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Comparative performance of *S*-adenosyl-L-methionine biosynthesis and degradation in *Pichia pastoris* using different promoters and novel consumption inhibitors

Xiaoqing Hu[a,b], Ju Chu[b], Siliang Zhang[b,*], Yingping Zhuang[b]

[a] *Key Laboratory of Industrial Biotechnology of Ministry of Education, and State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China*
[b] *State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China*

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ABSTRACT

The yeast *Pichia pastoris* is a widely used host for recombinant protein expression, and has recently been engineered for whole-cell biocatalysis. The inducible P_{AOX} and constitutive P_{GAP} promoters are commonly employed. In this study, the *S*-adenosyl-L-methionine (SAM) biosynthesis and degradation efficiency of two *P. pastoris* strains were compared, and novel inhibitors that suppress SAM degradation were characterized. The strains exhibited clear physiological differences. P_{GAP}-*Pichia* showed higher transcription and activity of SAM synthetase, and the rapid cell growth led to higher levels of spermidine synthesis from SAM. In contrast, P_{AOX}-*Pichia* synthesized higher levels of glutathione from SAM, and this strain responded to hydrogen peroxide formation during methanol utilization. Aristeromycin proved an efficient inhibitor of SAM degradation in P_{AOX}-*Pichia*; 0.02 mg/L led to a 36.36% reduction in the ratio of glutathionine:SAM, and SAM accumulation was enhanced by 7.74% to 11.83 g/L. Ethanol was an even more efficient inhibitor of SAM consumption in P_{GAP}-*Pichia*; 8 g/L resulted in a 73.68% decrease in the ratio of SPD:SAM, and SAM production was elevated by 54.55% to 0.17 g/L/h.

1. Introduction

Pichia pastoris is a robust expression system for recombinant proteins [1], since high cell density can be achieved and the expression of heterologous genes can be controlled by strong promoters that are tightly regulated at the transcriptional level. The most frequently used promoters include the promoter of alcohol oxidase gene (P_{AOX}) and the promoter of glyceraldehydes-3-phosphate dehydrogenase gene (P_{GAP}).

When under the control of the inducible P_{AOX}, heterologous gene expression is repressed when grown on glucose or glycerol, and induced upon addition of methanol [2]. While driven by the constitutive P_{GAP}, heterologous gene expression occurs along with glycerol uptake [3]. Previously, many proteins have been satisfactorily expressed using either P_{AOX} or P_{GAP}, and in some cases both promoters were tested for comparison. P_{AOX} performed better for expression of cellobiohydrolase [4], hepatitis B surface antigen [5] and human trypsinogen [6], while P_{GAP} was more attractive for production of marmoset α-1,3-galactosyltransferase, β-1,2-xylosyltransferase [7], human angiostatin [8], fructose-releasing exo-levanase [9], hPEPT1 and rPEPT2 [10]. These comparative studies focused on the protein yield and activity, and did not investigate the physiological features. Recently, *P. pastoris* has been engineered for use in whole-cell biocatalysis [11–15], in which appropriate heterologous gene clusters are incorporated for the production of specific products [16]. To date, information on the biocatalytic potential of P_{AOX} and P_{GAP} is scarce, and is addressed in this study for the first time.

In our previous work, inducible and constitutive *P. pastoris* strains were employed to overexpress methionine adenosyltransferase (MAT) and subsequently synthesize *S*-adenosyl-L-methionine (SAM) from ATP and L-methionine [17,18]. SAM is an important cosubstrate involved in methyl donor reactions, and has been used as an effective treatment for osteoarthritis, liver disease and other ailments. Accumulation of SAM is dependent on SAM biosynthesis and degradation. To enhance synthesis, we have previously developed two novel strategies to stimulate expression of MAT and generation of ATP [19,20]. Reducing SAM breakdown is another feasible strategy for maximizing SAM levels. Transmethylation, transsulfuration, and aminopropylation are the main

* Corresponding author. Tel.: +86 21 64253021.
E-mail address: juchu@ecust.edu.cn (S. Zhang).

