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Influence of some factors on autolysis of *Oenococcus oeni*

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Summary

***Oenococcus oeni* was able to autolyse after transfer to water at 40 °C. Cells harvested in the mid-log phase autolyse more rapidly than in other growth phases. Optimum conditions of pH (broad scale with two maxima at 4.0 and 7.0) and temperature (40 °C) have also been elucidated. Trypsin and EDTA appeared to enhance cell wall autolysis. The activity of several enzymes, namely an amidase or endopeptidase and a N-acetyl-ß-D-glucosaminidase, were shown to be involved in the autolytic process, thereby releasing soluble cell wall fragments and nitrogen compounds.**

K e y w o r d s : *Oenococcus oeni*, autolysis, N-acetyl-ß-D-glucosaminidase.

Introduction

Bacterial autolysis can be defined as a cell breakdown phenomenon due to the hydrolysis of various and specific bonds in their cell peptidoglycan by cell wall located enzymes, called autolysins (SHOCKMAN *et al.* 1996). Autolysins, *e.g.* ß-1,4-N-acetylmuramidases, ß-1,4-N-acetylglucosaminidases, N-acetylmuramyl-L-alanine amidases and peptidases are present in a wide variety of bacteria and might all be present in the cell (ROGERS *et al.* 1984). The role of these endogenous hydrolases is not limited to autolysis: they are also involved in cell surface growth, cell division and the peptidoglycan formation. These peptidoglycan hydrolases are potentially dangerous for the cell and need to be precisely regulated (CHAPOT-CHARTIER 1996; SHOCKMAN *et al.* 1996).

Autolysis can occur spontaneously after a stationary phase, when the culture medium is depleted of the available supply of nutritionally component(s) or after induction. Many studies have focused on strain dependence of autolysis, biochemical characterisation of the autolysins, effects of environmental parameters on the extent of autolysis (VEGARUD *et al.* 1983; ROGERS *et al.* 1984; LEMEE *et al.* 1994; DAKO *et al.* 1995). Various methods to induce bacteria cell autolysis have also been reported like down-shock-upshock treatment, transfer of cells to buffered solutions, use of detergent, rapid osmotic or EDTA shock treatments (CHAPOT-CHARTIER 1996).

In winemaking, malolactic fermentation occurs naturally after the alcoholic fermentation has ceased. In Burgundy wines, *0enococcus oeni* is the species most frequently associated with this second fermentation. Burgundy wines are mainly kept in barrels and can stay for some time on bacterial lees. Few studies have investigated the ability of *0. oeni* to autolyse (GARBAY and LONVAUD-FUNEL 1990). In contrast many studies have been conducted on yeast autolysis and the released products like proteins and glycoproteins which are associated with interesting enological properties, like aroma fixation or flavour increase FEUILLAT 1998). However a better knowledge of the release of different products such as nitrogen compounds or bacterial enzymes in the wine is of great interest because they can also have an impact on wine flavor and its organoleptic characters.

The present study investigates the conditions favouring autolysis of *0. oeni* and the specificity (of action) of the autolytic activity in these microorganisms.

Material and Methods

Choice of strain and growth conditions: Four strains of lactic acid bacteria *0. oeni* were selected from the laboratory collection. Two strains had shown to spontaneously autolyse in the growth medium just after the cells entered the stationary phase. From these two strains, the strain MV was chosen as a model because of its loss of A_{600} after the induction of autolysis of whole cells. Cultures of *0. oeni* MV were grown in a FT80 medium (GUILLOUX-BENATIER *et al.* 1998) and incubated at 25 °C. Growth was followed by measureing optical density at 600 nm using a Corning 253 spectrophotometer and viable cells were counted by plating on FT80 agar medium.

