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To link to this article: DOI:10.1016/J.FM.2009.09.008
URL: http://dx.doi.org/10.1016/J.FM.2009.09.008

To cite this version: Nehme, Nancy and Mathieu, Florence and Taillandier, Patricia (2010) Impact of the co-culture of Saccharomyces cerevisiae–Oenococcus oenion malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. *Food Microbiology* pp. 150-157. ISSN 0740-0020

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Impact of the co-culture of *Saccharomyces cerevisiae–Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction

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ABSTRACT

The present study was aimed to evaluate the impact of the co-culture on the output of malolactic fermentation and to further investigate the reasons of the antagonism exerted by yeasts towards bacteria during sequential cultures. The *Saccharomyces cerevisiae* D strain/*Oenococcus oeni* X strain combination was tested by applying both sequential culture and co-culture strategies. This pair was chosen amongst others because the malolactic fermentation was particularly difficult to realize during the sequential culture. During this traditional procedure, malolactic fermentation started when alcoholic fermentation was achieved. For the co-culture, both fermentations were conducted together by inoculating yeasts and bacteria into a membrane bioreactor at the same time. Results obtained during the sequential culture and compared to a bacterial control medium, showed that the inhibition exerted by *S. cerevisiae* D strain in term of decrease of the malic acid consumption rate was mainly due to ethanol (75%) and to a peptidic fraction (25%) having an MW between 5 and 10 kDa. 0.4 g l^{-1} of L-malic acid was consumed in this case while 3.7 g l^{-1} was consumed when the co-culture was applied. In addition, there was no risk of increased volatile acidity during the co-culture. Therefore, the co-culture strategy was considered effective for malolactic fermentation with the yeast/bacteria pair studied.

1. Introduction

Malolactic fermentation (MLF), an enzyme mediated decarboxylation of L-malic acid into L-lactic acid, is carried out by lactic acid bacteria belonging mainly to the *Oenococcus oeni* species. It usually occurs after alcoholic fermentation (AF) and is known to improve wine quality through deacidification, production of desirable flavors and aromas, and enhancement of microbial stability (Kunkee, 1984, 1991; Davis et al., 1985, 1988; Lonvaud-Funel, 1999, 2002; Bartowsky et al., 2002). However, this important secondary fermentation step in winemaking is often difficult to induce and control because of the harsh physicochemical conditions existing in wine, such as low pH (Britz and Tracey, 1990; Vaillant et al., 1995), high ethanol content (Capucho and San Romao, 1994; Vaillant et al., 1995) and low temperature (Britz and Tracey, 1990), in addition to the presence of some yeast inhibitory metabolites such as SO_2 (Henick-Kling and Park, 1994; Carreté et al., 2002; Osborne and Edwards, 2006) and medium chain fatty acids (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988; Capucho and San Romao, 1994). A few authors have suggested the implication of different proteins/peptides in MLF inhibition (Dick et al., 1992; Comitini et al., 2005; Osborne and Edwards, 2007) but without any convergent results. Therefore the success or failure of MLF is closely related to the choice of the yeasts and bacterial strains in a combination and the interactions that may occur between them. In the present work, we studied the effect of the inoculation strategy chosen on the occurrence of MLF by presenting an alternative strategy to the traditional sequential culture one: co-culture. Few authors have studied the effect of co-culture on the onset of MLF (Beelman and Kunkee, 1985; Krieger, 2002). While, during sequential culture, MLF started when AF was achieved, both fermentations were conducted simultaneously during the co-culture by inoculating the yeasts and the bacteria at the same time in a synthetic grape juice medium in a membrane bioreactor. The *Saccharomyces cerevisiae* D strain/*O. oeni* X strain combination was studied by applying both strategies in order to select the most suitable one for MLF. This strains combination was chosen because in a previous work we have shown that

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the yeast was very inhibitory towards the bacteria (Nehme et al., 2008). For a better understanding of the antagonism exerted by the *S. cerevisiae* D strain, the biochemical profile of the yeast fermented medium was established at the end of the AF and the presence of an inhibitory compound of protein nature was investigated.

2. Materials and methods

2.1. Strains and storage conditions

The *S. cerevisiae* D strain and the *O. oeni* X strain used in this work were kindly provided by Lallemand Inc. (Blagnac, France). Stock cultures of *S. cerevisiae* D strain were kept at 4 °C in YEPD (*Yeast Extract Peptone Dextrose*) agar composed of 20 g l^{-1} glucose, 10 g l^{-1} Yeast Extract (Oxoid, Hampshire-England), 20 g l^{-1} peptone and 20 g l^{-1} agar. *O. oeni* X strain was kept frozen at –20 °C in MRS (*Man Rogosa Sharpe*) broth (Biokar, Beauvais – France) containing 20% glycerol (v/v).

2.2. Growth media

2.2.1. Synthetic grape juice medium

The medium composition that simulated natural grape juice consisted of: glucose 100 g l^{-1}, fructose 100 g l^{-1}, Yeast Extract (Oxoid) 1 g l^{-1}, $(NH_4)_2SO_4$ 2 g l^{-1}, citric acid 0.3 g l^{-1}, L-malic acid 5 g l^{-1}, L-tartaric acid 5 g l^{-1}, $MgSO_4$ 0.4 g l^{-1} and KH_2PO_4 5 g l^{-1}, pH 3.5. The medium was autoclaved before use (120 °C, 20 min).

