Research article

Institute of Brewing & Distilling

Received: 19 August 2014 Revised: 7 October 2014 Accepted: 11 October 2014 Published online in Wiley Online Library: 9 January 2015

(wileyonlinelibrary.com) DOI 10.1002/jib.186

Flocculation, cell surface hydrophobicity and 3-OH oxylipins in the SMA strain of *Saccharomyces pastorianus*

Greg Potter,[1,2]* Suzanne M. Budge[1] and R. Alex Speers[2]

Three-hydroxy-oxylipins (3-OH oxylipins) have been previously detected in brewing yeast production strains at flocculation onset. In this work, the SMA strain of *Saccharomyces pastorianus* was characterized during growth in a miniature fermentation assay by measuring flocculation and cell surface hydrophobicity (CSH). Proportions of 3-OH oxylipin were also measured concurrently during growth in the miniature fermentation assay and a defined 3-OH oxylipin extraction protocol using ethyl acetate is presented along with a novel derivatization and gas chromatography–mass spectrometry (GC-MS) detection approach. When the SMA strain was grown in the assay, near maximal CSH and flocculation levels were achieved by a 36 h fermentation time. Under the same culture conditions, the oxylipin 3-OH decanoic acid (3-OH 10:0) was identified. This oxylipin could not be detected early in the fermentation, but elevated relative levels of 3-OH 10:0 were reached by 36 h, coinciding with increased CSH levels. It was previously presumed that the formation of 3-OH oxylipins at flocculation onset might increase the CSH. However, results from this study suggest that 3-OH 10:0 may not contribute to cell wall hydrophobicity. The flocculation behaviour of the SMA strain was also monitored in the presence of 3-OH 10:0, but exposure to this oxylipin did not impact the sedimentation of this yeast, suggesting that 3-OH oxylipins may not act as mediators of quorum sensing in this strain. Copyright © 2015 The Institute of Brewing & Distilling

Keywords: *Saccharomyces pastorianus*; flocculation; 3-OH oxylipin; cell surface hydrophobicity

Introduction

The current brewing yeast flocculation paradigm has identified three predominant factors that collectively control this phenomenon: zymolectin binding, hydrophobic interactions and surface charge neutralization *(1)*. The impact of hydrophobic interactions on yeast flocculation has been studied by numerous early researchers *(2–8)*. While past investigators used different methods to quantify cell surface hydrophobicity (CSH) with varying degrees of success, one method that was developed by Jibiki *et al*. *(9)* has been shown to give reproducible results *(10,11)*. This method is an adaptation of hydrophobic interaction chromatography and is called hydrophobic interaction chromatography for flocculation (HICF).

With HICF, it is assumed that components on the exterior of the cell are attracted to phenyl sepharose beads by hydrophobic interactions because the beads carry no charged groups and thus exhibit no electrostatic effect *(12)*. Yet there remains a lack of understanding as to what specific components on the surface of the yeast cell wall confer the hydrophobic effect. One would think that lipids in the cell and cell wall are drawn to the phenyl sepharose beads, but literature reports of cell wall lipid content, and thus hydrophobic wall constituents, vary. In an in-depth review of *Sacccharomyces cerevisiae* cell wall architecture by Lipke and Ovalle *(13)*, lipids are not mentioned at all as significant structural components. Despite that, Kock *et al*. *(14)* observed 3-OH oxylipin containing osmiophilic layers that migrated through the cell to the cell wall at flocculation onset. Klis *et al*. *(15)* noted that mature glycophosphatidylinositol (GPI) anchored cell surface proteins at the cell exterior contained a GPI anchor with a cleaved lipid moiety. The deposition of the GPI anchor lipid in the plasma membrane, in turn, may increase CSH. Alternatively, other studies have demonstrated that there are proteins at the cell surface of *S. cerevisiae* that impart a hydrophobic character to the cell exterior *(16–18)*.

While the main factor for increasing cell surface hydrophobicity remains undetermined, the 'flocculation paradigm' is further confounded by the myriad of strain genotypes routinely used in brewing science research *(19)*. In addition, some strains mutate and become less flocculent over successive generations *(20)*. Furthermore, production strains used in research are not necessarily 'pure' strains *(21,22)* and single populations of brewing yeast can have non-homogenous flocculation characteristics *(19)*. Owing to the lack of consistency and standardization, brewing scientists have often suggested that the field would benefit from selecting a small number of 'research' strains *(19)*. Recently, the SMA strain of *Saccharomyces pastorianus* has become a *de facto* research strain *(23)*. Even though trials have documented the flocculation characteristics of this strain by measuring the absorbance at 600 nm *(24)*, this yeast has not been examined using more conventional brewing yeast assays.

This communication, therefore, has three primary purposes: (a) to detail work that characterized the SMA test strain with

* Correspondence to: G. Potter, Process Engineering and Applied Science, Dalhousie University, Halifax, NS B3J 2X4, Canada. E-mail: gpotter@dal.ca

[1] Process Engineering and Applied Science, Dalhousie University, Halifax, NS, B3J 2X4, Canada

[2] The International Centre for Brewing and Distilling, School of Life Sciences, Heriot–Watt University, Riccarton, Edinburgh, EH14 4AS, UK

standard tests for brewing yeast flocculation *(23)* and cell surface hydrophobicity *(9)* during growth in the new miniature fermentation assay *(23)*; (b) to investigate the potential role of fatty acids and 3-OH oxylipins in cell hydrophobic interactions; and (c) to present a novel approach for 3-OH oxylipin extraction and detection based on several previously published works.

