donor in SAM synthesis. Pyruvate carboxylase (*PYC*) controls the metabolic step limiting the production of target carboxylic acids (Figure 1A). [24] Adenylate kinase encoded by *adk1* gene of *S. cerevisiae* converts AMP to ATP (Figure 1A). [25] Therefore, overexpression of *adk1* and *PYC* were conducted in strain C262 resulting in recombinants named C2621 and C262P respectively. Later, a 60-h fermentation of C262, C2621, and C262P was performed and their fermentation performances were compared. It was shown that SAM titer reached 1185.8 mg/L and 1222.0 mg/L at 24 h when overexpressing *adk1* and *PYC* increased by 10.7% and 14.2% respectively compared with C262 (Figure 3A).

The intracellular ATP supply of C2621 and C262P increased by 42.88% and 19.19% respectively comparing with C262 (Figure 3A). Despite the great increase of ATP supply caused by overexpression of *adk1*, it also showed a burden on cell growth, however, this phenomenon

309 did not appear in C262P with less ATP supply increase (Figure 3AB). Therefore, C262P was
310 chosen for further investigation.
311
312 **3.3 Enhancing SAM Synthesis by Downregulating SAM Further Metabolism Pathway**
313 SAM acts as a methyl radical donor [2] via the reaction where zymosterol is converted into
314 ergosterol serving as an important constituent of cytomembrane (Figure 1A). [26] This process
315 is controlled by a series of genes including *ergX* genes, which can be divided into two categories
316 regarding cell growth: essential genes and nonessential genes. Among these genes, *erg4* and
317 *erg6* are nonessential genes which means disrupting them will not affect cell growth generally.
318 Thus, Crispr-Cas9 technology was utilized to disrupt *erg4* and *erg6* in C262P and resulted in
319 two recombinants named C262P4 and C262P6. Then C262P, C262P4, and C262P6 were
320 cultured in a shake flask and their fermentation performances were compared.
321 Disrupting *erg6* brought a 10.39% SAM increase compared with C262P, reaching 1349.7
322 mg/L, while disrupting *erg4* not only did not show SAM increase but the cell growth of C262P4
323 throughout the whole fermentation process was greatly inhibited compared with C262P and
324 C262P6 (Figure 3CD). And the glucose consuming rate of C262P4 was also lower than that of
325 C262P and C262P6 (Figure 3E). Therefore, recombinant C262P6 was chosen for further
326 investigation.
327 **3.4 Enhancing SAM Synthesis by Downregulating Competing Pathways**
328 Cystathionine-β-synthase (CBS) encoded by *CYS4* gene can catalyze the synthesis of
329 cystathionine from homocysteine, leading to the reflux of cystathionine and reducing its flow
330 to the SAM precursor L-Met (Figure 1A). It was reported that disrupting *CYS4* in *P. pastoris*

has been shown to significantly increase SAM production, [18] but it also results in cysteine deficiency, requiring the addition of cysteine to the fermentation medium, which increases production costs. 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* resulting in a recombinant named C262P6S. Subsequently, C262P6 and C262P6S were cultured in a shake flask and their fermentation performances were compared. 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 (Figure 4B). The SAM titer of C262P6S reached 1551.9 mg/L, which was 15.0% higher than that of C262P6 (Figure 4A). Furthermore, the transcriptional level of *CYS4* with its original promoter and *SSA1* promoter was compared (Figure 4A). The results showed that the transcriptional level of *CYS4* with the *SSA1* promoter was 28.3% than that of *CYS4* with its original promoter.

3.5 Optimization of Medium

As both endogenous and exogenous L-Met can affect the SAM production of engineered strains, The concentration gradient of L-Met was set from 2 to 10 g/L and their influences on the SAM titer and cell growth 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. SAM titer started to increase as the L-Met concentration grew to 6 g/L and above, and the highest SAM titer reached 1769.3 mg/L when L-Met concentration was 10 g/L (Figure 4C). Since the precursor addition time also affects SAM production, a batch culture where 10 g/L L-Met was added at 0 h, 8 h, 12 h and 24 h was performed on strain C262P6S in

order to investigate the best precursor-adding time. As is shown, the SAM titer was the highest when L-Met was added at 0 h (Figure 4D).

