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How to improve the enzymatic worty flavour reduction in a cold contact fermentation

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Abstract

Although much more efficient at 28°C than at low temperature, enzymatic removal of Strecker aldehydes by brewer's yeast, *Saccharomyces cerevisiae*, is always limited to 60–85% of the initial concentration, whatever the fermentation conditions. This asymptotic reduction pattern leads to residual concentrations imparting the well-known unpleasant worty taste to alcohol-free beers. Low-energy binding to flavanoids is shown to hinder more complete enzymatic reduction in the cold contact fermentation process. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Alcohol-free beers; Cold contact process; Methional; Polyphenols; Reduction

1. Introduction

Even in a cold contact process conducted around 0°C (Schur, 1983), yeast can partially remove wort aldehydes such as 3-methylbutanal, 2-methylbutanal, and 3-methylthiopropionaldehyde (Collin, Montesinos, Meersman, Swinkels & Dufour, 1991; Debourg, Laurent, Goossens, Borremans, Van De Winkel & Masschelein, 1994; Peppard & Halsey, 1981; Perpète & Collin, 1999a). Yet, due to its very low flavour threshold, 3-methylthiopropionaldehyde produces an unpleasant worty aroma in most alcohol-free beers (Perpète & Collin, 1999b, c).

Monitoring of Strecker aldehyde concentrations during fermentation has revealed a similar reduction pattern, whatever the pitching rate or yeast strain (Perpète & Collin, 1999b). Enzymatic reduction is initially fast, but after a few hours it slows down quickly. Residual concentrations can reach 40% of the initial concentration in the wort. In a mixed-batch cold contact process, enzymatic reduction is in all cases much more pronounced for linear aldehydes than for branched ones (Perpète & Collin, 1999b). Similar results were described by Debourg et al. (1994) in an immobilized yeast system operating at 0 or 5°C.

The aim of this work was, therefore, to understand the discrepancy between both kinds of aldehydes and to explain why Strecker aldehyde concentrations follow this asymptotic pattern in cold contact fermentations. Yeast vitality was first suspected to be the limiting factor. We then considered the possibility that chemical binding to wort constituents might hinder enzymatic removal of aldehydes.

2. Materials and method

2.1. Reagents

Isobutanal (98%), 2-methylbutanal (95%), 3-methylbutanal (98%) and sodium bisulfite (p.a.) were purchased from Janssen Chimica (Geel, Belgium). Pentanal (>98%), 2-methylpentanal (98%) and (+)-catechin (>98) were from Sigma-Aldrich (Bornem, Belgium). Hexanal (98%), heptanal (98%) were from Fluka Chemika (Buchs, Switzerland).

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2.2. Strains

Bottom fermentation *Saccharomyces cerevisiae* MUCL28365 was provided by the MUCL collection of the Université Catholique de Louvain (Louvain-la-Neuve, Belgium).

2.3. Culture media

Cultures were grown in YPS medium (1% yeast extract, 0.5% peptone, 10% sucrose) at 28°C on a rotary shaker and collected in the exponential phase. Propagation was carried out in two steps with a pitching rate of 10^7 cells/ml each time. After collection and washing, the yeast was pitched at the same level in a 12°P (g extract/100 g wort) pure malt wort treated with 300 ppb of branched aldehydes and 100 ppb of linear aldehydes.

2.4. Cold contact sampling

Each sample was centrifuged at 4°C to remove yeast and immediately frozen in liquid nitrogen.

