JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 96, No. 2, 110–118. 2003

REVIEW

Flavor-Active Esters: Adding Fruitiness to Beer

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Received 9 October 2002/Accepted 25 March 2003

As they are responsible for the fruity character of fermented beverages, volatile esters constitute an important group of aromatic compounds in beer. In modern high-gravity fermentations, which are performed in tall cylindroconical vessels, the beer ester balance is often sub-optimal, resulting in a clear decrease in beer quality. Despite the intensive research aimed at unravelling the precise mechanism and regulation of ester synthesis, our current knowledge remains far from complete. However, a number of factors that influence flavor-active ester production have already been described, including wort composition, wort aeration and fermentor design. A thoughtful adaptation of these parameters allows brewers to steer ester concentrations and thus to control the fruity character of their beers. This paper reviews the current knowledge of the biochemistry behind yeast ester synthesis and discusses the different factors that allow ester formation to be controlled during brewery fermentation.

[**Key words:** aroma, flavor-active esters, volatile esters, fermentation, beer, alcohol acetyl transferase (AATase), *ATF1*, *ATF2*, isoamyl acetate]

I. INTRODUCTION: FLAVOR-ACTIVE ESTERS IN BEER

Volatile esters are only trace compounds in fermented beverages such as beer and wine, but they are extremely important for the flavor profile of these drinks (1–16). The most important flavor-active esters in beer are ethyl acetate (solvent-like aroma), isoamyl acetate (fruity, banana aroma), ethyl caproate and ethyl caprylate (sour apple), and phenyl ethyl acetate (flowery, roses, honey) (Fig. 1). Table 1 shows that in lager beers, only the concentration of isoamyl acetate reaches its threshold level (5, 17, 18). However, the presence of different esters can have a synergistic effect on the individual flavors, which means that esters can also affect beer flavor well below their individual threshold concentrations (14). Moreover, the fact that most esters are present in concentrations around the threshold value implies that minor changes in concentration may have dramatic effects on beer flavor (19). This problem has become very clear with the introduction of modern brewing practices, such as high-gravity brewing and the use of tall cylindroconical fermentors. Indeed, it is well known that the use of worts of high specific gravity results in a severe overproduction of acetate esters. The concentration of acetate esters after dilution of beers produced through ultra-high gravity fermentations (20 degrees Plato, °P) can be up to 75% higher than in beers produced with standard 12°P wort (19–21). In contrast to high-gravity brewing, fermentations

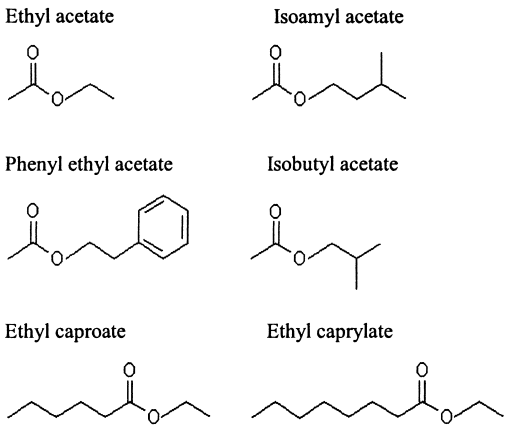

FIG. 1. Aroma-active esters in beer.

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TABLE 1. Threshold values, concentration range and average concentration of aroma-active esters in lager beer (5, 10, 16).

Component	Concentration range (mg/*l*)	Average concentration (mg/*l*)	Threshold level (mg/*l*)	Flavor description
Ethyl acetate	8–32	18.4	21–30	Fruity, solvent-like
Isoamyl acetate	0.3–3.8	1.72	0.6–1.2	Banana, pear
Ethyl caproate	0.05–0.3	0.14	0.17–0.21	Apple, aniseed
Ethyl caprylate	0.04–0.53	0.17	0.3–0.9	Apple
Phenyl ethyl acetate	0.10–0.73	0.54	3.8	Roses, honey, sweet

in tall 'Apollo' fermentors result in a decreased formation of esters, so that the produced beers lack desirable fruity tones (21). In order to obtain more control over ester production and counteract the negative consequences of high-gravity brewing and tall fermentors, intensive research has been carried out to elucidate the biochemical background of yeast ester formation.

