Contribution of 3-Methylthiopropionaldehyde to the Worty Flavor of Alcohol-Free Beers

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Alcohol-free beers are usually criticized for two major defects: a lack of fruity aroma and a strong worty flavor. 3-Methylbutanal and 2-methylbutanal are described in the literature as being predominantly responsible for the worty taste. Although detected in large amounts in most malt and wort extracts, both compounds have proven unable to confer worty taste to beers. In this work, we extracted volatiles from wort with a Likens−Nickerson microextractor. The resulting extract had a strong worty aroma. Following GC/MS and GC/olfactometry analysis, 3-methylthiopropionaldehyde turned out to be the key feature since it remained the most organoleptically active compound through extract dilution. In vitro assays showed that 3-methylthiopropionaldehyde is a substrate for several *Saccharomyces cerevisiae* reducing enzymes. Screening of various strains led us to conclude that the higher the 3-methylbutanal reductase activity, the higher the 3-methylthiopropionaldehyde reductase activity. Monitoring of 3-methylthiopropionaldehyde concentration through an in vivo cold contact process has also been undertaken.

Keywords: *Flavor; alcohol-free beer; reductase activity; worty aroma; methional*

INTRODUCTION

Alcohol-free beers are usually characterized by worty off-flavors and the lack of the pleasant fruity or ester aroma found in regular beers. Such defects may stem from a fermentation procedure that fails to reduce the chemical compounds responsible for the worty flavor and to produce fusel alcohols and esters.

Several carbonyl compounds are proposed to contribute to the worty off-flavor. According to Beal and Mottram (1994), 3-methylbutanal and 2-methylbutanal may be key contributors. When smelling a malt extract at the chromatographic sniffing port, assessors describe 3-methylbutanal as malty, chocolate-like, and almond-like while 2-methylbutanal is described as malty, cheesy, or estery apple.

Isobutanal, 3-methylbutanal, and 2-methylbutanal are efficiently removed under the fermentation conditions used for alcohol-free beer production (Collin et al., 1991; Perpète et al., 1994; Laurent et al., 1995). 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 flavorful compounds. Among these enzymes, alcohol dehydrogenase (Collin et al., 1991), aldehyde dehydrogenase, and aldoketoreductases (Laurent et al., 1995; Van Nedervelde et al., 1997) use either NADH or NADPH as cofactor. Perpète et al. (1997) have demonstrated high heterogeneity of reductase activity between yeast strains. More recently, a novel NADPH-dependent branched chain alcohol dehydrogenase was found under anaerobic conditions (Van Iersel et al., 1997).

In alcohol-free beers, levels of 3- and 2-methylbutanal (from 5 to 60 ppb; Perpète et al., 1994) are always far below the threshold values (600 ppb and more than 1 ppm, respectively; Meilgaard, 1975). Even when 2- and 3-methylbutanal are added at 2 ppm to lager beers, the worty flavor is not really detected, suggesting that other compounds contribute to the worty aroma. The aim of this paper is to identify such compounds by aroma extract dilution analysis (Grosch, 1994). The chosen extraction procedure led to highly representative extracts for wort and alcohol-free beer samples. Yeast reduction of the molecule identified as responsible for the worty flavor was measured both in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Chemicals. *Flavors*. 3-Methylbutanal (98%), 2-methylpentanal (98%), and 3-methylthiopropionadehyde (98%) were purchased from Aldrich Chemicals (Belgium). Dichloromethane (99.9%) from Romil (Belgium) was distilled twice before use.

Biochemicals. All biochemical products were purchased from Sigma (Belgium).

Wort and Beer Samples. A standard mashing of 100% pale type malt was carried out to obtain wort samples (Analytica EBC, F59, 1987). Alcohol-free beers (cold contact process; Schur, 1983) and regular beers were purchased from brewers.

Strains. All bottom and top fermentation *Saccharomyces cerevisiae* strains were provided by the BRAS collection of the Université Catholique de Louvain, Louvain-la-Neuve, Belgium. *Saccharomyces bayanus*, *Saccharomycodes ludwigii*, and *Candida boidinii* strains were provided by the MUCL collection of the Université Catholique de Louvain, Louvain-la-Neuve, Belgium. ADH0 *S. cerevisiae* (strain MC65-2A: MATα, *adh1*Δ, *adh2*, *adh3*, *adh4*::URA3, *trp1-289*) was obtained from Institut für Mikrobiologie, Universität Düsseldorf (Prof. M. Ciriacy).