2.2.2. Synthetic wine medium

This medium composition simulated wine, yet it was lacking yeast metabolites with the exception of ethanol: glucose 0.5 g l^{-1}, fructose 0.5 g l^{-1}, Yeast Extract (Oxoid) 0.5 g l^{-1}, $(NH_4)_2SO_4$ 0.2 g l^{-1}, citric acid 0.3 g l^{-1}, L-malic acid 4 g l^{-1}, L-tartaric acid 5 g l^{-1}, $MgSO_4$ 0.2 g l^{-1} and KH_2PO_4 2 g l^{-1}, pH 3.5. After autoclaving, 80 g l^{-1} ethanol (10% (v/v)) were added and the pH was readjusted to 3.5 using an 85% orthophosphoric acid solution. The medium was then sterilised by filtration through 0.2 μm membranes (Elvetec services, Meyzieu-France).

2.3. Inoculation strategies

2.3.1. Sequential culture strategy

All fermentation steps for both alcoholic and malolactic fermentations were carried out at 22 °C with stirring at 150 rpm in Erlen-Meyer flasks.

2.3.1.1. Alcoholic fermentation step. *S. cerevisiae* D strain was cultured in 400 ml of the synthetic grape juice medium at an initial concentration of 3 × 10^6cells ml^{-1} (direct cell counts under microscope using the Thoma hematocymeter). The yeast inoculum was beforehand prepared in two steps. First, a preculture of *S. cerevisiae* D strain was obtained by reactivating the stock culture in YEPD broth for 24 h. Second, the preculture was used to inoculate a low sugar concentration synthetic grape juice medium: glucose 50 g l^{-1} and absence of fructose. This step was carried out for 24 h and provided the yeast inoculum.

Yeast growth was followed during the AF and the biomass was measured by weighing cells after drying and was expressed in g l^{-1}.

2.3.1.2. Preparation of the yeast fermented medium for MLF. After completion of AF determined by total or cessation of sugar consumption (<2 g l^{-1}), the yeast fermented medium was subjected to different steps before inoculation of the malolactic bacteria. First, yeast cells were eliminated by centrifugation (2000 g for 20 min at 4 °C) and the supernatant was recuperated. Then, malic acid concentration was measured and readjusted to 5 g l^{-1}. Next, the pH was adjusted to 3.5 using a 10 mol l^{-1} NaOH solution. Finally, the yeast fermented medium was filtered in sterile conditions through 0.2 μm membranes (Elvetec services) and a volume of 200 ml was recuperated in an autoclaved Erlen-Meyer flask of 250 ml.

2.3.1.3. Malolactic fermentation step. The bacterial inoculum was prepared in two steps. First a preculture of *O. oeni* X strain was obtained by reactivating the stock culture in MRS broth with 3% ethanol (v/v) added. After 24 h, the preculture was used to inoculate the low sugar concentration synthetic grape juice medium (composition described in part 2.2 paragraph 2.2.1) with 6% ethanol (v/v) added and which provided the inoculum. 24 h later, the yeast fermented medium was inoculated with *O. oeni* X strain at an initial concentration of 2 × 10^6cells ml^{-1} (direct cell counts under microscope using the Petit Salumbeni hematocymeter). The MLF was followed until the cessation of malic acid consumption.

Bacterial growth was followed during the MLF and the biomass was determined by weighing cells after drying and was expressed in g l^{-1}.

2.3.2. Co-culture strategy using a membrane bioreactor

The membrane bioreactor (MBR) is a good tool for studying the microbial interactions between two microorganisms, which are kept in a homogenous liquid phase but physically separated by a membrane made of polysulfone hollow fibres of 0.1 μm porosity (Polymem SA, Fourquevaux, France) (Albasi et al., 2001). The liquid phase is continuously mixed between the two sides of the membrane by means of gas overpressure (0.5 bar); nitrogen in our case. The gas overpressure alternates from one vessel to the other via the solenoid valves, thus allowing alternating liquid flow between the two vessels. Air was introduced in the membrane side through a 0.2 μm filter membrane in sterile conditions for 30 min/day during the first three days of the co-culture at 1.8 vvm (volume of air/volume of liquid/minute) in order to ensure a good yeast growth (Fig. 1).

The yeasts and bacterial inocula used for the co-culture were obtained using the preculture steps described in part 2.3.1 (paragraphs 2.3.1.1, 2.3.1.3). The co-culture was conducted in the synthetic grape juice medium (total volume = 4 l) at 22 °C with stirring at 150 rpm. Yeasts and bacteria were inoculated into the MBR at the same time, each in one of the two vessels as shown in Fig. 1, at initial concentrations of 3 × 10^6 and 2 × 10^6cells ml^{-1} respectively.

2.4. Bacterial control cultures

As a reference for growth and malic acid degradation kinetics during the co-culture and sequential cultures, three control cultures of *O. oeni* X strain were carried out using the preculture steps described in part 2.3.1 (paragraph 2.3.1.3). These control cultures were grown at 22 °C with stirring at 150 rpm, starting with an initial concentration of 2 × 10^6cells ml^{-1} and were as follows:

- Bacterial control culture in the synthetic grape juice medium: this control was carried out in the MBR and in Erlen-Meyer flasks and gave similar results in both conditions. Therefore the mean values of the kinetic parameters (growth and malic acid consumption) were calculated and constituted control 1. It was used as control for co-cultures.
- Bacterial control culture 2 (Flask, synthetic wine): Culture of *O. oeni* X strain in 200 ml of the synthetic wine medium using a 250 ml Erlen-Meyer flask. It was used as control for sequential cultures.

