The influence of oxidized oils on fungal growth and lipid utilization

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This thesis is dedicated to my daughter Paballo Thatohatsi Rethabile Malebo.

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CHAPTER 1

Literature review

1.1 Motivation

Various types of oils are used today to fry food with. These include edible oils such as sunflower oil, soybean oil, palm oil, etc. During frying, usually at temperatures between 190 ºC and 200 ºC, various changes occur in the oil. These include the removal of antioxidants from the frying oil (Frankel 1998), hydrolysis, oxidation and polymerization (Warner 2002; Lalas 2008). These reactions are responsible for a variety of physical and chemical changes that may be observed in the oil during frying and that may eventually lead to the formation of oil breakdown products which include polar compounds (PCs) and polymerized triglycerides (PTGs) (Kock 2001).

According to South African regulations, oil that contains 16% and more PTGs and 25% and more PCs is considered harmful to human health. Oils containing breakdown products above these limits have been shown to cause cancer and diarrhoea in humans and animals (STOA Report 2000). In addition, a possible link suggesting these oils have adverse effects on HIV/AIDS progression especially in poor communities, has been reported (Kock 2006). Other studies have implicated reactive oxygen species (ROS), also present in these oils, as inhibitors of mitochondrial function which may also affect health negatively (Costantini et al. 2000; Lin et al. 2008).

Little is however known regarding the effect of oxidized oil on microorganisms although some studies have been carried out applying used frying oil as a growth substrate. These studies have focused on isolation of lipase-secreting bacteria (Haba et al. 1999) and also on the production of bio-surfactants by *Pseudomonas* spp. (Mercade et al. 1993; Fleurackers

2006). Other studies have indicated that repeatedly used frying oils are mutagenic towards *Salmonella* (Taylor et al. 1983; Saleh et al. 1986; Hageman et al. 1988).

In fungi, PTGs from oxidized oils were shown to have the ability to decrease riboflavin production by *Ashbya* (*Eremothecium*) *gossypii* (Park and Ming 2003). Furthermore, studies on oleaginous fungi concentrated on high value lipid production in *Mucor circinelloides* as well as citric acid production by *Yarrowia lipolytica* from fresh and oxidized sunflower oil (Venter et al. 2004; Joseph et al. 2005). In this study the oleaginous fungi *Cryptococcus curvatus* and *M. circinelloides* were grown on saturated palm oil with different levels of oxidative breakdown. The influence of these oils was then assessed on fungal growth, oil utilization and morphology. In addition the anti-mitochondrial activity of oxidized palm oil breakdown products on these fungi was assessed. These results were also compared to known anti-mitochondrial antifungals.

1.2 Fats and oils

Edible fats and oils (hereafter referred to as oils) are produced by plants, animals and microorganisms as bulk storage materials (Frankel 1998). They are lipids that contain different types of long chain fatty acids and are insoluble in water and soluble in organic solvents such as chloroform, alcohols and ethers (Ratledge and Wilkinson 1988a). They can be saturated (containing fatty acids without double bonds), mono-unsaturated (containing fatty acids with one double bond) or polyunsaturated (containing fatty acids with two or more double bonds) (Ratledge and Wilkinson 1988b). The number of double bonds present in fatty acids of oils determines their stability during frying i.e. the more unsaturated the oil, the less

stable it will be during frying. Long-chain fatty acids are characterized by a carbon chain with a methyl group at the ω-end and a carboxylic acid group at the α-end (Ratledge and Wilkinson 1988b). They are mainly found esterified to a glycerol molecule, as part of glycolipids, phospholipids and neutral lipids. The neutral lipids consist of monoglycerides, diglycerides, triglycerides as well as long-chain free fatty acids (Ratledge 1994).

The fatty acid composition of some major sources of oil is shown in Table 1 (Shukla 1994). Here, oils are grouped according to the predominance of saturated, mono- and polyunsaturated fatty acids. The majority of edible plant crop oils contain large amounts of mono- and polyunsaturated fatty acids whereas animal fats consist mainly of saturated oils (e.g. butterfat, beef tallow and lard). In this thesis, emphasis will be placed on saturated frying oils i.e. those that are used for the frying of various foods and derived from the plant *Elaeis guineensis*.

1.3 Types of edible oils

A number of plants are at present utilized for the production of edible oils. Forty different oilseed crops have been described, however, there are mainly 10 edible oil crops of commercial value. From this number, seven of these oil crops are seed crops and the remaining three are tree crops. Edible oil derived from oilseeds accounts for about 80% of the world's edible oil production. The remaining 20% is animal fat which include fish oils (Metzger and Bornscheuer 2006). Approximately 80% of these oils are for human consumption, *ca.* 6% is for animal feed and *ca.* 14% for the oleochemical industry.

Table 1. Fatty acid composition of the major oil sources (% w/w) (Shukla 1994).

Oil/ fat	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	20:0	16:1	18:1	20:1	18:2	18:3	S	M	P
Saturated																	
Beef tallow						3	24	19		4	43		3	1	46	47	4
Butterfat	4	2	1	3	3	11	27	12		2	29		2	1	63	31	3
Cocoa butter							26	35	1	35			3		62	35	3
Coconut oil		1	8	6	47	18	9	3			6		2		92	6	2
Lard						2	26	14		3	44	1	10		42	48	10
Palm-kernel oil		1	3	4	48	16	8	3			15		2		83	15	2
Palm oil						1	45	4			40		10		50	40	10
Mono-unsaturated																	
Olive oil							13	3	1	1	71		10	1	17	72	11
Peanut oil							11	2	1		48	2	32		14	50	32
Rapeseed oil							4	2			62		22	10	6	62	32
Poly-unsaturated																	
Corn oil							11	2			28		58	1	13	28	59
Cottonseed oil						1	22	3		1	19		54	1	26	20	55
Safflower oil							7	2			13		78		9	13	78
Soybean oil							11	4			24		54	7	15	24	61
Sunflower oil							7	5			19		68	1	12	19	69

4:0=butyric acid; 6:0=caproic acid; 8:0= caprylic acid; 10:0=capric acid; 12:0=dodecanoic acid; 14:0=myristic acid; 16:0=palmitic acid; 16:1=palmitoleic acid; 18:0=stearic acid; 18:1=oleic acid; 18:2=linoleic acid; 18:3=alpha-linolenic acid; 20:0=arachidic acid; 20:1=eicosenoic acid; S= total saturated fatty acids; M= total monounsaturated fatty acids; P= total polyunsaturated fatty acids.

However, these ratios are expected to change through the increasing use of oils for oleochemical purposes, especially for biodiesel production (Gunstone 2005; Gunstone et al. 2007). In South Africa the local supply of oils is unable to meet the demand due to limited crop sizes. Sunflower oil is mainly used in the food industry for frying and is consumed in large quantities followed by palm oil. Imported palm oil accounts for around 50% of imported oil followed by sunflower oil. The consumption of palm oil is at 26% and is second to first placed sunflower oil with consumption levels of 37% (Minal et al. 2003).

Plant crop oils are mainly preferred for frying because they are considered healthy in comparison to saturated oils of animal origin. However as a result of heat treatment, oils are broken down easily. Here unsaturated oils are most susceptible followed by saturated oils which are more stable. Therefore, when choosing oil for frying, thermal and oxidative stability is important, as these not only affect the quality of the oil but of the product as well (Matthäus 2007). Palm oil has been shown as one of the most stable oils (Minal et al. 2003). Studies by Fauziah and co-workers (2000) showed that palm oil was even more stable than high oleic sunflower oil during frying.

1.4 Palm oil

Palm oil is an edible vegetable oil which originates from the fruit *Elaeis guineensis* (which plays a major role in the commercial manufacture of palm oil) and *Elaeis oleifera* or *Elaeis odora* which constitute a minor part (Berger 2001; Matthäus 2007). It has been used for preparing food for over five thousand years (Mukherjee and Mitra 2009). It consists mainly of triglycerides of palmitic (16:0) and oleic (18:1) acids and is a semisolid at room

temperature. It has a characteristic fatty acid composition quite different from other edible oils; it contains almost equal amounts of saturated and unsaturated fatty acids. Palm oil has been previously classified as highly saturated oil and it was believed that its consumption raised blood cholesterol (Ebong et al. 1999). However, there is scientific evidence that suggests that palm oil raises blood cholesterol only when an excess of dietary cholesterol is presented in the diet (Mukherjee and Mitra 2009).

Palm oil is also different from other vegetable oils as it has a high amount of tocopherols and tocotrienols as well as beta-carotene and vitamin A which are potent antioxidants (Ebong et al. 1999). This increased content of natural antioxidants and moderate content of polyunsaturated fatty acids, gives palm oil more oxidative stability at high temperatures (Minal et al. 2005) which makes it a preferred oil for frying. It is also used for producing margarines and shortenings without applying hydrogenation, a process that may transform *cis* double bonds in unsaturated fatty acids to the unhealthy *trans* form. The presence of *trans* fatty acids in dietary oils have been reported to increase levels of total cholesterol (low density lipoprotein cholesterol and apolipoprotein A) and also decrease high density lipoprotein cholesterol concentrations (Ebong et al. 1999).

Consumption of palm oil can be in a fresh state and/or at various levels of oxidation. The beneficial role of fresh palm oil on animals and humans has been shown in various studies (Ebong et al. 1999). These include reduction in the risk of arterial thrombosis and atherosclerosis, inhibition of cholesterol biosynthesis and platelet aggregation and reduction in blood pressure. On the other hand, when used in an oxidized state, palm oil has been shown to affect physiological and biochemical functions of the body. It has been revealed

that consumption of oxidized palm oil induces reproductive toxicity and organ toxicity specifically of the lungs, kidneys, heart and liver (Ebong et al. 1999; Mukherjee and Mitra 2009). Furthermore, repeatedly heated palm oil can cause oxidative damage thereby prompting atherosclerosis (Adam et al. 2008).

When compared to other commercial oils such as soybean, groundnut, sunflower and rapeseed oil, palm oil performed most satisfactorily during frying (Matthäus 2007). In addition, palm oil demonstrated a lower increase of polymers, viscosity and foam formation than most of the other oils. The benefits of using palm oil for frying overshadow its shortcomings. In the harvest year 2004-2005, the world supply of palm oil increased to equal that of soybean oil at 33 million tonnes (Gunstone and Harwood 2007) making it one of the most popular oils used for frying.

1.5 Production of oxidized oils during frying

During frying (Fig. 1), food is immersed in frying oil at temperatures as high as 200 ºC, leading to several deteriorative chemical processes such as; hydrolysis (caused by moisture from the food), oxidation (due to oxygen entering the oil from the surface of the frying pan) and thermal alteration caused by high temperature (Warner 2002; Lalas 2008). These reactions are responsible for a variety of physical and chemical changes that may be observed in the oil during frying (Wanasundara and Shahidi 1995).

One of the first reactions that take place during frying is hydrolysis. Moisture from the food hydrolyzes the ester bonds of the triglycerides producing mono- and diglycerides, free fatty acids and glycerol. Glycerol is volatile at 150 - 200 ºC and is partially lost by evaporation. Free fatty acids and mono-glycerides emulsify the oil and this enhances oil hydrolysis (Wanasundara and Shahidi 1995; Choe and Min 2007). Heavy metals from the food and added spices, cause the free fatty acids to transform into soap compounds and this leads to foaming on the surface of the frying oil. Increased foaming raises the level of aeration and this result in an increase in oxidation (Kock and Coetzee 1998). Thermal oxidation follows the mechanism of autoxidation however the thermal oxidation rate is faster than autoxidation (Choe and Min 2007). Chemical reactions that occur during frying result in the formation of hydroperoxides. These are the primary products of lipid oxidation and are very unstable at frying temperatures. Their breakdown is also catalysed by the presence of transition metals such as iron and copper that enter foods via water or spices used in food preparation.

Copper and iron metals aid radical formation in oil by removing hydrogen protons from oil to form alkyl radicals by oxidation-reduction mechanisms of metals even at low temperatures (Choe and Min 2007). Oxidation reactions take place at an increased speed at high temperatures (Anelich 2000; Choe and Min 2007). It has also been reported that prolonged exposure of oils to high temperatures in the presence of oxygen and moisture leads to polymerization (Gurr and Harwood 1991; Chow and Gupta 1994; Stauffer 1996). The rate of hydroperoxide formation and decomposition has also been shown to increase during thermal oxidation. Breakdown of hydroperoxides is a complicated process that produces large amounts of materials that may have adverse biological effects. This breakdown proceeds by homolytic cleavage of hydroperoxides to form alkoxy radicals.

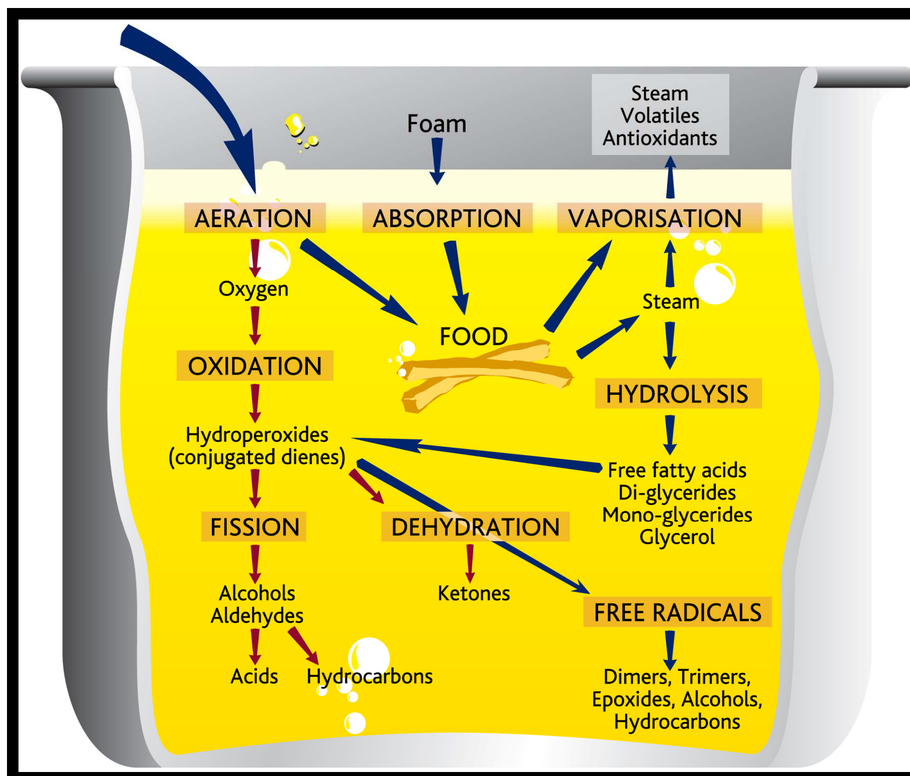

Fig. 1 Changes that occur during deep oil frying (Fritsch 1981; Frankel 1998)

These radicals can undergo carbon-carbon cleavage to form aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones. Hydroperoxides can also condense into dimers and polymers that can also break down to produce volatile compounds (Frankel 1998). Furthermore, free radicals have been reported to act as intermediates during the formation of secondary oxidation products (Kochhar and Gertz 2004). These free radicals ultimately form polymers, non-volatile oxy- and cyclic acids and volatile products such as saturated and unsaturated aldehydes, ketones, hydrocarbons, alcohols, acids and esters which eventually influence the quality of the heated oil (Anelich 2000).

Gupta (2005) suggested that products of oil oxidation are strong catalysts that cause further degradation of the oil during storage especially when the oil is abused during frying. It is also important to note that, when oil is extremely broken down, more of the oil will be absorbed by the food being fried than when fresh cooking oil is used. This will lead to more of this unhealthy broken down oxidized oil being consumed (Chow and Gupta 1994). In 2000, Anelich reported that as much as 40% (w/w) of broken down oil can be absorbed by specific food stuffs.

1.6 Biological activity of oxidized oils

The biological activity of oxidized oils has been studied comprehensively and various authors are in agreement that undesirable or harmful materials are formed during prolonged oxidation or high-temperature treatment. But, there is disagreement concerning the biological significance of such materials formed during experimental studies. This is because each study has its own experimental design and approach, particularly the extent of oxidation of oils used. An important aspect to consider when conducting such studies is the extent at which the oil has been heated and used, as this will play a vital role in establishing its biological activity i.e. if the oil has been extensively oxidized and therefore, abused or if it has been prepared under conditions similar to normal cooking practices (Chow 2007).

Oils that are extensively oxidized (thermally) or isolated fractions containing oxidation and degradation compounds such as hydroperoxides can interact with proteins, membranes and enzymes (Frankel 1998). These reactions with biological components are of concern as they affect vital cell functions. It has been reported that these oils can cause severe biological

effects such as growth retardation, diarrhoea and death in laboratory animals (Frankel 1998; Kock et al. 2002). Peroxidation of membranes and tissue lipids has been reported to have the ability to destroy the integrity of the cell membrane thus leading to leakage of cytoplasmic substances (Wu and Yen 2004). Furthermore interactions of oil breakdown products (especially free radicals) with proteins and DNA lead to damage of both these cellular components (Padney and Das 2006). This damage also affects the natural biological activities of proteins leading to toxicity of biological systems (Matsushita 1975).

Brandsch and co-workers (2009) further reported that dietary oxidized oils lowered activities of lipogenic enzymes in the liver leading to a decrease in the triglyceride content. This was also observed in the mammary glands. Furthermore, rats fed with heated oil developed liver damage and this was attributed to volatile compounds still found in the oil (Totani et al. 2008). Oxidized oils have been reported to promote or initiate carcinogenesis or mutagenesis (Chow 2007). In addition, vitamin E has been shown to partially protect cells against laboratory induced carcinogenesis which suggests that lipid peroxidation may play a role in carcinogenesis by damaging genetic material. Twenty percent polar compounds (i.e. total breakdown products) from oils used for normal frying, was shown to cause a small but significant reduction in growth and slight liver damage when included in diets of animals (Frankel 1998).