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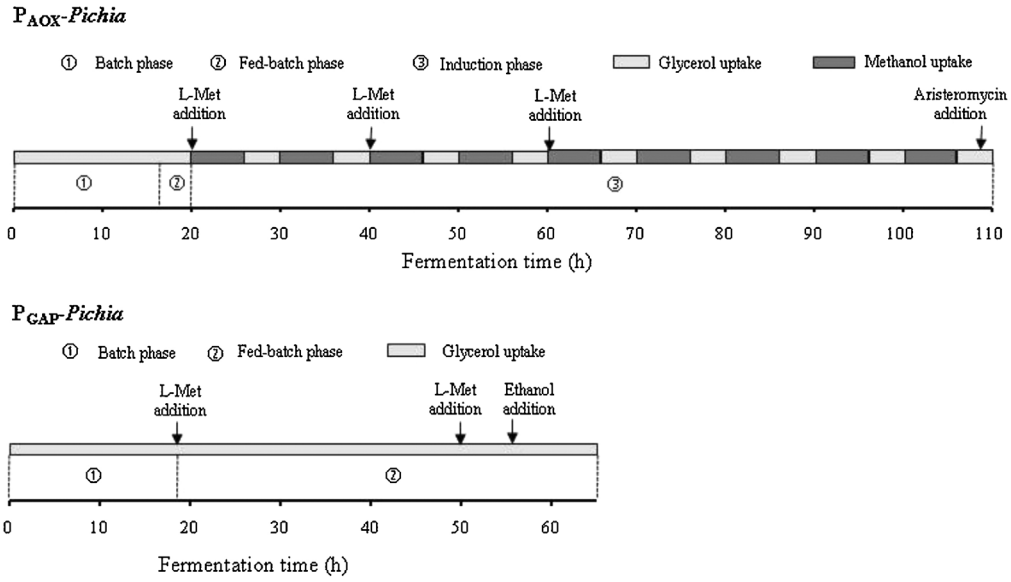

Fig. 1. Overall scheme for addition of carbon sources, L-Met and inhibitors during fermentation of the *Pichia pastoris* strains.

metabolic pathways that use SAM. As the primary methyl donor in the cell, SAM is converted to *S*-adenosyl-L-homocysteine (SAH) via a transmethylation reaction, and subsequently enters the transsulfuration pathway, leading to the synthesis of glutathionine [21]. SAM is also a precursor to the aminopropylation pathway, which leads to the synthesis of growth-stimulating polyamines such as spermidine (SPD) [21].

In this study, SAM biosynthesis in *P. pastoris* under the control of the inducible P_{AOX} and constitutive P_{GAP} were compared. Inhibitors were successfully used to enhance the ratio of SAM to glutathione and spermidine by decreasing SAM consumption, leading to significantly elevated SAM levels.

2. Materials and methods

2.1. Strains and media

P_{AOX}-*Pichia* [17] and P_{GAP}-*Pichia* [18] transformed with *mat* (MAT-encoding gene) were used throughout. buffered glycerol-complex medium (BMGY) [19] and basal salts medium (BSM) [20] were used for inoculum cultivation and fermentation, respectively.

