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Emerging developments and innovations in the construction of microbial cell factories for the improved production of S-adenosylmethionine

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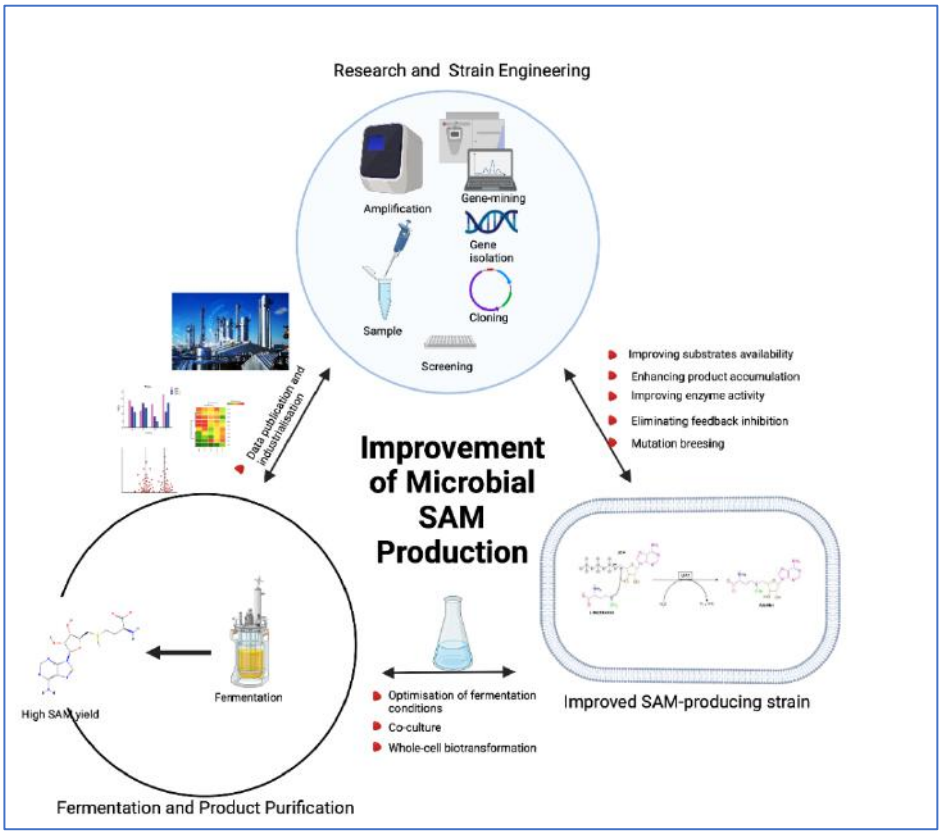
Abstract— *S-adenosylmethionine (SAM) is a metabolite of great biological and pharmacological significance. Its biochemical roles can only be described as central for life. It is produced in almost every organism, and its chemical structure enables it to participate in at least three classes of group transfer reactions as the group donor. It has been employed in treatment of osteoarthritis, depression, Alzheimer's disease, liver disease and fibromyalgia. Its enzymatic synthesis requires the participation of L-methionine and ATP as substrates and methionine adenosyltransferase the enzyme. Meeting the ever-increasing demand for the compound requires increasing production. A number of strategies have been employed to achieve sustainable SAM production. Principal focus has been on improving its production by microbial fermentation. Enzyme engineering, metabolic engineering, conditions optimisation are some of the biotechnological approaches the have been explored to achieve improved microbial SAM production. The successes achieved by the methods have mostly been mixed. Presently industrial SAM production is by yeast fermentation, applying high density cultures of Pichia pastoris or Saccharomyces cerevisiae. This review provides a concise report of the present achievements of methods that have been applied in Escherichia coli, P. pastoris and S. cerevisiae to achieve sustainable improvement in industrial SAM production, highlights contemporary strategies and also suggests potential methods that could be channelled to the same goal.*

Keywords— ***S-adenosylmethionine, microbial fermentation, methionine adenosyltransferase, bacteria, yeast.***

Graphical Abstract



Highlights:

- Coupling SAM production with supply of energy and/or substrate in a co-culture microbial consortium can enhance its production.
- Largely unexplored, mutation breeding exploring the unconventional space-flight microbial culturing can have beneficial effects on SAM production.
- Due to its high cost, strategies employing ATP generation or regeneration can greatly reduce the cost of SAM production.
- *Saccharomyces cerevisiae* expresses a SAM exporter that has heretofore remained largely unemployed in microbial construction for SAM production.
- We recommend system-wide metabolic engineering especially with relation to homogenous ATP availability, however, with consideration for strain viability alongside fermentation optimisation as valuable towards improving microbial SAM production.

I. INTRODUCTION

In all living organisms, reactions which require the transfer of methyl-, sulphur- or propylamine- groups occur, and very importantly, DNA methylation is a critical survival adaptation for most organisms. Such reactions require the participation of a group donor and a group recipient. S-adenosylmethionine (SAM) (Fig. 1), a small compound synthesized in almost every living organism serves as donor of these groups for most reactions. It is in fact able to donate all the groups surrounding its sulphur atom for participation in biochemical reactions requiring them. It is also a precursor in the production of glutathione [1], polyamines such as spermine and spermidine, some plant compounds (phytosiderophores and nicotianamine) that serve to chelate metal ions, and of ethylene, the important gaseous plant hormone responsible for some fruit ripening. SAM provides the 5′-deoxyadenosyl radicals which bear catalytic functions, generated as reaction intermediates by the radical SAM enzyme superfamily [2]. It is also the amino group donor in Gram-negative bacteria's manufacture of N-Acyl Homoserine Lactones (AHLs), where its amino group is employed to synthesize the homoserine lactone ring moiety of the chemical [3].

SAM physiologically functions in the synthesis of coenzyme Q10, creatine, melatonin, phosphatidylcholine, methylcobalamin, norepinephrine, and carnitine. It also participates in the metabolism of serotonin and

niacinamide [4]. Pharmacologically, it possesses anti-inflammatory activity [5] and has also been used in the therapy for disease including Alzheimer's disease [6], liver disease [7], depression [8, 9], fibromyalgia [5], osteoarthritis [10, 11], and colon cancer [12]. SAM has also been suggested to function in improvement of sleep quality [13], and in the regulation of lifespan in yeasts, flies and worms [14-16].

Due to the vast pharmacological applications of SAM, it has become expensive, requiring sustainable improved industrial production to keep it affordable. A variety of approaches to improve and provide efficient and sustainable production of the compound have been explored by different research groups. Chemical synthesis of SAM has applied reactions that chemically couple adenosyl homocysteine and the methyl group of a donor to produce biologically active SAM [17]. However poor diastereomeric excess, a key limitation of the method makes it unsustainable. Enzymatic synthesis presents as an alternative method for increasing production of SAM.