Finally, a fermentation verification of the engineered strain was performed on the medium that was acquired by Bayesian optimization. The OD_{600} value reached 46.5 at 36 h, which is 2.73 times higher than that of the recombinant's OD_{600} value under unoptimized conditions (Figure 4E). 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 (Figure 4E). Then the engineered strain was fermented in the original medium, medium acquired by orthogonal tests and medium acquired by Bayesian optimization, the OD_{600} and SAM titer were compared. Compared to medium acquired by orthogonal tests, SAM titer and OD_{600} increased 28.2% and 95.0% respectively in medium acquired by Bayesian optimization demonstrating the effectiveness of Bayesian 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 (Figure 4E).

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 enhanced by overexpressing the *adk1* gene and *PYC* gene. By utilizing CRISPR-Ca9 technology, 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 increasing SAM production. What's more, Bayesian optimization was firstly employed in SAM production to this date.

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. [29] Firtly, 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. [30] 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 lead 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. [5]*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

396 citric acid cycle. Besides, it links the high-capacity glycolytic pathway in *S. cerevisiae* to the
397 synthetic pathway of the desired product. [32] Xu conducted a heterologous expression of
398 pyruvate carboxylase (*PYC*) encoding gene from *R. oryzae* resulted in an increase in fumaric
399 acid titer in *S. cerevisiae*. [33] When overexpressing *PYC*, excess oxaloacetic acid will be
400 transported to mitochondria for glucose synthesis, providing a substrate supply for SAM
401 synthesis. As an agonist of *PYC*, AcCoA will increase when *PYC* is overexpressed, so that more
402 NADH will be produced from the tricarboxylic acid cycle. NADH can produce ATP molecules
403 under the catalysis of the *adk1* gene through the electron transport chain in mitochondria, and
404 some of the ATP will act as adenosine donors along with L-Met to form SAM. It was proven
405 that the cell growth was inhibited while intracellular ATP level increased to a certain level, [34]
406 which is identical to the case of overexpressing of *adk1*.
407 As a methyl donor, SAM participates in the pathway of ergosterol synthesis in *S. cerevisiae*.
408 Zhao measured the content of ergosterol after disrupting *erg4* in *S. cerevisiae* BY4741, and the
409 results showed that the content of ergosterol was only 33.8% of that of its parent strain. [35] It
410 was speculated that the non-prosperous ergosterol synthesis could be responsible for no
411 improvement of SAM production and greatly inhibited cell growth after the disruption of *erg4*.
412 Shobayashi detected the content of ergosterol after deleting *erg4* gene in *S. cerevisiae*, and no
413 ergosterol was not detected in the extracts of *erg4* disruptants whereas the intermediate
414 compound of ergosterol that originated from *erg4* mutation was. [15] Thus, it was speculated
415 that the deprivation of ergosterol caused by disrupting *erg4* incapacitates the usual synthesis of
416 cytomembrane, which inhibited the cell growth of the recombinants. However, substances that
417 supported the formation of cytomembrane in C262P4 in the later period of its fermentation

were yet to be confirmed. Thus, researches focused on ergosterol synthesis could investigate the dynamic metabolic process in recombinants where *erg4* is disrupted. When knocking out *erg6*, the process of zymosterol regenerating to ergosterol was entirely disrupted which incapacitates SAM to provide methyl for ergosterol synthesis while other sterols functioning as ergosterol like its intermediate compound replace its place in the cytomembrane enabling cells to grow.

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. The choice of sucrose instead of glucose could be rationalized by considering the dynamics of diauxic growth [36] 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.

