The role of lipids in the flocculation of *Saccharomyces cerevisiae*

by

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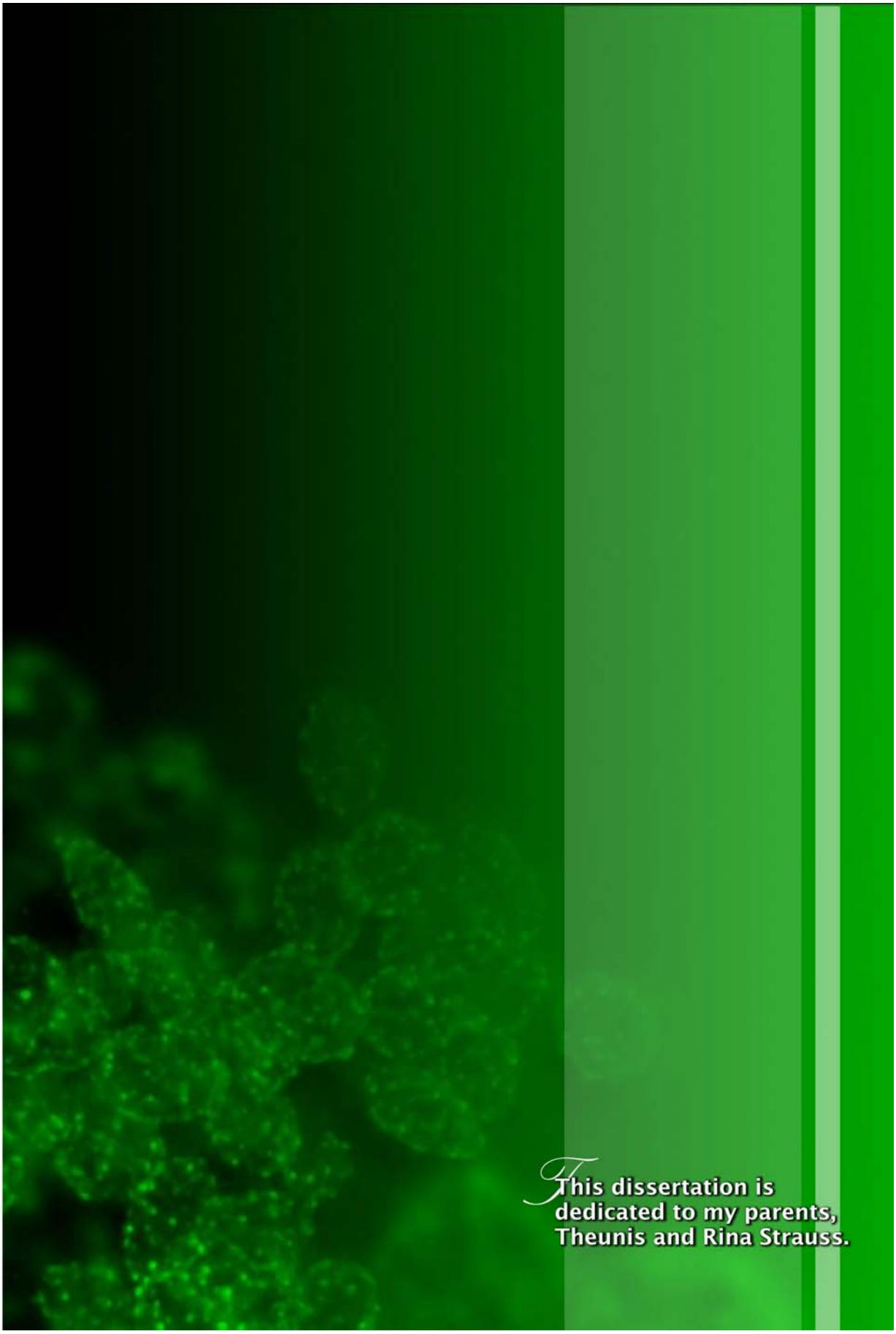
“The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka” but That’s funny…”

Isaac Asimov


This dissertation is
dedicated to my parents,
Theunis and Rina Strauss.

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Glory to* God *our creator, without whom none of this would have been possible.

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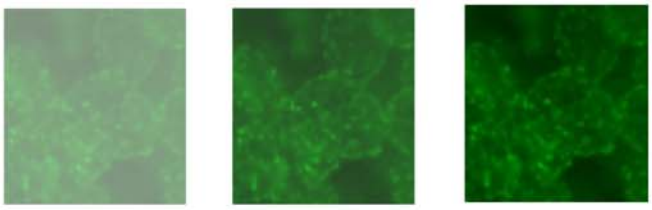
Chapter 1

Introduction



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1. MOTIVATION

Of the more than 600 species of known yeasts, *Saccharomyces cerevisiae* is the most widely used [1]. This yeast mainly finds application in the brewing of beer and production of wines. In these fermentation-based processes, suspended cells are normally separated from the medium through flocculation, before further processing may occur [2]. Lately, there has been considerable interest in flocculation as a natural and cheap method for cell separation in industrial fermentations. Although brewers and researchers have been exploring the mystery surrounding control of yeast flocculation for over a century, the causative mechanism of initiation is still unknown [2-5].

Currently, the lectin theory is accepted to explain flocculation [6]. On the basis of the lectin theory, flocculent *Sacch. cerevisiae* strains can be differentiated mainly into Flo1 and NewFlo flocculation phenotypes using sugar inhibition studies [7]. According to literature, a flocculent *Sacch. cerevisiae* strain demonstrates either Flo1 or NewFlo phenotype flocculation behavior, but never both [3,8]. During bioprospecting studies for flocculent *Sacch. cerevisiae* strains, a unique *Sacch. cerevisiae* strain was uncovered in this study, which demonstrated both Flo1 and NewFlo phenotype flocculation behaviour. Consequently the first aim of this study became to study these inverse flocculation patterns of this unique strain.

Next to the lectin theory, recent evidence pointed towards a possible role of lipids associated with the cell surfaces during flocculation [2,4]. This finding was further supported by Kock and co-workers who reported on the accumulation of hydrophobic carboxylic acids i.e. 3-hydroxy oxylipins (3-OH oxylipins) on the cell surfaces of flocculent *Sacch. cerevisiae* cells, during flocculation onset [9]. Furthermore, it has been proposed that these 3-OH oxylipins are produced through incomplete ß-oxidation, probably in the mitochondria of fungi [9,10]. Importantly, mitochondrial function has been implicated in the flocculation process [5,11-15]. Interestingly, it was found that the addition of low concentrations of aspirin (a known 3-OH oxylipins inhibitor) inhibited both 3-OH

oxylipins present on the surfaces of sexual cells as well as the ability of these cells to aggregate in *Dipodascopsis uninucleata* [16]. These results suggest that 3-OH oxylipins, present on the surfaces of flocculating vegetative cells of *Sacch. cerevisiae*, may play a role in flocculation [9]. Strikingly, 3-OH oxylipins were also found to play an important role in yeast cells to become infectious and to aggregate in biofilms during human infection [17-20].

As a result of the above mentioned, it became another aim of this study to determine the role of lipids, especially 3-OH oxylipins during the flocculation process of this unique strain. Here the lipid turnover during flocculation as well as the biological function of cell wall-associated oxylipins were studied. In addition, the involvement of these oxylipins in co-flocculation was also assessed.

2. BACKGROUND

There are three main mechanisms that give rise to clumps of yeasts. These are the formation of mating aggregates, chain formation and flocculation [4,14]. The most economically important of the three, i.e. flocculation, describes a reversible, asexual and calcium–dependant property, in which cells spontaneously adhere in clumps, dispersible by specific sugars or EDTA, which are subsequently removed from the medium by sedimentation [14].

Brewers and researchers have been exploring the mysteries of yeast flocculation since the time of Pasteur [4]. Therefore, the genetic, physiological, biochemical and colloidal aspects affecting yeast flocculation have been extensively studied. However, despite all these studies on yeast flocculation, many details of the process have been subjected to debate and remains to be elucidated. Therefore, the general view of the process is still cloudy.

Until the 1960's, the effect of different environmental factors on yeast flocculation was investigated. Following this, the understanding of yeast flocculation at molecular level has been broadened by various genetic studies since the mid-1970's. Today, even after extensive research, the mechanisms of

flocculent bond formation and flocculation onset are still poorly understood. For the past two decades the mechanism of lectin like cell-cell interactions has been accepted to explain yeast flocculation [6], although there are still matters to be resolved. One such a matter is the observed increase in cell surface hydrophobicity (CSH) during flocculation onset. Since the 1980's, CSH has been identified as the second most important factor responsible for flocculation onset [21-24]. Up till now, very little is known about the influence of environmental factors on CSH and the relationship between CSH and flocculation of yeast cells [2,4]. Therefore this aspect warrants further investigation.

Since it is impossible in a study like this to give a detailed account of all the literature concerning flocculation, this part will mainly concentrate on lectin mediated flocculation as well as CSH. For more in depth discussions on other aspects of yeast flocculation, the reader is referred to a number of reviews on this topic [2-5,14,25]. In order to understand why yeast flocculation is such a "hard to control" phenomenon, a brief discussion on the underlying biochemical, genetic and physical aspects that affect this phenomenon will be attempted.

According to the lectin theory, specific lectin-like proteins (zymolectins) present on the cell wall of flocculent cells selectively bind to α-mannose residues of mannoproteins present on the cell walls of adjacent cells [6]. Calcium ions are believed to maintain the correct configuration of the zymolectin binding site [14]. Interestingly, the co-flocculation of flocculent and non-flocculent yeasts, as well as co-flocculation of bacteria and *Sacch. cerevisiae* strains, is also established through zymolectin interaction similar to those present during yeast-yeast flocculation [26,27].

Investigations on the zymolectin sugar specificity of *Sacch. cerevisiae* strains have revealed at least two distinct forms of flocculation, the Flo1 and NewFlo phenotype [7]. The characteristics of Flo1 phenotype flocculation suggest a mannose-specific zymolectin binding, whereas NewFlo phenotype characteristics indicate a broader specificity, i.e. glucose, maltose and mannose specific zymolectins [7]. In addition, Flo1 phenotype strains demonstrate

constitutive flocculence, whereas NewFlo phenotype strains only start to flocculate during the late exponentional, early-stationary phase of growth [3,8].

Like any other protein, these zymolectins are encoded by specific genes, the so-called FLO genes (Fig. 1). The best known flocculation gene is FLO1 and almost all genetic studies on flocculation so-far have involved Flo1 phenotype flocculation [2-4]. Expression of the FLO1 gene and its homologues (FLO5 and FLO9) is known to cause flocculation of the Flo1 phenotype [2]. Interestingly, NewFlo phenotype yeast strains were found to contain a LgFLO1 gene, which is not found in Flo1 phenotypes [28-30]. Therefore, it is believed that LgFLO1 encodes for a zymolectin that binds both mannose and glucose residues, and is therefore responsible for the expression of NewFlo phenotype flocculation [28-30].

When these FLO genes become active, zymolectins are formed and flocculation can take place [14]. Therefore, it seems that any factor that can cause a stimulus to activate the FLO genes may in fact trigger the onset of flocculation. Unfortunately the situation is much more complex than this. The factors that affect flocculation onset can be divided into three categories according to their mode of action (Fig. 1A-C) [2]. Furthermore, it is important to note that some factors can act through more than one mechanism.

Firstly, the FLO family consists of several different FLO genes (Fig. 1A), each of which may be regulated through different complex mechanisms and may therefore be induced or repressed by different factors [3,31]. Due to the fact that the activation of these FLO genes is a highly strain specific character, flocculation expression is dependant on the genetic background of a specific strain.

The second mechanism includes all the environmental factors that affect FLO gene expression and activation (Fig. 1B) [2]. Since the FLO gene family is very unstable, these factors are known to cause great differences in the flocculation profile and response between different yeast strains and even between different generations of a specific strain [30,32,33]. Environmental

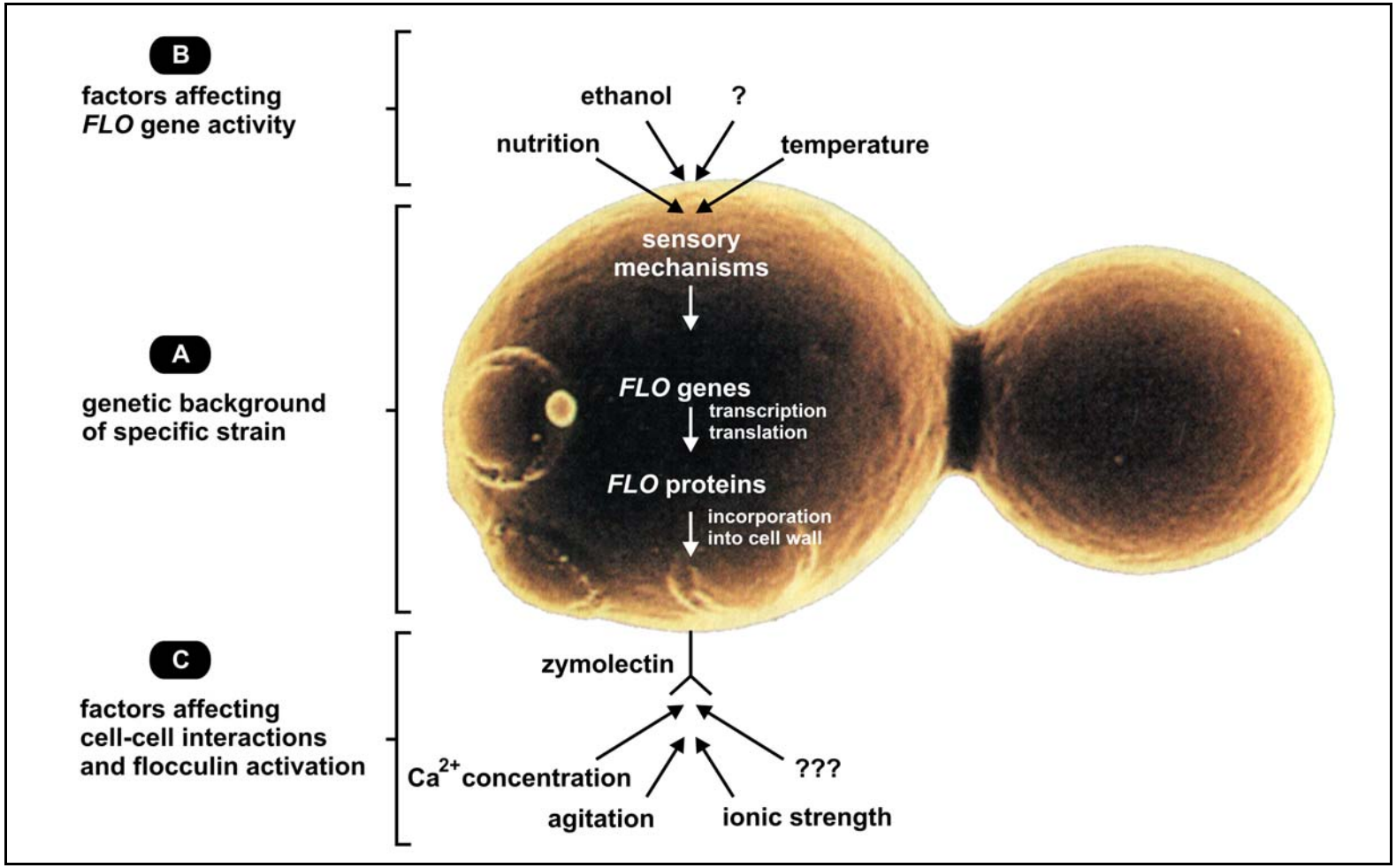

Fig. 1. Factors affecting flocculation in yeasts. Three categories (**A**, **B** and **C**) can be distinguished according to their mode of action. This figure is reproduced from [2].

factors that influence FLO gene expression include ethanol concentration and fermentation temperature. In addition, flocculation may also occur as a means of protection during adverse environmental conditions such as nutrient limitations [14,34].

Thirdly, factors that act upon the physical interactions (collisions) between yeast cells also play an important role in yeast flocculation, even though they do not influence the activity of the FLO genes (Fig. 1C) [2,5]. More specifically, factors that increase the collision rate between cells, e.g. agitation of the growth medium, may promote flocculation. Factors that decrease the cell surface charge in cell walls (i.e. decreased pH, cell age, phase of growth) or factors that increase the hydrophobic character of the yeast cell walls CSH (i.e. starvation, flocculation onset) also cause stronger flocculation, by facilitating cell-cell contact [5,14,21]. However, very little is known about the influence of environmental factors on CSH and the relationship between CSH and flocculation of yeast cells [2,4]. Therefore this aspect warrants further investigation.

Recent reports suggest that the noted increase in CSH during flocculation onset is probably due to an increase in cell wall carboxyl group density [4,24,35]. Here, a threshold value for carboxyl group density was observed. In cases where this threshold value was exceeded, flocculation was initiated [35]. Consequently, CSH has been identified as a major factor responsible for flocculation onset [21-24]. This observation was further supported by the accumulation of hydrophobic carboxylic acids i.e. 3-OH oxylipins on the cell surfaces of *Sacch. cerevisiae* strains during flocculation onset [9].

It has been proposed that 3-OH oxylipins are produced by incomplete β-oxidation probably in the mitochondria of fungi [9,10]. Strikingly, mitochondrial function also appeared to be important for flocculation since flocculation induction is repressed in the presence of uncouplers as well as glycolytic and respiratory inhibitors [14]. Furthermore, it was found that carcinogens acting on the mitochondruim also caused flocculation loss [14]. In addition, cells that carries

deletions in mitochondrial genes *oli1* and *oxi*2 have reduced or no flocculation ability [36].

From the above mentioned it can be proposed that lipids, possibly 3-OH oxylipins, could play a role in flocculation. However, little is presently known about the function of lipids, especially cell wall associated lipids in yeast flocculation. Therefore, the functional role of these compounds during flocculation needs to be further elucidated.

So far interesting patterns of distribution and function (i.e. adhesion) for 3-OH oxylipins in yeasts have been reported. This will now be discussed in detail in the form of a review that was published on invitation in Prostaglandins and other Lipid Mediators.

3. THE DISTRIBUTION OF 3-HYDROXY OXYLIPINS IN FUNGI

3.1. Abstract

One of the best-kept secrets by fungi especially yeast is the function of the different shapes and surface structures of their vegetative and sexual cells. They definitely do not produce these shapes (e.g. round, elongated, kidney, needle, hat, saturnoid, etc.) and surfaces (e.g. smooth, rough, hairy, warty, etc.) for our curiosity or to be classified, but surely produce these for their own benefit. This minireview will show that a large variety of 3-OH oxylipins are widely distributed in the fungal domain and closely associated with these surface ornamentations. In concert with nano-scale surface structures, they probably play a role in cell aggregation as well as spore release from sexual structures such as asci.

3.2. Introduction

Oxylipins are saturated and unsaturated oxidized fatty acids that are widely distributed in nature i.e. plants, animals and in some microorganisms as constituents of various complex lipids or as free carboxylic acids. These

compounds include the eicosanoids (e.g. prostaglandins, thromboxanes, leukotrienes, lipoxygenase products), many of which are pharmacologically potent compounds with important biological activities [36-40]. Also included are a large number of hydroxy oxylipins, which are formed either by lipoxygenase, dioxygenase or cytochrome P-450 mediated pathways, which have been reported in fungi [41-46]. These compounds carry one or more hydroxyl groups at carbon atoms 5, 7, 8, 9, 12, 13, 15 or 17 of the fatty acid molecule and are mostly formed from oleic or linoleic acid. In this minireview emphasis will be placed on the more recently uncovered 3-OH polyunsaturated oxylipins (with one hydroxyl group at carbon 3) especially regarding its distribution in fungi and possible biological function. The metabolic pathway and biological action of these compounds in mammalian cells will not be discussed and will be the topic of another review.

3.3. 3-Hydroxy oxylipins: chemical structure

The basic structure of 3-OH oxylipins comprises a hydrophilic carboxylate group (polar head) with a hydroxyl group at the C3 position (Fig. 2) [47]. The carbon chain can vary considerably in length and in the degree of desaturation. These compounds can also be present in two enantiomeric forms i.e. 3*R* and 3*S*. In some bacteria they can occur as polymers, which serve as energy source [48] or as part of Lipid A in the lipopolysaccharide (LPS) layer of Gram-negative bacteria that may act as potent endotoxins [49].

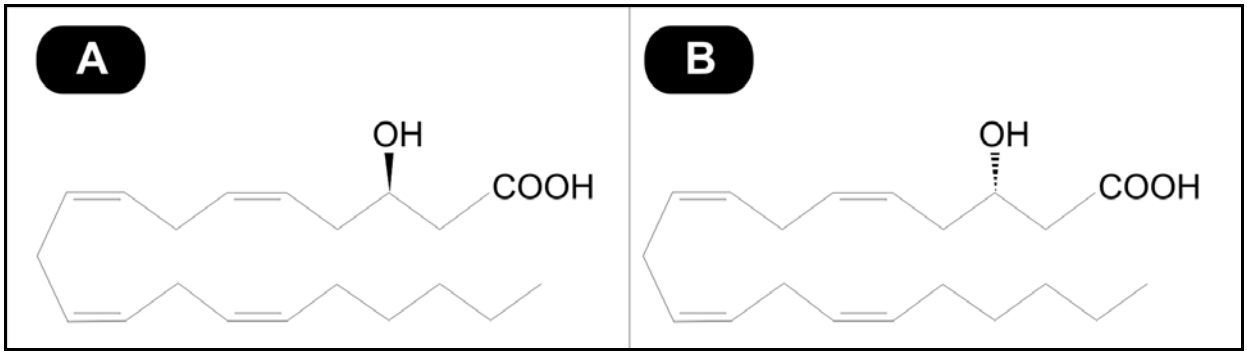

Fig. 2. The chemical structures of the enantiomers (**A**) *R*- and (**B**) *S*-3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE).