Extraction of Aromatic Compounds from Wort, Alcohol-Free Beer, and Lager Beer. To remove either sugars or high ethanol amounts, the first liquid/liquid extraction was operated on 25 g of sample with 50 mL of dichloromethane. A good separation between the organic and aqueous phase was achieved by centrifugation in hermetic tubes (Sorvall RC2-B centrifuge, GSA rotor, 3000*g*, 10 min). A modified Likens−Nickerson extraction procedure (Bouseta and Collin, 1995) was then applied to the organic phase and followed by concentration to 0.5 mL in a Danish-Kuderna vessel.

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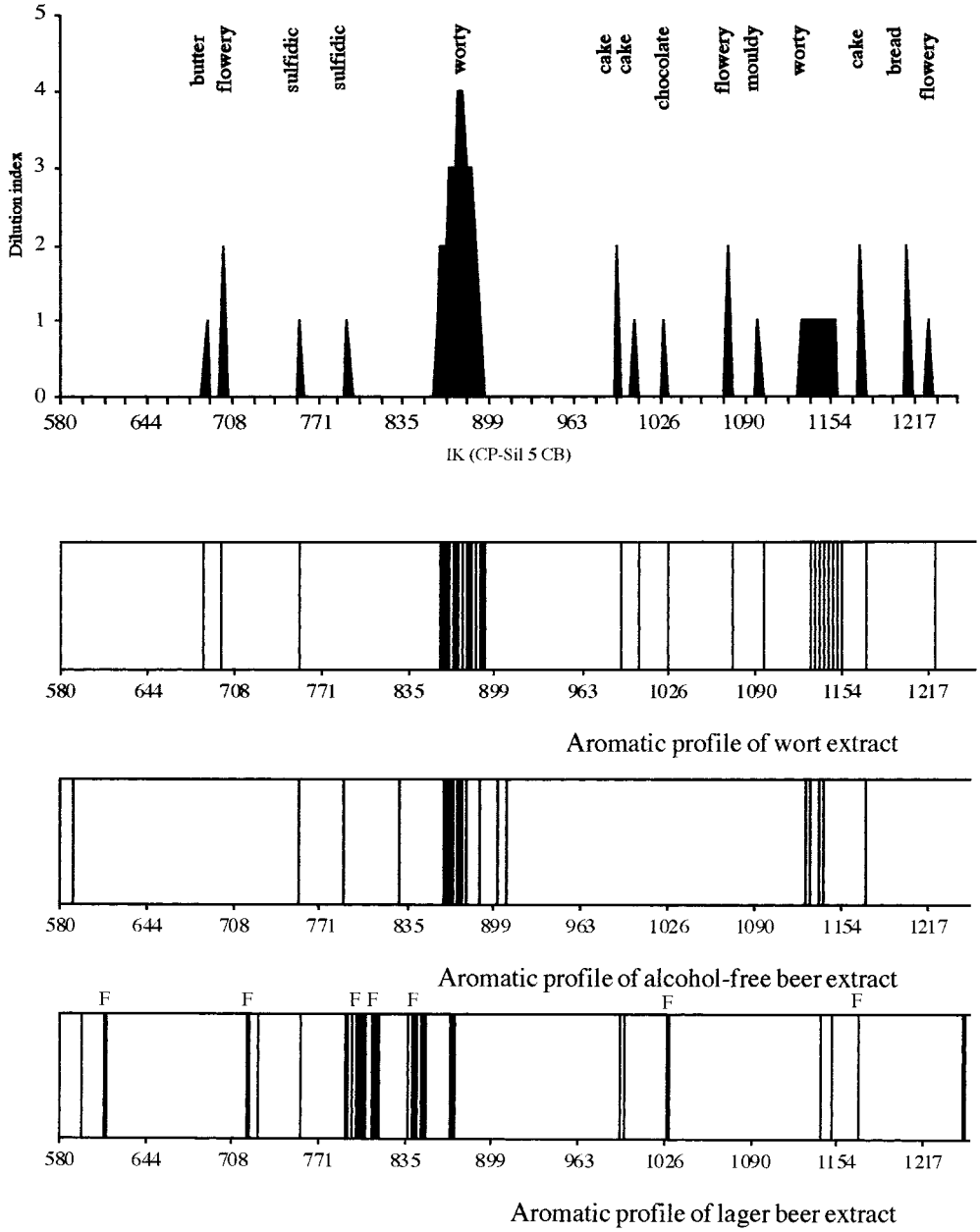

