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Full Length Research Paper

Optimization of overproducing S-adenosyl-L-methionine *Saccharomyces cerevisiae* S-W55 mutant utilizing unpolished rice from aging paddy by feeding L-methionine

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The medium and fed-batch fermentation by *Saccharomyces cerevisiae* S-W55 were optimized. The unpolished rice from aging paddy was used as major nutrient source to reduce the raw material cost of SAM fermentation. The unpolished rice saccharificate (URS) and yeast extract were employed as carbon source and nitrogen source, respectively. The dosages of URS and yeast extract in the medium compositions were optimized by response surface methodology (RSM). As a result, when the fermentation was carried out under the optimal conditions for URS (51.4 g/L) and yeast extract (4.74 g/L), the SAM yield reached 2.61 g/L. Some fed-batch processes by adding L-methionine (MET) were investigated. Adding MET into the fermentation broth at one time at the time of high cell density reaching 80 g/L could get better results with the optimal SAM concentration of 5.3 g/L and biomass yield of 89.1 g/L. By feeding MET into the fermentation broth at a feeding rate of 2 g/h for 5 h at the time of high cell density reaching 80 g/L, the optimal results were reached and the maximal SAM yield and biomass density were 5.82 and 90.2 g/L, respectively. It indicated that, the fed-batch at high cell density would be more propitious to the SAM biosynthesis.

Key words: Unpolished rice saccharificate, S-adenosyl-L-methionine, fermentation optimization, fed-batch fermentation, *Saccharomyces cerevisiae.*

INTRODUCTION

S-adenosyl-L-methionine (SAM) is the most important donor of methyl for many biochemical transmethylation reactions. This function determines its importance for the pharmaceutical industry and chemical therapy (Mincheva and Balutsov, 2002). It has received increasing interest in medical and pharmacological areas and several attempts have been made to produce it by conventional fermentation or enzymatic methods (He et al., 2006; Zhang et al., 2008). Microbiological synthesis is the most common method of SAM production. The yeast genera Saccharomyces and Candida are its main producers (Shimizu and Yamada, 1984; Shiomi et al., 1990).

A major concern in fermentation industry was to reduce the cost of raw materials which accounted for total production-cost. Utilization of cheap carbon sources was considered as an effective approach. Though many starchy materials from agriculture such as corn, rice and rice starch were used as carbon sources in many studies, the media costs were still high in relation to synthetic

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Abbreviations: SAM, S-Adenosyl-L-methionine; **URS,** unpolished rice saccharificate; **RSM,** response surface methodology; **MET,** L-methionine; **YE,** yeast extract.

media (Fukushima et al., 2004; Lee, 2007). Unpolished rice is a type of rice that had paddy hull removed during the processing but not the bran layer. Besides abundant starch, unpolished rice also contains greater amounts of dietary fibers, proteins, vitamins and minerals than polished rice (Das et al., 2008). In China, an agricultural country that produces paddy in large volumes and a considerable amount of aging paddy is mostly used in the feedstuff industry, which does not bring equivalent profit (Heerink et al., 2007; Liang et al., 2008). Thus, this cheap farm product containing abundant nutrients was used as a major nutrient source for SAM production in this study.

Though SAM was synthesized by ATP and L-methionine (MET) at the presence of S-adenosyl-L-methionine synthetase, some works indicated that the high concentrations of sugar and MET were disadvantageous for the growth and SAM biosynthesis by *Saccharomyces cerevisiae* (Lin et al., 2004; Liu et al., 2002). During the fermentation of yeast, ethanol might be formed either anaerobically under conditions of oxygen starvation or aerobically in the presence of high sugar concentration as a result of Crabtree effect. The formation of ethanol will prevent the yeast from reaching high cell density and decrease the yield of biomass on glucose. Thus, the batch fermentation is not a good choice for the production of yeast cell and its intracellular products. Some effective strategies were used to reduce the formation of ethanol and improve the yield of biomass and product on glucose during the fermentation (Lin et al., 2004; He et al., 2006; Zhang et al., 2008).

In this study, our objectives were to produce SAM by *S.cerevisiae* S-W55 utilizing unpolished rice from aging paddy, to optimize the dosage of unpolished rice saccharificate and yeast extract in the medium by response surface methodology (RSM), to optimize the fed-batch fermentation conditions for SAM in the fermentors and to obtain an effective strategy for large-scale production of those bioactive compounds by the bioprocess.