Materials and methods

SMA characterization

Determination of flocculation level and CSH of the SMA strain during growth in the miniature fermentation assay. The SMA yeast was cultured in the miniature fermentation assay and the initial inoculum was prepared according to the standard protocol *(23)*. However, a pre-made Pale Ale wort (Festa Brew, Vaughn, Ontario, Canada) with the following specifications was used in place of the congress wort: original gravity, 12.39–12.58°P (measured as 12.5°P); international bitterness units, 25; and real degree of fermentation, 66.4%. The pre-made wort had been previously portioned into 473 mL cups that were frozen in a −30 °C blast freezer. Prior to use, the contents of two 473 mL cups of microwave-defrosted wort were autoclaved for 20 min at 121 °C, and the sterile wort was cooled overnight at 4 °C for no more than 12 h.

Following the cold break, the wort was centrifuged at 1950 ***g*** for 15 min, and the supernatant was saved. Then 16.2 g of D-glucose was dissolved in 410 mL of wort supernatant instead of the 18 g specified in the protocol *(23)*. The mixture was aerated by bubbling it with medical-grade, compressed oxygen for 5 min to achieve a dissolved oxygen content of approximately 8.2 mg/mL (approximated from United States Environmental Protection Agency (2012) data). The wort was then pitched with the SMA yeast at a rate of 1.5×10^7 cells/mL based on a final volume of 450 mL and adjusted to this volume with sterile distilled H_2O if required. When the pitched wort had been thoroughly mixed, 15.0 mL aliquots were transferred to each of 30 sterile 20 mL test tubes that contained a sterile polytetrafluoroethylene boiling stone. All tubes were plugged with sterile sponge bungs and the rack containing the tubes was placed in a 21 °C water bath until sampling.

Density determination. Density was determined on a Plato scale (°P) using a handheld densitometer (Anton Parr DMA 35, Graz, Austria) at 12, 24, 36, 48 and 60 h fermentation time, and before each reading the sample was filtered as suggested in the standard method *(23)*.

Determination of CSH and flocculation level. At each sampling time (12, 24, 36, 48 and 60 h), the flocculation level was determined by the ASBC Flocculation Test *(23)*, except that the SMA strain was grown in the miniature fermentation assay protocol and initial 10 mL aliquots of fermenting wort were collected from two separate vortexed 20 mL test tubes. Further, cell pellets were always re-suspended in buffer by withdrawing and expelling through a Pasteur pipette approximately 10 times in addition to the recommended vortexing. CSH was determined using the method of Jibiki *et al. (9)* with samples also collected from 20 mL test tubes that were vortexed to re-suspend any settled yeast.

Hydrophobicity studies

Free fatty acid and 3-OH oxylipin content over the course of the miniature fermentation with a hexane/isopropanol extraction and an ethyl acetate (EA) extraction. The SMA yeast was grown using the ASBC miniature fermentation assay protocol *(23)* as described above. To collect yeast pellets at each sampling point (12, 24, 36, 48 and 60 h), four to six test tubes containing 15 mL each of fermenting wort were vortexed to re-suspend any settled yeast and the contents were transferred evenly to two sterile 50 mL centrifuge tubes. The 50 mL tubes were centrifuged for 2.5 min at 630 ***g*** and the supernatant was discarded. To each tube, 5 mL of sterile distilled water was then added and the pellets were re-suspended by vortexing. The tubes were then pooled into one sterile 50 mL centrifuge tube and again this tube was centrifuged for 2.5 min at 630 ***g***. The supernatant was discarded and the yeast pellet was stored at −30 °C until later use. Samples for the EA extraction were partially dried by temporary storage in a freeze-drying unit.

Hexane/isopropanol extraction. Lipids were extracted from the yeast pellets following a modified version of the Goettingen Centre for Molecular Biosciences hexane/isopropanol (HIP) extraction method *(25)*. To do this, 0.5 g of wet yeast that had been allowed to just thaw was aseptically added to a 40 mL durable solvent-cleaned glass tube. A 20 mL aliquot of *n*-hexane/2-propanol (3:2 v/v) was added to each glass tube. The tubes were sonicated for 4 min, shaken by hand for 15 s and centrifuged for 4 min at 160 ***g***. Following centrifugation, 12.5 mL of 6.7% (w/v) K_2SO_4, which had been prepared with triple chloroform-washed water, was added to the glass tube and the tube was shaken vigorously by hand for 2 min. After the mixture had settled, the resultant upper hexane layer was then transferred to a 10 mL solvent-cleaned test tube and was thoroughly dried by adding ~1.3 g of anhydrous Na_2SO_4 to each tube. The dried hexane-rich layer was subsequently transferred to a second clean 10 mL tube and the solvent was evaporated under streaming nitrogen. Between all aforementioned tube manipulations, the tube atmospheres were evacuated with streaming nitrogen.

Ethyl acetate extraction. Partially freeze-dried yeast (0.5 g) that had been allowed to just thaw was aseptically added to a 40 mL durable solvent-cleaned glass tube. Following this, 10 mL of triple chloroform-washed, distilled water was added to each tube along with a saturating amount of 4 g NaCl to help mitigate emulsion formation, as suggested by Salmon and Flower *(26)*. Each sample was then acidified with 5 μL of 0.25 M citric acid prepared from chloroform-washed distilled water and 10 mL of EA was added to every tube. All tubes were sonicated for 4 min.