2.5. Dynamic headspace gas chromatography–FID analytical conditions

A Hewlett Packard Model 5890 gas chromatograph equipped with a Chrompack Purge and Trap Injector, a flame ionisation detector and a Shimadzu CR3A integrator were used. Samples were injected into the chromatographic column in three steps as follows: (1) precooling of the trap (CPSIL 8 CB capillary column, 0.53 mm internal diameter ; film thickness, 5 μm): the trap was cooled at −95°C for 2 min in a stream of liquid nitrogen; (2) purging of the sample : the temperature of the purge vessel was set at 50°C. The sample was purged with helium gas (12 ml/min) for 15 minutes. The gas stream was passed through a condenser kept at −15°C by means of a cryostat (Colora WK 15) to remove water vapour and then through an oven at 200°C. The volatiles were finally concentrated in the cold trap maintained at −95°C (liquid nitrogen); (3) desorption of the volatiles: cooling was stopped, and the surrounding metal capillary was immediately heated to 220°C for 5 min. The carrier gas swept the trapped compounds into the analytical column. Analysis of samples was carried out on a 50 m×0.32 mm, wall-coated, open tubular (WCOT) CP-Sil5 CB (Chrompack, Antwerpen, Belgium) capillary column (film thickness, 1.2 μm). Oven temperature, initially kept at 36°C for 15 min, was programmed to rise from 36 to 120°C at 5°C/min then to 200°C at 10°C/min, remaining at the maximum temperature for 10 min thereafter. Helium carrier gas was used at a flow rate of 1.0 ml/min. Injection and detection temperatures were 200 and 220°C, respectively. All analyses were done in duplicate. The assessment of the technique reproducibility has been previously described (coefficients of variation under 10% for five analyses of the same standard mixture; Collin, Osman, Delcambre, El Zayat & Dufour, 1993).

2.6. Sulfite-binding assay

Increasing amounts of each aldehyde were added to 50 mM phosphate buffer (pH 4.5) with or without 10 ppm sulfite. By comparing the calibration slopes obtained by dynamic headspace analysis (see above) it was possible to estimate the relative recovery of aldehydes in the presence of sulfite.

2.7. Sulfite quantification

This was done on a Nucleogel Sugar 810H anion exclusion column (30.0 cm × 7.8 mm, particle size 10 μm — Macherey & Nagel, Düren, Germany) totally sulfonated for cation exchange. The separation process taking place on the surface of the resin particles involves three different principles: Donnan exclusion, steric exclusion, and adsorption (Kim, Park & Kim, 1987). The HPLC system was equipped with a 5 μl Rheodyne injection valve (COTATI, Berkeley, USA) and a Waters isocratic pump (Waters Corporation, Milford, USA). The detector was a Waters 464 amperometric detector (Waters Corporation, Milford, USA) with platinum working electrode set at 700 mV (time constant: 0.5 s, current range: 20 nA, reference electrode Ag/AgCl), connected to a Shimadzu CR6-A integrator. The eluent was 22.5 mM H_2SO_4 (pH 1.65) and the flow rate was 0.6 ml/min. This low pH allowed neutral sulfites to penetrate the 'Donnan membrane' of the column. For total sulfite detection, wort and beer samples were diluted 10 times in 100 mM boric acid solution (pH 9.0) containing 10 mM Titriplex III (pH adjusted with NaOH). Samples were then flushed through a Sep-Pak C18 cartridge (Waters Corporation, Milford, USA) preconditioned with 10 ml methanol and 10 ml boric acid solution (just described). The residual solution in the cartridge was expelled with air. The first 5 ml of sample was discarded and the next 3 ml collected for analysis.

2.8. Flavanoid binding assay

Aldehyde recovery was measured by dynamic headspace analysis (purge temperature: 20°C). Increasing amounts of 3-methylbutanal, 2-methylbutanal and pentanal were added at 20°C to 50 mM phosphate buffer (pH 4.5) with or without 100 ppm catechin. By comparing the calibration slopes obtained by dynamic headspace analysis (as described above, except that the purge vessel temperature was 20°C), it was possible to estimate the relative recovery of aldehydes in the presence of flavanoids.

3. Results and discussion

3.1. Physiological ability of yeast to reduce aldehydes after 5 h of cold contact fermentation

To test the ability of yeast to reduce aldehydes after 5 h of cold contact fermentation, 300 ppb 3-methylbutanal and 300 ppb 2-methylbutanal were added to partially fermented wort. Fig. 1 shows that even at the low temperature used, yeast was able to reduce the added aldehydes totally within 5 h.

