

## Article

# Deleting *Mig1* Combined with Introducing *MetK1* Improved S-Adenosyl-L-Methionine Production in *Saccharomyces cerevisiae*

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## Abstract

*Saccharomyces cerevisiae*, widely used in industrial fermentation, still suffers from inherent problems in the efficient utilization of carbon sources. Here, a strategy for alleviating glucose effect and improving S-adenosyl-L-methionine (SAM) production by deleting *Mig1* combined with introducing *MetK1* from *Leishmania infantum* was applied in *S. cerevisiae*. The deletion of *Mig1* improved glucose utilization by increasing the expression levels of genes related to glucose transport and glycolysis, thereby increasing the levels of glycolytic intermediates and increasing both the transcriptional levels of *ACS1* and *ALD6* and the activity of *ADH2*, which promotes the conversion of ethanol into acetyl-CoA. The deletion of *Mig1* also upregulated the transcripts of genes involved in the metabolism of precursor amino acids of SAM and ultimately responsible for the improvement in SAM synthesis. Finally, *MetK1* was introduced into yeast to redirect carbon flux toward SAM biosynthesis. As expected, the SAM production of the mutant Y*Mig1*Δ*PMetK1* reached 8.91 g/L in a 10 L fermenter, which was 72.3% higher than that of the parent strain *S. cerevisiae* CGMCC 2842 (5.17 g/L) reported in our previous studies. This study revealed that the strategy of alleviating glucose effect and redirecting carbon flux to nonethanol products by *Mig1* deletion combined with heterologous *MetK1* introduction possesses great potential for improving SAM synthesis in yeast cells.

**Keywords:** *Saccharomyces cerevisiae*; S-adenosyl-L-methionine; glucose effect; *Mig1*; *MetK1*



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## 1. Introduction

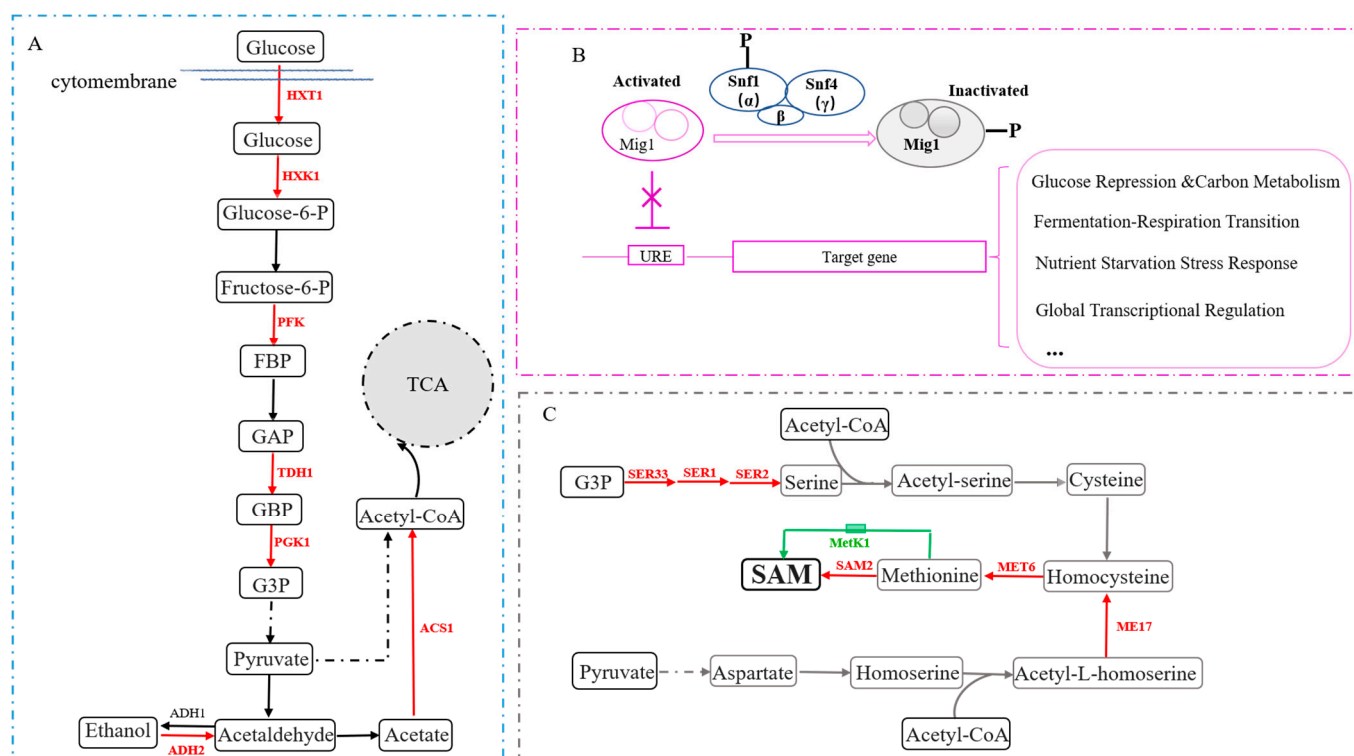
S-Adenosyl-L-methionine (SAM), synthesized from adenosine triphosphate (ATP) and methionine (Met) under the catalysis of methionine adenosyl-transferase (MAT) in living organisms [1–3], is involved in more than 40 biochemical reactions in the body and plays important roles in the synthesis of proteins, nucleic acids, neurotransmitters, phospholipids and vitamins [3,4]. Clinically, SAM is used to treat liver disease. SAM reduces the cholesterol/phospholipid ratio of the liver cell membrane, increases the fluidity of the liver cell membrane, and increases the activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase on the cell membrane to improve the dynamics of bile secretion and flow. It also increases glutathione levels, which help prevent intrahepatic cholestasis [5–8].

Researchers have focused on studies of the microbial production of SAM for many years, encompassing strategies of strain breeding, fermentation process control and optimization, metabolic engineering, and genome-scale engineering [2,9–11]. Our team has been deeply engaged in the microbial production of SAM. In our previous research, a high-SAM-yield yeast strain, *Saccharomyces cerevisiae* CGMCC 2842 (2842), which exhibited high ethionine resistance, was selected via an ethionine-resistant screening procedure coupled with ultraviolet irradiation. The SAM production of the mutant strain 2842 reached 1.7 g/L [12]. Despite the degradation that occurred after a decade of subculture, the SAM productivity of the mutant strain 2842 was still high (0.5 to 0.6 g/L) [2,12]. To increase SAM accumulation in 2842, a strategy based on yeast acetyl-CoA metabolism combined with the introduction of methionine adenosyl-transferase (encoded by the *MetK1* gene) from *Leishmania infantum* was carried out, and a recombinant strain, Ymls1ΔGAPmK, was obtained, which yielded 6.06 g/L SAM by fed-batch fermentation in a 10 L fermenter [13]. In another attempt in strain 2842, inositol pyrophosphate kinase gene *KCS1* and the malate synthase gene *MLS1* were deleted to alter the metabolism of inositol pyrophosphates and acetyl-CoA, after which the acetyl-CoA synthase gene *ACS2* and the SAM synthase gene *MetK1* from *L. infantum* were co-expressed, resulting in the recombinant strain Ymls1ΔKcs1ΔGAPmK, whose SAM production reached 2.89 g/L in a 250 mL flask and 8.86 g/L in a 10 L fermentation tank [14]. The above results establish that the yeast strain 2842 is a eukaryotic organism with great potential for the industrial production of SAM.

