LIPIDS AND ASCOSPORE MORPHOLOGY IN YEASTS

BY

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CHAPTER 1

Literature review

1.1 Motivation

The most predominant conventional techniques effectively employed in ascosporogenous yeast taxonomy involve, among others the morphology of vegetative cells and ascospores, mode of sexual reproduction, physiological characteristics and molecular methods (Kurtzman, 1998; Kurtzman and Robnett, 1998; Barnett et al. 2000). Probably one of the most conserved characters is ascospore morphology, which has been shown to be conserved at genus and sometimes, family levels. Through intriguing sexual reproduction processes, which include amphimixis and automixis (Van der Walt, 1999), yeasts produce a wide variety of ascospore shapes (e.g. round, elongated, kidney, needle, hat, saturnoid, etc.) and surface ornamentations (e.g. smooth, rough, hairy, warty, etc.) all enclosed within asci. These structures however are not produced by yeasts for the purpose of classification – they have their own "reason" for producing these. By using conventional light and transmission electron microscopic techniques (i.e. potassium permanganate used as sole fixative) in the past, an incomplete picture regarding ascospore morphology, especially regarding nano-scale ornamentations on the surfaces of meiospores was obtained. This is evident in the yeast *Dipodascopsis uninucleata* var. *uninucleata* where it was reported, using conventional transmission electron microscopy, to produce smooth bean-shaped ascospores without nano-

scale surface ornamentations (Kreger-van Rij and Veenhuis, 1974). Strikingly, when glutadialdehyde and osmium tetroxide were used as alternative chemical fixatives for transmission electron microscopy, nano-scale surface ornamentations (i.e. hooks) were uncovered surrounding the bean-shaped ascospores of this yeast (Kock et al. 1999). These hooks, which interlock with hooks of adjacent ascospores within the ascus, are involved in ascospore release and ordered re-aggregation upon release from the ascus (Kock et al. 1999).

It is documented in literature that during the onset of the sexual stage, fatty acid based lipids, especially the neutral lipids, accumulate in yeasts to become a major component of ascospores (Kock and Ratledge, 1993). These lipids are mainly used as energy source during adverse conditions as well as for the formation of new cells. In addition, fatty acid based lipids also serve as precursors for the production of 3-hydroxy oxylipins via incomplete ß-oxidation (Venter et al. 1997). These oxidized fatty acids have been shown to be part of the structure of nano-scale ornamentations on surfaces of ascospores of the yeast *D. uninucleata* var. *uninucleata* as demonstrated by the addition of acetylsalicylic acid – an inhibitor of 3-hydroxy oxylipin production and consequently hook formation on the surface of ascospores.

Consequently, the purpose of this study became the following: (i) To assess ascospore shape and nano-scale ornamentations and other ascus inclusions (i.e. lipids) using confocal laser scanning microscopy as well as transmission electron microscopy where glutadialdehyde and osmium tetroxide are used as alternative chemical fixatives instead of conventional potassium permanganate fixation. For this purpose some selected members of the families Eremotheciaceae, Lipomycetaceae,

Saccharomycetaceae, Saccharomycodaceae and Saccharomycopsidaceae were studied. (ii) To assess the possible role of ascospore shape and nano-scale surface ornamentations in ascospore movement in micron space. Here the possible function of fatty acid based lipids i.e. 3-hydroxy oxylipins on spore movement and release from asci will be investigated.

1.2 What are yeasts?

Yeasts are groups of unicellular fungi that belong to the phylum Dikaryomycota. Two major groups of yeasts are recognized, classified either as Ascomycetes or Basidiomycetes (Barnett et al. 2000). The yeasts are characterized by single cells that reproduce by budding from a narrow or broad base (e.g. *Saccharomyces*) or fission from a broad base (e.g. *Schizosaccharomyces*). In addition, pseudohyphae or true hyphae or both may be present (Kurtzman and Fell, 1998). Furthermore, during adverse conditions, ascomycetous yeasts are capable of undergoing sexual reproduction that leads to the formation of haploid ascospores of different shapes and nano-scale surface ornamentations. These ascospores, also known as meiospores, are all enclosed within asci. Furthermore, yeasts do not form their sexual states such as asci within or upon fruiting bodies such as apothecia, cleistothecia, etc. (Kurtzman and Fell, 1998). Moreover, some ascomycetous yeasts are characterized by the absence of sexual states and these are referred to as anamorphs (Van der Walt and Von Arx, 1985). In this study, emphasis will be placed on the ascomycetous yeasts.

1.3 Ascospore morphology

Ascospores are regarded as haploid cells, which are produced by reduction or meiotic division within an ascus (Yarrow, 1998). According to literature, ascomycetous yeasts are known to produce ascospores of different shapes (e.g. round, elongate, kidney, needle, hat, saturnoid, walnut, spindle-shaped with a whip like appendage, etc.) and nano-scale surface ornamentations (e.g. smooth, rough, hairy, warty, etc.) all carried within asci (Yarrow, 1998). These hyaline spores are produced through the process of amphimictic and automictic sexual reproductive cycles (Van der Walt, 1999) that take place within asci. Furthermore, the ascospore number within an ascus can vary significantly, i.e. from one to 150 ascospores and even more. The latter is produced through post meiotic mitosis. In addition, ascospores may be pigmented, sometimes exhibiting yellow, amber, brown or even reddish brown colours (Yarrow, 1998).

Ascospore morphology, including shape and nano-scale surface ornamentations is an important character in ascomycetous yeast taxonomy. This phenotypic character, known to be conserved especially at genus level, is currently used in the classification of more than 450 ascomycetous yeasts (Fig. 1) (Yarrow, 1998; Barnett et al. 2000).

1.3.1 Ascospore formation

Yeasts are known to undergo intriguing sexual cycles that lead to the formation of ascospores of different shapes and nano-scale surface ornamentations (Van der Walt, 1999). These sexual cycles include amphimictic and automictic life cycles forming ascospores within asci. Amphimictic life cycles implicate a mode of sexual

reproduction through mating of separate haploid gametes (Van der Walt, 1999). This sexuality is heterothallic i.e. reproduction through mating of separate haploid gametes which gives rise to diplontic (i.e. vegetative yeast cells are diploid) or haplontic (i.e. vegetative yeast cells are haploid) life cycles. During a diplontic life cycle, unicellular vegetative cells which are diploid give rise through meiosis directly to haploid mating types called gametes that in turn fuse to form diploid zygotes and eventually develop into diploid yeast cells. This type of life cycle is characteristic of *Saccharomyces cerevisiae*.

Some yeasts are characterized by automictic sexuality (Van der Walt, 1999). This implicates self-fertilizing, homothallic reproduction. This sexuality is expressed both in diplontic and haplontic life cycles. This implies that in diplontic species, the sexual stage is characterized by autodiploidization of meiotically derived haploid ascospores which will eventually produce diploid ascospores through the fusion of two post-meiotic, mitotically derived sister nuclei. This type of sexuality is found among others in the yeast *Hanseniaspora* and in some species of the yeast genus *Saccharomyces*.

Automictic sexuality in haplontic life cycles involves karyogamy (i.e. fusion of two nuclei) of two mitotically derived sister nuclei to form a diploid zygote. During vegetative reproduction, the mature yeast cell (i.e. mother cell) produces buds that eventually develop into mature cells. An immature bud or daughter cell may remain attached to the mother cell and eventually form the zygote that further develops through meiosis into an ascus containing haploid ascospores. In this case, the zygote is produced by the fusion of the two sister nuclei which are formed through mitosis of the mother cell nucleus. This behaviour is referred to as mother-daughter cell

conjugation or adelphogamy. This type of sexuality is found among others in the yeast genus *Schwanniomyces*.

Another type of sexual reproductive behaviour has been reported in yeasts that were presumed to lack sexual reproductive stages (Van der Walt, 1999). This was proven using X-ray inactivation studies. It was found that some diploid species show both haploid and diploid generations in the absence of meiotically derived ascospores. These investigations were conducted on the yeasts *Candida albicans*, *Candida magnoliae* and *Candida tropicalis*. Here, the site of meiosis is the chlamydospore (i.e. thick walled cells) forming an undifferentiated *dangeardien* (i.e. meioconidiophore). This investigation may have significant implications in the classification of the so-called anamorphic yeasts

According to Van der Walt (1999), amphimictic and automictic sexuality have important implications for the survival of yeasts. Amphimixis promotes genetic exchange as well as recombination thereby ensuring the diversity of the genome, which is important for the adaptation of organisms to new habitats. In contrast, automixis precludes genetic exchange, relinquishes the evolutionary advantage of genetic exchange and recombination while the advantage of meiosis in eliminating deleterious mutations is still retained. Automixis presumably constitutes a sexual strategy whereby the integrity and stability of the already successful genome is ensured. This extraordinary sexual reproductive behaviour may have evolved from the amphimictic sexual cycle and therefore may represent the more recently evolved yeasts (Van der Walt, 1999).

1.3.2 Ascospore morphology by electron microscopy

Ascospore morphology has proved to be valuable in yeast taxonomy (Barnett et al. 2000). Unfortunately, this phenotypic character has in the past mostly been investigated by only conventional light microscopy (Yarrow, 1998; Barnett et al. 2000). Conventional transmission electron microscopy was also applied using potassium permanganate as a sole chemical fixative (Kreger-van Rij and Van der Walt, 1963; Kreger-van Rij, 1966; Kreger-van Rij & Veenhuis, 1976; Kreger-van Rij, 1977; 1978a, b; 1979a, b; Van der Walt and von Arx, 1985). It is only during later years that glutaraldehyde and osmium tetroxide were used as alternative fixation reagents (Kock et al. 1999, Smith et al. 2000b, 2003; Bareetseng et al. 2004).

Conventional preparation techniques for electron microscopy involve a few basic steps. The first step is fixation of specimen in 1.5% potassium permanganate for one hour. This is followed by rinsing with distilled water to remove excess potassium permanganate with distilled water. The second step is dehydration by a series of acetone or ethanol concentrations, after which the specimen is either embedded for ultramicrotomy or dried by critical point drying for final electron microscopic observations. This method is known for its apparent high contrast or clarity of cells, highlighting cell membranes when viewed with the transmission electron microscope (Hayat, 1989). Hayat (1981) reported that this apparent high contrast of the membranes is ascribed to denaturation of the protein components of the membranes resulting in pronounced lipid content membrane structure. However, major problems are encountered when the conventional fixation method is employed. Significant swelling of the mitochondrion and the plastids can occur, leading to the overall swelling of the cell and resulting in the disappearance of the surface ornamentations.

In addition, major loss of other cytoplasmic components such as ribosomes, lipids especially neutral lipids, microtubules and nucleic acids also occur, leading to the loss of true structure and dimension of the cell wall. This was clearly demonstrated when the bean-shaped ascospores of the yeast *D. uninucleata* var. *uninucleata* were subjected to this method of fixation (Kreger-van Rij and Veenhuis, 1974). Here, this yeast was found to produce smooth walled bean-shaped ascospores without surface ornamentations when observed with the transmission electron microscope (Kreger-van Rij and Veenhuis, 1974).

Interestingly, when the alternative fixation technique with glutadialdehyde and osmium tetroxide was employed, this yeast was found to produce nano-scale surface hooks surrounding the bean-shaped ascospores when observed with the transmission electron microscope (Kock et al. 1999). These findings were obtained when this yeast was pre-fixed in 3% glutadialdehyde in a phosphate buffer solution at room temperature over-night. According to literature, glutadialdehyde, which is a five carbon dialdehyde, is known to be the best fixative reagent in preserving the fine structure of the biological specimen, due to its ability to form cross-links with the cellular components (Hayat, 1989). Furthermore, the cells can be left for many hours in glutadialdehyde solution without destroying too much cell structure and/or components. This procedure was followed by rinsing the specimen in the same buffer solution to remove the excess glutadialdehyde from the cells. Next, the cells were post-fixed in 1% osmium tetroxide for two hours at room temperature. According to literature, osmium tetroxide preserves the lipid fractions of the cell (Hayat, 1989) and also gives contrast, enabling visualization of the cell components when viewed with the transmission electron microscope. After the fixation procedure using

glutadialdehyde and osmium tetroxide, the cells were dehydrated stepwise through a series of acetone concentrations. This step was performed to remove excess water from the cells for ultimate embedding in water-immiscible resins. After sectioning material by ultramicrotomy, the sections were stained using uranyl acetate and lead citrate for transmission electron microscopic examination. A disadvantage of using this alternative fixation double technique is that osmium tetroxide can penetrate large specimens very slowly after pre-fixing with glutadialdehyde. This may result in poor fixation of internal tissue cells of plant and animal organs (Hayat, 1989). However, fixation of single cells such as ascospores will not be influenced by this slow penetration.

1.3.3. Function of ascospore structure

During the sexual stage, ascomycetous yeasts are capable of producing different shapes of ascospores and nano-scale surface ornamentations (Yarrow, 1998; Barnett et al. 2000). These different structures are generally applied in the classification of these yeasts (Barnett et al. 2000). However, ascomycetous yeasts do not produce these different shapes and nano-scale surface ornamentations for the purpose of classification or to benefit us. They have their own “reason” for doing this. So far, little is known about the functions of these structures in yeasts.

In 1999, Kock and co-workers obtained hints on the possible function of ascospore structure in the yeast *D. uninucleata* var. *uninucleata*. They suggested that hook surface ridges on ascospores probably assist in ascospore release. It therefore also became an aim of this study to further elucidate the function of these ascospore structures.

Fungi (Kingdom)
Ascomycota (Phylum)
Archiascomycetes (Class)
Protomycetales (Order)
Protomycetaceae (Family)
Mitosporic Protomycetales (Family)
Saitoella
Schizosaccharomycetales (Order)
Schizosaccharomycetaceae (Family)
Schizosaccharomyces
Euascomycetes (Class)
Oosporidium
Hemiascomycetes (Class)

Saccharomycetales (Order)

Candidaceae	**Dipodascaceae**	**Eremotheciaceae**
Aciculoconidium	*Dipodascus*	*Eremothecium*
Arxula	*Endomyces*	
Botryozyma	*Galactomyces*	
Brettanomyces	*Sporopachydermia*	
Candida	*Stephanoascus*	
Geotrichum	*Yarrowia*	
Kloeckera	*Zygoascus*	
Myxozyma		
Schizoblastosporion		
Sympodiomyces		
Trigonopsis		

Lipomycetaceae	**Metschnikowiaceae**	**Phaffomycetaceae**
Babjevia	*Clavispora*	*Phaffomyces*
Kawasakia	*Metschnikowia*	*Starmera*
Lipomyces		
Smithiozyma		
Zygozyma		

Saccharomycetaceae	**Saccharomycodaceae**	**Saccharomycopsidaceae**
Arxiozyma	*Hanseniaspora*	*Ambrosiozyma*
Citeromyces	*Nadsonia*	*Saccharomycopsis*
Debaryomyces	*Saccharomycodes*	
Dekkera	*Wickerhamia*	
Hansenula		
Issatchenkia		
Kazachstania		
Kluyveromyces		
Kodamaea		
Lodderomyces		
Pachysolen		
Pichia		
Saccharomyces		
Saturnispora		
Torulaspora		
Williopsis		
Zygosaccharomyces		

Fig. 1. Current classification of the ascomycetous yeasts. (Taken from Barnett et al. 2000).

1.4 Lipids

1.4.1 Lipid turnover in yeasts

Lipid turnover in yeasts is well established (Kock and Ratledge, 1993). During the onset of the sexual stage of the yeast *D. uninucleata* var. *uninucleata*, it was found that ungerminated ellipsoidal to reniform ascospores contained about 18 times more lipid than germinated cells. The lipid comprised of 58% (w/w) glycolipids, 28% (w/w) neutral lipids (mainly triacylglycerols) and 14% (w/w) phospholipids. During the germination of these spores, all three lipid fractions decreased but during the subsequent initiation of hyphal growth (i.e. active growth phase) the phospholipid fraction increased. This is probably due to the increase in membrane production. As the hyphae started to differentiate to form the sexual stage, the amount of neutral lipids increased significantly. This can be ascribed to the deposition or accumulation of lipids inside the ascospores to serve as reserve material upon germination.

Similar results were found during growth and development of the filamentous fungi *Mucor genevensis* (Pohl, 1999), *Blastocladiella emersonii* (Smith and Silverman, 1973), *Achlya* (Law and Burton, 1976) and the yeasts *Dipodascus ambrosiae* (Smith et al. 2003) and *D. tóthii* (Jansen van Vuuren et al. 1994). However, here the phospholipids decreased with a concomitant increase in the glycolipid fraction when the cells of *Dipodascus ambrosiae* entered the ascosporogenesis stage (Smith et al. 2003).

1.4.2 3-Hydroxy oxylipins

It has also been reported that certain lipids i.e. fatty acids can be transformed to *3R*-hydroxy oxylipins (Fig. 2) through incomplete ß-oxidation (Venter et al. 1997). These oxylipins are then deposited onto the surfaces of mainly ascospores (Kock et al. 2003), vegetative cells (Kock et al. 2000) as well as asexual spores of the *Mucorales* (Strauss et al. 2000).

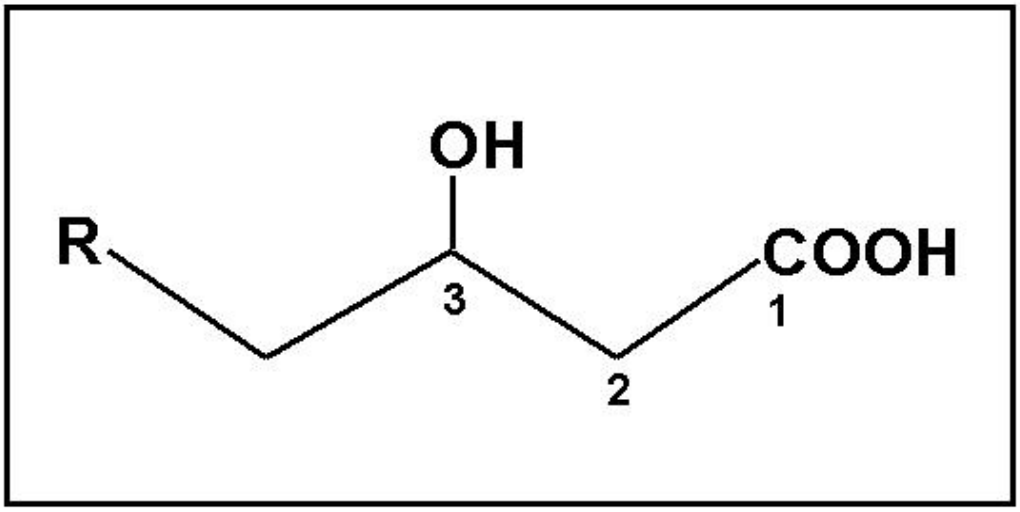

Fig. 2. The basic chemical structure of a 3-hydroxy oxylipin (fatty acid). R = carbon chain (saturated or unsaturated) with a hydroxyl group at position 3 (counted from carboxyl group).

1.4.3 The occurrence of 3-hydroxy oxylipins in fungi

The occurrence of oxylipins in plants (Granér et al. 2003) and animals (Noverr et al. 2003) has been well reported and they are known to be involved in cell signaling. Subsequently, evidence for the presence of these oxylipins such as 3-hydroxy oxylipins in fungi, particularly in yeasts has also been provided (Kock et al. 2003). In 1967, Stodola and co-workers reported the presence of saturated 3-hydroxy 16:0 and 18:0 in the gylcolipid fractions of the basidiomycetous yeasts *Rhodotorula graminis*

and *Rhodotorula glutinis* respectively. A year later, 3-hydroxy 16:0 was identified in the ascomycetous yeast *Saccharomycopsis malanga* (Versonder et al. 1968). In 1991, the first novel acetylsalicylic acid (aspirin) sensitive polyunsaturated 3-hydroxy oxylipin i.e. 3-hydroxy-5,8,11,14-eicosatetraenoic acid (*3R*- HETE or 3-OH 20:4) was uncovered in *D. uninucleata* var. *uninucleata* using techniques such as radio thin layer chromatography (TLC), [^{1}H] 2D-COSY nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS) and infra red (IR) spectroscopy (Van Dyk et al. 1991). Consequently, this oxylipin as well as other polyunsaturated 3-hydroxy oxylipins i.e. 3-OH 14:2, 3-OH 14:3, 3-OH 20:3, 3-OH 20:4, and 3-OH 20:5 were identified (Venter et al. 1997; Kock et al. 2003).

Furthermore, through immunofluorescence studies using polyclonal antibodies with high affinity towards 3-hydroxy oxylipins in general (i.e. specific against 3-hydroxy oxylipins of different chain lengths and desaturation), these compounds were found to be in close association with the surfaces and nano-scale ornamentations of liberated bean-shaped, aggregating ascospores of *D. uninucleata* var. *uninucleata* (Kock et al. 1999). Although these oxylipins were found to be biologically active when added to human neutrophils and tumor cells (Nigam et al. 1996; Kock et al. 2003), their biological function in yeasts was not clearly understood. However, in 1999, Kock and co-workers were the first to suggest the function of these oxylipins in yeast, particularly in *D. uninucleata* var. *uninucleata*. After extensive inhibition studies involving the addition of 0.1 mM and 1.0 mM acetylsalicylic acid, which inhibits the formation of 3-hydroxy oxylipins to *D. uninucleata* var. *uninucleata*, Kock and co-workers suggested that these oxylipins, in concert with the nano-scale surface hooks surrounding the ellipsoidal ascospores of this yeast, may be involved in the ordered

release of the ascospores from the ascus and subsequence re-aggregation of these spores (Kock et al. 1999).

Further bioprospecting studies showed that 3-hydroxy oxylipins are implicated in the aggregation of sexual spores of the ascomycetous yeasts families Lipomycetaceae and Dipodascaceae (Smith et al. 2000a, 2003). Moreover, these oxylipins were also detected on the surface or between flocculating vegetative cells of *Saccharomyces cerevisiae* (Kock et al. 2000). In the pathogenic yeast, *Candida albicans*, 3-hydroxy oxylipins were observed on the filamentous structures where they are involved in morphogenesis (Deva et al. 2000, 2001). The filamentous fungi *Absidia cylindrospora*, *Actinomucor elegans*, *Cunninghamella echinulata* and *Mortierella ramanniana* were also mapped for the distribution of 3-hydroxy oxylipins using immunofluorescence microscopy (Strauss et al. 2000). Here the presence of these oxylipins was observed on sporangia and columellae (Strauss et al. 2000). In addition, these oxylipins were also detected on collumellae, sporangia and dispersed sporangiospores of *Mucor genevensis* (Pohl, 1999).

1.5 Purpose of the study

On the basis of the preceding information, the purpose of this study became the following:

1. To assess the possible functions of ascospore structure (i.e. shape and nano-scale surface ornamentations) and associated 3-hydroxy oxylipins in selected yeasts (Chapter 2).

2. To further study ascospore shape and nano-scale surface ornamentations with accompanying oxylipins in selected representatives of the families Eremotheciaceae, Lipomycetaceae, Saccharomycetaceae, Saccharomycodaceae, Saccharomycopsidaceae and the yeast like fungus *Ascoidea*. This part is aimed at presenting clues to eventually elucidate the mechanical function of ascospore structure (Chapter 3).

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

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OBJECTIVE 1:

Assessment of the possible functions of ascospore shape, nano-scale surface ornamentation and oxylipins in selected yeasts.

CHAPTER 2

South African Journal of Science Vol. 100(5/6): 237-241

Revealing yeast spore movement in confined space

The Supplemented Online Movies 1, 2 and 3 are available on CD at the back of thesis. For detailed methodology, see Appendix at end of thesis.

Please note: The mathematical modelling was performed by Professor S.W. Schoombie from the Department of Mathematics and Applied Mathematics, University of the Free State, Bloemfontein, South Africa. The online movies and figures 2 and 3 were prepared by Kobus van Wyk.

Abstract

Some yeasts produce sexual spores (ascospores) in a variety of shapes and surface ornamentations. These intriguing structures have hitherto been used only in yeast classification. Here, we propose the likely primary function of spore shape and ornamentations, in water-driven movement, as aiding the dispersal of the spores from enclosed containers (asci). This interpretation of the mechanics involved might find application in nano-, aero- and hydro-technologies with the re-scaling of these structures.

Introduction

Through sexual reproduction,[1] some yeasts produce microscopic containers (asci) that enclose mainly water and spores (ascospores) of many different shapes and various nano-scale surface ornamentations. [2]Some spores are spherical with an equatorial ledge (like the planet Saturn), or resemble hats with a bole and brim, while others look like corkscrews, walnuts, spindles with whip-like appendages, needles, and hairy or warty balls. Until now, these structures were used to classify yeasts and little thought was apparently given to their possible function. [2]Here, we outline the role of spore shape and lubricated, nano-scale surface ornamentations in water-driven spore release.

The clue

The first clue regarding an explanation of the function of spore morphology came from a discovery we reported in 1991. Using radiolabelled thin-layer chromatography, [^{1}H]2D-COSY NMR, gas chromatography–mass spectrometry as well as infrared spectroscopy, we revealed that the polyunsaturated 3-hydroxy

oxylipin, 3*R*-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (3*R*-HETE or 3-OH 20:4), was sensitive to acetylsalicylic acid (aspirin). [3,4]This compound as well as other polyunsaturated 3-OH oxylipins, namely, 3-OH 14:2, 3-OH 14:3, 3-OH 20:3, 3-OH 20:4 and 3-OH 20:5, are produced by the yeast *Dipodascopsis uninucleata* probably through incomplete ß-oxidation. With the aid of polyclonal antibodies directed against 3-OH oxylipins (with different chain lengths and desaturation[5]), we observed that these oxylipins coat part of nano-scale (100–600 nm in diameter) ornamentations on the surfaces of many spore types. [3]The fact that hydroxy oxylipins have excellent lubricating properties and are today used in high-quality motor oils and lubricants for, amongst others, jet engines[6], gave rise to the following question. Do these compounds have a similar lubricating function on the surfaces of spores? If so, why?

Message in a bottle

We found a message concerning this question when studying spore release from bottle-shaped containers (asci) in the yeast *Dipodascopsis uninucleata*[3,5,7]. Here, spores with hooked surface ridges and linked in gear-like fashion within a bottle-shaped ascus, are covered in oily hydroxy oxylipins – resembling oil inside a gearbox. Strikingly, when a 3-hydroxy oxylipin inhibitor, acetylsalicylic acid, was added during the cultivation of this yeast, it inhibited the production of the oxylipin and subsequent release of spores in a dose-dependent manner[4,7]. We concluded that spore release is oxylipin-dependent probably also through the latter's lubricating ability. This interpretation prompted us to investigate the mode of spore release further from bottle-shaped containers in the yeasts in the genus *Dipodascus* as well as in *D. uninucleata.*

Those who have attempted to remove marbles or beans from an open bottle-shaped container will know that it is necessary, first, to invert and then shake the bottle in all directions (Fig. 1). In this way these objects are loosened, a prerequisite for sliding past each other under gravity for unhindered individual release through the narrow opening. When removing beans, it is necessary to shake the bottle even more vigorously, thereby also aligning them by chance near the narrow neck to pass, narrow end first, through the opening. Alignment prevents beans from turning sideways, which will block their release.

From literature[3,8] and microscopic observations, we found that *D. uninucleata* and *Dipodascus* have evolved sophisticated means that enable the dispersal of oxylipin-coated spherical and bean-shaped spores from asci without inverting or shaking them. In this case, instead of gravitational or centripetal pull, spores are pushed by turgor pressure towards the narrow opening and then ejected (Fig. 2).

Studies of *Dipodascopsis* spores,[3,5,7] which range from bean-like to ellipsoid in shape, suggest that oxylipin-coated, interlocked surface ridges and stretching across the length of the spore are responsible for their alignment. Here, spores inside the container are positioned side-by-side in a column of linked clusters with elongated sides attached by interlocking hooked ridges in gear-like fashion and orientated mainly with one end towards the opening. We conclude that the hooked ridges form turbine-like structures at both ends (Fig. 3, see also Appendix), causing propeller-like rotation when the spores are pushed by water pressure towards the ascus opening. This rotational movement loosens the spores (by the unlocking of the hooked ridges) near the container neck, which is necessary for their sliding past each other for eventual release. (Follow the effect of the rotation of one spore in gear-like fashion on the unlocking of attached neighbouring spores in the cross section shown in Fig.

2a; Supplementary Online Movie 1). Eventually, spores are released individually from the bottle-shaped ascus while rotating at about 1200 rpm at approximately 110 length replacements per second (Supplementary Online Movie 2). With some species of the genus *Dipodascus*,[8,9] compressible, oxylipin-coated sheathed surface structures and not gears, are used to separate and loosen spherical spores in a similar bottle-shaped container before individual release under turgor pressure (Fig. 2b). These spores simply slide past each other when pressed towards the opening. We presume that more complex mechanics are needed to allow the effective release of these variously elongated spores compared to spherical sheathed spores, for which alignment and rotation are unnecessary.

