Biogenic Amines Occurrence in Wine. Amino Acid Decarboxylase and Proteolytic Activities Expression by *Oenococcus oeni*

M. C. Leitão,[†] H. C. Teixeira,[†] M. T. Barreto Crespo,[†] and M. V. San Romão*[,†,‡]

Instituto de Biologia Experimental e Tecnológica (IBET) and Instituto de Tecnologia Química e Biológica (ITQB)-Universidade Nova de Lisboa, Apt. 12, 2781-901 Oeiras, Portugal, and Estação Vitivinícola Nacional, 2565-191 Dois Portos, Portugal

This work deals with the study of the proteolytic and amino acid decarboxylase activities of selected *Oenococcus oeni* isolates and the effect of yeast autolysis on biogenic amines production in wine. A total of 220 isolates of *O. oeni* were tested for decarboxylase and proteolytic activity. Only six isolates showed both activities, but only after a period of adaptation in a growth medium containing wine. The results reported on this paper show that proteolytic activity was dependent on medium composition and bacterial growth phase. It can be assumed that the ability of *O. oeni* to use wine peptides and to produce biogenic amines is not a constant characteristic of this species, and enzymatic system expression appears to be closely dependent on nutritional and energetical composition of the medium. It also seems to be strain dependent and not widespread among this bacterial community.

Keywords: *Oenococcus oeni; amino acid decarboxylase; proteases; biogenic amines*

INTRODUCTION

Yeast lysis in wine is known to favor lactic acid bacteria (LAB) development, following alcoholic fermentation (Guillout-Benatier et al., 1985). This is due to the release of vitamins and nitrogen compounds. Lonvaud-Funel et al. (1988) proposed that LAB stimulation can be attributed to the presence of several compounds of molecular mass higher than 12 000 Da.

Stimulation of LAB survival and growth in a wine fraction containing compounds having a molecular mass higher than 30 000 Da was observed in several isolates of *Oenococcus oeni* (results not shown). These facts suggest that LAB contain an enzymatic system that metabolizes the peptides accumulated during yeast autolysis and utilize the released amino acids as nutrients and/or energy sources. The amino acids can then be the precursors for biogenic amines (BAs) formation by the action of decarboxylases. The BAs most frequently identified in wine are histamine, tyramine, cadaverine, and putrescine, resulting from the decarboxylation of the amino acids histidine, tyrosine, lysine, and ornithine, respectively. The presence of BAs in food is considered undesirable due to their hazardous effects on human health (Bauza et al., 1995). It is however, important to note that the amounts of BA in wine are quite low in comparison to other fermented food products.

BA formation has been associated to malolactic fermentation (MLF) (Aerny, 1985); however, Delfini (1989), comparing the ability of several strains of *Leuconostoc* spp., *Lactobacillus* spp., and *Pediococcus* spp. to produce histamine, observed that only *Pediococcus damnosus* (*P. cerevisiae*) had the capability to produce significant amounts of histamine, while *Leuconostoc oenos* (*O. oeni*) strains were poor producers of BA.

Lafon-Lafourcade (1975) suggested that the accumulation of histamine occurs mainly as a result of bacteria growth in poor media, and Lounvaud-Funel et al. (1994) showed that histamine production by *O. oeni* is stimulated in media without glucose or malic acid and depends particularly on the histidine concentration of the media. Under those conditions, histidine decarboxylation contributed to an additional energy source for the bacteria as already demonstrated for other microorganisms (Molenaar, 1993).

Among lactic acid bacteria, *O. oeni* is the main species present in wine and the best adapted to carry out MLF at the low pH of wine (Wibowo et al., 1985). If BA formation is to be associated to MLF, it would be expected that *O. oeni* has the enzymes for breakdown of peptides and decarboxylation of amino acids present in wine at this stage. This paper examines the ability of selected strains of *O. oeni* to produce BA in wine based on their proteolytic and decarboxylase activities. It also investigates the effect of yeast autolysis on bacterial growth and biogenic amines production.

EXPERIMENTAL PROCEDURES

Bacteria. A total of 220 isolates of *O. oeni* was used. The isolates were obtained from the Instituto de Tecnologia Química e Biológica culture collection. Stock cultures were maintained at −80 °C in MRS culture medium (Difco, Detroit, MI), containing 50% glycerol (Merck, Darmstadt, Germany). The inoculum was prepared according to the experiments described below.