Although *S. cerevisiae* has been widely used in industrial fermentation, it still suffers from inherent problems in terms of the efficient and continuous utilization of carbon sources. *S. cerevisiae* exhibits the Crabtree effect (also referred to as glucose effect) [15]. The Crabtree effect tends to drive yeast metabolism toward the anaerobic pathway, which increases ethanol production while lowering the synthesis efficiency of the target product SAM because of the loss of carbon metabolic intermediates, energy and cofactors, which are required for the synthesis of SAM and the survival of yeast cells [3,16,17]. Beyond that, *S. cerevisiae* exhibits a transcriptional regulatory mechanism that is glucose repression. In terms of observable traits, it is manifested as the utilization of industrial carbon sources by yeast is strongly repressed when glucose and fructose are present. Continuous efforts have been made to address these issues. For instance, the production of ethanol can be reduced by an appropriate glucose-feeding strategy [18] or by deleting ethanol dehydrogenases (ADHs) or pyruvate decarboxylases (PDCs) [16,19,20]. Previous studies demonstrated that either deleting ethanol dehydrogenase genes or deleting pyruvate decarboxylase genes completely prevented the production of ethanol but also led to severe physiological defects in yeast [16,18–20]. For example, PDC-deficient strains cannot grow with glucose as the sole carbon source, and ADH-deficient strains exhibit weaker growth [16,19,20]. In our previous studies, a strategy of deleting the protein phosphatase 1 encoding gene *REG1* combined with overexpressing the catalytic subunit of the SNF1 encoding gene *SNF1* for weakening the Crabtree effect and glucose repression was applied to improve the biosynthesis of SAM in *S. cerevisiae* [3]. Thus, how to weaken the Crabtree effect and glucose repression in *S. cerevisiae* and redirect carbon flux is still a challenge for the industrial microbial production of SAM or other nonethanol target products.

The SNF1-Mig1 signaling pathway plays a crucial role in several physiological activities of yeast, such as the carbon metabolism, fermentation–respiration transition, global transcriptional regulation and nutrient starvation stress response, etc. (Figure 1B) [21–25]. In the Snf1-Mig1 glucose signaling pathway, the function of Mig1 (encoded by the *Mig1* gene) in the glucose repression has been well characterized. Mig1, as one of the main effectors of glucose repression, is a zinc finger protein that binds to the promoters of many genes and represses their transcription when glucose is added to the medium [26–28].

Deletion of the *Mig1* gene has been shown to alleviate glucose repression [29]. However, the influences of *Mig1* deletion on the synthesis of target products (SAM in this study), as well as its application in the construction of high-yield strains of SAM, remain unclear and need further investigation. In this study, we wondered whether weakening glucose repression through the deletion of *Mig1* would improve the production of SAM in a high-SAM-yield yeast strain 2842. To verify our hypothesis, a strategy for alleviating the glucose repression by deleting *Mig1* combined with introducing *MetK1* from *L. infantum* to redirect the amino acids to SAM synthesis was carried out (Figure 1). Data such as SAM production, glucose consumption, cell dry weight (DCW), key intermediate metabolites and gene transcriptional levels of related enzymes in the glycolysis pathway, ethanol accumulation, and precursor amino acid metabolism were analyzed and evaluated in this study.



**Figure 1.** Schematic representation of improving the production of SAM by *Mig1* deletion and *MetK1* introduction in *S. cerevisiae*. (A) The deletion of *Mig1* enhanced the glycolytic pathway. (B) The Mig1p plays a crucial role in many physiological activities of yeast. (C) The deletion of *Mig1* gene enhanced the biosynthesis metabolism of the precursor amino acids, and the precursor amino acids were effectively directed to the SAM synthesis pathway by the introduction of *MetK1* gene from *L. infantum*. The solid line represents a single step, and the dotted line represents multiple steps. The color of red represents the transcriptional levels of several genes enhanced by *Mig1* deletion; the green symbols (■) represents the introduction of *MetK1* gene from *L. infantum*. The purple cross (×) represents *Mig1* deletion.

## 2. Materials and Methods

### 2.1. Strains, Plasmids and Primers

All the strains, recombinant plasmids and sequences of primers used in this research are summarized in Table 1. The parent strain of *Saccharomyces cerevisiae*, CGMCC 2842 (2842), was preserved at the China General Microbiology Culture Collection Center (CGMCC, Beijing, China) [12]. The *Mig1* gene (GenBank ID: 852848) knockout strain was generated using the short flanking homology (SFH) replacement method [30]. The specific steps were as follows: To prepare the gene knockout cassette, the pUG6 plasmid was used as the tem-

plate, and PCR amplification was performed to obtain a gene knockout cassette containing the loxP-kanMx-loxP element, which carried a G418 resistance selection marker; for the traceless deletion of the resistance marker, the pSH65 plasmid, which carried bleomycin resistance, was transformed into the positive transformants, and Cre recombinase expression was induced by D-galactose to rescue the G418 resistance gene marker. In the experiment, two pairs of primers, A/B and C/D, were used to complete the PCR fragment amplification of the *Mig1* gene knockout cassette.

**Table 1.** Strains, plasmids and primers.

| Strains, Plasmids and Primers | Relevant Characteristics                                                                                                                  | Source                  |
|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|
| Strains                       |                                                                                                                                           |                         |
| 2842                          | <i>S. cerevisiae</i> CGMCC 2842                                                                                                           | Our previous study [12] |
| YMig1Δ                        | <i>S. cerevisiae</i> CGMCC 2842 derivative, <i>Mig1</i> Δ                                                                                 | This study              |
| YMig1ΔPMig1                   | <i>S. cerevisiae</i> YMig1Δ derivative, containing pGAL1-Mig1                                                                             | This study              |
| YPMetK1                       | <i>S. cerevisiae</i> CGMCC 2842 derivative, containing pGAL1-MetK1                                                                        | This study              |
| YMig1ΔPMetK1                  | <i>S. cerevisiae</i> YMig1Δ derivative, containing pGAL1-MetK1                                                                            | This study              |
| Plasmids                      |                                                                                                                                           |                         |
| pYES 2.0                      | 2μ, <i>URA3</i>                                                                                                                           | Invitrogen              |
| pYES-KanMX                    | pYES 2.0 derivative, 2μ, G418 resistance gene                                                                                             | Our previous study [12] |
| pGAL1-Mig1                    | pYES-KanMX derivative, 2μ, G418 resistance, expression of <i>Mig1</i> from <i>S. cerevisiae</i>                                           | This study              |
| pGAL1-MetK1                   | pYES-KanMX derivative, 2μ, G418 resistance, expression of <i>MetK1</i> , encoding methionine adenosyl-transferase from <i>L. infantum</i> | This study              |
| pUG6                          | Template plasmid containing loxP-KanMX-loxP elements                                                                                      | Euroscarf               |
| pSH65                         | Cre containing plasmid for loxP-KanMX-loxP cassette recycle                                                                               | Euroscarf               |
| Primers                       |                                                                                                                                           |                         |
| Mig1F                         | CCCAAGCTTATGCAAAGCCCCATATCCAATGAC                                                                                                         | This study              |
| Mig1R                         | GGGTACCTCAGTCCATGTGTGGGAAGGGCA                                                                                                            | This study              |
| A                             | CGAGAGTTGAGTATAGTGGAGACGACATACTACCATAGCCACAAGTGTCTAACGTTG                                                                                 | This study              |
| B                             | TACGCTGACAAGTTTTTGGCGGTGCAGATAAATCAAAAGATAGGCCACTAGTGGATCTG                                                                               | This study              |
| C                             | ATGCAAAGCCCATATCCAATGACACAAGTGTCTAACGTTGTACGCTGCAGGTGCACAAC                                                                               | This study              |
| D                             | CATAAGAAGTTTACCGTTGCCCTTCCACACATGGACTGATAGGCCACTAGTGGATCTG                                                                                | This study              |

A 1.5-kb nucleotide fragment containing the *Mig1* gene with *Hind* III/*Kpn* I restriction sites was amplified from the genomic DNA of the yeast strain 2842 through PCR and then inserted into the corresponding restriction site of pYES-KanMx to obtain pGAL1-Mig1. The *MetK1* gene (GenBank ID: 10966393) was codon-optimized to increase its expression efficiency in yeast cells. After optimization, it was obtained through artificial gene synthesis and introduced with *Hind* III/*Kpn* I restriction enzyme sites for subsequent cloning convenience, after which it was inserted into the corresponding site of pYES-KanMx to obtain the recombinant plasmid pGAL1-MetK1. The sequences of *MetK1* gene before and after codon optimization are presented in Appendices A and B, respectively.

