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Parameters Affecting Ethyl Ester Production by *Saccharomyces cerevisiae* during Fermentation[▽]

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Volatile esters are responsible for the fruity character of fermented beverages and thus constitute a vital group of aromatic compounds in beer and wine. Many fermentation parameters are known to affect volatile ester production. In order to obtain insight into the production of ethyl esters during fermentation, we investigated the influence of several fermentation variables. A higher level of unsaturated fatty acids in the fermentation medium resulted in a general decrease in ethyl ester production. On the other hand, a higher fermentation temperature resulted in greater ethyl octanoate and decanoate production, while a higher carbon or nitrogen content of the fermentation medium resulted in only moderate changes in ethyl ester production. Analysis of the expression of the ethyl ester biosynthesis genes *EEB1* and *EHT1* after addition of medium-chain fatty acid precursors suggested that the expression level is not the limiting factor for ethyl ester production, as opposed to acetate ester production. Together with the previous demonstration that provision of medium-chain fatty acids, which are the substrates for ethyl ester formation, to the fermentation medium causes a strong increase in the formation of the corresponding ethyl esters, this result further supports the hypothesis that precursor availability has an important role in ethyl ester production. We concluded that, at least in our fermentation conditions and with our yeast strain, the fatty acid precursor level rather than the activity of the biosynthetic enzymes is the major limiting factor for ethyl ester production. The expression level and activity of the fatty acid biosynthetic enzymes therefore appear to be prime targets for flavor modification by alteration of process parameters or through strain selection.

During fermentation the yeast *Saccharomyces cerevisiae* produces a broad range of aroma-active substances, which are vital for the complex flavor of fermented beverages, such as beer and wine. In particular, volatile esters are of major industrial interest because the presence of these compounds determines the fruity aroma of beer and wine (6, 7, 9, 20, 24, 26, 27, 30, 31, 36). Even small changes in the concentrations of these secondary metabolites can have large effects on the final sensorial quality of fermented beverages. There are two main groups of flavor-active esters in fermented beverages. The first group contains the acetate esters (in which the acid group is acetate and the alcohol group is ethanol or a complex alcohol derived from amino acid metabolism), such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), and phenyl ethyl acetate (roses, honey). The second group is the ethyl esters (in which the alcohol group is ethanol and the acid group is a medium-chain fatty acid [MCFA]) and includes ethyl hexanoate (anise seed, applelike aroma), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral odor). Of these two groups, the acetate esters have received the most attention, not because they are more important but because they are produced at much higher levels and therefore are easier to measure. Also, the genes involved in their synthesis were discovered first. By contrast, much less is known about ethyl ester production, despite the desirable applelike aromas of ethyl esters.

Aroma-active esters are formed intracellularly by fermenting yeast cells. Since they are lipid soluble, ethyl esters can diffuse through the cellular membrane into the fermenting medium. Unlike acetate ester excretion, which is rapid and complete, the transfer of ethyl esters to the fermenting medium decreases drastically with increasing chain length, from 100% for ethyl hexanoate to 54 to 68% for ethyl octanoate and 8 to 17% for ethyl decanoate (28). The rate of ethyl ester formation is dependent on three factors: the concentrations of the two cosubstrates (the acyl coenzyme A [acyl-CoA] component and ethanol) and the activity of the enzymes involved in their synthesis and hydrolysis. Hence, all parameters that influence substrate concentrations or enzyme activity may affect ethyl ester production.

Many variables are known to affect ester production, including the yeast strain used, the composition of the fermentation medium, and the fermentation conditions. The ester profile is highly strain dependent and strongly affects the perception of the flavor of the beer produced by a yeast. On the other hand,

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the regulation of the ester levels by environmental variables shows consistent trends for many strains. This has been reported for the effects of high-gravity brewing (1, 29, 47), stronger oxygenation of the wort (19, 39), and the unsaturated fatty acid (UFA) levels of the wort (19, 38–41) on acetate ester production. Also, from beer brewing practice it is well known that high-gravity brewing increases acetate ester production in industrial fermentations, and this is true for a wide range of brewer's strains (30). This indicates that adaptation of process parameters can be used to modify ester levels in a predictable way for a range of brewer's yeast strains. In contrast to the effects of these variables acetate ester production, their influence on ethyl ester production has never been studied in detail. It has been reported that not only the total ethyl ester production but also the relative proportion of each ethyl ester produced differs from strain to strain (30). For acetate ester production, the concentration of UFAs in the fermentation medium is the best-known regulator. A second important parameter, especially in breweries, is the carbon-to-nitrogen (C/N) ratio in the fermentation medium. For acetate esters, a carbon or nitrogen content higher than that in standard medium is correlated with greater acetate ester production. The influence of UFAs and the C/N ratio on ethyl ester production has not been investigated yet.

Apart from the yeast strain and the chemical composition of the medium, three other process characteristics affect ethyl ester production: aeration of the medium, hydrostatic pressure of the fermentation tank, and fermentation temperature. For both acetate and ethyl esters, aeration of the wort medium dramatically decreases the concentration of these esters. Only ethyl hexanoate is not affected (25). The influence of increasing hydrostatic pressure is attributed to the increase in dissolved carbon dioxide in the fermentation medium (16). However, as it is difficult to perform hydrostatic pressure experiments on a laboratory scale, no detailed studies have been carried out to analyze the influence on ester production. A final important fermentation variable is the temperature. According to Suomaleinen (36), an increase in the fermentation temperature releases higher levels of esters through more efficient excretion and/or enhanced autolysis of the yeast. An effect of the temperature on the thermodynamic equilibrium of ester solubility in cellular lipids and the aqueous medium is another possibly more likely explanation.