Methionine adenosyltransferase, a.k.a. MAT (E.C 2.5.1.6), otherwise referred to as S-adenosylmethionine synthase or S-adenosylmethionine synthetase is the enzyme that produces SAM from the substrates, L-methionine and ATP. Its catalysis occurs in a rare-kind two-step reaction in which while SAM is formed, the triphosphate chain of ATP is cleaved and then, the triphosphate is further hydrolysed to inorganic pyrophosphate (PPi) and inorganic phosphate (Pi) before the sulfonium product is released [18]. This enzyme has been exploited for enzymatic synthesis of SAM form L-methionine and ATP with varying fortunes in terms of productivity. Notable limitations of the enzymatic synthesis procedure is the requirement of too much effort to yield even small amounts of product [19] and product inhibition [17] when SAM accumulates to high amounts. MAT has been purified and characterised from several organisms including *Escherichia coli* [20], *Saccharomyces cerevisiae* [21], *Bacillus subtilis* [22, 23], *Solfolobus solfataricus* [24], *Leishmania infantum* [25], *Rattus norvegicus* [26] and *Homo sapiens* [27]. These enzymes have although only shown to have quite low specific activity. However, comparatively, MAT from the hyperthermophilic archaeon, *Pyrococcus furiosus* upon purification demonstrated significantly high activity, temperature and pH tolerance as well as it did upon treatment with organic solvent [28]. Also, the MAT from the thermo-acidophile, *Methanococcus jannaschii* has been characterised [29]. Thomas and Surdin-kerjan have reported that in their study that all eukaryotic organisms studied presented with multiple forms of the enzyme, which is believed to be well conserved through evolution [30]. In *S. cerevisiae*, two forms of the enzyme MAT I and MAT II have been reported. Overexpression of MAT in *B. subtilis* has reportedly led to methionine auxotrophy [22, 30].

Owing to the limitations of chemical synthesis and enzymatic synthesis, as well as the dawning of the age of metabolic engineering and synthetic biology with microbial fermentation of valuable products, the direction of recent research has now focused on using these tools to accelerate microbial fermentation of SAM. Efforts have applied several metabolic strategies including those directed at substrate and cofactor availability, improvement of enzyme availability and activity. A number of microbial strains for improved SAM production have also been engineered. Notably, *E. coli*, *Pichia pastoris*, *S. cerevisiae*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Candida utilis*, *Corynebacterium glutamicum*, *Scheffersomyces stipitis*, to mention but a few, have been used for extensive investigations towards improving microbial production of SAM. For specific direction, this review focus on trends involving *E. coli*, *S. cerevisiae* and *P. pastoris*. Several techniques have also been employed to improve SAM fermentation. Table 2 summarizes the strategies that have been employed to improve SAM production by industrially-relevant microorganisms. Most of the techniques have been based on enhancement of enzyme expression, improvement of enzyme specific activity, modulation and fine-tuning of enzyme expression, increasing ATP generation, elevation of substrate conversion ratio and blocking SAM consumption.

This review is intended to provide a condensed evaluation of recent results and progress towards sustainable increase in S-adenosylmethionine production by the application of metabolic engineering and synthetic biology.

1.1 Molecular Structure of SAM

Critical to its diverse functions is the molecular structure of SAM. The metabolite is a sulfonium compound. Its molecular structure (Fig. 1) is a ligation of the methionine structure and the adenosyl moiety of ATP via the sulphur atom of methionine, which subsequently acquires a positive charge, making it willing to donate all the groups surrounding it.

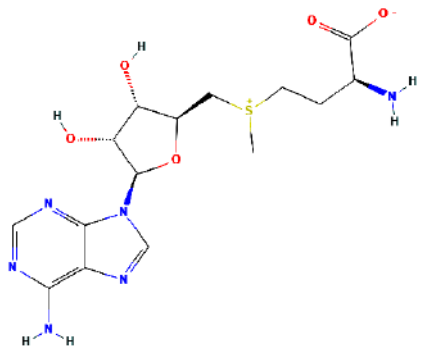


Fig 1. Molecular structure of SAM

1.2 Biosynthesis of SAM

The enzyme methionine adenosyltransferase (MAT) is a fascinating enzyme with diverse behaviours which can vary from organism to organism [31]. The enzyme requires methionine, ATP and water to form SAM, pyrophosphate and inorganic phosphate (Fig. 2a) in the presence of K^+ and Mg^{2+} ions as cofactors [31, 32] (Fig. 2a). MAT is highly conserved across species with up to 85% catalytic sequence amino acid sequence conservation reportedly seen between rat liver MAT and *E. coli* MAT [32]. Regulation of methionine adenosyltransferases vary across species, across organisms and across cells. The mammalian isozymes vary in their K_m for methionine. While MAT II has K_m of about 30 µM [27], it is about 100 µM for MAT I and that of MAT III about 1 mM [33]. In addition, MAT III seems to be greatly activated by DMSO through a mechanism that is unclear [33]. When MAT is overexpressed in *B. subtilis* methionine auxotrophy results [22, 30]. *S. cerevisiae* exhibits two isoforms of the enzyme which are regulated differently. While the isoform *sam1* (like most other MATs) has its activity repressed by accumulation of SAM, *sam2* is insensitive to this phenomenon [30]. In mammals, there are two MAT-coding genes, one of which is expressed exclusively in the liver, and the other in all tissues [33].

SAM synthesis catalysed by methionine adenosyltransferase (MAT) occurs in a reaction between L-methionine and ATP. It occurs in two reaction steps. SAM is produced in the first reaction step, but is however held by the enzyme until the second reaction step is completed. The SAM synthetic reaction is typically cytosolic, occurring via the one-carbon metabolic pathway that involves both methionine and folate cycles [34]. The generation of SAM via the methionine cycle (otherwise called the Activated Methyl Cycle) (Fig. 2b) can be facilitated by several amino acids, including serine, threonine and glycine initiating the folate cycle. During the process of SAM formation, methionine is transformed into a high energy reagent, carrying a sulfonium ion (positive charge) by its combination with the adenosyl moiety to ATP. In the synthesis of SAM, while methionine is a critical substrate, ATP, often regarded as co-factor in reactions wherein it participates, is also a substrate, and could become a rate-limiting substrate when supply of methionine is in excess [35]. In the activated methyl cycle (Fig. 2b) which ensures consistent regeneration of SAM, the methyl-transfer that generates S-adenosylhomocysteine (SAH) from SAM occurs in a typical S_N2-like fashion of nucleophilic substitution. In the reaction, at the expense of ATP, a high energy sulfonium compound is formed. ATP is completely dephosphorylated, and its 5'-deoxyadenosyl moiety transferred to a free electron pair carried by the sulphur atom. This is a resemblance to vitamin B_{12} adenosylation. Although the two innermost phosphate groups of ATP are released as inorganic pyrophosphate (PPi) and the outermost as inorganic phosphate (Pi), a compulsory reaction intermediate formed in the reaction is enzyme-bound tripolyphosphate (PPPi). The MAT enzyme exhibits tripolyphosphatase activity, which is significantly and specifically stimulated by low SAM levels [36]. In the reaction catalysed by the yeast MAT enzyme as described by Greene [37], there seems to be the initial formation of a ternary complex between the enzyme and both substrates, ATP and methionine prior to product formation.

