Journal of Industrial Microbiology & Biotechnology (2002) 29, 134–139

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Yeast adapted to wine: Nitrogen compounds released during induced autolysis in a model wine

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As important as the blend of base wines before bottling, one of the most important steps in the champagne-making process is the long ageing on lees. Two yeast strains of *Saccharomyces cerevisiae* MC001 and MC002, used in champagne wine production, were allowed to autolyse. After 8 days of autolysis, active dry yeasts adapted to wine released 1.7- to 1.8-fold more nitrogen compounds than nonadapted active dry yeast. The nitrogen content (total, proteins, peptides and amino) present in autolysates was measured for yeasts adapted to wine. The composition of free amino acids and amino acids constituting peptides showed no difference between the two strains of yeast used. Studies of intracellular proteolytic activity and release of peptides showed no correlation between these two phenomena. These results indicate that yeasts adapted to wine give results similar to those that occur in wine during ageing.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 134–139 doi:10.1038/sj.jim.7000291

Keywords: wine; autolysis; nitrogen compounds; peptides; yeast

Introduction

During the champagne-making process, quantitative and qualitative differences appear due to base wines, which are used for the blend, and to the length of the ageing process on lees. This last step is one of the most important, because of the yeast autolysis that releases many compounds into the wine and so contributes to the development of characteristic flavours [3,8,15].

This autolysis occurs just before cell death. It refers to the release of cell substances due to the action of endogenous enzymes [2]. Resulting products are peptides, amino acids, mannoproteins, polysaccharides, diglycerides, monoglycerides, fatty acids, oligonucleotides, nucleotides and nucleosides [2,3,8,10,15,26,27]. In the course of autolysis, there is no disruption of the yeast cell wall, but there are modifications in its structure [27]. In champagne, studies performed after 15 years of ageing on lees showed that membrane thickness is decreased (i.e., becoming equivalent to the distance between two adjoining cells) and that their lipid composition is modified [25,27]. During ageing on lees, dead yeast released compounds whose size is a function of the porosity of their cell wall.

According to Leroy *et al* [17], amino acid levels increase mainly during ageing on lees in champagne wine. The slow liberation of nitrogen compounds originates from the yeast, and as such results from the release of intracellular proteins and free amino acids that occurs during yeast autolysis in sparkling wine [9,22–24,26]. Peptidic nitrogen increases on contact with lees during ageing of sparkling wines [24], and peptides are released as oligopeptides (up to 10 amino acid residues [11]) as demonstrated by Martinez-Rodriguez and Polo [22] with a model wine system. Grape proteins present in wine are considered to be resistant to proteolytic enzymes present in the medium. Their decrease, observed during ageing, was ascribed to their low solubility in ethanol and/or to their precipitation by bentonite added in wines as a fining agent [19].

Protease A, located in the vacuoles of *Saccharomyces cerevisiae* is responsible for degradation of the proteins [14]. At the wine pH, only protease A can be active [13]. It is an important enzyme during the proteolytic process [20] and its action causes the release of peptides in the course of autolysis during ageing on lees. In champagne wine, its activity is maximum after 6 years of ageing [17].

Most of the results reported on nitrogen compound release and proteolytic activity in model system wines were obtained from active dry yeasts. In the "méthode champenoise," however, active dry yeasts are adapted to wine before use. This approach could have an important impact on wine quality since Feuillat and Charpentier [6] reported different sensory properties according to the method employed for yeast preparation.

The present study is part of research aimed at expanding current knowledge on nitrogen compounds released by yeast during autolysis, which is characteristic of wine ageing on lees (like sparkling wine and champagne). The aim of this research was first to determine differences in the amino nitrogen compounds released between active dry yeasts adapted or not to wine and, secondly, to study changes in some nitrogen compounds released in a model wine medium during induced autolysis using two different yeast strains adapted to wine.

Materials and methods

Reagents

HPLC-grade methanol, selenium catalyst, Folin-Ciocalteux reagent and acetonitrile were obtained from Carlo Erba (Rodano, Italy). Trifluoroacetic acid was obtained from Merck (Darmstadt, Germany). HLPC-grade water was purified using a Milli-Q®

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Received 19 December 2001; accepted 14 June 2002

system (Millipore, Saint-Quentin en Yvelines, France). All other reagents were of analytical grade unless otherwise noted.

Biological material and adaptation of yeasts to wine

S. cerevisiae strains MC001 and MC002 (Moët & Chandon collection) were donated by Moët & Chandon (Epernay, France) in active dry form and stored at 4°C.