5. CONFLICT-OF-INTEREST STAEMENT

The authors declare no commercial or financial conflict of interest.

6. ACKNOWLEDGMENTS

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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.

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author on reasonable request.

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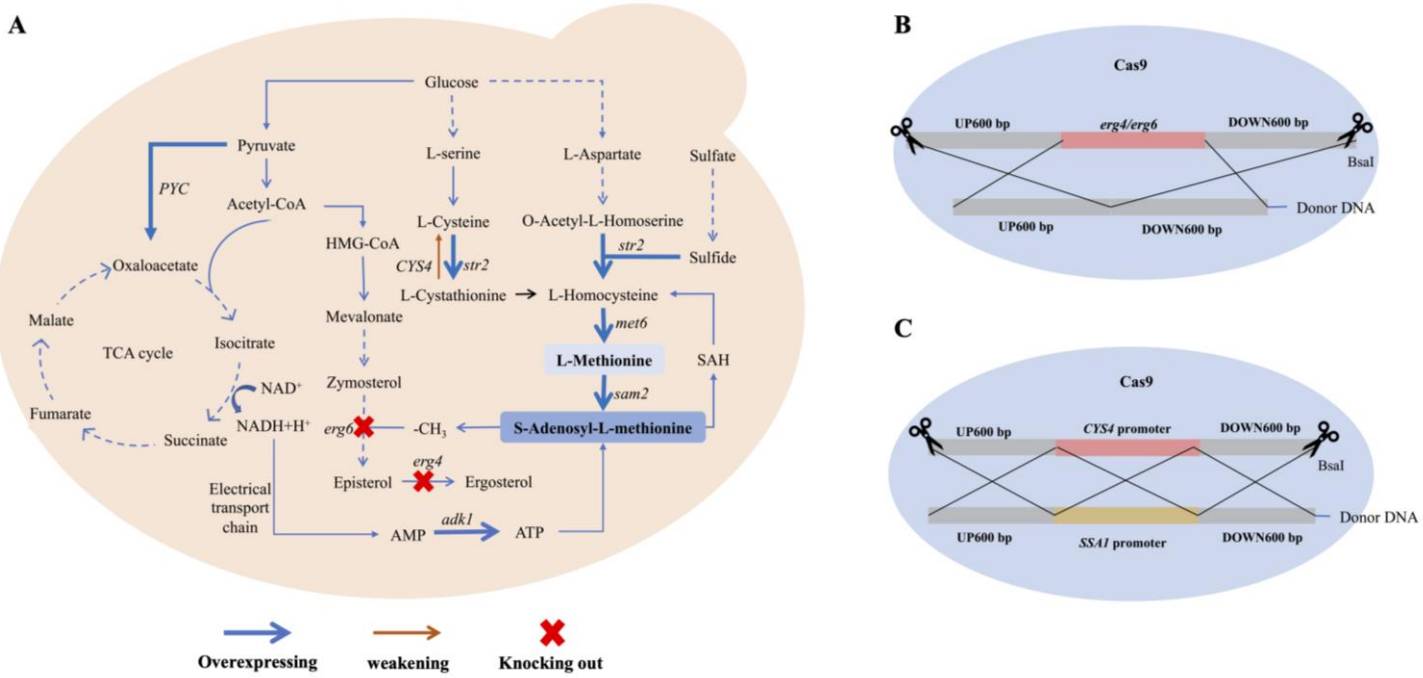
Tables

