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Yeast *ADHI* Disruption: A Way to Promote Carbonyl Compounds Reduction in Alcohol-Free Beer Production

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ABSTRACT

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Alcohol-free beers are usually characterized by worty off-flavors and the lack of the pleasant fruity or ester aroma found in regular beers. Enhancing yeast reduction of 3-methylthiopropionaldehyde, 3-methylbutanal, and 2-methylbutanal appears to be a good means of improving the organoleptic quality of alcohol-free beers. Upon screening of various *Saccharomyces cerevisiae* yeasts for in vitro reductase activity, a haploid *adh0* strain emerged as the most efficient yeast for nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-dependent Strecker aldehyde reduction. Tetrad analysis of diploids (*adh0* × 15D wild-type) demonstrated the predominant role of *adh1* mutation to enhance aldehyde reductase. Consequent to such alcohol dehydrogenase gene disruption, acetaldehyde accumulates, although partly oxidized into acetic acid by the NADP cofactor. We propose that regeneration of this cofactor in *adh1* strains can be improved by promoting NADPH-dependent aldehyde reductase activity.

Keywords: Alcohol dehydrogenase, Aldehyde reductase, *Saccharomyces cerevisiae,* Worty taste

RESUMEN

Normalmente, las cervezas sin alcohol se caracterizan por presentar sabores extraños que le confieren un carácter similar al del mosto y por la ausencia del agradable aroma a ésteres o frutal que se encuentra en las cervezas normales. Aumentar la reducción por parte de la levadura del 3-metil-tiopropionaldehido, 3-metilbutanal y 2-metilbutanal parece una forma adecuada de mejorar la calidad organoléptica de las cervezas sin alcohol. Tras examinar varias cepas de *Saccharomyces cerevisiae* en cuanto a la actividad reductasa que presentaban *in vitro*, una cepa haploide *adh0* destacó como la levadura mas eficiente para llevar a cabo la reducción de Strecker de aldehidos dependiente de NADPH. El análisis de las tétradas procedentes del cruce (*adh0* x 15D cepa salvaje) demostró el papel predominante de la mutación *adh1* en el aumento de la actividad Aldehido Reductasa. Como consecuencia de la mencionada disrupción del gen de la Alcohol Deshidrogenasa, se produce una acumulación de acetaldehido aunque éste se ve parcialmente oxidado a ácido acético por el cofactor NADP. Proponemos que la regeneración de este cofactor en las cepas *adh1* se puede mejorar fomentando la actividad Aldehido Reductasa dependiente de NADPH.

Several carbonyl compounds are proposed to contribute to the worty taste of alcohol-free beers. Among them, 3-methylbutanal is usually described as malty, chocolate-like, or almond-like and 2-methylbutanal contributes to a malty, cheesy, or estery apple aroma (3). More recently, 3-methylthiopropionaldehyde emerged through aroma extract dilution analysis (AEDA) as a key contributor to the worty off-flavor of alcohol-free beer (12).

To minimize this worty taste defect, yeast can be used as for regular beer with the subsequent removal of ethanol by physical processes (2). However, this requires extra costs and extra processing time; therefore, the cold contact process is often preferred (16). Fermentation at 0°C allows significant reduction of many carbonyls without increasing the ethanol level (13). Unfortunately, decreased nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-dependent reductase activity under these conditions leads to residual levels of 3-methylthiopropionaldehyde above this compound's threshold value of 1.7 ppb (8).

A new approach could be to use modified yeast that does not produce alcohol even at higher temperature. In *Saccharomyces cerevisiae,* four alcohol dehydrogenase (ADH) isozymes are known. The constitutive ADHI enzyme is recognized as the main catalyst of acetaldehyde reduction (9). The ADHII enzyme (5) and the mitochondrial ADHIII enzyme (14) convert ethanol to acetaldehyde. *ADHIV* is a cryptic gene giving rise to very low ADH activity (11).

