INFLUENCE OF NON-*SACCHAROMYCES* YEAST GROWTH ON THE METABOLISM OF NITROGENOUS COMPOUNDS IN LACTIC ACID BACTERIA FROM WINE

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***Abstract*-- A comparative study of the influence of *Hanseniaspora uvarum* metabolism on the growth and physiology of two lactic acid bacteria involved in vinification: *Lactobacillus hilgardii* 5w and *Oenococcus oeni* X_2L was carried out. At different yeast growth times in grape juice medium, fermented broth was inoculated with *L. hilgardii* or *O. oeni* and incubated for 48 h at 30°C. When *O. oeni* grown in the culture supernatant after 0, 3 and 8 hours of yeast growth (M1, M2 and M3 respectively), the growth rate and final biomass were not modified. With *L. hilgardii* a decrease of both parameters was observed. After 12, 24 and 34 h of yeast growth (M4, M5 and M6 respectively), the results for both microorganisms were similar: the growth rate increased and the final cellular mass decreased. When *O. oeni* was inoculated in M1, M2 and M3 media, an initial decrease of proteins directly related to amino acids production was observed. With *L. hilgardii* the amino acid and proteins concentrations diminished. In M4, M5 and M6 media *O. oeni* showed that the protein concentrations remained constant and the amino acid concentrations diminished. The results obtained with *L. hilgardii* were different with a higher protein consumption rate without significative amino acids modification. The amino acids increment in the first hours of *O. oeni* growth could regulate the protease production achieving the equilibrium between amino acids production and utilization. The high proteolytic activity in *L. hilgardii* could be responsible of the amino acids increase despite its consumption.**

***Keywords*-- Lactic acid bacteria, Non-*Saccharomyces* yeast, Wine.**

I. INTRODUCTION

Alcoholic and malolactic fermentations are the main processes that take place in wine winemaking. Yeasts transform sugars to ethanol, but at the same time play an important role in organic acid production and volatile end products in alcoholic beverages. The malolactic fermentation remains an imperfectly controlled process, since many nutritional and physic-chemical factors affect the growth and metabolism of lactic acid bacteria. Some of them depend on the yeast strain used, providing different amounts of amino acids, peptides and vitamins available acting as growth factors for lactic acid development, or on the presence of metabolic products which act as inhibitors. Several studies reviewed the importance of nitrogen from source, to transport, metabolic fate and influence on the fermentation carried out by the yeast *Saccharomyces cerevisiae.* Unfortunately, there is very little information about the nitrogen metabolism of non-*Saccharomyces* species of wine yeast. Recent quantitative studies on winemaking ecology showed that non-*Saccharomyces* species (especially *Hanseniaspora uvarum* (*Kloeckera apiculata*) and *Candida stellata*) survive during alcoholic fermentation at significant levels for longer periods than previously thought and grew to maximum populations of 10^6-10^7 cfu ml^{-1} (Fleet *et al*., 1984; Heard and Fleet, 1985; Pardo *et al*., 1989). Such growth was considered to be quantitatively significant and likely to influence the chemical composition of the wine.

We carried out a comparative study of the influence of *Hanseniaspora uvarum* metabolism on the growth and nitrogen physiology of two lactic acid bacteria involved in vinification. *Lactobacillus hilgardii* 5w, a detrimental microorganism, with negative ecological effect like hydrogen peroxide (Rodriguez and Manca de Nadra. 1995a, b) and histamine production (Farias *et al*., 1995; Farias *et al*., 1996), and *Oenococcus oeni* X_2L, a beneficial microorganism with optimal technological properties.

II. MATERIALS AND METHODS

A. Microorganisms and Culture Conditions

Lactobacillus hilgardii 5w, *Oenococcus oeni* X_2L and *Hanseniaspora uvarum* ca12 (*Kloeckera apiculata*) were isolated from Argentinean wines. Yeast and lactic acid bacteria were grown in basal medium containing per liter: yeast extract, 10 g; glucose, 5 g; tween 80, 1 ml and grape juice, 57 ml. When indicated, 1% KNO_3

was added to the culture medium. The pH was adjusted to pH 4.8 with 0.1 M HCl before sterilization by autoclaving for 15 min at 121°C.

