Selection and Characterization of L-Ethionine Resistant Mutants of *Trichosporon cutaneum*

Nelly Georgieva[a] and Zlatka Alexieva[b,*]

[a] University of Chemical Technology and Metallurgy, Kl. Ohridsky str. 8, 1756 Sofia, Bulgaria

[b] Institute of Microbiology, Bulgarian Academy of Science, Acad G. Bonchev str., bl. 26, 1113 Sofia, Bulgaria. Fax: +3592870109. E-mail: zlatkama@microbio.bas.bg

* Author for correspondence and reprint requests

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Trichosporon cutaneum R57 and its L-ethionine resistant mutant *NZ94* strain were investigated. The amino acid analyses of cell content of both strains were carried out. The pool of free methionine in the mutant strain is enhanced 16.5 times. The total amount of sulphur-containing amino acids in the mutant cells was significantly increased from 36.8 in the wild strain to 113.4 mg/g protein in the mutant strain. In the process of mutant strain cultivation there was found a high excretion of free methionine (259 μg/ml) in the medium. It was shown that the amino acid content of both wild and mutant strains would be helpful for formulating of new improved animal nutritional diets.

Key words: L-Ethionine, Methionine-enriched Mutant, *Trichosporon cutaneum*

Introduction

Wide spread amino acid usage as an important addition to fodder is one of the most important conditions for introducing of modern techniques in livestock. It permits a significant increase of easily assimilated fodder protein and as a result drops the expenses for fodder, yet keeps animal productivity at the same level.

The role of effectors and repressors of amino acid biosynthesis can be performed by some synthetic analogues of end products. The mutants, resistant to such compounds may change their regulatory characteristics, which usually leads to constitutive enzyme synthesis. That is the reason why metabolite analogues are widely applied for selection of microorganisms carrying regulatory mutations (Chattopadhyay *et al.*, 1991; Geeta and Singh, 2000; Mondal *et al.*, 1996*;* Umbarger, 1978). The genetic removal of the feedback controls the most direct and general method for the overproduction, and may be achieved by selecting mutants resistant to structural analogues of the amino acids (Wang-Jin *et al.*, 1990).

The sulfur-containing amino acid methionine is a limiting component in the diets of non-ruminant animals which must obtain it from their feedstaff. L-Methionine plays an important role in the cellular metabolism as an essential amino acid. As an approach to understand the regulation of methionine metabolism over-accumulating mutants have been isolated based on their resistance to selection by ethionine in different organisms (Barra *et al.*, 1996; Shen *et al.*, 2002).

L-Methionine overproduction by ethionine-resistant mutants has been reported for some yeast strains (Brigidi *et al.*, 1988; Dunyak and Cook, 1985; Mincheva *et al.*, 2002; Morzycka *et al.*, 1976). However, there has been no report on the production of L-methionine by *Trichosporon cutaneum* strains. The actual taxonomic affiliations of *Trichosporon cutaneum* based on biochemical and molecular analyses have been reported by Gueho *et al.* (1989, 1992) and Middelhoven *et al.* (2001).

This paper describes the isolation of an ethionine-resistant mutant of a filamentous yeast, *Trichosporon cutaneum R57*, and the essential amino acid productivity of methionine-enriched mutant cells.

Materials and Methods

Yeast strain and media

The basidiomycetes yeast strain *Trichosporon cutaneum R57*, registrated in National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC) N 2414, Bulgaria (Ivanova and Alexieva, 1996), was used in all experiments. The analyses carried out in Bulgarian Central Research Veterinarian Institute (Statement #5229/95) have proved that the investigated strain is not pa-

thogenic and has no toxic effects, so can be used as an additive to compounded fodder. The cultivation was carried out in a medium with following composition (g/l): 3.7 ml H_3PO_4 (diluted 1:10); 18.5 ml CH_3COOH (diluted 1:10); 4 ml 0.1 N NaOH; 2.7 ml NH_4OH; 0.3 g KCl; 0.15 g $MgSO_4 \cdot 7H_2O$; 0.042 g $Ca(H_2PO_4)_2$; 0.02 g tiamin·HCl; 0.002 g biotin. After autoclaving, a sterile glucose solution (10 g/l) was added to the buffered growth medium. Maltz agar (1.5%) was used as solid medium.