2.2. Inoculation and fermentation

Pichia was cultured at 30 °C in shake flasks and in a fermentor. 2 mL of a 2.5 g/L dry cell weight (DCW) *Pichia* glycerol stock was used to inoculate 50 mL BMGY, and cultivated for 12 h at 300 rpm to a cell density of 2.4 g/L DCW. The secondary inoculum was cultured for 11 h to a cell density of 4.1 g/L DCW, which was used to inoculate the 15-L fermentor (FUS, Guoqiang, China). Fermentation parameters such as air pressure in headspace, aeration and agitation rates, were identical to the previous report [20], while the feeding strategies were different for the two strains (Fig. 1). For P_{GAP}-*Pichia*, the inoculation and fermentation procedure were identical to strategy E [20]. Unlimited supplementation of glycerol was executed by pulsed feeding mode, and glycerol content throughout the fed-batch phase was maintained at 2%. For P_{AOX}-*Pichia*, the intermittent feeding of methanol and glycerol during the induction phase [19] was employed, with minor modifications as follows: (1) the glycerol batch phase lasted for 16 h until glycerol exhaustion, and the induction phase was initiated from 20 h to the end and (2) the feeding rate of PTM1-containing glycerol solution during fed-batch phase was 0.7 mL/L/h.

To facilitate SAM biosynthesis, sterile L-Met powder (Bafeng, China) was added to the fermentor to a final concentration of 15 g/L. The optimal L-Met feeding scheme for P_{GAP}-*Pichia* [20] and P_{AOX}-*Pichia* [19] were employed with some modifications; for P_{GAP}-*Pichia*, 7 g/L was added at 18 h and 8 g/L at 50 h; for P_{AOX}-*Pichia*, 5 g/L was added at 20 h, 40 h and 60 h (Fig. 1).

2.3. Effects of aristeromycin and ethanol supplement on SAM accumulation

For P_{AOX}-*Pichia* fermentation, aristeromycin was added at 0.02 mg/L at 108 h, only 2 h before cell harvesting. Influence on SAM, reduced glutathione (GSH) and oxidized glutathione (GSSG) abundance were investigated. For P_{GAP}-*Pichia* fermentation, ethanol was added at 8.0 g/L at 55 h, 10 h before cell harvesting (Fig. 1). Influences on SAM and SPD abundance were investigated.

2.4. Analytical methods

The relative transcription levels of *mat* in P_{AOX}-*Pichia* and P_{GAP}-*Pichia* were compared by RT-PCR, using actin as a control [22]. *Pichia* cells during the logarithmic phase were sampled to extract total RNA prior to reverse transcriptase-PCR. The relative quantification was determined from the crossing threshold (Ct) using the comparative Ct method.

MAT was extracted and enzyme activity was measured as described [19,23]. MAT expression was analyzed by SDS-PAGE, using previously purified MAT [24] as a control.

To determine DCW, *Pichia* cells were harvested by centrifugation at 5000 rpm for 10 min, washed three times with deionized water, and dried to a constant weight at 90 °C. SAM and SPD were extracted and assayed simultaneously according to Wagner et al. [25]. GSH and GSSG were determined by high performance liquid chromatography (HPLC) using a UV detector [26]. The ratios of GSSG/GSH and (GSH + GSSG)/SAM were calculated.

ATP was determined by HPLC [19]. Broth L-Met and glycerol were assayed as described [27,28]. Ethanol and methanol were determined as described [29]. Cellular H_2O_2 was determined according to the procedure of Patterson [30]. Catalase activity was measured by the method of Sibirnyi [31], and malondialdehyde (MDA) was assayed using the TBA method [32].

3. Results and discussion

The *mat* gene was placed under the control of the inducible P_{AOX} and constitutive P_{GAP} promoters. Transcription of *mat*, and cellular levels of SAM, and ATP and L-Met (two substrates for SAM synthesis) were determined. SAM is consumed during biosynthesis of glutathione and SPD, and levels of these SAM-dependent compounds were measured. Finally, mechanisms of SAM flux were investigated, and two inhibitors were characterized that maximize SAM accumulation by reducing SAM consumption.

