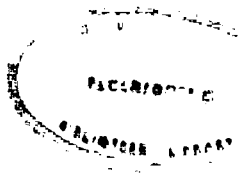

Utilisation of edible oils and GLA production by *Mucor* in the presence of acetate

by

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This dissertation is dedicated to my husband, Kobus Badenhorst

CONTENTS

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GLOSSARY

DAG	- diacylglycerol
EPO	- evening primrose oil
EPOeq	- EPO equivalent
FA	- fatty acid
GL	- glycolipid
GLA	- gamma-linolenic acid
MAG	- monoacylglycerol
MEL	- mannosylerythritol lipids
MMT	- million metric tons
NL	- neutral lipid
PC	- polar compound
PL	- phospholipid
PTG	- polymerised triglyceride
PUFA	- polyunsaturated fatty acid
SA	- South Africa
SCO	- single cell oil
TAG	- triacylglycerol
UCO	- used cooking oil

16:0	- palmitic acid
16:1	- palmitoleic acid
18:0	- stearic acid
18:1	- oleic acid
18:2	- linoleic acid
18:3 (ω3)	- alpha-linolenic acid
18:3 (ω6)	- gamma-linolenic acid

CHAPTER 1

INTRODUCTION

1.1 MOTIVATION

Plant oil containing gamma-linolenic acid (GLA) is currently being used in the cosmetic, food and pharmaceutical industries. This polyunsaturated fatty acid, which is a precursor for the vital cellular lipid hormones, is prescribed for the treatment of eczema. Not surprisingly, the biotechnological production of GLA containing oil, has been vigorously investigated in the past (Kock & Botha, 1993a). In 1994, it was discovered in our laboratory that *Mucor circinelloides* in the presence of acetate, is able to rapidly emulsify and utilise vegetable oil, while producing more biomass and GLA than when it was grown in the presence of vegetable oil as only carbon source. Our results are contrary to literature where it is generally believed that the fatty acids (FAs) recovered from fungi cultivated on a lipid substrate, reflect the chain length and degree of unsaturation present in the lipid substrate (Kendrick, 1991; Kendrick & Ratledge, 1996; Ratledge, 1989).

It is expected that due to legislation in 1996, large quantities of used edible oils and fats will accumulate in South Africa as waste each year. It is an offence to use or sell these oils and fats for human consumption if it contains 25 % or more polar compounds and/or 16 % or more polymerised triglycerides. Since frying establishments are not allowed to discard their used oils and fats by selling it to the public for consumption or dumping it into municipal drainage systems, it is important that these oils and fats are collected for re-use in another form (Kock *et al.*, 1997). Consequently, the aim of this study became to investigate the transformation of edible oils and fats to high value lipids such as GLA.

1.2 WHAT IS EDIBLE FAT OR OIL?

Edible fats and oils are lipids which are insoluble in water and soluble in organic solvents such as chloroform, alcohols and ethers (Ratledge & Wilkinson, 1988a). These compounds are bulk storage materials, which are produced by plants, animals and micro-organisms and contain fatty acid (FA) derivatives. These derivatives are mainly, but not entirely, mixtures of triacylglycerols (TAGs; Fig. 1A) and are known as oils or fats depending on whether they are liquid or solid at room temperature. Edible fats and oils also contain small amounts of other lipids such as diacylglycerols (DAGs; Fig. 1B), monoacylglycerols (MAGs; Fig. 1C), phospholipids (PLs; Fig. 1D) and free FAs (Fig. 1E) (Ratledge & Wilkinson, 1988b).

1.2.1 Types and features of fats and oils

Several plants are currently used for the production of edible oil. Although 40 different oilseeds have been described, there are mainly ten edible oil crops of commercial value. Seven of these oil crops are seed crops, namely cotton seed, groundnuts, rape seed, safflower seed, sesame seed, soybeans and sunflower seed. The remaining tree crops are coconut, olives and oil-palm (Shukla, 1994).

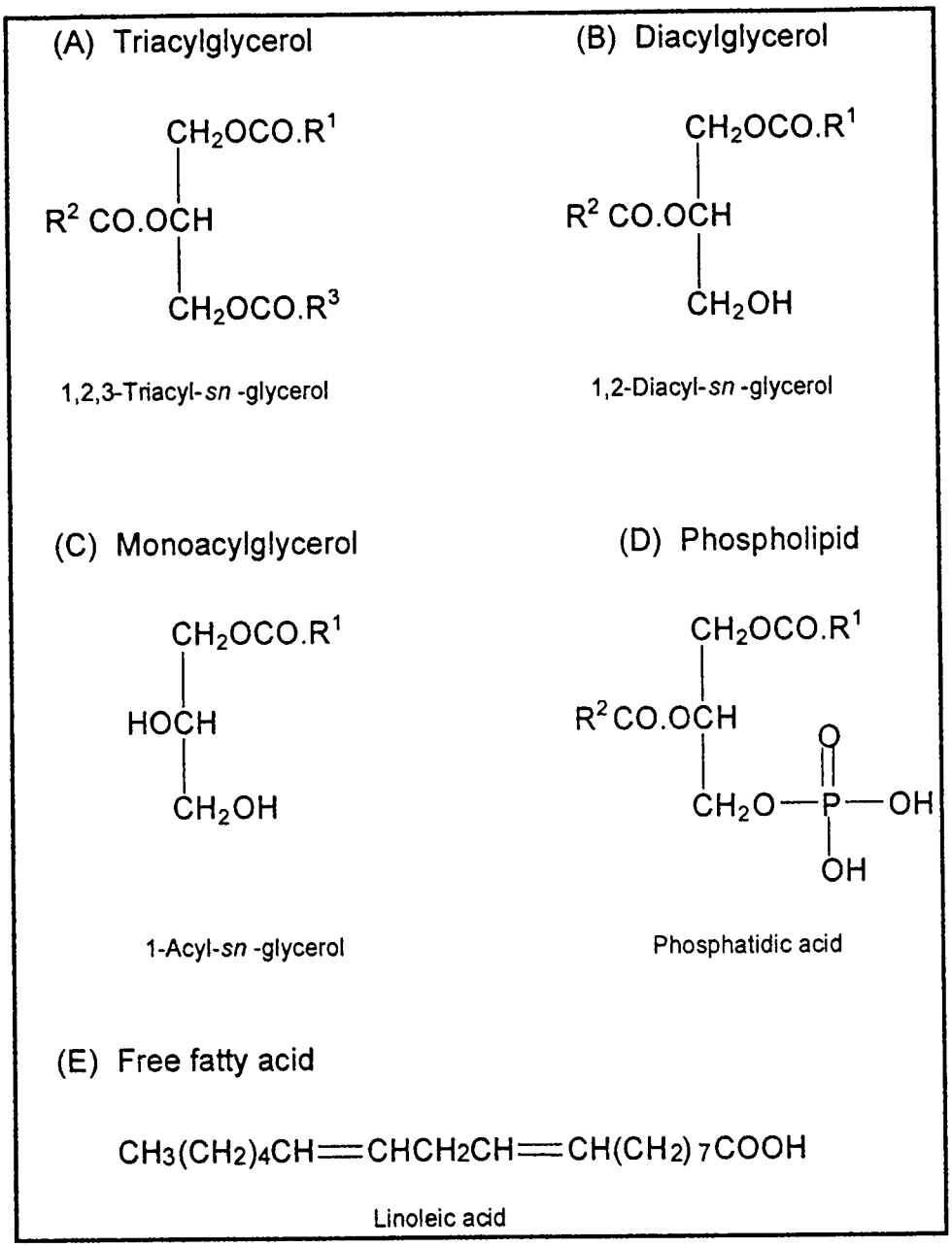


Fig. 1. Structures of fatty acid derivatives. R^1CO-, R^2CO-, R^3CO- represent fatty acyl groups (Ratledge & Wilkinson, 1988b)

Oilseed derived edible oil (i.e. vegetable oil) accounts for about 70 % of the world's edible oil and fat production. The remainder is animal fat (30 %), which include fish oils (2 %). Of the total oils and fats produced in the world, about 80 % are consumed as human food, 6 % are used as animal feed and 14 % are scheduled for the oleochemical industry (Shukla, 1994).

The FA composition of some major fats and oils is shown in Table 1 (Shukla, 1994). Here the fats and oils have been grouped according to the predominance of saturated (no double bond), mono-unsaturated (one double bond) and polyunsaturated (two or more double bonds) FAs. Examples of animal oils containing mainly saturated FAs are butterfat (63 % w/w), beef tallow (46 % w/w) and lard (42 % w/w).

The majority of vegetable oils contain large amounts of unsaturated FAs, e.g. olive oil has 71 % (w/w) oleic acid (18:1) and 10 % (w/w) linoleic acid (18:2), rapeseed oil 62 % (w/w) 18:1 and 22 % (w/w) 18:2, safflower oil 13 % (w/w) 18:1 and 78 % (w/w) 18:2 and sunflower oil 19 % (w/w) 18:1 and 68 % (w/w) 18:2. In general the most common FA is 18:1 and most common saturated FA is palmitic acid (16:0).

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

Oil or 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
Polyunsaturated																	
Corn oil							11	2			28		58	1	13	28	59
Cottonseed oil						1	22	3		1	19		54	1	26	20	55
Safflower							7	2			13		78		9	13	78
Soybean oil							11	4			24		54	7	15	24	61
Sunflower							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 mono-unsaturated fatty acids; P=total polyunsaturated fatty acids

1.2.2 Demand for edible fats and oils

According to Mielke (1992), the annual world demand for fats and oils is likely to increase by 32 %, i.e. from 80 million metric tons (MMT) in 1990 to 105 MMT annual consumption in the year 2000. In South Africa at present, 310 000 tons of vegetable oils are used in the food industry (Table 2).

Table 2. Edible oil and fat consumption and prices in South Africa for 1994 (Oil Seed Board, personal communication, 1995)

Type	Consumption (Tons)	Price (R) (Per Ton)
Sunflower oil	155 000	R 2 596.00
Soya oil	5 000	R 2 528.00
Groundnut oil	8 000	R 2 802.00
Cottonseed oil	3 800	R 2 608.00
Palmolein & Sunflower oil*	138 200	
TOTAL	310 000	

* Imported

Although the bulk of edible fats and oils are consumed as food in South Africa, significant quantities (approx. 7000 tons p.a.) are sold by major frying establishments at low cost, i.e. about R1200/ton, as used waste. These lipids are mainly re-used by smaller frying establishments, included in animal feed and for the production of low cost soap (Foodtek, personal communication, 1995).

1.3 HOW ARE FATS AND OILS UTILISED?

The utilisation of edible fats and oils by fungi is well documented (Lösel, 1989). Attention in this field of research has mainly been directed towards the attack of lipid-rich natural substrates by lipophilic fungi and the consequent production of extracellular lipases. These studies include investigation into lipase production by fungi in e.g. sunflower seed (Roberts *et al.*, 1987) as well as lipase activity in species such as *Rhizopus, Mucor* (Akhtar *et al.*, 1980), *Aspergillus* and *Syncephalastrum* which can utilise a wide variety of commercially available vegetable oils (Fermor & Wood, 1981).

Important studies in this field also include the isolation of thermophilic fungi from oil-palm kernels in Nigeria, which use fatty acids as carbon source. Ogundero (1981), by studying the degradation of oil-palm products by thermophilic fungi, showed that palmitic acid (16:0), the major FA in palm-oil, is a good carbon source for utilisation. It was also demonstrated in this study that fungal growth on stored rapeseed resulted in triacylglycerol (TAG) degradation.

1.3.1 Hydrolysis of triacylglycerols

Prior to the uptake of fats and oils by fungi, TAGs and other FA ester derivatives (Fig. 1) are first hydrolysed by fungal lipases to yield free FAs which can then be taken up by the cells. These fats and oils may either be

provided exogenously as growth substrates or endogenously by the cell's own stored TAGs which are consumed during starvation (Ratledge, 1989). All fungi, able to grow on fats and oils will produce lipases, especially when confronted with these compounds in a medium.

Hydrolysis of water-insoluble FA esters to free FAs is catalysed by lipases, also known as long-chain FA ester hydrolases. Fungal lipases are classified into three main types according to their reaction specificity, as shown in Fig. 2 (Ratledge, 1989). Firstly, non-specific lipases catalyse the total hydrolysis of TAGs to free FAs and glycerol. Secondly, 1,3-regiospecific lipases catalyse the hydrolysis reaction at the C-1 and C-3 positions of TAGs in order to yield free FAs, 2,3-diacylglycerol and 2-monoacylglycerol. The third type includes acyl-group specific lipases capable of catalysing the removal of a specific FA from a TAG.

1.3.2 Transport of long-chain fatty acids

In fungi, the uptake of FAs is by facilitated diffusion at low concentrations and by simple diffusion at high concentrations. A cytoplasmic membrane protein appears to be essential for long-chain FA transport. These long-chain FAs adsorb and pass unidirectionally through the membrane to become converted by acyl-CoA synthetase to acyl-CoA esters. This results in the minimisation of the inhibitory effects of free FAs in the cytoplasm (Finnerty, 1989).