3.4. 3-Hydroxy oxylipins in fungi: their distribution and possible function

The first report on the presence of these compounds in fungi came from Stodola et al. [50] who reported the identification of 3-D-OH 16:0 and 18:0 as components of the extracellular glycolipids of strains of the yeast *Rhodotorula graminis* and *R. glutinis.* 3-Hydroxy 6:0 and 8:0 were also found to be components of the glycolipids of the smut fungi, *Ustilago zeae* and *U. nuda* [51]. Furthermore, Vesonder *et al*. [52] discovered large quantities of extracellular 3-D-OH 16:0 in a yeast later identified as *Saccharomycopsis malanga* [53].

In 1988, an extensive bioprospecting program was launched to determine if yeasts can produce aspirin (acetylsalicylic acid) sensitive eicosanoids i.e. prostaglandins. The reason? These autacoids (chemically produced and very expensive) are administered to elicit several responses in humans e.g. labor induction [54] - therefore a cheaper biotechnological source for these compounds will have obvious advantages. Unfortunately, until now, (although radio TLC and RIA implicated its presence) it could not be unequivocally proven that prostaglandins (with characteristic cyclopentane ring) are present in any of the yeasts tested, even when the direct precursor arachidonic acid (20:4) was fed [54]. Strikingly, these studies uncovered the use of non-steroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid and ibuprofen as potent antifungals (Kock JLF & Coetzee DJ, RSA provisional patent, 7 June 1990, no. 90/4397). It was also discovered, using radio TLC in combination with H^1 2D-COSY NMR, gas chromatography mass spectrometry (EI & FAB) as well as IR spectroscopy analyses, that a novel acetylsalicylic acid sensitive 3-OH polyunsaturated oxylipin i.e. 3*R*-OH 5*Z*,8*Z*,11*Z*,14*Z* eicosatetraenoic acid (3*R*-HETE; Fig. 2) is produced by the yeast *D. uninucleata var. uninucleata* [55]. This only happened when fed with 20:4, a precursor for prostaglandin formation in humans [37]. Later studies show that this yeast is capable of producing a wide variety of novel 3-OH oxylipins when fed with different precursors i.e. 3-OH 20:3; 3-OH 20:5; 3-OH 14:2; 3-OH 14:3 [56,57]. Before this discovery, only the presence of saturated 3-OH oxylipins, with no referral to function, was reported in yeast by various researchers [40]. Later, the production of 3-HETE was reported by Kock *et al*. [58] in *D. tothii*, a close relative of *D. uninucleata* [59].

The first evidence concerning the biologic activity of 3*R*-HETE was presented in the early 1990's. It was reported that this compound affects signal transduction processes in human neutrophils and tumor cells in multiple ways [60], thereby rendering a biotechnological value to this compound. Of course curiosity also centered on the functions of this compound in fungi especially yeast! In order to determine this, the affects of different low concentrations of acetylsalicylic acid on the life cycle of this yeast when cultivated in synchrony were studied [61]. It was found that the most susceptible part of the life cycle towards this cyclooxygenase inhibitor was the sexual stage i.e. the liberation of ascospores from the ascus (Fig. 3). Coinciding with this, the production of 3*R*-HETE (found to occur mainly during sexual spore formation) was severely inhibited [62].

In 1998 the life cycle of this yeast was mapped for the production of 3*R*-HETE making use of polyclonal antibodies against this compound. This was the start of a new phase in this field of research. For this purpose, the oxylipin was first synthesized for antibody preparation [63,64]. The synthetic strategy for the production of 3*R*- and 3*S*-HETE, involved a convergent approach coupling a chiral aldehyde with a Wittig salt: these were derived from 2-deoxy-D-ribose and 20:4, respectively [63]. Next, antibodies against chemically synthesised 3*R*-HETE were raised in rabbits and then characterised by determining its titer, sensitivity and specificity [65]. It was found that the antibodies were specific for 3-OH oxylipins in general (cross-reactions occurred with different 3-OH oxylipins), which assisted in mapping 3-OH oxylipins with different chain lengths and desaturation. Immunofluorescence microscopy of cells of *D. uninucleata* at different stages of its life cycle (i.e. including both the vegetative asexual and sexual stages) indicated that these oxylipins are associated with the surfaces of adhering gametes (gametangiogamy, Fig. 3C) as well as liberated aggregating sexual spores also known as ascospores (Fig. 3E) [65]. Strikingly, according to transmission electron microscopy and oxylipin inhibition (addition of acetylsalicylic acid) studies, 3-OH oxylipin - associated ascospore ornamentation i.e. nano-scale hooks were responsible for ordered ascospore liberation (Fig. 4B, C) [16].

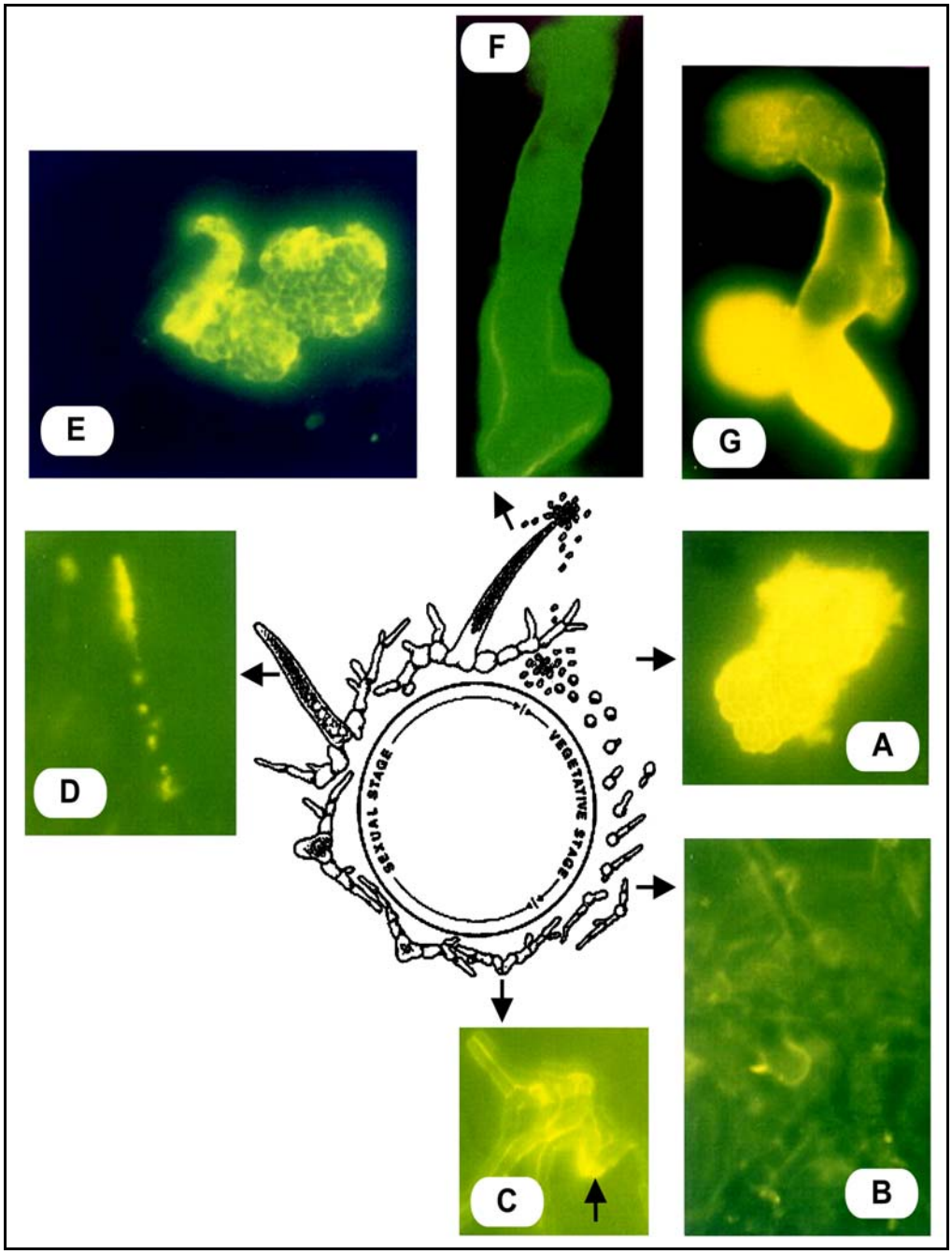

Fig. 3. The life cycle of *Dipodascopsis uninucleata* and distribution of 3-HETE visualized through immunofluorescence mapping. **A,** Liberated ascospores – high affinity for oxylipin antibody. **B,** Hyphae with cell wall – low oxylipin-antibody affinity. **C,** Gametangiogamy – tip of adhering gametes show high affinity for oxylipin antibody. **D,** Young ascus with cell wall. Ascospores in ascus show high affinity for oxylipin antibody. **E,** Liberated ascospores from ascus. Oxylipin antibodies attached to ascospores. **F,** Empty ascus protoplast: still with characteristic morphology. **G,** Deformed mature ascus protoplast containing highly fluorescing ascospores mainly at base (A, E, F, G: 10mm on photo = 10 μm cell size; B, C, D: 10 mm on photo = 25 μm cell size). Taken with permission from [65].

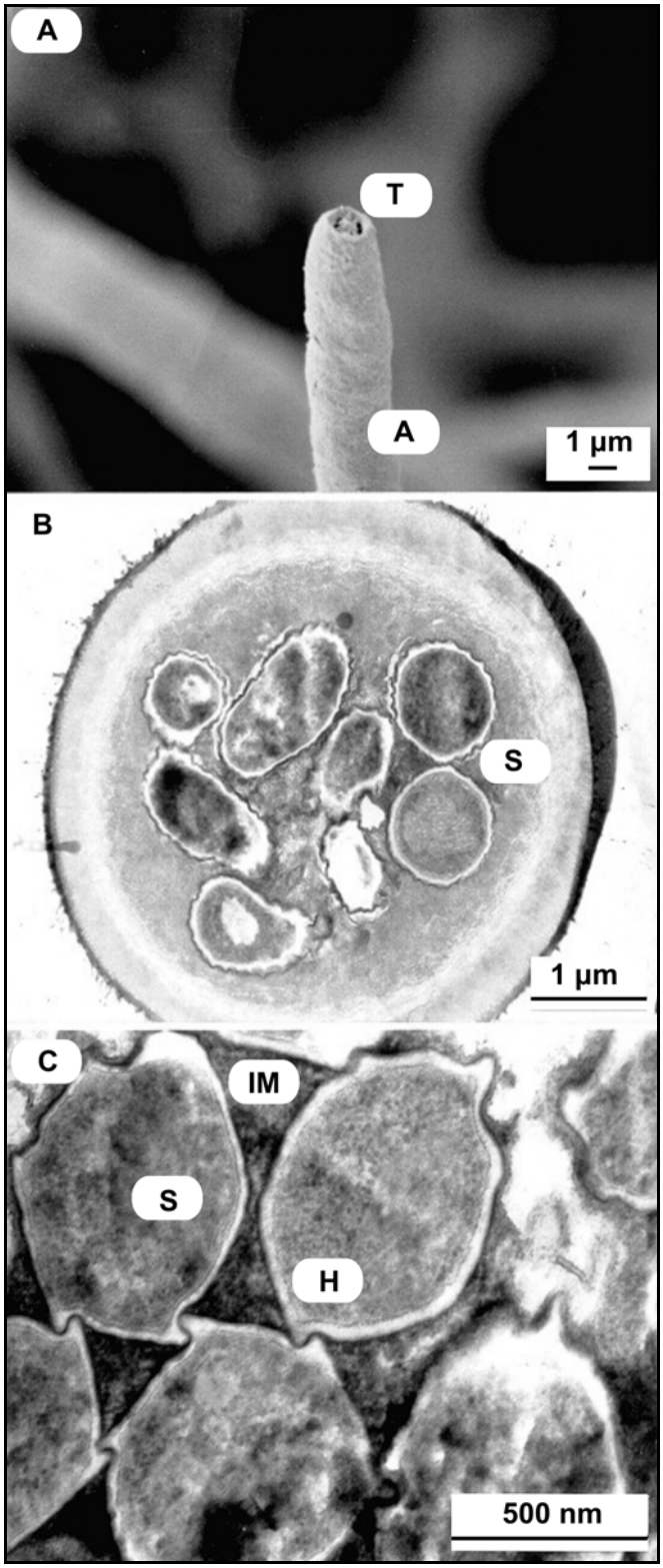

Fig. 4. Representative photomicrographs illustrating the effect of non-steroidal anti-inflammatory drugs (NSAIDs) i.e. 1 mM acetyl salicylic acid (ASA) on ascospore release and ultrastructure. **A,** Upper part of mature ascus (A) with partly closed tip (T). **B,** Ascospores (S) without defined hooks inside an ascus. **C,** Hooked (H) mature ascospores inside an ascus in the absence of ASA. Taken with permission from [16].

In the absence of acetylsalicylic acid, ascospores formed nano-scale hooks that are perfectly interlocked. In the presence of 1 mM acetylsalicylic acid these hooks were not formed (Fig. 4B) while the ascus tip did not open (Fig. 4A)- no ascospores were liberated in this case. It was also reported that the formation of these hooks is inhibited by even lower concentrations (0.1 mM) of acetylsalicylic acid [16].

After viewing many hours of video enhanced imaging displays [66] showing ascospore release as well as performing light- and electron microscopy, Kock and co-workers [16] concluded that surface ornamentation plays an important role in ascospore release and ordered reassembly outside the ascus of *D. uninucleata*. This is the first report elucidating the function of ascospore surface structures in fungi. Oil "lubricated" ascospores (the oil phase later found by Smith *et al*., [67] using immunogold labelling to contain 3-OH oxylipins) are at first perfectly interlocked through nano-scale surface hooks at the base of the ascus (Fig. 4C, 5F). As turgor pressure starts to increase, these spores disassemble (hooks unlock) as they are forced along a complex runway that tapers down as it reaches the ascus tip (Fig. 5A). At this stage only one spore at a time is forcibly propelled from the ascus through the tip (Fig. 5B) just to be trapped in an elegant orderly manner outside the ascus i.e. packet of spores (spores in unlock position, Fig. 5C, D) by a 3-OH oxylipin containing matrix that are released together with the spores to eventually form an interspore matrix attaching to the hooks (Fig. 5E).

It was also reported that 3-OH oxylipin-associated surface ornamentation was not limited to ascospores. It was found that flocculating vegetative cells of *Sacch. cerevisiae* contain 3-OH 8:0 and 3-OH 10:0 oxylipins, which are associated with "sticky" ornamentations that play a role during flocculation [9]. Relevant electron microscopy and immunofluorescence micrographs clearly show this phenomenon (Figs. 6A-G and 7). When flocculation starts, cell surfaces become wrinkled and produce protuberances (Fig. 6A, B). These protuberances were found to be osmiophilic layers protubing in a "ghost-like" fashion through the cell walls of the yeast (Fig. 6 E, F) to adhere to the cell wall of an adjacent yeast (Fig. 6G) to affect flocculation. These "ghost-like" protuberances were found to

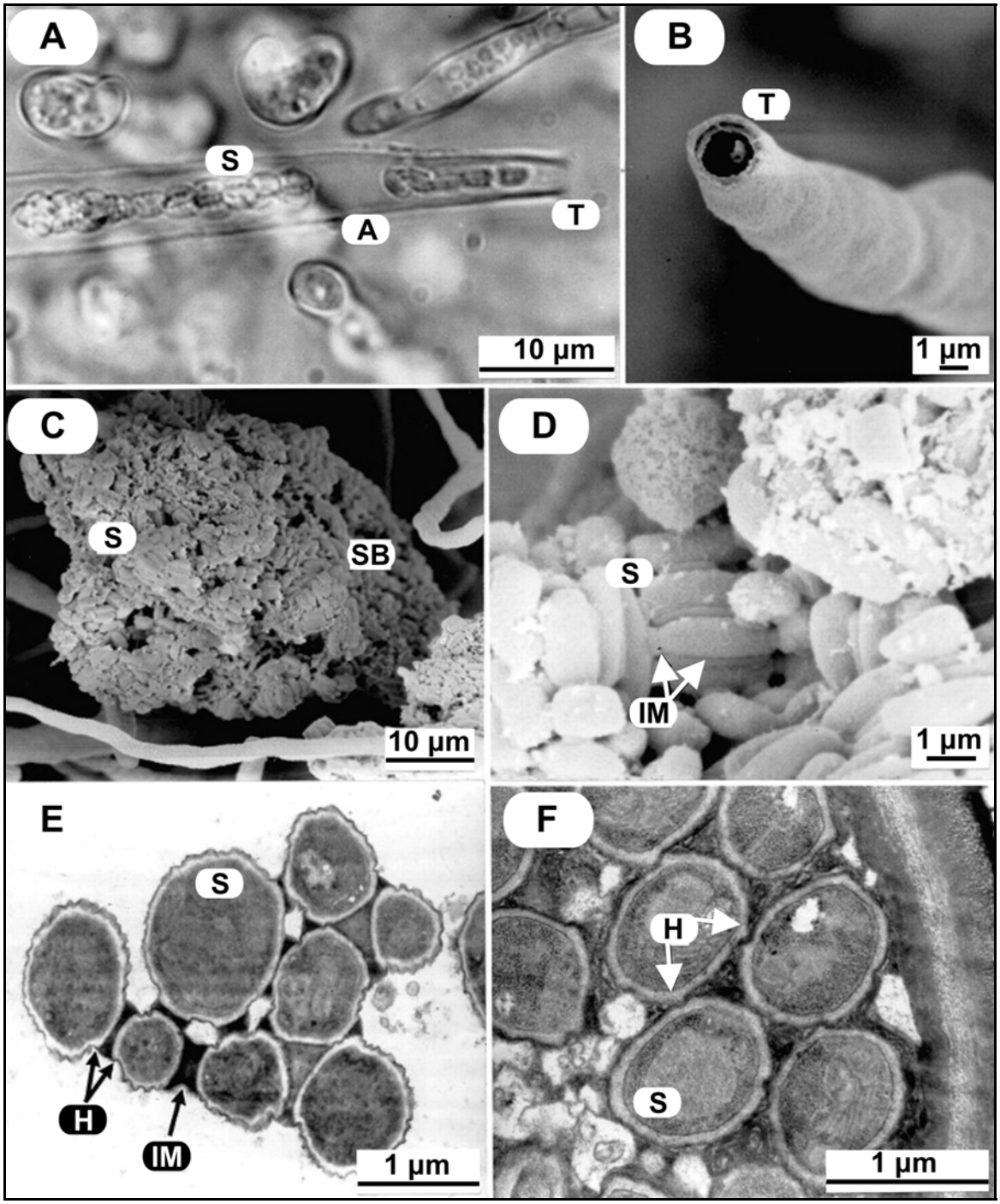

Fig. 5. Representative photomicrographs showing separation-aggregation phenomenon. **A,** Ascospores (S), in clusters inside an ascus (A) as observed by light microscopy are individually released after separation from the tip (T) of the ascus to re-aggregate in the medium. **B,** SEM of opened ascus tip. **C,** SEM of sticky aggregates (SB). **D,** More detailed SEM of sticky aggregates showing defined interspore matrix (IM). **E,** TEM of orderly re-aggregated hooked ascospores with pronounced interspore matrix. **F,** Clusters of hooked (H) ascospores before separation and release from ascus. Taken with permission from [16].

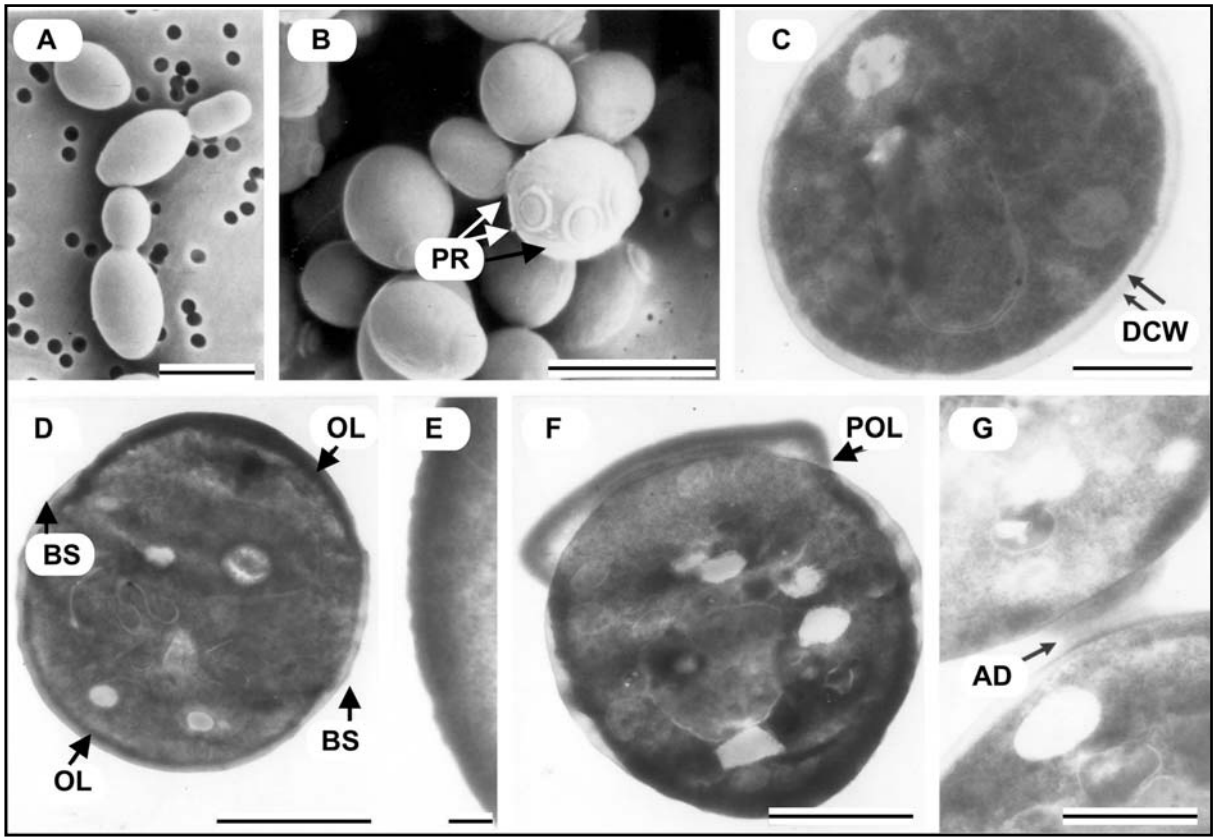

Fig. 6. Changes in cell wall protuberances as well as osmiophilic layer formation over the growth cycle of *Saccharomyces cerevisiae* ATCC 26602. **A,** A SEM of actively growing, non-flocculating cells. **B,** SEM photomicrographs showing protuberances (PR) on surface of mature yeast cells. **C,** TEM micrograph of immature young yeast cells indicating lack of osmiophilic layer and characteristic double layered cell walls (DCW). **D,** TEM micrograph indicating osmiophilic layer (OL) with budscar (BS). **E,** TEM micrograph of cell wall with closely associated overlapping osmiophilic layer. **F,** TEM micrograph indicating protubing osmiophilic layer (POL). **G,** TEM micrograph showing adhesion (AD) of POL to cell wall of neighboring cell. Scale bar, 5 μm in (A) and (B); 2 μm in (F); 1.5 μm in (D); 1 μm in (E) and (G); 0.5 μm in (C). Taken with permission from [16].