II. THE BIOCHEMICAL BACKGROUND OF YEAST ESTER PRODUCTION

Aroma-active esters are formed intracellularly by fermenting yeast cells. Being lipid-soluble, acetate esters rapidly diffuse through the cellular membrane into the fermenting medium. Unlike acetate esters, the proportion of the fatty acid ethyl esters transferred to the medium decreases with increasing chain length: 100% for ethyl caproate, 54–68% for ethyl caprylate, and 8–17% for ethyl caprate. Longer chain fatty acid ethyl esters all remain in the cell. It also seems that the distribution of esters between medium and cells is dependent on the yeast species used, with a higher proportion of the esters formed remaining in the cells of lager yeast (*Saccharomyces carlsbergensis*) (22, 23). Moreover, the distribution of fatty acid ethyl esters between cells and medium is temperature-dependent: more of each ester is retained at lower temperatures (12).

Volatile esters are the product of an enzyme-catalyzed condensation reaction between acyl-CoA and a higher alcohol (24, 25). Several different enzymes are involved in the formation of esters (26), the best characterized ones being the alcohol acetyl transferases I and II (AATase I and II; EC 2.3.1.84), which are encoded by the genes *ATF1* and *ATF2*, respectively (26–28). The enzymes Atf1p and Atf2p are at least partially responsible for isoamyl acetate and ethyl acetate production (28, 29). Other enzymes involved in ester production are Lg-Atf1p, an AATase found in lager yeast that is homologous to Atf1p (29), and Eht1p (ethanol hexanoyl transferase), an enzyme believed to catalyze the formation of ethyl hexanoate (17, 30–32). Furthermore, it has been shown that the balance between ester-synthesizing enzymes and esterases such as Iah1p, which hydrolyze esters, might be important for the net rate of ester accumulation (33). Residual esterase activity in final beer may also be responsible for the decrease in ester concentration as often monitored during storage (34).

Basically, two factors are important for the rate of ester formation: the concentration of the two substrates, acyl-CoA and fusel alcohol, and the total activity of the enzymes involved in the formation and breakdown of the respective ester (Fig. 2). Hence, all parameters that affect substrate concentrations or enzyme activity will influence ester production. The first models for the rate of ester synthesis during brewery fermentations focussed on the availability of the co-substrate acetyl-CoA as the main limiting factor. Parameters such as temperature, fatty acid addition, nitrogen and oxygen levels would exercise their influence on ester synthesis by changing the levels of acetyl-CoA. In brief, every factor that raises acetyl-CoA levels would also raise ester production. Oxygen, wort solids and wort lipids promote yeast growth and thus the usage of acetyl-CoA, leaving less acetyl-CoA available for ester production (24, 35, 36). However, this model fails to satisfactorily explain the influence of glucose or nitrogen addition and the lowering of top pressure, three factors that raise both yeast growth and ester production. Furthermore, Yoshioka and Hashimoto (37) found that the levels of acetyl-CoA were hardly affected by modification of the fermentation conditions. Other studies showed that the availability of the other co-substrate, higher alcohols, may be the main limiting factor for ester synthesis. It was found that supplementations of 3-methyl butanol to both normal and high-gravity worts increased the production of the corresponding acetate ester, isoamyl acetate (38, 39). Furthermore, it has been shown that mutants and transformants overproducing certain higher alcohols, also show a clear increase in the synthesis of the respective acetate ester (40, 41). For example, transformants overexpressing the cytosolic branched-chain amino acid

