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Lysis of yeast cells by *Oenococcus oeni* enzymes

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Oenococcus oeni **exhibited extracellular β (1→3) glucanase activity. This activity increased when cells were cultivated with glycosidic cell-wall macromolecules. In addition, the culture supernatant of the organism effectively lysed viable or dead cells of *Saccharomyces cerevisiae*. This lytic activity appeared in the early stationary phase of bacterial growth. Yeast cells at the end of the log phase of growth were the most sensitive. The optimum temperature for lysis of viable yeast cells was 40°C, which is very different from the temperatures observed in enological conditions (15–20°C). Moreover, the rate of the lytic activity was significantly lower in comparison with yeast cell wall-degrading activities previously measured in various other microorganisms. Therefore, yeast cell death that is sometimes observed during the alcoholic fermentation could hardly be attributed to the lytic activity of *O. oeni*.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 193–197.

Keywords: yeast cell lysis; *Saccharomyces cerevisiae*; *Oenococcus oeni*; β (1→3) glucanase

Introduction

Various microorganisms are capable of degrading different sorts of biopolymers such as chitin [14], wood polysaccharides [1] and agro-industrial residues [21]. Moreover, several different species of microorganisms also produce extracellular enzymes which can be used in the preparation of biologically active products with desirable properties [18,20].

Various microorganisms have been shown to accumulate enzymes in the culture media with lytic activities against yeast cell walls. In general, β-glucan fractions (the main structural component important for the rigidity of yeast cell walls) from yeast cell walls induced the highest yeast lytic activities. The ability to lyse cell walls of viable yeast is mainly associated with the production of β (1→3) glucanases, although β (1→6) glucanases and proteases with lytic activity have also been characterised [7,23]. Usually, the glucanase activity will not lyse viable yeast cells without either a thiol-reducing agent or a second enzyme activity [2,19]. Such studies have many applications in the yeast industry: production of yeast extract [22], production of commercial enzyme preparations such as zymolyase [13] and liberation of the yeast wall mannoproteins for structural analysis [25].

In winemaking, malolactic fermentation, the bacterial conversion of L-malic acid to L-lactic acid and carbon dioxide, usually occurs subsequent to the alcoholic fermentation, when certain strains of lactic acid bacteria (especially *Oenococcus oeni*) grow to sufficient cell numbers. But in some cases, slow or stuck alcoholic fermentations are observed in conjunction with uncontrolled growth of lactic acid bacteria [10,11]. Under these conditions an accelerated death rate of yeast cells was observed [15]. This stimulation of yeast autolysis could be due to bacterial glucanase activities. Moreover, cell-wall polyosides (glucans and mannoproteins) liberated by *Saccharomyces cerevisiae* during the alcoholic fermentation or autolysis stimulate growth of *O. oeni* and the production rates of different enzymatic activities such as aminopeptidase, α- and β-glucosidases and *N*-acetyl-β-glucosaminidase [8].

The present study was undertaken to examine the possibility that *O. oeni* secretes enzymes with significant lytic activity against yeasts into the culture medium and their possible action on yeast cells.

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Materials and methods

Chemicals

Laminarin was purchased from Sigma (Saint Quentin, Fallavier, France). Macromolecules (glucans and mannoproteins) released from cell walls of *S. cerevisiae* var. *cerevisiae* (strain isolated by our laboratory as Levuline Brg™ and commercialised by Oenofrance, Rueil-Malmaison, France) after 48 h of autolysis [5] were isolated by ethanol precipitation and dried with diethyl ether. Total carbohydrates were determined by the phenol–H_2SO_4 method [4] using glucose as a standard, and proteins were measured by the Lowry method using bovine serum albumin as a standard. All other reagents were analytical grade from commercial sources.