Moët and Chandon established a procedure for wine adaptation. Yeasts were first rehydrated according to the manufacturer's instructions. Cells were then washed and cultured (at 25°C) on Sabouraud's agar medium (Difco). Lastly, cells were accustomed to wine by serial culture in must and wine with increasing ethanol concentrations (up to 10% v/v). Cultures were then chilled at a rate of 2°C per day down to 4°C before use for the secondary "in bottle" fermentation and to facilitate yeast resistance to ethanol. Yeasts adapted to wine are named YGW (yeasts grown in wine) in this paper.

Autolysis conditions

Active dry yeasts (ADY) were rehydrated in water (10 g in 100 ml) at 30–35°C for 20 to 30 min with stirring, and washed three times with distilled water in order to remove additives used to maintain yeasts in active dry form and to reactivate the yeasts' metabolism before starting autolysis in model wine. Yeasts grown in wine were washed three times at 4°C with the autolysis model wine buffer (Feuillat [7], with minor modifications). After washing, each strain and preparation was seeded in the model wine buffer in individual flasks (3.7 and 3.5 g of ADY dry weight per 100 ml, and 2.7 and 2.8 g of YGW dry weight per 100 ml for MC001 and MC002, respectively). Model wine buffer contained ethanol (11.5%), malic acid (3 g/l), acetic acid (100 mg/l), potassium sulphate (100 mg/l) and magnesium sulphate (25 mg/l). Tartaric acid was not added to the medium (as mentioned in Ref. [7]) in order to prevent the salt's precipitation. pH was adjusted to 3.5 with NaOH. Autolysis was performed by incubating cell suspensions at 30°C. The medium was stirred at 200 rpm for 8 days. Everyday, homogenous samples of 100 ml were collected from a larger volume (made in a single batch) of autolysate under sterile conditions. They were then centrifuged for 10 min at 7500×*g* (4°C) to remove yeasts from collected samples. Autolysis was performed in duplicate for each strain.

Isolation of peptide fraction

During autolysis, the supernatant from each 100-ml sample was ultrafiltered on an Amicon apparatus (Amicon, Epernon, France) with a 10000-Da cut-off cellulose triacetate membrane. The ultrafiltrate (named peptide fraction in this manuscript) was concentrated three times under vacuum and stored at −20°C before analysis.

Intracellular Protease a Activity

For each centrifuged 100-ml sample, yeast pellets were washed in 0.1 M citrate buffer (pH 4.8) after centrifugation. Intracellular proteins and Protease A activity were measured. Protease A was extracted by breaking the cells with glass beads (0.45-mm) in a Braun homogeniser apparatus under CO_2 cooling. Cell debris was separated by centrifugation at 12000×*g* for 10 min at 4°C. Protease A activity of the extract was determined according to Jones [13].

Total nitrogen and protein analysis

Total nitrogen content was determined by the Kjeldahl method [16]. Proteins were measured according to the method of Lowry *et al* [18].

Amino acid analysis

Amino acids were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC), after precolumn derivatisation with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). The AQC reagent reacts rapidly with primary and secondary amino acids to yield highly stable derivatives that fluoresce strongly at 395 nm. The amino acid derivatisation was performed according to the specifications of the Waters AccQ-Tag method : 10 μl of sample, 20 μl of AQC reagent and 70 μl of 0.2 M borate buffer (pH 8.8) were mixed in a small tube. This solution was heated for 10 min at 55°C.

Separation was performed using an AccQ-Tag C_{18} column (150×3.9 mm I.D., 4-μm particle size; Waters-Millipore, Milford, MA) that was maintained at 37°C. A ternary gradient system was used. Eluent A consisted of Milli-Q water containing 19.04 g/l of sodium acetate, 1 mg/l of EDTA, and 0.975 ml of triethylamine. The eluent was titrated to pH 5.8 with phosphoric acid. Eluent B was acetonitrile and eluent C was Milli-Q water. The flow rate was set at 1 ml/min. The following gradient was used: initially, 100% A; 1 min, 1% B; 16 min, 3% B; 25 min, 6% B; 35 min, 14% B; 40 min, 14% B; 40–51 min, 60% B and 40% C; 51–54 min, 100% A; 54–100 min, 60% B and 40% C. The eluted AQC derivatives were detected by monitoring their fluorescence at 395 nm using an excitation wavelength of 250 nm. A standard solution of amino acids (0.1 mmol/l) was injected prior to each analysis run. One millimole per liter of α-aminobutyric acid was used as internal standard. Variation coefficient of the amino acid analysis was less

