

Linoleic Acid Supplementation of a Cropped Brewing Lager Strain: Effects on Subsequent Fermentation Performance with Serial Repitching

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ABSTRACT

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Most breweries collect yeast from a previous fermentation cycle for further use in a subsequent cycle. However, the cropped cells are deficient in membrane sterols and unsaturated fatty acids (UFAs) which are required for good fermentation performance in the next cycle. Consequently, the cellular levels of these compounds must be restored to obtain an optimal fermentation performance. There are currently three possibilities to satisfy this requirement. The common practice is aeration of the wort before pitching, thus providing oxygen needed for lipid synthesis during the first stages of fermentation. Oxygenation (aeration) of cropped yeast slurries is a second alternative. Finally, the addition of the required lipids to wort is sometimes suggested as an alternative to aeration. We examined a fourth possibility, namely the supplementation with UFA of cropped cells. Previously, we reported that the supplementation of stationary phase cropped brewer's yeast with linoleic acid is a good alternative to wort aeration. This conclusion resulted from results obtained with a well-defined stirred synthetic fermentation medium. We also showed that cells cropped from non-stirred tall-tube fermented malt wort incorporated linoleic acid into different cellular lipid fractions, when suspended and supplemented in fermented wort. Now, we report that such yeast, pitched in malt wort in non-stirred tall-tubes, showed growth and attenuation profiles comparable to unsupplemented yeast in pre-aerated wort. Moreover, the synthesis of acetate esters, which is known to be affected when UFAs are added directly to the wort, was not significantly affected. We hypothesize that the active uptake of linoleic acid during fermentation and its activation by coenzyme A (CoA) and phospholipid synthesis are responsible for the effects on ester synthesis, through repression of the alcohol acetyltransferase-encoding gene, *ATF1*. In supplemented cropped yeast, these reactions occur prior to fermentation, thus avoiding interferences with acetate ester synthesis. In serial repitching experiments with repeated linoleic acid supplementation of the cropped yeast, the fermentation performances of the yeast remained comparable to those of non-supplemented yeast in pre-aerated wort. However, due to a progressive increase of cellular UFA, negative effects on acetate ester synthesis appeared. Nevertheless, the supplement-

ation of cropped yeast with UFAs can be considered as an interesting alternative to wort oxygenation to restore optimal membrane functions.

INTRODUCTION

In traditional batch brewing practice, cropped yeast is reused in successive fermentation cycles. This results in a depletion of unsaturated lipids and a deterioration of growth under fermentation conditions. These lipids are essential membrane components. It has long been known that oxygen is required for sterol and unsaturated fatty acid (UFA) synthesis by *Saccharomyces cerevisiae* and that under strictly anaerobic conditions, yeast needs an exogenous supply of these membrane components in order to grow^{1,2}. Although they are abundant in malt, normal brewing processes prevent their passing into the wort, so that wort oxygenation is required for their synthesis^{18,19}. Wort oxygenation can be avoided by addition of selected lipids or lipids extracted from spent grains to wort^{6,21}. Wort or yeast enrichment with added UFAs are, however, known to decrease the synthesis of volatile esters during beer fermentation^{22,23}.

Acetate esters, such as ethyl acetate (solvent-like aroma) and isoamyl acetate (fruity, banana aroma), are recognized as important flavour-active compounds in beer. These esters are formed by the reaction of alcohol with acetyl coenzyme A catalyzed by alcohol acetyltransferase (AATase)^{12,13,15,26,27}. The AATase activity is repressed by both oxygen and linoleic acid¹². The AATase gene *ATF1* was cloned⁸ and it was shown that its expression is greatly reduced by aeration and by wort supplemented UFAs. *ATF1* transcription is directly repressed by UFAs¹⁰ and oxygen⁹. It was suggested that gene repression is the primary reaction leading to reduced AATase activity *in vivo*⁷. The significance of ester synthesis in yeast was reviewed by Mason and Dufour¹⁴ and Verstrepen et al.²⁴

We have shown previously that under certain conditions, linoleic acid does, however, not reduce the synthesis of the acetate esters¹⁶. After the supplementation with linoleic acid of cropped yeast suspended in a de-aerated medium, prior to pitching, we found that growth, attenuation, ethanol and ester profiles during a subsequent fermentation were similar to profiles obtained with unsupplemented cells pitched in a pre-aerated medium. In the control ester synthesis was affected when linoleic acid was added to the

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fermentation medium instead of to the cropped yeast. Consequently, we suggested that supplementation of cropped brewer's yeast with UFA could be a very interesting method to revitalize the yeast before its use in the next fermentation cycle. All previous experiments were, however, conducted under stirred fermentation conditions and with a synthetic medium. To better simulate real brewery conditions, we have now studied the fermentation by linoleic acid supplemented cropped yeast, using malt wort and tall tube non-stirred fermenters.

MATERIALS AND METHODS

Yeast strain and maintenance

All experiments were carried out with an industrial lager brewer's strain of *Saccharomyces cerevisiae* (*carlsbergensis*) KUL-CMBS 12 (Katholieke Universiteit Leuven, Centre for Malting and Brewing Science) maintained on wort agar (Difco Laboratories, Detroit, MI) at 4°C.

Growth and fermentation media

A synthetic medium described before¹⁶ was used for the first series of experiments. Wort medium (standard unhopped wort, 12°P) was prepared with 100% barley malt extract (Muntons Malt Products, Suffolk, UK) and polypropylene glycol 2000 (200 mg/L) (Sigma-Aldrich Chemie GmbH, Germany) as antifoaming agent. The media were sterilized by autoclaving at 1.0 kg/cm² and 121°C for 45 min.

Yeast propagation, fermentation and serial repitching

Fig. 1 summarizes the different steps. For yeast propagation, single yeast colonies were taken from stock plates and streaked on wort agar slants. After incubation at 27°C for 48 h, the slants were stored at 4°C. When required, 5 mL of sterile wort was added to a slant culture and the cells were brought into suspension by gently shaking. The suspension was inoculated in 150 mL of wort in cotton wool-plugged 250-mL Erlenmeyer flasks and incubated at 20°C for 48 h, on an orbital shaker at 150 rpm. Cells were harvested by centrifugation (1,400 g, 5 min) and pitched into fresh wort at a level of 1.25 g cell dry weight per litre (CDW/L) in a 7 cm diameter, 70 cm length, 2.5-L tall-tube glass fermenter containing 2 L of aerated wort (see section "Fermentation monitoring" for the cell dry weight measurement). After pitching, the headspace was flushed with nitrogen gas for 5 min at a flow rate of 1.5 L/min. The fermentation was allowed to proceed at 20°C without stirring and was terminated after the maximum attenuation was reached. The yeast cropped from this first fermentation cycle (crop I) was used to study the uptake of linoleic acid or for a second fermentation cycle in four different conditions: (1) cropped yeast was supplemented with linoleic acid and then pitched in de-aerated wort, (2) cropped yeast was pitched in pre-aerated wort, (3) cropped yeast was pitched in de-aerated wort supplemented with linoleic acid and (4) cropped yeast was pitched in de-aerated wort. To obtain pre-aerated conditions, before pitching, the wort was aerated to reach a dissolved oxygen concentration of 8 ppm, by flushing with sterile air at 20°C for 30 min and