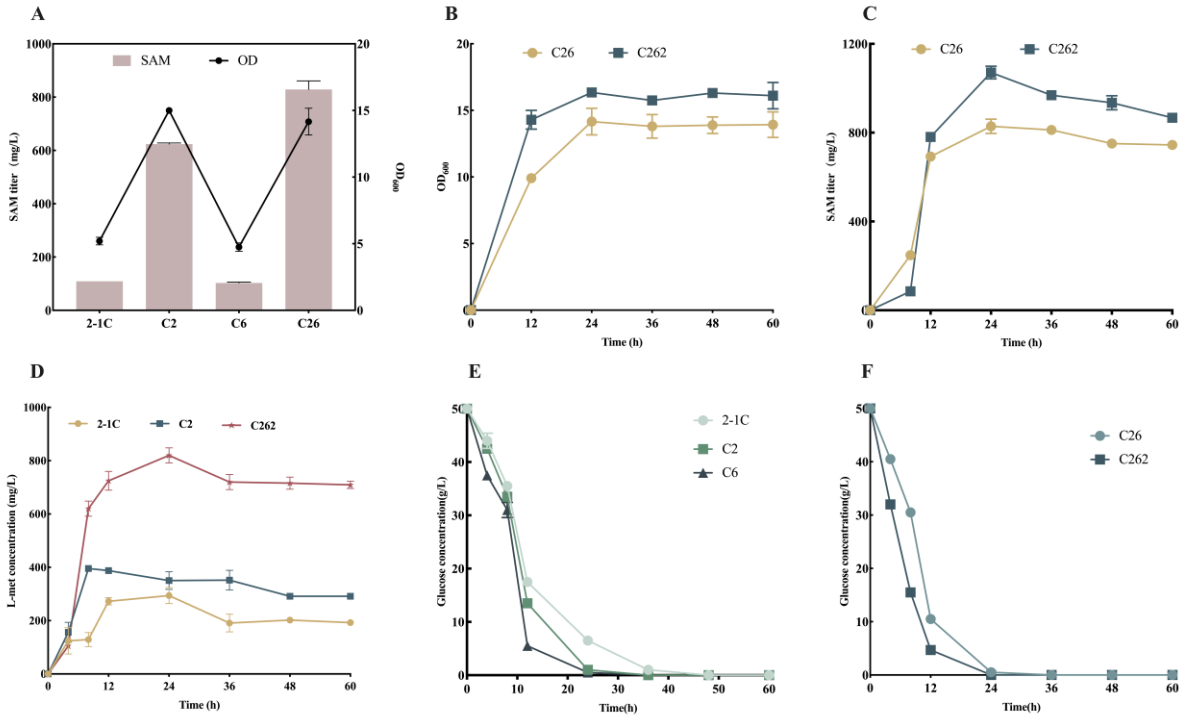
Table 1 Strains and plasmids used in this study

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
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

Figures



564 **Figure 1**. a. Schematic illustration of the metabolic engineering strategies involved in S-
565 adenosylmethionine biosynthesis in this study. *SAM2*, methionine adenosyltransferase, *met6*,
566 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, *str2*, cystathionine
567 gamma-synthase, *PYC*, pyruvate carboxylase, *erg6*, sterol 24-C-methyltransferase, *erg4*,
568 delta(24(24(1)))-sterol reductase, *CYS4*, cystathionine beta-synthase, *SAH1*,
569 adenosylhomocysteinase, TCA cycle tricarboxylic acid cycle, SAH S-adenosylhomocysteine;
570 b. Schematic illustration of knocking out *erg4* and *erg6* using gene editing technology; c.
571 Schematic illustration of replacing original promoter of *CYS4* with *SSA1* promoter using gene
572 editing technology.
573