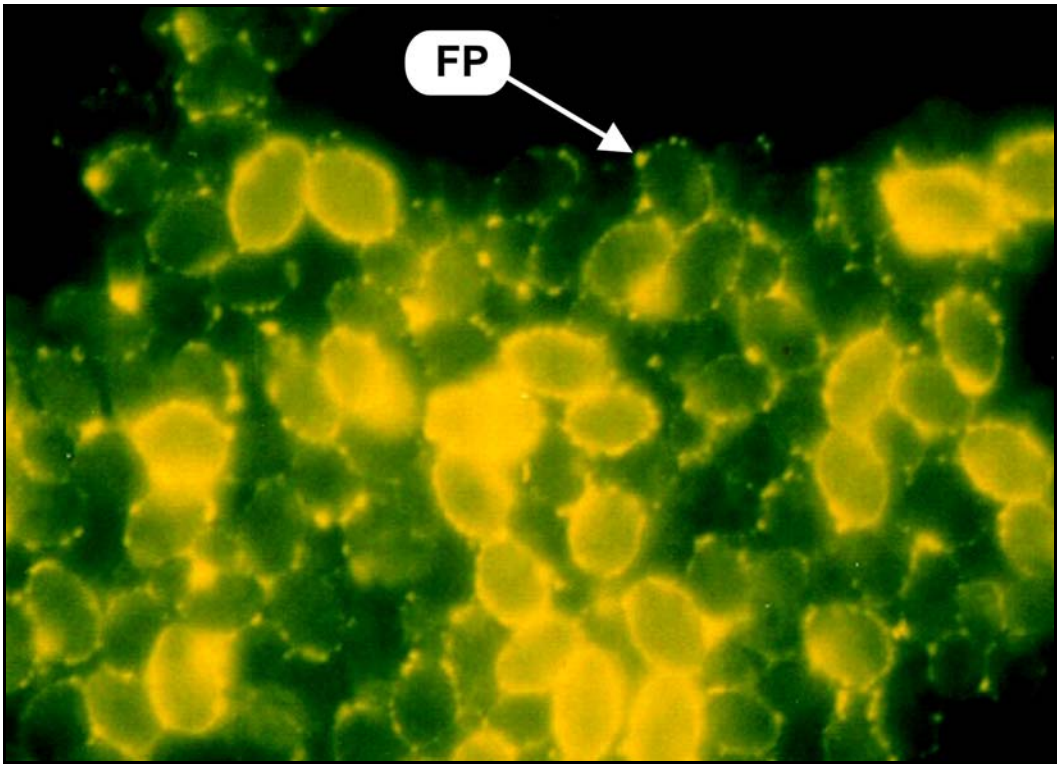

Fig 7. Fluorescing yeast cell micrograph showing high affinity of cell surface protuberances for oxylipin antibody. [8 mm = 5 μm]. Taken with permission from [9].

have a high affinity for the 3-OH oxylipin – antibody implicating its presence in these protuberances (Fig. 7).

Next, the *in situ* occurrence and localisation of 3-OH oxylipins in yeast and other fungi was mapped. Surprisingly, 3-OH oxylipins, many of which the complete chemical structures are only partially uncovered, were found to be produced by various yeasts and mucoralean fungi – in all cases these compounds were associated with the surfaces of aggregating vegetative - and sexual spores implicating an adhesive role probably through entropic based hydrophobic forces.

As found previously in *D. uninucleata*, fluorescence was associated with the closely associated sexual cells (ascospores) of all lipomycetaceous species [67] tested i.e. *Lipomyces doorenjongii, L kockii, L. kononenkoae, L. starkeyi, L. yamadae, L. yarrowii, Smithiozyma japonica* and *Zygozyma oligophaga*. However, in contrast to *D. uninucleata*, 3-OH oxylipins (i.e. 3-HETE) accumulated on the ascus tip of the closely related *D. tothii* and less were observed between

the aggregating ascospores, as observed by immunofluorescence microscopy [68]. The adhesive role of 3-OH oxylipins (i.e. 3-OH 16:0) was further illustrated in the yeast *S. malanga* where these compounds were observed by electron microscopy as micellar threads linking aggregating vegetative cells [69]. These oxylipins were also reported in the pathogenic yeast *Candida albicans.* The 3-OH oxylipin 3,18 – dihydroxy-5,8,11,14 eicosatetraenoic acid was observed on the surface of the filamentous structure and played a role in morphogenesis of this yeast [17,18].

Recently, 3-OH oxylipins were found to occur in the Mucorales [70]. For example, in *Pilobolus,* 3-OH 9:1 was observed on the sub-sporangial vesicle, as well as between aggregating sporangiospores with the aid of immunofluorescence microscopy and gas chromatography mass spectrometry analysis [71]. Furthermore, it was shown that *Mucor genevensis* was capable of transforming exogenously fed 20:4 to 3-OH 14:2 (5,8-tetradecadienoic acid or 3-HTDE) probably through a retroconversion metabolism [72]. In this fungus, 3-OH oxylipins were again mainly associated with the columellae, sporangia and aggregating sporangiospores (Fig. 8).

Strikingly in 2001, Noverr and co-workers [73] from the USA confirmed the discovery by Kock's laboratory [54] when they independently demonstrated that the pathogenic yeasts *Cryptococcus neoformans* and *C. albicans* produce immunomodulatory prostaglandins [73,74]. A review by Noverr and co-workers then followed in 2003 [19] acknowledging this discovery and the possible role of oxylipins as virulence factors [19]. Finally, in 2004 Alem and Douglas from the UK [20] demonstrated that infectious biofilms formed by *C. albicans* can be inhibited as much as 95% by aspirin. Strikingly, when prostaglandin E2 was added together with aspirin, the inhibitory effect of aspirin was abolished! They concluded that aspirin possesses potent antibiofilm activity *in vitro* and could be useful in combined therapy with conventional antifungal agents in the management of biofilm-associated *Candida* infections.

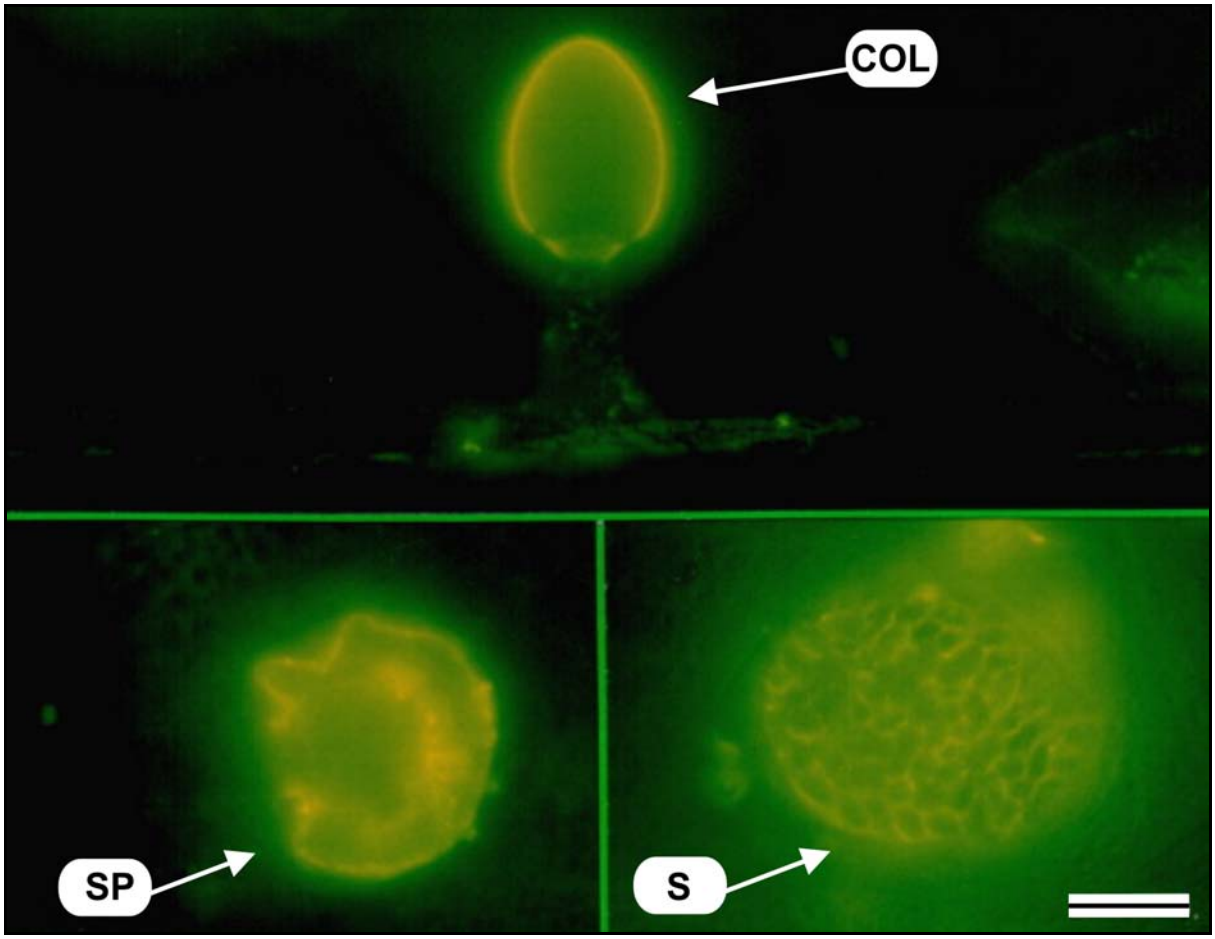

Fig. 8. Immunofluorescence micrographs of the zygomycotan fungus *Mucor genevensis* MUFS 038 showing fluorescing sporangium (SP), collumella (COL) and sporangiospores (S) (Bar = 5μm).

Recently, Kock and co-workers [75], proposed the likely primary function of oxylipin-lubricated yeast-meiospore shape and nano-scale ornamentations, in water-driven movement. Here, aspirin-sensitive 3-OH oxylipins act as prehistoric lubricants involved in the release mechanics of these spores from enclosed asci, probably for dispersal purposes. This interpretation may find application in nano-, aero- and hydro-technologies. In the same year plant pathologists from the University of Wisconsin (USA) proposed that oxylipins may play a role as regulators in sexual and asexual spore formation in *Aspergillus nidulans* [76].

Recently, clear-cut evidence for the role of 3-OH eicosanoids in candidiasis was presented [77]. Here, arachidonic acid (AA) is released from the infected or inflamed host tissue/cell and then converted by *C. albicans* to 3*R*-HETE, which serves as substrate for COX-2 (cyclooxygenase 2) in host

tissue/cell to produce pro-inflammatory 3-OH-PGE_2 (Fig. 9). Strikingly, in this investigation a cascade of novel bioactive 3-OH eicosanoids, produced from 3*R*-HETE via mammalian COX-2, was uncovered [77]. In addition, it was found that *C. albicans* selectively upregulates COX-2 in mammalian cells [78]. Prostaglandins produced by COX-2 are potent bioactive compounds in inflammatory diseases and cancer. Since both 3*R*-HETE and COX-2-produced 3-OH PGs are inhibited by acetylsalicylic acid, this research also suggests new targets for the control of yeast infection. It is therefore not surprising that today, NSAIDs such as ibuprofen are prescribed as potent topical antifungal medication [79].

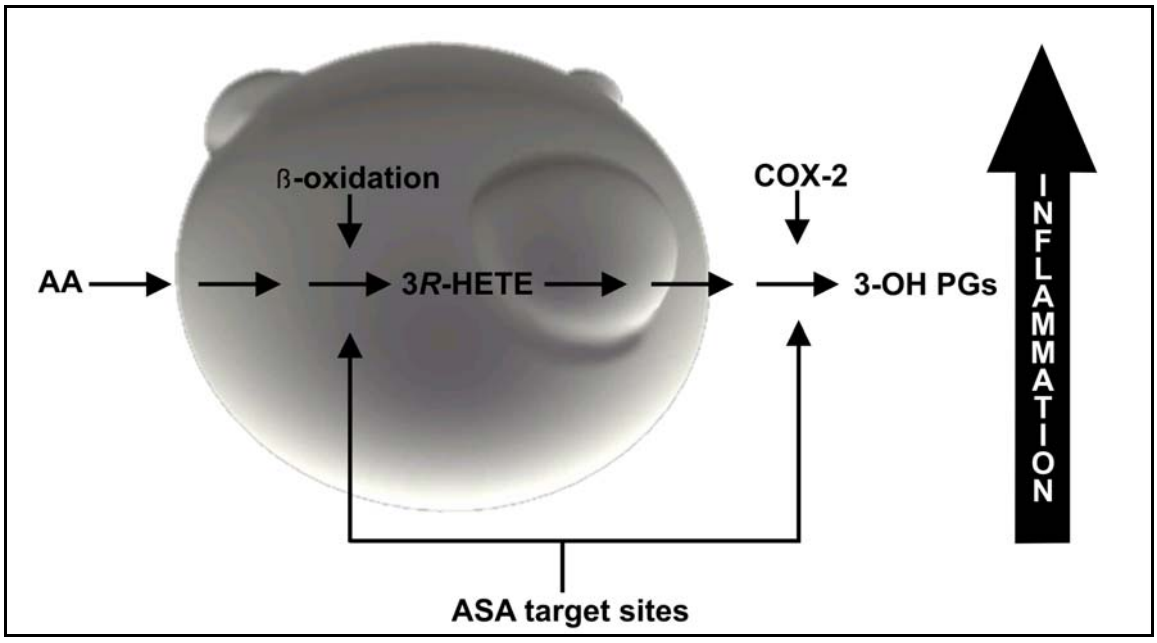

Fig. 9. Schematic representation of the role of 3-OH eicosanoids in candidiasis. AA = Arachidonic acid; 3*R*–hydroxy eicosatetraenoic acid = 3*R*-HETE; COX-2 = Cyclooxygenase 2; 3-OH-PG = 3*R*–hydroxy prostaglandins; ASA = Acetylsalicylic acid.

3.5. Conclusions

The studies on 3-OH oxylipins so far implicate a wide distribution of a large variety of these compounds in the fungal domain. As this mystery unfolds, it will be of interest to determine the taxonomic value of these oxylipins in this group of organisms. Especially the production of polyunsaturated 3-OH oxylipins e.g. 3-HETE and others seems to have taxonomic significance and literature suggests that these compounds may have the potential to be used as markers to identify for instance members of the yeast genus *Dipodascopsis*.

It can be concluded from this review that 3-OH oxylipins are apparently associated with structures that tend to aggregate. Consequently, a kind of adhesion property may be attributed to these compounds. Whether this occurs through entropic based hydrophilic forces and/or hydrogen bonds is yet to be verified [69,80,81]. Moreover, the 3-OH oxylipin structure may be of importance in the way adhesion occurs. For instance, in *S. malanga*, 3-OH oxylipin-threads are present as micellar–like units characterized by a hydrophilic outer layer and a more hydrophobic inner part [69]. This may be ascribed to a larger polar head of the 3-OH 16:0 (forming the micelle) compared to the hydrocarbon chain, hence this 3-hydroxy oxylipin are more likely to form thread-like micelles [80], which in turn may attach to the hydrophilic cell walls of the vegetative cells. This aspect however needs more clarification. Recently a novel 3-hydroxy oxylipin, 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid was identified in *C. albicans,* a pathogen in vulvovaginal candidiasis [17,18]. These researchers concluded that the administration of acetylsalicylic acid (aspirin) should be beneficial in the treatment of this disease by dual ways: (i) by inhibiting the 3-hydroxy oxylipin formation - mainly associated with the hyphal phase, and (ii) by inhibiting prostaglandin E_2 formation in the infected host tissue. Who knows what other secrets are locked up in the functional role of these chemical compounds? The role of these oxylipins in fungal cell aggregation may be of importance in medicine and the sedimentation of cells in biotechnological processes such as brewing where cells are at present mainly removed from culture by expensive centrifugation.

Inhibition studies so far on 3-OH oxylipins have partially unlocked the secret concerning the function of surface structures in yeast. The impact of further studies in this field will be followed with interest.

3.6. Acknowledgements/footnotes

The authors would like to thank the National Research Foundation (South Africa) as well as the Volkswagen Foundation, Germany (1/74643), for financial support.

4. PURPOSE OF RESEARCH

With this as background, the purpose of this study became the following:

1. To study the flocculation patterns of a unique *Sacch. cerevisiae* strain (UOFS Y-2330) capable of both Flo1 and NewFlo phenotype flocculation (Chapter 2).

2. To investigate the role of lipids, during inverse flocculation patterns of *Sacch. cerevisiae* UOFS Y-2330. Here, emphasis will be placed on the lipid turnover during the flocculation process (Chapter 3)

3. To elucidate the biological function of 3-OH oxylipins in the flocculation of *Sacch. cerevisiae* UOFS Y-2330 (Chapter 4).

4. To investigate co-flocculation of *Sacch. cerevisiae* UOFS Y-2330 with *S. pombe* strains (Chapter 5).

Please note: The chapters to follow are presented in the format depicted by the journal of submission. As a result repetition of some information could not be avoided.

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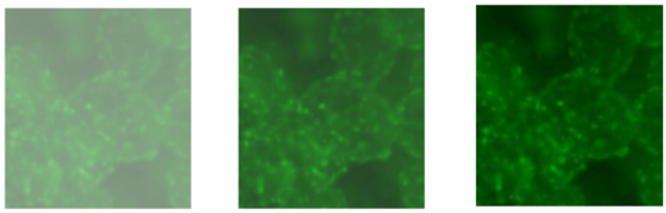
Chapter 2

Inverse flocculation patterns in *Saccharomyces cerevisiae* UOFS Y-2330



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ABSTRACT

An interesting yeast strain was uncovered which showed an inverse flocculation pattern when cultivated in chemically defined and complex media. When inoculated in a defined medium with glucose as a sole carbon source, this strain immediately flocculated strongly and lost this ability before stationary phase was reached. In a complex malt medium containing glucose, this yeast strongly flocculated throughout the exponential and stationary growth phases. This inverse pattern may be ascribed to a switch in sensitivity of the yeast to flocculate in the presence of glucose as well as pH level, which may, in turn, influence the availability of calcium ions. In both media, matured cells produced protuberances or "wrinkles" upon flocculation as observed by electron and immunofluorescence microscopy. These protuberances may be involved in cell adhesion during the flocculation process.

1. INTRODUCTION

In fermentation-based food and biotechnological processes, suspended cells are normally separated from the medium through flocculation before further processing. The processes for beer production are typical examples of this phenomenon.

In some yeasts (e.g. NewFlo phenotype), flocculation may develop only towards early stationary phase of growth[11] through the binding of zymolectins with cell wall sugar receptors of neighbouring cell surfaces[6]. It has been demonstrated that these receptors are available throughout the growth of yeasts[10]. The presence of glucose has been found to inhibit the flocculation of yeast by binding to specific lectins[12]. The lectins are produced and inserted into the cell wall at an early stage of growth and are probably activated later during fermentation[13]. It has been reported that flocculation onset coincides with the termination of budding and glucose limitations with a concomitant increase in cell surface hydrophobicity[14]. Also, in the case of beer fermentation, flocculent yeasts undergo several morphological changes before flocculation occurs at the end of

the process. These include for example, the deposition of chitin, an increase in the cell size, daughter cell retention and the occurrence of wrinkles on cell surfaces of matured cells[1]. The formation of wrinkles may resemble the hydrophobic oxylipin-associated "ghosting" phenomenon, that was reported previously in flocculent matured cells[8].

Moreover, it has also been reported that some yeasts (e.g. Flo1 phenotype) flocculate throughout the growth curve in all types of media[11]. In this case, glucose does not influence flocculation.

In this study, an inverse flocculation pattern is reported when *Saccharomyces cerevisiae* UOFS Y-2330 is grown in both complex and chemically defined glucose containing media. This phenomenon may shed additional light on yeast flocculation.

2. MATERIALS AND METHODS (See Appendix for details)

2.1. Strain used

Saccharomyces cerevisiae UOFS Y-2330 was used throughout this study and is held at the University of the Free State (UOFS), South Africa.

2.2. Growth experiments

Saccharomyces cerevisiae UOFS Y-2330 was inoculated from YM[17] agar slants into 250 ml conical flasks containing 50 ml of glucose–YNB defined medium (12 g/L glucose, 6.7 g/L YNB) and incubated at 30ºC while shaking (160 rpm) for 20 h until late exponential phase was reached. Appropriate volumes were then transferred to 500 ml side-arm conical flasks containing 100 ml of the same chemically defined medium to yield a final absorbency of 10 Klett units. The culture was incubated at 30ºC while shaking (160 rpm) for 30 h. Growth was measured at regular intervals by measuring changes in optical density using a Klett Summerson colorimeter (red filter). The degree of flocculation (i.e. % Δ floc)

was measured throughout growth by calculating the decrease in cell turbidity according to the method of Calleja and Johnson[4] by using the formula *Klett value at start of flocculation – Klett value after 5 min flocculation/Klett value at start of flocculation* while the pH was constantly monitored. This experiment was repeated when the yeast was grown for 22 h in complex media i.e. glucose-YM broth (12 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt) under similar growth conditions. All experiments were performed at least in duplicate. Materials from flocculating and non-flocculating cells grown on both complex and chemically defined medium were sampled for immediate immunofluorescence and electron microscopy analysis.