Drewke et al (7) have constructed *adh0* laboratory strains and Debourg et al (6) have confirmed radical depression of all nicotinamide adenine dinucleotide (reduced form) (NADH)-dependent reductase activities in fermentations conducted with such strains. Yet Perpète and Collin (12) have shown that NADPH-dependent reductase activity is enhanced, allowing lower residual levels of compounds liable to produce the worty off-flavor.

This article is devoted to clarifying possible relationships between NADH- and NADPH-dependent reductase activities in yeast. An *adh1* mutant was selected from tetrads issued from (15D × *adh0*) diploids. Yeast selection was performed with allyl alcohol, oxidized to toxic acrolein in the presence of ADH activity (15).

EXPERIMENTAL

Reagents

Biochemicals were purchased from Sigma-Aldrich (St. Louis). All chemicals were from Merck (Darmstadt, Germany) except for aldehydes from Janssen Chimica (Geel, Belgium).

Yeast Strains

Saccharomyces cerevisiae strains 15D (*Mat-a*, *leu2-3*, *ura3*) and BRAS223 were from the BRAS collection of the Université Catholique de Louvain, Louvain-la-Neuve, Belgium. The *adh0* mutant MC65-2A (*Mat-α*, *Δadh1*, *adh2*, *adh3*, *adh4::URA3*, *trp1-289*) and the ADHI-only mutant MC76-T10 (*Mat-α*, *adh2*, *adh3*, *adh4::URA3*, *trp1-289*) were obtained from the Institut für Mikrobiologie, Universität Düsseldorf (M. Ciriacy).

TABLE I
Allyl Alcohol-Resistance of Strains[a]

	Allyl Alcohol Content			
Strain	**0 m*M***	**0.1 m*M***	**1 m*M***	**5 m*M***
adh0	0.48	0.5	0.48	0.26
ADHI-only	+	+	+	–
15D	1.31	1.35	0.01	0.00
Diploid 2	1.32	1.32	0.05	0.00
Diploid 7	1.32	1.34	0.01	0.00
Diploid 8	1.34	1.37	0.02	0.00

[a] Cell population is expressed as optical density at 610 nm after 24 hr of fermentation.

[1] Corresponding author.

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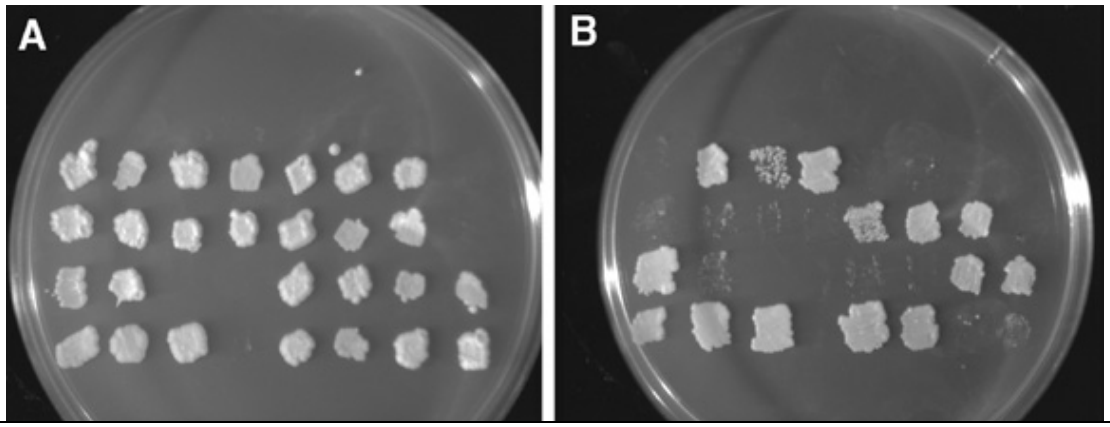

Control							
1	2	3	4	5	6	7	8
11	21	31	41	51	61	71	*
12	22	32	42	52	62	72	*
13	23	*	*	53	63	73	83
14	24	34	*	54	64	74	84

+ 5 mM allyl alcohol							
1	2	3	4	5	6	7	8
-	+	+	+	-	-	-	*
-	-	-	-	+	+	+	*
+	-	*	*	-	-	+	+
+	+	+	*	+	+	-	-

Fig. 1. Allyl alcohol resistance in cells derived from eight tetrads issued from diploid number 8. Position matrix is shown below the picture. * indicates non-viable cells.

