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Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance. Partial characterization of inhibitory compounds produced by yeasts

Lucía M. Mendoza[1], María C. Manca de Nadra[1,2] and Marta E. Farías[1,2,*]

[1]Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000, Tucumán, Argentina.

[2]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491, 4000, Tucumán, Argentina.

Running title: Inhibition of lactic acid bacteria by wine yeasts

* Corresponding author (telephone +54 381 4310465; fax +54 381 4005600; E-mail mfarias@cerela.org.ar).

Abstract

The interactions between wine yeasts and wine lactic acid bacteria were evaluated. The double-layer plate-growth method showed bacterial inhibition by yeasts as the most common interaction, being *Oenococcus oeni* more sensitive than *Lactobacillus hilgardii*. During sequential inoculations in the media fermented by *Saccharomyces cerevisiae* mc2 or *Candida pulcherrima* 3, showed strongest inhibition by S. *cerevisiae.* The inhibitory effect exerted by *S. cerevisiae* mc2 on *O. oeni* X_2L and *L. hilgardii* 5w growth could be due to a synergistic effect between fermentation metabolites and another compound(s) with a molecular size of 3-10 kDa. The last compound(s) was partially sensitive to protease and heat treatments, suggesting a peptidic nature structure. Although this yeast inhibited *O. oeni* growth, did not affect the malolactic activity. The peptidic inhibitory compound(s), heat and protease sensitive, showed a molecular weight between 3 and 10 kDa. Flow cytometry revealed different physiological responses of *O. oeni* and *L. hilgardii* to peptidic fraction. *C. pulcherrima* would exert its inhibitory effect on bacteria through low molecular weight metabolites.

Keywords: Wine yeasts, Lactic acid bacteria, Interaction, Inhibitory compound

1. Introduction

The winemaking process can consist of two main steps where alcoholic fermentation (AF) led by *Saccharomyces cerevisiae* species, is often followed by malolactic fermentation (MLF) carried out by lactic acid bacteria (LAB), mainly *Oenococcus oeni* species. The MLF which consists of the enzymatic decarboxylation of L-malic acid into L-lactic acid, is required during the vinification of most red wines and certain white and sparkling wine styles. This secondary fermentation diminishes wine acidity and improves taste, flavour and microbial stability (Kunkee, 1991; Henick-Kling, 1993; de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel & Bertrand, 1999). Nevertheless, this fermentation is often difficult to induce and control as many different factors may affect malolactic (ML) bacteria growth and activity. Consequently, selected starter cultures of wine LAB must be considered on the basis of their tolerance to the physico-chemical conditions of wine. Besides, the study of the interactions between yeast strains used to conduct AF and LAB strains is important for the comprehension of the bacterial ability to grow and carry out MLF.

The individual or synergistic effects of physicochemical factors on the occurrence of MLF has been investigated and the inhibition of this fermentation was correlated to low pH (Britz & Tracey, 1990; Vaillant, Formisyn & Gerbaux, 1995), temperature (Britz & Tracey, 1990) and nutrient depletion (Patynowski, Jiranek & Markides, 2002) as well as some inhibitory metabolites from yeasts such as ethanol (Vaillant et al., 1995; Capucho & San Romao, 1994), SO_2 (Carreté, Vidal, Bordons & Constanti, 2002) and medium chain fatty acids (Capucho & San Romao, 1994; Lonvaud-Funel, Joyeux & Desens, 1988).

Other studies have shown that the production of antibacterial peptides or proteins by yeasts may have an inhibitory effect on MLF. Dick, Molan and Eschenbruch (1992) reported the isolation of two antibacterial cationic proteins produced by *S. cerevisiae*. More recently, Comitini, Ferretti, Clementi, Mannazzu and Ciani (2005); Osborne and Edwards (2007); Nehme, Mathieu and Taillandier (2010) reported that some *S. cerevisiae* strains exerted inhibitory activity on ML bacteria by different proteinaceous or peptidic compounds.

Although *O. oeni* is the main LAB responsible for MLF, in wine other bacteria genera as *Pediococcus*, *Lactobacillus* and *Leuconostoc* are present in the process and can have positive or deleterious effects on the final product, depending on the bacterial species (Osborne & Edwards, 2005). The genus *Lactobacillus* is often involved in the spoilage of wines; particularly, the *Lactobacillus hilgardii* species has been identified as a major cause of spoilage of fortified wines (Couto & Hogg, 1994; de Revel, Capela & Hogg, 1994). This bacterium has negative ecological effects, such as hydrogen peroxide and biogenic amine production (Rodriguez & Manca de Nadra, 1995; Farías, Strasser de Saad & Manca de Nadra, 1996; Arena & Manca de Nadra, 2001).

In this context, interactive behaviors between the different species of LAB with positive and detrimental effects and the yeasts involved during winemaking need to be considered. The knowledge of the nature of yeast-bacteria interaction is important in optimal pair selection to conduce winemaking process. Also, the characterization of the nature of the inhibitory compounds involved in the antagonistic symbiosis could permit in the future to use these compounds as a biotechnological tool to biocontrol of the undesirable flora.

The objective of this study was to select yeast-LAB antagonistic pairs from a screening of the different interactions established between yeasts and LAB isolated from Argentine wines, to evaluate the influence of inhibitory yeasts on the growth and metabolism of LAB and to characterize the nature of the yeast antagonistic compounds.

2. Materials and Methods

2.1 Microorganisms

A total of 22 *Saccharomyces* and non-*Saccharomyces* yeast strains, previously isolated from grapes and musts of different regions in the Northwest of Argentina, were used. Yeasts have been identified according to conventional physiological and morphological tests described by Kurtzman and Fell (1998). The taxonomic identity of the yeasts isolated was subsequently confirmed by PCR–RFLP analysis of rDNA. All yeast strains were subcultured at 6-month intervals in YPD (Yeast extract Peptone Dextrose) agar slants and maintained at 4 ºC. Yeast strains were propagated in YPD broth pH 4.0 at 25 ºC for 24 h at least three times prior to experimental use.

Four strains of LAB isolated from Argentinean wines were utilized: *O. oeni* X_2L and ST, *L. hilgardii* 5w and X_1B (Strasser de Saad & Manca de Nadra, 1987). LAB strains were kept frozen at –20 ºC in MRS (Man Rogosa Sharpe) broth containing 20% glycerol (v/v). LAB strains were grown in MRS broth, pH 5.0 at 30°C for 24 h at least three times prior to experimental use.

All microorganisms were obtained from the Wine Yeasts and LAB Collection at the Microbiology Institute of the Biochemistry, Chemistry and Pharmacy Faculty (Tucumán National University, Argentina).

2.2 Growth media

Yeasts were grown in YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose, pH 4.0) and grape juice medium (500 ml l^{-1} grape juice, 1 g l^{-1} yeast extract, pH 3.7).