1.3 Regulation of Methionine Adenosyltransferases

Methionine adenosyltransferase is a highly conserved enzyme across the three domains of life. Even though there is evolutionary divergence at some point, reports have suggested that all extant MATs evolved from a common ancestry [38], with most organisms having the enzyme existing in multiple forms [31]. Regulatory mechanisms for the enzyme are however variable across species. SAM metabolism is in *E. coli* greatly controlled and is subject to feedback inhibition [39]. Holloway and colleagues in their 1970 publication [40] have reported the regulatory mechanisms for the enzyme in *E. coli*. In *E. coli*, MAT specific activity is reduced when methionine is added to growth medium, implying that the enzyme is rather repressible than inducible, suggesting that the enzyme is constitutively expressed. SAM biosynthesis is genetically regulated by *metJ*, a repressor. In conditions of its intracellular abundance, SAM binds to *metJ* which subsequently represses transcription of represses responsible for SAM biosynthesis [41]. However, ethionine, nor-leucine, and α-methylmethionine which are poor substrates or non-substrates for MAT have shown to be ineffective as repressors of the enzyme. The methionine analogue selenomethionine, which is a better MAT

substrate than methionine has also demonstrated a slightly higher repressive potential for the enzyme than even methionine. According to [37] tripolyphosphate is a non-competitive inhibitor of the yeast enzyme with respect to methionine at constant ATP concentration. By comparison, SAM is a much weaker inhibitor of the entire reaction than tripolyphosphate and is reportedly an uncompetitive inhibitor in relation to both ATP and L-methionine. While the substrate preference of this MAT is almost inelastic, with closely related L-methionine analogues unable to be activated by the enzyme owing to their very minimal, if any affinity to the enzyme, and thus negligible inhibitors of the enzyme. There are reports that one exception is S-trifluoromethyl-L-homocysteine. This compound exhibits a moderately good inhibition of the yeast enzyme [36]. Other inhibitors of the enzyme of both *E. coli* and yeast, according to the same report include 1-aminocyclopentanecarboxylic acid and 1-amino-3-methylcyclopentanecarboxylic acid [36].

II. CONSTRAINTS TO ENZYMATIC AND MICROBIAL S-ADENOSYLMETHIONINE PRODUCTION

A variety of microbial hosts such as *S. cerevisiae*, *P. pastoris* and *E. coli* have been engineered for SAM production. Despite some successes, there exists a number of bottlenecks hindering the SAM production by microbial or enzymatic strategies on a grand scale. The most concerning constraint to enzymatic SAM production is product inhibition. Reports have suggested severe inhibition of the MAT enzyme in systems accumulation the product to concentrations that exceed 0.1 mM [42-44]. Strategies that have been directed towards relieving this constrain include the use of high concentration of sodium *p*-toluenesulfonate (*p*TsONa) [45, 46]. In a recent report, Yin and colleagues have published that *p*TsONa concentrations up to 0.8M can completely alleviate product inhibition of the *E. coli* enzyme, and in one variant created by site-directed mutagenesis where the 303rd isoleucine residue was substituted for valine, only about 0.3 M *p*TsONa was required for complete alleviation of product feedback inhibition, and over 80% ATP conversion on a 40 mM- scale [45] was achieved.

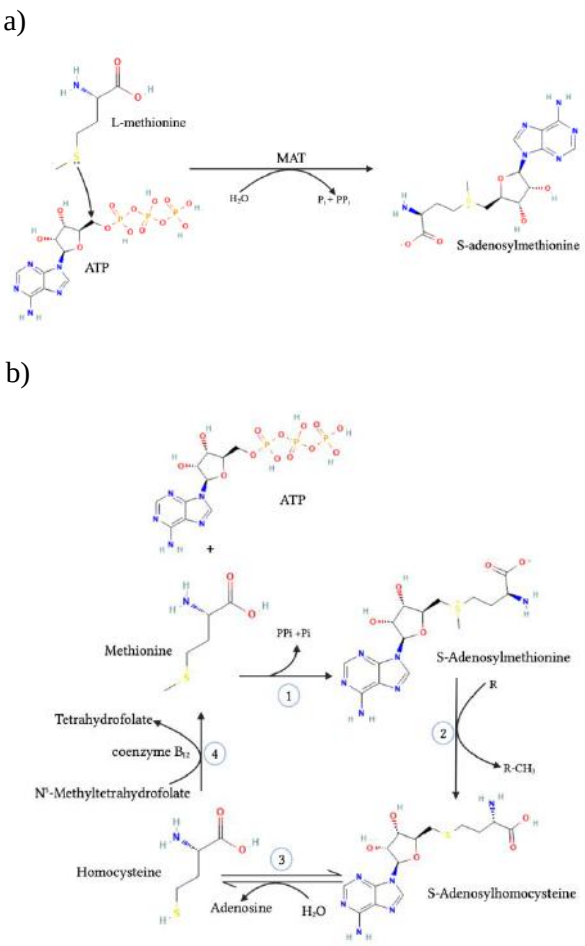


Fig. 2 Biosynthesis of SAM. a) Production of S-adenosylmethionine (SAM) from L-methionine and ATP, catalysed by Methionine adenosyltransferase (MAT) with release of inorganic phosphate (Pi) and inorganic pyrophosphate (PPi). b) Activated methyl cycle for the continuous cycling of SAM (1)methionine adenosyltransferase (2) various SAM-dependent methyltransferases (3) hydrolase (4) methionine synthase

Also, fermentative SAM production by yeast cells is hindered by uptake of L-methionine. When culture medium contains more than 1% of L-methionine, cell growth and transcription of MAT gene is repressed [47]. Expressing the enzyme using a strong promoter can easily manage this concern. Another constraint that requires mention is that when methionine is available in sufficient to high concentrations, ATP becomes a rate-limiting substrate for SAM synthesis [35].

Some other drawbacks of SAM fermentation associated with the use of yeast include very long fermentation time (at least 48 h and up to 120 h), which require very high energy consumption and often leads to yields not more than 15 g/L medium. Yeasts have a low L-met conversion rate [47]. Also, extraction and purification of SAM from

yeast and other downstream fermentation processing usually require some environmentally unfriendly procedures for cell disruption, as well as the excessive use of perchloric acid for product extraction.

III. STRATEGIES FOR IMPROVING MICROBIAL SAM PRODUCTION

3.1 Improving endogenous substrate availability and enhancing substrate uptake

Very often, the route to fermentative production of SAM is through the addition of L-methionine to the fermentation medium, with the utilisation of endogenous ATP as second substrate. [48] reported that when proper concentration is applied, the precursor methionine is favourable for improving production of SAM by *C. utilis*. Surprisingly however, in some microbial strains, especially yeast, cell growth and transcription of MAT genes is repressed when culture medium contains more than 1% methionine, as previously mentioned [47]. This MAT repression ultimately results in reduced SAM yield. A proposed mechanism for this yeast methionine uptake constraint is the repression, by excess methionine, of two out of seven important permeases that allow uptake of methionine into yeast cells, namely MUP1 and MUP3 [49]. In addition to this reported methionine uptake constraint, ATP the second substrate is important for a diverse variety of energy-dependent cellular functions, and in the abundance of methionine could become the rate-limiting substrate in the bioconversion of methionine to SAM [35]. When this happens, exogenous ATP supply might become necessary to sustain SAM production. Considering how expensive commercial ATP is, it is imperative to apply a cheaper alternative to its supplementation for more cost-effective SAM production. Several groups have attempted to improve microbial SAM production by increasing available endogenous ATP. There have been reports of improved microbial SAM production when either or both MAT substrates are endogenously generated by the cell. In one study with *S. cerevisiae*, Chen and colleagues reported that co-expressing *met6* (encoding yeast methionine synthase) and *sam2* (encoding yeast MAT2) combined with sodium citrate feeding; for increasing methionine availability, MAT expression and provision of auxiliary energy respectively. Their results showed the SAM production of the recombinant had increased by 2.34 folds from that of the wild-type, and sodium citrate feeding also led to 19% increase in SAM accumulation [50]. In a similar study aimed at improving the availability of ATP for SAM synthesis in *P. pastoris*, *Vitreoscilla* haemoglobin was expressed along with the MAT gene from *Streptomyces spectabilis.* Expression of the enzyme with the enhancement of ATP availability by increasing cellular oxygen delivery and oxidative respiration intracellularly resulted in higher MAT activity by a value of 27 times and an increase in SAM production about 19 times compared to wild-type [35]. Also, in *P. pastoris*, a recombinant overexpressing the *sam2* gene, methionine permease *mup1*, and adenylate kinase *adk1* for MAT, methionine uptake and ATP improvement respectively. While expressing the genes individually had only the *sam2* recombinant showing marked SAM production, there was great improvement of up to 77% with all three genes synergistically expressed [51]. To produce SAM in *C. glutamicum* without the addition of exogenous methionine, *thrB*, *metB*, *mcbR* and *Ncgl2640* were deleted, and *metK*, *vgb*, *lysCm*, *homm*, *metX* and *metY* were overexpressed in *C. glutamicum*ATCC13032. The recombinant strain was able to produce SAM as much as about 0.2 g/L after fermentation for 48 h [52]. Methionine biosynthesis in yeast typically requires consumption of acetyl-CoA in the cell cytosol which is mainly produced from acetate in a reaction which is catalysed by acetyl-CoA synthase (*acs2* encoded). Furthermore, there are also two enzymes of the glyoxylate which play significant roles in cytosolic acetyl-CoA homeostasis, namely malate synthase (encoded by *mls1*) and citrate synthase of the peroxisome (encoded by *cit2*). [53] demonstrated that over-expression of *acs2* and deletion of both *cit2* and *mls1* in a *S. cerevisiae* strain which has ethionine tolerance caused increase in levels of acetyl-CoA, and also elevated methionine level, and in turn SAM accumulation in the yeast strain was enhanced.

