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Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/148118 since 2016-07-13T09:25:53Z

Published version:
DOI:10.1016/j.lwt.2014.06.006

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The effects of extracellular pH and hydroxycinnamic acids influence the intracellular pH of *Brettanomyces bruxellensis* DSM 7001

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Abstract

Brettanomyces bruxellensis yeast produces ethyl phenols, which cause wine spoilage and severe economic losses. Ethyl phenols are produced by a class of polyphenols that are present in grapes and musts, called hydrocinnamic acids, which are capable of inhibiting yeasts and bacteria. The viability and intracellular pH changes in the B. bruxellensis DSM 7001, in response to extracellular pH, as well as to the presence of an energy source and hydroxycinnamic acids, have been investigated in this paper by means of Fluorescent Ratio Imaging Microscopy (FRIM). The results show that B. bruxellensis DSM 7001 is able to maintain viability and increase its pH gradient with decreasing external pH values, whereas it is not able to maintain a pH gradient at high external pH values (i.e. pH 8) and, as a consequence, dies. The growth inhibitory effects of ferulic and p-coumaric acid do not seem to be caused by a weak-acid inhibition mechanism, since both acids induce a similar, or even higher, intracellular acidification at a high external pH than at a low external pH. The results presented have to be confirmed by using other strains of B. bruxellensis in order to validate the outcomes obtained in this study.

Keywords

Brettanomyces bruxellensis, Fluorescent Ratio Imaging Microscopy (FRIM), hydroxycinnamic acid, weak acid inhibition mechanism
1. Introduction

Yeasts belonging to the genera Dekkera/Brettanomyces are considered the main causes of wine spoilage, especially of premium red wines matured in oak casks, and often lead to serious economic losses. The growth of B. bruxellensis, is currently a serious problem for winemakers, due to the resulting production of off-odours, which have been described as phenolic, animal, mousy, wet wool, medicinal, smoky and spicy (Oleofse et al., 2008).

The principal products responsible for wine spoilage by Dekkera/Brettanomyces spp. are volatile phenols, i.e. ethylphenols, acetic acid and tetrahydropyridines. The first evidence of ethylphenol production by B. bruxellensis was described by Heresztyń in 1986 after a gas chromatografich analysis of a wine fermented by this yeast. Even though other bacteria and yeast species are able to participate in this conversion, they cannot grow in wines. The sensory perception threshold of 4-ethylphenol ranges from as low as 350 to 1000 µg/L, depending on the type of wine (Suárez et al., 2007). The origin of volatile phenols is related to the sequential activity of two enzymes; the first one decarboxylates hydroxycinnamic acids (e.g. ferulic, p-coumaric and caffèic acids) into hydroxystyrenes (vinylphenols), which are then reduced to ethyl derivatives by the other enzyme (Suárez et al., 2007).

Hydroxycinnamic acids (HCAs) are endogenous components of grapes and are considered natural food preservatives, besides being precursors of volatile phenols. For this reason, ferulic acid has been used to prevent food spoilage since 1975. HCAs have been reported to inhibit the growth of a variety of organisms, including plants, fungi and bacteria (Campos et al., 2003). In 2010, Harris and colleagues examined the inhibitory effect of HCAs on the B. bruxellensis yeast, and showed that ferulic acid was the most powerful of all the compounds
tested in the study. However, the mechanisms underlining this inhibitory effect have never been studied.

Hydroxycinnamic acids may be considered as weak organic acids (Rosazza et al., 2005), and thus their inhibition mechanism may be postulated to resemble that of more common weak organic acids, such as acetic acid or lactic acid. The inhibition mechanism of weak organic acids is generally assumed to involve the passive diffusion of the undissociated acid (XCOOH), which remains uncharged, across the plasma membrane of the cell. If the the external pH is lower, more acid is present in undissociated form and is able to diffuse through the membrane, due to its unchanged form. Once inside the cell, undissociated acid dissociates because of its low pK, and the higher intracellular pH (pHi), thus protons are generated and intracellular acidification occurs. In order to counteract this acidification, the cell has to pump out protons by means of the energy requiring enzyme, plasma membrane H\(^+\)-ATPase, thereby leading to uncoupling of the energy generation from growth (Brul and Coote 1999). Moreover, the dissociation inside the cell generates an acid anion (XCOO\(^-\)). This anion tends to accumulate intracellularly in very high levels as it cannot readily diffuse from the cell that is being charged. This high anion accumulation may generate an abnormally high turgor pressure (Piper et al., 1994). Hydroxycinnamic acids are weak acids that cause inhibition of \textit{B.\textit{bruxellensis}} (Harris et al. 2010), but, to the best of our knowledge, the inhibition mechanism has so far not been proven to be related to the weak nature of the acid.