2.3. Immunofluorescence microscopy

Preparation and characterisation of antibody: Preparation and characterization of antibodies raised against synthetic 3-HETE[2] has already been described earlier in detail[7]. Interestingly, the polyclonal antibodies recognised specifically all long-chain fatty acids carrying a hydroxyl group at C-3 position.

Microscopy: Immunofluorescence of fungal cells was performed as described[7]. In order to maintain aggregated cell floc structure, antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Following adequate washing, the slides with fluorescing material were photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop microscope equipped for epifluorescence with a 50 W high pressure mercury lamp. The stained cells were compared with appropriate controls as described[7]. All experiments were repeated at least in duplicate.

2.4. Electron microscopy

Material for electron microscopy was chemically fixed (glutardialdehyde and osmiumtetroxide)[16]. Transmission electronmicrographs were taken with a Philips CM 100 (The Netherlands) TEM.

2.5. pH adjustment during growth

The strain was cultivated in a chemically defined medium as described previously. When the yeast de-flocculated completely after 30 h at pH 2.2, the pH was raised (reversed) by titration with 1M NaOH while the degree of flocculation was measured as described before. All experiments were repeated at least in duplicate.

2.6. Flocculation and dependence on ions

In a separate experiment, $CaCl_2.2H_2O$ was added to completely de-flocculated cells at pH 2.2 (see pH adjustment during growth section) at the following final concentrations: 0 mM, 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM and 0.10 mM. The degree of flocculation was measured each time upon addition of $CaCl_2.2H_2O$, while the pH 2.2 was maintained constant. All experiments were performed at least in duplicate.

2.7. Glucose determination

The residual glucose content of the supernatants was quantified at regular intervals over the growth cycle using a high-performance liquid chromatograph (HPLC) instrument (Waters, USA) equipped with a refractive index detector (Waters 2414, USA), and a Sugarpack 1 column (Waters), operating at 84°C with an eluent (deionised water) flow rate of 0.5 ml/min.

2.8. Measurement of flocculation using the Sugar Inhibition Modified Helm's test

In order to determine the effect of glucose (0.5 M) on yeast flocculation, the modified Helm's test of D'Hautcourt and Smart[5] was used on two day old cultures grown in previously described media, respectively.

2.9. Chemicals used

All chemicals and solvents used were of highest purity and obtained from major retailers.

3. RESULTS AND DISCUSSION

When *Sacch. cerevisiae* UOFS Y-2330 was grown in complex glucose containing medium, this yeast reached stationary growth phase after about 14 h (Fig. 1). The degree of flocculation (% Δ floc) also increased sharply towards late exponential phase (i.e. after 10 h), which is in accordance with literature for NewFlo phenotypes[6]. During this period, the pH decreased from pH 5.8 to pH 4.4 suggesting that hydrogen-ion concentration may play an important role in flocculation[3,9]. Also, the glucose concentration decreased dramatically after 8 h, which probably uplifted the competitive binding of this sugar with the available zymolectins, thereby increasing flocculation. This finding was confirmed with the modified Helm's sugar inhibition test where the addition of 0.5 M glucose to strongly flocculating cells grown in complex medium yield a percentage flocculation of only 13.1 +/- 6.7%. Experiments were compared with Flo1 and NewFlo phenotype controls.

When the yeast was grown in defined medium at pH 5.5 (Fig. 2), the cells reached stationary phase after about 16 h at a rather low pH 2.2. Strikingly, the yeast started to flocculate (visual inspection) immediately upon inoculation into the fresh medium, which suggests limited inhibition by glucose as a substrate[6]. This was followed by a rapid increase in flocculation up to 8 h until a pH of 2.7 was reached. The latter phenomenon may be ascribed to the increase in cell density during exponential growth phase bringing the cells in closer contact to affect more efficient flocculation[15]. Moreover, the flocculation was followed by a rapid total de-flocculation of the cells as these entered the stationary growth phase, i.e. from 8 h to 12 h, with a concomitant decrease in pH from 2.7 to 2.4 after 12 h. The pH dropped after 8 h of growth (from pH 5.5 to pH 2.7)

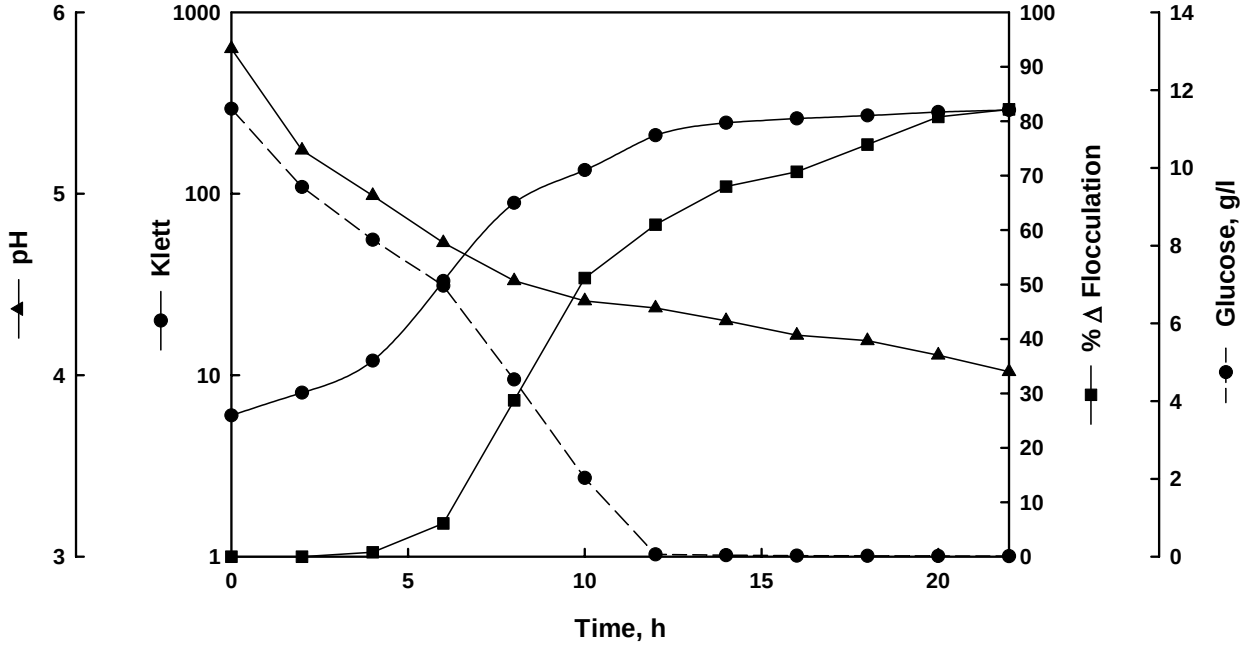

Fig. 1. Changes in growth (Klett-units), degree of flocculation, pH and glucose utilisation over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in complex medium. This experiment was repeated in duplicate and produced similar patterns.

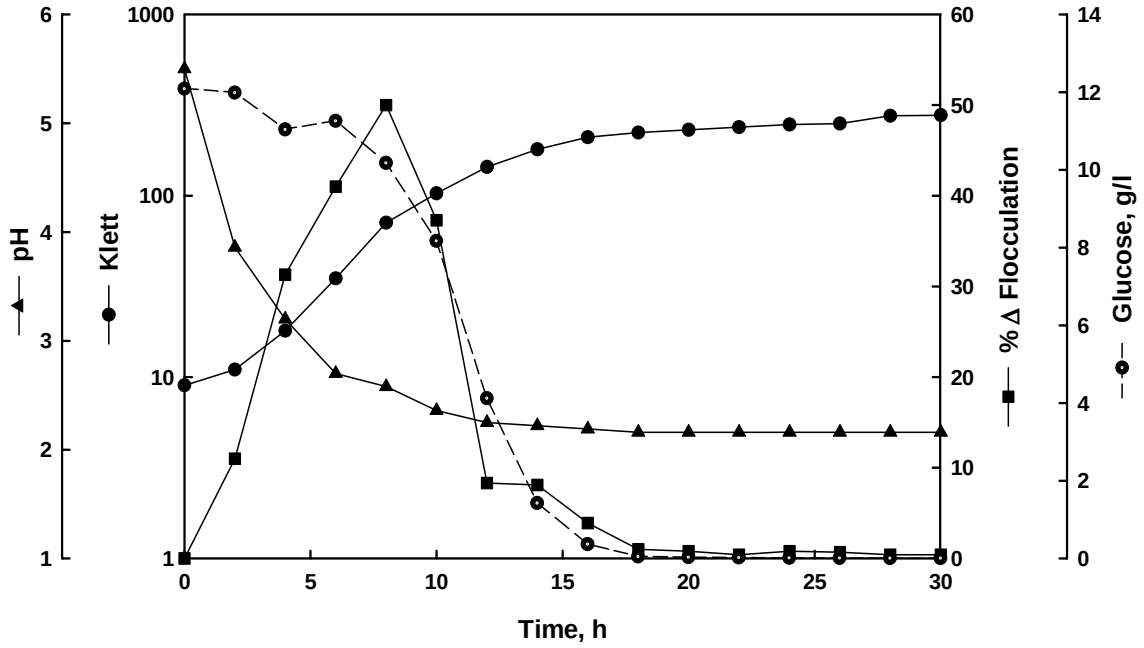

Fig. 2. Changes in growth (Klett-units), degree of flocculation, pH and glucose utilisation over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in chemically defined medium. This experiment was performed in triplicate and produced similar patterns.

and then steadily decreased to a value of pH 2.2 after 30 h. This drop in pH may be attributed to the low buffer capacity of the chemically defined medium. In this case only a small amount of glucose was utilized in the first 8 h. This finding was supported by the modified Helm's sugar inhibition test, in which the addition of 0.5 M glucose to strong flocculent cells grown in defined medium yield a percentage flocculation of as high as 62.1+/- 10.1%. Experiments were compared with Flo1 and NewFlo phenotype controls.

To determine whether flocculation was dependent on pH, it was decided to steadily increase the pH after 30 h of growth by titrating with 1 M NaOH and follow the changes in the degree of flocculation. Surprisingly, the degree of flocculation was almost perfectly reversed, with the slopes depicting the rate of de-flocculation and flocculation staying more or less the same (Fig. 3). In this case, the flocculation was switched on over a rather narrow pH range stretching from 2.5 to 2.6. From pH 3.8 to pH 10.7, the cells flocculated maximally and again de-flocculated rapidly at a narrow pH range of 10.7 to 11.0. The flocculation/de-flocculation phenomenon may be ascribed to the sensitivity of the zymolectin-cell wall sugar bonds to pH.

To shed more light on the mechanism of de-flocculation occurring at low pH, the calcium level was raised by addition of different concentrations of $CaCl_2.2H_2O$ to the de-flocculated culture while maintaining the pH 2.2 (Fig. 4). Calcium was chosen since it has been reported that these divalent cations play a significant role in flocculation, probably by influencing the zymolectin conformation[6]. Consequently, the degree of flocculation increased significantly with each addition of calcium ions suggesting that these may not be available for flocculation at low pH 2.2. This phenomenon is at present under investigation.

In order to determine if hydrophobic oxylipins were associated with the cell walls of flocculating cells, as previously reported by us[8], we applied specific 3-hydroxy-oxylipin antibodies in an immunofluorescence microscopic investigation. We found that these 3-hydroxy-oxylipins were present in both flocculent cells (i.e. after 8 h) and cells that lost this ability (i.e. after 20 h) when cultivated in chemically defined medium. In flocculating and de-flocculating cells, the oxylipins

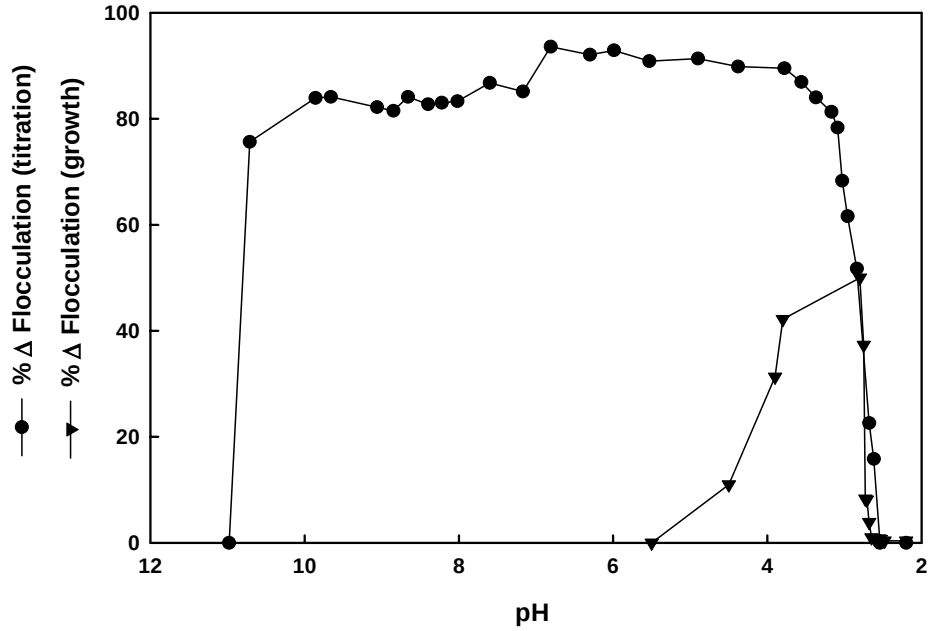

Fig. 3. Changes in the degree of flocculation over concomitant changes in pH from 5.5 to 2.2 when *Saccharomyces cerevisiae* UOFS Y-2330 was grown in chemically defined medium (read left to right on X-axis) followed by changes in the degree of flocculation when de-flocculated cells were titrated with 1 M NaOH, pH change from 2.2 to 11.0 (read right to left on X-axis). This experiment was performed in triplicate and produced similar patterns.

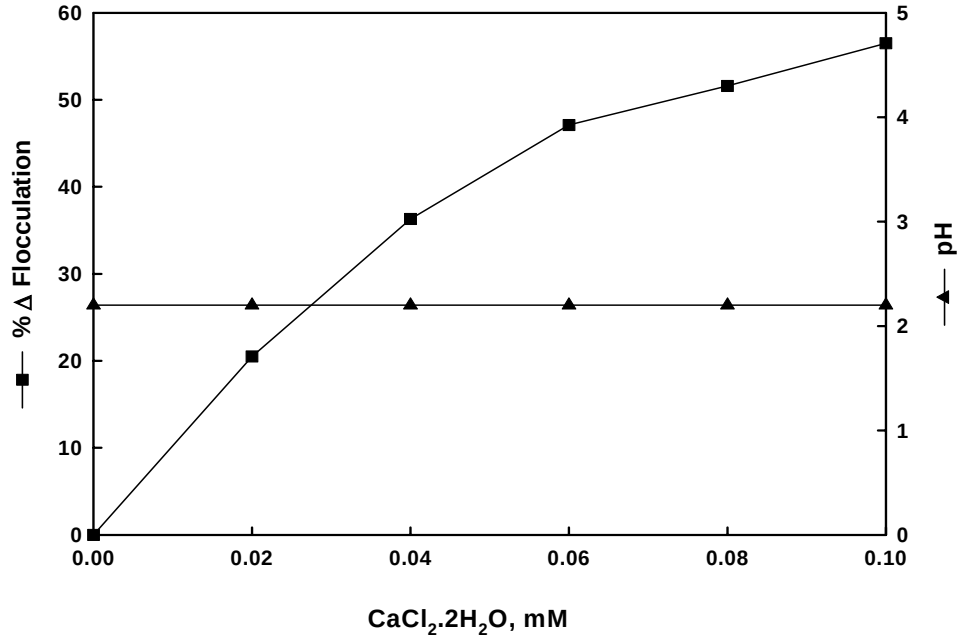

Fig. 4. Changes in the degree of flocculation in the presence of different concentrations of $CaCl_2$.$2H_2O$, while the pH was kept at about 2.2. This experiment was performed in duplicate and produced similar patterns.

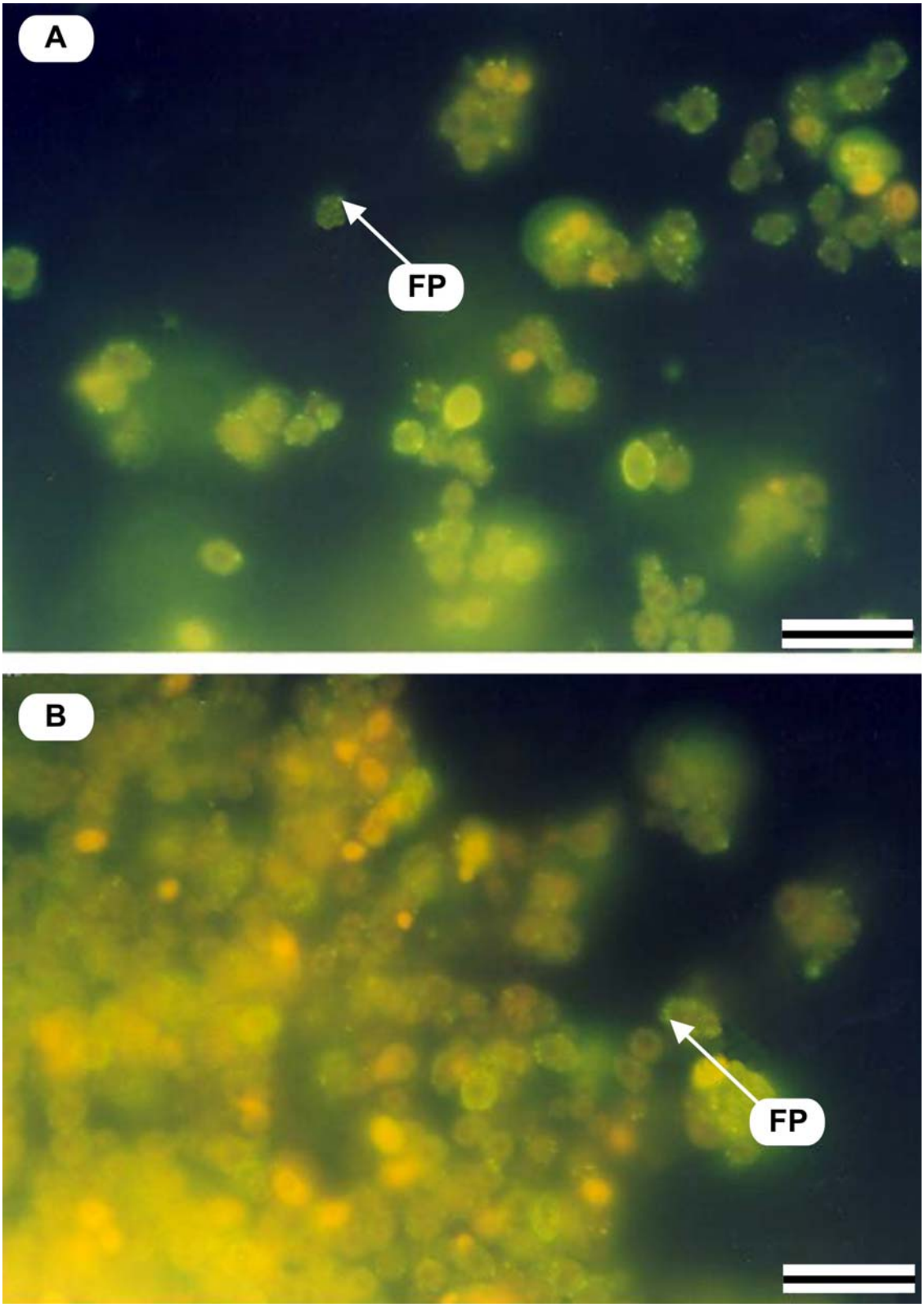

Fig. 5. Oxylipin specific immunofluorescence micrograph of flocculating cells when grown in chemically defined medium. Similar results were obtained for flocculating cells produced from complex medium. Fluorescing mature cells with oxylipin specific fluorescing protuberances on cell surfaces (FP). Scale bar represents 12 μm.

