Changes in Nitrogen Compounds in Must and Wine during Fermentation and Biological Aging by Flor Yeasts

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Urea, ammonium, and free amino acid contents were quantified in a must from *Vitis vinifera* cv. Pedro Ximenez grapes and in fermented wine and after a short aging of this wine by *Saccharomyces cerevisiae* race *capensis* yeast under variable oxygen availability conditions. The previous compounds were also determined in a wine in which the nitrogen source was depleted by the same race of flor yeast (old wine) and also following the addition of ammonium ion, L-glutamic acid, and L-proline. Under specific conditions such as low oxygen level and the absence of some nutrients, the yeasts release some amino acids including L-threonine, L-tryptophan, L-cysteine, and L-methionine to the medium. These amino acids must originate primarily in a de novo synthesis from ethanol that regenerates $NAD(P)^+$. On the basis of these results, the yeasts may be able to use amino acids not only as nitrogen sources but also as redox agents to balance the oxidation−reduction potential under conditions of restricted oxygen, when electron transport along the respiratory chain may be hindered or limited.

Keywords: *Amino acids; redox potential; fermentation; biological aging; flor yeast*

INTRODUCTION

Sherry wine of the "fino" type is produced in southern Spain (mostly in Jerez and the Montilla-Moriles region), using an original method that was developed over several decades. Young wine obtained in the fermentation of grape must by yeasts is allowed to age in American oak casks under a thick film (velum) formed by flor yeasts, which are *Saccharomyces cerevisiae* races that grow on the surface of wine with an ethanol content of 15.0−15.5% v/v and exhibit aerobic respiratory metabolism (*1*−*3*). The yeast velum plays two central roles in the aging process. Thus, the metabolism of flor yeasts influences the features of Sherry wine; also, the active consumption of oxygen and the isolating effect of the yeasts prevent oxidation of the wine, thereby providing a highly reductive medium (*4*).

S. cerevisiae can grow in a wide variety of nitrogen-containing media (*5*, *6*); the rates of consumption and metabolism of nitrogen compounds in such media are dependent on the particular yeast strain, its physiological status, and the physicochemical properties of the medium. Thus, *S. cerevisiae* can use amino acids both to synthesize proteins and as a nitrogen source: amino acids are degraded by yeast cells, and the nitrogen they contain is released usually, but not always, as ammonia and used to synthesize other nitrogen-containing cell constituents. The yeasts can also use the carbon in amino acids for synthetic purposes; these compounds thus act as carbon sources or are released into the medium (*6*).

Grape must contains a wide variety of nitrogen compounds (particularly ammonium ion, amino acids, peptides, and small polypeptides) that can be used as nitrogen sources by yeasts. 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 higher alcohols, which contribute to the aroma of wine and hence to its quality (*7*). Nitrogen deficiencies in grape must and juices can lead to a series of difficulties during vinification, as can limited yeast growth (*8*, *9*), and may result in sluggish or stuck fermentation (*9*−*11*) and in the release of hydrogen sulfide (*12*). Amino acids also play a prominent role in the formation of ethyl carbamate, a mild carcinogen encountered in fermented foods (*13*).

The influence of the nitrogen source on alcoholic fermentation has recently been the subject of much study (*8*, *14*−*17*). The biological aging process has also received some attention in this respect (*18*−*20*).

Nicotinamide adenine dinucleotides take part in a number of reactions that maintain the balance in the redox potential. Thus, the NADH surplus that originates mainly from the synthesis of amino acids (*21*) is converted into NAD^+ during the formation of glycerol in alcoholic fermentation (*14*).

In this work, we examined the consumption and release of amino acids, urea, and ammonium ion by a flor yeast, namely, *S. cerevisiae* race *capensis*, during alcoholic fermentation and biological aging under variable conditions. The release of some amino acids is interpreted as one of the effects exerted by the yeast cells to balance the intracellular redox potential. In addition to their well-known role as nitrogen sources, amino acids therefore appear to function as electron acceptors in order to oxidize excess NAD(P)H and maintain the oxidation−reduction balance when the electron transport chain is broken or limited.