To improve the amount of ATP available intracellularly for SAM production, Wang and colleagues [48] further reported that addition of amino acids oriented towards ATP production could improve accumulation of SAM and glutathione, which also stresses the fact that SAM biosynthesis could be enhanced by manipulating the ATP supply. Furthermore, sodium citrate has been reported to stimulated the production of ATP and further enhancing production of SAM by *C. utilis* [54]. A similar phenomenon has also been reported in *S. cerevisiae*[50]. [55] reports enhanced *E. coli* SAM production by using srRNAs for the control of ATP concentration. The SAM titre as well as the yield of the recombinant strain increased nearly two folds in comparison with control. In the study, genes involved in the synthesis of by-products requiring ATP in the pathways for SAM production were repressed. These genes include *argB*, *glnA* and *proB*. The highest SAM titre and yield were respectively obtained with strain Anti-*argB* (0.121 g/L) and the Anti-*glnA* strain (0.13 mg/g, 12 h). In another report, by combining PPK from *C. glutamicum*, and *V*Hb with *ydaO* motif riboswitch for sensing ATP riboswitch in separate experiments [56]

developed a dynamic ATP regenerating strain of *E. coli* which was able to accumulate SAM up to 82% more than the wild type. A recent publication has shown improved ATP and SAM synthesis by application of engineered multi-domain scaffold proteins in *E. coli* [57]. The study assembled an artificial protein scaffold having a CBM3 domain, CL-labelled proteins and IM proteins. This scaffold complex would serve to boost ATP production. The results demonstrated ATP production up to about 25 g/L with ADP concentration of 15 g/L; AMP 5 g/L using adenosine 12.5 g/L and sodium hexametaphosphate 40 g/L. Combined with the already established complex for ATP generation, SAM synthesis was achieved by another scaffold complex employing two CL-labelled methionine adenosyltransferases (CL9-MAT4 and CL9-MAT5). MAT4, in the presence of 0.5 g/L methionine was able to yield about 0.9 g/L SAM while MAT5 could produce 1.2 g/L.

Chen and Tan in 2018 also posits enhanced SAM production in *S. cerevisiae* by increasing ATP levels [42]. Here, they adopted ATP-regulation strategies which included manipulating the availability of NADH while also regulating the supply of oxygen. A variety of ATP-regulation systems were designed by employing NADH oxidase, *Vitreoscilla* haemoglobin and phosphite dehydrogenase. Improved SAM production was attained by combination of the system with overexpression of the *sam2* gene. With the collective system, the SAM titre of the resulting strain ABYSM-2 reached about 55 mg/L, amounting to about 67% increase relative to the control strain.

Oxygen supply and source of carbon limitations are causes of serious concern during fermentation when yeast cells grow rapidly. To improve the yield of SAM by recombinant *P. pastoris*, a technique which requires addition of 1 % n-heptane, to function as oxygen vector, and 1.2 % sorbitol, for supply of supplemental carbon has been described. The method led to significant improvement in SAM productivity (53.26 %) when 1.0 % n-heptane was added at 72 h, 1.2 % sorbitol at 72 h, 96 h, and 120 h of incubation, with 1.0 % methanol every 24 h during the fermentation process. This study confirms that carbon source supplementation and application of oxygen vectors is effective to overcome intracellular scarcity of energy by improving respiration and the subsequent ATP generation [58]. Table 1 summarises the substrates-focused improvements towards improving microbial SAM production.

3.2 Enhancing substrate conversion

Generally, a very simple way to improve bioconversion of substrate to product especially for a single-enzyme catalysed biotransformation such as that leading to SAM production would be increasing the cell saturation status of the enzyme: more enzyme equals more bioconversion. Typically, most methionine adenosyltransferases demonstrate great sensitivity to the product S-adenosylmethionine. They are inhibited when the product has accumulated to high concentration. Significantly, [20] had characterised the *E. coli* enzyme, stating that SAM is a competitive inhibitor against ATP (K_i = 0.01 mM) and a non-competitive inhibitor against methionine (K_i = 0.01 mM), and the other products pyrophosphate (PPi) and orthophosphate (Pi) also serve as non-competitive inhibitors against ATP (K_i, ppi = 0.4 mM & K_i, pi = 8 mM) and methionine (K_i, ppi = 0.4 mM & K_i, pi = 12 mM). Their publication suggests therefore that the *E. coli* enzyme is severely inhibited by SAM in incubations containing ≥ 1mM methionine. Notably, one variety of the MAT enzyme has been identified to resist inhibition by the product. This is the enzyme product of the *sam2* gene of *S. cerevisiae*. Several research groups have attempted to overcome this important problem, a cog towards enhanced microbial or enzymatic SAM production. A common strategy is the use of high concentration of a salt of an organic acid such as sodium *p*-toluenesulfonate (*p*TsONa) [45, 46]. In one of such studies that could be considered a pioneering one, Park and colleagues proved that when cell free catalysis is carried out in a system containing 400 mM *p*TsONa with 10 mM methionine, product inhibition was completely overcome. The effect of *p*TsONa in alleviating such product feedback inhibition was determined to be dependent on its concentration. And it was able to enhance permit the complete conversion of substrates to SAM within 5 hr when methionine concentration was about up to 30 mM and ATP 1.3 equivalents of that and $MgC1_2$ 2.6 equivalents [46]. Another strategy that has been employed to improve substrate conversion by MAT is enzyme engineering. Yin and colleagues have in 2017 reportedly improved the activity and substrate conversion strength of recombinant *E. coli* MAT overexpressed in the same organism by a site-directed mutagenesis wherein I303 residue was replaced with a less bulky valine residue. The resulting variant exhibited a significantly reduced susceptibility to product inhibition and therefore marked improvement in substrate conversion [45]. Also, in line with this strategy, semi-rationally modified the MAT enzyme from *E. coli* to produce a variant with triple amino acid substitution– Ile303Val/Ile65Val/Leu186Val– resulted in a 42-fold improvement in its K_i for ATP and a 2.08-fold improvement in the enzyme's specific activity in comparison with the native unedited MAT [59]. Its K_i for ATP was 0.42mM and specific activity was 3.78 ±0.19 U/mg. The increased K_i for ATP implies less susceptibility