MATERIALS AND METHODS

Maintenance and preculture of microorganism

S. cerevisiae S-W55, a SAM overproducing yeast, which was obtained as described by Wang and Xiao (2007), were maintained on the potato dextrose agar slants at room temperature. The preculture medium was composed of the following (Per liter): glucose 20, yeast extract 5, magnesium sulfate 2, potassium dihydrogen phosphate 3 and disodium hydrogen phosphate 1 (pH 5.0).

Samples of unpolished rice and unpolished rice saccharificate (URS)

The samples of aging and fresh paddies were harvested from Hubei Province of China in October 2006 and 2007, respectively. The unpolished rice was manufactured from the aging paddy by removing the paddy hull. The polished rice was manufactured from the fresh paddy by removing the paddy hull and bran layer. The sample of fresh corn was harvested from Hubei Province of China in October 2009. The URSs were prepared by the method of Lu et al. (2009).

Optimization of the dosage of URS and YE

The components of fermentation media (designed for this study) were (Per liter): URS and yeast extract (YE) will be optimized in this study, MET 10, $(NH_4)_2SO_4$, 3 g, K_2HPO_4, 5 g; KH_2PO_4, 10 g, $MnSO_4$, 0.1 g, $ZnSO_4$, 0.1 g, $MgCl_2$, 0.2 g; $CaCl_2$, 0.1 g (pH 5.0).

Response surface methodology (RSM) and central composite design (CCD) were applied to optimize the dosage of fermentation medium compositions (John et al., 2007). The software design-expert 7.0.0 (Stat-Ease Inc., USA) was used for experimental design, data analysis and quadratic model building. For statistical calculation, independent variables were coded as:

$$x_i=(X_i- X_{i0})/ \Delta x_i \quad (1)$$

Where, x_i represents the coded values for X_i (i = 1, 2, 3, 4, etc.), X_i is the experimental value of variable, X_0 is the mid-point of X_i (the results of steepest ascent and corresponding response experiments (data not shown) indicated X_{0URS}=50 g/L and X_{0YE}=5.0 g/L, respectively) and ≥ X_i is the step change in X_i (≥ X_{URS} = 5 and ≥ X_{MET} = 0.9).

For predicting the optimal point, a second-order polynomial equation was fitted to correlate the relationship between variables and response.

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{j=1}^{k} b_{ii} x^2 + \sum_{i}^{k} \sum_{j}^{k} b_{ij} x_i x_j \quad 2$$

Where, y is predicted response, x_i and x_j (I ≤ j) are coded variables, b_0, b_i, b_{ii}, b_{ij} are regression coefficients calculated from the experimental data by second-order multiple regression and k is the number of factors.

The experimental data were statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA). The fitted polynomial equation was then expressed in the form of three-dimensional surface plots to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study.

Fed-batch fermentation experiment design

Two liters of optimized medium were added to the 5-l bioreactor (Biostat 5, B. Braun, Germany) and sterilized at 121°C for 30 min. The medium was cooled down and the initial pH value of the medium was adjusted to pH 5.0. All fermentations were performed at 28°C.The pH was controlled at 5.0 through automated addition of ammonia, which was sterilized by filtering. Ten percent (v/v) activated inoculums incubated in optimized medium were used in all fermentations. The fermentation was conducted in a 5-l fermentor with on-line pH with DO detection and control. The culture conditions of temperature, agitation rate and the aeration rate were set at 28°C, 200 rpm at 2 h and up to 600 rpm at 8 h and 1 L /min, respectively. For fed-batch cultivation, the feeding URS and MET, with a concentration of 800 and 50 g/L, which were sterilized separately at 115°C for 25 min in an 1 L and a 250 ml bottles, was fed into the fermentor with a digital peristaltic pump (B.BRAUN, Germany) under computer control.

To improve the accumulation of SAM production in the cells, at the initial period of fermentation, the time of sugar consumed up, the time of cell density reaching 40 g/L, the time of high cell density

Table 1. Content of some amino acids (g/kg) in the corn and unpolished rice saccharificate.

Nutrient component	Corn	Unpolished rice	Unpolished rice saccharificate
Methionine	1.81	2.22	1.31
Leucine	10.21	7.06	1.82
Isoleucine	2.22	3.34	1.11
Lysine	3.23	3.58	1.12
Phenylalanin	3.57	4.42	1.12
Valine	4.01	5.12	1.55
Threonine	2.88	3.55	1.11
Histidine	2.23	2.21	1.13

(cell density over 80 g/L), MET (a precursor of SAM) were added into the fermentation broth at an amount of 10 g, at a feeding rate of 0.35 g/h in 30 h and 0.5 g/h in 30 h to keep high concentration of MET, which prevented SAM from being decomposed and at a feeding rate of 2 g/h of cells in 5 h and at an amount of 10 g, respectively. The feeding of URS was continued to provide necessary energy source and a few precursors for the synthesis of SAM, and the feeding rates of URS were controlled under the concentration 3 g/L of URS in the fermentation broth in all the fed-batch fermentation experiments.

