Fate of the worty flavours in a cold contact fermentation

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Abstract

3-Methylbutanal, 2-methylbutanal and 3-methylthiopropionaldehyde are described in the literature as preponderantly responsible for the worty taste of alcohol-free beers. Even in a cold contact process, such aldehydes are reduced through fermentation. The chemical removal of aldehydes by amino acids or proteins does not exceed 20% of the initial concentration, although such mechanisms appear much more effective at 28°C. The role of *Saccharomyces cerevisiae* brewer's yeast, in the reduction of wort aldehydes, is confirmed here. Only viable yeasts allow a significant decrease in carbonyl level. Unfortunately, residual concentrations are higher for Strecker aldehydes among which 3-methylthiopropionaldehyde is characterized by a very low flavour threshold. Effects of yeast strain, pitching rate and inhibitors have been assessed. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Two different methods are currently used to produce alcohol-free beers. One applies classical brewing processes followed by alcohol removal by techniques such as reverse osmosis, dialysis, or evaporation (Muller, 1990; Stein, 1993). The ethanol content can also be reduced by restraining fermentation. In 1983, Schur (1983) proposed a "cold contact process" (CCP) combining a long fermentation time with low temperature. No ethanol is produced under these conditions, although yeast exhibits moderate metabolism such as ester and fusel alcohol production or carbonyl reduction. CCP is currently applied to low-density worts in immobilized yeast reactors (Narziss, Back & Leibhard, 1991).

Beal and Mottram (1994) propose 3-methylbutanal and 2-methylbutanal as the main contributors to malt flavour. Yet both compounds have a high flavour threshold, and are therefore unable to confer, alone, the worty taste to beer. In a recent paper (Perpète & Collin, 1999), 3-methylthiopropionaldehyde emerged as the key compound responsible for the worty off-flavour in alcohol-free beers.

Since Peppard and Halsey's work (1981), yeast enzymes are known to be potentially responsible for

reduction of Strecker aldehydes and other linear aldehydes to less flavourful compounds. Among these enzymes, alcohol dehydrogenase (Collin, Montesines, Meersmann, Swinkels & Dutour, 1991), aldehyde dehydrogenase, and aldoketoreductases (Laurent, Geldorf, Van Nedervalde, Vandenbussche, Collin & Debourg, 1995; Van Nedervelde, Verlinden, Philipp & Debourg, 1997) use either NAD(H) or NADP(H) as cofactor. Perpète, Van Nedervelde, Vandenbissche, Collin and Debourg (1997) have demonstrated high heterogeneity of reductase activity between yeast strains. More recently, a novel NADP(H)-dependent branched chain alcohol dehydrogenase was found under anaerobic conditions (Van Iersel, Eppink, Van Berkel, Rombouts & Abee, 1997). Even in alcohol-free beer production (Collin et al., 1991; Peppard & Halsey, 1981; Van Nedervelde et al., 1997), the quantity of wort aldehydes can quickly drop when yeast is added. In a CCP immobilized yeast system, Collin et al. (1991) showed that the branched aldehydes 2-methylbutanal and 3-methylbutanal decreased to 70% of their initial concentration upon yeast addition.

The aim of the present work was to confirm the role of yeast enzymatic activities in reduction of wort aldehydes at 0°C. Effects of yeast viability, yeast strain, pitching rate, and protein synthesis inhibitors were also assessed. For experimental reasons, mainly 3-methylbutanal and 2-methylbutanal were monitored through our CCP fermentations. Only the first experiment includes

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the evolution of 3-methylthiopropionaldehyde, for which PFBOA (*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine) derivatisation and electron capture detection (ECD) are required. Indeed, the well known dynamic headspace method was revealed to be inefficient for the stripping of this sulfurous aldehyde.

2. Materials and methods

2.1. Reagents

Isobutanal (98%), 2-methylbutanal (95%), and 3-methylbutanal (98%) were purchased from Janssen Chimica (Geel, Belgium). 3-Methylthiopropionaldehyde (>95%) was from Acros Organics (New Jersey, USA). Hexanal (>98%), 2-methylpentanal (98%), and *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (98%) were from Aldrich Chemie (Steinheim, West Germany). Hexanal (98%), heptanal (98%) and actidione (cyclo-heximide, >98%) were from Fluka Chemika (Buchs, Switzerland). Methylene blue, CaO (95%), MgO (97%), Na_2SO_4 (99%), NaNO3 (99%), and NaCl (99,5%) were from Merck-Belgolabo (Overijse, Belgium).

