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Biosynthesis of flavour-active furanones by Saccharomyces cerevisiae during fermentation depends on the malt type used in medium preparation

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Extracts of a coloured malt contained 4-hydroxy-5-monomethyl-3(2H)-furanone (HMMF), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) but not 4-hydroxy-5(or 2)-ethyl-2(or 5)-methyl-3(2H)-furanone (HEMF). Extracts of a pale malt did not contain any of the furanones. HMMF and HDMF were produced by Saccharomyces cerevisiae during fermentation of both types of malt extract. About 0.09 mg HEMF l^{-1} was synthesised during fermentation of the coloured malt extract but none was produced with the pale malt extract. Final concentrations of HDMF (2.0 mgl^{-1}) and HEMF (0.09 mgl^{-1}) were in excess of their aroma threshold values in water (0.16 and 0.02 mgl^{-1}respectively) after fermentation of the coloured malt extract.

Introduction

Three chemically closely related compounds, 4-hydroxy-5-monomethyl-3(2H)-furanone (HMMF), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) and 4-hydroxy-5(or 2)-ethyl-2(or 5)-methyl-3(2H)-furanone (HEMF) have been identified in a number of foods. HMMF has a meaty/beefy flavour and a relatively high threshold value and its importance in flavour profiles is probably fairly restricted. Both HDMF and HEMF have sweet/caramel-like flavours and very low aroma thresholds of 0.16 mgl^{-1} and 0.02 mgl^{-1} in water respectively and are important in the flavour of fermented soy-bean products, cheeses, fruits, nuts, roasted and boiled beef, roasted coffee (Blank and Fay, 1996) and some beers (Sakuma *et al.*, 1996). Both compounds can be produced spontaneously through reaction between an amino acid, glycine or alanine, and a pentose sugar such as xylose (Blank and Fay, 1996) or by yeast fermentation. Both *Zygosaccharomyces rouxii* (Sasaki *et al.*, 1991; Hequet *et al.*, 1996; Hayashida *et al.*, 1997) and *Saccharomyces cerevisiae* (Sakuma *et al.*, 1996) can carry out this reaction. Sasaki (1996) has demonstrated that xylulose 5-phosphate is the likely precursor of HEMF in soy sauce fermentations but no suggestions have been made about the nature of the precursor(s) in beer. Sakuma *et al.* (1996) did, however, demonstrate that production in a beer fermentation was dependent on the yeast strain.

In addition to its importance in flavour, HEMF has been shown to have an anti-cancer activity in mice (Pariza, 1994) and it is possible that it could contribute to human health as part of a complex anti-oxidant mixture of food-derived compounds.

Methods and materials

Preparation of malt extracts

The malts used were commercially produced pale and coloured types. To prepare an extract, 75 g malt were finely ground using a Buhler Universal Laboratory Disc Mill (type DLFU) set with a 0.2 mm disc gap. The grist was stirred into 350 ml water at 65°C and maintained at this temperature for 60 min with stirring every 20 min. The mash was fitered through a Whatman No 2 paper and boiled for 50 min. After cooling the extract was clarified by centrifugation at 3000 × g for 10 min and the specific gravity adjusted to 1.065 with distilled water.

Yeast strain and fermentation conditions

Saccharomyces cerevisiae (strain KF-1 from the Kumamoto Industrial Research Institute collection) was grown and pre-cultured aerobically in 500 ml flasks with medium containing yeast extract (10 gl^{-1}), bacteriological peptone (20 gl^{-1}) and glucose (20 gl^{-1}) on a shaker at 28°C. For the experimental fermentations, cells were inoculated into 20 ml of test medium in 50 ml

screw-capped tubes at 10^7 cells ml^{-1} and fermentation was allowed to proceed at 28°C with the tubes loosely capped.

GC analysis

To extract furanones from malt extract, 0.1 ml 1-decanol solution (1.0 gl^{-1}) was added to 2 ml of extract which was then saturated with NaCl. Methyl acetate (3 ml) was added and the mixture shaken strongly for 10 min. The layers were separated by centrifugation at 2000 × g for 10 min and the organic layer used for GC analysis in a GC-MS HP6890 fitted with an HP-5 MS 0.25 mm × 30 m column. The temperature ran from an initial 40°C to 200°C at a rate of 3°C per min with a final hold at 200°C for 40 min. The carrier gas was He and the inonisation energy for the MS was 70eV. A calibration curve was constructed with HDMF and used for all three furanones. The minimum detectable level was 0.01 mgl^{-1} and linearity held from 0.02 to 50 mgl^{-1}.