Wine. Wine was obtained by grape must fermentation obtained from two *Vitis vinifera* varieties, cv. Fernão Pires, and Vital (provided by Estação Vitivinícola Nacional), with a

* To whom correspondence should be addressed. Instituto de Tecnologia Química e Biológica, Apartado 127, 2781-901 Oeiras, Portugal. Phone: 351-21-4469554. Fax: 351-21-4411277. E-mail: vsr@itqb.unl.pt.

[†] IBET and ITQB.

[‡] Estação Vitivinícola Nacional.

10.1021/jf991135v CCC: $19.00 © 2000 American Chemical Society
Published on Web 05/31/2000

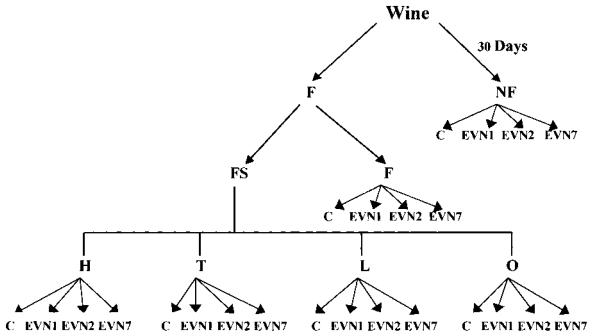

Figure 1. Wine samples preparation for the study of yeast autolysis effect on the cell growth and BA synthesis.

lyophilized preparation of *Saccharomyces cerevisiae* (FERMIROUGE, Gist Brocades, Holland).

Cell Growth. Cell concentration was monitored using absorbance readings at 600 nm. A calibration curve was defined using the dry weight of cells versus absorbance measurements.

Decarboxylase Activity. The stock cultures of *O. oeni* isolates were inoculated in FT80 culture medium (Cavin et al., 1989) in order to attain an initial absorbance of 0.5, measured at 600 nm, which is the cell concentration considered sufficient to start a new assay. The inoculated media were incubated at 30 °C, until early stationary phase, and screened for decarboxylase activity using the method described by Niven et al. (1981).

Proteolytic Activity. The decarboxylase positive isolates were screened for proteolytic activity. The isolates were cultivated as described above and adapted by subculturing in FT80 medium enriched with 10% wine. Proteolytic activity was evaluated by the method described by Smibert and Krieg (1994). One unit of proteolytic activity was defined as the increase of 0.001 (absorbance at 400 nm)/min/mL of culture medium (Gibb and Strohl, 1988).

To investigate the effect of medium composition on proteolytic activity, two culture media, FT80 and tomato juice (TJB) broth, Difco, were used. Enzymatic activity was assayed before and after three subcultures either in FT80 or TJB containing 10% wine. The enzymatic activity was evaluated along the bacterial growth.

Effect of Yeast Autolysis Either on Cell Growth or in Biogenic Amine Production. The wine was divided as shown in Figure 1. One part was filter sterilized in order to eliminate yeasts (filtered wine, F) while the second part was stored for 30 days in contact with yeast (nonfiltered wine, NF). The F was then divided in two parts (supplemented wine, FS, and nonsupplemented wine, F). FS was supplemented with 0.5 g L^{-1} of one of the following amino acids, either histidine (H), tyrosine (T), lysine (L), or ornithine (O) (Sigma) and F maintained its original composition.

The different wines (NF, F, and FS) were inoculated with the previously selected isolates, harvested at the end of the exponential growth phase, to a final cellular concentration of 0.1 mg mL^{-1} dry weight. For each wine, one part remained noninoculated, which constituted the different control wine (C), (Figure 1). Before and after bacterial growth**,** all the wines were analyzed for content of total and free amino acids, using the HPLC PICO TAG method proposed by Waters. Amines were quantified by the method described by Veciana-Nogues et al. (1995). Malic acid was quantified by enzymatic analysis using a kit commercialized by Boehringer Mannheim (Germany).

