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Continuous fermenter growth of a methionine-overproducing mutant of *Candida utilis*

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Summary. An ethionine resistant mutant of *Candida utilis* was found to maintain an expanded intracellular pool of free L-methionine in batch and continuous cultures. During glucose-limited growth in mineral salts medium in a continuous fermenter, the free L-methionine pool of the mutant was 40–80% higher than in batch cultures, and varied in the range of 25–30 μmoles/g dry cells (3.7–4.5 mg/g dry cells).

Introduction

A problem with food yeasts as sources of dietary protein is their relatively low content of the essential amino acid, L-methionine (Bressani 1969; Kihlberg 1972; Nelson et al. 1960). Supplementation with synthetic DL-methionine can raise the biological value of yeast protein to that of "complete" protein (Palmer and Smith 1971; Shaklady 1975), but the need for this step could be eliminated if a food yeast with a higher methionine content were available. Changes in growth conditions produce only minor alterations in overall amino acid composition (Alroy and Tannenbaum 1973, 1977), but significant modifications can be obtained genetically.

The methionine content of yeasts can be increased by expanding the intracellular pool of free methionine by regulatory mutations. Methionine overproduction by ethionine-resistant mutants has been reported for *Candida utilis* (Musilkova and Fencl 1964), *Saccharomyces cerevisiae* (Cherest et al. 1973) and *Saccharomycopsis lipolytica* (Morzyka et al. 1976).

It is known that total amino acid pools of wild-type yeasts are strongly growth-associated (Moat et al. 1969; Dawson 1965), and Brown and Stanley (1972) have found larger amino acid pools with glucose-limitation than nitrogen-limitation. Therefore, it seemed probable to us that the methionine content of such regulatory mutants would be maximized by use of continuous culture methods. To examine this point we isolated a methionine overproducing mutant of *Candida utilis* and studied its intracellular pool of free L-methionine during growth in a continuous fermenter.

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Materials and methods

Cultures. *Candida utilis* ATCC 8205 and *Pediococcus acidilactici* ATCC 8042 were from the collection of the Department of Microbiology, University of Maryland. Stocks of *C. utilis* and *P. acidilactici* were maintained on slants of Bacto-Sabouraud Dextrose Agar (Difco) and stabs of Bacto-Microassay Culture Agar (Difco), respectively.

Culture medium. Mineral salts-glucose medium for growth of *C. utilis* contained (per liter of deionized water): 1.0 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 \cdot 7 \cdot H_2O$; 1.4 g K_2HPO_4; 0.6 g KH_2PO_4; 0.8 mg $ZnSO_4 \cdot 7\,H_2O$; 0.8 mg $FeCl_3 \cdot 6\,H_2O$; 0.8 mg $NaMoO_4 \cdot H_2O$; 0.4 mg $MnSO_4 \cdot H_2O$; 0.08 mg $CuSO_4 \cdot 5\,H_2O$; 30 μg biotin. The final pH of the medium was pH 4.3. For batch cultures D-glucose was added to a concentration of 2 g/l

Isolation of ethionine resistant mutant ER-8. A washed cell suspension of *C. utilis* ATCC 8205 in deionized water was adjusted to 1×10^7 colony forming units per milliliter (CFU/ml), irradiated with ultraviolet light to produce a 99% decrease in viability, and spread on plates of mineral salts-glucose medium containing 30 mM DL-ethionine. A resistant colony was selected and re-streaked on the same medium. The resulting culture, designated ER-8, had an elevated intracellular content of free methionine. This mutant proved to be somewhat unstable, as after 12 serial subcultures at monthly intervals on media without ethionine, the culture no longer showed the original high methionine pool.

Batch cultures. Shake flask cultures were grown in 250-ml Delong flasks (Bellco Glass Co., Vineland, NJ) containing 50 ml of mineral salts glucose medium and inoculated with 0.5 ml of a 24-h shake flask culture in the same medium. Flasks were incubated at 37° C with shaking at 100 RPM in a waterbath shaker (Model 75 incubator shaker, Precision Scientific Co., Chicago, IL).

Batch fermenter cultures were grown in a 14-l fermentor (Model 43–100, Virtis Co., Gardiner, NY) containing 6 l of mineral salts glucose medium, with agitation at 200 RPM, aeration of one volume air/volume medium/min and a temperature of 37° C.