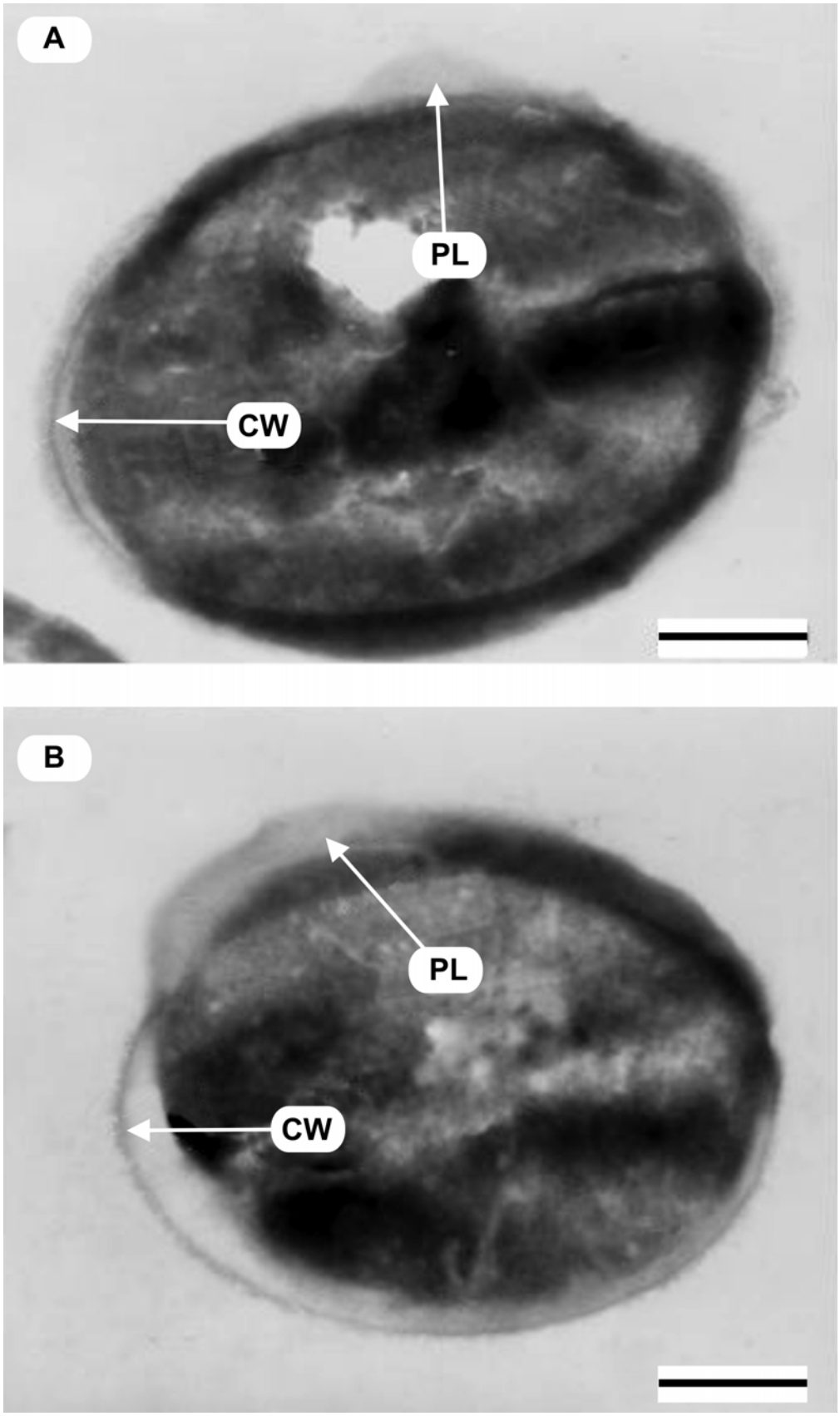

Fig. 6. TEM micrographs of *Saccharomyces cerevisiae* UOFS Y-2330. **A**, **B**: Cells showing characteristic cell walls (CW) and extensively protubing layers (PL). Scale bar represents 1 μm.

differentiated into localised fluorescing protuberances (Fig. 5) on the cell wall. 3-Hydroxy-oxylipins were also present between the flocculating cells suggesting a possible role in flocculation. Similar results were obtained when this yeast was grown in complex medium. These results are consistent with those reported previously by Kock and co-workers[8].

Consequently, these results prompted us to further investigate the fluorescing protuberances, which appeared similar to the wrinkles as described by Barker and Smart[1]. For this purpose, transmission electron microscopy (TEM) was performed on osmium tetroxide fixed yeast samples (see Materials and Methods). Again "ghost-like" osmiophilic protuberances were observed (Fig. 6) similar to that reported by Kock *et al*[8] . These protuberances extend randomly across any region of the cell wall (Fig. 6A, 6B) and may contain both oxylipins as well as activated lectins necessary for binding to cell walls of adjacent cells.

4. CONCLUSIONS

We uncovered in this study an interesting yeast, which showed an inverse flocculation pattern when cultivated in complex and chemically defined media. Seemingly, an apparent switch in sensitivity towards glucose and flocculation inhibition occurred. Can this be attributed to the presence of different sugar receptors on the cell walls of this yeast when grown in two different media? Is this yeast the possible ancestor of the Flo1 and NewFlo phenotype lines? This phenomenon remains the subject of further research.

In the chemically defined medium, a drop in pH was experienced over the growth cycle probably due to the low buffer capacity of this medium. The decrease in pH to below 2.5, probably caused the reversible breaking of the zymolectin-cell wall sugar bonds thereby affecting de-flocculation. Upon increasing the pH, these bonds were restored, thereby resulting in renewed flocculation of the cells. This may be related to the availability of divalent ions, such as calcium, that affect flocculation. Further studies on the physiological,

genetic as well as colloidal aspects of flocculation using this model strain may lead to important new insights in this fascinating phenomenon.

5. ACKNOWLEDGEMENTS

The authors would like to thank the South African Breweries, National Research Foundation, South Africa as well as the Volkswagen Foundation, Germany (1/74643), for financial support and Miss N.H. Mokone for practical assistance.

Supplementary Data

A three dimensional animation of a confocal laser scanning microscopy Z-stack, of flocculating *Saccharomyces cerevisiae* UOFS Y-2330 cells, clearly showing the distribution and dimensional arrangement of the fluorescing protuberances present on the cell walls. In order to view this the reader is referred to movie 1 on the CD at the back of the thesis.

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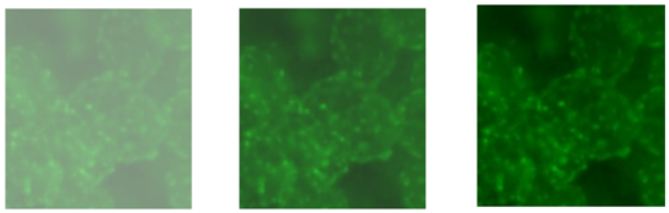
Chapter 3

Lipid turnover during inverse flocculation patterns in *Saccharomyces cerevisiae* UOFS Y-2330



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ABSTRACT

In this study we uncovered that *Saccharomyces cerevisiae* UOFS Y-2330 does not only demonstrate inverse flocculation, but is also characterised by two different lipid turnover patterns. During Flo1 phenotype flocculation, this yeast showed two neutral lipid accumulating stages (i.e. at 8 h and from 12 h). This is probably triggered by flocculation, which can be regarded as a survival mechanism where cells accumulate predominantly neutral lipids as reserve energy source - a similar mechanism is probably operative when cells enter stationary growth. Contrary to Flo1 behaviour, this strain in NewFlo phenotype mode demonstrates only a single lipid accumulation phase i.e. when cells enter stationary growth, which coincides with increase in flocculation. In addition, an increase in phospholipids was experienced during active growth in both flocculation behaviours i.e. Flo1 and NewFlo probably as a result of active membrane production.

1. INTRODUCTION

Flocculation is of prime importance to the beer industry and other biotechnological processes where it functions as a means to separate suspended cells from the medium, before further processing. In yeasts, flocculation may occur as a prelude to sexual reproduction or as a means of protection during adverse environmental conditions[18].

Even after extensive research, the exact mechanisms of flocculation onset and of flocculent bond formation are still poorly understood. Currently the Lectin Theory is accepted to explain flocculation[15], although an increase in cell wall carboxyl groups and thus cell surface hydrophobicity (CSH) at the onset of flocculation was observed[2]. Here, a threshold value for carboxyl group density was observed. In cases where this threshold value was exceeded, flocculation was initiated. Consequently, CSH has been identified as a major factor responsible for flocculation onset [1,20-22]. This observation was further supported by Kock et al.[13] who observed the accumulation of hydrophobic carboxylic acids

(3-hydroxy oxylipins) on the cell surfaces of a *Saccharomyces cerevisiae* strain during the initiation of flocculation.

It is proposed that 3-hydroxy oxylipins are produced by incomplete β-oxidation probably in the mitochondria of fungi[8]. Strikingly, mitochondrial function also appears to be important for flocculation since flocculation induction is repressed in the presence of uncouplers as well as glycolytic and respiratory inhibitors[17].

With this as background it became the purpose of this study to follow the lipid turnover during the growth cycle of a yeast strain showing both Flo1 and NewFlo behaviour[19]. This phenomenon may shed more light on the role of lipids in yeast flocculation.

2. MATERIALS AND METHODS (See Appendix for details)

2.1. Strain used

Saccharomyces cerevisiae UOFS Y-2330 was used throughout this study and is held at the University of the Free State (UFS), South Africa.

2.2. Growth experiments

Saccharomyces cerevisiae UOFS Y-2330 was cultivated as described by Strauss et al[19]. In short, cells were inoculated from YM[24] agar slants into 250 ml conical flasks containing 50 ml of glucose-YM broth (12g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract) and incubated at 30°C while shaking (160 rpm) for 20 h 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. These cultures were incubated at 30°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 turbidity[4] while the pH was constantly monitored in the cultures harvested. Several flasks to yield sufficient biomass and lipids were harvested at different time intervals as indicated in Fig. 1, followed by immediate freezing and freeze-drying.

This experiment was repeated when the yeast was grown for 20 h in chemically defined media i.e. glucose–YNB (Yeast Nitrogen Base, Difco Laboratories) medium (10 g/L glucose, 6.7 g/L YNB) under similar growth conditions. All experiments were performed at least in duplicate.

2.3. Harvesting of cells, lipid extraction and fractionation of extracted lipids

Cells were harvested by centrifugation at 9000 rpm for 10 min and then rapidly frozen at -70^{o}C followed by freeze-drying. Total lipid (TL) extraction was performed on freeze-dried cells according to Folch et al.[9]. In short, lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) and then washed with dH_2O. Next[12], the extracted lipids were dissolved in chloroform and applied to a column of activated silicic acid (by heating overnight at 110°C). Neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions were eluted from the column by successive applications of organic solvents of different polarities. The extracted TLs and lipid fractions were finally dissolved in a minimal volume of diethyl ether and transferred to pre-weighed vials followed by drying over a stream of nitrogen gas. In order to quantify the TL, NL, PL and GL fractions, samples were dried to a constant weight in a vacuum oven at 50^{o}C over P_2O_5 before they were finally weighed.

2.4. Fatty acid analysis

The fatty acid (FA) compositions of the different fractions were determined after transesterification by the addition of trimethyl sulphonium hydroxide (TMSOH) according to Butte[3]. Subsequently, the FA methyl esters were analysed using a Hewlett Packard 5890 gas chromatograph, equipped with a

Supelcowax 10 polar/capillary column (30m x 0.53mm) with nitrogen as carrier gas - set at a flow rate of 4 ml/min. The inlet temperature was set at 180^{o}C with the initial column temperature set at 145^{o}C - increasing at 3^{o}C/min, followed by the same temperature increment to a final temperature of 240^{o}C. The FA peaks were detected using a flame ionisation detector (FID) set at 300^{o}C. Peaks were identified using suitable standards.

2.5. Chemicals used

All chemicals and organic solvents used were of highest purity and analytical reagent grade and obtained from major retailers. All standards were from Sigma, while silicic acid (100 mesh) was from Aldrich.

3. RESULTS AND DISCUSSION

3.1. Lipid turnover in NewFlo phenotype behaviour

3.1.1. Changes in total lipid (TL) content

When *Sacch. cerevisiae* UOFS Y-2330 was grown in complex glucose containing medium, this yeast reached stationary growth phase after about 14 h (Fig. 1). The degree of flocculation (% Δ floc) increased sharply towards late exponential phase, which is in accordance with literature for NewFlo phenotypes[11] as well as that previously reported by us[19]. During the first 8 h of growth the TL content decreased from 25.3 mg/g dry biomass to a minimum value of 15.4 mg/g dry biomass, where-after it remained relatively constant for the next four hours. As this NewFlo yeast entered stationary phase (from 14h onwards), the TL content increased to a value of 26.1 mg/g dry biomass after 20 h of growth. When this experiment was repeated, similar patterns were observed.

3.1.2. Composition of total lipids (TLs) over the growth cycle

Changes in the NL content during growth were similar to those found for the TLs (Fig. 2). During the first 8 h of growth the cellular NLs decreased from 16.5 to 6.2 mg/g dry biomass probably to serve as energy source for growth, through oxidation of stored FAs[23] and/or PLs synthesis via diacylglycerol (DAG)[6]. Following this, the NLs started to accumulate from 8 h (6.2 mg/g dry biomass) to 20 h (17.4 mg/g dry biomass) of growth as the cells reached the stationary growth phase. These NLs are probably stored as energy source for later use[23].

Contrary to this pattern, the PL content increased for the first 8 h i.e. during active growth, where after it decreased i.e. as cells reach stationary phase (from 5.2 to 2.9 mg/g dry biomass). This may be ascribed to the demand for PLs needed for membrane development in active growing cells[12]. The GL fraction remained more or less the same throughout the growth cycle. When this experiment was repeated, similar patterns were observed.

Similar patterns were reported by various workers on the lipid turnover (i.e. NL and PL fractions) of *Achlya*[14], *Blastocladiella emersonii*[16], *Dipodascopsis uninucleata*[12] and *D. tothii* [10].

3.1.3. Fatty acyl composition

Changes observed in the major lipid classes over the growth cycle prompted an investigation into changes in their fatty acyl composition. All fractions contained 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid) and 18:2 (linoleic acid), while only 16:1 and 18:1 showed significant changes during the growth cycle (Fig. 3). In the NLs the percentage 16:1 decreased to a minimum value as this yeast approached the stationary phase (i.e. after 12 h), where after it started to increase. The opposite is true for 18:1. This phenomenon cannot be explained at present. Similar FA patterns were observed over the growth cycle in the other fractions (GL and PL) studied. When this experiment was repeated, similar patterns were observed.

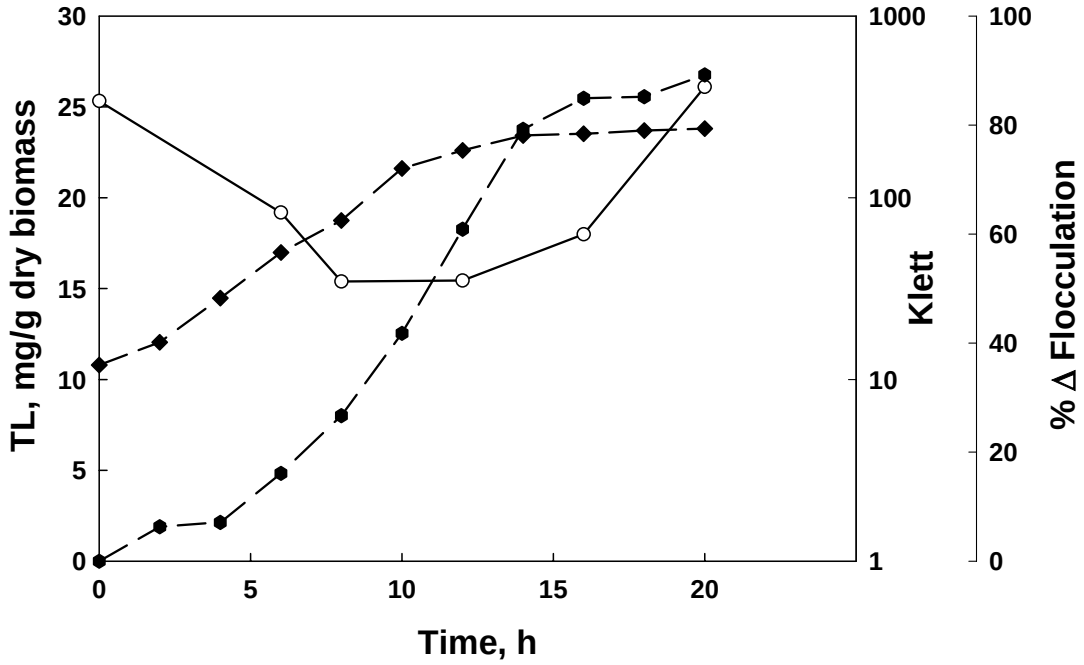

Fig. 1. Changes in growth (Klett), % Δ flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—○—): Total lipids (TL). This experiment was repeated and produced an SE < 5%.

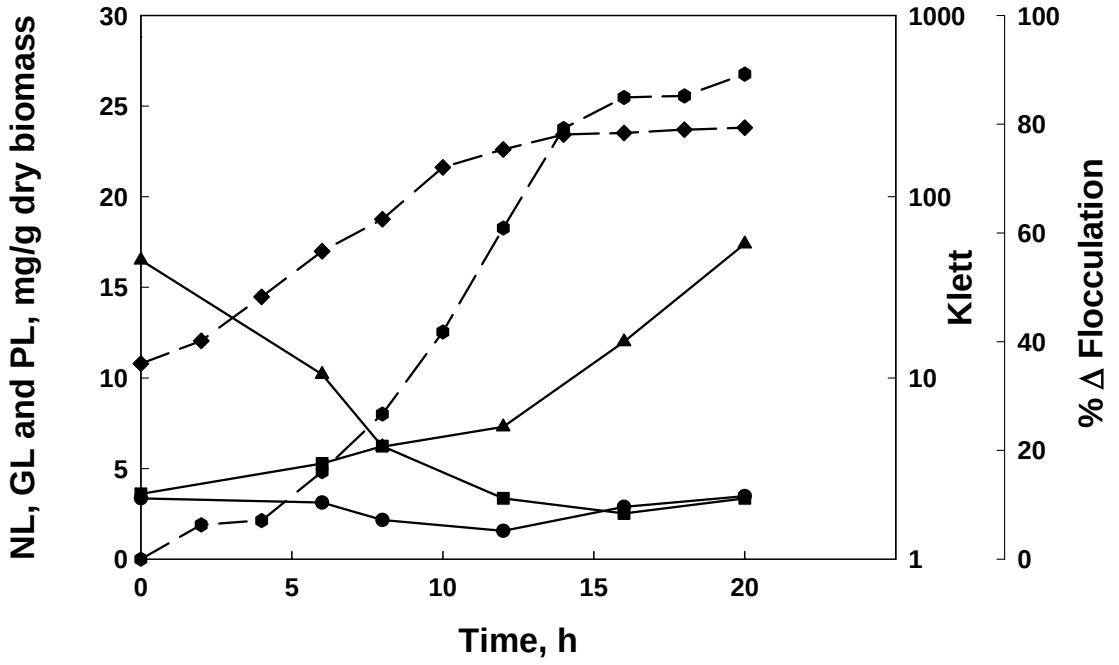

Fig. 2. Changes in growth (Klett), % Δ flocculation and different lipid fractions over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—▲—): Neutral lipids (NL); (—■—): Phospholipids (PL); (—●—): Glycolipids (GL). This experiment was repeated and produced an SE < 5%.

3.2. Lipid turnover in Flo1 phenotype behaviour

3.2.1. Changes in total lipid (TL) content

Here, the first 8 h of growth (flocculent phase) is characterised by actively growing and flocculent yeast cells (Fig. 4). After 8 h (start of non-flocculent phase) the still actively growing cells (in exponential phase) almost completely deflocculated in a 4 h period (from 8h to 12 h), where after they entered the stationary phase after 12 h of growth.

Changes in the intracellular TL content over the growth cycle are depicted in Fig. 4. During the first 6h of the flocculent phase, the TL content decreased from 14.6 to 12.9 mg/g dry biomass, where after it increased markedly to a maximum of 21.0 mg/g dry biomass during the last two hours of flocculation. Strikingly, the rapid deflocculating period (8 h to 12 h) was accompanied by a concomitant decrease in the TL content from 21.0 mg/g dry biomass to a minimum value of 9.9 mg/g dry biomass after 12 h. It was furthermore evident that as the cells entered the late exponential phase (i.e. after 12 h), another increase in the TL occurred from 9.9 to 16.1 mg/g dry biomass. When this experiment was repeated, similar patterns were observed.

3.2.2. Composition of total lipids (TLs) over the growth cycle

When the TLs extracted from the cells during different stages of growth were fractionated into three lipid classes by column chromatography, the results depicted in Fig. 5 were obtained. Changes in the TLs as well as NL and PL fractions followed similar patterns. The gradual decrease in NLs during the first 6 h of growth (from 9.1 to 5.6 mg/g dry biomass) may be ascribed to the fact that it was used as an energy source for growth through oxidation of stored FAs[23] and/or PL synthesis via diacylglycerol (DAG)[6]. Surprisingly, after 6 h, the cells started to accumulate NLs (i.e. from 5.6 to 9.4 mg/g dry biomass) after which (8 h) it decreased again to reach a minimum (3.9 mg/g dry biomass) after 12 h. Increase in NLs is probably a result of flocculation, which can be regarded as a survival mechanism or prelude to sexual reproduction[18].

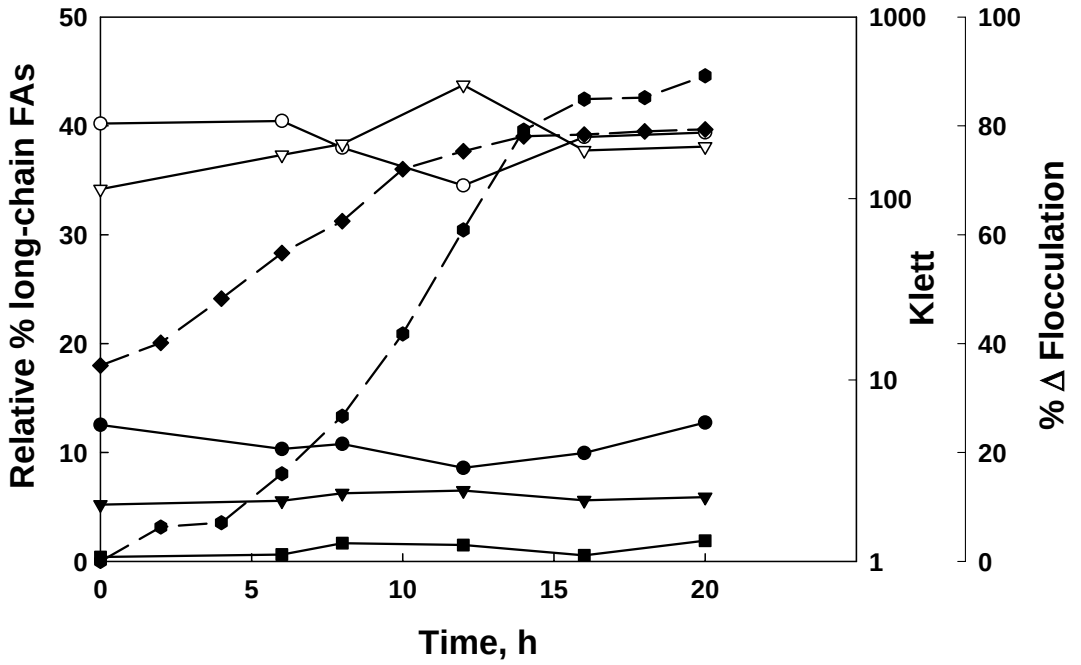

Fig. 3. Changes in growth (Klett), % Δ flocculation and % long-chain fatty acids of NLs over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a complex medium (NewFlo behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—●—): 16:0 (palmitic acid); (—○—): 16:1 (palmitoleic acid); (—▼—): 18:0 (stearic acid); (—▽—): 18:1 (oleic acid) and (—■—): 18:2 (linoleic acid). This experiment was repeated and produced an SE < 5%.