Induction and measurement of autolysis: Whole cells were harvested (7000 g, 20 min, 4 °C) in the middle of the exponential growth phase (except were indicated), washed twice with NaCl 155 mM and resuspended in sterile distilled water. The suspensions were then incubated at 40 °C (chosen for optimal temperature as shown in Fig. 3 a) without stirring. To determine the effect of pH on autolysis, the cell suspension was resuspended in various buffers: citrate 0.05 M, phosphate 0.1M (pH range 3.0-3.5), Na-acetate 0.1 M (pH range 4.0-5.5) and Na-phosphate 0.1 M (pH range 6.0-8.0). To determine the effect of temperature, the cell suspension was incubated at various temperatures (10-60 °C). Autolysis of whole cells was also measured in the presence of trypsin (0, 10 and 50 !JM, Sigma) or EDTA (0.1 mM).

In all cases, cell lysis was followed by measuring the decrease of turbidity at 600 nm with time using a Shimadzu UV160 spectrophotometer. The extent of autolysis (%) was

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displayed as the percent decrease of A_{600} as a function of time (24 or 26 h). Each test was performed in triplicate and the values given represent the average.

Biochemical assays: Autolysis of the cells was followed by measuring the solubilization of the cell material. Samples were incubated for various times and centrifuged (7000 *g*, 20 min, 4 °C). The supernatants were used for analyses. The liberation of the cell material was followed by measuring the absorbances of supernatants at 260 and 280 nm using a Shimadzu UV 160 spectrophotometer. Autolysis of the cell walls was also investigated chemically.

Protein contents were determined by the Lowry method using bovin serum albumin (BSA) as a standard. Free amino groups were determined by the reaction with 2,4-dinitrofluorobenzene as described by GHUYSEN *et al.* 1966 using leucine as a standard. Liberation of reducing groups was assayed by the Park-Johnson procedure as modified by GHUYSEN *et al.* (1966). Acetylhexosamines were determined by the Morgan-Elson reaction (30 min in borate buffer at 100 °C) as modified by GHUYSEN *et al.* (1966). N-acetyl-ß-D-glucosamine (NAG) was used as standard to quantify reducing groups and acetylhexosamines.

Quantitative amino acids and hexosamines (muramic acid and glucosamine) analyses were performed by reversed-phase HPLC after a precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters AccQ.Tag method). Supernatants were concentrated 5-fold by evaporation in vacuum and were analysed before and after hydrolysis in sealed tubes with 6 M HCl at 110 °C for 24 h. Hydrolysates were then evaporated to dryness under N_2 and resuspended in water. After injection into the analyser, derivates were quantified by fluorescence detection.

N-acetyl-ß-D-glucosaminidase activity: This activity was measured for cells and in the supernatants during autolysis according to the method previously described (GUILLOUX-BENATIER *et al.* 1993). To 2 ml of solution (bacterial biomass or supernatant) containing enzyme a 50 !Jl volume of a sterile aqueous solution of 4-methylumbelliferyl N-acetyl-ß-D-glucosaminide was added to the sample, yielding a final concentration of 1,5 !JM. The fluorescence of the 4-methylumbelliferone (4MU) produced by hydrolysis of the substrate was measured as a function of time over 20 to 30 min with a Hitachi F2000 fluorimeter.

Results and Discussion

Optimal conditions for autolysis of whole cells: The induction of autolysis in *0. oeni* cells by osmotic shock was investigated. When washed exponential-phase cells grown at 25 °C in FT80 medium were rapidly suspended in distilled water at 40 °C, a decrease in turbidity was observed (Fig. 1). Concomitantly, UV-absorbing material was observed. These facts were especially marked during the first 5 h while no further significant change was observed after prolonged incubation (26 h). Inhibition and/or degradation of the enzyme system responsible for autolysis may appear after a longer incubation period (LORTAL *et al.* 1989).

