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Isolation of a Tryptophan-Overproducing Strain Generated by EMS Mutagenesis of *Candida rugosa*

– Research Note –

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Abstract

To isolate a mutant strain which overproduces tryptophan, mutants of *Candida rugosa* were screened after EMS (ethyl methane sulfonate) mutagenesis. Fluorotryptophan, a tryptophan analogue, was used for selection of a tryptophan-overproducing mutant after mutagenesis. Among 50 mutants, several candidates were selected based on intracellular tryptophan content. Amino acid analysis results showed that C3 was the best strain because it had the highest amount of tryptophan among the mutants.

Key words: amino acid analog, *Candida rugosa*, tryptophan, EMS mutagenesis

INTRODUCTION

Amino acids have extensive industrial applications including uses as nutritional and food additives in bakeries and food industries (1). Tryptophan is an essential amino acid, but is limited in animal feed and human food. Tryptophan is one of the amino acids that are commercially produced by fermentation using genetically modified organisms. Many studies have reported the use of mutant bacteria to produce tryptophan (2-4). Yeasts such as *Saccharomyces cerevisiae* and *Candida* sp. have also been used for the production of tryptophan (5,6).

The regulation of amino acid biosynthesis can be studied by the use of amino acid analogues to select mutants which are altered in their ability to regulate the synthesis of the natural amino acids (7,8). Isolation of mutants resistant to amino acid analogues is an efficient method for separating and identifying amino acid overproducers (9). Thus, several amino acid analogues have been used to isolate mutants overproducing specific amino acids (10-12). Fluorotryptophan is a tryptophan analog known to inhibit cell growth because its chemical structure mimics tryptophan and hinders the activity of the tryptophan feedback-inhibited enzymes used for biosynthesis of the amino acid (10).

Although isolation of *Saccharomyces cerevisiae* mutants have been extensively studied, there are few studies on other yeasts such as *Candida rugosa*. Therefore, the purpose of this study was to generate mutants overproducing tryptophan using EMS mutagenesis of *Candida rugosa*.

MATERIALS AND METHODS

Strain and culture conditions

Wild-type of *Candida rugosa* was obtained from KCTC (Korea Collection for Type Culture, KCTC 7711, ATCC 20306). Cultivation was carried out with YM broth. YM broth contained yeast extract (3 g), malt extract (3 g), peptone (5 g), and dextrose (10 g) per liter. For solid media, 20 g agar was added per liter. SD minimal medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose) containing 0.023% proline as a sole nitrogen source was used for screening of mutants. Shaking flask experiments were performed at 24°C and 130 rpm.

Measurement of minimum inhibitory concentration (MIC) against tryptophan analogue

Sensitivity to fluorotryptophan of wild-type cells and the concentration of fluorotryptophan needed for the isolation of tryptophan-overproducing strain were determined by the minimum inhibition concentration (MIC) test. *Candida rugosa* grown on SD minimal medium containing 0.023% proline, were mixed with 1% soft agar and overlaid on the SD minimal plate containing 0.023% proline. The discs containing different concentrations (0.015 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.25 mM, 0.5 mM, 5 mM) of fluorotryptophan were

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placed on top of the agar layer containing cell culture. Growth inhibition by fluorotryptophan was indicated by the development of halos (Fig. 1).

Ethyl methane sulfonate (EMS) mutagenesis and screening of mutants

Candida rugosa cells (1×10^7 cells/mL, OD=0.5) were centrifuged at 3,000 rpm for 10 min and washed with sterile distilled water. After repeated washing, cells were resuspended in the sterile distilled water and treated with 4% EMS for 10 min, which was established by preliminary experimental results (data not shown). After treatment, the reaction was stopped by the addition of 10% sodium thiosulfate, and the cells were centrifuged at $14{,}240\times g$ for 1 min. The precipitate was washed twice with sterile distilled water, and cells were spread on SD-proline medium containing 2 mM fluorotryptophan. Fifty colonies, grown on the selection plate, were streaked onto YM and SD-proline minimal media, and 4 colonies which grew well on both YM and SD-proline minimal plates were selected.

Amino acid analysis

Cells were prepared for intacelullar tryptophan determination by first resuspending them in lysis buffer and then disrupting the cell walls using a bead beater with glass beads. Amount of free tryptophan in the lysis mixture was determined using HPLC (Waters Co., Milford, MA, USA) with a Pico-Tag detector system. An amino acid standard mixture was used for the quantitative analysis (Fig. 2). The harvested cells were treated with 4 M methanesulfonic acid at 110°C for 24 h for the determination of tryptophan content.