Figure 1. Aromatic profile of wort extract. Comparison of aromatic profile of wort, alcohol-free beer, and lager beer extract.

GC/Olfactometry and Aroma Extract Dilution Analysis. A Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250 °C and closed after 0.5 min was used. Analysis of the extract was carried out on a 50 m × 0.32 mm, wall-coated open tubular (WCOT) apolar CP-Sil5 CB capillary column (film thickness, 1.2 μm). The oven temperature, initially kept at 36 °C, was programmed to rise to 50 °C at 20 °C/min and kept at 50 °C for 10 min and thereafter increase from 50 to 200 °C at 5 °C/min and from 200 to 250 °C at 1 °C/min, remaining at the maximum temperature for 15 min. Helium carrier gas was used at a flow rate of 1 mL/min. A T-junction was used at the end of the capillary column so that one-half of the effluent was sent to a FID detector maintained at 250 °C and connected to a Shimadzu CR3A integrator while the other part went to the GC/odor-port kept at 250 °C. In that case, the eluent was diluted by a large air volume (20 mL/min) previously humidified in a water/$CuSO_4$ half-filled gallon jug. Aroma extraction dilution analysis was performed as described by Grosch (1994), 3 times (25 min each) by four assessors. No significant difference was detected between them.

Isolation of *S. cerevisiae* Acellular Extracts. A 20 g amount of fresh cells collected from a synthetic medium were crushed in a 50 mM HEPES buffer pH 7.5 containing 5 mM dithiotreitol and 10 mM PMSF using a MSK Braun shaker. After removing cell debris by centrifugation (Beckman L7-65 Ultracentrifuge, rotor A641, 100000*g*, 60 min), the supernatant was purified in a two-step ammonium sulfate precipitation (40−80%) and desalted 3 times for 45 min with the 30 kDa cutoff Centriprep (Amicon Inc., Beverly, MA). The so-obtained extracts contained more than 95% of the total reductase activity.

Assay Method. Aldehyde reductase activity was spectrophotometrically assayed at 25 °C by following the absorbance decrease at 340 nm of NADPH. The reaction mixture (1.5 mL) contained 0.1 M sodium phosphate buffer, pH 7.5, 100 μM NADPH, 100 μM substrate, and the acellular extract. The protein concentration was determined by the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

Gel Electrophoresis in Native Conditions. Polyacrylamide-gel electrophoresis was carried out in a 7.5−10% (w/v) gradient polyacrylamide gel at pH 8.5 (Van Iersel et al., 1997). Gels were stained for aldehyde reductase activity by incubation in the dark for 30 min at room temperature in 45 mL of 0.1 M sodium phosphate buffer, pH 8.3 containing 30 mg of $NADP^+$,

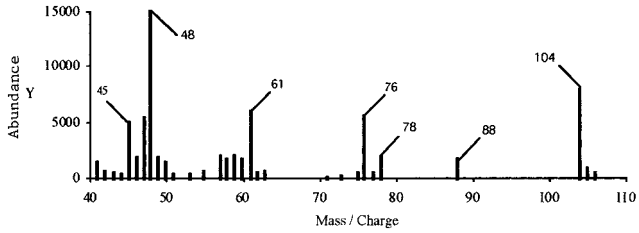

Figure 2. Mass spectrum of 3-methylthiopropionaldehyde.

