

Bioactive Oxylipins in *Saccharomyces cerevisiae*

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ABSTRACT

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Some strains of *Saccharomyces cerevisiae* (including strains used in fermentation processes) produce short chain (mainly 8 carbon) oxylipins and not potent inflammatory long chain (20 carbon) oxylipins such as prostaglandins. When acetylsalicylic acid (aspirin) was added to cultures of *Sacch. cerevisiae* UOFS Y-2330, flocculation was significantly inhibited as well as the production of 3-hydroxy 8:0 thereby linking flocculation and this oxylipin. Furthermore, no traces of 3-hydroxy 8:0 could be detected at the start of flocculation in this yeast. This research is based on (i) reports that yeasts in general can produce bioactive prostaglandins, (ii) findings suggesting a link between aspirin-sensitive prostaglandins and biofilm formation by *Candida albicans*, (iii) the discovery that the addition of low concentrations of aspirin abolish yeast biofilm formation and sexual cell aggregation and (iv) the recent discovery of a novel potent aspirin-sensitive pro-inflammatory 3-hydroxy prostaglandin E₂ synthesized by *Candida albicans* in conjunction with mammalian cells probably during candidiasis.

Key words: 3-hydroxy oxylipins, aspirin, *Candida albicans*, eicosanoids, flocculation, *Saccharomyces cerevisiae*.

INTRODUCTION

Oxylipins is the collective name for oxygenated lipids and include an important group of oxygenated 20 carbon compounds in mammals namely the eicosanoids. The latter are potent modulators of the immune response and play a role in many basic host physiological processes. Some eicosanoids such as the prostaglandins (PGs) may cause inflammation and are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as acetyl salicylic acid (aspirin)^{8,14}. The PGs are produced by aspirin-sensitive enzymes i.e. cyclooxygenase 1 (COX-1, a constitutive enzyme involved in hemostasis) and cyclooxygenase 2 (COX-2, an inducible enzyme expressed in inflammatory diseases and cancer)⁵.

Since the early 1990's, various aspirin-sensitive 3-hydroxy fatty acids (3-OH oxylipins) associated with nano-scale ornamentations on the cell walls of vegetative and sexual yeast cells were uncovered⁹. Here they serve as cell adhesives (flocculants) and to affect water-propelled movement in micron-space¹⁰. It was found that the addition of low concentrations of aspirin inhibited, in a dose dependent manner, both 3-OH oxylipins present on the surfaces of sexual cells of the yeast *Dipodascopsis* as well as the ability of these cells to aggregate⁹. These results also suggest that 3-OH oxylipins, present on the surfaces of flocculating cells of *Saccharomyces cerevisiae*, may play a role in flocculation¹².

Recently, clear-cut evidence for the role of 3-OH eicosanoids in candidiasis was presented⁵⁻⁷. Here, arachidonic acid (AA) is released from the infected or inflamed host tissue/cell and then converted by *Candida albicans* to 3R-hydroxy eicosatetraenoic acid (3R-HETE), which serves as substrate for COX-2 in host tissue/cell to produce pro-inflammatory 3-OH-PGE₂. Strikingly, in this investigation a cascade of novel bioactive 3-OH eicosanoids, produced from 3R-HETE via mammalian COX-2, was uncovered⁵. In addition, it was found that *C. albicans* selectively up-regulates COX-2 in mammalian cells⁷. Prostaglandins produced by COX-2 are potent bioactive compounds in inflammatory diseases and cancer. Since both 3R-HETE and COX-2-produced 3-OH PGs are inhibited by aspirin, this research also suggests new targets for the control of yeast infection.

Furthermore, the *ab initio* production of aspirin-sensitive eicosanoids such as PGs by yeasts has been suggested¹⁴. Alem and Douglas¹ demonstrated that low concentrations of aspirin drastically inhibit catheter-biofilm formation by *C. albicans*. Strikingly, when PGE₂ was added together with aspirin, the inhibitory effect of 25 µM or 50 µM aspirin was abolished. Unfortunately, only indirect immunological tests, mainly aimed at application in mammalian cells were performed to detect PGs in yeasts. Consequently, these may be error prone due to cross reactions with other molecules in yeasts. Only direct evidence, using methods such as gas chromatography-mass spectrometry (GC-MS) should be used to prove the presence of these compounds in yeasts. Such evidence does not exist at present.