Non-volatile compounds produced during the frying process are of more concern as they remain in the frying oil and promote further degradation of the oil. They are also absorbed by the food to eventually affect public health (Garibağaoğlu et al. 2007). Cyclic fatty acid

monomers produced during deep frying are considered to be toxic as they remain in the oil and are readily absorbed by the intestinal mucosa (Sebedio and Grandgirard 1989). Additionally, aldehydes (volatile compounds) such *trans-trans*-2,4-decadienal (*t-t*-2,4-DDE), 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) are considered to be high in toxicity, while *t-t*-2,4-DDE has been reported to damage DNA which might result in cancer (Wu et al. 2001; Wu and Yen 2004). 4-Hydroxynonenal (4-HNE) and malondialdehyde (MDA) are well recognized and found to be relatively toxic. Furthermore *t-t*-2,4-DDE was reported to induce oxidative stress and increase reactive oxygen species production in a dose dependent manner in cultured human bronchial epithelial cells (Chang et al. 2005)

In addition a possible link suggesting these oils have adverse effects on HIV/AIDS progression, especially in poor communities, has been reported (Kock 2006). In 2002 the Kock group hypothesized that oxidized oils may adversely affect HIV-infected subjects in the black South African population and may lead to oxidative stress and the progression of HIV-AIDS. Other studies have implicated ROS, also present in these oils, as inhibitors of mitochondrial function which may also affect health negatively (Costantini et al. 2000; Lin et al. 2008).

1.7 Biological effects on other organisms

The Ames test is widely used for testing genotoxicity of used cooking oil. This is a screening test that is used to assist in identifying chemicals that influence the structure of DNA. The test exposes *Salmonella* to chemicals and visualizes changes in their growth behaviour. A mutated strain of *Salmonella* that requires histidine for growth is used in the test. The ability

of this strain to grow in the absence of histidine indicates that the mutation has been reversed. Several researchers (Taylor et al. 1983; Saleh et al. 1986; Hageman et al. 1988) showed that repeatedly used frying oils were mutagenic using the Ames test. Even though the Ames test has been extensively used as a screening method, other tests such as the bacterial bioluminescence test (or mutatox test) have been developed for genotoxicity testing. Here, dark mutants of luminous bacteria are used to determine the ability of a tested compound to restore luminescence by inducing a mutation (Sun et al. 1998). Studies by Sun and co-workers demonstrated that heated soybean oil exhibited genotoxicity in the mutatox test.

Studies by Gamage et al. (1971) reported that oxidative damage of biomembranes may be induced by dietary oxidized oils. They reported that 1mM linoleate hydroperoxides added at the initial stage or early logarithmic phase completely inhibited growth of *Escherichia coli*. In addition, Park and Ming (2003) reported that the PTG content of rape seed oil (used as substrate) decreased riboflavin production by *Eremothecium gossypii*.

There have been different studies carried out by various authors using oxidized oils as carbon source for fungi and bacteria. However in these studies emphasis was placed on the production of high value lipids, isolation of lipase-secreting bacteria and bio-surfactant production. Pelesane and co-workers (2001) and Joseph et al. (2005) showed that gamma linolenic acid (GLA) could be successfully produced by *Mucor circinelloides* using oxidized sunflower oil (with PTGs above the South African regulatory limit) as carbon source. However, they showed that PTGs influenced biomass and GLA production as well as oil utilization and accumulation negatively. Other studies revealed that oxidized oils can be

successfully used to isolate lipase-secreting bacteria (Haba et al. 1999). In 2006 Fleurackers reported that oxidized oils can be successfully used for the production of bio-surfactants. Here the author showed that PTGs did not affect bio-surfactant production.

1.8 Utilization of oils by fungi

Oil utilization by fungi has been widely reported in literature by numerous authors (Mercade et al. 1993; Jeffery et al. 1997, Pelesane et al. 2001, Venter et al. 2004, Joseph et al. 2005; Fleurackers 2006; Sarubbo et al. 2006; Szczęsna-Antczak et al. 2006; Papanikolaou et al. 2007; Marsudi et al. 2008). Microorganisms require several modifications before they can utilize oils. These include uptake mechanisms as well as specific metabolic pathways for the breakdown of the oily substrate. In *Yarrowia lipolytica* and *Candida tropicalis*, cells were found to respond by releasing surface-active compounds and chemicals (such as lipases and bio-surfactants), changing the structure of their cell surface to transport water insoluble compounds (Käpelli et al. 1984). Papanikolaou and co-workers (2007) reported that the presence of fatty acids in culture medium stimulated lipase production in *Y. lipolytica* and the same phenomenon has been observed with other organisms.

Protrusions were found on the surface of cells grown in the presence of fatty acids and were implicated to play a role in the uptake of these compounds from the medium (Käpelli et al. 1984). These protrusions probably maximize cell-substrate contact by adhering to hydrophobic droplets in the medium and thereby facilitating uptake of hydrophobic substrates by the cell. Such cell surface protrusions in fungi have been reported in *C. tropicalis* and *Y. lipolytica* (Käpelli et al. 1984). According to Beopoulos and co-workers (2009), once taken

up by cells, fatty acids can remain unchanged or can be modified. Finally, fatty acids are incorporated in triglycerides and stored in intracellular compartments known as lipid bodies or they can be utilized for energy production purposes by the cell (Mlíčková et al. 2004).

External oil utilization by fungi begins when triglycerides are hydrolysed to yield free fatty acids (that can be easily utilized to produce biomass). This hydrolysis is catalysed by lipases (Shukla et al. 2007) which in fungi are classified into three main types according to the specific reaction they catalyze. Non-specific lipases catalyze the total hydrolysis of triglycerides to free fatty acids and glycerol, while, 1,3-regiospecific lipases catalyze the hydrolysis reaction at the C-1 and C-3 positions of triglycerides resulting in amongst others 2,3-diacylglycerol and 2-monoacylglycerol. The final type comprises of acyl-group specific lipases able to catalyze the removal of a specific fatty acid from a triglyceride (Ratledge 1989). In other studies, Loo and co-workers (2007) reported on the specificity of these lipases, when they demonstrated that lipases from *Geotrichum candidum* preferred unsaturated oleic acid above saturated palmitic acid during hydrolysis of palm olein.

Following hydrolysis of triglycerides by lipases, free fatty acids are taken up by the cell through simple diffusion at high concentrations and facilitated diffusion at low concentrations. In the latter case, fatty acids (especially long-chain fatty acids) bind to a protein carrier in the cytoplasmic membrane to facilitate transport across the membrane. Once inside the cytoplasm, fatty acids are changed to acyl-CoA esters by acyl-CoA synthetase. This minimizes the inhibitory effects of free fatty acids in the cytoplasm. Here, free fatty acids are converted back to triglycerides for storage in the cell, resulting in the fed

oil having a similar composition to the intracellular lipid formed. In some organisms intracellular lipids can also be elongated and desaturated (Finnety 1989; Ratledge 2004) to produce fatty acids with different chain lengths and desaturation. It was shown by Wynn and Ratledge (2000) that *de novo* fatty acid synthesis in certain fungi does not occur when cells are grown on oils since fatty acid biosynthesis is repressed.

Free fatty acids from fed oils can also be metabolized by fungi in the mitochondria to provide energy. This process is termed β-oxidation since it occurs through the sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl-CoA molecule. Since fatty acids in the form of acyl-CoA ester cannot pass through the mitochondrial membrane, a transport system as well as β-oxidation enzymes (i.e. epimerase and isomerase) are needed to initiate the pathway (Wynn and Ratledge 2000).

1.9 Purpose of research

With this information as background the aims of this study became, to assess if *M. circinelloides* and *C. curvatus* can utilize palm oil characterized by different levels of oxidative breakdown. Special attention will be given to lipid turnover, cell growth and the effects of breakdown products on cell morphology. The influence of these oils was then assessed on fungal growth, oil utilization and morphology. In addition the anti-mitochondrial activity of oxidized palm oil breakdown products on these fungi was assessed. These results were also compared to known anti-mitochondrial antifungals.

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CHAPTER 2

Inhibitory effects of palm oil breakdown products on *Cryptococcus curvatus* and *Mucor circinelloides*

Parts of this chapter have been submitted for publication in Archives of Microbiology

(Supplementary information is also included)

2.1 Abstract

The oleaginous fungi *Cryptococcus curvatus* and *Mucor circinelloides* were used to determine the effect of palm oil breakdown products, measured as polymerised triglycerides (PTGs) on lipid turnover, growth and morphology. In *Mucor circinelloides* we found after seven days of growth, a decrease in biomass, lipid utilization and accumulation at increased PTG levels, both at low and neutral pH. An increase in PTG concentration also influenced the morphology of *M. circinelloides*. Here, protrusions were observed on the surfaces of the fungal cell walls when grown on oil with 45% PTGs and not when the fungus was grown on fresh oil with 0.4% PTGs. In *C. curvatus* there was also a decrease in oil utilization and biomass production at increased PTG levels, at both low and neutral pH. Here an increase in oil accumulation was observed at low pH while it remained similar at neutral pH for all PTG levels tested. Hairy and warty protuberances on the cell surface were observed when *C. curvatus* was grown on oils with 15% and 45% PTGs respectively. We conclude that the changes observed in lipid turnover as well as morphology in both fungi are due to the presence of palm oil breakdown products.

Key words: *Cryptococcus curvatus*, Lipid turnover, Morphology, *Mucor circinelloides*, Polymerized triglycerides.

2.2 Introduction

When edible oils such as sunflower and palm oils are used repeatedly in frying processes, they break down to form various compounds such as free fatty acids, peroxides, aldehydes, free radicals and eventually, polymerised triglycerides (PTGs; Frankel 1998). PTG levels are used as indicator to assess the extent of oil breakdown (STOA Report 2000). According to South African and other international regulations, oil that contains 16% and more PTGs is considered harmful to human health since it will also contain toxic levels of other breakdown products (Kock et al. 1999; STOA Report 2000). Animal studies have shown that oils containing PTGs above these limits may cause, amongst others, growth retardation, cancer and diarrhoea (STOA Report 2000). In addition, a possible link suggesting that these oils have adverse effects on HIV/AIDS progression, especially in poor communities, has been reported (Kock 2006).

Adverse effects of oil breakdown products on fungi have also been reported. When the oleaginous fungus *Mucor circinelloides* was grown on unsaturated sunflower oil with increased levels of PTGs, biomass production as well as oil utilization and accumulation decreased at neutral and low pH levels (Joseph et al. 2005). PTGs in oxidized unsaturated rapeseed oil were also shown to decrease riboflavin production by the fungus *Ashbya* (*Eremothecium*) *gossypii* (Park and Ming 2003) and are therefore also considered to be anti-mitochondrial (Kock et al. 2009).

Until now, no work has been done on the effect of saturated palm oil breakdown products on fungi. Consequently, the aim of this study became to assess the effects of these oils on fungal

growth as well as oil utilisation and accumulation. This is also the first time that the effect of edible oil breakdown products on fungal cell morphology is assessed.

2.3 Materials and methods

Preparation of edible oils with varying amounts of PTGs

Fresh palm oil (PTG = 0.4% w w^{-1}) was heated to 200 °C while being stirred and aerated (air flow: 7 L min^{-1}, oil volume: 1 L) to reach PTGs levels of 15% and 45% (w w^{-1}) respectively. The levels of PTG were assessed by using the method of Beljaars (1993). Here, oils were dissolved in tetrahydrofuran (THF; Honeywell, Burdick and Jackson, Muskegon, MI, USA) and the polymers were determined by gel permeation chromatography using a Hewlett Packard HP 1047A liquid chromatograph (USA) equipped with a RD 1 detector (HP 1047 Refractive Index Detector, USA). PTGs are formed by carbon to carbon and/or carbon to oxygen linkages between mono-, di and triglyceride bound fatty acids as well as free fatty acids to produce dimeric or higher polymeric compounds.

Microorganisms and cultivation

Cryptococcus curvatus UOFS Y-0818T and *M. circinelloides* UOFS Y-2803 were first grown on YM (yeast malt agar) plates at 30 °C for four days and then transferred to 100 mL sterile growth media (pH 5.8) in 1 L conical flasks. The medium consisted of the following in g L^{-1}: fresh palm oil with PTG level of 0.4% (w w^{-1}), 30; sodium acetate, 10; yeast extract, 0.1; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.0; $CaCl_2.2H_2O$, 0.05; NH_4Cl, 1.28. Trace elements were

added to a final concentration (g L^{-1}) as follows: $FeSO_4.7H_2O$, 0.035; $MnSO_4.4H_2O$, 0.0007; $ZnSO_4.7H_2O$, 0.011; $CuSO_4.5H_2O$, 0.001; $CoCl_2.6H_2O$, 0.002; $Na_2MoO_4.2H_2O$, 0.0013; H_3BO_3, 0.002; KI, 0.00035; $Al_2(SO_4)_3$, 0.0005.

These cultures were then grown at 30 °C for seven days while being shaken at 160 rev min^{-1}. *C. curvatus* cultures were harvested by centrifugation (11180*g*) and *M. circinelloides* cultures by filtration (Whatman no. 1) at different time intervals (i.e. after 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h). All experiments were performed in triplicate. These experiments were repeated with palm oil containing 15% and 45% PTGs respectively. After harvesting, cells were extensively washed and immediately frozen and freeze-dried. Control experiments were carried out using the same medium as above with palm oil (40 g L^{-1}; PTG level = 0.4%, 15% and 45% w w^{-1}) as the only carbon source.

Lipid extraction

From the supernatant of each flask (pH < 4), extracellular lipids were immediately extracted with hexane (Radchem, Germiston, South Africa) until no lipids were detected (Kock et al. (1997). Lipids were also extracted from freeze-dried cells. Here cells from each flask were subjected to 150 mL chloroform/methanol (2:1 v v^{-1}) [Radchem, Germiston, South Africa] treatment as described by Folch et al. (1957). This was followed by two washes with distilled water and final evaporation of the organic phase under vacuum. The lipid fraction was dissolved in diethyl ether and transferred to pre-weighed vials. Finally samples were dried to constant weight in a vacuum oven over P_2O_5 at 55 °C.

Polymerized triglyceride analysis

Both intracellular and extracellular lipids were dissolved in THF. Polymers were determined by gel permeation chromatography as described by Beljaars (1993).

Acetic acid analyses

Acetic acid present in the supernatants in all experiments were determined using the method of Du Preez and Lategan (1978). Here, a Shimadzu gas chromatograph (Japan) with a flame ionisation detector and a 30 m x 0.25 mm, ID x 0.25 µm film thickness, SolGel column (USA) was used. The initial oven temperature was 50 ºC and held for 2.5 min and then increased at a rate of 20 ºC min^{-1} to 150 ºC. The inlet temperature was 250 ºC. The carrier gas was hydrogen at a flow rate of 1 mL min^{-1}. The samples and acetic acid standard (1 mL) were acidified with 0.3 mL of a 25% formic acid solution prior to analysis.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed according to van Wyk and Wingfield (1991). Fungal cells were first chemically fixed for 2 h using 3% (v v^{-1}; 1.0 mol L^{-1}) of a sodium phosphate buffered glutardialdehyde (Sigma-Aldrich, St. Louis, Mo., USA) solution at pH 7.0 and a similarly buffered solution (1% m v^{-1}) of osmium tetroxide (Sigma-Aldrich) for 1h. Next, all material was dehydrated in a graded series of ethanol solutions (50%, 70%, 90%, and 100% for 30 min per solution). These materials were then critical-point dried, mounted, and coated with gold to make it electrically conductive and finally examined with a Shimadzu SSX550 SEM (Japan).

Transmission electron microscopy

For transmission electron microscopy (TEM; van Wyk and Wingfield 1991), fungal cells were fixed using the same protocol described for SEM. After washing, the material was dehydrated in a graded acetone (Merck, Darmstadt, Germany) series (50 %, 70 %, 95 %, 2X 100 %). Dehydration lasted 30 min for each step. Next the material was embedded in an expoxy resin (Spurr 1969) and allowed to polymerise in an oven at 70 °C for 8 h. Thin sections were made using a LKB III Ultramicrotome (Stockholm, Sweden) and stained with uranyl acetate for 5 min and lead citrate for 1 min. Finally, these sections were viewed with a Phillips EM 100 transmission electron microscope (Eindhoeven, the Netherlands). These procedures were performed in at least triplicate.

2.4 Results and discussion

The effects of breakdown products on lipid turnover by both fungi after seven days of growth are shown in Tables 1 and 2 respectively. It was found that the pH increased to near neutrality when both fungi were grown on oil, at different PTG levels, in the presence of acetate and to below 4 in the absence of acetate. Acetate was almost completely utilised after 24 h of growth. Similar results were reported by Joseph et al. (2005) when *M. circinelloides* was grown on sunflower oil under similar conditions. Consequently, acetate may be used to maintain fungal cultures at neutral pH. Here sodium ions remain present in the medium while acetic acid is replaced with hydroxide and similar ions. This eventually leads to a pH increase since sodium hydroxide is a stronger base than sodium acetate (Ratledge et al. 2001).

Increased emulsification was also observed after 24 h of growth in both fungi in the presence of acetate and not in its absence. This may be ascribed to increased lipase activity in the neutral medium and the resulting fatty acids forming emulsifying soaps. It is interesting to note that the pH of the acetate-free media in which *Cryptococcus* was grown, was lower compared to media in which *Mucor* was grown. This is probably due to the production of additional organic acids by the former fungus.

Lipid turnover

Mucor circinelloides (Table 1)

Extracellular lipid (ECL) fraction: This fungus was capable of utilising palm oil at different levels of breakdown - measured as PTGs. Increased amounts of ECL remained in the pH neutral medium with PTG levels of 15% and 45% respectively when compared to fresh oil (PTG = 0.4%). This indicates decreased oil utilisation when compared to fresh oil (0.4% PTG). A similar trend is observed at low pH (pH 3.8) although much less oil was utilised by the fungus at all PTG levels. This may be ascribed to poor emulsification and therefore low lipase activity.

Biomass (BM) production: A significant ($P < 0.01$) dose dependent decrease in BM production occurred at increased levels of PTGs at both neutral and low pH conditions. This may be ascribed to the decrease in oil utilisation under these conditions probably caused by oil breakdown products.