The growth and production of 4-ethylphenol by \textit{D.\textit{bruxellensis}} in synthetic media with low glucose or fructose concentrations have been investigated by Barata et al. (2008), who showed that the behaviour is similar for both sugars. The production of 4-ethylphenol was detected at sugar concentrations of over 0.2 g/L and increased under higher sugar concentrations. The
process was closely correlated to the growth rate and to the amount of biomass produced. However, even in the presence of an energy source, it was demonstrated that the levels of volatile phenols did not increase in commercial red wines when *D. bruxellensis* was not growing, thus showing direct implications of an active growing population in phenolic taint development. The primary objective of rational winemakers should be to prevent actively growing populations and not to reduce *D. bruxellensis* to the lowest possible level. In fact, the complete absence of viable cells of *D. bruxellensis* is not easily achieved under wine making conditions, especially when oak ageing is used, because of the porous nature of the wood. As far as the growth and metabolite production of *B. bruxellensis* at different pH values is concerned, Blomkvist et al. (2010) showed that the growth rate was similar at pH 3 and 5, but that acetate production was higher at pH 5.

In this paper, the viability and intracellular pH changes of the spoilage yeast *B. bruxellensis* DSM 7001 in response to extracellular pH, as well as to the presence of an energy source and hydroxycinnamic acids, have been investigated by means of Fluorescent Ratio Imaging Microscopy (FRIM).

2. Materials and Methods

2.1 Yeast strain and media

*D. bruxellensis* DSM 7001 from DSMZ, German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) was grown on Yeast Peptone Dextrose agar (2% glucose, 1% yeast extract, 2% peptone, ) at 30°C.

2.2 Fluorescence staining of cells
5-(and -6)-carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester (CDCFDA-SE) (C1165, Molecular Probes Inc., Eugene, OR, USA) was used to stain the cells for pH measurements. CDCFDA-SE was dissolved in DMSO (Sigma Aldrich) to a final concentration of 4.5 mM. The cells were harvested in the exponential phase (OD = 0.3), washed twice in a citrate-phosphate buffer (pH 7.0), resuspended in a 990 µl citrate phosphate buffer (pH 7.0) to a final concentration of 1×10^7 cells/ mL to which 10 µl of 4.5 mM CDCFDA-SE was added. The cell suspension was incubated at 30°C for 30 min. The stained cells were harvested by means of centrifugation at 12,000 ×g for 5 min, washed twice with a citrate-phosphate buffer (pH 7.0) and resuspended in a YNB broth (Yeast Nitrogen Base (Difco) 6.7 g/L, Glucose 10 g/L), at the appropriate pH value, and a citrate-phosphate buffer (pH 7.0), kept on ice in the dark, prior to the microscopic analysis. Double staining with CDCFDA-SE and Propidium Iodide (PI, Sigma Aldrich), was performed for the viability measurements during exposure to the acid solution (p-coumaric acid, ferulic acid, lactic acid). Ten µl of 1.5 mM PI (dissolved in milliQ water) was added to each mL. The preparation was mixed thoroughly for 10 s, incubated for 10 min in the dark at 40 °C, and transferred to ice before the microscopic analysis.