With this as background it became the aim of this study to determine if oxylipins which include bioactive eicosanoids such as PGs and/or 3-OH eicosanoids are produced *ab initio* or from exogenous AA by some biotechnologically important strains of *Sacch. cerevisiae*. The ability of these yeasts to produce 3R-HETE from exoge-

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nously fed AA that may serve as precursor for bioactive COX-2-synthesized 3-OH eicosanoids in mammalian cells, were also assessed. Furthermore, the effect of aspirin on yeast flocculation and oxylipin production was investigated.

MATERIALS AND METHODS

Strains used

Saccharomyces cerevisiae (UOFS Y-2330) a known flocculent exposing both Flo1 and NewFlo flocculation behaviour¹⁵ as well as two strains used in fermentation processes (UOFS Y-1 and UOFS Y-3) and exposing NewFlo phenotype behaviour, were investigated. The yeast strains are held at the University of the Free State (UFS), Bloemfontein, South Africa.

Cultivation and oxylipin extraction in the absence of aspirin

Cultivation. All strains of *Sacch. cerevisiae* were cultivated as described by Strauss et al¹⁵. In short, cells were inoculated from YM¹⁸ agar slants into 250 mL conical flasks containing 50 mL of glucose-YM broth (12 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract) and incubated at 25°C while shaking (160 rpm) until late exponential phase was reached. Appropriate volumes were then transferred to several 500 mL side-arm conical flasks containing 100 mL of the same complex medium to yield a final absorbency of 10 Klett units (0.2 g dry weight/L). These cultures were incubated at 25°C while shaking (160 rpm) for 20 h. Growth was measured at regular intervals using a Klett Summerson colorimeter (red filter) in one of the flasks equipped with a side arm. The degree of flocculation (i.e. % Δ floc) in this culture was measured throughout growth by calculating the decrease in cell turbidity⁴. All experiments were performed in at least duplicate.

Extraction and analysis of *ab initio*-produced oxylipins by non-flocculating cells. In order to investigate the *ab initio* production and composition of 3-OH oxylipins associated with the surfaces of mainly non-flocculating cells, oxylipins in 6 h old cultures still containing cells (cultivated as described) were immediately extracted¹³ using 2× volume of ethyl acetate (Saarchem, Gauteng, South Africa) followed by evaporation of the organic phase. Extracts were derivatised (methylated and silylated) and subjected to GC-MS for analysis as described¹⁷. All experiments were performed in at least duplicate.

Extraction and analysis of *ab initio*-produced oxylipins by flocculating cells. Yeasts were cultivated as before until late stationary growth phase was reached i.e. after 20 h and cells flocculated maximally. This was followed by the immediate extraction of oxylipins, mainly associated with the cell surface and supernatant as described. Extracts were derivatised (methylated and silylated) and subjected to GC-MS for analysis¹⁷. All experiments were performed in at least duplicate.

Oxylipin production from exogenous AA. Growth experiments were repeated with the addition of AA (Sigma, St. Louis, U.S.A.) dissolved in a minimum volume of

ethanol¹⁷ to cultures at a final concentration of 100 mg/L at the onset of stationary growth phase i.e. after about 14 h. After 3 h of exposure, oxylipins were immediately extracted, derivatised and analysed as before. This was done to investigate the possible transformation of AA to PGs and 3-OH oxylipins especially 3R-HETE as found in *Dipodascopsis uninucleata*¹⁷. The same amount of ethanol without AA was added to control flasks and were subjected to the same cultivation, oxylipin extraction, derivatisation and GC-MS protocols. All experiments were performed in at least duplicate.

Oxylipins in growth medium. YM broth alone, without yeasts, was subjected to oxylipin extraction, followed by derivatisation and eventual GC-MS analysis as before. All experiments were performed in at least duplicate.

