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Comparative morphological characteristics of three *Brettanomyces bruxellensis* wine strains in the presence/absence of sulfur dioxide

Marli Louw [a], Maret du Toit [a], Hervé Alexandre [b], Benoit Divol [a,*]

[a] Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa
[b] UMR PAM, Institut Universitaire de la Vigne et du Vin Jules Guyot, Université de Bourgogne, 21078 Dijon Cedex, France

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ABSTRACT

The red wine spoilage yeast *Brettanomyces bruxellensis* has been the subject of numerous investigations. Some of these studies focused on spoilage mechanisms, sulfur dioxide tolerance and nutrient requirements. Pseudomycelium formation, although a striking feature of this species, has however been poorly investigated. Furthermore, literature regarding the induction mechanism of pseudomycelium formation in this yeast is limited and lacks clarity, as results published are contradictory. This study elucidates this phenomenon among strains from geographically different areas. Potential environmental cues were investigated, to attain a better understanding of this mechanism and its role as a survival strategy. SO_2 was previously reported to induce this morphological change however results obtained in this study did not support this. Nevertheless, the results obtained using scanning and transmission electron microscopy illustrate, for the first time in this yeast, deformity to the cell membrane and alterations to the fibrillar layers in SO_2 treated cells. In addition, the SO_2 exposed cultures displayed cell size variations, with cells displaying a decrease in length as well as delayed growth, with a prolonged lag phase. Fluorescence microscopy demonstrated a decrease in metabolic activity and the appearance of inclusion body-like structures in the cells following exposure to SO_2.

1. Introduction

Thanks to a subtle blended use of traditional know-how and scientific knowledge, wine production is an overall well-controlled process, but it is confronted with various challenges such as the growth of spoilage microorganisms during wine aging. Indeed, a few yeast and bacterial species possess the ability to alter wine composition, thereby negatively affecting its quality and sensorial properties (Bartowsky, 2009; Du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003). Microbial spoilage can induce significant financial losses. Spoilage yeasts may be responsible for the formation of biofilms, sediments, cloudiness (Dias et al., 2003; Mansfield et al., 2002; Tchobanov et al., 2008), gas and off-flavors such as volatile phenols, acetic acid and mousiness (Chatonnet et al., 1992, 1995, 1997; Echeverrigaray et al., 2013). These yeasts belong to several genera including *Hansenula, Candida, Pichia, Brettanomyces, Zygosaccharomyces, Schizosaccharomyces* and even some *Saccharomyces* strains (Echeverrigaray et al., 2013). Among them, *Brettanomyces bruxellensis* is the most notorious red wine spoilage yeasts (Boulton et al., 1996; Echeverrigaray et al., 2013; Loureiro and Malfeito-Ferreira, 2003; Oelofse et al., 2008; Suárez et al., 2007). The development of this yeast during wine production results in the formation of undesired compounds such as volatile phenols, acetic acid (Freer, 2002; Oelofse et al., 2009, 2010; Scheffers, 1961) and fatty acids (Licker et al., 1998; Malfeito-Ferreira et al., 1997; Rozès et al., 1992).

Controlling the development by *B. bruxellensis* is very difficult as this yeast is exceptionally well adapted to extreme environmental conditions, such as those occurring in wine (Hellborg and Piskur, 2009; Woolfit et al., 2007). Indeed, *B. bruxellensis* needs insignificant amounts of nutrients to sustain growth and is able to utilize an extensive range of metabolites as carbon and nitrogen sources (Conterno et al., 2006). Its ability to assimilate such a diverse group of compounds confers resilience to this yeast, by allowing it to adapt to its environment (Conterno et al., 2006; Curtin et al., 2012). Moreover, *B. bruxellensis* has also developed other mechanisms to survive in wine. Tolerance to both high ethanol concentrations (Barata et al., 2008) and varying sulfur dioxide (SO_2) concentrations (Curtin et al., 2012; Licker et al., 1998; Zuehlke and Edwards, 2013) has been reported.