3.1. MAT transcription and activity

Transcription levels of *mat* in both *Pichia* strains were compared using RT-PCR [22]. As expected, P_{AOX}-*Pichia mat* transcription was induced when methanol was added, P_{GAP}-*Pichia mat* transcription was sustained throughout the fermentation. Transcriptional levels of *mat* varied over time, and correlated closely with uptake of methanol for P_{AOX}-*Pichia* or glycerol for P_{GAP}-*Pichia* (data not shown). The highest levels were reached at 42 h and 54 h for

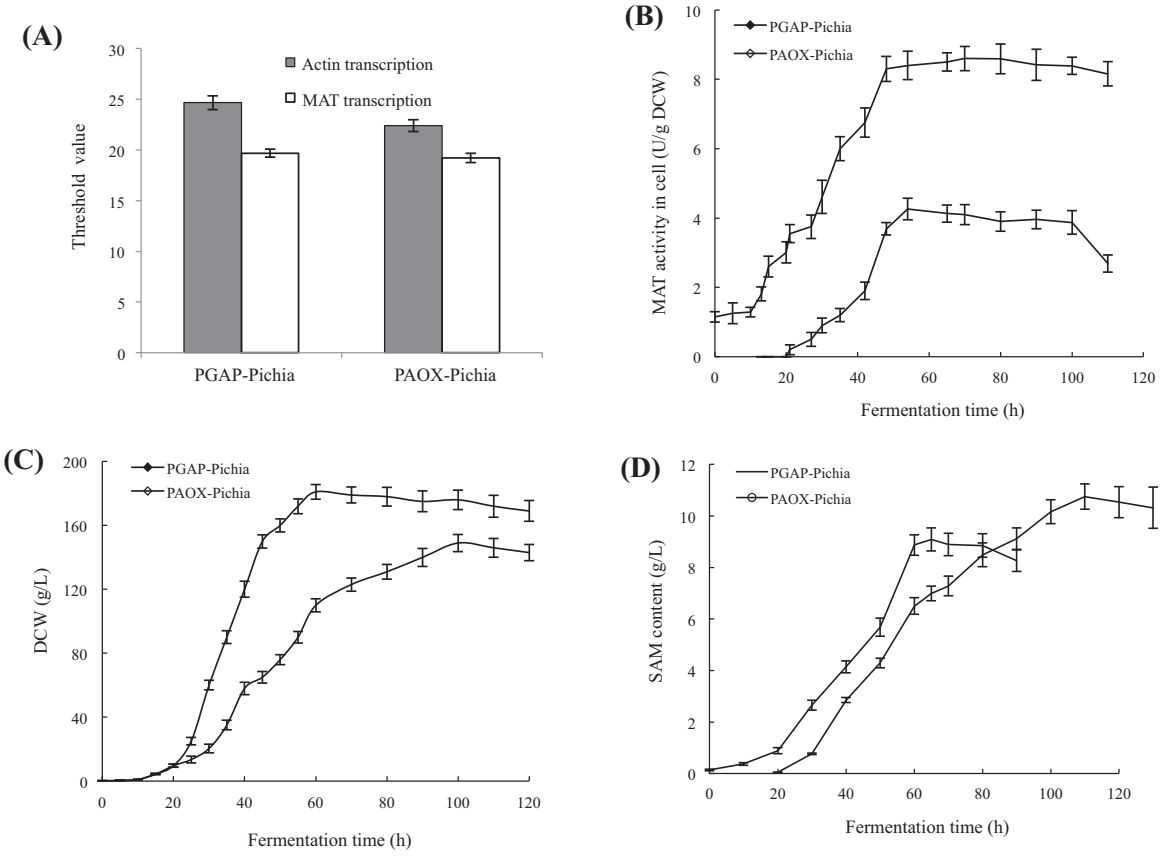

Fig. 2. Maximal *mat* transcription levels (A) and time courses of MAT activity (B), biomass level (C) and SAM accumulation (D).

P_{AOX}-*Pichia* and P_{GAP}-*Pichia*, respectively, around the rates of maximal carbon uptake (data not shown). Maximum *mat* RNA levels were 3.46-fold higher in the constitutive strain (Fig. 2A), indicating stronger *mat* transcription in P_{GAP}-*Pichia* under the fermentation conditions employed.