This experiment shows that the (poor) physiological state of the yeast cannot explain why Strecker aldehydes remain in a cold-contact-fermented wort at 40% of their initial concentration. This was confirmed in another experiment where a second pitching was done after 5 h with freshly propagated yeast (Fig. 1). The residual concentration was the same. This suggests that aldehyde interactions with the medium rather than failure of the yeast enzymes are responsible for stopping aldehyde reduction. Since dynamic headspace analysis conducted at 50°C can release the unreduced fraction, a chemical linkage more stable at low temperature should be involved.

As sulfites are produced in regular fermentations when growth is slow (Dufour, Carpentier, Kulakumba, Van Haecht & Devreux, 1989; Pickerell, Hwang & Axcell, 1991), we first suspected that they could be excreted very early in a cold contact fermentation. Sulfites are known to bind aldehydes (Chapon, Chapon & Djeuga, 1982) even at 4°C (Debourg et al., 1994), altering their ability to be reduced enzymatically (see fermentation experiments conducted at 15°C by Debourg et al., 1994).

3.2. Yeast reduction limited by retention in sulfitic complexes?

Dynamic headspace analysis (usual protocol conducted at 50°C) confirmed significant retention of 3-methylbutanal in sulfite-containing samples (69% of retention as shown in Fig. 2; $P < 0.05$ for all comparisons; F-tests for equality of slopes of several regression lines were executed in the SAS system; Sokal & Rohlf, 1969). Low retention at pH 9.0 confirmed the involvement of sulfite adducts. Very interesting was the difference between 3- and 2-methylbutanal, the latter being

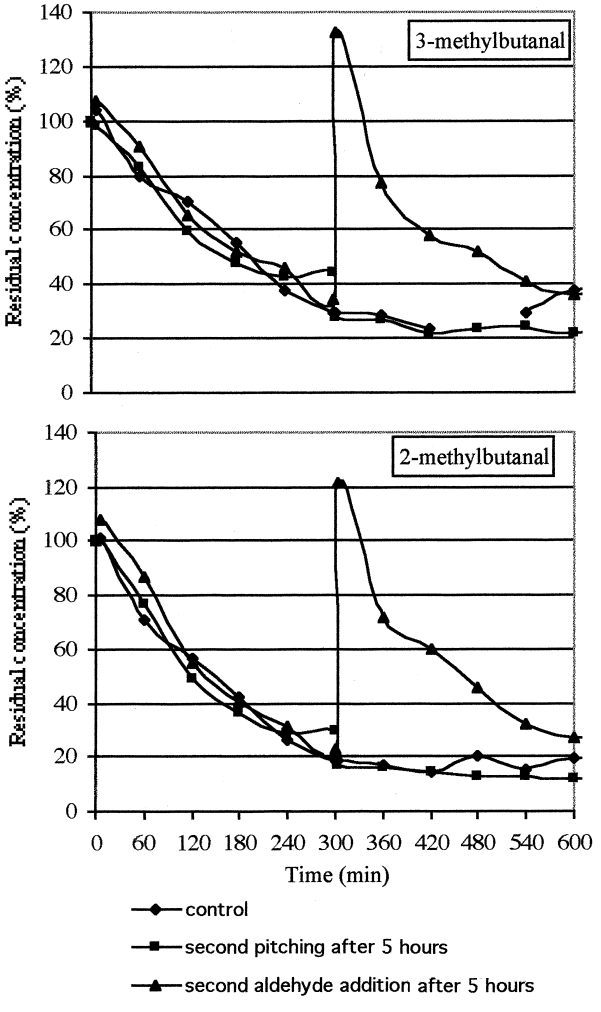

Fig. 1. Evolution of 3-methylbutanal and 2-methylbutanal (% of the initial concentration) in a 12°P wort (cold contact fermentation, pitching at 10^7 cells/ml, purge vessel at 50°C).

