POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2006, Vol. 15/56, No 4, pp. 445–452

EVALUATION OF THE EFFECT OF CARBAMYLPHOSPHATE SYNTHETASE AND UREASE ACTIVITIES IN SELECTED STRAINS OF WINE YEAST ON THE PRODUCTION OF ETHYL CARBAMATE

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Key words: ethyl carbamate, urease, carbamylphosphate synthetase, *Saccharomyces cerevisiae* yeast

The study was aimed at evaluating the enzymatic capacity of selected strains of wine yeast *S. cerevisiae* for the production of carbamylphosphate synthetase (EC 6.3.4.16) and urease (EC 3.5.1.5) as well as their effect on the production of ethyl carbamate in plum mashes.

The experimental material were strains of wine yeast: *Syrena, Tokay, Burgund, Bordeaux, Steinberg,* originating from the Pure Cultures Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź, and *Saccharomyces bayanus* yeast by Prochimica Varese SRL company (Italy). Distillery fruit mashes were prepared from plums var. Węgierka łowicka.

Under conditions of alcoholic fermentation, the highest activities of NH_4^+-dependent carbamylphosphate synthetase (45.43×10^{-3} U/mg protein) and urease (0.57 U/mg protein) were observed for the strain *Steinberg*. Irrespective of the activity (3.2–92.95 U/mg protein) of carbamylphosphate synthetase in the yeast strains examined, the concentration of ethyl carbamate in after-fermentation liquids was at a similar level (<0.01 mg/L).

Under conditions of a limited access of oxygen, the maximum activity of urease in *S. bayanus* yeasts (1.66 U/mg protein), was observed at the stage of preliminary fermentation (yeasts in the stationary phase of growth). Culture media of the *Steinberg* strain were found to demonstrate a relatively high degree of urea reduction under anaerobic conditions – 19% and a trace concentration of urethane (<0.01 mg/L).

A low urolytic activity (0.192 U/mg protein) of yeasts of the *Tokay* strain was reflected in a relatively high concentration of urethane (0.210 mg/L of 40% spirit) in plum spirit obtained with their participation.

Fermentation of plum mash with *S. bayanus* yeast applied at a dose of 0.5 g d.m./kg, resulted in a decrease in the concentration of ethyl carbamate by 44% (0.07 mg/L of 40% spirit), as compared to the dose of 0.1–0.3 g d.m./kg.

INTRODUCTION

Ethyl carbamate (EC), referred to as urethane, is a natural component of fermented foodstuffs: yoghurts, cheese, bread, tea, soybean sauce, and alcoholic beverages: beer, sake and wine. A source of urethane are bacteria participating in the production process of fermented foodstuffs [Ough, 1976; Mildau *et al*., 1987].

Urethane reaches a human body through the ingestion of alcoholic beverages and fermented food products. The intake of one liter of an alcoholic beverage with EC content of 7 mg in the case of a person weighing ca. 70 kg leads to the assimilation of 0.1 mg of EC per kg of body mass. In humans, an excessive dose of ethyl carbamate may cause damage to kidneys and liver as well as induce vomiting, coma and hemorrhages. In men, it is also likely to evoke potency disorders [Stoewsand *et al.*, 1991].

According to Canadian and German standards, the permissible content of ethyl carbamate is: 0.03 mg/L for white and red wines, and 0.4 mg/L for fruit vodkas and liquors [Christoph *et al*., 1987].

One of the major precursors of ethyl carbamate is urea which is produced as a result of catabolytic degradation of arginine – one of the dominating amino acids, occurring in grape juice, during metabolic transformations of *Saccharomyces cerevisiae* yeast. Ethanol produced during wine fermentation reacts with urea originating from fermenting grapes, which leads to the production of ethyl carbamate [Sumarada & Cooper, 1984].

Yeast of the species *Saccharomyces cerevisiae*, applied in the fermentation of fruit mashes, contain enzymes that may, to some extent, affect the content of urethanes. The first of them is urease (EC 3.5.1.5), *i.e.* urea amidohydrolase, whose activity is determined by Ni(II) ions. It catalyses the reaction of urea hydrolysis to CO_2 and two molecules of NH_3. The urolytic activity of wine yeast strains *Saccharomyces cerevisiae* is not sufficient for a complete hydrolysis of urea. An excess of that compound, accumulated in yeast cells, is released to the environment where it reacts with ethanol, which results in the production of ethyl carbamate. In order to reduce the quantity of the accumulating urea, and thus to decrease the level of urethane in wine, pitchings are enriched with "acid" urease isolated from *Lactobacillus fermentum* bacteria or *Schizosaccharomyces pombe* yeasts containing a mutated gene encoding that enzyme [Fujinawa, 1990; Pigeau, 2000].