Analytical methods

The samples of unpolished rice and saccharificate were analyzed after pretreatment (Das et al., 2008).The amount of reducing sugar was determined by the 3,5 dinitro salicylic acid method (Miller, 1959). The amino acids (except L-methionine) were measured by amino acid analyzer (Beckman-6300) referring to GB/T 5009.124-2003 of China. L-methionine was determined by high performance liquid chromatography (HPLC) with a C-18 column (COSMOSIL 250 mm×4.6 mm i.d.; 5 μm particle diameter, 250 Å average pore size) (Nacalai, Japan) with a mobile phase composed of acetonitrile-0.01M phosphoric acid (35:65, v/v) at a flow rate of 1.0 ml/min. Biomass was measured by dry cell weight (DCW).

At the end of the incubation period, 5 ml culture broth taken from the flask, after centrifuging and washed twice with water, SAM and related compounds in cells were extracted with 10% (w/v) perchloric acid for 1 h. Samples (20 μl) were analyzed according to the method of Shimizu et al. (1984) by HPLC, consisted of a solvent delivery system (L-7100, Hitachi, Japan) and a variable wavelength UV-Vis detector (at 254 nm, L 7420, Hitachi, Japan) equipped with a sample injector (7725i, Rneodyne, USA) fitted with a 20 μl sample loop. The chromatographic separations were carried out on Eclipse XDB-C18 column (250 mm × 4.6 mm i.d.; 5 μm particle diameter, 250 Å average pore size) (Agilent, USA) with a guard column holder. For qualitative study, the retention time of biotransformation products in the chromatograms were compared to SAM standard peaks.

SAM and L-methionine standards were purchased from Sigma. The results were statistically tested by analysis of variance, single classification (one-way ANOVA).

RESULTS AND DISCUSSION

Results of preparation of unpolished rice saccharificate

Reducing sugar was the major ingredients of the URS. The yield of reducing sugar reached 810 g/kg unpolished rice. The unpolished rice was rich in essential amino acids and B vitamins (Table 1). As shown in Table 1, the contents of amino acids of the unpolished rice were generally more than those of the corn. The amino acids and B vitamins were essential to the growth of some microorganism such as yeast and bacteria. The presence of amino acids, (especially MET in URS, about 1.31 g/kg URS) could be advantageous for the SAM accumulation as a precursor of SAM and lower the product cost.

Optimization of the dosage of URS and YE by RSM

The URS and YE were used as carbon and nitrogen sources, and growth factors of SAM production based on earlier mentioned experiments. Central composite design (CCD) was applied to find their appropriate dosages and predict maximum SAM concentration. The variables and responses of SAM production are listed in Table 2.

From the response obtained for 13 runs (Table 3) the analysis of variance for the quadratic model was calculated. The ANOVA showed that the model F-value of 31.18 implied the model was significant. Values of "p>F" <0.05 indicated model terms were significant. In this work, X_1, X_2, X_1X_2, X_1^2 and X_2^2 were significant model terms which implied that the change of concentrations of URS and yeast extract influenced SAM production directly.

The coefficient of determination (R^2) of the regression model by ANOVA was 0.9570, which indicated that 95.70% of the variation in the response could be explained by the model. The R^2 of 0.9570 was in reasonable agreement with the adjusted R^2 of 0.9263, which implied a good agreement between the experimental values and the predicted values of SAM production.

The experimental results of the CCD were fitted with the coded second-order polynomial function for the estimation of SAM production:

$$Y=2.512+0.16067X_1+0.1608X_2-0.0875X_1X_2-0.0485X_1^2-0.071X_2^2 \quad (4)$$

Where, Y is the response of SAM concentration, X_1 and

Table 2. Central composite design for optimization of two variables in experimental values for SAM production by *S. cerevisiae* S-W55.

Run	X_1 URS (g/L)	X_2 MET(g/L)	Y: SAM (g/L)
1	-1	-1	2.05
2	-1	1	2.55
3	1	-1	2.5
4	1	1	2.65
5	0	0	2.54
6	0	0	2.52
7	0	0	2.51
8	0	0	2.5
9	1.414	0	2.63
10	-1.414	0	2.11
11	0	1.414	2.55
12	0	-1.414	2.1

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model.