Intracellular lipid (ICL) fraction: The ICL content (oil accumulation) also decreased significantly ($P < 0.01$) with increased levels of oil breakdown levels at both pH levels.

Percentage PTGs in ECL fraction: It is interesting to note that the percentage PTGs increased significantly ($P < 0.01$) in the ECL fraction with an increase in PTG levels in the oil originally fed to the fungus. This was evident in both neutral and low pH media. This, points towards the inability of this fungus to utilize PTGs.

Percentage PTGs in ICL fraction: The percentage PTGs in the ICL fraction also increased with an increase in the PTG levels in the oil originally fed to this fungus. This may be ascribed to the uptake of these breakdown products by the fungus or by the *ab initio* formation of PTG in ICL fraction. Here, significantly more ($P < 0.01$) intracellular PTGs were formed in ICL fraction at a lower pH level when compared to ICL obtained at neutral pH. This cannot be explained at present.

Cryptococcus curvatus (Table 2)

Extracellular lipid (ECL) fraction: This fungus was also capable of utilising palm oil at different levels of breakdown. Here, residual ECL was dose dependent in media with increased levels of PTGs at both neutral and low pH. This indicates decreased oil utilisation as a result of increased levels of oil breakdown. At low pH, significantly ($P < 0.01$) more ECL remained in the medium compared to ECL in neutral pH media. This may again be ascribed to poor emulsification and therefore low lipase activity.

Biomass (BM) production: A decrease in BM production was evident at increased PTG levels when compared to a PTG level of 0.4% at both neutral and low pH. However a significant increase ($P < 0.01$) was observed from PTG levels of 15% to 45%. This may be ascribed to the formation of more inhibitory oxidation products at 15% PTGs than at 45% PTGs where (in the latter case) most oxidative intermediates transformed to PTGs (Frankel 1998).

Intracellular lipid (ICL) fraction: Interestingly, the ICL content remained similar at neutral pH while a dose dependant increase was found at low pH with an increase in PTG levels in the original oil fed to the yeast. This cannot be explained at present.

Percentage PTGs in ECL fraction: It is interesting to note that the percentage PTGs increased significantly ($P < 0.01$) in the ECL fraction in a dose dependent manner at both pH levels. This point towards selective accumulation of PTGs where the latter is not favoured for uptake probably due to toxicity.

Percentage PTGs in ICL fraction: Contrary to the results obtained in *M. circinelloides*, the percentage PTGs in the ICL fraction remained undetectable at all PTG and pH levels. This may be ascribed to a lack of uptake or intracellular production of these potentially toxic free radical induced polymers. This is probably a defence mechanism of this yeast (Frankel 1998).

Morphology

Mucor circinelloides (Fig. 1a-d)

Electron micrographs (SEM and TEM) clearly show a difference in hyphal cell wall morphology in the presence of 45% PTGs (in originally fed oil; Fig. 1c,d) compared to a PTG level of 0.4% (in originally oil; Fig. 1a,b) after seven days of growth. In the latter case the coenocytic hyphal cell wall surfaces are smooth (Fig. 1a,b) while in the 45% PTG-medium the surface demonstrated wart-like structures that gave the hyphae a wrinkled appearance (Fig. 1c,d). This was not observed when the fungus was grown in the absence of acetate where growth ceased after 48 h (see supplementary information).

Cryptococcus curvatus (Fig. 2a-f)

When this yeast was fed with palm oil containing PTG levels of 0.4%, 15% and 45% respectively in the presence of acetate, again an interesting change in cell wall surface morphology was observed by SEM and TEM analyses after seven days of growth. At 0.4% PTGs (in originally fed oil), the cell walls remained smooth (Fig. 2a,b) while hairy protuberances appeared at a 15% PTG level (in originally fed oil, Fig. 2c,d) and wart-like protuberances were visible at a PTG level of 45% (in originally fed oil, Fig. 2e,f). This was less pronounced when the fungus was grown in the absence of acetate.

This is the first time that cell wall protuberances in fungi are reported as a response to the presence of palm oil breakdown products. Is possible that these structures function in the uptake of palm oil breakdown products? This is probably similar to the cell wall protuberances found in *Candida tropicalis* that may serve as a possible mechanism for the uptake of hydrophobic substrates (Käpelli et al. 1984). In addition the observed cell shrinkage of *Mucor circinelloides*, the loss of intracellular structure of *Cryptococcus curvatus* as well as the blebb-like protruberances in both organisms may be indicators of apoptosis-like programmed cell death induced by the presence of palm oil breakdown products (Granot et al. 2003).

2.5 Conclusions

From this study it is clear that palm oil breakdown products, expressed as percentage PTGs, have a significant inhibitory effect on oil utilization, concomitant biomass production as well as lipid accumulation. In many cases this pattern is dose dependent with an increase in PTG content. Strikingly, interesting protuberances were observed on fungal cell walls of cultures

grown in the presence of elevated levels of oil breakdown. In future the function of these protuberances should be assessed as well as the different breakdown products formed in the heat treated palm oils.

It was previously reported that the production of riboflavin by the fungus *Eremothecium* may be used as indicator for the detection of compounds with anti-mitochondrial activity (Kock et al. 2009). Since PTGs also decrease riboflavin production by this fungus (Park and Ming 2003), we conclude that these oxidised oil compounds may also be anti-mitochondrial (Kock et al. 2009). Consequently the effect of PTGs and other oil breakdown products on mitochondrial activity should be assessed in the fungi studied as well as in animals and humans.

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2.8 Tables

Table 1. Effects of palm oil breakdown products on lipid turnover by *Mucor circinelloides* after seven days of growth

		% PTG[e]		
		0.4%	**15%**	**45%**
pH	+	6.8 (0.0)	7.0 (0.0)	7.0 (0.1)
	-	3.8 (0.0)	3.8 (0.2)	3.8 (0.1)
AA[a] (g L^{-1})	+	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
	-	-	-	-
BM[b] (g L^{-1})	+	16.3 (1.8)	9.6 (0.6)	8.3 (0.2)
	-	5.4 (0.6)	4.6 (0.3)	2.6 (0.3)
ECL[c] (g L^{-1})	+	11.6 (0.4)	16.0 (1.5)	14.0 (0.4)
	-	29.8 (3.7)	33.3 (1.8)	33.9 (0.4)
ICL[d] (%)	+	65.0 (1.9)	48.6 (1.7)	43.9 (2.1)
	-	56.0 (2.9)	51.7 (6.7)	19.9 (0.1)
% PTGs[e] (ECL)	+	0 (0.0)	2.4 (0.1)	28.1 (0.1)
	-	0 (0.0)	4.3 (0.1)	29.4 (0.1)
% PTGs[e] (ICL)	+	0 (0.0)	0.3 (0.0)	3.2 (0.1)
	-	0 (0.0)	9.8 (0.2)	9.7 (0.1)

[a]AA – acetic acid; [b]BM – biomass; [c]ECL – extracellular lipid content; [d]ICL – intracellular lipid content (% w w^{-1}); [e]PTGs – polymerized triglycerides (% w w^{-1}); + presence of AA; - absence of AA; ( ) – standard deviation.

Table 2. Effects of palm oil breakdown products on lipid turnover by *Cryptococcus curvatus* after seven days of growth

		% PTG[e]		
		0.4%	**15%**	**45%**
pH	+	7.2 (0.1)	7.0 (0.3)	6.8 (0.5)
	-	2.9 (0.2)	2.8 (0.1)	2.9 (0.1)
AA[a] (g L^{-1})	+	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
	-	-	-	-
BM[b] (g L^{-1})	+	13.1 (1.0)	6.0 (0.7)	10.3 (0.7)
	-	8.1 (1.4)	4.1 (0.1)	7.1 (0.1)
ECL[c] (g L^{-1})	+	1.2 (0.1)	10.6 (0.1)	12.0 (2.0)
	-	23.0 (0.2)	24.3 (1.9)	25.4 (0.4)
ICL[d] (%)	+	40.0 (1.4)	40.0 (0.8)	40.0 (2.9)
	-	42.0 (2.8)	51.3 (0.8)	53.4 (3.1)
% PTGs[e] (ECL)	+	0 (0.0)	16.5 (0.5)	48.0 (0.7)
	-	0 (0.0)	19.5 (0.2)	58.9 (0.1)
% PTGs[e] (ICL)	+	0 (0.0)	0.0 (0.0)	0.0 (0.0)
	-	0 (0.0)	0.0 (0.0)	0.0 (0.0)

[a]AA – acetic acid; [b]BM – biomass; [c]ECL – extracellular lipid content; [d]ICL – intracellular lipid content (% w w^{-1}); [e]PTGs – polymerized triglycerides (% w w^{-1}); + presence of AA; - absence of AA; ( ) – standard deviation.

2.9 Figures

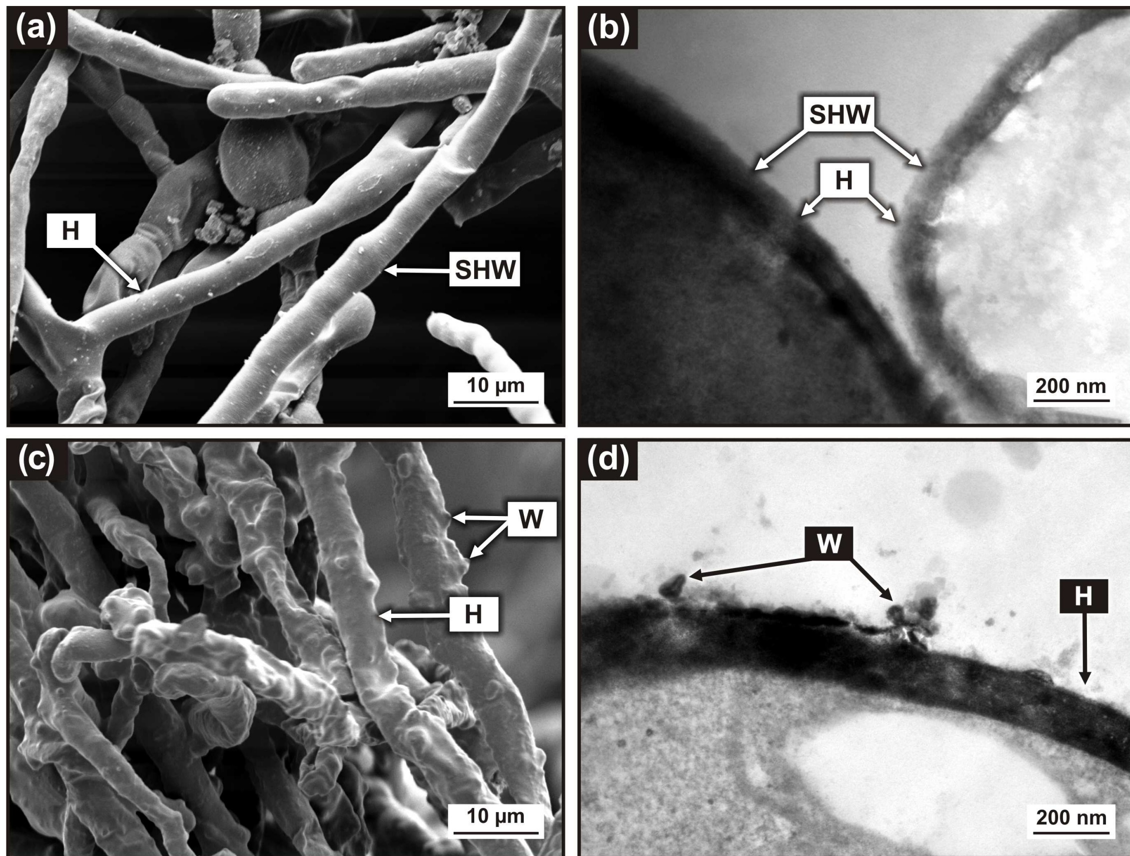

Fig. 1 Scanning (a) and transmission (b) electron micrographs showing smooth hyphal walls (SHW) of *Mucor circinelloides* after seven days of growth on fresh palm oil in the presence of acetate. Scanning (c) and transmission (d) electron micrographs showing wart-like (W) structures on hyphal (H) walls after seven days of growth on palm oil with 45% PTGs in the presence of acetate.

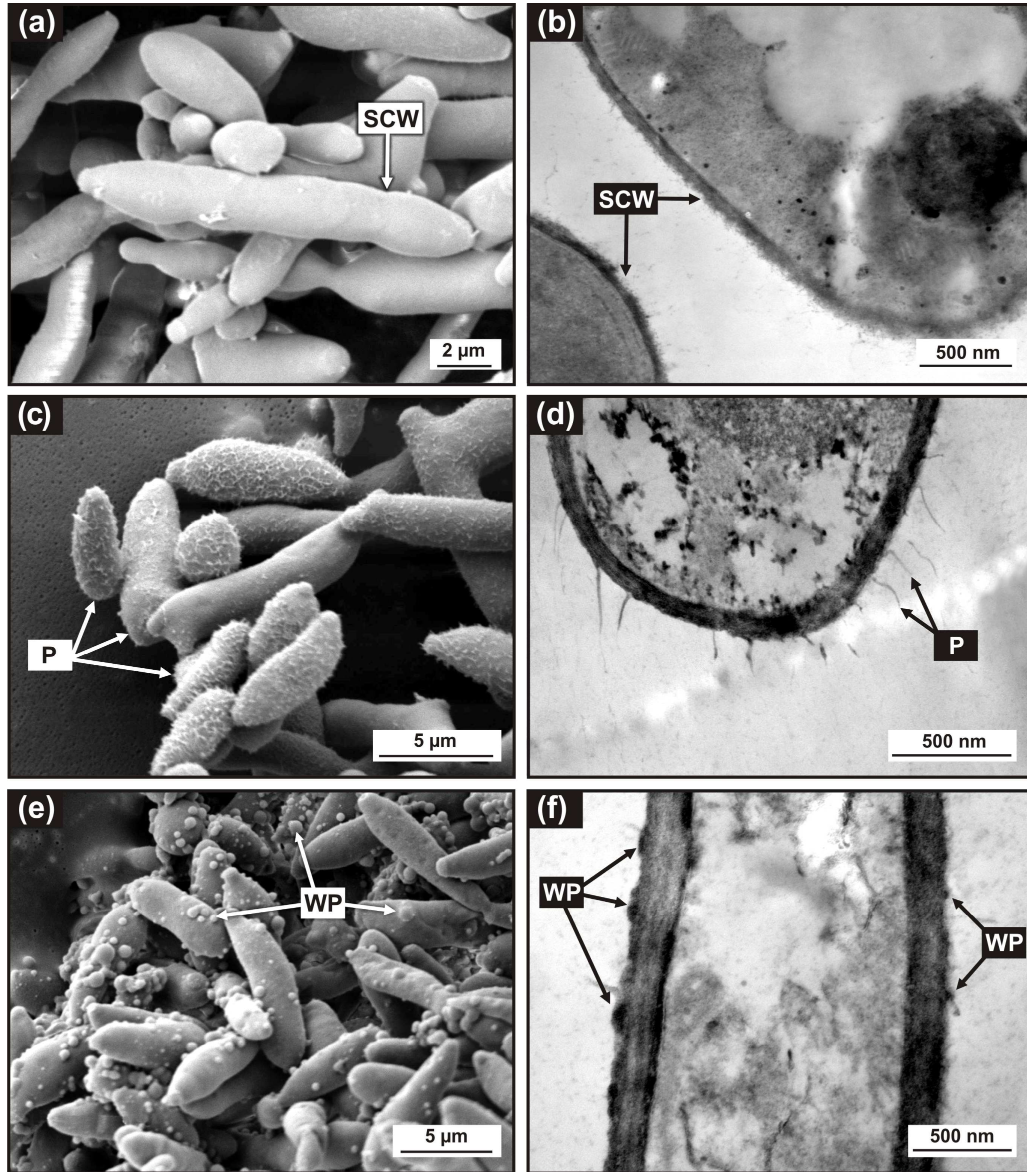

Fig. 2 Scanning (a) and transmission (b) electron micrographs showing smooth cell walls (SCW) of *Cryptococcus curvatus* after seven days of growth on fresh palm oil in the presence of acetate. Scanning (c) and transmission (d) electron micrographs showing hairy protuberances (P) on cells after seven days of growth on palm oil with 15% PTGs in the presence of acetate. Scanning (e) and transmission (f) electron micrographs showing wart-like protuberances (WP) on cell surface after seven days of growth on palm oil with 45% PTGs in the presence of acetate.

2.10 Supplementary Information

Results obtained from samples drawn after 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h are shown in Figs. 1- 10 and summarized in this section. All experiments were performed in triplicate and similar patterns were observed.

***Mucor circinelloides* (Figs. 1-5)**

pH and acetic acid utilization (Figs. 1,2): The pH increased to near neutrality at all PTG levels in the presence of acetate and decreased to less than 4 in the absence of acetate (Fig. 1). Acetic acid was almost completely utilized after 24h (Fig. 2). These results correspond with those reported by Joseph and co-workers in 2005.

Extracellular lipid (ECL) fraction (Fig. 3): Oil utilization was the highest at a PTG level of 0.4% in the presence of acetate when compared to PTG levels of 15% and 45%. This was also observed in the absence of acetate, where oil utilization was less at all PTG levels when compared to cultures grown in the presence of acetate. This may be due to decreased emulsification at low pH which probably affects lipase activity leading to a decrease in oil utilization.

Biomass (BM) production (Figs. 4a,b): A dose dependent decrease in biomass was observed with an increase in PTGs at both pH levels (Figs. 4a,b). This may be due to a decrease in oil utilization as PTGs increase. Interestingly, at PTG levels of 45%, in the absence of acetate, growth ceased after 48 h.

Intracellular lipid (ICL) fraction (Figs. 5a,b): There was ehanced oil accumulation (ICL content) when fresh oil (PTG = 0.4%) was fed to *M. circinelloides* than when oils with 15% and 45% PTGs respectively were fed to the fungus at both pH levels (Figs. 5a,b). Dose dependent effects were observed at both pH levels.