Cultivation and oxylipin extraction in the presence of aspirin

Saccharomyces cerevisiae UOFS Y-2330 was cultivated for 20 h, this time in the presence of aspirin (ASA) while growth and degree of flocculation were continuously measured as described. Aspirin (Sigma, Steinheim, Germany) was first diluted in a minimal volume ethanol and added to individual flasks at the start of cultivation to reach a final concentration of 1 mM. Next, the pH of the medium was adjusted to 5.6 by titration with 1 M NaOH. The yeast was cultivated for 20 h, until cells flocculated maximally. This was followed by the immediate extraction, derivatisation and GC-MS analysis of oxylipins as described. Control flasks contained the same amount of ethanol without ASA and were subjected to the same cultivation, oxylipin extraction, derivatisation and GC-MS protocols. All experiments were performed in at least triplicate.

Gas chromatography–mass spectrometry (GC-MS) analysis

A Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jose, California, U.S.A.) was used to record the EI mass spectra of the extracted 3-OH oxylipins and PGs present in all yeast cultures respectively. This instrument is equipped with a Finnigan Trace DSQ MS (Thermo Electron Corporation, San Jose, California, U.S.A.), with an HP-5-60m fused silica capillary column (0.32 μm i.d. and 0.1 μm coating thickness). Helium was used as a carrier gas at a constant flow of

Table I. Oxylipins produced *ab initio* and from arachidonic acid (AA) by flocculating cells of *Saccharomyces cerevisiae* UOFS Y-2330 and strains UOFS Y-1 and UOFS Y-3.

Yeast strain	3-Hydroxy oxylipins
UOFS Y-2330	
<i>Ab initio</i>	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
From AA	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
UOFS Y-1	
<i>Ab initio</i>	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
	3-OH 10:0 175; 259[M ⁺ -15 (CH ₃)]
From AA	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
UOFS Y-3	
<i>Ab initio</i>	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
From AA	3-OH 8:0 175; 246[M ⁺]; 231[M ⁺ -15 (CH ₃)]
YM	3-OH 12:1 175; 285[M ⁺ -15 (CH ₃)]