Continuous cultures. The chemostat-type computer controlled continuous fermenter system has been described (Hatch, Wilder and Cadman, 1979). This consists of a 14-l fermenter (Magnaferm, New Brunswick Scientific Co., New Brunswick, NJ) modified for aseptic continuous-flow operation. The basal salts solution was sterilized by passing through a 0.2 µm cartridge filter (Ultipore Filter, Poll Corp., Cortland, NY), and pumped into the fermenter, with monitoring of flow rates and liquid levels by differential pressure cells (Foxboro Co., Foxboro, MA) and regulation by pneumatic control valves. Glucose and ammonium sulfate solutions were added from separate reservoirs using metering pumps, with filtration throuth sterile 0.2 µm membrane filters (Nucleopore Co., Pleasonton, CA). For glucose-limited growth the glucose feed rate was adjusted to give a concentration equivalent to 0.925 g/l. The pH of the culture was monitored using a sterilizable electrode (Model 761-315B, Ingold Electrodes Inc., Lexington, MA) and a pH meter (Model 61, Radiometer, Copenhagen, Denmark). The pH was maintained by addition of filter sterilized (0.2-µm Teflon membrane filters, Millipore Corp., Bedford, MA) solutions of 1 N HCl and 1 N KOH. The fermenter was operated with an impeller speed of 200 RPM, aeration of one volume air/volume medium/min and a temperature of 30° C. For data collection and operation of the fermenter a process control system using a microcomputer (PDP 11/34, Digital Equipment Corp., Maynard, MA) was assembled.

Cell counts and dry weight measurement. Cell counts were obtained using a capillary flow laser microphotometer equipped with a 630-nm helium-neon laser (Cytofluorograph, Model FC 200R/FC4800A, Ortho Instruments, Westwood, MA).

To determine dry weights cells were centrifuged from 200 ml samples of culture, washed and resuspended in 0.1 vol distilled water, then dried to constant weight in an oven.

Glucose and ammonia determinations. Glucose concentrations in the chemostat cultures were measured using an enzyme electrode (Model 2520 and Model 25 Oxidase Meter, Yellow Springs Instruments, Yellow Springs, OH). The meter and probe were calibrated using a buffered D-glucose solution(100 mg/l).

Ammonia concentrations were measured using an ammonia-specific electrode (Model 95-10-00, Orion Research, Cambridge, MA) calibrated with standard of NH_4Cl and KOH.

Extraction of free amino acid pool. Procedures for extraction of free amino acids were adapted from those of Freeland and Gale (1947) and Dawson (1965). Cells were recovered from 40 ml of broth culture by centrifuging 10 min at 12,000 *g* (Sorval RC2-B centrifuge with SS34 rotor at 4° C), washed once in 40 ml of pH 7 buffer (Sorenson's; 0.026 M KH_2PO_4 and 0.040 M Na_2HPO_4), and resuspended in 10 ml of deionized water. The washed cell suspensions were heated 30 min at 100° C (boiling water bath) in a capped tube and cells removed by centrifugation.

Microbiological assay of L-methionine. The L-methionine content of the hot water extracts was measured by microbiological assay using *Pediococcus acidilactici* ATCC 8042 (Steele et al. 1949). Growth in Bacto-Methionine Assay Medium (Difco) was measured as turbidity at 620 nm.

Extraction and measurement of S-adenosylmethionine (SAM). The procedure for cold perchloric acid extraction of SAM was adapted from Cherest et al. (1973). Cells were centrifuged from 160 ml of culture (4° C, 10 min at 12,000 *g*), washed once in 40 ml cold deionized water and resuspended in 8 ml of cold 1.5 N perchloric acid. After shaking 1 h at 4° C, the cells were removed by centrifugation, and the extract was neutralized with 2 M $KHCO_3$. The extract was clarified by centrifugation, and SAM content was estimated by ion exchange chromatography and spectrophotometry after the method of Shapiro and Ehniger (1966). The extract was fractionated on a small column of Dowex 50W-X8 resin (J. T. Baker Chemical Co., Phillipsburg, N.J.) and the SAM content was calculated from the OD_{256} nm of the material eluted with 6 N H_2SO_4, assuming an extinction coefficient of 14,700.

HPLC of the amino acid pool. The method used for analysis of amino acids by ion-pair high performance liquid chromatography has been described (Radjai and Hatch 1980). Samples (20 µl) of the hotwater extracts were separated on a 30 cm × 3.0 mm µ Bondapack C_{18} column (Waters Associated, Milford, MA) using a dual pump liquid chromatograph (Waters Associates) with solvent programming and on-line post-column derivatization with O-phthaladehyde. The O-phthalaldehyde derivatives were measured by UV absorbance at 240 nm using a Model 440 UV detector (Waters Assoc.), and the identity of major peaks was determined by co-chromatography with amino acid standards.

Spectrophotometry. Measurements of turbidity and UV absorbance were made using an automatic recording spectrophotometer (Model 2400-3 Gilford Instrument Co., Oberlin, OH).