to product inhibition and a concomitant enhancement of SAM synthesis. The amount of SAM produced by this variant could reach 3.27 mM which is about twice that of the wild-type MAT when the concentration of substrates was 10 mM. Further optimization of the 104th residue of the variant, Asn104Lys by site-saturated mutagenesis, increased the enzyme's specific activity to about 6.02 U/mg at 37 °C. However, the amount of SAM accumulated by the resulting variant dropped to 2.68 mM with the same substrate concentration. When the endogenous *S. cerevisiae sam2* gene was integrated into the genome of an industrial *S. cerevisiae* strain in other to improve its SAM productivity while also barring product inhibition [30, 60], the application of a tri-phasic fed-batch fermentation module in 15 L fermenter led to 8.81 g/L SAM production after 52 h of cultivation. This result amounts to about 27.1 % better performance than the parent strain [61]. Similarly, overexpression of the same gene in *P. pastoris* led to the development of a strain that demonstrated a 17-fold higher MAT enzyme activity than the control [60]. [62] submits that an *E. coli* strain that could effectively produce SAM was constructed by overexpressing the *metK*. In shake flask and basic fermentation medium, the strain was able to produce about 34.5 mg/L of SAM.

Having expressed the enzyme, a report [25] has also described the characteristics of *Leishmania infantum* MAT (encoded by *metK1*). In contrast to the behaviour of the same enzyme from mammals and yeast, the activity of *Leishmania* MAT is only weakly subject to regulation by the product. To overcome possible feedback inhibition of MAT with the goal to improve SAM production, the *metK1* gene from *L. infantum* (codon-optimised) was expressed in *S. cerevisiae*. The resulting strain could accumulated up to 1.62 g/L of SAM, 2.45 times better than the untransformed wild type, and its calculated MAT activity was shown to be much greater in comparison with the wild type and a parallel strain which expressed *sam2* [53].

3.3 Application of unusual substrates

Production cost exerts heavy impact on sustainable SAM production. Thus, heralded by the constant quest to reduce cost of production, the use of cheaper or more readily available substrates presents an option for relieving this bottleneck. As has already been established, SAM is synthesised from methionine and ATP. Typically, most MATs prefer the L- isomer of methionine. DL-methionine, a racemic mixture consisting of both the D- and L- isomer has been used to successfully synthesise SAM, while also some promiscuous MAT enzymes with extended substrate specificity have been engineered or isolated. Based on a survey of the market in China, L-methionine was found to cost about 3-times more that of DL-methionine [63]. To reduce the cost of production, [64] by metabolic engineering developed an industrial yeast strain for conversion of D-methionine to L-methionine by disrupting D-amino acid-N-acetyltransferase (HPA3), and up-regulating expression of both *Trigonopsis variabilis* D-amino acid oxidase (DAAO) and *Rhodococcusjostii* L-phenylalanine dehydrogenase (L-PheDH) in the yeast strain. The resulting strain, when *sam2* was also overexpressed, could by such modification convert the cheaper racemic DL-methionine to SAM. When this strategy was combined with other genetic modifications to lessen SAM degradation, SAM was effectively produced to 10.3 g/L concentration by the resulting strain in 10 L fermenter when DL-methionine (16 g/L) was supplied. [65] also describes a method for producing SAM from pilot scale to industrial scale using DL-methionine as substrate by yeast fermentation applying *S. cerevisiae* SAM0801, a mutant displaying high MAT activity. Interestingly, while serving as a useful method for improved industrial SAM production, the method proves to also be effective for chiral separation of the racemic mixture of DL-methionine and thus, the by-product D-methionine might as well be treated as completely new product, effectively implying co-production of SAM and D-methionine. In a 5-L fermentation with 80 g/L of DL-methionine, the SAM concentration reached was about 13.74 g/L, with the rate of conversion of L-methionine being 32.15 %. Upon scaling up the process to more realistic industrial capacity in a 300-L pilot scale of the SAM-producing fermentation system, 10.76 g/L SAM was generated, with the rate of conversion of L-methionine approaching 30.22 %. The amount of D-methionine left behind in the fermentation broth was 76.89 %. This therefore implies the feasibility of this process for commercial scale SAM production.

3.4 Co-culture

When it comes to microbial production of useful chemicals, the monoculture strategy has been the mainstay. Production has traditionally focused on engineering a single microbial species for the total conversion of substrate to product. However, there are reports of the application of a divide-and-conquer strategy that involves modular co-culture which applies multiple microorganisms synergistically thriving in one system to enhance chemical biosynthesis. A key advantage of the latter strategy is that one can streamline genetic optimization of the individual modules to the requirements of the specific points of biosynthesis in the production process. Systems biology and certain integrated genetic engineering approaches on a single microbial strain would usually involve specific genes knockout or up-regulation which could concertedly

direct metabolic flux to production and withdraw nutrient from microbial thriving and formation of cell biomass, ultimately yielding detrimental effects on the life of the engineered microbe. Another feature of monoculture system that poses significant threat to productivity is that sometimes, some engineering strategies can lead to the development of redox imbalance to the detriment of cell survival [66]. This burden can be relieved by the latter modular approach that combines several engineered strains in a microbial consortium to achieve a final product while trying to maintain strains survival. Jones and Wang have a rich summary of the application of this model [67]. The co-culture approach has been successful in producing several chemicals of industrial interest [68, 69]. Despite this increasing popularity, there is scarcity of data reporting application of the method to SAM production.

A 2015 report [70] has suggested improved *E. coli* production of SAM in a co-culture system with *S. cerevisiae*. While *E. coli* was the producer strain overexpressing the MAT enzyme, the *S. cerevisiae* in the system provided the ATP substrate required for SAM production. The SAM concentration of the coupled system was able to reach about 1.7 g/L a 10-fold increase from the *E. coli* alone system which could only produce about 0.17 g/L. This points to the possible success of further exploration of the co-culture metabolic engineering model for application in the microbial production of SAM.

3.5 Optimization of fermentation conditions and feeding strategy

The effect of a variety of fermentation conditions and substrate feeding modes on the production of SAM by recombinant microbes have been investigated by several groups. Despite the diversity of methods tried, the goal remains to achieve sustainable improved production of SAM by the microbial machines at industrial capacity. Using recombinant *P. pastoris* harbouring shuffled genes for the MAT enzyme from *S. cerevisiae* and *E. coli*, Hu and colleagues [71, 72] explored a variety of L-methionine feeding strategies for improving SAM synthesis. They explored fed-batch and continuous L-methionine feeding in 5 L and 30 L bioreactors. The batch strategy involved addition of 15 g/L culture broth L-methionine supplementation independently delivered at three various times at a concentration of 5 g/L/20 hour from the 10^{th} to 50^{th} hour post-induction. Whereas, for continuous feeding of L-Methionine, a variety of concentrations of the substrate was applied. Concentrations of 8 g/L, 16 g/L, and 40 g/L were fed into the fermentation set-up continuously from 10 h post-induction at the same volumetric rate of 12.5 ml/L/h. They report that SAM concentration rose to much higher concentration when the continuous feeding mode was employed, in contrast to the fed-batch mode. Continuous feeding of 0.2 g/L/h L-methionine produced the greatest amount of SAM, about 8.5 g/L, which was about 49% better than what could be obtained by the batch- methionine addition strategy. In a 2008 report also using *P. pastoris* for SAM fermentation, Xiao-Qin Hu and their team examined how five modules of glycerol supply (based on limited and unlimited feeding modes) affected the production of SAM by *P. pastoris* overexpressing MAT under the P_{GAP} promoter [73]. They submit that SAM production was enhanced when glycerol concentration was kept at 2% by unlimited supplementation in fed-batch cultivation, reaching up to 9.26 g/L SAM volumetric accumulation, a yield of 0.058 g/g. Tofu yellow serofluid is a nutritionally rich, yet cheap microbial feedstock which makes it a reliable fermentation material. Its application in the fermentation of SAM is quite limited. However, one research group [74] in a recent publication have demonstrated its applicability as cheap nutrition source for SAM fermentation. The reported optimum fermentation medium with tofu yellow serofluid for SAM fermentation contained, 30 % yellow serofluid, 70 g/L glucose, 2.5 g/L ammonium citrate and 20 g/L L-methionine. Results showed high cell biomass, reaching 123.1 g/L dry cell weight, and SAM production reaching 16.14 g/L, with overall productivity of up to 1.05 g/L/h. It was also estimated that addition of tofu yellow serofluid to the fermentation was able to reduce the mean commercial SAM cost by about 31.9 % in contrast to fermentation without its addition.