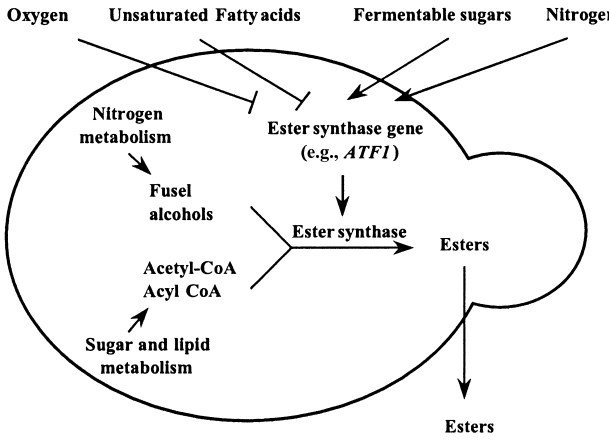

FIG. 2. Biochemical background of yeast ester production. Esters are synthesized from a fusel alcohol and an activated fatty acid (Acyl-CoA or Acetyl-CoA). The reaction is catalyzed by an ester synthase. The ester synthesis rate is determined by the concentration of available substrates and the total enzymatic activity. The substrate availability depends mainly on nitrogen, carbon and fatty acid metabolism, while the enzymatic activity is mainly determined by the activity of the corresponding genes. Thus, any factor that affects yeast metabolism and/or ester synthase gene activity, will influence the ester synthesis rate.

aminotransferase-encoding gene, *BAT2*, produce about 1.3-fold more isoamyl alcohol and 1.5 times more isoamyl acetate (41). This indicates that the availability of fusel alcohols indeed influences the production of the derived esters.

However, it is clear that the effects of some parameters on ester synthesis cannot be explained through higher alcohol availability alone. For example, high oxygen and unsaturated fatty acid levels are known to increase fusel alcohol production, but to decrease ester levels (19, 42, 43). A third model, which places the AATase enzyme in a central role, was developed by Yoshioka *et al*. (37, 44) and further elaborated by Malcorps *et al*. (45). It was shown that AATase activity follows a pattern very similar to that of ester production, and that the enzymatic activity is repressed by both oxygen and the supplementation of linoleic acid to the medium. After cloning of the AAT genes *ATF1* and *ATF2*, it was shown that *ATF1* gene transcription is directly repressed by unsaturated fatty acids and oxygen (46–48). It has been suggested that this regulation is mediated by the so-called Rox pathway (46, 47). However, recent work has demonstrated that *ATF1* and the Δ9 fatty acid desaturase-encoding gene, *OLE1*, are co-regulated through a so-called low-oxygen response element (LORE). This promoter element is activated under hypoxic conditions and selectively repressed by unsaturated fatty acids (49–55).

In addition, other studies have demonstrated that *ATF1* activity is also regulated through the protein kinases Sch9p and protein kinase A (PKA) (46, 56). These kinases play a central role in the transcriptional regulation of genes in response to changes in carbon, nitrogen and phosphate levels. The main targets of Sch9p are genes involved in cell growth, stress response and glycogen and trehalose metabolism (57–63). Furthermore, Verstrepen *et al*. (56) have demonstrated that genetically modified brewer's yeast strains overexpressing the *ATF* genes produce up to five times more isoamyl acetate and ethyl acetate in standard wort fermentations. This clearly demonstrates that ester synthesis in brewery fermentations is not limited by the availability of substrates, confirming the central role of AATase activity as proposed by Yoshioka *et al*. and Malcorps *et al*. (37, 44, 45). Moreover, as the total AATase activity seems to be limited mainly by *ATF* gene expression, the actual limiting parameter for acetate ester synthesis in brewer's yeast is the transcriptional activity of the *ATF* genes. Of course, in addition to *ATF* gene expression, the influence of substrate concentrations on ester production should not be neglected (56).

The different parameters that influence ester production provide several opportunities for brewers to exert a certain influence on ester concentrations in beer. However, as so many factors are involved in both the regulation of AATase activity (including multiple regulation mechanisms at the level of *ATF* expression) and in the regulation of substrate availability (including carbon, nitrogen and fatty acid metabolism), the control over ester formation is extremely complex and difficult to predict.