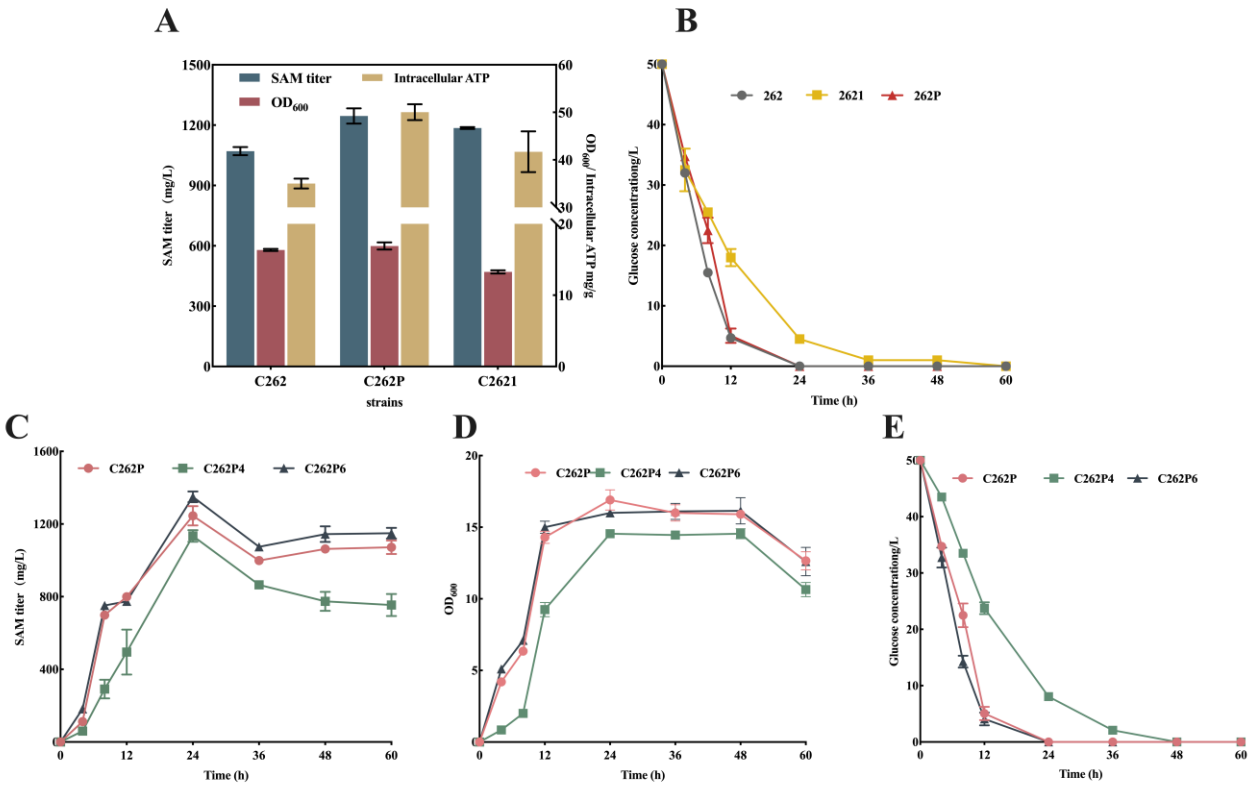


574 **Figure 2**. A. OD_{600} value and SAM titer of strain 2-1C, C2, C6 and C26; B. OD_{600} of strain

575 C26 and C262 throughout 60 h fermentation; C. SAM titer t of strain C26 and C262 throughout

576 60 h fermentation; D. L-Met content of 2-1C, C2 and C262; E. glucose consumption of strain

577 2-1C, C2 and C6; F. glucose consumption of strain C26 and C262.