Another uncommon nutrition source that has been used in the fermentative production of SAM is unpolished rice from aging paddy along with medium optimisation and *S. cerevisiae* fed-batch fermentation. In the study aimed to cut down overall SAM fermentation cost, the major source of nutrient provided was unpolished rice gotten from aging paddy. Carbon and nitrogen were supplied by the unpolished rice saccharificate (URS) and yeast extract, respectively. The adequate dosages of these nutrient sources were optimised using response surface methodology (RSM) and 51.4 g/L URS and 4.74 g/L yeast extract was determined as optimum and applied for fermentation. This led to SAM yield of 2.61 g/L. One-time methionine supplementation when cell density was 80 g/L led to SAM production of 5.3 g/L and cell biomass 89 g

Table 1: Summary of strategies for improving SAM production by substrates-focused modifications

Reference	Substrate targeted	Microbe employed	Strategy	Result
[50]	Methionine	*Saccharomyces cerevisiae*	co-expressing *met6* (encoding yeast methionine synthase) and *sam2* (encoding yeast MAT2) combined with sodium citrate feeding	2.34-fold increased SAM production. Sodium citrate feeding also led to 19% increase in SAM accumulation
[35]	ATP	*Pichia pastoris*	Expression of *Vitreoscilla*haemoglobin for improved oxygen delivery and MAT gene from *Streptomyces spectabilis*	19 times improved SAM production compared to wild type
[51]	Methionine, ATP	*P. pastoris*	Overexpressing the sam2 gene, methionine permease *mup1*, and adenylate kinase *adk1* for MAT, methionine uptake and ATP improvement respectively	77% improved SAM production with all three genes synergistically expressed
[52]	Methionine, ATP	*Corynebacterium glutamicum*	Deletion of *thrB*, *metB*, *mcbR* and *Ncgl2640* and overexpression of *metK*, *vgb*, *lysCm*, *homm*, *metX* and *metY*	196.7 mg/L (12.15 mg/g DCW)
[53]	Methionine, ATP	*S. cerevisiae*	Overexpressing *acs2* and deleting *mls1* and *cit2*	6.06 g/L SAM in 10-L fermenter by fed-batch fermentation
[54]	ATP	*Candida utilis*	Sodium citrate feeding	27.5 % increase compared to control
[55]	ATP	*E. coli*	Application of srRNAs for control of intracellular ATP concentration	2-fold increase in yield of SAM
[56]	ATP	*E. coli*	Combining PPK from *C. glutamicum*, and *V*Hb with *ydaO* motif riboswitch for sensing ATP	82% better SAM accumulation than wild type
[57, 75]	ATP	*E. coli*	Assembly of an artificial scaffold protein containing CBM3 domain, IM proteins and CL-labelled proteins for ATP improvement and scaffold protein construction comprising two CL-labelled methionine	1.2 g/L SAM accumulation

			adenosyltransferases (CL9-MAT4 and CL9-MAT5) to achieve SAM synthesis.	
[42]	ATP	*S. cerevisiae*	Introduction of NADH oxidase, phosphite dehydrogenase and *Vitreoscilla* hemoglobin in combination with *sam2* overexpression	67% increase in SAM accumulation
[58]	ATP	*P. pastoris*	Addition of 1 % n-heptane (oxygen vector) and 1.2 % sorbitol (supplemental carbon source)	53.26 % improvement in SAM productivity by adding 1.0 % n-heptane

However, slight improvement in SAM resulted when methionine was fed in the broth at 2 g per hour for 5 hours when the cell density had reached 80 g/L. With this method, SAM production reached 5.82 g/L and the cell biomass got up to 90.2 g/L. [76]. According to Yu and Zhu [77], *E. coli* overexpressing homologous *metK* had its SAM yield affected by pH, yeast extract and loaded volume. The study determined the optimal values of the parameters to be 7.5, 35 g/L and 30 mL, respectively. The final optimum medium condition was subsequently determined to be: 40 g/L peptone, 20 g/L glucose, 35 g/L yeast extract, 1.2 g/L $MgSO_4$, 10 g/L NaCl, 1 g/L L-methionine, rotation speed of 220 rpm, 30 mL loaded volume, inoculation of 1%, temperature and initial medium pH of 37 ºC and 7.5 respectively. Under these determined optimum conditions, the recombinant *E. coli* was able to produced SAM up to 128.2 mg/L during shake flask fermentation. In 5 L fermenter, cell biomass reached 60.4 g/L after 20 h of culture, and the SAM yield reached 300 mg/L after 8 h of induction [77].

3.6 Mutation breeding: UV mutagenesis, gene shuffling and spaceflight culture

In contemporary biotechnology, mutation breeding has been employed to improve performance of microbial strains in various biotechnological applications. UV mutagenesis, ARTP mutagenesis, DNA shuffling and spaceflight culture are some methods that have been used in introducing desirable genetic changes in microbes for strain improvement. UV mutagenesis has been used to significantly improve production of SAM by *S. cerevisiae* Sake K6 which overexpressed the *sam2* gene [43]. Yeast MTHFR (met13 gene product) is inhibited by SAM and requires NADPH to function, unlike plant MTHFRs, which are SAM-insensitive and rather require NADH. For ameliorating SAM feedback inhibition of MTHFR, expressing an chimeric MTHFR made up of the N-terminal domain of yeast MTHFR and C-terminal domain of Arabidopsis MTHFR, led to elevated methionine and SAM synthesis by 7- and 140-fold, respectively [2].

