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Elevated intracellular acetyl-CoA availability by *acs2* overexpression and *mls1* deletion combined with *metK1* introduction enhanced SAM accumulation in *Saccharomyces cerevisiae*

Hailong Chen, Yang Yang, Zhilai Wang, Jie Dou, Hui Wang, Changlin Zhou *

State Key Laboratory of Natural Medicines, School of Life Science & Technology, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, PR China

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ABSTRACT

S-Adenosyl-L-methionine (SAM), with diverse pharmaceutical applications, is biosynthesized from L-methionine and ATP. To enhance SAM accumulation in *Saccharomyces cerevisiae* CGMCC 2842 (2842), a new strategy based on yeast acetyl-CoA metabolism combined with introducing a methionine adenosyltransferase (*metK1*) from *Leishmania infantum*, was presented here. It was found that over-expressing *acs2* (encoding acetyl-CoA synthase) and deleting *mls1* (encoding malate synthase) increased SAM by 0.86- and 1.30-fold, respectively. To eliminate feedback inhibition of SAM synthase, a codon-optimized *metK1* was introduced into 2842, and an increase of 1.45-fold of SAM was observed. Subsequently, *metK1* and *acs2* were co-expressed in the *mls1* deleted strain, obtained the highly SAM-productive strain Y*mls1* △GAPmK, and 2.22 g/L of SAM accumulated, which was 3.36-fold that in 2842. Moreover, the Y*mls1* △GAPmK strain yielded 6.06 g/L SAM, which was 9.18-fold that in 2842, by fed-batch fermentation in a 10-L fermenter. Finally, the isolation and purification of SAM from yeast cell and preparation of SAM sulfate were preliminarily investigated. This study demonstrated that up-regulating *acs2* and deleting *mls1*, which elevated intracellular acetyl-CoA levels, effectively enhanced the intracellular methionine biosynthesis. The elevated intracellular acetyl-CoA levels ultimately enhanced SAM accumulation, whereas the introduction of *metK1* enhanced the redirection of acetyl-CoA to SAM biosynthesis in Y*mls1* △GAPmK strain.

1. Introduction

S-Adenosylmethionine (SAM), existing in all living organisms, serves as an activated group donor in a range of metabolic reactions, including methylations, *trans*-sulfuration and propylamine group donations [1]. Clinical applications include the treatments of osteoarthritis [2], fibromyalgia [3], depression [4], liver disorders [5] and Alzheimer's disease [6].

Generally, SAM is biosynthesized from methionine and ATP by catalysis of methionine adenosyltransferase (MAT). The addition of methionine to culture medium is essential for enhancing both SAM production and cell growth [7]. It was found that continuously feeding *Pichia pastoris* cultures with 0.2 g/L/h of L-methionine led to the highest SAM accumulation, which reached 8.46 g/L [8]. Wang reported that the precursor amino acids methionine and cysteine were favorable for improving intracellular SAM and GSH content in *Candida utilis*, under proper concentrations [9].

However, excess methionine represses yeast growth and transcription of genes encoding MAT. L-Methionine concentrations greater than 1% in the medium were shown to reduce SAM yield [10]. Moreover, MAT and methylenetetrahydrofolate reductase (MTHFR) are inhibited by abnormally high SAM accumulations. It is necessary to eliminate feedback inhibition of MAT and MTHFR with freshly synthesized SAM. Several mutations and genetic strategies to increase methionine adenosyltransferase activities were previously developed to enhance SAM biosynthesis. The methionine adenosyltransferase from *Saccharomyces cerevisiae* was overexpressed in *Saccharomyces sake* K6-1, which increased SAM production to 2.8 g/L [11]. A SAM-accumulating strain isolated through spaceflight culture *S. cerevisiae* H5M147 produced 86.9% more SAM than the wild strain, with an additional 43.0% SAM accumulation after expressing a heterologous methionine

* Corresponding author. Fax: +86 25 8327 1249.
E-mail address: cl_zhou@cpu.edu.cn (C. Zhou).