Table 1 Free amino acids and total nitrogen released after 8 days of autolysis (30°C) active dry yeasts and yeasts grown in wine

Amino acids (μmol/g idm)[a]	MC001 strain		MC002 strain	
	ADY	YGW	ADY	YGW
Asp	23	114	25	119
Glu	20	108	23	118
Ser	18	092	14	107
Asn	11	168	9	187
Gly	10	96	10	121
Gln	6	181	5	225
His	1	19	1	22
Thr	16	72	14	84
Arg	9	55	8	64
Ala	53	238	51	234
GABA[b]	28	126	30	116
Pro	15	38	20	42
Tyr	17	50	14	61
Cys	nd[c]	nd	nd	nd
Val	39	131	34	150
Met	16	49	14	54
Ileu	26	91	20	103
Leu	61	173	48	202
Lys	14	79	11	83
Phe	29	84	23	110
Sum of free amino acids	417	1964	372	2202
Total nitrogen (mg/g idm)	45	78	42	76

[a]idm: initial dry matter.
[b]GABA: γ-aminobutyric acid.
[c]nd: not detected by the HPLC method.

than 2%. Total amino acids (in the peptide fraction) were determined after hydrolysis of samples in glass tubes with 6 M HCl, under vacuum, at 110°C for 24 h.

Amino acids were named by their symbols established by IUPAC-IUB [12]. In Table 3, Asp and/or Asn and Glu and/or Gln are reported as Asx and Glx, respectively, because Asn and Gln are partially converted to Asp and Glu, respectively during acid hydrolysis.

Results and discussion

Comparison of amino nitrogen profiles of autolysates from ADY and YGW

Table 1 shows mean values of free amino acids and total nitrogen released after 8 days of autolysis for the two strains and the two preparations. Significant differences in the total amount of free amino acids released were found between ADY and YGW samples. MC001 and MC002 YGW strains released 4.7× and 5.9× more free amino acids than ADY, respectively. In a similar fashion, total nitrogen in autolysates increased significantly for the two YGW strains. During autolysis, free amino acid profiles were qualitatively different between YGW and ADY. For MC001 and MC002, Asn, Gly and Gln accounted for about 20% of all amino acids with YGW and only for 6% with ADY. Conversely, Pro, Tyr, Val, Leu and Phe accounted for about 35% of all amino acids with ADY, and only for 25% with YGW.

Martinez-Rodriguez and Polo [22] showed that the prevailing amino acids released by autolysis of ADY in a model wine, using a *S. cerevisiae* var. *bayanus*-like strain, were GABA, α-Ala, Phe and Leu [22]. In our work, the prevailing amino acids released by autolysis of ADY were Leu, α-Ala, Val, Phe and GABA, which is