Time courses of MAT activity were analyzed. Peak MAT activity was achieved at 54 h for P_{AOX}-*Pichia* and 70 h for P_{GAP}-*Pichia* (Fig. 2B), a lag of several hours behind the strongest transcriptional rates (data not shown). Peak MAT activity in P_{GAP}-*Pichia* (8.60 U/g DCW) was about twice that of P_{AOX}-*Pichia* (4.26 U/g DCW). MAT was purified from both strains, and the maximum content of purified enzyme from P_{GAP}-*Pichia* (16.63 mg/g DCW) again exhibited twice that from P_{AOX}-*Pichia* (7.91 mg/g DCW), presumably due to greater abundance resulting from the higher transcription level.

Differences in *mat* transcriptional levels between the two *Pichia* strains depended not only on the promoter, but also on the type and feeding rate of the carbon source. In preliminary tests, we compared MAT transcription levels of P_{GAP}-*Pichia* under glycerol limited-feeding and glycerol unlimited-feeding conditions. RNA levels of *mat* increased along with assimilation of glycerol (data not shown).

3.2. *Pichia* growth, ATP generation and SAM productivity

Different carbon sources resulted in different rates of growth, exponential phase, and ultimately biomass accumulation for both strains (Fig. 2C). For P_{AOX}-*Pichia*, biomass increased from the start of fermentation until the maximal level of 149 g/L was obtained at 100 h, while for P_{GAP}-*Pichia*, biomass increased more quickly, and reached 181 g/L at 60 h, 21.48% higher than the inducible strain. Glycerol was assimilated faster than methanol, explaining the higher growth rate and biomass accumulation observed with the constitutive strain.

ATP is the universal energy currency of the cell, and one of two substrates required for SAM synthesis. In this study, P_{GAP}-*Pichia* generated more ATP, as previously reported [19,20]. For P_{AOX}-*Pichia*, ATP levels were close to those of P_{GAP}-*Pichia* in the batch and fed-batch phases, but dropped sharply when the carbon source was switched to methanol at 20 h, and fluctuated during the induction phase as methanol and glycerol alternated [19].

L-Met, the other substrate required for SAM biosynthesis, was present in excess, ensuring SAM biosynthesis was initiated as soon as MAT was expressed (Fig. 2D). Even so, SAM accumulation differed between the strains, owing to slight differences in cell biochemistry and physiology. P_{GAP}-*Pichia* started to accumulate SAM in line with glycerol utilization, during the initial cultivation stages, while P_{AOX}-*Pichia* did not begin to synthesize SAM until fermentation had proceeded for 20 h. Upon cell harvesting, P_{AOX}-*Pichia* achieved SAM accumulation levels of 10.75 g/L, 18.26% higher than the constitutive strain. However, P_{GAP}-*Pichia* produced 0.14 g/L/h, 40% more efficient than the inducible strain, because total fermentation time (65 h) was nearly half that of P_{AOX}-*Pichia* (110 h).

3.3. Glutathione level

Not all the synthesized SAM accumulates in the cell; some is consumed during SAM-dependent biosynthesis of other active compounds depending on the requirements of the cell. Glutathione and SPD are two of the major cellular SAM-dependent compounds, and levels of these compounds, along with their ratios with SAM, were analyzed.

During fermentation, the strains displayed different time courses of glutathione/SAM accumulation (Fig. 3A). For P_{GAP}-*Pichia*,