2.2. Strains

Bottom fermentation *Saccharomyces cerevisiae* MUCL28365 was provided by the MUCL collection of the Université Catholique de Louvain (Louvain-la-Neuve, Belgium). Top fermentation *Saccharomyces cerevisiae* BRAS291 was provided by the BRAS 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 each of the two following CCP media: either a 12°P (g maltose/100 g wort) pure malt wort or a 50 mM phosphate buffer containing 300 ppb branched aldehydes, 100 ppb linear aldehydes, and 2205 ppm usual wort amino acids (Table 1).

Various ionic strengths were also tested. CaO (106 ppm), MgO (32 ppm), and Na_2SO_4 (7.5 ppm) were added to mimic the water used for brewing beers in Munich while CaO (530 ppm), MgO (154 ppm), Na_2SO_4 (91 ppm), NaCl (34 ppm), and KNO_3 (22 mg) were added to obtain the salt composition of Burton water.

2.4. Sampling

Each sample was centrifuged to remove the yeast and immediately frozen in liquid nitrogen.

Table 1
Amino acid composition of the phosphate buffer model solution

Amino acid	Concentration (ppm)
Proline	350
γ-Aminobutyric acid	350
Leucine	160
Arginine	150
Phenylalanine	150
Threonine	150
Valine	130
Alanine	120
Tyrosine	100
Lysine	95
Isoleucine	85
Serine	70
Glutamic acid	60
Histidine	60
Aspartic acid	55
Tryptophan	45
Glycine	40
Methionine	35
Total	2205

2.5. Dynamic headspace gas chromatography—FID analytical conditions

A Hewlett–Packard Model 5890 gas chromatograph equipped with a Chrompack Purge and Trap Injector (PTI), a flame ionization 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 (CPSIL8 CB capillary column, 0.53 mm internal diameter; film thickness, 5 μm): the trap was cooled for 1 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 min. 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 x 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 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, Elzayat & Dutour, 1993).

2.6. 3-Methylthiopropionaldehyde derivatisation and GC/ECD quantification

3-Methylthiopropionaldehyde derivatisation was performed with PFBOA O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine as described by Ojala, Kotiaho, Siirila and Sihvonen (1994). Quantification was carried out using a Chrompack CP9001 gas chromatograph equipped with a split injector (split vent 10 ml/min) maintained at 200°C. A 25 m x 0.32 mm, wall coated open tubular (WCOT) FFAP capillary column (film thickness, 0.3 μm) was used. Oven temperature, initially at 50°C was programmed to rise to 100°C at 10°C/min and kept at 100°C for 10 min, thereafter from 100 to 140°C at 1°C/min and from 140 to 250°C at 10°C/min, remaining at the maximum temperature for 15 min. Helium carrier gas was used at a flow rate of 1.3 ml/min. The effluent was sent to an ECD detector maintained at 250°C and connected to a Shimadzu CR4A Chromatopack integrator. ECD purge and make up nitrogen flow rates were 15 ml/min and 35 ml/min, respectively.

2.7. Heat inactivation of the yeast

MUCL28365 Saccharomyces cerevisiae strains grown in YPS medium were collected during their exponential growth phase and washed. The samples were then heated at 60°C for 20 min so as to inactivate the cells. Residual viability was below 1% according to a methylene blue staining assay (Lentini, 1993), and the cells proved unable to grow after heat treatment.

3. Results and discussion

3.1. Kinetics of aldehyde removal during a classical CCP process

A 12°P wort enhanced with 300 ppb 3-methylbutanal, 300 ppb 3-methylthiopropionaldehyde, 100 ppb pentanal, 100 ppb hexanal, and 100 ppb heptanal was pitched with Saccharomyces cerevisiae MUCL28365 at 10⁷ cells/ml. Aldehyde levels were monitored by PTI-GC (or PFBOA derivatisation - GC-ECD, in the case of 3-methylthiopropionaldehyde) during the first hours of fermentation at 0°C. As shown in Fig. 1, levels of all aldehydes decreased quickly in the wort. After 8 h of CCP fermentation, the 3-methylbutanal and 3-methylthiopropionaldehyde concentrations had dropped by only 60% of their initial levels, compared to the 85% decrease observed in a usual 28°C fermentation. Over the same period, linear aldehydes were almost completely removed. As depicted in Fig. 1, increasing the

pitching rate accelerated aldehyde removal over the first 2 h.