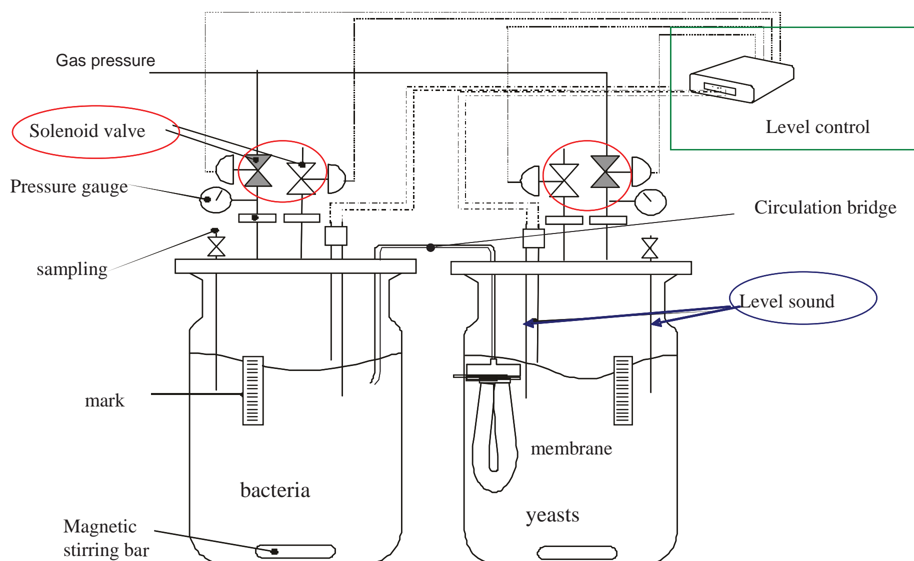

Fig. 1. Membrane bioreactor used for yeast/bacteria co-cultures.

2.5. Partial characterization of the extracellular anti-MLF compound(s)

2.5.1. Protease and heat treatments

At the completion of AF, the synthetic grape juice medium, so-called yeast fermented medium, was submitted to the following treatments:

- Treatment with 10 μg μl^{-1} of pepsin (Sigma P7012) for 1 h at 37 °C after adjusting the medium pH to 2 (optimal pH for pepsin activity) using an 85% orthophosphoric acid solution. The pH was brought back to 3.5 before bacteria inoculation.
- Heat treatment at 100 °C for 30 min.

In both cases, the treated fermented media were inoculated with *O. oeni* X strain at an initial concentration of 2×10^6cells ml^{-1} and the MLF was conducted in Erlen-Meyer flasks at 22 °C with stirring at 150 rpm.

2.5.2. Fractionation of the yeast fermented medium by ultrafiltration and dialysis

The yeast fermented medium was fractionated by ultrafiltration through Centricon Plus 70 centrifugal filter units (3500 g, 45 min, 4 °C) having cut-offs of 5 and 10 kDa (Millipore Corp., Billerica, MA-USA). This yielded 2 fractions containing compounds ≥5 kDa and ≥10 kDa. Each fraction was added to a modified MRS medium containing: 55.3 g l^{-1} of MRS, 4 g l^{-1} of L-malic acid, 10% ethanol (v/v), pH = 3.5. These fractions were finally 3.5 times concentrated in the modified MRS medium. The fraction lower than 5 kDa was dialysed for 24 h at 4 °C in 67 mmol l^{-1} phosphate buffer (pH = 4.8) using a 3.5 kDa Cellu-Sep dialysis tubing (MFP Inc., Texas-USA). This led to a fraction of MW between 3.5 and 5 kDa to which we added: 55.3 g l^{-1} of MRS, 4 g l^{-1} of L-malic acid, 10% ethanol (v/v), pH = 3.5. *O. oeni* X strain was inoculated into these three media at an initial concentration of 2×10^6cells ml^{-1} and the MLF was carried out at 22 °C with stirring at 150 rpm in Erlen-Meyer flasks. The modified MRS medium without the fractions of the yeast fermented medium was used as a control for this experiment.

2.6. Analytical methods

2.6.1. Sugar consumption by yeasts and bacteria

Sugar consumption was followed using the dinitrosalicylic colorimetric method (Miller, 1959) and results were expressed in g l^{-1}.

2.6.2. Malic acid degradation by yeasts and bacteria

L-malic acid concentration was determined using an enzymatic assay (Microdom, kit no 110 05 011 00, Taverny-France) and results were expressed in g l^{-1}.

2.6.3. Ethanol production by yeasts

Ethanol concentration was measured using the HPLC method. The column used was an Aminex@ HPX-87H Biorad having a cationic H^+ coverage thermostated at 40 °C and the solvent was a 5 mmol l^{-1} sulphuric acid solution at a flow rate of 0.4 ml min^{-1}. The HPLC was coupled to a refractive index detector. Results were expressed in g l^{-1}.

2.6.4. Acetic acid production by yeasts and bacteria

Acetic acid concentration was determined using an enzymatic assay (Boehringer Mannheim, kit no 10 148 261 035, Darmstadt-Germany) and results were expressed in g l^{-1}.

2.6.5. SO_2 production by yeasts

SO_2 concentration was evaluated using the Ripper iodimetric method (Recueil des méthodes analytiques de l'OIV, 1974). Results were expressed in mg l^{-1}.

2.6.6. Assimilable nitrogen consumption by yeasts and bacteria

The assimilable nitrogen in the medium, defined as the ammoniacal nitrogen (NH_4^+) in addition to the α-amino nitrogen, was measured using two enzymatic assays, one for the quantification of NH_4^+ (Microdom, kit no 110 05 037 00) and the other for the quantification of α-amino nitrogen (Microdom, kit no 110 10 110 00). Results were expressed in mg l^{-1}.

2.6.7. Fatty acids produced by yeasts

The fatty acids were measured using Gas Chromatography (Hewlet Packard HP 5890) with Hydrogen as vector gas and a capillary column SGE FFAP. The dectector was FID and the splitless mode was used. Octan-3-ol was used as internal standard. Results were contracted out in the Faculté D'Oenologie de l'Université Victor Segalen, Bordeaux 2 and results were expressed in mg l^{-1}.