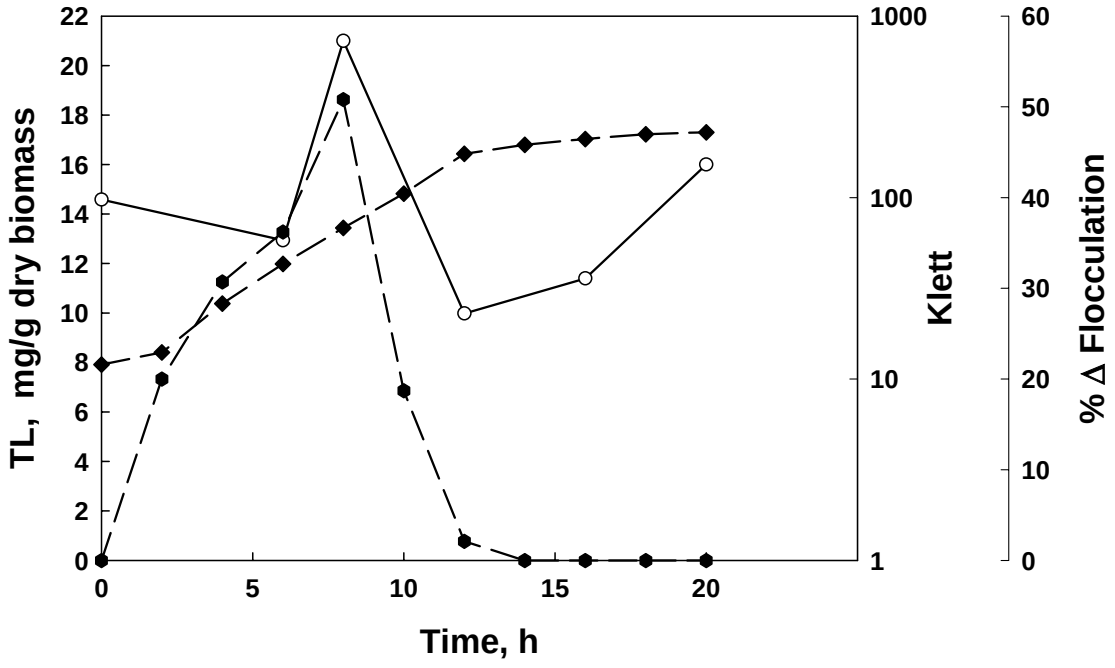

Fig. 4. Changes in growth (Klett), % Δ flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—○—): Total lipids (TL). This experiment was repeated and produced an SE < 5%.

As cells entered stationary growth phase, (from 12 h) the NLs increased from 3.9 to 10.2 mg/g dry biomass after 20 h. This may be ascribed to the tendency of yeasts to accumulate NLs during stationary growth phase as endogenous energy source for later utilisation[23].

The PL patterns observed were similar over the growth cycle as reported for NewFlo phenotype behaviour. The increased PL content during the first 6 h of flocculation (exponential growth phase) may be due to a bigger demand for membranes required for cell growth[12]. Similar patterns to that observed for the PL fraction were also evident for the GL fraction. This cannot be explained at present. When this experiment was repeated, similar patterns were observed for all lipid fractions.

3.2.3. Fatty acyl composition

When looking at the changes in FA composition of the different fractions over the growth cycle, interesting results were obtained. All the fractions contained 16:0, 16:1, 18:0, 18:1 and 18:2, but only 16:1 and 18:2 showed significant changes during the course of growth (Fig. 6). In the NLs, the percentage 16:1 decreased to a minimum value during the flocculent phase, where after it started to increase. The opposite trend was observed for the percentage 18:2, which increased to a maximum value during the first 8 h of flocculation, and then gradually decreased. We conclude that during the first 8 h of growth, 16:1 is probably converted to 18:1 via an elongase enzyme, which is then further desaturated to 18:2 via a Δ^{12} desaturase enzyme[5]. Furthermore, the demand for polyunsaturated FAs (PUFAs) seems to be higher during the exponential growth phase probably necessary to keep membranes fluid during active cell growth[12]. The FA patterns were similar in the GL and PL fractions studied. Similar patterns were observed when this experiment was repeated.

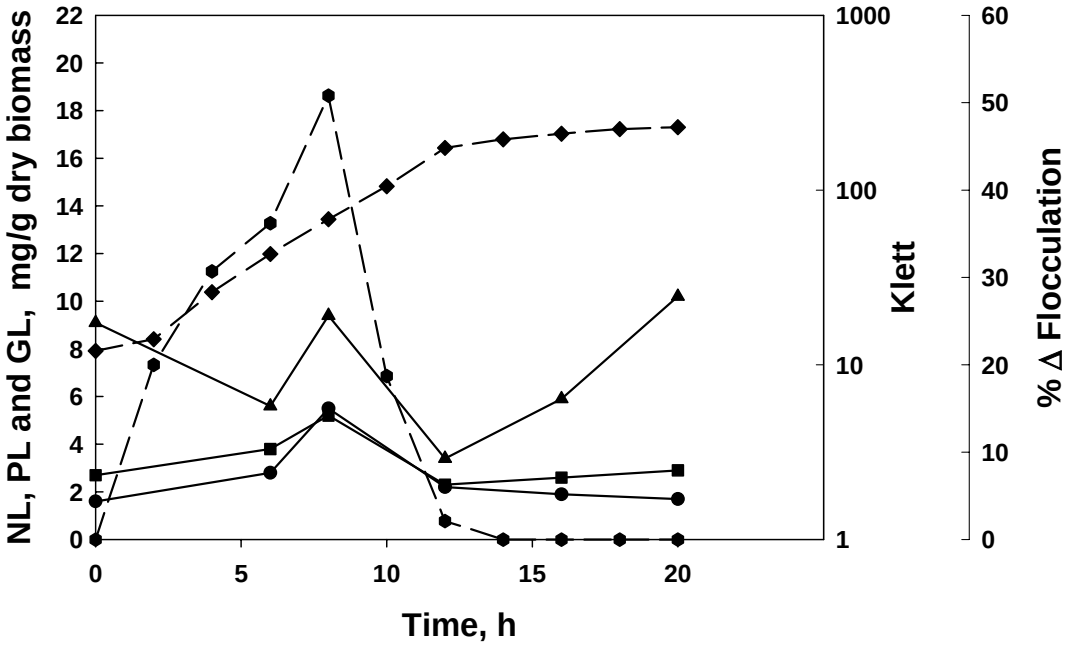

Fig. 5. Changes in growth (Klett), % Δ flocculation and total lipid content over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—▲—): Neutral lipids (NL); (—■—): Phospholipids (PL); (—●—): Glycolipids (GL). This experiment was repeated and produced an SE < 5%.

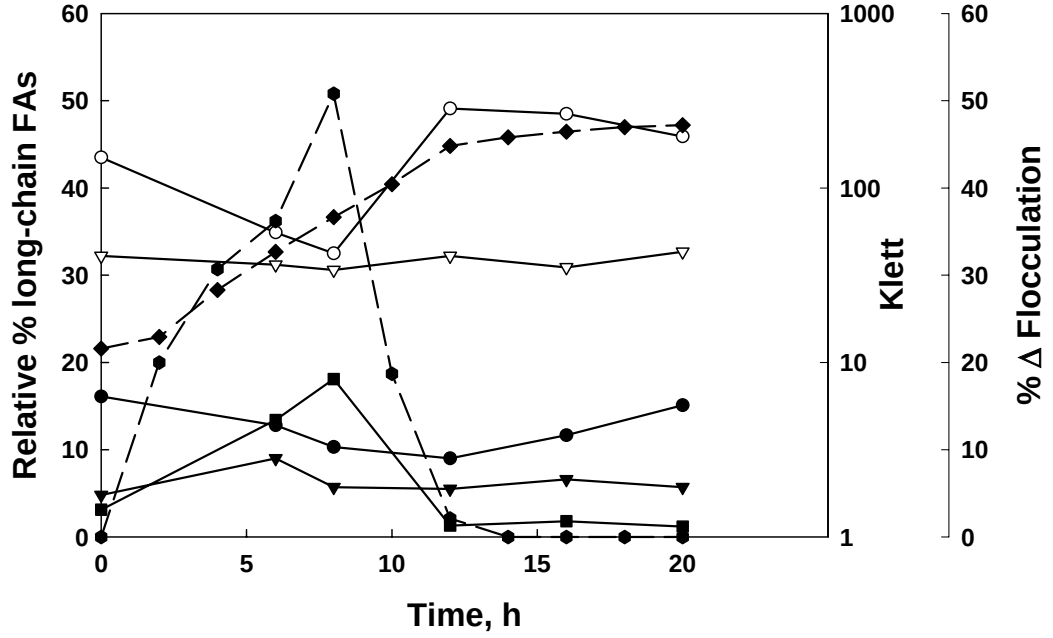

Fig. 6. Changes in growth (Klett), % Δ flocculation and % long-chain fatty acids of NLs over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330 when cultivated in a chemically defined medium (Flo1 behaviour). (--◆--): Optical density; (--●--): % Δ flocculation; (—●—): 16:0 (palmitic acid); (—○—): 16:1 (palmitoleic acid); (—▼—): 18:0 (stearic acid); (—▽—): 18:1 (oleic acid) and (—■—): 18:2 (linoleic acid). This experiment was repeated and produced an SE < 5%.

It is interesting to note that according to previously reported research[7] *Sacch. cerevisiae* cannot produce 18:2. The strain used in our study does not only deviate from other *Sacch. cerevisiae* strains by showing both Flo1 and NewFlo behaviours, but also in regard to its lipid metabolism.

4. ACKNOWLEDGEMENTS

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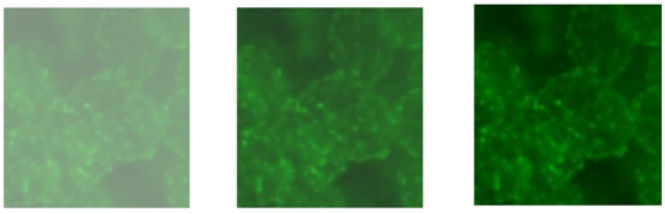
Chapter 4

Bioactive oxylipins in *Saccharomyces cerevisiae*



This chapter has been accepted for publication in the Journal of The Institute of Brewing and has been presented at the 24th International Specialized Symposium on Yeasts, Spain (2005).

ABSTRACT

We found that some strains of *Saccharomyces cerevisiae* (include 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 E_2 synthesized by *C. albicans* in conjunction with mammalian cells probably during candidiasis.

1. 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 uncovered[9]. Here they serve as cell

adhesives (flocculants) and to affect water-propelled movement in micron-space[10]. 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 aggregate[9]. These results also suggest that 3-OH oxylipins, present on the surfaces of flocculating cells of *Saccharomyces cerevisiae*, may play a role in flocculation[12].

Recently, clear-cut evidence for the role of 3-OH eicosanoids in candidiasis was presented[5,6,7]. Here, arachidonic acid (AA) is released from the infected or inflamed host tissue/cell and then converted by *Candida albicans* to 3*R*–hydroxy eicosatetraenoic acid (3*R*-HETE), which serves as substrate for COX-2 in host tissue/cell to produce pro-inflammatory 3-OH-PGE_2. Strikingly, in this investigation a cascade of novel bioactive 3-OH eicosanoids, produced from 3*R*-HETE via mammalian COX-2, was uncovered[5]. In addition, it was found that *C. albicans* selectively upregulates COX-2 in mammalian cells[7]. Prostaglandins produced by COX-2 are potent bioactive compounds in inflammatory diseases and cancer. Since both 3*R*-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 suggested[14]. Alem and Douglas[1] demonstrated that low concentrations of aspirin drastically inhibit catheter-biofilm formation by *C. albicans*. Strikingly, when PGE_2 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 3*R*-HETE from exogenously 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.

2. MATERIALS AND METHODS

2.1. Strains used

Saccharomyces cerevisiae (UOFS Y-2330) a known flocculent exposing both Flo1 and NewFlo flocculation behaviour[15] 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.

2.2. Cultivation and oxylipin extraction in the absence of aspirin

2.2.1. Cultivation

All strains of *Sacch. cerevisiae* was cultivated as described by Strauss et al[15]. In short, cells were inoculated from YM[18] 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 turbidity[4]. All experiments were performed in at least duplicate.

2.2.2. 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 extracted[13] using 2X 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 described[17]. All experiments were performed in at least duplicate.

2.2.3. 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 analysis[17]. All experiments were performed in at least duplicate.

2.2.4. 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 ethanol[17] 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 3*R*-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.

2.2.5. 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.

2.3. 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.

2.4. Gas chromatography – mass spectrometry (GC-MS) analysis

A Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jośe, California, USA) 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 Jośe, California, USA), 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 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 µl was injected at an inlet temperature of 230 °C at a split ratio of 1:50.

2.5. Chemicals used

All chemicals used were of highest purity grade.

3. RESULTS AND DISCUSSION

3.1. 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 PGE_2 and $PGF_{2\alpha}$ were reported in some yeasts including *Sacch. cerevisiae*[14]. In addition, our results suggest (Table 1) 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 1). 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 reported 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.

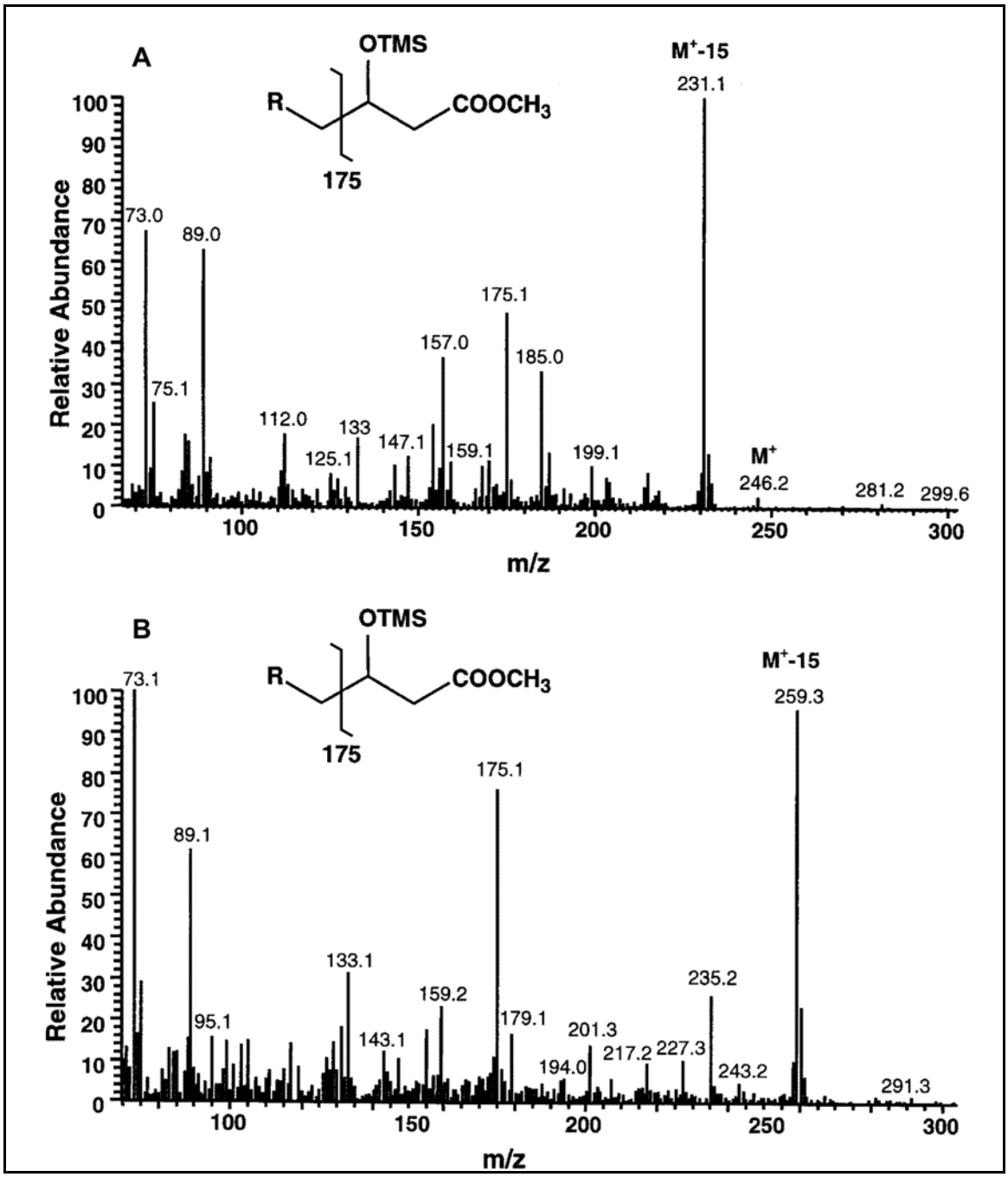


Fig. 1. Electron impact-mass spectra of methyl-trimethylsilylated 3-OH 8:0 (**A**) and 3-OH 10:0 (**B**). The characteristic m/z 175[17] was derived from the esterified carboxyl end.

3.2. 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 yeasts[9], aspirin inhibition studies were perform on strain 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.

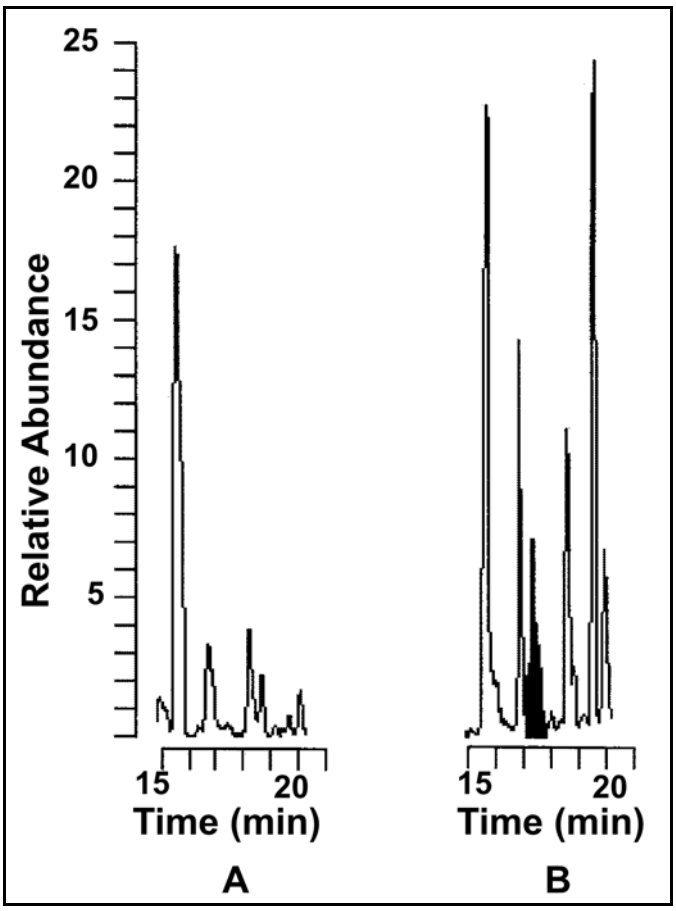


Fig. 2. Partial ion chromatograms of the methylated and trimethylsilylated extracts from cultures of *Saccharomyces cerevisiae* UOFS Y-2330 during the non-flocculent (6 h, **A**) and flocculent phases (20 h, **B**) of growth. Solid peak indicates 3-OH 8:0.

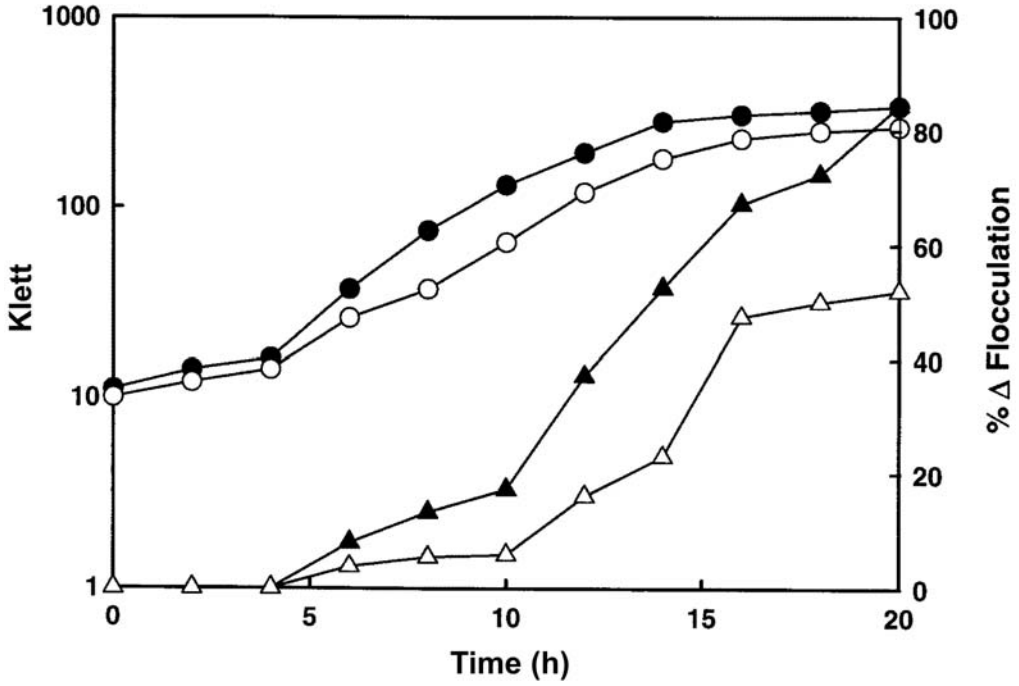


Fig. 3. Changes in growth (Klett units; circle) and degree of flocculation (% Δ flocculation; triangle) over the growth cycle of *Saccharomyces cerevisiae* UOFS Y-2330, when cultivated in a complex YM medium in the presence (hollow signs) and absence (solid signs) of 1 mM of acetylsalicylic acid (aspirin).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

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 cells[2]? 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.