***Cryptococcus curvatus* (Figs. 6-10)**

pH and acetic acid utilization (Figs. 6,7): The pH increased to near neutrality at all PTG levels in the presence of acetate and decreased to less than 3 in the absence of acetate (Fig. 6). Acetic acid was almost completely utilized after 24 h (Fig. 7) which was also observed with *M. circinelloides*. The pH in the absence of acetate was lower than that observed in *M. circinelloides* indicating production of more organic acids by this fungus.

Extracellular lipid (ECL) fraction (Fig. 8): In general, there was enhanced oil utilization in the presence of acetate than in its absence, with fresh oil (PTG = 0.4%) being utilized better than oxidized oils at PTG levels of 15% and 45% respectively. A dose dependent decrease in oil utilization was observed with an increase in PTGs at both pH levels.

Biomass (BM) production (Fig. 9a,b) Biomass production increased when fresh oil was fed to *C. curvatus* in the presence of acetate compared to oils at PTG levels of 15% and 45% (Fig. 9a). This was also observed in the absence of acetate (Fig. 9b). A decrease in biomass was observed at a PTG level of 15% from 144 h to 168 h of growth in the presence or absence of acetate. This decrease may be due to the presence of intermediary oxidized oil breakdown products which may be toxic (Frankel 1998). This also shows that *C. curvatus* is more sensitive to these breakdown products at this PTG level.

Intracellular lipid (ICL) fraction (Figs. 10a,b): The ICL fraction remained at about 40% at all PTG levels in the presence of acetate (Fig. 10a) after 168 h of growth. This phenomenon cannot be explained at the moment. A PTG dose dependent increase of the ICL fraction was observed in the absence of acetate (Fig. 10b). This cannot be explained at present.

2.11 Figures

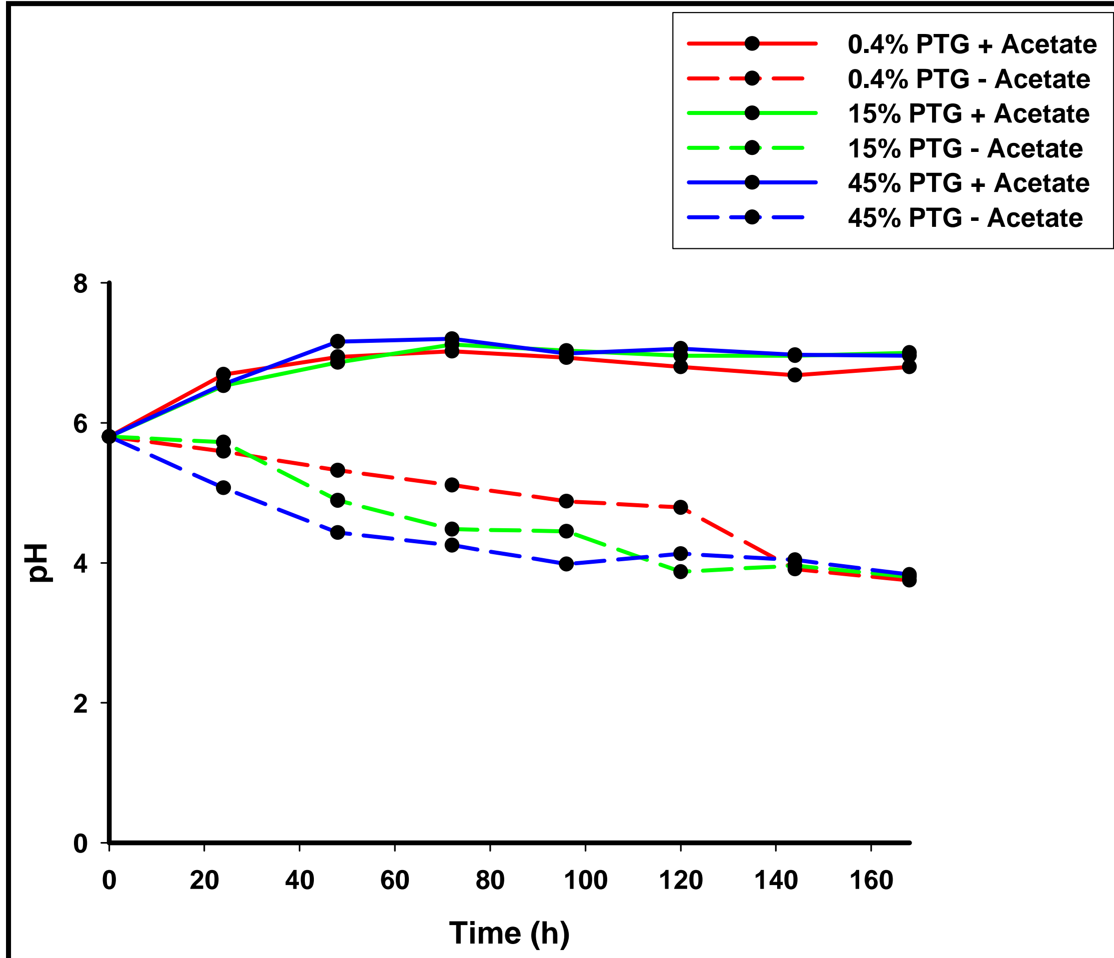

Fig. 1 Changes in pH of *Mucor circinelloides* grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence (solid line) and absence (broken line) of acetate respectively.

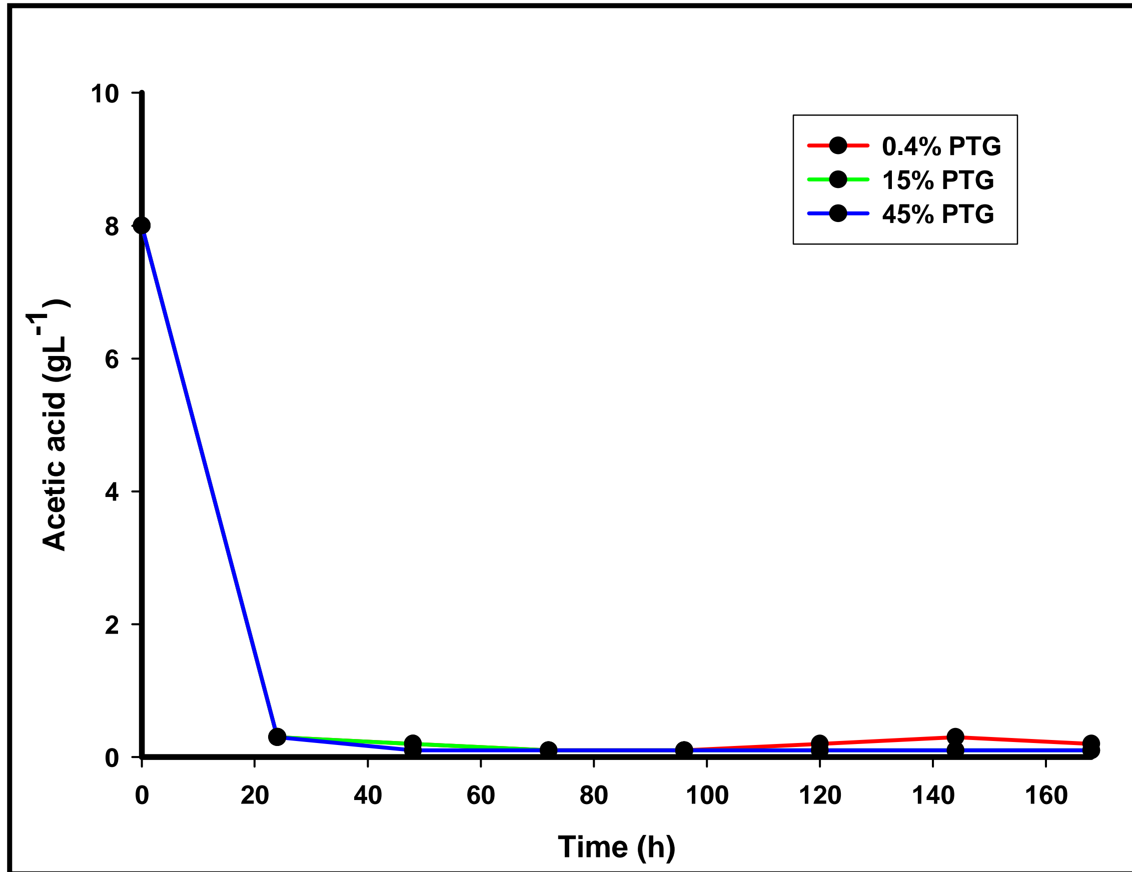

Fig. 2 Changes in acetic acid concentration of *Mucor circinelloides* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides respectively.

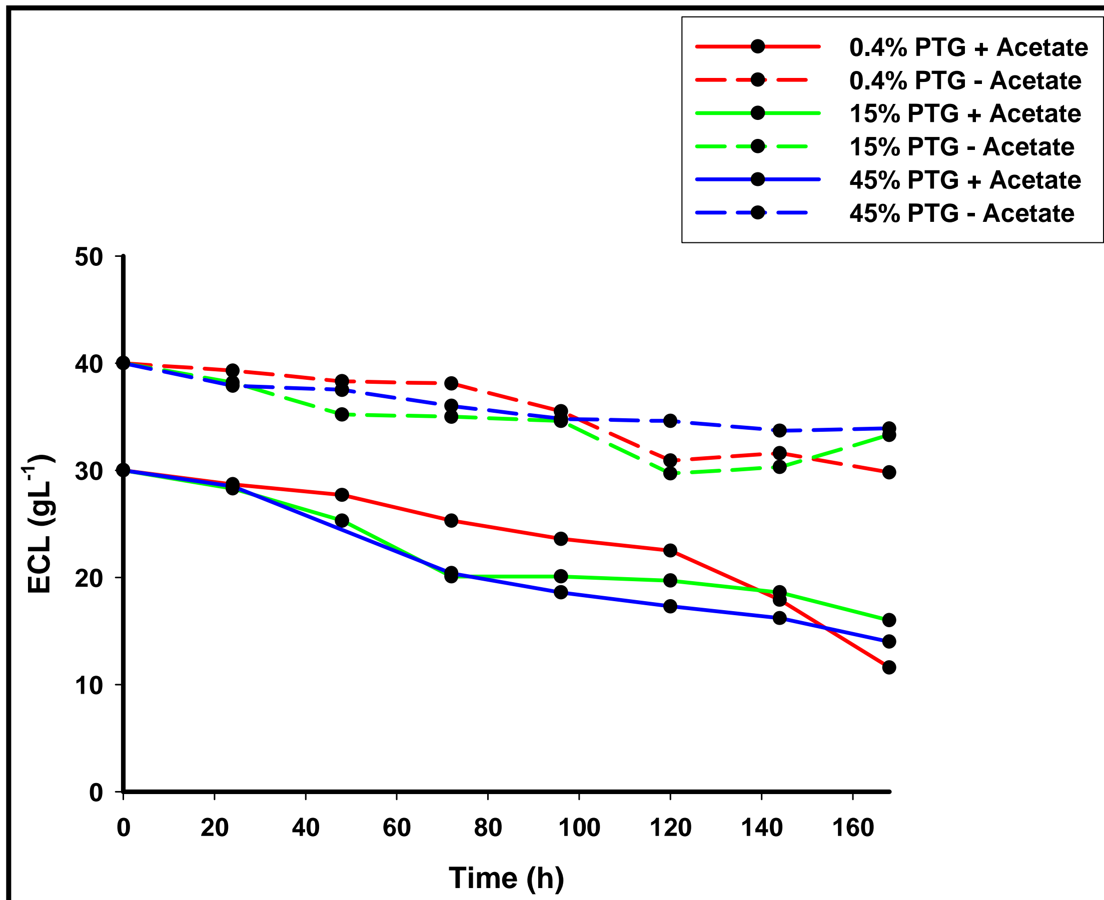

Fig. 3 Oil utilization by *Mucor circinelloides* grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence (solid line) and absence (broken line) of acetate respectively. ECL: extracellular lipid content.

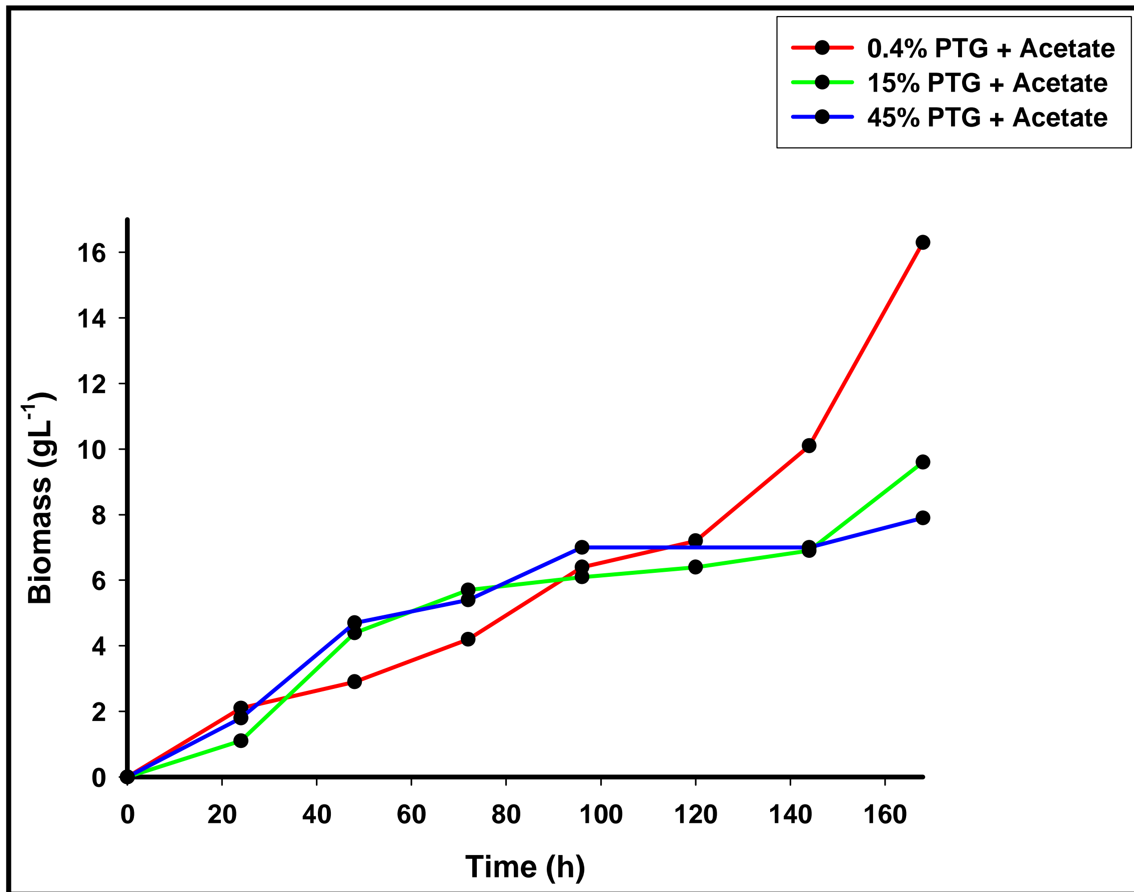

Fig. 4a Biomass production by *Mucor circinelloides* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence of acetate respectively.

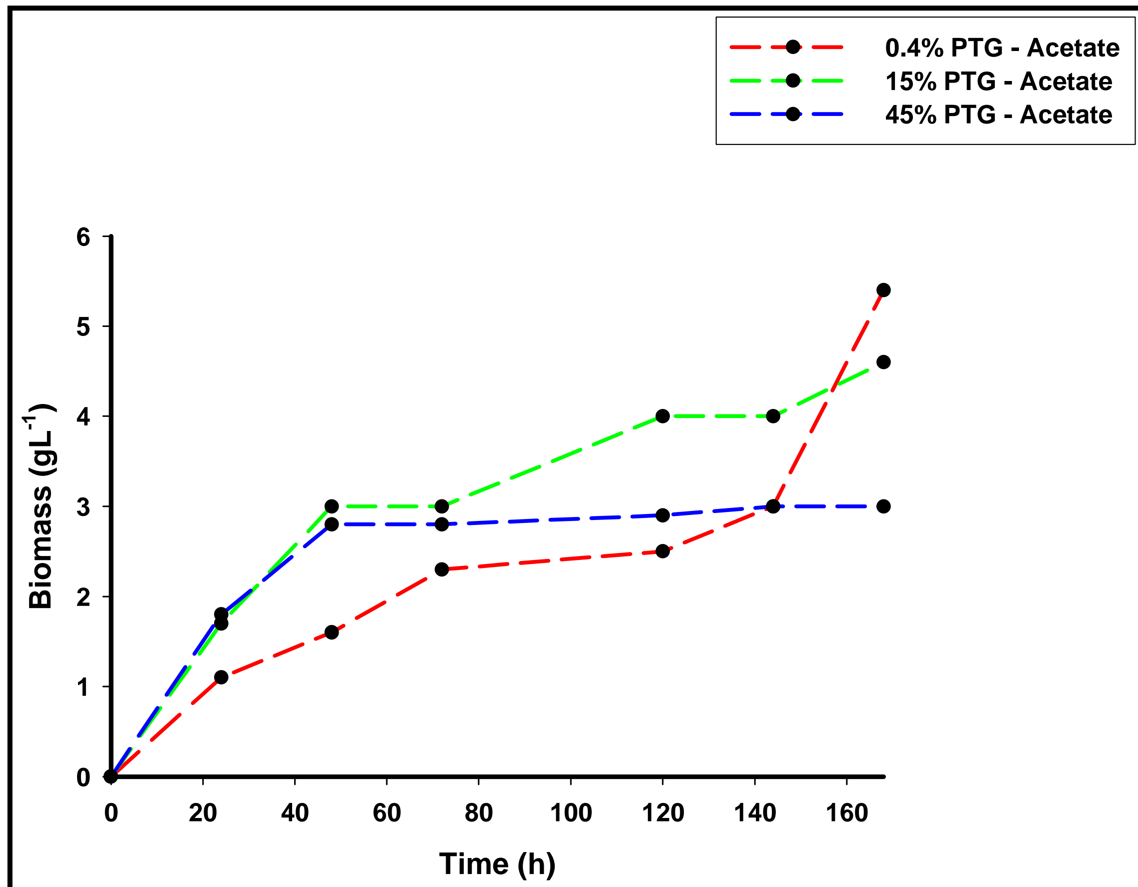

Fig. 4b Biomass production by *Mucor circinelloides* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the absence of acetate respectively.