III. GETTING A GRIP ON FRUITINESS: HOW TO CONTROL ESTER PRODUCTION

Yeast strain One of the most important factors affecting ester production is certainly the yeast strain. Not only the average ester production, but also the relative proportions of each individual ester produced, differs dramatically from strain to strain. Furthermore, the influence of fermentation parameters, such as oxygen and temperature, is highly dependent on the strain background (19, 20, 22, 64–66). Ramos-Jeunehomme *et al*. (67) suggested that the differences in ester production between distinct yeast strains are due to differences in AATase activity. Ideally, the production strain therefore should be selected from a pool of strains in order to find the strain that performs best in the particular circumstances of a certain production plant. However, in most practical cases, the yeast strain is not considered as a "variable", so that the use of different yeast strains can hardly be seen as a realistic manner to control ester synthesis, except maybe when totally "new" beers are developed.

Apart from the difference in ester production between distinct yeast strains, Watari *et al*. (68) have shown that the ester production of a specific strain may also be variable due to genetic drift during successive rounds of fermentation or cultivation on agar slants. These authors therefore recommend a complete periodic examination of the production strain, including genetic fingerprinting and standard fermentation trials.

Wort specific gravity and sugar profile While most of today's lager beers are produced by high-gravity brewing, it is well known that the fermentation of worts of high specific gravity often leads to unbalanced flavor profiles. The most common problem encountered when high-gravity brewing is applied is the relative overproduction of acetate esters. This results in over-fruity and solvent-like beers (19, 20, 65, 69, 70). Palmer and Rennie (71) observed a fourfold increase in ethyl acetate and isoamyl acetate production when the specific gravity was increased from 10.5°P to 20°P. Similarly, Anderson and Kirsop (20) found that the acetate ester concentration increased four- to eightfold when the specific gravity of the pitching wort doubled. This implies that, after dilution to 5% (v/v) ethanol, beers produced from high gravity worts still contain about two times more esters than beers produced with standard worts. However, the specific overproduction of acetate esters under high gravity conditions differs from strain to strain (72).

Apart from the total sugar content of the medium, which is reflected in the specific gravity, the relative amounts of different assimilable sugars in wort also have an influence on ester production. Generally, worts containing higher levels of glucose and fructose produce more esters than worts with high maltose contents (39, 69, 73–75). Compared to all-malt worts, worts supplemented with maltose syrups (final worts containing 30 vol% of syrups containing 70% maltose) show a reduction of 10% in ethyl acetate concentrations, and even up to 40% reduction in isoamyl acetate concentrations (75). Therefore, worts containing relatively high amounts of maltose are suited for high-gravity brewing, as the overproduction of acetate esters is diminished by the high maltose content (39). The reason for the difference in ester production between glucose- or maltose-grown cells is still unclear. It has been suggested that the metabolism of glucose produces higher amounts of acetyl-CoA, resulting in enhanced ester production (74). Another possibility is

that cells growing in glucose-containing media produce more fusel alcohols. Although some minor differences in higher alcohol formation have been found between glucose and maltose fermentations, it seems unlikely that this would explain the observed differences in ester production (74, 76). A third possibility is that glucose causes stronger expression of the ester synthase genes *ATF1* and/or *ATF2*, resulting in elevated ester synthase activities (56).

Wort nitrogen content The influence of nitrogen compounds on ester formation is very complex. Peddie (65) suggested that, in all-malt wort, the C:N ratio is low and oxygen is the main growth-limiting factor. While growth ceases because of oxygen depletion, metabolites including fusel alcohols and acetyl-CoA are still being formed, but cannot be used, resulting in the formation of esters as overspill products. Adversely, when adjuncts are used to produce wort, the C:N ratio is high, so that nitrogen may be a growth-limiting factor. When yeast growth ceases due to nitrogen depletion, the formation of metabolites such as acetyl-CoA may be reduced, resulting in a decreased overspill and thus lower ester production. However, as it has been demonstrated that the availability of acetyl-CoA is not the most important limiting factor, this theory cannot hold (37, 45).