After sonication, the tubes were shaken for 10 min on a Burrell 'wrist-action' bench-top shaker (Pittsburgh, PA, USA) and subsequently centrifuged for 10 min at 160 ***g***. Despite the addition of NaCl, after centrifugation an emulsion formed in some samples at the interface and extended into the EA layer. This was dispersed by adding ~1.5 mL cold acetone to the tube to precipitate protein and other macromolecular components of the emulsion *(26)*, swirling the tube and centrifuging for a further 4 min at the same speed. Once adequate phase separation occurred, the upper EA layer was transferred to a new clean 30 mL test tube. A second 10 mL aliquot of EA was then added to each 40 mL sample tube, and another extraction and centrifugation was carried out as before. The pooled lipid extracts in EA

were then dried using anhydrous sodium sulphate (~1.3 g) and the solvent removed under streaming nitrogen.

Production of trimethylsilyl esters and ethers for gas chromatography. To prepare trimethylsilyl (TMS) esters and ethers for gas chromatography (GC), 1 mL of Tri-Sil/BSA pyridine (Thermo Scientific Pierce, Rockford, IL, USA) reagent was added to the dried products of the HIP and EA extracts. The tube atmospheres were evacuated with streaming nitrogen and the closed tube was placed on a heat block at 68 °C for 20 min. A 3-OH 10:0 standard (Matreya LLC, Pleasant Gap, PA, USA) was also trimethylsilylated to determine the retention time and characteristic fragments of this oxylipin previously identified in production strains of brewing yeast by Strauss *et al*. *(27)*.

Analysis and identification of TMS esters and ethers using GC-MS. GC-MS analysis was adapted from a gas chromatographic protocol described by Molee *et al*. *(28)* to separate and quantify neutral lipids, including free fatty acids (FFA). This analysis was performed using a Trace GC Ultra fitted with a Varian FactorFour VF-1 ms column (100% dimethylpolysiloxane, 15 m × 0.25 mm i.d., 0.25 μm film thickness), a Polaris Q mass spectrometer and a Thermo Triplus AS autosampler. Programmed temperature vaporizing splitless injection was employed to minimize degradation of thermolabile TMS esters and ethers *(29)*. A 1.0 μL injection volume was used at an initial temperature of 130 °C for 0 min, followed by a ramp at 13 °C/s to 280 °C with a splitless time of 2 min. The GC oven temperature began at 100 °C and was held for 3 min. This was followed by an increase to 170 °C at 30 °C/min, then a ramp at 20 °C/min to a temperature of 250 °C and a final ramp at 7.5 °C/min to 320 °C, which was held for 9 min. Helium flowing at 1.0 mL/min was used as the carrier gas. The MS was operated in electron ionization mode (70 eV) and the mass spectrum was recorded from *m/z* 60 to 400. Compounds were identified using library matches with the NIST MS 2.0 Search Database and by retention time; peak areas were integrated with the Thermo Xcalibur™ Qual Browser (Waltham, MA, USA) and are expressed as percentage relative area of those compounds identified. Fatty acids were named following standard convention in the format *A*:*Bn-y*, where *A* denotes the total carbon chain length, *B* the number of double bonds and *y* the location of the double bond counted from the methyl end.

Results and discussion

SMA characterization

Determination of CSH and flocculation level of the SMA strain during growth in the miniature fermentation Assay. The SMA yeast strain exhibited a similar flocculation behaviour (Fig. 1) to another NewFlo strain tested with the ASBC Flocculation Test *(23)* in our laboratory, where a maximal flocculation level was not achieved until substantial sugar consumption had occurred *(11,12)*. However, the SMA yeast, characterized as a medium flocculent strain, was less flocculent early in the fermentation than the strain previously tested *(11,12)*. Similarly, the change in CSH during the fermentation was analogous to the previously tested NewFlo strain, but again, CSH values early in the fermentation were lower than those of the highly flocculent strain *(11,12)*. It should be noted that in the earlier studies *(11,12)* the yeast were grown in yeast extract, protein and dextrose broth (200 mL) and tall tube fermenters (1.22 m tall; 18.5 mm inner diameter), and not in the miniature fermentation assay. Despite this discrepancy in fermenter geometry, both NewFlo strains exhibited overall similar changes in flocculation and CSH behaviours during the fermentations.

Hydrophobicity studies

Free fatty acid content over the course of the miniature fermentation with a hexane/isopropanol extraction and TMS derivatization. In the GC method of Molee *et al*. *(28)*, neutral lipids, including monoglycerides, diglycerides, triglycerides, FFA and cholesterol, were resolved and quantified. In this study, when the trimethylsilylated products of the HIP extract were analysed, decanoic (10:0), dodecanoic (12:0), palmitoleic (16:1), palmitic (16:0), oleic (18:1n-9) and stearic (18:0) acids were identified (Fig. 2). Monoacylglycerols, ergosterol and squalene, were also tentatively identified based solely on spectral matches with the library (Fig. 2) but excluded from the data portrayed in Fig. 3. These results differ slightly from those of Strauss *et al*. *(30)* who did not detect 10:0 and 12:0 in a NewFlo strain; however, those authors employed a transesterification approach that does not derivatize FFA. Note that, while traces of 3-OH 10:0 were evident, they were not present in sufficient amounts to accurately integrate and/or quantify.

At all times, 16:1 and 16:0 were the most predominant fatty acids; however, their relative proportions changed over the fermentation (Fig. 3). Early in the fermentation 16:1 and 16:0 were present in similar relative amounts, while at 24 h 16:1 was less abundant and from 36 h onwards 16:0 was present in greater relative amounts. Interestingly, this flux corresponds to the onset of flocculation where flocculation of the SMA strain reached near maximal levels by 36 h fermentation time (Fig. 1) and could implicate the proportion of 16:1/16:0 as a marker of flocculence. Strauss *et al*. *(30)* also noted that the level of 16:1 decreased to a minimum as the cells approached stationary phase and increased thereafter but also could not offer any explanation for this phenomenon.