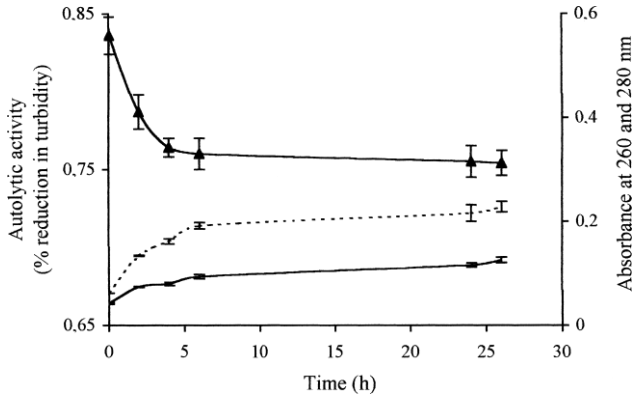

Fig. 1: Kinetics of autolysis of *Oenococcus oeni*. Exponential phase cells were grown at 25 °C in FT80 medium and autolysis was assayed as loss of A_{600} of the cell suspensions (.._) during incubation in distilled water at 40 °C and as measure of A_{260} (..) and A_{280} (-) of supernatants obtained after centrifugation.

During growth of *0. oeni* in the broth medium, bacteria were harvested and assayed for autolytic activity at the times indicated in Fig. 2. The harvesting of cells at different phases of growth modified greatly the extent of autolysis. The autolytic activity increased during the exponential phase of growth, reaching highest activities at the mid-log phase.

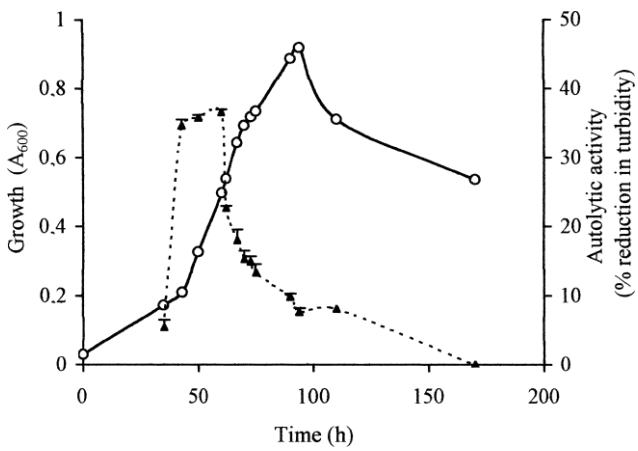

Fig. 2: Changes in autolytic activity (.._) during growth (o) of *Oenococcus oeni*. The organisms were grown at 25 °C in FT80 medium and bacterial growth was followed at 600 nm. The percentage of decrease in A_{600} after 24 h was used to compare autolytic activity.

Thereafter, the bacteria showed a marked decrease in autolytic activity. Therefore exponential phase cells at the mid-log phase were used further experiments. The potential ability of rapidly growing cells to lyse is not unique to *0. oeni* (NEUJAHR and LOGARDT 1973; NISKASAARI 1989) and supports the suggestion that autolysins play an important role in cell division and bacterial growth (CHAPOT-CHARTIER 1996). Moreover like for *L. lactis* (BUIST *et al.* 1998) or dairy propionibacteria (LEMEE *et al.* 1994; LORTAL *et al.* 1997), the culture of *0. oeni* showed a decrease of A_{600} just after the maximal growth correlated with low cell viability (<0.1 %). The absence of any stationary phase could be explained by spontaneous autolysis in the culture medium and suggests that nutrient starvation induces the autolytic system.

The effects of temperature and pH on autolysis of *0. oeni* cells are shown Fig. 3 a and b, respectively. The extent of autolysis was clearly dependent on the temperature at which cells were incubated (Fig. 3, a). A temperature of 40 °C appeared to be the most efficient to induce autolysis. Like for *L. fermenti* (NEUJAHR and LOGARDT 1973), the optimal temperatures for lysis and growth were different, 40 and 25 °C respectively. An interpretation of the autolytic activities at various pH values was difficult due to the very broad scale of optimum pH value. The autolytic activity was low at high and low pH values presumably due to denaturation of the autolysin(s). A large scale of optimal pH was also noted for *L. helveticus* without any explanation (LORTAL *et al.* 1989). Two maxima appeared, the first at pH 4 and the second at about pH 7; they possibly indicate optimal pH for two or more enzymes involved in the autolytic system. Previously we have found pH 4 being optimal for N-acetylglucosaminidase activity (GUILLOUX-BENATIER *et al.* 1993). Therefore we conclude that another autolytic enzyme with a more neutral pH optimum is involved.