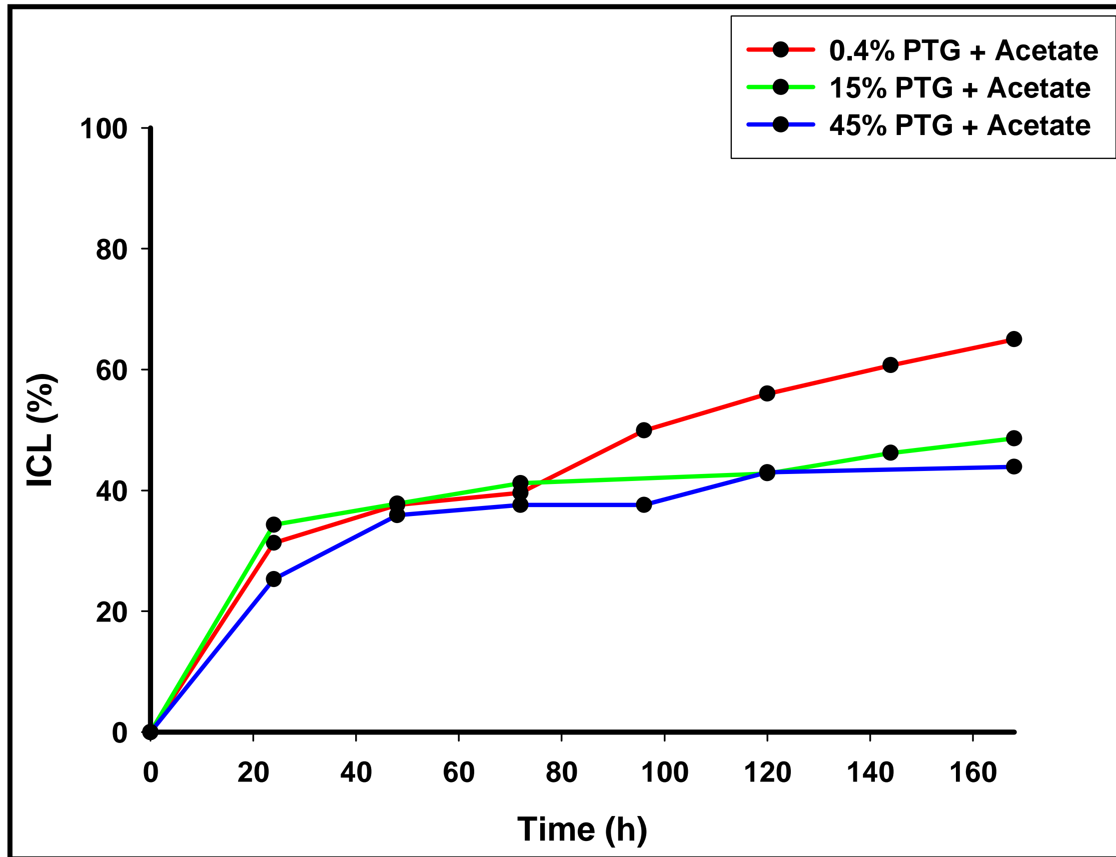

Fig. 5a Intracellular lipid (ICL) content of *Mucor circinelloides* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence of acetate respectively.

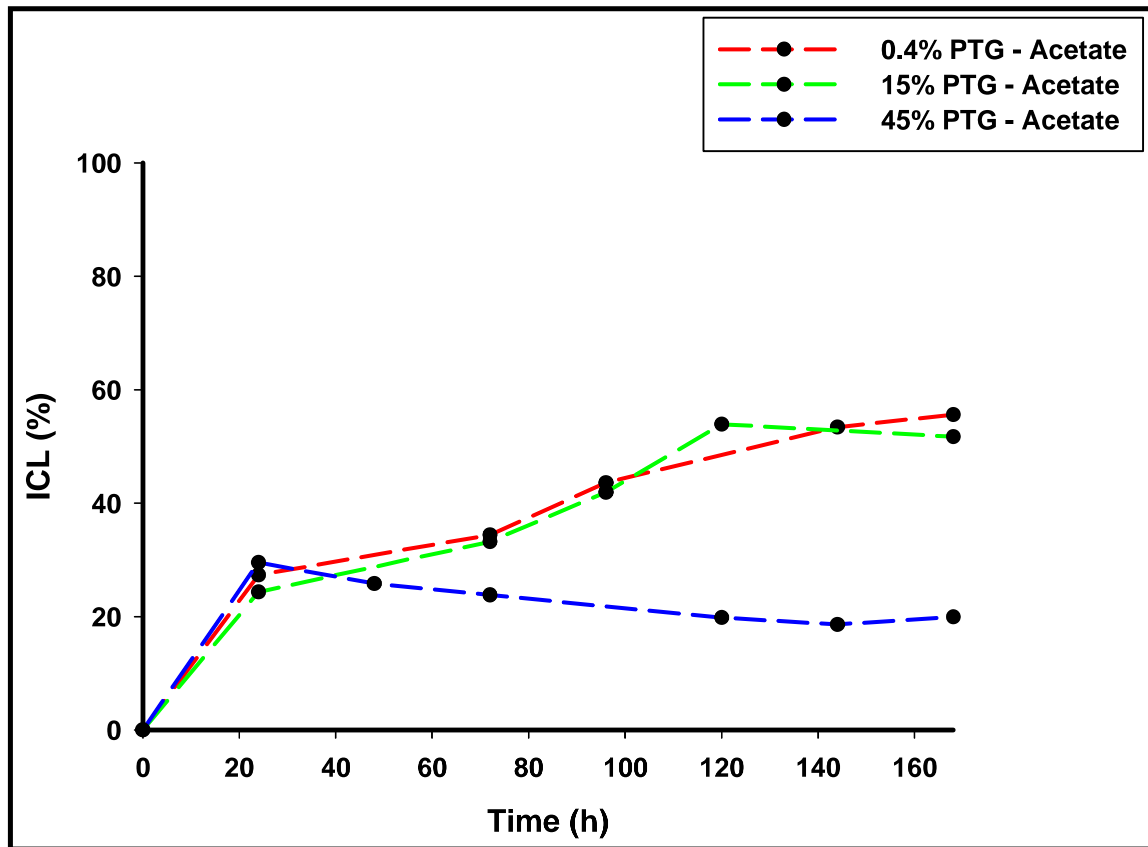

Fig. 5b Intracellular lipid (ICL) content of *Mucor circinelloides* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the absence of acetate respectively.

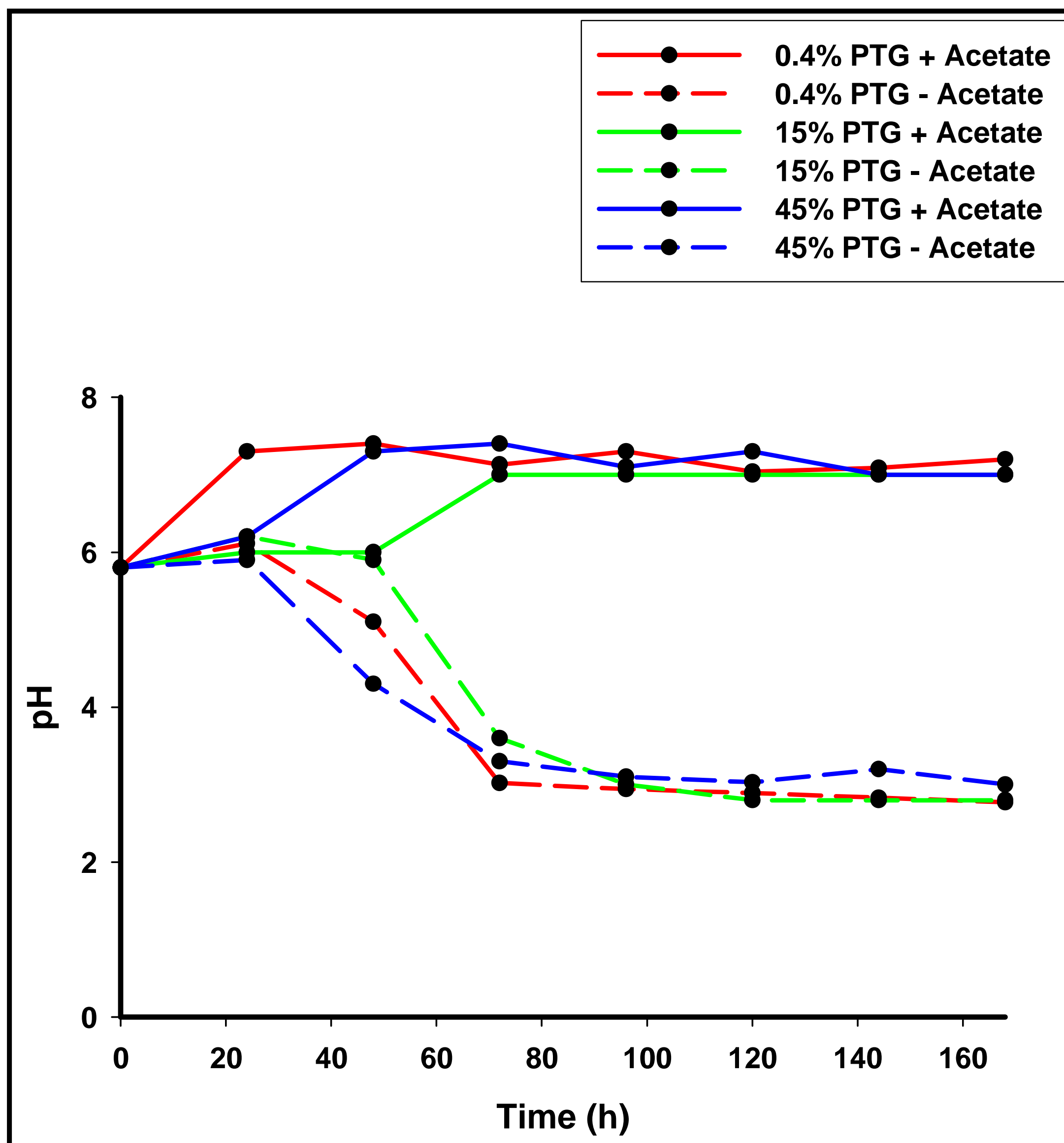

Fig. 6 Changes in pH of *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence (solid line) and absence (broken line) of acetate respectively.

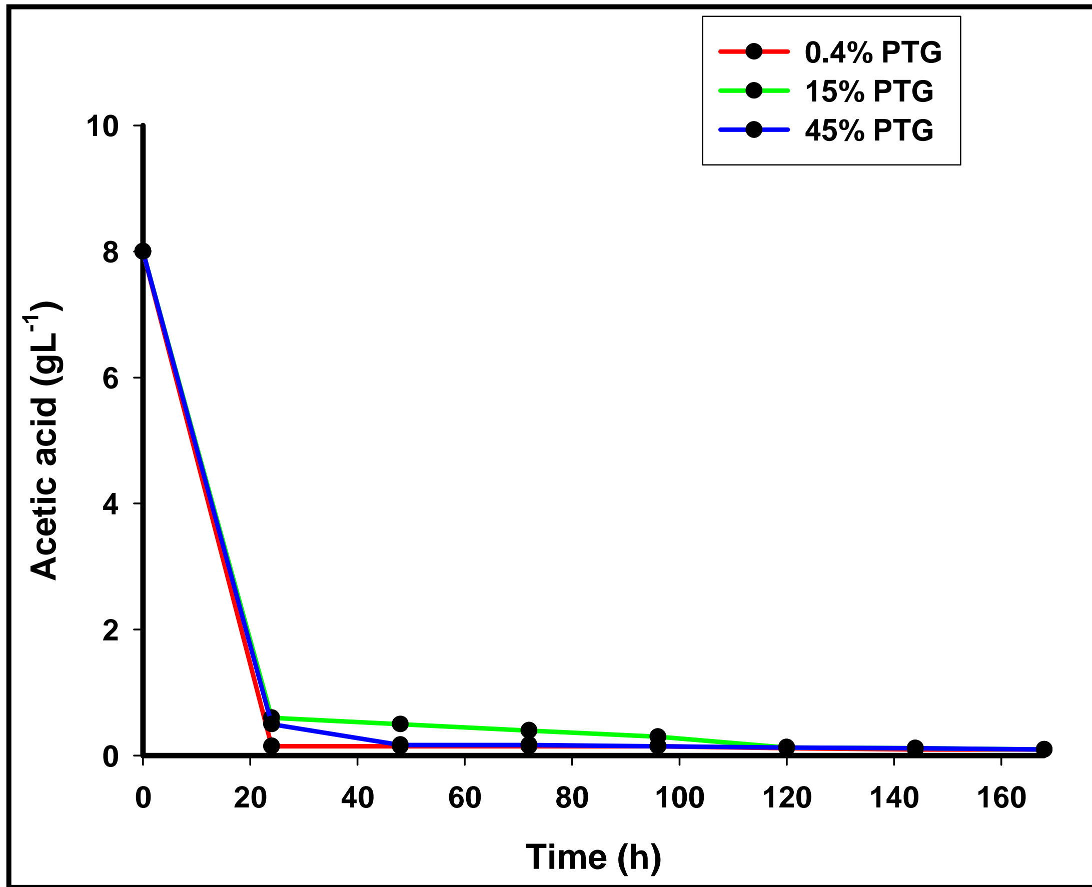

Fig. 7 Changes in acetic acid concentration of *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides respectively.

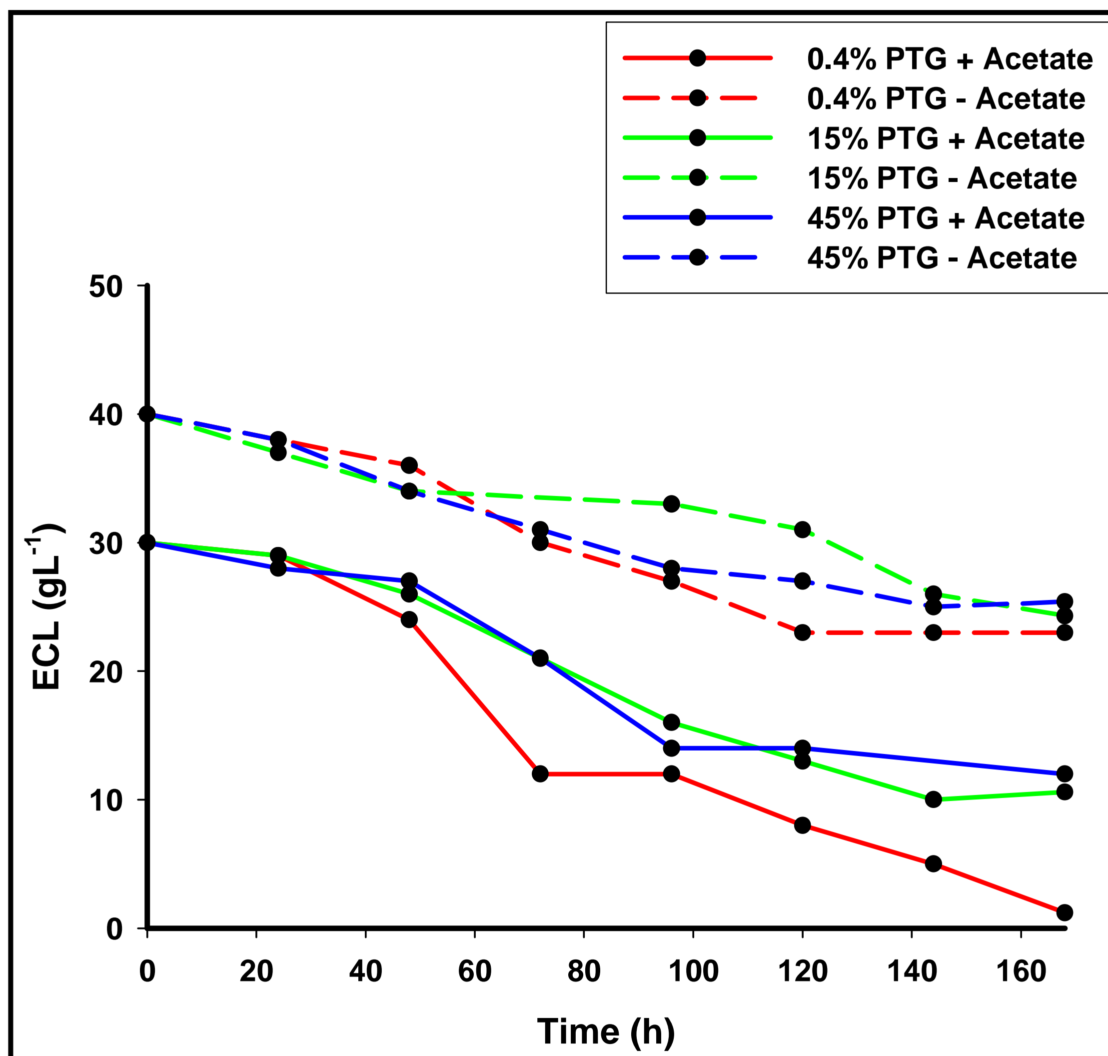

Fig. 8 Oil utilization by *Cryptococcus curvatus* grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence (solid line) and absence (broken line) of acetate respectively. ECL: extracellular lipid content.