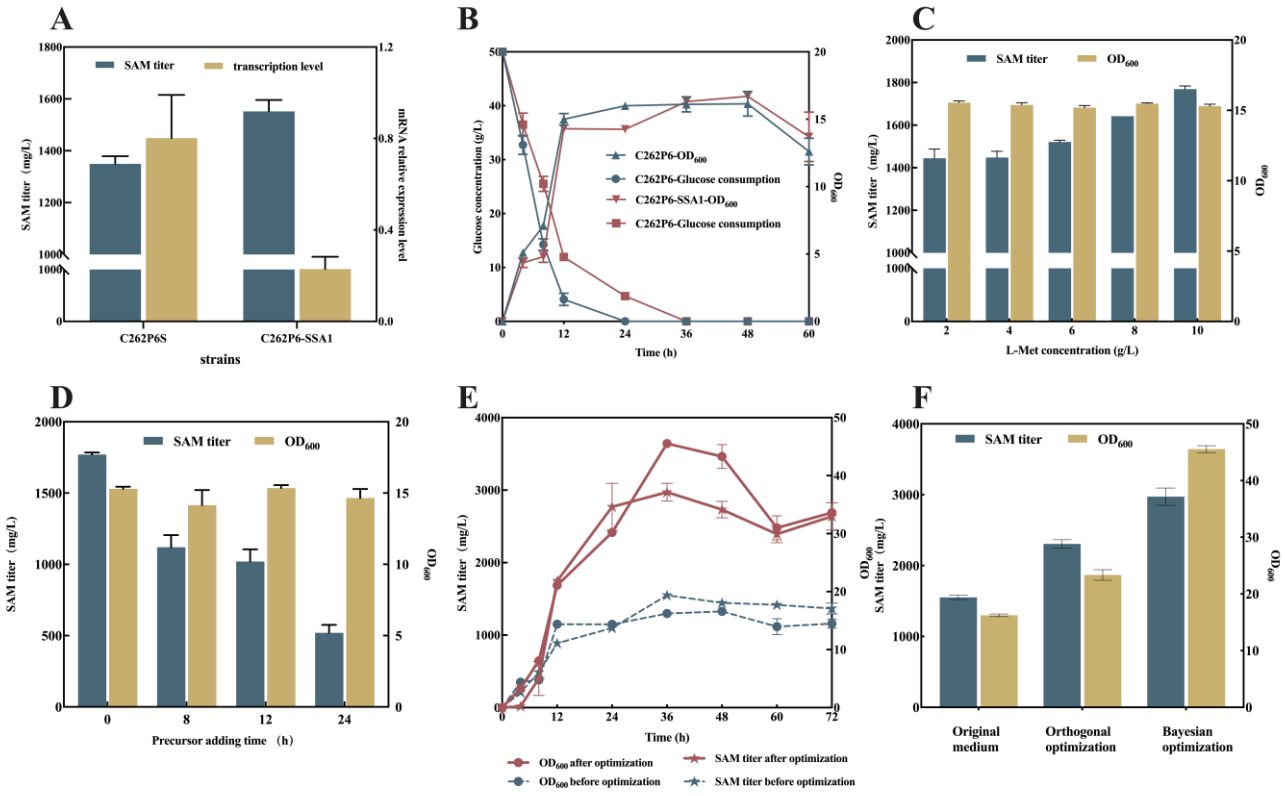
578 **Figure 3**. A. Illustration of SAM titer, Intracellular ATP and OD_{600} of engineered strain of

579 C262, C262P and C2621. B. Glucose consumption of engineered strain of C262, C262P and

580 C2621; C. SAM titer engineered strain of C262P, C262P4 and C262P6; D. OD_{600} value of

581 engineered strain C262P, C262P4 and C262P6; E. Glucose consumption of engineered strain

582 of C262P, C262P4 and C262P6.



583 **Figure 4**. A. Illustration of SAM titer of engineered strain C262P6-SSA1 and C262P6 and
584 the CYS4 mRNA relative expression level of corresponding strains; B. OD_{600} value and glucose
585 consumption and engineered strain C262P6-SSA1 and C262P6; C. Comparison chart of OD_{600}
586 and SAM titer of the recombinant with different exogenous L-Met supply D. Comparison chart
587 of OD_{600} and SAM titer of the recombinant with different L-Met adding time; E. Comparison
588 chart of OD_{600} and SAM titer of the recombinant cultured in optimized and unoptimized
589 medium; F. Comparison chart of OD_{600} and SAM titer of the recombinant cultured in original
590 medium, medium acquired by orthogonal tests and medium acquired by Bayesian optimization.