Yeast boomerangs

We next asked ourselves what the functions of other spore shapes and oxylipin-lubricated surface ornamentations might be. Using gas chromatography-mass spectrometry according to the method described in ref. 4, we discovered a saturated 3-OH 14:0 compound (mass fragments:175 [$CH_3O(CO)$-CH_2-CHO-TMSi]; 330 [M^+]; 315 [M^+ – 15]) in the yeast *Eremothecium ashbyii*. In order to map the oxylipin's location in the yeast, we applied the same antibodies and immunofluorescence microscopy on cells in sexual mode as described in ref. 5. The oxylipin was present as part of a V-shaped structure on sickle-shaped spores (Fig. 4a,b). With the aid of confocal laser scanning microscopy (Nikon TE 2000) to observe cells treated with antibody and fluorescine (FITC anti-rabbit IgG)[5], we concluded that the hydrophobic V-shaped structure was present as a mirror image on both sides at the blunt end of an otherwise hydrophilic spore as indicated by

differential ascospore staining[2]. Scanning electron microscopy, according to the method used in ref.10, showed this structure to be fin-like protuberances (Fig. 4c).

Next, we addressed the function of these fin-like structures and spore shape. Using microscopy, we discovered that spores are sometimes forced through the ascus with the spiked tip rupturing the ascus wall (Fig. 4d). Water pressure caused a boomerang movement when the blunt end was pushed forward with the spike leading the way in a circular motion. This happened only when micron-scale streams of water moved across the fins from the blunt end towards the tip of the spore. Mathematical modelling suggests a sharp build-up of pressure between fins, when water flows towards the blunt end and across the fins (from left to right in Fig. 5a, b). The pressure acts perpendicular to the fins (a, bottom). Because of their hydrophobic behaviour, there should be no viscous effects, that is, no forces acting parallel to the fins. Consequently, these pressures culminate in a resultant force across the spore, from left to right and slightly downwards, indicated by force vector F, thereby causing movement of the spore to the right. Since the line of force passes below the centre of mass at C(+), the spore will also tend to rotate anticlockwise, that is, in the direction of the spiked end, about C(+). In addition, there should be a tendency for water pressure to be greater at the left of the spore than on the right, since the spore gradually tapers towards the spike, that is, from the approximately 3-µm-diameter blunt end to the 2-nm-diameter spike. This shape should also enhance the boomerang effect. Furthermore, the shape of the fins (Fig. 5a) is such that they also act as hydrofoils when they start to move, creating a lifting force (as a result of a backward force on the slanted lower fins) on the spore, similar to the wings of an aircraft. Thus, the spore will start drifting to the right and slightly upwards (that is, closer to the cell wall), rotating anticlockwise until the spike reaches the ascus wall,

where the latter may be ruptured and the spore pushed out of the cell by water pressure. In addition, fins lend stability to the blunt end, that is, they resist rotation when pushed by water flow, causing the spiked tip to reach the cell wall at the speed required for rupturing. Fins are also constructed in such a way that, upon release through a self-punctured narrow opening, the spear-shaped end of the hydrophobic V structure exits first, thereby preventing spores becoming stuck to the cell wall. The relatively small height and width dimensions of the fins also support this argument, although the effective area offering water resistance is probably increased by their hydrophobic nature. We propose the formation of 'nanobubbles' through drying[11] at the fin–water interface, thereby increasing the relatively flat and thin fin surface area on the otherwise non-hydrophobic spore surface. This in turn increases the resistance of the fins to the water movement, which enhances overall spore stability and boomerang speed.

Conclusions

We believe that this report has only scratched the surface of water-driven spore movement in yeasts on a micrometre scale and that the mechanical implications of many spore shapes with a large number of different hydroxy oxylipin-lubricated, nano-scale surface ornamentations await similar explanation and elaboration.

Why did some yeasts evolve peculiar spore movement with the beneficial consequence, so far as we can see, to escape from closed or partially closed containers?[2] Of course, this would be important from a survival point of view, since without this ability, yeast spores would presumably not be able to disperse properly. The function of spore structure confined in persistent asci (that is, they seemingly do

not release spores from these enclosures)[2] should be investigated. We believe that if appropriate ultrastructural studies[10] are conducted on yeasts with the aim of exposing spore surface ornamentations and not merely membrane structure[2], clues can be gained to reveal the mechanics behind the motion of nano-sized particles in fluids.

This experience with yeasts might now be profitably applied to other, non-related cells of different shapes and ornamentations such as blood components and plant seeds dispersed by wind and water, as well as in nano-, aero- and hydro-technologies (see Appendix).

This work is supported by the National Research Foundation, South Africa, and the Volkswagen Foundation, Germany (1/74643). We thank Mark Vermeulen of Serengeti Moon, Christo Steyn and Kobus van Wyk for the illustrations.

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Fig. 1. Ever wondered how to remove marbles or beans through the narrow opening of a bottle without inverting or shaking it? This problem is solved by the yeast *Dipodascopsis uninucleata* var. *uninucleata* and by some species of *Dipodascus,* that produce bean-shaped, hooked spores (ascospores)[2] and spherical sheathed spores,[9] respectively.

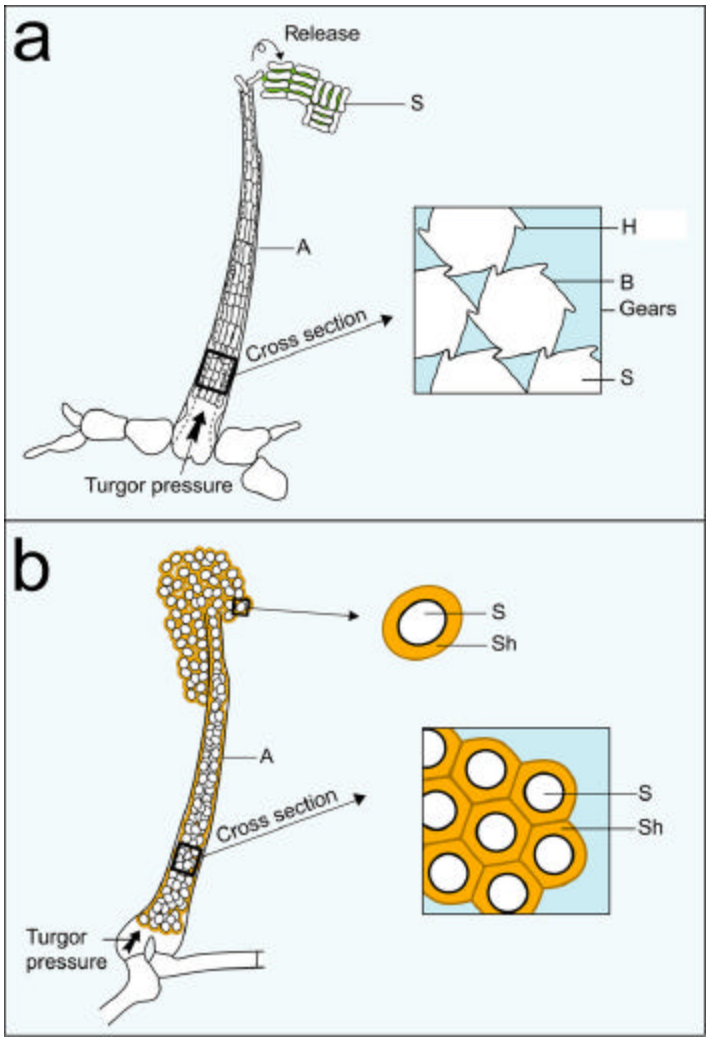

Fig. 2. Different spore release mechanisms in yeasts that ensure unhindered dispersal through narrow openings in bottle-shaped containers (asci). **a,** Rotational spore release in *Dipodascopsis uninucleata* UOFS Y-2000 (Supplementary Online Movie 1). In this case, hooked, gear-like surface structures, stretching across the length of the spores, play an important role in loosening spores for unhindered forced release through the opening. Key: A, ascus; B, blade; H, hooks; S, spore. **b,** Non-rotational spore release in *Dipodascus* sp. UOFS Y-1144. Here, flexible and compressible slimy sheaths enable spores to slip past each other when pressed towards the tapered tip without blocking the bottle-shaped container. Key: A, ascus; S, spore; Sh, sheath.

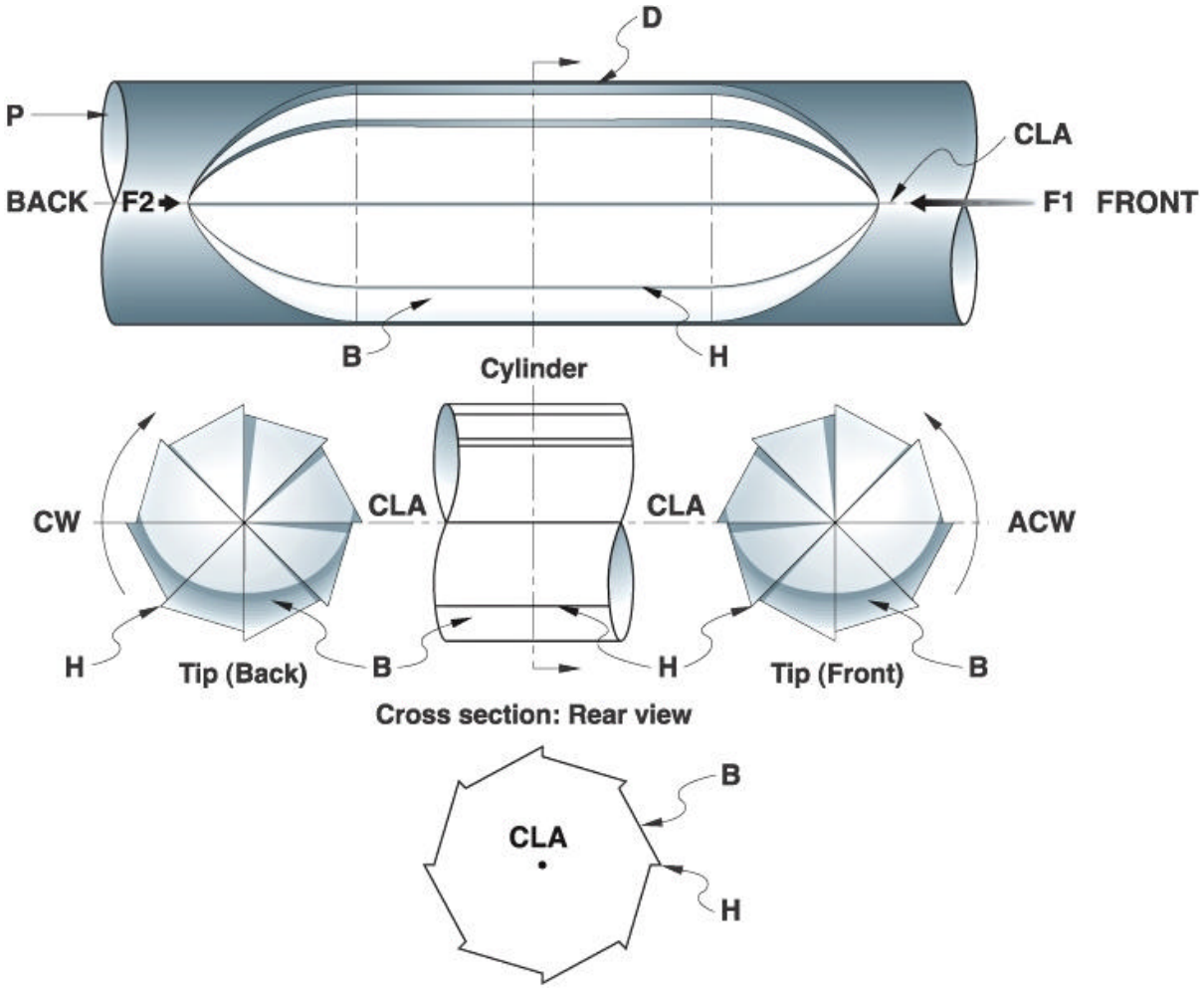

Fig. 3. A model, based on spore design and release in *Dipodascopsis uninucleata*, illustrating a device that we believe may keep (with minor changes) many kinds of pipelines clean while still in operation. Key: ACW, anticlockwise; B, blades; CLA, central longitudinal axis; D, device; F1, driving force; F2, backward force; H, hooked ridges; CW, clockwise; P, pipe.

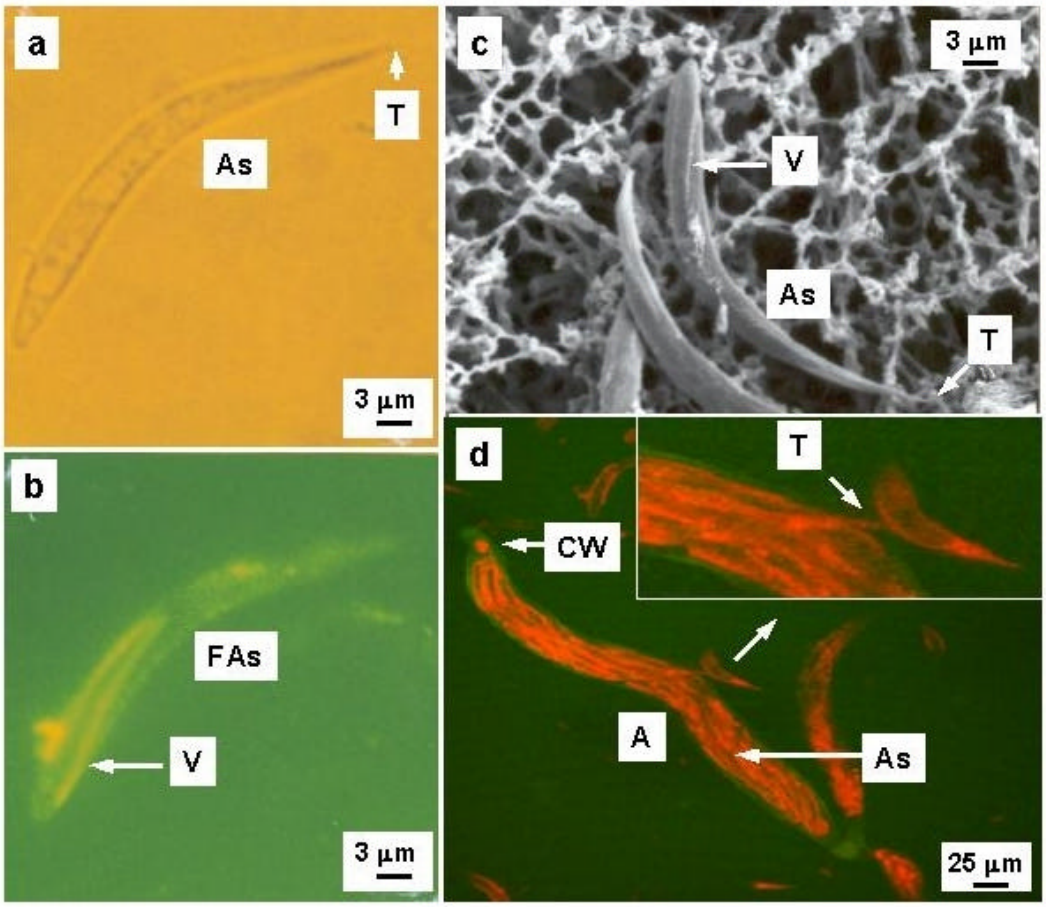

Fig. 4. Different images of sickle-shaped ascospores produced by *Eremothecium ashbyii* UOFS-Y 630 observed under various conditions: (a) light microscopy; (b) the subject in (a) viewed by immunofluorescence; (c) scanning electron micrograph; (d) confocal laser scanning micrographs indicating the spore's hydrophobic V-shaped fin structure (one leg of the V is approximately 600 nm x 600 nm x 20 µm, other leg is approximately 600 nm x 600nm x 17 µm) and in the process of being released (spiked tip first). Key: A, ascus; As, ascospore; CW, cell wall (green), surrounding ascospores (red) stained with fluorescing Orange G; FAs, fluorescing ascospore stained with FITC anti-rabbit IgG; T, tip of spike; V, fluorescing V-shaped fins stained with FITC anti-rabbit IgG in (b) and V-fins observed in (c) using scanning electron microscopy. Amorphous remnants [in (b) and (c)] are attached to fins probably as a result of rupturing after passage through a narrow, self-created hole in cell wall.

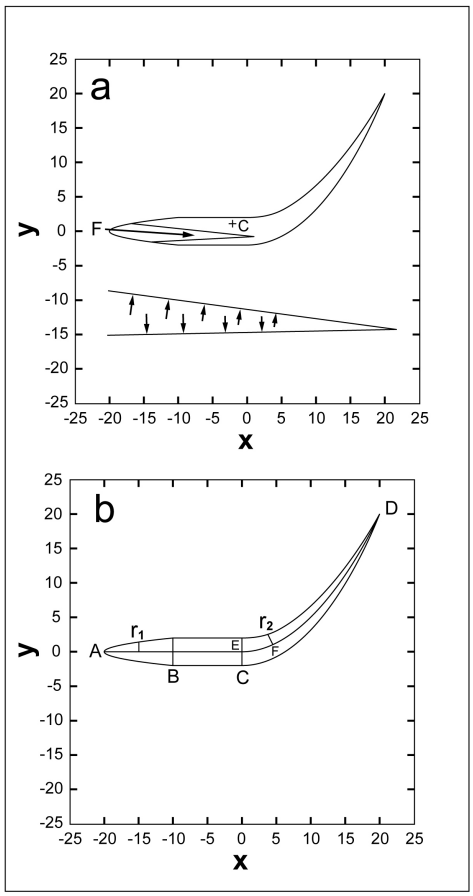

Fig. 5. A mathematical description, using elementary calculus, of a typical sickle-shaped spore of *Eremothecium ashbyii* UOFS-Y 630. (**a**) Movement of spore by water pressure across fins; (**b**) determination of the centroid. The tapered front part of the spore was modelled by a solid of revolution formed by rotating a parabola about a horizontal axis. The middle part of the spore is represented by a cylinder, while the curving tail was modelled by first describing its curved centre line as an arc of a parabola, and then assuming the cross section perpendicular to the parabola to be a circle, the radius of which decreases in proportion to the arc length along the parabola. The position of the centroid of the simulated spore was then calculated, and found to be situated at $x = -2.14$ and $y = 1.21$, as indicated in **a** (top). We assume this centroid to coincide more or less with the centre of mass C(+) of the spore.

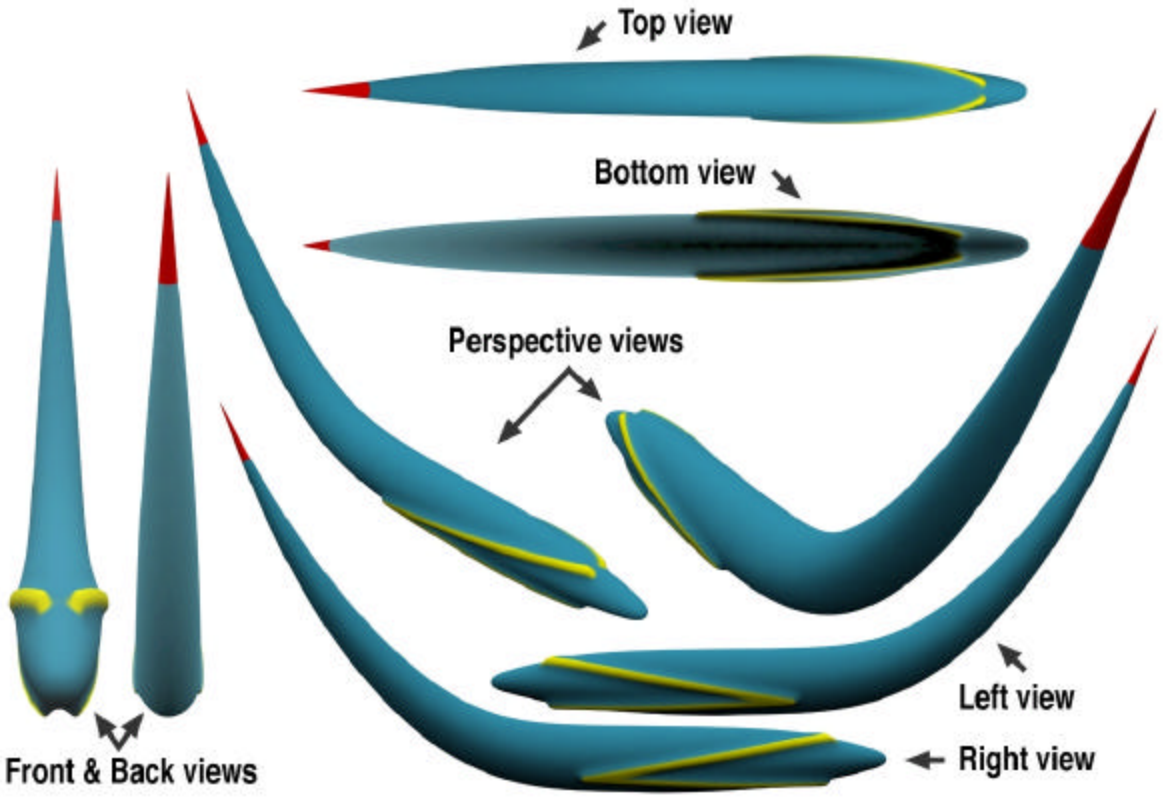

Fig. 6. A 3-D simulation, showing all sides, of the sickle-shaped spore of *Eremothecium ashbyii* UOFS Y-630.

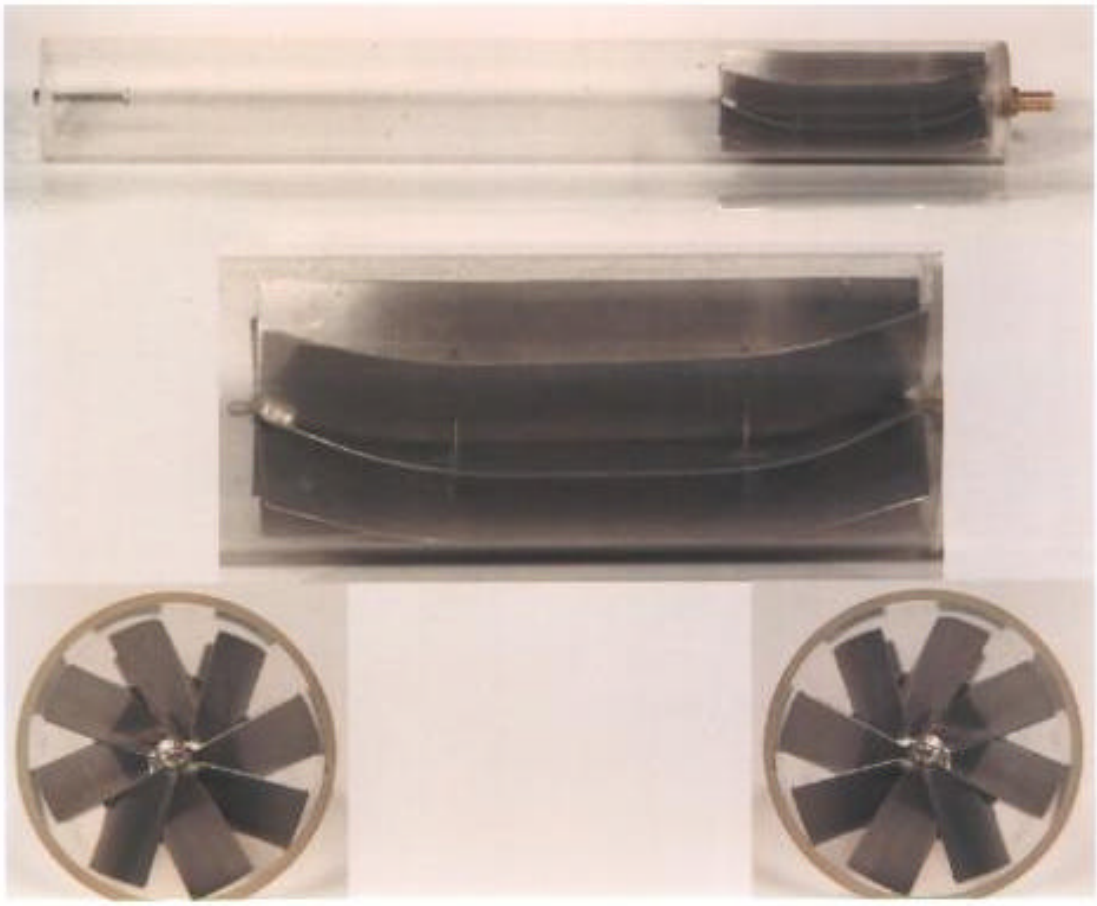

Fig. 7. An experimental pipe-cleaning devise (measuring 75 x 180 mm) adapted from the design in Fig. 3 and constructed by the authors. The apparatus used for testing the devise is shown at the top. Water pressure is applied to the pipe from the right-hand side.

Appendix

A basis for new technologies?

These observations of spore movements in confined spaces outlined above prompted us to scale-up spore structures and investigate some of their hydrodynamic properties, in particular to see if useful devices could be constructed and to learn if similar movements could be replicated when magnified.

Based on the design of *D. uninucleata* spores,[7] we devised a new generation of pipe-cleaning and drilling pigs (named after the screeching noise these metal devices make when forced through tight-fitting pipes). Here, we adapted spore shape and surface ornamentations to produce an instrument that we surmise can be used effectively (with minor changes to blade slant and orientation) to keep industrial pipelines clean (similar to pipe-cleaning pigs) while in operation, that is, by scraping and drilling movements (Fig. 3).

From mathematical modelling, we simulated movement of the ellipsoidal device (similar to spore shape) when pushed by fluid pressure through a close-fitting pipe (simulating ascus neck and forced spore movement) filled with moving liquid (simulating water movement through the ascus neck), while the latter caused enhanced rotation by turning both turbine-shaped ends of the device simultaneously in the same direction (simulating spore rotation), thereby exploiting the available liquid forces more effectively. Hooked ridges and blade orientation are shown on the device while moving, forced by liquid pressure, to the left through a close-fitting pipe (Fig. 3).

Rotation about a central longitudinal axis is the result of two forces. There are the driving force F1 and the backward acting force F2, caused when the device is pressed against the liquid present in the pipe. Both forces act simultaneously and in

concert perpendicular to the slanted blades on both turbine-like tips in propeller fashion. These forces result in a net resultant movement of the device to the left, guided by the pipe, as well as an increase in anticlockwise rotation observed from the front if forces are correctly balanced. This is the reason for the high rotational release of spores. Rotation will have a scraping effect, thereby removing any deposit on the inside of the pipes, while the rotating tip at the back will perform a clockwise drilling movement. This device may prove useful when used routinely, while liquid is pumped through pipes, to prevent them from clogging up. A scaled-up model (30 000 times spore size) positioned in a closely fitted pipe, and subjected to water pressure from one side, confirmed the behaviour anticipated (Fig. 7).

Other scaled-up models (10 000 times enlarged), simulating the sickle-shaped spore (Fig. 6) and subjected to water pressure from their blunt end, replicated the boomerang movement of spores observed within asci. The hydrophobic water-resistant properties of fins could not be tested, however, since these forces become significant only when exerted on small objects in restricted environments.[11] Furthermore, many spores packed into a micrometre-sized ascus may well behave differently. We propose also that a similar water-driven spore movement occurs within vascular bundles of plants where this yeast acts as a pathogen (Supplementary Online Movie 3).

This boomerang mechanism may in future inspire the design of environmentally driven miniature fleets of medical nanorobots as envisaged by some students of nanotechnology. Perhaps, this concept may prove useful in scouring clogged arteries through continuously blood-driven boomerang movements, thereby continuously sweeping the inside of artery walls with spiked ends without damaging them.

OBJECTIVE 2:

The assessment of ascospore shape and nano-scale surface ornamentations with accompanying lipids in different families of yeasts.

CHAPTER 3

3.1 Family: Eremotheciaceae

[(Parts of this chapter have been published in Antonie van Leeuwenhoek (In Press)]

Ascospores, 3-hydroxy oxylipins and lipid turnover in *Eremothecium sinecaudum*

Please note: All experiments except antibody preparation were performed by candidate.

Key words: Ascospore, confocal laser scanning microscopy, corkscrew, *Eremothecium sinecaudum*, 3-hydroxy oxylipins, lipid turnover

Abstract

3-Hydroxy oxylipins were uncovered on ascospores of *Eremothecium sinecaudum* using immunofluorescence microscopy. This was confirmed by gas chromatography-mass spectrometry. These oxylipins were observed only on ascospore parts characterised by nano-scale surface ornamentations simulating a corkscrew, as demonstrated by scanning electron microscopy. Conventional ascospore staining further confirms its hydrophobic nature. Using confocal laser scanning microscopy we found that the corkscrew part with spiky tip of needle-shaped ascospores may play a role in rupturing the ascus in order to affect its release. Through oxylipin inhibition studies we hypothesise a possible role for 3-hydroxy oxylipins in facilitating the rupturing process. In addition, a decrease in the neutral- and phospholipid fractions as well as an increase in the glycolipid fraction was experienced during the transition from asexual to sexual stages.

