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P-AMINOBENZOIC ACID (PABA) CHANGES FOLATE CONTENT IN CELL BIOMASS OF *SACCHAROMYCES CEREVISIAE*

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The folic acid intake is definitely lower than the recommended dietary allowance. This study shows the possibility of enhancing the synthesis of folates by the baker's yeast. *p*-Aminobenzoic acid (PABA) addition to the examined culture media at doses of 5–25 μg/cm^3 increases the content of folates, which in turn depends on the strain and PABA dose. The culturing of yeast with PABA might form the basis for the production of bakery products that are rich in folates and, consequently, might constitute an additional folate source in the everyday diet.

INTRODUCTION

Folic acid (vitamin B_9) is a completely oxidized compound which is produced using chemical synthesis [Hoffbrand & Weir, 2001]. A biologically-active form of folic acid is the polyglutamate of tetrahydrofolic acid, and its single-carbon derivatives occurring in cells are referred to as folates [Temple & Montgomery, 1984].

The main function of folate coenzymes in a human organism is either the acceptance or the release of single-carbon groups in many metabolic reactions [Blakely, 1969]. Therefore, they are indispensable for the proper functioning of the hematopoietic and nervous systems and for the development and metabolism of all the cells in the body. Deficiency of folic acid affects, first of all, the biosynthesis of nucleic acids by disturbing the biosynthesis of purine precursors. Because folic acid is indispensable for the production of red blood cells in bone marrow, its deficiency leads to anemia. Insufficient intake of folates increases the level of homocysteine in blood serum, which has been acknowledged as an independent risk factor for the development of cardiovascular diseases (venous thrombosis, myocardial ischemia, and apoplexy) [Graham *et al*., 1997; Rimm *et al.,* 1998; Brouwer *et al.,* 1999], neurodegenerative diseases – Alzheimer's and Parkinson's diseases [Seshadri *et al.,* 2002], and carcinomas, especially colon cancer [Moreiras *et al.,* 2005]. A high risk of folic acid deficiency occurs in all age categories but its deficiency poses especially severe consequences for pregnant women as it increases the incidence of spontaneous abortions [George *et al.*, 2002], decreases the birth weight of newborns, and may be the cause for the inborn neural tube defects in newborns [Vollset *et al.,* 2000; Molloy, 2005]. The folate content in the everyday food rations of people from various countries is determined by their nutritional pattern, and ranges from 95 μg to 562 μg [Gregory *et al.,* 1990; De Bree *et al.*, 1997]. In the member states of the European Union, the average daily intake of folic acid fluctuates between 100 and 200 μg [Jägerstad *et al.,* 2005], whereas the average daily intake of folic acid by women in the reproductive age accounts for approximately 250 μg [Buttriss, 2004]. A similar intake of folates has been shown in a study carried out in the United States of America [Report of the Food and Nutrition Board, Institute of Medicine, 1998]. These doses are definitely lower than the recommended levels. The recommended dietary allowance of folates for adults is 400 μg daily; hence, in a number of countries, attempts are undertaken to increase their content in the diet, mainly through supplementation of food products with folic acid. The application of baker's yeast containing increased folates in the bakery industry or intake of adequate folate supplements extend the possibilities of providing folates with an everyday diet.

p-Aminobenzoic acid (PABA) is a structural component of folic acid and its derivatives; thus it may be expected that the addition of this component to a culture medium is likely to significantly change the cellular metabolism of yeast in a manner enabling the enhanced biosynthesis of folates. This study was aimed at determining the effect of adding *p*-aminobenzoic acid to a culture of *Saccharomyces cerevisiae* with respect to its capacity to accumulate folates.

MATERIALS AND METHODS

Strains and media

The biological materials used in the study were the yeast strains *Saccharomyces cerevisiae*-2200 (Pure Cultures Collection of the Department of Biotechnology and Microbiology, Warsaw University of Life Science) and *Saccharomyces*

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cerevisiae CEN.PK 113-7D MAT(a) from the Lund Institute of Technology, Department of Applied Microbiology.

Experimental medium used in the study was a mineral medium [Verduyn *et al.*, 1992] containing 2% glucose. The defined mineral medium contained the following (per liter): $(NH_4)_2SO_4$, 5 g; KH_2PO_4, 3 g; $MgSO_4 \times 7H_2O$, 0.5 g; EDTA, 15 mg; $ZnSO_4 \times 7H_2O$, 4.5 mg; $CoCl_2 \times 6H_2O$, 0.3 mg; $MnCl_2 \times 4H_2O$, 1 mg; $CuSO_4 \times 5H_2O$, 0.3 mg; $CaCl_2 \times 2H_2O$, 4.5 mg; $FeSO_4 \times 7H_2O$, 3 mg; $NaMoO_4 \times 2H_2O$, 0.4 mg; H_3BO_3, 1 mg; and KI, 0.1 mg. The final vitamin concentrations per liter were as follows: biotin, 0.05 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; inositol, 25 mg; thiamine × HCl, 1 mg; and pyridoxine × HCl, 1 mg. A concentrated solution of PABA, sterilized by passing through a microbiological filter, Ministar (0.2 μm), was added to the culture media at doses that provided concentrations of 0.02, 1, 5, 10, 25, 50, 100, and 200 μg in 1 cm^3 of the medium. Mineral culture medium without PABA was used as controls.

