Research article

Effect of nitrogen sources on fermentation process and formation of hydrogen sulfide and ethyl carbamate by wine yeast

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Abstract: *The addition of nitrogen compounds during winemaking is required for the fermentation process to be conducted properly. These compounds are known to be essential to the vinification process, not only because they influence yeast growth but also because they affect the formation of main and by-products. The effect of nitrogen source on in vitro and in situ formation of hydrogen sulfide and ethyl carbamate was studied. Research material comprised two strains of wine yeast: Saccharomyces cerevisiae. In vitro model was carried out in a synthetic defined medium. In situ fermentations were carried out in musts prepared from apple concentration. The process of hydrogen sulfide formation was intensified in nitrogen deficiency. The presence of amino acids in a model substrate resulted in significant changes in the efficiency of formation of both compounds. Yeasts produced more H_2S in the presence of Cys, Phe, Gly, Glu, Ile, Thr, Pro, Leu, Trp, Val and less in the presence Ala, Arg, Asp, His, Ser, Met. The formation of ethyl carbamate was limited by the amino acids, except Arg, Asp and Lys, which during fermentation with Syrena yeasts caused an increase in the efficiency of formation of this compound. The Fermivit V preparation stimulated yeasts to form H_2S. In the presence of this preparation the Syrena yeasts formed more ethyl carbamate while Hefix yeasts formed about 3-fold less of this compound then in the presence diamonium phosphate.*

Keywords: *hydrogen sulphide, ethyl carbamate, wines, yeasts.*

Introduction

Musts used in wine-making are diverse with respect to the content of nitrogen compounds, and generally, the addition of these compounds is required for the fermentation process to be conducted properly. The average content of nitrogen compounds in grapes is 0.7%, while in apples it is 0.35% [1]. An additional problem during grape must fermentation is the fact that proline constitutes 35-65% of the overall grape juice amino acid pool. This amino acid is not

assimilated by yeast under fermentation conditions. Apple musts contain only slight amounts of proline (about 2%), while aspartate, which can constitute up to 60% of the overall amino acids pool is usually the main source of nitrogen [2]. Bely *et al*. [3] stated that a minimum of 140 mg L^{-1} FAN (free amino nitrogen represented by amino acids and ammonium ion) was needed for satisfactory fermentation. Ammonium ion and amino acids are the basic yeast-assimilable nitrogen compounds (YANC). Ammonium ion contained in the medium diminishes the effectiveness of transport of non-preferable nitrogen sources by nitrogen catabolic repression. This repression depends on the action of 3 proteins (GLN3p, URE2p, GAP1p). GLN3p and URE2p are necessary for the transcription of many genes affecting the alternative nitrogen assimilation pathways. GAP1p is general amino acid permease responsible for the transport of amino acids through the cytoplasmic membrane. If ammonium ions is depleted by yeasts, cells take amino acids from the substrate in the amount determined by biosynthetic requirements [4-7]. However, in oxygen-free conditions, yeast cells are incapable of biodegradation of proline. This amino acid can be transported into the cell by general amino acid permease (GAP1p) and specific amino acid permease (PUT4p). Proline is converted into glutathione, however, for this conversion to take place the presence of oxygen is necessary. In principle, all exogenous sources of nitrogen which are transported into the cell can be used to form NH_4^+ or glutamate [7]. Nitrogen availability can also affect many aspects of yeast metabolism, including the formation of volatile and non-volatile compounds [8-9].

Deficiencies in assimilable nitrogen compounds in a medium is the common cause of H_2S overproduction. Formation of H_2S by yeast during wine fermentation is undesirable due to its low odor threshold. The rate of H_2S formation seems to be regulated by cellular demands for sulfur amino acids and maintenance of intracellular nitrogen pools [10].