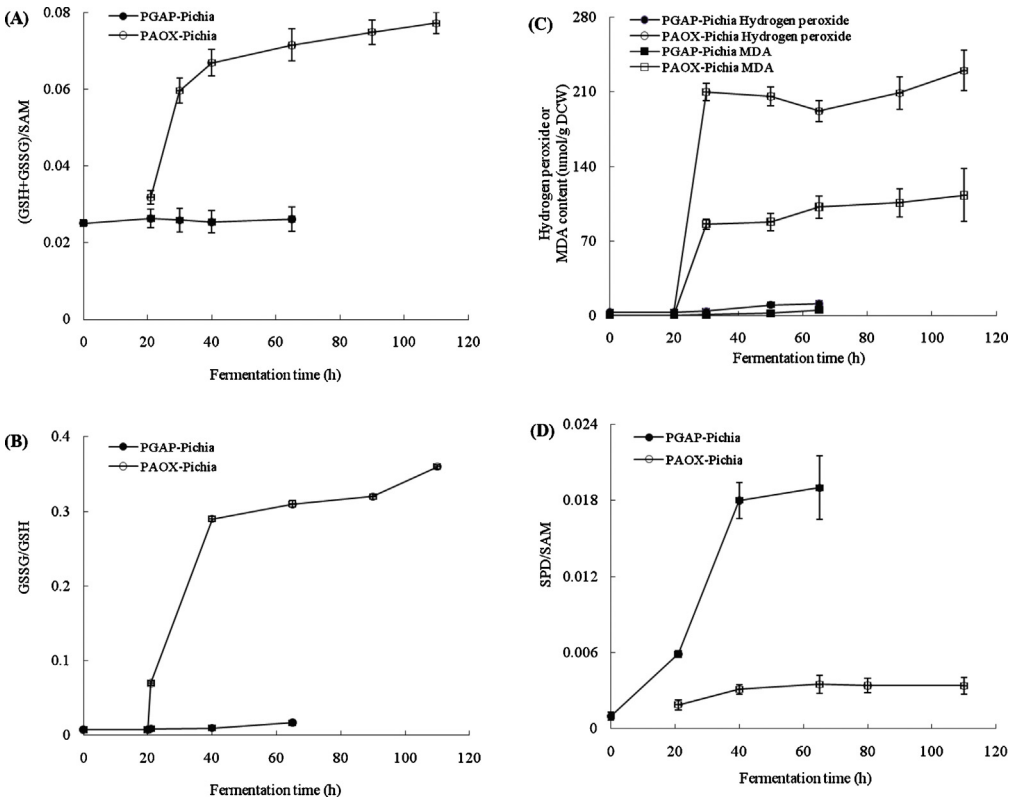

Fig. 3. Ratios of (GSH + GSSG)/SAM (A) and GSSG/GSH (B), accumulation levels of hydrogen peroxide and SMD (C), and ratio of SPD/SAM (D).

(GSH + GSSG)/SAM remained stable around 0.25–0.26 (0–65 h), while for P_{AOX}-*Pichia*, this increased from 0.31 at 21 h to 0.77 at 110 h. P_{AOX}-*Pichia* displayed higher (GSH + GSSG)/SAM than P_{GAP}-*Pichia*, suggesting more SAM had entered the transsulfuration pathway to produce glutathione. During the fermentation process of P_{AOX}-*Pichia*, the upward sloping curve of (GSH + GSSG)/SAM was closely correlated with a shift in cellular glutathione redox potential, as indicated from the GSSG/GSH ratio. For P_{GAP}-*Pichia*, the time course of GSSG/GSH was flatter in comparison (Fig. 3B). For P_{AOX}-*Pichia*, a similar curve was obtained before methanol induction (0–20 h), while a steeper curve was displayed later in the fermentation (20–110 h). The rise of GSSG/GSH indicated that the cellular redox potential gradually shifted to a more oxidizing state during the methanol induction phase. An explanation may be that the antioxidant GSH was produced at the cost of consuming SAM, in order to maintain a healthy redox balance under these metabolic conditions.