The plasmids pGAL1-Mig1 and pGAL1-MetK1 and the gene knockout cassettes were subsequently transformed into the parent strain 2842 or YMig1Δ using the lithium acetate method with G418 resistance selection, as described in previous studies [3,30]. All of the recombinant mutants were verified by DNA sequencing.

## 2.2. Culture Conditions

All the glycerol-preserved yeast strains were streaked onto YPD plates and cultured at 30 °C for 20 h. The single colonies were picked and inoculated into 50 mL of YPD liquid medium and then shaken at 30 °C and 200 rpm for 20 h. To maintain the stability of the recombinant plasmid, 250 mg/mL G418 was added to all the YPD media and plates to apply selective pressure. Two percent of the preculture was inoculated into 50 mL of O-medium and shaken at 30 °C and 200 rpm for 48 h. After 16 h of culture, 2% D-galactose was added to induce the expression of the target gene. All shake flask experiments were performed in triplicate. YPD medium contained 1.0% peptone, 2.0% yeast extract and 2.0%

glucose. The YPD plate medium contained YPD medium plus 2.0% agar. The fermentation medium (named O-medium) was composed of glucose (50 g/L), peptone (10 g/L), yeast extract (5 g/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g/L), Met (1.5 g/L),  $\text{KH}_2\text{PO}_4$  (4 g/L),  $\text{K}_2\text{HPO}_4$  (2 g/L), and at pH 6.0. The batch fermentation experiments were repeated three times.

### 2.3. The SAM Production of the Mutant Strain YMig1 $\Delta$ PMetK1 by Fed-Batch Fermentation

To assess the SAM productivity of the mutant strain YMig1 $\Delta$ PMetK1, a batch fed-batch fermentation process was carried out in a 10 L fermenter (B. Braun Biotech, Melsungen, Germany) with O-medium. A 500 mL inoculum was transferred into the fermenter. The culture conditions were 30 °C and pH 6.0, with 30% dissolved oxygen. When the glucose concentration decreased below 5 g/L (at 16 h of fermentation), the molasses was fed into the fermenter at a rate of 1.2 g/L/h for 72 h. The cell growth (DCW), glucose consumption and SAM production accumulation were monitored every 4 h.

### 2.4. The Biomass and Carbon Source Consumption in the Fermentation Broth Assays

The biomass and carbon source consumption in the fermentation broth were sampled and measured every 4 h. The detections were carried out in accordance with the method previously reported by Chen et al.: the biomass was measured using the dry cell weight (DCW) method [12,13]; the determinations of carbon source consumption were carried out using the 3,5-dinitrosalicylic acid (DNS) method [3,31].

### 2.5. Amino Acid Analyses

Cells were harvested after 36 h of fermentation, and sample preparation for amino acid analysis was conducted following methods described in previous studies [3,32–34]. The concentrations of SAM, Met, serine (Ser), aspartic acid (Asp) and cysteine (Cys) were determined using a Shimadzu LC-10A high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) coupled with a Megres C18 column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm; Hanbon Sci. & Tech., Huaian, China). Quantitative analysis of peak areas was carried out on the basis of standard calibration curves prepared with authentic standards of SAM, Met, Ser, and Asp (Sangon Biotech, Shanghai, China), in accordance with previously reported protocols [3,32–34]. For Cys quantification, a precolumn derivatization HPLC method was adopted as described in the literature [34].

### 2.6. Intracellular Glycolytic Intermediate Analysis

The samples for intracellular glycolytic intermediate analysis were prepared according to the methods described in previous studies. The sample processing and quantitative assay of glucose 6-phosphate (G6P) were carried out according to the methods previously reported by Chen et al. and Zhu et al. [3,35]. The sample processing and quantitative assay of 1,6-diphosphate fructose (FBP) was performed according to the methods previously reported by Chen et al. [3]. The sample processing and quantitative assay of pyruvic acid (PY) was performed according to the methods previously reported by Saavedra et al. [36]. The sample processing and quantitative assay of acetyl-CoA were performed according to the methods previously reported by Chen et al. [13].

### 2.7. Analysis of the Transcriptional Levels of Several Glycolytic Genes

For quantitative assay of the transcriptional levels of genes related to sugar metabolism, total RNA was prepared and evaluated by the agarose gel electrophoresis and Eppendorf Bio-Photometer Plus (Hamburg, Germany). The RNA from each sample was reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, Takara-Bio Inc, Dalian, China) following the manufacturer's instructions. The primers used to quantify the transcriptional levels of the hexose transporter-encoding gene *HXT1*, the hexokinase

1-encoding gene *HXK1*, the hexokinase 2-encoding gene *HXK2*, the 6-phosphofructokinase 1-encoding gene *PFK1*, the glyceraldehyde-3-phosphate dehydrogenase-encoding gene *TDH1*, the 3-phosphoglycerate kinase-encoding gene *PGK1*, the pyruvate kinase-encoding gene *PYK1*, the aldehyde dehydrogenase 6-encoding gene *ALD6* and the acetyl-CoA synthase 1-encoding gene *ACS1*, and the reference gene *ACT1* were based on the primer sequences reported in the previous study by Chen et al. [3] and were listed in Table 2. Gene transcription was quantified by real-time qPCR on a StepOnePlus instrument (ABI, USA) by using the  $2^{-\Delta\Delta CT}$  algorithm.

**Table 2.** The primers used in Q-PCR analyses of the gene transcription [3].

| Gene         | Sense Primer          | Antisense Primer       |
|--------------|-----------------------|------------------------|
| <i>HXT1</i>  | tgtgccattggtggtatcgt  | accaccgacacctaaccag    |
| <i>HXK1</i>  | tactgggtgtcaacggtgctt | gttcgtcgacagcaacatcg   |
| <i>HXK2</i>  | ctgctccaatggccatcaac  | aaggtttgttgccctggtct   |
| <i>PFK1</i>  | tggtcttgcggttccatcg   | aaggtttgttgccctggtct   |
| <i>TDH1</i>  | ttgaggttgtgctgtcaacg  | gcttgcgtcatgggaaacag   |
| <i>PGK1</i>  | aggcttctgcccagggttc   | cagcacgttgtggcaagtc    |
| <i>PYK1</i>  | cgactcagatgctggattca  | ccgtttctccagaaagcataa  |
| <i>ACS1</i>  | gctgcaaaggataaggatgg  | gcctcaattcagcggtaga    |
| <i>ACS2</i>  | ctgctgttgcggtattcca   | tgtgttctgcacaccttca    |
| <i>SER33</i> | ctcgtcgtgaagcatt      | acttgtggaactctactt     |
| <i>SER1</i>  | acaactcagcctataatacaa | ccaatgcctcatataatatctt |
| <i>SER2</i>  | cctatcgtagacggacag    | caccatacaacttgcttca    |
| <i>MET17</i> | aaatggattggtggtcatgg  | gaaagaggcaaatgggttca   |
| <i>MET6</i>  | tggaagctgcccgtatcaag  | gcaactctgaaagcttcggc   |
| <i>SAM2</i>  | tgaatccgtcgggtgaaggtc | agctgtttcacaggcaacct   |
| <i>ACT1</i>  | acgctcctcgtgctgtcttc  | gttcttctggggcaactctca  |