MCFAs are the main precursors for ethyl ester biosynthesis. During alcoholic fermentation, short-chain fatty acid and MCFA intermediates are prematurely released from the cytoplasmic fatty acid synthase (FAS) complex (21, 37). Because MCFAs are synthesized in the FAS complex, it is likely that the control mechanisms operating on fatty acid synthesis are also involved in control of MCFA formation. The key enzyme in the regulation of fatty acid biosynthesis is acetyl-CoA carboxylase (35, 45). According to Dufour et al. (8), feedback regulation of this enzyme triggers the release of MCFAs from the FAS complex (8). During fermentation, long-chain saturated fatty acids accumulate and start to inhibit acetyl-CoA carboxylase, which causes the release of unfinished MCFAs from the FAS complex (2, 8). On the other hand, Furukawa et al. (14) claimed that instead of the decreased elongation of fatty acids promoting the release of MCFAs, the increase in expression of fatty acid synthesis genes enhances MCFA formation. These workers showed that overexpression of the *FAS1* and *FAS2* fatty acid synthetic genes triggered more MCFA formation.

A second possible parameter affecting ethyl ester production is the activity of the acyltransferase enzyme. For acetate ester synthesis, the responsible enzymes, biochemical pathways, and regulation are already well defined (8, 22, 42, 43). Only recently, however, our research group discovered the major enzymes responsible for ethyl ester synthesis in yeast (8, 33). Formation of ethyl esters in yeast is catalyzed by two acyl-CoA: ethanol *O*-acyltransferases, Eeb1 and Eht1 (33). Eeb1 is the main enzyme, while Eht1 plays a minor role. Whereas deletion of both *EEB1* and *EHT1* resulted in a severe decrease in ethyl ester production, overexpression of the *EEB1* or *EHT1* gene from laboratory yeast in a laboratory strain did not result in an increase in the production of ethyl esters (33). On the other hand, overexpression of the *EHT1* allele from wine yeast in a wine yeast strain has recently been shown to cause a clear increase in ethyl ester production (17). This apparent discrepancy can be ascribed either to differences between the alleles from the two strain backgrounds or to the different genetic backgrounds of the host strains. Evidence has been reported that industrial wine yeast strains produce esters and other flavor compounds more efficiently than laboratory strains and are therefore more suitable for studies on these processes (17, 33).

In this study we investigated the influence of important fermentation parameters on the production of ethyl esters by an industrial ale brewer's yeast strain. The results clearly highlight the importance of the precursor concentration for the production of ethyl esters, whereas the activity of the ethyl ester biosynthetic enzymes does not appear to be a limiting factor. Since overexpression of fatty acid synthesis genes results in a higher precursor concentration and thus greater ethyl ester production, our work identified a valuable new target for flavor modification by alteration of fermentation process parameters or strain selection.

MATERIALS AND METHODS

Microbial strains and culture conditions. All plasmids and yeast strains used in this study are listed in Table 1. Yeast cultures were routinely pregrown at 30°C in YPD medium (2% [wt/vol] glucose, 2% peptone, 1% yeast extract) (3) in an orbital shaker at 200 rpm. For selection of yeast transformants containing the integrated overexpression construct with *SMR1-410* as a selectable marker (which provides resistance to sulfometuron methyl [46]), minimal synthetic defined medium was used, which contained 0.67% yeast nitrogen base without amino acids and 2% glucose and was supplemented with 60 mg/liter of sulfometuron methyl (E. I. Du Pont de Nemours) (4).

DNA manipulations. Standard procedures for isolation and manipulation of DNA were used (3). Restriction enzymes, T4 DNA ligase, and Expand high-fidelity DNA polymerase were used as recommended by the supplier. Yeast transformation was carried out using the lithium acetate method (15).

Construction of the overexpression strains. Plasmids pEHT1s and pEEB1s were constructed by insertion of the appropriate open reading frames (ORFs) into the XhoI restriction site in the *PGK1* overexpression cassette of the ps vector (Table 1) (the *EHT1* and *EEB1* PCR products were cut with XhoI). The following primers were used for amplification of DNA fragments by PCR: for the *EHT1* ORF, XhoI-*EHT1*-ORF-F (TGCCTCGAGATGTCAGAAGTTTCCAAAGCC) and XhoI-*EHT1*-ORF-R (TTGCCTCGAGTCATACATATTCATCAAAC); and for the *EEB1* ORF, XhoI-*EEB1*-ORF-F (TTGCCTCGAGATGTTTCGCCGTACTATC) and XhoI-*EEB1*-ORF-R (TTGCCTCGAGTTATAAAACTAACTCATCAAAG) (the XhoI restriction sites are underlined). The plasmids were checked for correct integration of *EHT1* and *EEB1* by PCR. Before transformation, all vectors were linearized in the mutated *ILV2* gene; ps (the empty vector), pEHT1s, and pEEB1s were linearized with BlpI. Colonies were analyzed

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
CMBS SS01	Industrial ale yeast	CMBS collection[a]
CMBS SS01/ps	Industrial ale yeast, *SMR1-410 PGK1$_p$-PGK1$_t$*	This study
CMBS SS01/p*EHT1*s	Industrial ale yeast, *SMR1-410 PGK1$_p$-EHT1-PGK1$_t$*	This study
CMBS SS01/p*EEB1*s	Industrial ale yeast, *SMR1-410 PGK1$_p$-EEB1-PGK1$_t$*	This study
Plasmids		
ps (empty vector, Yip)	*bla LEU2 SMR1-410 PGK1$_p$-PGK1$_t$*	18
pEHT1s	*bla LEU2 SMR1-410 PGK1$_p$-EHT1(CMBS SS01)-PGK1$_t$*	This study
pEEB1s	*bla LEU2 SMR1-410 PGK1$_p$-EEB1(CMBS SS01)-PGK1$_t$*	This study

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further by PCR and subsequent restriction analysis to confirm that the *PGK* overexpression constructs were integrated into the genomic *ILV2* gene.