3.2. Evolution of wort aldehydes without yeast

As shown in Fig. 2, removal of free aldehydes from a 12°P wort was very low when no yeast was added. The decrease did not exceed 20% of the initial concentration. Since the effect occurred in the first 30 min of the experiment, chemical binding with amino acids or proteins was suspected. This kind of effect has been described in wort for various aldehydes, e.g., trans-2-nonenal (Noël & Collin, 1995). In order to confirm such binding, aldehyde levels were monitored for 4 h in a phosphate buffer containing the usual wort amino acids.

Fig. 3 shows that chemical removal of aldehydes by amino acids was very low under CCP conditions. This contrasts with the 25 and 35% removal observed at 20°C for branched and linear aldehydes, respectively.
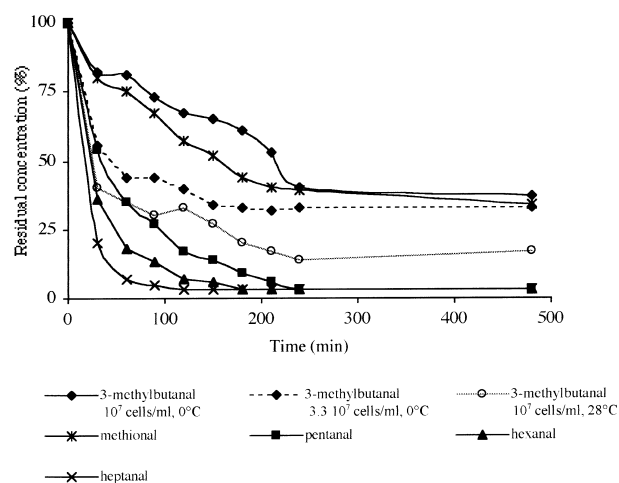

Fig. 1. Evolution of aldehydes (% of the initial concentration) in a 12°P wort pitched with 10⁷ cells/ml or 33.10⁶ cells/ml under CCP conditions.
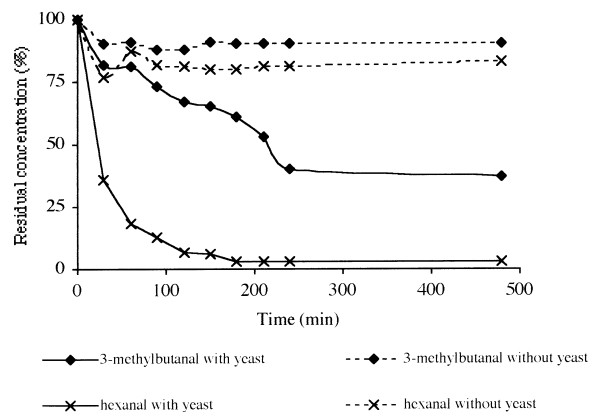

Fig. 2. Evolution of 3-methylbutanal and hexanal concentration (% of the initial concentration) in a 12°P wort under CCP conditions. Comparison between a pitched wort (10⁷ cells/ml) and a non-pitched wort.
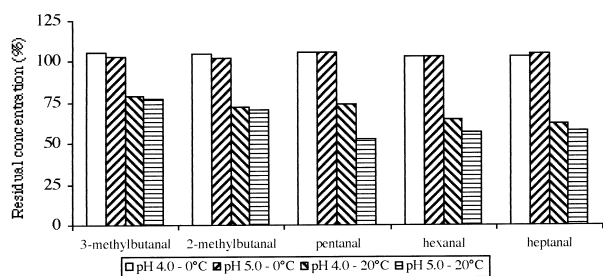
Fig. 3. Aldehyde removal from phosphate buffer containing amino acids at 0°C and 20°C.

Table 2
Chemical binding of aldehyde (% of the initial concentration) after 4 h in (A) a phosphate buffer containing 2205 ppm amino acids or (B) a 12°P industrial wort

Temperature	(A)		(B)	
	0°C	20°C	0°C	20°C
3-Methylbutanal	2	21	2	90
2-Methylbutanal	2	29	2	75
Hexanal	2	36	2	84
Heptanal	1	39	19	> 95

As shown in Table 2, binding is even more efficient in an industrial wort where proteins may also be involved in the interaction.