## 2.8. Enzymatic Assays

The yeast cells were sampled every 4 h, centrifuged at 8000 rpm for 5 min and then washed twice with a phosphate-buffered (0.1 mol/L, pH 7.0) solution. Cell-free extracts were prepared by ultrasonication for a total duration of 10 min (0.5 s on and 10 s off), an ultrasound power of 300 W and a frequency of 22 kHz [13]. ADH2 activity was analyzed through the protocols described by Mauricio et al. and Johansson and Sjöström [37,38]. The crude extract was incubated at 60 °C for 15 min for thermal denaturation of ADH1. ADH2 activity was subsequently measured in cuvettes with 3 mL of reaction mixture containing 20 µL of crude extract, 1 mmol/L NAD<sup>+</sup>, 2 mmol/L β-mercaptoethanol, 70 mmol/L aminourea and 100 mmol/L pyrophosphate buffer (pH 8.5). The reaction was started by the addition of 100 mmol/L ethanol. The NADH production rate was calculated by monitoring the change in absorbance at 340 nm; 1 unit of enzyme activity was defined as the amount of enzyme that formed 1 µmol of NADH per min at 25 °C.

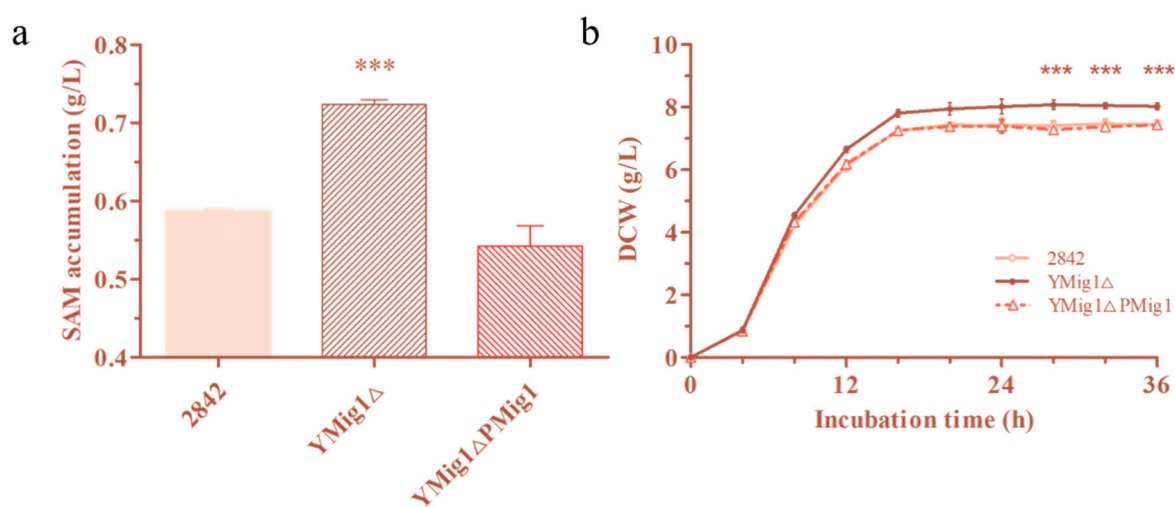
## 2.9. Statistical Analysis

All experiments were performed in triplicate, and results are presented as mean ± standard deviation (mean ± SD). Data analysis was carried out with GraphPad Prism 5.0 (San Diego, CA, USA). One-way analysis of variance (one-way ANOVA) was used for statistical comparison. Asterisks indicate significant differences between recombinant strains and the parental strain: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### 3. Results and Discussion

#### 3.1. Deletion of *Mig1* Increased SAM Accumulation and DCW in *S. cerevisiae*

To evaluate whether knocking out the *Mig1* gene would increase the accumulation of SAM in yeast, the accumulation of SAM and the DCW of the *Mig1*-deleted strain YMig1Δ were monitored at 48 h. The accumulation of SAM in YMig1Δ reached 0.72 g/L, which was 22% greater than that of the parent strain 2842 (0.59 g/L of SAM) (Figure 2a). Like the increase in SAM production, the biomass of the mutant strain was 8% greater than that of the parent strain (Figure 2b). To verify that this phenomenon was caused by *Mig1* gene knockout, the *Mig1* gene was reintroduced into the mutant strain YMig1Δ, which led to a decrease in SAM production and DCW. It was demonstrated that the knockout of *Mig1* significantly increased the accumulation of SAM and DCW in *S. cerevisiae*.

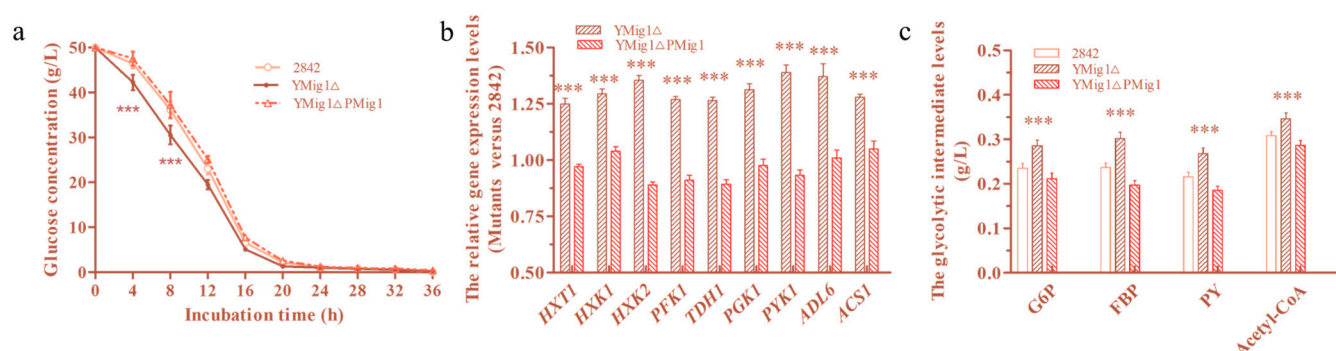


**Figure 2.** Deletion of *Mig1* increased SAM accumulation and DCW in *S. cerevisiae*. (a) The SAM production of the mutant strains at 48 h. (b) The DCW of the mutant strains was significantly improved. \*\*\* indicates  $p < 0.001$ .