MATERIALS AND METHODS

Yeast Strain. The strain of *S. cerevisiae* race *capensis* (*22*) used in this study is typical of flor yeasts from the Montilla-Moriles region. It was isolated and selected by the authors'

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group because of its efficient growth in wine and its special features (*19*, *23*−*25*).

Culture Medium and Growth Conditions. Alcoholic fermentation was studied in must from *Vitis vinifera* cv. Pedro Ximénez grapes with a fermentable sugar content of 250 ± 15 g/L. The pH of the must was adjusted to 3.2 with tartaric acid, and 60 mg/L SO_2 was added. Biological aging was examined in the fermented must after 3 months, in a young wine with a 15% v/v ethanol content that was obtained from Pedro Ximenez grapes and supplied by Bodegas Alvear, and also in an old wine obtained in the laboratory, from the later one after three successive velum formations and the respective removal of the yeast film until no velum was formed, with the aim of impoverishing the wine of nitrogen sources (particularly L-proline). Subsequently, the old wine was supplied with vinic alcohol to 15.5% v/v ethanol and subjected to four different aging experiments, namely, as such (with no addition) for control purposes and with the addition of an 11 mM concentration of L-proline (initial concentration observed in the young wine), L-glutamic acid, or ammonium ion, respectively.

Fermentation was conducted under semiaerobic conditions, biological aging of young wine under both semiaerobic and semi-anaerobic conditions, and biological aging of old wine under semiaerobic conditions. The semi-anaerobic condition was established by fitting the flask with a mercury valve that allowed gases out but not in, and the semiaerobic condition was established by allowing the fermenting grape must to stand in a flask stoppered with a cotton plug. Prior to use, the must and the two types of wine were sterilized by filtration through a Seitz-Supra EK filter from Seitz (Bad Kreuznach, Germany). Two liter flasks were filled with 1.8 L of must, and the wines were placed in 100 mL Erlenmeyer flasks at the same surface/volume ratio as in the celler barrels (0.016 cm^{-1}). The fermentation and aging temperatures were 28 and 20 °C, respectively. The inocula used in both the fermentation and the aging experiments contained 1×10^6 viable cells/mL.

Analyses. Yeast cells were isolated from the medium by filtration through Millipore filters of 0.22 μm pore size and nitrogen-containing compounds analyzed as follows: urea and ammonium in wine were determined using the enzymatic method (urea/ammonia UV method, catalog no. 542946) of Boehringer Mannheim (Germany); free amino acids in wine were quantified essentially according to the method of Botella et al. (*18*), by measuring the absorbance at 254 nm of their dansyl derivatives, which were previously separated by high-performance liquid chromatography (HPLC) using a 15 m × 0.4 cm reversed-phase column packed with Spherisorb ODS2 resin of 5 μm particle size from Tracer Analítica (Barcelona, Spain). Twenty microliters of L-norleucine (5 mmol/L) was added as internal standard.

All of the results are given as mean values with the standard deviations for three replicate experiments. Where no error bar is shown, the standard deviation was too small to be plotted.

RESULTS

Changes in Nitrogen Compounds in Wine during Fermentation and Biological Aging under Semiaerobic Conditions. L-Proline and L-arginine, which accounted for 38.2 and 25.4%, respectively, of total free amino acids, were the major species in the must, followed by ammonium ion, GABA, L-threonine, and L-glutamic acid (Figure 1). Fermentation of this must resulted in extensive consumption of all these compounds except L-proline, which was released to the medium to some extent. The main nitrogen source under these conditions was found to be L-arginine (Figure 1). On the other hand, L-proline was the main nitrogen source for the yeasts during the short biological aging of the wine (Figure 2). The amino acids released during fermentation were basically L-leucine and, to a lesser extent, L-cystine, L-methionine, and L-ornithine (Figure 1); those released during biological aging were L-tryptophan, L-cystine, L-methionine, and L-ornithine (Figure 2).