Trypsin caused a considerable increase in the extent of autolysis in exponential phase cells of *0. oeni*. After 24 h of incubation, the decreases in A_{600} were 14, 44 and 53 % in the presence of 0, 10 and 50 !JM trypsin, respectively. This suggests a latent autolytic activity in *0. oeni* similar to *S. faecalis* (SHOCKMAN *et al.* 1967). The higher rate of cell wall autolysis may be due to an activation of endogenous autolysins, which could enhance the dissolution process (NISKASAARI 1989). The addition of EDTA (0.1 mM) had the same effect with regard to the extent of autolysis: 27 % with EDTA and only 14 % for the control. The stimulation of autolysis by a chelating agent such as EDTA has already been observed with other gram-positive bacteria (LORTAL *et al.* 1989). EDTA possibly removes bivalent cations with an inhibitory effect on autolysis from the medium.

Mode of action of the autolytic system: Cells were grown in FT80 medium and harvested at the mid log phase. Cells were washed, suspended in water and incubated at 40 °C for 26 h. Specific biochemical residues appeared in parallel to cell wall autolysis (Tab. 1). Proteins were released during the decrease of A_{600}. The clear increase in reducing groups, which were not reactive in the Morgan-Elson reaction, strongly suggests the occurrence of only one glycosidase, an N-acetylglucosaminidase. This glycosidase cleaves the linkages between the N-acetylglucosaminyl and the N-acetylmuramyl residue in the polysaccharide chains of the peptidoglycan. In addition free amino groups are also released in the supernatant during autolysis which reflects the presence of an amidase or endopeptidase activity in the autolytic system of *0. oeni*. Thus it corresponds to the autolytic systems in related organisms (CHAPOT-CHARTIER 1996).

The N-acetylglucosaminidase activity was tested both in the supernatant and in the bacterial biomass at different times during incubation (Tab. 1). This enzymatic activity was never detected in the extracellular medium but could be measured during the first 4 h of incubation only in the bacterial biomass. Autolysins of *0. oeni*, like those of *S. thermophilus* (SANDHOLM and SARIMO 1981), appeared to be tightly bound to the cell and were not released during lysis.

If the degradation of peptidoglycan by specific endogenous hydrolases is possible, it can be expected that peptidoglycan fragments would be released in the extracellular medium in the course of autolysis. Thus the amino acid content of the supernatant was determined after incubation of whole cells of *0. oeni* in water at 40 °C for 24 h. Analyses were carried out before and after acid hydrolysis of the supernatant (Tab. 2). After autolysis a free amino acid content of 217 nM in the supernatant indicated that free amino acids had been released during lysis. Moreover we observed an increase in the amino acid content after acid hydrolysis of 494 nM. This indicates a liberation of proteins or peptides in the medium during cell wall lysis. The absence of free muramic acid and glucosamine residues in the supernatant before acid hydrolysis was strongly correlated only with