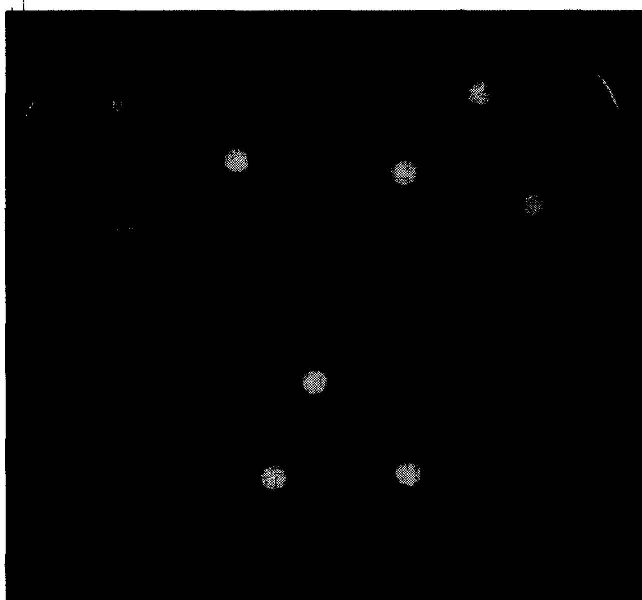

Fig. 1. Sensitivity of *Candida rugosa* to fluorotryptophan. A, control; B, 0.015 mM; C, 0.025 mM; D, 0.05 mM; E, 0.1 mM; F, 0.2 mM; G, 0.25 mM; H, 0.5 mM; I, 5 mM.

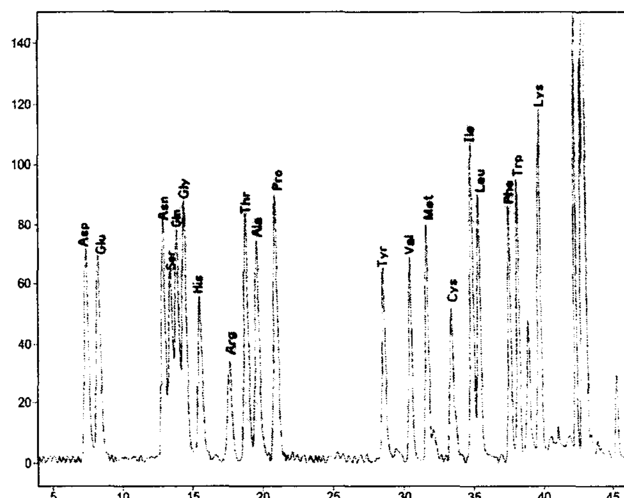

Fig. 2. HPLC chromatogram of 20 amino acid derivatives using a Pico-tag column.

RESULTS AND DISCUSSION

Toxic amino acid analogues are known to block essential functions of amino acids in cells (13). Use of resistance to these analogues has led to a method to isolate mutants which overproduce the specific amino acids (8,14,15). Tryptophan-overproducing mutants may overcome the inhibitory effect of fluorotryptophan due to competition between tryptophan and the analogue for the t-RNA binding site (5). Sensitivity to fluorotryptophan of wild-type cells was examined by MIC test. Growth inhibition by fluorotryptophan indicated by halos which could be visually observed (Fig. 1). Based on the MIC test results, 2 mM of fluorotryptophan was used as the optimal concentration for the isolation of tryptophan-overproducing strains.

To generate mutants overproducing tryptophan, EMS mutagenesis was employed. Optimal conditions for EMS mutagenesis were determined by examining the survival rate of cells treated at different EMS concentrations. Preliminary experiments showed that treatment with 4% EMS for 10 min was the best mutagenesis condition (data not shown), based on the survival rate, where a 40% of survival rate was observed.

After treatment of *Candida rugosa* cells with 4% EMS for 10 min, fifty colonies grown on the selection plate were streaked onto both YM medium and SD minimal plates containing 0.023% proline. To determine the content of tryptophan, the mutant cells were harvested, and amino acid analysis was performed by HPLC (Waters Co., Milford, MA, USA) using a Pico-tag detector system. An HPLC chromatogram of 20 amino acid stan-

Table 1. Relative amount of intracellular tryptophan produced by the wild type and mutants

Strains	Peak area
	Tryptophan
Wild type	1486305
C3	2131689
C4	1753358
C11	1551442
C12	1649043

Table 2. Amount of tryptophan produced by the wild type and the mutant (unit: μg/cell culture mL)

	Intracellular accumulation in the cell	Harvested cell
	Tryptophan	Tryptophan
Wild type	7.28×10^{-2}	1.51×10^{-1}
C3	9.9×10^{-2}	2.35×10^{-1}

dards is shown in Fig. 2. The results of the amino acid analysis of the samples showed that the intracellular contents of tryptophan in the mutant C3, C4, C11, C12 needed further analysis (Table 1). Among the four candidate mutants, repeated experiments clearly indicated that C3 was the best strain in terms of production of tryptophan in the cell.