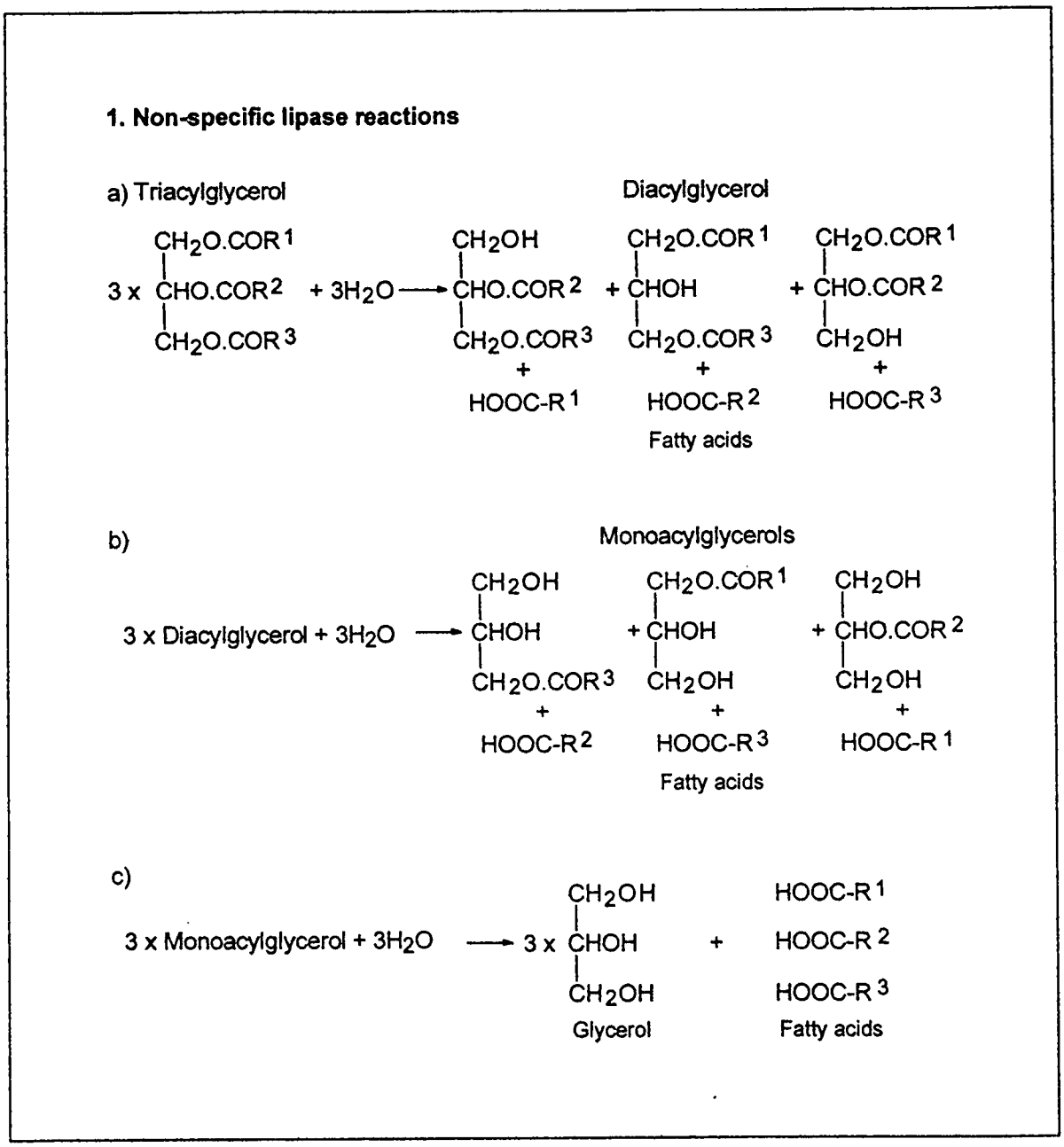
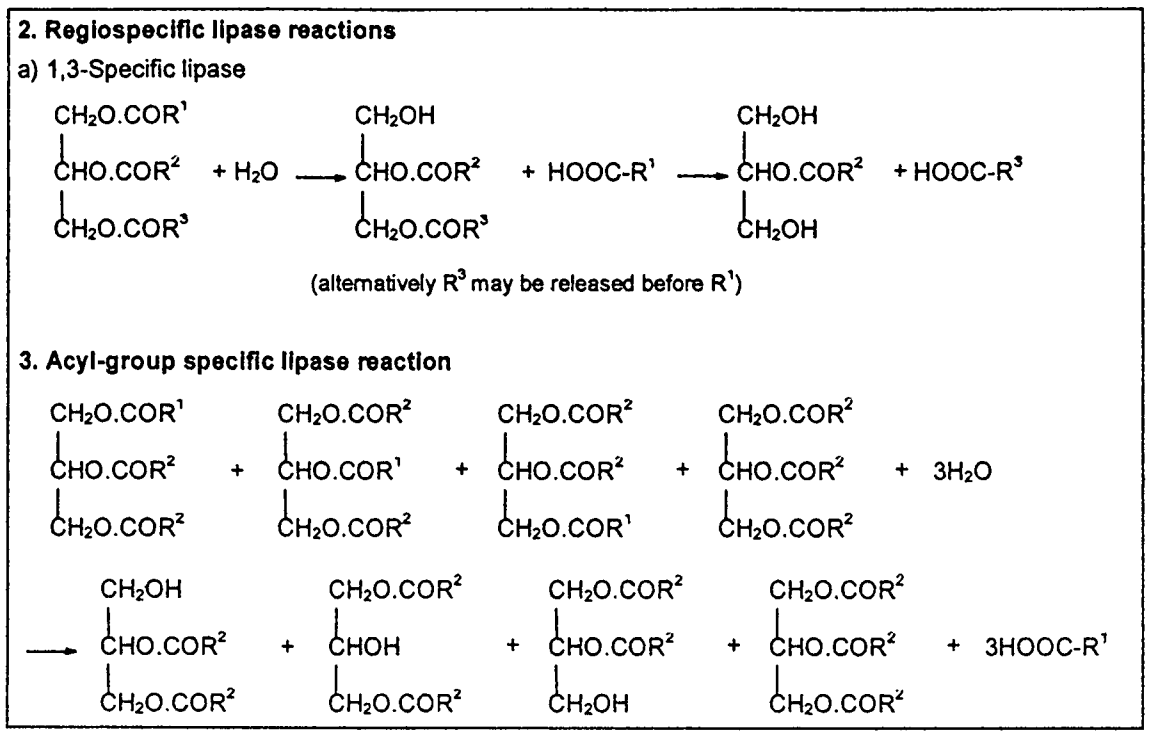


Fig. 2. The classification of fungal lipases (Ratledge, 1989)

Fig. 2. Continued



1.3.3 Oxidation of fatty acids

Yeasts and fungi are generally able to grow on C12 or longer chain FAs as the sole source of carbon and energy. Their growth on FAs requires the co-ordinated induction of the β-oxidation enzymes plus a FA transport system. The reactions involved in the cyclic 2-carbon shortening of a FA during β-oxidation include an inducible enzyme system as well as epimerase and isomerase which are involved in the β-oxidation of unsaturated FAs (Finnerty, 1989).

The activation of FAs to acyl-CoA esters by acyl-CoA synthetase represents the initial step in FA oxidation (Fig. 3). It was found that acyl-CoA synthetase is a loosely membrane-bound enzyme which exhibits broad substrate specificity. Acyl-CoA synthetases are found inside and on the outer membrane of mitochondria. After their activation, the long-chain FAs penetrate the inner mitochondrial membrane only in combination with carnitine, which facilitate transport of acyl-groups through the mitochondrial membrane.

In β-oxidation, two carbons are cleaved at a time from acyl-CoA molecules, starting at the carboxyl end and the 2-carbon units formed are acetyl-CoA (Fig. 3). After the penetration of the acyl moiety through the mitochondrial membrane via the carnitine transporter system and the formation of acyl-CoA, there follows the removal of two hydrogen atoms from the 2 and 3 carbon atoms, catalysed by acyl-CoA dehydrogenase (Fig. 3). This results in the formation of Δ^2-trans-enoyl-CoA. This is followed by the addition of water in order to saturate the double bond and form 3-hydroxyacyl-CoA, catalysed by the enzyme Δ^2-enoyl-CoA hydratase. This 3-hydroxyacyl-CoA undergoes further dehydrogenation on the 3-carbon, catalysed by 3-hydroxyacyl-CoA dehydrogenase to form the corresponding 3-ketoacyl-CoA compound. Finally, 3-ketoacyl-CoA is split at the 2,3 position by thiolase to form acetyl-CoA and an Acyl-CoA derivative containing two carbons less than the original acyl-CoA molecule that underwent oxidation. The acyl-CoA formed in the cleavage reaction re-enters the oxidative pathway and in this way long-chain FAs may be degraded completely to acetyl-CoA (i.e. C2 units). As acetyl-

CoA can be oxidised to CO_2 and water via the citric acid cycle, the complete oxidation of FAs is achieved (Mayes, 1990a).

Long-chain unsaturated FAs move through β-oxidation in a similar manner than saturated FAs, with the exception of two enzymes namely enoyl-CoA isomerase and 3-hydroxyacyl-CoA epimerase. The normal β-oxidation enzymes, oxidise mono-unsaturated FAs (e.g. *cis*-octadec-9-enoic acid (18:1)) to *cis*-dodec-3-enoic acid (12:1). This is a non-metabolisable intermediate and, is therefore, isomerised to *trans*-dodec-2-enoyl-CoA by enoyl-CoA isomerase, a normal substrate for enoyl-CoA hydratase (Finnerty, 1989).

During β-oxidation, polyunsaturated FAs (e.g. *cis*,*cis*-octadec-9,12-dienoic acid (18:2)) are oxidised to *cis*,*cis*-dodec-3,6-dienoic acid, which is then isomerised to *trans*,*cis*-dodec-2,6-dienoic acid by enoyl-CoA. This substrate undergoes oxidation to *cis*-oct-2-enoyl-CoA, which is converted to D-3-hydroxyoctanoyl-CoA. However, 3-hydroxyacyl-CoA dehydrogenase is specific for the L configuration and 3-hydroxyacyl-CoA epimerase convert this substrate into L-3-hydroxyoctanoyl-CoA. This allows the resumption of β-oxidation (Finnerty, 1989).

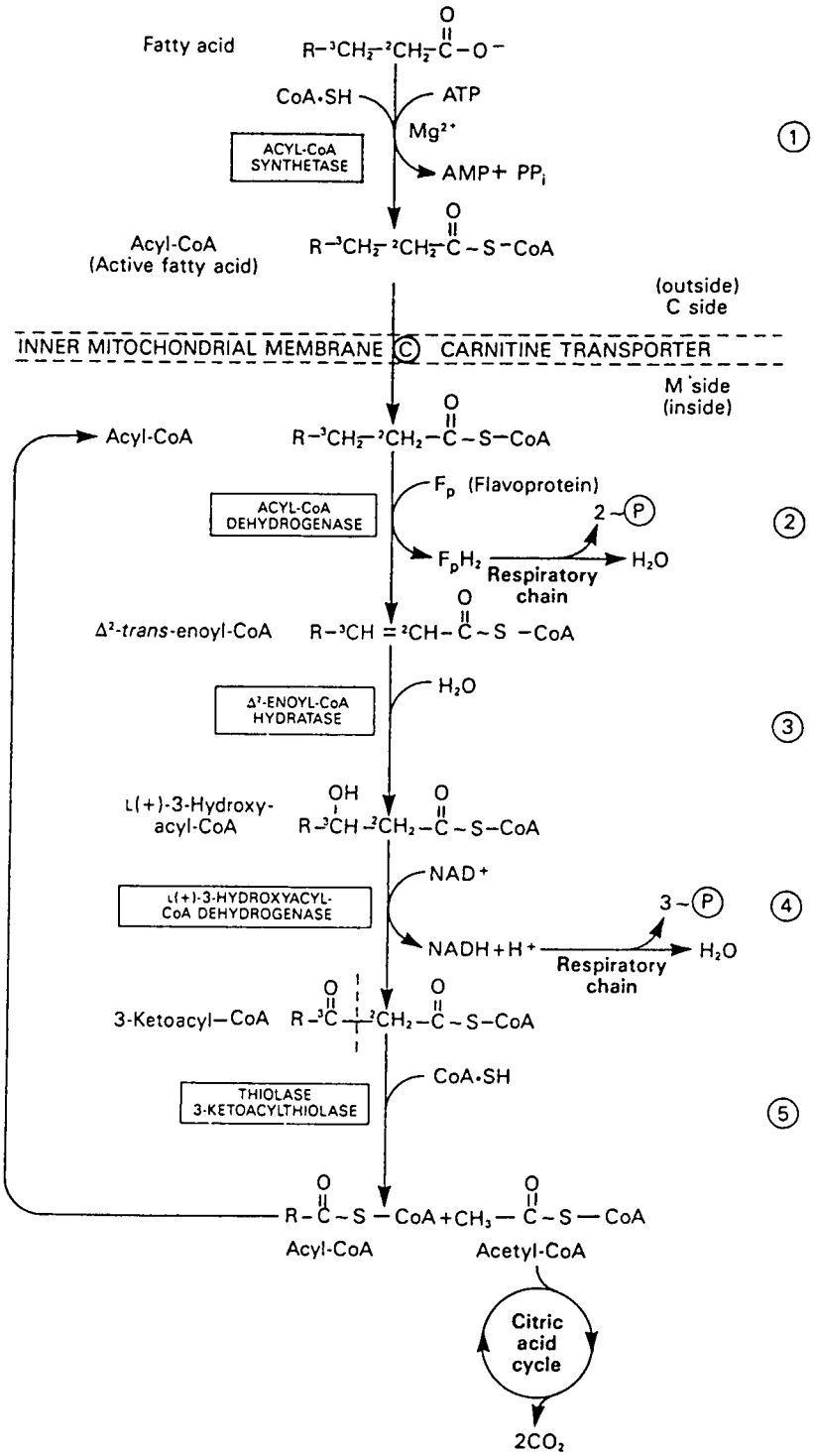


Fig. 3. β-Oxidation of fatty acids. Long-chain acyl-CoA is cycled through reactions 2-5, acetyl-CoA being split off each cycle by thiolase (reaction 5). When the acyl radical is only 4 carbon atoms in length, 2 acetyl-CoA molecules are formed in reaction 5 (Mayes, 1990a).

1.3.4 Production of fungal lipids from fats and oils

According to literature many micro-organisms are able to utilise fats and oils as sole carbon source (Koritala *et al.*, 1987; Ratledge, 1989). In order to obtain good growth, it is important that the pH of the medium be maintained near neutrality in order for the extracellular lipase activity and uptake of the fatty acid anion to be optimal (Tan & Gill, 1985a; Tan & Gill, 1985b). At low medium pH the lipid substrates may not be hydrolysed and if they are, the toxicity of the resulting free FAs (rather than their salts) becomes too high (Bell, 1971; Hunkova & Flench, 1977). Another problem encountered to obtain FA uptake, is the failure to achieve adequate dispersal of a FA into an emulsified form (Tan & Gill, 1985c).

According to literature, a general rule can be made regarding the lipid substrate and accumulation within fungi. Within broad limits, the FAs recovered from fungi, after cultivation on a lipid substrate, reflect the chain length and degree of unsaturation present in the lipid substrate. This phenomenon has been used to increase the stearic acid (18:0) content of fungal triglycerides in order to improve their cocoa butter-like features by presenting extracellular 18:0 to fungi (Ratledge, 1989). In 1996, Kendrick and Ratledge demonstrated that extracellular oil in the medium caused repression of fatty acid desaturation as well as elongation in filamentous fungi. The results of experiments by separate research groups, all using *Yarrowia lipolytica* with different lipid substrates, are shown in Table 3.

Table 3. Fatty acyl composition of *Yarrowia lipolytica* after growth on various fats and oils (Ratledge, 1989)

Oil used	Lipid Analysed	Relative % (w/w) of fatty acyl groups					
		16:0	16:1	18:0	18:1	18:2	18:3
Bonefat	Fed	25	3	13	40	13	1
	Recovered	16	6	11	35	32	tr
Corn	Fed	12	-	2	25	62	0.5
	Recovered	11	6	2	36	45	-
Linseed	Fed	7	-	4	20	15	54
	Recovered	7	3	7	36	18	29
Mixed soapstocks	Fed	10	tr	4	41	32	2
	Recovered	4	6	2	38	47	2
Olive	Fed	13	2	3	69	11	1
	Recovered	12	3	3	70	13	1
Palm	Fed	30	7	2	45	11	5
	Recovered	26	7	8	47	10	2
Rapeseed	Fed	7	-	1	56	24	8
	Recovered	3	9	1	55	25	7
Soybean	Fed	10	-	4	22	56	9
	Recovered	8	-	4	24	58	6

tr = trace

In these experiments, the presented triacylglycerols were hydrolysed, probably extracellularly, and the FAs then re-esterified once they were inside the cell. According to Table 3, certain modifications of individual FAs occurred. The most striking example is when linseed oil, containing high percentages of α-linolenic acid (18:3 ω3), was fed. The recovered lipids from *Yarrowia lipolytica* grown on this substrate had 29 % (w/w) 18:3 (ω3). The yeast most probably could not tolerate the high percentage of 18:3 (ω3) and either failed to incorporate it or reduced it to linoleic acid (18:2), oleic acid

(18:1) and stearic acid (18:0). With other fats and oils presented as substrate, there was a good correlation between what was fed and what was recovered (Ratledge, 1989).

Kendrick (1991) grew four oleaginous fungi in a range of triglycerides containing oils as sole carbon source in an attempt to promote higher amounts of polyunsaturated FAs. According to his results, the lipid contents of oil-grown cultures were significantly higher than the glucose-grown counterparts. This was not due to residual oil being associated with the mycelia as the mycelia were extensively washed with distilled water or even chloroform. Other authors (Aggelis *et al.*, 1991a; Aggelis *et al.*, 1991b; Čertík *et al.*, 1997), observed similar results.

Generally, the fungi in the above studies produced extractable oil with a FA profile similar to that of the lipid substrate. In contrast to these results, two Japanese groups have succeeded in stimulating the increased production of polyunsaturated FAs by growth of fungi (i.e. *Conidiobolus* spp. and *Mortierella* spp.) on oils as sole carbon source (Kendrick, 1991; Yamada *et al.*, 1992). Aggelis *et al.* (1991a & 1991b) found that *Mucor circinelloides* CBS 172.27, cultured on sunflower oil contained more than 65 % oil with a gamma-linolenic acid (GLA) content of 17.4 %.

1.4 INCORPORATION OF FATTY ACIDS FROM EDIBLE OIL INTO MOULD LIPIDS

Many fungi, known as oleaginous fungi, are capable of accumulating 20 % or more of their biomass as lipids. According to Aggelis *et al.* (1991a, 1991b), oleaginous micro-organisms cultured on media containing oil as carbon source accumulate reserve lipids by mechanisms different from those encountered when glucose is used as substrate.