2.6.8. L-lactic acid and D-lactic acid produced by bacteria

L-lactic acid and D-lactic acid were determined using enzymatic assays (Microdom, kits no 110 05 020 00 and no 110 05 025 00 respectively, Taverny-France) and results were expressed in g l^{-1}.

2.6.9. Citric acid consumed by yeasts and bacteria

Citric acid was determined using an enzymatic assay (Microdom kit no 110 05 036 00, Taverny-France) and results were expressed in g l^{-1}.

3. Results

3.1. Comparison of the MLF results obtained with the S. cerevisiae D strain/O. oeni X strain combination using both co-culture and sequential culture strategies

Fig. 2 shows the kinetics of MLF during both the sequential culture and the co-culture. It is clear that the growth of *O. oeni* X strain and its malic acid consumption were improved when the co-culture strategy was applied. 3.7 g l^{-1} of malic acid were consumed within 500 h.

Two control experiments of the MLF were carried out. The first was bacterial control culture 1 (synthetic grape juice) which was used as a control of the MLF during co-culture. The second was bacterial control culture 2 (Flask, synthetic wine) and was used as a control of the MLF during sequential culture. Their results are reported in Table 1, which also gives the kinetic parameters of the MLF during the two inoculation strategies.

The comparison of the biomass productivities obtained using the two strategies showed that the growth rate of *O. oeni* X strain was multiplied by 2.6 when the co-culture was applied. However, the maximal biomasses reached were practically the same. In addition, the malic acid consumption rate was 3.55 times faster when the co-culture was used. Although the malic acid consumption was improved in this case, the malic acid was not completely consumed.

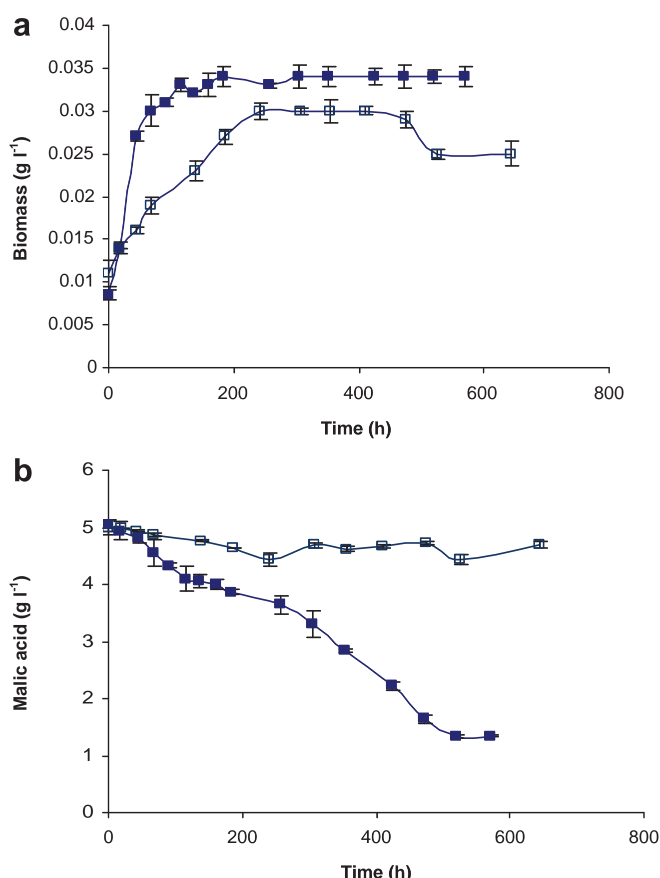

Fig. 2. Variation of the biomass of *O. oeni* X strain (a) and of its malic acid consumption (b) during co-culture (■) and sequential culture (□). Each value is the mean of triplicate experiments ± SD.

The specific average malic acid consumption rate was 2.82 times higher during the co-culture, which means that the conditions provided by this strategy were more favourable to the bacterial metabolism. Even though the co-culture strategy was advantageous, the biomass productivity and the malic acid consumption rate were still respectively 14 and 2.5 times lower than those obtained with the bacterial control culture 1 (synthetic grape juice). The comparison of the MLF results obtained during the sequential culture and its corresponding bacterial control culture 2 (Flask, synthetic wine) showed that the biomass productivity and the malic acid consumption rate in the yeast fermented medium were respectively 10.6 and 3 times weaker than in the wine synthetic medium. This suggests that the greater bacterial inhibition observed in the yeast fermented medium was due to other yeast inhibitory metabolites produced by *S. cerevisiae* D strain in addition to ethanol. Another hypothesis could be nutrient depletion.

3.2. Biochemical characteristics of the co-culture of S. cerevisiae D strain/O. oeni X strain and of the pure cultures of these strains in the MBR

The concentrations of L-malic acid and citric acid consumed as well as L-lactic acid, D-lactic acid and acetic acid produced during the AF and the MLF in the MBR are reported in Table 2. These important acids affect wine quality.

The decarboxylation of 1 mol of L-malic acid by *O. oeni* gives 1 mol of L-lactic acid (Renault et al., 1988; Cox and Henick-Kling, 1989). Therefore, we can assume that the 25 mmol l^{-1} of L-lactic acid obtained during the co-culture resulted from the consumption of 25 mmol l^{-1} of L-malic acid by the bacteria. The remaining 2.5 mmol l^{-1} of L-malic acid were therefore consumed by the yeasts, which indeed corresponds to the amount consumed by *S. cerevisiae* D strain during its pure culture in the MBR.