Introduction

In 1991, the first acetylsalicylic acid sensitive 3-hydroxy polyunsaturated oxylipin i.e. 3*R*-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (3*R*- HETE or 3-OH 20:4) was uncovered (Van Dyk et al. 1991). This compound as well as other novel 3-OH oxylipins i.e. 3-OH 14:2, 3-OH 14:3, 3-OH 20:3, 3-OH 20:4 and 3-OH 20:5 are produced by the yeast *Dipodascopsis uninucleata* probably through incomplete ß-oxidation. In medical studies 3*R*-HETE exerts potent biological effects (Kock et al. 2003). But what are the functions of these compounds in yeasts?

To find out, we developed polyclonal antibodies against 3-hydroxy oxylipins with different chain lengths and desaturation to map their distribution. We observed 3-hydroxy oxylipins associated with ascospores of different shapes and nano-scale surface ornamentations produced by *Dipodascopsis uninucleata* var. *uninucleata* (Kock et al. 1997) as well as representatives of *Lipomyces* and *Dipodascus* (Smith et al. 2000a; 2003). Strikingly, these compounds were also present on the ascus tip of *Dipodascopsis tóthii* (Smith et al. 2000b) and protuberances of flocculating cells of *Saccharomyces cerevisiae* (Kock et al. 2000).

When inhibiting the production of 3-hydroxy oxylipins in *D. uninucleata* var. *uninucleata* by adding low concentrations of acetylsalicylic acid (0.1 to 1.0 mM), ascospore release through narrow asci openings was also inhibited (Kock et al. 1999). This enabled us to decode the mechanics of ascospore release in this yeast. We conclude that 3-OH oxylipins probably function as lubricant that facilitates ascospore release.

In this study, we map the distribution of these oxylipins in the yeast *Eremothecium sinecaudum*, a pathogen of mustard seeds (de Hoog et al. 1998). Particular attention is paid to its association with ascospore surface ornamentations and possible function.

Materials and methods (See Appendix for detail)

Strain used and cultivation

Eremothecium sinecaudum UOFS Y-17231, held at the University of the Free State, Bloemfontein, South Africa, was cultivated on YM (Wickerham, 1951) agar slants and

transferred to 250 ml conical flasks containing 50 ml YM medium (shaking at 160 rpm) at 25 ^{O}C until sexual stage was reached. The ability to produce asci containing ascospores was followed microscopically (Axioskop, Zeiss, Germany).

Oxylipin inhibition

In order to determine the sensitivity of the sexual stage to acetylsalicylic acid (Sigma), this oxylipin inhibitor (Kock et al. 1999), suspended in ethanol (>99 % pure), was added to the liquid culture described above at different concentrations i.e. 0.0 mM (control), 0.1 mM and 1.0 mM final concentration at the start of growth. In all cases equal amounts of ethanol was added. The effect of the different concentrations of acetylsalicylic acid on ascospore formation and release was compared microscopically (Axioskop, Zeiss, Germany) when the sexual stage could be observed in the control.

Staining of spores

Ascospores were stained according to the method proposed by Yarrow (1998) to expose hydrophobic surfaces.

Immunofluorescence microscopy (Kock et al. 1998)

Synthesis of 3-hydroxy oxylipins: These were first synthesized for antibody preparation. The synthetic strategy for the production of 3*R*- and 3*S*-hydroxy oxylipins (i.e. 3-hydroxy- 5,8,11,14-eicosatetraenoic acid or 3-HETE), involved a convergent approach coupling chiral aldehyde with Wittig salt: these were derived from 2-deoxy-D-ribose and arachidonic acid, respectively.

Preparation and characterisation of antibody: Briefly, antibodies against chemically synthesised 3*R*-HETE were raised in rabbits and characterised by determining its titre, sensitivity and specificity. Cross-reactivity was only experienced with 3-hydroxy oxylipins of different chain lengths and desaturation. Hence, in our study the immunoreactivity indicates solely the presence of 3-hydroxy oxylipins.

Microscopy: Immunofluorescence of yeast cells was performed as described (Kock et al. 1998) and includes treatment with primary antibody against 3-hydroxy oxylipins as well as FITC-conjugated secondary antibody. In order to maintain cell 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 (Germany) microscope equipped for epifluorescence with a 50 W high-pressure mercury lamp. The stained cells were compared with appropriate controls. Corresponding photomicrographs without fluorescence were also taken with the same microscope.

Electron microscopy

Material for scanning electron microscopy (SEM) was chemically fixed (glutaraldehyde and osmium tetroxide) according to Van Wyk and Wingfield (1994). SEM micrographs were taken with a Jeol 6400 WINSEM (Japan).

Confocal laser scanning microscopy

Cells were suspended in 10 % Orange G (Yarrow, 1998) solution on a microscope slide and subsequently analysed with a Nikon TE 2000, confocal laser scanning microscope (CLSM).

Total lipid extraction

During asexual (i.e. two days) and sexual stages (i.e. six days), cells were scraped off from the medium, frozen, freeze dried and finally weighed. Lipids were extracted using chloroform:methanol (2:1, v/v) (Kock & Ratledge, 1993) and the organic phase washed (Folch et al. 1957). The organic phase was finally evaporated and the lipid material was dried in an oven at 50 ^{O}C over P_2O_5 over night and finally weight.

Lipid fractionation

Total lipid samples were fractionated according to the method of Kock & Ratledge (1993). In short, the total lipid samples were dissolved in chloroform and applied to a column (140 mm x 20 mm) of activated silicic acid (i.e. heating in an oven at 110 ^{O}C over night). The neutral-, glyco- and phospholipid fractions were eluted from the column by applying different solvents with different polarities. The fractionated lipid samples were dried in an oven over P_2O_5 at 50 ^{O}C overnight and finally weight. All lipid samples were stored under N_2 gas at –20 ^{O}C.

Fatty acid determination

All lipid fractions were transesterified with trimethylsulphonium hydroxide (TMSOH) as described by Butte (1983). The fatty acid methyl esters were analysed by gas chromatography (GC) with a flame ionisation detector and Supelcowax 10 capillary

column (30 m x 0.75 mm). The initial column temperature of 145 ^{O}C was increased by 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C, respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Gas chromatograph-mass spectrometry (GC-MS)

Extraction of oxylipins followed by methylation, silylation and GC-MS analysis were performed according to Van Dyk et al. (1991).

Chemicals used

All chemicals used were of highest purity grade and obtained from reputable dealers.

Results and discussion

According to literature, needle-shaped ascospores of *Eremothecium sinecaudum* are characterised by a smooth surface at one end (blunt end) and a part characterised by a surface with nano-scale concentric ridges on the opposite end. These parts are separated by an equatorial plate collar (Holley et al. 1984; De Hoog et al. 1998). The strain used in this study showed a similar ultrastructure (Fig. 1a,b). Here ascospores just released from asci, are shown with concentric circles in corkscrew orientation with attached sharp tip as well as a smooth blunt end separated from the former by a collarette (Fig. 1a). When these ascospores germinate (Fig. 1b), the smooth blunt end swells to eventually form new cells while the corkscrew part is still visible.

The presence of 3-hydroxy oxylipins on ascospores of *Eremothecium sinecaudum* was mapped by exposing this yeast to antibodies against these compounds (Kock et al. 1998). Strikingly, only the corkscrew ridges of the ascospore showed a high affinity towards the antibodies, i.e. fluoresced (Fig. 2a,b) compared to the germinating ascospore part, vegetative cells and pseudohyphae (Fig. 2a). Using gas chromatography-mass spectrometry (Fig. 3a,b), a 3-hydroxy oxylipin with characteristic ion at 175 (indicating hydroxyl group at position 3 of fatty acid) was identified (Van Dyk et al. 1991). The complete structure of this compound is still to be elucidated. Furthermore, only the corkscrew part stained green with conventional ascospore staining thereby further confirming its hydrophobic nature (Yarrow, 1998).

When Orange G was added to the culture, again only the corkscrew part fluoresced (Fig. 2c). This staining method, visualized by confocal laser scanning microscopy assisted us in exposing a method of ascospore release from oval-shaped asci i.e. corkscrew tip piercing through asci walls followed by release (Fig. 2d). Here a schematic representation of the corkscrew part, protubing through the cell wall of the ascus is presented.

As was observed in *D. uninucleata* (Kock et al. 1999), the sexual stage of *Eremothecium sinecaudum* was also sensitive towards the 3-hydroxy oxylipin inhibitor. No release of ascospores could be observed in the presence of either 0.1 mM or 1.0 mM acetylsalicylic acid. The sexual stage was totally inhibited in the presence of 1.0 mM acetylsalicylic acid. We conclude that 3-hydroxy oxylipins are probably necessary for ascospore release and may play a role in facilitating the corkscrew movement through the ascus wall. Strikingly, hydroxy oxylipins are today

essential for making high-quality motor and cutting oils and lubricants for amongst others jet engines (Johnson, 1999).

Yeasts definitely do not produce these striking hydrophobic nano-scale surface ornamentations for our curiosity, benefit or to be classified. Is it possible that ascospores first drill through asci walls by corkscrew drilling actions facilitated by these hydrophobic oxylipins before release? If so, how is the drilling motion achieved?

During the life cycle of *Eremothecium sinecaudum*, i.e. after two days (asexual stage) and six days (sexual stage), the percentage total lipid content increased slightly from 4.8 % (w/w) - 5.0 % (w/w) (Table 1). These results are similar to those reported in literature for *Dipodascopsis uninucleata* var. *uninucleata* (Kock & Ratledge, 1993) and *Dipodascopsis tóthii* (Jansen van Vuuren et al. 1994) and *Dipodascus ambrosiae* (Smith et al. 2003). However, an interesting change was evident in the total lipid composition (i.e. neutral-, phospho- and glycolipids) over the life cycle (i.e. from asexual to sexual stages) (Table 1). The neutral lipids decreased from 7.7 % (w/w) to 3.1 % (w/w) when the sexual stage was reached. This phenomenon may be due to the utilisation of the neutral lipids present in the vegetative cells to form the corkscrew like ascospores. These results are contrary to those obtained in literature where normally the neutral lipids are stored in the sexual structures as energy source for germination (Kock & Ratledge, 1993; Jansen van Vuuren et al. 1994; Pohl, 1999; Smith et al. 2003). Also, the phospholipids decreased from 35.0 % (w/w) to 31.3 % (w/w) with a concomitant increase in the glycolipids from 57.4 % (w/w) to 65.6 % (w/w) when the sexual stage was attained. A decrease in the phospholipids as cells

reach the sexual stage may be due to a cessation in membrane production. An increase in the glycolipids as the life cycle approaches the sexual phase may be ascribed to the formation of cell walls containing this lipid fraction and surrounding the spore. Similar observations were also experienced during the life cycle of *Dipodascus ambrosiae* (Smith et al. 2003).

When the fatty acid content of the different lipid fractions (i.e. neutral-, phospho- and glycolipids) were investigated over the life cycle (i.e. asexual to sexual stages), we found that all the lipid fractions contained palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) but not linolenic acid (18:3) (Table 2). Palmitic acid (16:0) showed an increase in all the lipid fractions when *Eremothecium sinecaudum* entered the sexual stage. Also, 16:1 experienced an increase in the neutral lipids except in the phospho- and glycolipids, where this fatty acid decreased towards the sexual reproductive stage (Table 2). Stearic acid (18:0) increased notably in the neutral lipids as well as to a minor extend in the phospholipids and remained more or less the same in the glycolipids when the sexual stage was expressed. Oleic acid (18:1) on the other hand, only increased in the neutral lipids when the sexual stage was reached. The polyunsaturated fatty acid 18:2 decreased significantly in all the lipid fractions during the production of ascospores. This cannot be explained at present.

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Table 1: Changes in lipid content and lipid composition during the asexual and sexual stages of *Eremothecium sinecaudum* UOFS Y-17231

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
2	A	4.8	7.7	35.0	57.4
6	S	5.0	3.1	31.3	65.6

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %

Table 2: Changes in percentage fatty acid composition of the different lipid fractions during the asexual to sexual stages of *Eremothecium sinecaudum* UOFS Y-17231

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	21.6 (A)	18.7 (A)	25.3 (A)
	24.7 (S)	25.9 (S)	30.7 (S)
16:1	9.8 (A)	21.5 (A)	18.5 (A)
	14.0 (S)	18.1 (S)	16.8 (S)
18:0	5.6 (A)	1.9 (A)	3.4 (A)
	10.3 (S)	2.5 (S)	3.3 (S)
18:1	37.5 (A)	55.0 (A)	51.7 (A)
	43.1 (S)	53.0 (S)	48.8 (S)
18:2	24.6 (A)	2.3 (A)	0.8 (A)
	5.3 (S)	0.6 (S)	0.4 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %

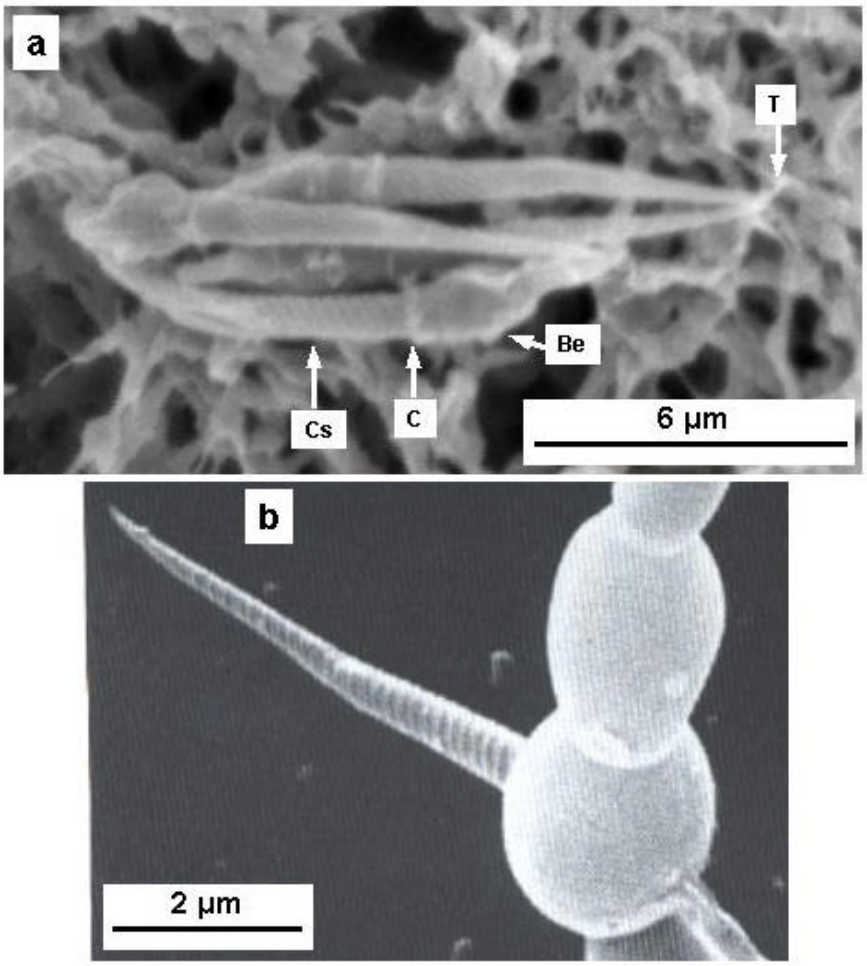

Fig. 1: Scanning electron micrograph of *Eremothecium sinecaudum* showing a needle-shaped ascospore with blunt end (Be), collarette (C), germinating ascospore and nano-scale surface ridges in corkscrew orientation (Cs). (a) Ascospores released from ascus before germination. Clearly visible are the blunt-end (Be), collarette (C) and corkscrew part (Cs) ending in a sharp tip (T). (b) Comparison with released germinating ascospore of *Eremothecium sinecaudum* strain studied by Holley et al. (1984). Corkscrew part ending in the tip is clearly visible.

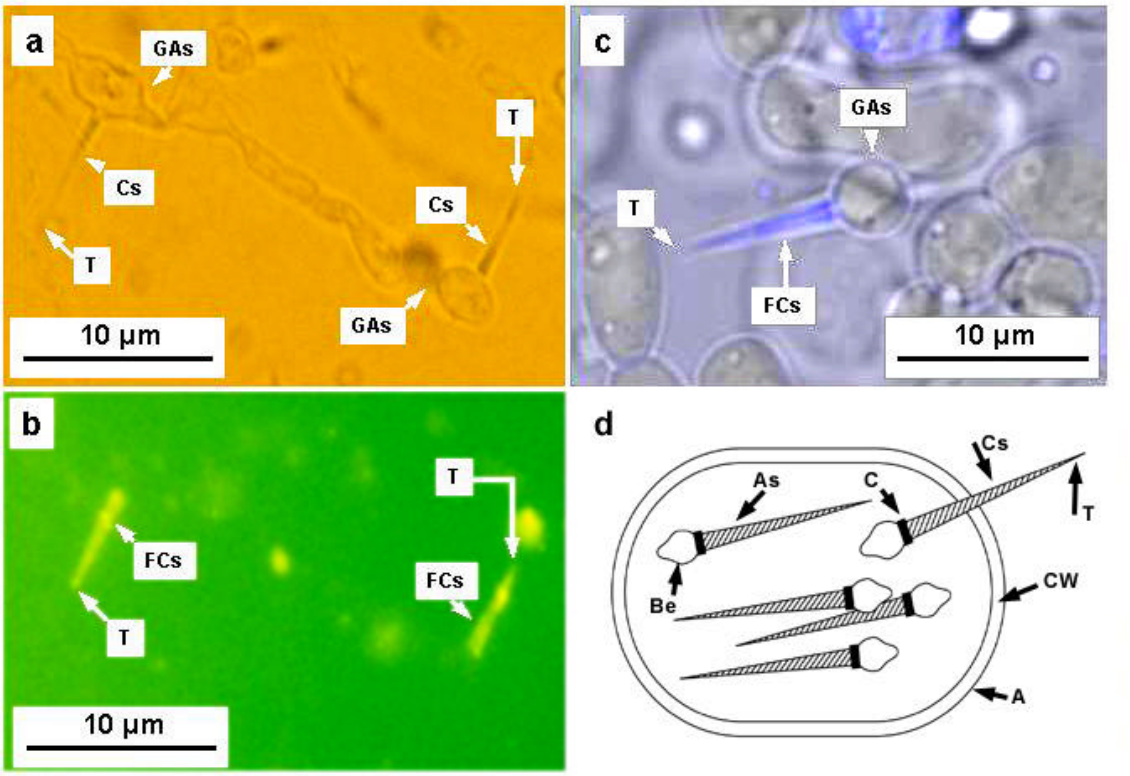

Fig. 2: Immunofluorescence and confocal laser scanning micrographs of *Eremothecium sinecaudum* showing fluorescing corkscrew-like structures (FCs). (a) Light micrograph showing corkscrew (Cs) with sharp tip (T) as well as germinating part (GAs). (b) Corresponding fluorescence micrograph showing fluorescing corkscrew part (FCs) with tip (T). (c) Fluorescing corkscrew part (FCs) visualized by Orange G as studied by confocal laser scanning microscopy. The germinating ascospore part (GAs) as well as vegetative cells does not stain. (d) Schematic presentation based on confocal laser scanning micrograph showing corkscrew (Cs) protubing through ascus (A) wall (CW), tip (T) first. As, ascospore; Be, blunt end; C, collarette.

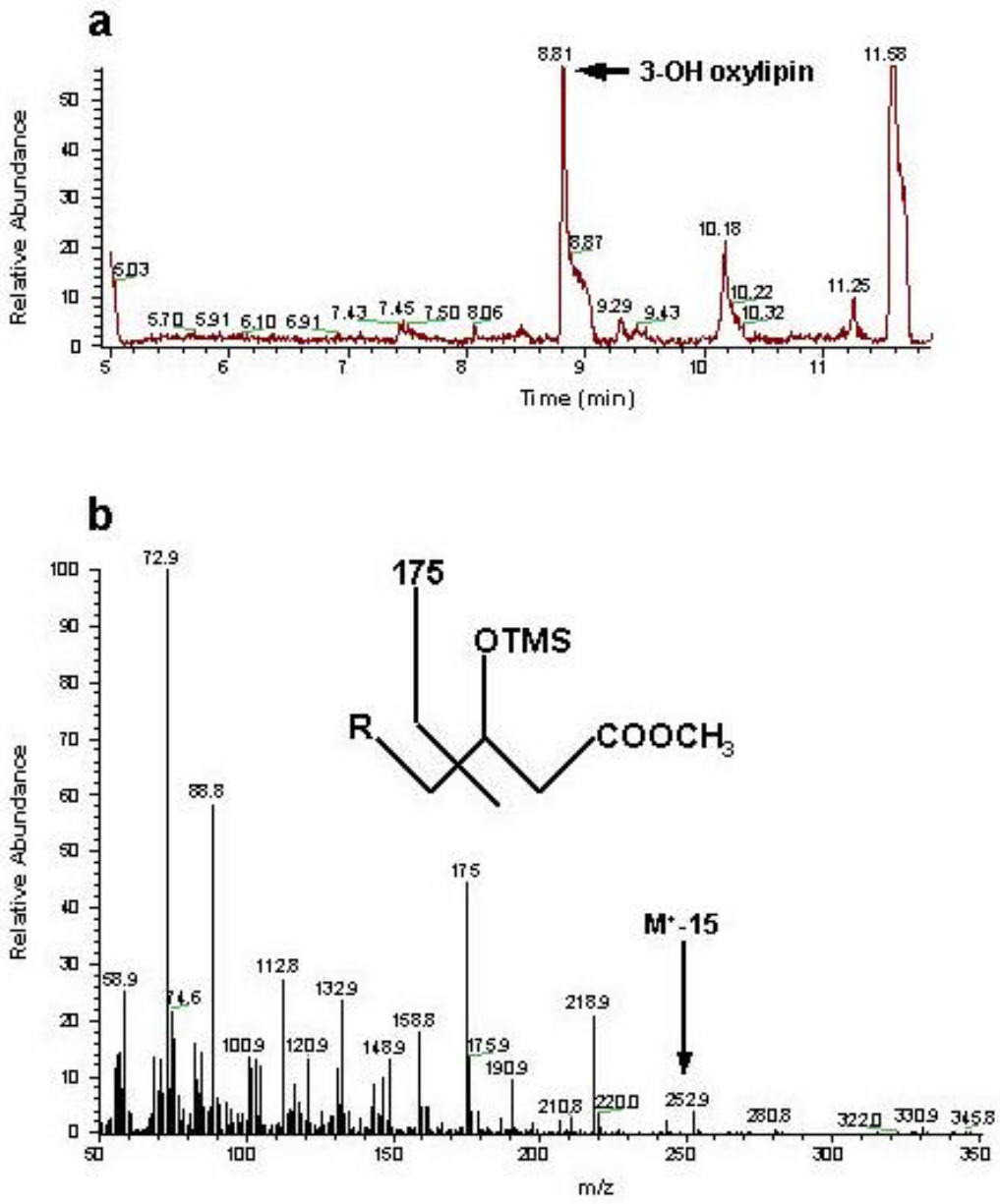

Fig. 3: Ion chromatogram (a) and mass spectrum (b) of a methyl-trimethylsilylated 3-OH oxylipin in *Eremothecium sinecaudum.*

3.2 Family: Lipomycetaceae

(Parts of this chapter have been published in Antonie van Leeuwenhoek85: 187-189)

3.2.1 Variation in functional ascospore parts and lipid turnover in the ascomycetous yeast *Dipodascopsis uninucleata*

Key words: ascospore morphology, *Dipodascopsis uninucleata* var. *uninucleata, Dipodascopsis uninucleata* var. *wickerhamii*, hooks, lipid turnover

Abstract

A variation in functional ascospore morphology was detected using electron microscopy (EM) (where glutaraldehyde and osmium tetroxide were used as fixatives) in two varieties of the yeast *Dipodascopsis uninucleata* i.e*. D. uninucleata* var. *uninucleata* and *D. uninucleata* var. *wickerhamii.* It was found that the latter produces ascospores characterized by the absence of small surface hooks which have been implicated in the release and re-assembly of ascospores in *D. uninucleata* var. *uninucleata*. These varieties are closely related on the basis of their mode of sexual reproduction, ascospore morphology as observed under the light microscope, physiological characteristics, as well as the extent of divergence in the variable D1/D2 domain of the large subunit 26S ribosomal DNA. However, opposite trends regarding the lipid turnover between the varieties were found in the phospho- and glycolipid fractions over their life cycles (i.e. from asexual to sexual stages).

Introduction

It is known that yeasts are able to produce a wide array of ascospores with different morphological shapes i.e. round, hat-shaped, needle-shaped, reniform, etc. as well as different surface ornamentations (Yarrow 1998). The functions of these different shapes and surface structures are however unclear, although it can be speculated that they may assist in ascospore dispersal by some unknown mechanisms.

In 1999, Kock and co-workers described a function for nano-scale hooks associated with the bean-shaped ascospores of *D. uninucleata* var. *uninucleata.* This study reports variation in these functional ascospore parts as well as lipid turnover among members of this species.

Materials and methods (See Appendix for detail)

Strain used and cultivation

Dipodascopsis uninucleata var. *wickerhamii* UOFS Y-1129 obtained from the University of the Free State, Bloemfontein, South Africa, was used throughout this study. This yeast was cultivated on solid YM (Yeast-malt) agar medium (Wickerham 1951) at 25 ^{O}C for 10 days to reach its sexual stage.

Electron microscopy (EM)

These cells were chemically fixed according to Van Wyk and Wingfield (1994). Scanning electron micrographs were taken with a Joel 6400 WIN-SEM (Japan) and transmission electron micrographs were taken with a Phillips CM 100 (Delft, The Netherlands TEM) (Van Wyk & Wingfield 1994).

Intracellular lipid extraction

The freeze-dried cells of *D. uninucleata* var. *wickerhamii* at their asexual and sexual stages were subjected to intracellular lipid extraction the same way as the cells *of D. uninucleata* var. *uninucleata* (Kock & Ratledge 1993). Then, the organic phase was washed twice with distilled water (Folch et al. 1957).

Lipid fractionation (Kock & Ratledge 1993)

The lipid content obtained from the freeze-dried cells of *D. uninucleata* var. *wickerhamii* during asexual and sexual stages was fractionated into neutral- (NL), phospho- (PL) and gylco- (GL) fractions. These fractions were eluted from the column of activated silicic acid (i.e. heating at 110 ^{O}C oven overnight) by applying different solvents with different polarities. After evaporation, these fractions were dried at 50 ^{O}C over P_2O_5 overnight and then weighed. These fractions were finally stored under a blanket of N_2 gas for further analysis.

Fatty acid determination

The fractionated lipid samples were first dissolved in a minimal volume of chloroform and transesterified by adding trimethylsulphonium hydroxide (TMSOH) for fatty acid analysis using gas chromatography (GC) (Butte 1983). The GC was equipped with a flame ionisation detector and Supelcowax 10 capillary column (30 m x 075 mm). The initial column temperature of 145 ^{O}C was increased from 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Chemicals used

All chemicals used were of highest purity grade and obtained from reputable dealers.

Results and discussion

In 1999, Kock and co-workers reported on the presence of an interspore matrix and interesting interlocking small hooks associated with the bean-shaped ascospores of *D. uninucleata* var. *uninucleata* as observed with the scanning electron microscope (SEM) and the transmission electron microscope (TEM) (Figure 1 A, B). After extensive inhibition studies, where the formation of the nano-scale hooks was inhibited by the addition of 1.0 mM acetylsalicylic acid, they proposed that the interspore matrix and the small hooks may play an important role in the ordered separation as well as ordered aggregation of the ascospores (during release from the ascus). When the ascospore ultrastructure of *D. uninucleata* var. *wickerhamii* was investigated in the same way, no small hooks could be observed on the smooth surfaces of the ellipsoidal ascospores (Figure 1 C, D). Also, no ordered aggregation of ascospores showing a well-demarcated interspore matrix could be seen (Figure 1 C). These observations further demonstrate the differences between these two varieties which have been otherwise found to be similar on the basis of sexual reproduction, ascospore morphology as observed under the light microscope, as well as the extent of divergence in the variable D1/D2 domain of the large subunit 26S ribosomal DNA (Kurtzman & Robnett 1998; Smith & De Hoog 1998). Also, both varieties have been isolated from the fruit fly (Smith & De Hoog 1998).

Furthermore, during the life cycle of *D. uninucleata* var. *wickerhamii* (i.e. from asexual to sexual stages), it was found that the total lipid content extracted from the freeze-dried biomass decreased from 5.1 % (w/w) to 1.9 % (w/w) (Table 1). These results are contrary to those reported for *D. uninucleata* var. *uninucleata* (Kock & Ratledge 1993). When the intracellular total lipid content was fractionated into neutral-, phospho- and glycolipid fractions during transition from asexual to sexual stages, the following results were obtained (Table 1). The neutral lipids (NL) increased from 36.9 % (w/w) to 42.2 % (w/w), which may be attributed to the production of the lipid rich ascospores. Here, the stored NLs (triacylglycerides) will be utilized as energy source during germination to form vegetative cells. Similar results were also found during the NL turnover of *D. uninucleata* var. *uninucleata* (Kock & Ratledge 1993). The PLs of *D. uninucleata* var. *wickerhamii* decreased while the GLs increased during the sexual stage (Table 1).