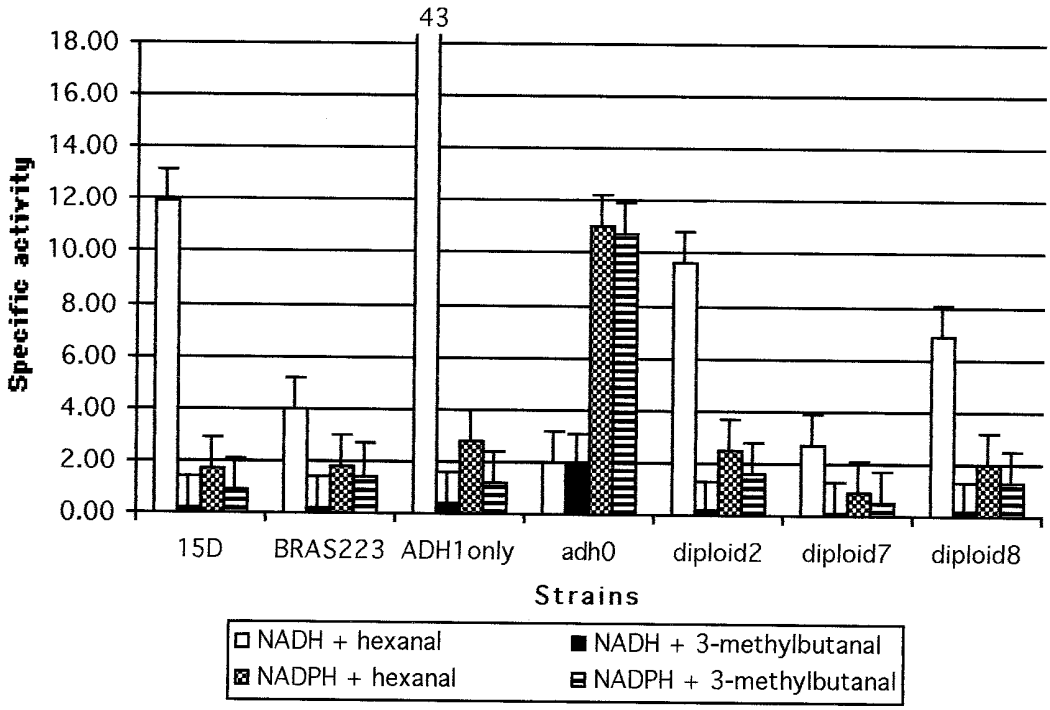

Fig. 2. Aldehyde reductase activities of 15D, BRAS223, *adh0* , ADHI-only, and diploid strain extracts. Specific activities are expressed as $\times 10^{-5}$ U/mg proteins (U = µmol of oxidized cofactor per hour at 25°C).

Media

For fermentations, yeasts were grown in yeast-extract-peptone-sucrose medium (YPS; 1% yeast extract, 0.5% peptone, 10% sucrose). Diploid selection was carried out on minimal synthetic medium (yeast nitrogen base [YNB] without tryptophan and uracil; 0.67% yeast nitrogen base without amino acid, 10% glucose supplemented with 0.5% casamino acid, 0.5% adenine, 1% histidine, 1% lysine). Potassium acetate (1%) plates were used for diploid strain sporulation. Yeast-extract-peptone-dextrose-sorbitol medium (1% yeast extract, 2% bactopeptone, 2% glucose, 18.2%