Fermentation experiments. Yeast precultures were shaken overnight at 30°C in test tubes with 5 ml of YPD medium. After 16 h of growth, 1 ml of an overnight culture was used to inoculate 50 ml of filtered standard wort medium (12° Plato) in 250-ml Erlenmeyer flasks, and this second preculture was shaken at 30°C until stationary growth phase (optical density at 600 nm, 10) was reached. Cells were washed with sterile, distilled water and used to inoculate 350 ml of fresh, prewarmed (20°C) wort medium to an optical density at 600 nm of 0.4. Static fermentation was carried out at 20°C in flasks with water locks placed on top in order to create semianaerobic conditions mimicking full-scale fermentations. Samples used for chromatographic analysis were taken when fermentation was completed and were immediately cooled on ice in an airtight container. Standard 12° Plato unhopped wort was prepared with 100% barley malt and filtered over a lauter tun in a pilot-scale brewery. For sterilization, the wort was autoclaved for 10 min at 121°C before use. The pH of the wort was 5.2, and the free amino nitrogen (FAN) content was 160 ppm.

For fermentations with different carbon levels and a fixed nitrogen level, a synthetic medium mimicking industrial wort was used; this medium contained (per 350 ml) 0.595 g yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ and 1.029 g balanced peptone no. 1 (Lab M), which corresponded to 150 mg liter^{-1} FAN, as well as different concentrations of maltose (4, 8, 12, 16, and 20%). For fermentations with different nitrogen levels and a fixed carbon level, another synthetic medium was used; this medium contained 8% maltose, 0.595 g yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ per 350 ml, and 0.343, 0.686, 1.029, 1.37, or 1.716 g balanced peptone per 350 ml (corresponding to 50, 100, 150, 200, and 250 mg liter^{-1} FAN).

Fatty acid analysis. For fermentation with addition of UFAs, the UFA concentration in the wort was analyzed. Fatty acids in the medium were extracted by a modification of the Chen method (5). The lipids of the wort medium were extracted twice with an equal volume of chloroform-methanol (3:1, vol/vol) containing 300 mM HCl to acidify the preparation and 250 ppm heptadecanoic acid as an internal standard. After extraction, 1 g of Na_2SO_4 was added to eliminate all the water from the extract. Filtering the extract over glass wool eliminated the Na_2SO_4. Samples were evaporated to dryness under a nitrogen flow. The lipids in the extract were saponified with 10 ml of an equal-volume mixture of KOH (1 N) and methanol in a 30-ml capped Pyrex tube at 100°C for 30 min. After cooling, the saponified mixtures were acidified with 1 ml of 6 N HCl, which was followed by extraction with *n*-hexane. The extract was evaporated to dryness. The fatty acids were methylated by incubation of the dried extract with 1 ml of boron trifluoride in methanol (14% solution; Sigma Chemical Co., St. Louis, MO) at 100°C for 10 min. After cooling, 6 ml of NaCl-saturated water was added, and the fatty acid methyl esters were extracted with 300 μl of toluene. Gas chromatographic analysis was done with a Varian 3300 instrument (Varian Association, Inc., Walnut Creek, CA) equipped with an Alltech Heliflex AT-225 capillary column (length, 30 m; inside diameter, 0.32 mm; film thickness, 0.25 μm; Alltech Associated, Inc., Deerfield, IL) and a flame ionization detector. The oven temperature was increased from 100 to 200°C at a rate of 10°C per min and then kept at 200°C for 6 min. The injection port was kept at 250°C, and the detector was kept at 230°C. The carrier gas was helium.

Headspace GC-FID analysis. Headspace gas chromatography coupled with flame ionization detection (GC-FID) was used for measurement of acetate esters and ethyl esters in the fermentation products. Samples (5 ml) were collected in 15-ml precooled glass tubes, which were immediately closed and cooled on ice. The samples were then analyzed with a calibrated Autosystem XL gas chromatograph equipped with a headspace sampler (HS40; Perkin-Elmer, Wellesley, MA) and with a CP-Wax 52 CB column (length, 50 m; inside diameter, 0.32 mm; layer thickness, 1.2 μm; Chrompack, Varian, Palo Alto, CA). Samples were heated for 25 min at 60°C in the headspace autosampler. The injection block and flame ionization detector temperatures were kept constant at 180 and 250°C, respectively; helium was used as the carrier gas. The oven temperature was held at 50°C for 5 min, then increased to 200°C at a rate of 5°C per min, and finally held at 200°C for 3 min. The results were analyzed with Perkin-Elmer Turbochrom Navigator software.