LAB were cultured in MRS medium containing 52 g l^{-1} MRS broth supplemented with 150 ml l^{-1} of tomato juice, pH 4.0. All culture media components were supplied by Merck.

2.3 Double-layer plate-growth method

In order to evaluate yeasts-bacteria interactions the double-layer plate-growth method was used. A layer of MRS agar (10 ml) was poured into a Petri dish. After solidification a top layer of MRS agar (15 ml) containing about $3x10^6$ cells ml^{-1} of bacteria, grown in MRS broth, was poured onto the bottom layer. A yeast culture grown for 48 h in YPD broth was seeded onto top layer by means of a 2-cm diameter "home-made" circular inoculator, to form two rings of yeast inoculum that were 1 cm distant from each other and to get a concentration effect within the yeast circle. Plates were incubated in microaerophilic conditions at 25 ºC during 2 and 7days for *L. hilgardii* and *O. oeni*, respectively (Lemaresquier, 1987). Yeast activity on LAB is expressed as null activity (without modification of bacterial growth); stimulatory (increased bacterial growth around the ring of yeast inoculums) or inhibitory (inhibition halo observed around yeast growth). The magnitude of the positive interaction was correlated with enhanced growth surrounding the rings of yeasts. The magnitude of the negative interaction was correlated to the diameter of inhibition zones.

2.4 Alcoholic fermentations

Fermentations were carried out in Erlenmeyer flasks containing 200 ml of grape juice medium (80 g l^{-1} of sugars, 4 g l^{-1} of malic acid, pH 3.7). *S. cerevisiae* mc2 and *Candida pulcherrima* 3 were inoculated with 10^6 cells ml^{-1} of 14 h pre-cultures grown in the same medium. Fermentations were conducted with an agitation of 150 g at 25 ºC during 2 and 6 days, to evaluate two different yeast fermentation stages.

2.5 Sequential inoculation of lactic acid bacteria in media fermented by wine yeasts

Samples were aseptically taken after 2 and 6 days of the AF and cells were removed by centrifugation (5,000 g for 10 min at 4 ºC). The supernatant was used as fermented medium for sequential inoculation of *O. oeni* and *L. hilgardii*. Unfermented grape juice medium was used as control. To avoid bacterial inhibition caused by depletion of nutrients, all media were supplemented with a concentrated solution of MRS components. After addition of this solution all media yielded a concentration of nutrients at least equal to that found in the original MRS broth medium.

Then, media were adjusted to pH 3.7 with 0.1 mol l^{-1} NaOH and were sterilized by filtration through 0.2 µm membranes (Millipore) and 100 ml of each medium was recuperated in an autoclaved Erlenmeyer flask. Bacteria were inoculated in the filtered yeast-fermented media at initial populations of $3x10^6$ cells ml^{-1} and suspensions incubated at 30°C during 96 h for *O. oeni* and 32 h for *L. hilgardii* (the incubation time corresponds to the stationary growth phase of each bacterium). Enumeration of LAB was determined by measuring OD_{560nm} or by viable plate count on MRS agar medium, pH 6.0. Biomass was also determined by weighing cells after dryness and was expressed in g l^{-1}.

2.6 Analytical determinations

2.6.1 Determination of fermentation metabolites

The ethanol concentrations produced by wine yeasts in grape juice medium was measured by enzymatic method of alcohol dehydrogenase (Bernt & Gutmann, 1974), while free and total SO_2 was determined by titration with iodine (Zoecklein, Fugelsang, Gump & Nury, 1999).

2.6.2 Determination of malic acid concentration

The L-malic acid concentration was determined in the cell free supernatants by enzymatic assay (kit no. 139068 R-Biopharm AG, Darmstadt, Germany).

2.7 Determination of the nature of inhibitory compounds produced by wine yeasts

In order to study the antibacterial effect of ethanol and SO_2, both fermentation metabolites were added to unfermented medium at concentrations produced by the inhibitory yeasts.

The heat sensitivity of the inhibitory compounds was examined by heating the fermented media (6 days by *S. cerevisiae* mc2 or *C. pulcherrima* 3) at 100 ºC for 5, 10 and 20 min. To determine the approximate size of the inhibitory compounds produced by the antagonistic yeasts, 6 days fermented broths were fractionated by ultrafiltration (3000g, 45 min, 4 ºC) through Amicon 3, 10 and 30 kDa centrifugal filter units (Millipore Corp). This yielded three times concentrated fractions containing compounds ≥ 3 kDa, ≥10 kDa, or ≥30 kDa. In order to assess the antibacterial effect, 3 ml of each concentrated fraction, adjusted to pH 3.7 and supplemented with MRS components, were inoculated with LAB at an initial concentration of $3x10^6$ cells ml^{-1}. When the malic acid utilization was evaluated, the fraction was added with 4 g l^{-1} L-malic acid.

Furthermore, to elucidate the kinetics of bacterial inhibition, the LAB were inoculated in fractions with different concentrations of the inhibitory compounds with a molecular

weight (MW) between 3 and 10 kDa (1.5, 3.0, 4.5, 6.0, 7.5 and 9.0 fold concentrated through a 3 kDa Amicon filter). The effect of each concentration on bacterial growth was estimated with non-linear regression analyses.

In addition, to evaluate the proteinaceous nature of the inhibitory compounds, fractions with antibacterial activity of both wine yeasts were treated with each of the following proteases: 80 U ml^{-1} trypsin and 10 U/ml protease XIV (Sigma, Saint Louis, MO, USA). The enzyme treatments were carried out at 37 ºC during 1 h, the pH was adjusted at 7.5 with NaOH solution. These conditions were utilized for both enzymes.

2.8 Flow cytometric analysis of bacterial viability

Percentage of viable, injured and dead bacteria were obtained by the use of BD Cell Viability Kit (BD, Biosciences). The kit contains thiazole orange (TO) solution to stain all cells and propidium iodide (PI) to stain dead cells. Live cells have intact membranes and are impermeable to dyes such as PI, which leaks into cells with compromised membranes. TO is a permeant dye and enters all cells, live and dead, to varying degrees. LAB cells precultured in MRS broth were grown to exponential phase (24 h for *O. oeni* X_2L and 12 h for *L. hilgardii* 5w) in unfermented media and 6 days yeast-fermented media. Also, both LAB were exposed to yeast inhibitory fraction with peptidic compounds. Cell samples of LAB were diluted to approximately 10^6 cells ml^{-1} in staining buffer (physiologic phosphate-buffered saline containing 0.01% Tween 20). It was added 5.0 µl of each dye solution to 500 µl of cell suspension. The final staining concentrations were 420 nmol l^{-1} for TO and 43 µmol l^{-1} for PI. The samples were vortexed and incubated for at least 5 min at room temperature.