Source	Degree of freedom	Sum of square	Mean square	F-value	p-value prob > F
model	5	0.49	0.098	31.18	0.0001
X_1	1	0.21	0.21	65.72	<0.0001
X_2	1	0.21	0.21	65.82	<0.0001
X_1X_2	1	0.031	0.031	9.74	0. 0168
X_1^2	1	0.016	0.016	5.21	0.0489
X_2^2	1	0.035	0.035	11.16	0.0124
Residual	7	0.022	0.003143		
Lack of fit	3	0.021	0.00684	18.49	0.0083
Pure error	4	0.00148	0.00037		
Cor total	12	0.51			

C.V. %, 2.30; R^2,0.9570; Adj R^2,0.9263.

X_2 are the concentrations of URS and YE, respectively.

The effect of URS, yeast extract and their interactions on SAM concentration is shown in Figure 1. It was evident that SAM production increased with increasing URS concentration. A similar trend was observed in yeast extract. However, the two variables showed a synergistic effect on SAM production, and further increase of SAM concentration could not be achieved when the two variables increased unceasingly. The surface plots of yield indicated that the SAM concentration could not exceed 2.65 g/L.

Using the desirability function for point prediction of the design expert 7.0.0 software, the optimal conditions for URS (51.4 g/L) and yeast extract (4.74 g/L) were obtained. The predicted maximum response of SAM concentration was 2.647 g/L.

Validation of the model was carried out in 250 ml Erlenmeyer flasks under the optimal conditions to confirm the predicted response. As a result, the SAM concentration reached 2.61 g/L, which neared to the maximum SAM concentration of 2.647 g/L in CCD experiment, while the dosages of raw materials were obviously decreased.

Adding MET into the medium at the initial period of fermentation

At the initial period of fermentation, MET was added into the fermentation medium directly at the concentration of 10 g and the fermentation kinetics curves were shown in Figure 2. As Figure 2 deposited from 2 to 30 h, a quick increase of SAM production was observed and then followed by a rapid decline till the end fermentation. The increase of biomass and SAM were both accompanied with a mass of energy consumption because SAM was synthesized with ATP and L-methionine (MET) at the presence of S-adenosyl-L-methionine synthetase, so SAM might be decomposed to produce energy when the yeast grew very fast which led to the lack of energy. The maximal yield of SAM production was 2.51 g/L and biomass yield at this moment was 42 g/L, which was

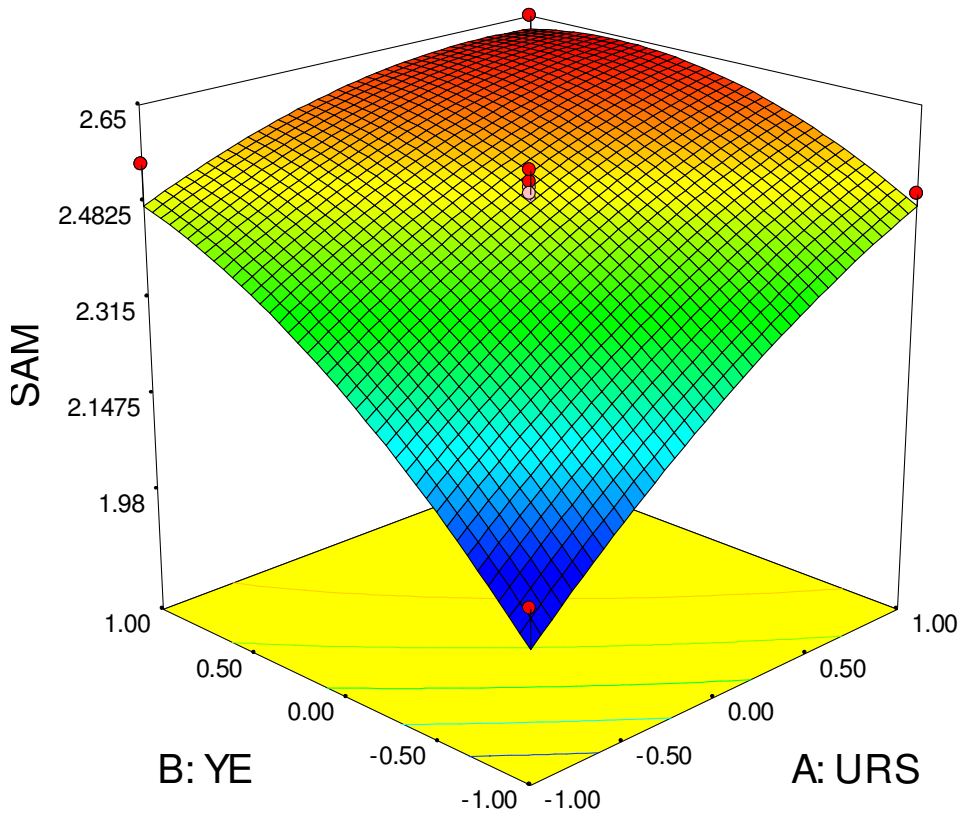

Figure 1. Response surface and contour plots showing the interaction of URS and yeast extract on SAM production by *S. cerevisiae* S-W55.