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adenosyltransferase [12]. Kant et al. observed that methionine adenosyltransferase is the primary limiting factor and its overexpression is essential to increase the SAM productivity [13]. Moreover, strategies using strong promoters or DNA shuffling were employed to improve MAT activity [8]. All experiments confirmed that over-expressing methionine adenosyltransferase, especially heterogeneous MAT genes using strong promoters, increased SAM accumulation in both *P. pastoris* and *S. cerevisiae* [8,10]. To release MTHFR from feedback inhibition, expressing chimera-1, which comprised the yeast MTHFR N-terminal domain and the *Arabidopsis* MTHFR C-terminal domain increased SAM and L-methionine by 140- and 7-fold, respectively [14]. Thus, genetic approaches were as powerful as fermentation optimization at redirecting the metabolic flux toward the biosynthesis of SAM. However, despite all of these approaches, SAM yields still fall short, so a novel method for producing SAM is necessary.

In our previous studies, a highly SAM-productive yeast strain, *S. cerevisiae* 2842 (CGMCC No. 2842), which exhibited higher ethionine resistance, was selected with an ethionine-resistant screening procedure coupled with ultraviolet irradiation. Ethionine is a methionine analogue, and the mutant strain, 2842, exhibited high ethionine resistance. It was hypothesized that improvement of methionine metabolism would be beneficial for the accumulation of SAM in yeast strain 2842.

In yeast, methionine biosynthesis usually consumes cytosolic acetyl-CoA. Cytosolic acetyl-CoA is mainly produced from acetate under catalysis of acetyl-CoA synthase (ACS2) encoded by *acs2*. In addition, there are two glyoxylate cycle (GYC) genes, *mls1* encoding cytosolic malate synthase (MLS1) and *cit2* encoding peroxisomal citrate synthase (CIT2). It was found that the acetyl-CoA consumed by CIT2 in peroxisomes is formed by ACS1 from acetate, and MLS1 can convert the cytosolic acetyl-CoA into C4 organic acids for oxidation [15]. A number of metabolic engineering studies have focused on increasing the intracellular acetyl-CoA levels for enhancing chemical molecules and fuel biosynthesis that usually consumes acetyl-CoA. For example, over-expressing *acs1* and *acs2* increased acetyl-CoA synthase activity three- to six-fold and increased intracellular acetyl-CoA two- to five-fold, whereas the disruption of the glyoxylate shunt by deleting *cit2* or *mls1* could increase acetyl-CoA levels [16,17]. Moreover, disruption of competing pathways and introducing heterologous biosynthetic pathways with lower energy requirement and higher catalytic efficiency were also carried out to enhance acetyl-CoA availability [18].

Herein, to prove that elevating intracellular acetyl-CoA levels would enhance SAM accumulation, *acs2* up-regulation, *cit2* and *mls1* deletion were first carried out. Besides, methionine adenosyltransferase is the primary limiting factor in the biosynthesis of SAM. MAT is negatively regulated by its product SAM in the micromolar range in most of mammalians sources, as well as in *S. cerevisiae* and *Escherichia coli*. Therefore, eliminating feedback inhibition of methionine adenosyltransferase and improving its activity are essential to increase the SAM productivity. Rosa et al. expressed and characterized methionine adenosyltransferase (encoded by *metK1*) from *Leishmania infantum*. It was found that unlike the mammalian and yeast enzymes, *Leishmania* MAT activity is weakly regulated by its product [19]. To eliminate feedback inhibition and enhance SAM biosynthesis, the codon-optimized *metK1* from *L. infantum* was evaluated in yeast strain 2842 and was then introduced into the recombinant strain that over-expressed *acs2* and deleted *mls1*, obtaining the highly SAM-productive strain Y*mls1* △GAPmK (Fig. 1). Moreover, the ACS2 and MAT activities, as well as intracellular acetyl-CoA and methionine levels, were measured. Finally, the SAM accumulation capacity of Y*mls1* △GAPmK was assessed in a 10-L fermenter.