RESULTS AND DISCUSSION

Decarboxylase and Proteolytic Activities. The 220 isolates did not show any of the chosen enzymatic activities when grown after being stored at −80 °C, as

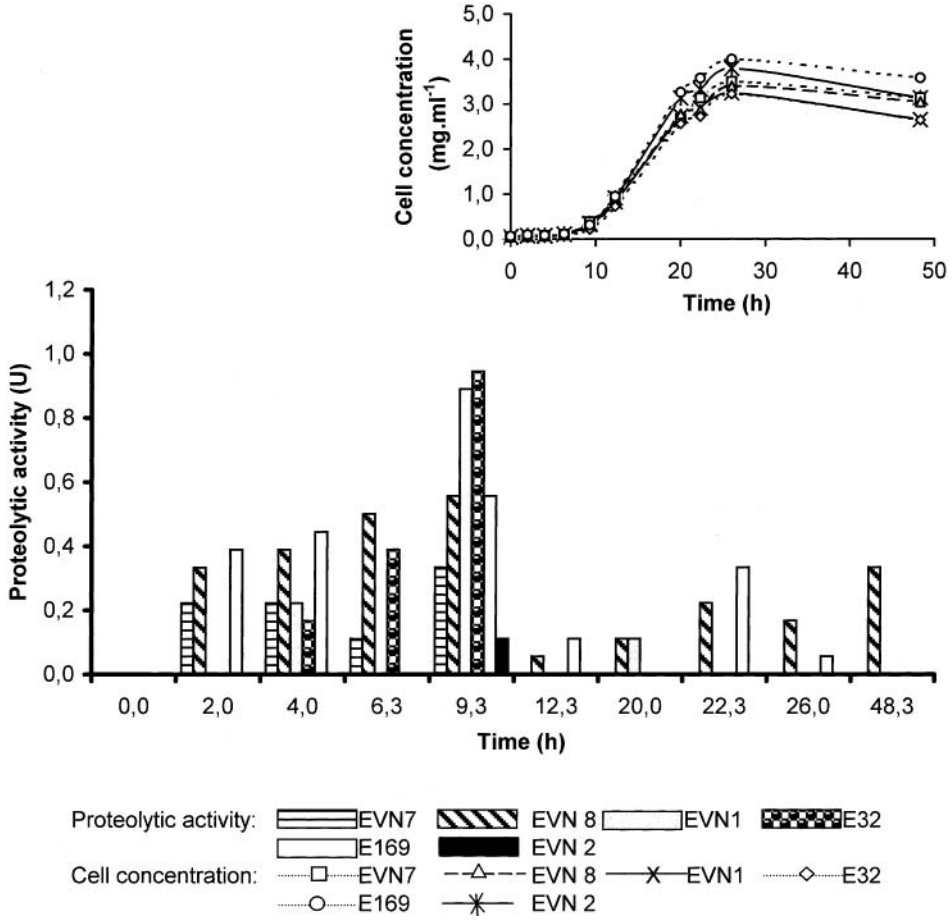

Figure 2. Growth and extracellular proteolytic activity of *O. oeni* isolates after adaption in medium FT80 containing 10% wine.