When the fatty acid composition of the different lipid fractions was analysed, using gas chromatography, the results in Table 2 were obtained. The percentage saturated fatty acids (SAFAs), i.e. 16:0 plus 18:0 decreased in the NLs, PLs and GLs when the sexual stage was reached. The monounsaturated fatty acids (MUFAs), i.e. 16:1 plus 18:1 stayed more or less the same in the NL in both stages (Table 2). These fatty acids decreased in the PL and increased in GL upon reaching the sexual stage (Table 2). Similar trends were also obtained for MUFAs turnover in the GL fraction of *D. uninucleata* var. *uninucleata* (Kock & Ratledge 1993) except in the PL fraction. The polyunsaturated fatty acids, i.e. 18:2 plus 18:3 (PUFAs) increased significantly in the PL from 7.4 % to 34.7 % during transition from asexual to sexual stages. This

change can be explained since the PUFAs are required to maintain the cell membrane fluidity during the sexual stage.

Conclusions

1. This study reports a variation in functional ascospore parts within members of the same yeast species. In another study, variation in the ascospore shape in *Kodamaea ohmeri* has been reported. However, no function could be linked to the two ascospore shapes i.e. hat-shaped or round found when different strains of this species were paired (Kurtzman 1998).

2. In addition, variation in lipid turnover, i.e. intracellular total lipid content, PL and GL was experienced during the life cycles of *D. uninucleata* var. *uninucleata* and *D. uninucleata* var. *wickerhamii*. On the other hand, similar trends were found during NL turnover between the two varieties.

Acknowledgements

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Table 1. Changes in lipid content and lipid composition during the asexual and sexual stages of *Dipodascopsis uninucleata* var. *wickerhamii* UOFS Y-1129

Time (days)	Stages of life cycle	% Lipid content	% Lipid fractions		
			NL	PL	GL
2	A	5.1	36.9	42.7	20.4
10	S	1.9	42.2	27.4	30.3

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 2. Changes in percentage fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Dipodascopsis uninucleata* var. *wickerhamii* UOFS Y-1129

Time	Steges of life cycle	% SAFAs			% MUFAs			% PUFAs		
		NL	PL	GL	NL	PL	GL	NL	PL	GL
2	A	41.8	46.1	31.8	37.8	46.6	36.4	20.4	7.4	31.9
10	S	28.3	22.2	24.2	37.0	43.1	40.4	34.7	34.7	35.5

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, SAFAs = Saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFAs = Polyunsaturated fatty acids. Similar trends were observed when this experiment was done in triplicate. SE < 5%.

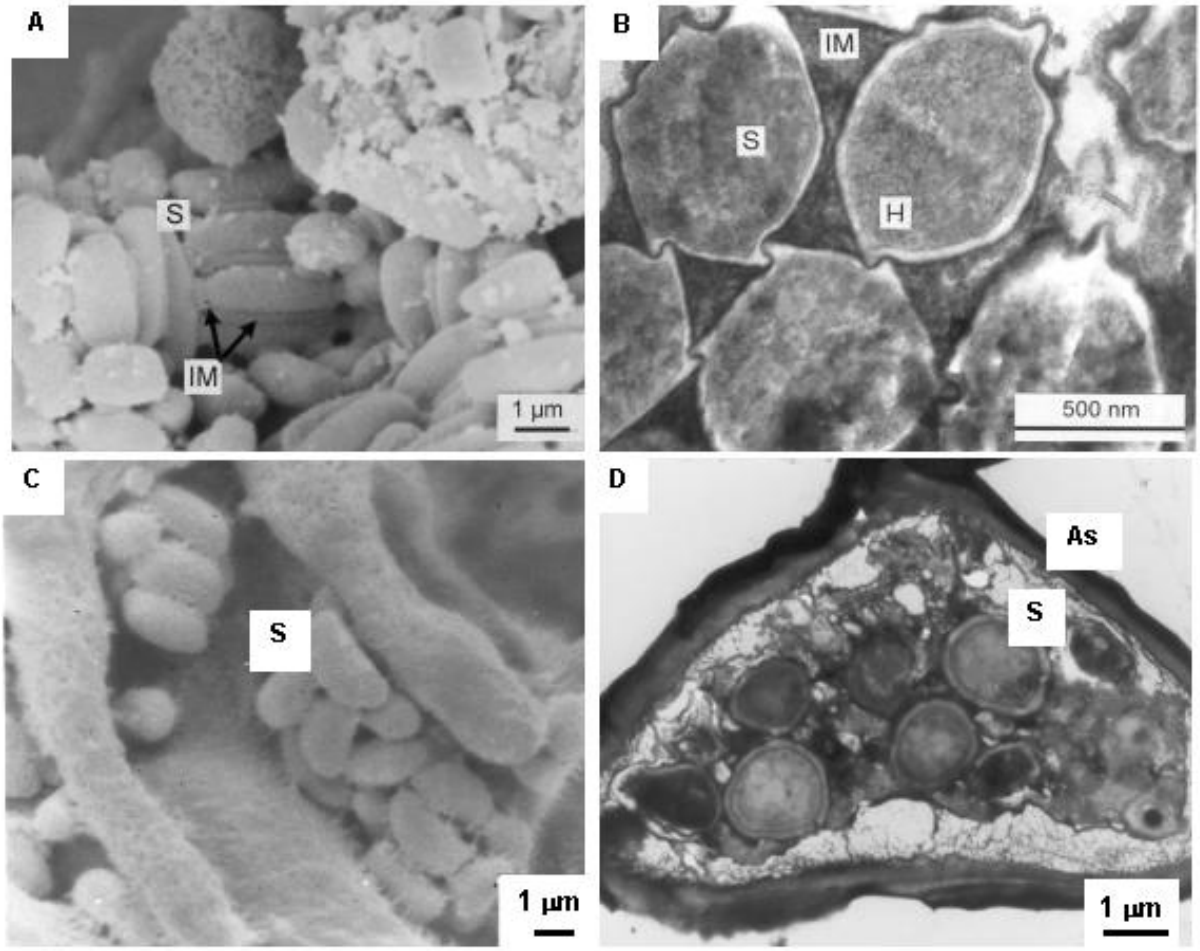

Figure 1. Representative electron micrographs of *Dipodascopsis uninucleata*. A, SEM of *Dipodascopsis uninucleata* var. *uninucleata* showing interspore matrix (IM) between the ascospores (S) (Taken from Kock et al. 1999). B, TEM of *Dipodascopsis uninucleata* var. *uninucleata* showing hooks (H) on the ascospore (S) surface (Taken from Kock et al. 1999). C, SEM of *Dipodascopsis uninucleata var. wickerhamii* showing ascospores (S) without interspore matrix. D, TEM of *Dipodascopsis uninucleata* var. *wickerhamii* showing smooth walled ascospores (S) without hooks inside ascus (As).

3.2 Family: Lipomycetaceae

3.2.2 Ascospores and lipid turnover in selected species of the genus *Lipomyces* Lodder & Kreger-van Rij

Key words: Ascospore, fatty acids, lipid turnover, *Lipomyces*, transmission electron microscopy, yeast

Abstract

Smooth and ridged walled ascospores are reported in *Lipomyces kononenkoae* and *Lipomyces tetrasporus* respectively, when observed under the transmission electron microscope using glutaraldehyde and osmium tetroxide as chemical fixatives. Furthermore, in both yeasts, membrane bound lipid sacs surrounding the ascospores inside the ascus were found. This is the first report of the presence of these structures in the genus *Lipomyces* using transmission electron microscopy. No such structures could be observed when the conventional fixation method with potassium permanganate was used as sole chemical fixative. Furthermore, interesting changes regarding the total lipids, neutral lipids, phospholipids and glycolipids were encountered towards the sexual reproductive stages of these yeasts.

Introduction

Ascospore morphology of the lipomycetaceous yeast, *Dipodascopsis uninucleata* var. *uninucleata*, was investigated using transmission electron microscopy and

potassium permanganate as sole chemical fixative (Kreger-van Rij and Veenhuis, 1974). Using this method of fixation, smooth walled and bean-shaped ascospores without surface ornamentations were observed and thus confirmed the findings using conventional light microscope (Smith, 1998). Surprisingly, when glutaraldehyde and osmium tetroxide were used as alternative chemical fixatives, interesting nano-scale surface ornamentations (i.e. hooks) were uncovered which surround the bean-shaped ascospores of this yeast (Kock et al. 1999). These lubricated 3-hydroxy oxylipin covered surface hooks are involved in the ordered release and subsequent re-aggregation of these spores after discharge from the elongated ascus (Kock et al. 1999) (Chapter 2).

It is known that lipids usually occupy a major part of the ascospore. During sexual stage, the lipid metabolism is mainly directed towards the production of neutral lipids that accumulate within ascospores (Kock and Ratledge, 1993). This is mainly used as energy deposit during survival and the onset of germination.

Hence, it became the purpose of this study to investigate ascospore morphology and other ascus inclusion of selected members of the genus *Lipomyces* using transmission electron microscopy (i.e. with glutaraldehyde and osmium tetroxide fixation). In addition, the lipid turnover of these yeasts was investigated over their respective life cycles, i.e. asexual and sexual stages. This study does not include oxylipin mapping in the genus *Lipomyces* since this has been addressed in detail by Smith et al. (2000).

Materials and methods (See Appendix for detail)

Strains used and cultivation

L. kononenkoae UOFS Y-2135 T and *L. tetrasporus* UOFS Y-1112 were obtained from the culture collection of the University of the Free State, Bloemfontein, South Africa. These yeasts were cultivated for 23 and 21 days respectively on solid YM (yeast-malt) medium (Wickerham, 1951) at 25 ^{O}C until sexual stage. This material was used to study ascospore morphology as well as lipid turnover. All the experiments were performed in triplicate.

Transmission electron microscopy (TEM)

During the sexual stage, cells were immediately fixed overnight in 3 % aqueous glutadialdehyde in 0.1 M phosphate buffer and 1 % osmium tetroxide in 0.1 M phosphate buffer for two hours at room temperature. After washing with the same buffer, the material was dehydrated through a graded acetone series (i.e. of different concentrations) and then, the material was embedded in epoxy (Spurr, 1969) and set in a silicone mould (14 mm long x 6 mm wide x 4 mm deep). After polymerisation at 70 ^{O}C for eight hours, the material was cut on a LKB ultramicrotome with a glass knife and finally stained in a saturated solution of uranyl acetate (10 min) and lead citrate (five min). Transmission electron micrographs were taken with a Phillips CM 100, (The Netherlands) (TEM) (Van Wyk and Wingfield, 1994).

Lipid extraction

During the asexual and sexual stages, cells were scraped from the YM medium, rapidly frozen, freeze-dried and weighed. Lipids were extracted according to the method of Kock and Ratledge (1993). Briefly, pre-weighed freeze-dried cells were

dissolved overnight in a mixture of chloroform:methanol (2:1, v/v) and then washed twice with distilled water (Folch et al. 1957). The organic phase was evaporated. Lipid samples were dried in an oven at 50 ^{O}C over P_2O_5 overnight and finally weighed.

Lipid fractionation

Briefly, the lipid samples were dissolved in a minimal volume of chloroform and applied to a clean column (140 mm x 20 mm) of activated silicic acid. Next, appropriate volumes of trichloroethane, acetone and methanol were applied to a column to elute neutral, glyco- and phospholipids fractions respectively. These lipid fractions were subsequently dried in an oven over P_2O_5 at 50 ^{O}C and finally weighed. All lipid fractions were then stored under a blanket of N_2 at –20 ^{O}C (Kock and Ratledge, 1993).

Fatty acid determination

All lipid samples were transesterified by the addition of trimethylsulphonium hydroxide (TMSOH) as performed by Butte (1983). The fatty acid methyl esters were analysed with a gas chromatograph (GC) equipped with a flame ionisation detector and Supelcowax 10 capillary column (30 m x 075 mm). The initial column temperature of 145 ^{O}C was increased from 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Chemicals used

All chemicals and solvents used were of highest purity grade and obtained from reputable dealers. Silicic acid (100 mesh) was obtained from Aldrich (Germany).

Results and discussions

Ascospore morphology

Using TEM (glutadialdehyde-osmium tetroxide fixation) we found that both *L. kononenkoae* (smooth globose ascospores) and *L. tetrasporus* (ridged globose ascospores) produced similar ascospores regarding shape and surface ornamentations compared to that reported in literature (Nieuwdorp et al. 1974; Smith and Batenburg-van der Vegte, 1984; Smith et al. 1995). Strikingly, in both yeasts, membrane bound lipid sacs, surrounding the ascospores, were observed only with glutaraldehyde-osmium tetroxide fixation (Figs 1 and 2). The possible function of these lipid sacs may be linked to providing energy during germination of these spores. In contrast, when these yeasts were subjected to potassium permanganate fixation alone, these lipid sacs could not be detected under TEM (Nieuwdorp et al. 1974; Smith and Batenburg-van der Vegte, 1984; Smith et al. 1995). This could probably be due to the loss of these lipid sacs during fixation using potassium permanganate.

Changes in total lipid content over the life cycle

The significant changes in total lipid content of *L. kononenkoae* (Table 1) and *L. tetrasporus* (Table 3) over the life cycle (i.e. from asexual to sexual stages) were profound. During the sexual stage of *L. kononenkoae*, i.e. after 23 days, a drastic decrease in the percentage intracellular total lipid content of the vegetative cells

occurred from 11.6 % (w/w) to 5.0 % (w/w). A similar pattern [decrease from 13.8 % (w/w) to 5.2 % (w/w)] was also experienced for *L. tetrasporus* when it reached the sexual reproductive stage (Table 3). This decrease in the intracellular lipids may be due to utilisation of these lipids during energy production to form sexual spores. However, these results are in contrast with the total lipid turnover of *Dipodascopsis uninucleata* var. *uninucleata* and *Dipodascus ambrosiae*, where there was an increase in the total lipid contents as the yeasts entered their sexual reproductive stage (Kock and Ratledge, 1993; Smith et al. 2003). Consequently, a clear distinction can be drawn between *L. kononenkoae* and *L. tetrasporus*, on the one hand and *Dipodascopsis uninucleata*.

Changes in the composition of the intracellular lipid content

The percentage neutral lipid (NL) fraction of *L. kononenkoae* decreased from 78.2 % (w/w) to 49.1 % (w/w) while the percentage phospholipid (PL) as well as gylcolipid (GL) fractions increased significantly as the cells entered sexual stage (Table 1). Similar trends were also obtained for these lipid fractions as cells of *L. tetrasporus* entered the sexual stage (Table 3). These results are in contrast to those obtained for *Dipodascopsis uninucleata* var. *uninucleata* and *Dipodascus ambrosiae* except for the GL fraction, which also increased during the sexual stage (Kock and Ratledge, 1993; Smith et al. 2003). A decrease in the NLs during the sexual stage may be due to the utilisation of these lipids as energy source to produce membranes containing PL and cell walls containing GL necessary for ascospore development. This may account for the increase in PL and GL fractions in both yeasts.

Changes in the fatty acid composition

Since significant changes of the NLs, PLs and GLs occurred over the life cycles of *L. kononenkoae* and *L. tetrasporus*, we next decided to investigate the changes in the fatty acid composition over the life cycle of these lipid fractions. Both yeasts were characterised by palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) in their lipid fractions throughout the vegetative and sexual stages (Tables 2 and 4). Interestingly, *L. tetrasporus* (Table 4) was characterized by a high percentage of 18:0 and lower 18:1 in the PL fraction throughout its life cycle while the opposite was true for *L. kononenkoae* (Table 2). This cannot be explained at this stage. Furthermore, an increase in 16:0 and a decrease in 16:1 were experienced in the GL fraction when these yeasts reached their sexual reproductive stages. During the life cycles of these yeasts, the relative percentage of 18:1 was more abundant in all the lipid fractions with the exception of this fatty acid in the PL fraction in *L. tetrasporus*. During the life cycles of these yeasts 18:2 increased in the NL and decreased in the GL fractions of these yeasts. Small amounts of 18:3 were detected in the NL and PL fractions of these yeasts throughout their life cycles as compared to the GL fraction. However, this fatty acid in the latter lipid fraction decreased in *L. kononenkoae* (Table 2) and increased in *L. tetrasporus* when reaching the sexual stages (Table 4).

Conclusions

1. In this study two types of ascospore wall structures were found in *Lipomyces kononenkoae* and *Lipomyces tetrasporus*, i.e. smooth and ridged respectively. These results further confirm those already reported in literature and underline the value of ascospore morphology in the genus *Lipomyces* as identification marker.

However, the functions of these ascospore shapes and surface ornamentations are still not clear and should be investigated especially since it has previously been reported that 3-hydroxy oxylipins are associated with the latter structures (Smith et al. 2000).

2. Strikingly, in both yeasts, lipid sacs were found surrounding the ascospores inside the ascus. This is the first report of the presence of these structures in these yeasts using TEM when glutadialdehyde and osmium tetroxide were used as chemical fixatives. These lipid sacs could not be observed when potassium permanganate fixation was performed alone. This may be attributed to the loss of these structures during fixation using permanganate.

3. The decrease in total lipids and NLs as the yeasts approached sexual stage is intriguing and contrary to literature. This phenomenon should be further investigated.

Acknowledgements

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Table 1: Changes in lipid content and lipid composition during the asexual and sexual stages of *Lipomyces kononenkoae* UOFS Y-2135 T

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
7	A	11.6	78.2	17.2	4.6
23	S	5.0	49.1	31.1	19.8

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 2: Changes in percentage fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Lipomyces kononenkoae* UOFS Y-2135 T

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	33.0 (A)	24.0 (A)	26.7 (A)
	32.4 (S)	24.2 (S)	38.5 (S)
16:1	5.2 (A)	11.3 (A)	6.2 (A)
	5.8 (S)	10.2 (S)	2.2 (S)
18:0	3.1 (A)	0.6 (A)	8.0 (A)
	3.0 (S)	0.8 (S)	7.8 (S)
18:1	53.0 (A)	44.2 (A)	42.8 (A)
	48.6 (S)	50.4 (S)	41.7 (S)
18:2	5.5 (A)	18.9 (A)	14.1(A)
	9.7 (S)	13.8 (S)	8.7 (S)
18:3	0.2 (A)	0.9 (A)	3.0 (A)
	0.5 (S)	0.7 (S)	1.1 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid, 18:3 = Linolenic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 3: Changes in lipid content and lipid composition during the asexual and sexual stages of *Lipomyces tetrasporus* UOFS Y-1112

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
8	A	13.8	79.5	15.0	5.5
21	S	5.2	55.5	32.8	11.7

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 4: Changes in fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Lipomyces tetrasporus* UOFS Y-1112

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	36.0 (A)	21.6 (A)	21.2 (A)
	23.7 (S)	22.5 (S)	40.9 (S)
16:1	6.1 (A)	11.1 (A)	10.8 (A)
	4.0 (S)	10.4 (S)	4.3 (S)
18:0	3.0 (A)	50.3 (A)	5.3 (A)
	3.1 (S)	53.8 (S)	11.6 (S)
18:1	49.1 (A)	14.1 (A)	41.8 (A)
	59.9 (S)	11.7 (S)	32.3 (S)
18:2	5.3 (A)	2.7 (A)	14.6 (A)
	8.7 (S)	1.5 (S)	7.6 (S)
18:3	0.6 (A)	0.1 (A)	1.7 (A)
	0.6 (S)	0.1 (S)	3.2 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid, 18:3 = Linolenic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

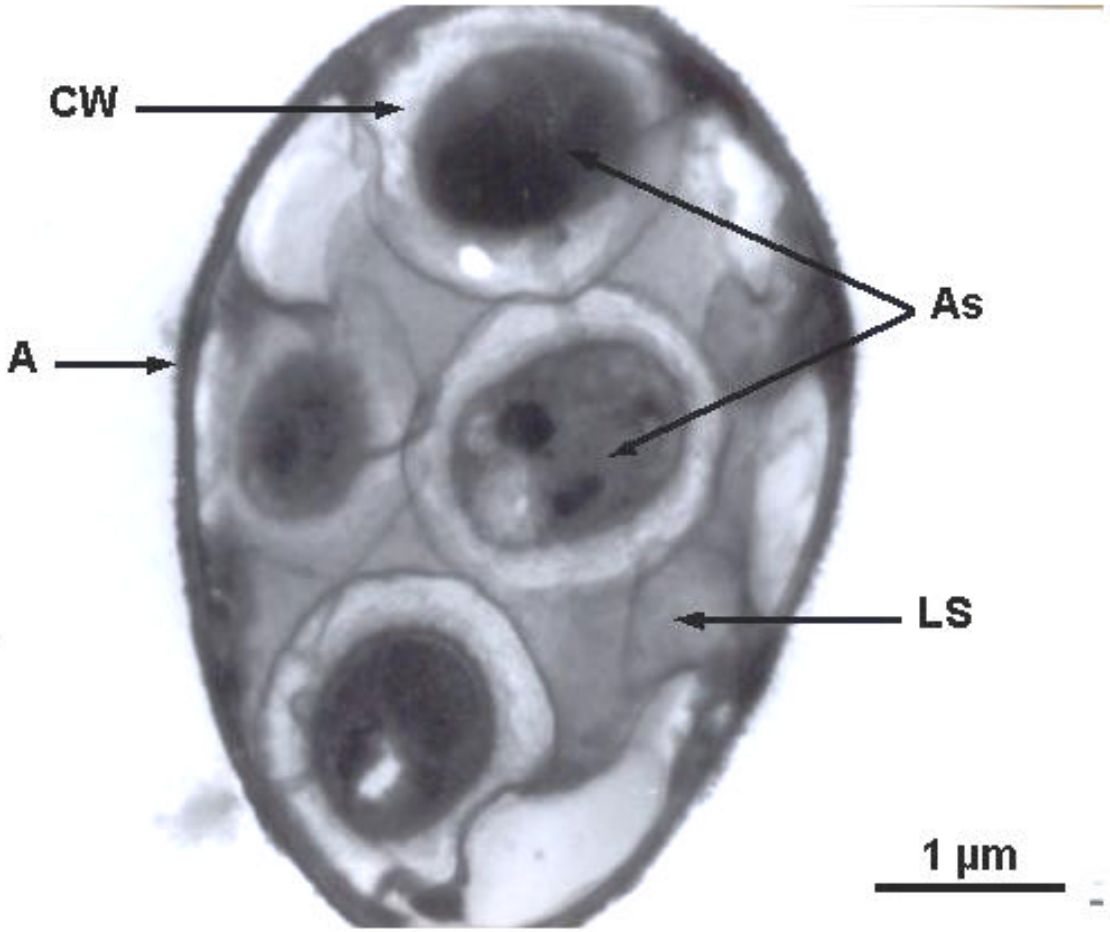

Fig. 1. Transmission electron micrograph of *L. kononenkoae* obtained when glutadialdehyde and osmium tetroxide were used as chemical fixatives showing an ascus (A) containing smooth walled globose ascospores (As) with cell wall (CW). All ascospores (As) are embedded between lipid sacs (LS).

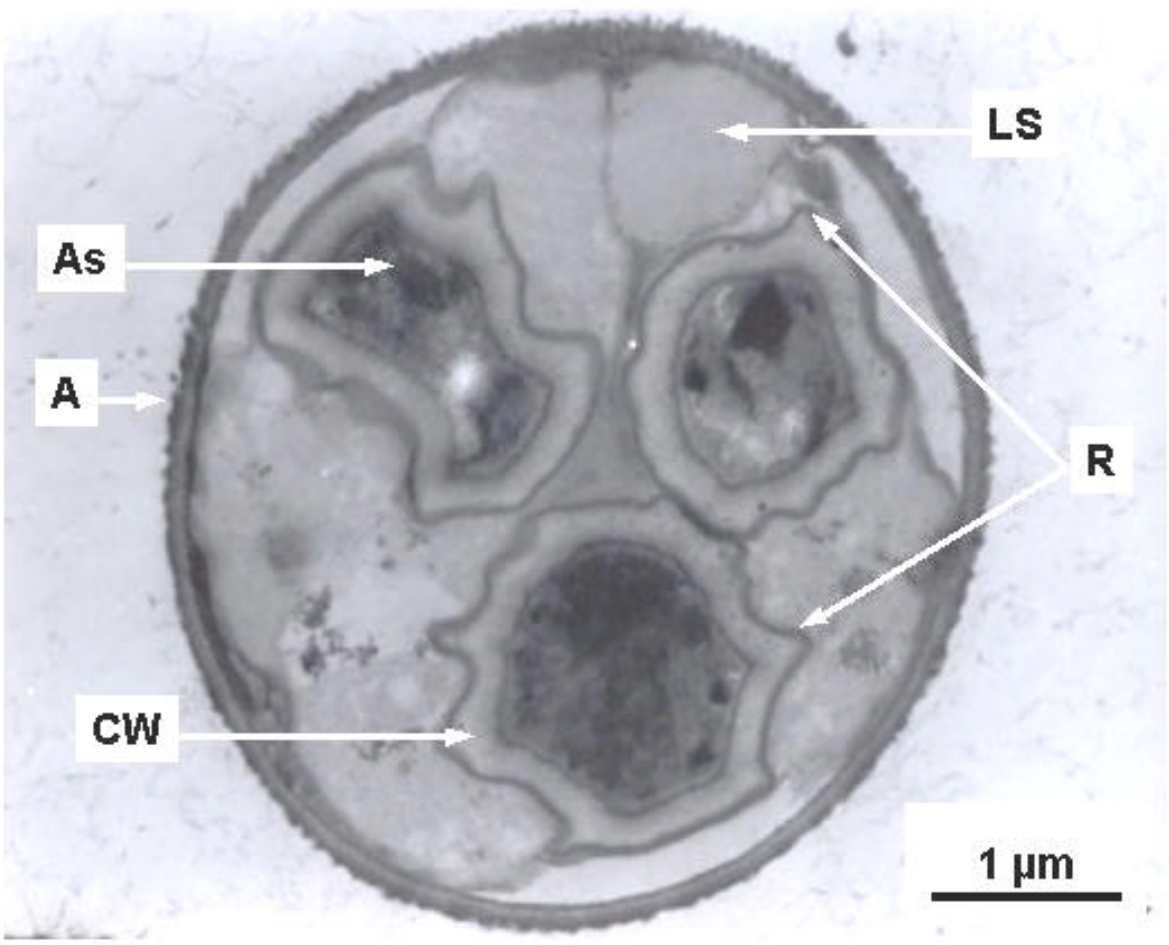

Fig. 2. Transmission electron micrograph of *L. tetrasporus* obtained when glutadialdehyde and osmium tetroxide were used as chemical fixatives. The TEM shows an ascus (A) containing globose ascospores (As) with surface ornamentations (ridges, R) associated with the cell wall (CW). Ascospores (As) are imbedded between well-demarcated membrane covered lipid sacs (LS).

3.3 Family: Saccharomycetaceae

(Parts submitted for publication in Systematic and Applied Microbiology)

Ascospores, 3-hydroxy oxylipins and lipid turnover in the ascomycetous yeast *Saturnispora saitoi*

Running title: 3-Hydroxy oxylipins in *Saturnispora saitoi*

Abstract

The distribution of 3-hydroxy oxylipins in *Saturnispora saitoi* was mapped using immunofluorescence microscopy. Fluorescence was observed on aggregating ascospores, indicating the presence of 3-hydroxy oxylipins on the surface or between ascospores. The presence of these oxylipins was confirmed using gas chromatography-mass spectrometry. Furthermore, ultrastructural studies using scanning and transmission electron microscopy on ascospores revealed a clear equatorial ledge surrounding oval-shaped ascospores. In addition, a decrease in the neutral and phospholipid lipid fractions was experienced during the sexual reproductive stage. The glycolipid fraction on the other hand, increased during this part of the life cycle.

Key words: Ascospores - electron microscopy - 3-hydroxy oxylipins - immunofluorescence - lipid turnover - *Saturnispora saitoi* - ultrastructure - yeast

Introduction

The distribution of 3-hydroxy oxylipins in yeast was previously reported using polyclonal antibodies as well as gas chromatography-mass spectrometry analysis [8, 16]. Consequently, these compounds were found to be associated with aggregating sexual as well as asexual vegetative cells in the yeasts *Candida albicans* [3, 2], *Dipodascopsis uninucleata* var. *uninucleata* [7], Dipodascaceae [14], *Lipomyces* [15], *Saccharomyces cerevisiae* [9], *Saccharomycopsis malanga* [12] and others [6, 13]. Since 3-hydroxy oxylipins show potent biological activities in medical studies [6] and importance in ascospore formation and movement in micron-space, bioprospecting studies are undertaken to expose rich sources of these compounds for eventual biotechnological production and decoding of function.