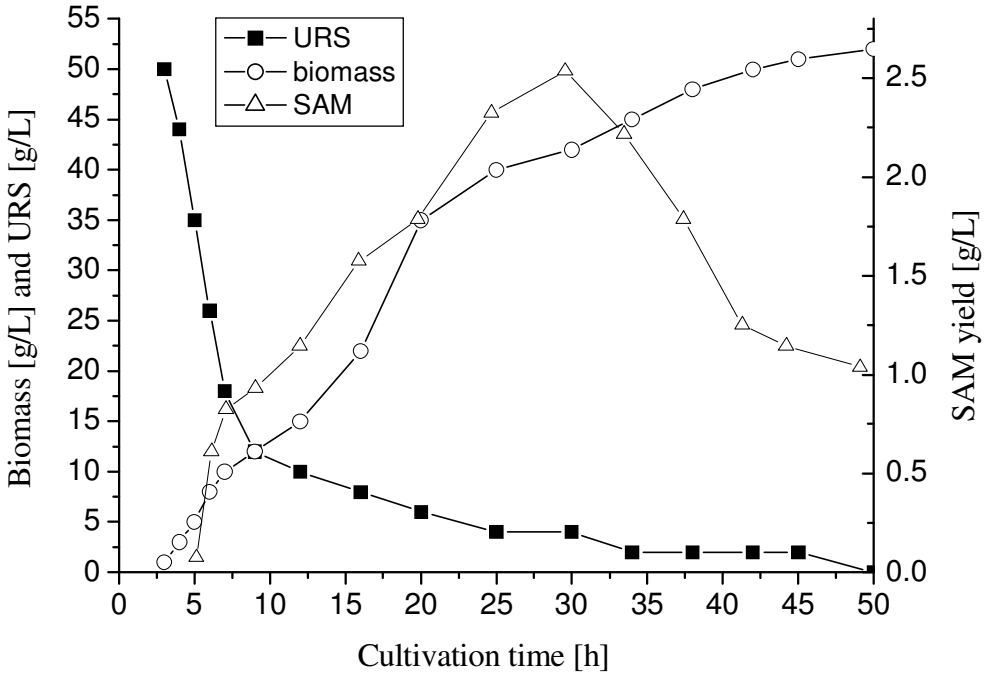

Figure 2. Time profiles of biomass, URS and SAM concentration of *S. cerevisiae* S-W55 by adding 10 g MET in initial medium in a 5-l fermentor.