stirring at 500 rpm. For de-aerated conditions, oxygen was removed from the wort by flushing with nitrogen gas for 10 min at 20°C and stirring at 500 rpm. The content of dissolved oxygen was measured by means of a dissolved oxygen meter (Oxi 340-A/SET, Weilheim, Germany). For de-aerated conditions with wort supplemented with linoleic acid (Sigma Chemical Co., St. Louis, MO), the acid in 2 mL of ethanol was added to a final concentration of 15 mg/L. An equal amount of ethanol (2 mL) was added in the other fermentations. After pitching, nitrogen gas was passed over the medium for 5 min at a flow rate of 1.5 L/min. All fermentations were maintained at 20°C. At regular time intervals, 100 mL samples were removed for

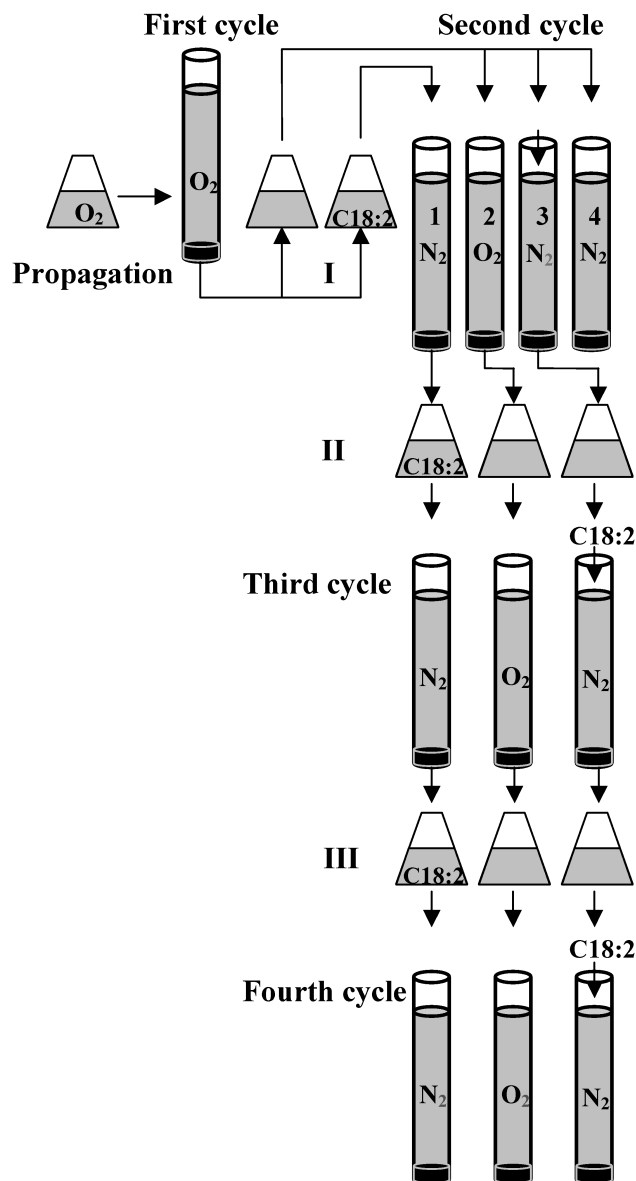


Fig. 1. Schematic overview of serial repitching under non-stirred tall tube fermentations: (1) cropped yeast was supplemented with linoleic acid and then pitched in de-aerated wort, (2) cropped yeast was pitched in pre-aerated wort, (3) cropped yeast was pitched in de-aerated wort supplemented with linoleic acid and (4) cropped yeast was pitched in de-aerated wort. I, II and III indicated the 1st, 2nd and 3rd supplementation of cropped yeast with linoleic acid, respectively.

analysis. At the end of the second fermentation cycle under the three different conditions the yeasts were cropped (crop II). The cells were used for a third fermentation cycle under the three different conditions as in the previous cycle. The yeasts from the third cycle (crop III) were used for a fourth fermentation cycle in the same way (see Fig. 1).

Linoleic acid supplementation of cropped yeast

Yeast was cropped from the bottom of the tall-tube fermenters and re-suspended in part of the fermented wort, at a concentration of 25 g cell wet weight per litre (CWW/L). Linoleic acid was added to a final concentration of 6 mg/g CWW using 1 mL of an ethanolic solution per 1 L of fermented medium. Ethanol (1 mL without linoleic acid) was added as a control to yeast suspensions to be used in fermentation conditions with non-supplemented yeast. All cell suspensions were incubated at 20°C with magnetic stirring at 150 rpm. Nitrogen gas was passed continuously over the media at a flow rate of 30 mL/min to avoid any entry of oxygen. After a contact time of 24 h with or without linoleic acid, the cells were harvested by centrifugation (1,400 g, 5 min), washed twice with sterile cold (4°C) water and then used for fermentations.

Fermentation monitoring

Yeast growth as cell dry weight was followed by measuring the turbidity of 10-fold diluted cell suspensions at 600 nm against initial wort medium and relating the values to dry weight equivalents. Dry weight was determined by vacuum filtration of suitable volumes of suspensions through pre-weighed filters (0.22 µm PTFE Phenomenex AFO-0513), washing and drying at 105°C for 24 h. For analysis, yeast cells were harvested by centrifugation (1,400 g, 5 min), washed with 30 mL of 1% (v/v) tergitol (Sigma Chemical Co., St. Louis, Mo.), then with 30 mL of 0.5% (v/v) tergitol, and finally with 30 mL of distilled water to remove adsorbed fatty acids. Yeast pellets were weighed and stored at -70°C for further analysis. The apparent attenuation of clarified media was measured using a digital density meter (Paar DSA 48 + SP-1, Anton PAAR KG, Graz, Austria). Volatile compounds (ethyl acetate, isoamyl acetate, propanol, isobutanol and isoamyl alcohol) were determined by headspace gas chromatography (Perkin-Elmer Autosystem XL, Wellsley, MA) and flame ionization detection.

Table I. Linoleic acid and unsaturated fatty acid (UFA) levels in pitching yeast obtained after a pre-incubation in fermented synthetic medium, with and without linoleic acid. The results are based on a single experiment.