In the case of glucose and other carbohydrates, the accumulation of reserve lipids starts after the depletion of certain nutrients (e.g. nitrogen) from the culture medium (Botham & Ratledge, 1979). This is in contrast to the studies of Aggelis *et al.* (1991a, 1991b), where some oleaginous micro-organisms (e.g. *Mucor* spp.) accumulated significant quantities of lipids when grown on a culture medium containing vegetable oil as carbon source, regardless of the nitrogen concentration in the medium. When nitrogen becomes exhausted from the medium or reaches a very low concentration in continuous cultures, the cells are faced with a surfeit of carbon. The carbon source continues to be assimilated under nitrogen limited conditions and the cells then become obese and convert excess carbohydrates to lipids. As the carbohydrates continue to be metabolised, the intracellular concentrations of various key intermediates change (Fig. 4).

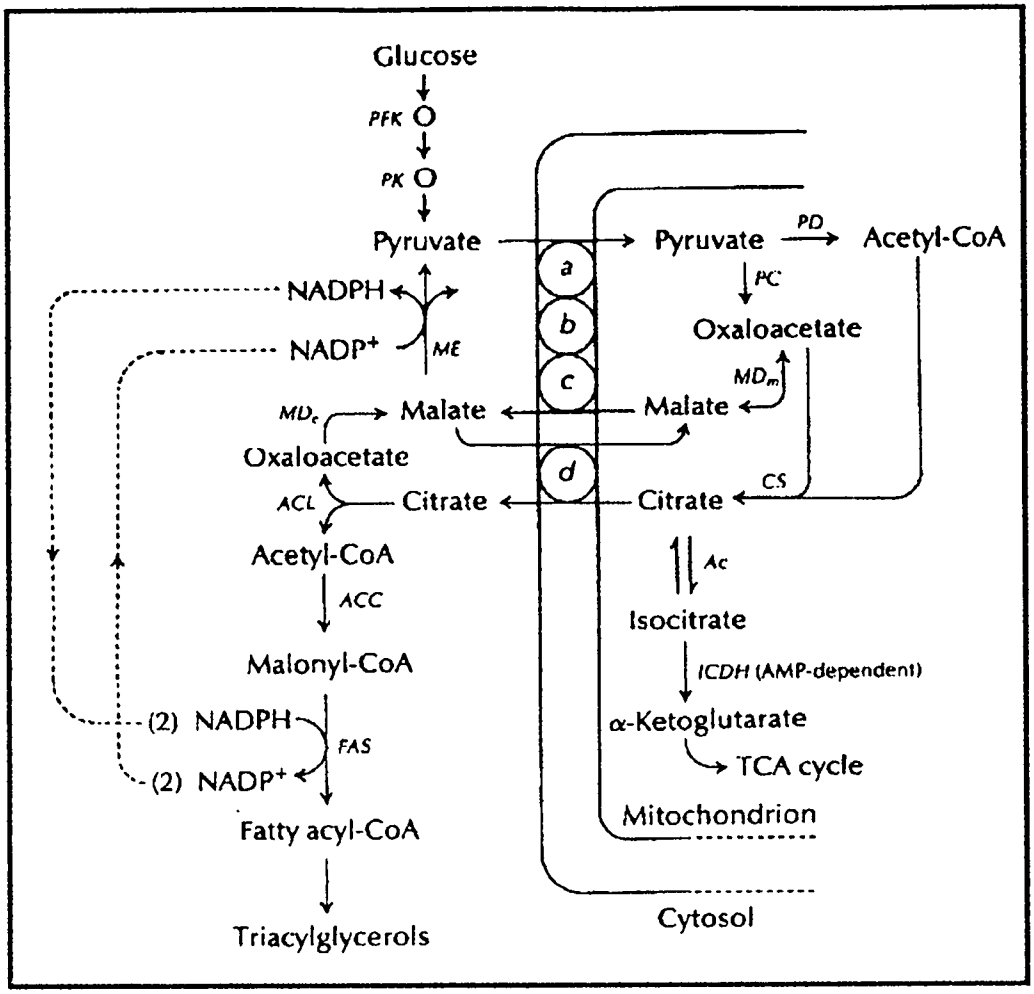


Fig. 4. Biochemical pathway for triacylglycerol (TAG) biosynthesis in oleaginous yeasts. Mitochondrial transport processes: *a*, *b*, and *c* interlinked pyruvate-malate translocases; *d*, malate/citrate translocase. Enzymes: *PFK*, phosphofructokinase; *PK*, pyruvate kinase; *PD*, pyruvate dehydrogenase; *PC*, pyruvate carboxylase; MD_m, malate dehydrogenase (mitochondrial); MD_c, malate dehydrogenase (cytosolic); *CS*, citrate syntase; *Ac*, aconitase; *ICDH*, isocitrate dehydrogenase; *ACL*, ATP:citrate lyase; *ME*, malic enzyme; *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthetase complex (Ratledge, 1989).

In this situation a rapid decrease in AMP concentration occurs which is due to the activation of AMP deaminase which converts AMP to IMP and NH_3. The immediate metabolic consequence of this decrease in AMP concentration is on the operation of NAD^+-isocitrate dehydrogenase (Fig. 4) within the mitochondrion of the cell. In oleaginous fungi this enzyme is wholly dependent for activity upon the presence of AMP. As a consequence, isocitrate can no longer be effectively metabolised through the citric acid cycle and both this compound and citric acid accumulate. In non-oleaginous fungi NAD^+-isocitrate dehydrogenase is more active in the absence of AMP so that citrate does not accumulate. As rapidly as it accumulates, citrate is transported across the mitochondrial membrane in exchange for malate. Malate is thought to arise in the cytosol in exchange for the uptake of pyruvate in the mitochondrion.

It is considered that the principal key to oleaginicity resides in the possession of ATP:citrate lyase by oleaginous micro-organisms. This enzyme catalyses the irreversible cleavage of citrate into acetyl-CoA and oxaloacetate. Oxaloacetate is converted in the cytosol to malate and then to pyruvate by the malic enzyme producing NADPH. NADPH is necessary as reducing power for lipid synthesis from acetyl-CoA (Weeks *et al.*, 1969; Ratledge, 1989).

1.5 EFFECT OF FATTY ACIDS ON THE MALIC ENZYME

When grown on oil as sole carbon source, as opposed to glucose, cytosolic malic enzyme activity of *Entomophthora exitalis* and *Mucor circinelloides* was found to decrease or even disappear as shown in Table 4 (Kendrick, 1991).

Table 4. Effect of carbon source (3 % w/w) on fungal malic enzyme activity (Kendrick, 1991)

Fungus	Carbon source	Malic enzyme activity (nmol/min/mg)
Entomophthora exitalis	Glucose	40.4
	Safflower	0
	Sesame oil	0
	Triolein	9.9
Mucor circinelloides	Glucose	54.3
	Safflower	6.9
	Sesame oil	0
	Triolein	16.9

The putative role of malic enzyme in lipogenesis is to provide NADPH for FA biosynthesis (Botham & Ratledge, 1979) and FA desaturation. Under conditions of NADPH limitation induced by growth on oils, the fungi no longer have the ability to synthesise FAs or desaturate them further. The fungi therefore can only incorporate those FAs presented to them directly into cell lipids without modification. This may explain the similarity between the FA profiles of the oil carbon source and the fungal lipids.

1.6 BIOTECHNOLOGICAL VALUE OF UTILISING FATS AND OILS

According to most literature (Bati *et al.*, 1984; Tan & Gill, 1985a; Koritala *et al.*, 1987; Kendrick, 1991) the FAs recovered from fungi, after growing on edible oils and fats, reflect the chain length and degree of unsaturation present in the original substrate. Consequently, the possibility of utilising these lipids as substrates for the production of high value polyunsaturated FAs, i.e. gamma-linolenic-, arachidonic-, eicosapentaenoic- and docosahexaenoic acid (Ratledge, 1994) seems to be limited. Exceptions to this belief have been published (Kendrick, 1991; Yamada *et al.*, 1992) where it was found that arachidonic acid (20:4) producing *Mortierella* strains can accumulate detectable amounts of eicosapentaenoic acid in their mycelia when grown in a medium containing 18:3 (ω3). Under optimal conditions, *M. alpina* converted 5.1 % (w/w) of 18:3 (ω3) present in the added linseed oil into 1.35 g/l (41.5 mg/g dry mycelia) eicosapentaenoic acid when grown at room temperature. It was also reported that the production of polyunsaturated FAs can be stimulated when *Conidiobolus* spp. are grown on oils as sole carbon source (Ratledge, 1994).

Fats and oils are frequently used as carbon sources in antibiotic fermentations (Bader *et al.*, 1984) while waste fats, including fish oils, have been used as substrates for the production of single cell protein (Ratledge, 1989). These carbon sources can also be used in the production of a wide variety of biosurfactants (Banat, 1995).

1.7 BIOSURFACTANTS

The production of biosurfactants by bacteria has been well established (Fiechter, 1992; Georgiou *et al.*, 1992). These micro-organisms are capable of producing a variety of biosurfactants. These include glycolipid surfactants (Fig. 5) produced by *Pseudomonas* or *Arthrobacter*, amino-acid containing lipid biosurfactants (Fig. 6) formed by *Bacillus licheniformis*, biosurfactants containing polysaccharide-lipid complexes such as Emulsan produced by *Acinetobacter calcoaceticus* and protein-like substances such as Serraphobin produced by *Serraphobin marcescens.*

There is a large industrial demand for chemically synthesised surfactants from mainly petroleum which are widely used in industry, agriculture and food processing. The market value for soaps and detergents were as high as US$ 12.8×10^9 in 1990 with an expected annual increase of 5.9 %. Of this, surfactants accounted for about 30 % of the total market value. Biosurfactants are, however, unable to compete with chemical surfactants due to high production costs. This can be blamed on inefficient bioprocessing, poor strain productivity and the need to utilise expensive substrates to produce these substances. Biosurfactants will only be able to compete with chemically produced surfactants if (1) strains can be manipulated such that cheaper substrates may be used and (2) process technology is improved to enhance product recovery (Fiechter, 1992).

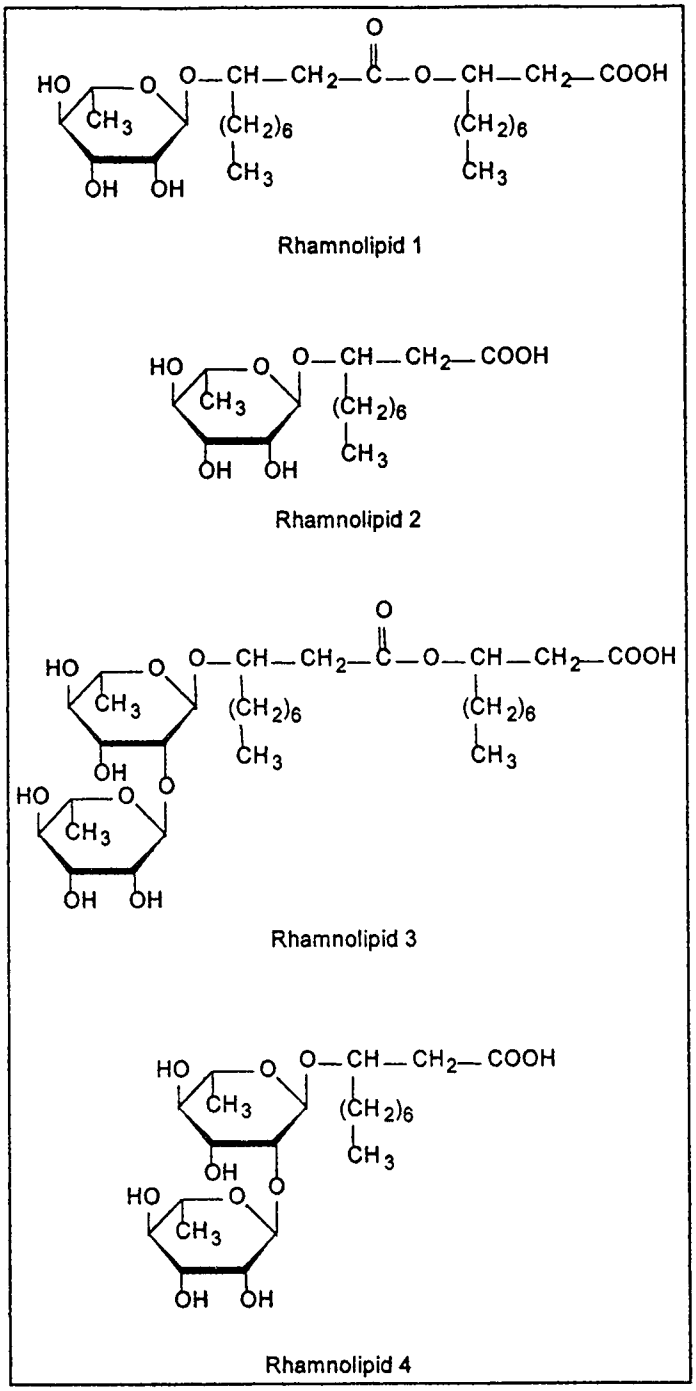


Fig. 5. Glycolipids i.e. rhamnolipids, produced by *Pseudomonas aeruginosa* (Fiechter, 1992)

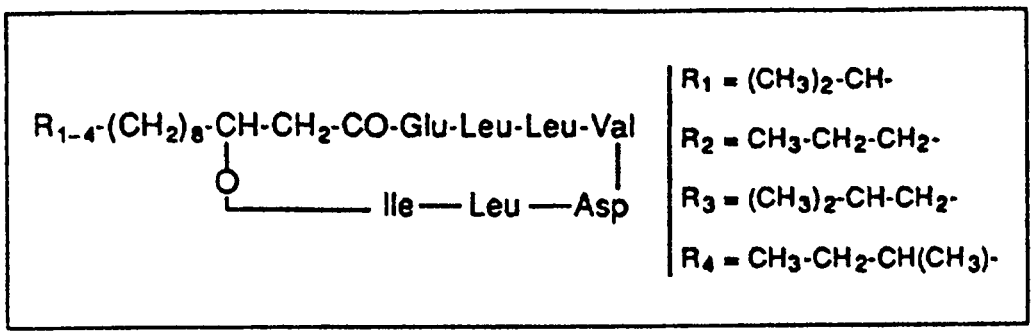


Fig. 6 Surface-active lipopeptide of *Bacillus licheniformis* (Fiechter, 1992)

1.7.1 What is a biosurfactant?

Biosurfactants can be defined as surface-active molecules produced by living cells - in the majority of cases, by micro-organisms (Fiechter, 1992). The surfactant character of these molecules is due to their amphipathic nature. Part of the molecule is hydrophobic, or water-insoluble, and part is hydrophilic, or water-soluble (Fig. 7A) (Georgiou *et al.*, 1992). Biosurfactant molecules tend to associate at interfaces of different polarity such as oil/water (Fig. 7D) or in micelles (Fig. 7C), lowering the surface tension (Fiechter, 1992).

The major role of biosurfactants is to render water-immiscible substrates more available for utilisation by micro-organisms by reducing the surface tension at the phase boundary and thereby emulsifying the substrate. Biosurfactants are also involved in the adhesion of micro-organisms to water-

immiscible substrates, thereby bringing the organism in close contact with the substrate (Käppeli & Fiechter, 1976).