2.3 Fluorescence microscope set-up

The microscope set-up was the same as that described in (Guldfeldt and Arneborg, 1998), and consisted of an inverted epifluorescence microscope (Zeiss Axiovert 135 TV, Carl Zeiss, Oberkochen, Germany) and a fibre-connected monochromator (Monochromator B, TILL Photonics) with a 75 W xenon lamp, to provide excitation at pH sensitive and insensitive wavelengths of 488 and 435 nm for CDCFDA-SE, respectively, and at 536 nm for PI. The microscope was equipped with a Zeiss Fluar 40x lens (numerical aperture 1.3), a dichroic mirror (510 nm), and an emission band pass filter (515-565 nm). No neutral-density filter was
used in the experiments. In order to collect the fluorescence emission of PI, a long pass filter was used that allowed higher wavelengths than 610 nm to pass (Zeiss model LP 610). Images of the fluorescence emissions were collected using a cooled charge-coupled device camera (CCD) (CoolSnapfx, Photometrics, Birkerød, Denmark) and were stored on a computer using MetaMorph 7 (Universal Imaging Corp., West Chester, Pa.).

2.4 Perfusion set-up and exposure to acidulants

Microscopic glass slides were rinsed by soaking overnight in an ethanol/HCl solution (ethanol, 70% v/v, HCl, 1% v/v). Subsequently, the slides were rinsed with milliQ water, air-dried, and coated with concavalin A (Sigma-Aldrich). A perfusion chamber was constructed by attaching a Coverwell™ perfusion chamber gasket (19 mm X 6 mm, 0.5 mm deep, Invitrogen, Molecular probes) onto the glass slide. A small piece of silicon tube, with a diameter of 1.60 mm X 1.60 mm, was glued onto the inlet hole (Watson Marlow Limited, Falmouth, Cornwall, England). A silicon tube with a diameter of 4.80 mm X 1.60 mm was used for the outlet hole. The stained cell suspension was placed in the perfusion chamber and allowed to immobilize for 5 minutes. The chamber was subsequently mounted onto the fluorescence microscope, and the cells were focused on under bright-field illumination. The acidulant solutions were perfused through the inlet of the perfusion chamber at a rate of 170 µL/min using a modified Alitea XV pump (Microlab Aarhus A/S, Aarhus, Denmark) and removed from the outlet of the chamber using a 101U pump (Watson Marlow, Wilmington, Mass.). The perfusion started with a YNB medium at the appropriate pH value prior of each experiment in order to wash out the cells that were not attached to the glass slides. Two images were acquired prior to the addition of 100 mg/L of the acidulants dissolved in water, which entered the chamber after 2 minutes from the beginning of the experiments. Images
were acquired at 488 nm and 435 nm (for pH<sub>i</sub>) at 60 s intervals for 30 min, while images were acquired at 536 nm (for viability) at the beginning and at the end of each experiment.

2.5 Determination of pH<sub>i</sub> and viability

A data analysis of the stored images was performed using the ImageJ 1.37v software programme (http://rsb.info.nih.gov/ij/). Intracellular pH was determined, by means of Fluorescence Ratio Imaging Microscopy (FRIM), of the CDCFDA-SE stained cells as described in (Mortensen et al. 2006).

The fluorescence ratios were converted to pH<sub>i</sub> using an in vivo calibration curve. CDCFDA-SE stained <i>B. bruxellensis</i> cells were incubated with ethanol (70% v/v) at 30 °C for 15 min to achieve cell death and full loss of membrane integrity. Subsequently, the cells were harvested by means of centrifugation at 12,000 × g for 5 min, resuspended in YNB media with a pH ranging from 3 to 8, and the pH<sub>i</sub> was allowed to equilibrate (2 mins) with the external pH. The calibration curve was generated by plotting the fluorescence ratios against their corresponding pH equilibrated cell values. A third degree polynomial model (Microsoft Excel 2003) was fitted to the experimental data and used for the pH<sub>i</sub> calculations (Fig. 1).

Viability was determined for the same single cells that were used for the pH<sub>i</sub> determinations, by counting the number of PI-stained cells. In each experiment, around 70-100 cells were analysed.

3. Results and discussion

Strategies for the control and prevention of <i>Brettanomyces/Dekkera</i> contamination of wine are important for wine quality. Thus, equipment that comes into contact with juice, must, and wine should ideally be subjected to routine and rigorous sanitisation practices. Maintaining appropriate wine parameters, such as pH and sulphur dioxide, can minimise yeast growth
(Harris et al., 2010). However, this is not always enough to avoid unpleasant yeast growth (Campolongo et al., 2010). In this preliminary work, the behaviour of B. bruxellensis was first studied at different pH in order to understand what happens if fermentable sugars are not present in the media, then the short-term response of yeast to hydroxycinnamic acids was examined.