Incuba- tion time (h)	Linoleic acid supplemented condition			Unsupple- mented condition
	Linoleic acid (mg/g CDW)	Linoleic acid (% of total fatty acids)	UFA (% of total fatty acids)	UFA (% of total fatty acids)
0	0	0	56.8	56.8
6	0.9	4.2	57.5	56.3
12	2.3	8.1	63.2	57.2
24	3.9	11.5	66.9	57.7

Lipid analysis

Total fatty acids composition of yeast cells was determined as described previously¹⁶. For analysis of distinct lipid fractions, lipids were extracted from whole cells by a modification of the procedure of Swan and Watson²⁰. Cell pellets (0.5 g CWW) transferred into 30 mL capped Pyrex tubes were suspended in 5 mL of 80% (v/v) ethanol and incubated at 80°C for 20 min in a water bath, to deactivate lipolytic enzymes and to split lipid-protein linkages. The suspensions were then sonicated for 1 min. The broken cells were separated by centrifugation (1,400 g, 5 min) and the supernatant was transferred into a 30 mL capped Pyrex tube. To the residue, 10 mL of a chloroform/methanol (3:1, v/v) mixture, containing 3 µg/L of N,N'-diphenyl-1,4-phenylenediamine (Merck-Schuchardt, Hohenbrunn, Germany) as antioxidant, was added, and the tube was shaken for 30 min at room temperature (20°C). After centrifugation, the residue was once more extracted with chloroform/methanol (3:1, v/v). The supernatant fractions were combined and washed twice with 5 mL aqueous 0.88% (w/v) KCl. The solvent phase was collected and evaporated to dryness under a flow of nitrogen gas at room temperature. The lipid extract was dissolved in 300 µL of chloroform/methanol (3:1, v/v) and stored at -20°C. Thin-layer chromatography (TLC) of the lipid fractions was performed using silica gel polyester coated plates (Alltech Associated, Inc., Deerfield, IL, USA) and petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v) as solvent. To locate the lipid fractions on the plates, a lipid standard mixture was run in parallel on the same plate. The standard lipids were detected after spraying with a 0.2% (v/v) ethanolic solution of Rhodamine 6G (Alltech Associated, Inc., Deerfield, IL USA) and observation under UV-light. The fatty acid composition of lipid fractions was analysed in the same way as for total yeast lipids.

RNA extraction and Northern blot analysis

The expression level of the *ATF1* gene was monitored during the first 12 h of fermentation with supplemented cells or in cells with supplemented wort. Cell culture samples of 20 mL were added to 30 mL of ice-cold water and rapidly further cooled on ice. The cells were pelleted and washed once with ice-cold water and then stored at -70°C. Cells suspended in 1 mL ice-cold water were transferred to a 2-mL Eppendorf tube with screw-cap. Cells were pelleted at 4,000 g for 15 sec and re-suspended in 0.5 mL extraction buffer (1mM EDTA, 0.1M LiCl, 0.1M Tris-HCl pH 7.5). Then 0.5 mL of glass beads (0.45 mm diameter) and 0.5 mL of phenol chloroform isoamyl alcohol (PCI) mixture (25/24/1 citrate-buffered, water-equilibrated phenol pH 4.2/chloroform/isoamyl alcohol), containing 1% (w/v) sodium dodecyl sulfate (SDS), were added. The tube was shaken at 6 m/sec for 20 sec using a Fastprep FP120 (BIO101) and then centrifuged at 4,000 g for 10 min at 2°C. The aqueous upper layer was transferred to a clean Eppendorf tube. The PCI extraction was repeated. To the extract, 1/10 vol 40% (w/v) potassium acetate (pH 5.5) and 2 vol of 99.9% (v/v) ethanol were added. After mixing and storage at -20°C for at least 1 h, the RNA was pelleted by centrifuging at 14,000 rpm for 10 min. The RNA pellet was washed with 70% (v/v) ethanol and dissolved in 40 µL DEPC-treated double distilled water.

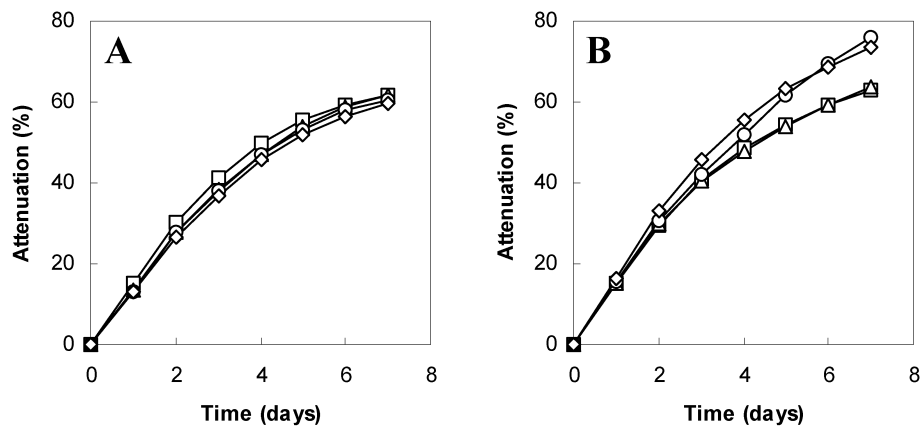


Fig. 2. Changes in attenuation during fermentation of synthetic medium with unsupplemented (A) and linoleic acid supplemented yeast (B). Symbols: pre-incubation time of pitching yeast (□) 0 h, (△) 6 h, (○) 12 h, and (◇) 24 h. The results are based on a single experiment.

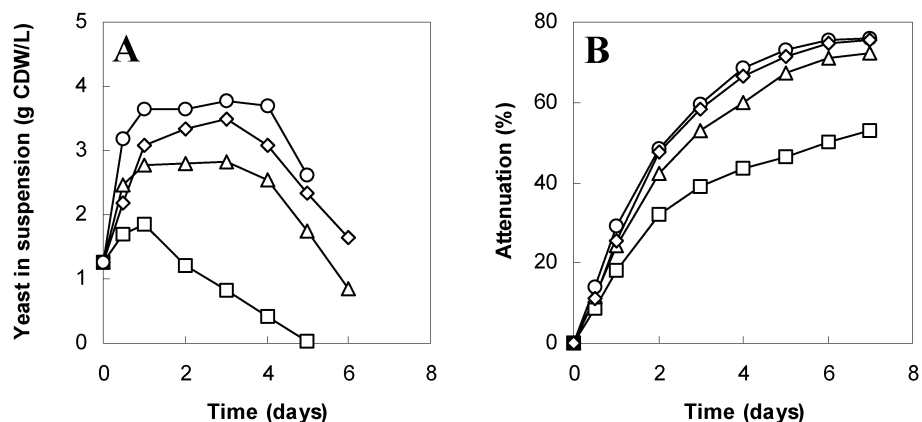


Fig. 3. Changes in suspended yeast (A) and wort attenuation (B) during fermentation under different conditions. Symbols: (□) de-aerated wort, (△) de-aerated wort with linoleic acid addition, (○) de-aerated wort with linoleic acid supplemented yeast, and (◇) pre-aerated wort. The results are based on a single experiment. (CDW = cell dry weight.)