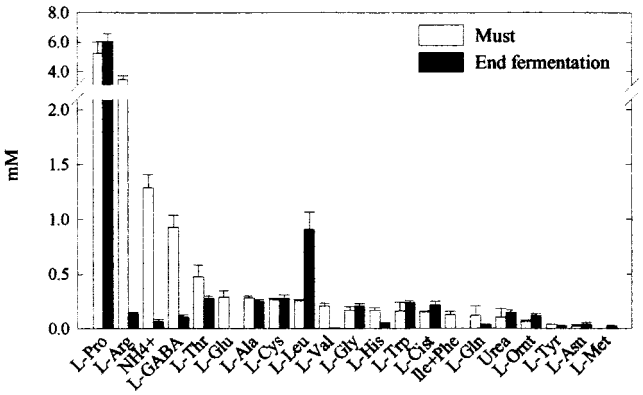

Figure 1. Changes in nitrogen compounds during semiaerobic fermentation.

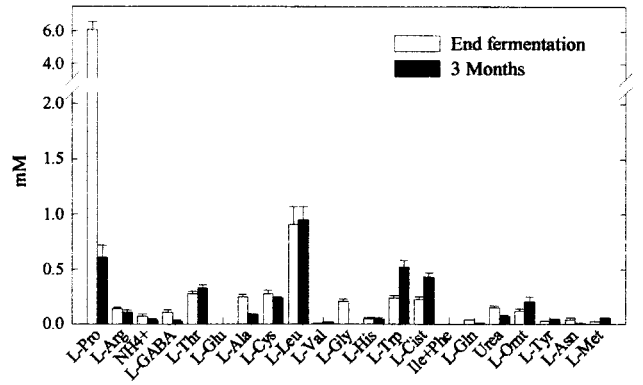

Figure 2. Changes in nitrogen compounds during a short biological aging period in semiaerobic conditions.

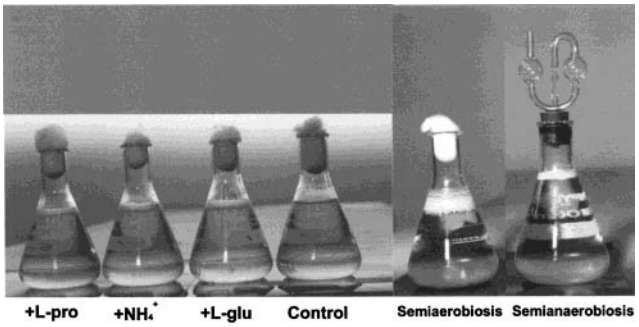

Figure 3. Flor velum formed by *S. cerevisiae* race *capensis* after 2 months of wine aging: old wine with additions (L-proline, ammonium, L-glutamic acid) and no additions; young wine in semiaerobic and semi-anaerobic conditions.

Changes in Nitrogen Compounds in a Young Wine during Biological Aging under Semi-anaerobic Conditions. Under these conditions, *S. cerevisiae* race *capensis* cells formed a thin velum (Figure 3). There was virtually no consumption of amino acids; rather, significant amounts of L-proline, L-threonine, L-cysteine, L-tryptophan, L-methionine, urea, L-phenylalanine, and L-isoleucine were released into the wine (Figure 4).

Changes in Nitrogen Compounds in Nutrient-Depleted Old Wine during Biological Aging under Semiaerobic Conditions. Simultaneously with the previous experiments, the formation of velum and changes in the nitrogen-containing compounds in nutrient-depleted wine were studied. The old wine used as

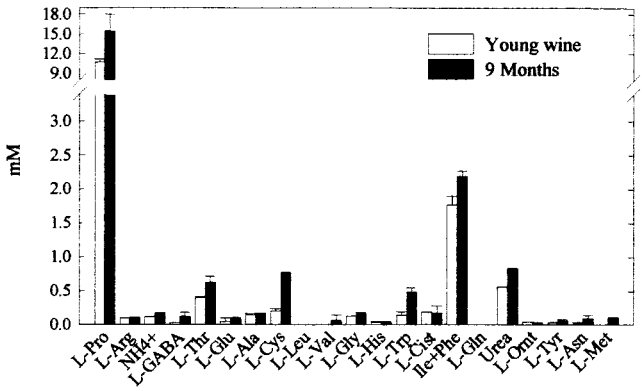

Figure 4. Changes in nitrogen compounds during a biological aging period in semi-anaerobic conditions.