Yeast cells were transferred from solid medium to 10 ml of the liquid synthetic medium for preculture. The preculture was cultivated at 28 °C for 18 h with reciprocal shaking (220 rpm). The culture broth of 1 ml was then inoculated into a 500 ml-shaking flask containing 50 ml of synthetic medium. The cultivation was carried out at 28 °C for 48 h on a reciprocal shaker at 220 rpm (Ivanova and Alexieva, 1996).

L-Amino acid assay

The cells were harvested in the stationary phase of growth and washed twice with distilled water by centrifugation. 2 g of the pellet (140 mg cells as dry cell weight) were resuspended in 10 ml distilled water and heated for 20 min at 100 °C (boiling water bath) in a capped tube. The pool amino acid content was assayed in the supernatant derived by centrifugation (8,000 × *g* for 20 min). The protein bound amino acids were determined after hydrolysis with 6 N HCl. Amino acid analyses were performed on a Hitachi KLA-5 Automatic Analyzer (Yoshiki and Wang-Jim, 1988). The method for assessment of the biological protein value on the base of amino acid composition has been accepted by FAO/WHO and is applied in most laboratories for food protein evaluation (Ribarova and Shishkov, 1986). The equation used in this method is:

$$\text{amino acid score} = \frac{\text{mg essential amino acid/g protein} \cdot 100}{\text{mg essential amino acid/g standard protein}}.$$

The experiments for determination of amino acid content were performed in quintuple.

Results

Our experiments with *Trichosporon cutaneum R57* established that the strain had a natural ability to tolerate relatively high concentration of L-ethionine (600 μg/ml on solid medium; 1 mg/ml in liquid synthetic medium).

By multistep selection mutants resistant to different L-ethionine concentrations have been obtained. We isolated a mutant resistant to 55 mg/ml L-ethionine. In the process of experiments morphological and growth differences arose in the mutants expressing resistance to concentrations higher than 1 mg/ml. On rich solid medium including L-ethionine, the colonies had a smaller size and did not exhibit the flour-like surface, typical for the parent strain. Microscopic analyses showed that the cells resistant to high concentration of L-ethionine were smaller than the parent strains after 48 h of cultivation in liquid synthetic medium. We could not find any formation of pseudomycel which is typical for the wild strain.

Among the ethionine-resistant mutants tested, *Trichosporon cutaneum NZ94* showed the highest contents of pool and total methionine. Table I shows a comparison of the L-methionine content of mutant strain *NZ94* with that of the wild strain. The mutant strain had a 16.5 times increased content of total methionine compared to the wild type strain, and an increased part of the total methionine in the mutant strain corresponded to the increase in the methionine pool. The results of our protein analyses of the bound amino acids are also shown in Table I. Our analysis of the medium content after wild strain cultivation showed no traces of amino acids. It is noteworthy that we found an amount of 259 μg/ml free methionine in the medium.

The amino acid balance of the total content of the investigated strains was calculated by accepting the lysine value as 100%. The essential amino acid patterns are presented by Fig. 1.

Table I. Essential amino acids content in a wild and mutant strain of *Trichosporon cutaneum.*

Amino acids	Amino acid content [mg/g protein]	
	T. cutaneum R57	*T. cutaneum NZ94*
Valine	55.8 ± 0.04	70.0 ± 0.01
Isoleucine	40.7 ± 0.01	40.3 ± 0.04
Leucine	75.4 ± 0.04	83.8 ± 0.04
Lysine	78.4 ± 0.03	69.9 ± 0.01
Methionine	17.4 ± 0.01	100.6 ± 0.02
Cysteine + Cystine	19.5 ± 0.04	12.8 ± 0.04
Threonine	59.2 ± 0.03	51.4 ± 0.04
Tryptophan	13.6 ± 0.01	13.0 ± 0.01
Tyrosine	30.8 ± 0.01	42.9 ± 0.01
Phenylalanine	39.1 ± 0.01	46.8 ± 0.01