The amount of acetic acid produced during both the co-culture and the pure culture of *S. cerevisiae* D strain was practically the same which indicates that this acid was mainly a metabolite of the AF. This is confirmed by the weak production of acetic acid during the pure culture of *O. oeni* X strain although 67 mmol l^{-1} of sugar (12 g l^{-1}) were consumed in this case (Table 3). Moreover we checked that neither the yeasts nor the bacteria strains used in this study were able to catabolise citric acid.

The D-lactic acid obtained during the co-culture resulted only from the sugar consumption by *O. oeni* X strain since it was not detected in the yeast pure culture.

Table 3 gives the biochemical data of the fermented media at the end of the co-culture of *S. cerevisiae* D strain/*O. oeni* X strain and at the end of the pure cultures of these strains in the MBR: consumed nitrogen and produced fatty acids and sulphur dioxide.

The behaviour of *S. cerevisiae* D strain was similar during its pure culture and its co-culture with *O. oeni* X strain, indicating that the yeast was not negatively affected by the presence of the bacteria. Minimal concentrations of sugar and alpha amino nitrogen were consumed by *O. oeni* X strain during its pure culture, showing that this strain was not very demanding from a nutritional point of view when compared with the yeast.

3.3. Partial characterization of an extracellular anti-MLF compound produced by S. cerevisiae *D strain*

In order to investigate the chemical nature of potential inhibitory metabolites produced by the yeast other than ethanol, fatty acids and sulphur dioxide we carried out two different experiments using the yeast fermented synthetic grape juice medium: denaturing treatments (heat and protease treatments) and size evaluation (fractionation by ultrafiltration and dialysis).

Table 1
Kinetic parameters of MLF carried out by *O. oeni* X strain during its co-culture and its sequential culture with *S. cerevisiae* D strain and during its control cultures.

	Bacterial control culture 1[e]	Bacterial control culture 2[f]	Co-culture of *S. cerevisiae* D strain/*O. oeni* X strain, MLF phase	Sequential culture of *S. cerevisiae* D strain/ *O. oeni* X strain, MLF phase
Initial biomass (g l^{-1})	0.012	0.013	0.009	0.011
Maximal biomass (g l^{-1})	0.8	0.29	0.034	0.03
Biomass productivity (g l^{-1} h^{-1})[a]	28.8×10^{-4}	8.5×10^{-4}	2.07×10^{-4}	0.8×10^{-4}
Initial malic acid (g l^{-1})	5.1	4	5.03	5
Final malic acid (g l^{-1})	0.06	0	1.34	4.63
Malic acid consumption rate (g l^{-1} h^{-1})[b]	18×10^{-3}	6×10^{-3}	7.1×10^{-3}	2×10^{-3}
Specific average malic acid consumption rate (g g^{-1} h^{-1})[c]	22.5×10^{-3}	21×10^{-3}	209×10^{-3}	74×10^{-3}
Duration of experiment (h)[d]	286	695	520	187

[a] Biomass productivity (g l^{-1} h^{-1}) is defined as the biomass formed (g l^{-1}) at the end of the growth phase divided by the time (h) without taking the lag phase and the stationary phase into consideration.
[b] Malic acid consumption rate (g l^{-1} h^{-1}) is defined as the malic acid consumed (g l^{-1}) divided by the duration of experiment (h).
[c] Specific average malic acid consumption rate (g g^{-1} h^{-1}) = [malic acid consumed (g l^{-1})/duration of experiment (h)]/biomass (g l^{-1}) present at the end of MLF.
[d] Duration of experiment considers only the time until the cessation of malic acid consumption (h).
[e] Bacterial control culture 1: Culture of *O. oeni* X strain using the synthetic grape juice medium.
[f] Bacterial control culture 2: Culture of *O. oeni* X strain in the synthetic wine medium using an Erlen-Meyer flask.

3.3.1. Protease and heat treatments

Fig. 3 shows that the growth of *O. oeni* X strain was improved and the malic acid was completely consumed after heat and pepsin treatments of the yeast fermented medium. These results were indicative of the protein nature of the inhibitory compound synthesized by *S. cerevisiae* D strain. The comparison of these results with those obtained with bacterial control culture 1 (synthetic grape juice) showed that the inhibition due to the proteinaceous compound constituted a part of the whole inhibition. In fact, after heat and pepsin treatments, the maximal biomasses reached were still lower and the duration of the malic acid consumption was still much longer. Moreover, *O. oeni* X strain displayed similar behaviour in both treated media and wine synthetic medium (bacterial control culture 2), suggesting that the remaining inhibition in the treated media was mainly due to ethanol.

Table 4 gives the kinetic data of the MLF in the treated media, the untreated yeast fermented medium and the corresponding controls.

Table 4 shows, that after heat and pepsin treatments, the maximal biomasses reached and the biomass productivities were close and were strongly improved when compared with those obtained in the untreated yeast fermented medium. Although these two growth parameters were improved, they were still lower than those obtained with bacterial control culture 1 (synthetic grape juice). In addition, the malic acid was completely consumed in both treated media and the malic acid consumption rates were close, with a slightly higher value in the pepsin treated medium. The MLF results obtained in the treated media and the synthetic wine medium (bacterial control culture 2) showed some similarities. In fact, the maximal biomasses reached were close even though the biomass productivity in the synthetic wine medium was slower due to the lag phase. The malic acid consumption rates were also close and the malic acid was completely consumed in these media. In addition, the inhibition percentages, calculated as the reduction of the malic acid consumption rate in comparison to the control 1, were practically the same. This underlines that the remaining inhibition after heat and pepsin treatments was mainly due to ethanol. Therefore, if we assume that the untreated yeast fermented medium exhibited 100% of the whole inhibition which indeed corresponded to 89%, the 67% due to ethanol and calculated with control 2 would subsequently represent 75% of the whole inhibition. Consequently the metabolites of protein nature would be responsible of the remaining 25% of the whole inhibition. Finally, the specific average malic acid consumption rates of the treated media and the bacterial control cultures were practically the same. This shows that the longer duration required for total malic acid consumption in the treated media and the wine synthetic medium was related to an inhibition of the bacterial growth rather than an inhibition of the malic acid consumption.