To measure ethanol during fermentation, 1 ml of sample and 4 ml aqueous n-butanol solution (10 ml l^{-1}) were mixed and 2 µl injected into a GC-FID equipped with a CP Silica 5CB column 10 m × 0.32 mm, DF 1.2 um, operating at 80°C with helium as carrier gas.

Results and discussion

A hot-water extract was prepared from a pale and a coloured malt as described in the Methods. After adjustment of the specific gravities the colours of the extracts were 10 and 50 EBC units respectively. The furanone content of the samples was measured, yeast added and fermentation allowed to proceed at 28°C. Analysis for furanones and specific gravity was carried out daily for three days by which time fermentation was complete. The experiment was repeated but with inclusion of an additional tube for each of the malt types containing 200 g NaCl l^{-1}. The results at the end of the fermentations (averages of duplicate analyses) are shown in Table 1. Ethanol concentrations in the second experiment were 15 gl^{-1} in the pale malt extract and 25 gl^{-1} in the coloured malt extract but none could not be detected in the sample with added NaCl.

The results indicate that whilst there was distinct quantitative variation between the replicate experiments, with the second experiment always yielding lower concentrations, fermentation of an extract of coloured malt gave much higher concentrations of all three furanones than fermentation of a pale malt extract. The relative formation of HDMF and HEMF in the coloured malt extract was similar in both experiments with 16 to 19 times more HDMF being produced than HEMF. HMMF was found in the extracts of both pale and coloured malt and its concentration increased over the fermentation period. When alcoholic fermentation was prevented by adding NaCl, HMMF production was much less in the pale malt extract and its concentration actually declined in the coloured malt extract. HMMF appears to be produced during both malting and fermentation but never approached its aroma threshold concentration in the current experiments.

HDMF was not found in the extracts of pale malt but occurred in coloured malt extracts at concentrations over the aroma threshold value. It was produced during fermentation in both media but to a very much greater extent when the coloured malt was used. With pale malt the final concentrations were in the area of the aroma threshold value but this value was exceeded ten or more times with the coloured malt. Neither pale nor coloured malt extracts contained HEMF. It was produced during fermentation of the coloured malt and reached concentrations three to five times the aroma threshold value but was never detected with the pale

Table 1 The concentration of furanones in fermented and unfermented malt extracts

Malt	Compound	Concentration (µg l^{-1})				
		Expt 1		Expt 2		
		Initial Extract	After Day 3	Initial Extract	After Day 3	After Day 3*
P	HMMF	55	246 (191)	13	84 (71)	46 (33)
C	HMMF	359	1012 (653)	311	800 (489)	277 (–34)
P	HDMF	ND	314 (314)	ND	123 (123)	24 (24)
C	HDMF	544	2358 (1814)	333	1693 (1360)	290 (–43)
P	HEMF	ND	ND (0)	ND	ND (0)	ND (0)
C	HEMF	ND	113 (113)	ND	70 (70)	ND (0)

P: pale malt; C: coloured malt; *: fermentation includes 200 g NaCl l^{-1}; ND: not detectable. See Methods for details of analytical procedure and detection limits. Data in parentheses indicate change in concentration during fermentation.

malt. These results are compatible with those of Sakuma *et al.* (1996) who found that, when using a 200 L pilot brewery and a standard cereal grist, their wort contained HDMF but not HEMF. Two different strains of yeast were used for subsequent fermentation and one resulted in increased HDMF concentration and the appearance of low concentrations of HEMF in the ratio of about 10:1 whilst the other strain produced neither of the two compounds.

The results indicate that final concentrations of HDMF and HEMF, but not HMMF, could be above their aroma thresholds at the end of fermentation of malt extracts and so have to be seen as potential flavour compounds contributing towards "sweet", "malty" and "caramel" perceptions of beer flavour. Our results clearly show that the method of malt preparation is a very important factor in determining the final concentration of both HDMF and HEMF after fermentation and it will be important to elucidate the conditions which affect formation of these two compounds and their precursors during malting and kilning. Taken in conjunction with the report of Sakuma *et al.* (1996) that brewing yeast strains vary in their ability to produce furanones, our work indicates that the appearance of these flavour notes in beer needs to be seen not as the result of a single part of the production process but as a consequence of conditions used from malting through to fermentation and are thus, at the moment, likely to vary significantly from one beer type and brewery to another.

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