In this study, we mapped the distribution of 3-hydroxy oxylipins in the ascomycetous yeast *Saturnispora saitoi* while ascospore ultrastructure was elucidated.

Materials and Methods (See Appendix for detail)

Strain used and cultivation

Saturnispora saitoi UOFS Y-1243, held at the University of the Free State, Bloemfontein, South Africa, was used throughout the study and cultivated on YM (Yeast-malt) agar slants [18], transferred to YM agar medium and incubated at 25 ^{O}C for 12 days until sexual stage was reached. Thereafter, the cells were subjected to immunofluorescence, and electron microscopic studies while cell extracts were subjected to gas chromatography-mass spectrometry and lipid analysis.

Immunofluorescence microscopy

The synthesis of 3-hydroxy fatty acids as well as preparation and characterisation of antibodies against these oxylipins were according to KOCK et al. [8]. Antibody and fluorescine labelling as well as eventual immunofluorescence microscopy were performed as described [8].

Electron microscopy

This was performed according to VAN WYK and WINGFIELD [17]. In short, cells were chemically fixed in 3 % glutadialdehyde (over night) and 1 % osmium tetroxide (two hours) at room temperature. Electron micrographs were taken with a Joel 6400 WINSEM (Japan) SEM and a Philips CM 100 (The Netherlands) TEM.

Lipid extraction

During asexual (i.e. after two days) and sexual (i.e. after 12 days) reproductive stages, cells were scraped off from the medium, frozen, freeze dried and finally weighed. Lipid extraction was performed according to the method of KOCK and RATLEDGE [5] using chloroform:methanol (2:1, v/v) and washed twice with distilled water [4]. The organic phase was evaporated and the lipid material was dried in an oven at 50 ^{O}C over P_2O_5 over night and weighed.

Lipid fractionation

Lipid samples were fractionated into three lipid fractions (i.e. neutral, phospho- and glycolipids) over a clean column (140 mm x 20 mm) of activated silicic acid. These fractions were eluted respectively from the column by applying successively different solvents with different polarities [5]. After evaporation, the

fractionated lipid samples were dried in an oven over P_2O_5 at 50 ^{O}C overnight, weighed, stored under a blanket of N_2 gas and eventually stored at –20 ^{O}C for fatty acid analysis.

Fatty acid determination

Lipid fractions were subjected to transesterification by adding trimethylsulphonium hydroxide (TMSOH) [1]. The fatty acid methyl esters were analysed by using gas chromatography (GC) with a flame ionisation detector and Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 ^{O}C was increased by 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C, respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Gas chromatography-mass spectrometry (GC-MS)

Extraction of oxylipins followed by methylation, silylation and GC-MS analysis were performed according to VAN DYK et al. [16].

Chemicals used

All chemicals used were of highest purity grade and obtained from reputable dealers.

Results and discussion

When subjecting cells of *Saturnispora saitoi*, treated with fluorescine labelled antibodies (i.e. fluorescine anti rabbit IgG), to immunofluorescence microscopy, mainly the ascospores fluoresced (Fig. 1a, b). By closer inspection we found that these compounds are associated with surfaces of ascospores (Fig. 1b). The presence of these oxylipins was confirmed by gas chromatography-mass spectrometry (Fig. 4). Here a characteristic mass ion at 175 (indicating hydroxyl group at position 3 of fatty acid) was identified [16] thereby indicating the presence of a 3-hydroxy oxylipin. The complete structure of this compound is still to be elucidated.

Since 3-hydroxy oxylipins are associated with the surfaces of these ascospores, we decided to study the ultrastructure of these ascospores and especially the nano-scale ornamentations, which are covered with oxylipins. Consequently, we found that oxylipins are attached to cell walls of oval-shaped ascospores, each surrounded by a sub-equatorial ledge (Figs 2, 3). This is in accordance to literature where similar observations were made regarding ascospore morphology of the closely related *Saturnispora ahearnii* using scanning electron microscopy [10].

The function(s) of these oxylipins in ascospore formation, aggregation and release from asci are now under investigation.

Since, electron microscopic studies have revealed striking ultrastructural results regarding ascospore morphology, we decided next to investigate the lipid

composition of cells in asexual and sexual stages. During ascosporogenesis, the percentage intracellular total lipid content increased from 5.3 % (w/w) to 7.8 % (w/w) (Table 1). This may be ascribed to the storage of these lipids inside yeast ascospores as energy reserve for survival during adverse conditions as well as for germination into new cells [11]. These findings are similar to those reported for *Dipodascopsis uninucleata* var. *uninucleata* [5] and *Dipodascus ambrosiae* [14]. The composition of the intracellular total lipid material, which comprises of neutral lipid (NL), phospholipid (PL) and glycolipid (GL) was also analysed throughout the different stages of the life cycle (i.e. from asexual to sexual stages). When the sexual stage was reached, a small decrease in the NL and PL fractions was experienced with a concomitant increase in the GL fraction (Table 1). An increase in the GL fraction at this stage may be attributed to the formation of additional cells walls containing GLs necessary for saturn-shaped ascospore formation.

Using gas chromatography, all the lipid fractions studied in the asexual and sexual stages, contained palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). The percentage 16:0 decreased throughout the life cycle (i.e. from asexual to sexual stages) in all the lipid fractions (Table 2). The same is true for 18:1 and the abundant polyunsaturated fatty acid 18:2. On the contrary, the percentage 16:1 increased in the NL and GL fractions when this yeast formed sexual spores. The percentage 18:3 increased significantly in all the lipid fractions when the sexual stage was reached (Table 2).

Acknowledgements

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Table 1: Changes in lipid content and lipid composition during the asexual and sexual stages of *Saturnispora saitoi* UOFS Y-1243

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
2	A	5.3	67.6	22.6	9.8
12	S	7.8	66.1	18.6	15.2

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %

Table 2: Changes in percentage fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Saturnispora saitoi* UOFS Y-1243

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	18.2 (A)	22.6 (A)	21.4 (A)
	13.2 (S)	21.2 (S)	16.8 (S)
16:1	17.0 (A)	14.1 (A)	5.7 (A)
	20.2 (S)	13.6 (S)	17.9 (S)
18:0	2.9 (A)	2.3 (A)	7.1 (A)
	1.8 (S)	3.1 (S)	3.2 (S)
18:1	26.1 (A)	23.0 (A)	32.6 (A)
	24.0 (S)	21.7 (S)	24.9 (S)
18:2	35.5 (A)	37.8 (A)	30.2 (A)
	31.5 (S)	30.8 (S)	29.5 (S)
18:3	0.3 (A)	0.2 (A)	2.9 (A)
	9.2 (S)	9.7 (S)	7.7 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %

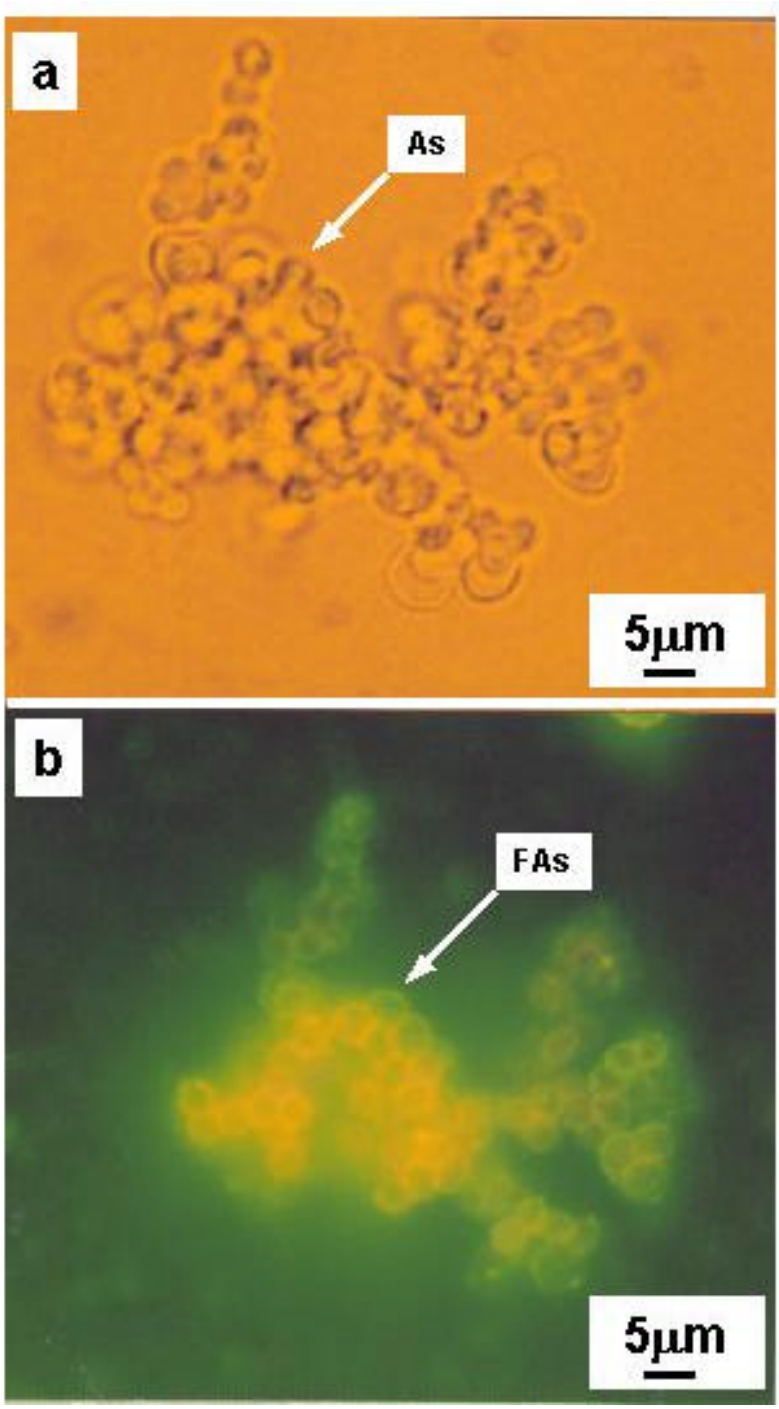


Fig. 1: Light and immunofluorescence micrographs of *Saturnispora saitoi* showing aggregating saturn-shaped ascospores. (a) Light micrograph showing saturn-shaped ascospores (As) aggregating after released from the ascus. (b) Immunofluorescence micrograph showing fluorescing ascospores (FAs) after treatment with an antibody specific to 3-hydroxy oxylipins.

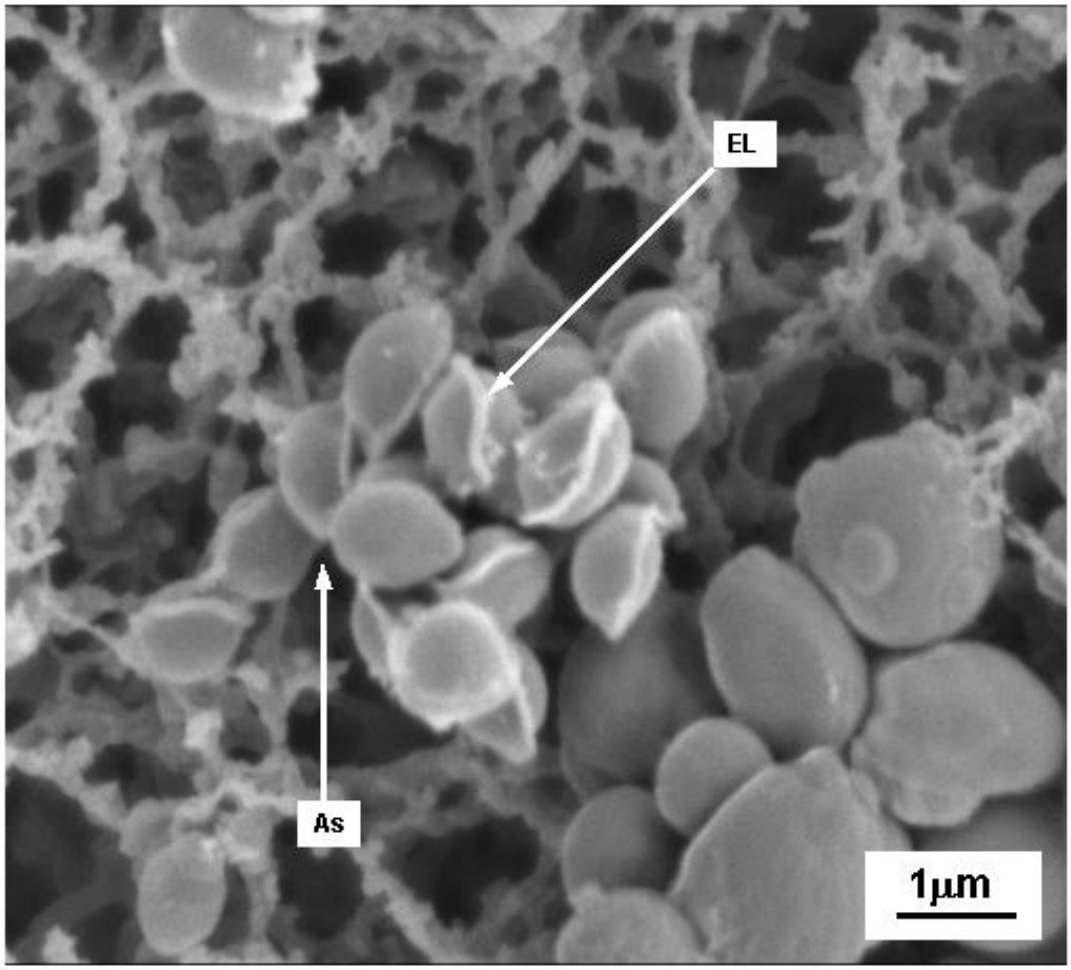


Fig. 2: Scanning electron micrograph of *Saturnispora saitoi* showing aggregating saturn-shaped ascospores (As) with sub-equatorial ledge (EL).

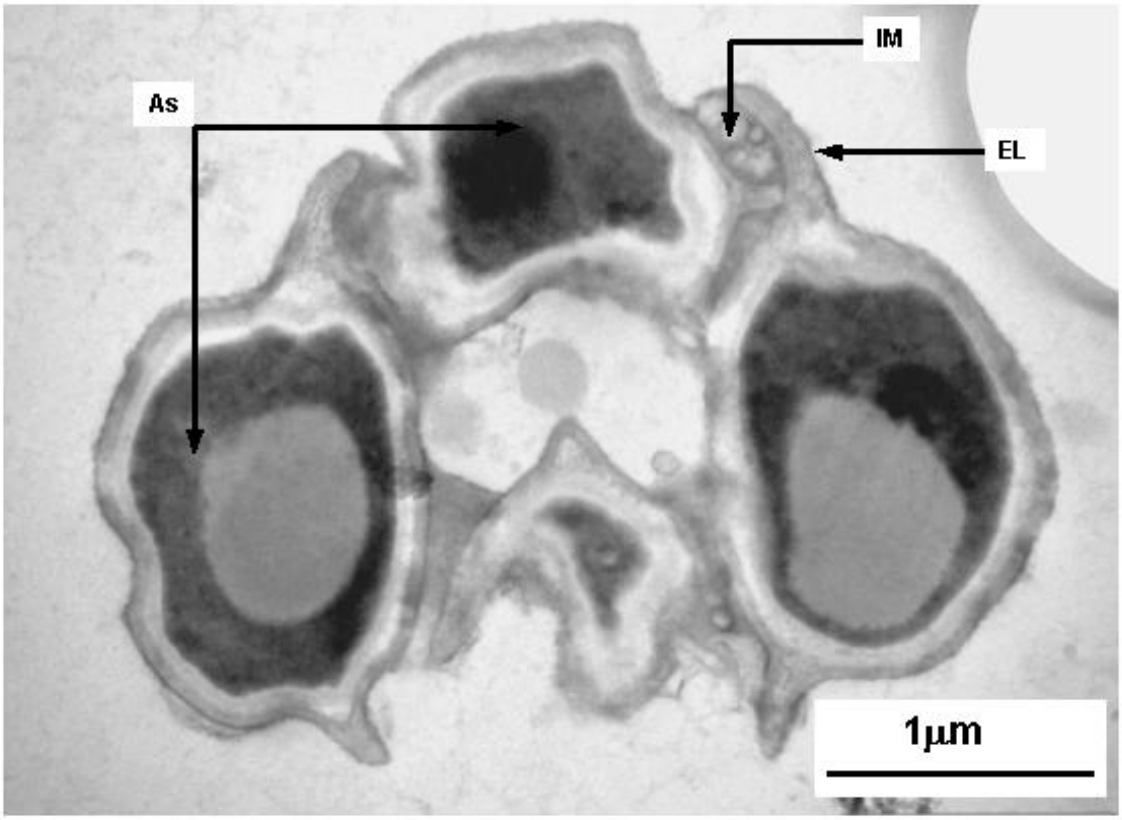
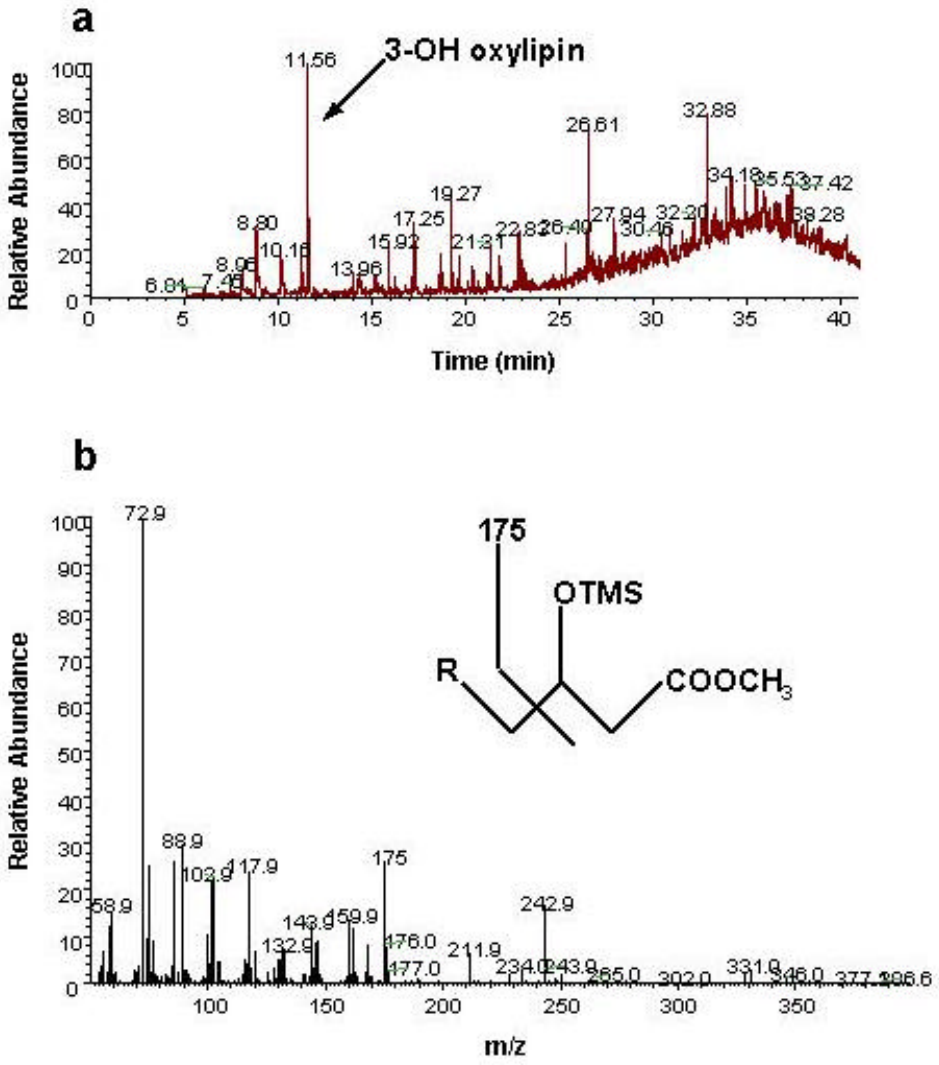


Fig. 3: Transmission electron micrograph of *Saturnispora saitoi* showing a cross section through saturn-shaped ascospores (As) each with a sub-equatorial ledge (EL) and interspore matrix (IM) between ascospores.



Figures 4: Total ion (a) and mass spectrum (b) of methyl-trimethylsilylated 3-hydroxy (OH) oxylipin on ascospores of *Saturnispora saitoi*.

3.4 Family: Saccharomycodaceae

(Submitted for publication in Antonie van Leeuwenhoek)

Ascospores, 3-hydroxy oxylipins and lipid turnover in the ascomycetous yeast genus *Nadsonia* Sydow

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Key words: Ascospore ultrastructure, fatty acids, 3-hydroxy oxylipin turnover, lipid turnover, *Nadsonia,* yeast

Abstract

Ascospore ultrastructure of the two species of the genus *Nadsonia* Sydow, i.e. *N. commutata* and *N. fulvescens*, were studied using transmission electron microscopy (using glutadialdehyde and osmium tetroxide as fixatives). Spherical ascospores with warty and hairy-like ornamentations were revealed in *N. commutata* and *N. fulvescens* respectively. Furthermore, a thick capsule was observed surrounding the ascospores of *N. fulvescens*. In addition, opposite trends regarding lipid turnover of the two species i.e. from asexual to sexual stages, were found in the neutral and phospholipid fractions. Interestingly, the presence of 3-hydroxy oxylipins was detected using gas chromatography-mass spectrometry during the asexual and sexual stages. The complete structure of 3-OH 9:1 could be determined in *Nadsonia commutata* during asexual stage. These results further highlight the differences in ascospore surface ornamentations, lipid and 3-hydroxy oxylipin turnover in these species.

Introduction

The genus *Nadsonia* Sydow comprises of two species i.e. *N. commutata* and *N. fulvescens* . These species have intriguing sexual cycles that result in *N. commutata* producing one ascospore within a mother cell while *N. fulvescens* also forms one ascospore, this time within a daughter cell (Miller and Phaff 1998). In addition, clear differences exist in their lipid composition, where *N. commutata* produces high amounts of linoleic acid (18:2) while *N. fulvescens* has lower amounts of this polyunsaturated fatty acid (PUFA) (Golubev et al. 1989).

At present no detailed transmission electron microscopic results are available on the ascospore ultrastructure of these yeasts. In addition, limited data is available on the lipid composition, especially when the sexual cycle is expressed. This is of particular interest since according to literature significant changes in lipid composition take place towards ascosporogenesis (Kock and Ratledge 1993).

Hence, it became the aim of this paper to report on the ascospore ultrastructure of these yeasts through transmission electron microscopy using glutadialdehyde and osmium tetroxide as fixatives (Van Wyk and Wingfield 1994). In addition, lipid and 3-hydroxy oxylipin turnover during transition from asexual to sexual stage were determined.

Materials and methods (See Appendix for detail)

Strains used and cultivation

Nadsonia commutata UOFS Y-2332 and *N. fulvescens* UOFS Y-0705 were obtained from the University of the Free State, Bloemfontein, South Africa and used throughout this study. These yeasts were cultivated on YM (Yeast-malt) agar medium (Wickerham 1951) for 24 and 14 days at 4 ^{O}C and 25 ^{O}C respectively to reach their respective sexual stages. The asexual stages were studied after six and four days respectively.

Transmission electron microscopy

After reaching the sexual stage as observed with the light microscope, these yeasts were immediately subjected to transmission electron microscopy preparation by chemical fixation in 3 % glutadialdehyde (primary fixative) overnight and 1 % osmium tetroxide (secondary fixative) for two hours at room temperature (Van Wyk and Wingfield 1994). This was followed by dehydration, embedding, ultramicrotomy and staining. Prepared sections were viewed using a Phillips CM 100 (The Netherlands) transmission electron microscope according to Van Wyk and Wingfield (1994).

Lipid extraction

During the asexual and sexual stages, the cells were scraped from the medium, rapidly frozen, freeze dried and weighed. Lipids were extracted using chloroform:methanol (2:1, v/v) (Smith et al. 2003) and washed (Folch et al. 1957). The organic phase was finally evaporated and the lipid material was dried in an oven at 50 ^{O}C over P_2O_5 overnight and finally weighed.

Lipid fractionation

Lipid samples were fractionated according to the method of Smith et al. (2003). In brief, the lipid samples were dissolved in a minimum volume of chloroform and applied to a column (140 mm x 20 mm) of activated silicic acid. The neutral, glyco- and phospholipid fractions were eluted from the column by applying different solvents with different polarities. The fractionated lipid samples were dried in an oven over P_2O_5 at 50 ^{O}C overnight and finally weighed. All lipid samples were stored under N_2 gas at –20 ^{O}C.

Fatty acid determination

All lipid fractions were dissolved in chloroform and transesterified with trimethylsulphonium hydroxide (TMSOH) as described by Butte (1983). The fatty acid methyl esters were analysed by gas chromatography (GC) with a flame ionisation detector and Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 ^{O}C was increased by 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C, respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

3-Hydroxy oxylipin analysis

Immunofluorescence microscopy : This was done according to the method performed by Kock et al. (1998). Briefly, cells of *N. commutata* and *N. fulvescens* at their asexual (i.e. after six and four days respectively) and sexual stages (i.e. after 24 and 14 days respectively) were subjected to primary antibody (one hour) against 3-hydroxy oxylipins as well as FITC-conjugated secondary antibody (one hour in the

dark). In order to maintain cell structure, antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Following adequate washing with a buffer, the slides with fluorescing material were photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop (Germany) microscope equipped for epifluorescence with a 50 W high-pressure mercury lamp. The stained cells were compared with appropriate controls. Corresponding light photomicrographs were also taken with the same microscope.

Gas chromatography-mass spectrometry: Cells of *N. commutata* and *N. fulvescens* at their asexual (i.e. after six and four days respectively) and sexual stages (i.e. after 24 days and 14 days respectively) were subjected to 3-hydroxy oxylipin extraction as performed by Smith et al. (2003). Briefly, cells were suspended in 100 ml of distilled water and the pH was decreased to below pH 3 by the addition of minimum volume of 3 % formic acid. Next, 3-hydroxy oxylipins were extracted from the cells using 2x volumes of ethyl acetate (200 ml). After evaporation as described (Nigam, 1987), extracts were subsequently methylated (1 hour) and trimethyl-silylated (1 hour) at room temperature. Finally, the extracts were dissolved in 400 µl chloroform:hexane (4:1 v/v) and injected for gas chromatography-mass spectrometry (GC-MS) - 3-hydroxy oxylipin analysis. A Finnigan Trace GC Ultra gas chromatograph (San Jose, California) equipped with a HP5 (60 m x 0.32 mm) fused silica capillary column, coupled to a Finnigan Trace DSQ MS, (San Jose, California) was used. Helium was used as a carrier gas at 1.0 ml/min. The initial oven temperature was 110 ^{O}C. This was increased at 5 ^{O}C/min to a final temperature of 280 ^{O}C. The GC-MS was auto-tuned for *m/z* 62 to 512. A sample volume of 1 µl was introduced at an inlet temperature of 230 ^{O}C and a split ratio of 1:60.

Chemicals used

All chemicals used were of highest purity grade and obtained from reputable dealers. Silicic acid (100 mesh) was obtained from Aldrich (Germany).

Results and discussion

The transmission electron micrograph (TEM) of *N. commutata* (Fig. 1) reveals a spheroidal ascospore with warty walls inside a mother cell (ascus). Interestingly, the TEM of *N. fulvescens* shows relatively long surface hairy structures surrounding the spheroidal ascospore inside the daughter cell (ascus) (Fig. 2). Furthermore, a thick capsule, onto which these surface ornamentations are attached, was observed in *N. fulvescens* (Fig. 2) but not in *N. commutata* (Fig. 1). The functions of this capsule and hairy structures are unknown.

During the life cycle of *N. commutata*, i.e. after six (asexual stage) and 24 days (sexual stage) of reproductive growth, the intracellular lipid content decreased from 14.6 % (w/w) to 7.8 % (w/w) (Table 1). A similar trend was also observed during the life cycle of *N. fulvescens* (Table 3). A decrease in these lipids during the life cycles of these yeasts may be attributed to their utilisation as energy source to form ascospores. These results are opposite to those reported in literature for the life cycle of *Dipodascopsis uninucleata* var. *uninucleata* (Kock and Ratledge 1993).

When studying the different lipid fractions over the life cycles of these yeasts, interesting changes were observed. Firstly, the neutral lipid (NL) fraction of *N. commutata* decreased from 82.1 % (w/w) to 72.5 % (w/w) when the sexual stage was reached (Table 1). This may be due to the utilization of the NLs present in the

vegetative cells in order to provide energy for the production of ascospores. On the other hand, the phospholipid (PL) and the glycolipid (GL) fractions increased remarkably during this part of the cycle (Table 1). This may be due to the production of additional cell walls (containing glycolipids) and membranes (containing phospholipids) during ascosporogenesis. In *N. fulvescens*, there was an increase in the NL and GL fractions with a concomitant decrease in the PL fraction over the life cycle i.e. from asexual to sexual modes of reproduction (Table 3). An increase in the NL during the sexual stage may be attributed to the production of lipid rich ascospores serving as reserve material (Pohl 1999). A decrease in the PL during sexual stage cannot be explained at this stage. The lipid composition of only ascospores should now be analysed.