Fatty acid and 3-OH oxylipin content over the course of the miniature fermentation with an EA extraction and TMS derivatization. In a previous communication *(31)* we reported that we were unable to detect 3-OH oxylipins in the SMA yeast strain using a HIP extraction and an acid-catalysed transesterification. This method results in the formation of methyl esters of both free and esterified FA. Kock *et al*. *(32)* subsequently suggested that we employ an approach that targeted FFA specifically to find 3-OH oxylipins. In earlier works by these authors and coworkers *(14,27)*, they utilized a two-step derivatization where 3-OH oxylipins were first methylated at the carboxylic acid functional group with diazomethane and then trimethylsilylated at the β-hydroxyl group with an appropriate reagent. While this technique has been used in numerous earlier applications, diazomethane has increasingly fallen into disuse for safety reasons. Interestingly, there is also a suggestion that it may result in partial transesterification of glycerol esters *(33)*. Instead, we elected to trimethylsilylate the total lipid extract, which would add a TMS group to the carboxylic acid of FFA and to β-hydroxyl groups of any 3-OH oxylipins present. This derivatization technique has the advantage of being unreactive with esterified FA; thus, only FFA and other structures bearing a hydroxyl group will undergo reaction. Trimethylsilylation has been used by previous researchers to resolve FFA from other

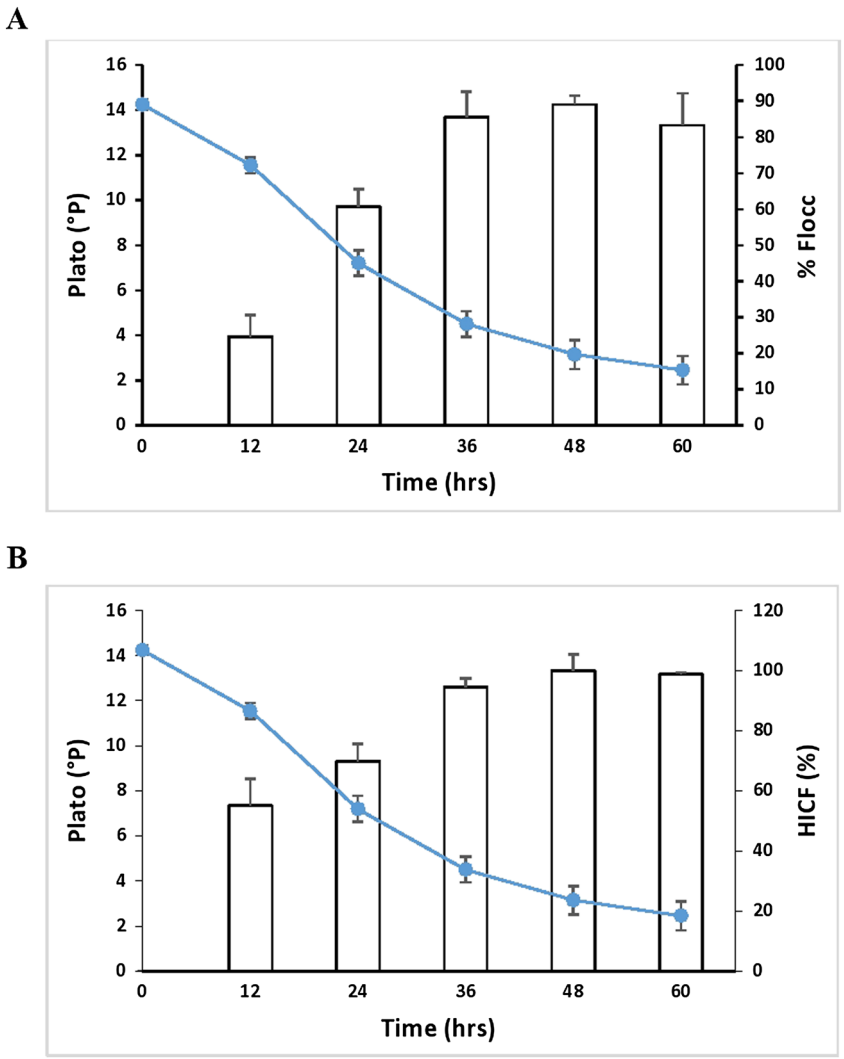

Figure 1. Fermentation properties of the SMA yeast strain grown in the miniature fermentation assay *(23)*. Fermentations were conducted in duplicate and error bars represent one standard deviation from the mean. 1A. Flocculation level measured with the ASBC Flocculation Test *(23)* (bar graph) and fermentation progress measured on the Plato (°P) scale (line). IB. Cell Surface Hydrophobicity determined by the method of Akiyama-Jibiki *et al. (9)* (bar graph) and fermentation progress measured on the Plato (°P) scale (line). This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

lipid species *(34)*, with the drawbacks that TMS esters and ethers do not store well and are prone to hydrolysis *(35)*.