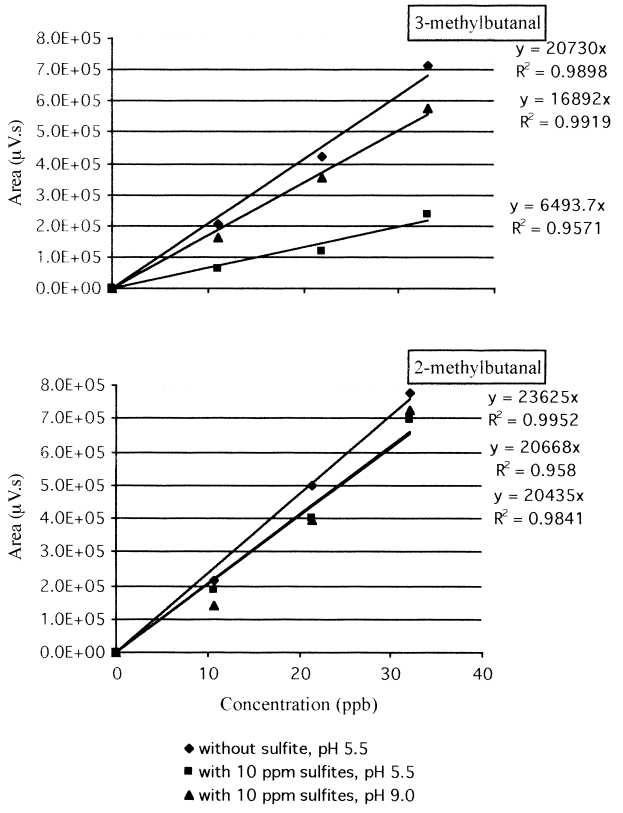

Fig. 2. Effect of sulfites on 3-methylbutanal and 2-methylbutanal retention in a dynamic headspace system.

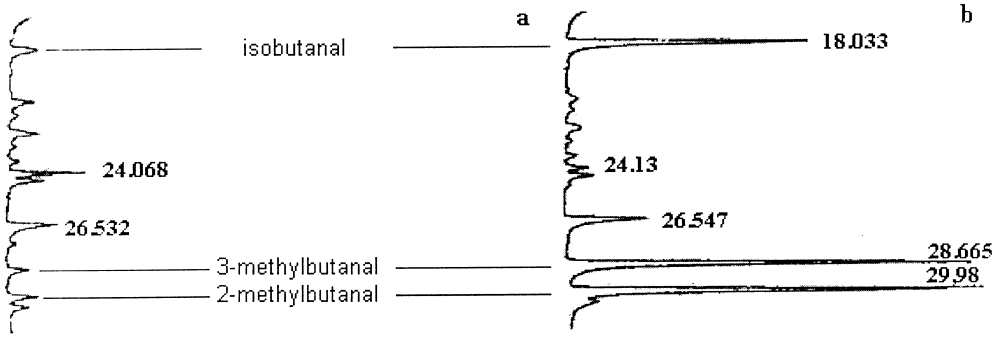

Fig. 3. Dynamic headspace analysis chromatograms in (a) presence and (b) absence of 10 ppm sulfites (purging at 2°C).

too hindered to interact well with sulfites ($P > 0.05$ for all comparisons; F-tests for equality of slopes of several regression lines were executed in the SAS system; Sokal & Rohlf, 1969). Similar results were obtained for isobutanal (data not shown). The retention effect was pronounced for both compounds when headspace analysis was done at 2°C, as shown qualitatively in Fig. 3 (no quantitative data due to too-low recovery factors at this temperature).

It would thus seem that, through a cold contact fermentation performed at 0–1°C, sulfite-aldehyde linkages are tight enough to prevent complete reduction of some aldehydes by yeast. This was confirmed in the following experiment where we enhanced a 12°P wort with 3-methylthiopropionaldehyde, 3-methylbutanal, 2-methylbutanal (300 ppb of each aldehyde), and 10 ppm sulfite before pitching with *Saccharomyces cerevisiae* MUCL28365 at 10^7 cells/ml. Aldehyde levels were monitored during the first hours of fermentation at 0°C. As shown in Fig. 4, the level of 3-methylbutanal did not drop as did the 2-methylbutanal level, confirming that