Throughout the life cycle of *N. commutata* (Table 2) and *N. fulvescens* (Table 4), all the lipid fractions contained palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). *Nadsonia commutata* contained a significantly higher amount of 18:2 in its major lipid fraction (NL) compared to *N. fulvescens* in both reproductive stages. This is similar to differences reported for total fatty acid composition between these species by Golubev et al. (1989). In *N. commutata*, the percentage 18:2 increased when the sexual stage was reached i.e. from 15.2 % to 23.8 % when compared with *N. fulvescens* where small amounts of these PUFAs were detected and remained similar i.e. about 1.2 %. This cannot be explained at present. It is also interesting to note that the PUFA content (18:2 plus 18:3) decreased in the PL fractions of both yeasts i.e. from 38.1% to 31.7 % (*N. commutata*, SE < 5 %) and 45.5 % to 36.9 % (*N. fulvescens* , SE < 5 %). This may be ascribed to the necessity for membranes to be

more fluid when produced during the growth phase (Kock and Ratledge 1993). Strikingly, in both species, the 3-hydroxy oxylipins were detected using GC-MS over the two stages (i.e. asexual and sexual reproductive stages) (Table 5). Only the complete structure of a 3-OH 9:1 could be elucidated during asexual stage of *Nadsonia commutata* (Fig. 3). Each oxylipin identified has a characteristic ion at m/z 175 [$CH_3O(CO).CH_2.CHO.TMSi$] indicating a hydroxyl group at position 3 of the fatty acid (Van Dyk et al. 1991). The function of oxylipins in these yeast species is unknown. Using immunofluorescence microscopy, the presence of oxylipins could not be detected. This may be due to the fact that ascospores, which in other yeasts usually fluoresce (Kock et al. 2003), are enveloped in asci.

To conclude, the results obtained in this study, correlate with other phenotypic characters employed previously in literature to separate these species on the basis of growth conditions, mode of ascospore formation, certain physiological characters, amino acid sequences, electrophoretic patterns of enzymes, DNA relatedness and fatty acid profiles (Golubev et al. 1989; Botha and Kock 1993).

Acknowledgements

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Table 1: Changes in lipid content and composition during the asexual and sexual stages of *Nadsonia commutata* UOFS Y-2332

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
6	A	14.6	82.1	14.8	3.1
24	S	7.8	72.5	21.4	6.1

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was performed in triplicate. SE < 5 %.

Table 2: Changes in percentage fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Nadsonia commutata* UOFS Y-2332

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	18.4 (A)	9.6 (A)	30.7 (A)
	13.1 (S)	12.4 (S)	28.6 (S)
16:1	38.3 (A)	34.3 (A)	17.2 (A)
	39.8 (S)	35.8 (S)	18.3 (S)
18:0	1.3 (A)	0.6 (A)	7.5 (A)
	0.8 (S)	0.8 (S)	7.9 (S)
18:1	26.7 (A)	17.4 (A)	21.6 (A)
	22.3 (S)	19.2 (S)	21.5 (S)
18:2	15.2 (A)	31.8 (A)	21.6 (A)
	23.8 (S)	29.3 (S)	22.6 (S)
18:3	0.1 (A)	6.3 (A)	1.3 (A)
	0.8 (S)	2.4 (S)	1.1 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 3: Changes in lipid content and lipid composition during the asexual and sexual stages of *Nadsonia fulvescens* UOFS Y-0705

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
4	A	9.2	69.4	23.1	7.4
14	S	7.0	74.7	16.6	8.6

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was performed in triplicate. SE < 5 %.

Table 4: Changes in percentage fatty acid composition of the different lipid fractions during the asexual and sexual stages of *Nadsonia fulvescens* UOFS Y-0705

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	6.1(A)	12.5 (A)	20.8 (A)
	7.0 (S)	10.7 (S)	19.8 (S)
16:1	24.5 (A)	15.3 (A)	8.6 (A)
	23.9 (S)	13.9 (S)	9.1 (S)
18:0	38.6 (A)	0.7 (A)	6.4 (A)
	38.1 (S)	0.8 (S)	6.4 (S)
18:1	29.4 (A)	26.2 (A)	37.5 (A)
	29.7 (S)	37.6 (S)	33.8 (S)
18:2	1.2 (A)	43.7 (A)	24.0 (A)
	1.2 (S)	35.6 (S)	27.7 (S)
18:3	0.1 (A)	1.8 (A)	2.6 (A)
	0.1 (S)	1.3 (S)	3.3 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 5: 3-Hydroxy oxylipin turnover in *N. commutata* and *N. fulvescens*

	3-Hydroxy metabolite	
Yeast	Asexual stage	Sexual stage
N. commutata	3-OH 9:1 175; 243 [M^+ - 15]; 258 [M^+]	3-OH ?
N. fulvescens	3-OH ?	3-OH ?

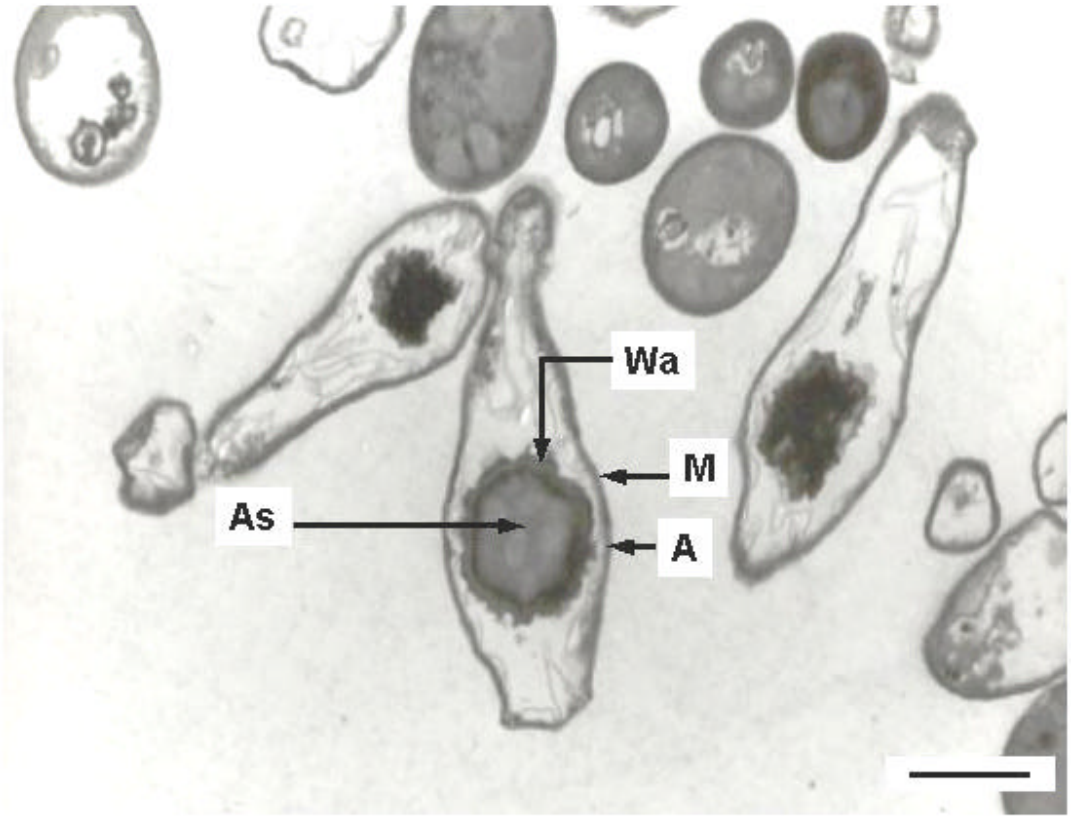

Figure 1: Transmission electron micrograph of *Nadsonia commutata* showing an ascus (A) formed by a mother cell (M) containing one spheroidal ascospore (As) with warty walls (Wa). Bar = 1 μm

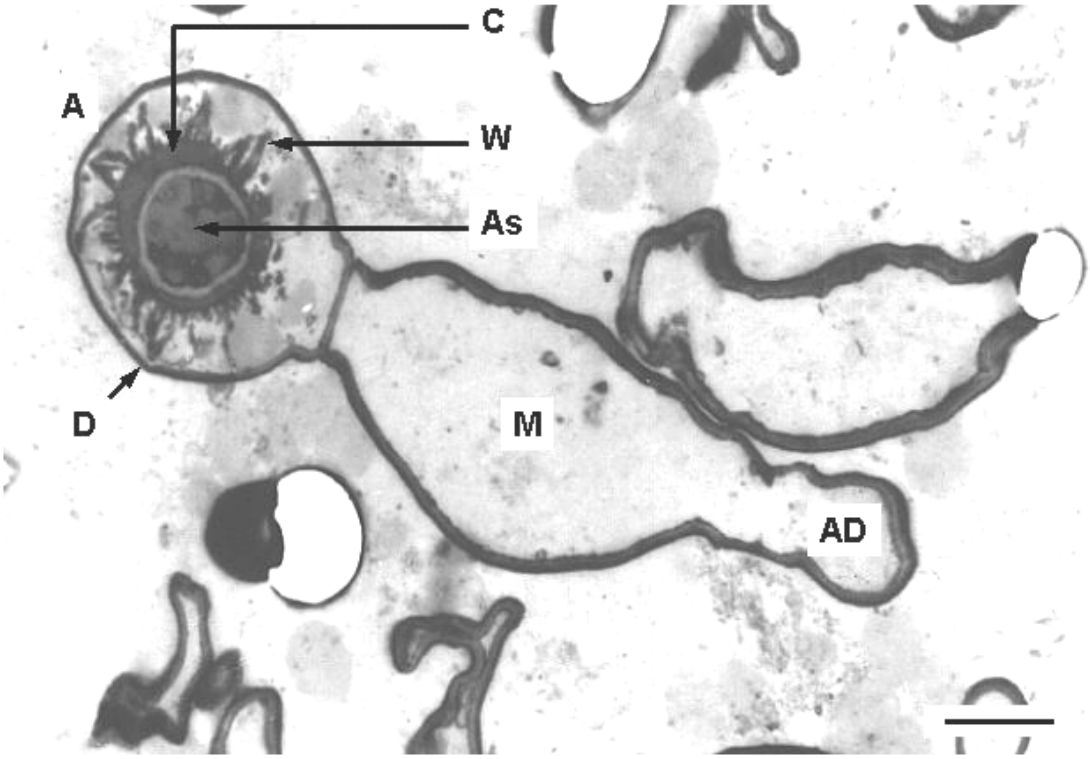

Figure 2: Transmission electron micrograph of *Nadsonia fulvescens* showing an ascus (A) formed by a daughter cell (D) and containing one spheroidal ascospore (As) with hairy structures (W). Each ascospore is surrounded by a thick capsule (C). Bar = 1 μm

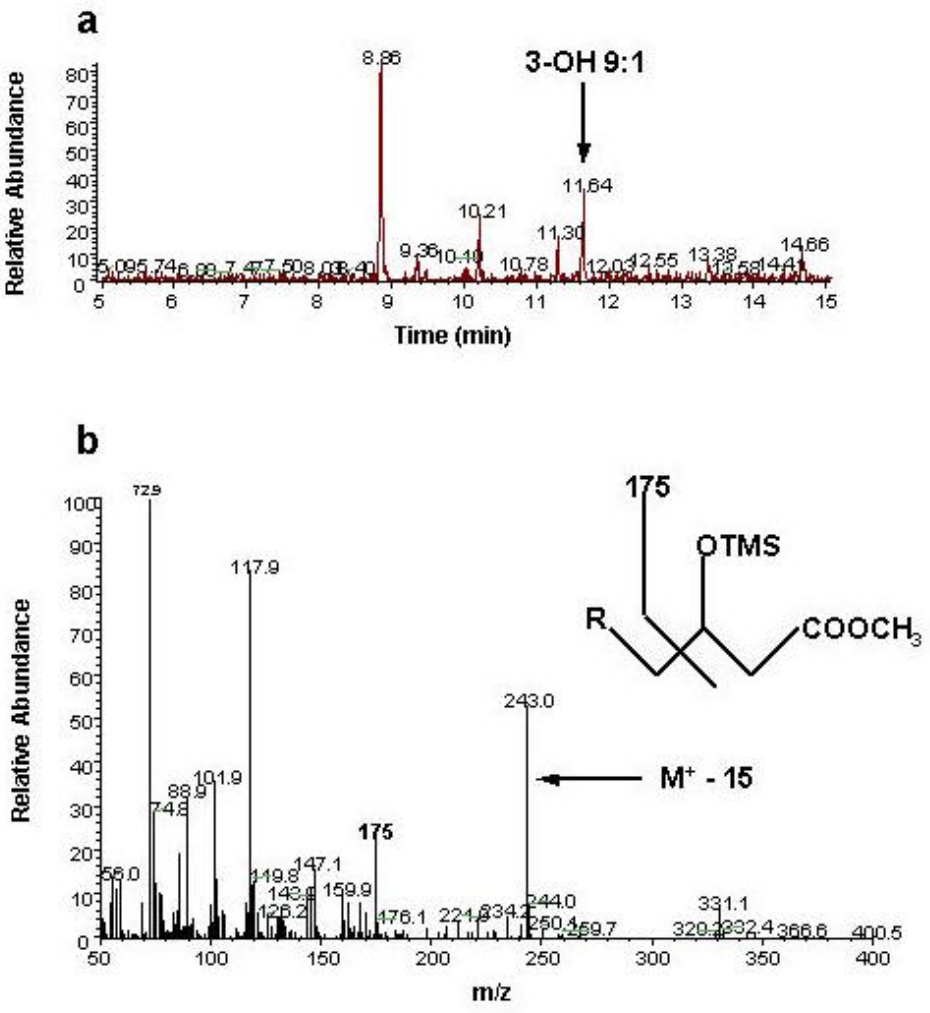

Figure 3: Ion chromatogram (a) and mass spectrum (b) of a methyl-trimethylsilylated 3-OH 9:1 in *Nadsonia commutata* detected by gas chromatography-mass spectrometry during asexual stage.

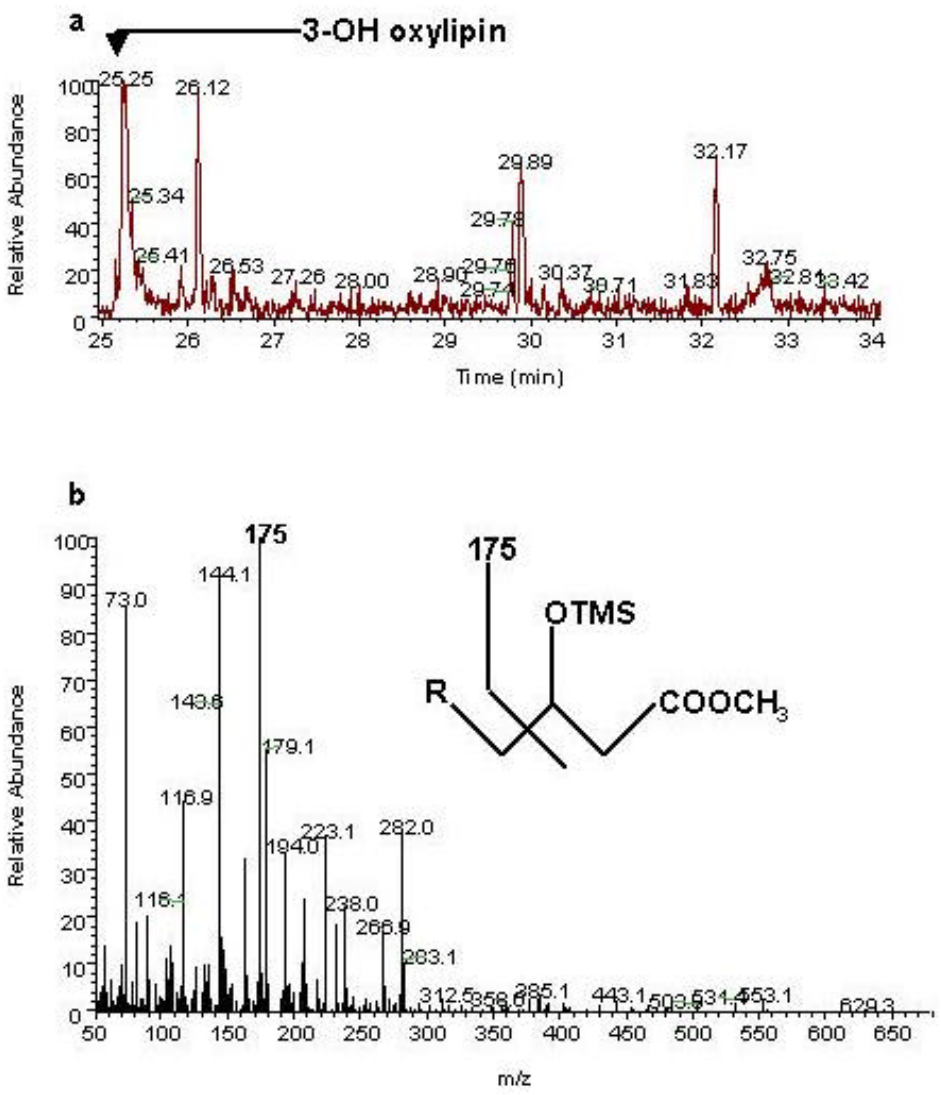

Figure 4: Ion chromatogram (a) and mass spectrum (b) of a methyl-trimethylsilylated 3-OH oxylipin in *Nadsonia commutata* detected using gas chromatography-mass spectrometry during sexual stage.

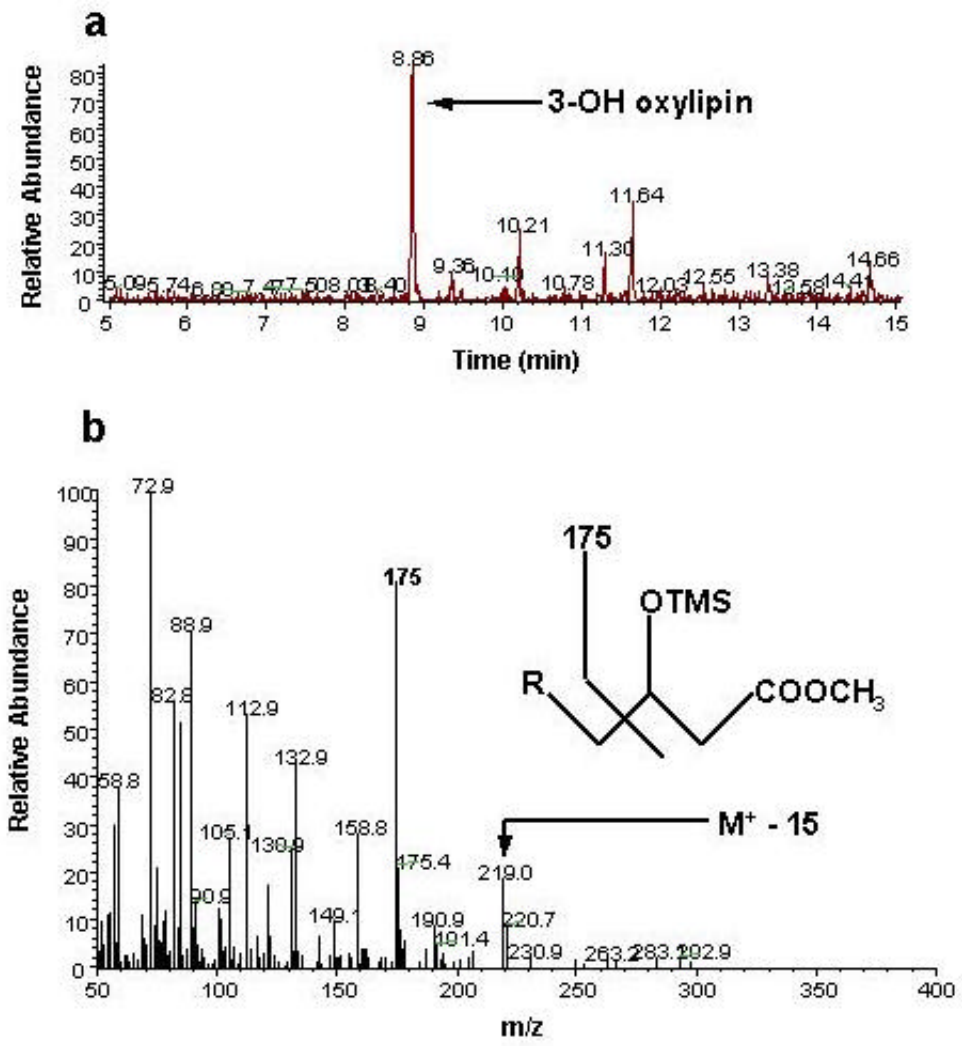

Figure 5: Ion chromatogram (a) and mass spectrum (b) of a methyl-trimethylsilylated 3-OH oxylipin in *Nadsonia fulvescens* detected using gas chromatography-mass spectrometry during asexual stage.

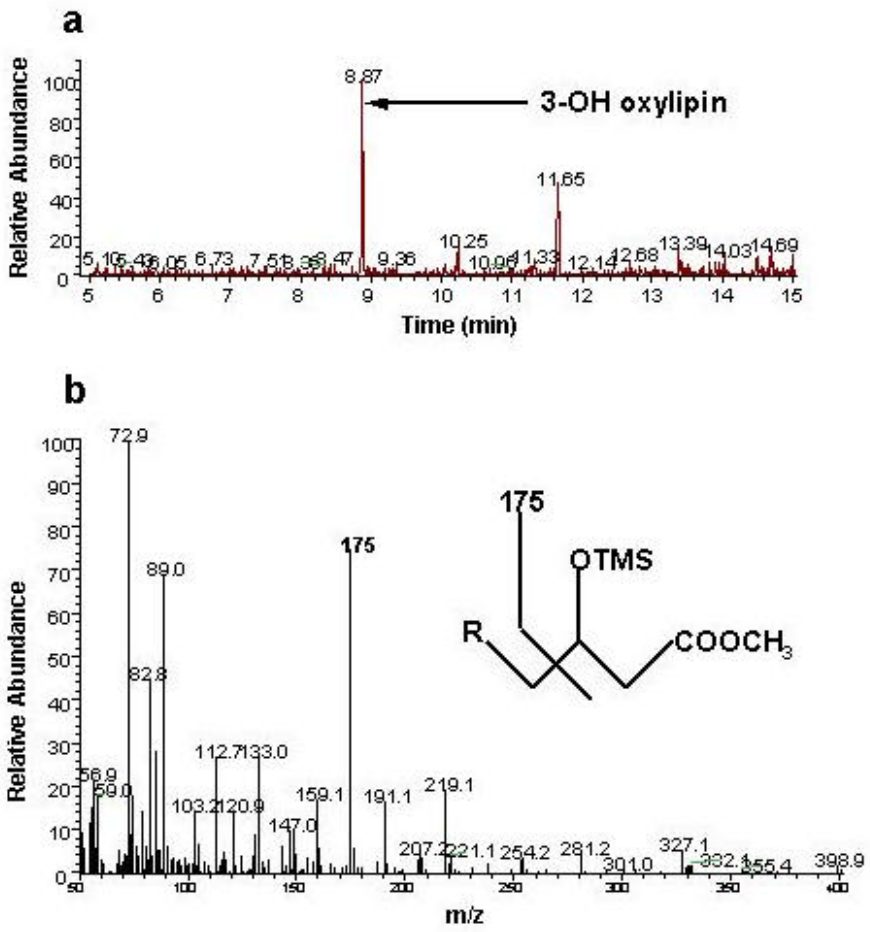

Figure 6: Ion chromatogram (a) and mass spectrum (b) of a methyl-trimethylsilylated 3-OH oxylipin in *Nadsonia fulvescens* detected using gas chromatography-mass spectrometry during sexual stage.

3.5 Family: Saccharomycopsidaceae

[Parts of this chapter have been published in Antonie van Leeuwenhoek (In Press)]

NOTE

Ascospores and lipid turnover in *Ambrosiozyma platypodis* Van der Walt

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Key words*:* *Ambrosiozyma platypodis*, double brimmed ascospore, lipid turnover, transmission electron microscopy, yeast

Abstract

Using transmission electron microscopy with glutaraldehyde and osmium tetroxide as chemical fixatives, hat-shaped ascospores with two brims each were uncovered in the yeast *Ambrosiozyma platypodis*. This is the first report on such structures. A notable increase in the neutral lipid fraction was found during the sexual stage as compared to the asexual stage. The phospholipid fraction decreased with the glycolipids remaining constant in both asexual and sexual stages.

Note

The use of transmission electron microscopy (TEM) in the study of yeast ascospore ultrastructure is well reported (Yarrow 1998, Kock et al. 1999; Smith et al. 2000, 2003; Bareetseng et al. 2004). With this method as well as light microscopy a wide variety of ascospore shapes with different nano-scale ornamentations has been reported (Yarrow 1998). Some are spherical with an equatorial ledge, others are characterized by a bowl and a brim, others again resemble walnuts, spindles with whip-like appendages, needles, hairy and warty balls and many more.

In this paper, we report for the first time on the ultrastructure of a peculiar hat-shaped ascospore, characterized by two brims, in the yeast *Ambrosiozyma platypodis* as well as the lipid composition of these spores.

Ambrosiozyma platypodis UOFS Y-0857 was obtained from the University of the Free State, Bloemfontein. This yeast was cultivated on YM (Yeast-malt) agar (Wickerham 1951) at 25 ^{0}C for 10 days until sexual stage was reached. During this stage, cells were subjected to transmission electron microscopic (TEM) studies and lipid analysis.

During their sexual stage, cells were chemically fixed according to the method performed by Kock et al. (1999). In short, cells were first fixed in a buffered aqueous solution of 3 % glutadialdehyde overnight followed by fixation in 1 % osmium tetroxide for four hours. Cells were dehydrated using a series of acetone concentrations, embedded in epoxy and finally, ultramicrotomy was performed. Ultra

thin sections containing the material were stained using uranyl acetate and lead citrate and then observed using a Phillips CM 100 transmission electron microscope.

During asexual (three days) and sexual stages (10 days), cells of this yeast were frozen, freeze-dried and weighed. Total lipid extraction was carried out by suspending the cells in a mixture of chloroform:methanol (2:1, v/v) overnight (Smith et al. 2003). The organic solvent containing total lipids, was washed twice with distilled water (Folch et al. 1957) and finally evaporated. Total lipids were transferred to pre-weighed vials using minimal volume of diethyl ether. After evaporation, lipid samples were dried in an oven at 50 ^{O}C over P_2O_5 over night and finally weighed. These lipids were subsequently fractionated into neutral, phospho- and gylcolipid fractions using trichloroethane, acetone and methanol respectively, over activated silicic acid column (140mm x 20mm) chromatography (Smith et al. 2003). After evaporation, the fractionated lipid samples were dissolved in diethylether and transferred to pre-weighed vials. The fractionated lipid fractions were finally dried in an oven over P_2O_5 at 50 ^{O}C overnight, weighed and finally stored under N_2 gas at – 20 ^{O}C.

Fatty acid determination of the fractionated lipid samples was done according to Butte (1983). In short, fractionated lipid samples were first dissolved in a minimal volume of chloroform and transesterified with trimethylsulphonium hydroxide (TMSOH) (Butte 1983). These were eventually subjected to gas chromatography analyses (GC) with a flame ionization detector and Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 ^{O}C was increased by 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, increased to 240 ^{O}C at

the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C, respectively. Nitrogen was used as a carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards. All chemicals used were of highest purity grade and obtained from reputable dealers.

According to literature (Smith 1998), *Ambrosiozyma platypodis* produces hat-shaped ascospores characterized by a bowl and distinctive ledge or brim as observed under the light microscope. No mention was made of the intriguing double brims attached to these ascospores i.e. one short and blunt (approximately 150 nm x 300 nm) and the other much longer and thinner (approximately 50 nm x 500 nm). These hat-shaped ascospores are released from a spheroidal ascus by an unknown mechanism to produce a well defined cluster of attached ascospores. Our results show that these ascospores are grouped in such a way that the longer brims attach to the bowl of the ascospores and also to each other.

What is the function of these surface ornamentations? This is of interest especially since it has been shown that nano-scale hooks and 3-hydroxy oxylipins on surfaces of reniform ascospores of *Dipodascopsis uninucleata* var. *uninucleata* are responsible for rotational release of ascospores through narrow asci openings and re-assembly in ordered clusters outside the ascus (Kock et al. 1999).