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The most effective activity of urease in wine occurs at pH 3–4. That pH range is favourable for the functioning of bacterial urease, whereas urease originating from *Schizosaccharomyces pombe* yeasts exhibits activity at neutral pH. Hence, the application of yeast urease for urea reduction in wines is possible after the modification of the gene of that enzyme.

The second enzyme likely to affect the production of urethane is carbamylphosphate synthetase (EC 6.3.4.16). It catalyzes the synthesis reaction of carbamylphosphate which, in the urea cycle, reacts with L-ornithine and produces L-citrulline. Ough *et al.* [1988] claim that EC may be produced in the process of alcoholic fermentation once the reaction is disturbed. A competent reaction would be the production of carbamate by the attachment of an ethyl group to carbamylphosphate which loses a phosphate group and does not detach the carbonyl group, as in the case of L-citrulline production.

In eukaryotic organisms, compared to the prokaryotic ones, the synthesis of carbamylphosphate can proceed with the participation of two synthetases. One of them is specific to the arginine pathway (CPS-A), whereas the other – to the pyrimidine pathway (CPS-P). Both these enzymes are regulated independently by two specific genes. Carbamylphosphate synthetase, catalyzing the synthesis reaction of carbamylphosphate necessary for the production of citrulline in the urea cycle, is located in a mitochondrial matrix, whereas that participating in the synthesis of pyrimidines occurs in the cytosol. In cytosolic synthesis a nitrogen donor is glutamine, whereas in the mitochondrial synthesis – NH_4^+ ions. In *Saccharomyces cerevisiae* yeast, the CPS-A is built of two subunits: a small one encoded by CPA1 gene (45 kDa) and a big one encoded by CPA2 (124 kDa), and is located in the cytosol, unlike in the other eukaryotes [Davis, 1986].

The study was aimed at evaluating the enzymatic capacity of selected strains of wine yeast for the production of carbamylphosphate synthetase (EC 6.3.4.16) and urease (EC 3.5.1.5) as well as their effect on the production of ethyl carbamate in plum mashes.

MATERIAL AND METHODS

Yeasts and their culture conditions. Experiments were carried out with the following strains of wine yeast *(Saccharomyces cerevisiae)*: *Syrena, Tokay, Burgund, Bordeaux, Steinberg,* originating from the Pure Culture Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź, and *Saccharomyces bayanus* yeast by the Prochimica Varese SRL company (Italy). Yeast strains were stored on YPG slants at a temperature of 4°C.

Activation of yeast was carried out after they have been re-inoculated from the slant to 30 mL of a liquid YPG medium, within 24-h static cultures. In the next stage, inoculum was prepared from 48-h shaken cultures of yeast in respective model culture media. The multiplied yeasts (5% vol.) were used for the inoculation of the model culture media (250 mL).

To determine the activity of yeast urease under aerobic conditions, the propagation was carried out in medium according to Ghasemi *et al*. [2004], that contained (per 1 L of a solution): 1.3 g of urea, 20 g of glucose, 0.5 g of $MgSO_4 \times 7H_2O$, 0.348 g of K_2HPO_4, 13.3 g of KH_2PO_4, 0.3 g of $CaCl_2$, and 0.032 g of $NiSO_4 \times 7H_2O$. To assay the activity of carbamylphosphate synthetases, under analogous conditions, use was made of a modified medium according to Serre *et al*. [2004], containing (per 1 L of a solution): 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 1 g of L-glutamine, 5 g of Peptobak, 8 g of glucose, 0.5 mg of $ZnSO_4 \times 7H_2O$, 0.1 mmol/L of $CaCl_2$, 1 mmol/L of $MgSO_4 \times 7H_2O$, and 10 mg of tryptophan. Media pH accounted for 4.8 units. The shaken cultures were run at a temperature of 28–30°C until reaching the stationary phase of growth.

Under anaerobic conditions, the cultures were run in a modified culture medium according to Romano [1976], containing (per 1 L of a solution): 80.0 g of glucose, 2.0 g of KH_2PO_4, 0.25 g of $CaCl_2$, 0.25 g of $MgSO_4 \times 7H_2O$, 1.0 mg of $ZnSO_4 \times 7H_2O$, 0.5 mg of $FeCl_3 \times 6H_2O$, 0.1 mg of KJ, and 0.1 mg of $CuSO_4 \times 5H_2O$, supplemented either with urea (1.3 g/L) to determine urease activity or with glutamine (0.5 g/L) and NH_4Cl (0.5 g/L) to determine the activity of carbamylphosphate synthetase; pH of the solution was adjusted with 0.5 mol/L citric acid up to the value of 3.8. Sterile medium was supplemented with the following doses of vitamins (per 1 L): 25 μg of biotin, 300 μg of thiamine, 300 μg of niacin, 300 μg of Ca-pantothenate, 300 μg of HCl-pyridoxine, and 25 mg of mesoinositol. The vitamin complex was added in the form of a concentrated solution (filtration – Milex®-GV with pore size of 0.22 μm). Under conditions of reduced oxygen access, the cultures were run at a temperature of 28–30°C, for 72 h.