The response of *B. bruxellensis* to SO_2, a commonly used preservative, has been extensively studied. Various coping mechanisms have been identified; these include sulfur reduction, acetaldehyde production, active sulfur efflux and ability of this yeast to enter a viable but not culturable (VBNC) state (Agnolucci et al., 2010; Divol et al., 2012; Duckitt, 2012; Du Toit et al., 2005). The VBNC state is characterized by the ability of the cells to remain viable while temporarily losing their

* Corresponding author.
E-mail address: divol@sun.ac.za (B. Divol).

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ability to proliferate on solid culture medium (Serpaggi et al., 2012; Vigentini et al., 2013). These strategies are nevertheless not specific to *B. bruxellensis*.

Another possible adaptation is the morphological changes observed among strains of *B. bruxellensis,* forming pseudomycelium structures, which seem to be highly strain dependent (Aguilar Uscanga et al., 2000; Conner and Beuchat, 1984; Dickinson, 1996; Echeverrigaray et al., 2013). Pseudomycelium growth is characterized by cell elongation, due to the mechanism controlling cell division becoming impaired (Dickinson, 2008; Morris, 1958). This results in a delay in development during the G2/M phase (Dickinson, 2008; Morris, 1958; Song and Kumar, 2012) leading to prolonged apically directed polarized growth, with no disconnection after cytokinesis resulting in the formation of pseudohyphae (multi-cellular filaments), that do not possess multi nuclei unlike true hyphae (Cullen and Sprague, 2012; Dickinson, 1996, 2008; Kron et al., 1994; Rua et al., 2001; Song and Kumar, 2012). These filaments therefore only resemble true hyphae observed in fungi (Trinci, 1974). Pseudomycelium formation in budding yeast is reported to be induced by nutrient scarcity and sporadically oxidative stress (Li et al., 2011; Sierra-Campos et al., 2009). The triggers for this morphological adaptation have also been reported to be linked to the ploidy of the yeast (Cullen and Sprague, 2012; Gancedo, 2001; Lo and Dranginis, 1997; Song and Kumar, 2012; Zaragoza and Gancedo, 2000). Indeed, nitrogen limitation induces pseudohyphae formation in diploid cells (Rua et al., 2001), while glucose deprivation induces this phenomenon in haploid cells (Cullen and Sprague, 2012; Song and Kumar, 2012). In fungi such as *Candida albicans* and *Aspergillus fumigatus*, this phenomenon is of significant interest as the morphogenetic switch between budding cells and pseudomycelium is related to pathogenicity in these fungi (Gastebois et al., 2009; Netea et al., 2008). In *Saccharomyces cerevisiae*, pseudomycelium has been reported to help the organism scavenge for food during nutrient deprivation as a survival mechanism (Kron et al., 1994; Roberts and Fink, 1994; Wright et al., 1993). Subsequently, it has been meticulously studied especially in *C. albicans* and *S. cerevisiae*, as they serve as model organisms to investigate the environmental cues and respective pathways responsible for this cell modification (Kuriyama and Slaughter, 1995; Sudbery, 2011). Pseudomycelium development has also been reported and studied in other yeasts, such as *Schizosaccharomyces pombe, Pichia guillermondi* and *Yarrowia lipolytica* (Cullen and Sprague, 2012; Echeverrigaray et al., 2013; Gancedo, 2001), but in *B. bruxellensis*, this phenomenon is poorly understood and investigated, although it was first reported in 1958 (Morris, 1958). Pseudomycelium has been observed during previous studies (Aguilar Uscanga et al., 2000; Dickinson, 1996) and more recently studies reported that *B. bruxellensis* exhibit pseudomycelium formation upon exposure to SO_2 (Vigentini et al., 2013). These results are however inconsistent, as other authors observed pseudomycelium only in the absence of SO_2 (Echeverrigaray et al., 2013). These discrepancies in literature could be due to the high degree of strain variance and SO_2 tolerance, associated with *B. bruxellensis*.

This study investigated the cell morphology of three *B. bruxellensis* strains isolated from geographically distinct areas and its evolution over time, under typical growing and stress conditions induced by the presence of SO_2. It also aimed to document the impacts of SO_2 on *B. bruxellensis* cells using a range of microscopic tools.