Another compound which may be formed in fermented foods and beverages is ethyl carbamate (EC). In 2007, the International Agency for Research on Cancer (IARC) reclassified ethyl carbamate recently from possible carcinogenic to humans (group 2B) to probably carcinogenic to humans (group 2A). It was noted that experimental evidence suggests great similarities in the metabolic pathways of the activation of ethyl carbamate in rodents and humans, and the formation of proximate carcinogens that are DNA-reactive and are thought to play a major role in ethyl carbamate-induced carcinogenesis in rodents and probably also in human cells. It is well-known that ethyl carbamate is formed in a reaction of urea with ethanol and for this reason the supplementation of must subjected to fermentation with urea-containing nutrients is prohibited [11]. However, *S. cerevisiae* yeasts are capable of forming urea during the degradation of arginine cathalyzed by arginase encoded by the *CAR1* gene. During this reaction arginine is converted into ornithine, while urea is a by-product [12]. There are currently no harmonized maximum levels for ethyl carbamate in the European

Union. However, some Member States and Third Countries recommend maximum levels for ethyl carbamate in alcoholic beverages. In 1986, Canada was the first country to introduce maximum levels (30µg L^{-1} in wine; 100µg L^{-1} in fortified wine; 150µg L^{-1} in distilled spirits; 200µg L^{-1} in sake; 400µg L^{-1} in fruit brandy). The Czech Republic closely followed the Canadian limits. In France the maximum levels for ethyl carbamate are defined in distilled spirits (150µg L^{-1}) and in fruit brandy (1000µg L^{-1}), and in Germany only in fruit brandy (800µg L^{-1}).

Considering widespread use of ammonium addition and active dry yeasts in vineyards, it is necessary to know the effect that such addition may have on the aroma and safety of the wine. The objective of this study was to provide a characterization of the combined effect of yeast and nitrogen supplementation on the fermentation process and formation of hydrogen sulfide and ethyl carbamate.

Materials and methods

Yeasts strains

The research material consisted two strains of wine yeast: *Saccharomyces cerevisiae* strain Syrena (from the Culture Collection of the Institute of Fermentation Technology and Microbiology ŁOCK 0201) and *Saccharomyces bayanus* isolated from the commercial preparation Hefix®2000 (Erbslöh Geisenheim AG, Germany).

Fermentation conditions

In vitro model investigations were carried out in a synthetic defined medium of the following composition: 150 g L^{-1} glucose, 6 or 0.1 g L^{-1} $(NH_4)_2SO_4$, 2 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $CaCl_2$, 0.25 g L^{-1} Mg SO_4 x $7H_2O$, 1 mg L^{-1} $ZnSO_4$ x $7H_2O$, 0.5 mg L^{-1} $FeCl_3$ x $6H_2O$, 0.1 mg L^{-1} KI, 0.1 mg L^{-1} $CuSO_4$ x $5H_2O$, 25 µg L^{-1} biotin, 300 µg L^{-1} thiamine, 300 µg L^{-1} nicotinic acid, 300 µg L^{-1} calcium pantothenate, 300 µg L^{-1} pyridoxine HCl, 25mg L^{-1}*myo*-inositol. The pH of the medium was brought to 3.6 by means of 0.5M citric acid solution. A culture incubated for 24h in the same medium was used at a rate of 5% (v/v) as an inoculum for the experimental culture. When ammonium ion was depleted, cultivations containing 0.1 g L^{-1} $(NH_4)_2SO_4$ were supplemented with particular amino acids (Ala, Arg, Asp, Cys, Phe, Gly, Glu, His, Ile, Tyr, Ser, Pro, Met, Lys, Leu, Trp and Val) or $(NH_4)_2SO_4$ in an amount corresponding to 10 mM ammonium ions. Fermentations were carried out at 26°C in 5 replicates. Following incubation, the optical absorbance at 600 nm (OD_{600}) was recorded using an CECIL CE2041 spectrofotometer (CECIL Instruments, Cambridge, England). The OD_{600} values were converted to cfu mL^{-1} and dry mass of yeasts using a calibration curves. The cell numbers were fitted to the Gompertz equation using an Excel add-in, DMFit 2.1 (Institute of Ford Research, Norwich, UK):
L(t) = A + C exp{-exp[-B (t – M)]}

The following growth parameters were estimated: maximum specific growth rate μ_{max} = BC/e; lag time t_{Lag} = M - (1/B); and maximum population density $\log(N_{max})$ = A + C.

All the growth curves were drawn using the Gompertz equation in OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA).

In situ fermentations were carried out in 3 L bottles containing 2L of musts prepared from apple juice concentrate. Pitchings of apple wines were prepared from a commercial apple juice concentrate, which was diluted with water to obtain apple must of 10°Bx. Must consumption for pitchings reached 70%. The pitchings were sweetened with sucrose to a level that enabled obtaining wine proof of 12% v/v alcohol. To enrich pitchings with nitrogen, 0.3 g L^{-1} $(NH_4)_2HPO_4$ or 0.4 g L^{-1} of a Fermivit V (Institut Oenologique de Champagne, Epernay, France) preparation was added. A culture incubated for 24h in the same medium was used at a rate of 5% (v/v) as an inoculum for the experimental culture. Fermentations were conducted at 26°C in 5 replicates.

