The effect of hydroxycinnamic acids on growth and H\(^+\)-ATPase activity of the wine spoilage yeast, *Dekkera bruxellensis*

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Hydroxycinnamic acids are lipophilic compounds naturally present in grape must, and proposed to have antimicrobial properties. Consequently, microorganisms that grow in media containing these acids must have efficient adaptation mechanisms. In *Saccharomyces cerevisiae* hydroxycinnamic acids enter into the cell where they are deprotonated causing a decrease in internal pH, this variation in the intracellular pH is counteracted by an increase in the activity of the H\(^+\)-ATPase pump Pma1p. *Dekkera bruxellensis* however, is able to transform hydroxycinnamic acids into volatile-less toxic derivates, a mechanism used by few yeast species. Nonetheless, *D. bruxellensis* could also have an adaptation mechanism similar to that of *S. cerevisiae*. Our results showed that hydroxycinnamic acids caused a longer lag phase during *D. bruxellensis* growth, particularly when supplementing media with ferulic acid. Additionally, extracellular pH decreased while Pma1p activity increased during lag phase in media supplemented with p-coumaric acid. These results suggest the existence of a complementary mechanism of resistance to hydroxycinnamic acids in *D. bruxellensis* which involves the H\(^+\)-ATPase pump Pma1p.

**Key words:** *Dekkera bruxellensis*, H\(^+\)-ATPase Pma1p, p-coumaric acid.

**INTRODUCTION**

Hydroxycinnamic acids (HCAs) are the most important group of polyphenols present in wine. These compounds which are initially esterified with tartaric acid are released into the grape juice by the action of cinnamoyl esterase enzymes naturally found in the grape must. Within hydroxycinnamic acids, the most important compounds are cafeic, p-coumaric and ferulic acid (Vrhovšek, 1998). These molecules are weak acids with a lipophilic character and show antioxidant and antimicrobial properties. In addition, these compounds are precursors of volatile phenols (4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, 4-vinylguaiacol) that impact negatively on wine sensory properties.

Since wine pH hydroxycinnamic acids are protonated, they can freely diffuse into the cell where they release protons affecting the cellular capacity to maintain pH homeostasis, blocking the transport of substrates, and finally inhibiting growth (Piper et al., 2001; Beales, 2004). Some studies have reported a H\(^+\)-ATPase enzyme (Pma1p) in *S. cerevisiae* which pumps protons to the extracellular media in response to increased concentration of weak acid in the culture media (Chambel et
al., 1999; Viegas et al., 1998). Thus, Pma1p constitutes a support mechanism to counteract the decrease in internal pH caused by the presence of weak acids (Sá-Correia et al., 1989; Viegas and Sá-Correia, 1995; Viegas et al., 1995; Carmelo et al., 1997). Indeed, octanoic acid and cinnamic acid increase the activity of Pma1p in S. cerevisiae and extend the duration of the lag phase (Viegas et al., 1998; Chambel et al., 1999). During this extended period cells adapt to the toxic effects of weak acids, and the duration of lag phase would depend on the concentration of these acids.

Thus, for S. cerevisiae there is a direct co-relation between the antimicrobial effects of weak acids, the duration of lag phase and the activity of H+ -ATPase pump Pma1p. D. bruxellensis responds to hydroxycinnamic acids toxicity by metabolizing these compounds into less toxic volatile metabolites (Dias et al., 2003). Nevertheless, we have observed that p-coumaric acid affects D. bruxellensis growth extending lag phase duration. Similarly to described Curtin et al. (2012) and Piskur et al. (2012), we found that D. bruxellensis L1359 has a Pma1p protein, which is involved in a mechanism of adaptation to hydroxycinnamic acids, and seems to be activated during lag phase in the presence of these acids.

MATERIALS AND METHODS

Strains and culture conditions

D. bruxellensis L1359 was obtained from the strain collection of the Applied Microbiology and Biotechnology Laboratory of the Universidad de Santiago de Chile.