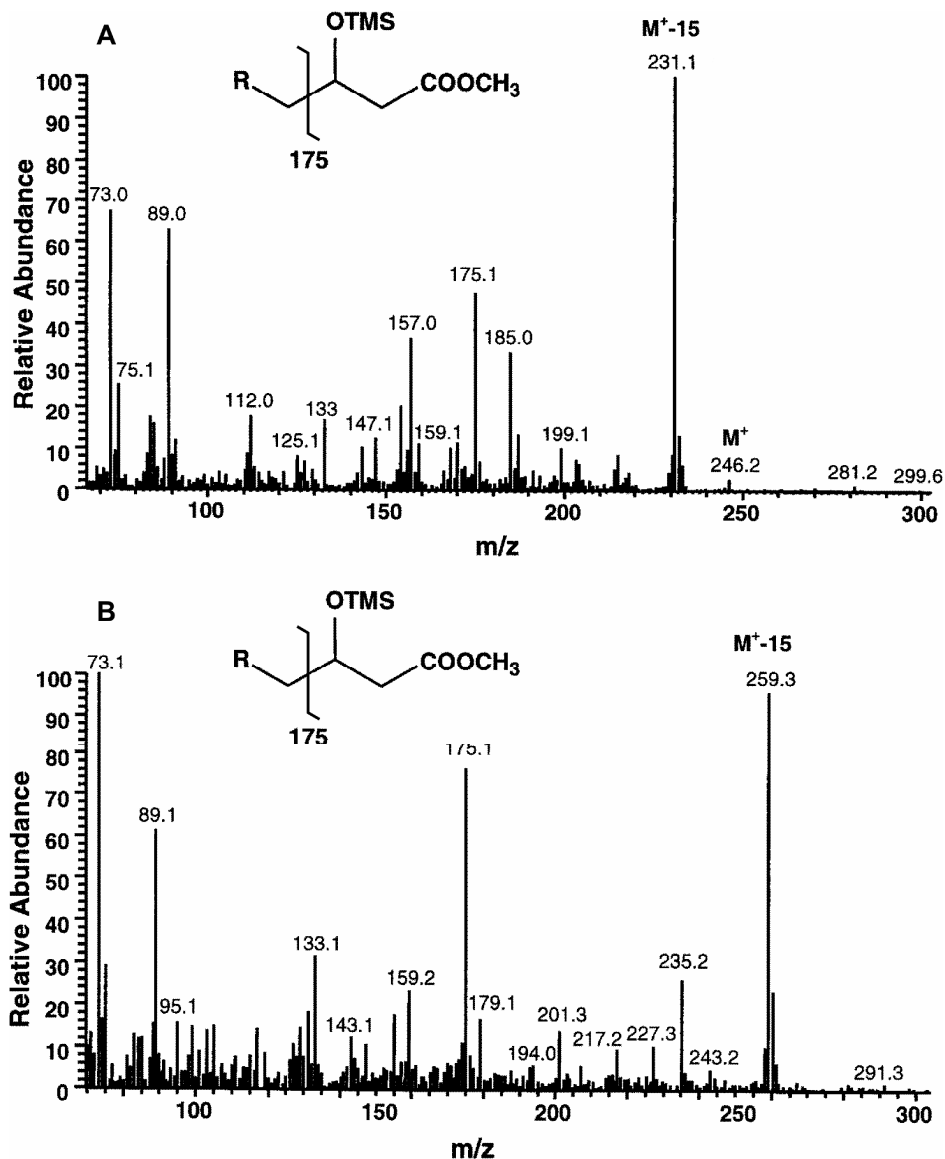


Fig. 1. Electron impact-mass spectra of methyl-trimethylsilylated 3-OH 8:0 (A) and 3-OH 10:0 (B). The characteristic m/z 175¹⁷ was derived from the esterified carboxyl end.

1 mL/min. The initial oven temperature of 110°C was held for 2 min before it was increased by 3°C/min to a final temperature of 300°C. The MS was auto-tuned to m/z 50–1050. A sample volume of 1 μ L was injected at an inlet temperature of 230°C at a split ratio of 1:50.

Chemicals used

All chemicals used were of highest purity grade.

RESULTS AND DISCUSSION

Oxylipin production in the absence of aspirin

In this study we could not find evidence of any PGs in the three flocculating yeast strains studied (Table I) that were produced *ab initio* or from AA. This is contrary to the findings based on immunological assays (Radio Immuno Assay and Enzyme-linked Immunosorbent Assay)

where the presence of particularly PGE₂ and PGF_{2 α} were reported in some yeasts including *Sacch. cerevisiae*¹⁴. In addition, our results suggest (Table I) that the *Sacch. cerevisiae* strains are incapable, under the conditions tested, to produce 3*R*-HETE (*ab initio* or from exogenously fed AA) necessary for the synthesis of inflammatory COX-2-produced 3-OH PGs in mammalian cells. Since no known inflammatory eicosanoids or COX-2 precursors were detected, our results affirm the GRAS-status of the biotechnologically important *Sacch. cerevisiae* strains tested.

Using GC-MS, only 3-OH oxylipins with less than 20 carbons (ranging from 8 to 10 carbons) were found to be associated with all yeasts tested after 20 h of growth (Table I). All the yeast strains, produced mainly 3-OH 8:0 *ab initio* or upon addition of AA, while strain UOFS Y-1 also produced 3-OH 10:0 *ab initio* (Figs. 1, 2). This is in accordance with results reported earlier in another strain of *Sacch. cerevisiae*¹². Since 3-OH 8:0 has only been re-

ported in *Sacch. cerevisiae* so far^{9,12}, the conserved status and value of this novel oxylipin as a taxonomic marker for *Sacch. cerevisiae* strains should be assessed.