Amino acid analysis showed that the amount of intracellular tryptophan in the mutant C3 increased 1.4-fold, compared with the wild type (Table 2). For total tryptophan content of cells, harvested cells were treated with 4 M methanesulfonic acid at 110°C for 24 h and its tryptophan amount was determined. Results indicated that harvested cells had an increase in tryptophan content by 1.6-fold (Table 2). The productivity of tryptophan remained relatively low compared to the microbial production of other amino acids due to the complex multiple regulations in the biosynthetic pathway of tryptophan (6).

In summary, we established a method to isolate tryptophan-overproducing mutants of *Candida rugosa* using a tryptophan analogue for selection from among cells altered by EMS-generated mutagenesis, and isolated a tryptophan-overproducing mutant, C3.

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REFERENCES

1. Farfan MJ, Calderon IL. 2000. Enrichment of threonine content in *Saccharomyces cerevisiae* by pathway engineering. *Enzyme Microbial Technol* 26: 763-770.
2. Kurahashi O, Tokozeki K, Nakamori S, Yamanaka S, Enei H. 1987. Production of L-tryptophan by 5-fluorotryptophan and indolmycin resistant mutants of *Bacillus subtilis* K. *Agric Biol Chem* 51: 231-235.
3. Terasawa M, Fukushima M, Kurusi Y, Yukawa H. 1990. L-tryptophan production by the application of high expressed tryptophanase in *Escherichia coli*. *Process Biochem* 25: 172-175.
4. Tsunekawa H, Azuma S, Okabe M, Okamoto R, Aiba S. 1992. Acquisition of a sucrose utilisation system in *Escherichia coli* K-12. Derivatives and its application to industry. *Appl Environ Microbiol* 58: 2081-2088.
5. Moller A. 1994. L-tryptophan production from anthranilic acid by amino acid auxotrophic mutants of *Candida utilis*. *Process Biochem* 29: 521-527.
6. Xiu ZL, Zeng AP, Deckwer WD. 1998. Multiplicity and stability analysis of microorganisms in continuous culture: Effects of metabolic overflow and growth inhibition. *Biotechnol Bioeng* 57: 251-261.
7. Arfin SM, Gantt JS. 1983. Amino acid analogs as tools for the study of amino acid metabolism in mammalian cells analog. *Trends Biochem Sci* 8: 163-164.
8. Martinez-Force E, Benitez T. 1992. Selection of amino-acid overproducer yeast mutants. *Curr Genet* 21: 191-196.
9. Alix JH. 1982. Molecular aspects of the *in vivo* and *in vitro* effects of ethionine, an analog of methionine. *Microb Rev* 46: 281-295.
10. Ramos C, Calderon IL. 1992. Overproduction of threonine by *Saccharomyces cerevisiae* mutants resistant to hydroxynorvaline. *Appl Environ Microbiol* 58: 1677-1682.
11. Riccardi G, Sora S, Ciferri O. 1981. Production of amino acid by analog-resistant mutants of the cyanobacterium *Spirulina platensis*. *J Bact* 147: 1002-1007.
12. Martinez-Force E, Benitez T. 1992. Changes in yeast amino acid pool with respiratory versus fermentative metabolism. *Biotech Bioeng* 40: 643-649.
13. Rincon AM, Benitez T. 2001. Improved organoleptic and nutritive properties of bakery products supplemented with amino acid overproducing *Saccharomyces cerevisiae* yeasts. *J Agric Food Chem* 49: 1861-1866.
14. Gasent-Ramirez JM, Benitez T. 1998. Lysine-overproducing mutants of *Saccharomyces cerevisiae* baker's yeast isolated in continuous culture. *Appl Environ Microbiol* 63: 4800-4806.
15. Gasent-Ramirez JM, Castrejon F, Querol A, Ramon D, Benitez T. 1999. Genomic stability of *Saccharomyces cerevisiae* baker's yeast. *Syst Appl Microbiol* 22: 329-340.

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