B. Sequential Cultures in Liquid Medium.

A stoppered 250-ml flask, containing 200 ml of basal medium, was inoculated with a yeast strain and incubated at 30°C. At different times (0, 3, 8, 12 , 24 and 34 h), 20 ml of fermented broth was collected and the number of viable cell counts determined. The broth was filtered aseptically and dispensed into 75-ml flask to which 10% of basal medium culture of the *Lactobacillus hilgardii* 5w or *Oenococcus oeni* X_2L were added and incubated for 3 d at 30°C. 5ml-aliquots of culture were taken at different time intervals and the number of viable cell counts was determined. The yeast and bacteria culture supernatants were utilized for analytical measurements.

C. Analytical Measurements

Proteolytic activity and amino acids determination: At different times of growth, cells were centrifuged and the supernatant was used for proteolytic activities determinations. Using autoclaved grape juice as substrate, amino acids were determined by the modified Cd-ninhydrin method (Doi *et al.*,1981). After 1 h of incubation at 30°C, the reaction was stopped by the addition of 0.65 ml of 24% trichloroacetic acid (TCA). A sample of TCA supernatants (20-100 µl, depending on the concentration of amino acids) was added with 1.7 ml Cd-ninhydrin reagent (0.8 g ninhydrin were dissolved in a mixture of 80 ml 99.5% ethanol and 10 ml acetic acid, followed by the addition of 1 g $CdCl_2$ dissolved in 1 ml of distilled water). The mixture was heated at 84°C for 5 min, then cooled and the absorbance at 507 nm determined. The absorbance for each sample was adjusted to the value produced by 1 mmol l^{-1} of leucine solution. The molar absorption coefficient of leucine $\&_M$(M^{-1} cm^{-1}) was obtained from: $\&_M$= absorbance (1 mol l^{-1} sample) x (total vol/sample vol)x 10^3.

The amino acid concentration was determined as follow:

$$[\text{mmol } l^{-1}] = \frac{\text{Absorbance x dilution}}{\&_M \text{ leucine}}$$

Protein determination: The proteins of the samples were determined by the reaction with Coomassie Brilliant Blue G-250 (Bradford, 1976). Calibration was carried out using bovine serum albumin (BSA) (Sigma Chemical Company).

III. RESULTS AND DISCUSSION

Figure 1 shows *Hanseniaspora uvarum* ca12, growing in basal medium. The end of exponential growth phase was achieved in 12 h incubation at 30°C. After 34 h growth, a decrease in the cell number was observed (from 3.1 x 10^7 cfu ml^{-1} in 24 h to 5.0 x 10^6 cfu ml^{-1} in 34 h incubation).

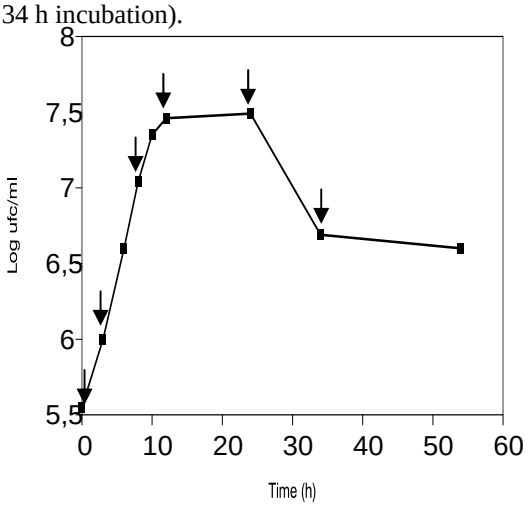

Fig. 1. Growth of *Hanseniaspora uvarum* in basal medium. Narrows indicate time to which the supernatant was taken for bacteria sequential inoculation. (0 h: M1; 3 h: M2; 8 h: M3; 12 h: M4; 24 h: M5 and 34 h: M6).