The lipid turnover of *Ambrosiozyma platypodis* was investigated in order to determine the lipid composition when this yeast enters sexual stage (Table 1). The percentage intracellular total lipid content of this yeast remained more or less constant during both phases (Table 1). Opposite results were reported during the intracellular total

lipid turnover of *Dipodascus ambrosiae* (Smith et al. 2003). Notable changes were evident when the lipid composition was investigated in both the asexual and sexual stages. The NL fraction increased significantly from 28.9 % (w/w) to 40.0 % (w/w) when *Ambrosiozyma platypodis* entered sexual stage (Table 1). Similar findings were reported for *Dipodascopsis uninucleata* var. *uninucleata* (Kock & Ratledge 1993) and *Dipodascus ambrosiae* during their NL turnover (Smith et al. 2003). The significant increase in the NL fraction in the sexual stage compared to the asexual stage, may be attributed to the production of lipid rich double-brimmed hat-shaped ascospores by *Ambrosiozyma platypodis* .

The fatty acid composition was also analysed using gas chromatography during the asexual and sexual stages of this yeast. The lipid fractions analysed comprised of palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Table 2). Although almost no change in 16:0 was found in the NL fraction when the two stages were compared, this fatty acid experienced a change in the PL and GL fractions, where it decreased slightly and increased respectively (Table 2). Palmitoleic acid (16:1) decreased drastically in the PL fraction during sexual stage and increased in the NL fraction during sexual stage. Stearic acid (18:0) increased significantly in the NL fraction during the sexual stage. Oleic acid (18:1) increased in the PL fraction during ascosporogenesis. The abundant percentage of the polyunsaturated fatty acid 18:2 in NL and PL fractions increased during the sexual stage, while it decreased in the GL fraction. The opposite trend was found for the percentage 18:3, i.e. it decreased in the NL and PL fractions during sexual stage (Table 2). In future, the presence of especially 3-hydroxy oxylipins should be mapped and chemically analysed.

Acknowledgements

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Table 1: Changes in lipid content and lipid composition during the asexual and sexual stages of *Ambrosiozyma platypodis* UOFS Y-0857

Time (days)	Stages of life cycle	% Lipid content (w/w)	% Lipid fraction (w/w)		
			NL	PL	GL
3	A	2.0	28.9	41.2	29.9
10	S	1.9	40.0	39.4	29.6

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

Table 2: Changes in percentage fatty acid composition of the different lipid fractions during asexual and sexual stages of *Ambrosiozyma platypodis* UOFS Y-0857

Fatty acid	Lipid fractions		
	NL	PL	GL
16:0	14.6 (A)	21.4 (A)	24.3 (A)
	14.6 (S)	19.7 (S)	28.7 (S)
16:1	4.2 (A)	10.1 (A)	14.9 (A)
	6.3 (S)	5.7 (S)	13.9 (S)
18:0	2.4 (A)	4.5 (A)	13.0 (A)
	4.2 (S)	4.7 (S)	11.6 (S)
18:1	21.8 (A)	17.5 (A)	29.3 (A)
	22.3 (S)	22.7 (S)	30.0 (S)
18:2	38.1 (A)	30.0 (A)	15.7 (A)
	40.0 (S)	33.5 (S)	13.4 (S)
18:3	19.0 (A)	16.5 (A)	2.8 (A)
	17.0 (S)	13.6 (S)	2.4 (S)

A = Asexual; S = Sexual; NL = Neutral lipid; PL = Phospholipid; GL = Glycolipid, 16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid. Similar trends were observed when this experiment was done in triplicate. SE < 5 %.

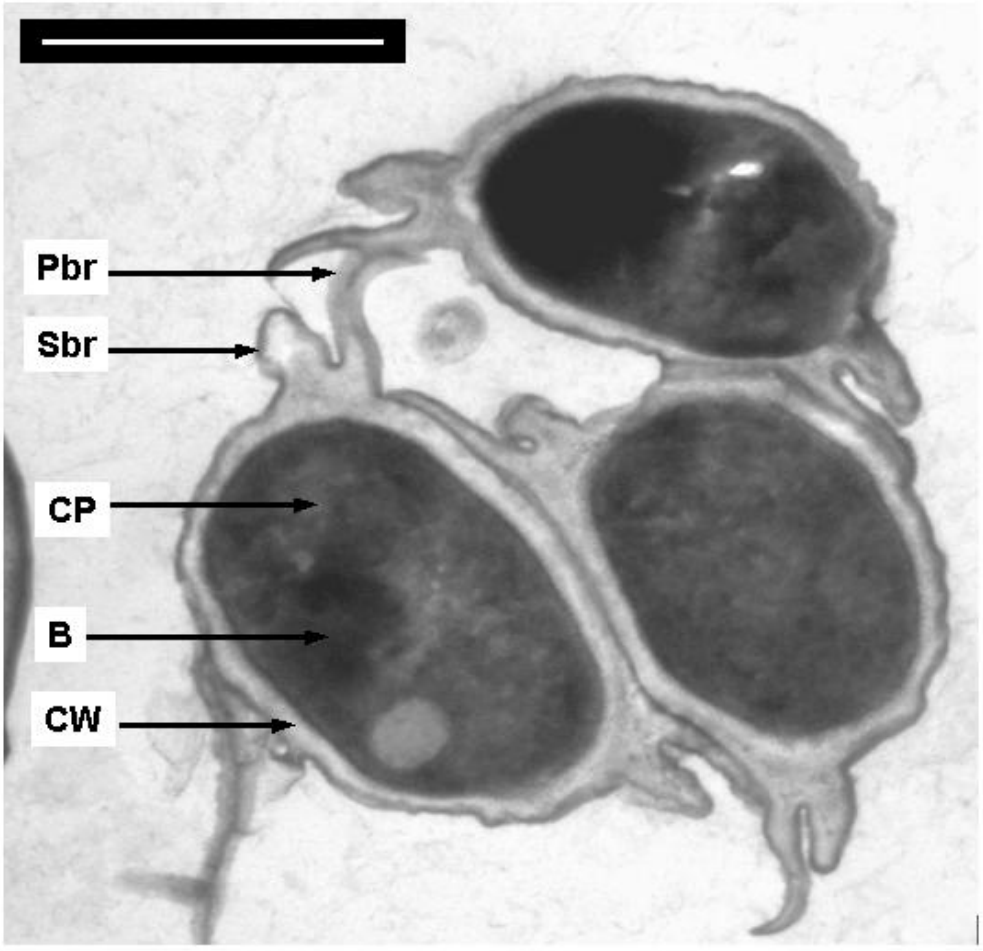

Fig. 1. Transmission electron micrograph of a cluster of released hat-shaped ascospores by *Ambrosiozyma platypodis*. Ascospores are characterized by a bowl (B), a relatively thick cell wall (CW), cytoplasm (CP) as well as a longer and thinner primary brim (Pbr) compared to a shorter and thicker secondary brim (Sbr). Bar = 1 µm

3.6 Yeast-like fungus: *Ascoidea*

(Accepted for publication in Canadian Journal of Microbiology)

NOTE

Ascospores and 3-hydroxy oxylipins in *Ascoidea africana* Batra & Francke-Grosmann

Abstract: Immunofluorescence microscopy exposed the presence of novel 3-hydroxy oxylipins on the surfaces of aggregated hat-shaped ascospores of *Ascoidea africana*. These were confirmed by gas chromatography-mass spectrometry analysis. Only the complete structure of a novel 3-hydroxy 10:1 could be determined.

Key words: Ascoidea africana, ascospore, 3-hydroxy oxylipins, immunofluorescence microscopy, yeast.

Note

Bioprospecting studies using immunofluorescence microscopy as well as gas chromatography-mass spectrometry have uncovered various 3-hydroxy oxylipins in fungi. These are oxygenated fatty acids of various chain lengths and desaturation and containing a hydroxyl group at the third carbon as counted from the carboxylic

group e.g. 3-hydroxy 8:0, 3-hydroxy 9:1, 3-hydroxy 10:0, 3-hydroxy 14:2, 3-hydroxy 14:3, 3-hydroxy 20:3, 3-hydroxy 20:4, etc. (Kock et al. 2003). These compounds have been found in yeasts representing the families Lipomycetaceae and Dipodascaceae as well as some filamentous fungi. These oxylipins were also found to be of biotechnological value since they show potent biological activities in signal transduction processes of human neutrophils and tumour cells (Nigam et al. 1996). In addition, it was recently reported that 3-hydroxy oxylipins probably also serve as a prehistoric lubricant that covers ascospore surfaces and assists in ascospore release from asci (Kock et al. 2004).

As a further expansion of this bioprospecting project, we report the presence of various 3-hydroxy oxylipins, associated with the surfaces of released and aggregated hat-shaped ascospores *of Ascoidea africana* using immunofluorescence microscopy and gas chromatography-mass spectrometry analysis (GC-MS). In addition, the ascospore ultrastructure of this yeast is reported.

Ascoidea africana UOFS Y-1217 T was obtained from the University of the Free State and investigated throughout this study. This yeast was maintained on YM (yeast-malt) agar medium (Wickerham 1951) at 25 ^{O}C until sexual stage was reached. Immunofluorescence studies, GC-MS and transmission electron microscopy (TEM), were then performed. All experiments were done in triplicate.

Antibodies used for immunofluorescence microscopy were raised in a rabbit and characterized according to its titer, sensitivity and specificity (Kock et al. 1998). Cells in their sexual stage were prepared for immunofluorescence studies as

described by Kock et al. (1998). Briefly, cells were suspended in a buffer in 2 mL plastic tubes and treated with primary antibody. This was incubated for one hour at room temperature and immediately washed with a phosphate buffer. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) was added and incubated in the dark for one hour at room temperature. Followed by adequate washing, fluorescing material was prepared on microscope slides and photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop (Germany) microscope equipped for epifluorescence with a 50 W high-pressure mercury lamp (excitation filter: Blue, 460 nm). The fluorescing cells were compared with appropriate controls. Corresponding light photomicrographs were also taken with the same microscope.

Transmission electron microscopy was performed according to the method of Van Wyk and Wingfield (1994). Cells in the sexual phase showing asci with ascospores under the light microscope, were immediately fixed overnight in 3 % aqueous glutadialdehyde in 0.1 M phosphate buffer and then in 1 % osmium tetroxide in 0.1 M phosphate buffer for two hours at room temperature. After washing with the same buffer, the material was dehydrated through a graded acetone series (i.e. of different concentrations) and then the material was embedded in epoxy and set in a silicone mould (14 mm long x 6 mm wide x 4 mm deep). After polymerisation at 70 ^{O}C for eight hours, the material was cut on a LKB ultramicrotome with a glass knife to obtain ultra thin sections containing the material, which were finally recovered on a Formvar coated grid. These sections were subsequently stained in saturated solution of uranyl acetate (10 min) and lead citrate (five min). Transmission electron micrographs were taken with a Phillips CM 100, (The Netherlands) (TEM) (Van Wyk and Wingfield, 1994).

Cells in their sexual stage were subjected to 3-hydroxy oxylipin extraction. This was done by suspending the cells in 100 mL distilled water after which the pH was decreased to below pH 4 by the addition of 3 % formic acid. Lipids from cells were extracted with two volumes of ethyl acetate (200 mL) and the organic solvent was evaporated (Van Dyk et al. 1991). Extracted lipids were methylated and silylated, dissolved in a mixture of chloroform:hexane (4:1, v/v) and eventually analyzed by GC-MS (Venter et al. 1997). A Finnigan Trace GC Ultra gas chromatograph (San Jose, California) equipped with a HP5 (60 m x 0.32 mm) fused silica capillary column, coupled to a Finnigan Trace DSQ MS, (San Jose, California) was used. Helium was used as a carrier gas at 1.0 $mL.min^{-1}$. The initial oven temperature was 110 ^{O}C. This was increased at 5 $^{O}C.min^{-1}$ to a final temperature of 280 ^{O}C. The GC-MS was auto-tuned for *m/z* 62 to 512. A sample volume of 1 µL was introduced at an inlet temperature of 230 ^{O}C and a split ratio of 1:60. All chemicals used in this study were of highest purity grade and obtained from reputable dealers.

When the sexual stage of *Ascoidea africana* was treated with an antibody specific to 3-hydroxy oxylipins and visualized with FITC-conjugated secondary antibody and immunofluorescence microscopy, the hat-shaped ascospores inside the ellipsoidal ascus fluoresced (Fig. 1a,b). In addition, released aggregating ascospores outside the ascus also fluoresced (Fig. 2a,b) indicating the presence of oxylipins on the surfaces of these hat-shaped ascospores i.e. consisting of a bole and brim (Fig. 3).

The identity of the 3-hydroxy oxylipins associated with the hat-shaped ascospores of *Ascoidea africana* was determined by GC-MS (Fig. 4). Here, three

peaks with mass spectra characterized by (m/z) 175 [$CH_3O(CO).CH_2.CHO.TMSi$] were identified, indicating a 3-hydroxyl group at carbon 3 (Van Dyk et al. 1991). One of these 3-hydroxy oxylipins, as determined from the mass spectrum, was characterized by an M^+-15 of 257 implying a mother ion (M^+) of 272. We conclude this oxylipin to be a 3-hydroxy 10:1, which is the first report of such a structure in yeast (Fig 4b). However, the chemical structure of the other 3-hydroxy oxylipins could not be determined (Figs 5 and 6). Further chemical studies are underway to elucidate these structures and the function of these oxylipins in yeast.

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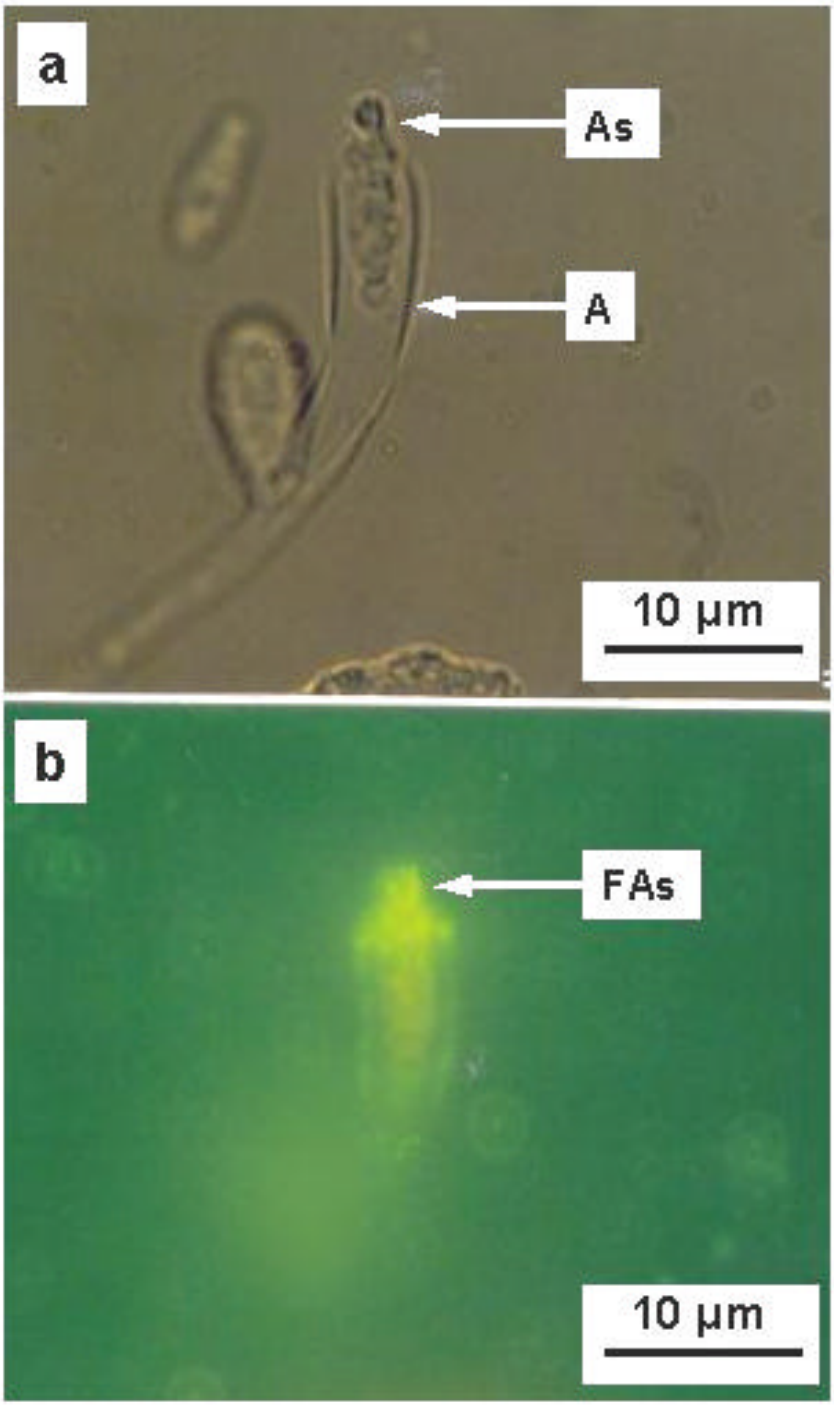


Figure 1: Light and immunofluorescence micrographs of an ascus (A) containing hat-shaped ascospores (As) of *Ascoidea africana*. (a) Light micrograph of ascus (A) containing hat-shaped ascospores (As). (b) Corresponding immunofluorescence micrograph of ascus (A) containing fluorescing hat-shaped ascospores (FAs).

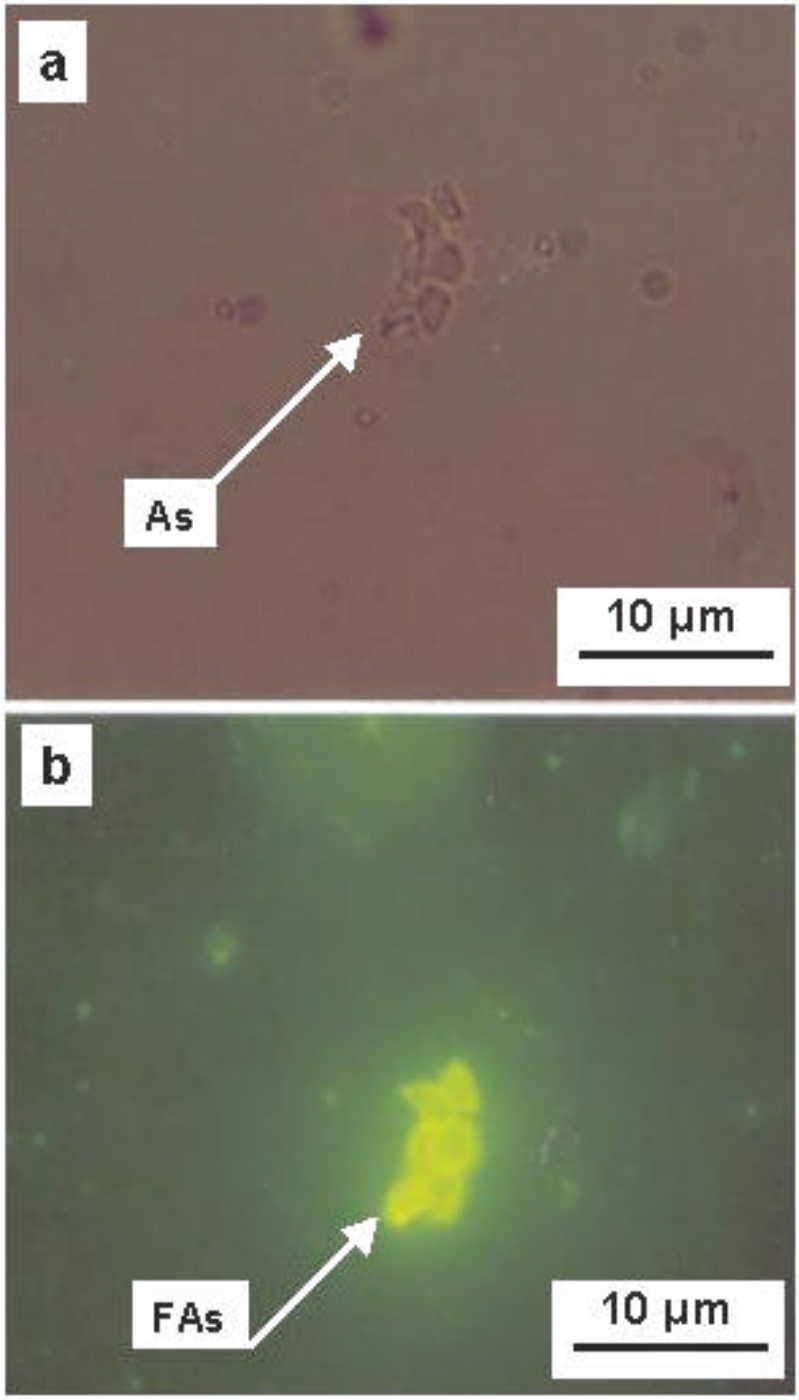


Figure 2: Light and immunofluorescence micrographs of liberated hat-shaped ascospores (As) of *Ascoidea africana*. (a) Light micrograph of liberated hat-shaped ascospores (As). (b) Corresponding immunofluorescence micrograph of liberated fluorescing hat-shaped ascospores (FAs).

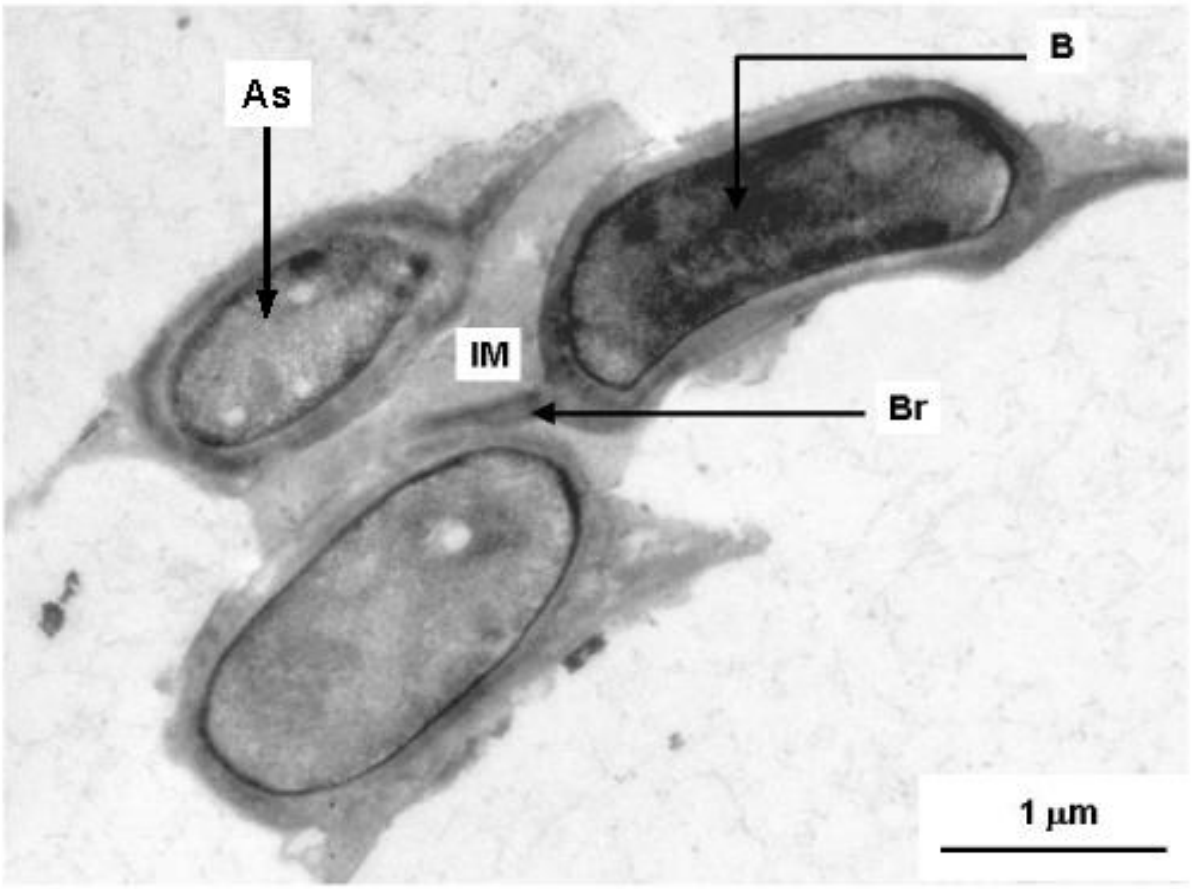
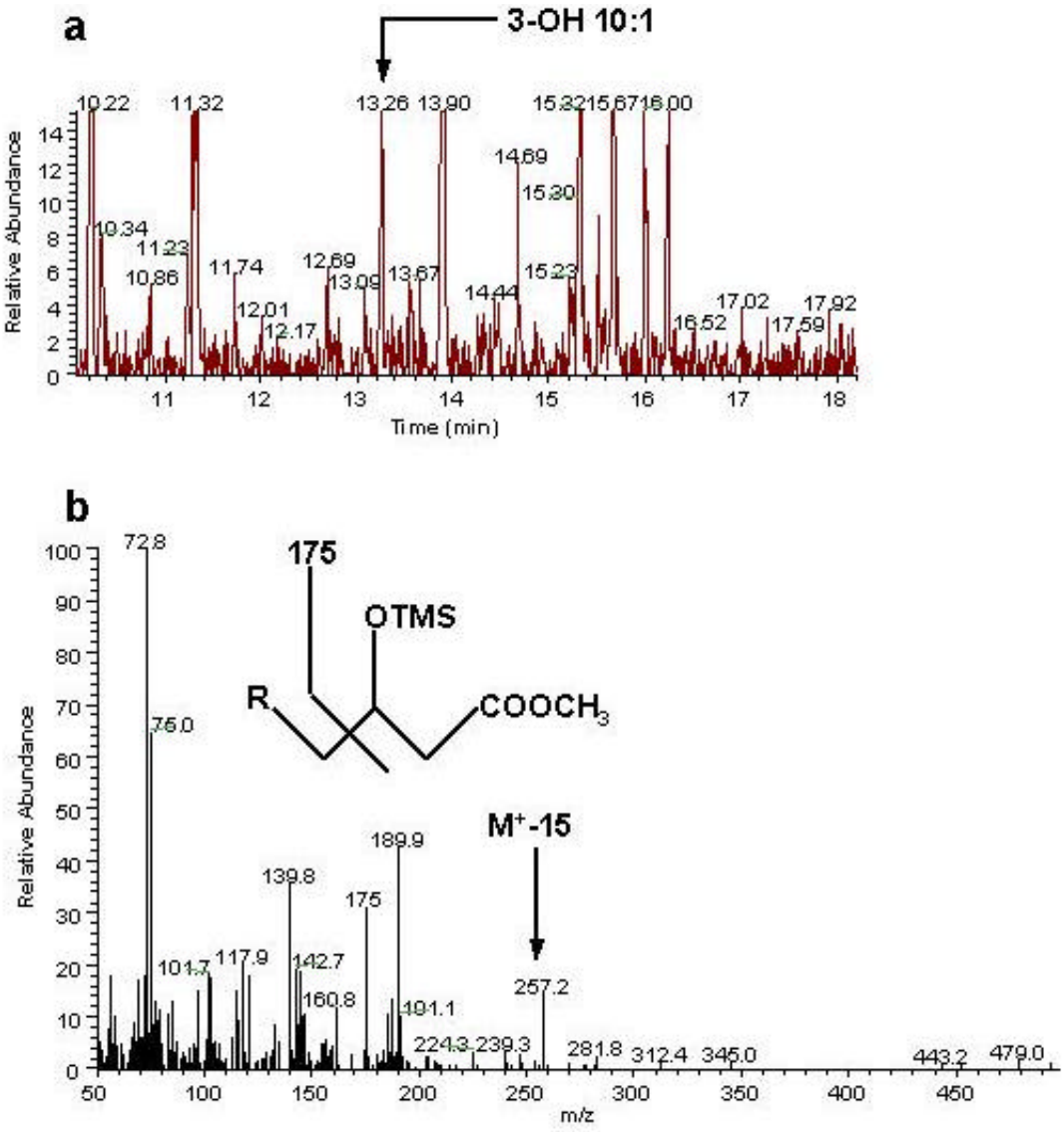
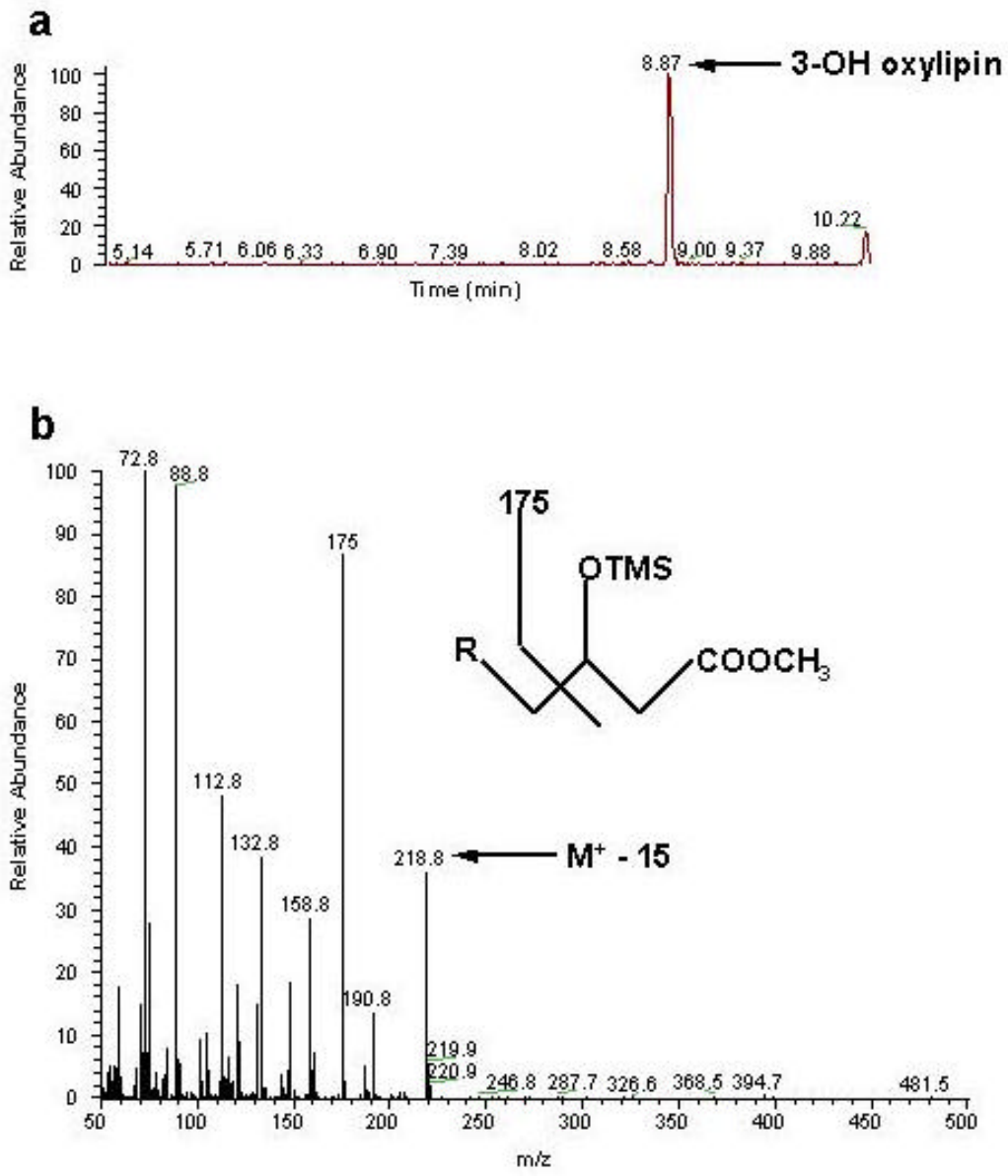