Quantitative PCR. The levels of *EHT1* and *EEB1* expression were determined using quantitative PCR. CMBS SS01 yeast cells were grown for 12 h at 30°C in 50 ml of 2% YPD medium to which the appropriate MCFA was added. Extraction of the RNA of pelleted cells was performed with Trizol (Invitrogen), used according to the manufacturer's instructions. For each sample, 1 μg of total RNA was subject to reverse transcription (RT) using an RT system (A3500; Promega, Madison, WI). Concentrations were determined, and samples were diluted to obtain a concentration of 100 ng μl^{-1}. The 25-μl PCR mixture consisted of 12.5 μl Platinum SYBR green quantitative PCR SuperMix-UDG with ROX (Invitrogen) and 1.25 μl of each primer (500 nM). Five microliters of cDNA was added to each reaction mixture. The PCR program consisted of an initial denaturation for 2 min at 95°C and amplification using 50 cycles of 15 s at 95°C and 1 min at 60°C. The actin gene (*ACT1*) was used as the reference gene, because the expression of this gene was found to be relatively stable. The PCR primers were all designed with the PRIMER EXPRESS software (PE Applied Biosystems, Cheshire, United Kingdom) according to the PE Applied Biosystems guidelines. The primer sequences used for RT-PCR analysis (5′ to 3′) are as follows: EHT1-qPCR-FW, TGGCTCTCCCCGATCA; EHT1-qPCR-RV, AGGCGTGA ACATATAGAAAGATGGA; EEB1-qPCR-FW, TCGTACACACTTGGGAC AAGTTG; EEB1-qPCR-RV, CAGTCCTTGTTAGAAATTGTGTTAAAG TTC; ACT1-FW, CGTCTGGATTGGTGGTTCTA; and ACT1-RV, GTGGTG AACGATAGATGGAC. The expression levels were analyzed with ABI Prism (Sigma). The levels of expression of the different genes were all normalized with respect to the levels of *ACT1* expression.

RESULTS

The industrial ale strain CMBS SS01 was used to evaluate the influence of three important industrial parameters on ethyl ester production during alcoholic fermentation in yeast. The industrial factors investigated were (i) the concentration of UFAs in the fermentation medium, (ii) the carbon-to-nitrogen ratio of the fermentation medium, and (iii) the fermentation temperature.

Increasing the level of UFAs in the fermentation medium results in a decrease in ethyl ester production. UFA content is one of the most important parameters affecting acetate ester production, but its influence on ethyl ester formation remains unknown. The lipid (including UFA) content of wort can be adapted relatively easily by the brewer through specific

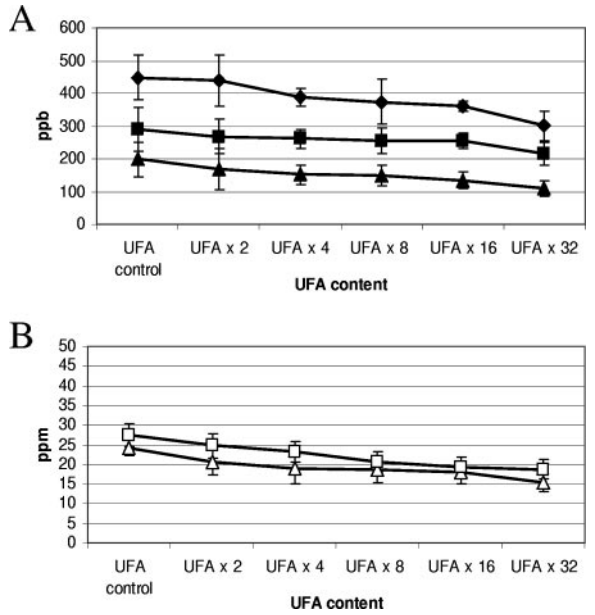

FIG. 1. Ethyl ester production by CMBS SS01 after fermentation of wort with different UFA contents. The UFA content of the standard wort was used as the control, and it was increased 2-, 4-, 8-, 16-, and 32-fold by addition of a pure UFA mixture containing oleic, linoleic, and linolenic acids at a ratio of 1:5.4:1.5. (A) Concentrations of ethyl hexanoate (◆), ethyl octanoate (■), and ethyl decanoate (▲). (B) Concentrations of ethyl acetate (△) and isoamyl acetate (□). The isoamyl acetate concentrations were multiplied by 10.

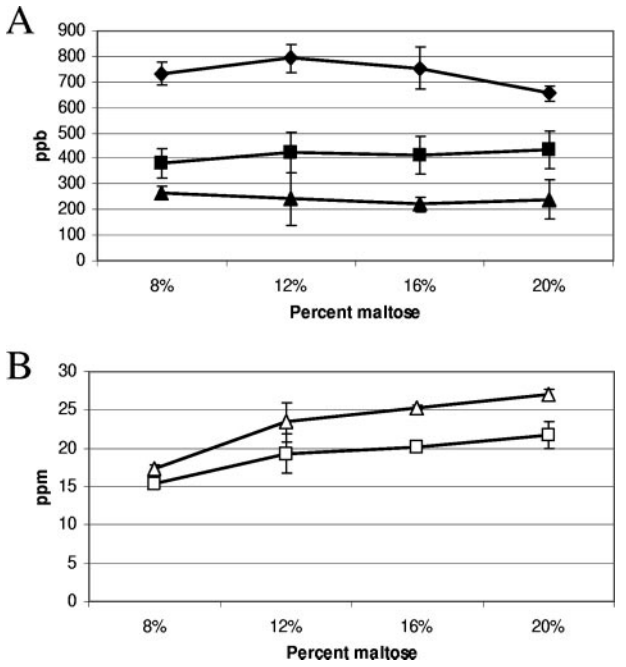

FIG. 2. Ethyl ester production by CMBS SS01 after fermentation of a synthetic medium with different carbon contents. The nitrogen content of the medium was kept constant (150 mg liter^{-1} FAN), while the carbon content was varied from 4 to 20% maltose. (A) Concentrations of ethyl hexanoate (◆), ethyl octanoate (■), and ethyl decanoate (▲). (B) Concentrations of ethyl acetate (△) and isoamyl acetate (□). The isoamyl acetate concentrations were multiplied by 10.