Determination of biomass growth. Biomass growth was determined with the spectrophotometric method, by measuring turbidity at a wave length of $\lambda = 540$ nm, as compared to the control sample (culture medium). The results obtained were converted into g d.m./L with the use of a previously plotted standard curve depicting the relationship $A_{540} = f$ (g d.m./L).

Preparation of a cell-free extract to assay the activity of selected enzymes. Biomass obtained from cultures under aerobic and anaerobic conditions was centrifuged (3000 × g, 30 min) at a temperature of 4°C, and then rinsed with a phosphate buffer with pH 7.0 in the case of determining urease activity or a buffer with pH 7.5 (50 mmol/L Tris-HCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol) in the case of determining the activity of carbamylphosphate synthetase. Next, the cells were suspended in a respective buffer (at buffer to biomass ratio of 2:1, v/v). Yeast were subjected to disintegration with sterile sand (ø=0.45–0.50 mm) in ice until reaching a 99% degree of destruction (microscopic evaluation). The crude extract was centrifuged (12000 × g, 30 min) at a temperature of 4°C to remove sand and cell remnants.

Total protein. Protein content of the cell-free extracts obtained was determined colorimetrically according to Lowry [1951], against a standard curve plotted for crystalline albumin of bovine serum. The essence of the assay is colorimetric determination of a colour product of Folin reagent reaction with a copper-protein complex.

Determination of the activity of carbamylphosphate synthetase. The principle of determination consists in the

conversion of sodium bicarbonate with activated carbon [C^{14}] with the use of NH_4^+ ions (originating from NH_4Cl or glutamine) and ATP to carbamylphosphate which – upon the activity of hydroxylamine – is hydrolysed to hydroxyurea [Abdelal *et al*., 1975].

A 0.5 mL portion of the reaction mixture containing: 100 mmol/L of triethanolamine buffer with pH 8.0, 100 mmol/L of KCl, 12 mmol/L of ATP, 16 mmol/L of $MgCl_2$, 10 mmol/L of $NaH^{14}CO_3$ (0.1 μCi/μmol), 10 mmol/L of glutamine or 100 mmol/L of NH_4Cl, and the examined cell-free extract, was incubated at a temperature of 37°C for 15 min. The reaction was stopped by adding 50 μL of 1.2 mol/L hydroxylamine hydrochloride. Next, the sample was heated for 10 min in a boiling water bath. After cooling, 0.1 mL of 60% trichloroacetic acid was added and the sample was mixed for 10 min to remove released $^{14}CO_2$. A 0.5 mL portion of the sample was transferred to a test-tube containing 7 mL of a scintillating mixture Insta-Gel Plus (Canberra-Packarol) and its radioactivity was measured using a LKB Wallac 1219 measuring instrument by Rackbet. The control sample contained the denaturated enzyme.

The activity that catalysed the synthesis of 1 μmole of carbamylphosphate at a temperature of 37°C within 1 h was accepted as a unit of carbamylphosphate urease activity. The values of activity obtained were referred to protein content of the sample examined.

The activity of carbamylphosphate **(N)**, expressed in U/mg of protein, was calculated according to the following scheme:

$$A=\frac{T}{E} \quad [dpm]$$

where: *A* – radioactivity *(dpm-desintegration per minute)*, *T* – the number of impulses read out from the measuring instrument *(cpm-counts per minute)*, and *E* – coefficient determined for the sample without $NaHCO_3$ = 0.961.

$$A'=\frac{A}{2.2\times10^6} \ [\mu Ci] \qquad \mu Ci=2.2\times10^6 \text{ dpm}$$

(μCi – *micro curie)*

$$n=\frac{A'}{a} \ [\mu mol]$$

$$n=k\cdot t\cdot N$$

$$N=\frac{n}{k\cdot t} \ [U]$$

where: *a* ($NaH^{14}CO_3$) – specific radioactivity = 0.1 μCi/ μmol, *n* – the quantity of carbamylphosphate produced in the reaction (μmol), *k* – enzymatic reaction rate constant (μmol/h × unit), *t* – time of the enzymatic reaction (h), and N – the number of enzyme units (units).

The activity values obtained were referred to protein content of the samples examined: N=U/mg of protein.

Determination of urease activity. The urolytic activity was assayed with the method of Weatherburn [Ghasemi *et al*., 2004] that involves: the hydrolysis reaction of urea under the influence of urease present in the cell-free extract analysed, the production of a colour compound of ammonia with the reaction mixture sodium phenol-nitroprussiate in the presence of a basic solution of sodium hypochlorite, and next a colorimetric comparison of the colour of the sample examined with that of standard solutions with known concentrations of ammonia (prepared from $(NH_4)_2SO_4$), at a light wave length of λ=630nm.