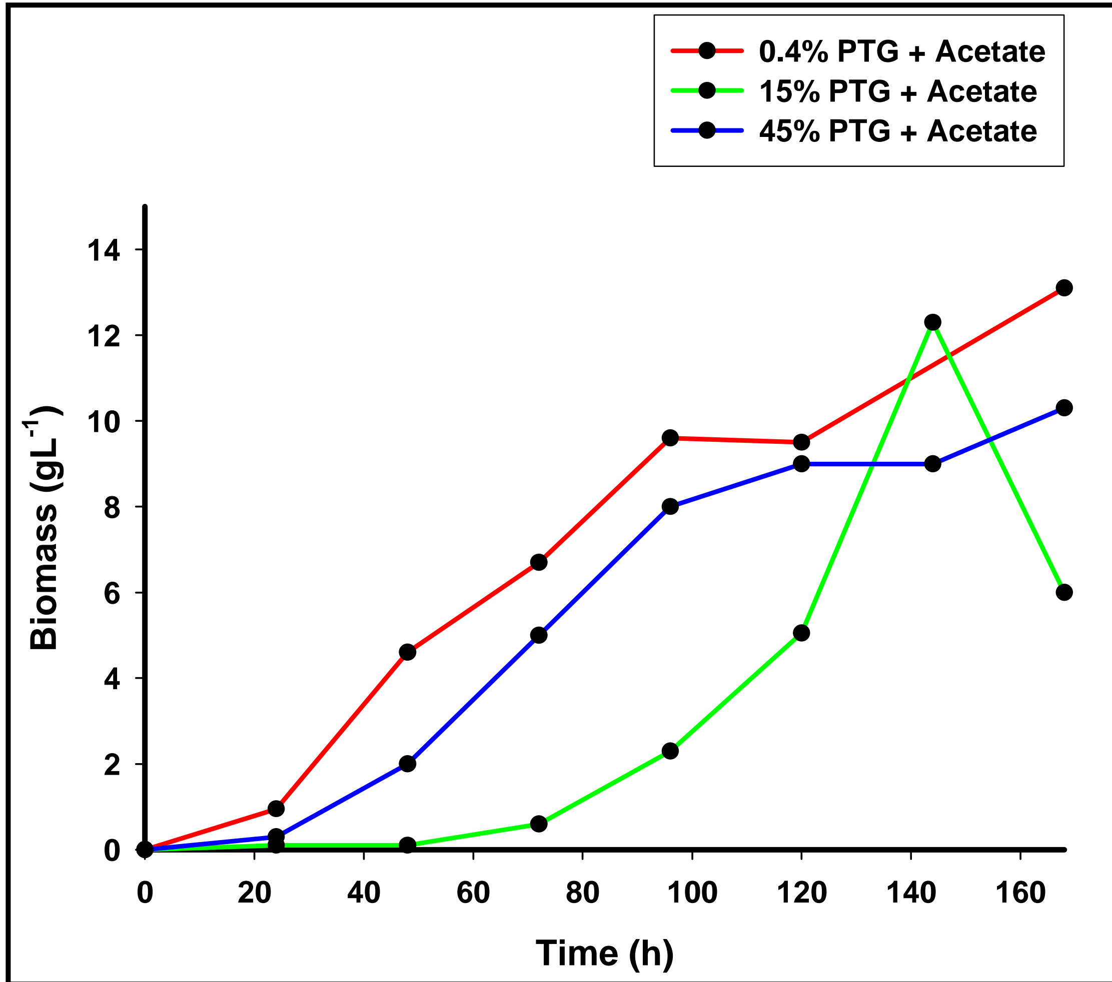

Fig. 9a Biomass production by *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence of acetate respectively.

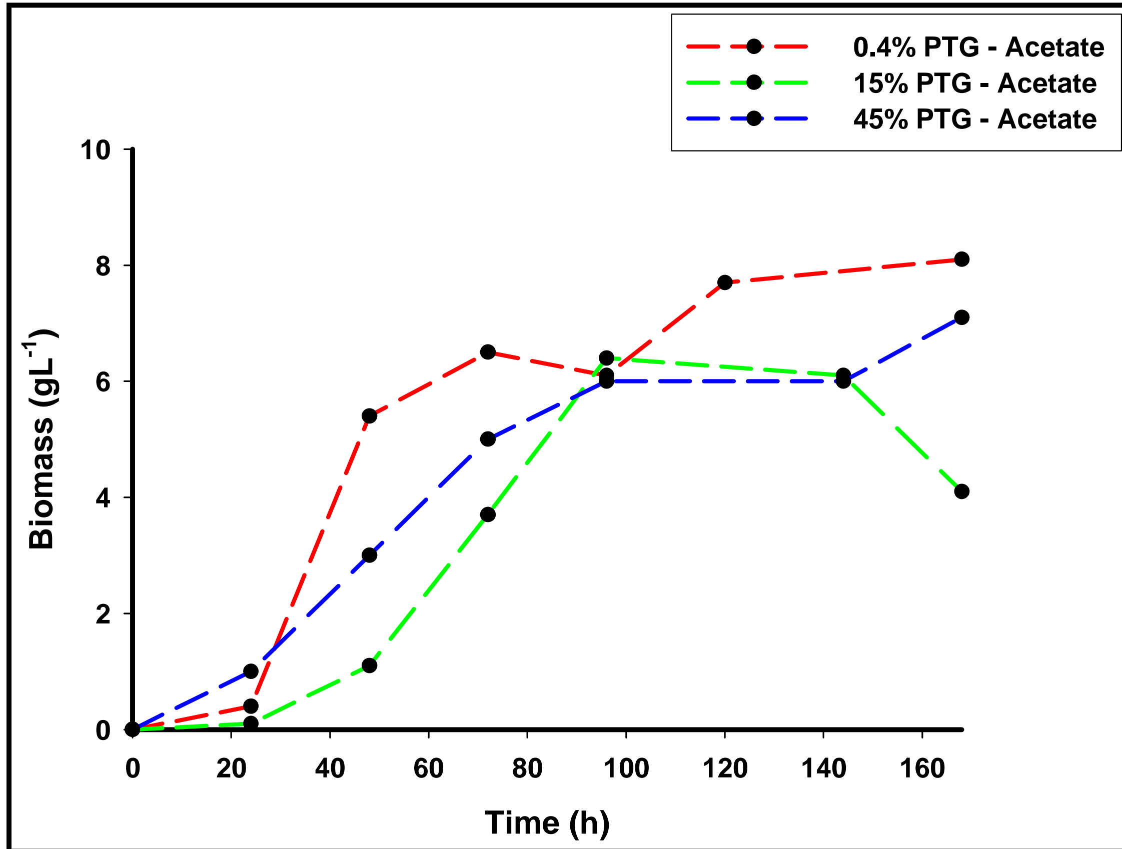

Fig. 9b Biomass production by *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the absence of acetate respectively.

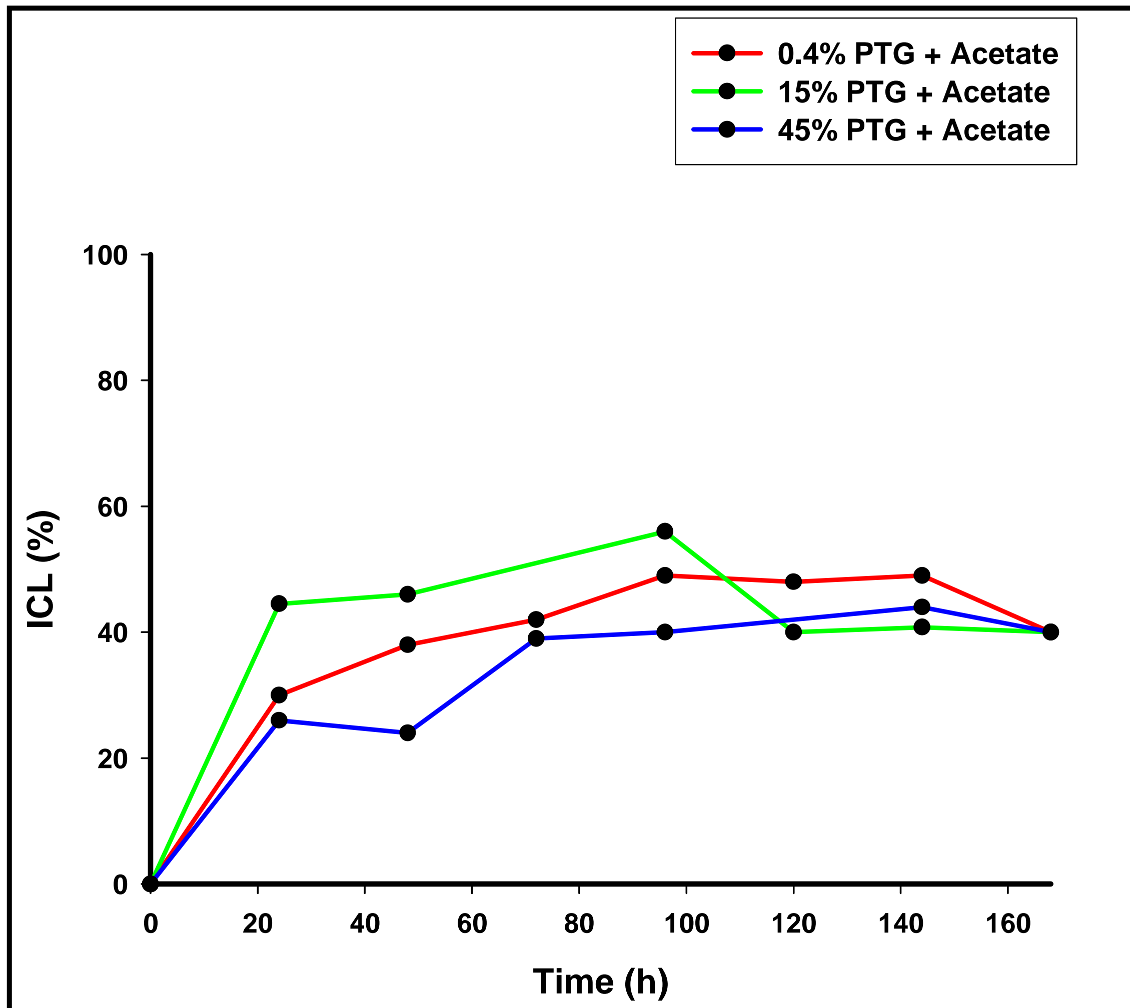

Fig. 10a Intracellular lipid (ICL) content of *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the presence of acetate respectively.

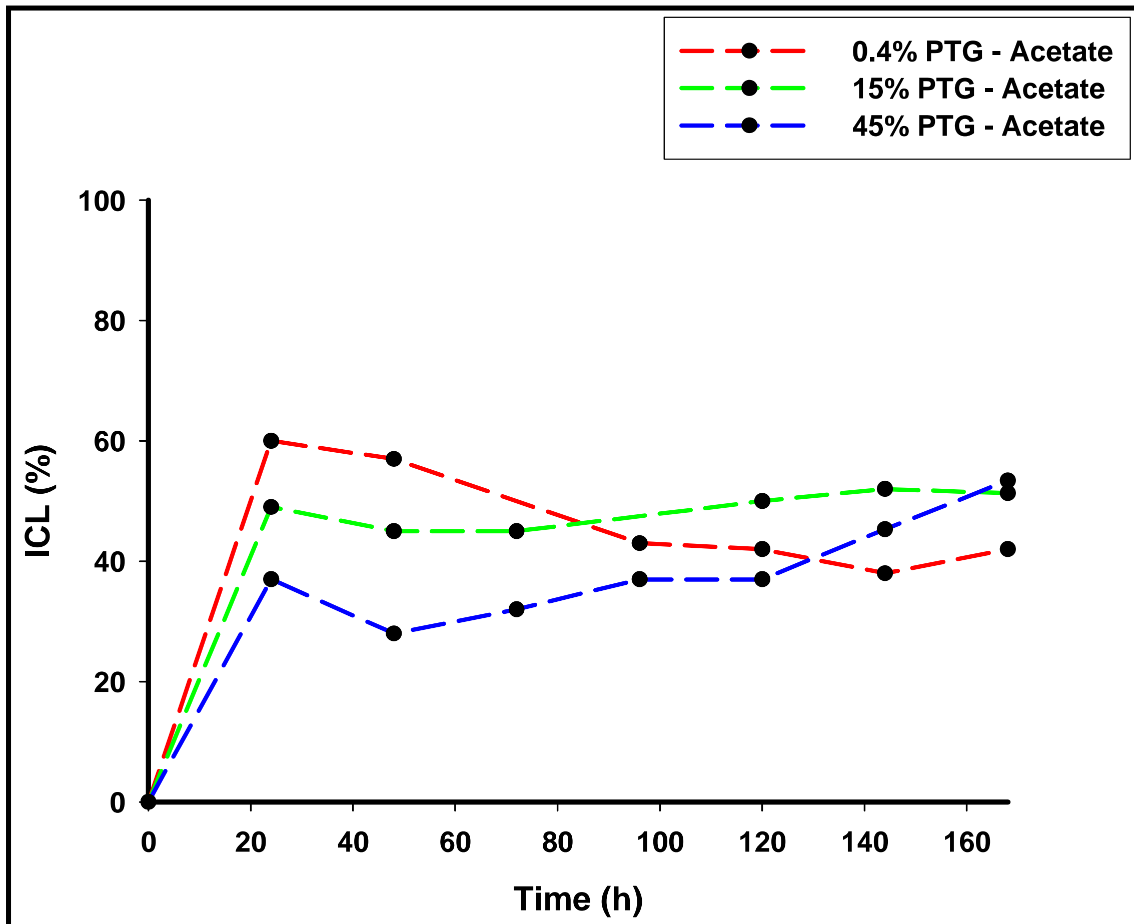

Fig. 10b Intracellular lipid (ICL) content of *Cryptococcus curvatus* cultures grown in shake flasks for seven days at 30 °C containing 0.4%, 15% and 45% polymerized triglycerides in the absence of acetate respectively.

CHAPTER 3

Anti-mitochondrial activity of oxidized palm oil breakdown products

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3.1 Abstract

Oxidized oil breakdown products such as aldehydes are major sources of reactive oxygen species (ROS). Studies have shown that ROS has anti-mitochondrial action. It was also reported that acetylsalicylic acid (ASA), an anti-inflammatory and anti-mitochondrial drug, targets structure development and functions of yeasts, needing elevated levels of mitochondrial activity. Using antibody probes we previously reported that sporangia of *Mucor circinelloides* also contain increased mitochondrial activity yielding high levels of 3-hydroxy (OH) oxylipins. This was however not found in *Mortierella alpina* (subgenus *Mortierella*). In this study we report that oxidized palm oil breakdown products and ASA also targets sporangium development of *M. circinelloides* selectively while hyphae, with lower levels of mitochondrial activity, are more resistant. Similar results were obtained when the anti-inflammatory compounds benzoic acid, ibuprofen, indomethacin and salicylic acid were tested. Here, oxidized oils and anti-inflammatory, anti-mitochondrial drugs exerted similar effects on this dimorphic fungus as found under oxygen limited conditions. Interestingly, sporangium development of *M. alpina* was found not to be selectively targeted by these compounds. *Mortierella alpina*, which could not exhibit dimorphic growth under oxygen limitation conditions, was also more sensitive to the anti-inflammatory drugs when compared to *M. circinelloides.* These results prompt further research to assess the applicability of these anti-mitochondrial antifungals to protect plants and animals against *Mucor* infections.

Key words: Anti-inflammatory compounds, Anti-mitochondrials, Mitochondrial activity, *Mucor circinelloides*, Sporangia.

3.2 Introduction

During frying, oil undergoes deteriorative changes due to hydrolysis, oxidation and eventually polymerization (Frankel 1998). Oil oxidation breakdown products such as aldehydes have been reported as major sources of reactive oxygen species (ROS) exhibiting anti-mitochondrial action in biological systems (Costantini et al. 2000; Lin et al. 2008). Action of ROS on the mitochondria was reported to lead to the opening of the permeability transition pore (PTP) resulting in a decrease in mitochondrion function ($\Delta\psi_m$) due to an increased proton influx through the pore (Szewcyk and Wojtczack 2002).

Furthermore, oxylipin studies exposed a link between the anti-inflammatory drug acetylsalicylic acid (ASA), mitochondrial activity and sexual and asexual reproduction in respiratory and fermentative yeasts (Kock et al. 2007). Here, yeast structure and function, which are dependent on increased mitochondrial activity, were found to be more sensitive to ASA than those parts with less mitochondrial activity. Therefore, ASA probably targets yeast structures and functions with enhanced mitochondrial activity. It was found that yeasts with an additional anoxic fermentative energy generating metabolism are more resistant to ASA compared to yeasts that are only respiratory driven (Leeuw et al. 2007; Swart et al. 2008). Here, fermentation probably provides sufficient energy for cell metabolism. It is therefore not surprising that ASA addition simulates anoxic growth conditions in most of these yeasts. Oxidized oils are therefore also expected to target structures with increased mitochondrial activity.

The association of increased mitochondrial levels with certain structures of the Mucorales has been reported. Here, increased mitochondrial activity expressed as 3-OH oxylipin production has been reported in sporangia of various members of the Mucorales i.e. *Absidia, Actinomucor, Cunninghamella*, *Mortierella* (subgenus *Micromucor*), *Mucor*, *Pilobolus* and *Rhizomucor* (Kock et al. 2001; Strauss et al. 2000). Strikingly, no increase in these oxylipins was found in sporangia of *Mortierella alpina* (subgenus *Mortierella*) (Strauss et al. 2000).

In this study the conserved status of the ASA yeast inhibition phenomenon was further assessed i.e. ASA targeting structures with enhanced mitochondrial activity. Consequently, the influence of ASA, oxidized oils and other anti-inflammatory drugs -, also known for their anti-mitochondrial activity in mammalian studies (Al-Nasser 2000; Costantini et al. 2000; Norman et al. 2004; Somasundaram et al. 2000; Tanaka et al. 2005) -, on sporangium development in *M. circinelloides* and *M. alpina* was researched.

