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Optimised quantification of the antiyeast activity of different barley malts towards a lager brewing yeast strain

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ABSTRACT

The brewing of beer involves two major biological systems, namely malted barley (malt) and yeast. Both malt and yeast show natural variation and assessing the impact of differing malts on yeast performance is important in the optimisation of the brewing process. Currently, the brewing industry uses well-established tests to assess malt quality, but these frequently fail to predict malt-associated problem fermentations, such as incomplete fermentations, premature yeast flocculation (PYF) and gushing of the final beer product. Antimicrobial compounds, and in particular antiyeast compounds in malt, may be one of the unknown and unmeasured malt factors leading to problem fermentations.

In this study, the adaptation of antimicrobial assays for the determination of antiyeast activity in malt is described. Our adapted assay was able to detect differing antiyeast activities in nine malt samples. For this sample set, malts associated with PYF during fermentation and gushing activity in beer showed high antiyeast activity. Both PYF and gushing are malt quality issues associated with fungal infection of barley in the field which may result in elevated antimicrobial activity in the barley grain. Also, two more malts that passed the normal quality control tests were also observed to have high antiyeast activity and such malts must be considered as suspect. Based on our results, this assay is a useful measure of malt quality as it quantifies the antiyeast activity in malt which may adversely impact on brewery fermentation.

1. Introduction

The presence of antimicrobial compounds or factors in grain and in particular barley is well established (Broekaert et al., 1992, 1995; Leah et al., 1991; Mendez et al., 1990; Molina et al., 1993a; Molina and Garcia-Olmedo, 1993; Mundy and Rogers, 1986; Ponz et al., 1983). However, in the brewing industry the impact of the presence of such compounds in barley or the malt prepared from barley (the major raw material for beer brewing) on the brewing process has not been investigated as discussed by Van Nierop et al. (2006). The impact, if any, may be direct or indirect. The direct impact would be the inhibition of yeast metabolism during fermentation by antiyeast compounds from the malt. The indirect impact may be much more complex, whereby the occurrence of microbial colonisation/infection on grain and the defence/stress response to that by the barley is indicative of a range of other malt quality aspects. These quality aspects may not necessarily affect yeast, but may lead to mycotoxin contamination (Spicher, 1989; Wolf-Hall and Schwarz, 2002; Wolf-Hall, 2007), introduction of off-odours (Spicher, 1989), inconsistency in brewhouse performance leading to slower processing and higher beer colours (Prentice and Sloey, 1960; Bol et al., 1985) and flavour instability (Guido et al., 2007; Etchevers et al., 1977) and haze in beer (Etchevers et al., 1977).

Malt analyses used in the brewing industry often fail to adequately describe the malt performance in the brewing process (Axcell et al., 1984). In particular, these analyses frequently fail to predict problem fermentations such as incomplete or the so-called "stuck" fermentations (inhibition of glucose uptake, yeast metabolism and/or proliferation), premature yeast flocculation (PYF) and gushing of the final beer product. This is probably because yeast strains used in fermentation are not included in the current protocols of The American Society of Brewing Chemists, Institute of Brewing and European Brewery Convention. There, therefore, is a need for more predictive analyses to complement the current analyses.

Problem malts are sometimes associated with microbial colonisation/infection of barley, leading to the production of antimicrobial compounds by the grain (Van Nierop et al., 2006).

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These compounds include plant defence peptides (Broekaert et al., 1992, 1997; García-Olmedo et al., 1998) such as those from the thionin (Florack and Stiekema, 1994) and defensin (originally called γ-thionins) families (Broekaert et al., 1995; Pelegrini and Franco, 2005), as well as lipid transfer proteins (LTPs) (García-Olmedo et al., 1995; Kader, 1996). Thionins (Ponz et al., 1983), plant defensins (Mendez et al., 1990; Bruix et al., 1993) and non-specific LTP (ns-LTP) (Molina and Garcia-Olmedo, 1993) are found in barley seeds, probably to protect the seed and seedlings during microbial infection. These cationic antimicrobial peptides (thionins and defensins) and ns-LTPs act on membranes and the membrane activity mostly leads to target cell lysis. They are relatively small (5 and 9–10 kDa, respectively) and quite stable due to multiple disulphide bridges, therefore may survive during the malting and brewing process and could potentially influence the yeast and thus fermentation.