4. ACKNOWLEDGEMENT

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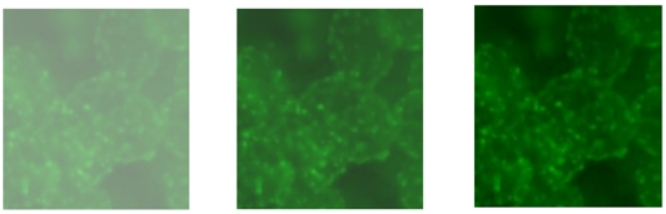
Table 1. Oxylipins produced *ab initio* and from arachidonic acid (AA) by flocculating cells of *Saccharomyces cerevisiae* UOFS Y-2330 and strains UOFS Y-1 and UOFS Y-3.

Yeast strain	3-Hydroxy oxylipins	
UOFS Y-2330		
Ab initio	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		
Ab initio	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		
Ab initio	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)]

Chapter 5

Oxylipin associated co-flocculation in yeasts



This chapter has been submitted for publication in Prostaglandins and other Lipid Mediators.

ABSTRACT

According to the lectin-theory, the yeast *Schizosaccharomyces pombe* lacks the specific receptors (α-mannans) necessary to facilitate co-flocculation with *Saccharomyces cerevisiae* species. In this study we demonstrate oxylipin associated co-flocculation between *Sacch. cerevisiae* and *S. pombe* strains using differential cell staining, immunofluoresence and ultrastructural studies. Using a 3-hydroxy (OH) oxylipin specific antibody coupled to a fluorescing compound, 3-OH oxylipins were found to be present on the cell surfaces of *Sacch. cerevisiae* and *S. pombe*. The presence of 3-OH oxylipins was confirmed using gas chromatography-mass spectrometry. Whether these 3-OH oxylipins play a role in affecting co-flocculation of *Sacch. cerevisiae* with *S. pombe* cells through entropic-based hydrophobic interactions and/or hydrogen bonds still needs to be verified.

1. INTRODUCTION

Flocculation is the phenomenon whereby yeast cells spontaneously adhere in clumps and sediment rapidly from the medium in which they are suspended [1]. This phenomenon is of great interest in brewing and many other fermentation industries.

For the past two decades, the mechanism of lectin type cell-cell interaction [2,3] has been proposed to explain flocculation. Cell surface hydrophobicity has been identified as the second major factor responsible for flocculation onset [1,3-7]. This observation was further supported by the accumulation of hydrophobic carboxylic acids i.e. 3-hydroxy (OH) oxylipins on the cell surfaces of *Saccharomyces cerevisiae* strains during flocculation onset [8-10].

Co-flocculation is the adherence of non-flocculent to flocculent cells and the subsequent sedimentation of the non-flocculent cells with the flocs [11]. According to the lectin-theory, for yeast to flocculate/co-flocculate, both parts of the flocculent bond, mannans and lectins, must be present [3]. Unlike *Sacch.*

cerevisiae, the carbohydrate component of the cell walls of *Schizosaccharomyces pombe*, involved in flocculation, consists of galactomannans [12]. The outer structure of these galactomannans has a α-1, 2 linked galactose attached to a poly-α-1, 6 linked mannose backbone [13]. Specific receptors (α-mannans) necessary to affect co-flocculation of *S. pombe* with *Sacch. cerevisiae* are not readily available due to the shielding of the mannose residues by the galactose side branches [14]. Consequently, on the basis of the lectin-theory one should not expect *Sacch. cerevisiae* and *S. pombe* to co-flocculate. In addition, *S. pombe* is also often used as a negative control in co-flocculation studies of *Sacch. cerevisiae* species [11,15].

In 2003, we uncovered a *Sacch. cerevisiae* strain, which demonstrated inverse flocculation patterns (NewFlo and Flo1), when cultivated in complex and chemically defined glucose containing media respectively [10]. Strikingly, this strain was found to co-flocculate with *S. pombe* strains. In this study we demonstrate co-flocculation between *Sacch. cerevisiae* UOFS Y-2330 and *S. pombe* as well as the presence of various 3-OH oxylipins, probably also involved in this process.

2. MATERIALS AND METHODS (See Appendix for details)

2.1. Strains used

Yeast strains used in this study, are listed in Table 1 (A - F).

2.2. Cultivation

Yeast strains (Table 1: A, C - F) were cultivated as described [10]. In short, cells were inoculated from YM [16] 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 at 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. These cultures were incubated at 25 °C while shaking (160 rpm) for 20 h. *Saccharomyces cerevisiae* UOFS Y-2330 demonstrated NewFlo phenotype flocculation under these conditions [10,17]. Following this, cells were harvested (8000 rpm for 10 min) for microscopic investigations, while whole cultures (cells with supernatant) were used for chemical analysis. All experiments were performed at least in duplicate.

Saccharomyces cerevisiae UOFS Y-2330 (Table 1: B) was grown in chemically defined media i.e. glucose–YNB (Yeast Nitrogen Base, Difco Laboratories) medium (12 g/L glucose, 6.7 g/L YNB) under similar growth conditions in order to facilitate Flo1 phenotype flocculation [10,17]. After 20 h, cells were harvested as before for morphological investigations and chemical analysis (i.e. cells with supernatant). All experiments were performed at least in duplicate.

2.3. Morphological investigations

2.3.1. Sample preparation for microscopy

Harvested wet yeast cells (Table 1: A - F) were collected and resuspended in their respective supernatants in a ratio 1:1 (w/v). Next, all flocculating cells (Table 1: A - D) were deflocculated by decreasing the pH below 2.7 with 1 M HCl [10]. For each yeast, equal amounts of these non-flocculent suspensions were mixed in a 1:1 ratio (w/w; Table 1: G - N). Non-flocculating cells of both species were thoroughly mixed and the pH of the mixtures increased to 5.5. At this pH, cells of *Sacch. cerevisiae* (Table1: A – D) flocculate optimally [10]. Subsequently, mixtures that showed co-flocculation under light microscopy (cells of different species adhere together) were subjected to differential cell staining, immunofluorescence and ultrastructural studies.

2.3.2. Differential cell staining studies

Co-flocculating cells of *Sacch. cerevisiae* UOFS Y-2330 and *S. pombe* strains (Table 1: G - J), were stained with a 1% Orange G (Molecular Probes, Eugene, OR, U.S.A.) aqueous solution for 5 min at room temperature (21 °C). Stained cells were rinsed twice with dH_2O. These cells were resuspended in 1% Acridine Orange (Molecular Probes, Eugene, OR, U.S.A.) and counterstained for 5 min at 21 °C. After staining, these cells were again rinsed twice with dH_2O and mounted on a microscope slide with a cover slip. Fluorescing material was photographed using a Nikon TE 2000 confocal laser scanning microscope (CLSM; Nikon, Tokyo, Japan). All experiments were repeated at least in duplicate.

2.3.3. Immunofluorescence studies

Specific 3-OH oxylipin antibodies, chemically synthesised against *R* and *S* isomers of 3-OH-5Z,8Z,11Z,14Z-eicosatetraenoic acid [18,19], were raised in rabbits and characterised as described [20]. Immunofluorescence of co-flocculating cells (Table 1: G - J) were performed as described [20]. This included treatment with the primary antibody against 3-OH oxylipins as well as FITC conjugated secondary antibody. In order to maintain aggregated cell floc structure, antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Following adequate washing, cells were mounted on a microscope slide with a cover slip. Fluorescing material was photographed using a Nikon TE 2000 CLSM. All experiments were repeated at least in duplicate.

2.3.4. Ultrastructural studies

Co-flocculating cells (Table 1: G, I) were chemically fixed, using 3% glutardialdehyde (Merck, Darmstadt, Germany) and 1% osmiumtetroxide [21] (Merck). Transmission electron micrographs were taken with a Philips (FEI) CM 100 (Amsterdam, The Netherlands) transmission electron microscope (TEM).

2.4. Chemical analysis

2.4.1. Extraction and derivatisation of ab initio-produced 3-OH oxylipins

In order to investigate the *ab initio* production and composition of 3-OH oxylipins associated with the surfaces, oxylipins of 20 h old whole cultures (cultivated as described; Table 1: A, B, E, F) were immediately extracted [22] using 2X volume of ethyl acetate (Saarchem, Gauteng, South Africa). Following this, samples were methylated and silylated (Me-TMSi) using diazomethane [Prepared from Diazald (Aldrich, Schnelldorf, Germany)] and bis-(trimethylsilyl) trifluoroacetamide (BSTFA; Merck, Germany). Finally, the samples were reconstituted in 400 μl chloroform:hexane (4:1, v/v; Merck, Germany) followed by gas chromatography – mass spectrometry (GC-MS) analysis [23]. All experiments were performed in at least duplicate.

2.4.2. Oxylipins in growth medium

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

2.4.3. Gas chromatography – mass spectrometry

A Finnigan TraceGC ultra gas chromatograph (Thermo Electron Corporation, San Jośe, California, USA) was used to record the Electron Impact (EI) mass spectra of the extracted 3-OH oxylipins present in the yeast cultures. This instrument is equipped with a Finnigan Trace DSQ MS (Thermo Electron Corporation, San Jośe, California, USA), with an 60m-HP-5 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 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. A sample volume of 1 μl was injected at an inlet temperature of 230 °C at a split ratio of 1:50.

3. RESULTS AND DISCUSSION

3.1. Morphology

Co-flocculation was clearly visible as observed with light microscopy (results not shown), in aggregates containing a mixture of *S. pombe* (non-flocculent) and *Sacch. cerevisiae* UOFS Y-2330 (flocculent) cells of both the Flo1 and NewFlo phenotype (Table 1: G - J). In agreement with literature, control experiments with cells of *Sacch. cerevisiae* NCYC 1364 and NCYC 1026, failed to co-flocculate with the *S. pombe* strains studied [11,15] (Table 1: K – N). These results prompted us to further confirm this co-flocculation phenomenon through differential staining methods using CLSM (Fig. 1). Cells of *Sacch. cerevisiae* UOFS Y-2330 and *S. pombe* strains showed differing affinities to Acridine Orange and Orange G which made differential fluorescence possible - needed to distinguish between the two species (Fig. 1). The reason for this differential staining is still unclear. Similar results were obtained for all co-flocculating combinations studied (Table1: G, I, J; results not shown).

In 2000, Kock and co-workers reported that flocculating vegetative cells of *Sacch. cerevisiae* contain oxylipin associated “sticky” protuberances that possibly play a role in flocculation. Using a 3-OH oxylipin specific antibody coupled to a fluorescing compound (FITC anti-IgG), additional investigations by CLSM were performed to map the presence, distribution and dimensional arrangement of these compounds during co-flocculation (Fig. 2). Here, fluorescence was observed on the cell surfaces and in between co-flocculating cells, implicating a possible role of 3-OH oxylipins in co-flocculation. Fluorescing protuberances randomly extended across the cell walls of both species, probably to attach to the cell surfaces of the neighboring cells [10]. Similar results were obtained for all co-flocculating combinations studied (results not shown, Table1: G - I).

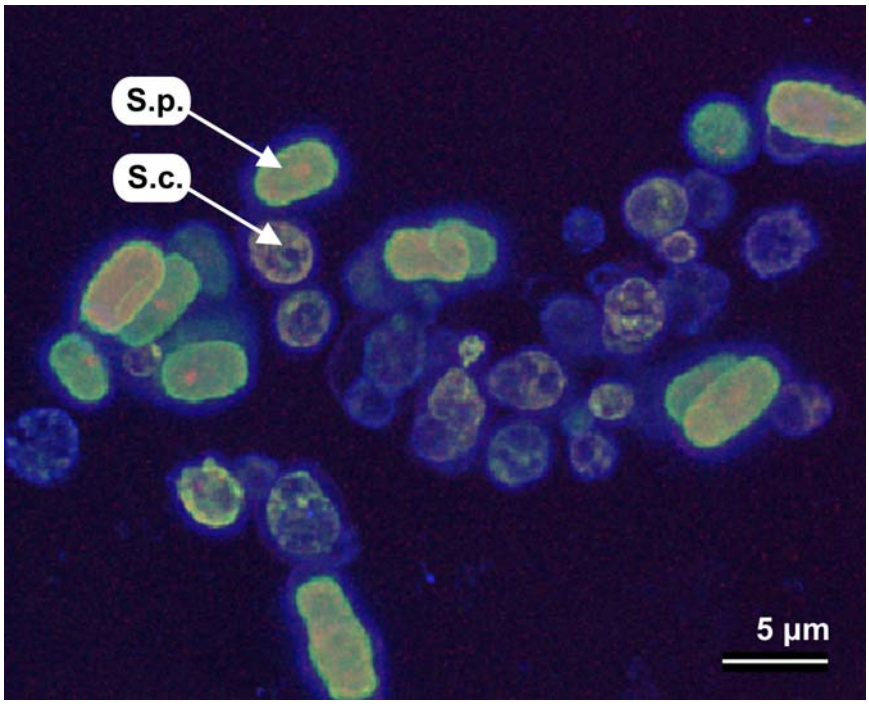


Fig. 1. Confocal laser scanning micrograph of differentially stained cells demonstrating co-flocculation between *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Table 1: H). *Saccharomyces cerevisiae* (S.c.; round) and *S. pombe* (S.p.; cylindrical) cells showed differing affinities to Acridine Orange and Orange G. The cytoplasm of *S. pombe* cells stained yellow-green, whereas the cytoplasm of *Sacch*. *cerevisiae* remained blue.

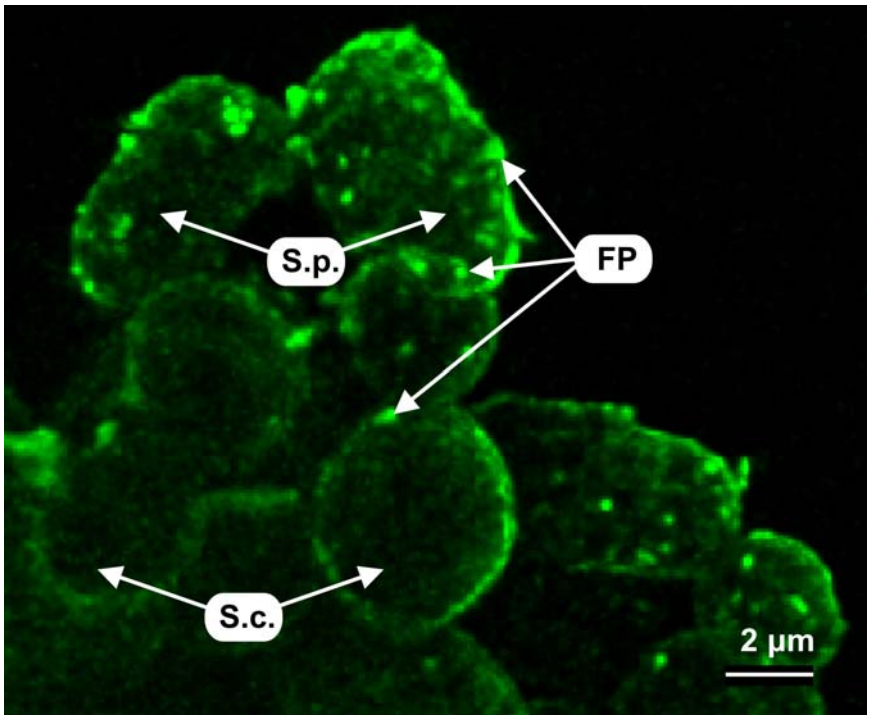


Fig. 2. Oxylipin specific immunofluorescence micrograph of co-flocculating *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cells (Table 1: J). Oxylipin specific fluorescing protuberances (FP) were observed on the cell surfaces of *Sacch. cerevisiae* (S.c.; round) and *S. pombe* (S.p.; cylindrical).

These results prompted us to further investigate the fluorescing protuberances found on these yeasts during co-flocculation (Table 1: G - J). For this purpose, TEM was performed on osmium tetroxide fixed yeast samples to determine the cell ultrastructure of fluorescing protuberances as observed using CLSM. Here, osmiophilic protuberances were observed which migrated through the cell walls of *Sacch. cerevisiae* (cell wall less granular) in order to attach to adjacent *S. pombe* cells (cell wall more granular; Fig. 3). This is similar to the results obtained by Kock and co-workers [8] where they reported "ghost like" protuberances responsible for attaching flocculating cells of *Sacch. cerevisiae* during flocculation. Using immunogold labelling, these authors demonstrated the presence of 3-OH oxylipins in these protubing osmiophilic layers. Similar results were obtained for co-flocculating combination I (Table 1; results not shown).

3.2. Chemical studies

The presence of 3-OH oxylipins as observed by CLSM and TEM were confirmed using GC-MS analysis. All these oxylipins produced a base peak of *m/z* 175 [$CH_3O(CO).CH_2.CHOSi(CH_3)_3$] in their mass spectra, which is characteristic of hydroxylation of fatty acids at C-3 [23] (Table 2).

Using GC-MS, 3-OH 8:0 (NewFlo phenotype) and 3-OH 6:0, 3-OH 12:0 and 3-OH 14:1 (Flo1 phenotype) were found to be associated with cultures of *Sacch. cerevisiae* UOFS Y-2330 tested after 20 h of growth (Table 2). Kock and co-workers [8] also found 3-OH 8:0 to be associated with flocculating NewFlo phenotype *Sacch. cerevisiae* cells. In S. *pombe* CBS 5557 we detected 3-0H 9:1, whereas *S. pombe* CBS 356T contained 3-OH 11:0 and 3-OH 15:0 (Table 2). According to literature [8] and results obtained in this study, YM broth contains 3-OH 12:1 (Table 2). Whether these 3-OH oxylipins play a role in affecting co-flocculation of *Sacch. cerevisiae* to *S. pombe* cells by for instance entropic-based hydrophobic interactions and/or hydrogen bonds, still needs to be verified [8,9,24-26].

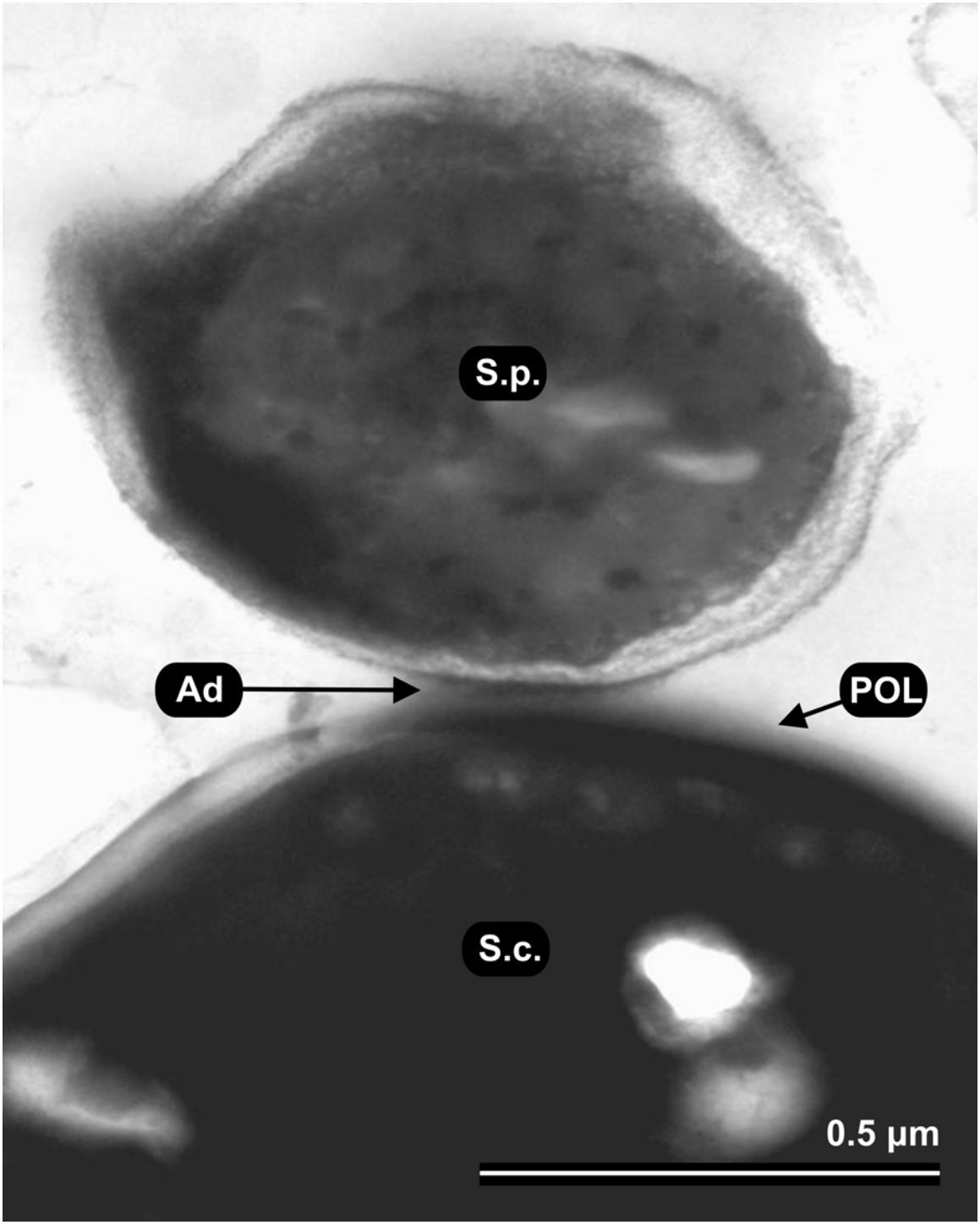


Fig. 3. Transmission electron micrograph (TEM) showing adhesion (Ad) of the protubing osmiophilic layer (POL) of *Saccharomyces cerevisiae* (S.c.) to the cell wall of the neighbouring *Schizosaccharomyces pombe* (S.p.) cell (Table 1: G).