#### 3.2. The Improvement in SAM Accumulation Was Attributed to the Knockout of *Mig1* Enhancing Glucose Utilization in *S. cerevisiae*

The responses of glucose metabolism to the knockout of *Mig1* in the yeast strain were investigated. Compared with the 2842 strain and the YMig1ΔPMig1 strain, the YMig1Δ mutant strain was able to consume glucose in the medium more rapidly (Figure 3a). It was consistent with the results of the previous study reported by Yan et al. and Alipourfard et al., and the knockout of *Mig1* significantly improved glucose utilization and biomass production [39,40]. To clarify the reasons for the accelerated sugar metabolism, the transcriptional levels of several genes related to glucose transport and glycolytic metabolism were quantitatively determined at 48 h by qPCR. The relative transcription levels of the hexose transporter-encoding gene *HXT1*, the hexokinase 1-encoding gene *HXK1*, the hexokinase 2-encoding gene *HXK2*, the 6-phosphofructokinase 1-encoding gene *PFK1*, the glyceraldehyde-3-phosphate dehydrogenase-encoding gene *TDH1*, the 3-phosphoglycerate kinase-encoding gene *PGK1*, the pyruvate kinase-encoding gene *PYK1*, the aldehyde dehydrogenase 6-encoding gene *ALD6* and the acetyl-CoA synthase 1-encoding gene *ACS1* were significantly upregulated by the knockout of *Mig1*. In addition, the reintroduction of the *Mig1* gene into the mutant strain YMig1Δ downregulated the transcription levels of the aforementioned related genes (Figure 3b). The knockout of *Mig1* upregulated the transcription level of glucose transporter encoding gene *HXT1* which can increase the transport of glucose into the yeast cells, previously discussed by Yan et al. [39]. The increases in the transcriptional levels of genes related to the glycolysis pathway, such as the *HXK1*, *HXK2*,

*PFK1*, *TDH1*, *PGK1*, and *PYK1* genes, help to yeast cell utilize glucose more quickly and convert it into energy and metabolic intermediates, which has also been confirmed by the previous study of Chen et al. [3].



**Figure 3.** The knockout of *Mig1* enhanced glucose utilization in *S. cerevisiae*. (a) The glucose consumptions of the mutant strains. (b) The relative transcriptional levels of several genes related to glucose transport and glycolytic metabolism at 48 h. (c) The intracellular levels of several glycolytic intermediates at 48 h. \*\*\* indicates  $p < 0.001$ .

In addition, several intermediate metabolites of the glycolytic pathway were also quantitatively analyzed. Corresponding to the aforementioned upregulation of gene expression, the accumulation levels of intermediate metabolic products of glycolysis, such as G6P, FBP, PY and acetyl-CoA, in the YMig1Δ strain reached 0.29 g/L, 0.30 g/L, 0.27 g/L and 0.35 g/L, which were 20.8%, 25.0%, 22.7% and 12.9% greater than those in the 2842 strain, respectively (Figure 3c). These findings were consistent with the expected results, as reintroducing the *Mig1* gene also reduced the accumulation levels of these glycolytic metabolites in the yeast cells (Figure 3c). The results demonstrated that the intracellular levels of G6P, FBP, PY and acetyl-CoA were also significantly increased by the deletion of the *Mig1* gene. Additionally, both the yields of SAM per glucose consumed and per biomass formed were calculated, as shown in Table 3. Both the yields of SAM per glucose consumed and per biomass formed were significantly improved by the knockout of the *Mig1* gene. In short, the knockout of *Mig1* enhanced the activity of the glycolysis pathway and ultimately led to increases in the intracellular levels of glycolytic intermediates, which are necessary for the survival of yeast and amino acid metabolism.

**Table 3.** The SAM yields (48 h) were improved by *Mig1* deletion combined with *MetK1* introduction in yeast.

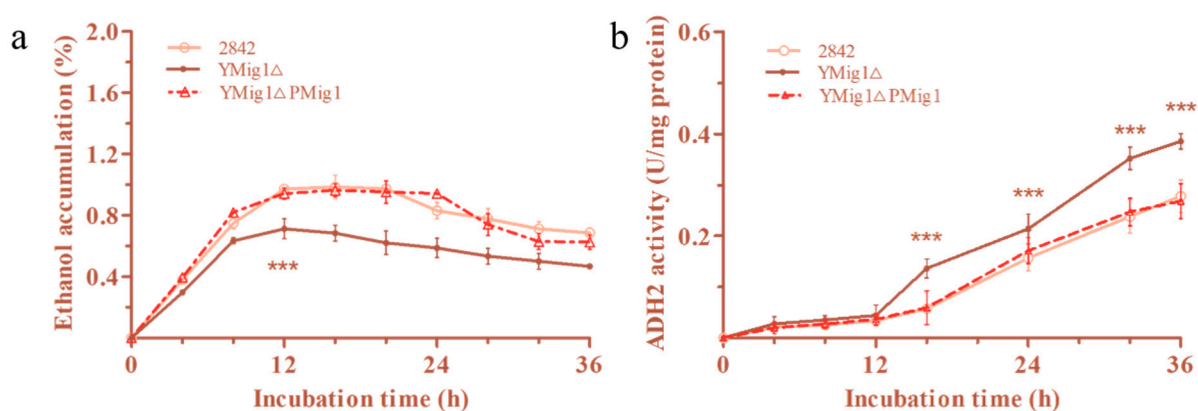
|                             | 2842          | YMig1Δ            | YMig1ΔPMig1   | YMig1ΔPMetK1      |
|-----------------------------|---------------|-------------------|---------------|-------------------|
| SAM (g/L)                   | 0.587 ± 0.002 | 0.724 ± 0.006 *** | 0.543 ± 0.025 | 1.511 ± 0.003 *** |
| DCW (g/L)                   | 7.45 ± 0.120  | 8.03 ± 0.110 *    | 7.43 ± 0.110  | 7.37 ± 0.110      |
| Glucose consumed (g/L)      | 50.000        | 50.000            | 50.000        | 50.000            |
| SAM yield to DCW (mg/g)     | 78.790        | 90.160 ***        | 73.080        | 205.020 ***       |
| SAM yield to glucose (mg/g) | 11.740        | 14.480 ***        | 10.860        | 30.220 ***        |

\* indicates  $p < 0.05$ , \*\*\* indicates  $p < 0.001$ .

### 3.3. Knocking Out the *Mig1* Gene Promoted the Conversion of Ethanol into Acetyl-CoA in *S. cerevisiae*

Since *S. cerevisiae* is a glucose effect-positive cell, it produces a significant amount of ethanol when there is an adequate supply of glucose in the fermentation broth, while the knockout of *Mig1* has been shown to alleviate glucose effect [29,30]. To investigate the response of ethanol metabolism in yeast to the knockout of *Mig1*, the ethanol accumulation

and the enzyme activity of ADH2 in the mutant strain YMig1 $\Delta$  were preliminarily analyzed every 4 h. The maximum accumulation level of ethanol in the fermentation broth of YMig1 $\Delta$  reached 0.71%, which was 28.3% and 28.4% lower than those of the wild-type strain 2842 and the strain YMig1 $\Delta$ PMig1, respectively (Figure 4a). During the whole fermentation process, the ethanol production level of the YMig1 $\Delta$  strain was clearly lower than that of the 2842 strain and the YMig1 $\Delta$ PMig1 strain. Alipourfard et al. have also reported that knocking out *Mig1* gene in *S. cerevisiae* reduced ethanol accumulation by 24% [23]. This is precisely the result of the fermentation–respiration transition achieved through the SNF1-Mig1 signaling pathway in *S. cerevisiae*. In accordance with the decrease in ethanol accumulation, the enzyme activity of ADH2 in the *Mig1*-knockout strain YMig1 $\Delta$  was significantly greater than that in the wild-type strain (Figure 4b). Consistent with these findings, the gene transcription levels of *ALD6* and *ACS1* (shown in Figure 3b) and the intracellular level of acetyl-CoA (shown in Figure 3c) were significantly elevated by the knockout of *Mig1*.