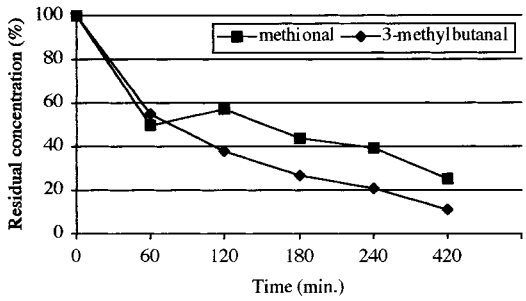

Figure 3. Evolution of aldehyde concentrations during a cold contact process. Initial concentrations (100%) are 405 and 501 ppb for 3-methylbutanal and 3-methylthiopropionaldehyde, respectively.

60 μL of 3-methylbutanol or 3-methylthiopropanol, 25 mg of Nitro Blue Tetrazolium, and 2.5 mg phenazine methosulfate.

Aldehyde Derivatization and GC/ECD Quantification. Aldehyde derivatization was performed with PFBOA *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine as described by Ojala et al. (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 × 0.32 mm, wall-coated open tubular (WCOT) FFAP capillary column (film thickness, 0.3 μm) was used. The 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 and thereafter increase 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 makeup gases were 15 and 35 mL/min nitrogen, respectively.

RESULTS AND DISCUSSION

Key Odorants in Wort and Alcohol-Free Beer. An aroma extract dilution analysis was undertaken on a representative dichloromethane extract derived from an alcohol-free beer (cold contact process). Each flavor descriptor indicated in the aromatic profile (Figure 1) comes from a consensus vocabulary. For all four assessors, the most persistent flavor was detected at IK-(CPSil-5 CB) = 862 and described as "worty", "cake-like", and "potato-like". Mass spectrometry revealed the presence of 3-methylthiopropionaldehyde (methional) at this retention time (Figure 2). Assessors also detected a less-persistent wort-like aroma at IK(CPSil-5 CB) = 1150 which has not been identified.

3-Methylthiopropionaldehyde is a Strecker aldehyde derived from methionine. Therefore, its synthesis could occur during malt kilning, wort mashing, wort boiling, or hot wort stand. By comparison with 3- or 2- methylbutanal, moreover, less of it might be lost from the kettle, as a result of its higher boiling point. Although easily synthesized by heating methionine with glucose or maltose, 3-methylthiopropionaldehyde can also be quickly degraded to other potent odorants such as methanethiol (Ballance, 1961; Yu and Ho, 1995). Very different flavor threshold values have been published in the literature for 3-methylthiopropionaldehyde according to the matrix (0.05 ppb in sunflower oil, 1.7 ppb in water, Grosch, 1994; 250 ppb in regular beer, Meilgaard, 1975). This sulfur compound has already been identified as a key flavor compound in many foods such as corn tortillas (Buttery and Ling, 1995), boiled trout (Milo and Grosch, 1993), cheese (Christensen and Reineccius, 1995; Milo and Reineccius, 1997), and self-prepared yeast extracts (Münch et al., 1997). In all cases, it is particularly persistent in aroma extract dilution analyses and described as cooked or boiled potatoes. Hop varieties such as Bullion or Northern Brewer can also contain amounts of 3-methylthiopropionaldehyde, as indicated by Anderson and Howard (1974).

Figure 1 shows the aromatic profile of an undiluted wort extract compared to extracts obtained from an alcohol-free beer and a lager beer. The profiles of wort and alcohol-free beer appear very similar, particularly in the 3-methylthiopropionaldehyde region. On the other hand, expectedly, regular beer is characterized by other compounds defined as fruity, flowery, or sulfurous.

Evolution of 3-Methylthiopropionaldehyde Concentration during Fermentation. A 12 °Plato wort enhanced with 300 ppb of 3-methylthiopropionaldehyde