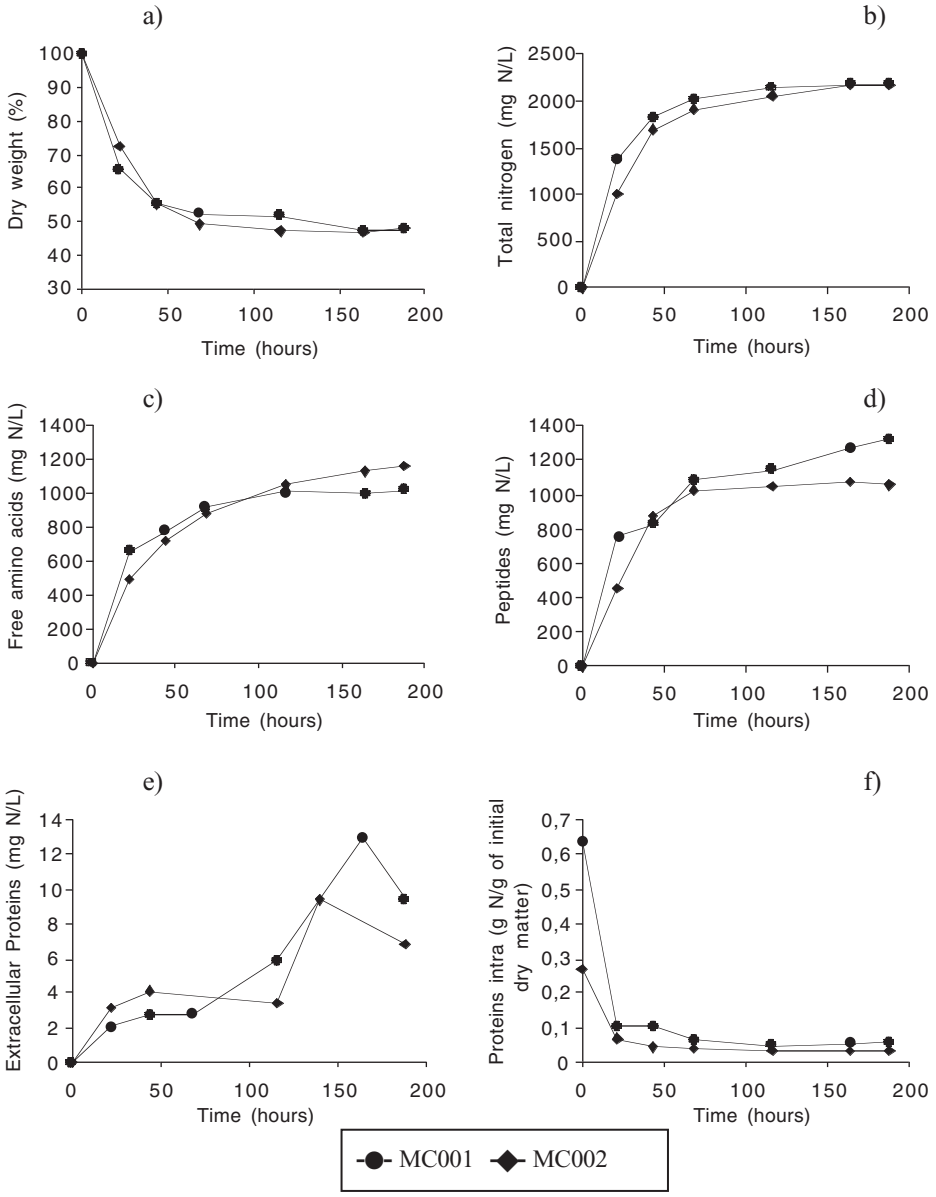

Figure 1 Changes in dry weight (a), total nitrogen (b), free amino acids (c), peptides (d), extracellular proteins (e) and intracellular proteins (f) content during autolysis of MC001 and MC002 yeasts grown in wine.

Table 2 Evolution of intracellular proteolytic activity during autolysis of MC001 and MC002 yeasts grown in wine

Intracellular Proteolytic activity[a]	Time (h)						
	0	22	44	68	116	164	188
MC001	7.7	25.1	46.7	112.4	165.6	151.0	164.9
MC002	14.8	15.8	72.8	60.9	91.0	140	143.6

[a]Specific activity (μg Tyr/min/mg protein).

in agreement with the above results obtained under the same experimental conditions. With YGW, α-Ala, Gln, Asn, and Leu were predominant. These profile changes might be due to different amino acid synthesis by YGW, which is related to their growth stage.

Adaptation to wine seems to influence amino acid synthesis and/or the release of nitrogen compounds in to the medium due to a higher level of synthesis in the cell. Adaptation of yeast to wine may have a key role in enzymatic activities (particularly for Protease A) that are responsible for the release of cellular components.

Change of nitrogen compound contents during induced autolysis for the MC001 and MC002 YGW strains

During autolysis of YGW, dry matter swiftly decreased by about 40% in the first 2 days (Figure 1a) and then reached a plateau close to 45% of the initial dry matter. A consequence of autolysis was thus a decrease in total dry matter of the lees [10]. Such a loss of biomass under our experimental conditions was different from that observed by Fornairon-Bonnefond *et al* [10] and Martinez-Rodriguez *et al* [23] with ADY. These authors showed a 17% to 19% decrease in dry matter after 21 days of autolysis at 28°C, and a