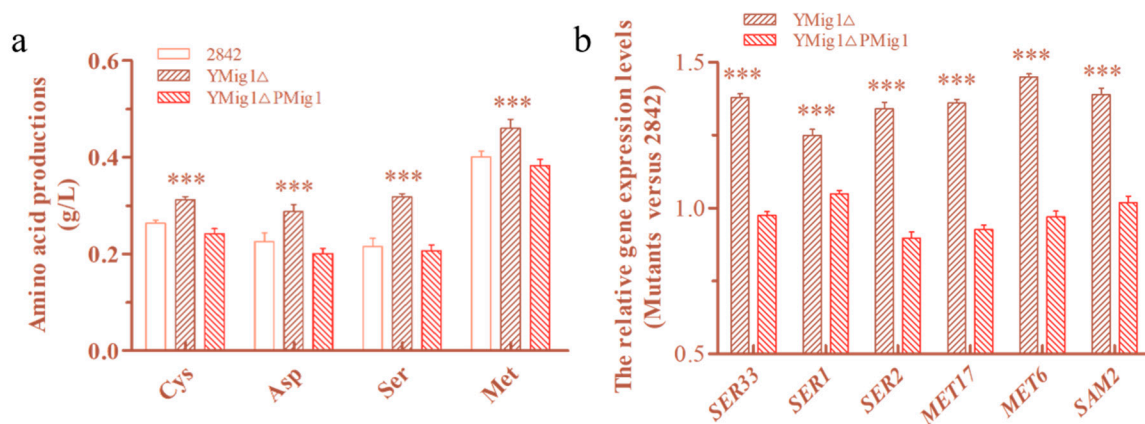
**Figure 4.** Knocking out the *Mig1* gene promoted the conversion of ethanol into acetyl-CoA in *S. cerevisiae*. (a) The ethanol accumulations in the fermentation broth of the mutants. (b) The ADH2 activities of the mutants. \*\*\* indicates  $p < 0.001$ .

Alcohol dehydrogenase 2 (ADH2) is responsible for catalyzing the conversion of ethanol into acetaldehyde, which in turn is transformed into acetyl-CoA under the joint catalytic action of aldehyde dehydrogenase 6 (ALD6) and acetyl-CoA synthase 1 (ACS1) [3,22,25,41]. The reduction in ethanol accumulation in the fermentation broth (Figure 4a) and the increase in the accumulation level of acetyl-CoA (Figure 3c) within yeast cells also confirmed that the knockout of *Mig1* enhanced the metabolic process of converting ethanol into acetyl-CoA, which serves as a precursor material for the tricarboxylic acid cycle and SAM-related precursor amino acid synthesis metabolism, which may explain the improvement in SAM synthesis.

#### 3.4. The Biosynthesis Metabolism of the Precursor Amino Acids Was Enhanced by the Knockout of *Mig1* in *S. cerevisiae*

In yeast, the metabolism of Ser, Asp and sulfur-containing amino acids is closely related [3,13,42]. The biosynthesis metabolism of the precursor amino acids was analyzed as illustrated in Figure 5a. Compared with the parental strain 2842, the *Mig1* deletion strain YMig1 $\Delta$  exhibited significantly elevated intracellular levels of all four precursor amino acids: Asp, Ser, Cys and Met. In contrast, the reintroduction of *Mig1* in the *Mig1* deletion background strain fully reversed this phenotype and reduced all the amino acid concentrations to levels comparable to or below those of strain 2842 (Figure 5a). Consistent with the metabolic data, the results of the qRT-PCR analysis revealed that YMig1 $\Delta$  significantly upregulated the expression of key genes involved in the Ser (*SER1*, *SER2* and

SER33), Met (MET17 and MET6), and SAM (SAM2) biosynthetic pathways (Figure 5b). This upregulation was completely abrogated in the mutant strain YMig1ΔPMig1, in which all target genes were downregulated to near-baseline levels (Figure 5b).



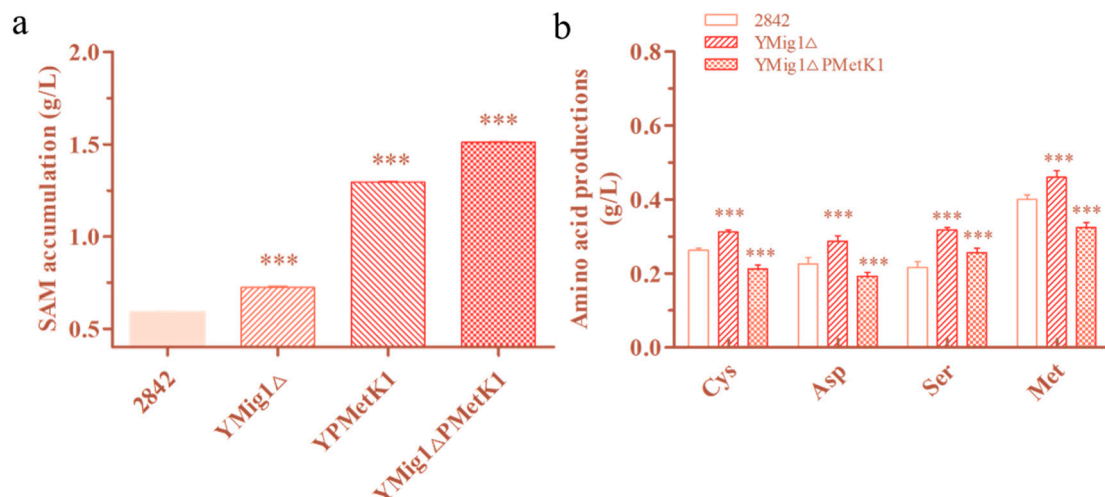
**Figure 5.** The biosynthesis metabolism of the precursor amino acids was enhanced by the knockout of *Mig1* in *S. cerevisiae*. **(a)** The precursor amino acid accumulations of the mutants. **(b)** The relative expression levels of several genes related to amino acid metabolism of the mutants. \*\*\* indicates  $p < 0.001$ .

The enzymes encoded by genes of *SER1*, *SER2* and *SER33* together catalyze the conversion of G3P to Ser, which is a key node in the sulfur-containing amino acid biosynthesis pathway [3,43,44]. The enzymes encoded by genes of *MET17*, *MET6* and *SAM2* are responsible for the synthesis of homocysteine, Met and SAM [13]. As expected, the intracellular levels of Ser, Asp, Cys and Met, which are precursor amino acids for the biosynthesis of SAM, were ultimately increased. Collectively, these results demonstrated that the *Mig1* gene played an important role in sulfur amino acid and SAM biosynthetic pathways in *S. cerevisiae*, with its deletion increasing the biosynthesis of the precursor amino acids of SAM. Meng et al. discovered that overexpression of *SNF1* significantly upregulated the transcriptional levels of genes such as *SER2*, *SER3*, *SER33*, *MET17*, *MET6* and *SAM2* [44]. Nurcholis et al. have reported a novel Mig-dependent regulatory mechanism governing histidine biosynthesis in *Kluyveromyces marxianus*. The transcriptome sequencing (RNA-Seq) analysis demonstrated that Mig1 functions as a global regulator, directly or indirectly participating in the expression regulation of over 1000 differentially expressed genes [45]. These findings inspired us to further investigate whether the SNF1-Mig1 pathway directly or indirectly modulates the gene expression of *SER2*, *SER3*, *SER33*, *MET17*, *MET6* and *SAM2*. Further RNA-Seq will help identify additional target genes controlled by the SNF1-Mig1 pathway.