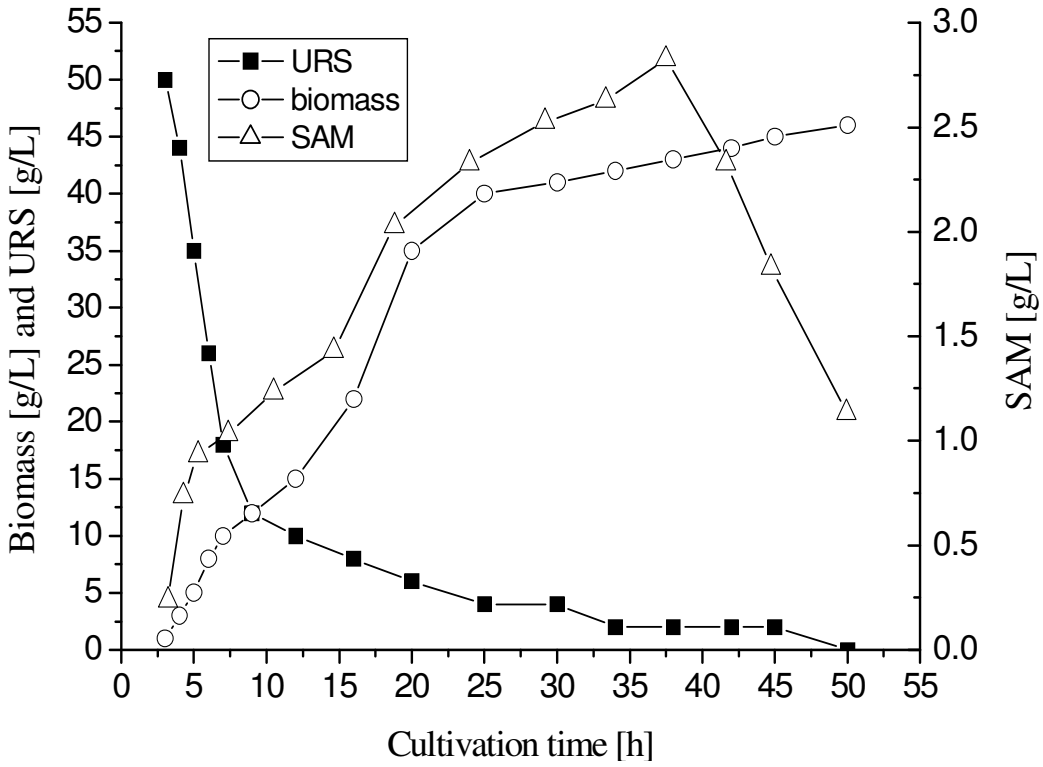

Figure 3. Time profiles of biomass, URS and SAM concentration of *S. cerevisiae* S-W55 by feeding MET at a feeding rate of 0.35 g/h in 30 hat the time of sugar consumed up in a 5-l fermentor.

40.1% lower than the result of not adding MET (data not shown). It indicated that MET could inhibit the growth of *S. cerevisiae* S-W55.

Feeding MET at the time of sugar consumed up at a feeding rate of 0.35 g/h in 30 h.

When the concentration of sugar was lower (2 g/L), MET was added into the medium at a feeding rate of 0.35 g/h in 30 h till the end fermentation (shown in Figure 3). From Figure 3, the maximal SAM production concentration of 2.83 g/L was obtained at 38 h, while the relevant biomass concentration was 43 g/L. In this case, the maximal biomass was 38% lower than the result without MET adding into and the SAM degrading very obviously. It indicated that MET still was disadvantageous for the yeast growth and the higher concentration of S-adenosyl-L-methionine synthetase was obtained to reach higher SAM production in the condition of higher density by *S. cerevisiae* S-W55.

Feeding MET at the time of cell density reaching 40 g/L at a feeding rate of 0.5 g/h in 30 h

From Figures 2 and 3, the growth of *S. cerevisiae* S-W55 was inhibited with MET added into the broth at the early period of the fermentation, so we fed MET at the time of higher cell density (reaching 40 g/L) at a feeding rate of 0.5 g/h in 30 h to keep superabundant MET concentration in order to prevent SAM from decomposing till the end fermentation. As deposited in Figure 4, the SAM was biosynthesized quickly and the increase of biomass after feeding was also rapid. The maximal SAM concentration could reach 3.5 g/L and the final biomass was 69 g/L, which were both much higher than the former results in this study.

Adding MET into the fermentation broth at one time at the time of high cell density

Based on the stated results, 10 g MET was added into the fermentation broth at one time at the time of high cell density reaching 80 g/L. As shown in Figure 5, SAM production was accumulated very slowly without adding MET. When the biomass reached 80 g/L, the SAM concentration was only 1.11g/L. After adding MET, SAM was increased very quickly. The maximal SAM yield was 5.3 g/L after 16 h of adding MET and the final biomass density was 89.1 g/L. But SAM concentration began to decline from the peak remarkably and its yield was only 4.3 g/L at the end of the experiment. It indicated that, the process of adding MET should be employed because the higher concentration of MET was disadvantageous to the