Probes were labelled with [α P³²]dCTP (Amersham Biosciences, Buckinghamshire, UK) using the High prime kit from Roche Diagnostics (Mannheim, Germany). Northern blots were made of separated total RNA fractions (18 μ g total RNA/lane) in gels containing 1% (w/v) agarose in 50 mM boric acid, 1 mM sodium citrate, 5 mM NaOH, pH 7.5, and 1% (w/v) formaldehyde. RNA was transferred by capillary blotting to a Hybond-N membrane (Amersham Biosciences, Buckinghamshire, UK) using 10 \times SSC (sodium chloride-sodium citrate) buffer. These blots were hybridized with labelled probes of the coding region of *ATF1* and *ACT1*. The blots were analysed using the Fujix BAS-1000 phosphorimager and the PCBAS 2.0 software (Raytest, Straubenhardt, Germany). Relative expression levels were determined using TINA V9.0 software (Raytest, Straubenhardt, Germany).

RESULTS

Non-stirred tall-tube fermentation of synthetic medium

Yeasts were propagated in a stirred synthetic medium¹⁶, collected at stationary phase and pre-incubated with or

Table II. Characteristics of final beer after 7 days of fermentation under 4 different conditions: N = de-aerated wort, NL = de-aerated wort with linoleic acid addition, L = de-aerated wort with linoleic acid supplemented yeast, and A = pre-aerated wort. The results are based on a single experiment.

Characteristics	Fermentation conditions			
	N	NL	L	A
Maximum attenuation (%)	52.7	72.1	75.7	75.5
Alcohol content (% v/v)	3.24	4.05	4.58	4.56
Total esters (mg/L)	14.5	20.2	25.9	27.5
Ethyl acetate	13.8	19.4	24.7	26.2
Isoamyl acetate	0.66	0.83	1.19	1.30
Total higher alcohols (mg/L)	65.9	102.4	108.3	106.1
Propanol	7.0	12.3	12.7	12.7
Isobutanol	9.2	14.1	15.8	14.9
Isoamyl alcohol	49.7	76.0	79.8	78.5

without linoleic acid. At different incubation times, cells were harvested, washed and pitched in de-aerated medium in non-stirred tall-tube fermenters. Table I shows that the content of linoleic acid and total unsaturated fatty acids (C16:1 + C18:1 + C18:2) of the supplemented cropped

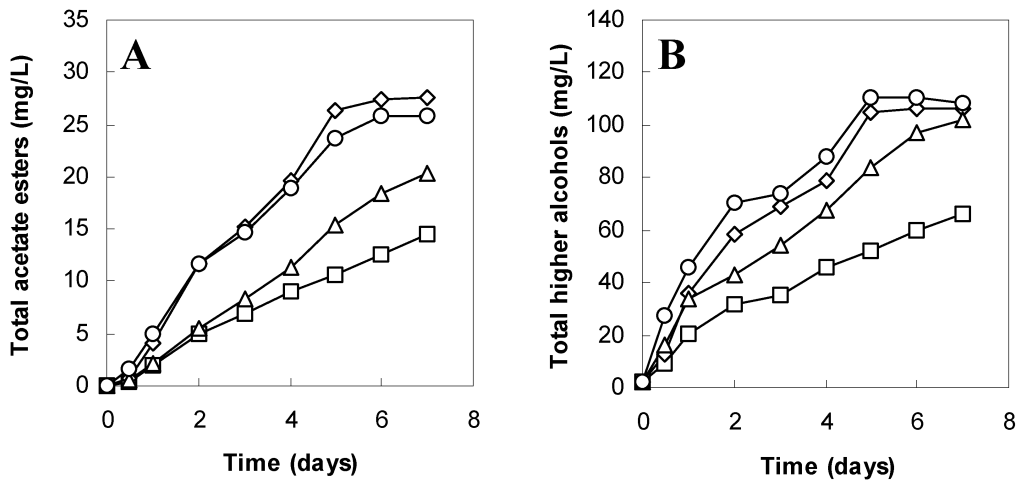


Fig. 4. Total acetate esters (A) and total higher alcohols (B) during fermentation under different conditions. Symbols: (□) de-aerated wort, (△) de-aerated wort with linoleic acid addition, (○) de-aerated wort with linoleic acid supplemented yeast, and (◇) pre-aerated wort. The results are based on a single experiment.

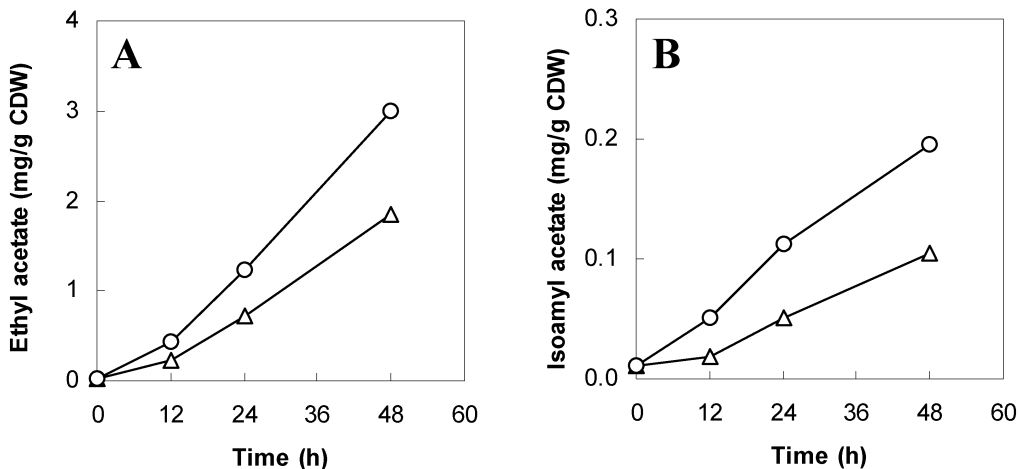


Fig. 5. Yields on biomass of ethyl acetate (A) and isoamyl acetate (B) during fermentation of de-aerated wort with linoleic acid addition (△) and de-aerated wort with linoleic acid supplemented yeast (○). The results are based on a single experiment.

yeast increased with time of incubation with linoleic acid. The total content of unsaturated fatty acids (UFAs) did not change in unsupplemented conditions. The fermentation performance of the yeast is shown in Fig. 2. All fermentations with non-supplemented yeast attenuated to around 60% after 7 days (Fig. 2A). In contrast, the attenuation rates increased to 75% with yeast pre-incubated with linoleic from 12 or 24 h. (Fig. 2B). A pre-incubation time of 6 h was without effect. It was decided to use a pre-incubation time of 24 h for the experiments with wort medium.