A 0.2-mL portion of a cell-free yeast extract was added to the reaction mixture prepared in 100 mmol/L phosphate buffer, pH 8, with the addition of urea (50 mmol/L). So prepared sample was incubated for 30 min at 37°C. After the incubation, a 0.2-mL portion of the mixture was collected and 2 mL of Na_2HPO_4 and a mixture of phenol with nitroprussiate were added, the incubation was run once again for 30 min at a room temperature. The control sample contained the denaturated enzyme.

The activity leading to the release of 1 μmole of NH_3 within 1 h, under determination conditions, was accepted as a unit of urease activity. The activity values obtained were referred to protein content of the sample examined.

Fruit mashes. Mashes were prepared from plums var. Węgierka łowicka, whose chemical composition was assayed according to standards binding in the fruit-vegetable industry [PN-90/A-75101] (Table 1).

TABLE 1. Chemical composition of plums.

Specification	Unit	Content
Dry matter	(g/100 g)	13.60±0.20
Extract	(°Blg)	12.20±0.20
Acidity	(g of malic acid/100 g)	0.90±0.05
Reducing sugars	(g of glucose/100 g)	6.38±0.20
Total sugars	(g of invertase/100 g)	7.16±0.30
Ash	(g d.m./100 g)	2.59±0.05
Protein	(g d.m./100 g)	2.64±0.03
pH	-	3.70±0.01

To prepare mashes for fermentation, defrosted and stone-deprived plums were comminuted with skin. Comminuted stones in the amount of 5% of their total mass and nitrogen medium $(NH_4)_2HPO_4$ at a dose of 0.2 g/kg were added to fruit pulp.

Dried wine yeast *Saccharomyces bayanus* were rehydrated before being added to mashes. The dose of inoculum reached 0.1–0.5 g/kg of the pulp.

To prepare inoculum, pure cultures of yeasts from agar YPG slant were re-inoculated under sterile conditions to 30 mL of malt wort with density of 10°Blg and pH 5. The preliminary culture was run in a thermostat, at a temperature of 30°C for 24 h. The second stage of the culture proceeded in 0.5-L flasks, each containing 150 mL of medium, pH 5. Those cultures were run for 48 h at a temperature of 28–30°C. Multiplied yeasts, in the form of cream, were added at a dose of 0.3 g d.m./kg of fruit pulp.

Fermentation of the mashes (*ca.* 3 kg) was carried out in flat-bottomed flasks with a volume of 4 L, covered with fer-

mentation tubes, at a temperature of 28–30°C, with occasional stirring and measurement of carbon dioxide loss as an index of fermentation dynamics. The process was continued until the mass of flasks measured in few-hour intervals was not subject to changes.

The assay of fermentation indices was carried out according to methods accepted in the distilling industry. The concentration of urea in the after-culture liquids and in attenuated mashes was determined with the use of enzymatic tests by R-Biopharm.

In order to separate ethanol from the attenuated mashes, distillations were run and the raw spirits with the proof of 14.5–27.5% vol. were subject to concentration in a distillery apparatus equipped with a birectifier, up to the proof of 40-45% vol.

Determination of ethyl carbamate content. The content of ethyl carbamate in after-culture liquids and raw spirits was determined with the method of gas chromatography coupled with mass spectrometry [Conacher *et al*., 1987]. A sample of the after-culture liquid (after having separated yeast biomass) was collected in a dose of 50 mL, whereas a sample of the spirit examined was collected in the amount providing ethanol concentration not higher than 10% vol. after filling up to the volume of 50 mL. Next, double extraction was carried out with 75 mL of methylene chloride. The extract obtained was filtered through a filter paper Watman 1 and dried by passing through a glass column filled with 40 g of anhydrous Na_2SO_4 (filling height 0.2 m; diameter 0.015 m). Next, 5 mL of ethyl acetate were added to the extract and concentrated in an evaporator at a temperature of 28°C, to a volume of *ca*. 2 mL. The residue after evaporation was transferred quantitatively into a test-tube by three-fold rinsing the evaporator's flask with 1 mL of ethyl acetate, and filled up to the volume of 5 mL. The ethyl carbamate extract in ethyl acetate was injected in 2-μL portions onto a chromatographic column. Use was made of a 6890N gas chromatograph (Agilent) with a flame-ionization detector (FID) and SSL injector.

Separation of components was carried out with the use of a capillary column filled with a stationary phase DB-WAX 123–7032 with the length of 30 m, internal diameter of 0.32 mm and film thickness of 0.50 μm.

Conditions of the GC analysis were as follows: temperature program -50°C (25 min) > 5°C / min > 250°C (10 min); injector temperature -260°C; detector temperature -250°C; flow rate of carrier gas (helium) – 1 mL/min, splitless.