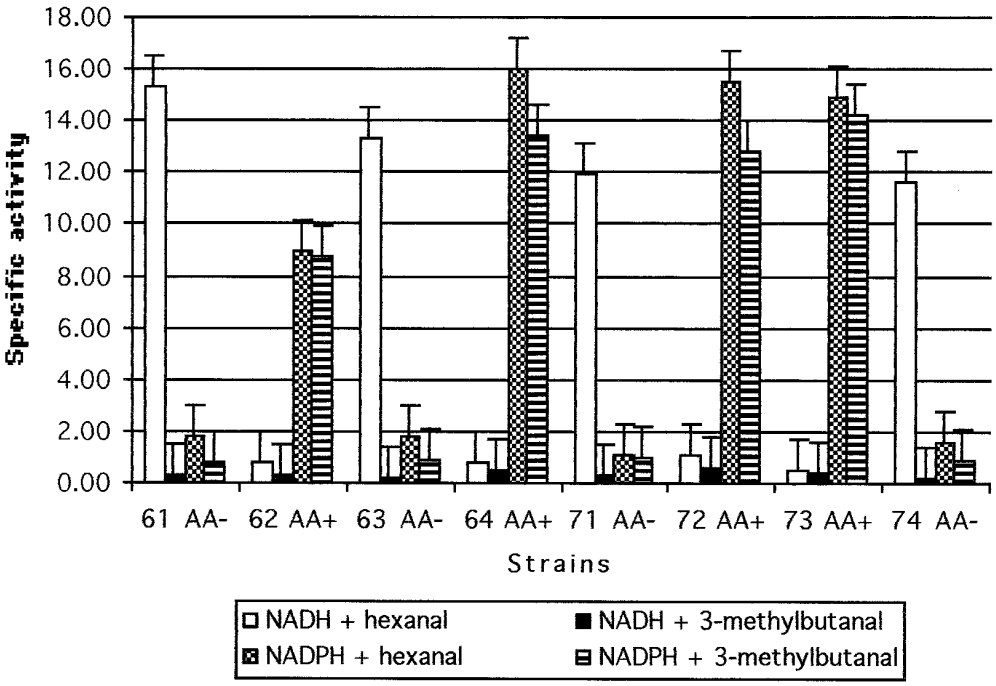

Fig. 3. Aldehyde reductase activities of colonies from tetrads 6 and 7. Specific activities are expressed as $\times 10^{-5}$ U/mg proteins (U = µmol of oxidized cofactor per hour at 25°C). AA indicates allyl alcohol resistance.

sorbitol) was used as dissection medium. For resistance tests, autoclaved YPS (20 min at 121°C) was supplemented with 0.1, 1, or 5 m*M* allyl alcohol.

Yeast Genetics

Yeast crosses and tetrad analyses were done according to standard methods (17). Tetrads were isolated with a micromanipulator (De Fonbrune, France).

Aldehyde Reductase Assay

Cell-free extracts were obtained according to Perpète and Collin (12). Aldehyde reductase activity was assayed spectrophotometrically at 25°C by monitoring the absorbance decrease at 340 nm due to NADPH or NADH oxidation (12).

Quantification of Acetaldehyde, Ethanol, and Acetate

Acetaldehyde and ethanol were monitored in the fermentation supernatant by static headspace gas chromatography as described by Alvarez et al (1). Acetate was quantified by using enzymatic test kits (Boeringher Mannheim, Germany) (4).

RESULTS AND DISCUSSION

Selection of *adh1* Mutants with Allyl Alcohol

An allyl alcohol concentration of 1 m*M* inhibited the growth of the wild-type strain 15D, allowing an efficient selection of our mutants (Table I). Indeed, the *adh0* mutant grew in YPS containing 5 m*M* allyl alcohol, indicating the predominant role of ADHI in acrolein excretion. The combined *adh2*, *adh3*, and *adh4* mutations of ADHI-only cells made them resistant to allyl alcohol at concentrations up to 1 m*M*; this resistance probably reflects the absence of ADHIII activity.

All of our diploids (15D × *adh0*) proved very sensitive to 1 m*M* allyl alcohol. However, when diploid 8 was used to produce tetrads (five correctly developed tetrads in control medium; Fig. 1), 50% of the segregants proved resistant to 5 m*M* allyl alcohol.