3.1 Correlation between pH gradient and viability without energy source

The results of our investigation of the survival of B. bruxellensis DSM 7001 at different pH in a simple citrate buffer without any energy source, have shown that the yeast is able to maintain a rather unconstant pH gradient, ranging from 4.1 to 1.3 pH units, for one hour, without any viability losses, when the external pH changes from 3 to 5, while the capability of maintaining a pH gradient suddenly decreases for higher pH (Fig. 2A). Furthermore, our data indicate a good adaption of the yeast to a sudden change in external pH, without any losses of viability after 1 hour (Fig. 2A).

The correlation between pH gradients and viability after 1 and 24 hours suggests the existence of an adaptation process of the yeast, which adjusts the internal pH in order to survive. After 1 hour of incubation, the yeast still maintains an internal pH of about 6.5, which is similar to the values of the resuspension buffer before the change (Fig.2A).

After 24 hours, the yeast has lowered the pHi, adjusting it to similar values to the external pH, has minimized the energetic requirements to counteract the external pH and has maintained complete vitality and metabolic activity. These results indicate the efforts of the yeast to survive to sudden pH changes and the existence of an adaptation process.

Information on the vitality and the behaviour of Brettanomyces at different pH is still lacking in the literature. The results obtained in this study allow us to speculate that this particular ability could be related to a particularly efficient system of H⁺ pumps and transporters.
Alternatively, a particular membrane lipid composition could be involved, as highlighted in the proteome analysis conducted by Woolfit et al. (2007).

### 3.2 Relationship between pH_{i} and pH_{e}

Summarizing the results presented in Fig. 2, the existence of a positive pH gradient at low pH values and a negative pH gradient at high pH values can be noticed, thus demonstrating that the cells are trying to maintain an internal pH value that is close to their physiological value, ranging from values of 5 to 5.5. These results agree with the pH indicated for the preparation of specifically formulated media for Brett cultivation, which usually have pH values of between 5 and 5.5 (Couto et al., 2005).

As shown in Fig 2A and 2B, if the pH is increased from 3 to 5, the pH gradient increases, thus confirming a range of internal pH of between 6 and 7. The present results indicate that the yeast in limited energy conditions, after 1 and 24 hours at low pH, is still able to adjust the internal pH to values close to the physiological pH.

### 3.3 Correlation between pH gradient and viability with energy source

Viability is higher in the presence of an energy source. Only at a high pH, such as 8, does the yeast die, with 10% and 54% of dead cells after 1 hour and 24 hours of exposure, respectively (Fig. 2C and 2D). These data underline the well-know capacity of this yeast to resist and actively grow in harsh environments, such as in dry wines, even with a low sugar concentration (Barata et al., 2008).

When glucose is used as an energy source, the pH gradients are similar after 1 and 24 hours of incubation, especially when the external pH is between 3 and 5. These data agree with the previously mentioned results, which indicate that the physiological pH of the yeast is between 5 and 6. Such a statement has never before been reported in literature.
Brettanomyces bruxellensis DSM 7001, in presence of an energy source, is able to resist for 24 hours at pH 8, as indicated in Fig. 2D, where the mortality (54%) is lower than in the data obtained without an energy source (94%). No data are available on the viability of this species at high pH. At pH 5 and 6, the cells try to counteract the external pH, but pH 7 and 8 seem to be critical for the yeast to survive during nutrient limitation (Palkova et al., 2009). Cell survival during starvation requires preservation of plasma membrane integrity and the ability to generate an electrochemical gradient at the plasma membrane. Consequently, these results may suggest that in order to achieve an efficient cleaning and sanitisation of winemaking equipment, it serves no purpose to use weak acids to which the spoilage yeast is highly resistant, but rather soda or highly basic solutions should be used for equipment decontamination even though the efficiency of such treatments should be checked.