3.3. Evolution of aldehydes in the presence of inactivated yeast

A 12°P wort enhanced with aldehydes was pitched with heat-inactivated yeast. As shown in Fig. 4, removal of 3-methylbutanal and hexanal, respectively, reached only 15 and 25% of the initial concentration over the first 8 h of this CCP experiment. As in the case of chemical binding, the disappearance occurred immediately after pitching. Viable yeast thus appears necessary for reducing the aldehyde level.

3.4. Cycloheximide and ionic strength effects

When 4 ppm cycloheximide was added to the pitching wort, the aldehyde removal rate was unaffected (Fig. 5), even though this inhibitor of protein synthesis effectively inhibits yeast growth in YPS medium at 28°C. This suggests that enzymatic removal of aldehydes does not require de novo enzyme synthesis.

Next, various ionic strengths (Munich- or Burton-like water) and yeast strains (BRAS291 top-fermentation yeast and MUCL28365 bottom-fermentation yeast) were tested under our CCP conditions. In all cases, 60% of the initial 3-methylbutanal and 99% of the hexanal were reduced after 8 h of fermentation. Neither the choice of the *Saccharomyces cerevisiae* strain nor the
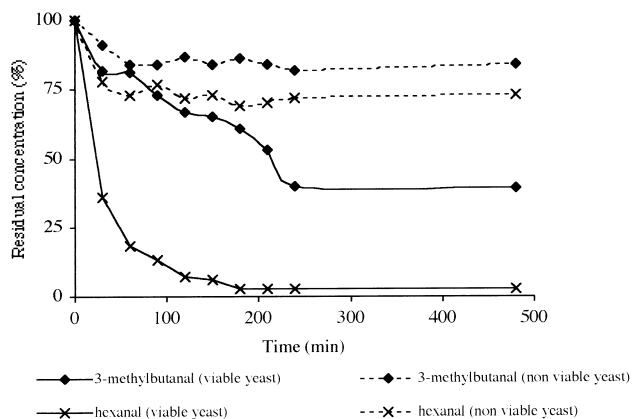

Fig. 4. Evolution of 3-methylbutanal and hexanal concentration (% of the initial concentration) in a 12°P wort pitched with 10^7 cells/ml under CCP conditions. Comparison between heat-inactivated and viable yeast.
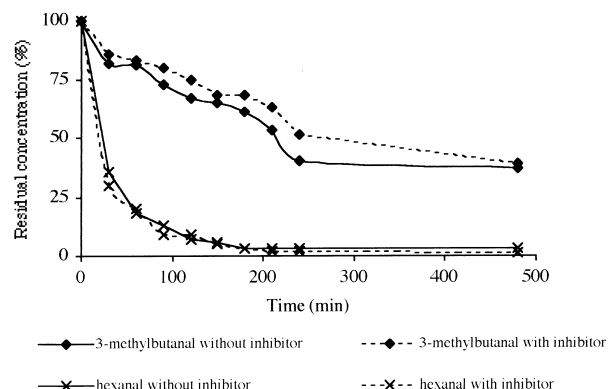

Fig. 5. Evolution of 3-methylbutanal and hexanal concentration in a 12°P wort pitched with 10^7 cells/ml under CCP conditions. Effect of adding 4 ppm cycloheximide.

ionic strength proved a key factor for improving worty flavour removal.

4. Conclusions

In CCP fermentations, removal of worty off-flavours can be partly achieved by enzymatic reduction (up to 60% of the initial concentration for Strecker aldehydes). Although much more efficient at 20°C, chemical removal of aldehydes by amino acids or proteins leads, under CCP conditions, to no more than a 20% reduction in aldehyde levels. Both chemical binding and enzymatic reduction are more pronounced for linear aldehydes than for branched ones. Consequently, residual concentrations of the latter, especially ones with a low odour threshold value, may be sufficient to impart the well-known worty off-flavour (e.g.: 3-methylthiopropionaldehyde flavour threshold in water = 1.7 ppb; Grosch, 1994).