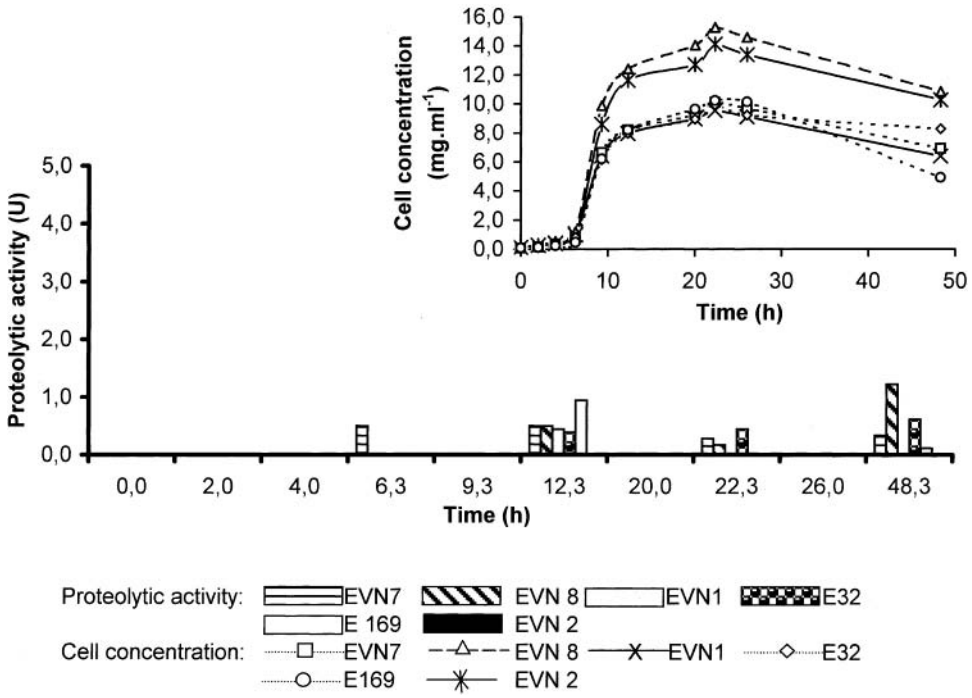

Figure 3. Growth and extracellular proteolytic activity of *O. oeni* isolates in medium TJB.

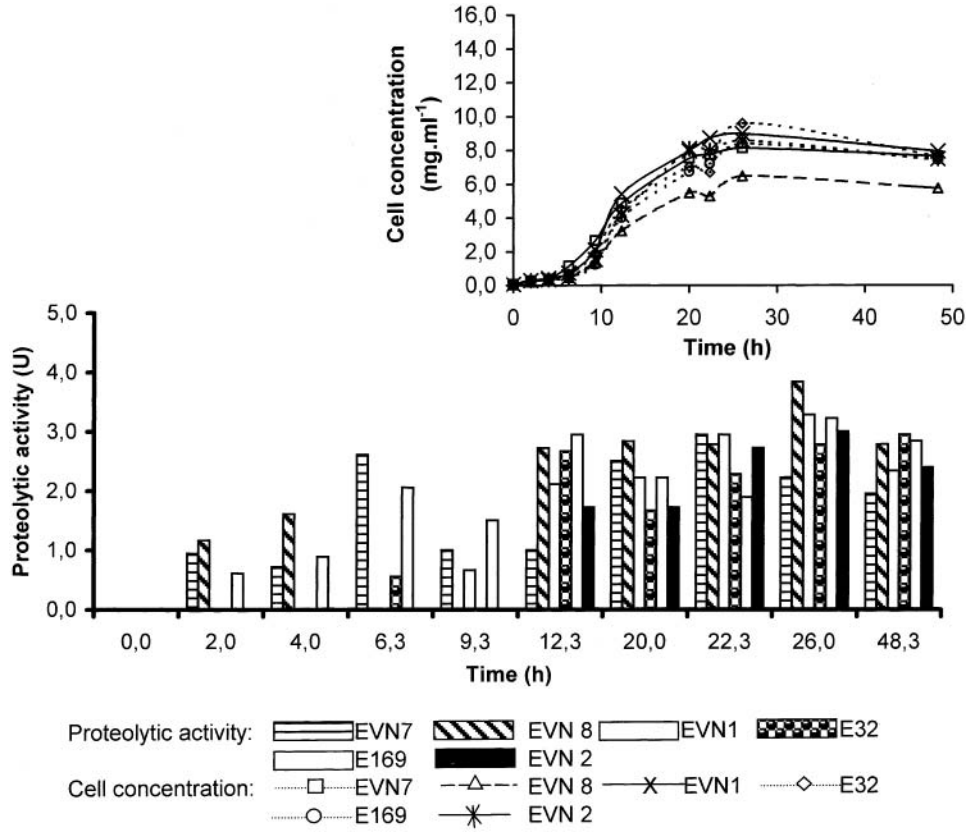

Figure 4. Growth and extracellular proteolytic activity of *O. oeni* isolates in medium TJB containg 10% wine.

they had not been recently isolated from their natural habitat, wine. Therefore, they were subcultured three times in FT80 medium enriched with 10% wine, and a second screening was performed as described in materials and methods. Six isolates, EVN1, EVN2, EVN7, EVN8, E32, and E169, recovered their decarboxylase activity. The results show that *O. oeni* stored in laboratory culture media are able to recover their amino acid decarboxylase activity by subculturing them in the presence of wine. Only these six isolates were investigated for proteolytic activity.