Table 2
Consumption and production of acids during the co-culture of *S. cerevisiae* D strain/ *O. oeni* X strain and during the pure cultures of these strains in the MBR using the synthetic grape juice medium.

	Co-culture of *S. cerevisiae* D strain/ *O. oeni* X strain	Pure culture of *O. oeni* X strain (control 1)	Pure culture of *S. cerevisiae* D strain in the MBR[a]
L-malic acid consumed (mmol l^{-1})	27.5	37	2.54
L-lactic acid produced (mmol l^{-1})	25 (±0.6)	37 (±1.1)	0
Acetic acid produced (mmol l^{-1})	16.2 (±0.17)	1.5 (±0.03)	16 (±0.17)
D-lactic acid produced (mmol l^{-1})	6 (±0.2)	21 (±0.4)	0
Citric acid consumed (mmol l^{-1})	0	0	0

[a] The pure culture of *S. cerevisiae* D strain was conducted in the MBR using the synthetic grape juice medium and the same conditions used for the co-culture.

Table 3
Production of ethanol, SO_2 and fatty acids by *S. cerevisiae* D strain and consumption of sugar and nitrogen by *S. cerevisiae* D strain and *O. oeni* X strain during their co-culture and their pure cultures in the MBR.

	Co-culture of *S. cerevisiae* D strain/*O. oeni* X strain	Pure culture of *S. cerevisiae* D strain in the MBR	Pure culture of *O. oeni* X strain (control 1)
Sugar consumed (g l^{-1})	200.43	200.7	12
Ethanol produced (g l^{-1})	84 (±3.4)	86 (±3)	0
Initial alpha amino nitrogen (mg l^{-1})	74 (±2)	74 (±1.72)	74 (±2.4)
alpha amino nitrogen consumed (mg l^{-1})	58.1	61.6	10.7
Initial ammoniacal nitrogen (mg l^{-1})	451 (±11)	451 (±11.6)	451 (±10.5)
Ammoniacal nitrogen consumed (mg l^{-1})	330.5	330	0
Free SO_2 (mg l^{-1})	9 (±0.6)	9 (±0.8)	–
Total SO_2 (mg l^{-1})	20 (±2)	20 (±1.6)	–
Octanoic acid (mg l^{-1})	0.7	0.7	–
Decanoic acid (mg l^{-1})	<0.1	<0.1	–
Dodecanoic acid (mg l^{-1})	<0.1	<0.1	–
Yeast biomass formed (g l^{-1})	2.53 (±0.04)	2.43 (±0.06)	–
Duration of AF (h)	134	127	–

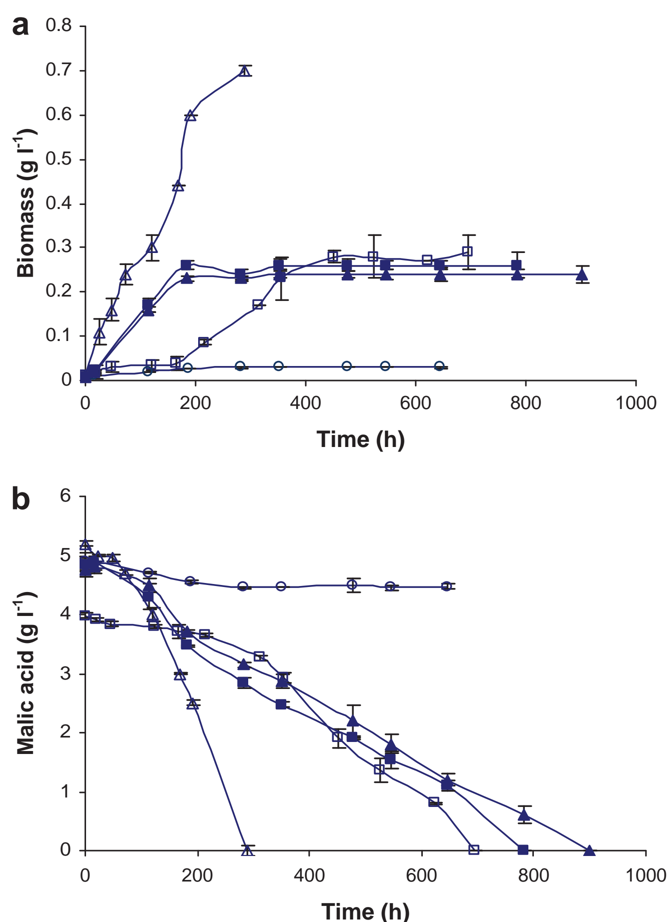

Fig. 3. Variation of the biomass of *O. oeni* X strain (a) and of its malic acid consumption (b) in the treated and untreated yeast fermented media and in the bacterial control cultures. (■) Pepsin treatment, (▲) Heat treatment, (○) Untreated yeast fermented medium, (△) Bacterial control culture 1: Culture of *O. oeni* X strain in the synthetic grape juice medium, (□) Bacterial control culture 2: Culture of *O. oeni* X strain in the wine synthetic medium. Each value is the mean of triplicate experiments ± SD.

3.3.2. Fractionation of the yeast fermented medium by ultrafiltration and dialysis

The inhibitory activity of the different MW fractions prepared from the yeast fermented medium was tested in a modified MRS medium inoculated with *O. oeni* X strain. Results were compared with those for a modified MRS control. The MRS medium was chosen because it is very favourable for the growth and metabolism of lactic acid bacteria from a nutritional point of view. Therefore any inhibition observed would be only due to the MW fraction introduced.