Identity of ethyl carbamate was confirmed based on the presence of m/z 62, 74 ad 89 ions, using a GC 8000 gas chromatograph (Fissons) coupled with an MD 800 mass spectrometer. The capillary column and analytical conditions were as mentioned above, whereas energy of ionization reached 70 eV. The concentration of ethyl carbamate in the samples examined was determined based on a calibration curve.

Mathematical methods. Standard deviation, being a measure of an average uncertainty of measurements, and Pearson's coefficient of correlation (r), determining an analysis of correlations between results obtained, were calculated with the use of Origin 6.0. software.

RESULTS AND DISCUSSION

A yeast enzyme participating in the synthesis of carbamylphosphate – a precursor of urethane – is carbamylphosphate synthetase (CPS-A). A major donor of nitrogen in the synthesis of carbamylphosphate, taking part in the urea cycle, are NH_4^+ ions, the source of which can be ammonium salts: NH_4Cl, $(NH_4)_2SO_2$, $(NH_4)_2HPO_4$ and others. In contrast, carbamylphosphate participating in the synthesis of pyrimidines is produced in the presence of carbamylphosphate synthetase (CPS-P) that utilizes nitrogen from glutamine. In *Saccharomyces cerevisiae* yeast both the synthetases are located in cytosol [Davis, 1986]. It can be supposed, therefore, that under conditions of alcoholic fermentation, both the carbamylphosphate produced with NH_4^+-dependent and glutamine-dependent synthetase may be subject to esterification with ethanol.

Characteristics of the activity of those enzymes in selected strains of wine yeasts, depending on the culture conditions and growth phase, was presented in Tables 2 and 3. The highest activity of NH_4^+-dependent synthetase, ranging from 39.39×10^{-3} U/mg protein (aerobic conditions) to 45.43×10^{-3} U/mg protein (anaerobic conditions), was found for yeasts of *Steinberg* strain. In the case of the glutamine-dependent carbamylphosphate synthetase, its highest activity was reported for the strains: *Steinberg* (28.48×10^{-3} U/mg protein) and *Tokay* (29.28×10^{-3} U/mg protein) – under aerobic conditions as well as for *S. bayanus* (92.95×10^{-3} U/mg protein) under the anaerobic ones. The lowest activity of both synthetases, irrespective of culture conditions, was demonstrated by *Bordeaux* strain, *i.e.* $(3.20\text{-}5.52) \times 10^{-3}$ U/mg protein.

TABLE 2. Activity of carbamylphosphate synthetase in selected strains of wine yeasts.

Yest strain	Aerobic conditions		Anaerobic conditions		EC (mg/L)
	Activity of carbamylphosphate synthetase A×10^{-3} ($^{(1)}$U/mg protein)				
	NH_4^+-dependent	Glutamine-dependent	NH_4^+-dependent	Glutamine-dependent	
Bordeaux	5.52±0.25	2.48±0.15	3. 20±0.17	4.12±0.21	< 0.01
Burgund	8.43±0.28	9.03±0.29	7.18±0.24	4.78±0.23	<<0.01
Steinberg	39.39±1.50	28.48±1.20	45.43±2.25	17.78±0.52	< 0.01
Syrena	14.35±0.32	26.15±1.11	10.3±0.35	15.75±0.49	< 0.01
Tokay	15.13±0.45	29.28±1.20	14.25±0.31	34.3±1.50	< 0.01
S. bayanus	21.44±1.08	67.93±2.90	21.17±0.89	92.95±4.20	< 0.01

$^{(1)}$U=(μmole of carbamylphosphate/h) (yeast from the stationary phase of growth)

TABLE 3. Changes in the activity of carbamylphosphate synthetase in wine yeast *Saccharomyces bayanus.*

Culture period (h)	Aerobic conditions		Anaerobic conditions		
	Activity of carbamylphosphate synthetase A×10^{-3} ([1]U/mg protein)				EC (mg/L)
	NH_4^+-dependent	Glutamine-dependent	NH_4^+-dependent	Glutamine-dependent	
24	20.33±0.98	55.33±2.30	25.31±1.10	92.95±4.21	0.00
48	21.44±1.08	67.93±2.90	21.17±0.89	92.48±4.20	<<0.01
72	19.79±0.71	61.12±2.60	12.53±0.40	33.61±1.50	<0.01

[1]U=(μmole of carbamylphosphate/h)

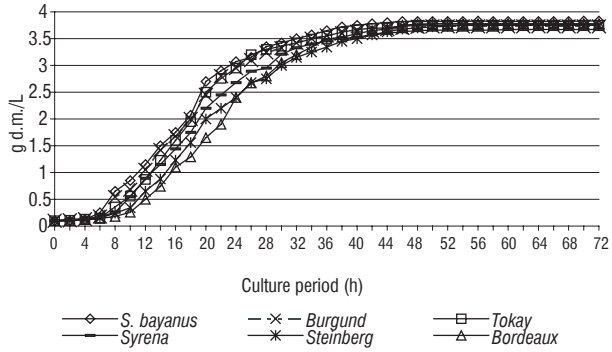

FIGURE 1. Growth curves of wine yeast under aerobic conditions.