For all aldehydes, the reductases responsible for removal proved constitutive, since cycloheximide did not affect the reduction rate. Neither ionic strength optimization nor yeast strain selection appears promising as a means of improving the reduction efficiency. A more interesting approach would probably be to decrease the amount of free and bound Strecker aldehydes before CCP fermentation, for instance by improving the malt kilning and wort clarification steps. Another promising approach would be the use, at higher temperature, of mutants characterized by high reductase (complete removal of the worty flavour) and low alcohol dehydrogenase activities (low level of ethanol even at 10°C).

References

Beal, A. D., & Mottram, D. S. (1994). Compounds contributing to the characteristic aroma of malted barley. *Journal of Agricultural and Food Chemistry, 42*, 2880–2884.

Collin, S., Montesinos, M., Meersman, E., Swinkels, W., & Dufour, J.P. (1991). Yeast dehydrogenase activities in relation to carbonyl compounds removal from wort and beer. In *Proceedings of the 23rd European Brewery Convention Congress* (pp. 409–416). Oxford, UK: IRL Press.

Collin, S., Osman, M., Delcambre, S., El Zayat, A. I., & Dufour, J. P. (1993). Investigation of volatile flavour compounds in fresh and ripened Domiati cheeses. *Journal of Agricultural and Food Chemistry, 41*, 1659–1663.

Grosch, W. (1994). Determination of potent odourants in food by aroma extract dilution analysis (AEDA) and calculation of odour activity value (OAVs). *Flavour and Fragrance Journal, 9*, 147–158.

Laurent, M., Geldorf, B., Van Nedervelde, L., Dupire, S., Debourg, A. (1995). Characterisation of the aldoketoreductase yeast enzymatic systems involved in the removal of wort carbonyls during fermentation. In *Proceedings of the 25th European Brewery Convention Congress* (pp. 337–344). Oxford, UK: IRL Press.

Lentini, A. (1993). A review of the various methods available for monitoring the physiological status of yeast: yeast viability and vitality. *Ferment, 6*, 321–327.

Muller, R. (1990). The production of alcohol-free beers by limited fermentations. *Ferment, 3*, 224–230.

Narziss, L., Back, W., & Leibhard, M. (1991). Optimization of biological processes for the production of alcohol-free beer using suitable cultures. *Brauwelt International, I*, 52–57.

Noël, S., & Collin, S. (1995). Trans-2-nonenal degradation products during mashing. In *Proceedings of the 25th European Brewery Convention Congress* (pp. 483–490). Oxford, UK: IRL Press.

Ojala, M., Kotiaho, T., Siirila, J., & Sihvonen, M. L. (1994). Analysis of aldehydes and ketones from beer as *O*-(2,3,4,5,6-penta-fluorobenzyl)hydroxylamine derivatives. *Talanta, 41*, 1297–1309.

Peppard, T. L., & Halsey, S. A. (1981). Malt flavour—transformation of carbonyl compounds by yeast during fermentation. *Journal of the Institute of Brewing, 87*, 386–390.

Perpète, P., Van Nedervelde, L., Vandenbussche, J., Collin, S., & Debourg, A. (1997). Evidence of heterogeneity in 3-methylbutanal reduction enzymes of *Saccharomyces cerevisiae*. *ASBC Newsletter, 57*, 29.

Perpète, P., & Collin, S. (1999). Contribution of 3-methylthiopropionaldehyde to the worty flavour of alcohol-free beers. *Journal of Agricultural and Food Chemistry* (accepted).

Schur, F. (1983). Ein neues verfharen herstellung von alkoholfreien bier. In *Proceedings of the 19th European Brewery Convention Congress* (pp. 353–360). Oxford, UK: IRL Press.

Stein, W. (1993). Dealcoholization of beer. *Master Brewers Association of the Americas. Technical Quaterly, 30*, 54–57.

Van Iersel, M. F. M., Eppink, M. H. M., Van Berkel, W. J. H., Rombouts, F. M., & Abee, T. (1997). Purification and characterization of a novel NADP-dependent branched chain alcohol dehydrogenase from *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology, 63*, 4079–4082.

Van Nedervelde, L., Verlinden, V., Philipp, D., & Debourg, A. (1997). Purification and characterization of yeast 3-methylbutanal reductases involved in the removal of wort carbonyls during fermentation. In *Proceedings of the 26th European Brewery Convention Congress*, (pp. 447–454). Oxford, UK: IRL Press.