Flow cytometric analysis was performed using a BD FACS Calibur flow cytometer (Becton, Dickinson and Co., United States) with an air-cooled argon ion laser (488 nm at

15 mW). This standard instrument was equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and three fluorescence detectors (FL1, 525 nm; FL2, 585nm; FL3, 620 nm). Data were stored as list mode files and analyzed off-line using the FCS Express version 3 software (Beckman Coulter). A combination of FSC and SSC was used to discriminate bacteria from background. TO fluoresced primarily in FL1 and FL2 while PI fluoresced primarily in FL3. Therefore, the best discrimination of live and dead populations was on an FL1 vs. FL3 plot. Also, the software allows quantifying the number of the different cell populations as percentage.

2.9 Statistical analysis

One-way analysis of variance (ANOVA) was used to test the effect of inhibitory compounds produced by wine yeasts during AF on growth parameters of LAB, and Tukey test was used for multiple mean comparisons. All statistical analyses were performed with Statistica software version 7.

3. Results

3.1 Screening of yeast activity on lactic acid bacteria by the double-layer plate-growth method

The present work evaluated interactions between several wine yeast strains and two strains of *O. oeni*, X_2L and ST, with optimal MLF performance as well as two *L. hilgardii* strains, 5w and X_1B, both with verified detrimental properties to the health quality of wines.

Results of screening with the plate method are shown in Table 1 and yeast activity on LAB is expressed as null, stimulatory or inhibitory. Four strains of *S. cerevisiae* showed inhibitory activity against *O. oeni* X_2L and ST while the remaining strains exhibited null

effect on ML bacterial growth. Non-*Saccharomyces* yeasts such as *C. pulcherrima* 3, *C. stellata* 7, *C. parapsilosis* 6, *Pichia fermentans* 8 and *Metschnikowia pulcherrima* 2(J)3 also showed inhibitory activity against both *O. oeni* strains.

However, strains of *Hanseniaspora/Kloeckera* exerted null or a stimulatory effect on the ML bacteria. From all apiculate non-*Saccharomyces* yeasts tested, 44% and 33% exerted a stimulatory effect on *O. oeni* X_2L and ST, respectively.

Moreover, a strong stimulatory effect of the yeast *Cryptococcus* spp. was observed on *O. oeni* strains.

With regard to *L. hilgardii* bacteria, only the 5w strain was inhibited by different strains of *S. cerevisiae* (mc2, 4, 5 and ATCC 39600). None of the wine yeasts showed any effect on *L. hilgardii* X_1B.

Further studies were conducted with *S. cerevisiae* mc2 and *C. pulcherrima* 3, which showed the strongest inhibition on *O. oeni* X_2L and *L. hilgardii* 5w in order to assess negative interactions.

***3.2 Sequential inoculation of Oenococcus oeni* X_2L *and Lactobacillus hilgardii* 5 w *in fermented media by Saccharomyces cerevisiae* mc2 *and Candida pulcherrima* 3**

To quantify the negative interactions the strategy adopted sequential inoculations which simulate the natural winemaking process. The yeasts carried out AF in grape juice medium for 2 and 6 days and the LAB were sequentially inoculated in these fermented media. Two different stages of the AF, middle (2 days) and final (6 days), were chosen with the aim to assess the time to which the yeasts exerted more significant inhibitory effect on LAB growth.Table 2 shows the growth kinetics and the MLF performance of both LAB strains in the yeast-fermented broths. An inhibition of *O. oeni* X_2L growth

determined by classical counting plate method was observed in the media fermented by both yeasts during 2 days. However, in this phase of AF, the inhibitory effect exerted by *S. cerevisiae* mc2 was higher than *C. pulcherrima* 3. In 6 days medium fermented by *S. cerevisiae*, the kinetic behavior of *O. oeni* was similar to that observed after 2 days of AF. In fermented broths, viable cells of *O. oeni* showed a diminution of about 3 log cycles with regard to the control (unfermented media). Also, whereas in the control media the bacterium did not show a loss of viability, in 2 and 6 days *S. cerevisiae* fermented media it was observed negative relative growth of 17.5% and 21.8%, respectively. However, the inhibitory effect of *C. pulcherrima* was higher with increasing fermentation time. The ML bacterium lost 11.9% of its viability in 6 days fermented yeast broth, whereas no cell death was observed in 2 days fermented medium (relative growth 5.9%).

S. cerevisiae exerted an inhibitory effect on the growth rate and cell population of *L. hilgardii* in both phases of the AF, but bacterial inhibition was stronger in 6 days fermented medium. Antimicrobial activity of *C. pulcherrima* against *L. hilgardii* growth was only observed in 6 days fermentation medium. In all yeast-fermented broths that showed inhibitory effect, *L. hilgardii* did not show any negative relative growth, indicating the absence of bacterial cell death.

Figure 1 shows the MLF performance of *O. oeni* X_2L in yeast-fermented broths. The bacterium completed the MLF slower in media fermented by wine yeasts compared to unfermented medium. However, a higher specific demalication rate was observed in 2 and 6 days media fermented by *S. cerevisiae* mc2 (Table 2), whereas *C. pulcherrima* 3 did not significantly modify the specific malic acid consumption rate of *O. oeni*. Even if

L. hilgardii showed a weak ML activity, similar behavior was observed when the bacterium was cultured in yeast fermented broths.

3.3 Evaluation of the nature of compounds inhibiting LAB growth

To further comprehend bacterial inhibition, yeast-fermented media were subjected to analyses in order to search for possible inhibitory molecules.

Ethanol and SO_2 production by wine yeasts was examined in 2 and 6 days fermented media (Table 3). Then, the two fermentation products were added to the unfermented medium at concentrations corresponding to the average of those determined in the supernatants. Table 4 shows that the growth rate of *O. oeni* was reduced by 10, 15 and 24% after addition of 40 g l^{-1} ethanol, 30 mg l^{-1} total SO_2 (5mg l^{-1} of free SO_2) and both compounds, respectively, to the unfermented medium. Separately the fermentation compounds did not show a noticeable inhibitory effect on the final cell population of *O. oeni*. However the combined effect of ethanol and SO_2 diminished the amount of viable cells of almost one log cycle. Even though the relative growth of *O. oeni* decreased approximately 50% by the presence of ethanol and SO_2, cell death was not observed under these conditions.

Regarding ML activity of *O. oeni*, only addition of ethanol increased the specific demalication rate of this bacterium. However, when SO_2 and ethanol were added together, this effect was reversed.

Addition of the metabolites displayed a slight inhibition of the growth parameters of *L. hilgardii*. The growth rate decreased 8.6 and 14% by the presence of ethanol, SO_2 and both metabolites, respectively, whereas the final biomass and malic acid consumption were not significantly affected.

These results revealed that ethanol, SO_2 or both metabolites together were not the only contributing factors in bacterial inhibition at the concentration tested.

In order to pursue characterization of the inhibitory metabolites produced by wine yeasts during fermentation and with the aim to assess the nature of the antibacterial compounds, the supernatants of *S. cerevisiae* and *C. pulcherrima* were subjected to heat and protease treatments.