Fig. 4 shows that the difference between the growth of *O. oeni* X strain in the presence of the fraction having an MW between 3.5 and 5 kDa and its growth in the modified MRS control was minimal. It was lower in the presence of the fraction having an MW $\geq$ 10 kDa and was totally inhibited in the presence of the fraction having an MW $\geq$ 5 kDa. The malic acid consumption was complete and presented the same kinetic profile in the presence of the fractions having an MW between 3.5 and 5 kDa and $\geq$10 kDa as well as in the control. However we did not observe any malic acid consumption in the presence of the fraction having an MW $\geq$ 5 kDa. These results led us to the conclusion that the extracellular anti-MLF compound produced by *S. cerevisiae* D strain was of protein nature and presented an MW between 5 and 10 kDa.

4. Discussion

In the present work, the co-culture was proposed as an alternative inoculation strategy to the traditional sequential culture one. It was studied using an interesting tool for the comprehension of the yeasts/bacteria interactions on a laboratory scale; the MBR. We previously checked that co-cultures of yeasts/bacteria pairs carried out in one vessel of the MBR gave the same results as those obtained when the microorganisms were physically separated (data not shown). In industrial conditions, co-cultures are usually conducted with direct cell contact. The study was realized in a synthetic grape juice medium in order to obtain a reproducible medium with a controlled and non limiting composition. In the case of an inhibitory yeast strain, such as *S. cerevisiae* D strain, the advantage of the co-culture strategy over the sequential one could be due to the presence of the bacteria from the beginning in a medium lacking the yeast inhibitory metabolites and rich in nutrients, unlike the yeast fermented medium used for the sequential cultures. Even though these metabolites gradually occurred during the AF, which lasted for 134 h of the co-culture (Table 3), the bacteria had the time to grow better and especially to better consume malic acid (Fig. 2 and Table 1). Some of the yeast inhibitory metabolites produced by *S. cerevisiae* D strain, such as ethanol, SO_2 and fatty acids, were measured at the end of the co-culture (Table 3). These inhibitory compounds are known to limit bacterial growth and to reduce the ability of bacteria to catabolise malic acid at different levels depending on their concentrations and the medium composition and pH (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988; Capucho and San Romao, 1994; Henick-Kling and Park, 1994; Guerzoni et al., 1995; Guzzo et al., 1998; Carreté et al., 2002). Although, in our case, the fatty acids and SO_2 concentrations were lower than the ones found in the literature for stopping growth and MLF (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988;

Table 4
Kinetic parameters of the MLF conducted in the treated and untreated yeast fermented media used for sequential cultures and in the bacterial control cultures.

	Bacterial control culture 1[b]	Bacterial control culture 2[c]	Untreated yeast fermented medium	Pepsin treatment	Heat treatment
Initial biomass ($g\ l^{-1}$)	0.012	0.013	0.011	0.009	0.009
Maximal biomass ($g\ l^{-1}$)	0.8	0.29	0.03	0.26	0.24
Biomass productivity ($g\ l^{-1}\ h^{-1}$)	288×10^{-5}	85×10^{-5}	8×10^{-5}	137×10^{-5}	121×10^{-5}
Initial malic acid concentration ($g\ l^{-1}$)	5.1	4	5	4.85	4.75
Final malic acid concentration ($g\ l^{-1}$)	0.06	0	4.63	0	0
Malic acid consumption rate ($g\ l^{-1}\ h^{-1}$)	18×10^{-3}	6×10^{-3}	2×10^{-3}	6.2×10^{-3}	5.3×10^{-3}
Inhibition percentage of malic acid consumption rate (%)[a]	0	67	89	65	70
Specific average malic acid consumption rate ($g\ g^{-1}\ h^{-1}$)	22.5×10^{-3}	21×10^{-3}	74×10^{-3}	24×10^{-3}	22×10^{-3}
Duration of experiment (h)	286	695	187	784	900

[a] The inhibition percentage of malic acid consumption rate is defined as the reduction of malic acid consumption rate within a pair in comparison to a control (bacterial control culture 1 in our case) and is calculated as follows: [1 − (malic acid consumption rate within a pair/malic acid consumption rate of the control)] × 100.
[b] Bacterial control culture 1: Culture of *O. oeni* X strain in the synthetic grape juice medium.
[c] Bacterial control culture 2: Culture of *O. oeni* X strain in the synthetic wine medium using an Erlen-Meyer flask.