When lipids were extracted from the SMA yeast strain at 12, 24, 36, 48 and 60 h growth with an EA extraction and trimethylsilylated as described, 3-OH 10:0 was detected. In other studies investigating 3-OH oxylipins in yeasts, methylation and methylation plus trimethylsilylation produced characteristic spectral peaks in GC-MS when the molecule fragmented at the β-carbon (Table 1). This fragmentation pattern was also observed in this study and we identified a spectral peak at 232 *m/z* as indicative of 3-OH oxylipins with two TMS moieties. At 12 h of fermentation, there were chromatographic peaks that eluted at the retention time of 3-OH 10:0, but the peak area was below our detection limit (Fig. 4). The onset then of detectable levels of 3-OH 10:0 at 24 h of fermentation was 12 h before elevated flocculation levels were achieved (Fig. 1). This observation could imply that these compounds play some role in brewing yeast flocculation, as has been suggested by both Kock *et al. (14)* and Strauss *et al. (27)*.

In our previous letter *(31)* we hypothesized that 3-OH oxylipins that formed at the cell exterior might have increased the CSH leading to increased flocculence. Indeed, other authors have suggested that these short-chained hydroxylated fatty acids on the cell surface could impart a hydrophobic effect *(36)*. Yet parts of this work would indicate that these short-chained 3-OH oxylipins, such as 3-OH 10:0, may not be hydrophobic after all, particularly as free molecules not associated with the cell. In this study, the 3-OH 10:0 standard was not soluble in non-polar hexane, and required a solvent of more intermediate polarity, such as chloroform, ethanol or methanol, for dissolution. Similarly, when we employed the HIP extraction to extract lipids from the SMA strain, we noted poor recovery of 3-OH 10:0 because this molecule is more strongly solvated by isopropanol and the subsequent isopropanol–water mixture than hexane. Furthermore, with the HIP extraction we could obtain positive mass spectral identifications for 3-OH 10:0 but the chromatographic peaks were so minimal they could not be accurately integrated.

Other researchers who have used a HIP extraction to detect oxylipins in plant material reported that jasmonic acid, a 12 carbon fatty acid containing a pentane ring and carbonyl functionality, partially partitioned into the lower isopropanol layer in

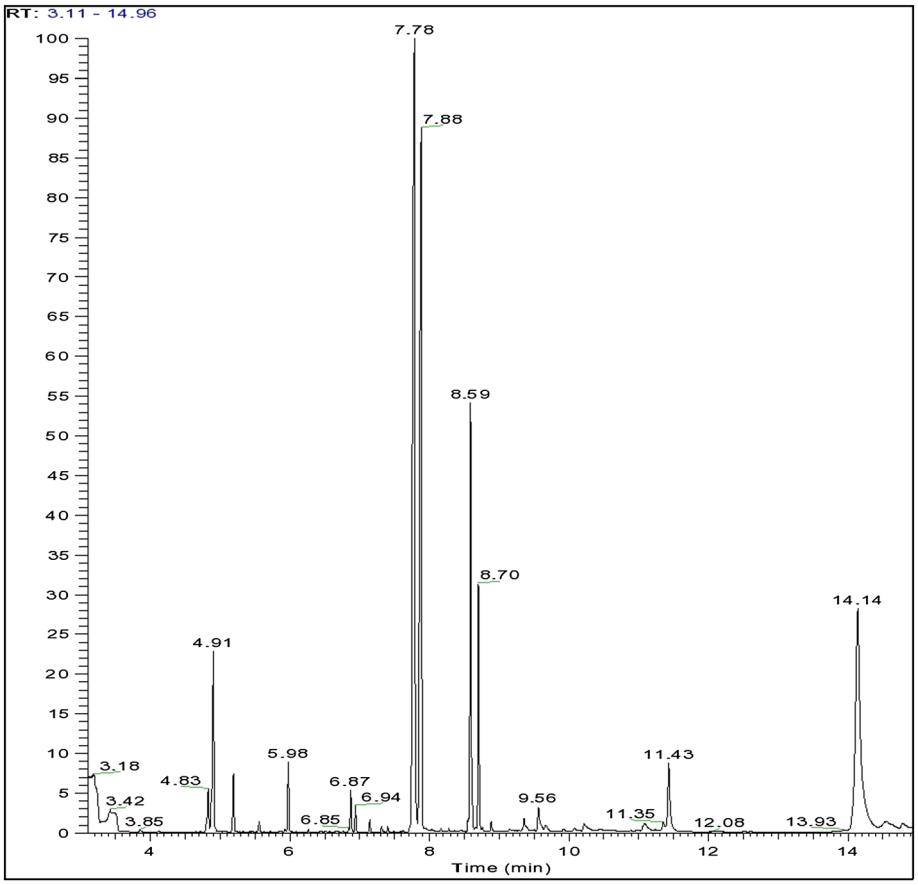

Figure 2. A chromatogram showing resolution of neutral lipids extracted from the SMA strain with a HIP extraction and derivatized as TMS esters, except for squalene. Neutral lipids detected included 10:0 (4.91), 12:0 (5.98), 16:1 (7.78), 16:0 (7.88), 18:1n-9 (8.59), 18:0 (8.70), squalene (11.43) and ergosterol (14.14). This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