Heat treatment of the yeast-fermented media at 100 ºC showed a partial reversion of the inhibitory effect on bacterial growth in direct relation to the heating time (Table 5). Viability of *O. oeni* was partially restored when both fermented media were heated for 20 min. Relative growth increased compared to untreated yeast-fermented media. The lower antagonistic effect of both yeasts on *L. hilgardii* 5w was partially reversed after 20 min of heat treatment.

When the yeast-fermented media were fractionated according to MW, it was observed that *S. cerevisiae* fractions with metabolites less than 3 kDa and between 3 and 10 kDa showed antimicrobial action against *O. oeni* (Figure 2A), whereas *C. pulcherrima* exerted inhibitory activity only with fractions containing compounds less than 3 kDa (Figure 2B). Figure 3 presents the effect of protease treatment on the different yeast fractions with antimicrobial activity against the ML bacterium. The inhibitory effect of the *S. cerevisiae* and *C. pulcherrima* fractions with compounds of MW ≤ 3 kDa was not affected by addition of trypsin or protease XIV, while the antibacterial activity of the fraction of *S. cerevisiae* with MW between 3 and 10 kDa decreased after addition of each enzyme. In addition, the inhibitory activity of the fractions of *S. cerevisiae* and *C. pulcherrima* was partially reverted by heat treatment during 20 min.

Similar results were obtained for *L. hilgardii* regarding antimicrobial activity of the different fractions and the effect of proteases and heat treatment when the strain was sequentially inoculated (data not shown).

In addition, LAB viability exposed to 3-10kDa inhibitory fraction of *S. cerevisiae* containing compounds of peptidic nature was evaluated by flow cytometry. The live cells decreased 66.5 and 34.2% for *O. oeni* and *L. hilgardii*, respectively (Table 6). Among the damaged cells, the most of *O. oeni* was labeled with PI (dead cells), whereas *L. hilgardii* showed 29.37% of the TO-PI labeled cells (injured cells). The peptidic fraction exerted a more pronounced inhibitory effect on both LAB in regards to the fermented media. Furthermore, the effect of different concentrations of inhibitory fractions of *S. cerevisiae* containing peptidic compound(s) with size 3-10kDa on LAB growth was evaluated. The growth rate of *O. oeni* (Figure 4A) and *L. hilgardii* (Figure 4B) decreased with increasing concentrations of antibacterial compound(s). However, the inhibitory effect on the growth rate of the bacteria did not increase further after the fraction was concentrated 6 times or more, possibly due to the saturation of the cell receptor sites for the antagonistic compound(s).

4. Discussion

LAB are responsible for the MLF in wine, which reduces acidity, changes the organoleptic properties and stabilizes the wine, with an overall positive effect. However, uncontrolled growth of LAB and the corresponding fermentation may cause spoilage, depending on the type of wine and bacterial species involved. *O. oeni* is generally used as a MLF starter because of its acid tolerance, flavor profile and production of proteases (Rodriguez & Amberg, 1990; Farías & Manca de Nadra, 2000). Unfortunately, this

species can also spoil wine if it grows at an early stage and starts to ferment hexoses. *L. hilgardii* is one of the most frequent wine-related spoilage bacteria, and its uncontrolled growth can lead to increased volatile acidity, formation of adverse odors or flavors and production of biogenic amines. Therefore, it is especially important to effectively control MLF and undesirable bacterial spoilage (Osborne & Edwards, 2005; Enrique, Manzanares, Yuste, Martínez, Vallés & Marcos, 2009).

In the present work, screening carried out with the double-layer plate-growth method showed that different genera of wine yeasts can exert null, stimulatory or inhibitory effect on bacterial growth. Four *S. cerevisiae* strains and several non-*Saccharomyces* yeasts inhibited growth of *O. oeni*. These results are in accordance with previous publications (Arnink & Henick-Kling, 2005; Comitini & Ciani, 2007). The authors indicated that among the interactive mechanisms that can occur between wine yeasts and bacteria, the ability of certain yeast strains to inhibit ML bacteria has been the most commonly reported, whereas stimulation and neutralism are less frequent. Of the two *L. hilgardii* strains assayed, only 5w was negatively sensitive to yeast activity.

The semi-quantitative method utilized in the previous screening for the effect of different yeasts on LAB cannot always successfully be extrapolated to natural media (Arnink & Henick-Kling, 2005; Taillandier, Tataridis & Strehaiano, 2002). Therefore, in order to study the inhibitory activity exerted by the two yeasts (*S. cerevisiae* mc2 and *C. pulcherrima* 3) on bacterial growth, sequential fermentations were carried out. These yeasts were selected due to the strongest inhibition on both LAB and by its oenological relevance. *S. cerevisiae*, is the main yeast to carry out the AF and *C. pulcherrima*, a non-*Saccharomyces* yeast dominant at the beginning of fermentation with a positive impact

on the wine aroma (Zohre & Erten, 2002; Rodríguez, Lopes, Barbagelata, Barda & Caballero, 2010). The classical plate-counting method revealed that both yeasts inhibited growth kinetics of LAB, and *O. oeni* X_2L was found to be more sensitive than *L. hilgardii* 5w. *S. cerevisiae* mc2 showed more bacterial inhibition at both AF stages than *C. pulcherrima* 3, and after 6 days of fermentation the antagonistic effect towards LAB growth was higher. These findings are consistent with those by Larsen et al. (2003), who found that when *O. oeni* was inhibited by a yeast strain, inhibition was generally the highest during the middle stages of the AF. This suggests that certain bound forms of SO_2 produced by *S. cerevisiae* may be involved in the inhibition of *O. oeni*. Osborne and Edwards (2007) reported that *S. cerevisiae* inhibited ML bacteria during almost the entire AF.

Despite the fact that growth of *O. oeni* was inhibited by *S. cerevisiae* mc2 and *C. pulcherrima* 3, these wine yeasts did not modify the ability of the bacteria to carry out MLF. Even more, the specific demalication rate by the ML bacterium was higher during sequential inoculation in *S. cerevisiae*-fermented broth. This behavior could be related to an increase in the permeability of the cytoplasmic membrane of the living *O. oeni* cells, allowing major passive transport of organic acid across the membrane caused by the higher ethanol concentration produced by this yeast. *C. pulcherrima* 3, a lower ethanol-producing strain, did not significantly modify the specific malic acid consumption rate of *O. oeni*.

Previous studies have revealed similar results. Arnink and Henick-Kling (2005) observed that inhibition of *O. oeni* growth due to interactions with *S. cerevisiae* did not necessarily

inhibit MLF. Nehme, Mathieu and Taillandier (2008) found that *O. oeni* X strain, which growth was inhibited by *S. cerevisiae* C, showed a rise in the demalication rate.