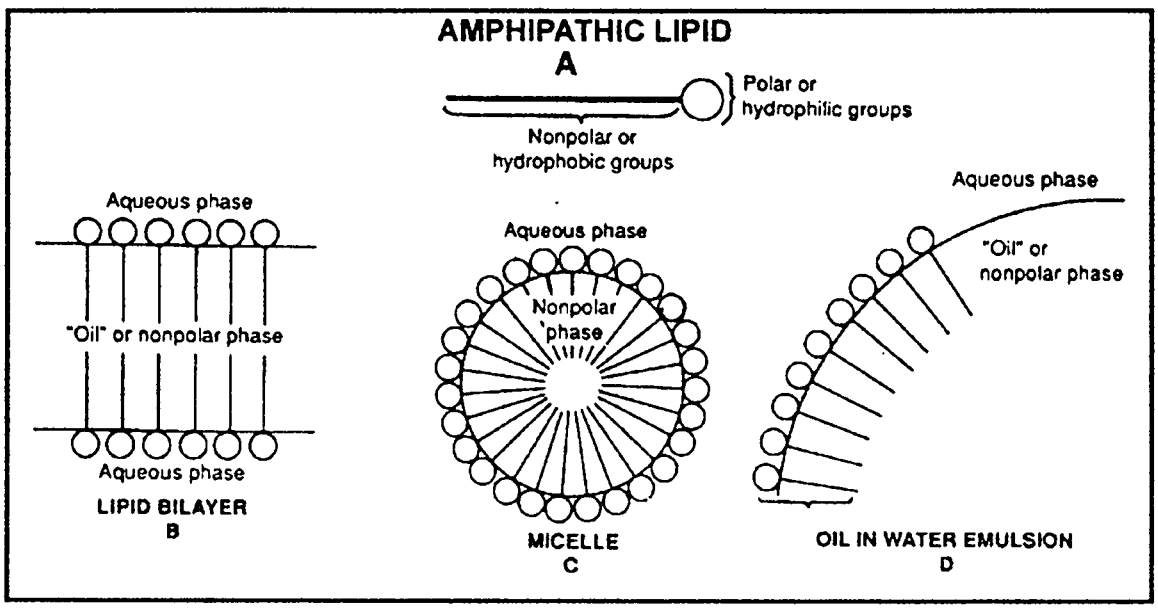


Fig. 7. Formation of lipid membranes, micelles and emulsions from amphipathic lipids (Mayes, 1990b).

Various types of biosurfactants are produced and include glycolipids (Matsuyama *et al.*, 1990), lipopeptides (Arima *et al.*, 1968; Matsuyama *et al.*, 1985; Matsuyama *et al.*, 1986; Neu *et al.*, 1990), polysaccharide-protein complexes (Zajic *et al.*, 1977; Persson *et al.*, 1988), phospholipids (Jones & Starkeyi, 1961; Beebe & Umbreit, 1971) and neutral lipids (Cooper *et al.*, 1979; MacDonald *et al.*, 1981) including fatty acids (Rosenberg, 1986). The types of biosurfactants produced depend on the type of carbon source,

nutritional limitations, aeration, temperature and pH as well as the microbial strain used (Fiechter, 1992).

Only a few examples exist (mainly applications in the petroleum industry, for example Emulsan) where the production of biosurfactants is economically viable. This is due to the high cost of production, unacceptability of many production strains by the public (for example *Pseudomonas aeruginosa*), and the high degree of purity required by the food, cosmetics and pharmaceutical industries. These compounds have the potential for being applied to emulsification processes, phase separation, wetting, foaming, solubilisation, de-emulsification and viscosity reduction in high viscosity crude oils. Consequently, there are many areas such as agriculture, building and construction, food and beverage industries, as well as pharmaceutical and petroleum industries where chemically synthesised surfactants can be replaced by biosurfactants. Biosurfactants are also superior to chemically produced surfactants due to their different physical properties, the ability to be produced on renewable substrates, their ability to be modified to meet required needs, and most importantly their biodegradability (Fiechter, 1992).

1.7.2 Biosurfactant production by fungi

The production of biosurfactants by fungi is well established (Boulton, 1989). The type, quality and quantity of these substances are influenced by the carbon source, concentrations of N, P, Mg, Fe and Mn ions in the medium as

well as cultivation conditions such as pH, temperature, agitation and dilution rate (Georgiou *et al.*, 1992; Khire & Khan, 1994). Various biosurfactants have been isolated and characterised in fungi and are summarised in Table 5.

Candida bombicola CBS 6009 was found to produce high concentrations sophorose lipids during stationary phase when grown on rapeseed-oil and glucose as major carbon sources (Gobbert *et al.*, 1984; Davila *et al.*, 1992). Yields as high as 340 g/l were obtained in these experiments. In addition, two types of biosurfactants i.e. mannosylerythritol lipids (MEL-A and MEL-B) were produced by resting cells of the yeast *Candida antarctica* T-34 in a medium containing soybean oil as carbon source (Kitamoto *et al.*, 1993). At critical micelle concentrations both types (MEL-A and MEL-B) are capable of reducing the surface as well as interfacial tension dramatically, i.e. to 28 mN/m and 2 mN/m respectively. In addition MEL, also exhibited antimicrobial activity against Gram-positive bacteria.

It was also reported that *Candida apicola* is capable of producing increased concentrations of sophorose lipids as biosurfactant when the ammonium sulphate concentration in the medium is increased (Desai & Desai, 1993). These authors have concluded that the extracellular biosurfactant production is associated with the carbon to nitrogen ratio in the growth medium. It was also reported that *Penicillium herqueii* produces lipophilic surfactants when grown on sucrose containing medium, especially during foam formation. According to literature, lipophilic surfactants enhanced and reduced foaming

in bacterial and yeast cultivations respectively (Bahr *et al.*, 1991; Reiling *et al.*, 1986).

Lipases from the yeast *Candida cylindracea* and the mould *Rhizopus delemar* were utilised to produce rhamnolipids as biosurfactants from soy phospholipids as well as sardine oil (Mutua & Akoh, 1993). *Candida tropicalis* was found to produce a polysaccharide-fatty acid complex (Käppeli & Fiechter, 1977; Pines & Gutnick, 1986).

The yeast *Torulopsis petrophilum* was reported to produce glycolipid-type and protein biosurfactants (depending on the substrate) capable of stabilising water/oil emulsions (Cooper & Paddock, 1983). *Candida lipolytica*, on the other hand, was shown to produce a 27.6 kDa complex protein-like surfactant called Liposan, which also contains 83 % carbohydrates. This complex is capable of stabilising water/oil emulsions (Cirigliano & Carman, 1985). It was reported that *Torulopsis magnoliae* produced sophorolipids when fed with fatty acids, hydrocarbons or glucose (Asselineau & Asselineau, 1978). Boulton (1989) also reported the production of sophorose in *Candida bogoriensis*.

Table 5. Biosurfactants produced by fungi

Micro-organism	Carbon-source	Biosurfactant	References
Candida antarctica T-34	Soybean-oil	Mannosylerythritol lipids (MEL-A and MEL-B)	Kitamoto *et al.*, 1993
Candida apicola	Carbohydrates	Sophorose lipids	Desai & Desai, 1993
Candida bogoriensis	Hydrocarbons	Sophorose lipids	Boulton, 1989
Candida bombicola CBS 6009	Rapeseed-oil fatty acids and glucose	Sophorose lipids	Davila *et al.*, 1992
Candida cylindracea	Sardine-oil	Rhamnolipids	Mutua & Akoh, 1993
Candida lipolytica	Hexadecane	Liposan	Cirigliano & Carman, 1985
Candida tropicalis	Alkanes	Polysaccharide-fatty acid complex	Käppeli & Fiechter, 1977
Penicillium herqueii	Sucrose	lipophilic lipids	Reiling *et al.*, 1986
Rhizopus delemar	Sardine-oil	Rhamnolipids	Mutua & Akoh, 1993
Torulopsis magnoliae	Fatty acids and hydrocarbon	Sophorolipids	Asselineau & Asselineau, 1978
Torulopsis petrophilum	Hydrocarbons	Protein emulsifier	Cooper & Paddock, 1983

1.8 HIGH VALUE LIPIDS IN FUNGI

The exploitation of micro-organisms in the production of single cell oil (SCO) is not a new idea. However, due to the inability of current biotechnology to compete against the low cost of oil production from agricultural seed, only two processes ever reached commercial realisation (Kyle & Ratledge, 1992).

According to Ratledge (1993), there are potentially three markets which SCO products may influence. These include cocoa butter, gamma-linolenic acid

(GLA) and some polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid and arachidonic acid. For many years, oil containing between 7 % and 12 % GLA (Robert *et al.*, 1992; Ratledge, 1994), has been produced from seeds of the evening primrose (*Oenothera biennis*) (Cisowski *et al.*, 1993; Redden *et al.*, 1995). Other sources of GLA-rich oils include borage (*Borago officina*) (Raederstorff & Moser, 1992; Barre *et al.*, 1993; Redden *et al.*, 1995) containing between 19 % and 25 % (w/w) GLA (Ratledge, 1994) and black currant (*Ribes nigrum*) (Barre & Holub, 1992) which contains 17 % (w/w) GLA (Ratledge, 1994). As a relatively high priced PUFA, GLA is an obvious target for SCO production (Ratledge, 1993).

GLA is a precursor for lipid hormones in humans (i.e. prostaglandins E_2, $F_{2\alpha}$ and I_2, leucotrienes and thromboxanes) (Kock & Botha, 1993a). These GLA-rich oils is at present a prescribable medicine for the treatment of eczema (Berth-Jones & Graham-Brown, 1993; Fiocchi *et al.*, 1994; Harwood *et al.*, 1994).

In 1992, the United Kingdom and European market for oils rich in GLA was between 250-400 tons per year. Similar sales were observed in Japan, at a price ranging between $30 and $60 per kg (Kyle & Ratledge, 1992). A strain of *Mortierella isabellina* is currently used for commercial production of GLA-rich oils by Idemitsu Co. Inc., in Japan, using glucose as carbon source (Ratledge, 1993). *Mucor circinelloides* f. *circinelloides* was also applied by J. and E. Sturge Ltd. in Selby, Yorkshire (U.K.) in the production of GLA-rich

oils from glucose. Japan is the only country pursuing this biotechnological route at present (Ratledge, 1993).

Kock and Botha (1993b) patented an invention relating to a new biotechnological route where GLA-rich oils are produced by growing an FDA (U.S. Food and Drug Administration) approved *Mucor circinelloides* f. *circinelloides* on acetic acid as sole carbon source, at sub-toxic levels. The overall productivity (i.e. biomass density, biomass yield, GLA yield, lipid yield, lipid content and GLA content) in GLA-rich oil production was similar to that where glucose was used as sole carbon source. The possibility therefore exists of utilising a lower-cost substrate, namely acetic acid, in order to replace the more expensive glucose used in conventional processes.

The commercial feasibility of a new process will depend on the market, price and acceptability of fungi as a source of high-value lipids (Kock & Botha, 1993a). However, interest in producing SCO containing GLA continues, as this is the most useful starting material for producing purified GLA, at up to 90% purity. The only problem is in identifying a market for such a preparation. It is possible that only a pharmaceutical application would justify the costs. Highly purified GLA-oils have not yet been shown to have any advantage over the original oils and as they tend to be used for the treatment of marginal disorders, rather than life-threatening diseases, it may be some time before these processes become viable (Ratledge, 1993).

1.9 PURPOSE OF RESEARCH

With this as background, the purpose of the study became to evaluate the possibility of converting used cooking oil to high value oils containing GLA. In order to achieve this, the following aspects were investigated:

1.9.1 The situation of frying oil and fat abuse in South Africa. Emphasis is placed on the availability of this high energy substrate in South Africa (Chapter 2).

1.9.2 Next, fungi capable of effective utilisation of edible oils were selected. All experiments were performed in the presence and absence of acetate (Chapter 3).

1.9.3 Having observed enhanced emulsification and subsequent increased lipid utilisation in the presence of acetate, the researcher proceeded with investigations aimed at elucidating this phenomenon (Chapter 4).

1.9.4 Finally, edible oil utilisation and GLA production by *Mucor* in bioreactors were investigated in order to elucidate the effect of pH change and acetic acid utilisation on oil utilisation (Chapter 4).

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CHAPTER 2

FRYING OIL AND FAT ABUSE IN SOUTH AFRICA - A REVIEW

Part of this chapter has been published as:

Fryer oil initiative for SA: restaurant waste oils now available in SA for animal feeds.

AFMA MATRIX 6, 23-25

2.1 INTRODUCTION

"The biological and toxicological properties of fats and oils have been extensively investigated. There is general agreement that undesirable or harmful materials are formed during storage and usage." "Furthermore, oxidation products of fats and oils are capable of exerting adverse biological effects. A large number of publications, including recent reviews, dealing with the biological and toxicological effects of oxidised fats and oils are available" (Chow & Gupta, 1994).

Oxidised materials in oils and fats increase during prolonged heating at high temperatures causing these lipids to become more polar. This may result in the accumulation of large amounts (more than 10 % by mass) of these oils and fats in some fried foods. Also, the nutritive value of fats and oils is decreased during extended frying due to the loss of essential fatty acids and fat soluble vitamins. Other abusive practices include the addition of mineral oils in order to increase oil volume and the indiscriminate refining of abused oils by adding, for instance, substances such as lime and bleaching agents. These "treated" oils are then recirculated into the human food chain in an uncontrolled manner (Second National Symposium On Abused Cooking Oils, 1996). In Spain 600 people died in 1981 and more than 20,000 became ill as a result of the uncontrolled distribution of unlabelled refined cheap toxic cooking oils (in this case rapeseed oil) in plastic containers (Mitchell, 1987). Consequently, many countries have regulations prohibiting the use of

adulterated and over-oxidised oils and fats in the frying of foods (Firestone, 1993).

2.2 FRYING OILS AND THEIR ABUSE IN SOUTH AFRICA

Frying oils are typical lipids consisting mainly of triglycerides. Sunflower oil is used extensively in South Africa and contains large concentrations polyunsaturated fatty acids, such as linoleic acid (18:2), which is an essential fatty acid and necessary in the diet of humans and animals. This fatty acid, however, is also susceptible to oxidation producing a large array of compounds, which include among others polymers, cyclic monomers, low molecular weight products such as malondialdehyde and 4-hydroxyalkenals etc. Some of these compounds have been found to be extremely toxic to mice and rats while others showed diverse activities in human *in vitro* studies (Chow & Gupta, 1994).

The production of these oxidised compounds is initiated when fryer oil triglycerides are hydrolysed at high temperatures in the presence of moisture. This results in the formation of free fatty acids such as linoleic acid which are easily oxidised to form products such as hydroperoxides, free radicals, polymers and low molecular weight products. Many of the dimers and polymers formed are also referred to as polymerised triglycerides (PTGs) and the degradation products as polar compounds (PCs). The production of these compounds is enhanced by overheating (above 200 °C), extended heating

and repeated usage of the same oils and fats. This results in the darkening of the oil, bad odours and taste (rancid), excessive foaming, increase in oil viscosity, etc. (Kock *et al.*, 1996).

