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Isolation and characterisation of *Candida* sp. mutants enriched in S-adenosylmethionine (SAM)

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Abstract - The ability to accumulate S-adenosylmethionine (SAM) of 572 yeast strains isolated from environmental sources were surveyed. An S-adenosylmethionine enriching strain S42-12, identified as *Candida* sp., was chose to develop a SAM-accumulating mutant successfully. The final SAM-accumulating mutant strain YQ-5 was isolated by UV radiation or by NTG treatment using ethionine selection and nystatin selection method. The mutant strain YQ-5 accumulated 112.1 mg per gram biomass, was 3.14-fold higher than the original strain S42-12. When cultivated in the optimal medium with a favourable fermentation conditions, SAM content of the mutant strain reached at 1740 mg L^{-1}. Trend of SAM and ergosterol contents and methionine adenosyltransferase activity of SAM-accumulating mutants during fermentation were analysed. The results suggested that one of the reasons why the mutants accumulated SAM in significantly high amounts may be the lower consumption of SAM for ergosterol biosynthesis, other than improvement of methionine adenosyltransferase activity.

Key words: S-adenosylmethionine, isolation, ribosomal DNA, *Candida* sp., mutant.

INTRODUCTION

S-Adenosylmethionine (SAM) is an important metabolite and as a universal methyl group donor participates in many biochemical reactions involving DNA, proteins, polysaccharides and polyamines (Tabor and Tabor, 1984). SAM's involvement in the process of methylation helps maintain phospholipids in the cell membrane, regulates the activities of various enzymes, and helps maintain the action of several hormones and neurotransmitters that affect mood, including dopamine and serotonin (Bottiglieri and Hyland, 1994). Although SAM has been available as a prescription medication to improve many diseases such as depression, osteoarthritis, fibromyalgia, and liver disorders (Lieber and Packer, 2002) about two decades ago, it is expensive and out of reach for most people at this time. The reason is that there is no a more efficient way to produce it, also industrial-scale production of SAM is very limited (Chen *et al*., 2004). Several authors have reported production of SAM using yeast strains. Shiozaki *et al*. (1984) reported that a group of *sake* yeasts (*S. cerevisiae*) accumulated SAM to a high concentration compared with other yeasts and some other microorganisms. Mincheva and Balutsov (2002) reported the isolation of lactose-utilising *Kluyveromyces lactis* which accumulated SAM intracellularly in whey media. However, there have been few reports concerning efficient improvement of SAM production in yeasts by mutagenesis method.

In *Saccharomyces cerevisiae*, one of the pathways in which SAM be consumed is the ergosterol biosynthesis reaction. Shobayashi *et al*. (2006) demonstrated that nystatin-resistant mutants with deficiencies in ergosterol biosynthesis stimulated SAM accumulation in *sake* yeasts, and used positive selection method for the isolation of SAM-accumulating *sake* yeasts, their results suggested that the same phenomenon may be also seen when the nystatin selection method used with other yeasts. Mincheva and Balutsov (2002) isolated a SAM-accumulating yeast by selecting ethionine-resistant *Kluyveromyces lactis* mutants.

In this study, the ability to accumulate SAM in a variety of yeast strains isolated from environmental sources was surveyed, and the most potent strain was used to develop a SAM-accumulating mutant successfully by ethionine resistance and nystatin selection one after the other, also trend of the SAM contents, ergosterol contents and the methionine adenosyltransferase activity in the mutants cells were analysed.

MATERIALS AND METHODS

Materials. N-methyl-N-nitro-N'-nitrosoguanidine (NTG) was purchased from Tokyo Kasei kogyo co. ltd, Japan. DL-ethionine was from Alfa Aesar, USA. S-Adenosylmethionine and nystatin were obtained from Sigma, USA. HPLC water was produced with a Milli-Q Water Purification System (Millipore). All other chemicals used were of analytical grade and commercially available.

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Isolation of yeasts and culture conditions. Various soil or water samples from dairy factories, food factories, sewage plant, orchard, Chinese cheese including *Douchi* (traditional Chinese fermented soybean) and *Rufu* (traditional Chinese fermented soybean curd) were collected.
To isolate yeasts, 1 g or 1 mL of each sample was suspended in 100 mL of distilled water. After vigorously shaking the suspension for 30 min, 0.2 mL of the supernatant was spread onto agar plates containing isolation medium, which was composed of 5% glucose, 0.05% yeast extract, 0.1% urea, 0.25% KH_2PO_4, 0.05% Na_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4$, 0.0033% Rose Bengal, pH 5.5 and was solidified with 1.8% agar. The plates were incubated at 30 °C for 24 h; the colonies that appeared were picked up and inoculated on YPD agar slant containing 2% glucose, 2% tryptone, 1% yeast extract, 1.8% agar (Yarrow, 1998).