Although the biochemical basis of the antagonistic interaction between wine yeasts and LAB may be unclear, several factors, or their combination, including the production of bioactive yeast metabolites, could be involved. Ethanol and SO_2 have been reported to be responsible for this antagonistic behavior (Capucho & San Romao, 1994; Carreté et al., 2002). The influence of these fermentation metabolites on bacterial growth was evaluated by addition of both compounds to control medium at the same concentrations produced by inhibitory yeasts. Only the combination of ethanol and SO_2 showed significant inhibition of LAB. However, this antagonistic effect was lower than that produced by *S. cerevisiae* mc2 and *C. pulcherrima* 3 on sequentially inoculated LAB. Even if addition of the combined metabolites produced a decrease in the bacterial relative growth no cell death was observed. However, in 6 days media fermented by *S. cerevisiae* and *C. pulcherrima*, ML bacterium showed negative relative growth, indicating a loss of viability.

Other products of yeast metabolism, including medium chain fatty acids such as decanoic and dodecanoic acids may also be inhibitory to ML bacteria. Although these effects are highly dependent upon the type and concentration of fatty acids present in the medium (Edwards & Beelman, 1987; Lonvaud-Funel et al., 1988; Capucho & San Romao, 1994). The sensitivity to fatty acids of strains of LAB utilized in this work, *O. oeni* X_2L and *L. hilgardii* 5w, was previously evaluated by Manca de Nadra and Strasser de Saad (1991). The authors determined that both strains were only inhibited to high concentration of dodecanoic acid (20 mg/l), whereas decanoic acid did not affect the bacterial growth. Our

previous studies indicated that yeast strains used in this work are poor fatty acid producers.

Another possible explanation for inhibition of LAB growth proposed by certain authors is the depletion of nutrients by wine yeasts during AF (Gilis, Dupuy & Strehaiano, 1996; Nygaard & Prahl, 1997). However, in the present work, prior to sequential inoculation with LAB, the yeast-fermented media were supplemented with MRS components to rule out the lack of nutrients (see Materials and Methods).

The fermented media were fractionated and *S. cerevisiae* mc2 showed two fractions with antibacterial inhibition. The fraction with metabolites ≤ 3 kDa showed a decrease in activity after heating, but was not affected by protease treatment. Whereas the fraction with a MW between 3 and 10 kDa was partially sensitive to protease and heat exposure, suggesting that the yeast produced inhibitory compound of peptidic nature. Previous studies have indicated that the inhibitory activity of specific *S. cerevisiae* strains against *O. oeni* is partly due to the natural production of uncharacterized peptides or proteins during AF (Dick et al., 1992; Comitini et al., 2005; Osborne & Edwards, 2007; Nehme et al., 2010). The estimated MW (3-10 kDa) of the peptidic antibacterial compounds produced by *S. cerevisiae* mc2 is in agreement with those reported by some authors. Nehme et al. (2010) found that 25 % of the whole inhibition exerted by *S. cerevisiae* D on *O. oeni* X strain is due to a peptidic fraction with MW between 5 and 10 kDa. Also, Osborne and Edwards (2007) indicated that *S. cerevisiae* strain RUBY.ferm inhibited an *O. oeni* strain through a peptide of 5.9 kDa. However, other authors reported that *S. cerevisiae* produced proteinaceous compounds with MW greater to 10 kDa (Dick et al., 1992; Comitini et al., 2005).

On the other hand, when *O. oeni* was grown in the peptidic fraction a decrease in the demalication rate was observed but no residual acid it was detected at the final of the bacterial growth (data not shown). These results are in disagreement with previous publications (Comitini et al., 2005; Osborne & Edwards, 2007; Nehme et al., 2010). The authors reported that *O. oeni* did not consume the malic acid in presence of peptidic or proteinaceous compounds.

Flow cytometric analysis indicated that *O. oeni* cells exposed to proteic inhibitory fraction (3 and 10 kDa) of *S. cerevisiae* showed a more pronounced drop of the viability in regards to *L. hilgardii*. The size of the subpopulation of cells in an intermediate state of membrane damage (double-stained cells with TO-PI) was higher for *L. hilgardii* indicating a progressive change in the physiological status of this bacterium. Moreover, the subpopulation corresponding to dead cells, stained only with PI, was almost ten times smaller in comparison with *O. oeni*. The subpopulations heterogeneity observed between both bacteria could reveal different physiological responses to antimicrobial peptidic compound produced by yeast. Others authors (Herrero, Quirós, García & Díaz, 2006; da Silveira, San Romao, Loureiro-Dias, Rombouts & Abee, 2002) reported similar results for LAB species exposed to stress conditions.

On the other hand, the inhibitory fraction of *C. pulcherrima* 3 with low-size compounds was heat sensitive. This yeast strain would exert its antibacterial activity by production of fermentation metabolites and/or other uncharacterized compound(s). One of them could be pulcherrimic acid, a dibasic acid synthesized by certain strains of *C. pulcherrima* with inhibitory activity against bacteria and moulds (MacWilliam, 1959; MacDonald, 1965; Sipiczki, 2006). However, more specific information on the possible production of

pulcherrimic acid by the *C. pulcherrima* strain assayed in this study and the activity of this acid on LAB is required. When the 3-10 kDa fraction of *S. cerevisiae* mc2 was concentrated, the antagonistic effect on the LAB growth rate increased until a 6x-concentrated fraction. Bacterial inhibition was similar in higher concentrated fractions, indicating that the protease sensitive compound(s) showed typical saturation kinetics. Comitini et al. (2005) reported that the inhibitory effect exerted by proteinaceus factor(s) of *S. cerevisiae* F63 on *O. oeni* is dose-dependent, which would suggest the presence of a receptor on bacterial cells.

In conclusion, the antagonistic effect on LAB by 22 Argentine wine yeasts assayed in the current study was predominant, mainly among *Saccharomyces* yeasts. Some apiculate non-*Saccharomyces* yeasts showed a stimulatory effect on *O. oeni* strains, which is of biotechnological importance due to the present trend to include this yeast in winemaking to improve the wine flavor.

The inhibitory effect exerted by *S. cerevisiae* mc2 on growth of *O. oeni* X_2L and *L. hilgardii* 5w could be due to a synergistic effect between fermentation metabolites and another compound(s) with a MW of 3-10 kDa and peptidic nature. These compounds inhibited bacterial growth but did not affect the ability of *O. oeni* X_2L to consume malic acid. Peptidic substance(s) produced by *S. cerevisiae* mc2 also inhibited the LAB growth rate showing saturation kinetics and exerted different physiological responses of both bacteria.

Fermentation metabolites and other compounds with low MW produced by *C. pulcherrima* 3 could be the substances that exerted the antagonistic effect on LAB.