This conclusion is supported by previous observations that showed intracellular reactive oxygen species are elevated during methanol metabolism in P_{AOX}-*Pichia* [33]. In this study, H_2O_2 levels increased rapidly to 210 μmol/g 10 h after methanol induction, and then fell slightly during the following 30 h (Fig. 3C). This fall may be due to the inducible expression of catalase. Between 65 h and the end of the fermentation, H_2O_2 levels rose again, and peaked at 230 μmol/g. H_2O_2 results in oxidative damage, and this was investigated in more detail by measuring the intracellular levels of MDA, a reliable indicator of lipid peroxidation [32]. Both MDA and H_2O_2 increase earlier in the fermentation and reach much higher levels in P_{AOX}-*Pichia* (Fig. 3C), suggesting much higher oxidative stress and damage in this strain. H_2O_2 and other reactive oxygen species (ROS) change the redox potential within the cell, leading to the destruction of proteins, nucleic acids and membranes, and ultimately to cell death by apoptosis [34]. The glutathione redox system in *P. pastoris* has been shown to play an important role in scavenging H_2O_2, by synthesizing large amounts of GSH and converting it to GSSG to protect against ROS [35]. In *Saccharomyces cerevisiae*, glutathione protects proteins from oxidation by a process called glutathionylation [36]. Additionally, glutathione has been reported to regulate gene expression and enzymatic activities [37].

3.4. SPD level

SPD is an indispensable growth factor for many microorganisms, and can be biosynthesized from SAM. In this study, the SPD/SAM ratio increased over time to a maximum 1.90% in P_{GAP}-*Pichia* (Fig. 3D) In contrast, P_{AOX}-*Pichia* maintained a lower SPD/SAM ratio of 0.42% throughout the fermentation, implying more SAM was consumed to synthesize SPD in P_{GAP}-*Pichia*. The physiological roles of polyamines at the molecular level were elucidated in plants. Polyamines are involved in a variety of fundamental cellular processes, including transcription, RNA modification, protein synthesis, modulation of enzyme activity, and cell cycle regulation [38]. Important polyamines such as SPD are widespread in living cells, and accumulates at high levels in actively proliferating cells [39]. Our data for P_{GAP}-*Pichia* was consistent with these observations, with higher *mat* transcription (Fig. 2A), MAT activity (Fig. 2B), growth rate (Fig. 2C) and energy generation (data not shown), but with a subsequently higher demand for SPD biosynthesis.

3.5. Inhibition of SAM degradation

In addition to enhancing SAM biosynthesis, suppression of SAM degradation represents a previously unexplored method of maximizing SAM production by biocatalysis.

Guided by the physiological characteristics of the strains in the earlier experiments, putative SAM degradation inhibitors were obtained and tested (Fig. 4). Including ethanol in the fermentation

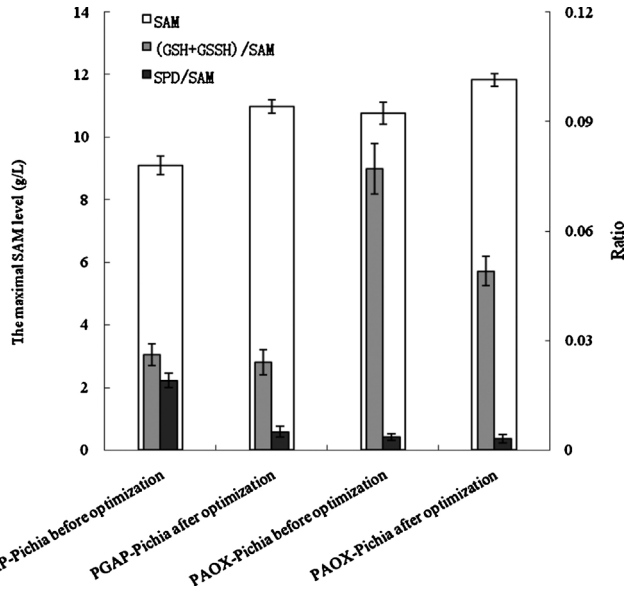

Fig. 4. SAM accumulation, and levels of total glutathione and SPD relative to SAM before and after optimization.