Saccharomyces cerevisiae UOFS Y-2330 does not only deviate from other *Sacch. cerevisiae* strains by demonstrating different flocculation behaviors [10] and lipid metabolisms [17], but also with regards to its ability to co-flocculate with *S. pombe* strains. This further highlights the unique position of this strain.

4. ACKNOWLEDGEMENTS

The authors wish to thank the National Research Foundation, South Africa and Volkswagen Foundation, Germany (1/74643) for financial support.

Supplementary Data

A three dimensional animation of a confocal laser scanning microscopy Z-stack, demonstrating co-flocculation of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cells (Table 1: H). *Saccharomyces cerevisiae* (round) and *S. pombe* (cylindrical) cells showed differing affinities to Acridine Orange and Orange G. The cytoplasm of *S. pombe* cells stained yellow-green, whereas the cytoplasm of *Sacch*. *cerevisiae* remained blue. In order to view this the reader is referred to movie 2 on the CD at the back of the thesis.

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Table 1
Strains used and flocculation patterns on various media

Experiment Number	Yeast	Strain Number	Growth media	Flocculation type
A	*Sacch. cerevisiae*	UOFS Y-2330	YM-glc	NewFlo
B	*Sacch. cerevisiae*	UOFS Y-2330	YNB-glc	Flo1
C	*Sacch. cerevisiae*	NCYC 1026	YM-glc	NewFlo
D	*Sacch. cerevisiae*	NCYC 1364	YM-glc	NewFlo
E	*S. pombe*	CBS 356T	YM-glc	-
F	*S. pombe*	CBS 5557	YM-glc	-
G	(A) x (E)			Co-floc
H	(A) x (F)			Co-floc
I	(B) x (E)			Co-floc
J	(B) x (F)			Co-floc
K	(C) x (E)			-
L	(C) x (F)			-
M	(D) x (E)			-
N	(D) x (F)			-

UOFS: University of the Free State, Bloemfontein, South Africa; NCYC: National Collection for Yeast Cultures, Norwich, United Kingdom; CBS: Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands; YNB-glc: Yeast Nitrogen Base-glucose; YM-glc: Yeast Malt-glucose; Co-floc: Cells of different species adhering together; NewFlo: flocculation occurs towards the stationary growth phase; Flo1: flocculation occurs throughout growth; *Sacch. cerevisiae*: *Saccharomyces cerevisiae*; *S. pombe*: *Schizosaccharomyces pombe*

Table 2
Oxylipins present in *Schizosaccharomyces pombe* CBS 356T, *S. pombe* CBS 5557 and *Saccharomyces cerevisiae* UOFS Y-2330 after 20 h of growth

Yeast	Strain Number	Growth media	Flocculation Type	Metabolite	Fragments
Sacch. cerevisiae	UOFS Y-2330	YM-glc	NewFlo	3-OH 8:0	175; 231 [M^+-15 (CH_3)]
Sacch. cerevisiae	UOFS Y-2330	YNB-glc	Flo1	3-OH 6:0	175; 203 [M^+-15 (CH_3)]
				3-OH 12:0	175; 287 [M^+-15 (CH_3)]
				3-OH 14:1	175; 313 [M^+-15 (CH_3)]
S. pombe	CBS 356T	YM-glc	-	3-OH 11:0	175; 273 [M^+-15 (CH_3)]
				3-OH 15:0	175; 329 [M^+-15 (CH_3)]
S. pombe	CBS 5557	YM-glc	-	3-OH 9:1	175; 243 [M^+-15 (CH_3)]
YM-glucose broth		YM-glc		3-OH 12:1	175; 285 [M^+-15 (CH_3)]

UOFS: University of the Free State, Bloemfontein, South Africa; NCYC: National Collection for Yeast Cultures, Norwich, United Kingdom; CBS: Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands; YNB-glc: Yeast Nitrogen Base-glucose; YM-glc: Yeast Malt-glucose; Co-floc: Cells of different species adhering together; NewFlo: flocculation occurs towards the stationary growth phase; Flo1: flocculation occurs throughout growth; *Sacch. cerevisiae*: *Saccharomyces cerevisiae*; *S. pombe*: *Schizosaccharomyces pombe*

Appendix

LIPID EXTRACTION

(Performed by candidate: C.J. Strauss)

Several flasks to yield sufficient biomass and lipids were harvested (12400 *g* for 10 min) at different time intervals and then rapidly frozen at -70 ^{o}C overnight. The frozen cells were then freeze-dried and weighed. Next, total lipid (TL) extraction was performed on freeze-dried cells according to Folch et al. (1957). In short, lipids were extracted from crushed freeze-dried cells with a mixture of 150 ml chloroform and methanol (2:1, v/v). In order to collect the organic phase, the cells were filtered, using Whatman no.1 filter paper. The collected organic phases were then added to separating funnels, washed twice with 25 ml dH_2O and left to clear. Thereafter, the organic phases were collected in a round bottom flasks and evaporated under a vacuum using a Heidolph VV 2011 rotary evaporator. Subsequently, the lipid samples were transferred to a pre-weighed vial using diethyl ether and left to evaporate. In order to quantify the TL fraction, samples were dried to a constant weight in a vacuum oven at 50 $^{\circ}$C over phosphorus pentoxide (P_2O_5) before they were finally weighed.

LIPID FRACTIONATION

(Performed by candidate: C.J. Strauss)

The extracted TLs were dissolved in chloroform and applied to a column (140 mm x 20 mm) of activated silicic acid (by heating overnight at 110 ºC). Neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions were then eluted from the column by successive applications of organic solvents of different polarities.

Volumes of trichloroethane (150 ml), acetone (100 ml) and methanol (100 ml) were applied to the column to elute NL, GL and PL fractions respectively (Kock and Ratledge, 1993). These fractions were collected in respective round bottom flasks. Following this, the organic phases were evaporated under a vacuum using a Heidolph VV 2011 rotary evaporator. The lipid fractions were finally dissolved in a minimal volume of diethyl ether and transferred to pre-weighed vials followed by drying over a stream of nitrogen gas. In order to quantify the NL, PL and GL fractions, samples were dried to a constant weight in a vacuum oven at 50 ºC over P_2O_5 before they were finally weighed.

FATTY ACID DETERMINATION

(Performed by candidate: C.J. Strauss)

The fatty acid (FA) compositions of the different fractions were determined after transesterification by the addition of 200 µl trimethyl sulphonium hydroxide (TMSOH) according to Butte (1983). Subsequently, the FA methyl esters were analysed using a Hewlett Packard 5890 gas chromatograph, equipped with a Supelcowax 10 polar/capillary column (30m x 0.53mm) with nitrogen as carrier gas - set at a flow rate of 4 ml/min. The inlet temperature was set at 180 °C with the initial column temperature set at 145 ºC - increasing at 3 °C/min, followed by the same temperature increment to a final temperature of 240 °C. The FA peaks were detected using a flame ionisation detector (FID) set at 300 °C. Peaks were identified using suitable standards.

SYNTHESIS OF 3-HYDROXY OXYLIPINS AND ANTIBODIES

(Performed in Germany by Prof. S. Nigam and his research group)

3-Hydroxy-5,8,11,14,-eicosatetraenoic acid (3-HETE) was chemically synthesized for antibody preparation as previously described. The synthetic strategy for the synthesis of 3*R* and 3*S* hydroxy arachidonic acid (AA) involved a convergent approach coupling chiral aldehyde with a Wittig salt (Bhatt et al. 1998; Groza et al. 2002). These compounds were derived from 2-deoxy-D-ribose and AA respectively.

Antibodies against 3-HETE was raised in rabbits according to the following protocol: The carboxyl group of 3-R HETE was conjugated to amino groups of bovine serum albumin (BSA) via the N-succinimidyl ester method. For initial injection one mg of this conjugated protein was emulsified in an equal volume of Fraud's complete adjuvant, and in incomplete adjuvant for successive injections. A female New Zealand white rabbit was injected subcutaneously in its back with this emulsion every second week, for about three months. After treatment the blood was collected from the carotid artery, left for two hours at room temperature and centrifuged (1200 *g* for 20 min at 4 ºC). Finally, sera were purified by Biogenes, Berlin (Kock et al. 1998).

PREPARATION AND CHARACTERISATION OF ANTIBODY

(Performed in Germany by Prof. S. Nigam and his research group as well as Prof. J.L.F. Kock)

The 3-HETE raised antibody was characterized in terms of its titer, sensitivity and specificity. As radiolabelled [^{14}C]-3-HETE is not commercially available, a biological tracer was prepared in small amounts by the biotransformation of [^{14}C]-arachidonic acid (AA) to [^{14}C]-3-HETE using the yeast *Dipodascopsis uninucleata*. Subsequently, radio labeled [^{14}C]-3-HETE was purified using Radio High Performance Liquid Chromatography (HPLC). Determination of the antibody titer resulted in approximately 30 % labeled 3-HETE at a dilution of 1:1000 in the absence of unlabeled 3-HETE. The sensitivity (minimum detectable amount) of 3-HETE was 30 pmol as determined by 10 % displacement of radioactivity by unlabeled 3-HETE from the zero point. Finally the specificity of the antibody was analyzed, using various structurally related compounds to determine the possible cross-reactions with the antibody. The antibody exhibited cross-reaction of < 0.5 % with 5-, 12-, or 15-HETE, while significant cross reactivity was observed against other 3-hydroxy (OH) oxylipins of different chain lengths and degrees of desaturation. No immunoreactivity against free fatty acids was observed. Thus, in our studies immunoreactivity solely indicates the presence of 3-OH oxylipins.

CONFOCAL LASER SCANNING MICROSCOPY

(Performed by candidate: C.J. Strauss)

Differential cell staining studies

Co-flocculating cells of *Sacch. cerevisiae* UOFS Y-2330 and *S. pombe*, were stained with a 1 % Orange G (Molecular Probes, Eugene, OR, U.S.A.) aqueous solution for 5 min at room temperature (21 ºC). Stained cells were rinsed twice with dH_2O. These cells were resuspended in 1 % Acridine Orange (Molecular Probes, Eugene, OR, U.S.A.) and counterstained for 5 min at 21 ºC. After staining, these cells were again rinsed twice with dH_2O and mounted on a microscope slide with a cover slip (0.1 mm). Fluorescing material were photographed using a Nikon TE 2000 confocal laser scanning microscope (CLSM; Nikon, Tokyo, Japan).

Immunofluorescence studies

(Performed by candidate: C.J. Strauss)

Immunofluorescence of flocculating and co-flocculating cells were performed as described (Kock et al. 1998). In order to maintain aggregated floc structure, antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Yeast cells were harvested after 20 h of growth by centrifugation (9820 *g* for 10 min). A very small amount of cells were suspended in phosphate buffer solution (PBS) and centrifuged to wash the cells. These cells were then treated with the 30 µl (1:2 v/v) of the primary antibody against 3-OH oxylipins for one hour at room temperature (21 ºC). After adequate washing with PBS, 30 µl (1:2 v/v) of Fluorescein (FITC)-conjugated Affinipure Goat Anti-Rabbit IgG (Jackson Immunoresearch Laboratories, U.S.A.) was added and incubated for one hour in the dark. Following adequate washing, cells were mounted in Dabco (Aldrich Chemical Company, U.S.A.) on a microscope slide. Dabco, a free radical scavenger, helps to sustain the fluorescence. Fluorescing material were photographed using a Nikon TE 2000 CLSM. In addition, fluorescing material was also photographed with a Zeiss Axioscope (Germany) microscope, equipped

for epifluorescence with a 50 W high pressure mercury lamp (exitation filter: Blue, 460 nm), using a Kodak Gold Ultra 200 film.

TRANSMISSION ELECTRON MICROSCOPY

(Performed by candidate: C.J. Strauss)

Transmission electron microscopy (TEM) was performed according to the method of Van Wyk and Wingfield (1991). Material for TEM was chemically fixed using 3 % gluteraldehyde (3 h) and 1 % osmiumtetroxide (1 h) in a 0.1 M sodium phosphate buffer. After rinsing with the same buffer, the fixed cells were dehydrated using an acetone series (50 %, 70 %, 95 %, 2X 100 %). Each dehydration step lasted for 30 min. Material for TEM was subsequently impregnated in epoxy resin (Spurr 1969) and set in a silicone mold (14 mm long x 6 mm wide x 4 mm deep). The cells embedded in epoxy were polymerized at 70 ºC in an oven for eight hours. After polymerization, ultramicrotomy were performed with a LKB Ultratome III, and the ultra-thin sections (60 nm) collected on 200 mesh Formvar copper coated grids. Finally the sections were stained with a saturated solution of uranyl acetate for 10 min and lead sitrate for five min. Transmission electron micrographs were taken with a Philips CM 100 (The Netherlands).

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Summary

Although beer production is one of the oldest biotechnologies in the world, a major constraint in brewing remains controlling flocculation. Evidence points towards a possible role of lipids, associated with the cell surfaces, as a major factor responsible for flocculation. Therefore, the aim in this study became to evaluate the contribution of lipids, especially oxylipins, in the flocculation of *Saccharomyces cerevisiae* UOFS Y-2330.

Saccharomyces cerevisiae UOFS Y-2330 was selected as a model, since it was found to demonstrate both Flo1 and NewFlo phenotype flocculation behaviour, when cultivated in different media. In a defined medium with glucose as a sole carbon source, this strain immediately flocculated strongly and lost this ability before stationary phase was reached. In a complex medium containing glucose, this yeast strongly flocculated towards the stationary growth phase without losing this ability during this phase. This inverse pattern may be ascribed to a switch in sensitivity of the yeast to flocculate in the presence of glucose as well as pH level, which may, in turn, influence the availability of calcium ions. In both media, matured cells produced protuberances upon flocculation as observed by electron and immunofluorescence microscopy, which may be involved in cell adhesion. This was followed by further investigations into the role of lipids over the growth cycle of this yeast. Here, it was uncovered that *Sacch. cerevisiae* UOFS Y-2330 does not only demonstrate inverse flocculation, but is also characterised by two different lipid turnover patterns. During Flo1 phenotype flocculation, this yeast showed two neutral lipid accumulating stages (i.e. at 8 h and from 12 h). This is probably triggered by flocculation, which may be regarded as a survival mechanism where cells accumulate especially neutral lipids as reserve energy source - a similar mechanism is probably operative when cells enter stationary

growth. Contrary to Flo1 behaviour, this strain in NewFlo phenotype mode demonstrates only a single lipid accumulation phase i.e. when cells enter stationary growth, which coincides with the increase in flocculation. In addition, an increase in phospholipids was experienced during active growth in both flocculation behaviours, probably as a result of active membrane production. These results prompted us to investigate the possible role of oxylipins present on the cell surfaces during the flocculation process. It was found that some strains of *Sacch. cerevisiae* (include 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 aspirin was added to cultures of *Sacch. cerevisiae* UOFS Y-2330, flocculation was significantly inhibited as well as the production of 3-hydroxy (OH) 8:0 thereby linking flocculation and this oxylipin. Furthermore, no traces of 3-OH 8:0 could be detected before flocculation onset in this yeast. Next, the involvement of these oxylipins in co-flocculation was assessed. According to the lectin-theory, the yeast *Schizosaccharomyces pombe* lacks the specific receptors necessary to facilitate co-flocculation with *Sacch. cerevisiae* species. In this study we demonstrate oxylipin associated co-flocculation between *Sacch. cerevisiae* UOFS Y-2330 and *S. pombe* strains using differential cell staining, immunofluorescence and ultrastructural studies. Using a 3-OH oxylipin specific antibody coupled to a fluorescing compound, 3-OH oxylipins were found to be present on the cell surfaces of *Sacch. cerevisiae* and *S. pombe*. The presence of 3-OH oxylipins was confirmed using gas chromatography-mass spectrometry. Whether these 3-OH oxylipins play a role in affecting co-flocculation of *Sacch. cerevisiae* with *S. pombe* cells through possibly entropic-based hydrophobic interactions and/or hydrogen bonds still needs to be verified. Studies on the physiological, genetic as well as colloidal aspects of flocculation using this model strain may lead to important new insights in this fascinating phenomenon as well as applications in industry.

Opsomming

Alhoewel bierproduksie een van die oudste biotegnologiese prosesse ter wêreld is, bly die beheer oor flokkulasie ‘n groot probleem. Data dui daarop dat lipiede wat met die seloppervlaktes gedurende flokkulasie geassosieer word, moontlik ‘n groot bydraende faktor tot flokkulasie kan wees. Die doelstellings van hierdie studie is dus om die bydrae van lipiede, veral oksielipiene, tot die flokkulasie proses in *Saccharomyces cerevisiae* UOFS Y-2330 te evalueer.

Saccharomyces cerevisiae UOFS Y-2330 is as model geselekteer, aangesien daar bevind is dat dit inverse flokkulasie patrone tydens kweking in verskillende media vertoon. In ‘n gedefinieerde medium met glukose as enigste koolstofbron, het hierdie stam dadelik sterk begin flokkuleer, maar het egter hierdie vermoë verloor voordat die stasionêre fase bereik is. In ‘n komplekse medium wat glukose bevat, het hierdie gis sterk begin flokkuleer aan begin van die stasionêre fase, sonder om hierdie vermoë gedurende hierdie fase te verloor. Hierdie inverse patroon kan moontlik toegeskryf word aan ‘n omskakeling in die sensitiwiteit van hierdie gis om by verskillende glukose-konsentrasies te flokkuleer asook pH-vlakke, wat gevolglik die beskikbaarheid van kalsium moontlik kan beïnvloed. In beide media, soos waargeneem deur elektron- en immunofluoresensiemikroskopie, het volwasse selle uitstulpings tydens flokkulasie getoon, wat moontlik betrokke kan wees in seladhesie. Dit is gevolg deur verdere ondersoeke na die rol van lipiede oor die groeisiklus van hierdie gis (op komplekse – en sintetiese media). Dit is ontdek dat *Sacch. cerevisiae* nie slegs inverse flokkulasie-patrone demonstreer nie, maar ook deur twee verskillende omskakelingspatrone van lipiede gekenmerk word. Gedurende Flo1-fenotipe flokkulasie, het hierdie gis twee neutrale akkumuleringstadiums in lipiede getoon (naamlik by 8 h en vanaf 12 h). Dit word waarskynlik deur flokkulasie

ontlok, wat as ‘n oorlewingsmeganisme beskou word, waar selle – veral neutrale lipidiede – as ‘n reserwe energiebron akkumuleer. ‘n Soortgelyke meganisme is waarskynlik teenwoordig wanneer selle die stasionêre groeifase binnegaan. In teenstelling met die Flo1-gedrag, demonstreer hierdie stam tydens NewFlo fenotipe gedrag slegs ‘n enkele akkumuleringsfase van lipiede, naamlik wanneer hierdie selle stasionêre groei binnegaan, wat met die toename in flokkulasie ooreenstem. Daar is ook ‘n toename in fosfolipiede ondervind gedurende aktiewe groei in beide flokkulasie fenotipes, waarskynlik as gevolg van aktiewe membraanproduksie. Hierdie resultate het ons geïnspireer om die moontlike rol van oksielipiene te ondersoek, wat op die seloppervlaktes gedurende flokkulasie teenwoordig is. Daar is bevind dat sommige stamme van *Sacch. cerevisiae* (insluitend stamme wat in fermentasieprosesse gebruik word) kortketting-oksielipiene (hoofsaaklik 8 koolstof lank) en nie potente inflammatoriese langketting-oksielipiene (20 koolstof lank), soos prostaglandiene produseer nie. Nadat aspirien by kulture van *Sacch. cerevisiae* UOFS Y-2330 gevoeg is, is daar bevind dat flokkulasie aansienlik geïnhibeer word, asook die produksie van 3-hidroksie (OH) 8:0 - en dus word flokkulasie aan hierdie oksilipien gekoppel. Boonop kon geen bewyse van hierdie oksilipien opgespoor word net voor die begin van flokkulasie in hierdie gis nie. Vogende is die betrokkenhied van hierdie oksielipiene in ko-flokkulasie ondersoek. Na aanleiding van die lektienteorie, het die gis *Schizosaccharomyces pombe,* ’n gebrek aan spesifieke reseptore wat noodsaaklik is om ko-flokkulasie met *Sacch. cerevisiae*-spesies te fasiliteer. In hierdie studie demonsteer ons oksilpien-geassosieerde ko-flokkulasie tussen *Sacch. cerevisiae* UOFS Y-2330 en *S. pombe* -stamme deur gebruik te maak van differensiële selkleuring, immunofluoresensie en ultrastrukturele studies. Deur ‘n 3-OH oksilipien spesifieke teenliggaam gekoppel aan ‘n fluoresserende verbinding te gebruik, is bevind dat 3-OH oksielipiene op die selwande van *Sacch. cerevisiae* en *S. pombe* teenwoordig is. Dit is bevestig deur gaskromatografie – massa spektrometrie. Of hierdie 3-OH oksielipiene ‘n rol speel in ko-flokkulasie van *Sacch. cerevisiae* met *S. pombe* selle deur moontlike entropies-gebaseerde hidrofobiese interaksies en/of waterstofbindings, moet nog bevestig word. Studies op die fisiologiese, genetiese asook kolloïdale aspekte van flokkulasie op hierdie modelstam, kan moontlik tot nuwe insigte in hierdie fassinerende verskynsel lei, asook toepassings in die bedryf.

Keywords

Co-floccculation, Fatty acids; Industry; Inverse flocculation; Lipids; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*; 3-Hydroxy oxylipins; Yeast

Sleutelwoorde

3-Hidroksie-oksielipiene; Gis; Industrie; Inverse flokkulasie; Ko-flokkulasie; Lipiede; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*; Vetsure