In addition, SAM is unstable both in solution and in crystalline form. SAM undergoes cleavage to 5′-methylthioadenosine and homoserine lactone, or hydrolysis to adenine and *S*-pentosylmethionine [20]. Research about SAM stabilization has been directed towards the preparation of SAM salts, such as SAM sulfate and chloride, which are stable under special temperature, humidity and pH conditions [21]. Because there is a sulfonium existing in SAM molecular and the amino is easily dissociated in the acidic environment, the SAM sulfate was easily prepared by precipitating the sulfonium compound with sulfuric acid solution [21]. In this study, the isolation and purification of SAM from yeast cell and preparation of SAM sulfate were preliminarily investigated.

The SAM accumulation of the final mutant strain Y*mls1* △GAPmK in fed-batch fermentation achieved 6.06 g/L, which is 9.2-fold of that in parent strain 2842. Although the SAM was yield lower than those reported in literatures [7,12,32]. However, this study demonstrated that elevation of acetyl-CoA levels through over-expressing *acs2* and deleting *mls1*, combined with introduction of *metK1*, significantly enhanced SAM biosynthesis, which showed considerable potential for developing highly SAM-productive strains based on intracellular acetyl-CoA and methionine metabolism.

2. Materials and methods

2.1. Plasmids and strains construction

All plasmids strains and oligonucleotides used are summarized in Table 1. *S. cerevisiae* CGMCC 2842 was used as the parent strain, obtained from the China General Microbiology Culture Collection Center (Beijing, China). For the construction of *mls1* and *cit2* deleted strains, the loxP-KanMX-loxP method, and the short flanking homology regions (SFH) replacement method, were used for successive deletion of these genes [22–24]. SFH replacement cassettes were obtained by PCR amplification using the plasmid pUG6 as the template. The primer was composed of the internal (3′) 19 bases annealing to KanMx and the external (5′) 40 bases corresponding to the target gene. The primer pairs of A and B, C and D were used for deletion of *mls1*, and primer pairs of E and F, G and H were used for deletion of *cit2*. The G418 resistance gene marker was rescued by transforming the plasmid pSH65 with bleomycin resistance into positive transformants and inducing Cre recombinase expression by D-galactose [22].

A 2.4 kb *Bam* HI/*Eco* RI fragment including *acs2* (GenBank: NC_001144.5) and its terminator, and a 1.5 kb *Not* I/*Pae* I fragment including *sam2* (GenBank: NC_001136.10), were PCR-amplified from the chromosomal DNA of 2842. The *metK1* (GenBank: NC_009414.2) with *Not* I/*Pae* I was synthetic after yeast codon optimization. All fragments were inserted into the corresponding sites of pYES-KanMx to obtain pGAL1-*acs2*, pGAL1-*sam2* and pGAL1-*metK1*. To co-express *acs2* and *metK1*, a promoter pPGK1 (GenBank: FJ415226.1), with *Eco* RI/*Not* I amplified using primer pairs pPGK1F and pPGK1R, was incorporated into pGAL1-*acs2*. Then, a *Not* I/*Pae* I digested *metK1* was inserted into pGAL1-*acs2*-pPGK1 to obtain pGAL1-*acs2*-pPGK1-*metK1*. All plasmids were transformed into 2842 using the lithium acetate method with G418 resistance selection [25]. All of the engineered strains were verified by DNA sequencing.

2.2. Culture conditions

All yeast strains were streaked onto YPD plates from glycerol stocks and incubated at 30 °C for 20 h. Single colonies were transferred into 50 mL YPD medium and incubated for 20 h at 30 °C and 200 rpm. To ensure the plasmid was not lost in recombinant strains, 250 μg/mL G418 was added into YPD plates and medium to maintain a selection pressure. YPD medium comprised 1.0% peptone, 2.0% yeast extract and 2.0% glucose. The YPD plate medium