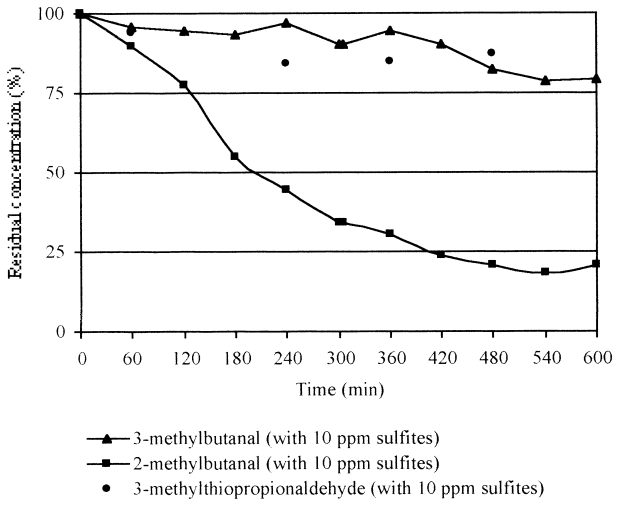

Fig. 4. Evolution of 3-methylthiopropionaldehyde, 3-methylbutanal and 2-methylbutanal (% of the initial concentration) in a 12°P wort containing 10 ppm sulfites (cold contact fermentation, pitching at 10^7 cells/ml, purge vessel at 50°C).

this aldehyde was not sufficiently free at 0°C for enzymatic reduction (80% of the initial concentration remained after 5 hours of fermentation, as opposed to 30% for 2-methylbutanal). PFBOA derivatisation of some samples (Perpète & Collin, 1999a) confirmed that 3-methylthiopropionaldehyde, the compound imparting the worty off-flavour, has the same reduction profile as 3-methylbutanal. Since the yeast reduction pattern was the same for all Strecker aldehydes in the previous experiments, we conclude that sulfites are not responsible in our fermentations for insufficient yeast reduction. Furthermore, the HPLC data shown in Table 1 confirm that only ppb amounts of sulfite are produced in our cold contact fermentations. Our data obtained in the presence of added sulfite could be relevant, however, in industrial processes where antioxidants are added during mashing or boiling.

Other wort constituents must thus be responsible for hindering enzymatic reduction. As low-energy interactions have been described between polyphenols and wine aroma (Dufour & Bayonove, 1999), we considered this possibility in the case of worts. As well documented for haze proteins (McMurrough, Madigan & Kelly, 1996; Bamforth, 1999), aldehydes might bind flavanoids more easily at a sufficiently low temperature. About 150–300 ppm polyphenols can be found in a typical 12°P wort. Anthocyanogens constitute the most important fraction with 30–100 ppm while minor constituents such as catechin and epicatechin account for about 10 ppm (Moll, 1991).

3.3. Yeast reduction power limited by aldehyde-flavanoid complexes?

As shown in Fig. 5, dynamic headspace chromatography as routinely applied in this laboratory allows quantification of both free and catechin-bound aldehydes. On the other hand, only free aldehydes are purged at room temperature. Surprisingly, both branched aldehydes showed here a similar retention pattern (21–23% retention at 20°C), pentanal remaining unaffected by the presence of catechin (data not shown).

A cold contact experiment conducted on a 12°P wort enhanced with 300 ppb 3-methylbutanal, 300 ppb 2-methylbutanal, 100 ppb pentanal and 100 ppm catechin confirmed the determining impact of polyphenols in low-temperature fermentations. As shown in Fig. 6, linear aldehydes were not affected by the additional 100 ppm of catechin, but residual concentrations of Strecker aldehydes increased by 20% as compared to the control.