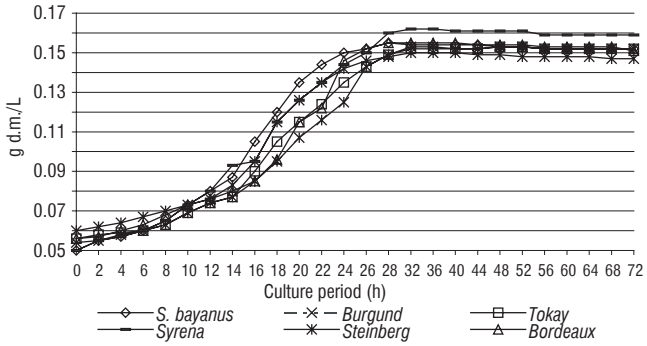

FIGURE 2. Growth curves of wine yeast under anaerobic conditions.

The evaluation of the enzymatic capacity of *S. bayanus* yeast in different growth phases demonstrated that under aerobic conditions the highest activity of NH_4^+-dependent synthetase (21.44 × 10^{-3} U/mg protein) and glutamine-dependent synthetase (67.93 × 10^{-3} U/mg protein) was demonstrated by yeast in the early stationary phase of growth (Table 3, Figure 1). Analogously, under conditions of alcoholic fermentation, the activity of synthetase, both the NH_4^+ and glutamine-dependent one, was the highest after the first 24 h of fermentation (in the early stationary phase) and accounted for 25.31 × 10^{-3} and 92.95 × 10^{-3} U/mg protein, respectively (Table 3, Figure 2).

Irrespective of the activities of carbamylphosphate synthetases (3.2–92.95 U/mg protein) in the examined strains of yeast, the concentration of ethyl carbamate in the after-culture liquids was at a similar level (<0.01 mg/L).

Literature provides scant reports on the activity of carbamylphosphate synthetases in wine yeast strains and their effect on the production of ethyl carbamate. Christoph *et al*. [1987] and Ough [1988] postulated that a precursor of urethane might be carbamylphosphate – produced by some strains of yeasts and moulds from ammonia ions at the presence of ATP – which under conditions of alcoholic fermentation reacts with ethanol, thus producing ethyl carbamate. Still, the authors have not carried out any extensive studies in that area.

Activity of urease

Amongst the investigated yeast strains, the highest activity of urease under aerobic conditions, *i.e.* 1.228 U/mg protein, was reported for the strain *Steinberg,* whereas the lowest one, reaching from 0.400 to 0.470 U/mg protein, for *Tokay, Syrena* and *Burgund*, respectively.

Under conditions of alcoholic fermentation, the activity of that enzyme in yeasts of *Steinberg* strain was subject to a considerable decrease, down to the value of 0.569 U/mg protein. However, culture media of that strain were characterized by the greatest decline in the concentration of urea, namely 27% under aerobic conditions and 19% under conditions of a limited access to oxygen. In addition, the liquid after the culture of those yeasts under fermentation conditions contained the lowest concentration of urethane (<0.01 mg/L) (Table 4).

Analogous cultures were run, under aerobic conditions

TABLE 4. Activity of urease in selected strains of wine yeast.

Yeast strain	Aerobic conditions		Anaerobic conditions		
	Urease activity ([2]U/mg protein)	Urea (g/L)	Urease activity ([2]U/mg protein)	Urea (g/L)	EC (mg/L)
Bordeaux	0.756 ± 0.014	1.05±0.01	0.420 ± 0.008	1.12±0.02	0.02
Burgund	0.470 ± 0.008	1.20±0.04	0.301± 0.005	1.15±0.03	0.02
Steinberg	1.228 ± 0.021	0.95±0.02	0.569 ± 0.012	1.05±0.01	<0.01*
Syrena	0.428 ± 0.009	1.15±0.03	0.367 ± 0.006	1.12±0.02	0.02
Tokay	0.400 ± 0.008	1.15±0.04	0.192 ± 0.004	1.25±0.04	0.05

[2]U=(μmole of NH_3/h) (yeast from the stationary phase of growth); * the concentration of 0.01 was used to calculate Pearson's correlation coefficient (r)

TABLE 5. Changes in the urolytic activity in wine yeast *Saccharomyces bayanus*.