Non-stirred tall-tube fermentation of malt wort

Yeast growth and fermentation performance. After a first wort fermentation in tall tubes, yeast was harvested and subsequently incubated with and without linoleic acid for 24 h. The yeasts were then pitched in fresh wort under four different conditions as described in the materials and methods section (Fig. 1). Fig. 3 indicates that in conditions of de-aerated wort fermented by unsupplemented yeast, the yeast biomass in suspension and the attenuation were low. The addition of linoleic acid to the de-aerated wort

resulted in an increase in the concentrations of suspended yeast and more complete attenuation. The highest attenuations were obtained with pre-aerated wort and especially with de-aerated wort pitched with linoleic acid supplemented cells. An attenuation of 76% was reached for de-aerated conditions in the presence of linoleic acid supplemented cells compared to only around 55% when non-supplemented cells were used.

Volatiles formation. Fig. 4 shows the evolution of total acetate esters (ethyl acetate + isoamyl acetate) and total higher alcohols (propanol + isobutanol + isoamyl alcohol) during the fermentations. The rate and extent of volatiles production were the lowest under wort de-aerated conditions. The highest production rate was found in pre-aerated fermented wort and in de-aerated wort in the presence of supplemented yeast. The use of wort supplemented with linoleic acid resulted in a significant reduction in the production of volatiles. Table II shows the concentration of individual esters and higher alcohols in the final beer. A 27% reduction of esters was monitored in the beer when linoleic acid is added to the wort, compared to the beer

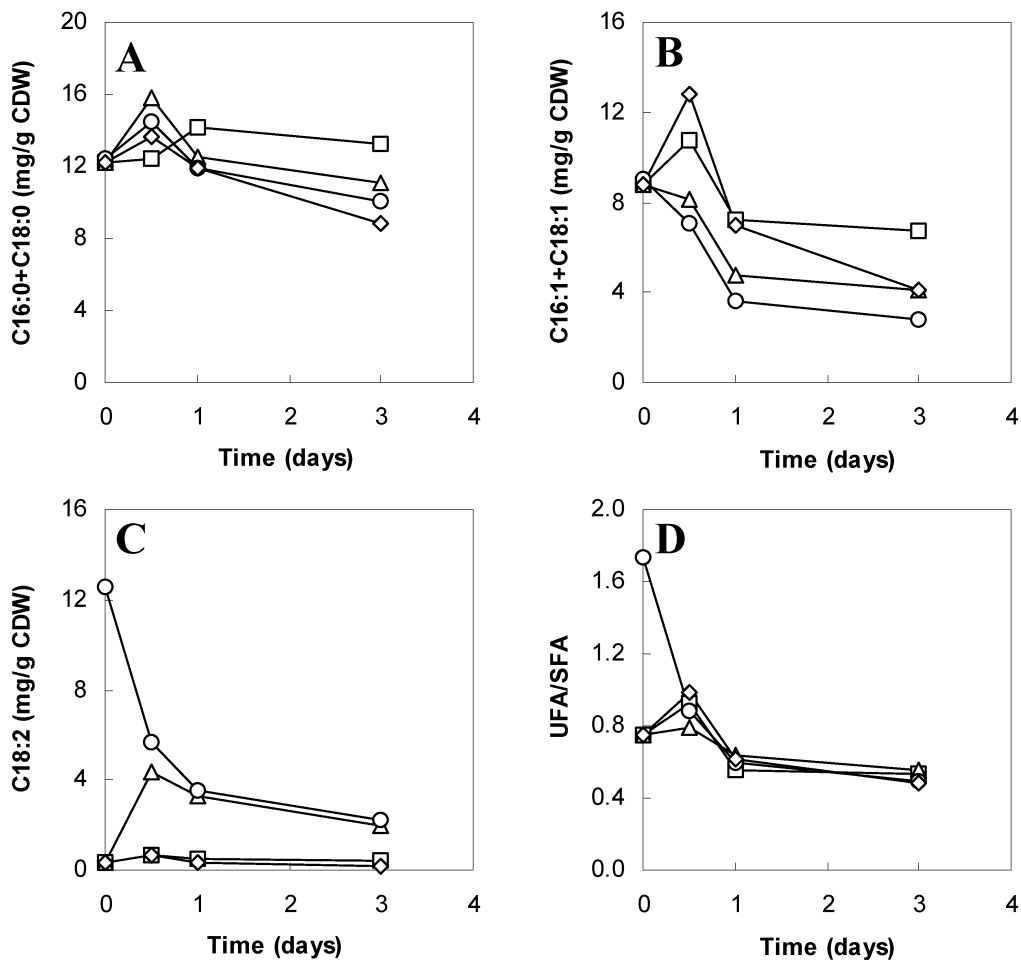


Fig. 6. Changes in cellular levels of saturated C16:0 and C18:0 acids (A), unsaturated C16:1 and C18:1 acids (B), C18:2 acid (C), and in ratios of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) (D) during fermentation under different conditions. Symbols: (□) de-aerated wort, (△) de-aerated wort with linoleic acid addition, (○) de-aerated wort with linoleic acid supplemented yeast, and (◇) pre-aerated wort. The results are based on a single experiment. (CDW = cell dry weight.)

obtained with linoleic acid-enriched pitching yeast. The reduction was observed for both ethyl acetate and isoamyl acetate. A small reduction of around 6% was found when enriched pitching yeast was used, compared to yeast grown in pre-aerated wort. The final level of higher alcohols was not significantly affected by the presence of linoleic acid, either in the wort or in the pitching yeast. Fig. 4, however, shows that the higher alcohol production rate was reduced after 1 day of fermentation of linoleic acid supplemented wort.

Fig. 5 shows the synthesis of the esters per g CDW of suspended yeast during the first two days of fermentation. The results indicate that the synthesis of acetate esters was delayed during the first 12 h when linoleic acid was added to the wort. Later, the synthesis proceeded, but at a lower rate than in wort pitched with supplemented yeast.

Fatty acid composition of yeast during fermentation.

The most abundant cellular fatty acids of non-linoleic acid supplemented pitching cells were mainly palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1). In addition, trace amounts of linoleic acid (C18:2) coming from wort were found. The changes in cellular levels of long-chain fatty acids during

fermentations under different conditions are given in Fig. 6. For saturated fatty acids (C16:0 + C18:0), there was an increase after 12 h followed by a continuous decrease, except under de-aerated conditions (Fig. 6A). For unsaturated fatty acids (C16:1 + C18:1), there was a large increase during the first 12 h, under pre-aerated conditions, followed by a large decrease (Fig. 6B). A temporary increase was, however, also observed under de-aerated conditions. In conditions where linoleic was present (either after addition to the wort or after cropped yeast supplementation), the levels of C16:1 + C18:1 decreased; somewhat more rapidly using the supplemented yeast. Fig. 6C shows that the addition of 15 mg/L linoleic acid to the de-aerated wort resulted in an increase in cellular levels of linoleic acid to a maximum of 4.4 mg/g CDW after 12 h of fermentation. Later, the content slowly decreased. With supplemented cropped cells, the pitched yeast contained 12.6 mg/g CDW of linoleic acid. During the first 12 h of fermentation, the content rapidly decreased, most likely due to the higher growth rate under these conditions (see Fig. 3). After 1 day, the linoleic acid contents were the same in conditions of supplemented wort and supplemented cells. After 1 day, the ratio of total unsaturated to

saturated fatty acids was the same for all fermentation conditions, although the absolute concentrations of the different fatty acids were different (Fig. 6D).