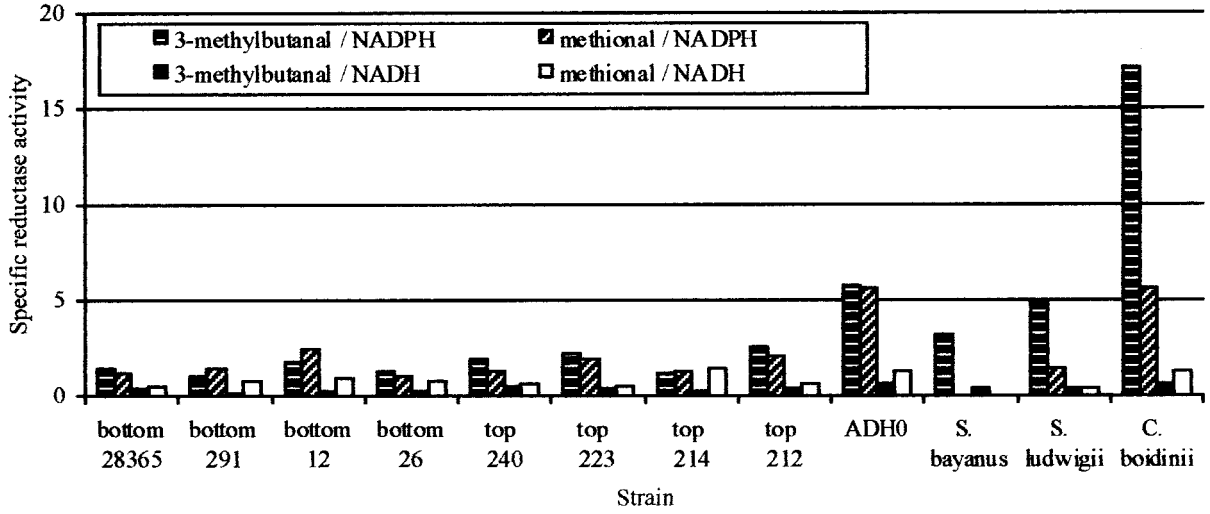

Figure 4. Kinetic activities of yeast strain extracts. Specific activities are expressed as 10^{-5} U/mg proteins (U = μmol of oxidized cofactor per hour at 25 °C).

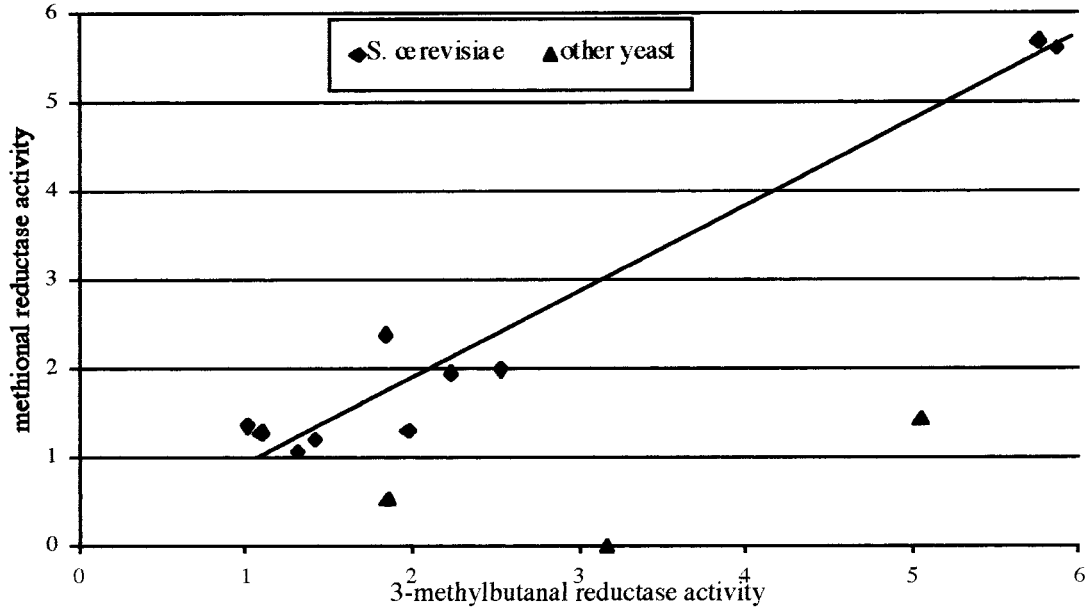

Figure 5. Correlation between 3-methylthiopropionaldehyde and 3-methybutanal reductase activities in the presence of NADPH. Regression factor is 0.96. Specific activities are expressed as 10^{-5} U/mg (U = μmol of oxidized cofactor per hour at 25 °C).

and 3-methylbutanal was pitched with a *S. cerevisiae* strain (10×10^6 cells/mL). Fermentation was carried out under cold contact conditions at 0−1 °C. As dynamic headspace revealed not to be efficient for 3-methylthiopropionaldehyde analysis, PFBOA derivatization was used in that case to quantify carbonyl compounds during the first hours of fermentation. Both 3-methylthiopropionaldehyde and 3-methybutanal were extensively removed from the wort, as shown in Figure 3, leading to 45 and 127 ppb of 3-methylbutanal and 3-methylthiopropionaldehyde, respectively, after 5 h.