2.3 LEGISLATION AGAINST THE USE OF ABUSED FRYING OILS IN SOUTH AFRICA

Surveys conducted as part of this Ph.D. project in collaboration with the Health Departments of different municipalities, as well as surveys by the National Department of Health, have shown that many frying establishments in South Africa are using badly oxidised oils and fats in the frying of their foods (Fig. 1). According to Dr. T. van de Venter (Director: Food Control of the Department of Health), a sample drawn from a certain South African frying establishment contained as high as 74.7 % polymerised triglycerides, with the legal limit being below 16 %. According to him approximately 12 % of the 729 oil and fat samples drawn in a national survey (1996) by health officials from frying establishments across South Africa did not comply with specifications and were unsafe according to analyses by the Department of Health's Forensic Chemistry Laboratory in Pretoria. Dr van de Venter stated the following: *"The survey was not an unbiased epidemiologically correct exercise so we are not claiming that almost 12 % of the cooking oil that is in use is unsafe. The fact that so many samples were in non-compliance is, however, a cause of concern."* These surveys have led to the publication of the Edible Fats and Oil Regulations in terms of the Foodstuffs, Cosmetics

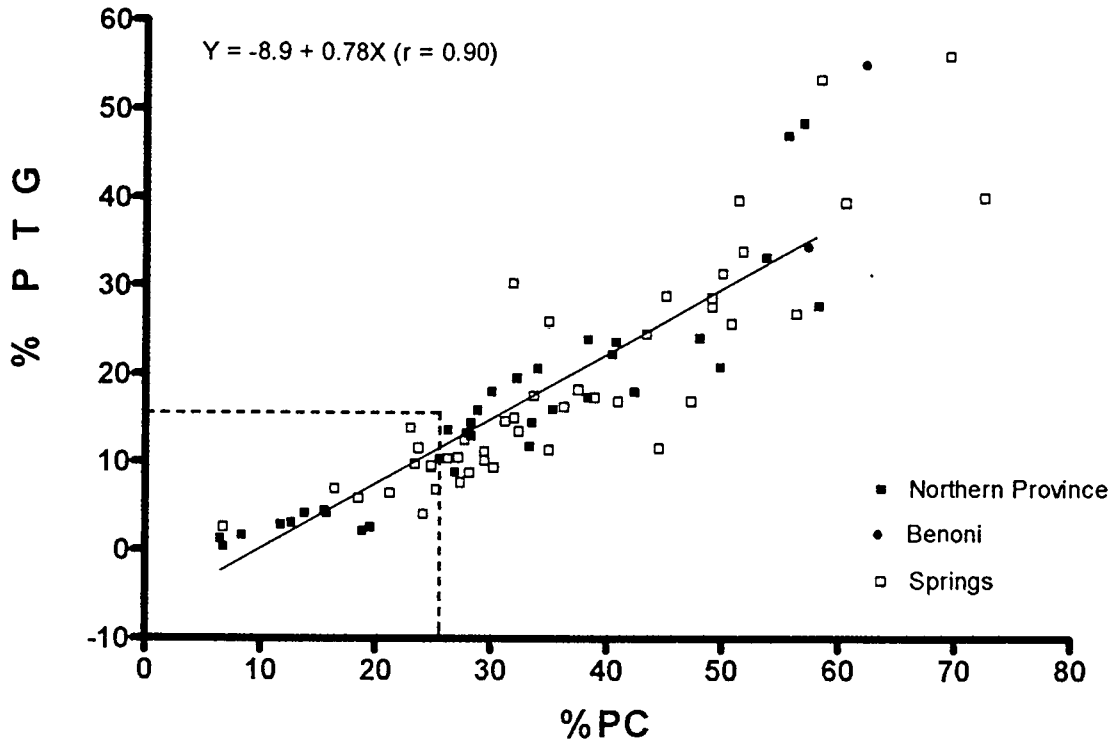

Fig. 1. The quality of frying oil and fat samples drawn (n = 81) by environmental health officials in the Northern Province, as well as Gauteng Towns i.e. Benoni and Springs. PC = polar compounds; PTG = polymerised triglycerides. Polar compounds means monoglycerides, diglycerides and free fatty acids as well as oxidative degradation products of these compounds and their parent triglycerides as determined by column chromatography (Official Methods of Analysis of the AOAC, 1990). Polymerised triglycerides means the degradation products formed by carbon to carbon and/or carbon to oxygen linkages between triglyceride-bound fatty acids which produce dimeric or higher polymeric triglycerides, as determined by gel permeation chromatography (Beljaars, 1993).

and Disinfectants Act 1972 (Act 54 of 1972), on 16 August 1996 (Second National Symposium On Abused Cooking Oils, 1996).

These include the following: *"For the purposes of section 2 (1) (b) (i) of the Act, in so far as it applies to foodstuffs, edible fats and oils used for the frying of food are hereby deemed to be harmful or injurious to human health, unless they contain less than –*

(a) 16 % polymerised triglycerides; and /or

(b) 25 % polar compounds".

2.4 ACCUMULATION OF RESTAURANT WASTE OILS AND FATS

Since frying establishments in South Africa are not allowed to sell these oils for human consumption or, in most cases, discard used frying oils and fats by dumping into municipal drainage systems, these substances will now start to accumulate at these premises. Consequently, it is of the utmost importance that competent oil renderers come forward to collect these oil and fat wastes for recycling to, for instance, animal feed. Renderers must make sure that only legally used oils and fats are collected which are still within the limits set out by legislation. If used restaurant oils and fats are oxidised excessively, free radicals are formed which, when mixed into animal feed, can cause the deterioration of oil and fat into unhealthy degradation products which will negatively influence the shelf life of these products. In South Africa it is estimated that more than 50,000 tons/year of oils and fats are used for the

frying of food. Of this, at least 60 % should accumulate as used oils and fats if the situation in the United States of America is used as reference model (Haumann, 1990).

2.5 THE U.S.A. SOLUTION

More than 440,000 commercial food service operations exist in the United States of America. These companies are the main source of waste oils and fats and purchase an estimated 0.8 to 0.9 million tons of frying oils and fats each year. From these companies, approximately 1.1 million tons of waste oils and fats are collected annually by the rendering industry and independent grease peddlers. The increased waste fat and oil yield is due to "invisible" fat originating from foods being fried, and the addition of water. These waste oils and fats are then specially processed to yield approximately 0.7 million tons of yellow grease for incorporation into animal and poultry feeds, including pet foods. It is estimated that 95 % of the yellow grease produced from restaurant waste oils is later incorporated into animal feed and pet foods and the remaining 5 % distributed to the soap industry. The purpose of adding yellow grease to these feeds is mainly to provide energy through its caloric value, reducing dust levels and increasing lubricity during pelletizing operations. Yellow grease contains two and a quarter times the energy value of carbohydrates such as corn (on a weight per weight basis).

In the U.S.A. metropolitan areas, renderers usually pay frying establishments for waste oils and fats according to a formula based on the weight of waste grease collected and the selling price of the finished yellow grease product. In some areas, frying establishments pay for grease collection or give the grease away for free, as they have no other legal way of discarding oil wastes. In general, about 2 to 4 U.S. cents per kilogram are paid for waste restaurant oils and fats. Processed yellow grease is worth 20 to 33 U.S. cents per kilogram. The following suggests the quality specifications for yellow grease in the U.S.A.: total fatty acids, 90 %; free fatty acids, 15 %; moisture, 1.0 %; impurities, 0.5 %; unsaponifiables, 1.0 %; total MIU, 2.0 %; iodine value, 58-79; AOM (hours), 20 (NRA, 1992).

2.6 OTHER POTENTIAL USES OF USED OILS AND FATS

The idea of recycling used oils and fats has its origin during the Second World War, when households were requested to save their bacon drippings for conversion to machinery lubricants. These oils and fats have also found application as part of a concrete mix in order to help prevent cracks; in spraying plants to decrease water loss through evaporation resulting in less irrigation; a potential alternative diesel fuel source; soaps and dispersing agent in biodegradable detergents (Haumann, 1990). It is also possible to use these oils as a cheap carbon source in the production of high value essential lipids such as gamma-linolenic acid (GLA). Consequently, the rest of the thesis will be dedicated to this aspect. It is important to note that used

frying oils and fats, which are discarded before reaching regulatory limits, are not toxic. All used cooking oil (UCO) used in experiments to follow fall into this category.

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CHAPTER 3

EDIBLE OIL UTILISATION BY FUNGI IN THE PRESENCE OF ACETATE

The first part of this chapter has been published as:

Enhanced sunflower oil utilisation and gamma-linolenic acid production by *Mucor circinelloides* f. *circinelloides* CBS 108.16 in the presence of acetate

World Journal of Microbiology & Biotechnology 13, 357-358

3.1 ENHANCED SUNFLOWER OIL UTILISATION AND GAMMA-LINOLENIC ACID PRODUCTION BY *MUCOR CIRCINELLOIDES* F. *CIRCINELLOIDES* CBS 108.16 IN THE PRESENCE OF ACETATE

3.1.1 INTRODUCTION

When micro-organisms are cultivated on plant and animal oils, little change usually occurs in the fatty acid profile of the added oil. In this case, micro-organisms generally appear to terminate lipid synthesis as well as desaturation and elongation reactions (Ratledge, 1989; Kendrick & Ratledge, 1996). With this as background and the current interest in the production of polyunsaturated fatty acids (PUFAs) by fungi, we report an increase in gamma-linolenic acid (GLA) production and enhanced utilisation of sunflower oil by *Mucor circinelloides f. circinelloides* CBS 108.16 when grown on this oil in the presence of sodium acetate.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Micro-organisms and Growth Conditions

The strain *Mucor circinelloides f. circinelloides* CBS 108.16 was transferred from 4-day-old YM slants (incubated at 30 °C) to 100 ml sterile growth medium in 1 l conical flasks. The sunflower oil medium contained (g/l):

sunflower oil, 40; 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 of (g/l): $FeSO_4.7H_2O$, 0.035; $MnSO_4.4H_2O$, 0.007; $ZnSO_4.7H_2O$, 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. The pH was adjusted to 5.8. The mixed medium was similar to that described previously but contained 30 g/l sunflower oil and 10 g/l sodium acetate as carbon sources. All experiments were performed at least in triplicate and the flasks were incubated at 30 °C for 7 days while shaking at 160 rev/min.

3.1.2.2 Assays

After cultivation, cells were harvested by filtration (Whatman no. 1), washed extensively as described by Kendrick & Ratledge (1996) and then immediately frozen and freeze dried. This was followed by lipid analyses which included determining the lipid content of cells, fractionation of lipids, and fatty acid analyses as previously described (Roux *et. al.*, 1994).

3.1.3 RESULTS AND DISCUSSION

The fungus produced similar amounts of lipid when grown on sunflower oil alone or on a combination of sunflower oil and sodium acetate (Table 1). It is unlikely that the high lipid content was caused by oil adsorption to surface layers since all cells were thoroughly washed and appeared clean under the microscope. Strikingly, when sodium acetate was added to the medium

containing sunflower oil, a drastic increase in lipid utilisation and consequent biomass production occurred (Table 1). This phenomenon could not be explained and was the subject of further research.

When the fungus was grown on sunflower oil alone, the fatty acid profiles of the fungal neutral lipids were, as expected (Kendrick & Ratledge, 1996), predominantly similar to that of the original substrate (Table 2). Only small amounts of GLA (18:3 ω6) were formed, even though high proportions of the immediate precursor of GLA (i.e. linoleic acid, 18:2 ω6) was present in the original substrate. Consequently, this fungus had a much decreased capacity for GLA production when grown on sunflower oil alone. In the presence of both sunflower oil and sodium acetate, a significant increase in the GLA proportion of the fungal neutral- and phospholipids occurred. These results indicated that an increase in the Δ^6 desaturation occurred in the phospholipid fraction when sodium acetate was added, which was probably responsible for the increased amounts of this fatty acid in the neutral lipid fraction.

To conclude, evidence exists to suggest that the Δ^6 desaturation reaction is less repressed when sodium acetate is added to the medium containing sunflower oil. This discovery may give new insight in the utilisation of plant oils as a possible source for the production of high value fatty acids such as GLA. Further research is underway to elucidate the mechanisms underlying this phenomenon.

Table 1. Growth yield, lipid accumulation and lipid utilisation by *Mucor circinelloides* f. *circinelloides* CBS 108.16 grown on sunflower oil and on a mixture of sunflower oil and sodium acetate.

Substrate	Cell Yield (g dry wt/l)	Lipid (% w/w dry wt)	Lipid utilisation (% w/w of original oil)
Sunflower oil (40 g/l)	9.3	50.0	33.5
Sunflower oil (30 g/l) + Sodium acetate (10 /l)	19.1	50.8	95.4

Values represent means of at least three repetitions. Standard deviation was <10% of the mean in all cases.

Table 2. Fatty acyl profiles of neutral lipid (NL), sphingo- plus glycolipid (S+G) and phospholipid (PL) fractions from *Mucor circinelloides f. circinelloides* CBS 108.16 grown on sunflower oil in combination with sodium acetate.

		Relative % fatty acyl groups						
Substrate	Fraction	16:0	16:1	18:0	18:1	18:2	γ18:3	α18:3
Sunflower oil (40 g/l)	S + G	8.8	0.2	6.6	19.9	58.2	0.4	0.1
	PL	16.7	1.6	7.7	20.1	42.5	5.7	0.1
	NL	9.8	0.4	8.7	19.5	59.1	0.6	0.1
Sunflower oil (30 g/l) +	S + G	11.9	0.2	15.9	18.6	48.1	0.5	0.2
Sodium acetate (10 g/l)	PL	16.5	2.3	8.7	24.7	33.0	9.6	0.3
	NL	14.9	0.9	4.7	19.0	53.5	4.6	0.2
Original oil		7.4	0.2	6.0	19.0	63.9	0.4	0.1

Values represent means of at least three repetitions. Standard deviation was <10% of the mean in all cases.

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3.2 THE BIOTRANSFORMATION OF USED COOKING OIL TO ESSENTIAL LIPIDS

3.2.1 INTRODUCTION

Evening Primrose oil (EPO), which is extracted from the seeds of the evening primrose plant, is characterised by the presence of 7 % to 12 % (w/w) gamma-linolenic acid (18:3 ω6 or GLA) as well as high concentrations linoleic acid (18:2 ω6) (Graham, 1984). Gamma-linolenic acid is an essential precursor for the production of prostaglandins, which is necessary for the maintenance of all living cells in mammalian tissues. For this reason, EPO is at present a prescribable medicine for the treatment of skin disorders such as eczema and about 800 tons are sold annually on the world market (Ratledge, 1994).

According to literature (Kendrick, 1991), zygomycetous fungi such as *Mucor* produce the Δ^6-desaturase enzyme, making it possible to convert 18:2 (ω6) to GLA. Since most cooking oils, especially those consisting of sunflower oil, contain substantial amounts (about 70 %) of 18:2 (ω6) (Shukla, 1994) it seems possible to convert the much cheaper cooking oil to oils containing GLA by utilising *Mucor.* However, according to literature (Kendrick, 1991), fatty acids derived from used cooking oil in the growth medium inhibit the Δ^6-desaturase as well as the *ab initio* synthesis of lipids in *Mucor,* which in turn results in the accumulation of oil in this fungus which is similar to that of cooking oil.

Recently, it was discovered in our laboratory that when acetate was added to a fungal growth medium containing lipids as sole carbon source, the Δ^6-desaturase inhibition by the resulting free fatty acids seemed to be partially uplifted resulting in the production of oil containing unsaturated fatty acids such as GLA by *Mucor*. With this as background, the aim of this study became to evaluate the possibility of converting cooking oil as well as linseed oil in the presence of acetate to high value oils containing GLA.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Fungal strains studied. Seven strains of *Mucor circinelloides*, i.e. CBS 203.28, CBS 108.16, PPRI 5511, PPRI 5512, PPRI 5514, PPRI 5513 and PPRI 5515, and one strain of *Mucor rouxii* CBS 416.77 were examined. The PPRI cultures were obtained from the culture collection of the University of the Orange Free State in Bloemfontein, South Africa and the CBS culture from the Centraalbureau voor Schimmelcultures in Baarn, The Netherlands.

3.2.2.2 Cultivation. The eight *Mucor* strains used in this study were transferred from 4 day old YM (yeast extract and malt extract agar) slants (incubated at 30 °C) to sterile 100 ml growth medium contained in 1 l conical flasks. The basal medium consisted of the following in g/l: yeast extract, 0.1; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.00; $CaCl_2.2H_2O$, 0.05; NH_4Cl, 1.28. The pH was set at 5.8.

In order to determine the effects of used cooking oil (UCO) and sodium acetate alone and in combination on lipid production, the following experiments were performed: To one set of experiments (Run 1), 30 g/l non-toxic UCO (sunflower oil used continuously for 96 h in the frying of potato

chips at 190 °C and still within regulatory limits) was added to the basal medium. In another set of experiments (Run 2) both 10 g/l sodium acetate as well as 30 g/l UCO were added while in another set of experiments (Run 3) only 10 g/l sodium acetate was added to the basal medium. All experiments were performed at least in duplicate and the flasks incubated at 30 °C for 7 days while shaking (160 rev/min).

These experiments were repeated for fresh cooking oil (sunflower oil not yet used in frying) as well as linseed oil where UCO was replaced by 30 g/l of each of these oils. Only *Mucor circinelloides* CBS 108.16 was used in these experiments. After cultivation, cells were harvested by filtration (Whatman no. 1), washed with distilled water and then immediately frozen and freeze dried.

In order to determine the effects of different concentrations of UCO on lipid production in *Mucor circinelloides* CBS 108.16, 0, 10, 20, 30, 40, 50, 60, and 100 g/l UCO were added in combination with 10 g/l sodium acetate to the same basal medium as described earlier. All experiments were performed at least in duplicate. Cultivation and harvesting were similar as described previously.

3.2.2.3 Lipid extraction. In all experiments the corresponding supernatants were first acidified to pH 4 with 1 N HCl before lipids were extracted with 4 volumes (50 ml each) hexane. These were then evaporated to dryness and dried to constant weight over P_2O_5 in a vacuum oven at 55 °C and then weighed.

Lipids were extracted from freeze dried cells as described by Kendrick & Ratledge (1992). These include extraction with chloroform/methanol (2:1 v/v) according to Folch *et al.* (1957), followed by two washes with distilled water

and final evaporation of the organic phase under vacuum. Following this, the lipid material was dissolved in diethyl ether and then transferred to preweighed vials. Before lipids were weighed, samples were dried to constant weight in a vacuum oven over P_2O_5 at 55 °C. Samples were stored at –20 °C under a blanket of N_2.