Culture period (h)	Aerobic conditions		Anaerobic conditions		
	Urease activity ($^{(2)}$U/mg protein)	Urea (g/L)	Urease activity ($^{(2)}$U/mg protein)	Urea (g/L)	EC (mg/L)
24	0.834 ± 0.018	1.05±0.01	1.660 ± 0.035	1.12±0.02	0.00
48	2.431 ± 0.033	0.97±0.005	0.920± 0.027	1.05±0.01	<0.01*
72	0.675 ± 0.012	0.95±0.005	0.402 ± 0.009	1.03±0.01	0.02

$^{(2)}$U=(μmole of NH_3/h) (yeast from the stationary phase of growth); * the concentration of 0.01 was used to calculate Pearson's correlation coefficient (r)

and those of a limited access to oxygen, for *Saccharomyces bayanus* yeasts (Table 5). In the course of the process, the activity of urease was examined in different phases of growth, which enabled finding out that under aerobic conditions its highest activity (2.431 U/mg protein) was demonstrated by the yeast in the early stationary phase (48 h) (Table 5, Figure 1). Under conditions of alcoholic fermentation, the maximum urolytic activity (1.66 U/mg protein) occurred at the stage of preliminary fermentation (yeasts in the stationary phase) (Table 5). Hence, it can be concluded that the increasing concentration of ethanol inhibited the activity of urease.

A correlation between the concentration of ethyl carbamate produced in fermentation media (y) and activity of urease (x) (Table 4) was described by a linear function y=f(x), in the form of: y=0.0059−0.0093x. The Pearson's correlation coefficient reached r=−0.8618. Its absolute value indicates a statistically significant correlation, whereas the negative sign of correlation indicates that with an increase in the activity of urease a decrease in urethane concentration can be expected.

It should be emphasized, however, that the examined strains of yeast were characterized by a low urolytic activity. The results obtained confirm the thesis advanced by Pigeau [2000] that urease activity of wine yeast *Saccharomyces cerevisiae* impairs the complete hydrolysis of urea.

Ethyl carbamate in the plum spirits obtained

The chromatographic analysis of raw spirits, obtained from fermented plum mashes, indicated differences in the contents of ethyl carbamate depending on the strain of yeast applied in the process.

In the case of *Tokay* yeast, exhibiting urolytic activity under fermentation conditions at a level of 0.192 U/mg protein (Table 4), the concentration of ethyl carbamate in plum distillate obtained with them reached 0.210 mg/L of 40% spirit (Table 6).

The application of yeast of the *Steinberg* strain, that demonstrated over twofold higher urolytic activity under anaerobic conditions, *i.e.* 0.569 U/mg protein (Table 5), resulted in a decrease in urethane content to 0.040 mg EC/L of 40% spirit. Simultaneously, the fermented mash, the spirit was made of, was characterized by the lowest concentration of urea (0.008 g /L).

Statistical methods applied for the description of relationships between the activity of yeast enzymes and the quantity of urethane produced, under experimental conditions, failed to demonstrate any significant correlation between the concentration of ethyl carbamate in the sample examined (Table 6) and the activity of carbamylphosphate synthetase (Table 2) (Pearson's coefficient r=-0.3188).

A correlation between the concentration of ethyl carbamate in plum spirits (y) (Table 6) and the activity of urease (x) (Table 4) was described with a linear function, in the form of: y=0.2390−0.3813x, whereas the coefficient of correlation reached r=-0.7713, as compared to that computed for model samples, *i.e.* r=-0.8618. However, both these values are indicative of a statistically significant correlation between the evaluated variables.

TABLE 6. Effect of strains of yeast used for the fermentation process on the content of urea in fermented mashes and that of ethyl carbamate in plum distillates.

Yeast strain*	Urea (g/L of mash)	Ethyl carbamate (mg/L of 40% spirit)
Bordeaux	0.018±0.001	0.110±0.009
Burgund	0.040±0.002	0.087±0.004
Steinberg	0.008±0.0005	0.040±0.002
Syrena	0.028±0.002	0.043±0.003
Tokay	0.020±0.001	0.210±0.014

*inoculum dose – 0.3 g d.m./L

It can be supposed, then, that a reduced synthesis of urethane in fruit mashes is facilitated by the selection of yeast strains characterized by a relatively high urolytic activity.

While determining the above-mentioned correlation, no consideration was given to the results obtained with the contribution of *S. bayanus* yeast, since – despite the highest urolytic activity – plum distillates obtained with those yeast (0.3 g d.m./kg of mash) demonstrated a high content of urethane (0.125 mg/L of 40% spirit) (Tables 4 and 7). It was also observed that after the fermentation of mash with yeast addition of 0.5 g d.m./kg, the content of ethyl carbamate in the spirit reached 0.070 mg/L of 40% spirit and was lower by *c*a. 44% than its concentration in distillates obtained after the fermentation with the lowest doses of yeast – 0.1 and 0.3 g d.m./kg. It can be assumed that increasing yeast inoculum may contribute to the reduction in the amount of urethane produced under fermentation conditions.

Assessment of the results obtained points to significant differences in the contents of ethyl carbamate in plum spirits and in after-fermentation liquids (model media), despite the

TABLE 7. Effect of *S. bayanus* yeast inoculum on the content of urea in fermented mashes and that of ethyl carbamate in plum distillates.