D. bruxellensis growth was evaluated in microtiter plates sealed with gas permeable membranes. Briefly, colonies from YPD agar were inoculated into YPD media (0.5% peptone, 0.5% yeast extract, 4% glucose, pH 6.0) and grown overnight at 28°C with shaking (150 rpm). This culture was then inoculated in 200 µL of Synthetic Dextrose Minimal Medium (SD) (glucose 20 g/L, YNB 6.7 g/L, pH 4.3 (unbuffered)) containing different concentrations of hydroxycinnamic acids at a cell density of 1 x 10⁶ cells/mL. SD media contained 0, 25, 50, 75 and 100 mg/L of p-coumaric acid, caffeic acid and ferulic acid (Sigma-Aldrich, USA), each condition was replicated thrice. The pH value of the media was not modified by the presence of HCA. Plates were maintained at 28°C for three days, with 10 s of agitation (500 rpm) every hour. Growth was monitored by measuring optical density at 600 nm using Elix 808 multiplates reader (BioTek, USA) coupled to the Gen5 program (BioTek, USA).

The specific growth rate (µ) was estimated from the slope of the growth curve during exponential phase according to the equation: 
\[ \mu = \frac{x_0}{x_0 + \mu t}, \]
where: \( x_0 \) and \( x_0 + \mu t \) correspond to the biomass concentration or the optical density (OD) at time t (h) and t = 0, respectively (Barata et al., 2008). The \( R^2 \) values of the curves were 0.996 or higher in all cases.

Lag phase duration was determined mathematically according to Buchanan and Cygnarowicz (1990) as the time when the second derivative of the logarithm of the growth curve reaches a maximum value.

Evaluation of extracellular acidification during yeast growth

Extracellular acidification was evaluated as described by Chambel et al. (1999). Yeast colonies were grown overnight in YPD media at 28°C with shaking (150 rpm). Cultures were then inoculated in 1 L of SD media (control) or SD media supplemented with 100 mg/L of p-coumaric acid. SD media were inoculated at 5 x 10⁵ cell/ml and incubated at 28°C with shaking (150 rpm). All cultures were performed in triplicate. Cell counts were determined using a Neubauer chamber as described previously (Becker et al., 1999). Samples (5 ml) were taken periodically during yeast growth, centrifuged at 3000 xg for 5 min and pH was determined from the supernatant using a pH meter (HI 2221 Calibration Check Ph/ORP Meter, Hanna Instruments, USA).

Determination of acetic acid during growth by HPLC

The following acetic acid was done using the technique of high performance liquid chromatography (HPLC) (Shimadzu Scientific Instruments, Colombia, MD, USA). The ion exchange column Bio-Rad HPX-87H was used, a mobile phase of sulfuric acid (5 mmol/L), at a flow rate of 0.4 mL/min, IR and UV detector at 200 nm, at 55°C (Ross et al., 2009). Detection limit was 0.05 g/L.

Plasma membrane ATPase Pma1p activity assay

The activity of the plasma membrane ATPase Pma1p was estimated from the rate of phosphate production after ATP hydrolysis (Baykov et al., 1988). First, cell cultures were grown in SD media (supplemented and unsupplemented with p-coumaric acid) as described above. Culture samples (100 ml) were taken during lag phase (12 h for unsupplemented and 48 h for supplemented), exponential phase (48 h for unsupplemented and 144 h for supplemented) and stationary phase (168 h for unsupplemented and 216 h for supplemented) and centrifuged at 3000 xg for 2 min at room temperature. After centrifugation, cell pellets were resuspended in 800 µL of SD medium for 5 min at room temperature with occasional agitation. Cells were then disrupted with glass beads (0.5 mm; Sigma, St. Louis, USA) to obtain crude membrane suspensions as previously described by Serrano (1983)

To avoid interfering ATP hydrolysing or phosphatase activities specific inhibitors were used for the enzymatic assay. Thus, plasma membrane ATPase activity was assayed in crude membrane suspensions using 50 mmol/L of buffer MES (2-(N-morpholino)ethanesulfonic acid) pH 5.7, 10 mmol/L MgSO4, 50 mmol/L KCl, 0.2 mmol/L ammonium heptamolybdate (phosphatase inhibitor), 5 mmol/L NaN3 (ATPase mitochondrial inhibitor), 100 mmol/L KNO3 (vacuolar ATPase inhibitor) and 2 mmol/L ATP (Sigma, St. Louis, USA). Under these conditions, ATPase activity could be attributed predominantly to plasma membrane H+ -ATPase Pma1p. Phosphate released by Pma1p activity was then quantified according to Baykov et al. (1988). Phosphate forms a bright green complex with malachite green in acid conditions which can be followed spectrophotometrically at 630 nm. Plasma membrane ATPase specific activity (U/mg) was calculated from the rate of phosphate production and was expressed as micromoles of phosphate released per min (U) per mg of protein. Protein concentration in crude membrane suspensions was determined according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis of the data