Bacteria and cultivation conditions

O. oeni isolated in our laboratory and marketed as Malolactine O (Oenofrance) was used in all experiments. Cells were grown at 20°C in an FT 80 liquid medium, pH 3.5 [9]. Growth was estimated by measures of optical density at 600 nm in a Corning Model 253 spectrophotometer. For the determination of glucanase activities, lactic acid bacteria were grown in a modified FT 80 liquid medium containing yeast nitrogen base sterile solution (6.7 g l^{-1}, Difco reagent) instead of yeast extract. Wine yeast-

Table 1 β (1–3) glucanase activity of *O. oeni* cultivated with and without the presence of yeast macromolecules, in biomass (mg reducing sugar mg^{-1} biomass h^{-1}) and in culture supernatant (mg l^{-1} reducing sugar per 1 ml of enzyme solution after 1 h) during bacterial growth

Growth	− Yeast macromolecules		+ Yeast macromolecules	
	Biomass	Supernatant	Biomass	Supernatant
Start of log phase	0.17	81	0.12	177
Mid-log phase	0.94	103	0.58	196
End of log phase	0.30	148	0.14	204
Stationary phase	0.16	78	0.09	94

precipitated macromolecules were added to 1 g l^{-1} into the culture medium.

Preparation of enzyme solution from culture of O. oeni

During cultivation, the lactic acid bacteria were harvested, centrifuged (8000×*g*, 30 min, 4°C) and washed twice with a saline solution (155 mM NaCl). The cells were then resuspended in appropriate buffer for enzyme activity determination. The culture supernatants were concentrated by ultrafiltration on a Millipore membrane 5000 Da at 4°C. The concentrates were diluted with appropriate buffer for enzyme activity determination and kept at 4°C before utilisation. A 10-fold concentration of the supernatants needs only 2.5 h and was sufficient to determine the lytic activity against yeast cells (see Results). Further concentration of the bacterial supernatant could result in a great loss of yeast lytic activity due to enzyme denaturation. In fact, we observed a total loss of yeast lytic activity in the supernatant after 12 h at 4°C (data not shown). Based on these data, we used culture supernatants of *O. oeni* that were concentrated 10-fold by ultrafiltration.

Yeast cell preparations

Yeast cells at various growth stages were prepared as follows: *S. cerevisiae* was maintained on agar slants of Wickerham medium. Cells were grown at 25°C in a medium containing per liter of distilled water (in g): 75 glucose, 85 fructose, 1.15 NH_4Cl, 2 tartaric acid, 10 DL-malic acid, 11.7 yeast carbon base (Difco, Fisher Scientific, Elancourt, France). The medium was sterilised by filtering it through a Sartorius 0.22-μm pore size membrane. The yeasts were grown in conical flasks with an empty/full ratio of 0.25 for semi-aerobic conditions. Yeast cells were harvested periodically during cultivation, centrifuged (8000×*g*, 15 min, 4°C), washed twice with 155 mM NaCl and resuspended with 0.1 M potassium phosphate buffer (pH 7.5) containing 2.4 M KCl and 10 mM mercaptoethanol. Viable and total cells were determined on Malassez cells after coloration with methylene blue [6].

Lytic activity against yeast cells

Lytic activities were determined using two preparations of yeasts: yeast cells of *S. cerevisiae* at various growth stages (viable yeast cells assay) and cells prepared in the same manner with an addition of 10 mM sodium azide in the buffer solution (dead yeast cells assay). A mixture of 1 ml of *O. oeni* enzyme solution, 0.5 ml of yeast cell suspension at various concentrations (from 0.25×10^8 to 5.3×10^8 cells ml^{-1}) and 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5 containing 2.4 M KCl and 10 mM mercaptoethanol) was incubated at 30°C for 2 h with gentle shaking. To 1 ml of this mixture, 2 ml of water was added. Dilution with water induced the lysis of osmotically sensitive yeast cells caused by the enzyme [19]. After exactly 5 min at 20°C, the absorbance of the mixture was measured at 600 nm. The lytic activity was estimated from the extent of turbidity reduction, which was calculated according to the following equation:

$$\text{Percent decrease in turbidity} = \frac{(A_0 - A_t) - (A_{r0} - A_{rt})}{A_0 - (A_{r0} - A_{rt})} \times 100$$

where A_0=absorbance of the reaction mixture at time zero, A_t=absorbance of the reaction mixture at time t (t=2 h) and r indicates a reference tube which does not contain enzyme but the buffer of the enzyme solution.

All experiments were performed twice. Lytic activity was expressed as percentage of turbidity diminution for 1 ml of enzyme solution after 2 h.