Results and discussion

A methionine-overproducing mutant, strain ER-8, was isolated from an ultraviolet-irradiated culture of *Candida utilis* ATCC 8205 and shown to grow as well as the parent culture in shake flasks of mineral salts-glucose medium (Table 1). Exponentially growing cells of ATCC 8205 contained no detectable free L-methionine, but S-adenosylmethionine (SAM) was present (Table 1). In contrast, the intracellular pool of the ethionine-resistant mutant ER-8 contained a greatly increased level of free L-methionine, which amounted to 2.08 mg/g of dry cells, i.e., 13.4 µmoles/g dry (Table 1). There was no detectable free L-methionine in the spent growth medium from either

Table 1. Elevated intracellular pool of free L-methionine in exponentially growing cells of the ethionine-resistant mutant *Candida utilis* ER-8

Culture	Specific growth rate (hr^{-1})		Intracellular pool[a] (µmoles/g dry cells)	
	No addition[a]	With 50 mM DL-ethionine	SAM	L-Methionine
ATCC 8205	0.48	0.08	3.4	< 0.5[b]
ER-8	0.46	0.36	0.49	13.4

[a] Exponentially growing cells in shake flask cultures of mineral salts-glucose medium without ethionine

[b] Undetectable

culture. The amount of SAM found in cells of ER-8 was less than in the parent culture.

The free L-methionine pool of ER-8 is comparable to that reported for other ethionine resistant mutants. Morzycka et al. (1976) found a methionine pool of 11.4 μmoles/g in an ethionine-resistant mutant of *Saccharomycopsis lipolytica* (*Candida lipolytica*). Cherest et al. (1973) reported methionine pools of 14 and 16 μmoles/g in *Saccharomyces cerevisiae* with mutations in the *eth*-2 and *eth*-10 loci, respectively. Methionine overproduction in these mutants has been associated with altered regulation of enzymes specific for the methionine pathway, such as homocysteine synthase. It is presumed that a similar mutation has occurred in ER-8.

It has been shown previously that overproduction of methionine by yeasts is not necessarily accompanied by an increased pool of its metabolically "active" derivative, S-adenosylmethionine (Morzyka et al. 1976; Cherest et al. 1973). Synthesis of SAM from L-methionine and ATP is catalyzed by methionine adenosyltransferase (ATP- L-methionine-S-adenosyltransferase, EC 2.5.1.6), and lowered activity of this enzyme has been shown to enhance resistance to ethionine (Mertz and Spence 1972). This suggests the possibility of some impairment of methionine adenosyltransferase in ER-8.

In wild-type *C. utilis* there is a small growth-associated pool of free amino acids (Dawson 1965), and the L-methionine pool of ER-8 also clearly was growth associated. However, free L-methionine was present in ER-8 even after growth had ended (Table 2). During active growth in a batch fermenter of mineral salts-glucose medium the intracellular free L-methionine content of ER-8 was about 17 μmoles/g of dry cells. Even 42 h after growth had ceased, the cells still showed a free L-methionine pool of 3.4 μmoles/g.

To examine the effect of growth rate on the methionine pool, ER-8 was grown with limiting glucose in the mineral salts medium using a computer controlled 14 l chemostat-type continuous fermenter as described by Hatch et al. (1979). The actual glucose concentrations present in the chemostat fermenter were measured at dilution rates of 0.2, 0.3, and 0.4 h^{-1}, and found to be 2.0 mg/l, 4.0 mg/l, and 10 mg/l, respectively. Based on a reciprocal plot of these values, the μ_{max} for ER-8 was estimated to be 0.54 h^{-1} and the K_s to be 2.87 mg/l.

As shown in Table 3, cells grown with limiting glucose in the continuous fermenter had a L-methionine pool that was 40–70% larger than that of batch fermenter cells. Possibly this increase may reflect a lack of catabolite repression or the altered N : C ratio resulting from the low glucose conditions. No attempt was made to measure the total amino acid pool, and it is possible that conditions favoring greater methionine production also involve expansion of the total amino acid pool. This would be expected from the results of Brown and Stanley (1972), who have reported the expansion of the amino acid pool of wild-type yeast during glucose-limited growth. Changes in the dilution rate produced the expected changes in cell size and cell numbers, and caused a small increase in the overall free L-methionine content on a dry weight basis. Increasing the dilution rate from 0.2 h^{-1} to 0.4 h^{-1} resulted in a drop in cell numbers, but the average dry weight of a cell increased by about 53%. At the same time the free L-methionine content per gram of dry cells increased by about 21%. Increased cell size at faster dilution rates has been reported for other organisms (Jagadish et al. 1977; Tyson et al. 1979).

In earlier batch culture experiments there had been some suggestions of an increased free methionine content at higher pH values (data not shown). The effect of pH was tested by operating the continuous fermenter with limiting glucose at a constant dilution rate of 0.2 h^{-1} and adjusting the pH of the medium (Table 4). Although the cells growing at pH 5 had a somewhat higher methionine content that those at pH 3.5, the difference is not convincing.