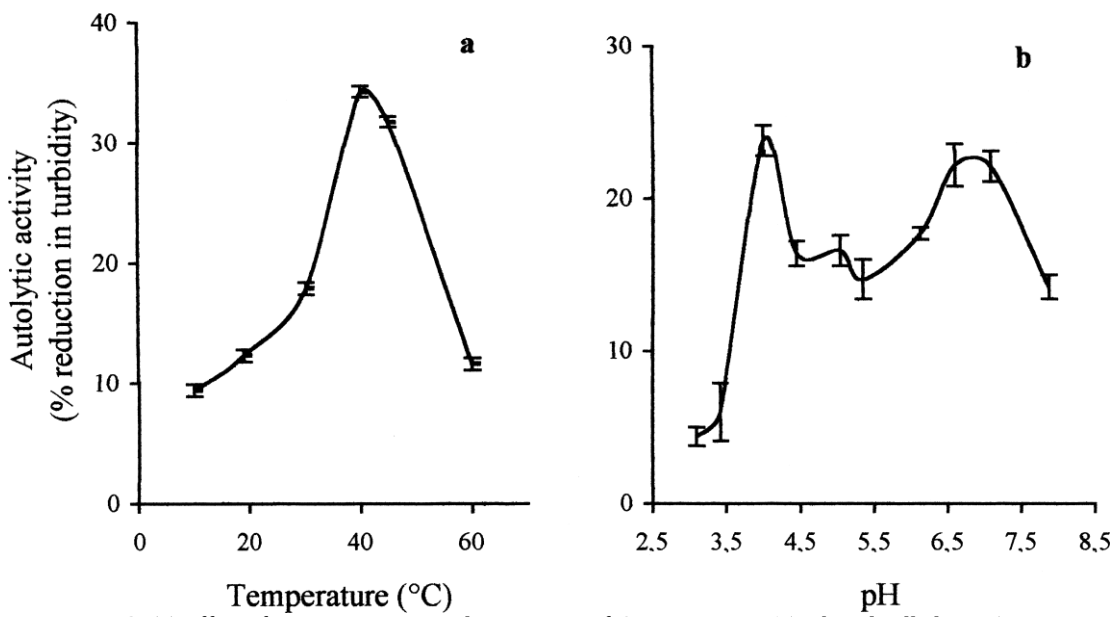

Fig. 3: (**a**) Effect of temperature on autolytic activity of *Oenococcus oeni* (24 h in distilled water); (**b**) effect of pH on autolytic activity of *Oenococcus oeni* (24 h in various buffers at 40 °C).

Table 1

Autolysis (26 h) of *Oenococcus oeni* in water at 40 °C followed by a reduction in turbidity and the release of several products

	t = 0	t = 2 h	t = 4 h	t = 6 h	t = 26 h
$A_{600\ nrn}$	0,836 (0,012)	0,780 (0,021)	0,761 (0,006)	0,763 (0,014)	0,754 (0,008)
Reducing groups (!JM NAG)	9,8 (0,6)	18,3 (0,9)	23,2 (0,8)	26,7 (0,9)	32,6 (0,8)
Hexosamines with Morgan-Elson reaction (!JM NAG)	0	0	0	0	0
Free amino groups (mM Leu)	0,15 (0,03)	0,45 (0,03)	0,71 (0,09)	0,62 (0,03)	0,58 (0,09)
Proteins ($mg \cdot l^{-1}$ BSA)	8,3 (1,3)	7,9 (2,3)	10,4 (1,3)	-	18,7 (1,4)
N-acetylglucosaminidase activity in bacterial biomass ($nmole\ 4MU \cdot min^{-1} \cdot g^{-1}$)	54,9 (3,8)	28,3 (2,3)	2,4 (1,5)	0	0

Number in brackets correspond to standard deviation

Table 2

The amino acid and hexosamine content after autolysis of *Oenococcus oeni* (24 h, 40 °C in water) before and after acid hydrolysis of supernatants

	Content (nM) Supernatant before hydrolysis	Supernatant after hydrolysis
Muramic acid	0	10
Glucosamine	0	13
Asp	0	25
Glucosamine	41	73
Ser	3	35
Gly	23	46
GLn	2	0
His	6	10
Thr	31	32
Arg	2	7
Ala	18	68
Gaba	8	0
Pro	2	9
Tyr	1	3
Val	8	18
Met	0	2
0rn	28	46
Ileu	0	6
Leu	2	10
Lys	42	78
Phe	0	3
Total	217	494

the action of one glycosidase: the N-acetylglucosaminidase. Thus, in this study, it was possible to demonstrate that *0enococcus oeni* is able to autolyse. Further investigations are needed to determine the nature of the released products, the enzymes and their real impact on wine flavor and organoleptic characters.

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