3.3 Materials and methods

Strains used and cultivation

Mortierella alpina (subgenus *Mortierella*) UOFS 2802 and *Mucor circinelloides* var. *circinelloides* UOFS 2803 were used in the study and is preserved at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa. The fungus was streaked out on yeast malt (YM) agar (Wickerham 1951) and cultivated at 25 ^{0}C in Petri dishes until asexual sporulation in sporangia was observed.

Bio-assay preparation and anti-inflammatory drug application

The bio-assay is based on the agar diffusion method where activity of anti-inflammatory drugs is measured along a concentration gradient across the agar plate (i.e. from position of addition) by observing the growth inhibition-zone (i) and changes in density of sporangia formation towards outside of plate (i.e. towards lower inhibitor concentrations).

Cells from both fungi were scraped from above YM-agar grown cultures and suspended in sterilized dH_2O from where 0.2 ml were streaked out on YM–agar (0.5% agar m/v) contained in Petri dishes to produce a uniform lawn that completely covers the agar surface. Next, a well of 0.5 cm in diameter and depth was constructed aseptically in the middle of the agar plate followed by the addition (46 µl) of the following anti-inflammatory compound solutions (Mackowiak 2000; Pina-Vaz et al. 2000; Sagone and Husney 1987): ASA (aspirin: Sigma, Steinheim, Germany), ibuprofen (Sigma-Aldrich, Steinheim, Germany), indomethacin (Sigma, Steinheim, Germany), salicylic acid (The British Drug Houses Ltd., Poole, England) and benzoic acid (The British Drug Houses Ltd., Poole, England) – compound concentration: 2 g compound in 25 ml 96% ethanol (Merck, Gauteng, South Africa). In addition, controls were constructed by the addition of similar amounts of 96% ethanol to wells. All plates were incubated at 25 ^{0}C until an inhibition zone (i), cream to yellow zone devoid of sporangia (if observed) as well as zone with sporangia could be observed (usually after 48 h). Each zone was then subjected to microscopic analysis using a light microscope (Axioplan, Zeiss, Göttingen, Germany) coupled to a Colorview Soft Imaging System (Münster, Germany).

Breakdown products extraction

Ethanol was used to extract breakdown products from oxidized oil (at PTG levels of 0.4%, 15% and 45%) using the method of Anelich (2000). Briefly, 200 ml ethanol was used to extract breakdown products from 50 ml of oils at different PTG levels. The extract was evaporated and resuspended in ethanol – compound concentration 2 g compound in 25 ml 96% ethanol and applied to bioassays as described before.

Mitochondrion mapping

This was performed according to Ncango et al. (2008). In short, sporulating cells obtained from 48 h grown cultures of *Mortierella alpina* and *Mucor circinelloides* var. *circinelloides* on YM plates at 25 ^{0}C were washed with PBS in a 2 ml plastic tube to get rid of agar and debris and then treated with 31 µl Rhodamine 123 (Rh123; Molecular Probes, Invitrogen Detection Technologies, Eugene, Oregon, U.S.A.). Cells were treated for 1 h in the dark at room temperature after which cells were washed again with PBS to remove excess stain. These were fixed on microscope slides in Dabco (Sigma-Aldrich) and viewed with a confocal laser scanning microscope (Nikon TE 2000, Japan). Rh123 is a cationic lipophilic mitochondrion stain used to map mitochondrion function ($\Delta\psi_m$) selectively. This is ascribed to the highly specific attraction of this cationic fluorescing dye to the relative high negative electric potential across the mitochondrion membrane in living cells (Armstrong 2006; Costantini et al. 2000; Johnson et al. 1980). With this dye, a high mitochondrion $\Delta\psi_m$ is signified by a yellow-green fluorescence (collected at 450 nm), while a low mitochondrion $\Delta\psi_m$ is signified by a red fluorescence collected at 625 nm.

Quantitative measurement of metabolic state

The XTT colorimetric assay was used to determine the activity of mitochondrion dehydrogenases, an indicator of metabolic activity (Bachmann et al. 2002; Moss et al. 2008; Strauss et al. 2007), in cells of *M. ciricinelloides* scraped from the zone containing both hyphae and sporangia (H+SP) and zone containing mainly hyphae (H), respectively (Fig. 2). This was only performed for *Mucor* since *Mortierella* did not show selective sporangium inhibition zones or selective increase in mitochondrial activity as determined by Rh123 staining. Equal amounts (0.5 g) of cells from respective zones were suspended in 5 mL phosphate buffer. Following this, 2.5 ml XTT [2.5 g XTT (Sigma Chemicals, St. Louis, Mo.) in 1 L Ringer's lactate solution] and 400 µl menadione (Fluka, 1 mM in acetone) were added to each of these replicates. The cells were incubated at 37 ºC for 3 h in the dark. A hundred and fifty micro liter of the formazan product in the supernatant was transferred to wells of a 96-well flat bottom polystyrene microtiter plate (Corning Incorporated, NY, USA). Plates were analyzed by measuring the formazan product in the supernatant spectrophotometrically in terms of optical density at 492 nm using a Labsystems iEMS reader (Thermo BioAnalysis, Helsinki, Finland). This was repeated on cells grown under oxygen limitation and normal oxic conditions as described. Experiments were performed in at least triplicate. No growth was obtained for *M. alpina* under oxygen limitation conditions and this experiment could therefore not be performed.

Oxygen inhibition studies

Bio-assays for both fungi were placed in an Oxoid anaerobic jar (2.5 L; Oxoid, Cambridge, UK) and cultivated at 25 ^{0}C for 48 h. The Anaerocult A system (Merck, Darmstadt,

Germany) was used to remove most oxygen. An anaerotest test-strip (Merck, Darmstadt, Germany) moistened with a drop of water, was included to monitor the anaerobic atmosphere in the sealed jar. Control bio-assays were placed in normal atmosphere all at 25 ^{0}C for 48 h.

3.4 Results and discussion

Since ASA, also a mitochondrial inhibitor (Kock et al. 2004, 2007), was found to preferentially inhibit structures (cells within cells i.e. asci) with elevated levels of mitochondrial activity in yeasts, it was decided to assess if the same is true for sporangia ("cells within cells") in *Mucor circinelloides* var. *circinelloides* as well as *Mortierella alpina* (subgenus *Mortierella*).

Mitochondrion activity mapping

In 1998, we developed 3-OH oxylipin antibody probes from chemically synthesized 3-OH oxylipins (Bhatt et al. 1998) specific for these oxylipins irrespective of carbon chain length and level of desaturation (Kock et al. 1998). When added to cultures of different *Mucor* species, including *Mucor circinelloides* var. *circinelloides*, we found that sporangia of all strains tested contain elevated levels of 3-OH oxylipins when compared to the asexual cells (Kock et al. 2001; Strauss et al. 2000). However, *Mortierella alpina* (subgenus *Mortierella*) was an exception to this rule showing no increased levels of these oxylipins in sporangia i.e. hyphae and sporangia contain similar low amounts of oxylipins.

Since these oxylipins are produced by mitochondria (Ciccoli et al. 2005; Venter et al. 1997) we decided to stain sporangia of *Mucor circinelloides* var. *circinelloides* as well as *Mortierella alpina* (subgenus *Mortierella*) with Rh123, a mitochondrion transmembrane potential ($\Delta\psi_m$) probe (Johnson et al. 1980; Ludovico et al. 2001; Ncango et al. 2008) to assess the levels of mitochondrial activity. With the aid of confocal laser scanning microscopy we found that the sporangia of *Mucor* indeed showed a much higher affinity for the stain compared to the hyphae (Fig. 1*a,b*) while sporangia of *Mortierella* did not preferentially stain compared to hyphae i.e. hyphae and sporangia contain similar mitochondrial activity (results not shown).

Effect of anti-inflammatory drugs

When the anti-inflammatory drugs ASA, benzoic acid, ibuprofen, indomethacin and salicylic acid were added to the *Mucor* bio-assays (Figs. 2,3; Table 1), similar inhibition zones were formed followed by a sporangium inhibition zone and then followed on the outskirts by hyphae plus sporangium zone. Similar results were found for all anti-inflammatory drugs tested. Interestingly, when looking at the plates of *Mucor* macroscopically, different structured inhibitory patterns (circles) were found for the different drugs tested over concentration gradients while the ethanol control was not inhibitory to growth or sporangium development (Fig. 3; Table 1). However, sporangium development of *Mortierella* was not selectively inhibited when these drugs were added. Here only an inhibitory zone (no growth) and growth zone with sporangia already formed on the border of inhibition zone could be observed (Fig. 4).

The inhibition of sporangium development by ASA via mitochondrial inhibition in *Mucor* was also reflected in the XTT assay that indicates mitochondrial dehydrogenase activity (i.e. metabolic rate; Fig. 5). Here it is clear that the zone containing only hyphae and no sporangia is characterized by about half the mitochondrial activity when compared to the same amount of cells consisting of both hyphae and sporangia. This supports the increased levels of mitochondrial activity in sporangia found previously with Rh123 (Fig. 1). No such experimentation could be performed on *Mortierella alpina* since no selective sporangium inhibition zone could be observed.

Effect of oxidized oil breakdown products

We found that oxidized oil breakdown products exerted the same effects as those observed for the anti-inflammatory drug ASA (Fig. 2). In *M. circinelloides* selective inhibition of the sporangia was observed (Figs. 2,3) which was not the case with *M. alpina* (similar to ASA effect Fig. 2).

Oxygen inhibition studies

Since sporangium development in *Mucor* seems to be dependent on increased mitochondrial activity, we next decided to perform mitochondrial inhibition tests by limiting oxygen (anoxic) supply to cultures of *Mucor*. Here again no sporangia but only hyphae and single cells were observed in *Mucor* when compared to oxic conditions (Fig. 6). This is in accordance with results obtained in the anti-inflammatory and oxidized oil breakdown products oxic experiments (Figs. 2, 3). Strikingly, similar experiments on *Mortierella alpina*

show that both sporangium and hyphal development have similar sensitivities towards oxygen depletion. Here both these growth stages were inhibited under anoxic conditions and no preference towards hyphal and single cell growth was observed as was the case in *Mucor* (results not shown).

3.5 Conclusions

It was found that known anti-mitochondrial and anti-inflammatory compounds such as ASA, ibuprofen, indomethacin, salicylic- and benzoic acid inhibit both hyphal and sporangium development in *Mucor circinelloides* and *Mortierella alpina*. Interestingly, similar anti-mitochondrial activity was also observed when oxidized oil breakdown products were used in the bio-assay. In *Mucor*, sporangium development was most sensitive probably due to its increased dependence on mitochondrial activity when compared to hyphal cells. On the other hand, hyphae may also be more resistant to these mitochondrial inhibitors since they may also obtain energy through alternative anoxic fermentation. In *Mortierella*, sporangia do not show increased levels of mitochondrial activity compared to hyphae and are not selectively inhibited by anti-mitochondrial compounds or oxygen limitation. This may be ascribed to similar susceptibilities of both sporangia and hyphae to these mitochondrial inhibitors. Inhibition zones formed by anti-mitochondrials in *Mucor* (Fig. 3) were much smaller compared to *Mortierella* (Fig. 4) which may be ascribed to the additional fermentative metabolism present in the former (Orlowski 1991).

Anti-inflammatory drugs may have a dual action in combating fungi that also cause disease while at the same time decreasing the host inflammatory response. This may be of value

especially when treating *Mucor* infections in humans (Iwen et al. 2007; Ribes et al. 2000). It is interesting to note that salicylic acid also enhances postharvest disease resistance in certain fruit (Cao et al. 2006; Zeng et al. 2006). Is it possible that this anti-inflammatory drug also has a dual action in plants – combating disease while increasing the plant's disease resistance?

Oxidized palm oil (45% PTG) extracts exerted similar anti-mitochondrial effects compared to ASA. This supports the results obtained (Chapter 2) for *M. circinelloides* when fed with these oils. Here, mitochondria are affected negatively thereby inhibiting energy production necessary for growth, lipid utilization and accumulation (Wynn and Ratledge 2000). Is it possible that heavily oxidized oils may find application as anti-mitochondrial antifungal agents? This should next be assessed.

3.6 Acknowledgements

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3.8 Table

Table 1. The influence of anti-inflammatory drugs on growth and sporangium development of *Mucor circinelloides* var. *circinelloides* UOFS 2803 after 48 h of growth at 25 ^{0}C.

	Zones		
Compound	**I**	**H**	**H+SP**
Acetylsalicylic acid	√	√	√
Benzoic acid	√	√	√
Ibuprofen	√	√	√
Indomethacin	√	√	√
Salicylic acid	√	√	√
Oxidized oil	√	√	√
EtOH	X	X	√

Note: I, Inhibition zone; H, Hyphal zone; H+SP, Hyphae + Sporangia zone; EtOH, Ethanol; √, zone present; X, zone not present.

3.9 Figures

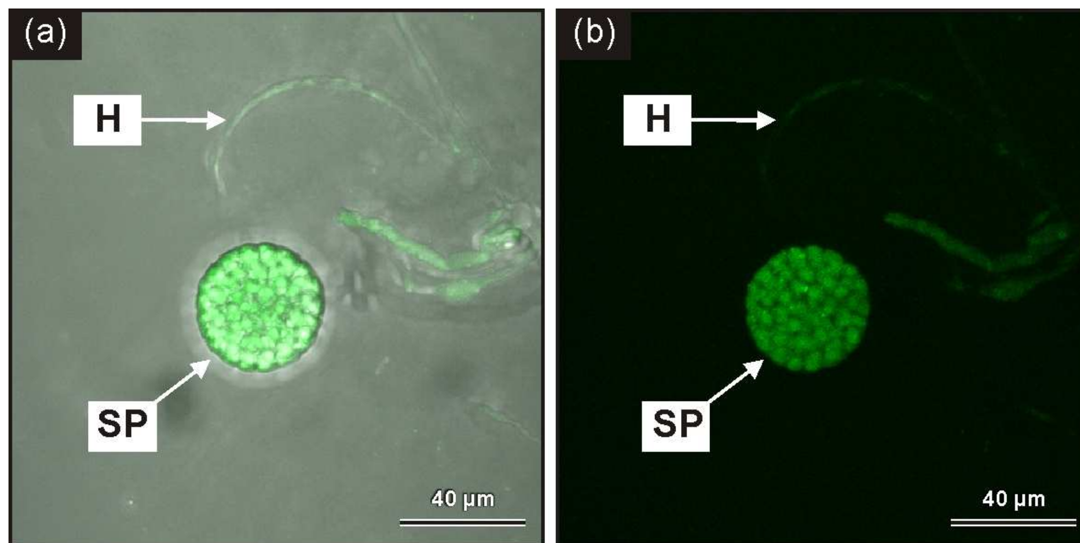

Fig. 1 Confocal laser scanning micrograph of fluorescing structures of *Mucor circinelloides* var. *circinelloides* UOFS 2803 after rhodamine 123 treatment. (a) Light superimposed on laser microscopy. (b) Only laser micrograph. H, hyphae; SP, sporangium.

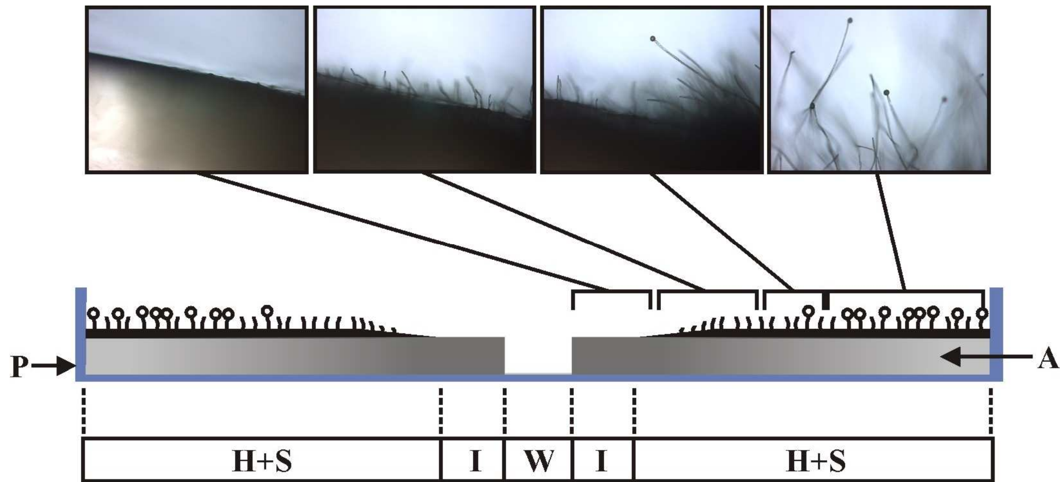

Fig. 2a A diagrammatic view of a Petri dish (P) displaying a well (W), inhibition zone (I) and growth zone containing at first only hyphae (H) and eventually hyphae and sporangia (H+SP) as the aspirin concentration decreases across the agar. Light micrograph inserts display this trend in *Mucor circinelloides* var. *circinelloides* UOFS 2803.

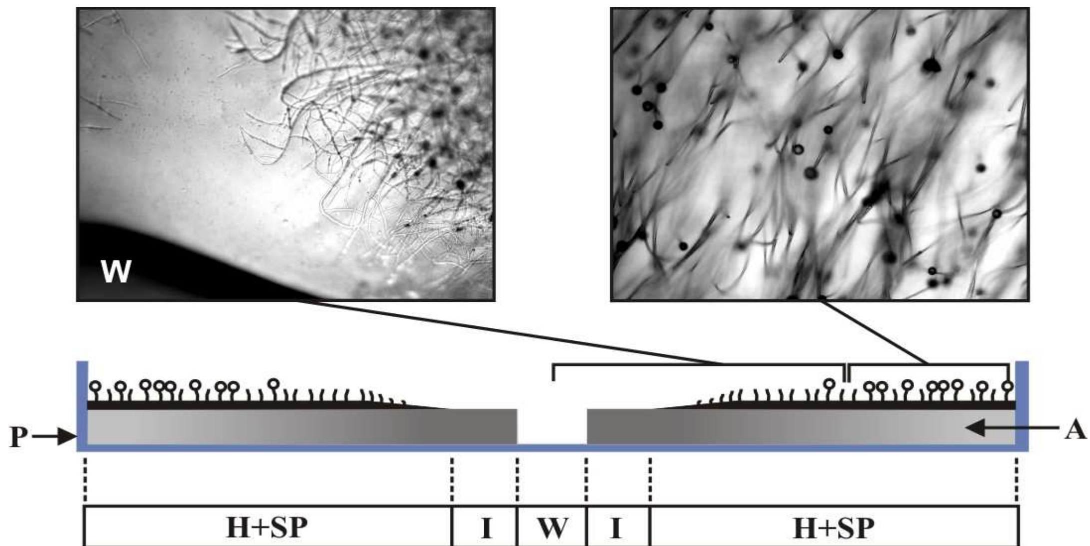

Fig. 2b A diagrammatic view of a Petri dish (P) displaying a well (W), inhibition zone (I) and growth zone containing at first only hyphae (H) and eventually hyphae and sporangia (H+SP) as the oxidized palm oil concentration decreases across the agar. Light micrograph inserts display this trend in *Mucor circinelloides* var. *circinelloides* UOFS 2803.