Concentration of ammonium ions

Ammonium nitrogen occurs partly in the form of ammonium ions and partly as molecular ammonia. A pH-dependent equilibrium exists between the two forms. In strong alkaline solutions ammonium nitrogen is present almost entirely as ammonia. This reacts with hypochlorite ions to form monochloramine, which reacts with a substituted phenol to form a blue indophenol. This was determined photometrically. Measurements were made using Spectroquant tests (Merck, Darmstadt, Germany). The method is analogous to DIN 38406, E5, Iso 7150/1, APHA 4500-NH$_3$ D, and EPA 350.1.

Concentration of hydrogen sulfide

Hydrogen sulfide reacts with dimethyl-p-phenylenediamine and iron (III) ions to form methylene blue. This was determined photometrically. Measurements were made using Spectroquant tests (Merck, Darmstadt, Germany). The method is analogous to EPA 376.2, US Standard Method 4500-S^{2-}, and ISO 10530.

Concentration of ethyl carbamate

The sample was subjected to extraction using methylene chloride, sodium sulfate and ethyl acetate. Ethyl carbamate was determined by gas chromatography. A Carlo-Erba Instruments chromatograph with a FID detector with SSL sample injector was used. To separate components, a capillary chromatographic column packed with a Stobilwax stationary phase was used (30 m x 0.32 mm ID, 0.25 µm film). The following conditions were used: temperature 60-250°C; temperature increase 4°C min^{-1}; injector temperature 270°C; detector temperature 260°C; carrier gas flow (nitrogen) 1.2mL min^{-1}; Split 50:1; analysis time 30 min.

Free amino acids

The determination of amino acids of the samples studied was made using ABI 420A automatic analyzer (Applied Biosystems Inc., Foster City, CA, USA) coupled with an ABI 130A liquid chromatograph. This system makes use of pre-column derivatization by means of phenol isothiocyanate in heptane (Edman's reagent) in a medium of diisopropylethylamine supplied in gaseous phase. A set of reagents and derivatization protocol recommended by the apparatus manufacturer were used. Samples of a volume of up to 30 µL were deposited once. Samples of a larger volume (up to 100 µL) were deposited in portions of 30-40 µL each with drying in a stream of warm air. An HPLC analysis was performed by means of a PTC-C18 RP column of dimension 220 x 2.1 mm (Brownlee Columns, ABI) using a flow of 300 µL min^{-1} and standard buffers: buffer A – 50 mM sodium acetate, 3% acetonitrile, pH 5.4; buffer B – 32 mM sodium acetate, 70% acetonitrile, pH 6.1. Calibration was made using Amino Acid Standards H of PIERCE make (Rockford, IL, USA). Chromatograms were recorded at a wavelength setting of 254 nm of the UV detector.

Fermentation products

Fermentation products were analyzed using high-performance liquid chromatography (HPLC) (Finnigan Surveyor HPLC System ThermoScientific, Waltham, MA, USA). HPLC analysis was performed under the following conditions: Aminex HPX87H column (300x7,8mm, Bio-Rad, Hercules, CA, USA); sample 10µL; eluent, 5mM H_2SO_4; flow rate 0.6 mL min^{-1}, column temperature, 60°C; Surveyor Plus RI detector. The fermentation products were identified based on comparison of retention times with those of the standard. The concentration of products in samples were determined based on calibration curve.

Statistical analysis

All fermentations were repeated fivetimes and determinations were made for tree parallel samples. Using OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA) standard deviation was determined and Student's *t* test was made, assuming a significance level of 0.05. The results in the tabulated form are presented as mean±standard deviation (M±SD).