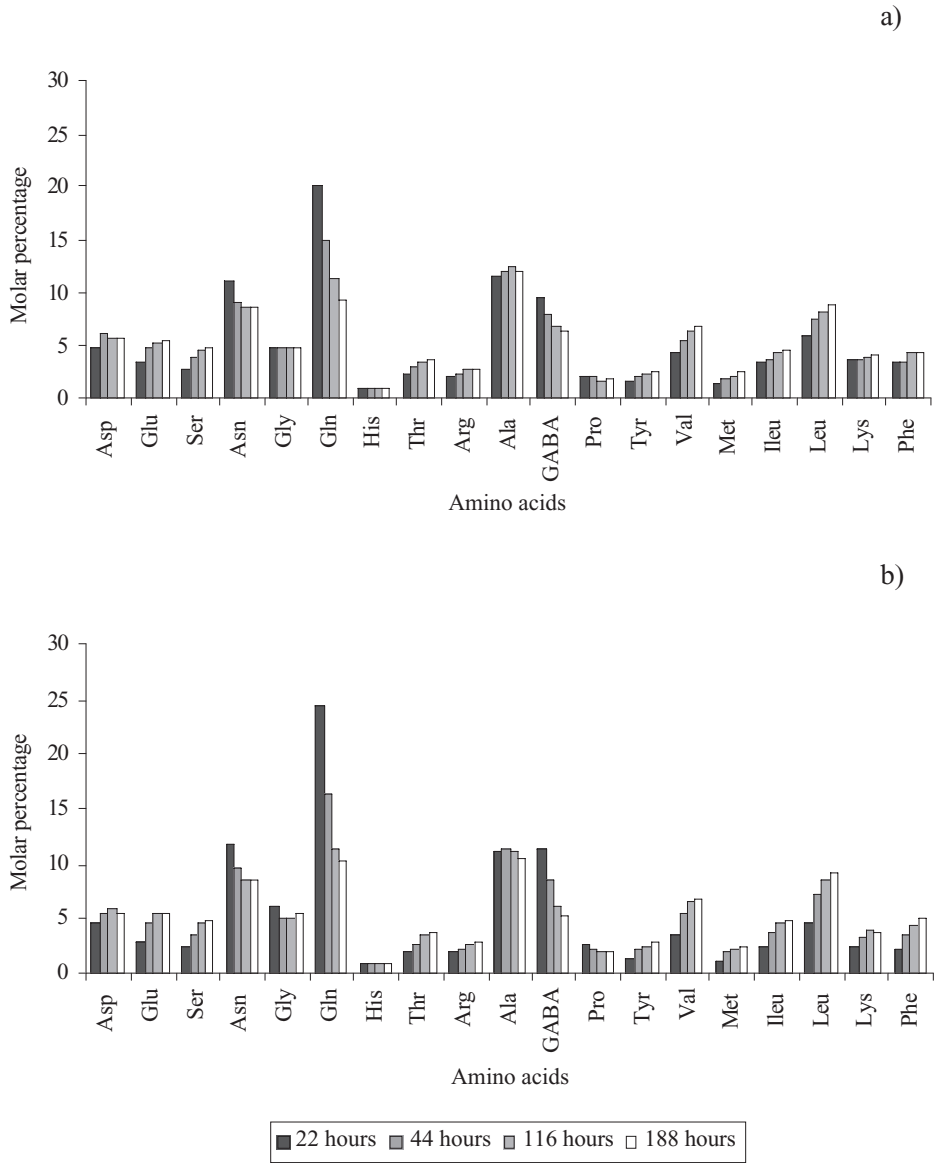

Figure 2 Changes in free amino acids (expressed as a molar percentage) during autolysis of MC001 (a) and MC002 (b) yeasts grown in wine. GABA: γ-aminobutyric acid. Cys was not determined.

Table 3 Amino acid distribution (expressed as a molar percentage) in peptides present in MC001 and MC002 autolysates after 188 h of autolysis

Amino acid	MC001 strain	MC002 strain
Asx	6.20	5.53
Glx	12.87	10.73
Ser	6.44	6.22
Gly	12.15	12.46
His	3.16	2.94
Thr	6.87	7.08
Arg	4.92	4.70
Ala	8.46	8.17
Pro	5.77	9.16
Tyr	3.91	3.22
Val	5.64	5.83
Ileu	5.13	5.67
Leu	6.01	5.23
Lys	6.98	9.41
Phe	3.89	2.25
Met	–	0.26

31% to 42% decrease after 216 h of autolysis at 30°C, respectively. Our results are more comparable with those obtained by Leroy *et al* [17] who observed that lees from champagne wine represent only 50% of the initial dry content at the beginning of ageing.