### 3.5. Introducing the *MetK1* Gene from *L. infantum* into the YMig1Δ Strain to Redirect Precursor Amino Acids into the SAM Synthesis Pathway

The results of this study indicated that the knockout of the *Mig1* gene led to an increase in SAM synthesis in yeast but still resulted in a high level of precursor amino acid accumulation. To further direct these precursor amino acids into the SAM synthesis pathway, the *MetK1* gene from *L. infantum*, reported in our previous study was introduced into the mutant strain YMig1Δ. As shown in Figure 6, the SAM accumulation of YMig1ΔPMetK1 reached 1.51 g/L, which was 52.3% and 60.9% greater than that of the YMig1Δ strain (0.72 g/L SAM) and 2842 strain (0.59 g/L SAM), respectively (Figure 6a). Consistent with our hypothesis, the intracellular levels of related amino acids in the YMig1ΔPMetK1 strain also significantly decreased (Figure 6b). Additionally, the yield of SAM to glucose and

the yield of SAM to biomass of the mutant strain YMig1ΔPMetK1 reached approximately 205 mg/g and 30 mg/g, which were 160.2% and 157.4% greater than those of the parent strain 2842 (Table 3). It was demonstrated that increasing the intracellular levels of relevant amino acids and directing them into the SAM synthesis pathway through the introduction of *MetK1* is a feasible strategy.



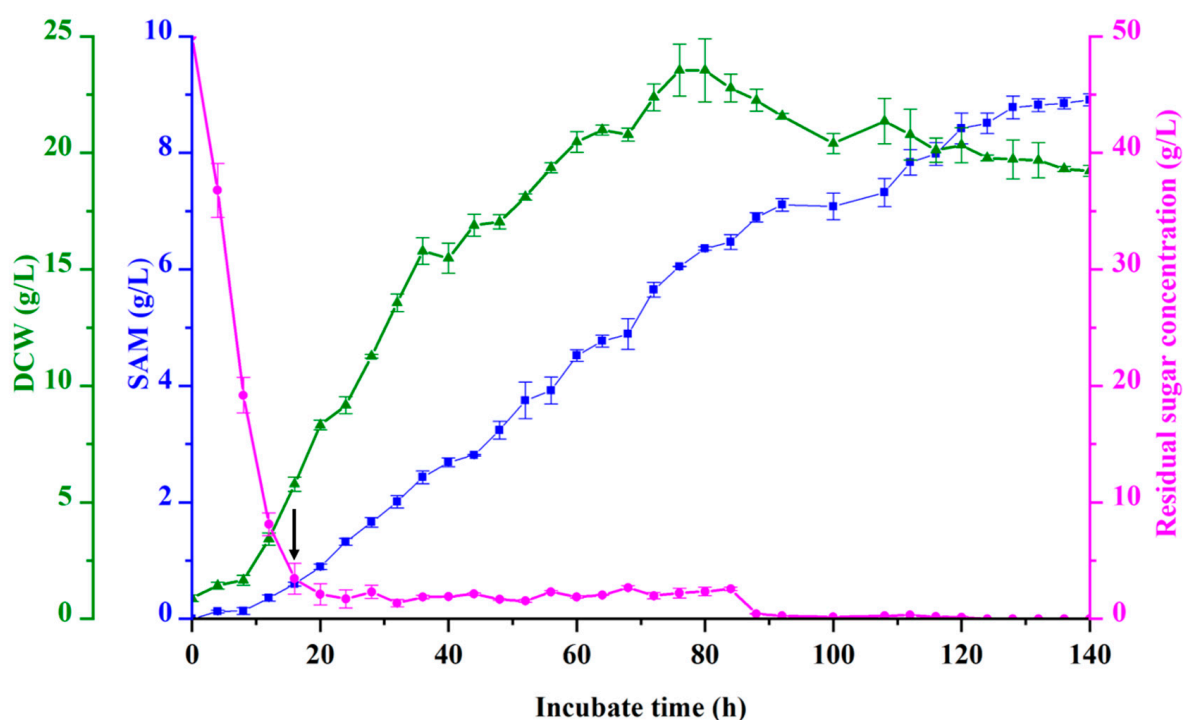
**Figure 6.** Introducing the *MetK1* gene from *L. infantum* into the YMig1Δ strain to redirect precursor amino acids into the SAM synthesis pathway. (a) The SAM production of the mutant strains at 48 h. (b) The precursor amino acid accumulations of the mutants at 48 h. \*\*\* indicates  $p < 0.001$ .

### 3.6. The SAM Production of the Mutant Strain YMig1ΔPMetK1 by Fed-Batch Fermentation

To assess the SAM productivity of the mutant strain YMig1ΔPMetK1, a batch fed-batch fermentation process was carried out in a 10 L fermenter with 7 L O-medium. The fermentation kinetics curve is shown in Figure 7. During the initial stage of fermentation, the concentration of glucose in the medium decreased significantly with increasing culture time. Within 16 h, it rapidly decreased below 5 g/L from the initial concentration. At this point, the molasses was fed into the fermenter at a rate of 1.2 g/L/h for 72 h. The biomass of strain YMig1ΔPMetK1 peaked at approximately 80 h (approximately 23.5 g/L); after 80 h, it entered the stable phase, with the biomass stabilizing and showing slight fluctuations, and a slight decrease occurred in the later stage (>120 h). The concentration of SAM products increased simultaneously with increasing cell biomass. After entering the stable phase, the rate of SAM synthesis slowed and reached 8.91 g/L at approximately 140 h, which was, respectively, 72.3% and 7.6% higher than that of the parent strain 2842 (5.17 g/L) and that of YREG1ΔPSNF1 (8.28 g/L) reported in our previous studies [3].

Although the SAM production of YMig1ΔPMetK1 is still not satisfactory, there are still several points that are worth discussing. Firstly, our previous research has shown that the multiple effects of *REG1* deletion and *SNF1* overexpression improved SAM production in *S. cerevisiae*, which demonstrated that it was feasible to improve the synthesis of SAM through SNF1-Mig1 pathway to enhance the utilization of carbon sources and redirect carbon flux to SAM biosynthesis pathway in *S. cerevisiae* [3]. Therefore, in this study, we initially focused on the global transcriptional regulatory factor Mig1. It was found that deleting the Mig1 encoding gene of *Mig1* effectively regulated the glucose effect, reduced ethanol accumulation, and enhanced the utilization of carbon sources and the synthesis of precursor amino acids, thereby improving the synthesis of SAM in *S. cerevisiae*. Secondly, the SNF1-Mig1 signaling pathway is extremely complex and involves many regulatory factors, such as Mig2, Mig3, Ssn6, Tup1 and SIP4. To further increase the yield of the target product SAM, our future research will focus on the roles of other regulatory factors in this

signaling pathway in the SAM biosynthesis. Thirdly, due to the limitations of objective conditions, we selectively studied the transcriptional levels of several relevant key genes based on previous research. This might have restricted our understanding of the role the SNF1-Mig1 pathway plays in SAM synthesis. Transcriptome sequencing may help us conduct a more comprehensive and in-depth study of this issue. Finally, further control of the fermentation process and optimization of fermentation conditions are necessary for the improvement of SAM production of YMig1 $\Delta$ PMetK1. One more point is that the molasses used in the fermentation process is also worth discussing. Molasses is a by-product of the sugar industry, and its composition varies considerably with raw materials (e.g., sugarcane, sugar beet, starch) and processing technologies. Generally, it comprises sugars (48–50%), minerals (8–10%), a small amount of proteins and nitrogenous compounds (3–6%), water, and trace vitamins. Owing to its low cost, molasses is widely utilized in fermentation processes. Although the component ratios of this industrial by-product fluctuate among different batches, its complex composition brings unique merits to fed-batch fermentation, which accounts for its extensive application in various fermentation systems. For the sake of scientific rigor, we intend to adopt glucose instead of molasses in our subsequent research, which will allow us to reliably characterize the inherent SAM biosynthesis performance of the yeast strain. In short, the strategy of deleting *Mig1* combined with introducing *MetK1* exhibited great potential for improving the production of SAM in yeast. It also provided new insight into research of nonethanol products biosynthesis by weakening glucose effect through the SNF1-Mig1 pathway.