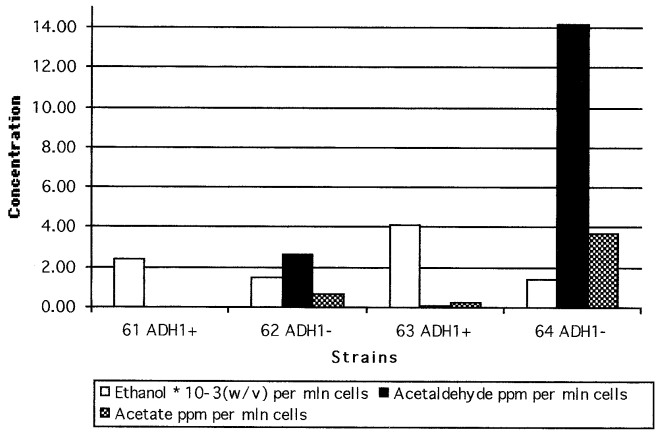

Fig. 4. By-products analysis of spores issued from tetrad 6.

These data confirm that the absence of a single gene (*ADHI*) is responsible for this resistance to allyl alcohol in the highly resistant segregants.

Enhancement of NADPH-Dependent Aldehyde Reductase Activity in *adh1* Mutants

By comparison with 15D, BRAS223, or the ADHI-only mutant, the *adh0* mutant predictably showed a significantly lower NADH-dependent hexanal reduction rate, but also a strongly enhanced NADPH-dependent aldehyde reduction rate. Our (15D × *adh0*) diploids displayed high NADH-dependent aldehyde reductase activity (Fig. 2). Aldehyde reductase activity was also measured in extracts of segregants derived from tetrads 6 and 7. All segregants resistant to allyl alcohol at high concentration (strains 62, 64, 72, and 73) could reduce aldehydes very efficiently in the presence of NADPH (Fig. 3). From our experiments, the *adh1* mutation thus emerges as responsible for improved reduction of

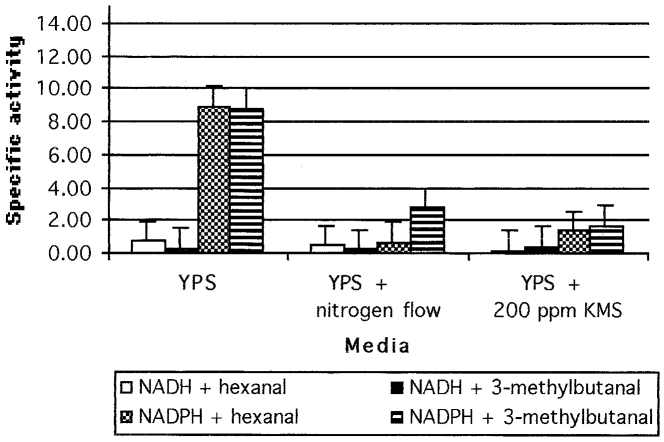

Fig. 5. Effect of nitrogen flow and potassium metabisulfite on aldehyde reductase activity in the *adh1* strain 62. Specific activities are expressed as $\times 10^{-5}$ U/mg proteins (U = µmol of oxidized cofactor per hour at 25°C).

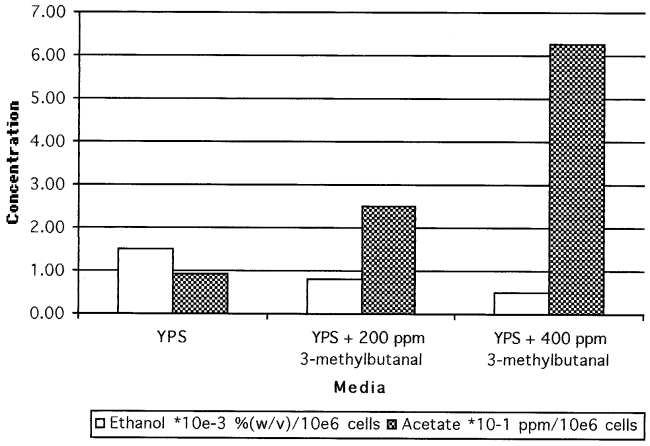

Fig. 6. Effect of 3-methybutanal on by-products levels of *adh1* strain 64.