Table 1 shows the growth kinetic parameters obtained when *Lactobacillus hilgardii* 5w and O*enococcus oeni* growth in the filtered (0.2 µm porous size membrane) basal culture medium, after *Hanseniaspora uvarum* growth at 30°C. When *Oenococcus oeni* was grown in the culture supernatant after 0, 3 and 8 hours of yeast growth (M1, M2 and M3), the growth rate and final biomass were similar (approximately 0.035 h^{-1} and 3 x 10^8 cfu ml^{-1}, respectively). The growth rate of *Lactobacillus hilgardii* in the basal medium without yeast growth was 0.11 h^{-1}, while the growth rate after 8 h of yeast growth was 0.055 h^{-1}. As the time of yeast growth was increased from 0 to 8 h, there was a corresponding decrease in the bacterium growth from 2.5 x 10^7 to 1.25 x 10^7 cfu ml^{-1}. After 12, 24 and 34 h of yeast growth (M4, M5 and M6), an increase of the growth rate with diminution of the final cellular mass of both microorganisms was observed.

When *Oenococcus oeni* was inoculated in M1, M2 and M3 media, an initial decrease of proteins directly related to amino acids production was observed (Fig. 2A). Farías *et al*. (1996); Manca de Nadra *et al*. (1999) and Farías and Manca de Nadra (2000) characterized and purified extracellular proteases produced by *Oenococcus oeni* isolated from wine. Protein consumption during the first 12 h growth could be related to time of protease production by *Oenococcus oeni.* Its activity is observed up to 24 h growth as

showed by amino acids increase. Approximately 14 mmol l^{-1} amino acids were produced by *O. oeni* after 48 h growth in the different media. The protein consumption rates were 0.07, 0.05 and 0.09 μg l^{-1} h^{-1} in M1, M2 and M3 media respectively.

Table 1. Kinetic of *Lactobacillus hilgardii* growth in media obtained after *Hanseniaspora uvarum* growth.

Culture Media[a]	μ_{max} (h^{-1}) x 10^{-2}		Final biomass (log cfu ml^{-1})	
	O.oeni	***L.hilgardii***	***O.oeni***	***L.hilgardii***
M1	3.5±0.1	11.0±0.4	8.45±0.10	7.40±0.07
M2	3.2±0.1	10.0±0.6	8.50±0.09	7.25±0.05
M3	3.7±0.2	5.5±0.5	8.60±0.08	7.10±0.04
M4	4.5±0.2	6.0±0.2	7.61±0.10	6.80±0.10
M5	5.0±0.3	6.5±0.3	7.71±0.12	6.60±0.05
M6	5.2±0.2	10.5±0.3	7.56±0.09	7.00±0.12

[a] Culture media after 0 h (M1), 3 h (M2), 8 h (M3), 12 h (M4), 24 h (M5) and 34 h (M6) *Hanseniaspora uvarum* growth at 30°C.

Values are means of three independent experiments ± Standard Deviation.

With *Lactobacillus hilgardii* the amino acid and protein concentrations diminished (Fig. 2B). Approximately 5 mmol l^{-1} amino acids were consumed after 48 h growth, independently of the media. Protein consumption rates were 0.009, 0.005 and 0.01 μg l^{-1} h^{-1} in M1, M2 and M3 respectively. L. *hilgardii* showed proteolytic activity from 24 h.

After 12, 24 and 34 h yeast growth (M4, M5 and M6 media), *Oenococcus oeni* showed that the protein concentrations remained constant and the amino acid concentrations diminished (Fig. 3A). The amino acids increment in the first hours of *Oenococcus oeni* growth could regulate the protease production achieving the equilibrium between amino acids production and utilization.

The results obtained with *Lactobacillus hilgardii* were different showing a higher protein consumption rate (0.03, 0.04 and 0.03 μg l^{-1} h^{-1} in M4, M5 and M6 media respectively) without significative modification of amino acids concentration (1 mmol l^{-1}) (Fig. 3B). In the same conditions, an increase in ammonia concentration from amino acids was observed (unpublished data). From these results it is possible to infer that the amount of liberated amino acids by protease action is higher than its utilization.