decreased the SPD/SAM ratio from 1.9% to the final 0.5% in P_{GAP}-*Pichia*. This elevated final SAM levels from 9.09 g/L to 10.98 g/L. The selection of alcohol as an inhibitor of SAM conversion to SPD was inspired by the lower SPD/SAM ratio observed in P_{AOX}-*Pichia* under methanol induction. In preliminary tests, methanol addition also led to a significant reduction in the SPD/SAM ratio in P_{GAP}-*Pichia*. Methanol was a poor choice due to its toxicity, but ethanol exhibits the desired inhibition without the toxicity. The inhibitory activity of ethanol is possibly explained by reduction in growth rate, which led to decreases in glycerol uptake, ATP generation and biomass (data not shown). Together, these appear to reduce the demand for polyamine biosynthesis. Timing is important; ethanol should be supplemented 10 h before harvesting cells. If added too early, very slow growth will lead to poor biomass accumulation, whilst no inhibition of SPD biosynthesis will occur if ethanol was added too late.

Aristeromycin is a known inhibitor of SAH hydrolase, and since this enzyme is involved in the biosynthesis of glutathione from SAM, it seemed logical to see if aristeromycin could be used to block this SAM consumption pathway. As expected, addition of aristeromycin led to a significant decrease in the (GSH + GSSG)/SAM ratio, from 7.7% to 4.9% in P_{AOX}-*Pichia*, with a negligible effect on SPD/SAM (Fig. 4). Consequently, SAM levels were increased from 10.75 g/L to 11.83 g/L in this strain. Aristeromycin presumably inhibits SAM-dependent transmethylation, which is involved in cellular processes, and so should be included in sublethal doses. Although not tested, higher concentrations would lead to cell death. Again, timing is important; supplementation should be carried out 2 h before harvesting cells. If added earlier, it would slow growth, leading to poor biomass and low levels of SAM.

The two *Pichia* strains exhibited differences in SAM level to inhibition. P_{AOX}-*Pichia* accumulated 7.74% more SAM, while P_{GAP}-*Pichia* achieved a 54.55% higher SAM productivity of 0.17 g/L/h.

In previous reports, overexpression of heterologous genes led to a significant rerouting of carbon and energy resources in *Pichia*. Increased glucose feeding rate alters metabolic flux and affects protein synthesis directly [40]. Increased flux through the tricarboxylic acid cycle during the protein expression phase has also been reported, indicating increased energy demand [41]. In this study, it was further revealed that inducible and constitutive *Pichia* strains exhibited different requirements for glutathione and SPD, both of which were formed at the expense of SAM consumption.

During whole-cell biocatalysis, reaction efficiency is not only influenced by the intrinsic properties of the enzymes catalyzing the desired reactions, but also by the physiological state of the cell. Redox potential, growth rate, and energy state can all be highly influential over the final product yield. This study illustrates the importance of investigating the contribution of the physiological state when engineering heterologous hosts for whole-cell biocatalysis. Other physiologically relevant factors influencing the biosynthetic potential of the *Pichia* host strains remain to be elucidated.

4. Conclusion

To compare the biocatalytic capabilities of inducible P_{AOX}-*Pichia* and constitutive P_{GAP}-*Pichia* strains, SAM biosynthesis and degradation efficiency were investigated. The physiological differences affecting SAM biosynthesis in the strains were studied. In P_{AOX}-*Pichia*, more SAM was converted to glutathione in response to H_2O_2-induced oxidative stress resulting from growth on methanol. In P_{GAP}-*Pichia*, more SAM was converted to SPD to satisfy the requirement of rapidly growing cells. Aristeromycin and ethanol were used to suppress SAM consumption, decreasing GSH and SPD biosynthesis, respectively, in a novel approach to maximize SAM production. P_{AOX}-*Pichia* accumulated SAM levels of 11.83 g/L, 7.74% higher than the constitutive strain, but P_{GAP}-*Pichia* was 54.55% more efficient, producing SAM levels of 0.17 g/L/h.

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