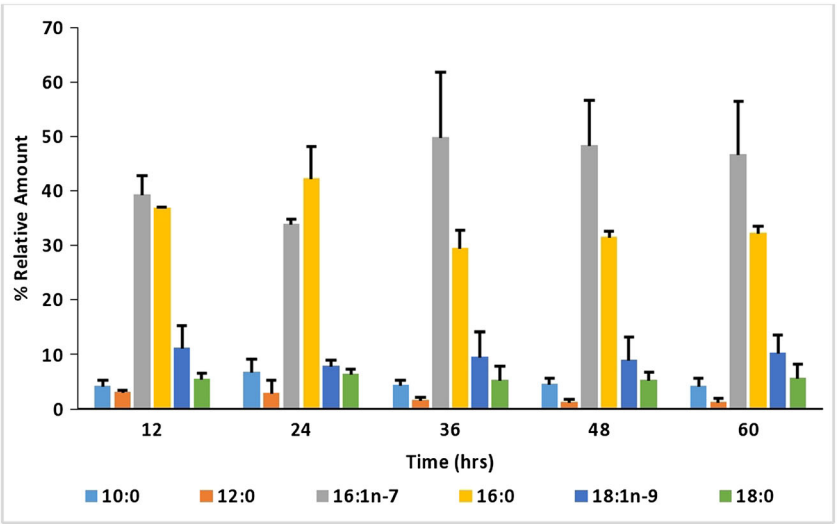

Figure 3. Relative amounts of FFA in the SMA yeast strain during growth in the miniature fermentation at 12, 24, 36, 48 and 60 hours growth time. Lipids were extracted from the yeast pellets with a HIP extraction. Fermentations and measurements were conducted in duplicate and error bars represent one standard deviation from the mean. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

addition to the upper hexane-rich layer with the majority of other lipids (personal communication, Dr Ivo Feussner, University of Goettingen), preventing their complete extraction. Thus, the extraction efficiency of these compounds was reduced. We encountered the same problem when the recovery of 50 μg of the 3-OH 10:0 standard was tested in the HIP extraction. Chappell *et al*. *(37)* also reported that the use of *n*-hexane as the extraction solvent gave low recoveries of γ-hydroxy butyric acid during a liquid–liquid extraction, further supporting the idea of polar oxylipins and their minimal role in determining CSH.

Table 1. Past research on 3-OH oxylipins in yeasts and the diagnostic peaks produced in GC-MS by fragmentation at the β-carbon

Authors	Derivatization at carboxyl end	Derivatization at β-hydroxyl group	*m/z* of characteristic fragment	*m/z* of base peak
Vesonder *et al. (41)* and van Dyk *et al. (42)*	Methylation	None	103	103
Van Dyk *et al. (42)*	Methylation	Trimethylsilylation	175	175
Strauss *et al. (27)*	Methylation	Trimethylsilylation	175	231/73
Current study	Trimethylsilylation	Trimethylsilylation	232	147

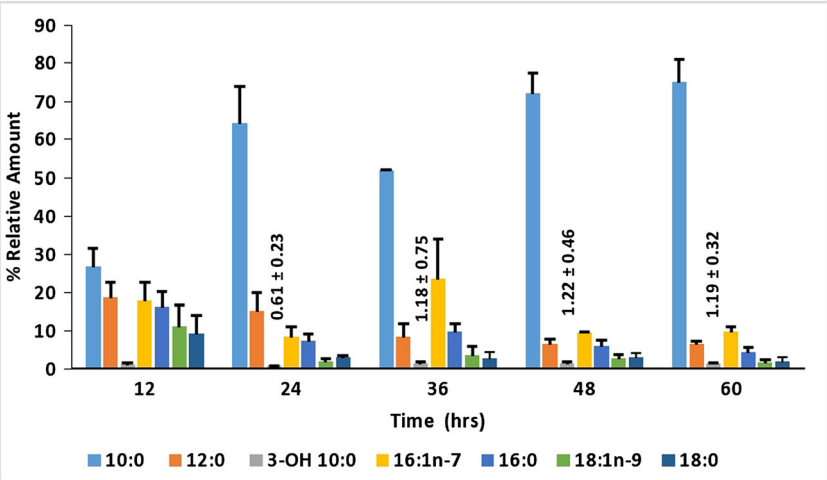

Figure 4. Relative amounts of FFA in the SMA yeast strain during growth in the miniature fermentation at 12, 24, 36, 48 and 60 hours growth time. Lipids were removed from the yeast pellets with an EA extraction. Fermentations and measurements were conducted in duplicate and error bars represent one standard deviation from the mean. This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

Given then that these short-chained 3-OH oxylipins may not be particularly hydrophobic, there are several other reports in the literature that could provide insight as to their function in *S. cerevisiae* and *S. pastorianus* flocculation. Specifically, oxylipins have been implicated as potent mediators of quorum sensing in the fungi *Candida albicans (38)* and *Aspergillus nidulans (39)*. With this in mind, we also monitored the level of flocculation in a 15 mL test tube of a 24 h shake flask culture of the SMA strain in the presence of 25 μg/mL 3-OH 10:0, a concentration in excess of those used by Nigam *et al. (38)* and Affeldt *et al. (39)*. It was notable that the rate of sedimentation in the tube with 3-OH 10:0 did not differ from a control between 0 and 60 min, monitored at 5 min intervals. However, in future work the expression of flocculation controlling FLO genes should be monitored during exposure to 3-OH 10:0 as Nigam *et al. (38)* noted an up-regulation of gene transcripts in *C. albicans* within a 30 min subjection to a 3-OH oxylipin.

In an earlier study, Lafon-Lafourcade *et al. (40)* noted the production of octanoic (8:0) and decanoic (10:0) acids during wine must fermentation. These authors demonstrated that cell counts were reduced by the addition of these acids to the fermenting medium. Interestingly, they showed that the addition of cell ghosts to the ferment reduced the concentration of these short-chained fatty acids and increased the cell count. This suggests that accumulation of 8:0 and 10:0 (and their 3-OH oxylipins) in the extracellular environment in threshold concentrations could have an inhibitory effect. Whether this is an inhibitory effect or some unknown function, in this work we were unable to conclusively demonstrate that oxylipins play a particular role in brewing yeast flocculation. However, this study does present a defined EA extraction and GC-MS detection protocol for 3-OH oxylipins in *S. pastorianus*. We believe that these methods, in conjunction with approaches to more specifically probe the cell wall architecture, will help to better elucidate the specific role of 3-OH oxylipins in brewing yeast flocculation.