Dose of *S. bayanus* yeast inoculum (g d.m./kg)	Urea (g/L of mash)	Ethyl carbamate (mg/L of 40% spirit)
0.1	0.022±0.001	0.129±0.011
0.3	0.020±0.001	0.125±0.010
0.5	0.005±.0003	0.070±0.003

application of the same yeast strains in the fermentation process. They result from a number of factors, including a low urolytic activity of the strains analysed hindering a complete reduction of urea. In addition fruit mashes, especially those from stone fruits, *e.g.* plums, contain cyanogenic glycosides whose hydrolysis leads to the release of hydrogen cyanide. Under conditions of alcoholic fermentation, that compound may be transformed to ethyl carbamate.

A comparison of the results obtained with those cited by Christoph *et al.* [1987], demonstrates that the concentration of ethyl carbamate in raw plum spirits fluctuated at a low level (0.040–0.210 mg/L of 40% spirit), thus meeting the requirements of German and Canadian standards established for distillates made of stone fruits (0.4 mg/L).

CONCLUSIONS

1. Evaluation of the enzymatic capacity of NH_4^+ and glutamine-dependent carbamylphosphate synthetase of the selected strains of wine yeast indicated that under experimental conditions applied, irrespective of differences between their activities (3.20–92.95 U/mg of protein), the concentration of after-fermentation liquids was at a similar level, lower than 0.01 mg/L.

2. No statistically significant effect of the activity of that enzyme was observed on urethane content of plum distillates.

3. Analyses of the activity of urease point to its contribution to the reduced synthesis of ethyl carbamate; the examined strains of wine yeast were characterized by a low urolytic activity, not sufficient for a complete hydrolysis of urea.

4. Increasing the yeast inoculum in the fermentation culture media is likely to affect a decreased production of urethane.

5. The content of ethyl carbamate in mashes made of stone fruits is determined, to a great extent, by the presence of cyanogenic glycosides.

ACKNOWLEDGEMENTS

The study was financed by the State Committee for Scientific Research (KBN) under a research Project No. 2P06T 007 26.

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Received November 2005. Revision received February and accepted June 2006.

OCENA WPŁYWU AKTYWNOŚCI SYNTETAZY KARBAMYLOFOSFORANU I UREAZY W WYBRANYCH SZCZEPACH DROŻDŻY WINIARSKICH NA TWORZENIE KARBAMINIANU ETYLU

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Celem pracy była ocena uzdolnień enzymatycznych wybranych ras drożdży winiarskich *S. cerevisiae* do tworzenia syntetazy karbamylofosforanu (EC 6.3.4.16) i ureazy (EC 3.5.1.5) oraz ich wpływu na powstawanie karbaminianu etylu w zacierach śliwkowych.

Materiał do badań stanowiły szczepy drożdży winiarskich: *Syrena, Tokay, Burgund, Bordeaux, Steinberg,* pochodzące z Kolekcji Czystych Kultur Instytutu Technologii Fermentacji i Mikrobiologii PŁ oraz drożdże *Saccharomyces bayanus* firmy Prochimica Varese SRL (Włochy). Do sporządzenia gorzelniczych zacierów owocowych wykorzystywano śliwki odmiany Węgierka łowicka.

W toku przeprowadzonych eksperymentów wykazano, że najwyższą aktywnością syntetazy karbamylofosforanu NH_4^+-zależnej (45.43×10^{-3} U/mg białka) i ureazy (0.57 U/mg białka), w warunkach fermentacji alkoholowej, odznaczał się szczep *Steinberg*. Niezależnie od aktywności (3.2–92.95 U/mg białka) syntetaz karbamylofosforanu w badanych szczepach drożdży, stężenie karbaminianu etylu w cieczach pofermentacyjnych było na zbliżonym poziomie (<0.01 mg/L).

W warunkach ograniczonego dostępu tlenu, maksymalna aktywność ureazy w drożdżach *S. bayanus* (1.66 U/mg białka), przypadała na etap zafermentowania (drożdże w fazie stacjonarnej). W podłożach, w których hodowano szczep *Steinberg*, odnotowano stosunkowo wysoki stopień redukcji mocznika w warunkach beztlenowych – 19% oraz śladowe stężenie uretanu (<0.01 mg/L).

Niska aktywność ureolityczna (0.192 U/mg białka) drożdży rasy *Tokay,* znalazła odzwierciedlenie w relatywnie dużym stężeniu uretanu (0.210 mg/L spirytusu 40%) w spirytusie śliwkowym, otrzymanym z ich udziałem.

Fermentacja zacieru śliwkowego drożdżami *S. bayanus*, w ilości 0.5 g s. s. /kg, wpłynęła na obniżenie zawartości karbaminianu etylu o 44% (0.07 mg/L spirytusu 40%) w stosunku do dawki 0.1–0.3 g s. s./kg.