β (1→3) glucanase activity

This activity was assayed by incubating 0.1 ml 2% (w/v) laminarin in sterile 0.1 M potassium phosphate pH 6 buffer with 1 ml of enzyme solution (bacterial biomass or 10-fold concentrated supernatant in the same buffer) at 55°C for 60 min. The reaction was stopped in an ice-bath. As controls, 0.1 ml of laminarin solution in 1 ml buffer and 1 ml of enzyme solution with 0.1 ml of buffer were incubated and cooled to correct for the reducing sugars in the substrate and the enzyme solution. Reducing sugar equivalents were measured in both the original and the control solutions by the colorimetric method

Table 2 Influence of enzyme solution concentration on lytic activity against yeast cells

	Yeast cell suspension (cells ml^{-1})					
	4×10^8				2×10^8	
Supernatant concentration (enzyme solution)	×1	×5	×10	×20	×1	×10
Lytic activity[a]	1.3	1.7	3.1	4.7	2.0	7.1

[a]Lytic activity was measured by the percentage of turbidity diminution for 1 ml of enzyme solution after 2 h on viable yeasts cultivated in a fermentation medium.

using 3,5-dinitrosalicylic acid reagent [3] with glucose as a standard. The activity tests containing bacterial cells were first filtered through a Sartorius 0.22-μm pore size membrane before the absorbances were measured. The precision was estimated at 5%.

Results and discussion

Glucanase activity in cultures of O. oeni

Among enzymes causing yeast cells to lyse, glucanases are the most important. Therefore, the presence of β (1→3) glucanase activity was tested in bacterial cultures. *O. oeni* was cultivated in modified FT 80 medium (without yeast extract) and incubated at 25°C in the absence and in the presence of cell-wall macromolecules obtained after autolysis of *S. cerevisiae*. These macromolecules contained 95.1% total carbohydrates (glucans and mannoproteins) and 4.9% proteins. β (1→3) glucanase activity was determined both in bacterial cells and in the bacterial supernatant. Results are given in Table 1. β (1→3) glucanase activity was present both in the culture supernatant obtained from bacteria and in the bacterial cells in different growth phases. The activity measured on cells was due to release of glucanase from bacteria during the 60 min of incubation. Thus, identical results (±5%) were obtained with bacteria incubated with laminarin for 60 min and with bacteria incubated without substrate for 60 min, then eliminated by centrifugation and after adding laminarin to this supernatant and incubated for a further 60 minutes. Therefore, this bacterial β (1→3) glucanase is an extracellular enzyme. This activity appeared greater during growth in log phase than in stationary phase but the highest extracellular glucanase activity was always obtained in the medium supplemented with glycosidic cell-wall macromolecules. This bacterial enzymatic activity, secreted by *O. oeni* into the culture medium during growth, might be able to lyse yeast cells after glucan degradation of the yeast cell walls.

Development of a lytic assay for O. oeni

To test this hypothesis, we have assessed the lytic activity of the supernatant of a bacterial culture against yeast cells. The data reported in Table 2 show that yeast lytic activity was greater when the bacterial culture supernatant (corresponding to enzyme solution) was concentrated. Lytic activity increased with more concentrated enzyme solutions for the two yeast cells levels (2×10^8 or 4×10^8 cells ml^{-1}). Therefore *O. oeni* appears to be capable of secreting enzymes into the culture medium that can hydrolyse yeast cell walls.

Several dilutions of yeast cell suspension were used to test the influence on lytic activity. Yeast cells were harvested at the end of the log phase corresponding to the middle of sugar fermentation and diluted in phosphate buffer to give concentrations from 0.25×10^8 to 5.3×10^8 cells ml^{-1}. Results are shown in Figure 1. The rate of lytic activity increased linearly with increasing yeast cell concentrations from 0.5×10^8 cells ml^{-1} to 3×10^8 cells ml^{-1}. Increasing the yeast cell concentration further did not result in any increase in the rate of lytic activity. A yeast concentration of 2×10^8 cells ml^{-1} was used for further experiments.