2. Materials and methods

2.1. Yeast strains, growth parameters and sampling

Three strains of *B. bruxellensis* were used during the course of this study (Table 1). According to an ISS-PCR fingerprinting performed by Dr. Ileana Vigentini according to the method described in Vigentini et al. (2012), the genetic profile of strain AWRI 1499 shares >43% similarity with those of IWBT Y121 and LO2E2 and those of the latter two strain share >57% similarity between themselves (data not shown). They were maintained on yeast peptone dextrose (YPD) (Biolab Diagnostics, Wadeville, South Africa) with 1.5% agar added when appropriate and incubated at 30 °C. Plates were stored at 4 °C.

Table 1
Brettanomyces bruxellensis strains used during this study.

Collection	Strain number	Source	Reference
IWBT	Y121	South African wine	Oelofse (2008)
ITV	LO2E2	Burgundian wine	Serpaggi et al. (2012)
AWRI	1499	Australia wine	Curtin et al. (2012)

IWBT: Institute for Wine Biotechnology, Stellenbosch University, South Africa; AWRI: Australian Wine Research Institute, Adelaide, Australia; ITV: Institut Technique de la Vigne et du Vin, Beaune, France.

For the cell growth experiment, single colonies were inoculated into 5 mL YPD and incubated for 24 h at 30 °C. The pre-cultures were inoculated into Erlenmeyer flasks containing 100 mL YPD to a concentration of 1×10^6 cells/mL. The YPD medium was adjusted to pH 3.5 with tartaric acid. Flasks were saturated with nitrogen gas, to ensure an anaerobic environment. Cultures were grown with shaking (130 rpm) at 25 °C.

All experiments, unless otherwise stated, were carried out in triplicate, and the analysis on each sample was performed in triplicate. Cultures were sampled every 4 h for a total of at least 100 h. Cell growth was estimated spectrophotometrically at 600 nm using a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer). Furthermore, two-milliliter samples were taken at each sampling interval and centrifuged for 5 min at 13,200 rpm. The supernatant was transferred to a sterile microcentrifuge tube and stored at −20 °C for further analysis. Another 1 mL sample was taken for microscopy.

The same culture conditions were maintained for the SO_2 stress experiment. SO_2 was added to the cultures through a side port at concentrations of 0.2, 0.4 and 0.6 mg/L molecular SO_2, after the flasks have been saturated with nitrogen gas and sampling performed as described above. The molecular SO_2 concentrations were obtained from total SO_2 concentrations calculated using a previously determined equation from Duckitt (2012).

2.2. Sample analysis

D-Glucose and acetic acid concentrations were quantified from the supernatant using the Arena 20XT automated enzymatic kit robot (Thermo Electron Oy, Finland), with the following enzymatic kits: Enzytec™ *Fluid* Acetic Acid Id-No: 5226 and Enzytec™ *Fluid* D-Glucose Id-No: 5140 (Thermo Fisher Scientific, Oy, Finland).

2.3. Microscopy

2.3.1. Light microscopy

Cells were visually inspected with the Olympus IV81 Widefield Fluorescent Microscope Imagining Station for both light and fluorescent images at 20× and 100× magnification (Central Analytical Facility, Stellenbosch University). All photographs taken were further analyzed with the Olympus Cell^R Imaging Software, scale bar set at 20 μm and 100 μm. In particular, cell measurements in μm were performed for length, width and area of the cells.

Viability staining and fluorescent microscopy.

Two fluorochromes were used to discriminate between viable and dead cells by means of fluorescent microscopy. Cell viability was determined by staining living cells with fluorescein diacetate (FDA, Sigma-Aldrich, St. Louis, MI) and dead cells with propidium iodide (PI, Sigma-Aldrich). The same microscope as described above was used equipped with the FITC and TxRed filters in order to visualize green and red fluorescence, respectively. FDA is a lipophilic, non-fluorescent substrate that is cleaved by cellular esterase within living cells, releasing green fluorescence. Cells with intact membranes are able to retain the green