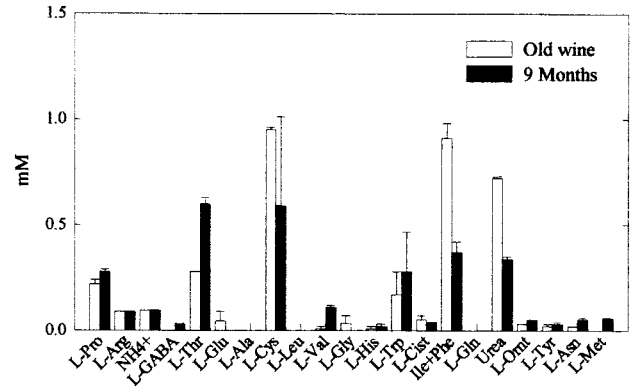

Figure 5. Changes in nitrogen compounds in nutrient-depleted old wine.

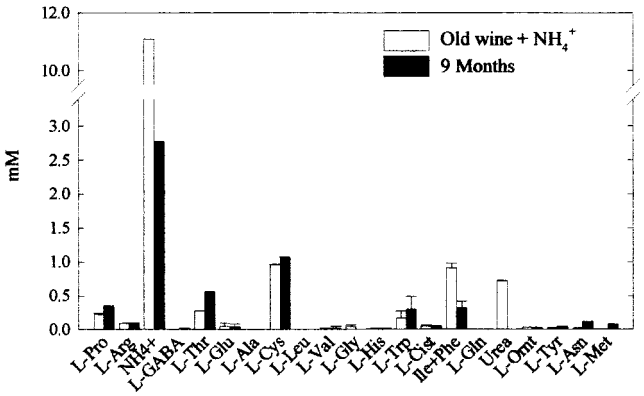

Figure 6. Changes in nitrogen compounds in nutrient-depleted old wine with added ammonium ions.

control, which was supplied with no additives, formed no velum; rather, it exhibited a ring around the liquid meniscus in the flask and slight turbidity in the bulk medium. The addition of an 11 mM concentration of ammonium ion, L-glutamic acid, or L-proline resulted in the formation of velum by race *capensis* yeast cells; the yeast film, however, was never so extensive or strong as that formed on the young wine (Figure 3). Yeast cells used part of the different additives (Figures 6 and 8) except L-glutamic acid, which was fully depleted (Figure 6). The addition of L-glutamic acid and, to a lesser extent, L-proline resulted in substantial release of ammonium into the wine (Figures 7 and 8). It is remarkable that in the case of the depleted wine and the wine having added ion ammonium or L-glutamic acid, the yeast cells exhausted the urea from the

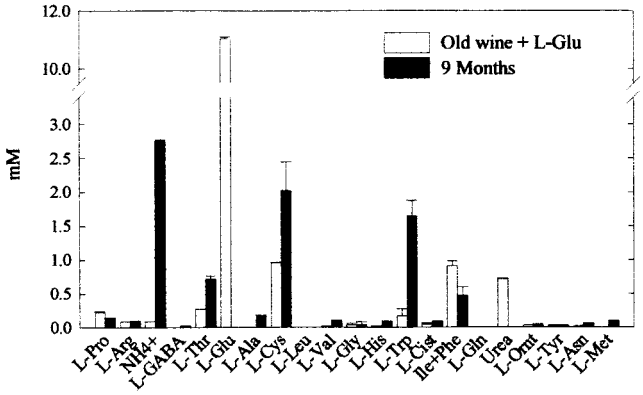

Figure 7. Changes in nitrogen compounds in nutrient-depleted old wine with added L-glutamic acid.