changes in the filtration process. Cloudy worts can be obtained by filtration over a lauter tun, while a membrane filter is capable of producing a clear wort in terms of turbidity while the solid content (in particular, larger molecular substances, like lipids) is retained (34). To investigate the influence of the UFA level in the medium, first the UFA content of standard low-UFA wort, used as the basis in our fermentations, was analyzed. The standard wort was then supplemented with increasing amounts of a pure UFA mixture so that the UFA content was increased 2-, 4-, 8-, 16-, or 32-fold. The UFA mixture contained oleic, linoleic, and linolenic acids at the same ratio as the standard wort (1:5.4:1.5). The concentrations of these UFAs, determined after the wort was autoclaved, were 0.34 mg liter^{-1} oleic acid, 1.82 mg liter^{-1} linoleic acid, and 0.50 mg liter^{-1} linolenic acid (averages of three independent analyses; the standard deviations were typically about 10% and did not exceed 20%). Fermentations were carried out at 20°C in 350 ml of standard wort with water locks placed on top as described in Materials and Methods. When the fermentations were complete, samples were taken for volatile compound determination. Ethyl and acetate ester concentrations were analyzed by headspace GC-FID (Fig. 1). Each fermentation experiment and the subsequent analysis were repeated three times. Biomass production and fermentation efficiency were the same for all the fermentations (results not shown).

Addition of UFAs resulted in a decrease in the production of all ethyl esters analyzed (Fig. 1A). The concentration of ethyl decanoate decreased the most. In wort containing 32 times the UFA content of standard wort, a 50% decrease in the ethyl decanoate concentration was observed. The decreases in the ethyl hexanoate (33%) and ethyl octanoate (25%) concentrations were smaller than the decrease in the ethyl decanoate concentration. However, changes in the ethyl hexanoate concentration of similar magnitude have previously been shown to affect flavor perception (23). To compare the influence of UFAs on ethyl ester production and the influence of UFAs on acetate ester production, the latter was also analyzed. Figure 1B shows that acetate ester production also decreased when more UFAs were present. The decreases in the ethyl acetate (37%) and isoamyl acetate (32%) concentrations were in the same range as the decrease in the ethyl hexanoate concentration. This is consistent with the results of Fujii et al. (10), who showed that UFAs lower acetate ester production by repressing the *ATF1* gene, which is responsible for the major part of acetate ester synthesis (10, 12, 13).

Carbon and nitrogen contents of the fermentation medium affect the production of specific ethyl esters. A second important medium parameter, especially in breweries, is the carbon-to-nitrogen ratio of the fermentation medium. To obtain worts with a set of predetermined C/N ratios, synthetic wort was used. The nitrogen content of wort which can be "consumed" or "assimilated" by the yeast is called FAN, whereas the carbon or total sugar content of the wort is reflected in the specific gravity of the wort. The amount of sugar which can be assimilated by the yeast is called the fermentable sugar.

For the first series of fermentations, the nitrogen content of the medium was kept constant (150 mg liter^{-1} FAN), while the carbon content was varied from 8 to 20% maltose. Figure 2A shows that the ethyl hexanoate concentration decreased

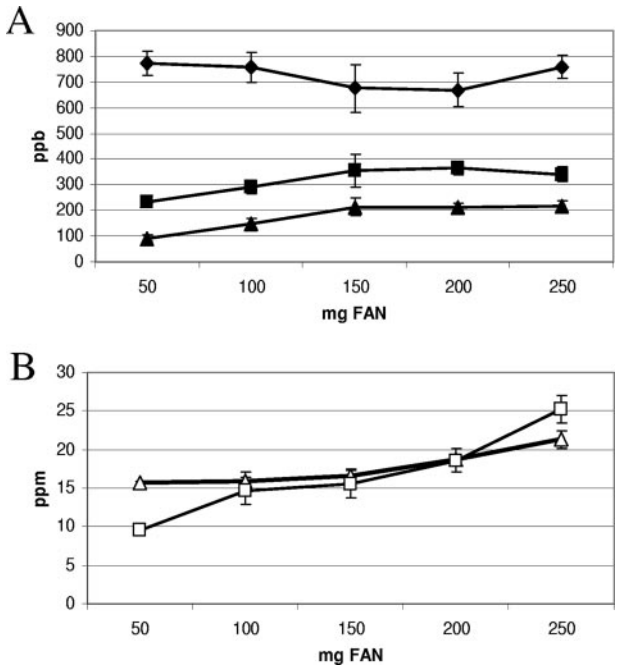

FIG. 3. Ethyl ester production by CMBS SS01 after fermentation of a synthetic medium with different nitrogen contents. The carbon content of the medium was kept constant (8% maltose), while the nitrogen content was varied from 50 to 250 mg liter^{-1} FAN. (A) Concentrations of ethyl hexanoate (◆), ethyl octanoate (■), and ethyl decanoate (▲). (B) Concentrations of ethyl acetate (△) and isoamyl acetate (□). The isoamyl acetate concentrations were multiplied by 10.