Culture conditions

Yeast inoculum was prepared by inoculating the medium with a 24-h pure culture of the examined yeast strain, collected from an YPD slant with an inoculation loop. The inoculum culture was incubated for 24 h at a temperature of 28°C in a reciprocating shaker (SM-30 Control E. Büchler, Germany), at 200 rpm. The specific culture was inoculated (10%v/v) and incubated for 24 h at a temperature of 28°C in the reciprocating shaker, also at 200 rpm. On termination of the culture growth, the biomass was centrifuged three times at 1075×*g* for 10 min (Centrifuge MPW-365, Poland); after each centrifugation, the biomass was rinsed with deionized water. The air in the wet yeast biomass was removed using nitrogen, and the mass obtained was frozen and stored at a temperature of –80°C for a period not exceeding 2 months.

Determination of total folate content

Yeast homogenate (1 g of yeast suspended in 20 cm^3 of 0.1 mol/L phosphate buffer (pH 6.1) containing 1% ascorbic acid (POCH, S.A.) and 0.1% 2-mercaptoethanol (Sigma)), was cooked for 10 min, and then rapidly cooled in ice. Subsequently, the yeast extract was incubated with 3 cm^3 of conjugase (Kidney acetone powder, porcine–Sigma) and 1 cm^3 of α-amylase (Sigma) for 3 h at a temperature of 37°C. The extract was cooked to denature enzymes and centrifuged at 8050×*g* at a temperature of 4°C and then purified and concentrated on strong anion exchange columns. Consolidation and purification of the yeast extract were carried out according to the methods described by Vahteristo *et al.* [1996]. Folates were determined by the HPLC method using a Phenomenex Synergi UU Hydro RP-80-A column (250×4.6 mm; 2U micron), a UV–HP 35900 interface detector, and an HP-1046A programmable fluorescence detector. The mobile phase was 33 mmol/L H_3PO_4 (pH 2.3), and the temperature was set at 25°C.

Determination of free folate content

Determination of free folates was carried out by a microbiological method that followed the methodology described by Iwatani [2003]. Yeast cells (1 g) were homogenized in a phosphate buffer (0.1 mol/L, pH 6.1) containing 0.15% ascorbic acid (POCH, S.A.), cooked for 10 min, and rapidly cooled in ice. After sterilization, the extract was inoculated with the previously prepared inoculum of *Lactobacillus rhamnosus* (American Type Culture Collection 7469) bacteria and cultured for 18 h at a temperature of 37°C. The strain of *Lactobacillus rhamnosus* bacteria was prepared for inoculation according to the methodology elaborated by Tamura [1990], and its growth was assayed on the basis of absorbance measurements at a wavelength of 546 nm (Spectronic 20, Genesys, USA).

Statistical analysis

For statistical analysis, the Statgraphics Plus Version 4.1 software was used. Most of the results were subjected to the statistical analysis using the one-way or multifactor analysis of variance (Multifactor Anova). The least significant differences were determined according to Tukey's method as the honestly significant difference. A significance level of $p=0.05$ was adopted in calculations.

RESULTS

Folate content in *S. cerevisiae* CEN.PK biomass

The biomass of *S. cerevisiae* CEN.PK yeast obtained from the control mineral medium (Figure 1) contained 1464 μg of total folates in 100 g dry matter (d.m.). The highest content of folates –3256 μg/100g d.m. – was recorded in the biomass obtained from the mineral medium containing 5 μg of PABA/cm^3. Biomass obtained from the medium containing 100 and 200 μg PABA/cm^3 was characterised by the lowest content of total folates, although it was not significantly different from that noted for the biomass from the control medium. The folate contents in the cell biomass of CEN.PK yeast incubated in the other experimental mineral media (those containing 0.02, 1, 5, 10, 25, and 50 μg PABA/cm^3) were significantly different from the folate content of the biomass obtained from the control medium (without PABA). Among the identified forms of folates, 5-methyl tetrahydrofolate (5-CH_3-THF) and tetrahydrofolate (THF) appeared to be predominant. 5-CH_3--THF constituted 59 (medium with 10 μg of PABA/cm^3)