New and often interesting or desirable phenotypes have been successfully developed through exposure of cells to Ultraviolet (UV) irradiation, lithium chloride and ethyl methane sulfonate [78, 79]. Ethionine is able to regulate the synthesis of sulphur-containing amino acids and is also an analogue of methionine that is competitive with it in protein composition [80]. Employing the budding yeast as chassis and introducing genetic variations by UV irradiation, [81] achieved a high SAM-accumulating strain which was isolated by ethionine-resistance screening which was quick and effective for their selection. The strain demonstrated improved SAM production and MAT activity by about 4.3-fold and 2.7-fold compared to each of their starting strains. SAM production by the mutant *S. cerevisiae* CGMCC 2842 reached 6 g/L in a 15-L fermenter following 36-h fed-batch fermentation. *In vitro* recombination has also been employed towards improving microbial production of SAM. Enhanced MAT activity and SAM production by recombinant *P. Pastoris* expressing a chimeric MAT gene that consisted of the gene from *E. coli*, *S. cerevisiae* and *S. spectabilis* recombined by DNA shuffling for enhancement of MAT activity, and in turn, accumulation of SAM has been reported [71]. The recombinant strain which carried the shuffled gene demonstrated greatly improved MAT activity at least 200% more and SAM accumulation at least 103% more than any recombinant with a native MAT gene merely overexpressed. Upon scaling up fermentation to a 500-L bioreactor, SAM production of up to 6.14 g/L was recorded. A genetic recombination strategy that increased SAM production alongside that of S-adenosylhomocysteine in sake yeast has been reported [82]. *Sah 1* is required for growth in yeast, and a *Sah1-1*

mutant engineered in two steps via genetic recombination showed increased SAM production 0.1 g/g DCW compared with wild type, 0.04 g/g DCW [82, 83]. Most industrial strains of yeast exhibit diploidy and are often sporulation- defective. Consequently, genetic engineering and/or manipulation of the strains are somewhat thought to be less manageable. The diploid Japanaese sake yeast is one of such strains. To make it more tractable for genetic manipulation, variants carrying homozygous deletion of *ura3*, showing homozygousity for either MATα or MATa, and homozygousity for either the *his3* or the *lys4* mutation were engineered. Gene targeting was enhanced in the *ura3*-null genotype variant. To enable their overexpression constitutively, the TDH3 promoter was placed upstream of six yeast genes known to participate in the control of flavour. Tetraploid strains simultaneously overexpressing two different genes were combinatorially constructed by allowing mating between homozygous MAT allele carries with non-complementary auxotrophic mutants and selecting by the resulting tetraploidy. Furthermore, by replacing one wild-type allele and subsequent disruption of the other, a recessive mutant *sah1-1* known to SAM in high amounts was introduced into the diploid sake yeast strain. The strain resulting from the construct was determined to produce much higher quantities of SAM compared with the wild-type

During mutation breeding, it is imperative to apply mutation techniques with high mutation rate and ability to introduce a broad range of mutations. Spaceflight environment represents a complex environmental state, involving the interplay of a variety of forces, including, *inter alia*, microgravity, space magnetic fields, cosmic radiation, high charge and high energy (HZE) particles and high vacuum [84, 85]. Within the last five decades, several natural or simulated spaceflight experimental procedures have been intentionally performed on terrestrial life on the basis of spaceflight biological research platform [84, 86]. DNA mutation [87-89], changes in gene expression [90, 91], changes in the production profiles of secondary metabolites [92, 93], physiological and morphological characteristics change [94, 95] are some observed modifications due to spaceflight. A high SAM-accumulating *S. cerevisiae* strain has been isolated through spaceflight culture. Upon isolation, this strain was reported to synthesize SAM 86.89% more than the control strain ground. By application of Amplified fragment length polymorphism (AFLP) analytical method, it was confirmed that demonstrated that genetic variations had occurred between spaceflight cultured strain and the control on ground. By means of recombinant DNA technology, a heterologous MAT gene was inserted into the spaceflight strain genome. This generated a recombinant with 42.98% improved SAM production. On further optimisation of cultivation conditions using Taguchi and one-factor-at-a-time methods, the final strain produced SAM up to 7.76 g/L in shake flask, representing about 536% improvement from the initial ground control strain. In fermenter cultivation, about 9.64 g/L SAM resulted [96].

3.7 Whole-cell biotransformation

In classical fermentation, a palpable demerit is that while being hopeful of great productivity due to channelling of pathways and overexpression of key enzyme(s), often, the microbial strain could convert some substrate into biomass if they remain in growing state. To overcome this perceived limitation, cells could be used to make products in biotransformation or bioconversion in their resting state. In recent years, biocatalysis has gained much attention as an environmentally friendly and sustainable way to synthesize valuable organic compounds [97]. The application of whole-cells for biocatalysis is an area that is still gaining popularity in synthetic biology. Besides its offering of a channel for the conversion of substrates to product without diversion to growth and cell maintenance, whole-cell application for biocatalysis or biotransformation also provides the advantages of reduced undesired reactions, easier products purification, and less environmental harm [98]. The application of whole-cells in resting phase for biotransformation or bioconversion outmanoeuvres the requirement of intracellular enzyme purification for catalysis and also provides a more favourable environment for the functioning of enzymes. An advancement of the method is the use of immobilised whole-cells. The immobilization of cells for biocatalysis can enhance product purification and allow for several re-uses of the biocatalysts, thus significantly cutting down the production cost. Despite these merits, this method remains largely unexplored for the production of SAM as there is scarcity of data reporting its application. However, one recent publication where *E. coli* cells overexpressing a variant of its MAT enzyme was used for whole-cell biotransformation, high activity was demonstrated by both the mobilised and immobilised cells, and immobilised cells kept about 90% of the initial activity they demonstrated after being reused five times, and 67% being reused 10 times. In addition, the cells, immobilised magnetically could produce SAM using 40-mM ATP effectively even when re-used 10 times, with a very efficient rate of conversion of ATP greater than 95%. There was 100% ATP conversion within the first four cycles within four hours while in the fifth to tenth cycles, complete conversion required about 5-6 hours [45].

3.8 Enhancing product accumulation by metabolic engineering

A study of the metabolic fluxes towards and away from SAM would reveal that besides driving substrates towards SAM production, it is also imperative to keep SAM from being used up if high intracellular accumulation of SAM is to be achieved. Homocysteine is a critical metabolite in the flux map of SAM metabolism. This compound has three alternative fates that somewhat directly imparts SAM: it could be converted to methionine, or S-adenosylhomocysteine or to cystathionine by cystathionine-ß-synthase. The latter takes homocysteine away from the SAM, and therefore is of negative influence towards accumulation SAM. In a bid to enhance *P. pastoris* capacity to produce SAM, He and colleagues knocked-in the *S. cerevisiae sam2* gene and knocked out the CBS gene [47]. The result was a significant rise in SAM accumulation by the recombinant strain which accumulated SAM up to 3.6 g/L at shake flask level, and 13.5 g/L in 5 L fermenter. In order to produce SAM in without addition of the principal substrate methionine, [52] has described a metabolic engineering strategy that utilises the metabolic system of the popular amino acids-producing *C. glutamicum.* The resulting strain from SAM-centred gene deletions and knock-ins was able to produce 196.7 mg/L SAM (12.15 mg/g DCW) after 48 h. Similarly, the SAM productivity of *B. amyloliquefaciens* has been significantly improved by metabolic engineering [99]. In the publication, four genes thought to affect SAM production, including *S. cerevisiae sam2, E. coli metA* and *metB* and *B. amyloliquefaciens mccA* were applied together in engineering the SAM synthetic pathway. This resulted in a 1.42-fold SAM titre increase in the recombinant. A subsequent coupling of this pathway with the TCA cycle by deleting the succinyl-coA synthetase gene (*sucC*) led to a 0.59-fold increase in SAM titre, producing up to 107.74 mg/L SAM. Further expression of *sam2* in the strain generated 0.65 g/mL SAM in semi-continuous batch fermentation, a comparatively high SAM yield when methionine is not applied in fermentation medium.

Also, in *E. coli*, deletion of *metJ* alongside overexpression of genes that play part in the pathway for biosynthesis of methionine led to a 33% increase in SAM concentration [100].