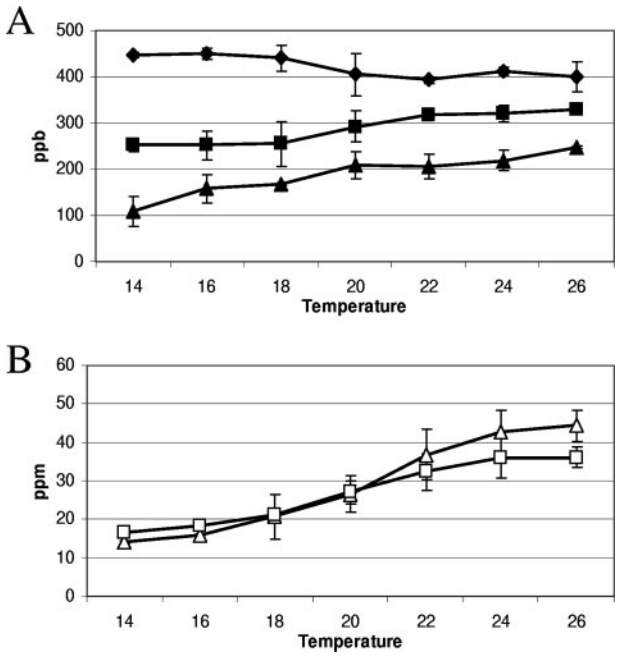

FIG. 4. Ethyl ester production by CMBS SS01 after fermentation of wort at different temperatures. The fermentation temperature was varied from 14 to 26°C. (A) Concentrations of ethyl hexanoate (◆), ethyl octanoate (■), and ethyl dec noate (▲). (B) Concentrations of ethyl acetate (△) and isoamyl acetate (□). The isoamyl acetate concentrations were multiplied by 10.

slightly at high maltose concentrations. The ethyl octanoate and ethyl decanoate concentrations did not vary significantly with the different carbon contents of the fermentation medium. We also compared these effects with the influence of a higher carbon-to-nitrogen ratio on acetate ester production. Figure 2B shows that the acetate ester levels increased about 50% when the carbon content of the fermentation medium was enhanced. This result fits with the previous finding that *ATF1* gene expression is induced by addition of maltose to carbon-starved cells (42). *ATF1* encodes the enzyme responsible for the major part of acetate ester synthesis (11, 44). As biomass production and fermentation efficiency were the same for all the fermentations, the results show the effect of the higher sugar concentration used. However, the sugar consumed was the same in every fermentation, but the residual sugar concentration was higher when higher maltose concentrations were used.

In a second series of fermentations, the carbon content of the medium was kept constant (8% maltose), while the nitrogen content was varied from 50 to 250 mg liter^{-1} FAN. The biomass production and fermentation efficiency were the same for all the fermentations carried out in these experiments. The ethyl hexanoate concentration showed only slight variation with increasing FAN levels (Fig. 3A). On the other hand, the ethyl octanoate and ethyl decanoate concentrations increased slightly with FAN concentrations between 50 and 150 mg liter^{-1}, but they remained virtually constant with further increases in the FAN concentration. Again, the ethyl octanoate and decanoate patterns are similar to each other. In the brewing industry 150 mg liter^{-1} FAN is used as the standard FAN content. A lower FAN content is almost never used. Hence, only the ethyl hexanoate concentration increases when more nitrogen is present in the regular fermentation medium. We also compared the results with the effect of increasing the FAN level on acetate ester production. As Fig. 3B shows, both the ethyl and isoamyl acetate concentrations increased when more nitrogen was available in the fermentation medium. These results fit with the previous demonstration that *ATF1* gene expression also increases with increasing FAN content of the fermentation medium (42).

Fermentation temperature affects ethyl ester production. To investigate the effect of temperature on ethyl ester formation, we carried out fermentations at temperatures ranging from 14 to 26°C. The biomass production and fermentation efficiency were the same for all the fermentations (data not shown), because the fermentations were carried out until the same attenuation was reached. As the ethyl ester concentration was measured at the end of fermentation, the effect of a higher temperature was due the effect of the temperature itself and not due to better yeast growth at 26°C than at 14°C. The results indicate that ethyl octanoate and decanoate production increased with increasing temperature, whereas ethyl hexanoate production decreased but only up to a temperature of about 20°C (Fig. 4A). Ethyl hexanoate production was apparently affected differently by several fermentation parameters than the production of ethyl octanoate and decanoate. For the production of ale beers, the brewing industry uses higher fermentation temperatures, and the temperature interval between 20 and 26°C is the most relevant. In this interval, the ethyl

TABLE 2. Headspace GC-FID measurement of volatile esters produced by *EHT1* and *EEB1* overexpression strains

Compound	Concn (mg liter^{-1})[a]		
	CMBS SS01/ps	CMBS SS01/pEHT1s	CMBS SS01/pEEB1s
Ethyl hexanoate	0.62	0.59	0.63
Ethyl octanoate	0.33	0.34	0.33
Ethyl decanoate	0.13	0.12	0.12
Ethyl acetate	16.49	16.68	17.83
Isoamyl acetate	1.57	1.57	1.65

[a] The data are averages from three independent fermentations. The standard deviations were typically about 10% and did not exceed 20%.

hexanoate concentration remains more or less constant, while the concentrations of ethyl octanoate and decanoate still increase slightly. Hence, increasing the temperature above 20°C affects only the ethyl octanoate and decanoate levels and not the ethyl hexanoate level.