FT80 precultured bacteria only showed proteolytic activity following culturing in the presence of wine (Figure 2). The expression of proteolytic activity of TJB precultured bacteria was not dependent on the presence of wine in the medium; however, activity was increased in medium containing wine (Figures 3 and 4), and was always higher than in FT80 precultured bacteria. It was

Table 1. Amino Acids Consumption (%) by the Isolate EVN1, in Filtered (F) and Nonfiltered Wines (NF)[a]

	amino acids													
wine	Asp	Glu	Ser	Gly	His	Arg	Thr	Tyr	Val	Met	Ile	Leu	Phe	Lys
F	57	26	17	17	40	0	11	45	0	18	0	0	0	0
NF	42	8	0	0	23	4	0	6	16	0	11	4	21	10

[a] Asp, aspartic acid; Glu, glutamic acid; Ser, serine; Gly, glycine; His, histidine; Arg, arginine; Thr, threonine; Tyr, tyrosine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Lys, lysine.

observed that, in TJB cultures, proteolytic activity is higher during the late exponential to stationary phase of bacterial growth (Figure 4) while, in FT80, it is higher during the lag and early exponential phase (Figure 2). These results suggest that the expression of this enzymatic system is dependent on medium components and on the bacteria growth phase.

The results show that proteolytic activity increases when nutrients of high-energy content are exhausted (late exponential to stationary phase). The same behavior was also found for malolactic enzyme activity in *O. oeni* (Salema et al., 1996). Decarboxylase activity is expressed after peptide breakdown, allowing the cell to use the additional energy produced during amino acid transport (Molenaar et al., 1993). Results suggest that *O. oeni* does not need to express these activities when media composition allows an easier strategy for cell survival.

Effect of Yeast Autolysis on the Cell Growth and Biogenic Amines Production. From the six *O. oeni* isolates selected above, three, showing higher decarboxylase and proteolytic activities, were selected for this part of the work. The isolates were EVN1, EVN2, and EVN7. These isolates were grown in F and NF. Bacterial growth was very low in both cases. However, after 7 days of culture, all the malic acid was totally metabolized in both wine samples, meaning that cells were metabolically active. In the assay with wine supplemented with amino acids, similar results were observed. As expected, the NFs were richer in some peptidic amino acids: tyrosine, valine, isoleucine, leucine, phenylalanine, and lysine (results not shown). Table 1 shows the consumption of several amino acids for isolate EVN1, in either the F or the NF. Amino acid consumption was not significant for the other two isolates.

Despite using decarboxylase positive bacteria, no significant levels of BA were detected either in the presence of yeast debris (NF) or in its absence (F), even 25 days after MLF was accomplished. Only very low concentrations of histamine were formed, particularly in the NF. This fact can be attributed to pH and/or ethanol inhibition of enzymatic activities as the culture media used for decarboxylase detection had a pH value of 6 and did not contain ethanol, while the pH value of wine was 3.5.

No legal limits are established for histamine content in wine, but the values recorded were lower than the recommended German maximum limit (2 mg L^{-1}) (Mafra et al., 1997). No BAs were detected in wine supplemented with amino acids. These results do not agree with those of Ingargiola and Bertrand (1991) who reported an increase of BA concentration in wine due to an increase in its nitrogen content.

The results observed clearly show that BA formation cannot be directly related to the MLF of wine, which is in accordance with the findings of other authors (Lafon-Lafourcade, 1975; Delfini, 1989). It can be assumed that the ability of *O. oeni* to use wine peptide and to produce biogenic amines is not a constant characteristic of this species, and it seems to be strain dependent. The enzymatic system expression appears to be closely dependent on nutritional and energetical composition of the medium.

Even in the presence of *O. oeni* strains possessing both amino acid decarboxylase activity and proteolytic activity, it can be concluded from this study that wine is a medium which limits the formation of biogenic amines. The synthesis of significant amounts of these compounds does not normally occur under common and careful wine-making conditions. However, according to previous works, low concentrations of biogenic amines can be present in wine, depending on the colonization by undesirable bacteria, like *Pediococcus* sp., originating from the grapes and/or wineries, and also on the adopted viticultural and enological practices.