3.2.2.4 Fractionation of extracted lipids. The extracted lipids were dissolved in chloroform and then applied to a column (140 mm x 20 mm) of silicic acid, activated by heating overnight. Neutral, sphingo- and glycolipids (as a combined fraction), as well as polar lipids, were eluted by successive applications of organic solvents as described by Kendrick & Ratledge (1992). Final solvent removal and storage were as for whole lipid extracts.

3.2.2.5 Fatty acid analysis. Lipids were dissolved in chloroform and methylated by the addition of trimethylsulphonium hydroxide (TMSH) as described by Butte (1983). The fatty acid methyl esters were analysed by gas chromatography (GC), with a flame ionisation detector and a supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 °C was increased by 3 °C/min to 225 °C and, following a 10 min isothermal period, was then increased to 240 °C at the same rate. The inlet and detector temperatures were 170 °C and 250 °C, respectively. Nitrogen was used as carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

3.2.2.6 Acetic acid analysis. Residual acetic acid present in the supernatants of all experiments performed after 7 days of growth, was determined by GC as described by Du Preez & Lategan (1978).

3.2.2.7 Chemicals. All organic chemicals and solvents used were of analytical reagent grade and obtained from major retailers. Silicic acid (100 mesh) was from Aldrich. Lipid standards were from Sigma.

3.2.3 RESULTS AND DISCUSSION

3.2.3.1 Production of GLA containing oils by *Mucor* strains

When UCO in combination with sodium acetate were added to the growth medium, a clear stimulatory effect on GLA production could be seen in all mucoralean fungi tested when compared to experiments where only UCO or sodium acetate was used as carbon source (Fig. 1). *Mucor circinelloides* CBS 108.16 proved to produce the most GLA (820 mg GLA/l) followed by *Mucor rouxii* CBS 416.77 (600 mg GLA/l) and then *M. circinelloides* PPRI 5511 (398 mg GLA/l), PPRI 5512 (336 mg GLA/l), CBS 203.28 (320 mg GLA/l), PPRI 5514 (315 mg GLA/l), PPRI 5513 (190 mg GLA/l) and PPRI 5515 (95 mg GLA/l). It was also found that *Mucor circinelloides* CBS 108.16 accumulated oil with a similar GLA concentration to that of EPO. All the other strains tested produced oils containing less than 4.2 % GLA (w/w).

Similar results were obtained (826 ± 0.09 mg GLA/l) when *Mucor circinelloides* CBS 108.16 was grown in the presence of both fresh cooking oil and sodium acetate. When these experiments were repeated with linseed oil and sodium acetate as sole carbon sources, much less GLA was produced (351 ± 0.02 mg GLA/l) as well as fungal oil with a low GLA content (2.5 ± 0.30 %, w/w). This may be explained by a possible inhibitory effect of the large concentrations of alpha-linolenic acid (18:3 ω3) present in linseed oil (Shukla, 1994) on GLA production from 18:2 (ω6). Again a clear stimulatory effect on

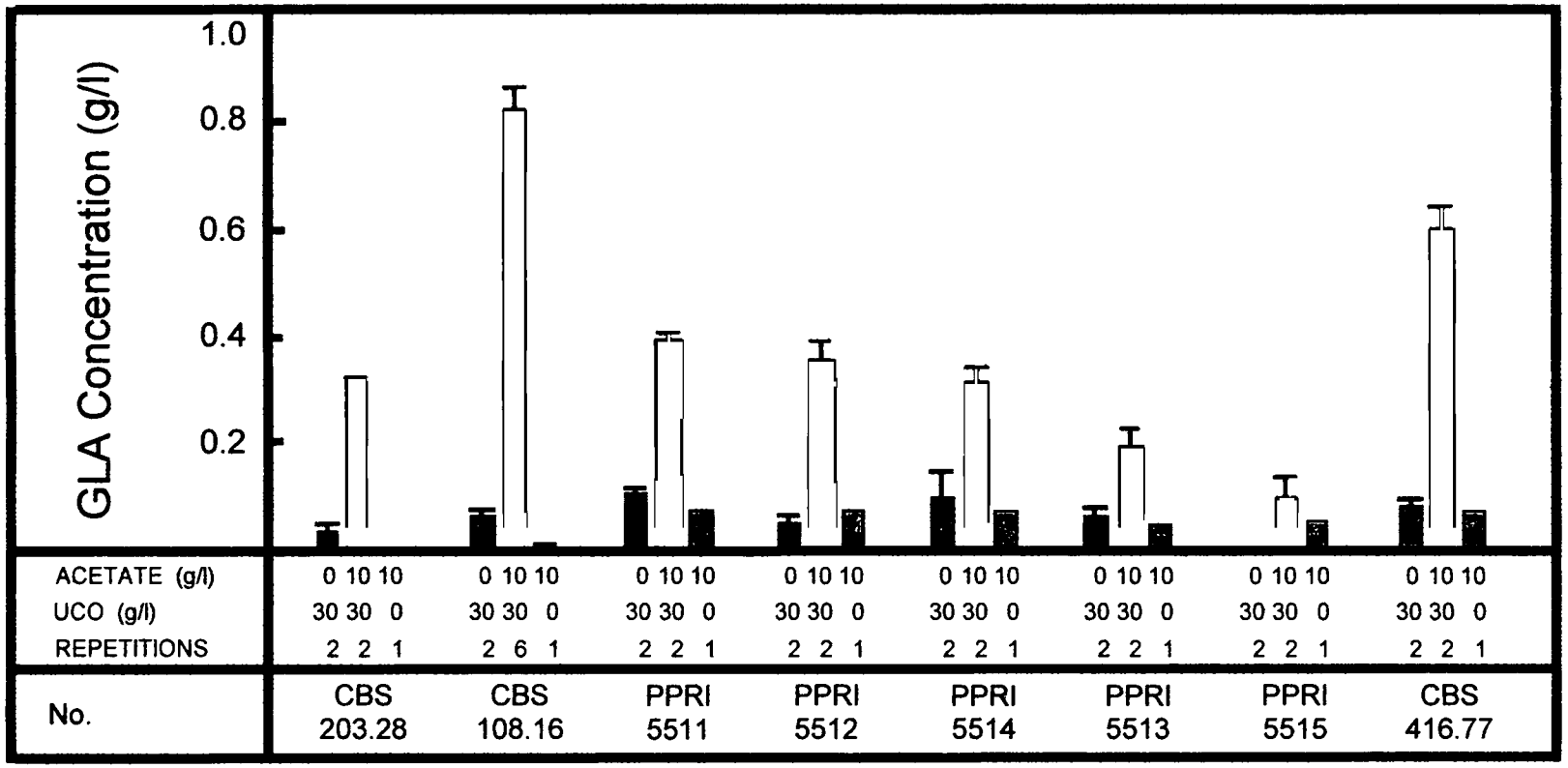

Fig. 1. Production of gamma-linolenic acid (GLA) by 8 *Mucor* strains grown on used cooking oil (30 g/l) and sodium acetate (10 g/l). Bars indicate variation.

GLA production (351 ± 0.02 mg GLA/l) was evident when both linseed oil and sodium acetate were used as carbon sources, compared to when only linseed oil (15 ± 0.001 mg GLA/l) or sodium acetate (9 ± 0.00 mg GLA/l) was added.

3.2.3.2 Lipid production by *Mucor circinelloides* CBS 108.16

Because of the results above, lipid production by *Mucor circinelloides* CBS 108.16 grown on UCO will be described in more detail (Figs. 2A to G). According to Fig. 2A, it is clear that the combination of UCO and sodium acetate stimulated biomass production to about 22 g/l compared to the much lower values obtained when only UCO (about 11 g biomass/l) or sodium acetate (about 1 g biomass/l) was used as sole carbon source.

It is important to note that the cells contained extremely high concentrations of crude oil (Fig. 2B) when grown on only UCO (about 70 %, w/w) as well as on the combination of UCO and sodium acetate (about 68 %, w/w). In both cases, these oils contained more than 90 % (w/w) NLs (Fig. 2C). Much less crude oil and NLs were produced when only sodium acetate was used as carbon source. Consequently, a significantly enhanced NL production (about 14 g/l) by this fungal strain was achieved when grown on both sodium acetate as well as UCO (Fig. 2D). It is also important to that the NL composition in this case was similar to that of EPO, containing about 6 % GLA (Fig. 2E) as well as high concentrations of 18:2 (about 40 %). When UCO was added to the growth medium as sole carbon source, a significant decrease in NL production occurred (to about 7 g/l) and in this case the fatty acid composition of this fraction was similar to that of UCO originally fed to the medium containing a very low GLA percentage (about 2 %, Fig 2E). This is in accordance with literature (Kendrick, 1991), where it is proposed that the free fatty acids derived from oils used as substrate, inhibit both lipid synthesis *ab*

initio as well as Δ^6-desaturase responsible for GLA production inside the fungus. Eventually these free fatty acids are re-esterified to glycerol and leads to the accumulation of fungal oil similar to UCO, which was originally fed to the medium.

Consequently, according to Fig. 2F, the presence of both sodium acetate and UCO in the growth medium enhanced GLA production by this fungus significantly (to 820 mg GLA/l) when compared to the very low concentrations of GLA produced in the presence of UCO only (about 60 mg GLA/l) and sodium acetate only (about 9 mg GLA/l).

Note that the combination of these carbon sources not only significantly enhanced biomass, NL and GLA production, but it also improved the utilisation of the UCO which was added to the growth medium (Fig. 2G). In this case about 98 % of oil added to the medium was consumed compared to only about 60 % in the absence of sodium acetate.

It was also found that the acetic acid fraction was totally depleted in all experiments conducted after 7 days of cultivation. Consequently, the combination of both carbon sources resulted in a GLA yield of 0.021 g GLA/g substrate utilised as well as a substantial EPO equivalent (EPOeq.) yield of as high as 0.35 g EPOeq./g substrate utilised. Similar results as are illustrated in Fig. 2 (A to G) were obtained when fresh cooking oil was used in combination with sodium acetate as carbon sources.

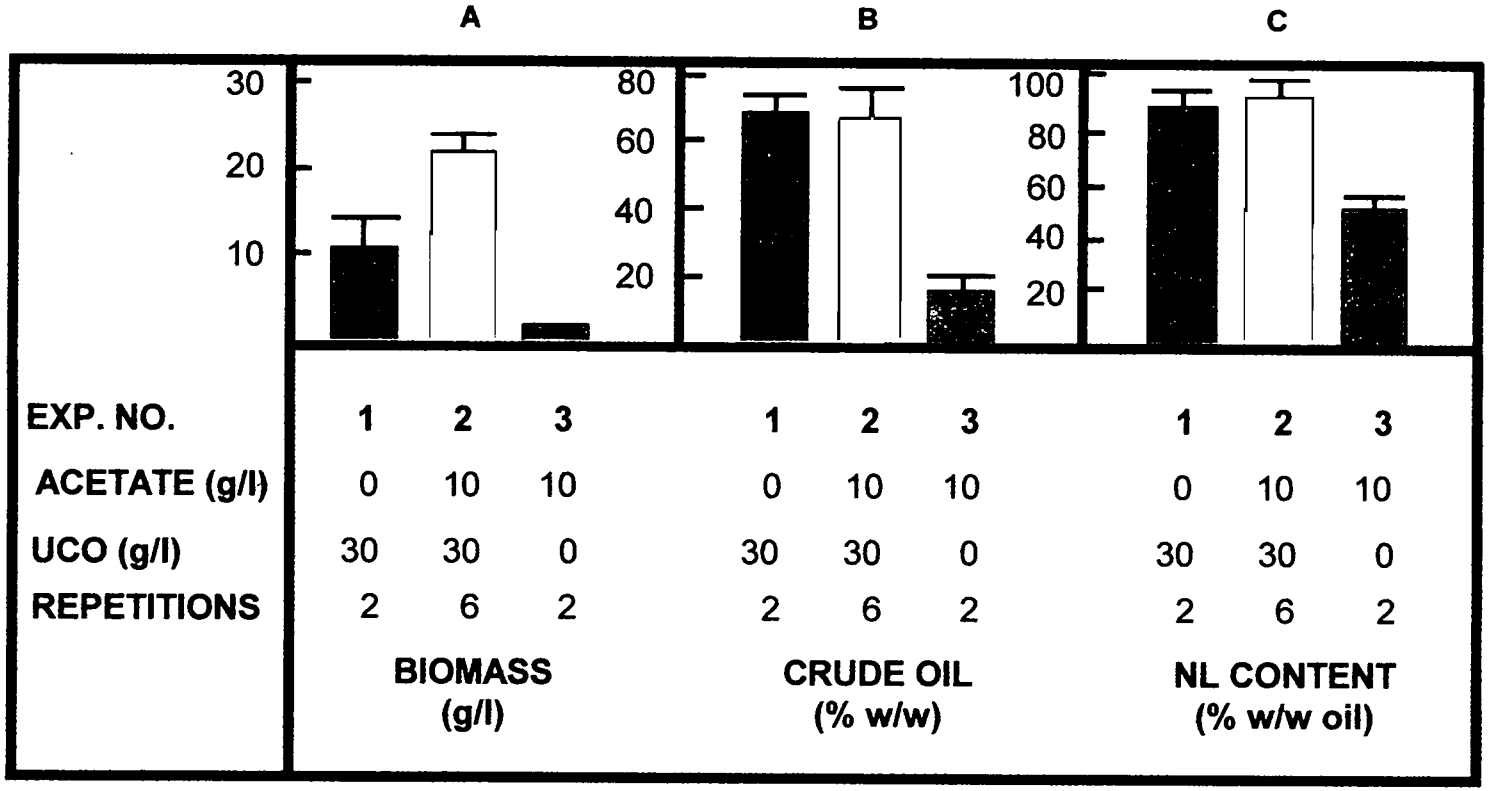

Fig. 2. Effect of sodium acetate (10 g/l) in combination with used cooking oil (30 g/l) on biomass and lipid production by *Mucor circinelloides* CBS 108.16. (A) Biomass; (B) Crude oil; (C) Neutral lipid (NL) content. Bars indicate variation.