Figure 8. Changes in nitrogen compounds in nutrient-depleted old wine with added L-proline.

medium (Figures 6 and 7). In some cases, yeast cells also released variable amounts of L-threonine, L-tryptophan, L-cysteine, and L-methionine (Figures 5−8).

DISCUSSION

The two major amino acids in must are L-arginine and L-proline, in addition to ammonium ions (*17*, *26*). Much of the ammonium, L-arginine, and other amino acids are used by yeasts during alcoholic fermentation. However, L-proline is used only to a limited extent under enological conditions owing to the inhibition of proline permease by nitrogen catabolites (*27*, *28*) and to a restricted supply of molecular oxygen, which is essential for the activity of proline oxidase (*29*, *30*).

When the must is inoculated, yeast cells are under high stress owing to the high concentrations of sugars (glucose and fructose) present, which result in a high osmotic pressure, the acid pH of the medium (~3.2), and the limited availability of oxygen (<10 mg/L), which is rapidly consumed by the cells; this places the medium under semi-anaerobic conditions, where yeast cells exhibit fermentative metabolism. Under such conditions, the initial cell levels of NAD^+ and NADPH exceed those of NADH and $NADP^+$, respectively (*31*). Biosynthetic metabolism (e.g., amino acid biosynthesis) produces excess NADH (*21*), so cells must reoxidize NADH and balance the redox potential by, for example, producing glycerine (*14*), ethanol, higher alcohols, and other compounds. We believe that the production and release of some amino acids by yeast cells under certain conditions are intended to maintain the oxidation−reduction balance. Thus, the reactions most markedly favored during alcoholic fermentation were found to be