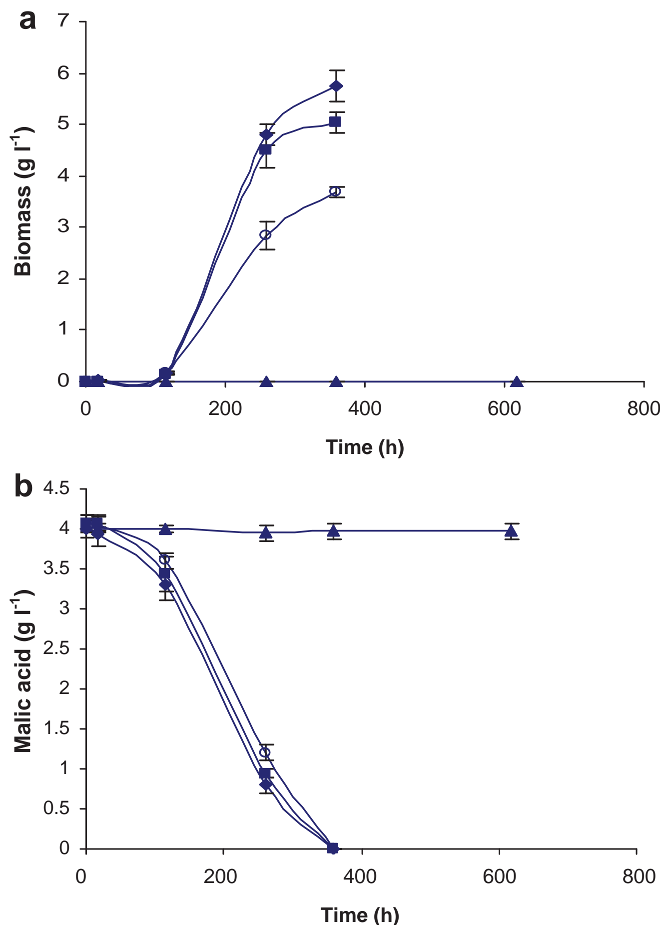

Fig. 4. Variation of the biomass of *O. oeni* X strain (a) and of its malic acid consumption (b) in the presence of three different MW fractions from the yeast fermented medium and in the control. (■) MW between 3.5 and 5 kDa, (▲) MW ≥ 5 kDa, (○) MW ≥ 10 kDa, (◆) Control: modified MRS medium. Each value is the mean of triplicate experiments ± SD.

Guzzo et al., 1998), they may have acted synergistically with ethanol and other potentially inhibitory compounds. This could explain the inhibition of *O. oeni* X strain observed when compared to its pure culture in the MBR (Table 1, bacterial control culture 1). Concerning the nitrogen consumption, our analyses confirmed that *O. oeni* did not consume ammoniacal nitrogen during its pure culture; therefore this substrate was only consumed by yeasts during the co-culture (Table 3). However, nitrogen from alpha amino acids is essential for bacterial metabolism and survival, but extremely low levels are required, as low as 0.7 mg N l^{-1} in the case of Tyrosine (9.1 mg l^{-1} of tyrosine) (Remize et al., 2006). Our measurements also showed that low levels were necessary since the consumption of only 10.7 mg N l^{-1} was sufficient to ensure good growth and complete malic acid consumption during the pure culture of *O. oeni* X strain in the MBR (Table 3). Therefore, this amount was available at the beginning of the co-culture and the bacterial inhibition was not due to a lack of nutrients but rather to the progressive appearance of the yeast inhibitory metabolites which prevented it from taking full advantage of them.

Despite its advantages, the co-culture strategy has not been widely adopted by winemakers so far because they have always feared producing high acidity in wine resulting from a large consumption of sugar by *O. oeni*, a facultative heterofermentative lactic acid bacterium (Kandler, 1983; Garvie, 1986). However, the results obtained showed weak and acceptable concentrations of D-lactic acid and acetic acid during both the co-culture and the pure culture of *O. oeni* X strain (Table 2). In addition, the pure culture showed that sugar consumption by this strain was weak (Table 3). Furthermore, the acetic acid produced during the co-culture was mainly a metabolite of the AF (Table 2). These results are in agreement with the findings of Beelman and Kunkee (1985) who noted that, in the case of some yeasts/bacteria pairs, the production of acetic acid by bacteria was weak or even non-existent when MLF and AF were conducted simultaneously. Therefore the risk of excessive volatile acidity was excluded, at least for the strain tested.

The synthetic grape juice medium fermented by *S. cerevisiae* D strain was very inhibiting towards *O. oeni* X strain during the sequential culture (Fig. 2, Table 1). The concentrations of ethanol, SO_2 and fatty acids in this medium were measured at the end of the AF and were the same as those produced by *S. cerevisiae* D strain during the co-culture (Table 3). As previously mentioned, these concentrations were lower than the ones found in literature to cease MLF. Therefore, they cannot alone entirely clarify the drastic inhibition of MLF in this case. Besides, nutrient depletion was excluded from inhibitory factors as the addition of MRS to the yeast fermented medium before inoculation of the bacteria did not reduce the inhibition (data not shown), a finding also reported by Larsen et al. (2003), Comitini et al. (2005) and Osborne and Edwards (2007). All this encouraged us to search for new inhibitory metabolites of protein nature which could explain the decrease of the bacterial activity. Results obtained after heat and pepsin treatments (Fig. 3 and Table 4) and after ultrafiltration and dialysis of the yeast fermented medium (Fig. 4) revealed the presence of an inhibitory peptidic fraction having an MW between 5 and 10 kDa and responsible for 25% of the whole inhibition. It mainly acted with ethanol to strongly inhibit growth and malic acid consumption (Table 4). While the proteinaceous compound characterized by Comitini et al. (2005) and produced by the yeast strain F63 presented an MW greater than 10 kDa, the peptide characterized by Osborne and Edwards (2007) and produced by the yeast strain RUBY. Ferm had an MW of 5.9 kDa. Therefore, we can infer from these results that the inhibitory peptides are most likely strain dependant. In addition, since the co-culture strategy gave the better result with this pair we suggest that the inhibitory peptidic fraction did not build up enough during the early stages of the AF, which allowed the bacteria to better perform MLF.

Finally, the co-culture strategy may be very interesting for winemakers since, with the pair tested, it improved the bacterial growth and malic acid consumption without risk of off-flavours (aroma and flavour defects). Therefore, this strategy is worth scaling up as it will save time and improve the MLF output. However, the choice of the yeast and bacterial strains within a pair is still an important criterion to control since, with some of the other pairs tested, no improvement of the MLF was detected (data not shown). Further work is required to determine the exact nature of the inhibitory peptidic fraction, when it is produced, its mode of action and the ability of different yeast strains to produce it.

Acknowledgment

The authors would like to thank Lallemand Inc. for their financial support and for providing the strains.

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