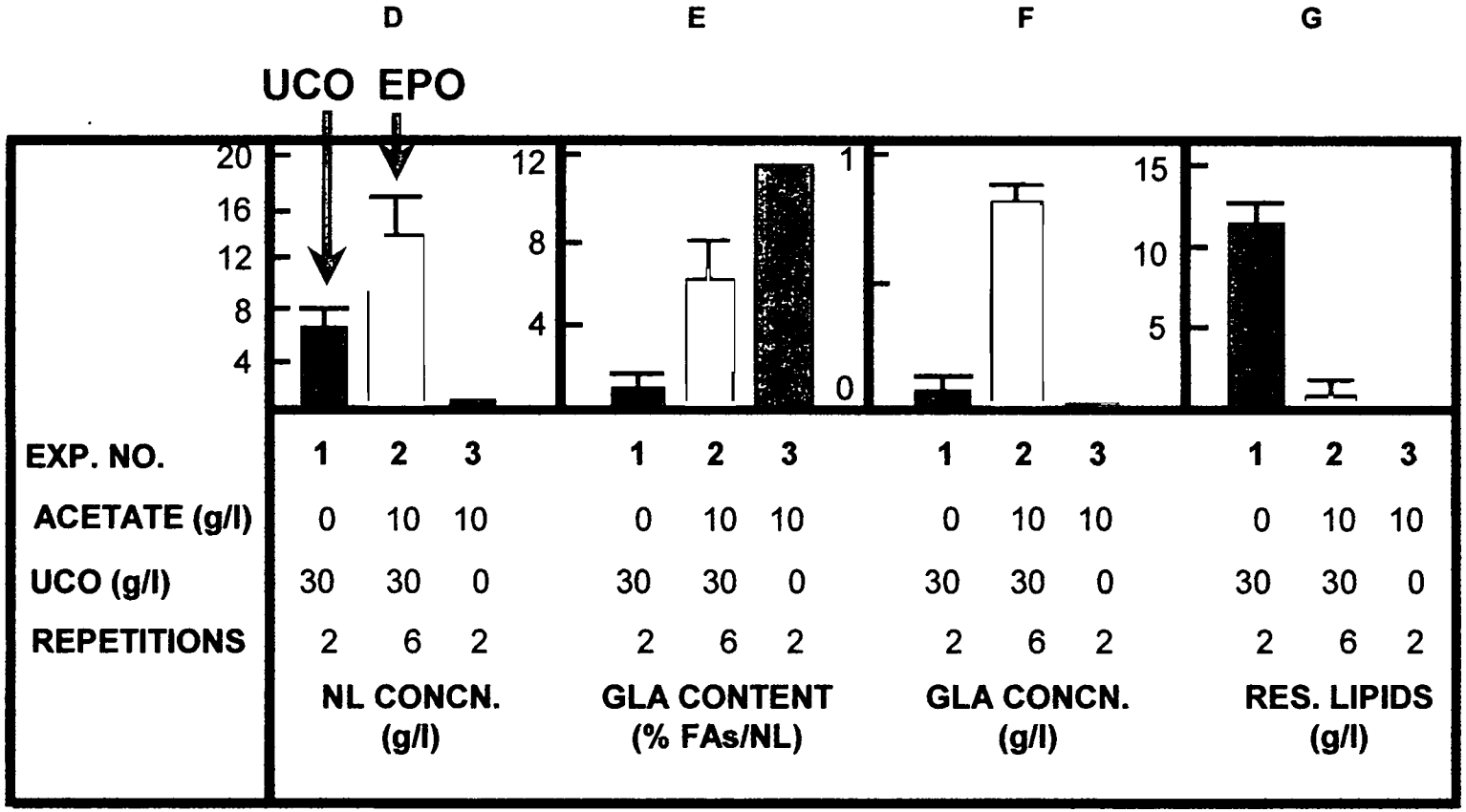

Fig. 2. Continued. (D) NL concentration; (E) Gamma-linolenic acid (GLA) content; (F) GLA concentration; (G) Residual lipids; UCO = Used Cooking Oil; EPO = Evening Primrose Oil. Bars indicate variation.

3.2.3.3 Effect of different UCO concentrations in the presence of sodium acetate on biomass and lipid production in *Mucor circinelloides* CBS 108.16

In these experiments different concentrations of UCO in the presence of 10 g sodium acetate/l were added to separate experiments to determine the effect of UCO concentrations on biomass as well as lipid utilisation and production by *Mucor circinelloides* CBS 108.16. An almost linear increase in biomass concentration (from about 8 to 41 g/l) was experienced with an increase in UCO (from 10 to 60 g/l) (Fig. 3). When 100 g UCO/l was added, the biomass increased to 48 g/l. The NL concentration increase followed a similar trend and reached a maximum when 100 g UCO/l was added (about 35 g/l). It is important to note that the GLA content already reached an optimum (48 mg GLA/g dw) when only 10 g UCO/l was added.

When increased amounts of this oil were added, a dramatic decrease in GLA content occurred (to as low as 19 mg GLA/g dw) which coincided with a drop in GLA percentage in the fungal oil (to as low as 3 %, w/w). Maximum yields for both GLA production (0.021 g GLA/g carbon substrate) and EPO equivalent production (0.35 g EPOeq./g carbon substrate) were achieved when 30 g UCO/l in the presence of sodium acetate was added to the growth medium.

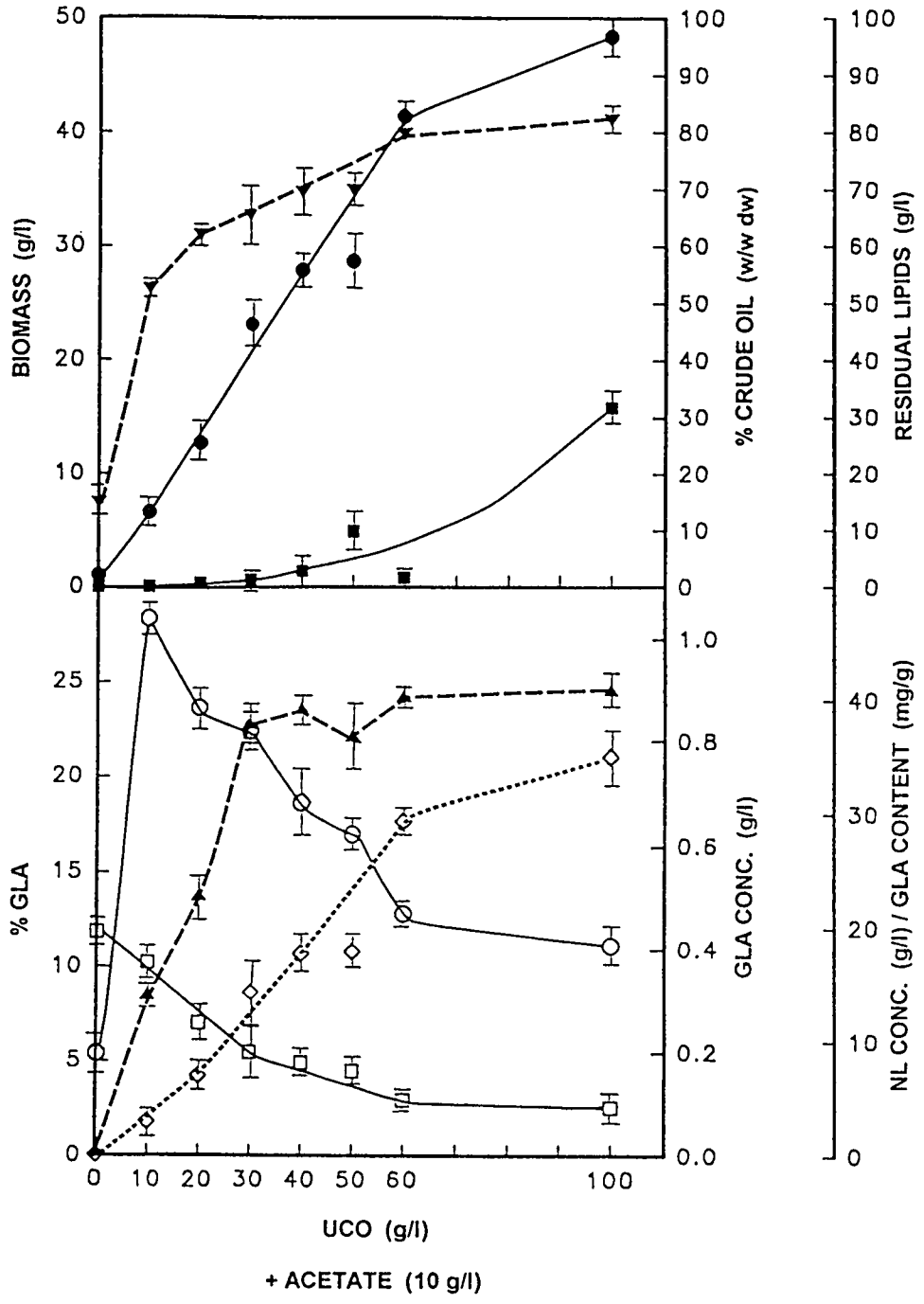

Fig. 3. Effects of different used cooking oil (UCO) concentrations in the presence of sodium acetate on biomass, lipid production as well as lipid utilisation by *Mucor circinelloides* CBS 108.16. Bars indicate variation. Each point represents the mean of duplicate cultures. (●), Biomass; (▼), % Crude oil; (■), Residual lipids; (▲), GLA concentration; (◇), NL concentration; (O), GLA content; (□), % GLA.

On the basis of these results the following conclusions were drawn:

1. Sodium acetate in combination with edible oils had a large stimulatory effect on edible oil utilisation as well as biomass, NL and GLA production in several mucoralean strains. Increased GLA production was possibly due to the partial upliftment by acetate of the inhibitory effect of free fatty acids on malic enzyme responsible for producing the reducing power (NADPH) necessary for Δ^6-desaturase activity (Kendrick, 1991).

2. A large portion of UCO was converted to EPO equivalents (yield = 0.35 g EPOeq./g carbon substrate utilised) by *Mucor circinelloides* CBS 108.16 when sodium acetate in combination with UCO were used as carbon substrates. This poses the possibility of producing EPO equivalents from a waste product.

3. Maximum yields for GLA production (0.021 g GLA/g carbon substrate) and EPO equivalent production (0.35 g EPOeq./g carbon substrate) were obtained when 30 g UCO/l in combination with 10 g sodium acetate/l were used as carbon sources.

3.2.4 ACKNOWLEDGEMENTS

We thank P.J. Botes for his invaluable technical assistance. This work was funded by the Foundation for Research Development and the Sasol Centre for Biotechnology.

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CHAPTER 4

EFFECT OF ACETATE AND pH ON THE LIPID COMPOSITION OF *MUCOR CIRCINELLOIDES* GROWN ON SUNFLOWER OIL

This chapter has been accepted for publication in Systematic and Applied Microbiology

4.1 INTRODUCTION

Recently we described a substantially improved utilisation of sunflower oil by *Mucor circinelloides* f. *circinelloides* in the presence of sodium acetate. This utilisation was accompanied by a doubling of the biomass production and an enhancement of the intracellular gamma-linolenic acid (GLA) content as compared to growth conditions with sunflower oil as sole carbon source (Jeffery *et al.*, 1997). Because of the current interest in the production in polyunsaturated fatty acids (PUFAs) by fungi (Kock & Botha, 1995), we investigated the effect of sodium acetate on changes in the extracellular and intracellular lipid composition during growth on sunflower oil. Here we report on some characteristics of the acetate-supported utilisation of sunflower oil by this fungus.

4.2 MATERIALS AND METHODS

4.2.1 Shake flask cultivations

4.2.1.1 ***Micro-organism, growth and harvesting:*** *Mucor circinelloides* f. *circinelloides* CBS 108.16 was transferred from 4 day old YM (yeast extract and malt extract agar) slants (incubated at 30 °C) to sterile 100 ml medium in 1 l conical flasks. The medium consisted of the following (g/l): sunflower oil, 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 and trace elements as described elsewhere

(Du Preez & Van der Walt, 1983). The pH was adjusted to 5.8. Shake flasks, incubated at 30 °C for 7 days at 160 r/min, were drawn at different time intervals as indicated in Fig. 1 for analysis. All experiments were performed at least in triplicate. Following cultivation, cells were harvested by filtration using Whatman no. 1 filters, washed extensively as described elsewhere (Kendrick & Ratledge, 1996) and then immediately frozen and freeze-dried. As control, the same medium as above was used with the exception that the sunflower oil was increased to 40 g/l and the sodium acetate omitted.

4.2.2 Bioreactor cultivations

4.2.2.1 ***Inoculum:*** Petri dishes with 4 % Sabouraud dextrose agar (SDA agar) were inoculated by streaking out spores from fresh SDA agar slants and incubated at 30 °C. After 2 days the spores were washed off with 0.05 M KH_2PO_4 containing 0.1 % Tween 80 and spore counts done in a Fast-read 10 counting chamber (Davies Diagnostics, Randburg). This spore suspension was used to inoculate 1 l conical flasks, each with 250 ml medium, to a concentration of approximately 1 x 10^5 spores/ml. The medium contained (g/l): glucose, 20; citric acid, 0.15; $(NH_4)_2SO_4$, 2.5; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 5; $CaCl_2.2H_2O$, 0.04; yeast extract, 3 and the pH was adjusted to 6. The flasks were incubated on a rotary shaker for 24 h at 30 °C, 160 r/min and 1 l used to inoculate the bioreactor.

4.2.2.2 ***Cultivation:*** The fungus was grown at 30 °C for 168 h in a 14 l stirred tank reactor (Chemap, CF 3000) fitted with three disk turbine impellers and containing 9 l medium. The dissolved oxygen tension (DOT) was

monitored with an Ingold (Urdorf) polarographic pO_2 electrode and maintained at or above 20 % of saturation by automatic adjustment of the stirrer speed with 1 vvm aeration throughout the cultivation. The pH was monitored with an Ingold pH electrode. Three different experiments were conducted. In the first experiment the medium contained (g/l): sunflower oil, 30; anhydrous sodium acetate, 10; citric acid, 0.25; yeast extract, 0.1; $(NH_4)_2SO_4$, 1.6; KH_2PO_4, 7.5; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05 and trace elements as above. The initial pH was adjusted to 5.8 with 5 M KOH. In the second experiment the sodium acetate was omitted from the medium and the pH gradually increased during the course of the cultivation from pH 5.8 to pH 8.4 with 5M KOH, according to a pH profile set to mimic the natural increase in pH (due to acetic acid assimilation) observed in the first experiment. In the third experiment the conditions were identical to the second experiment, except that the pH was not controlled but allowed to decrease due to ammonia assimilation. The bioreactor and medium were sterilized *in situ*, with the exception of the sunflower oil, which was autoclaved separately and aseptically added to the sterile reactor. All samples for lipid analyses were harvested as described above.

4.2.3 Analytical procedures

4.2.3.1 ***Extraction and fractionation of lipids:*** These were performed according to the methods described by Kock *et al.* (1997). Briefly, extracellular lipids, present in the supernatant (pH < 4) were immediately extracted after harvesting with hexane until almost no lipids could be detected. Intracellular lipids were extracted from the freeze-dried cells using

chloroform/methanol (2:1, by vol.) (Folch *et al.*, 1957). The neutral (NL), glyco- (GL) and phospholipid (PL) contents were obtained for both extracellular and intracellular lipids by applying organic solvents of various polarities to a column of activated silicic acid. Column separation efficiency was determined by TLC (Kock & Ratledge, 1993). The various fractions were dissolved in diethyl ether and transferred to pre-weighed vials and dried to a constant weight in a vacuum oven at 50 °C over P_2O_5.

4.2.3.2 ***Fatty acid analysis:*** Trans-esterification of extracellular and intracellular lipids as well as methylation of extracellular free fatty acids were performed by the respective addition of trimethyl sulphonium hydroxide (TMSH) and diazomethane followed by gas chromatographic analysis (Kock *et al.*, 1997).

4.2.3.3 ***Acetic acid analysis:*** The acetic acid content of the supernatant was determined using a Hewlett Packard gas chromatograph equipped with a flame ionization detector and a 30 m x 0.53 mm Nukol capillary column. The initial oven temperature of 120 °C was increased at a rate of 6 °C/min to 165 °C. The inlet and detector temperatures were 135 °C and 250 °C, respectively. The carrier gas was nitrogen at a flow rate of 5 ml/min. The samples and acetic acid standard (1 ml) were acidified with 0.3 ml of a 25 % formic acid solution prior to analysis.

4.2.3.4 ***Dry weight determination:*** Dry mycelial biomass was gravimetrically determined in duplicate by filtration of 10 ml aliquots through pre-dried and

pre-weighed glass microfibre filters (Whatman GF/A), washed with distilled water and dried at 105 °C in an infrared drying oven (Mettler LP 16).

4.2.3.5 ***Chemicals:*** All organic chemicals and solvents used were of analytical reagent grade and obtained from major retailers. Silicic acid (100 mesh) was obtained from Aldrich (Germany). NL and PL standards, as well as the fatty acid standards were obtained from Sigma (Germany).

4.3 RESULTS AND DISCUSSION

In the presence of a substrate mixture of sunflower oil plus sodium acetate, the fungus grew much better than with sunflower oil as sole carbon source, reaching the stationary phase within 72 h (Fig. 1). At this point the biomass of about 23 g dry weight/l contained as much as 65 % (w/dry weight) lipids, which was much higher as compared to cells grown on sunflower oil alone. Further incubation resulted in a slight reduction in the lipid content to approximately 54 % (Fig. 1), which may have been due to a switch-over of energy metabolism to a utilisation of endogenous lipids, since the sunflower oil was already exhausted after 120 h (Fig. 2A) and acetate after 72 h (Fig. 3A). In the presence of sodium acetate the sunflower oil was nearly completely (97.5 %) assimilated during cultivation, whereas in its absence only one-third of the oil was utilised even after 168 h (Fig. 2A).