We also compared these results with the influence of temperature on acetate ester production. Acetate ester production gradually increased with increasing fermentation temperature for temperatures between 14 and 26°C (Fig. 4B). Thus, in the important temperature interval between 20 and 26°C, the acetate ester level clearly increased with increasing temperature. As it is known from brewing practice that temperature affects the flavor of beer (30, 32, 43), this observation could lead to appreciable sensory differences.

Overexpression of the *EHT1* and *EEB1* alleles from an ale brewer's yeast does not alter ethyl ester production. Conflicting results have been reported for the effect of *EHT1* overexpression on ethyl ester production. Saerens et al. (33) showed that overexpression of neither *EHT1* nor *EEB1* in a laboratory yeast strain enhanced ethyl ester production. In this case the *EHT1* and *EEB1* alleles were also derived from a laboratory strain. On the other hand, Lilly et al. (17) showed that overexpression in the VIN13 wine yeast strain of an *EHT1* allele derived from wine yeast resulted in a clear increase in ethyl ester production. We cloned the *EHT1* and *EEB1* alleles from the industrial ale strain CMBS SS01 behind the strong *PGK* promoter and transformed the same strain with the overexpression plasmids or the empty plasmid as a control. Fermentations with the control strain (CMBS SS01/ps) and with *EHT1* and *EEB1* overexpression strains (CMBS SS01/pEHT1s and CMBS SS01/pEEB1s) were carried out as described in Materials and Methods. When the fermentations were complete, samples were removed and used for volatile compound determination. Table 2 shows the results of a headspace GC-FID analysis. The *EHT1* and *EEB1* overexpression strains produced the same amounts of ethyl hexanoate, octanoate, and decanoate as the empty vector control strain. As previously reported, acetate ester production was also not significantly affected by *EHT1* or *EEB1* overexpression. This means that overexpression of the *EHT1* or *EEB1* gene derived from the industrial ale strain CMBS SS01 did not result in an increase in the production of ethyl esters, as previously observed for overexpression in a laboratory strain of the *EHT1* or *EEB1* allele derived from a laboratory strain (33). The effect previously observed in wine yeast (17) might have been due to the *EHT1* wine yeast allele being different, to the specific genetic background of the wine yeast strain, or to the different fermentation conditions used for beer and wine production.

Precursor concentration is the limiting factor for ethyl ester synthesis. The previous observation that addition of MCFA precursors to the fermentation medium resulted in greater ethyl ester production (33), as opposed to overexpression of the ethyl ester synthesis genes, suggested that precursor concentration might be a limiting factor for ethyl ester synthesis. To obtain more evidence for this possibility, we investigated the effect of MCFA addition on the expression of *EEB1* and *EHT1*. CMBS SS01 yeast cells were grown for 12 h at 30°C in 2% YPD medium to which 0.01% hexanoic acid (C_6), 0.001% octanoic acid (C_8), 0.001% decanoic acid (C_{10}), or 0.001% dodecanoic acid (C_{12}) was added. After the cells were harvested and RNA was extracted, the levels of *EHT1* and *EEB1* expression were determined using RT-PCR as described in Materials and Methods. Figure 5A shows the level of *EEB1* expression relative to that of *ACT1*, which was used as an endogenous control. *EEB1* expression seemed to be upregulated significantly when C_8 was added to the fermentation medium, whereas addition of C_6, C_{10}, or C_{12} had no effect. The same effect was observed for *EHT1* expression, but, although significant, the increase observed after C_8 addition was much smaller than the increase observed for *EEB1* expression (Fig. 5B). Addition of C_6, C_{10}, or C_{12} had no significant effect on the expression of *EHT1*. These results show that the stimulation of ethyl ester synthesis by addition of C_6, C_{10}, or C_{12} precursors cannot be explained by induction of higher ethyl ester synthase

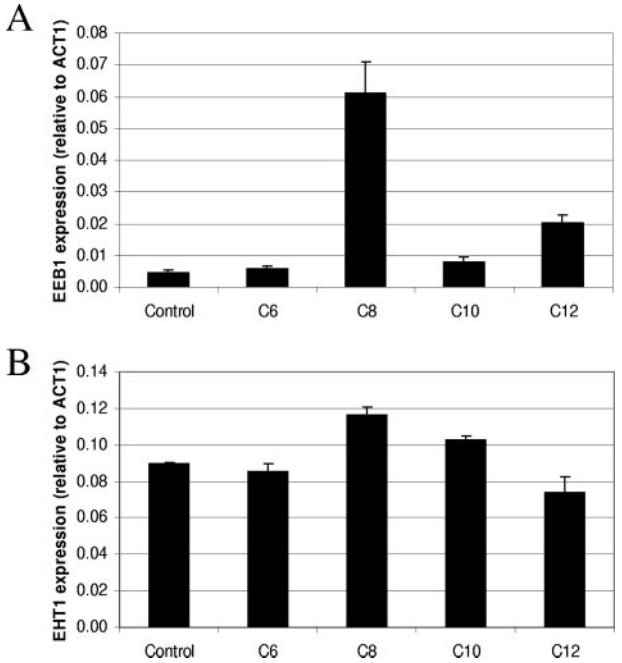

FIG. 5. Levels of expression of *EEB1* (A) and *EHT1* (B) in the CMBS SS01 yeast strain after addition of MCFAs to the fermentation medium. CMBS SS01 yeast cells were grown for 12 h at 30°C in 50 ml of 2% YPD medium to which hexanoic acid (C_6), octanoic acid (C_8), decanoic acid (C_{10}), or dodecanoic acid (C_{12}) was added. For the control, only water was added. The levels of *EEB1* and *EHT1* mRNA were normalized by using the actin level as an internal control. The error bars indicate the standard deviations for three repeats.