Results and Discussion

Based on the preliminary studies we had found that for the both investigated yeast strains, 1.5mM of NH_4^+ is the optimum dose if conditions of the deficiency of this ion are to be obtained during cultivation. The depletion of ammonium ions had exponential character (Figure 1).
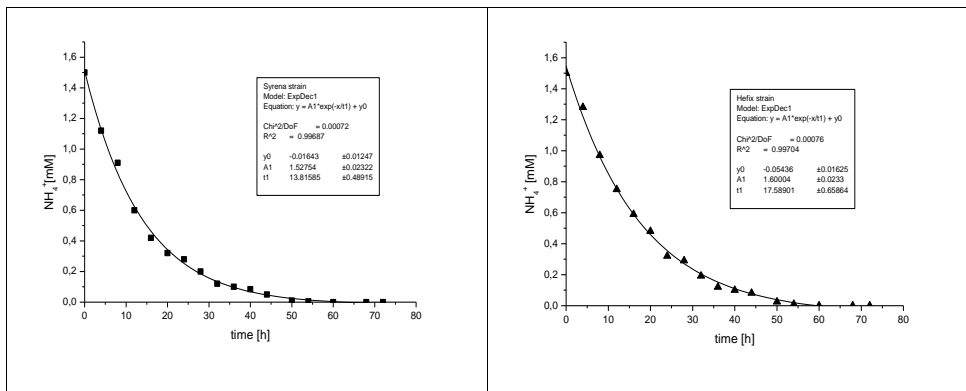
Figure 1. Dynamics of ammonia ion utilization in synthetic defined medium ■
Syrena yeasts; ▲ Hefix yeasts

The ammonium ion was completely utilized after about 60h of fermentation. To exactly determine the effect of NH_4^+ concentration on the parameters of growth of the wine yeasts under study, we analyzed the growth parameters obtained during fermentation in the medium containing 1.5 and 90 mM of NH_4^+. The Gompertz function appropriately describes the growth of the yeasts, which was confirmed by the high values of the R^2 coefficient, ranging from 0.9666 to 0.9988. It was found that the lag phase was longer in a statistically significant manner during fermentation under depletion condition only for the Syrena yeasts. For both populations the nitrogen depletion caused a reduction in the maximum specific growth rate (μ_{max}). The reduction was greater during fermentation with Syrena (6-fold) then Hefix (3.6-fold) yeasts. Also, the final population density was significantly reduced during fermentation in the medium containing 1.5 mM of NH_4^+ (Table 1). Similar effects, resulting probably from disturbances in hexoses transport, had been described by Varela *et al.* [13], Pizarro *et al.* [14] and Vilanova *et al.* [9].

Table 1. Ammonium ions concentration effects on Syrena and Hefix yeasts
growth parameters (mean±SD)

Strain	Initial concentration of NH_4^+ [mM]	μ_{max} [h^{-1}]	t_{Lag} [h]	$\log(N_{max})$
Syrena	1.5	0.0026*±0.0003	22.4*±1.4	7.08*±0.13
	90	0.0155±0.0007	0.3±0.05	7.61±0.11
Hefix	1.5	0.0059*±0.0004	0.5±0.05	7.24*±0.10
	90	0.0212±0.0011	0.5±0.02	7.73±0.23

μ_{max} – growth rate; t_{Lag} – lag time, $\log(N_{max})$ – maximum population density, * – statistically significant differences compared to 90 mM NH_4^+ supplementation at a probability of $p < 0.05$

The dependence between the overproduction of hydrogen sulfide by wine yeasts and the qualitative and quantitative composition of the nitrogen compounds in a fermentation medium has been receiving particular attention recently. Based on our studies we have confirmed that the process of H_2S formation was considerably intensified when there was a deficiency of nitrogen compounds (Tab. 2). Under these conditions the efficiency of H_2S formation increased about 4-fold in the case of Hefix yeasts and over 5-fold for the Syrena strain (in relation to the culture supplemented with ammonium sulfate). At the same time, H_2S efficiency values obtained during fermentation with the Syrena yeasts were considerably higher than those obtained by the Hefix, irrespective of process conditions. While tracing the formation of H_2S by *S. cerevisiae* yeasts, Stratford and Rose [15] also observed about 2-fold increase in the efficiency of formation of this compound in the case of nitrogen deficiency. The authors thought the high productivity of H_2S was caused by the complete depletion of ammonium ions contained in the medium. Similar conclusions were drawn by Henschke and Jiranek [16], Jiranek *et al.* [17] and Ugliano *et al.* [18]. Mendes-Ferreira et al. [10] found that the trigger levels at which an inverse relationship exists between the initial nitrogen present in the medium and total H_2S production varied among yeast strains.