The effect of temperature on lytic activity is shown in Figure 2. Activity measurements at 40°C resulted in maximum lytic activity (8.2% of turbidity diminution for 1 ml of enzyme solution after 2 h). Above this optimum temperature a considerable loss of lytic

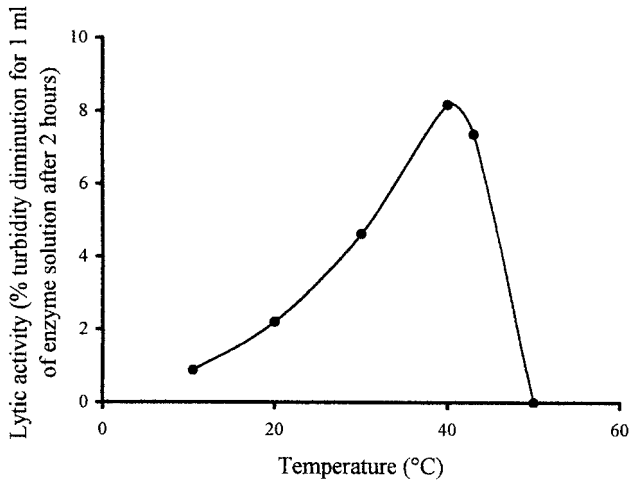

Figure 2 Influence of temperature on yeast cell-lytic activity by *O. oeni*.

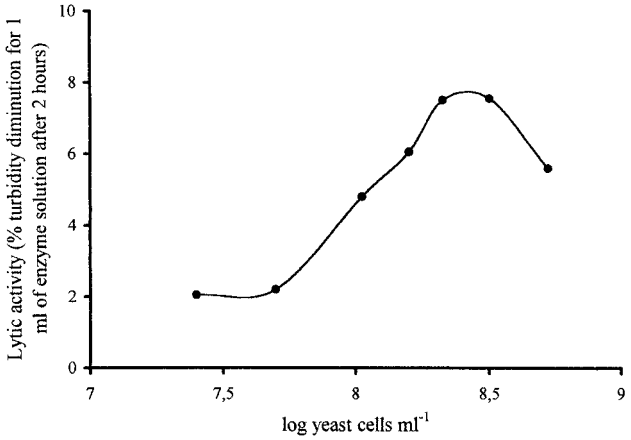

Figure 1 Influence of yeast concentration on yeast cell-lytic activity by *O. oeni*.

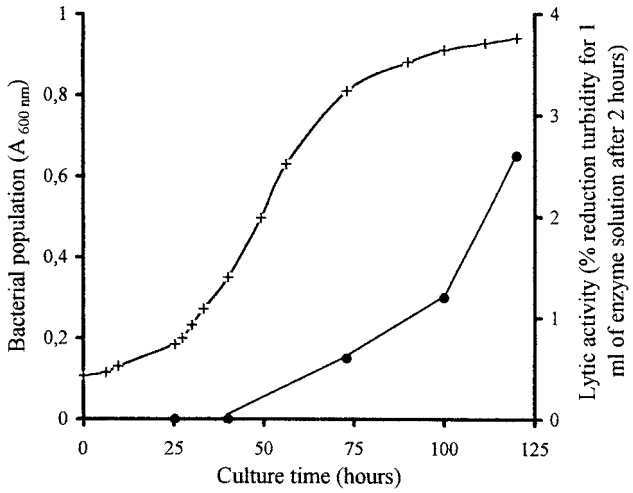

Figure 3 Time course production of the yeast cell-lytic activity by *O. oeni*.

Table 3 Lytic activity by *O. oeni* on yeasts cultivated in a fermentation medium and harvested at different growth phases

	Yeast population (cells ml^{-1})			Lytic activity on 2×10^8 cells ml^{-1} (% of turbidity diminution per 1 ml of enzyme solution after 2 h)		
	Mid-log phase	End of log phase	Stationary phase	Mid-log phase	End of log phase	Stationary phase
Viable yeasts ($-NaN_3$ in buffer)	0.1×10^8	0.4×10^8	3.1×10^8	0	4.1	1.5
Dead yeasts ($+NaN_3$ in buffer)	1.6×10^7	3.8×10^7	24.7×10^7	0.6	5.6	4.6

activity was observed with no activity at 50°C. However, more than 20% of maximal activity was still evident at 20°C.