Oxylipin production in the presence of aspirin

In order to determine if aspirin inhibits 3-OH oxylipin production as well as cell flocculation as reported in other yeasts⁹, aspirin inhibition studies were performed on strain

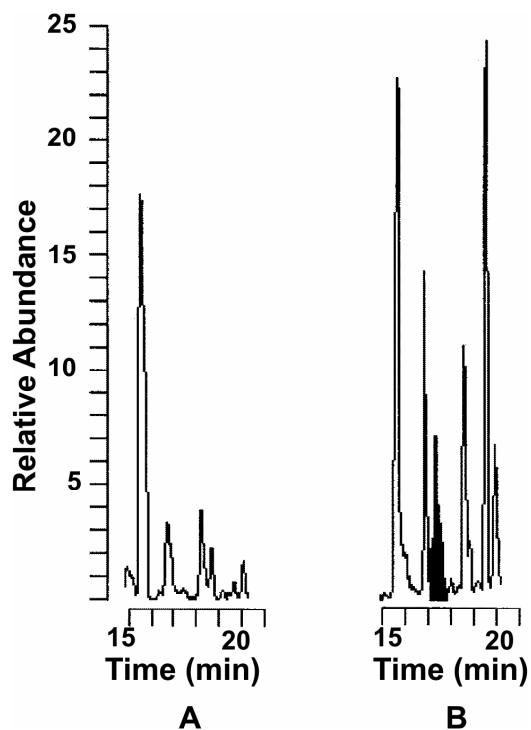


Fig. 2. Partial ion chromatograms of the methylated and trimethylsilylated extracts from cultures of *Saccharomyces cerevisiae* UOFS Y-2330 during the non-flocculent (6 h, A) and flocculent phases (20 h, B) of growth. Solid peak indicates 3-OH 8:0.

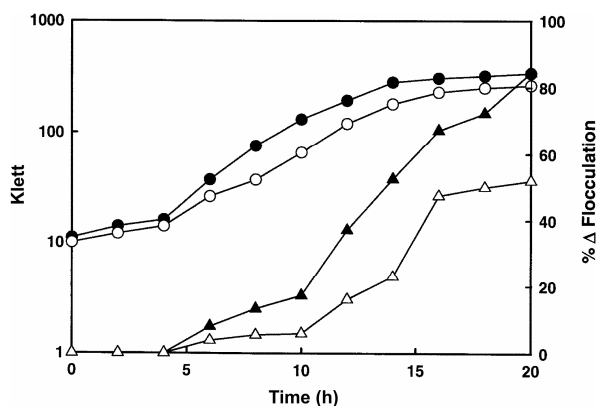


Fig. 3. Changes in growth (Klett units; circle) and degree of flocculation (% Δ flocculation; triangle) over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330, when cultivated in a complex YM medium in the presence (hollow signs) and absence (solid signs) of 1 mM of acetylsalicylic acid (aspirin). This experiment was performed in duplicate and produced similar patterns.

UOFS Y-2330. We found that, in the presence of 1 mM aspirin, the production of 3-OH 8:0 was totally inhibited while a 30% (SE < 5%) reduction in the % Δ flocculation occurred (Fig. 3). Strikingly, this corresponds with the absence of 3-OH 8:0 in poorly flocculating cells obtained after 6 h of growth (Fig. 2). These results clearly show a link between 3-OH 8:0 and the flocculation of cells in strain UOFS Y-2330. This is in accordance with that reported in the yeast *Dipodascopsis uninucleata*^{3,9,11} where sexual cell aggregation is linked to the production of polyunsaturated longer chain 3-OH oxylipins such as 3-HETE. Strikingly, both cell aggregation and 3-OH oxylipin production in *D. uninucleata* is inhibited by aspirin in a dose dependant manner. We found that the addition of ethanol without aspirin to control cultures in this study showed no effect when compared to cultures grown in the absence of both aspirin and ethanol.

Our findings therefore not only affirmed the GRAS-status of the biotechnologically important *Sacch. cerevisiae* strains, but also found a strong link between the presence of 3-OH 8:0 and flocculation of *Sacch. cerevisiae* UOFS Y-2330. Is it possible that 3-OH 8:0 is a conserved characteristic with an important function in all flocculating *Sacch. cerevisiae* strains? What is the biological role of this fascinating oxylipin together with lectins^{15,16} in the flocculation process? What is the effect of oxylipins on cell morphology as observed during the ageing of these cells²? Will it be possible to partially control yeast flocculation in fermentation processes by using compounds similar to aspirin and 3-OH 8:0? It is hoped that answers to these questions will help to partially control yeast flocculation in fermentation processes by using compounds similar to aspirin and 3-OH 8:0.

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