Table 2. Effect of nitrogen source on the hydrogen sulphide and ethyl carbamate formation by Syrena yeasts

Nitrogen source	Efficiency of H_2S [μmol per g dry weight]		Efficiency of EC [μmol per g dry weight]	
Yeast strain	Syrena	Hefix	Syrena	Hefix
$(NH_4)_2SO_4$	0.676±0.030	0.135±0.011	1.008±0.078	1.980±0.120
Ala	0.389*±0.015	0.041*±0.002	0.416*±0.015	0.546*±0.032
Arg	0.201*±0.008	0.019*±0.001	1.702*±0.059	1.785±0.114
Asp	0.292*±0.010	0.060*±0.003	1.545*±0.068	1.669*±0.096
Cys	16.687*±0.798	1.054*±0.042	0.254*±0.007	0.169*±0.010
Phe	1.720*±0.044	0.157±0.008	0.417*±0.017	0.511*±0.026
Gly	2.168*±0.093	0.309*±0.013	0.709*±0.025	0.8688±0.052
Glu	0.928*±0.020	0.146±0.008	0.848*±0.044	0.932*±0.055
His	0.342*±0.016	0.084*±0.003	0.201*±0.009	0.244*±0.011
Ile	0.749*±0.027	0.146±0.007	0.452*±0.010	0.482*±0.030
Tyr	0.595*±0.027	0.233*±0.010	0.147*±0.004	0.09±0.003
Thr	0.980*±0.035	0.313*±0.011	0.665*±0.023	0.35±0.02
Ser	0.615*±0.030	0.103*±0.005	0.310*±0.011	0.19±0.01
Pro	0.971*±0.040	0.180*±0.008	0.329*±0.014	0.12±0.004
Met	0.518*±0.021	0.036*±0.002	0.157*±0.006	0.19±0.01
Lys	1.738*±0.058	0.074*±0.003	1.250*±0.036	0.62±0.03
Leu	1.018*±0.040	0.203*±0.007	0.361*±0.012	0.25±0.01
Trp	1.000*±0.044	0.518*±0.020	0.242*±0.009	0.11±0.004
Val	1.593*±0.065	0.211*±0.010	0.133*±0.004	0.16±0.01
Depletion conditions	3.815*±0.152	0.574*±0.020	0.126*±0.007	0.18±0.01

* – statistically significant differences compared to 10 mM NH_4+ supplementation at a probability of $p < 0.05$

It is supposed that the intensification of H_2S formation during the deficiency of nitrogen results from the inhibition of formation of O-acetylhomoserine. This compound binds adverse sulfide to supplement the intracellular pool of sulfur amino acids. Another cause might be a reduction in the content of metabolites formed from methionine: methionyl-tRNA and S-adenosylomethionine, which inhibit sulfite reductase [16, 19].

In natural media not only NH_4^+ but also nitrogen-containing organic compounds can be used as a source of nitrogen for yeasts. For this reason, we made an attempt to assess the effect of particular amino acids on the H_2S formation by the yeasts. Effect of individual amino acids on the ability to form H_2S depends on yeast strains. We have found that 11 amino acids (Cys, Phe, Gly, Glu, Ile, Thr, Pro, Lys, Leu, Trp, Val) caused an increase in the liberation of H_2S by the Syrena strain. Two of them (Glu, Ile) did not cause changes in the efficiency of H_2S formation by Hefix yeasts and one (Lys) caused the reduction of this parameter. The production of H_2S was higher when the medium was supplemented with lysine during fermentation with Syrena and lower with the Hefix yeast. The use of six amino acids (Ala, Arg, Asp, His, Tyr, Met) allowed the efficiency of formation of H_2S to be reduced during fermentation with both yeasts strains. We observed that the highest increase in the quantity of H_2S was obtained after enriching the medium with cysteine. It is worth noting that in this case also the Syrena yeast showed a stronger response to the presence of cysteine in the medium (over 24-fold increase in the efficiency of H_2S formation). This amino acids can be degraded during the fermentation by cysteine desulfhydrolase, thus becoming a direct source of H_2S. The other sulfur amino acids – Met – is responsible for the inhibition of sulfite reductase, hence the reduction in the amount of H_2S in its presence was observed. Aspartate is utilized by yeasts to form O-acetylhomoserine, the precursor of methionine. Serine, which during our experiment brought about a marked reduction in the intensity of H_2S overproduction, is the precursor of O-acetylserine, a compound causing the repression of SRS enzymes [19-20]. It is interesting to note that the efficiency of H_2S formation decreases in the presence of arginine. This amino acid is the direct precursor of urea. In a yeast cell, urea can be further converted into NH_4^+ or can undergo secretion into the fermentation medium. The formation of H_2S was considerably less efficient in the presence of arginine for Hefix than Syrena yeast. At the same time, in the case of the Hefix strain the efficiency of formation of ethyl carbamate did not increase in relation to the sample supplemented with $(NH_4)_2SO_4$, which occurred during fermentation conducted with Syrena yeast. This can mean that urea is a more effective intracellular nitrogen source for the Hefix strain than for Syrena.