3.9 Sundry Technologies

These days, genes can be switched on and off in similar fashion to light switch control by a simple circuit. One report [101] suggests the effectiveness of a constructed genetic circuit that reports the concentration of SAM available intracellularly in *S. cerevisiae*, simply by applying the *metJ-metO* system and SAM feedback regulation. To construct the circuit, *metJ* was fused with transcriptional activator domain (AD) B42 in addition to a reporter gene incorporated downstream of *metO*, a methionine operator. The SAM-*metJ*-B42 complex will usually bind to *metO* when SAM is present, leading to the activation of the downstream reporter gene by B42. This circuit has the ability to perceive SAM concentrations as little as 5 µM. The study reports that with this circuit enabling high-throughput screening for SAM production, the authors were able to identify that SAM production improved in *S. cerevisiae* by 3.3-fold when *gal11* was overexpressed. CRISPR technologies have exposed almost every field in biotechnology to a world of unending possibilities. Dong and their colleagues [102] reportedly applied one such CRISPR-based technologies to improve SAM production of industrial *S. cerevisiae*. They describe MAGIC (multi-functional genome-wide CRISPR), a method by which transcription was simultaneously activated and interfered with, and genes deleted. They combined this method with Umeyama and collegue's circuit, and by several cycles of transformation of guide RNA libraries into yeast harbouring the SAM circuit, they identified new mutable targets for improving SAM accumulation by the yeast strain. Upregulating RPS18B, SNZ3, and RFC4 improved SAM production by 2.2- and 1.6-fold respectively, in laboratory strain and industrial yeast strain.

IV. CONCLUSION AND RECOMMENDATION

The existing wide applications of SAM necessitates the need for its sustainable production. Presently, its market capacity is quite unsatisfactory and requirement constantly increasing, hence it is unable to quench existing demand. Herein, we have described several strategies that have been employed to boost production of SAM which can translate into commercial scale sustainable production. These strategies comprise system metabolic engineering and mutation breeding, encompassing carbon source and nutrients feeding, endogenous substrates generation, and energy supply, enzyme evolution and conditions optimisation, system metabolic engineering. While successes have been mixed, previous and ongoing studies on engineering of microbial cells for SAM production suggests great promise for sustained availability of the product in the near future, as it is expected that application of existing and novel strategies can fuel success. While most research has focused on improving accumulation, there is scarcity of data on improving SAM efflux. *S. cerevisiae* has been reported to possess a high-affinity S-adenosylmethionine permease, a SAM transmembrane transporter encoded by *sam3* gene [103]. Successful

exploration and application of this transporter to improve product efflux could prove to be game-changing in improving microbial SAM production. Many reports have applied chemicals and surfactants such as Cetyl trimethyl ammonium bromide (CTAB), ethyl acetate, hexane and triton X-100 to enhance cell permeability and efflux of the product. Most of these chemicals are potentially toxic both to the cells and to the handlers. Transporter engineering and/or application of environmentally safe cell cytoplasm permeabilizers in contrast to toxic surfactants currently in use, could represent a potential defining moment in SAM production research as it can serve two purposes. While eliminating the arduous task of intracellular SAM extraction, it also has the potential to ameliorate product feedback inhibition of the MAT enzyme. Breakthrough in enhancing product efflux could be tantamount to unprecedented success in enhancing largescale SAM production by the application of microbial cell factories. *P. pastoris* and *S. cerevisiae* have the capacity to accumulate SAM contents to high amounts because of their possession of large vacuoles which are able to accommodate sequestration of SAM within and could hence provide effective starting strains for improved SAM production. In addition, a potentially useful starting strain for SAM fermentation might be one that produces high amounts of substrates available for conversion to product. This might be cost-saving when such strains are applied as starting strains or as part of a SAM-producing microbial consortium. Wilke [104] has summarised microbes engineered

Table 2: General strategies employed to improve the production of SAM by industrially relevant microbes

Microbe	Strategy	SAM Titre Attained	Reference
Escherichia coli	Control of intracellular ATP concentration using ATP-sensing riboswitch	1.23 mg/L	[105]
E. coli (coupled with *Saccharomyces cerevisiae*)	Overexpression of *Pichia pastoris* MAT in *E. coli* and co-culture with *S. cerevisiae*	1.7 g/L	[70]
E. coli	Magnetically immobilised recombinant cells overexpressing a variant of the E. coli MAT enzyme	3.8 mM	[45]
S. cerevisiae	Metabolic engineering to overcome SAM decarboxylation, glycogen branching and ergosterol biosynthesis combined with a pseudo-exponential fed-batch cultivation of a SAM-producing strain	12.47 g/L	[106]
P. pastoris	Overexpression of MAT from *S. cerevisiae*	0.74 g/L (20-fold increase)	[47]
S. cerevisiae	Overexpression of homologous MAT	7.76 g/L (flask); 9.64 g/L (fermenter)	[96]
P. pastoris	Maintaining 2% broth glycerol concentration to increase glycerol consumption rate by enhanced oxygen transfer rate in broth	9.26 g/L (77.39% increase)	[73]
S. cerevisiae	Overexpression of homologous MAT, and ERC1 to enhance ethionine tolerance	2 g/L	[107]
P. pastoris	Overexpression of MAT from *S. cerevisiae* combined with gene shuffling	6.14 g/L	[71]
P. pastoris	Modification of promoter for driving overexpression of MAT from *S. cerevisiae*	11.04 g/L	[108]
P. pastoris	Alternative feeding of methanol and glycerol in strain transformed with MAT from *S. cerevisiae*	13.24 g/L (34.3% increase)	[109]
P. pastoris	Continuous L-methionine feeding at 0.5 g/L/h to prevent heterogenous L-methionine inhibition while sustaining substrate supply	8.46 g/L (48.9% increase)	[72]

P. pastoris	*cbs* knockout	13.5 g/L (2.8-fold increase	[47]

for effective methionine production, some with theoretical yield greater than 100 g/L methionine.

CRISPR technology and its derivative have had tremendous impact on biotechnology. Its derivative, CRISPRi is a potent tool for gene regulation by synthetic circuits. The potentials of CRISPRi as a technology tool to develop regulatory networks of genes is limitless. With this tool and further advancement of new technologies in the field of system metabolic engineering, achieving optimal SAM production on and industrial scale can now be possible by application of CRISPRi in the construct of regulatory circuits for its precursors especially the versatile high energy compound ATP which cells require for viability. Also as has previously been highlighted, another derivative of CRISPR named MAGIC holds great potential for gene deletion and the simultaneous activation and manipulation of transcription and thus useful application for system metabolic engineering for improving SAM production by microbial species. Furthermore, application of omics technologies such as metabolomics, transcriptomics and proteomics can reveal physiological information about a engineered microbes which can be applied for insights into hidden constraints and development of methods to relieve such constraints.

Finally, sustainable industrial production of SAM requires both system-wide engineering and also some culture and fermentation conditions optimization for best results. However, care must be taken not to affect cell viability. We expect that adoption and implementation of some of the strategies proffered in this review might to a great extent exert positive effects on the construction of microbial cell chasses for the sustainable production of SAM and in turn increase its market availability while also reducing price.

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ABBREVIATIONS

AFLP: Amplified fragment length polymorphism

ARTP: Atmospheric Room Temperature Plasma

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeat

CRISPRi: CRISPR interference

CTAB: Cetyl Trimethyl Ammonium Bromide

DCW: Dry Cell Weight

MAGIC: multi-functional genome-wide CRISPR

MAT: methionine adenosyltransferase

MTHFR: methylenetetrahydrofolate reductase

***p*TsONa:** Sodium *p*-toluenesulfonate

RSM: Response Surface Methodology

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

srRNA: small regulatory RNA

URS: Unpolished Rice Saccharificate

UV: ultraviolet

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