Statistical comparisons were made using the Student's t-test or analysis of variance (ANOVA) as indicated and considered significant differences at p ≤ 0.05. This analysis was carried out using Statgraphics Plus, version 5.1 (StatPoint Technologies, Warrenton, Virginia, USA).
RESULTS AND DISCUSSION

Inhibition of yeast growth by hydroxycinnamic acids

The effect of hydroxycinnamic acids on *D. bruxellensis* growth depends on their concentration (Baranowski et al., 1980). In this work, we assessed the effect of several concentrations (0-100 mg/L) of *p*-coumaric acid, caffeic acid and ferulic acid on the cell growth of *D. bruxellensis* L-1359. At concentrations of 25 mg/L of HCA there was a small but significant increase on growth rate as compared to the control (Table 1). In contrast, concentrations of 75 mg/L or more affected negatively *D. bruxellensis* growth rate. This inhibition of cell growth can be expected since HCAs act as antimicrobial agents (Baranowski et al., 1980). Similar results have been reported previously showing that ferulic acid at 388 mg/L inhibited the growth of several *D. bruxellensis* strains (Harris et al., 2008). Positive effects of HCAs on the growth rate of different isolates of *Dekkera/Brettanomyces* spp. have also been reported (Godoy et al., 2009). These findings suggest that the effect of HCAs on cell growth might be strain-dependent.

Ferulic acid showed the most negative effect on growth rate (Table 1). Baranowski et al. (1980) reported that the inhibitory capacity of HCAs is proportionally inverse to its polarity, making ferulic acid the most inhibitory of the acids assayed in this study.

The addition of HCAs also altered the length of the lag phase for *D. bruxellensis* (Table 2). At 100 mg/L *p*-coumaric acid extended the duration of the lag phase from 2 (control) to 15 h (Table 2), while ferulic acid and caffeic acid increased lag phase to 16.5 and 12 h, respectively. Similar results showing a longer lag phase have been reported for *S. cerevisiae* growing in media supplemented with *p*-coumaric acid (Baranowski et al., 1980) and for *D. bruxellensis* exposed to *p*-coumaric acid (Dias et al., 2003) and ferulic acid (Harris et al., 2008, 2010).

Evaluation of extracellular acidification during yeast growth

Weak acids can enter the cell undissociated and once inside dissociate affecting intracellular pH and potentially cell metabolism. Thus, to maintain intracellular homeostasis the yeast cell requires mechanisms that can reduce the concentration of protons in the cytoplasm. It has been reported that *S. cerevisiae* can decrease extracellular pH during the first hours of cell growth (lag phase) when exposed to weak acids such as cinnamic acid (Chambel et al. 1999) and sorbic and acetic acids (Stratford et al., 2013). These results suggest that the proton pump Pma1p is stimulated when *S. cerevisiae* is cultured in the presence of weak acids.

Similarly, in this study extracellular pH decreased during lag phase when *D. bruxellensis* was grown in media containing 100 mg/L of *p*-coumaric acid (Figure 1). Although a similar decrease was initially observed for the control, extracellular pH then increased to pH 4.3 and remained stable up to 100 h to decrease again to pH 4.1 (Figure 1). In media containing *p*-coumaric acid extracellular pH decreased steadily to pH 3.8 during lag phase (96 h). Subsequently, extracellular pH increased to similar values than the control (pH 4.1). Sigler and Hofer (1991) suggested that while the production of organic acids during yeast growth contributes to extracellular acidification, this only occurs during exponential phase. In this work, according to HPLC data, *D. bruxellensis* supplemented with *p*-coumaric acid.