Table 2. Free L-methionine pool of *Candida utilis* mutant ER-8 during batch fermenter growth in mineral salts-glucose medium

Incubation time (h)	Glucose remaining (mg/l)	Cell crop (g dry cells/l)	Specific growth rate (h^{-1})	Intracellular free L-methionine	
				mg/g dry cells	μmoles/g dry cells
8	633	0.296	0.39	2.5	16.8
10	183	0.60	0.39	2.5	16.8
30	10	0.84	0	0.83	5.6
72	10	0.84	0	0.50	3.4

Table 3. Intracellular free L-methionine content of *Candida utilis* mutant ER-8 during glucose-limited growth in a continuous-flow fermenter[a]

Dilution rate (h^{-1})	Cell titer (log_{10}cell count/ml)	Cell dry weight (g/cell × 10^{12})	Cell crop (g dry cells/l)	Free L-methionine pool	
				μg/cell × 10^8	μmoles/g dry cells
0.2	7.6160	7.13	0.294	2.60	24.5
0.3	7.5185	8.16	0.269	2.93	24
0.4	7.2480	10.90	0.193	4.73	29

[a] Mineral salts medium (pH 4.3) with limiting glucose

Table 4. Effect of pH on the free L-methionine pool of mutant ER-8 growing in a continuous-flow fermenter[a]

pH	Cell titer ($\log_{10}$ cell count/ml)	Cell dry weight (g/cell × 10^{12})	Cell crop (g dry cells/l)	Free L-methionine pool (μmoles/g dry cells)
3.5	7.6128	6.68	0.274	26.5
5.0	7.6128	6.48	0.268	31

[a] Mineral salts medium with limiting glucose at a dilution rate of 0.2 h^{-1}

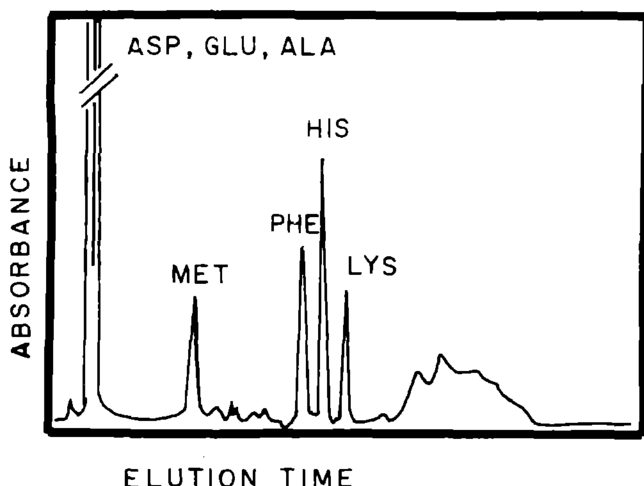

Fig. 1. HPLC of free amino acids in cells of *Candida utilis* mutant ER-8 during glucose-limited growth in the continuous fermenter at a dilution rate of 0.3 h^{-1}

This experiment does at least demonstrate that a high methionine content is attainable at low pH values where bacterial contamination problems are minimized.

Because methionine was measured by microbiological assays, it was desirable to confirm the identity of the bioactive material. In preliminary trials with paper chromatography of extracts of batch culture cells (data not shown), both the parent culture and ER-8 showed the four amino acids that Dawson (1965) had identified as predominant in the wild-type *C. utilis* pool: alanine, glutamic acid, aspartic acid, and histidine. In addition, ER-8, but not ATCC 8205, showed an amino acid with the same Rf value as the L-methionine standard. These results were confirmed using high performance liquid chromatography. Extracts of cells of ER-8 growing in the continuous fermenter with glucose limitation showed a peak with an elution time corresponding to L-methionine (Fig. 1). As expected there were large peaks corresponding to alanine, glutamic acid and aspartic acid, along with a smaller peak for histidine. In addition, the ER-8 pool showed significant peaks corresponding to phenylalanine and lysine. Dawson (1965) observed phenylalanine in the wild-type *C. utilis* pool under certain conditions, particularly at low dilution rates.

Our results demonstrate that use of glucose-limited growth in a chemostat-type continuous fermenter is important for obtaining significant levels of free L-methionine in the metabolic pool of an ethionine-resistant mutant of *C. utilis*. Assuming no change in the composition and amount of cellular protein in such mutants, the observed large methionine pool of 25–30 μmoles/g (i.e., 0.35–0.44% by weight) would considerably improve the nutritional value of the yeast. These results can be compared with those of Okanishi and Gregory (1970), who reported a total methionine content (protein plus pool) of about 0.34% by weight for a wild-type *C. tropicalis*, and an increase to 0.48% for a "methionine-rich" mutant.

The mutant used in our study unfortunately has proven to be somewhat unstable, so it is not particularly suitable for production of high-methionine food yeast. Also; it seems likely that mutants with higher methionine levels can be obtained by repeated rounds of mutation and selection.

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