This study highlights the importance of evaluating the interactions between wine yeasts and LAB in order to control MLF and avoid bacterial spoilage. Also, the control of *L. hilgardii*, potentially detrimental during winemaking, is also important to remark. However, further research is required in order to characterize fully the inhibitory compounds produced by *Saccharomyces* and non-*Saccharomyces* yeasts, taking into account their possible future utilization for undesirable flora biocontrol.

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Figure Captions

Figure 1. Utilization of L-malic acid by *O. oeni* X_2L in unfermented medium (♦), media fermented by *S. cerevisiae* mc2 (■) and *C. pulcherrima* 3 (Δ) for 2 (A) and 6 (B) days. Data are mean values of three experiments.

Figure 2. Growth of *O. oeni* X_2L in unfermented medium (♦), media fermented by *S. cerevisiae* mc2 (A) and *C. pulcherrima* 3 (B) fractionated and containing compounds with MW ≤ 3 kDa (□), ≥3 kDa (Δ), ≥10 kDa(◊) and ≥30 kDa (○). Data are mean values of three experiments.

Figure 3. Effect of the trypsin addition to fractions with antibacterial activity. Growth of *O. oeni* X_2L in unfermented medium (♦); fraction ≥3 kDa of *S. cerevisiae* mc2 without treatment (■) and treated with protease (□); fraction ≤ 3 kDa of *S. cerevisiae* mc2 without treatment (▲) and treated with protease (Δ); fraction ≤ 3 kDa of *C. pulcherrima* 3 without treatment (●) and treated with protease (○). Data are mean values of three experiments.

Figure 4. Influence on the growth rate of *O. oeni* X_2L (A) and *L. hilgardii* 5w (B) of increasing concentrations of the inhibitory fraction containing compounds with MW between 3 and 10 kDa produced by *S. cerevisiae* mc2. Experiments were performed in duplicated.

Table 1. Screening of yeast-bacteria interactions by double-layer plate-growth method.

Wine yeast strains	**Lactic acid bacteria strains**			
	O. oeni X_2L	*O. oeni* ST	*L. hilgardii* 5w	*L. hilgardii* X_1B
***Saccharomyces* yeasts**				
S. cerevisiae mc2	---	--	--	**N**
S. cerevisiae ATCC 36900	--	--	-	**N**
S. cerevisiae 4	-	-	-	**N**
S. cerevisiae 5	-	-	-	**N**
S. cerevisiae 42	**N**	**N**	**N**	**N**
S. cerevisiae 48	**N**	**N**	**N**	**N**
Non-*Saccharomyces* apiculate yeasts				
Kloeckera apiculata mc1	+	**N**	**N**	**N**
Kloeckera apiculata 1	+	+	**N**	**N**
Kloeckera apiculata 2	+	+	**N**	**N**
Kloeckera apiculata II	**N**	**N**	**N**	**N**
Kloeckera apiculata A	**N**	**N**	**N**	**N**
Kloeckera apiculata B	**N**	**N**	**N**	**N**
Kloeckera africana mf	**N**	**N**	**N**	**N**
Hanseniaspora uvarum C	**N**	**N**	**N**	**N**
Hanseniaspora uvarum I	+	+	**N**	**N**
Non-*Saccharomyces* yeasts				
Cryptococcus spp. B3	+++	++	**N**	**N**
Pichia fermentans 8	--	-	**N**	**N**
Pichia guilliermondii 9	**N**	**N**	**N**	**N**
Candida pulcherrima 3	--	--	--	**N**
Candida stellata 7	--	-	**N**	**N**
Candida parapsilosis 6	-	-	**N**	**N**
Metschnikowia pulcherrima 2J3	--	-	**N**	**N**

N: null activity

Code of intensity of observed stimulation: +, weak; ++, moderate; +++, strong

Code of intensity of observed inhibition: -, weak (inhibition zone of 2-3 mm); --, moderate (inhibition zone 4-5 mm); --- strong (inhibition zone >5 mm).

Table 2. Effect of inhibitory yeasts on growth parameters and malolactic activity of lactic acid bacteria in sequential fermentations.

		***O. oeni* X$_2$L**			
	Control	*S. cerevisiae*	*C. pulcherrima*	*S. cerevisiae*	*C. pulcherrima*
		2 days of fermentation		6 days of fermentation	
Final biomass (cfu ml^{-1})	1.2x10^8	4.1x10^5	1.6x10^7	2.1x10^5	9.8x10^5
μ (h^{-1}) *	0.21±0.02^a	0.09±0.01^b	0.18±0.02^a	0.08±0.01^b	0.13±0.02^c
Relative growth (%)$^\&$	18.8±0.9^a	-17.5±1.3^b	5.9±1.1^c	-21.8±1.4^d	-11.9±1.6^e
Malic acid consumption rate (g l^{-1} h^{-1})	0.16±0.01^a	0.12±0.01^b	0.15±0.01^a	0.10±0.015^b	0.12±0.01^b
Specific demalication rate (g g^{-1} h^{-1}) $^\pi$	0.47±0.01^a	0.54±0.01^b	0.48±0.01^{ac}	0.56±0.02^b	0.50±0.01^c
		***L. hilgardii* 5w**			
Final biomass (cfu ml^{-1})	3.0x10^8	9.8x10^7	2.8x10^8	5.9x10^7	1.6x10^8
μ $_{max}$ (h^{-1})	0.36±0.02^a	0.28±0.01^b	0.36±0.02^a	0.22±0.02^c	0.32±0.01^d
Relative growth (%)	30.5±1.2^a	22.9±1.3^b	30.0±1.1^a	19.5±1.6^c	26.1±2.1^d
Malic acid consumption rate (g l^{-1} h^{-1})	0.06±0.01^a	0.05±0.01^{ab}	0.06±0.01^a	0.03±0.01^b	0.05±0.01^{ab}
Specific demalication rate (g g^{-1} h^{-1}) $^\pi$	0.13±0.01^a	0.12±0.01 ^{a}	0.13±0.01 ^{a}	0.15±0.01 ^{a}	0.14±0.01 ^{a}

*Maximum specific growth rate (h^{-1}).

$^\&$Relative growth (%) = (X_t - X_0)/ X_0 x100 where X_0: initial viable cell number; X_t: viable cell number after 96 h for *O. oeni* and 32 h for *L. hilgardii*. Negative values indicate cellular death.

$^\pi$Specific demalication rate (g g^{-1} h^{-1}) = Malic acid consumed (g l^{-1}) / duration of MLF (h^{-1}) / biomass (g l^{-1}) present at this time.

Mean values with different superscript letters within the horizontal line are significantly different according to the Tukey test ($p \leq 0.05$).

Data are mean values of three experiments ± standard deviation.

Table 3. Concentration of fermentation metabolites produced by wine yeasts during alcohlic fermentation.