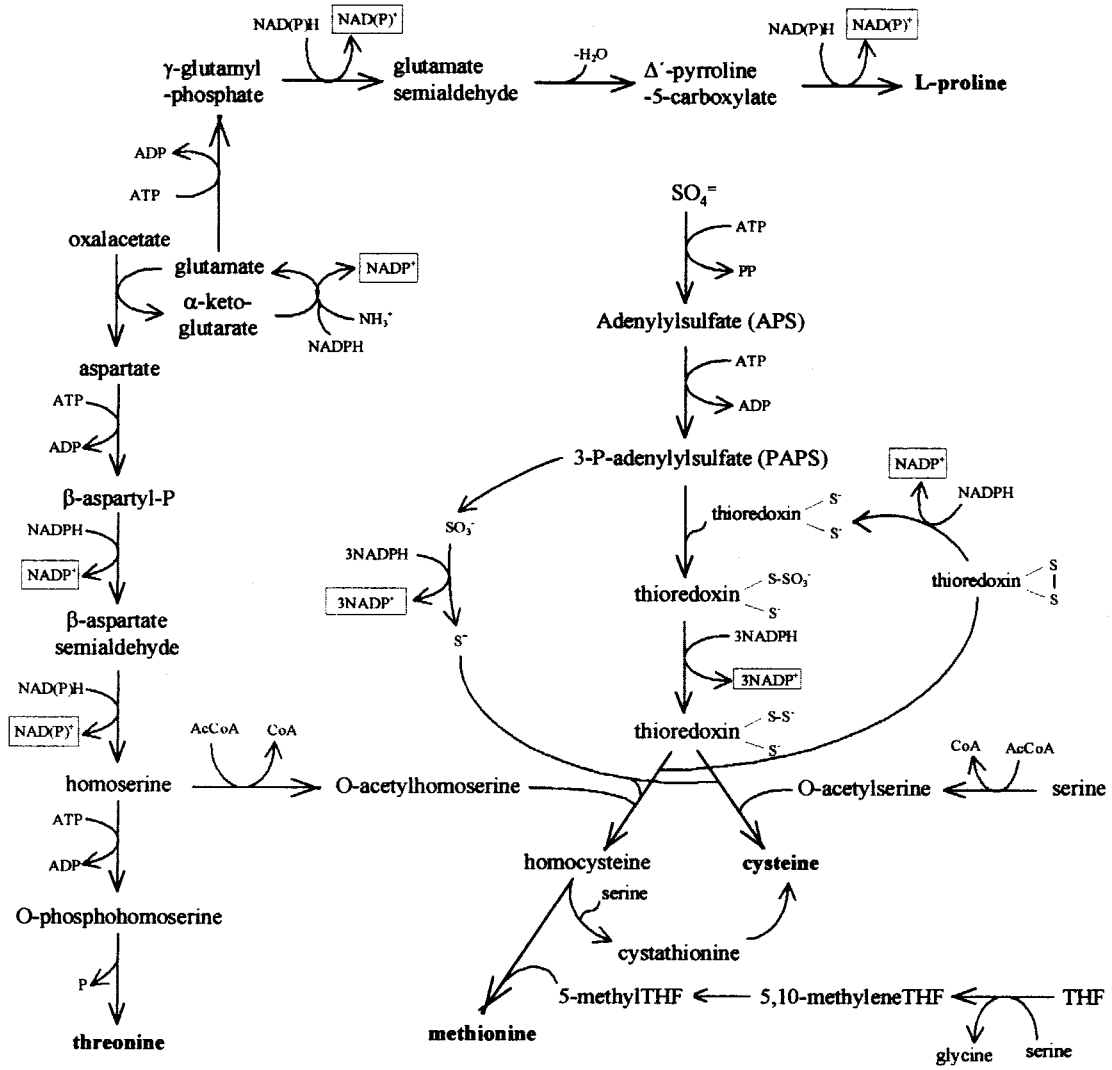

Figure 9. Biosynthesis of mainly amino acids producing $NADP^+$ (*5*, *14*, *32*).

those allowing $NAD(P)^+$ to be regenerated and included the formation of ethanol and higher alcohols (results not shown), as well as the syntheses of L-proline, L-cysteine, and L-methionine, which produce excess $NADP^+$ (Figure 9).

Once alcoholic fermentation is finished, "fino" wine is obtained by biological aging; yeast cells are also under high stress in the new medium by virtue of its high ethanol content (15.5% v/v) and acidity (pH 3.2) and its low dissolved oxygen concentration. Flor yeasts rapidly grow and form a film on the wine surface, again using dissolved oxygen in the medium by switching from fermentative metabolism to aerobic respiratory metabolism; once formed, the film hinders or blocks access of oxygen, thereby establishing a strongly reducing medium (*25*, *33*). The targets of cell metabolism under these conditions are thus the oxidized coenzymes required to maintain the intracellular redox balance. These conditions favor reduction reactions, so the metabolism may be aimed at minimizing the formation of NAD(P)H and ensuring a surplus of $NAD(P)^+$. In previous work (*25*), we hypothesized that the electron transport chain must somehow be broken or limited during biological aging, so alternative mechanisms such as the production of ethanol and higher alcohols must exist for $NAD(P)^+$ to be regenerated; this is supported by the high alcohol dehydrogenase activity observed under these conditions. Thus, using continuous cultures, Keulers et al. (*34*) found that the conversion of ethanol into acetaldehyde and that of the latter into acetate might be two sources of regulation for NAD^+ to be available, a key species with a view to maintaining the redox balance. On the basis of our own observations, NADH levels exceed NAD^+ levels, the NADPH concentration oscillated, and that of $NADP^+$ decreased during the formation of the flor film. Once the velum was formed, NAD^+ levels exceeded those of NADH, and $NADP^+$ levels exceeded those of NADPH. This reveals that reduction reactions are favored after the flor film is formed, when the electron transport along the respiratory chain is inefficient because of the limited availability of oxygen (*25*).