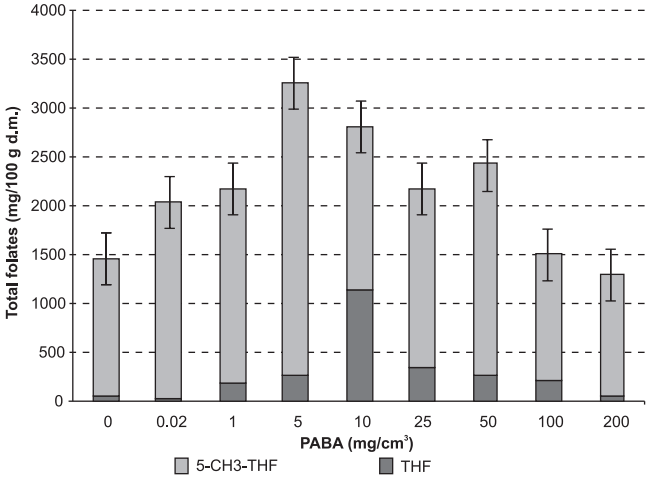

FIGURE 1. Content of total folates (THF and 5-CH_3-THF) in biomass of *S. cerevisiae* CEN.PK yeast obtained from 24-h culture in the mineral medium with PABA addition.
I – NIR = 534, (acc. to Tukey, one-way analysis of variance, $\alpha = 0.05$).

to 98% (medium with 0.02 μg of PABA/cm^3) of the total folates estimated.

The content of free folates in the biomass of CEN.PK yeast was found to depend on the PABA concentration in the mineral medium (Table 1) and ranged from 118 to 264 μg/100 g d.m. PABA addition in doses ranging from 0.02 to 50 μg/cm^3 of mineral medium significantly increased the free folate content in the cell biomass of CEN.PK yeast as compared to their content estimated in the biomass originating from the control medium. Higher concentrations of PABA in the medium (100 and 200 μg/cm^3) inhibited the production of folates, although the difference was not significant with respect to the control sample. Biomass obtained from the medium containing 5 μg of PABA/cm^3 was characterised by the highest content of free folates.

Folate content in *S. cerevisiae* 2200 biomass

Total folate content of the cell biomass of *S. cerevisiae* – 2200 yeast cultured in the mineral medium (Figure 2) was determined by the addition of PABA to the medium, and the values ranged from 1430 to 3543 μg/100 g d.m. The medium containing 10 and 25 μg of PABA/cm^3 yielded a biomass of yeast that was characterised by the highest content of folates. This strain of yeast was also found to accumulate a higher amount of the methylated form of tetrahydrofolate compared to the nonmethylated form. THF constituted barely 5 to 29% of the total folates, with the highest content recorded in the biomass obtained from the medium containing 25 μg of PABA/cm^3. The lowest concentration of folates was observed in the biomass obtained from the medium supplemented with a PABA dose of 200 μg/cm^3; yet, it was not significantly different from that assayed in the control sample.

S. cerevisiae – 2200, cultured in the mineral medium containing various doses of PABA, produced 115 to 181 μg of free folates/100 g d.m. (Table 1). The differences in the free folate content between the biomass of *S. cerevisiae*-2200 from the control mineral medium and that from the experimental media with 0.02, 5, 10, 25, 50, 100, and 200 μg/cm^3 of PABA were not significantly different. A significant increase of free folates in the biomass was evoked by a PABA dosage of 1 μg/cm^3 in the experimental medium. Conversely, the lowest concentration of free folates was observed in the biomass obtained from the culture grown in the mineral medium containing 200 μg of PABA/cm^3.

TABLE 1. Content of free folates (microbiological method) determined in biomass of *S. cerevisiae* CEN.PK and 2200 yeast from the mineral medium containing PABA (mean±SD).

PABA (μg/cm^3)	Free folates (μg/100 g d.m.)	
	CEN.PK	2200
0	152±7^{a*}	122±16^{A*}
0.02	200±18^{b}	139±41^{A}
1	201± 37^{b}	181±41^{B}
5	264±49^{c}	158±37^{A}
10	214± 8^{b}	154±37^{A}
25	208±25^{b}	143±20^{A}
50	238±12^{c}	128±27^{A}
100	121±28^{a}	124±33^{A}
200	118±34^{a}	115±13^{A}

* The same letter index denotes a lack of significant difference.