For production of SAM, the isolates were inoculated in the 100 mL Erlenmeyer flasks containing 20 mL of FM fermentation medium at 30 °C for 48 h with shaking, the fermentation medium contained 0.3% yeast extract, 0.3% urea, 0.25% KH_2PO_4, 0.01% $K_2HPO_4 \cdot 3H_2O$, 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% L-methionine. A yeast strain, S42-12, was finally selected as a producer enriched in SAM.

Taxonomic identification of strain S42-12. Cellular DNA was isolated by previously described method (Philippsen *et al*., 1991). The 18S rDNA was amplified using primers EF3 (5′- TCCTCTAAATGACCAAGTTTG -3′) and EF4 (5′- GGAAGGGRTGTATTTATTAG -3′) (Smit *et al*., 1999). Another set of primers, NL-1 (5′-GCATATCAAAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman *et al*., 2001), were used to amplify the 26S rDNA D1/D2 region. The amplification was carried out by polymerase chain reaction (PCR) and the amplified product was then purified and sequenced (Shanghai GeneCor Biotechnologies Co., Ltd., P. R. C). The nucleotide sequences obtained was compared with those of all known yeast species available at the GenBank database.

Mutagenesis and screening method. The yeast strain S42-12 was cultivated in YPD medium at 28 °C for 18 h, then cells were collected and washed twice with 0.85% NaCl solution and twice with citrate buffer (0.067 mol/L sodium citrate and 0.03 M citrate acid, pH 5.5). The washed cells were suspended in citrate buffer and treated with 500 μg/mL NTG for various time in dark. The treated cells were washed twice with citrate buffer and suspended in the same buffer. The suspension (200 μL) was spread on the solid MM medium containing DL-ethionine (Alfa Aesar, USA) or nystatin (Sigma, USA). The plates were incubated at 28 °C for 4-8 days in dark.

Cells were treated with UV as follow. The strain was cultivated at 28 °C for 18 h, and cells were collected and washed twice with 0.85% NaCl solution. The washed cells were suspended in the same solution and transferred to the plates at a density of 10^7 cells per mL. After UV irradiation a few seconds, the treated cells were transferred to the solid YPD medium containing 50 μg/mL nystatin. The plates were incubated at 28 °C for 4-8 days in dark.

Determination of SAM of the yeast cells. The yeast cells were cultivated in fermentation medium; cells were collected and washed twice with ice-chilled sterilised, distilled water, and extracted with 2-4 volume of 1.5 N perchloric acid for 1 h at room temperature. Then it was followed by centrifugation, the amount of SAM in the supernatant extracted was analysed using C18 column (4.6 x 250 mm, 5 μm, Shimadzu Corporation, Kyoto, Japan) with a Shimadzu LC-6A pumping system (Shimadzu). The column eluate (flow rate, 1 mL/min) was composed of ammonium formate buffer (0.01 mol/L, pH 3.0).

Determination of ergosterol of the yeast cells. Yeast cells were cultivated in fermentation medium as described above, cells harvested were washed twice with ice-chilled sterilised, distilled water, and treated with 30 mL of alcoholic potassium hydroxide solution (20% KOH and 40 mL of 100% ethanol) in 85-90 °C water bath for 3 h under N_2 gas, and then allowed to cool to room temperature. The sterols were extracted with 20 mL of petroleum ether and followed by vigorous vortex mixing for 3 min. The petroleum ether layer was then determined according to Arthington-Skaggs *et al*. (1999).

RESULTS

Isolation of yeasts

In the first step, 572 strains were isolated from 69 different samples with isolation medium. According to morphology colonies on YPD agar medium and cells form under inverted microscope (Leica, DM IL, Germany), the 572 strains being capable of growing on isolation medium should belong to yeast. In the second step, the intracellular SAM content of those strains was determined. As shown in Table 1, among 572 strains of yeasts tested, 25 strains of them exhibited accumulation of intracellular SAM of 20-35.7 mg/g of dry cell, which accounted for 4.37% only of the tested yeast strains. About 88.64% of them exhibited accumulation of intracellular SAM under 15 mg/g of dry cell. This result suggests that the level of SAM accumulated is varied within quite wide ranges among the strains tested. The strain named S42-12 exhibited the highest accumulation of SAM with 35.7 mg per gram biomass; we chose this strain as the parental strain for isolating higher SAM-accumulating mutants. Strain S42-12 was isolated from silage sample of dairy factories.