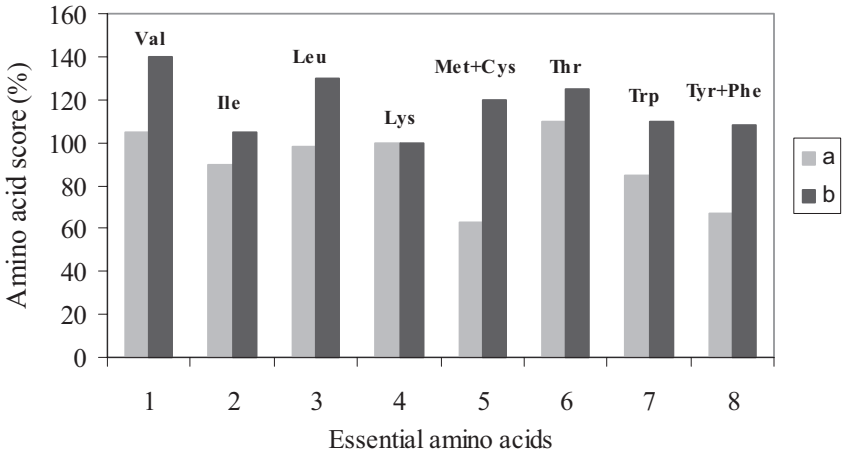

Fig. 1. Amino acid balance in *T. cutaneum* biomass: a) *T. cutaneum R57* and b) the mutant *T. cutaneum NZ94*.

Discussion

L-Methionine overproduction by ethionine-resistant mutants has been reported for *Candida utilis* (Dunyak and Cook, 1985), *Saccharomyces cerevisiae* (Brigidi *et al.*, 1988; Shiio, 1982), *Kluyveromyces lactis* (Mincheva *et al.*, 2002), *Saccharomycopsis lipolytica* (Morzycka *et al.*, 1976), *n*-paraffin utilizing yeast *Candida petrofilum* (Nuesch *et al.*, 1978), *Candida boidini 2201* (Wang-Jin *et al.*, 1990). Until now there are no data in the literature on such mutants with the *Trichosporon* genus.

Compared to other amino acids, the content of L-methionine in microorganisms is fairly low because its biosynthesis is strictly controlled by feedback regulation (Umbarger, 1978). *Trichosporon cutaneum* had been proved as a protein producer. Sulfur-containing amino acids in it were established in amounts higher than it is usual for other yeasts, known as protein producers (Wang-Jin and Yoshiki, 1988). The high level of methionine biosynthesis and consequent endurance to L-ethionine were among the reasons to choose the *T. cutaneum R57* strain for our work. The total amount of S-containing amino acids of mutant strain *T. cutaneum NZ94* (113.4 mg/g protein) was above 3-fold higher than that of the wild strain. We assumed that one of the probable explanation for the increase of methionine content (almost 3-fold) in the cells protein might be a synthesis stimulation of specific proteins containing more methionine in their structures (see Table I).

Generally, plant fodders have low concentrations of sulphur-containing amino acids. That is the reason why some additives (amino acid mixtures, yeast protein, etc.) are used for optimizing plant fodders' amino acid content. The strain *NZ94* possessed a high content of methionine as well as an appropriate threonine/lysine and tryptophan/lysine ratio. These data showed that the *Trichosporon cutaneum NZ94* strain would be helpful for formulating of new improved animal nutritional diets.

According to Degussa standards (the world leader of synthetic methionine production) for amino acid balance in formulated fodder, used for pig nutrition, the amino acid content of both wild and mutant strains secures optimum performance by so-called "ideal protein" (Tanner and Schmidtborn, 1980). However, the mutant strain biomass percentage in the fodders would be less than that of the wild-type strain and so the formulated feeds should be considerably cheaper.

The excretion of methionine into the medium by the *Trichosporon cutaneum NZ94* strain might be appropriate for rapid selection technique to be developed. This ability of the mutant indicated as well an opportunity for improving the strain as a methionine producer.

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