Food Research International 51 (2013) 670–678

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Overview of *Dekkera bruxellensis* behaviour in an ethanol-rich environment using untargeted and targeted metabolomic approaches

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ARTICLE INFO

Article history:
Received 20 September 2012
Accepted 22 January 2013

Keywords:
Dekkera bruxellensis
Metabolomics
Ethanol
GCMSMS
UPLC–MS
Metabolite profile

ABSTRACT

Dekkera bruxellensis is a yeast known for its ability to produce ethyl phenols from hydroxycinnamic acid in wine, affecting the quality of its flavour. In wine, *D. bruxellensis* is not responsible for producing ethanol, however it is able to survive and sometimes also to grow in the presence of large amounts of ethanol. Because of its endurance, *D. bruxellensis* poses a serious threat to the wine industry and can cause substantial financial losses. In order to analyse yeast activity in the presence of different amounts of ethanol, the metabolic profile of a *D. bruxellensis* strain isolated from wine was outlined in defined chemical conditions in model wines. The metabolic profile of model wines with 10%, 11%, and 12% ethanol after *D. bruxellensis* growth was studied. Several ethyl esters and phenyl ethanol, together with 4-ethyl guaiacol, were produced in significantly higher amounts in response to the increase in ethanol stress. It was shown how the cell metabolism of specific compounds increased in response to a higher ethanol content, although yeast cell growth was limited.

1. Introduction

Dekkera bruxellensis (teleomorph of *Brettanomyces bruxellensis*) is a yeast isolated from different fermented foods (Barnett, Payne, & Yarrow, 2000; Kurtzman & Fell, 1998) often impacting its flavour development. It is commercially used for the production of Belgium lambic or gueze beer (Verachtert & Dawoud, 1984; Martens, Iserentant, & Verachtert, 1997; Vanbeneden, Gils, Delvaux, & Delvaux, 2008) and belongs to the natural microflora of sourdough (Meroth, Hammes, & Hertel, 2003). It has also been identified as a major contaminant in fuel ethanol fermentation (Beckner, Ivey, & Phister, 2011; De Souza Liberal et al., 2007). *D. bruxellensis* has long been recognised as a widespread contaminant of wine and has been associated with wines which exhibit off-flavours described as 'burnt plastic', 'BAND-AID®', 'horse sweat', 'barn yard', and 'mousy' (Chatonnet, Dubourdieu, Boidron, & Pons, 1992, Chatonnet, Viala, & Dubourdieu, 1997). Characteristic products of *D. bruxellensis* in wine are also pyridine derivatives (Snowdon, Bowyer, Grbin, & Bowyer, 2006), acetic acid alone, and ethyl acetate (Freer, Dien, & Matsuda, 2003). The spoiling feature most studied regards its ability to produce 4-ethylphenol and 4-ethylguaiacol from hydroxycinnamic acid precursors and *p*-coumaric and ferulic acids respectively (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). This "horse sweat" taint is one of the major threats for ageing bulk or bottled red wines and has been a major challenge for winemakers in the last few decades (Malfeito-Ferreira, 2011). Characterisation of the metabolic response of this yeast to wine composition can make a significant contribution to winemaking. The ability of *D. bruxellensis* to grow in wine, albeit sometimes at low cell density, shows its capacity to withstand combined stresses such as low nutrient availability, SO_2, low pH, and ethanol.

Several investigations have been carried out to characterise factors controlling the production of chemicals responsible for the characteristic flavours associated with *D. bruxellensis* (Brock, Conterno, Lavin, Acree, & Henick-Kling, 2006; Ciani & Ferraro, 1997; Heresztyn, 1986, Rodrigues, Gonçalves, Pereira-da-Silva, Malfeito-Ferreira, & Loureiro, 2001), but the published results appear to be conflicting as regards the pyridoxine effect (Rose & Harrison, 1971; Uscanga, Delia, & Strehaiano, 2000). According to Dias, Pereira-da-Silva, Tavares, Malfeito-Ferreira, and Loureiro (2003) *D. bruxellensis* and *D. anomala* are the only species capable of producing 4-ethylphenols. Conterno, Joseph, Arvik, Henick-Kling, and Bisson (2006), showed *D. bruxellensis* strains producing less than 4 ng ml^{-1} of ethyl phenols under certain conditions. Whilst capable of growing in the presence of an ethanol concentration of over 10%, ethanol influences *D. bruxellensis* growth (Dias et al., 2003), depending on the strain (Conterno et al., 2006).