Identification of strain S42-12

In the analysis of 18S rDNA, a fragment of 1446-bp was obtained by PCR amplification (GenBank accession number EF489418). The BLAST search result through GenBank revealed that 18S rDNA sequences of the yeast had the highest similarity to *Candida tropicalis* with 99.93% identity, and the strain also showed similarity to *Candida sojae* and *Candida viswanathii*, with 99.72 and 96.65% identity respectively. The 26S rDNA D1/D2 region sequences (564 bp, GenBank accession number EF489417) of the yeast showed a 99.2% similarity with 26S rDNA of *Candida tropicalis* and 95.3% similarity with the 26S rDNA of *Candida albicans*. As a consequence, strain S42-12 was identified as *Candida* sp.

Selection of DL-ethionine-resistant mutants and their SAM contents

First, strain S42-12 was mutated by N-methy-N-nitro-N′-

nitrosoguanidine (NTG), and the ethionine-resistant mutants were selected on the MM medium containing DL-ethionine. Next, the SAM contents of the ethionine-resistant strains were measured to isolate SAM-accumulating mutants.

To test the ethionine resistance of the original strain S42-12, cell suspension was spread on solid MM medium containing 0.06, 0.1, 1 and 2% DL-ethionine respectively. Like most yeast species, it is sensitive to 0.1% DL-ethionine. Therefore, strain S42-12 was treated with NTG treatment, and selected on the solid MM medium containing 0.1% DL-ethionine. 98 mutants were obtained, and named YA-1~YA-98 respectively. These ethionine-resistant mutants were screened for SAM-accumulation; the highest level of intracellular SAM (66.8 mg/g dry cell weight) was produced by strain YA-89, which was 1.87 times as much as that of S42-12. Test of ethionine resistance showed that strain YA-89 had a high level-resistance to ethionine (above 3% ethionine). Thus, it is very necessary to choose other selection method to further improve *S*AM-accumulation.

Selection of nystatin-resistant mutants and their SAM contents

Cells of strain YA-89 were spread on the plates contained 10 μg/mL nystatin after being treated with NTG for 30 min and the death rate was 80.95%. The SAM contents of the nystatin-resistant mutants were determined, and only three of 35 nystatin-resistant mutants from YA-89 accumulated more SAM than the parental strain (Fig. 1), these strains were named YN-13, YN-14, YN-34, respectively. The highest level of intracellular SAM was produced by strain YN-34 (71.6 mg/g dry cell weight).

Strain YN-34 was once more mutated by UV irradiation with subsequent selection on solid YPD medium containing 50 μg/mL of nystatin. The survival rates of strain YN-34 Cells which were treated with UV irradiation for 30 seconds, 60 seconds and 90 seconds were 11.13%, 3.96% and 0.01% respectively, and 30 seconds were used for mutagenesis. Strain YN-34 yielded 41 nystatin-resistant mutants, and named YQ-1~YQ-41 respectively. The frequency of the appearance of the nystatin-resistant mutants was 1.4×10^{-6}. Four of 41 nystatin-resistant mutants from strain YN-34 were isolated with high SAM-production (Fig. 2). The mutant YQ-5 strain accumulated 1.57-fold more SAM (112.1 mg/g dry cell weight) than the level in the parental strain YN-34. It is seem that UV treatment was more efficient for improvement of SAM production than the NTG in this study.

Culture conditions for the SAM production by the mutant strain

The effects of various carbon source and nitrogen source on SAM production were studied (the data in detail not showed). Results revealed that 8% sucrose was the most favourable carbon source for SAM production by mutant YQ-5 strain. The best nitrogen source is tryptone (1.5%). Also the effects of inorganic salts on intercellular accumulation of SAM were studies. In conclusion, the optimised fermentation medium for SAM production was composed of 0.1% KH_2PO_4, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.01% $FeSO_4 \cdot 7H_2O$, 0.01% $CoCl_2$, 0.01% $CuSO_4 \cdot 5H_2O$, 0.03% H_3BO_3, 2% yeast extract, 8% sucrose, 1.5% tryptone, 0.75% L-methionine, pH 6.0. After incubation for 48 h in the optimal fermentation medium resulted in SAM production at 1740 mg L^{-1}, and the biomass reached at about 15.5 g cell dry weight per litre medium.