Conclusions

Earlier studies of 3-OH oxylipins in *S. cerevisiae* successfully detected these compounds by methylation with diazomethane or by diazomethane methylation followed by trimethylsilylation. Owing to the danger and scarcity of diazomethane, other derivatization approaches were considered. In this work, 3-OH oxylipins were detected in the SMA strain of *S. pastorianus* with an EA extraction, trimethylsilylation of the total lipid extract and with attention to a diagnostic 232 *m/z* fragment. Previous reports by our laboratory hypothesized that 3-OH oxylipin formation at the onset of flocculation may increase CSH. However, this study suggested that free 3-OH 10:0 in particular is not very hydrophobic. To understand the precise role of 3-OH oxylipins in brewing yeast flocculation, the methods we present must be combined with novel techniques to target the cell wall architecture.

Acknowledgements

Discovery grants from the Natural Sciences and Engineering Research Council of Canada to S.M.B. and R.A.S. supported this work. The authors would also like to acknowledge assistance from the Heriot–Watt Global Research Leader programme given to R.A.S.

Conflict of interest statement

The authors have declared no conflict of interest.

References

1. Speers, R. A. (2012) A review of yeast flocculation, in *Proceedings of the 2nd International Brewers Symposium: Yeast Flocculation, Vitality and Viability* (Speers, R. A. Ed.), Master Brewers Associations of the Americas, St Paul, MN.
2. Iimura, Y., Hara, S., and Otsuka, K. (1980) Fatty acids as hydrophobic substance on cell surface of film strain of *Saccharomyces*, *Agric. Biol. Chem. 44*, 1223–1229.
3. Kamada, K., and Murata, M. (1984) On the mechanism of brewer's yeast flocculation, *J. Agric. Soc. Jpn 48*, 2423–2433.
4. Amory, D. E., and Rouxhet, P. G. (1988) Flocculence of brewery yeasts and their surface properties: Chemical composition, electrostatic charge and hydrophobicity, *J. Inst. Brew. 94*, 79–84.
5. Mozes, N., Amory, D. E., Leonard, A. J., and Rouxhet, P. G. (1989) Surface properties of microbial cells and their role in adhesion and flocculation, *Colloids Surf. 42*, 313–329.
6. Mestdagh, M. M., Rouxhet, P. G., and Dufour, J. P. (1990) Surface chemistry and flocculation of brewery yeast, *Ferment 3*, 31–37.
7. Straver, M. H., Kijne, J. W., and Smit, G. (1994) Induced cell surface hydrophobicity influences flocculation of brewer's yeast in a flocculation assay, *Colloid. Surf. B: Biointerfaces 2*, 173–180.
8. Straver, M. H., and Kijne, J. W. (1996) A rapid and selective assay for measuring cell surface hydrophobicity of brewer's yeast cells, *Yeast 12*, 207–213.
9. Jibiki, M., Ishibiki, T., Yamashita, H., and Eto, M. (1997) A rapid and simple assay to measure flocculation in brewer's yeast, *Tech. Q. Master Brew. Assoc. Am. 34*, 278–281.
10. Jin, Y., Ritcey, L. L., Speers, R. A., and Dolphin, P. J. (2001) Effect of cell surface hydrophobicity, charge, and zymolectin density on the flocculation of *Saccharomyces cerevisiae*, *J. Am. Soc. Brew. Chem. 59*, 1–9.
11. Speers, R. A., Wan, Y., Jin, Y., and Stewart, R. J. (2006) Effect of fermentation parameters and cell wall properties on yeast flocculation, *J. Inst. Brew. 112*(3), 246–254.
12. Jin, Y.-L. (1999) Flocculation of brewing yeast: Effects of cell surface hydrophobicity, zymolectin and environmental factors. MSc thesis, Dalhousie University, Halifax, Canada.
13. Lipke, P. N., and Ovalle, R. (1998) Cell wall architecture in yeast: New structure and new challenges, *J. Bacteriol. 180*, 3735–3740.
14. Kock, J. L. F., Venter, P., Smith, D. P., Van Wyk, P. W. J., Coetzee, D. J., Pohl, C. H., Botha, A., Ridel, K.-H., and Nigam, S. (2000) A novel oxylipin-associated 'ghosting' phenomenon in yeast flocculation, *Antonie Van Leeuwenhoek 77*, 401–406.
15. Klis, F. M., Boorsma, A., and De Groot, P. W. J. (2006) Cell wall construction in *Saccharomyces cerevisiae*, *Yeast 23*, 185–202.
16. Smit, G., Straver, M. H., Lugtenberg, B. J. J., and Kijne, J. W. (1992) Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant, *Appl. Environ. Microbiol. 58*, 3709–3714.
17. Guo, B., Styles, C. A., Feng, Q., and Fink, G. R. (2000) A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating, *Proc. Natl. Acad. Sci. U. S. A. 97*, 12158–12163.
18. Kang, S., and Choi, H. (2005) Effect of surface hydrophobicity on the adhesion of *S. cerevisiae* onto modified surfaces by poly (styrene-ransulfonic acid) random copolymers, *Colloids Surf. B 46*, 70–77.