Another possible explanation for the influence of nitrogen compounds on ester formation is the link between nitrogen metabolism and the production of higher alcohols. It has been shown that addition of valine, leucine and isoleucine strongly increased the production of the corresponding fusel alcohols isobutanol, isoamyl alcohol and amyl alcohol (77–79). Of course, the higher levels of fusel alcohols may in turn lead to enhanced ester production, as shown by Calderbank and Hammond (38). Theoretically, this raises the possibility of steering the production of specific esters by manipulating the formation of the corresponding higher alcohols through the concentration of the corresponding amino acids in the pitching wort. Engan (79) showed that the level of isoamyl acetate can indeed be changed by the addition of certain amino acids, such as leucine and, to a lesser extend, isoleucine.

In addition, it has been suggested that nitrogen and carbon compounds affect transcription of the *ATF1* gene (46). This hypothesis is supported by the fact that *ATF1* transcription is mediated through Sch9p, a protein kinase known to be involved in glucose and nitrogen sensing (46, 56, 57, 59, 61, 80–82).

Wort oxygen and lipid content The formation of volatile esters is repressed by dissolved oxygen in the fermenting medium (20, 25, 36, 43, 45–47, 70, 83–88). Some researchers have suggested that oxygen exerts its influence through its effect on yeast growth and thus acetyl-CoA availability (20). However, more recent work provided strong evidence that oxygen directly represses the expression of the AATase-encoding genes *ATF1* and *ATF2* (48). This implies that no ester-synthesizing enzymes are formed as long as oxygen is present in the growth medium. On the other hand, when the oxygen content of the pitching wort is extremely low (*e.g.*, <1 mg/*l*), ester concentrations also drop because of insufficient yeast growth. Therefore, ester production is maximal at a certain oxygen level, and drops when the initial oxygen content is higher or lower (38). Hence, wort aeration or oxygenation is a powerful tool to control ester production during brewery fermentations, although in practice it is not always possible or desirable to change the oxygen content of the pitching wort.

Unsaturated fatty acids in the fermenting medium also repress ester formation through direct repression of *ATF* gene transcription (20, 45, 47, 48, 87, 89, 90). The lipid (including unsaturated fatty acid) content of wort can be adapted relatively easily through specific changes in the filtration process. Cloudy worts containing relatively high lipid concentrations therefore offer a possible solution for ester overproduction in high-gravity fermentations, although the use of cloudy worts may have a negative effect on beer flavor and resistance to ageing (20, 89, 91).

An interesting way to increase ester production was developed by Moonjai *et al*. (92). These authors have shown that it is possible to reduce the need for wort aeration by supplementing cropped yeast with unsaturated fatty acids prior to pitching. After washing (in order to remove excess unsaturated fatty acids), the supplemented yeast cells can be pitched in low-oxygen worts, which results in elevated ester production without reducing fermentation kinetics.

Yeast growth factors It has been suggested that yeast growth factors may influence ester production. For example, high levels of pantothenate, which is required for CoA synthesis, may lead to an increased ethyl acetate production (19). Hodgson and Moir (93) showed that ester production is also enhanced when zinc is added to the medium, due to stimulation of higher alcohol formation. However, these observations have not yet been reported in full-scale brewing practice.