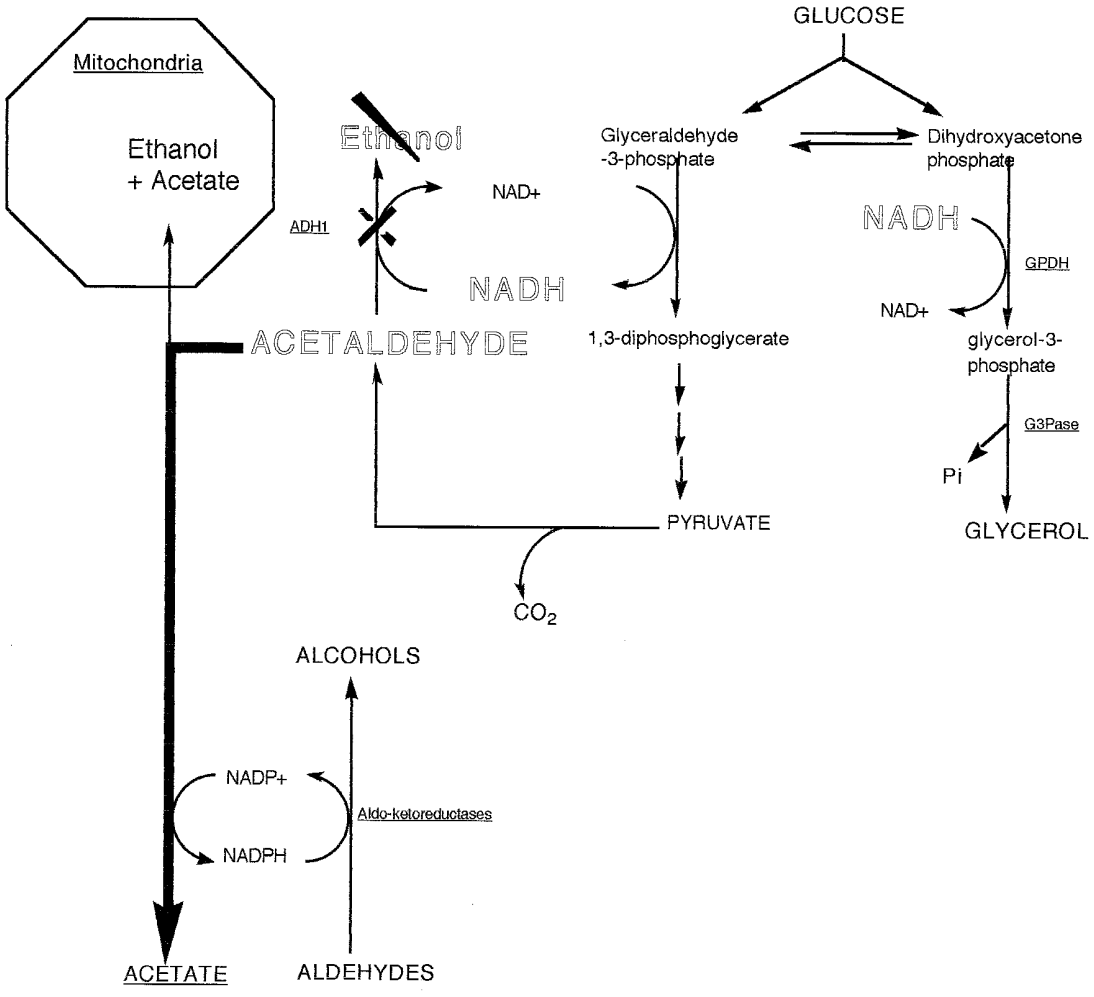

Fig. 7. Model proposed for nicotinamide adenine dinucleotide phosphate (reduced form) cofactor regeneration.

branched aldehydes. In experiments where $ADHI^+$ and $ADHI^-$ strains were compared, NADPH-3-methylbutanal reductase activity was the most markedly enhanced (the multiplying factors were 13.61, 8.77, 2.04, and 0.05 for NADPH-3-methylbutanal, NADPH-hexanal, NADH-3-methylbutanal, and NADH-hexanal reduction, respectively).