Figure 1 also shows changes in total nitrogen (Figure 1b), free amino acids (Figure 1c) and peptides (Figure 1d) released in the extracellular medium as well as changes in extracellular (Figure 1e) and intracellular proteins (Figure 1f) observed during induced autolysis for the two strains. In Figure 1d, the concentration of peptides was computed from the difference in amino acid concentrations before and after hydrolysis of the peptide fraction. A large increase in total nitrogen occurred in the first day for both strains (62% and 46% increase for MC001 and MC002, respectively). Variations in free amino acids and peptides were almost similar to that of total nitrogen. After 1 day of autolysis, 60% and 38% of free amino acids and 57% and 43% of peptides were released for MC001 and MC002, respectively. These results are slightly different from those obtained by Martinez-Rodriguez and Polo [22] who found that 71% of free amino acids were released after 24 h of autolysis by using ADY (with a strain closely related to MC002). Under our experimental conditions, this ratio was obtained after 44 and 68 h of autolysis for MC001 and MC002, respectively. Extracellular proteins increased during the first 2 days of autolysis, reached a plateau after 44 h, then increased again from 68 to 164 h for MC001 and from 116 to 140 h for MC002. This observation may be due to the size of the pores in the yeasts cell walls that is known to increase during autolysis, or to the release of cell-wall proteins. After reaching their maximum values, the quantities of extracellular proteins decreased for both strains, an effect probably due to the release of a proteolytic activity from yeasts in the autolysate. This evolution is somewhat different from that observed by Martinez-Rodriguez *et al* [23]. They reported a strong release of extracellular proteins (with maximum quantities equivalent to ours) within the first 24 h of autolysis and then a slow decrease due to enzymatic breakdown. In our case, most compounds were released within the first 116 h with no subsequent changes. MC002 released more free amino acids in the autolysate than MC001, an effect probably caused by a stronger proteolytic activity released in the medium by MC002 as hypothesized by Alexandre *et al* [1].

A fraction of the amino acids released during the first 22 h of autolysis may be explained by the exsorption of yeast internal contents that occurs during the first 4 h [21,22], which can be accompanied by cell wall peptides [4]. Thus, half of the total nitrogen was found in the autolysate after 22 h of autolysis. The reduction of intracellular proteins after 68 h would suggest that proteolytic activity is released in the medium as reported by Leroy *et al* [17] and Martinez-Rodriguez and Polo [22]. In our study, such an activity was not detected in autolysates of the two strains under investigation. We noted, however, a strong correlation ($R>0.95$) between peptide release, free amino acid release and intracellular proteolytic activity until 116 h of induced autolysis (Table 2). During this period, formation of peptides and free amino acids would mainly occur in cells. At times longer than 116 h, all nitrogen metabolites were released into the extracellular medium.

Figure 2 reports the molar percentage of each amino acid at different times of autolysis for each strain. After 22 h, Glu, α-Ala, Asp and GABA were the most prevalent free amino acids released in the medium for both strains. In the course of autolysis, the distribution of free amino acids was fairly similar for the two strains. Leu significantly increased in the medium during autolysis with both strains and became one of the most prevalent free amino acids at 188 h, even though it was not at 22 h. In the same manner Ser and Val significantly increased but were not prevalent. On the other hand, prevalence of GABA and Gln greatly decreased in the course of the 8 days of autolysis at the expense of most other amino acids.

In the study of Rodriguez-Martinez and Polo [22], the distribution of free amino acids was quite different. α-Ala, GABA and Leu were prevalent as reported in our work, although contents of Phe, Glu and Arg were different. However, the distribution of amino acids during autolysis under our experimental conditions was similar to data obtained with champagne wine [5].

Changes in free amino acids and in the distribution of amino acid residues obtained in this work are in good agreement with those of Alexandre *et al* [1] who investigated the effect of fermentation on autolysis. Peptides released in the medium (Table 3) were mainly composed of Gly, α-Ala, Glx, Lys, Asx, Pro, Thr and Ser. In our case, the distribution of amino acid residues was virtually identical to that of Desportes' work [5]. Except for Lys and Pro, which were more frequent in peptides released during autolysis with MC002, all amino acids constituting peptides were distributed in the same order for MC001 and MC002 during autolysis. Consequently, only the measurements performed at 188 h are shown in Table 3.

In conclusion, there is no strain effect on the composition of free amino acids and of peptides released during autolysis with MC001 and MC002. ADY suspended in autolysis medium were used in most of the published studies [9,21–23]. This does not reflect cell physiology at the end of the alcoholic fermentation. Our results show that adaptation of yeast to wine leads to data very similar to what has been observed in champagne wine. Thus, yeast preparation could play a key role in the characteristics of wines ageing on lees.

Acknowledgements

The authors thank Bruno Duteurtre (Director of the Laboratoire de Recherche Moët and Chandon) and Francis Duchiron (Director of the Laboratoire de Microbiologie Industrielle of the Université de Reims Champagne-Ardenne, France) for access to their technical equipment. They gratefully acknowledge Jocelyne Guerreau for her

technical assistance and Matthieu Kaltenbach for proofreading this manuscript.

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