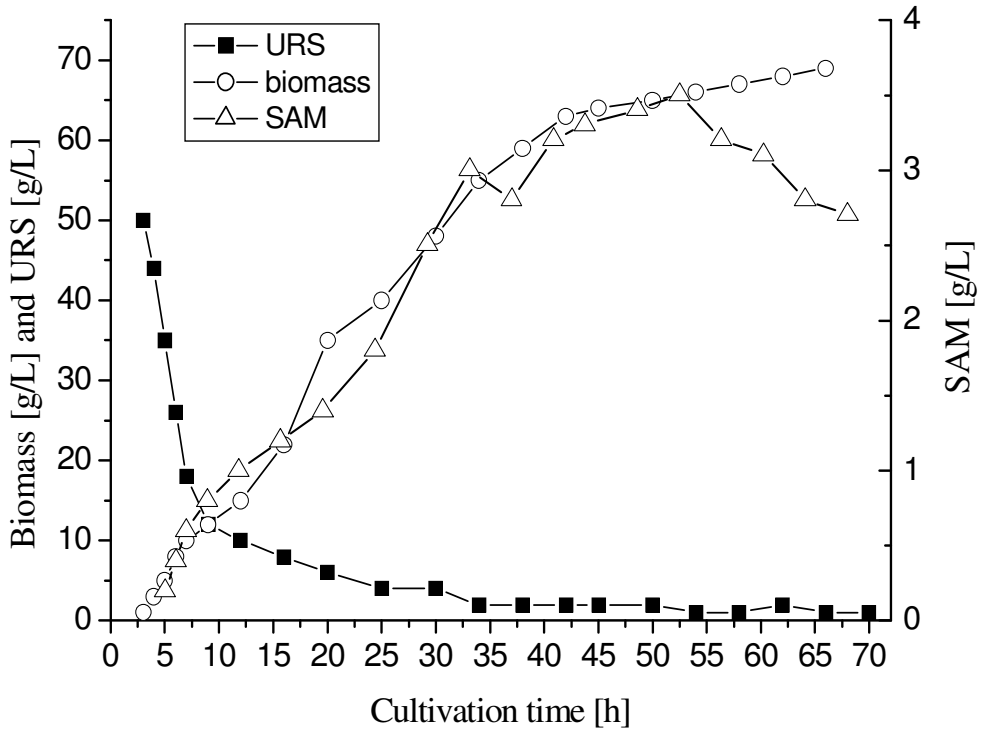

Figure 4. Time profiles of biomass, URS and SAM concentration of *S. cerevisiae* S-W55 by feeding MET at a feeding rate of 0.35 g/h in 30 h at the time of cell density reaching 40 g/L in a 5-l fermentor.

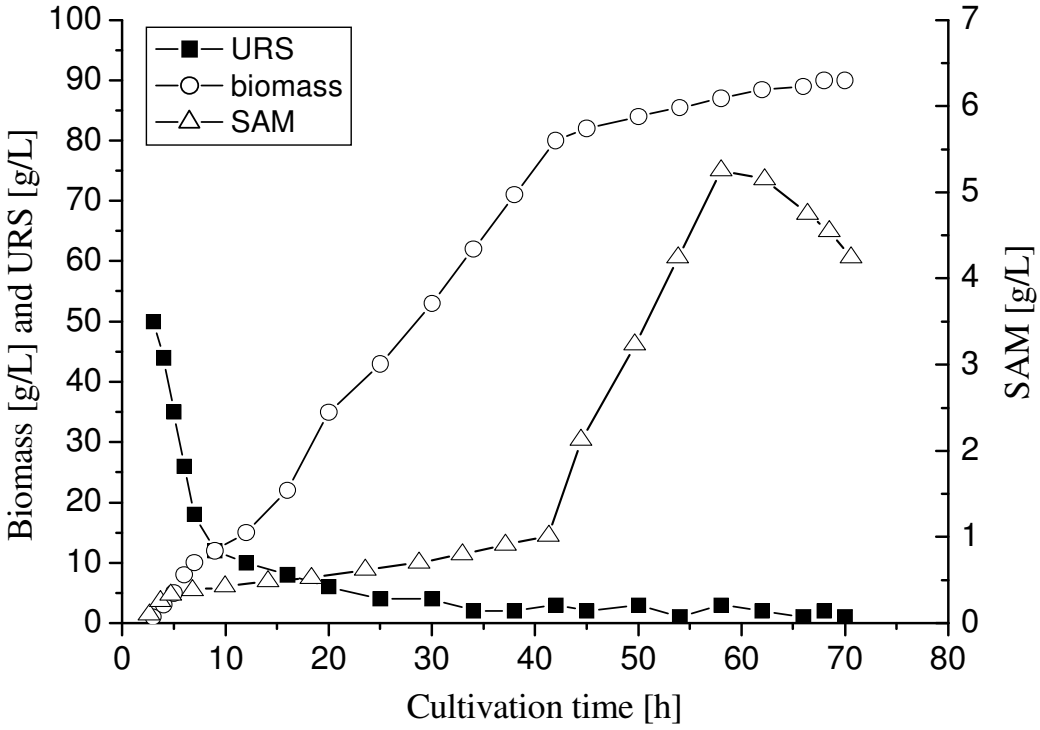

Figure 5. Time profiles of biomass, URS and SAM concentration of *S. cerevisiae* S-W55 by adding 10 g MET into the fermentation broth at one time at the time of cell density reaching 80 g/L in a 5-l fermentor.