activity at the level of transcription. Also, the induction of *EHT1* by C_8 appeared to be too small to provide an explanation for the previously observed (33) strong stimulation of ethyl ester production by addition of C_8.

DISCUSSION

In this study we investigated the influence of important fermentation parameters on the production of ethyl esters by an industrial ale brewer's yeast strain during fermentation of an unhopped and autoclaved wort or of artificial media designed to mimic brewer's wort. In most cases, ethyl ester production was affected the same way as acetate ester production, except when the carbon content of the fermentation medium was increased. In this case, only acetate ester production increased, while ethyl ester production did not change. The ethyl hexanoate concentration appears to be differentially affected by several fermentation parameters compared to the ethyl octanoate and decanoate concentrations. An exception is the addition of UFAs, which results in a decrease in the concentration of all ethyl esters. Since a higher level of nitrogen in the medium or an increase in the fermentation temperature causes a stronger increase in acetate ester production than in ethyl ester production, variation of these parameters in industrial beer brewing can be used to control the ester profile.

Changes in any of the industrial parameters investigated had an effect on the level of ethyl esters in the fermentation product. Together, addition of UFAs to the fermentation medium and increasing the fermentation temperature affect ethyl ester production. Addition of UFAs to the medium downregulates ethyl ester production, while increasing the fermentation temperature results in higher levels of all ethyl esters except ethyl hexanoate. For the production of ale beers, brewers use higher fermentation temperatures, and the temperature interval between 20 and 26°C is the most relevant. In this interval, only ethyl octanoate and decanoate levels increase, while the ethyl hexanoate level remains more or less constant. A possible explanation for this result is that an increase in the fermentation temperature releases higher levels of ethyl octanoate and decanoate into the medium (36). Virtually 100% of the ethyl hexanoate is released, whereas 50% of the ethyl octanoate and almost 100% of the ethyl decanoate are retained in the cells. Increased temperatures affect the thermodynamic distribution coefficient, favoring ester accumulation in the aqueous medium.

To understand why these external parameters, especially the nutrient composition of the medium, influence the production of MCFA esters, it is necessary to understand what controls the formation of these flavor compounds at the cellular level. The rate of ethyl ester formation is dependent on three factors: (i) the concentration of the two substrates, (ii) the total activity of the enzymes responsible for the synthesis, and (iii) the hydrolysis of the ethyl esters. Enhancing enzyme activity by overexpression of the ester synthesis genes does not affect ethyl ester production, since overexpression of the *EHT1* or *EEB1* allele derived from the industrial ale strain CMBS SS01 did not result in an increase in the production of ethyl esters. This was also observed previously for overexpression in a laboratory strain of the *EHT1* or *EEB1* allele derived from a laboratory strain (33). On the other hand, both Eht1 and Eeb1 also display esterase activity in addition to ester synthesis activity (33). This might provide an alternative explanation for the absence of an increase in the final ester levels. To evaluate whether ethyl ester synthesis is controlled by the substrate level directly, or possibly through elevated expression of *EEB1* or *EHT1*, the effect of MCFA addition to the fermentation medium on *EEB1* and *EHT1* transcriptional expression was investigated. Only C_8 was able to induce expression of *EEB1* and *EHT1*, while C_6, C_{10}, and C_{12} did not affect *EEB1* or *EHT1* expression. We have previously shown that addition of C_6 or C_8 to the fermentation medium causes a strong increase in the formation of the corresponding ethyl ester (33). Here we demonstrated that one of these two precursors, C_6, is not able to induce expression of either *EEB1* and *EHT1*. If we assume that the C_6 and C_8 precursors do not specifically inhibit the esterase activity of the Eht1 and Eeb1 proteins (compared to their ester synthesis activity), these two results together suggest that the cellular precursor concentration is rate limiting for ethyl ester synthesis.

The control of flavor ester levels in alcoholic beverages is often problematic. Insufficient flavor ester synthesis or aberrant flavor ester profiles are quite common in beer and wine fermentations (43). Our observation that alteration of the sugar and FAN levels affects acetate ester accumulation, while ethyl ester concentrations are not altered, is in agreement with the well-established practice in breweries of controlling flavor production by altering the sugar/FAN ratio. By adding more sugar to the fermentation medium, acetate ester levels can be increased without changing the ethyl ester levels, thus providing a way to adapt the flavor profile. All the other fermentation parameters investigated affect acetate and ethyl ester formation in similar ways, except for ethyl hexanoate production. Hence, by altering these parameters, beneficial adaptation of the flavor profile may also be possible. In strain selection, specific attention to the levels of expression of other genes (e.g., fatty acid synthesis genes [14]) appears to be more relevant than the levels of expression of the *EEB1* and *EHT1* genes for final ethyl ester levels.

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