Heresztyn (1986) described the production of ethyl phenols by *D. bruxellensis* in grape juice for the first time. Based on their findings, Chatonnet et al. (1992) suggested that the first stage involves phenolic acid decarboxylation by cinnamate decarboxylase followed by a reductase (vinylphenol reductase). Cavin et al. (1997) and Barthelmebs, Divies, and Cavin (2000) studied the *p*-coumarate decarboxylase gene in *Lactobacillus*. Subsequent conversion of vinyl phenol to ethyl phenols

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http://dx.doi.org/10.1016/j.foodres.2013.01.049

can be performed by *D. bruxellensis* or *Pichia guillermondii* (Dias et al., 2003) but not by *Saccharomyces cerevisiae*, and only *D. bruxellensis* would reduce the vinyl phenols in wine. Unfortunately, no information is available on the genetic regulation of ethyl phenol production in *D. bruxellensis* (Godoy, Martinez, Carrasco, & Ganga, 2008). The genome of *D. bruxellensis* has been sequenced and appears to be quite complex (Curtin, Borneman, Chambers, & Pretorius, 2012; Piškur et al., 2012) therefore using a metabolomic approach may help to better define the yeast's contribution to wine flavour.

Despite numerous papers on *D. bruxellensis* volatile production, the metabolites analysed are limited to a few known compounds studied using targeted analysis (Licker, Acree, Waterhouse, & Ebeler, 1999; Romano, Perello, de Revel, & Lonvaud-Funel, 2008). However, the recent development of wide-range metabolic profiling techniques allows the application of a comprehensive "metabolomic" approach to investigate the *D. bruxellensis* metabolism. Metabolomics has already been used to study yeast strains of *S. cerevisiae* during must alcoholic fermentation (Son et al., 2009) and to study the metabolic response to ethanol in *S. cerevisiae* (Li et al., 2012). To our knowledge, the metabolomic approach has never been applied for studying *D. bruxellensis* growing in an ethanol-rich environment.

In this study we combined targeted and untargeted mass spectrometry (MS) based metabolomics to reveal compounds characterising the response of *D. bruxellensis*, during growth with low nutrient availability and different ethanol concentrations modelling the wine environment. By studying the extracellular metabolome only a small part of the total yeast metabolome is revealed, our aim being to better understand *D. bruxellensis* behaviour in response to ethanol. Targeted and untargeted metabolomic approaches (GC–MS and LC–MS) were applied to investigate the growth and metabolic products of *D. bruxellensis*, in response to the ethanol content of a synthetic wine. Multivariate statistical techniques were used on the untargeted data to select metabolic markers associated with yeast growth at defined ethanol concentrations. Target analysis of the ethyl phenols described was used to verify the consistency of the results of untargeted analysis.

2. Material and methods

2.1. Yeast strain and inoculum

The experiments were carried out using the CE116 *D. bruxellensis* strain isolated from wine, previously genetically and physiologically characterised (Conterno et al., 2006). The pure yeast culture stored at −80 °C was streaked on YPG agar, incubated at 22 °C until clear visible single colonies were observed. Cells from a single colony were picked, inoculated in 10 ml of YPG broth, and incubated at 22 °C (not shaken). When the population grew to approximately 10^8 cell ml^{-1} the cells were added to a wine model medium containing 10% ethanol. The wine model medium was inoculated with 3×10^4 cell ml^{-1}. At the onset of the logarithmic phase of growth, the cells were counted using a hemacytometer and a proper amount of cell suspension was used to inoculate the next medium (3×10^4 cell ml^{-1}).