Time course of production of lytic activity by O. oeni

O. oeni was cultivated in FT 80 medium at 25°C for 5 days. Culture supernatants were collected at several points during growth. Lytic activity was tested on viable yeast cells at 2×10^8 cells ml^{-1}. Changes in the lytic activity on viable yeast cells in the culture medium during bacterial growth are shown in Figure 3. During the log growth phase of *O. oeni*, no activity against yeast cells in the concentrated supernatant was detected. As for *Arthrobacter luteus* [12], this activity appeared in the medium in the early stationary phase and then increased significantly. The highest lytic activity, which corresponds to a reduction of turbidity about 2.6% for 1 ml of enzyme solution after 2 h, was obtained after 120 h in the stationary phase. However the highest β (1→3) glucanase activity was observed during the log phase of *O. oeni*. This suggests that β (1→3) glucanase is not the only enzyme involved in yeast lysis. Other bacterial enzymatic activities could be implicated in the yeast lytic process, such as mannanases, *N*-acetyl-glucosaminidase, and β (1→6) glucanase.

Susceptibility of lysis of viable and dead yeast cells at various stages of growth

The susceptibility of viable or dead yeast cells (see Materials and methods) corresponding to various stages of yeast growth was examined using a bacterial cell-free concentrated (×10) supernatant (Table 3). The lytic activity was tested on yeast cells at 2×10^8 cells ml^{-1}. The extent of lysis was estimated as the percentage decrease in turbidity. A difference in the lytic activity was observed between dead and viable yeast cells corresponding to various growth stages. Dead yeast cells appeared more sensitive to lysis. The maximum extent of lysis was seen with yeasts (dead or viable) harvested in the mid-log phase. In accordance with other studies [13], older cells appear to be more resistant to lysis than cells in the exponential growth phase. In comparison with yeast cell wall-degrading activities measured in various other microorganisms, the lytic activity of *O. oeni* is significantly lower [19]. *O. oeni* induced a decrease in turbidity of only 4–6% after 2 h with 1 ml of enzyme solution from a 10-fold concentrated supernatant when using dead or viable yeast cells. *A. luteus*, by contrast, induced a decrease in turbidity of 69% (after 2 h for 1 ml of enzyme solution obtained without any concentration) when heat-treated yeast cells were used, and a 22% decrease when viable yeast cells were used [13].

In a common environment, growing bacteria accelerated the death phase of the yeast growth cycle [11,15]. Metabolic compounds produced by bacteria have often been examined for their possible inhibitory effects. However, the faster death rate of the yeast could be also due to the release into the culture medium of bacterial lytic activities. Thus, our results show that *O. oeni* can hydrolyse cells of *S. cerevisiae* but with a limited action. Furthermore all the lytic activity measurements were done under optimal pH (7) and temperature (30°C) conditions, which are significantly different from enological conditions (pH 3, 15–20°C). Therefore, yeast cell death that sometimes occurs during alcoholic fermentation is not likely to be due to the lytic activity of *O. oeni*. However, the extracellular production of β (1→3) glucanase by this microorganism presents a real interest in winemaking. Malolactic fermentation generally occurs during yeast-lees contact in white Burgundy wine production. During this contact, it is well known that release of glycosidic cell-wall macromolecules occurs as a result of yeast autolysis [5]. It has been demonstrated that mannoproteins present some interesting enological properties such as protein stability [24], tartaric stability [17] and aroma fixation [16]. It is possible that the bacterial β (1→3) glucanase activity could enhance the release of yeast cell wall mannoproteins by increasing the hydrolysis of the cell wall glucans.