19. Speers, R. A., Tung, M. A., Durance, T. D., and Stewart, G. G. (1992) Biochemical aspects of yeast flocculation and its measurement: A review, *J. Inst. Brew. 98*, 293–300.
20. Sato, M., Watari, J., and Shinotsuka, K. (2001) Genetic instability in flocculation of bottom-fermenting yeast, *J. Am. Soc. Brew. Chem. 59*, 130–134.
21. Speers, R. A. (1991) Rheological and colloidal properties of brewing yeast suspensions, PhD thesis, University of British Columbia, Vancouver, BC.
22. Speers, R. A., Durance, T. D., Odense, P., Owen, S., and Tung, M. A. (1993) Physical properties of commercial brewing yeast suspensions, *J. Inst. Brew. 99*, 159–164.
23. American Society of Brewing Chemists. (2013) Methods of Analysis, 14th ed., Yeast-11B Absorbance method, -14 Miniature fermentation assay, The Society, St Paul, MN.
24. Lake, J. C., Speers, R. A., Porter, A. V., and Gill, T. A. (2008) Miniaturizing the fermentation assay: Effects of fermentor size and fermentation kinetics on detection of premature yeast flocculation, *J. Am. Soc. Brew. Chem. 66*, 94–102.
25. Albrecht-von-Haller Institute for Plant Sciences-Oxylipin Profiling Database. (2005) HIP extraction. http://www.oxylipins.uni-goettingen.de/methods.php?path=hip_f&caption=HIP%20Extraction (last accessed October 2014)
26. Salmon, R. A., and Flower, R. J. (1982) Extraction and thin layer chromatography of arachidonic acid metabolites, *Meth. Enzymol. 86*, 477–493.
27. Strauss, C. J., Kock, J. L. F., Van Wyk, P. W. J., Lodolo, E. J., Pohl, C. H., and Botes, P. J. (2005) Bioactive oxylipins in *Saccharomyces cerevisiae*, *J. Inst. Brew. 111*, 304–308.
28. Molee, W., Bouillier-Oudot, M., Auvergne, A., and Babilé, R. (2005) Changes in lipid composition of hepatocyte plasma membrane induced by overfeeding in duck, *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 141*, 437–444.
29. Sparkman, O. D., Penton, Z. E., and Kitson, F. G. (2011) *Gas Chromatography and Mass Spectrometry: A Practical Guide*, Academic Press, Oxford.
30. Strauss, C. J., Kock, J. L. F., Viljoen, B. C., Botes, P. J., Hulse, G., and Lodolo, E. (2004) Lipid turnover during inverse flocculation in *Saccharomyces cerevisiae* U0FS Y-2330, *J. Inst. Brew 110*, 207–212.
31. Potter, G., Speers, R. A., and Budge, S. M. (2013) 3-OH oxylipins in *Saccharomyces cerevisiae*, *J. Inst. Brew 119*, 85.
32. Kock, L., Swart, C., and Pohl, C. (2013) Response: 3-OH oxylipins in *Saccharomyces cerevisiae*, *J. Inst. Brew 119*, 201.
33. Ord, W. O., and Bamford, P. C. (1967) The thin layer chromatographic separation of fatty acid methyl esters and glycerides according to the chain length and unsaturation, *Chemistry and Industry*, London, 277–278.
34. Myher, J. J., and Kuksis, A. (1984) Determination of plasma total lipid profiles by capillary gas–liquid chromatography, *J. Biochem. Biophys. Meth. 10*, 13–23.
35. Kuksis, A., Meyher, J. J., Marai, L., and Geher, K. (1976) Estimation of the plasma free fatty acids as the trimethylsilyl (TMS) esters, *Anal. Biochem. 70*, 302–312.
36. Leeuw, N. J., Kock, J. L. F., Pohl, C. H., Bareetseng, A. S., Sebolai, O. M., Joseph, M., Strauss, C. J., Botes, P. J., Van Wyk, P. W. J., and Nigam, S. (2006) Oxylipin covered ascospores of *Eremothecium coryli*, *Antonie Van Leeuwenhoek 89*, 91–97.
37. Chappell, J. S., Meyn, A. W., and Ngim, K. K. (2004) The extraction and infrared identification of *gamma*-hydroxy butyric acid (GHB) from aqueous solutions, *J. Forensic Sci. 49*, 1–8.
38. Nigam, S., Ciccoli, R., Ivanov, I., Scezpanski, M., and Deva, R. (2011) On mechanism of quorum sensing in *Candida albicans* by 3(*R*)-hydroxytetradecaenoic acid, *Curr. Microbiol. 62*, 55–63.
39. Affeldt, K. J., Brodhagen, M., and Keller, N. P. (2012) *Aspergillus* oxylipin signalling and quorum sensing pathways depend on G-protein coupled receptors, *Toxins, 4*, 695–717.
40. Lafon-Lafourcade, S., Geneix, C., and Riberau-Gayon, P. (1984) Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by ghosts, *Appl. Environ. Microbiol. 47*, 1246–1249.
41. Vesonder, R. F., Wickerham, L. J., and Rohwedder, W. K. (1968) 3-D-Hydroxypalmitic acid: A metabolic product of the yeast NRRL Y-6954, *Can. J. Chem., 46*, 2628–2629.
42. Van Dyk, M. S., Kock, J. L. F., Coetzee, D. J., Augustyn, O. P. H., and Nigam, S. (1991) Isolation of a novel arachidonic acid metabolite 3-hydroxy-5, 8, 11, 14-eicosatetranoic acid (3-HETE) from the yeast *Dipodascopsis unicleata* UOFs-Y128, *FEBS J. 283*, 195–198.