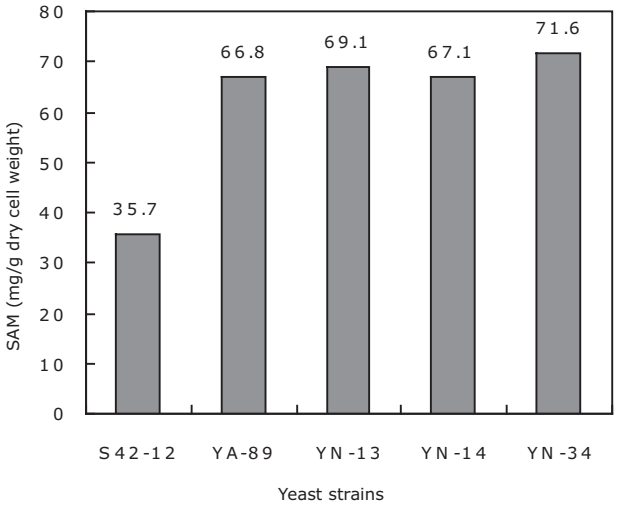

FIG. 1 - SAM content of nystatin-resistant mutants isolated from strain YA-89 treated with NTG. Cells were cultured in FM medium for 48 h at 30 °C.

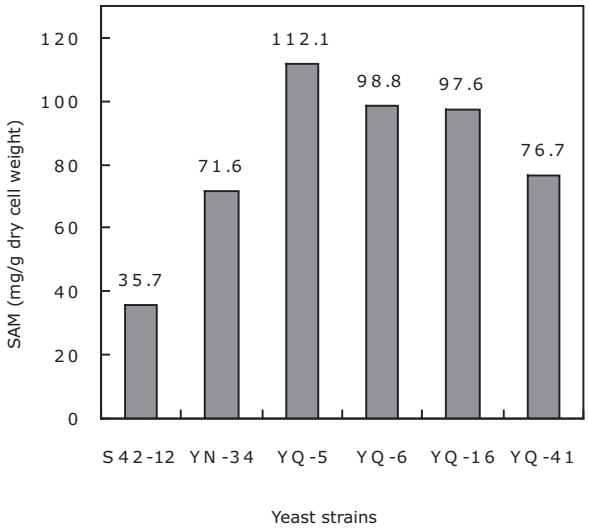

FIG. 2 - SAM content of nystatin-resistant mutants isolated from strain YN-34 treated with UV irradiation. Cells were cultured in FM medium for 48 h at 30 °C.

Trend of SAM and ergosterol contents and methionine adenosyltransferase activity of SAM-accumulating mutants during fermentation

Trend of SAM and ergosterol contents and methionine adenosyltransferase activity of SAM-accumulating mutants were investigated during fermentation. Mutant YA-89 strain and YQ-5 strain were compared as to SAM contents, ergosterol contents and methionine adenosyltransferase activity with the original strain S42-12 during fermentation (showed as Fig. 3).

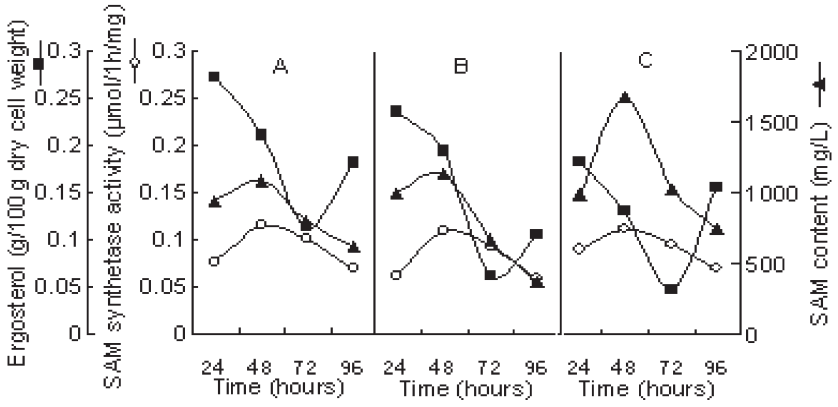

FIG. 3 - SAM contents, ergosterol contents and methionine adenosyltranferase activity of S42-12 (A), mutant strain YA-89 (B) and YQ-5 (C). A suspension of cells (0.1mL) of each yeast was incubated in 250 mL–flask containing 25 mL of the optimal fermentation medium, cultivation was carried out at 28 °C and 250 rpm. SAM contents, ergosterol contents and methionine adenosyltranferase activity were determined every 24 h during 4 days.