Figs. 7 and 8 represent the percentage distribution of long chain fatty acids in the fractions: total cellular lipids, phospholipids and free fatty acid. In linoleic acid supplemented pitching yeast (Fig. 7), linoleic acid accounted for 37% of the total cellular fatty acids, for 23% of the acids in the phospholipid fraction and for 40% of the free fatty

acid pool. After 12 h of fermentation, the percentage of linoleic acid in the total lipid fraction was much reduced (Fig. 7A), to almost half the initial level. This decrease can be attributed to a reduction of the linoleic acid percentage of the free acid pool (Fig. 7C). Consequently, also the percentage of unsaturated fatty acid decreased in both fractions. The linoleic acid percentage in phospholipids was not changed. However, the percentage of unsaturated fatty acids in this fraction increased. This may be due to

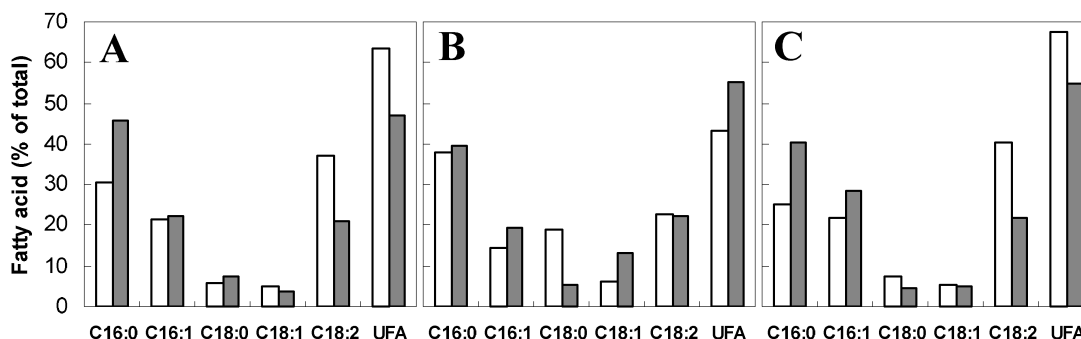


Fig. 7. Percentage distribution of long chain fatty acids and unsaturated fatty acids (UFAs) in total cellular lipids (A), in phospholipids (B) and in free fatty acids pool (C) before (open bars) and after (filled bars) 12 h of fermentation with C18:2 supplemented cropped yeast. The results are based on a single experiment.

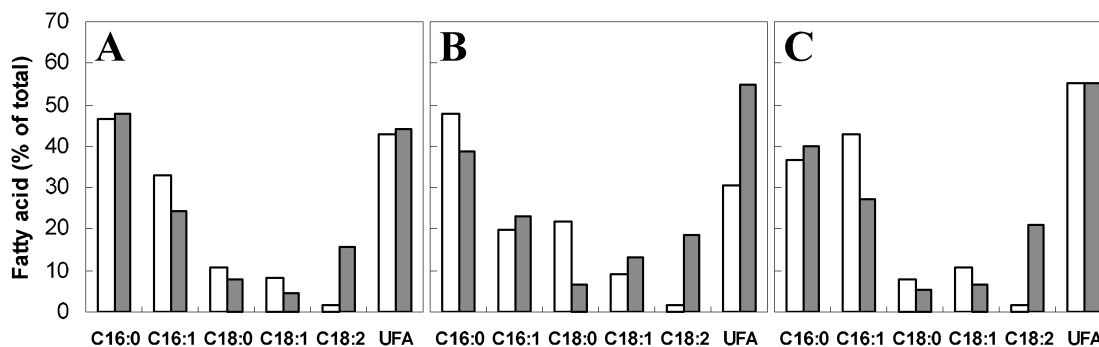


Fig. 8. Percentage distribution of long chain fatty acids and unsaturated fatty acids (UFAs) in total cellular lipids (A), in phospholipids (B) and in free fatty acids pool (C) before (open bars) and after (filled bars) 12 h of fermentation with addition of C18:2 to wort. The results are based on a single experiment.

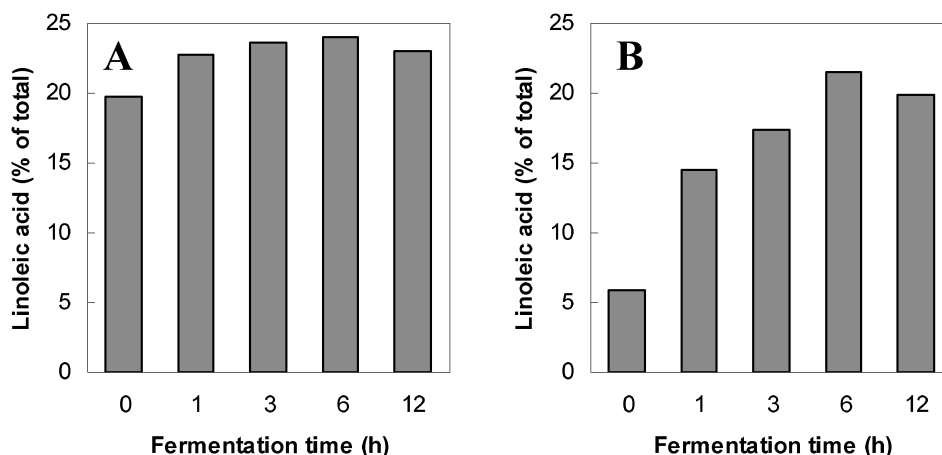


Fig. 9. Level of linoleic acid (C18:2) in phospholipids during first 12 h of fermentation with C18:2 supplemented yeast (A) and with addition of C18:2 to wort (B). The results are based on a single experiment.

higher percentages in C16:1 and C18:1 in phospholipids (Fig. 7B).

Fig. 8 shows the distribution in the lipid fractions when wort was supplemented with linoleic acid. After 12 h, lin-

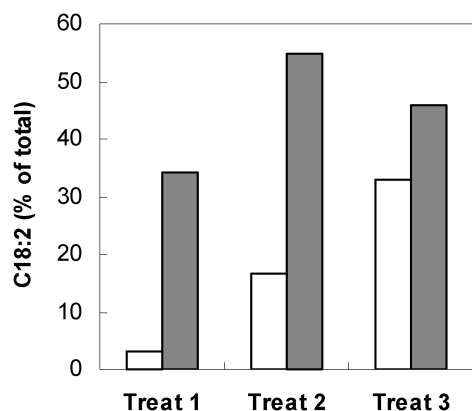


Fig. 10. Level of linoleic acid (C18:2) in total cellular lipids before (open bars) and after (filled bars) supplementation with linoleic acid, after a first (treatment 1), a second (treatment 2) and a third (treatment 3) fermentation cycle. The results are based on a single experiment.

oleic acid accounted for 16% of the total cellular fatty acids and 20% of the free acids. Contrary to the conditions with supplemented pitching yeast, the phospholipid fraction showed an increase in C18:2 from around 2% to 17% of the acids. Fig. 9 shows the changes in the levels of linoleic acid in phospholipids during 12 h of fermentation using supplemented cells or supplemented wort. Especially with supplemented wort, a significant incorporation of linoleic acid occurred.