As shown in Figures 2C and 2D, the capability of yeast to resist a high pH is increased compared to the condition without any energy source, and that after 24h, the yeast is able to maintain a good pH gradient, even at pH 7 and 6, with percentages of dead cells of 3% and 2%, respectively. These results are in agreement with those reported in literature for S. cerevisiae. Imai and Ohno (1995) demonstrated that the intracellular pH has a clear relationship with the proton extrusion activity, which is an important process in cell deactivation that ultimately leads to cell death (Imai and Ohno, 1995).

3.4 Effects of hydroxycinnamic acids on pH

The inhibitory effect of HCAs has been extensively demonstrated in literature (Harris et al., 2010), but the mechanisms that cause this inhibition have, to the best of our knowledge, never been studied. Since HCAs may be considered to be weak organic acids, having almost the same pKa values (i.e. 4.58 for ferulic acid and 4.64 for coumaric acid), we asked ourselves whether the inhibitory effect was due to the classical weak organic acid inhibition mechanism.
Our data clearly indicate that neither for ferulic acid nor for coumaric acid this seems to be the case (Fig. 3). The intracellular acidification caused by ferulic acid is more pronounced at pH 5.5 than at pH 3.5, whereas for coumaric acid, it is more or less similar. Moreover, due to the above mentioned and to the fact that the two acids cause different intracellular acidification patterns at a given extracellular pH, our data suggest that the two acids have different inhibition mechanisms (Fig. 3). This issue, however, requires further investigation.

4. Conclusions

In conclusion, our results show that *B. bruxellensis* DSM 7001, regardless of the presence of an energy source, is able to maintain viability and increase its pH gradient with decreasing external pH values, whereas at high external pH values (i.e. pH 8), it is not able to maintain a pH gradient and dies. Moreover, our data suggest that the growth inhibitory effect of ferulic and *p*-coumaric acid is not caused by a weak-acid inhibition mechanism, since both acids induce a similar, or even higher, intracellular acidification at a high external pH than at a low external pH. These data may contribute to finding appropriate cleaning and sanitation solutions in the wine industry that specifically target this significant spoilage yeast. The limitation of this study is the use of only one *B. bruxellensis*, thereby more strains should be tested in order to validate the results reported in this study. However, it should be underlined that this is the first time, such an approach has been used in order to understand the physiology of this spoilage yeast when exposed to different external pH values and hydroxycinnamic acids, and for this reason it should be considered as a pioneering study giving new insight in the possible control of *B. bruxellensis* in wine making.
References


Figure legends

Figure 1. Calibration curve describing the relationship between the 488 nm/435 nm ratio values and internal pH of individual cells of *B. bruxellensis* DSM 7001. The curve was constructed as described in Materials ans Methods, and the ratio values are averages of 70–100 single cells. The error bars represent the standard error of the mean.

Figure 2. Response to external pH and presence of an energy source. The data presented in Figs. A and B were obtained for *B. bruxellensis* in a phosphate-citrate buffer. The data presented in Figs. C and D were obtained for *B. bruxellensis* in a YNB media. The pHi values are averages of 70–100 individual cells and error bars represent the standard error of the mean. The columns present the percentage of dead cells at each pH value with error bars representing the standard deviation. Numbers indicate the ΔpH between external and internal pH.

Figure 3. Hydroxycinnamic acid short-term response. The presented data were obtained for *B. bruxellensis* in a YNB media. pHi indicates the evolution of the internal pH, after perfusion at a rate of 170 µl/min of YNB (◊), YNB with 100mg/L of ferulic acid (❏) or YNB with 100mg/L of p-coumaric acid (Δ), at pH 3.5 (Fig.A) and 5.5 (Fig. B). Images were taken every minute. The values are averages of 70–100 individual cells. The error bars represent the standard error of the mean.
Figure 1

The graph shows a plot of pH against the ratio 488/436, with a fitted curve given by the equation:

\[ y = 0.0024x^3 - 0.0491x^2 + 0.6519x + 1.1077 \]

and an \( R^2 \) value of 0.9896.
Figure 2

A. Citrate-phosphate buffer - 1h

B. Citrate-phosphate buffer - 24h

C. YNB - 1h

D. YNB - 24h
Figure 3