The use of amino acids selected allowed a substantial reduction in the relative efficiency of ethyl carbamate formation to be obtained for the majority of the fermentation. Only Syrena yeast in the presence of arginine, aspartate, and lysine formed this compound in greater quantities than in the presence of $(NH_4)_2SO_4$

(Tab. 2). The causes of an increase in the efficiency of ethyl carbamate in the presence of Arg were described in the paragraph above. Aspartate participates in an ornithine cycle during which it condenses with citrulline, and in successive reactions fumarate and arginine are formed.

Due to the affirmed strong effect of the type of nitrogen source on the formation of H_2S and ethyl carbamate, fermentations of apple musts were enriched with diammonium posphate and Fermivit V. We analyzed the apple musts and Fermivit V preparation to determine the content of free amino acids that could potentially be a nitrogen source for the yeasts. The must obtained from apple juice concentrate contained the largest quantities of aspartate and glutamate. It had a relatively small content of sulfur amino acids (Tab. 3). Fermivit V preparation turned out to be a rich source of free amino acids and it was very abundant in glutamate. It should be stressed that there is a high content of cysteine, which – as we have found in earlier studies – stimulates the formation of H_2S by studied yeasts. We found no glycine, histidine, tyrosine, methionine, isoleucine and phenylalanine. These amino acids were present in small quantities in the apple must (Tab. 3).

Table 3. Content of free amino acids in apple must and Fermivit V preparation

Amino acid	Apple must	Fermivit V
	mg per 100g	mg per 1g
Asp	3.24±0,23	8.98±0.90
Glu	2.79±0.32	56.16±0.26
Gly	0.10±0.01	ND
His	0.54±0.04	ND
Arg+Thr	0.66±0.03	17.29±1.64
Ala	0.90±0.06	24.47±0.03
Pro	0.38±0.04	6.39±0.53
Tyr	0.90±0.09	ND
Val	0.55±0.05	3.59±0.27
Met	0.09±0.002	ND
Cys	0.08±0.003	16.15±1.03
Ile	0.21±0.01	ND
Leu	0.10±0.004	5.13±0.30
Phe	0.15±0.01	ND
Lys	0.08±0.003	2.08±0.13

ND – not detected

Fermivit preparation as a nitrogen source allows fermentation to be reduced by several days; from 27 to 22 days in the case of the process conducted with Syrena yeast and to 24 days in the presence of Hefix yeast. This is very favorable from an economical point of view. Addition of Fermivit clearly stimulated the formation of glycerol by the yeasts investigat
ed. Its amount in wines obtained in the presence of Fermivit was higher by 0.93 and 1.43 g L^{-1} in wines fermented with Hefix and Syrena yeast, respectively (Table 4). According to Ugliano *at al.* [18] and Vilanova *et al.* [9], glycerol production depends strongly not only on the Yeasts Asssimilable Nitrogen

(YAN) concentration but also on the yeast strain used. About 4-10% of the carbon source during fermentation is converted by yeast into glycerol [21]. The literature data show that glycerol is not the component affecting the aroma of wines. However, this triol imparts certain other sensory qualities. It has a slightly sweet taste, and owing to its viscous nature, also contributes to the smoothness, consistency, and overall body of wine [22]. Acetaldehyde, as an intermediate product of ethanol fermentation, is formed from pyruvate and then converted into ethyl alcohol. At a concentration of over 100 mg L^{-1} this compound imparts an aroma of "sour green apples" to wines [23]. The yeasts used during investigations formed varied quantities of acetaldehyde. Fermivit V preparation stimulated its production in both cases. Romano *et al.* [24] claim that an increase of the acetaldehyde content entails an increase of acetic acid concentration in wine. This dependence was confirmed by our studies (Tab. 4). In all the wines we determined slight amounts of lactic acid. Its content was higher after enriching the pitchings with the Fermivit V preparation. It may be formed as a result of the conversion of malic acid which occurs with participation of bacteria or as a by-product of the alcoholic fermentation [25]. The strain investigated formed slight amounts of succinic acid. The content of this metabolite in wines also increased in the presence of Fermivit V. Low quantities of methanol, which is a product of the enzymatic decomposition of pectins, were also found in the wines. The analysis of the ethanol content and fermentation by-products has shown that the use of the Fermivit preparation leads to an intensification of yeast metabolism.