On the basis of the results of Cooper (*5*) and Jones and Fink (*32*), on amino acid metabolism by *S. cerevisiae*, we developed a simplified scheme for the biosynthesis of major amino acids resulting in especially marked reoxidation of NAD(P)H in order to explain our results (Figure 9). Thus, the increase in L-proline levels in the medium during fermentation and during biological aging under semi-anaerobic conditions and increases in the L-threonine, L-tryptophan, L-methionine, and L-cysteine levels during biological aging under semi-aerobic conditions may be a result of intermediates in the syntheses of these compounds acting as electron

acceptors in the reoxidation of NAD(P)H coenzymes. When yeast cells grew in the young wine, excess NADH produced in the degradation of L-proline (*5*, *6*) may be used in the formation of higher alcohols. In must or wine containing abundant amino acids, excess NAD(P)H produced in, for example, the degradation of L-proline or L-arginine might be oxidized in the formation of higher alcohols from ketoacids of the corresponding amino acids. On the other hand, in amino acid-deficient must or wine, excess NAD(P)H from the biosynthesis of amino acids might be diverted to the production of specific amino acids such as L-threonine, L-cysteine, L-methionine, and L-tryptophan, which would regenerate $NAD(P)^+$ as a result (*14*) (Figure 9) because electron transport along the respiratory chain is hindered under these fermentation or biological aging conditions (*25*). The formation of ethanol and higher alcohols, and changes in amino acids under the different conditions studied, can be ascribed to an increased efficiency in maintaining the potential balance (*14*).

The fact that no flor film was formed on the old wine used as control can be ascribed to the nitrogen source being depleted; in fact, the addition of ammonium, L-proline, or L-glutamic acid facilitated formation of the film. In no case, however, was film formation or consumption of proline, when added to the medium, as marked as in the young wine (*19*), possibly as a result of yeast growth being slowed or even halted by a deficiency in some growth factor.

Glutamic acid is central to the biosynthesis of amino acids and is therefore an appropriate choice for a single amino acid as the nitrogen source. Ammonium is one other nitrogen-rich source. Addition of either compound to the wine raised the total consumption of urea. Ever since this compound has been known to influence the formation of ethyl carbamate (*35*), reducing its presence in wine has been a desirable goal (*20*, *31*). Further research into the action of these additives should be conducted with a view to effectively reducing any urea in wine.

Our findings are in agreement with previous results. Thus, our data are consistent with those of Albers et al. (*14*), who showed product formation by *S. cerevisiae* under anaerobic conditions is affected by the particular nitrogen source. Our results are also consistent with those of Omori et al. (*36*), who suggested that the activation of leucine or phenylalanine biosynthesis in *S. cerevisiae* mutants leads to increased production of glycerol. Michnik et al. (*37*) and Remize et al. (*38*) used genetic engineering to obtain various wine yeast strains that overexpressed a *GPD1* gene encoding a glycerol-3-phosphate dehydrogenase; the yeast cells resulted in increased production of glycerol at the expense of ethanol. Surprisingly, they also accumulated large amounts of other byproducts such as acetate, acetoin, 2,3-butanediol, and succinate. These byproducts must have accumulated as a result of the redox potential, which was altered by the diversion of the carbon flux from ethanol to glycerol, being balanced.

In conclusion, under specific conditions, the accumulation of glycerol, higher alcohols, acetate, 2,3-butanediol, acetoin, succinate (*5*, *14*, *38*−*40*), and some amino acids can be ascribed to regulation of the metabolism succeeding in the most efficient balancing of the redox potential. Also, the redox potential of the medium appears to be an important factor in controlling these compounds.

The present study is a previous work which aimed to explain that the consumption and release of some amino acids observed under different conditions by us and reported by different authors in previous papers are the result of the reoxidation of NAD(P)H by *S. cerevisiae* cells in order to maintain a normal redox balance.

We are currently conducting aging experiments involving flor yeasts and synthetic wines containing various amino acids to examine the influence of the amino acids on the aging process and correlating them with the formation of higher alcohols and other compounds such as glycerol, acetaldehyde, and acetoin. In this way, we may acquire new knowledge of their effect on the intracellular redox potential with a view to accelerating biological aging of wine.

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