Serial repitching

Linoleic acid supplementation to cropped yeast. Linoleic acid supplementations of the cropped yeasts after successive fermentation cycles were conducted as before. Fig. 10 shows the percentages of C18:2 in cellular lipids before and after each supplementation. After the first supplementation (after the first fermentation cycle), the level of linoleic acid increased from 3% to 34% of total fatty acids. Supplementation of the yeast cropped after a second fermentation cycle showed an increase from a residual 15% to 55%. Supplementation of the cropped yeast after a third fermentation cycle showed an increase from a residual 33% to 46%.

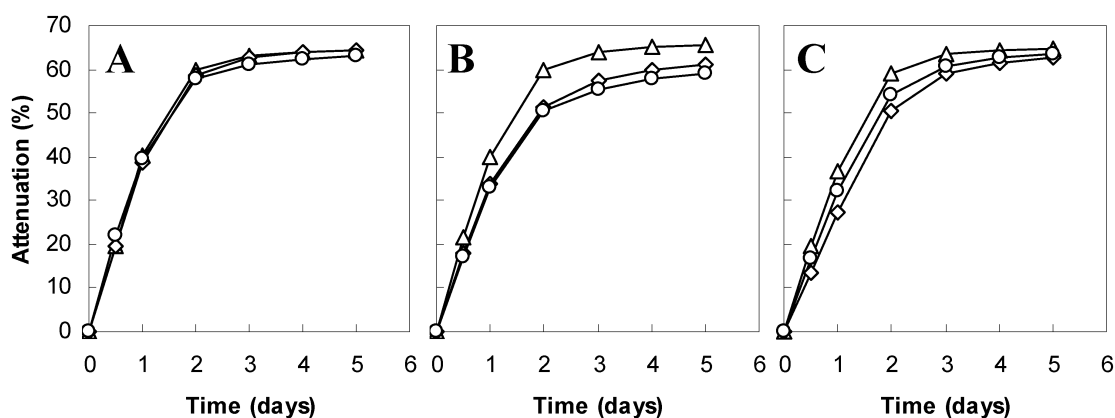


Fig. 11. Changes in wort attenuation during the second (A), third (B) and fourth (C) fermentation cycle under different conditions. Symbols: (Δ) de-aerated wort with linoleic acid addition, (\circ) de-aerated wort with linoleic acid supplemented yeast, and (\diamond) pre-aerated wort. The results are based on a single experiment.

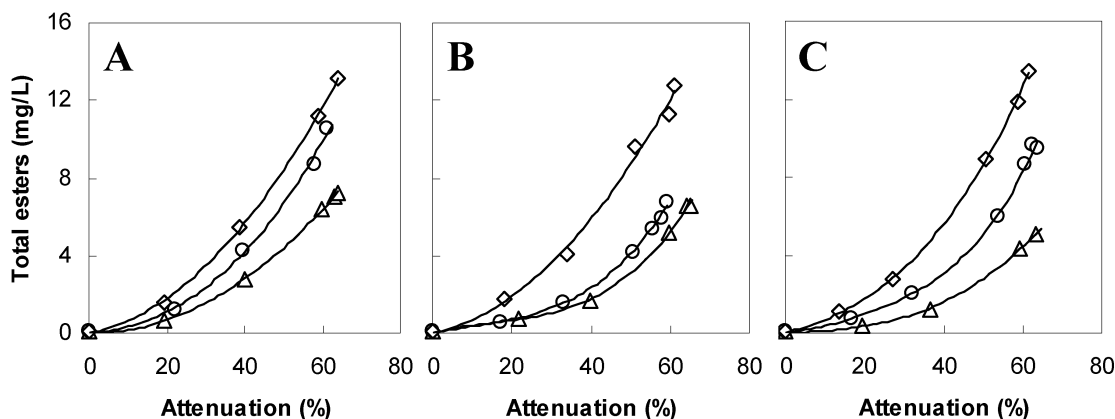


Fig. 12. Formation of total acetate esters as a function of attenuation during the second (A), third (B), and fourth (C) fermentation cycle under different conditions. Symbols: (Δ) de-aerated wort with linoleic acid addition, (\circ) de-aerated wort with linoleic acid supplemented yeast, and (\diamond) pre-aerated wort. The results are based on a single experiment.

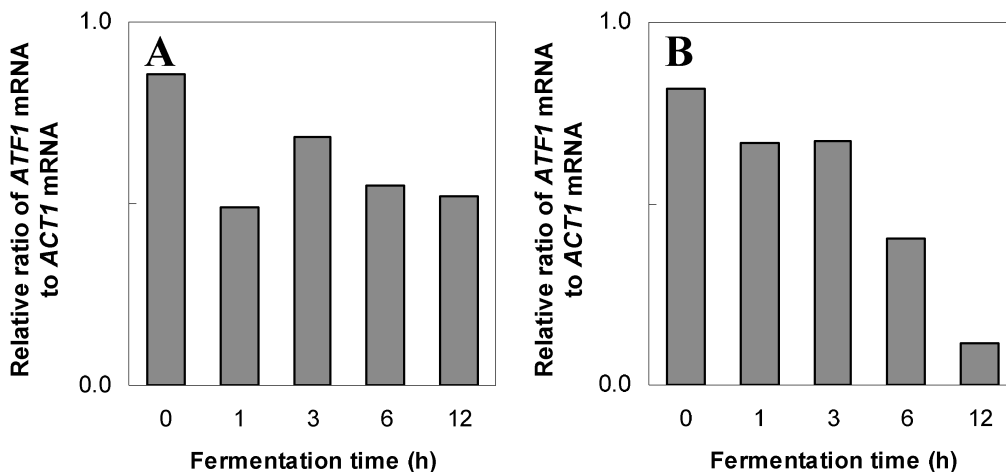


Fig. 13. Reduction in the level of *ATF1* mRNA by C18:2 supplemented yeast (A) and by C18:2 addition to wort (B). The results are based on a single experiment.

Yeast fermentation performance. The effects of linoleic acid supplementation to serial repitched yeast on its fermentation performance were compared to the effects obtained in conditions of using pre-aerated wort and de-aerated wort with linoleic acid supplementation, and unsupplemented pitching yeast. The results are given in Fig. 11. In the second fermentation cycle, under all three conditions, the fermentation performances did not differ significantly. During the third fermentation cycle, the maximum attenuations and the attenuation rates were slightly lower in pre-aerated wort and de-aerated wort with supplemented pitching yeast. However, the same results were obtained for pre-aerated wort and wort with supplemented pitching yeast. During the fourth fermentation cycle, the maximum attenuation was the same in all three conditions, although the rate was slightly higher with supplemented wort.