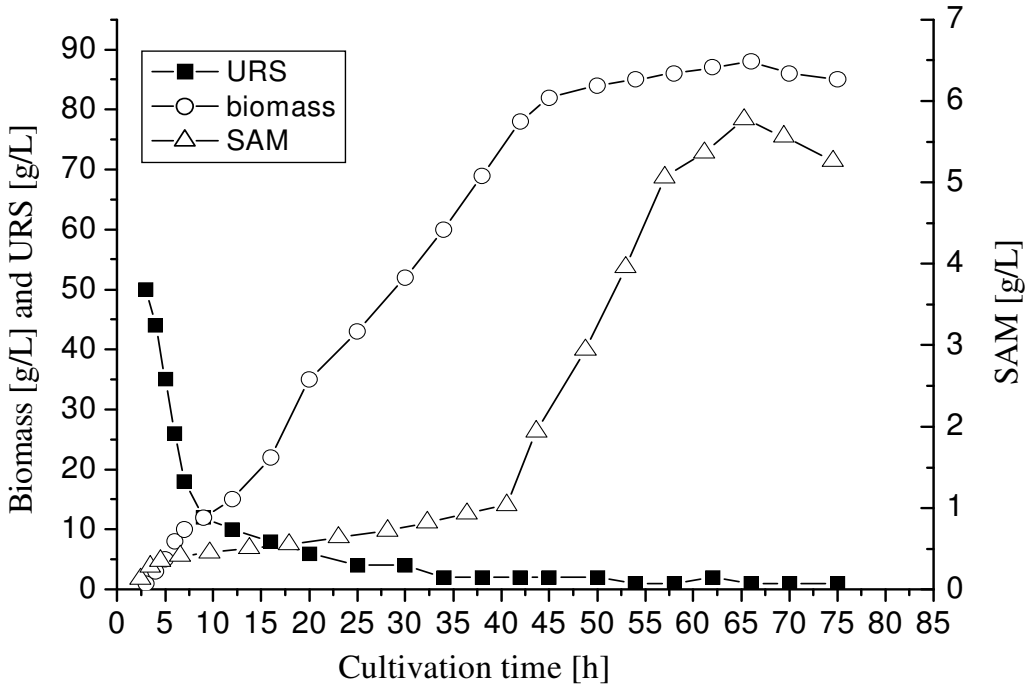

Figure 6. Time profiles of biomass, URS and SAM concentration of *S. cerevisiae* S-W55 by feeding MET at a feeding rate of 2 g/h in 5 h at the time of cell density reaching 80 g/L in a 5-l fermentor.

SAM biosynthesis at the later stage of fermentation.

Feeding MET at the time of high cell density at a feeding rate of 2 g/h for 5 h

Subsequently, MET was fed into the fermentation broth at a feeding rate of 2 g/h for 5 h at the time of high cell density reaching 80 g/L. The time profiles are shown in Figure 6. It was clearly stated that, the adding of MET could improve the SAM accumulation significantly. At 66 h the optimal SAM concentration was 5.82 g/L and the decomposition trend of SAM was not very visible when compared with other cases in this study. With feeding MET, the cell density was increased very slowly and the maximal biomass concentration reached 90.2 g/L. It demonstrated that, the higher SAM yield could be reached at high-cell-density by adding substrate of MET.

Conclusion

The reducing sugar was the primary ingredients of the URS prepared from the unpolished rice whose contents of amino acids and B vitamins were very abundant. The URS, amino acids and B vitamins were essential to the growth and SAM biosynthesis of *S. cerevisiae* S-W55. After medium optimization, the SAM concentration reached 2.61 g/L in 250 ml Erlenmeyer flasks under the optimal conditions, which was close to the maximum SAM concentration of 2.647 g/L in CCD experiment while the dosages of raw materials were obviously decreased.

As a precursor of SAM, adding MET or not had a marked impact on the biomass and SAM concentration by *S. cerevisiae* S-W55. Adding MET into the fermentation broth at one time, at the time of high cell density reaching 80 g/L could get better results with the optimal SAM concentration of 5.3 g/L and biomass yield of 89.1 g/L. By feeding MET into the fermentation broth at a feeding rate of 2 g/h for 5 h at the time of high cell density reaching 80 g/L, the optimal results were reached and the maximal SAM yield and biomass density were 5.82 and 90.2 g/L, respectively. It indicated that the fed-batch at high cell density would be more advantageous to the SAM biosynthesis, because the yeast could adapt the slow present of a small quantity of MET in the broth under high cell density which means the presence of more S-adenosyl-L-methionine synthetase.

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