Table 1
Sulfite level measured by HPLC through a cold contact fermentation

Time (h)	1	3	5	7	8
Total sulfites (ppb)	130	230	130	< 10	< 10

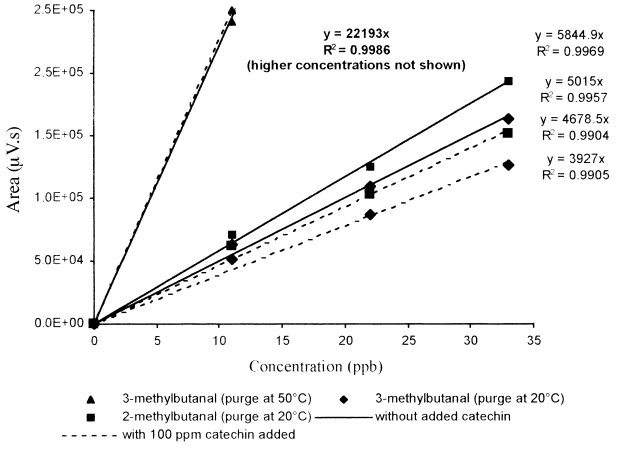

Fig. 5. 3-Methylbutanal and 2-methylbutanal calibration curves with purging at either 20 or 50°C in presence or absence of catechin ($P \leqslant 0.05$ for all comparisons; F-tests for equality of slopes of several regression lines executed in the SAS system; Sokal & Rohlf, 1969).

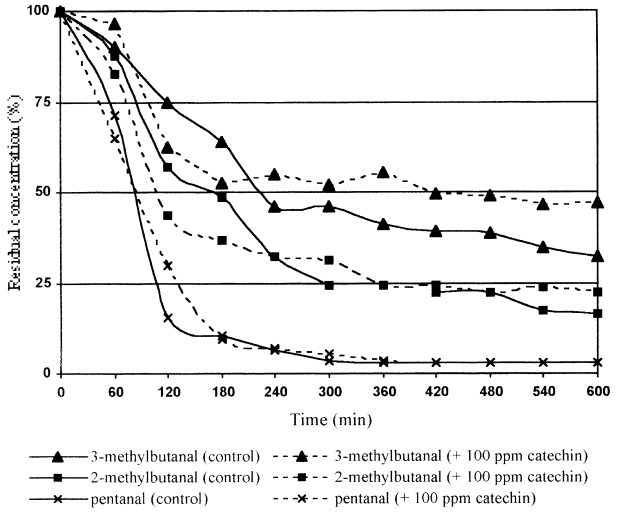

Fig. 6. Evolution of 3-methylbutanal and 2-methylbutanal (% of the initial concentration) in a 12°P wort containing 100 ppm catechin (cold contact fermentation, pitching at 10^7 cells/ml, purge vessel at 50°C).

A student test applied after 600 min fermentation statistically confirmed our conclusions. Indeed, t-values calculated between 3-methylbutanal (44.04) and 2-methylbutanal (43.61) data, with and without catechin, are in all cases above 31.82 (signification at 0.01%). On the other hand, a t-value = 4.00 is calculated for pentanal with and without catechin, indicating no binding for linear aldehydes (t-value must be $\geqslant 6.31$ for a signification at 0.05%).

4. Conclusion

In conclusion, flavanoids emerge as probably responsible for the different behaviours of Strecker and linear aldehydes in cold contact fermentations. Linear aldehydes, less bound to flavanoids, can be removed quickly by yeast from the wort, but a significant fraction of the 2- and 3-methylbutanal is not available for enzymatic reduction at low temperature. This points to two possible strategies for low-alcohol beer production: either working at higher temperature with genetically modified yeasts or decreasing the polyphenol level in the wort. It can be emphasized that formol (aldehyde structure) has often been added in the kettle to remove wort anthocyanogens in regular beer productions. Nowadays, specific filters and PVPP (McMurrough et al., 1996; Bamforth, 1999) are usually used just before bottling. We advise use of this filter aid just after wort cooling in the case of low-alcohol beer production. An additional advantage of this filtration would be removal of part of the aldehydes. For immobilized yeast systems, we also advise minimization of the period between wort cooling and fermentation in order to decrease the polyphenol-bound fraction. More basic studies are needed, however, to determine which kind of interaction takes place and which polyphenols are the most involved.

Acknowledgements

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