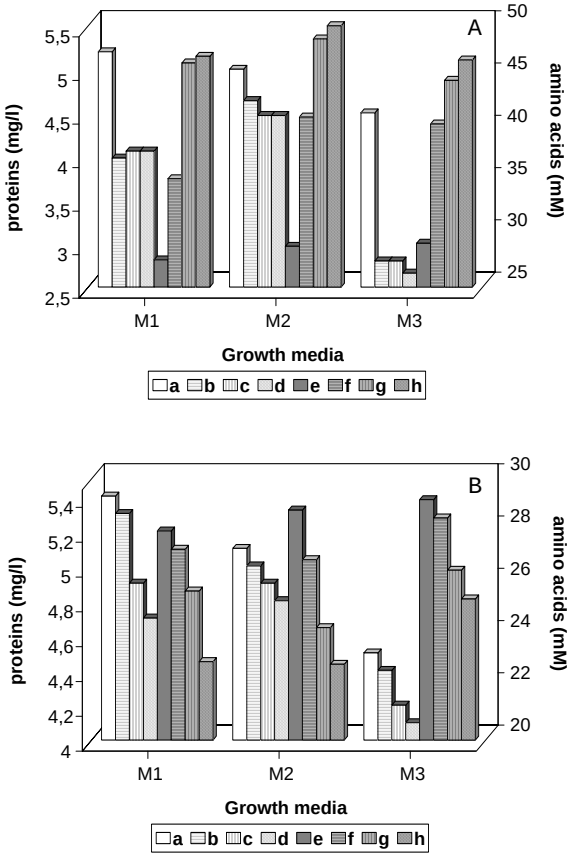

Fig. 2. *Oenococcus oeni* **(A)** and *Lactobacillus hilgardii* **(B)** growth in the media obtained after 0, 3 and 8 hours (M1, M2 and M3 respectively) of yeast growth. (□) Protein and (■) amino acid concentrations during 0 h (a, e), 12 h (b, f), 24 h (c, g) and 48 h (d, h) bacteria growth.

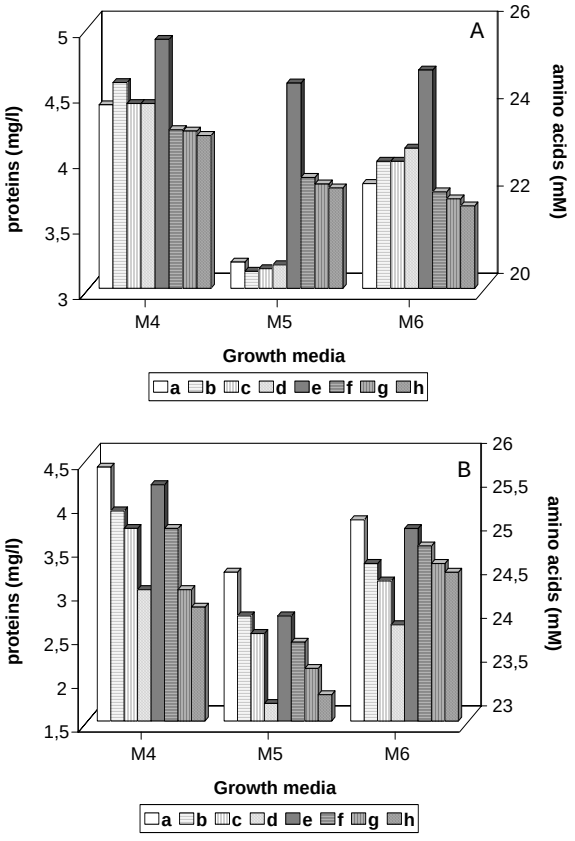

Fig. 3. *Oenococcus oeni* **(A)** and *Lactobacillus hilgardii* **(B)** growth in the media obtained after 12, 24 and 34 hours (M4, M5 and M6 respectively) of yeast growth. (□) Protein and (■) amino acid concentrations during 0 h (a, e), 12 h (b, f), 24 h (c, g) and 48 h (d, h) bacteria growth.

IV. CONCLUSIONS

We can conclude that the modifications produced by *Hanseniaspora uvarum* growth in the media affect both growth and nitrogen metabolism of *Oenococcus oeni* and *Lactobacillus hilgardii*. The different response of the lactic acid bacteria could be related to the different nutritional requirements and enzymatic regulatory mechanism of each microorganism.

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