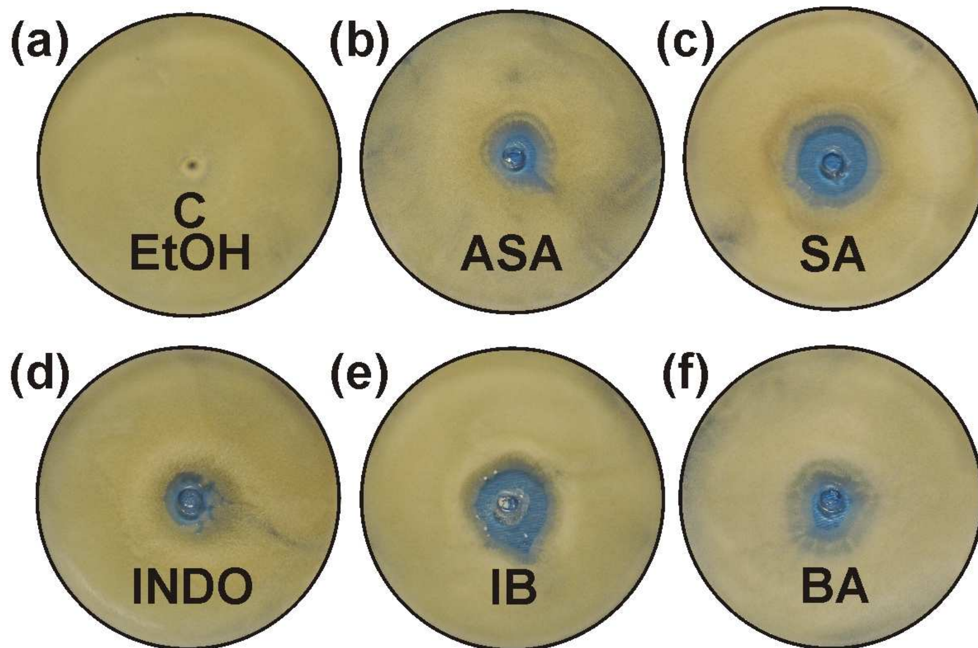

Fig. 3 Bio-assays with central well showing the effects of anti-inflammatory drugs on growth of *Mucor circinelloides* var. *circinelloides* UOFS 2803. (a) Ethanol (EtOH) control (C); (b) Acetylsalicylic acid (ASA) treatment; (c) Salicylic (SA) acid treatment; (d) Indomethacin (INDO) treatment; (e) Ibuprofen (IB) treatment; (f) Benzoic acid (BA) treatment.

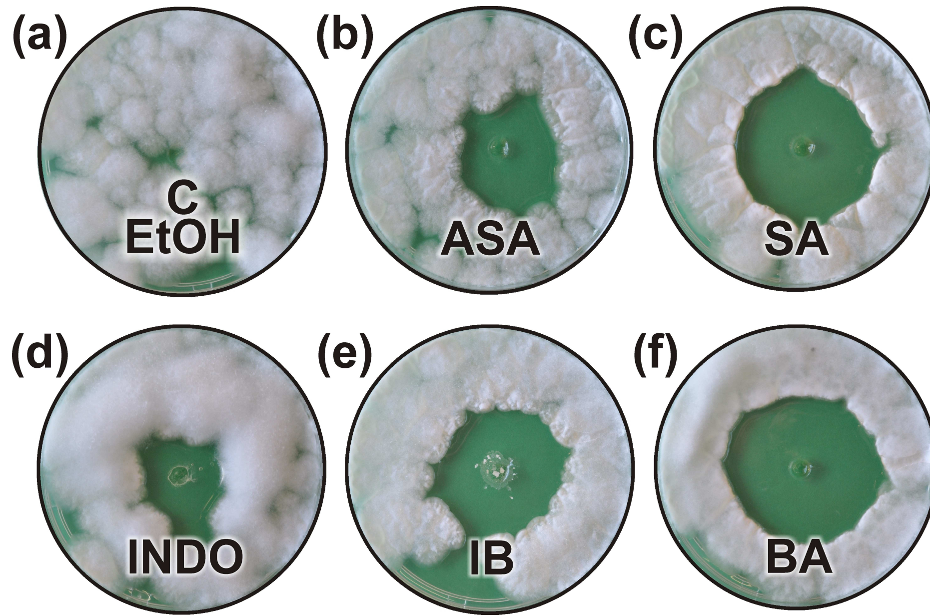

Fig. 4 Bio-assays with central well showing the effects of anti-inflammatory drugs on growth of *Mortierella alpina* UOFS 2802. (a) Ethanol (EtOH) control (C); (b) Acetylsalicylic acid (ASA) treatment; (c) Salicylic (SA) acid treatment; (d) Indomethacin (INDO) treatment; (e) Ibuprofen (IB) treatment; (f) Benzoic acid (BA) treatment.

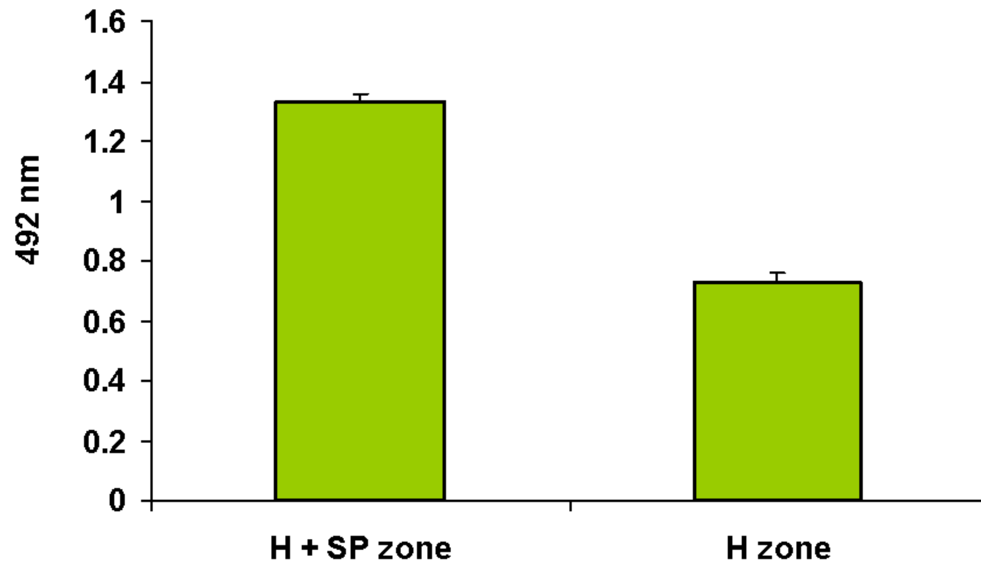

Fig. 5 XTT formazan signal (measured at 492 nm) produced by *Mucor circinelloides* var. *circinelloides* UOFS 2803 when present in hyphal and sporangium (H+SP) mode and only hyphal (H) mode at a concentration of 0.1 g ml^{-1} cells. The error bars indicate standard errors of the means.

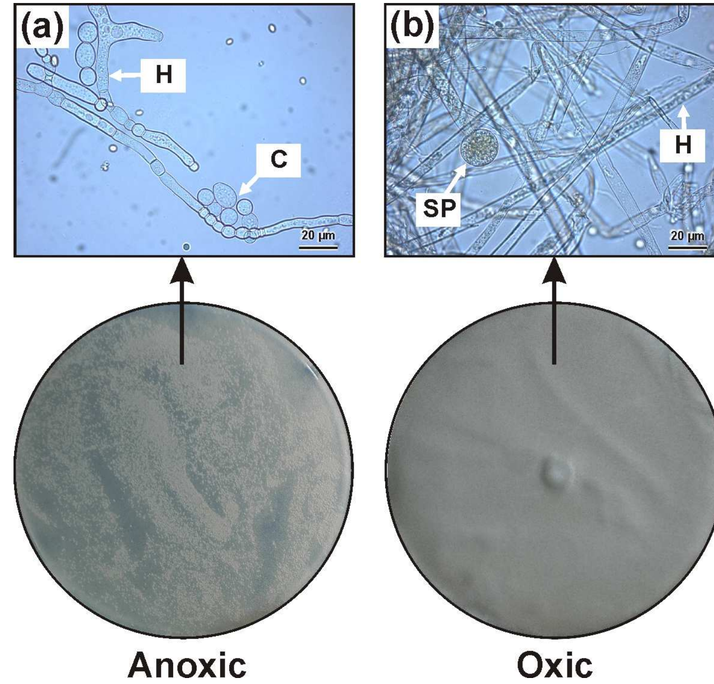

Fig. 6 The influence of oxygen limitation on hyphal and sporangium formation in *Mucor circinelloides* var. *circinelloides* UOFS 2803 when cultivated on agar plates at 25 ^{0}C for 48 h. (a) Growth under oxygen limitation (anoxic) conditions. (b) Growth under normal atmospheric (oxic) conditions. Both macroscopic and microscopic images are shown. H, Hyphae; C, single cells; SP, Sporangia.

Summary

Edible oils such as sunflower oil, soybean oil and palm oil are used today in the frying of food. During the frying process, various changes such as removal of antioxidants, hydrolysis, oxidation and polymerization occur in these oils. These reactions are responsible for a variety of physical and chemical changes observed in the oil during frying and may lead to the formation of breakdown products which include polar compounds (PCs) and polymerized triglycerides (PTGs). South African regulations state that oils that contain 16% and more PTGs and 25% and more PCs are harmful to human health. These oils may cause cancer and diarrhoea in humans and animals. However, little is known regarding the effect of oxidized oils on fungi. The oleaginous fungi *Cryptococcus curvatus* and *Mucor circinelloides* were used to determine the effect of palm oil breakdown products, measured as PTGs on lipid turnover, growth and morphology. In *Mucor circinelloides* we found, after seven days of growth, a decrease in biomass, lipid utilization and accumulation at increased PTG levels, at low and neutral pH. An increase in PTG concentration also influenced the morphology of *M. circinelloides*. Protrusions were observed on cell surfaces when grown on oil with 45% PTGs and not when the fungus was grown on fresh oil with 0.4% PTGs. In *C. curvatus* there was also a decrease in oil utilization and biomass production at increased PTG levels, at low and neutral pH. An increase in oil accumulation was observed at low pH while it remained constant at neutral pH for all PTG levels tested. Hairy and warty protuberances on cell surfaces were observed when *C. curvatus* was grown on oils with 15% and 45% PTGs, respectively. It is concluded that the changes observed in lipid turnover and morphology in both fungi are due to the presence of palm oil breakdown products. Oxidized oil breakdown products such as aldehydes are major sources of reactive oxygen species (ROS). Studies have shown that ROS has anti-mitochondrial action. It was also reported that acetylsalicylic acid (ASA), an anti-inflammatory and anti-mitochondrial drug, targets structure development and functions of yeasts, needing elevated levels of mitochondrial activity. Using antibody probes

it was previously reported that sporangia of *Mucor circinelloides* also contain increased mitochondrial activity yielding high levels of 3-hydroxy (OH) oxylipins. This was however not found in *Mortierella alpina* (subgenus *Mortierella*). In this study, it is reported that oxidized palm oil breakdown products and ASA also targets sporangium development of *M. circinelloides* selectively while hyphae, with lower levels of mitochondrial activity, are more resistant. Similar results were obtained when the anti-inflammatory compounds benzoic acid, ibuprofen, indomethacin and salicylic acid were tested. Here, oxidized oils and anti-inflammatory, anti-mitochondrial drugs exerted similar effects on this dimorphic fungus as found under oxygen limited conditions. Interestingly, sporangium development of *M. alpina* was found not to be selectively targeted by these compounds. *Mortierella alpina*, which could not expose dimorphic growth under oxygen limitation conditions, was also more sensitive to the anti-inflammatory drugs when compared to *M. circinelloides.* These results prompt further research to assess the applicability of these anti-mitochondrial antifungals to protect plants and animals against *Mucor* infections. It is concluded that indications exist that oxidized palm oil breakdown products target mitochondrial function. This may explain the inhibitory effect of these compounds on fungal growth, lipid turnover and altered cell wall morphology.

Key words: Anti-inflammatory compounds, Anti-mitochondrials, *Cryptococcus curvatus*, Lipid turnover, Mitochondrial activity, Morphology, *Mucor circinelloides*, Oxidized oils, Polymerized triglycerides, Sporangia.

Opsomming

Eetbare olies, soos sonneblom-, soja- en palmolie is van die olies wat vandag in die braai van voedsel gebruik word. Gedurende die braaiproses vind verskeie veranderinge, soos die verwydering van anti-oksidante, hidrolise, oksidasie en polimerisering, in hierdie olies plaas. Hierdie reaksies is verantwoordelik vir 'n verskeidenheid fisiese en chemiese veranderings wat in die olie tydens die braaiproses waargeneem word en wat mag lei tot die vorming van afbraakprodukte, insluitend polêre komponente (PKs) en gepolimeriseerde trigliseriede (PTGs). Suid-Afrikaanse wetgewing bepaal dat olies wat 16% en meer PTGs en 25% en meer PKs bevat, skadelik vir menslike gesondheid is. Hierdie olies mag kanker en diariëe in mense en diere veroorsaak. Daar is egter nog min bekend oor die effek van geoksideerde olies op fungi. In hierdie studie, is die oleogene fungi *Cryptococcus curvatus* en *Mucor circinelloides* gebruik om die effek van palmolie-afbraakprodukte, gemeet as PTGs, op lipiedomset, groei en morfologie te bepaal. Na sewe dae van groei, het ons in *Mucor circinelloides* 'n afname in biomassa, lipiedverbruik en –ophoping by verhoogde PTG-vlakke gevind, by lae en neutrale pH. 'n Toename in PTG-konsentrasie het ook die morfologie van *M. circinelloides* beïnvloed. Uitsteeksels is op die seloppervlakte waargeneem na groei op olie met 45% PTGs en nie nadat die fungus op vars olie met 0.4% PTGs gegroei is nie. Daar was ook 'n afname in olieverbruik en biomassa produksie by *C. curvatus* by verhoogde PTG-vlakke, by lae en neutrale pH. 'n Toename in olie-ophoping is by lae pH waargeneem, maar by neutrale pH het dit konstant gebly vir alle getoetsde PTG-vlakke. Harige en vratagtige uitsteeksels is op die seloppervlaktes waargeneem nadat *C. curvatus* op olies met onderskeidelik 15% en 45% PTGs gegroei is. Ons lei af dat die veranderinge in lipiedomset en morfologie wat by beide fungi waargeneem is, die gevolg is van die teenwoordigheid van palmolie-afbraakprodukte. Afbraakprodukte soos aldehiede is hoofbronne van reaktiewe suurstofspesies (RSS). Studies het aangetoon dat RSS antimitochondriale werking het. Dit is bekend dat asetielsalisielsuur (ASS), 'n antiïnflammatoriese en antimitochondriale middel, die ontwikkeling van strukture

en funksies in giste, wat afhanklik is van verhoogde vlakke van mitochondriale aktiwiteit, teiken. Dit is voorheen getoon dat sporangia van *Mucor circinelloides* ook verhoogde mitochondriale aktiwiteit het, wat lei tot hoë vlakke van 3-hidroksie oksilipiene toon. In hierdie studie is bevind dat ASS ook sporangiumontwikkeling in *M. circinelloides* selektief teiken, terwyl hifes, met laer mitochondriale aktiwiteit, meer weerstandbiedend is. Soortgelyke resultate is verkry toe die antiïnflammatoriese verbindings, benzoësuur, ibuprofen, indometasien en salisielsuur getoets is. In hierdie studie is geoksideerde olies (met hoë PTG-vlakke) ook ingesluit. Hierdie olies is beweerde inhibitore van mitochondriale aktiwiteit. Hier het antiïnflammatoriese middels en geoksideerde olies soortgelyke effekte op hierdie dimorfe fungus gehad as toestande van suurstofbeperking. Merkwaardig is bevind dat sporangiumontwikkeling in *Mortierella alpina* nie selektief deur hierdie verbindings geteiken word nie. *Mortierella alpina*, wat nie dimorfies onder suurstofbeperking groei nie, was ook meer sensitief vir antiïnflammatoriese middels in vergelyking met *M. circinelloides.* Hierdie resultate verg verder navorsing om die toepaslikheid van hierdie antimitochondriale antifungale middels om plante en diere teen *Mucor* infeksies te beskerm, te bepaal. Hierdie studie impliseer dat geosideerde palmolie-afbraak produkte, mitochondriale funksie teiken. Dit kan moontlik die inhibitoriese effek van hierdie komponente op fungale groei, lipiedomset en veranderde selwandmorfologie verklaar.

Sleutelwoorde: Anti-inflammatoriese verbindings, Antimitochondriale middels, *Cryptococcus curvatus*, Lipiedomset, Mitochondriale aktiwiteit, Morfologie, *Mucor circinelloides*, Geoksideerde olies, Gepolimeriseerde triglisiriede, Sporangia