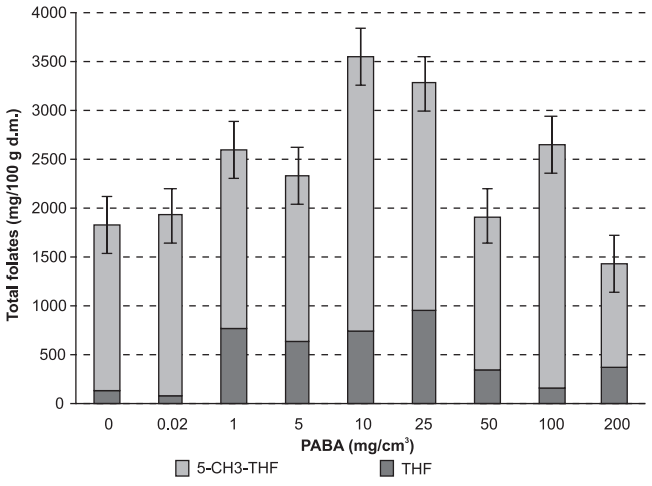

FIGURE 2. Content of total folates (THF and 5-CH_3-THF) in biomass of *S. cerevisiae* 2200 yeast obtained from 24-h culture in the mineral medium with PABA addition.
I – NIR = 562, (acc. to Tukey, one-way analysis of variance, α = 0.05).

DISCUSSION

The mean content of total folates in yeast biomass obtained from the control mineral medium reached 1464 μg/100 g d.m. for the CEN.PK strain and 1827 μg/100 g d.m. for the 2200 strain, respectively. These results are, however, remarkably lower compared with the findings of Hjortmo *et al.* [2005]. According to those authors, different strains of *Saccharomyces cerevisiae* cultured in a medium with a composition similar to that of the mineral medium contained 4000 to over 14,000 μg of total folates/100 g d.m. Explicit elucidation of such high differences is difficult. This might have resulted from the fact that those authors applied conjugase of a different origin and an additional enzyme, *i.e.* proteinase. The fact that the study was carried out on different yeast strains is also of significance to the experimental results obtained.

Comparability of the results of folate determination is disputable. In 2005, over twenty laboratories from various countries, which carry out routine assays of folate content, determined concentrations of folates in identical test-food products [Puwastien *et al.*, 2005]. Although an identical method of extraction and analysis (HPLC) was used, the differences in results reached over 200% in some cases. The authors therefore postulated an urgent need for elaborating the reference materials.

In analysing the results obtained herein, significant differences were observed in the total folate content of the biomass obtained from control media and experimental ones (containing *p*-aminobenzoic acid). Significant changes have been obtained especially in the biomass obtained from experimental media that contained 1, 5, 10, and 25 μg of PABA/cm^3. The application of these doses of PABA to the mineral medium resulted in greater accumulation of total folates

in the cell biomass of both CEN.PK and 2200 yeast. Worthy of notice is the fact that application of the highest PABA dose significantly diminished the capacity of both strains for accumulating folates.

p-Aminobenzoic acid addition to medium did not affect the type of folate forms produced. The cell biomass of yeast obtained from the control and experimental media was characterised by the presence of two forms of folates, namely THF and 5-CH_3-THF. In each case, the prevalence of methylated tetrahydrofolate was observed, which is consistent with the results reported by Hjortmo *et al.* [2005]. According to Seyoum & Selhub [1998], approximately 77.4% of the quantity of folates determined in baker's yeast was constituted by the methylated forms, whereas 19.8% was constituted by the THF form. Those authors estimate that predominance of some of the forms of folic acid derivates in the biomass may be determined by the genus of yeast, because none of the methylated forms was found to prevail in the group of "nonbaker's" yeast. Some strains showed a high level of THF whereas others were rich in 5-CH_3-THF.

The folates determined with the biological method without previous treatment of the examined sample with conjugase are the mono- and diglutamic forms [Goli & Vanderslice, 1992], which is the result of a dependency between the growth of test bacteria *Lactobacillus rhamnosus* used in the assay and the length of glutamine chains of folates investigated. Application of the HPLC method requires the use of conjugase, and the assayed folates constitute the total of free folates and polyglutamic forms. Comparing the contents of free folates and total folates in the biomass of both yeast strains obtained in the experimental media, it may be observed that the biomass is richer in the polyglutamic forms. Depending on the PABA concentration, the content of free folates as compared to that of total folates ranged from 8 to 10% for CEN.PK strain and from 5 to 8% for 2200 strain. These results correspond with the findings reported by other authors [Hjortmo *et al.*, 2005].

CONCLUSIONS

The results obtained in this study point to the possibility of practically using the capacity of yeast biomass for the enhanced biosynthesis of folates. It may be applied in the bakery industry as a starter inoculum or in any other method to increase the content of folates in bakery products. It should be emphasized that baker's yeast synthesize folates during the rising of bread dough [Jägerstad *et al.*, 2005]. Yeast, with an increased content of folates, could be the basis for the so-called "nutritional yeast" in the form of a supplement or feed additive.

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