Acetaldehyde Level Modulates the Increase of NADPH-Dependent Aldehyde Reduction

Several studies have shown accumulation of acetaldehyde and acetate in cultures of *adh1* mutants (7,20). To confirm this phenotype with our yeasts, we grew strains 61, 62, 63, and 64 and measured by-product levels after 48 hr. Because *adh1* cells grew

slowly (final biomass was 10× lower than that obtained with the wild-type), all by-product concentrations were divided by the number of cells (Fig. 4). All *adh1* strains (62 and 64) displayed the same phenotype (i.e., a low ethanol level and higher acetaldehyde and acetate concentrations). Cells lacking ADHI predictably accumulated acetaldehyde, partly oxidized to acetate. Several aldehyde dehydrogenases (ALDH) may catalyze acetaldehyde oxidation in vivo, the most important being ALDHI. ALDHI is essentially NADP-dependent and located in the cytosol, whereas ALDHII can use either NADP or NAD and appears to be a mitochondrial enzyme (19). In the *adh1* mutant, the surplus acetaldehyde probably stimulates ALDHI activity, leading to NADPH accumulation. To maintain a stable redox balance, the cells might promote some NADPH-dependent activities such as worty aldehydes reduction.

To check these relationships, we added 200 ppm of potassium metabisulfite (KMS) (10) or a 20 ml/min nitrogen flow to cultures of the *adh1* mutant strain 62 in order to decrease the acetaldehyde level. We then assessed the influence of the additives after 24 hr of culture in YPS at 28°C. In the presence of KMS or nitrogen, the NADPH-dependent aldehyde reduction rate was reduced (Fig. 5). Similar results were obtained for strain 64. In all cases, the ethanol, acetaldehyde, and acetate levels were significantly decreased (e.g., 1.03×10^{-3}%, 56.22 ppm/10^6 cells, and 16.77 ppm/10^6 cells compared to 2.45×10^{-3}%, 86.95 ppm/10^6 cells, and 34.17 ppm/10^6 cells for ethanol, acetaldehyde, and acetate, respectively, for strain 64 with or without KMS). The lower ethanol level is explainable by reduced acetaldehyde dismutation (18) in the presence of KMS or nitrogen.

Addition of 3-methylbutanal Enhances Acetate Production in *adh1* Cells

To promote cofactor regeneration, we added 200 or 400 ppm of 3-methylbutanal to a culture of strain 64. Ethanol and acetate were measured after 24 hr. The addition of 3-methylbutanal caused increased acetate production, whereas only a residual level of ethanol was measured (Fig. 6). High concentrations of 3-methylbutanal probably stimulate cofactor regeneration through NADPH oxidation, thus increasing oxidation with respect to dismutation of acetaldehyde (Fig. 7).

CONCLUSION

An *adh1* mutation induces a double phenotype: low ethanol production and enhanced worty aldehyde reduction. To regenerate the NADP cofactor, such mutants seem to enhance NADPH-dependent aldehyde reduction. The addition of branched aldehydes could improve the cofactor regeneration while nitrogen flow or KMS decrease the acetate level. At the moment, however, a genetic aberration cannot be excluded to explain this apparent metabolic shift.

The results of tetrad analysis of diploids (*adh0* × 15D wild-type) demonstrate the predominant role of the *adh1* mutation in enhancing aldehyde reductase activity. As a result of disruption of this alcohol dehydrogenase gene, acetaldehyde accumulates, although probably partly oxidized to acetic acid by the NADP cofactor.

The next step in this work should be to construct a brewer's yeast lacking ADHI activity. Brewer's yeasts are usually polyploid; therefore, it will be necessary to integrate several plasmids, one at each *ADHI* locus.

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