2.2. Media

Yeast–peptone–glucose (YPG) broth was prepared with 5 g l^{-1} of yeast extract (Oxoid), 10 g l^{-1} of bacto-peptone (BD), and 20 g l^{-1} of glucose. YPG agar also contained 20 g l^{-1} of technical agar (Oxoid SpA, Milan, Italy).

The wine model media were prepared as for the "synthetic reference wine" described by Costello, Henschke, and Markides (2003) and modified as follows: amino acids were added as a nitrogen source by adding a total of 488 mg l^{-1} (each amino acid was added at the same ratio indicated by Costello et al., 2003); 1 g l^{-1} of both glucose and fructose were added; pentose ribose, rhamnose, arabinose, xylose, galactose and trehalose were added using 100, 50, 200, 200, 100, and 150 mg l^{-1} respectively; no purines or pyrimidines were added; *p*-coumaric, ferulic, and caffeic acids were added using 4.6, 4.5, and 5.3 mg l^{-1} respectively. All the reagents used were purchased from Sigma (Milan, Italy) or Carlo Erba (Milan, Italy).

Ethanol was added to the four media E10, E11, E12, and E13 to reach a final concentration of 10, 11, 12 and 13% (v/v) respectively.

2.3. Cell enumeration and growth

The number of cells was measured using microscopy (bright light), by counting using a Bürker hemocytometer. Cell growth was monitored by measuring the cell suspension optical density at 600 nm (OD_{600}) using a U2000 spectrophotometer (Hitachi, Brugherio, Italy).

2.4. Fermentation conditions

The four media, E10, E11, E12, and E13 were tested in series: the cells grown in E10 (lowest amount of ethanol), were used to inoculate E11 and so on (i.e. from E11 to E12 and from E12 to E13).

Six 120 ml glass vials were filled with 100 ml of sterile-filtered medium and closed with a Teflon faced butyl septa (Supelco, Milan Italy) and an aluminium crimp (Supelco, Milan Italy). After adding the proper inoculum volume, the closure was crimped and the vials incubated at 22 °C.

2.5. Sampling and sample treatment

To monitor cell growth, samples were taken under sterile conditions every 24 h, using a 2.5 ml disposable syringe.

For the cell count and the metabolomic analysis, samples of the yeast suspension were taken at the time of inoculum, at the beginning of the exponential phase of growth (MID: OD_{600} reached about 0.1 Abs), and when the yeast culture reached the stationary phase of growth (END: 36 h after OD_{600} had a second inflexion point). Three of the six vials were analysed at MID time and the three remaining vials were analysed at the END time. Each vial was considered as a replicate.

At MID and END, cell enumeration was carried out, after dilution when necessary. After enumeration, MID suspension was also used to inoculate the next medium to be tested. The residue was filtered with a 0.22 μm membrane filter unit (Millipore, Bedford, MA), divided into around 20 ml aliquots and stored in a brown glass vial at −20 °C until further analysis.

2.6. Untargeted analysis

2.6.1. GC/MS/MS analysis

Volatile metabolites were extracted from sample headspace and concentrated on 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fibre using a TriPlus™ autosampler (Thermo Fisher Scientific, Milan, Italy). Since different ethanol contents cause variations in fibre trapping efficiency (Robinson et al., 2009), prior to the analysis, samples containing 11% and 12% ethanol were diluted to reach a 10% ethanol content. In order to have the same dilution factor in all the samples, E10 samples were diluted with an appropriate volume of 10% ethanol aqueous solution. The fibre was preconditioned before the analyses, according to the manufacturer's instructions, performing two blank injections at a temperature of 270 °C. Prior to the injections, the samples were stirred (250 rpm) at 40 °C for 10 min and the fibre was then exposed to the headspace. Volatiles, after 30 min of absorption, were thermally desorbed in splitless mode for 5 min in the GC injector port held at 250 °C of a TSQ Quantum GC triple stage quadrupole (Thermo Fisher Scientific, Milan, Italy) equipped with a split/splitless injector. Separation was achieved on a *forte* SolGel-Wax fused-silica capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; SGE Analytical Science Pty Ltd., Victoria, Australia). The GC oven temperature programme applied was as follows: start at 40 °C for 4 min, then 40–150 °C at 5 °C min^{-1}, stable