ABBREVIATIONS USED

BA, biogenic amine; HPLC, high-performance liquid chromatography; LAB, lactic acid bacteria; MLF, malolactic fermentation; MRS, Man, Rogosa, and Sharp.

ACKNOWLEDGMENT

Authors are grateful to Dr. Rogério Tenreiro from Faculdade de Ciências, Universidade de Lisboa, for permission to use bacteria isolated by his team.

LITERATURE CITED

Aerny, J. Origine de l'histamine dans les vins. Connaissances actuelles. *Bull. O.I.V.* **1985**, *656*−*657*, 1019−1061.

Bauza, J.; Blaise, A.; Teissedre, P. L.; Cabanis, J. C. Les amines biogènes du vin. Metabolisme et toxicité. *Bull. O.I.V.* **1995**, *767*−*768*, 42−68.

Cavin, J. F.; Prevost, H.; Lin, J.; Divies, C. Medium for screening *Leuconostoc oenos* strains defective in malolactic fermentation. *Appl. Environ. Microbiol.* **1989**, *55*, 751−753.

Delfini, C. Ability of wine malolactic bacteria to produce histamine. *Sci. Aliments* **1989**, *9*, 413−416.

Gibb, G. D.; Strohl, W. R. Physiological regulation of protease activity in *Streptomyces peucetius*. *Can. J. Microbiol.* **1988**, *34*, 187−190.

Guillout-Benatier, M.; Feuillat, M.; Ciolfi, B. Contribution à l'étude de la dégradation de l'ácide L-malique par les bactéries lactiques isolées du vin: Effet stimulant des autolysats de levures. *Vitis* **1985**, *24*, 59−74.

Ingargiola, M. C.; Bertrand, A. Origine des amines dans les vins. *FV - O.I.V.* **1991**, *896*.

Lafon-Lafourcade, S. L'histamine des vins. *Connaiss. Vigne Vin* **1975**, *2*, 103−115.

Lounvaud-Funel, A.; Joyeux, A. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. *J. Appl. Bacteriol.* **1994**, *77*, 401−407.

Lonvaud-Funel, A.; Masclef, J. P.; Joyeux, A.; Paraskevopoulos, Y. Étude des interactions entre levures et bactéries lactiques dans le moût de raisin. *Connaiss. Vigne Vin* **1988**, *22*, 11−24.

Mafra, I.; Sol, C.; Herbert, P.; Alves, A. Biogenic amines in Portuguese wines. In *Proc. I Symp. Appl. Chim. Anal. aux vins spiritueux* **1997**, 338−340.

Moolenaar, D.; Bosscher, J. S.; Ten Brink, B.; Drissen, A. J. M.; Konings, W. N. Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *J. Bacteriol.* **1993**, *175*, 2864−2870.

Niven, C. F.; Jeffrey, M. B.; Corlett; D. A. Differential plating method for quantitative detection of histamine producing bacteria. *Appl. Environ. Microbiol.* **1981**, *41*, 321−322.

Salema, M.; Lolkema, J. S.; San Romão, M. V.; Loureiro-Dias, M. C. The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. *J. Bacteriol.* **1996**, *178*, 3127−3132.

Smibert, R. M.; Krieg, N. R. Phenotypic characterisation. In *Methods for General and Molecular Bacteriology*; Phillip, G., Murray, R. G. E., Willis, A., Krieeg, N. R., Eds.; American Society for Microbiology: Washington DC, 1994; pp 607−654.

Veciana-Nogues, M. T.; Hernandez-Jover, T.; Marine-Font, A.; Vidal-Carou, M. C. Liquid chromatography method for determination of biogenic amines in fish and fish products. *J. AOAC Int.* **1995**, *78*, 1045−1050.

Wibowo, D.; Eschenbruch, R.; Davis, C. R.; Fleet, G. H.; Lee, T. H. Occurrence and growth of lactic acid bacteria in wine. A review. *Am. J. Enol. Vitic.* **1985**, *36*, 302−313.

Received for review October 19, 1999. Revised manuscript received April 11, 2000. Accepted April 11, 2000. This work was partially supported by the PAMAF Program, Project 2053 and by PIDDAC Program, Project 706/95.

JF991135V