In order to examine residual antimicrobial activity in barley malt, which may not be detected by current malt analyses, a reliable and sufficiently sensitive antimicrobial assay, using brewing yeast as an indicator organism, is required. Various methods for the evaluation of antimicrobial activity already exist. Some focus on the assessment of the microbial membrane by examining change in membrane permeability (Terras et al., 1993; Thevissen et al., 1996, 1997, 1999), membrane lysis (Jewel et al., 2002) or nutrient uptake (Wetter et al., 2003). Other methods focus on microbial growth itself, which is by far the most commonly used and least cumbersome. Microbial growth or inhibition is quantified by a number of assays. Some of the oldest antimicrobial activity assays measure the growth inhibition zones when the organism is grown in the presence and absence of an antibiotic on solid media, mostly agar (disk diffusion and gel diffusion assays) (Barry et al., 1979; Cooper and Woodman, 1946; Detmar et al., 1974; Ericson, 1960; DiCuollo et al., 1965; Du Toit and Rautenbach, 2000; Lehrer et al., 1991; Raahave, 1974). The increase or decrease in cell count can be measured by an electronic impedance counter (Eng and Valenstein, 1989), by counting the surviving cells in terms of colony forming units (CFU) or by measuring the turbidity/optical density in the presence or absence of antimicrobial compounds (Skyttä and Mattila-Sandholm, 1991; Amsterdam, 1996; Boeira et al., 1999). Growth inhibition assays that utilised optical density measurements were adapted to microtitre plates (Broekaert et al., 1990; Espinel-Ingroff et al., 1991; Hancock, 1997; Steinberg and Lehrer, 1997; Du Toit and Rautenbach, 2000). These micro-assays, collectively known as microbroth dilution or microdilution assays, require far less sample material and allow for simultaneous testing of many different samples or samples over a range of concentrations in a microtitre plate format. Many different test organisms such as Gram-positive and Gram-negative bacteria, different types of fungi including yeasts (Boeira et al., 1999; Espinel-Ingroff, 2006; Kubo et al., 2003; Kubo and Himejima, 1992; Okada et al., 1970; Thevissen et al., 2000; Wetter et al., 2003) are used in these broth assays. Hadacek and Greger (2000) compared different types of anti-microbial assays and concluded that the microtitre plate micro-dilution-type method offered the greatest potential of all the bioassays.

The microbroth dilution assays referenced above were used as a basis for the development of a suitable antiyeast assay to determine the impact of antimicrobial compounds on a lager brewing strain of *Saccharomyces cerevisiae* (renamed *S. pastorianus* by Barnett, 2004; Rainieri et al., 2006) from the culture collection of The South African Breweries Ltd. The ultimate aim was the application of this method to the investigation of malt antiyeast compounds and their possible impact on malt quality and the brewing process.

2. Materials and methods

2.1. Malt extract preparation

A range of commercial lager-type two row barley malts were milled to a flour consistency in a 3100 Perten hammer mill with a 0.2 mm sieve (Huddinge, Sweden). Duplicate 5 g aliquots were weighed out in disposable 50 mL centrifuge tubes (Sterilin, Staffs, UK). An acid extraction of the milled samples was done according to Okada et al. (1970) and Okada and Yoshizumi (1970) with slight modifications. Sulphuric acid (BDH, Poole, England) (30 mL, 0.05 M) was added to each tube and the mix was incubated on ice for 3 h with vigorous shaking every 15 min to re-suspend all the malt. The tubes were centrifuged at 4000*g* for 15 min at room temperature. The supernatant was dialysed at 4 °C against distilled water using 1 kDa cut-off dialysis tubing (Spectro/Por®, Rancho, Dominguez, California, USA) pre-blocked by soaking tubing in 2% casamino acid (Sigma, St. Louis, USA) for 3 h at room temperature. Post-dialysis, the content of the dialysis tubing was made up to 45 mL with distilled water and centrifuged at 6500*g* for 10 min at 4 °C. The supernatant was filtered though a 0.45 μm acetate syringe filter (Osmonics, Warren, Indiana, USA) and 4.5 mL aliquots were dispensed into 10 mL BD Vacutainer® tubes (Preanalytical Solutions, Plymouth, USA) before freeze drying (Labconco, Kansa City, Montana, USA). The dried aliquots were centrifuged at 3000*g* for 3 min to collect the dry material at the bottom of the tube for easier re-suspension. Dried samples were stored at −20 °C until use and then re-suspended in 1000 μL acetonitrile/water (25:75 v/v) (acetonitrile-HiPerSolv™, BDH, Poole, England). Finally, the samples were centrifuged at 6500*g* for 15 min before using the supernatant in the assay. The same extraction procedure was applied to 0.05 M sulphuric acid without the malt as a negative reagent control.

The antiyeast assays on the extracts were performed according to the optimised protocol described below. The concentration of the malt extracts used in the assay was related back to the amount of grain it was extracted from. For example, each aliquot or tube of extract contains extract from 0.5 g malt. This was re-suspended in 1000 μL of acetonitrile/water (25:75 v/v), hence the concentration was 0.5 g malt extracted per mL of the assay solution. The actual extract mass was therefore not considered because we standardised the amounts used in dose–response assays in terms of the quantity of malt used to produce the extract. Only 20 μL was used per well in the assay (undiluted or in doubling dilutions) and the total volume per well was 100 μL. The highest concentration in the well was therefore 0.01 g malt extracted per 100 μL or 0.1 g/mL. Any deviation from these concentrations was noted where applicable.

2.2. Culturing of brewing yeast

A lager brewing strain of *Saccharomyces cerevisiae* (*S. pastorianus*) supplied by The South African Breweries Ltd. was propagated by inoculating 15 mL MYGP (3 g each of malt and yeast extract, 5 g peptone and 10 g glucose [Biolab Diagnostics, Midrand, South Africa] dissolved in 1 L water and autoclaved 15 min under pressure to attain 121 °C) with cryogenically stored yeast at 1×10^6 cells/mL in a 100 mL Erlenmeyer flask. The yeast was allowed to grow for between 7.5 and 10 h (25 °C) while shaking (150 rpm) to attain log phase yeast. The yeast was then diluted in MYGP to the required cell concentration for the assay. Yeast counts were determined by using a Hawksley–Cristallite haemocytometer with improved Neubauer ruling (Boeco, Hamburg, Germany). Yeast growth was monitored by measuring the increase in light dispersion (light scatter) at 600 nm using a microtitre plate reader (Bio-Rad model 450, Richmond, CA,