Fermentation temperature Generally, increased fermentation temperatures in the range of 10–25°C lead to increased ester production (19, 65). For example, Engan and Aubert (94) have shown that up to 75% more esters are produced at 12°C than at 10°C. Similarly, Titica *et al*. (95) found a 40% to 50% increase in ester formation when the fermentation temperature was raised from 10°C to 16°C. However, different esters may show different temperature dependencies. Some studies show that ethyl acetate and phenyl ethyl acetate are produced maximally at 20°C, whereas maximal production of isoamyl acetate and ethyl caprylate occurs at 15°C (19, 83). This temperature dependency is not valid for all yeast strains, however, and some strains may show different temperature dependencies for the production of certain volatile esters (Verstrepen, unpublished data). Interestingly, ester production is not only dependent on the mean fermentation temperature, but is also influenced by the temperature profile throughout fermentation. Sablayrolles and Ball (78) showed that more esters are produced when fermentation is started at relatively high temperatures, and subsequently decrease after the maximal CO_2 production rate occurs. The reason for the temperature-dependence of ester synthesis is as yet unknown, although it has been suggested that temperature affects AATase activity and/or formation (32, 65). Furthermore, it is well known that the formation of higher alcohols is also temperature dependent (96), so that changes in temperature may cause changes in the availability of fusel alcohols that are neces-

sary for ester formation (38, 83). Another parameter that is often neglected is the fact that aroma-active esters are volatile, which implies that a certain amount of esters will inevitably evaporate from the fermenting medium. Obviously, this process will occur more intensely at higher temperatures. The effect of evaporation, of course, will be most important when ester concentrations are high and formation is low, as for example towards the end of the fermentation process.

Pitching rate and drauflassen Maule (97) observed a reduction in ester synthesis when the pitching rate is increased fourfold. For modest increases in pitching rate, only slight differences, mainly in ethyl acetate production, could be monitored. Accordingly, D'Amore *et al*. (98) suggest that, for high gravity fermentations (25°P), the optimal pitching rate is about 35.10^6 cells per ml, compared to 15.10^6 at 12°P. Wackerbauer *et al*. (99) observed that ester synthesis is not only dependent on pitching rate, but is also influenced by the use of the so-called 'drauflassen' technique, in which batches of fresh (mostly oxygen-free) wort are added to actively fermenting yeast. This observation was later confirmed by Malcorps *et al*. (45), who showed that the higher ester production when drauflassen is applied is due to higher and prolonged AATase enzymatic activity.

Fermentor design and top pressure As one of the results of mergers, acquisitions and large-scale production, it has become economically desirable to build larger fermentors. However, depending on the shape of the fermentor, serious problems are encountered when volumes reach about 10,000–12,000 hectoliter (21). Larger fermentors lead to poor yeast growth, poor diacetyl reduction and, most importantly, poor ester production. A good example are the so-called 'Apollo' fermentors, in which isoamyl acetate levels decrease from 4 ppm at 1 m depth to 0.3 ppm at 18 m depth (21). The influence of fermentor design on flavor production is largely attributed to increased carbon dioxide due to the higher hydrostatic pressure in tall fermentors. Excessive dissolved carbon dioxide generally leads to an inhibition of yeast growth and metabolism, presumably because of the inhibition of vital decarboxylation reactions (21, 100–104). As decarboxylation reactions are also necessary for the formation of both fusel alcohols and acetyl-CoA, it is believed that the influence of carbon dioxide on ester production is due to the inhibition of substrate formation. The inhibitory effect of carbon dioxide on ester production is found in most strains, but it has been shown that the specific response differs from strain to strain (105). While the inhibition of ester formation is mostly unwanted, pressure can, in some cases, be applied as an effective way of reducing excessive ester production, for example in high-gravity brewing or when higher fermentation temperatures are applied. A useful empirical formula to estimate the appropriate pressure is given by the equation P (in bar) = °C/10. For example, when fermentation is performed at 19°C, pressure is allowed to build up to 1.9 bar (0.9 bar overpressure). At 2 bar, dissolved carbon dioxide doubles and ester levels drop by 50% or more when compared to non-pressurized fermentations (21). Titica *et al*. (95) confirmed the effect of top pressure and developed a more complex mathematical model to predict and control ester production based on carbon dioxide emission.