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References

1 Araujo A and OP Ward. 1990. Extracellular mannanases and galactanases from selected fungi. *J Microbiol* 6: 171–178.
2 Bielecki S and S Galas. 1991. Microbial β-glucanases different from cellulases. *Crit Rev Biotechnol* 10: 275–303.
3 Bruner RL. 1964. Determination of reducing value 3,5-dinitrosalicylic acid method. *Chem Anal* 19: 67–71.
4 Dubois M, A Gillesk, JJ Hamilton, H Rebersp and F Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350–356.
5 Feuillat M, M Freyssinet and C Charpentier. 1989. L'élevage sur lies des vins blancs de Bourgogne: II. Evolution des macromolécules: polysaccharides et protéines. *Vitis* 28: 161–176.
6 Fink H and R Kuhles. 1933. Beiträge zur Methylenblaufärbung der Hefelzellmembran: II. Mitteilung Eine verbesserte Färbeflüssigkeit zur Erkennung von toten Hefezellen. *Hoppe Seyler's Z Physiol Chem* 218: 65–66.
7 Fleet GH and HJ Phaff. 1974. Lysis of yeast cell walls: glucanases from *Bacillus circulans* WL-12. *J Bacteriol* 119: 207–219.
8 Guilloux-Benatier M, HS Son, S Bouhier and M Feuillat. 1993. Activités enzymatiques: glycosidases et peptidase chez *Leuconostoc oenos* au cours de la croissance bactérienne. Influence des macromolécules de levures. *Vitis* 32: 51–57.
9 Guilloux-Benatier M, Y Le Fur and M Feuillat. 1998. Influence of fatty acids on the growth of wine microorganisms *Saccharomyces cerevisiae* and *Oenococcus oeni*. *J Ind Microbiol Biotechnol* 20: 144–149.
10 Huang YC, CG Edwards, JC Peterson and KM Haag. 1996. Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. *Am J Enol Vitic* 47: 1–10.

11 King SW and RB Beelman. 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. *Am J Enol Vitic* 3: 53–60.
12 Kitamura K, T Kaneko and Y Yamamoto. 1972. Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*: I. Isolation of lytic strain and studies in its lytic activity. *J Gen Appl Microbiol* 18: 57–71.
13 Kitamura K, T Kaneko and Y Yamamoto. 1974. Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*: II. Purification and properties of an enzyme, zymolyase, which lyses viable yeast cells. *J Gen Appl Microbiol* 20: 323–344.
14 Latzko F and W Hampel. 1995. Enzyme formation by a yeast cell wall lytic *Arthrobacter* species: chitinolytic activity. *Appl Microbiol Biotechnol* 44: 185–189.
15 Lonvaud-Funel A, Ph Masclef, A Joyeux and Y Paraskevopoulos. 1988. Etude des interactions entre levures et bactéries lactiques dans le moût de raisin. *Connaiss Vigne Vin* 22: 11–24.
16 Lubbers S, B Leger, C Charpentier and M Feuillat. 1993. Effet colloide-protecteur d'extrais de parois de levures sur la stabilité tartrique d'une solution hydroalcoolique modèle. *J Int Sci Vigne Vin* 27: 13–22.
17 Lubbers S, C Charpentier, M Feuillat and A Voilley. 1994. Influence of yeast walls on the behavior of aroma compounds in a model wine. *Am J Enol Vitic* 45: 29–33.
18 Malek MA and DR Berry. 1995. Isolation and partial characterization of yeast mannan hydrolysing enzymes from bacterial isolates. *Microbios* 83: 229–241.
19 Mann W, TW Jeffries and JD Macmillan. 1978. Production and ecological significance of yeast cell wall-degrading enzymes from *Oerskovia*. *Appl Environ Microbiol* 36: 594–605.
20 Petruccioli M and F Federici. 1988. Extracellular enzyme production in species of the genus *Penicillium*. *Mycologia* 80: 726–728.
21 Rao M, C Mishra, R Seera, MC Srinivasan and VV Deshpande. 1983. *Penicillium janthinellum* as a source of fungal biomass protein from lignocellulosic waste. *Biotechnol Lett* 5: 301–304.
22 Ryan E and OP Ward. 1988. The application of lytic enzymes from *Basidiomycete aphyllophoroles* in production of yeast extract. *Pr Biochem* 12–16.
23 Vrsanska M, P Biely and Z Kratky. 1977. Enzymes of the yeast lytic system produced by *Arthrobacter* GJM-1 bacterium and their role in the lysis of yeast cell walls. *Z Allg Mikrobiol* 17: 465–480.
24 Waters EJ, P Pellerin and JM Brillouet. 1994. A *Saccharomyces* mannoprotein that protect wine from protein haze. *Carbohydr Polym* 23: 185–191.
25 Zlotnik H, MP Fernandez, B Bowers and E. Cabib. 1984. *Saccharomyces cerevisiae* mannoproteins from an external cell wall layer that determines wall porosity. *J Bacteriol* 159: 1018–1026.