### Table 1. Specific growth rate (µ *10^-3 (h^-1)) of the strain *D. bruxellensis* L1359 grown in SD media containing different concentrations of hydroxycinnamic acids.

<table>
<thead>
<tr>
<th>Medium/HCA (mg/L)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p</em>-Coumaric acid</td>
<td>32 ± 0.46</td>
<td>33 ± 0.25</td>
<td>34 ± 0.21</td>
<td>28 ± 0.25</td>
<td>27 ± 0.31</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>32 ± 0.46</td>
<td>37 ± 0.25</td>
<td>30 ± 1.53</td>
<td>27 ± 0.06</td>
<td>26 ± 0.21</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>32 ± 0.46</td>
<td>33 ± 0.15</td>
<td>32 ± 0.12</td>
<td>28 ± 0.25</td>
<td>27 ± 0.15</td>
</tr>
</tbody>
</table>

All results were expressed as means of three replicates.

### Table 2. Lag phase duration (h) of the strain *D. bruxellensis* L1359 grown in SD media containing different concentrations of HCA.

<table>
<thead>
<tr>
<th>HCA (mg/L)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p</em>-Coumaric acid</td>
<td>2.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
<td>3.5 ± 0.7</td>
<td>7.5 ± 0.7</td>
<td>15.0 ± 0.0</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>2.0 ± 0.0</td>
<td>3.5 ± 0.7</td>
<td>4.0 ± 0.0</td>
<td>8.5 ± 0.7</td>
<td>16.5 ± 0.7</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>2.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
<td>3.0 ± 0.0</td>
<td>5.5 ± 0.7</td>
<td>12 ± 0.7</td>
</tr>
</tbody>
</table>

All results are expressed as means of three replicates.
produced acetic acid during exponential phase (Figure 2) which is in agreement with literature (Geros et al., 2000; Leite et al., 2012), and in lag phase, acetic acid production was not detected.

These findings suggest that the pH decrease observed during lag phase in media supplemented with p-coumaric acid might be the result of H⁺-ATPase activity, similar to what has been observed in S. cerevisiae (Chambel et al., 1999).

Quantification of Pma1p activity in the presence of p-coumaric acid

Pma1p activity was quantified on membrane protein extracts from cultures grown in SD media or SD media containing p-coumaric acid. Since Pma1p activity was estimated from the rate of phosphate production after ATP hydrolysis, different compounds were used to inhibit other enzymes capable of hydrolyzing ATP or molecules
containing phosphate groups (see materials and methods). During lag phase *D. bruxellensis* showed increased Pma1p activity in media containing *p*-coumaric acid. However, during exponential and stationary phases Pma1p activity was similar for both growth conditions (Figure 3).

On the other hand, the observed increase in activity may also be due to increased amount of protein, however more studies are needed to prove this hypothesis.

The H⁺-ATPase pump Pma1p has been associated with cellular homeostasis regulating internal pH and therefore helping cell growth (Serrano, 1989). This H⁺-ATPase plays a critical role in cell adaptation to stress conditions caused by weak acids such as sorbic acid (Holyoak et al., 1996), octanoic acid (Viegas et al., 1998), cinnamic acid (Chambel et al., 1999), succinic and acetic acids (Carmelo et al., 1997) and hydroxycinnamic acids (Harris et al., 2008, 2010).

It has been estimated that during *S. cerevisiae* growth the H⁺-ATPase Pma1p pump uses 10-15% of the total ATP produced by the cell (Gancedo and Serrano, 1989). However, in the presence of weak acids, the activity of Pma1p increases in response to the reduction of internal pH, which is vital for re-establishing homeostasis. Since this process demands a large amount of energy, up to 60% of the total ATP produced (Serrano, 1989; Holyoak et al., 1996), the high energy demand reduces ATP concentration until growth is minimal (Holyoak et al., 1996; Piper et al., 1997). This could explain the longer lag phase observed for *D. bruxellensis* when grown in media containing *p*-coumaric acid. Therefore, our results suggest the existence of an early adaptation mechanism involving H⁺-ATPase pump Pma1p, which deals with the inhibitory effects of *p*-coumaric acid and potentially other hydroxycinnamic acids.

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