	Ethanol (g l^{-1})		**Total SO_2 (mg l^{-1})**		**Free SO_2 (mg l^{-1})**	
	2 days*	6 days	2 days	6 days	2 days	6 days
S. cerevisiae mc2	29.8±1.3[a]	38.9±1.1[a]	16.5±0.6[a]	29.1±0.8[a]	2.8±0.1[a]	5.4±0.2[a]
Candida pulcherrima 3	14.6±1.1[b]	28.5±0.9[b]	12.2±0.4[b]	21.6±0.5[b]	2.1±0.2[b]	3.2±0.2[b]

* Time of incubation at 25 ºC in grape juice medium.

Mean values with different superscript letters within each column are significantly different according to the Tukey test ($p \leq 0.05$).

Data are mean values of three fermentations ± standard deviation.

Table 4. Influence of addition of fermentation products on growth and metabolic kinetics of *O. oeni* and *L. hilgardii*.

	***O. oeni* X_2L**			
	Control	Ethanol	SO_2	Ethanol and SO_2
Final biomass (cfu ml^{-1})	$1.2x10^8$	$8.7x10^7$	$7.9x10^7$	$2.3x10^7$
μ_{max} (h^{-1})*	$0.21±0.02^a$	$0.19±0.01^{ab}$	$0.18±0.01^b$	$0.15±0.01^c$
Relative growth (%)&	$18.8±0.9^a$	$16.7±1.4^a$	$16.1±1.8^a$	$8.2±1.6^b$
Malic acid consumption rate (g l^{-1} h^{-1})	$0.16±0.01^a$	$0.17±0.01^a$	$0.14±0.01^{ab}$	$0.13±0.01^b$
Specific demalication rate (g g^{-1} h^{-1}) π	$0.47±0.01^a$	$0.53±0.02^b$	$0.44±0.01^c$	$0.43±0.01^c$
	***L. hilgardii* 5w**			
Final biomass (cfu/ml)	$3.0x10^8$	$2.1x10^8$	$2.4x10^8$	$1.1x10^8$
μ_{max} (h^{-1})	$0.36±0.01^a$	$0.33±0.01^{bc}$	$0.34±0.01^{ab}$	$0.31±0.01^c$
Relative growth (%)	$30.5±1.2^a$	$28.0±1.5^a$	$28.9±1.3^a$	$23.7±1.1^b$
Malic acid consumption rate (g l^{-1} h^{-1})	$0.06±0.01^a$	$0.05±0.01^a$	$0.06±0.01^a$	$0.05±0.01^a$
Specific demalication rate (g g^{-1} h^{-1})	$0.13±0.01^a$	$0.14±0.01^a$	$0.13±0.01^a$	$0.12±0.01^a$

* Maximum specific growth rate (h^{-1})

&Relative growth (%) = $(X_t - X_0)/ X_0 \times 100$ where X_0: initial viable cell number; X_t: viable cell number after 96 h for *O. oeni* and 32 h for *L. hilgardii*. Negative values indicate cellular death.

πSpecific demalication rate (g g^{-1} h^{-1}) = Malic acid consumed (g l^{-1}) / duration of MLF (h^{-1}) / biomass (g l^{-1}) present at this time.

Mean values with different superscript letters within the horizontal line are significantly different according to the Tukey test ($p \leq 0.05$).

Data are mean values of three experiments ± standard deviation.

Table 5. Effect of heat treatment on inhibitory activity exerted by yeasts against lactic acid bacteria.

	Relative growth (%)*								
	Control medium	*S. cerevisiae* fermented media				*C. pulcherrima* fermented media			
		Untreated	Heat treated			Untreated	Heat treated		
			5 min	10 min	20 min		5 min	10 min	20 min
O. oeni X_2L	18.8±0.9	-21.8±1.4	-17.6±1.1	-12.1±1.5	0.3±0.6	-11.9±1.6	-10.1±1.1	-7.3±1.3	1.4±0.7
L. hilgardii 5w	30.5±1.2	19.5±1.6	21.4±1.3	22.3±1.1	23.7±1.2	26.1±2.1	26.6±1.8	27.4±1.3	28.6±1.5

*Relative growth (%) = $(X_t - X_0)/ X_0 \times 100$ where X_0: initial viable cell number; X_t: viable cell number after 96 h for *O. oeni* and 32 h for *L. hilgardii*. Negative values indicate cellular death.

Data are mean values of three experiments ± standard deviation.

Table 6. Percentage (%) of lactic acid bacteria viability evaluated by flow cytometry in different culture conditions.

	O. oeni X_2L			*L. hilgardii* 5w		
	Live	Injured	Dead	Live	Injured	Dead
Unfermented media	99.11±1.5	0.78±0.03	0.11±0.01	98.23±2.7	1.65±0.05	0.12±0.01
Fermented media*	89.32±2.0	5.94±0.3	4.74±0.2	95.65±1.5	2.74±0.07	1.61±0.04
Fraction 3-10 kDa&	32.57±0.5	8.19±0.5	59.24 ±2.5	64.05±1.8	29.37±1.1	6.58±0.3

Viability at 0 h = 100%

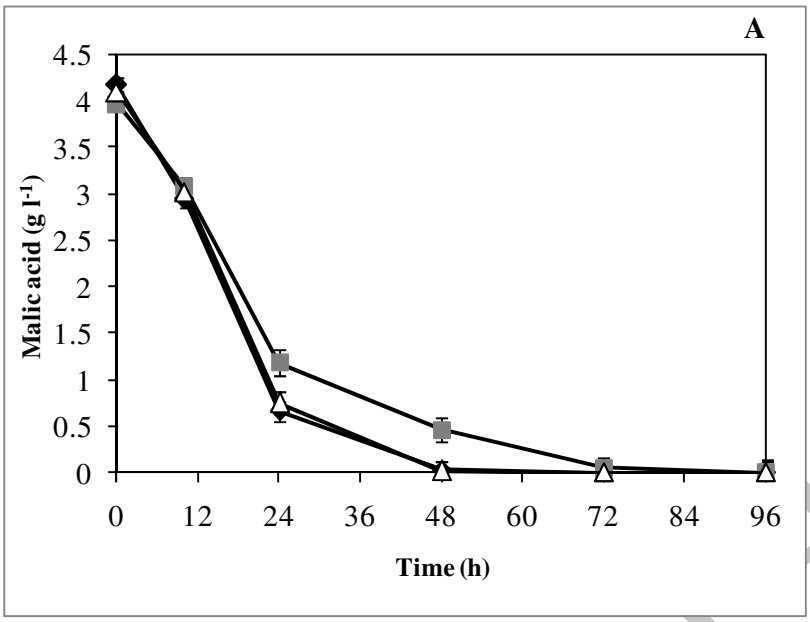
L. hilgardii 5w and *O. oeni* X_2L were incubated at 30 ºC during 12 and 24 h, respectively.

*Media fermented by *S. cerevisiae* mc2 during 6 days at 25 ºC.

&Fraction containing peptidic compounds with weight between 3 and 10 kDa obtained from yeast-fermented media by ultrafiltration.