Genetic modification *ATF1* and *ATF2* were first deleted and overexpressed by Fujii *et al*. (27, 29, 106) and Nagasawa *et al*. (28), respectively. It was found that deletion of *ATF1* reduces isoamyl acetate production by 80% and ethyl acetate production by 30%. *ATF2* deletion has similar but smaller effects on ester production (28; Verstrepen *et al*., unpublished results). Accordingly, overexpression of these genes in sake yeast led to a tenfold increase in ethyl acetate production and a thirtyfold increase of isoamyl acetate formation. Similarly, Lilly *et al*. (107) showed increased ester concentrations in wines produced with genetically modified yeast overexpressing the *ATF1* gene. Verstrepen *et al*. (56) have overexpressed *ATF1* and *ATF2* in a commercial brewer's strain. The pilot-scale beers produced with an *ATF1*-overexpressing strain contained five times more acetate esters than the beers produced with the wild-type strain. Overexpression of *ATF2* led to smaller increases in isoamyl acetate formation and no significant changes in ethyl acetate levels (56). These results indicate that it is possible to use genetic modification in order to create new yeast strains with desirable ester production characteristics. In addition, the highly elevated ester levels obtained with the overexpression strains clearly indicate that ester synthesis during brewery fermentations is not strictly limited by substrate availability. Indeed, it can be concluded that not the substrate concentration, but rather the expression level of the *ATF* genes, is one of the main controlling factor that affects ester synthesis during wort fermentations, as first suggested by Yoshioka *et al*. (37, 44) and later elaborated by Malcorps *et al*. (45).

CONCLUSIONS

Acetate ester formation in brewer's yeast is controlled mainly by the expression level of the AATase-encoding genes. In addition, changes in the availability of the two substrates for ester production, higher alcohols and acyl-CoA, also influence ester synthesis rates. Thus, any factor that influences the expression of the ester synthase genes and/or the concentrations of substrates will affect ester production accordingly. Brewers therefore have a broad range of different ways at their disposal to control acetate ester production. A thoughtful use of these techniques allows the counteraction of the negative effects on ester production of some modern brewing practices, such as high-gravity brewing and the use of large fermentors. While the optimal technique is dependent on many factors and may therefore differ from case to case, some parameters are more easily adapted and allow a more selective control than others. Perhaps the most convenient and selective way to reduce ester production is by applying tank overpressure, if necessary in combination with (slightly) lower fermentation temperatures, low wort free amino nitrogen (FAN) and glucose levels and elevated wort aeration or wort lipid concentrations. However, care has to be taken in order to avoid excessive yeast stress due to low FAN concentrations or high dissolved carbon dioxide levels, as this may lead to decreased fermentation performance. Enhancing ester production is slightly more complicated. Of course, if it is possible to reduce overpressure or

wort aeration, this may offer a good solution. In other cases, worts rich in glucose and nitrogen combined with higher fermentation temperatures and lower pitching rates or application of the drauflassen technique may prove helpful. The easiest way to get a grip on beer fruitiness is perhaps by using genetically modified variants of the production strain. Indeed, using strains with different expression profiles of the *ATF* genes makes it possible to selectively enhance or decrease the production of aroma-active acetate esters. However, this requires both further research and a drastic change in public perception concerning the use of genetically modified organisms in food production (for reviews on the use of genetically modified yeasts for the production of fermented beverages, see Ref. 108–114). In addition, it has to be noted that currently almost nothing is known about the factors influencing the synthesis of other esters, such as ethyl caproate and caprylate, so that further research in this area is certainly needed.

ACKNOWLEDGMENTS

Kevin Verstrepen is a Research Assistant of the Fund for Scientific Research–Flanders (Belgium) (FWO-Vlaanderen). K.J. Verstrepen greatly acknowledges the Fund for Scientific Research–Flanders (FWO Vlaanderen) for the financial support of his research. This work was supported by grants from the Fund for Scientific Research–Flanders (FWO project G.0082.03), the Research Fund of the KU Leuven (OT/03/40 and BIL02/34) to F.R. Delvaux and J.M. Thevelein.

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