**Figure 7.** The SAM accumulation capacity of the mutant strain YMig1 $\Delta$ PMetK1 by fed-batch fermentation. Black arrows represent the feeding initiation at 16 hours.

#### 4. Conclusions

This study revealed that the deletion of *Mig1* could increase the transcriptional levels of genes related to glucose transport and glycolysis, improve glucose utilization, increase the levels of glycolytic intermediates, increase the transcriptional levels of *ACS1* and *ALD6* and increase the activity of *ADH2*, which can increase the conversion of the accumulated ethanol in the fermentation broth into acetyl-CoA. The deletion of *Mig1* also increased the

transcriptional levels of genes related to the metabolism of SAM precursor amino acids and sulfur-containing amino acids, which was ultimately responsible for the improvement in SAM synthesis. In short, the strategy of deleting *Mig1* combined with introducing *MetK1* from *L. infantum* shows great potential for improving SAM synthesis in yeast cells.

**Author Contributions:** H.C. and H.R. designed the research; G.Z. and W.X. performed the experiments; H.C., G.Z. and X.G. analyzed the data; H.C., W.C. and W.X. wrote and prepared the original draft; X.G. and F.S., writing—review and editing; L.X. and F.S., project administration; H.C. and L.X., funding acquisition. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

|     |                          |
|-----|--------------------------|
| SAM | S-adenosyl-L-methionine  |
| DCW | cell dry weight          |
| SFH | short flanking homology  |
| Met | methionine               |
| Ser | serine                   |
| Asp | aspartic acid            |
| Cys | cysteine                 |
| G6P | glucose 6-phosphate      |
| FBP | 1,6-diphosphate fructose |
| PY  | pyruvic acid             |

## Appendix A

The sequences of *MetK1* gene before and after codon optimization:

ATGTCTGTCCACAGCATTCTCTTCTCCTCCGAGCACGTGACGGAGGGCCATCCAGAC-  
CAAGC TGTGCGATCAGGTATCCGACGCTGTGCTTGACGCGTGCCCTCGCCGGCGAC-  
CCGTTCTCGA AGGTTGCGTGCGAGTCGTGCGCAAGACGGGCATGGTGATGGT-  
GTTTCGGCGAGATCACGA CAAAGGCAGTGCTAGACTACCAGAAGATCGTCCGCAA-  
CACGATCAAGGACATTGGCTTC ATTCCGCGGACAAGGGTCTGGACTACGAGTCGT-  
GCAATGTGCTGGTTGCGATTGAGCAGC AGTCGCCGGACATCTGCCAGGGTCTGGGC-  
AACTTCGATAGCGAGGATCTCGGCGCTGGCG ACCAGGGCATGATGTTTCGGCTACG-  
CGACGGACGAGACGGAGACGCTGATGCCGCTGACG TACGAGCTGGCCCGCGGC-  
CTTGCGAAGAAGTACAGCGAGCTTCGCCGCTCCGGCAGCCT GGAGTGGGCTCG-  
CCCGGACGCGAAGACGCAGGTGACGGTGGAGTACGACTACGACACG CGCGAGGG-  
CAAGCAGGTGCTGACGCCAAGCGCGTGGCGGTGGTGCTGATCTCTGCGCA GCAC-  
GACGAGCACGTGACCAACGATAAGATCAGCGTGATCTGATGGAGAAGGTGATC  
AAGGCTGTGATCCCTGCGAACATGCTGGACGCGGAGACGAAGTACTGGCTGAACC-  
CATC TGGCCGCTTCGTGCGCGGTGGGCCGCATGGCGACGCCGCTGACTGGCC-

GAAAGATCA TCGTAGACACGTACGGCGGCTGGGGCGCGCACGGCGGTGGCGC-  
 CTTCTCCGGTAAGGAC CCTTCGAAGGTGGACCGCTCCGCCGCGTACGCCGCGCGAT-  
 GGATCGCGAAGTCGATCGT TGCAGGTGGCCTGGCGCGCCGCTGCCTTGTGCAGCTC-  
 GCGTACGCCATCGGTGTAGCGGA GCCGCTGAGCATGCACGTGGAGACGTACGGCA-  
 CCGGCAAGTACGATGACGCGAAGCTGC TGGAGATCGTGAAGCAGAACTTCAAGCT-  
 GCGCCCGTACGACATCATCCAGGAGCTGAACC TGCGCCGCCCATCTACTACGAT-  
 ACGTCGCGCTTCGGCCACTTCGGCCGTAAGGACGAGTT GGGCACGGGCGGGTTCA-  
 CTTGGGAGGTGCCAAAGAAGATGGTCGAGTAA

## Appendix B

The sequences of MetK1 gene before and after codon optimization

ATGTCTGTTCACTCTATTTTATTCTCTTCAGAACATGTCACTGAGGGTCACCCTGACA-  
 AGTT GTGTGACCAAGTCTCTGACGCTGTTTTGGACGCTTGTGGCTGGTGACCCAT-  
 TTTCTAAG GTCGCGTGCGAGTCTTGCGCAAAAACTGGTATGGTTATGGTTTTCGGT-  
 GAAATTACTACTA AAGCTGTTTTAGATTATCAAAA AATTGTTAGAAACACAATAAA-  
 GGATATTGGTTTTGATT CTGCTGATAAAGGTTTG GATTATGAATCTTGTAACGTTTTG-  
 GTTGCTATTGAACAACAGTC TCCTGATATTTG TCAAGGTTTGGGTAATTTGACTCA-  
 GAGGACTTGGGTGCTGGTGATCAA GGTATGATGTTCCGTTATGCTACTGATGAACT-  
 GAAACATTAATGCCTTTAACATACGAAT TAGCTAGAGGTTTGGCTAAAAAGTATTCT-  
 GAATTGAGAAGGTCAGGTTCTTTGGAGTGGG CAAGACCAGATGCTAAGACTCAAG-  
 TTAGTGTGAATACGATTACGACACTAGAGAGGGT AAGCAGGTCTTGACTCCAAA-  
 GAGGGTCGCTGTCGTTTTGATTTCTGCTCAACATGATGAA CATGTTACTAATGATAA-  
 AATATCAGTTGATTTAATGGAAAAGGTTATTAAAGCAGTTATT CCAGCTAACATGT-  
 TAGATGCTGAAACAAAATACTGGTTGAACCCATCAGGTAGATTCGTT AGGGGTG-  
 GACCACATGGAGACGCTGGTTTGACTGGAAGAAAGATTATTGTTGACACTTA TGGT-  
 GGTGTTGGGTGCTCATGGTGGTGGTCTTTCTCAGGTAAAGACCCGTCTAAGGTGCA  
 CAGGTCAGCTGCTTACGCTGCTAGATGGATTGCAAAGTCTATAGTTGCTGGTGGTTT-  
 AGC AAGAAGGTGCTTGGTCCAGTTAGCATAACGCTATTGGTGTCGCTGAGCCATTGT-  
 CTATGCA CGTTGAAACATACGGTACAGGTAAATATGACGATGCTAAATTGTTGGAA-  
 ATTGTTAAAC AAAATTTCAAGTTAAGACCATATGATATTATTCAAGAATTAAATTTG-  
 AGAAGGCCAATTT ACTACGATACATCTAGGTTTGGTCATTTCGGTAGGAAGGACGA-  
 GTTGGGAACAGGTG T TCACTTGGGAAGTTCCTAAGAAAATGGTCAATAA

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