Ester synthesis. The formation of acetate esters in terms of the attenuation percentage is given in Fig. 12. In the second fermentation cycle, the rate of total esters formation was the lowest in linoleic acid supplemented wort. During the third fermentation cycle, the rate and the extent of formation was much higher in pre-aerated wort. During the fourth fermentation cycle, the highest ester concentration was in pre-aerated wort and the lowest was in wort with added linoleic acid. Intermediate results were found with C18:2 supplemented pitching yeast.

Effect of linoleic acid on *ATF1* gene expression in relation to *ACT1* gene

The addition of unsaturated fatty acids to the fermentation medium has been reported to cause a reduction in AATase activity and a repressed *ATF1* gene expression. We compared the expression level of the *ATF1* gene in supplemented yeast and yeast in supplemented wort during the first 12 h of tall-tube fermentations. Fig. 13 shows a slight reduction of the *ATF1* mRNA level within 1 h for both conditions possibly because the trace amounts of oxygen to which the yeast is inevitably exposed during pitching. Later, the level of mRNA remained unaffected in linoleic acid supplemented yeast, whereas in fermentation with supplemented wort, *ATF1* expression levels dropped

to almost zero, as was expected due to the high concentration of unsaturated fatty acids.

DISCUSSION

In common brewing practice, cropped yeast cells are re-used in several consecutive fermentation cycles. However, to obtain satisfactory yeast growth and wort attenuation in a next fermentation cycle, oxygen or specific lipids are required in the wort. Previously, we have suggested that, with respect to fermentation performance and especially ester synthesis, supplementation of cropped brewer's yeast with the unsaturated fatty acid linoleic acid is a good alternative to wort aeration¹⁶. The experiments were, however, conducted with a stirred synthetic medium, in order to obtain reproducible experimental conditions. The experiments were now repeated using malt wort and non-stirred tall tube fermentation conditions in order to approach real brewing conditions.

In a first experiment, it was confirmed that with the synthetic medium under non-stirred tall tube conditions, the linoleic acid supplemented cells showed rates of fermentation similar to the rates previously found under stirred conditions¹⁶. The fermentation performance was improved by pre-incubation of cropped cells with linoleic acid. It was also shown that the incorporation of linoleic acid in the supplemented cells increased with longer incubation times. In addition, it was found that cells cropped from a wort fermented medium in tall tubes and re-suspended in fermented wort also took up linoleic acid. The incorporation of lipids was even higher than the incorporation monitored with cells suspended in fermented synthetic medium.

To study the fermentation performances of linoleic acid-supplemented cells, a wort fermentation with supplemented cropped yeast was compared to fermentations using either pre-aerated wort, or wort supplemented with C18:2 or de-aerated wort. It was found that the linoleic acid supplemented cropped yeast showed growth and attenuation profiles comparable to yeast in pre-aerated wort. Interestingly, the level of the two important acetate esters, ethyl acetate and isoamyl acetate, was almost not affected,

compared to levels in pre-aerated wort. When linoleic acid was added to the wort, the synthesis of the esters, especially isoamyl acetate, showed a 12-h lag phase. In addition, the rate of ester synthesis remained much lower throughout the fermentation. Consequently, the ester content of the final beer was much reduced (20 mg/L versus 25–27 mg/L in pre-aerated wort or wort with supplemented cropped yeast). This confirms the negative effects on ester synthesis by unsaturated fatty acids added to wort as reported previously^{12,22,26}.

In contrast, the rate of ester synthesis in fermentations with cells pre-incubated with linoleic acid was unaffected. Hence, the effect of supplemented wort must be due to linoleic acid uptake during active fermentation or the reactions involved in its activation by coenzyme A (CoA) and its incorporation into cellular lipids. When the concentration of fatty acids is high, *Saccharomyces* yeasts take up fatty acids by simple diffusion. At low concentrations the entry uses a facilitated transport system³. Free fatty acids are powerful detergents, and immediately after entry into the cell they are esterified to CoA derivatives. This reaction is catalyzed by the fatty acyl-CoA synthetases encoded by the *FAA1-FAA4* genes¹¹. In a recent study, Choi and Martin⁴ established that these enzymes are, in fact, essential for the import of long-chain saturated and unsaturated fatty acids. Thus, when these acids are added to the fermenting medium, they are transported across membranes along with their conversion to the CoA derivatives and subsequently incorporated into membrane and/or storage lipids. Only unsaturated fatty acids present in the fermenting medium have been shown to affect ester production. This seems due to the repression of the *ATF1* gene. UFA-mediated transcriptional repression of *ATF1* genes was relieved by deletion of the *FAA* gene^{5,10}. This suggests that the transcription of the *ATF1* gene is no longer repressed by unsaturated fatty acids when these acids cannot enter the cells and cannot form unsaturated acyl-CoA derivatives. In addition, the work of Malcorps and Dufour¹² and Verstrepen et al.²⁵, showed that the expression levels of *ATF1* largely determine the rate of acetate ester production during wort fermentation. Hence, the high levels of acetate ester formation in de-aerated medium with supplemented cells can be attributed to the higher *ATF1* expression under these conditions.

Analysis of yeast lipid fractions indicates that linoleic acid in supplemented cropped yeast is found mainly in the free acid pool, but also in neutral lipids and in phospholipids. About 20% of linoleic acid that is taken up after supplementation is found in the phospholipids fraction and about the same percentage is found in phospholipids in fermenting cells in supplemented wort. These observations confirm our previous studies¹⁷. However, as mentioned before, ester synthesis is only inhibited in fermentations with supplemented wort, and not in fermentations with supplemented cells. This indicates that the intermediate products of unsaturated fatty acid metabolism, e.g. linoleoyl-CoA, may act as repressor of *ATF1* transcription.

Although supplementation of cropped brewer's yeast with linoleic acid is a good alternative to wort aeration with respect to its fermentation performance, our results strongly suggest that care must be taken in serial repitchings of the supplemented yeast, as it was found that this

may lead to a reduction in ester synthesis. Repeated linoleic acid supplementation of cropped yeast resulted in the accumulation of excessive amounts of the acid, later leading to a repression of ester synthesis during subsequent fermentations. This is also apparent in the study by Thurston et al.²³ where pitching yeasts were enriched with linoleic acid addition (1 g/L) during propagation and the level of linoleic acid reached up to 70% of total fatty acids. Further research is needed to comprehend the underlying mechanisms involved in the regulation of fatty acid metabolism and ester synthesis by the use of molecular biological techniques and biochemical studies. Moreover, a more extensive comparative study, under the same experimental conditions, may be needed to better understand the relations between ester synthesis and the synthesis of unsaturated fatty acids, either in conditions of pre-oxygenated wort or cells, or in conditions of supplemented wort or cells.

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