Data are mean values of two experiments ± standard deviation.

Fig. 1



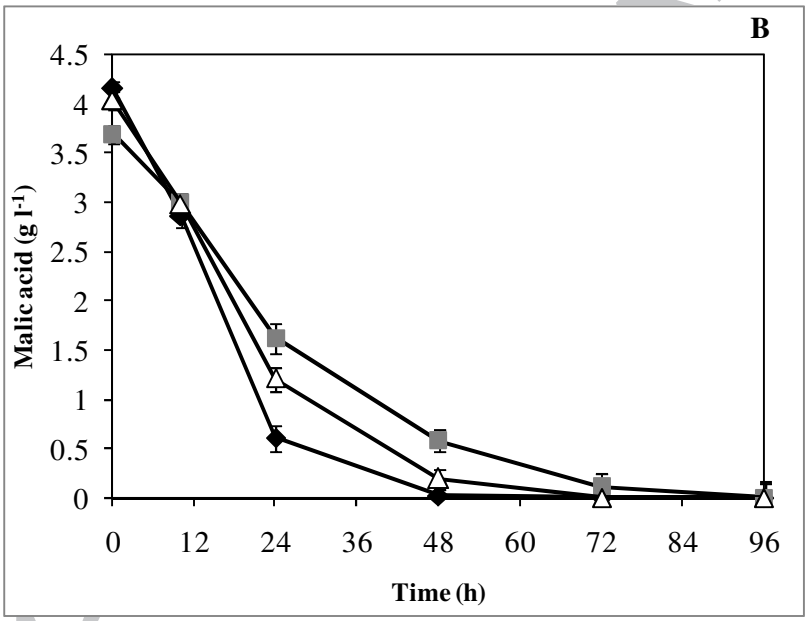
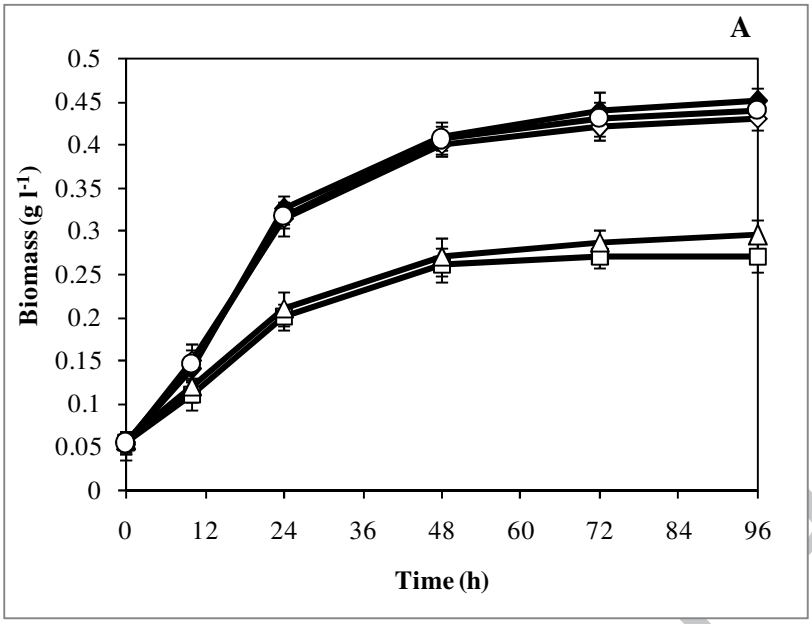


Fig. 2



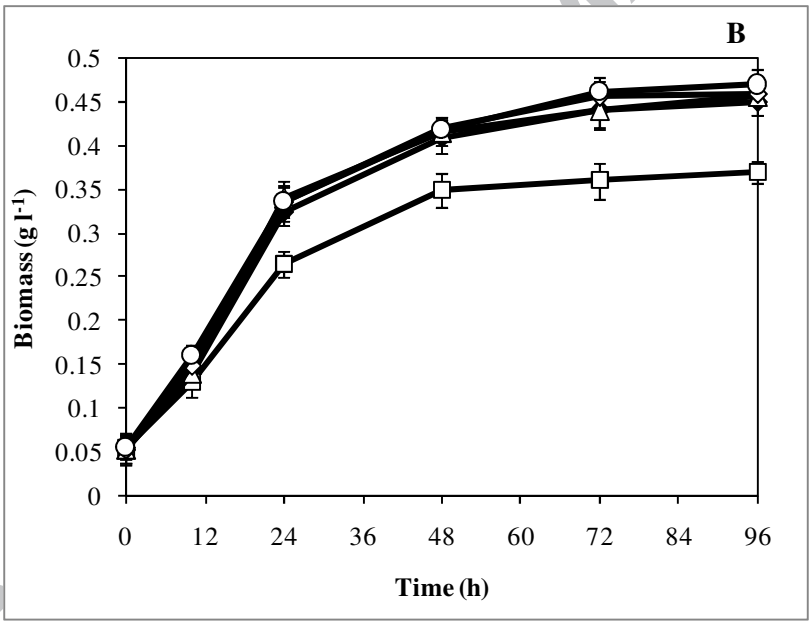


Fig. 3

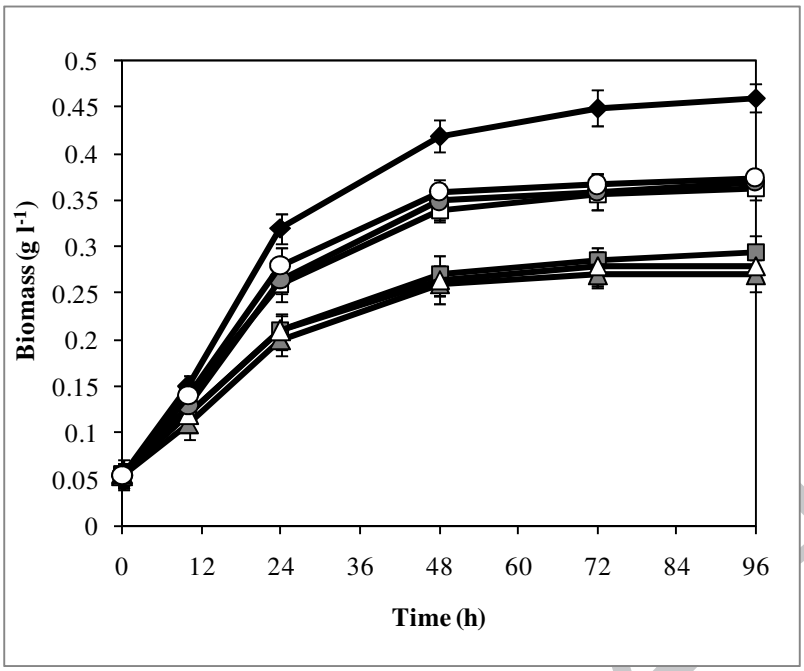


Fig. 4