However, since flavor thresholds can be very similar in water and alcohol-free beers, we may assume that the residual concentration is sufficient to impart the worty off-flavor of methional.

3-Methylthiopropionaldehyde as Yeast Substrate. It is generally accepted that yeast enzymes catalyze the reduction of worty off-flavor aldehydes to more neutral alcohols (Peppard and Halsey, 1981; Collin et al., 1991; Laurent et al., 1995). Figure 4 shows the reducing activity of brewer's yeast strains measured with either NADH or NADPH as the cofactor. From this chart, it appears that activities against 3-methylbutanal and 3-methylthiopropionaldehyde correlate in all *S. cerevisiae* strains (regression factor of 0.96 for NADPH-related reducing activities, as shown in Figure 5).

However, this correlation applies only to *S. cerevisiae* strains (e.g., *S. bayanus* displays no 3-methylthiopropionaldehyde reductase activity at all). Our results suggest that 3-methylthiopropionaldehyde and 3-methylbutanal are probably substrates of the same enzyme in *Saccharomyces cerevisiae*. This hypothesis is strengthened by the results of gel electrophoresis under native conditions (Figure 6). The band pattern was the same in all *S. cerevisiae* strains for both substrates, while *S. bayanus* showed no activity band when 3-methylthiopropionaldehyde was used. Multiplicity and heterogeneity between yeast strains can also be pointed out, as previously suggested by Van Nedervelde et al. (1997) and Perpète et al. (1997).

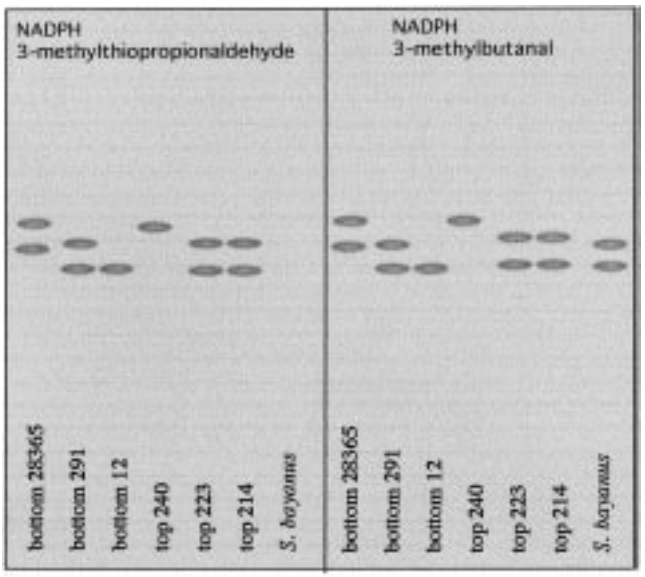

Figure 6. Polyacrylamide-gel electrophoresis of yeast acellular extracts in native conditions, using a specific staining for reductase activities. Each band of the gel shows a NADPH-dependent reductase activity.

CONLUSIONS

3-Methylthiopropionaldehyde emerges as the key compound responsible for the worty off-flavor in alcohol-free beers. The difficulty of extracting this compound by the usual headspace technique may explain why previous works did not evidence it. From the kinetic data and the results of gel electrophoresis under native conditions, it appears that reduction of 3-methylthiopropionaldehyde and 3-methylbutanal may be catalyzed by the same enzymes. In vivo experiments confirm that more than 75% of both aldehydes is reduced after 5 h of cold contact fermentation. To improve the organoleptic properties of alcohol-free beers, it now seems essential to assess the occurrence of synergetic interactions with 3- or 2-methylbutanal and with sulfur-containing degradation products issued from methional. Retention of such compounds by ethanol could also

explain organoleptic differences between alcohol-free and regular beers.

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