The results revealed that the yield of SAM increased gradually from 24 to 48 hours, and reached its maximum at 48 hours, after which began to decrease along with the methionine adenosyltransferase activity. Methionine adenosyltransferase catalyses transadenosylation of methionine yielding SAM. It clearly demonstrated that there is a positive correlation between the SAM accumulation and methionine adenosyltransferase activity during fermentation. Although YA-89 strain and YQ-5 strain accumulated more SAM compared with original strain S42-12, there were no obvious difference in methionine adenosyltransferase activity among them (about 0.113 μmol/hour/mg dry cells).

Ergosterol content of the three strains decreased gradually until at the 72nd hour, and then began to increase gradually. At 72 hours, YA-89 strain produced ergosterol with 0.63 mg/g dry cell weight, YQ-5 strain produced ergosterol with 0.47 mg/g dry cell weight, and the original strain S42-12 produced 1.13 mg/g dry cell weight of ergosterol. These results clearly demonstrated that there is a negative correlation between the SAM accumulation and ergosterol content during fermentation; also support the hypothesis of Shobayashi *et al*. (2006) that reduction of ergosterol biosynthesis decreases SAM consumption and leadings to further accumulation of SAM.

DISCUSSION

Surveys of accumulation of intracellular SAM were reported by Schlenk and Depalma (1957) with several common yeasts and Shiozaki *et al*. (1984) with 178 yeasts. In present study, we thoroughly determined the accumulation of intracellular SAM among 572 yeast strains that directly isolated from environmental sources (Table 1). Our result suggests that the level of SAM accumulated is varied within quite wide ranges among the strains tested, and few of yeast strains from environmental sources exhibited accumulation of intracellular SAM with high content. Previous reports showed the yeast genera *Saccharomyces* is main producer of SAM, our studies showed that the yeast belongs to genus *Candida* can accumulate much intracellular SAM, and mutagenic treatment of the *Candida* sp. strain S42-12 cells gave rise to the mutant strain YQ-5, the content of SAM in *Candida* mutants reached at 1740.0 mg L^{-1}, which also has a rather high level of SAM accumulation compared with other yeast genera.

To improve *S*AM-accumulation level of *Candida* sp. strain S42-12, SAM-accumulating mutants were isolated by UV radiation or by NTG treatment using ethionine selection and nystatin selection method. One of the pathways in which SAM participates is the ergosterol biosynthesis reaction in *Saccharomyces cerevisiae*. And nystatin is an antifungal agent that interacts with ergosterol in the cell membrane. Thus, nystatin-resistant mutants may yield much less ergosterol, accordingly consumed much less SAM to biosynthesize ergosterol, and as a result accumulated more SAM. Shobayashi *et al*. isolated nystatin -resistant mutants to obtain SAM-accumulating strains of *Saccharomyces cerevisiae* (Shobayashi *et al*., 2006). In present study, the method has been applied to isolate SAM-accumulating mutant of *Candida* sp with nystatin, and SAM-accumulating mutants were isolated successfully and is very useful for further researches of the production of SAM.

TABLE 1. Investigation on SAM content of yeast strains*

Level of SAM content	SAM content (mg/g)	Number of strains	$\frac{\text{Number of strains}}{\text{Total number of strains}} \times 100$
High level	20~35.7	25	4.37
Medium level	15~20	40	6.99
Lower level	5~15	263	45.98
Low level	5<	244	42.66

* The strains were cultured in FM fermentation medium for 48 h at 30 °C.

Ethionine-resistant mutants often have a damaged methionine adenosyl transferase gene, cannot catalyse L-methionine and ATP yielding SAM, and accumulate free intracellular L-methionine. However, there was no obvious increase or decreases in methionine adenosyltransferase activity in the *S*AM-accumulation mutants compared with the original strain in present study (showed as Fig. 3). Thus, one of the reasons why the mutants accumulated SAM in significantly high amounts may be the lower consumption of SAM for ergosterol biosynthesis, other than improvement of methionine adenosyltransferase activity.

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