Table 4. Analysis of wines obtained with studied yeasts

Strain		Syrena		Hefix	
Nitrogen source		$(NH_4)_2HPO_4$	Fermivit V	$(NH_4)_2HPO_4$	Fermivit V
Glucose		ND	0.20*±0.01	ND	3.83*±0.11
Fructose	g L^{-1}	1.30±0.05	1.92*±0.05	19.34±0.10	17.72*±0.12
Malic acid		7.80±0.28	8.03±0.27	8.06±0,52	8.13±0,69
Glycerol		5.35±0.17	6.78*±0.29	4.43±0.18	5.40*±0.19
Acetaldehyde	mg L^{-1}	139.5±4.6	281.6*±10.9	92.5±3.4	229.9*±8.1
Succinic acid		281.4±9.8	548.7*±20.9	351.5±9.5	551.38±18.7
Lactic acid		79.5±3.1	123.6*±4.8	91.2±2.7	99.5*±3.5
Acetic acid		177.7±6.8	319.7*±9.2	83.1±3.7	204.2*±7.1
Methanol	% v/v	0.16±0.01	0.54*±0.01	0.11±0.01	0.21*±0.01
Ethanol		12.61±0.04	13.38*±0.37	12.84±0.35	12.56±0.36

* – statistically significant differences compared to NH_4^+ supplementation at a probability of $p < 0.05$

As was determined during model studies, the kind of nitrogen source can have an effect on the formation of hydrogen sulfide and ethyl carbamate. This phenomenon was fully confirmed during must fermentation. The Fermivit V preparation stimulated the yeasts to form hydrogen sulfide. The final content of

this compound in the wines obtained with Hefix and Syrena yeasts increased by 20 and 27 µM, respectively, in relation to the wines obtained with an addition of diammonium phosphate (Table 5). This is probably caused by a the high content of cysteine in Fermivit, whereas in the apple must there were only slight quantities of this amino acid. Using the assumed dose of Fermivit V preparation, we provided 53.3 µM of this amino acid. It should be emphasized that Syrena yeast in musts also formed substantially greater quantities of hydrogen sulfide than Hefix.

Changes in the ethyl carbamate in the wines depending on the nitrogen source were correlated with the results obtained during model studies. The Syrena yeast formed greater amounts of ethyl carbamate in the pitchings enriched with Fermivit V. This preparation contained relatively large quantities of arginine and aspartate, and their presence brought about an increase in the efficiency of ethyl carbamate formation by Syrena strain during fermentation of the model medium. Nitrogen-containing organic compounds decreased the efficiency of ethyl carbamate formation in the model medium fermented with the Hefix strain. This was confirmed in apple musts, where the Fermivit V preparation caused a threefold decrease in the concentration of ethyl carbamate in the wines (in relation to the wines obtained with participation of diammonium phosphate).

Table 5. Effect of a nitrogen source on the formation of ethyl carbamate (EC) and hydrogen sulphide by studied yeasts

Strain	Nitrogen source	H_2S [µM]	EC [µM]
Syrena	$(NH_4)_2HPO_4$	12.40±0.38	0.592±0.020
	Fermivit V	39.25*±1.14	0.688*±0.037
Hefix	$(NH_4)_2HPO_4$	6.67±0.14	0.747±0.044
	Fermivit V	26.63*±1.01	0.247*±0.009

* – statistically significant differences compared to NH_4^+ supplementation at a probability of $p < 0.05$

Conclusions

This study shows that the initial nitrogen concentration and composition of a nitrogen source strongly modulate wine composition in a strain-dependent manner. Winemakers need to carefully select the kind of nitrogen source for the yeast starter culture. This will allow to modulate wine flavor and to minimize concentrations of undesirable metabolites such as hydrogen sulfide and ethyl carbamate.

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