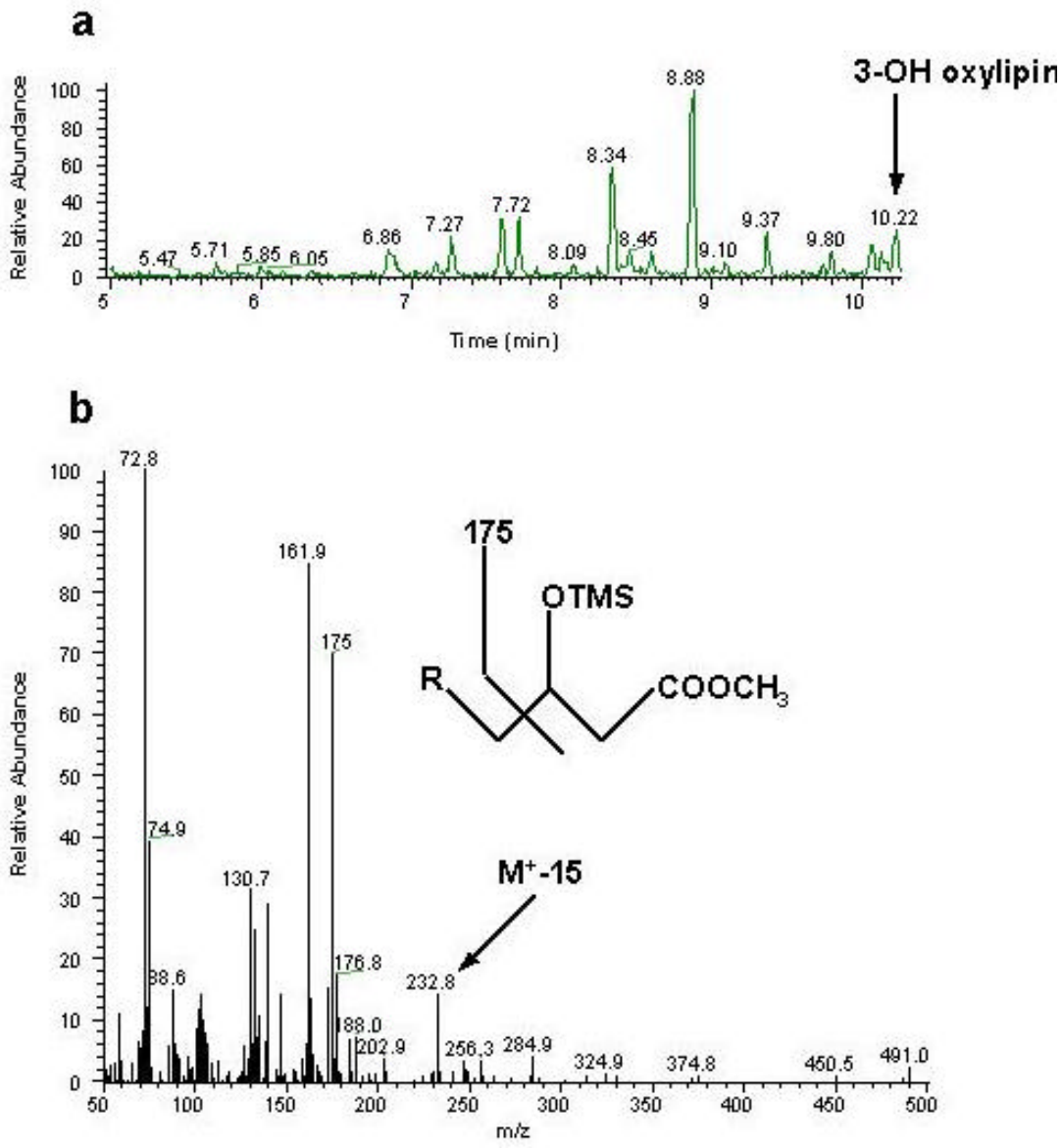
Figure 3: Transmission electron micrograph of hat-shaped ascospores (As) of *Ascoidea africana*, showing a bole (B) and brim (Br). An interspore matrix (IM) between the ascospores is visible.



Figures 4: Total ion (a) and mass spectrum (b) of methyl-trimethylsilylated 3-hydroxy (OH) oxylipin 10:1 on ascospores from *Ascoidea africana*.



Figures 5: Total ion (a) and mass spectrum (b) of methyl-trimethylsilylated 3-hydroxy (OH) oxylipin on ascospores from *Ascoidea africana*.



Figures 6: Total ion (a) and mass spectrum (b) of methyl-trimethylsilylated 3-hydroxy (OH) oxylipin on ascospores from *Ascoidea africana*.

APPENDIX

Lipid extraction (Performed by Candidate – A.S. Bareetseng)

During asexual and sexual stages, yeast cells were scraped from YM agar medium and immediately frozen at -70^{O}C overnight. The frozen cells were then freeze-dried overnight and weighed. The pre-weighed freeze dried cells were crushed in a mortar using pestle and suspended in 150 ml chloroform:methanol (2:1 v/v) overnight (Kock & Ratledge, 1993). The cells were filtered using Whatman no. 1 filter paper and the organic phase collected and added to a separating flask. The organic phase was subsequently washed twice with 25 ml distilled water (Folch *et al.,* 1957) and left to clear in fume cabinet. Thereafter, the organic phase was added to round bottom flasks and finally evaporated under vacuum using a Heidolph VV 2011 rotator evaporator. Lipid samples were transferred to clean pre-weighed vials using a minimal volume of diethyl ether and left in a fume cabinet until the solvent has evaporated. The intracellular lipid samples were dried in an oven at 50^{O}C over phosphorus pentoxide (P_2O_5) overnight and finally weighed.

Lipid fractionation (Performed by Candidate – A.S. Bareetseng)

The intracellular lipid samples were dissolved in a minimal volume of chloroform and applied with a glass pipette to a clean column (140 mm x 20 mm) of activated (i.e. heated at 110^{O}C overnight) silicic acid. Next, appropriate volumes of trichloroethane (150 ml), acetone (100 ml) and methanol (100 ml) were applied to a column to elute

neutral-, glyco- and phospholipid fractions respectively (Kock & Ratledge, 1993; Smith et al. 2003). These fractions were collected in respective round bottom flasks. The organic phases were evaporated under vacuum using a Heidolph VV 2011 rotator evaporator. These fractions were transferred to their respective pre-weighed vials using a minimal volume of diethyl ether and left in a fume cabinet to evaporate. Finally, the fractionated lipid fractions were dried in an oven over P_2O_5 at 50^{O}C and weighed. All lipid fractions were then stored under a blanket of N_2 at –20^{O}C (Kock & Ratledge, 1993).

Fatty acid determination

The fractionated lipid samples were subjected to gas chromatography (GC) to determine their fatty acid composition. This was done by dissolving each lipid fraction in a minimal volume of chloroform. Then 200µl of each sample was transferred to a GC-vial where it was transesterified by the addition of 200µl trimethylsulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed with a GC equipped with a flame ionisation detector and Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 ^{O}C was increased from 3 ^{O}C/min to 225 ^{O}C and, following a 10 min isothermal period, then increased to 240 ^{O}C at the same rate. The inlet and detector temperatures were 170 ^{O}C and 250 ^{O}C respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

Synthesis of 3-hydroxy oxylipins and antibodies (Performed by Prof. S. Nigam and his group in Germany)

The 3-hydroxy oxylipin (3-HETE) was synthesised for antibody production (Kock *et al.,* 1998). The synthetic strategy for the production of 3*R*- and 3*S*-hydroxy oxylipins (i.e. 3-hydroxy- 5,8,11,14-eicosatetraenoic acid or 3-HETE), involved a convergent approach, coupling a chiral aldehyde with Wittig salt. These were derived from 2-deoxy-D-ribose and arachidonic acid (AA), respectively. The antibodies against chemically synthesised *3R*-HETE were raised in a rabbit according to this approach: The carboxyl group of *3R*-HETE was conjugated to amino groups of bovine serum albumin (BSA) through a N-succinimidyl ester bond. One mg of this conjugated protein was emulsified in an equal volume of Freund's complete adjuvant for the first injection or incomplete adjuvant for later injections. Every second week, for three months, the protein emulsion was injected subcutaneously on the back of a white female New Zealand docile rabbit. After treatment, the blood was collected from the carotid artery of the rabbit, left for two hours at room temperature and then centrifuged at a speed of 1200 x g at 4^{O}C for 20 minutes. The sera were finally purified by Biogenes in Berlin (Kock *et al.,* 1998).

Preparation and characterisation of antibody (Performed by Prof. S. Nigam and his group in Germany)

The antibodies raised in the rabbit against chemically synthesised *3R*-HETE, were characterised by determining titre, sensitivity and specificity. Since the radio- labeled [^{14}C]-3-HETE is not commercially available, the tracer was biologically prepared by transforming the radio-labeled arachidonic acid [^{14}C]-AA (sp. Act. 52 mCi/mmol) to radio-labeled [^{14}C]-3-HETE using the yeast *Dipodascopsis uninucleata*. The radio-

labeled [^{14}C]-3-HETE was eventually purified using radio-HPLC. The titer of the antibody was determined and gave a binding of approximately 30% radio-labeled 3-HETE at a dilution of 1:100 in the absence of unlabeled 3-HETE. The minimum detectable amount of *3R*-HETE was 30pmol as determined by 10% displacement of the radioactivity by unlabelled 3-HETE from the zero point of approximately maximum binding of labeled 3-HETE. Finally, the specificity of the antibody was analysed using various structurally related compounds to determine the possible cross-reactions with the antibody. The antibody gave 0.5% cross-reactivity with 5-, 12- or 15-HETE while cross-reactivity was only observed with 3-hydroxy oxylipins of different chain lengths and desaturation. However, immunoreactivity was not detected using other commercially available hydroxylated fatty acids and free fatty acids when immufluorescence microscopy was employed. Hence, in our study the immunoreactivity indicates solely the presence of 3-hydroxy oxylipins.

Immunofluorescence microscopy (Performed by Candidate – A.S. Bareetseng)
Immunofluorescence of yeast cells was performed as described (Kock et *al.,* 1998) and includes treatment with primary antibody (30 µl: one hour at room temperature) against 3-hydroxy oxylipins as well as FITC-conjugated secondary antibody (30 µl: one hour at room temperature in the dark). In order to maintain cell 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 (Germany) microscope equipped for epifluorescence with a 50 W high-pressure mercury lamp (excitation filter: Blue, 460 nm). The stained cells were compared with appropriate controls. Corresponding light photomicrographs were also taken with the same microscope.

Confocal laser scanning microscopy (Performed by Candidate – A.S. Bareetseng) Yeast cells at their sexual stage were stained with 10% Orange G solution or 3-OH oxylipin antibody on a microscope slide for 5 minutes at room temperature (Yarrow, 1998). The slides were finally visualized with Nikon TE 2000, confocal laser scanning microscope (CLSM), Japan.

Gas chromatography-mass spectrometry (GC-MS) Performed by Candidate – A.S. Bareetseng)

Cells in their sexual stage were subjected to 3-hydroxy oxylipin extraction. This was done by suspending the cells in 100 ml distilled water after which the pH was decreased to below pH 4 by the addition of 3% formic acid. Lipids from cells were extracted with two volumes of ethyl acetate (200 ml) and the organic solvent was evaporated (Van Dyk *et al.,* 1991). Extracted lipids were methylated and silylated, dissolved in a mixture of chloroform:hexane (4:1, v/v) and eventually analyzed by GC-MS (Venter et al. 1997). A Finnigan Trace GC Ultra gas chromatograph (San Jose, California) equipped with a HP5 (60 m x 0.32 mm) fused silica capillary column, coupled to a Finnigan Trace DSQ MS, (San Jose, California) was used. Helium was used as a carrier gas at 1.0 ml/min. The initial oven temperature was 110^{O}C. This was increased at 5^{O}C/min to a final temperature of 280^{O}C. The GC-MS was auto-tuned for *m/z* 62 to 512. A sample volume of 1µl was introduced at an inlet temperature of 230^{O}C and a split ratio of 1:60. All chemicals used in this study were of highest purity grade and obtained from reputable dealers.

Electron microscopy Performed by Candidate – A.S. Bareetseng)

Scanning and transmission electron microscopy were performed according to the method of Van Wyk and Wingfield (1994). Yeasts cells in sexual stage were immediately fixed overnight in 3% aqueous glutaraldehyde in 0.1 M phosphate buffer and then in 1% osmium tetroxide in 0.1 M phosphate buffer for two hours at room temperature. After rinsing twice with the same buffer, the material was dehydrated through a graded ethanol and/or acetone series (50%, 70%, 95%, 2 x 100% 30 min per step). The cells were eventually dried using critical point drying (Polaron E 3000 C.P.D, UK) for scanning electron microscope and/or embedded in epoxy and set in a silicone mould (14 mm long x 6 mm wide x 4 mm deep) for scanning and/or transmission electron microscopic observation respectively. The cells for scanning electron microscopy were also coated with 150 nm gold (Biorad E 5000 Sputter Coater, UK) to make them electrically conductive during scanning microscopic observations. The cells, which were embedded in epoxy (Spurr, 1969) and set in a silicone mould, were polymerised at $70^{O}C$ in an oven for eight hours. After polymerization, the epoxy block containing the cells was cut on a LKB ultramicrotome with a glass knife to obtain ultra thin sections containing the cells. The sections were finally recovered on 200 mesh Formvar coated grid and stained in a saturated solution of uranyl acetate for 10 minutes and lead citrate for five minutes for visualisation under the transmission electron microscope. Scanning electron micrographs were taken with a Joel 6400 WINSEM (Japan) Scanning electron microscope. Transmission electron micrographs were taken with a Phillips CM 100, (The Netherlands) (TEM) (Van Wyk and Wingfield, 1994).

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SUMMARY

Some yeasts produce sexual spores (ascospores) in a variety of shapes and surface ornamentations. These intriguing structures have hitherto been used only in yeast classification. In this study the likely primary function of ascospore shape and ornamentations with associated lipids in water-driven movement as aiding the dispersal of ascospores from enclosed containers (asci), is proposed. This interpretation might find application in nano-, aero- and hydro-technologies with the re-scaling of these structures. Through sexual reproduction, some yeasts produce microscopic containers (asci) that enclose ascospores of many different shapes and various nano-scale surface ornamentations. Some spores are spherical with an equatorial ledge (like the planet Saturn), or resemble hats with a bole and brim, while others look like corkscrews, walnuts, spindles with whip-like appendages, needles, and hairy or warty balls. Until now, these structures were used to classify yeasts and little thought was apparently given to their possible functional role. From literature and microscopic observations, it was found that the yeasts *Dipodascopsis uninucleata* and *Dipodascus* have evolved sophisticated means that enable the dispersal of oxylipin-coated spherical and bean-shaped spores from bottle-shaped containers (asci) without inverting or shaking them. Here, spores are pushed by turgor pressure towards the narrow opening and then ejected. Studies of *Dipodascopsis* suggest that oxylipin-coated, interlocked hooked ridges on the surfaces and stretching across the length of bean- to ellipsoidal-shaped spores are

responsible for the alignment of the latter. Here, spores inside the container are positioned side-by-side in a column of linked clusters with elongated sides attached by interlocking hooked ridges in gear-like fashion and orientated mainly with one end towards the opening. It was concluded that hooked ridges form turbine-like structures at both ends, causing propeller-like rotation when the spores are pushed by water pressure towards the ascus opening. This rotational movement loosens the spores (by the unlocking the hooked-ridges) near the container neck, which is necessary for sliding past each other for eventual release. Eventually, spores are released individually from the bottle-shaped ascus while rotating at about 1200 rpm at approximately 110 length replacements per second. With some species of the genus *Dipodascus*, compressible oxylipin-coated sheathed surface structures and not gears are used to separate and loosen spherical spores in a similar bottle-shaped container before individual release under turgor pressure. These spores simply slide past each other when pressed towards the opening. It is presumed that more complex mechanics are needed to allow the effective release of bean- to ellipsoidal-shaped ascospores compared to spherical sheathed ascospores, for which alignment and rotation are unnecessary. Using gas chromatography-mass spectrometry, it was discovered that a saturated 3-OH 14:0 (mass fragments:175 [$CH_3O(CO)$-CH_2-CHO-TMSi]; 330 [M^+]; 315 [M^+-15]) is produced by the yeast *Eremothecium ashbyii*. In order to map the oxylipin's location in the yeast, antibodies (against these oxylipins) and immunofluorescence microscopy on cells in sexual mode was employed. The oxylipin was present as part of a V-shaped structure on sickle-shaped spores. With the aid of confocal laser scanning microscopy to observe cells treated with antibody and fluorescine (FITC anti-rabbit IgG), it was concluded that the hydrophobic V-shaped structure was present as a mirror image on both sides at the blunt end of an

otherwise hydrophilic spore as indicated by differential ascospore staining. Scanning electron microscopy showed this structure to be fin-like protuberances. Next, the function of these fin-like structures and ascospore shape was addressed. Using microscopy, it was discovered that spores are sometimes forced through the ascus with the spiked tip rupturing the ascus wall. Water pressure caused a boomerang movement when the blunt end is pushed forward with the spike leading the way in a circular motion. This happens only when micron streams of water move across the fins from the blunt end towards the tip of the spore. It is believed that this part of the study has only scratched the surface of water-driven ascospore movement in yeasts on a micrometer scale and that the mechanical implications of many spore shapes with a large number of different hydroxy oxylipin-lubricated, nano-scale surface ornamentations await similar explanation and elaboration. Why did some yeasts evolve peculiar spore movement with the beneficial consequence, so far as we can see, to escape from closed or partially closed containers? Of course, this should be important from a survival point of view since without this ability, yeasts will probably not be able to disperse properly. It is believed that if appropriate ultrastructural studies (using glutadialdehyde and osmium tetroxide as fixatives) are conducted on yeasts aimed at exposing ascospore surface ornamentations and not merely membrane structure, conducted in the past, clues can be gained to reveal the mechanics behind the motion of nano-sized particles in fluids. Consequently a further aim of this study became to assess ascospore structure (using above ultrastructural method) especially nano-scale ornamentations with associated lipids especially oxylipins in various unrelated yeasts. These were obtained for the yeasts *Eremothecium sinecaudum* (ascospores corkscrew-shaped and coated with oxylipins), *Dipodascopsis uninucleata* var. *wickerhamii* (smooth surfaces without

oxylipins), *Lipomyces kononenkoae* (smooth ascospore surface with lipid sacs), *L. tetrasporus* (ridged ascospore surface with lipid sacs), *Saturnispora saitoi* (Saturn-shaped ascospores covered with oxylipins), *Ascoidea africana* (hat-shaped ascospores covered with oxylipins), *Ambrosiozyma platypodis* (double brimmed hat-shaped ascospores), *Nadsonia commutata* (ascospore surface warty; cells contain oxylipins) and *N. fulvescens* (ascospore surface hairy-like; cells contain oxylipins). Interesting patterns regarding lipid turnover (i.e. total-, neutral-, phospho-, glycolipids and associated fatty acids) were found when asexual and sexual stages of above yeasts are compared.

OPSOMMING

Sommige giste produseer geslagtelike spore (askospore) met ‘n verskeidenheid vorms en oppervlakornamentasies. Hierdie fassinerende strukture is tot dusver slegs vir klassifikasie gebruik. In hierdie studie word die waarskynlike primêre funksie van askospoorvorm en –ornamentasie met geassosieerde lipiede in water gedrewe beweging voorgestel as hulpmiddele in die verspreiding van askospore vanuit geslote houers (aski). Hierdie interpretasie mag, met geskikte skaalveranderinge van strukture, toepassings vind in nano-, aero- en hidrotegnologie. Sommige giste produseer, d.m.v. geslagtelike voortplanting, mikroskopiese houers (aski) wat askospore met verskillende vorms en nanoskaalornamentasies omhul. Sommige spore is rond met ‘n ekwatoriale lys (soos die planeet Saturnus), of lyk soos hoede met ‘n bol en rand, terwyl ander weer soos kurktrekkers, okkerneute, spoele met sweepagtige aanhangsels, naalde en harige of vratagtige balle lyk. Tot op hede is hierdie strukture gebruik om giste te klassifiseer en min aandag is blykbaar gegee aan hul moontlike funksie. Uit literatuur en mikroskopiese waarnemings is gevind dat die giste, *Dipodascopsis uninucleata* en *Dipodascus* gesofistikeerde metodes ontwikkel het wat die verspreiding van oksilipienbedekte, ronde en boontjievormige spore vanuit bottelvormige houers (aski) moontlik maak sonder om hulle om te keer of te skud. Spore word d.m.v. turgordruk na die nou opening gestoot en dan vrygestel. Studies op *Dipodascopsis* stel voor dat oksilipienbedekte, gekoppelde haakagtige riwwe wat oor die lengte van die oppervlakte van die boontjievormig tot

elipsoïdiale spore strek, verantwoordelik is vir die oriëntasie van die spore. Spore in die houer word sy aan sy in ‘n kolom van aaneengeskakelde groepe geposisioneer met hulle langkante geheg deur gekoppelde vratagtige riwwe. Die afleiding is gemaak dat die haakagtige riwwe turbine-agtige strukture aan beide kante tot vorm. Dit veroorsaak skroef-agtige rotasies wanneer die spore deur waterdruk na die askusopening gedruk word. Hierdie rotasie maak die spore los van mekaar (d.m.v. die ontsluiting van die haakagtige riwwe) naby die houer se nek. Dit is noodsaaklik om spore toe te laat om verby mekaar te gly vir uiteindelike vrystelling. Uiteindelik word die spore een vir een uit die bottelvormige askus vrygestel terwyl hulle teen ongeveer 1200 rpm met ongeveer 110 lengteverplasings per sekonde beweeg. In sommige spesies van die genus *Dipodascus* , word oksilipienbedekte, saampersbare skedes en nie ratte nie, gebruik om ronde spore los van mekaar te maak in soortgelyke bottelvormige houers voor vrystelling d.m.v turgordruk. Hierdie spore gly eenvoudig verby mekaar wanneer hulle na die opening gedruk word. Dit word aanvaar dat meer komplekse meganismes nodig is vir die effektiewe vrystelling van boontjievormige tot ellipsoïdale askospore in vergelyking met ronde spore met skede waar rigting en rotasie nie nodig is nie. Met behulp van gas chromatografie-massa spektrometrie is ontdek dat ‘n versadigde 3-OH 14:0 (massa fragmente:175 [$CH_3O(CO)$-CH_2-CHO-TMSi]; 330 [M^+]; 315 [M^+-15]) deur die gis *Eremothecium ashbyii* geproduseer word. Om die oksilipien se posisie in die gis te bepaal, is teenliggaampies (teen die oksilipien) en immunofluoresensie mikroskopie op selle in die geslagtelike fase gebruik. Die oksilipien was teenwoordig as deel van ‘n V-vormige struktuur op sekelvormige spore. Met behulp van konfokale laser skandeer mikroskopie op selle behandel met die teenliggaampies en fluoresien (FITC anti-konyn IgG), is afgelei dat die hidrofobiese V-vormige struktuur teenwoordig is as ‘n

spieëlbeeld aan albei kante van die stomppunt van ‘n andersins hidrofiliese spoor. Skandeerelektronmikroskopie het aangetoon dat die struktuur ‘n vinagtige uitsteekstel is. Vervolgens is die funksie van hierdie vinagtige structure bepaal. Mikroskopie het aangetoon dat die spore soms deur die askus geforseer word met die skerppunt wat die askuswand breek. Waterdruk veroorsaak ‘n boomerangbeweging met die skerppunt voor, wanneer die stomppunt voorentoe gedruk word. Dit gebeur slegs wanneer mikronstrome water oor die vinne beweeg vanaf die stomppunt na die skerppunt van die spoor. Hierdie deel van die studie is bloot die punt van die ysberg wat watergedrewe askospoorbeweging in giste op ‘n mikronmeterskaal betref. Die meganiese implikasies van baie spoorvorme met verskillende hidroksie oksilipiengesmeerde, nanoskaal oppervlakornamentasies moet op soortgelyke wyse bestudeer en verduidelik word. Hoekom het sommige giste hierdie spoorbeweging, met die voordelige gevolg van vrystelling in geslote of gedeeltelik geslote houers ontwikkel? Dit is natuurlik belangrik uit ‘n oorlewingsoogpunt, aangesien giste sonder hierdie vermoë nie behoorlik sal kan versprei nie. Indien deeglike ultrastrukturele studies (met glutadialdehied en osmiumtetroksied as fikseermiddels) op giste gedoen word met die doel om askospoorstruktuur en –ornamentasie te ondersoek, en nie bloot membraanstruktuur nie, kan leidrade gekry word aangaande die meganika agter die beweging van nanogrootte deeltjies in vloeistowwe. Gevolglik het dit ‘n verdere doel van die studie geword om askospoorstruktuur, veral nanoskaal ornamentasie en geassosieerde lipiede, in verskeie onverwandte giste te evalueer. Die volgende resultate is verkry vir die giste *Eremothecium sinecaudum* (askospore kurktrekkervormig en bedek met oksilipiene), *Dipodascopsis uninucleata* var. *wickerhamii* (gladde oppervlaktes sonder oksilipiene), *Lipomyces kononenkoae* (gladde askospore met lipiedsakke), *L.*

tetrasporus (geriffelde askospore met lipiedsakke), *Saturnispora saitoi* (Saturnusvormige askospore bedek met oksilipiene), *Ascoidea africana* (hoedvormige askospore bedek met oksilipiene), *Ambrosiozyma platypodis* (hoedvormige askospore met dubbel randte), *Nadsonia commutata* (vratagtige askospooroppervlakte, selle bevat oksilipiene) en *N. fulvescens* (harige askospooroppervlakte, selle bevat oksilipiene). Interessante patrone aangaande lipiedomset (d.i. totale-, neutrale-, fosfo-, glikolipiede en geassosieerde vetsure) is gevind toe geslagtelike en ongeslgtelike fases van die giste vergelyk is.

Key words: Ascospores; confocal laser scanning microscopy; electron microscopy; fatty acids; gas chromatography-mass spectrometry; 3-hydroxy oxylipins; immunofluorescence microscopy; lipid turnover; ultrastructure; yeasts

Sleutelwoorde: Askospore; konfokale laser skandeer mikroskopie; elektron mikroskopie; vetsure; gas kromatografie-massa spektrometrie; 3-hidroksie oksielipiene; immunofluoresensie mikroskopie, lipied omskakeling; ultrastruktuur; giste