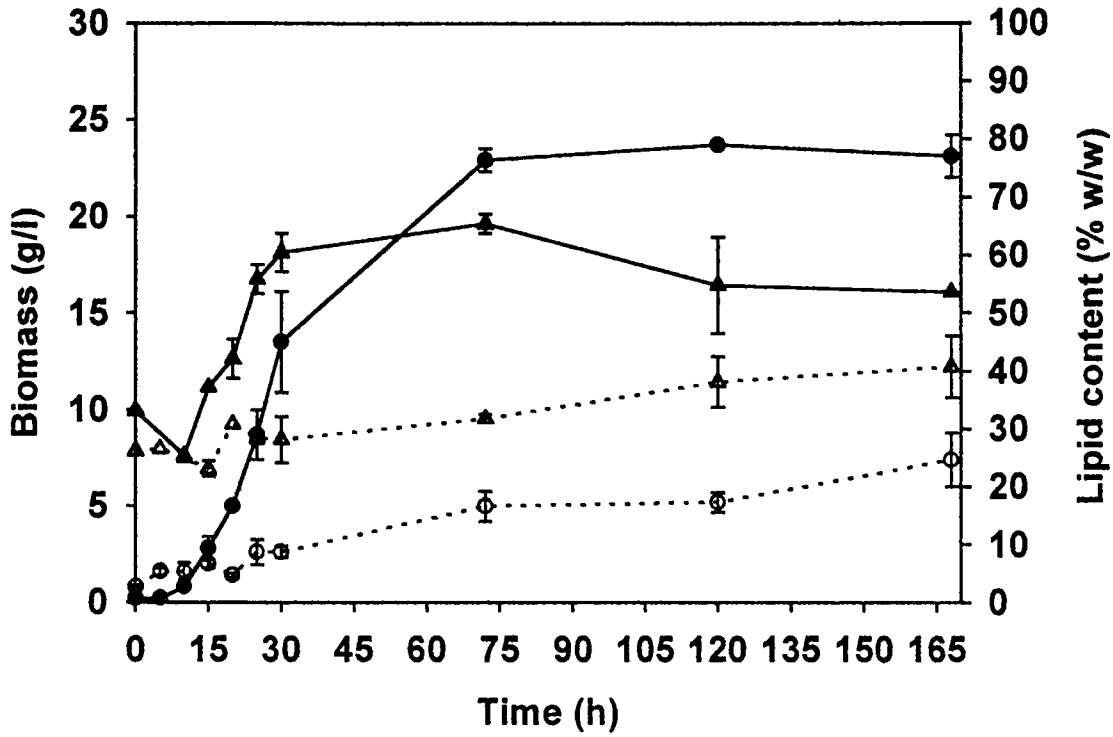

Fig. 1. Biomass production (●/○) and intracellular lipid content (▲/△) in shake flasks of cells grown on the mixed substrate (_____) and only sunflower oil (--------). Bars indicate standard deviation.

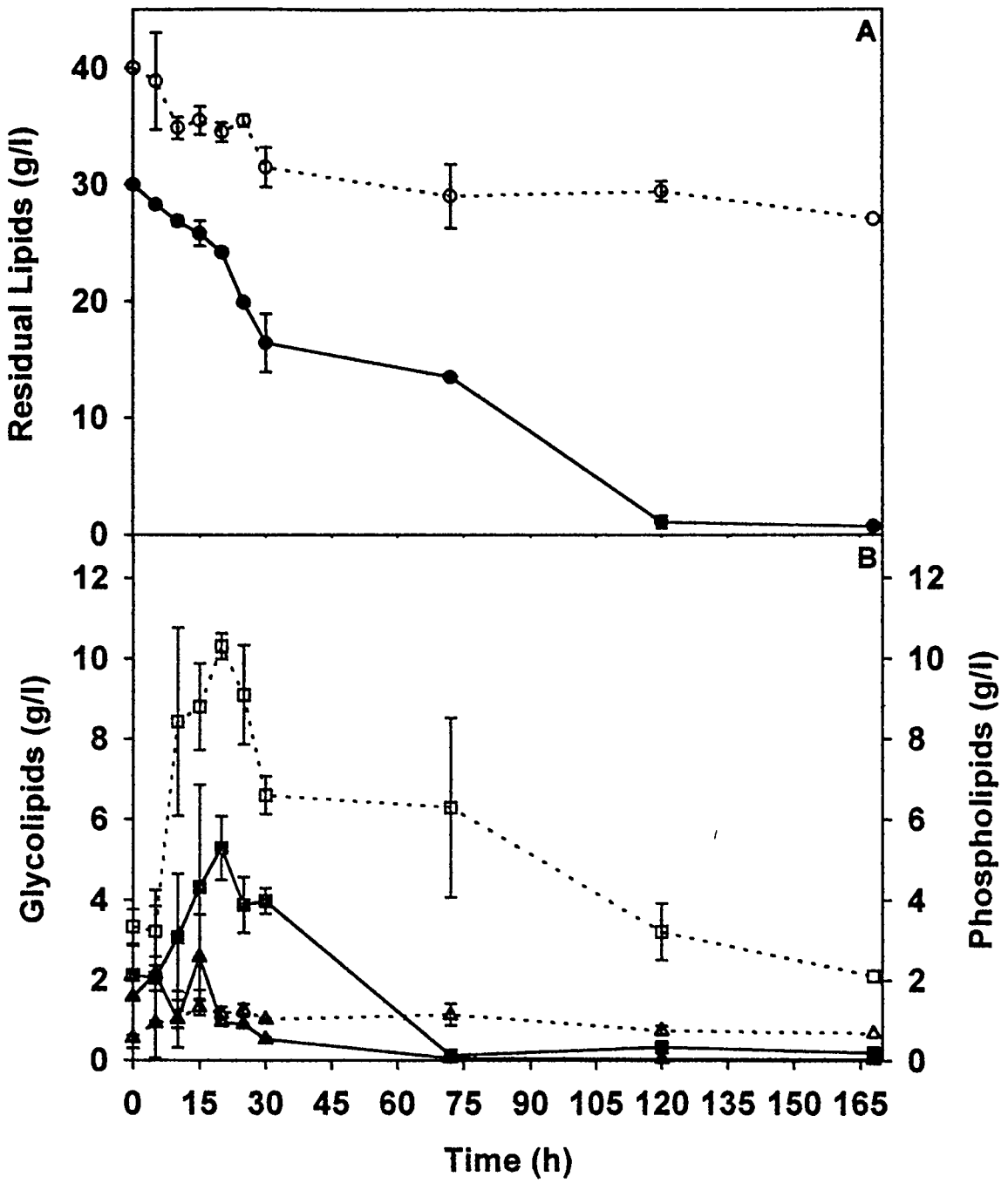

Fig. 2. Utilisation of sunflower oil by cells grown in shake flasks on the mixed substrate (_____) and only sunflower oil (-------). Bars indicate standard deviation. A: concentration of extracellular residual lipids (g/l; ●/○); B: Glycolipid (GL) (■/□) and phospholipid (PL) (▲/△) concentrations (g/l) in the culture.

A considerably higher accumulation of extracellular GLs in the medium (probably excreted by cells) was observed after 20 h of growth in the presence of sunflower oil alone as compared to growth in the combined substrate mixture (Fig. 2B). The cellular percentages of all lipid classes (w/w of total cellular lipids) did not differ significantly after growth with or without acetate. On both substrates, the cellular NL fractions reached a maximum after 30 h of cultivation (about 85 % w/w) and subsequently remained constant. The cellular GL fractions decreased on both substrates from about 15 % w/w after 30 h to about 9 % w/w after 168 h of growth. The PL fractions remained low (less than 3 % w/w) during the cultivation period in cells grown on both substrates (data not shown).

The change in extracellular free fatty acid concentrations exhibited different kinetic patterns during cell growth with or without acetate. While in the presence of acetate a transient sharp maximum was observed after 25 h of growth, followed by a sharp decrease, in the absence of acetate these fatty acids gradually increased to reach a plateau at the relatively high level of about 6 g/l after 30 h (Fig. 3B). These data suggest that cell growth in the absence of acetate is limited by the metabolism of free fatty acids rather than by lipase activity which may in turn be a consequence of the decline in pH.

In the presence of sodium acetate, but not in its absence, the fungus apparently exhibited a preference for the utilisation of unsaturated fatty acids, since the initial percentage of saturated fatty acids in the extracellular lipids increased from 18 % to approximately 45 % (Fig. 4). Since no accumulation

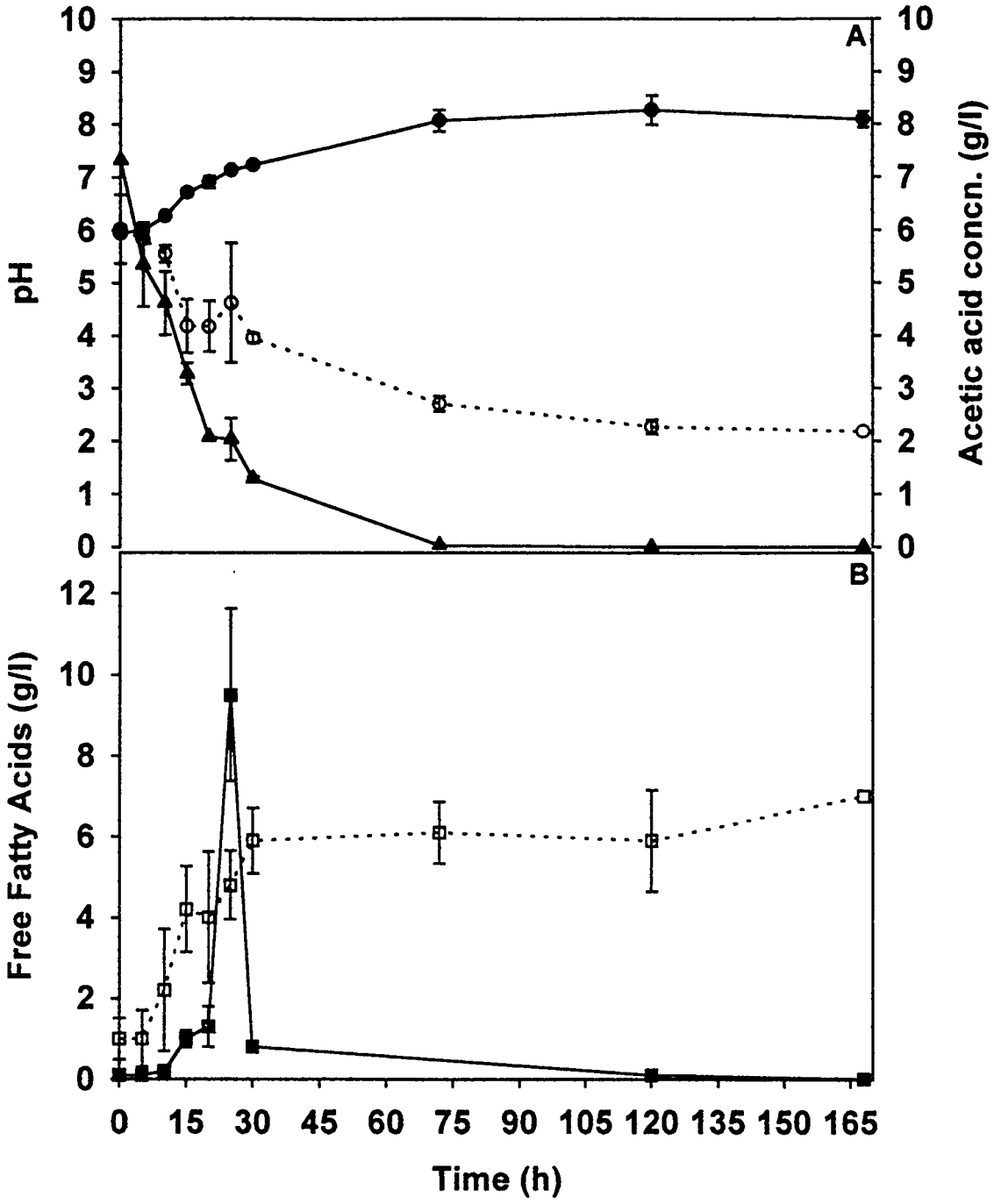

Fig. 3. A: Changes in pH (●/○) and acetic acid (▲) concentration as well as B: extracellular free fatty acids (■/□), of cultures grown in shake flasks on the mixed substrate (_____) and only sunflower oil (--------). Bars indicate standard deviation.

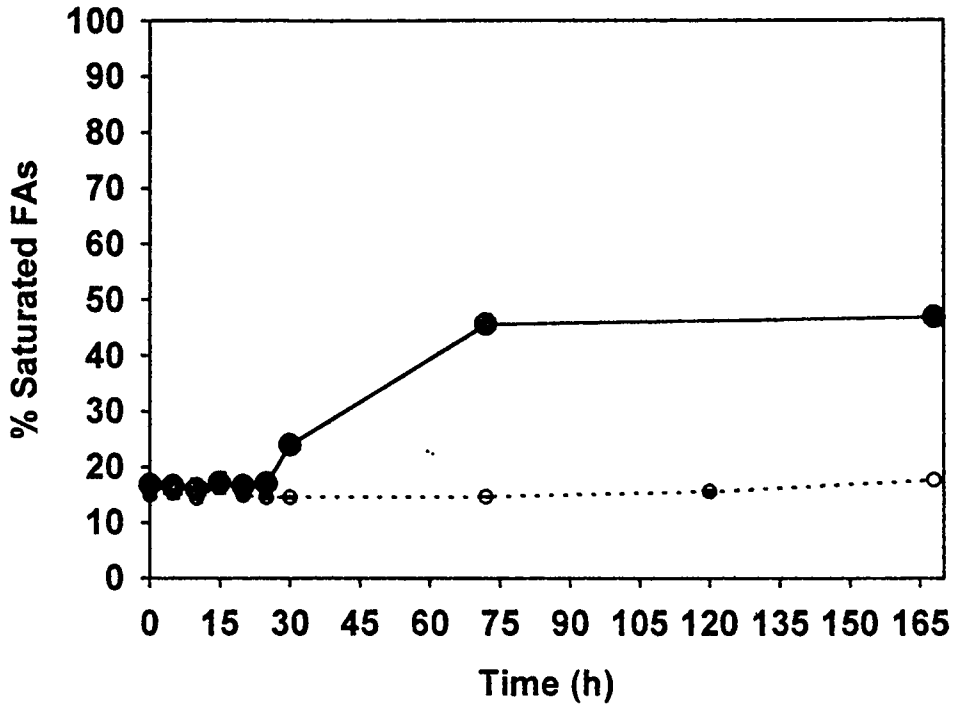

Fig. 4. Changes in extracellular saturated fatty acids (expressed as a percentage of the total extracellular fatty acids) of cultures grown in shake flasks on the mixed substrate (●_____●) and only sunflower oil (○-------○). FAs = fatty acids. Bars indicate standard deviation.

of free fatty acids occurred in the supernatant during the stationary growth phase (Fig. 3B), it is conceivable that the fungal lipase preferentially cleaved the unsaturated fatty acid residues so that mono- and diacylglycerols containing saturated fatty acid residues accumulated. Moreover, in the presence of sodium acetate, the content of GLA in the cellular lipids reached a minimum of 1 % in the NL fraction during the growth phase, followed by a continuous rise up to 5.2 % (w/w) (Fig. 5). Here again the pattern turned out to be different in the absence of acetate, in which case the GLA content remained low.

During the metabolism of the combined substrate mixture, the sunflower oil was observed to be fully dispersed, probably emulsified (i.e. no oil droplets were visible at the surface of the growth medium when motionless for 5 min) within the first 20 to 25 h (data not shown). This effect was not seen in the absence of acetate. The reason for the acetate-supported emulsification remains to be clarified. Glycolipids (Fiechter, 1992; MacDonald *et al.*, 1981) may be of minor importance since their extracellular concentration was found to be higher in the absence of acetate (Fig. 2B). The free fatty acids released by the fungus may contribute to some extent to the emulsification since they form acid soap dimers at pH values above 7 (Verhagen *et al.*, 1978).

Collectively, our results demonstrate that the simultaneous metabolism of sodium acetate dramatically improved the utilisation of sunflower oil by *Mucor circinelloides* probably via a drastic shift of pH to about 8.0. This pH range appeared to favour both emulsification of the oil and its cleavage by fungal

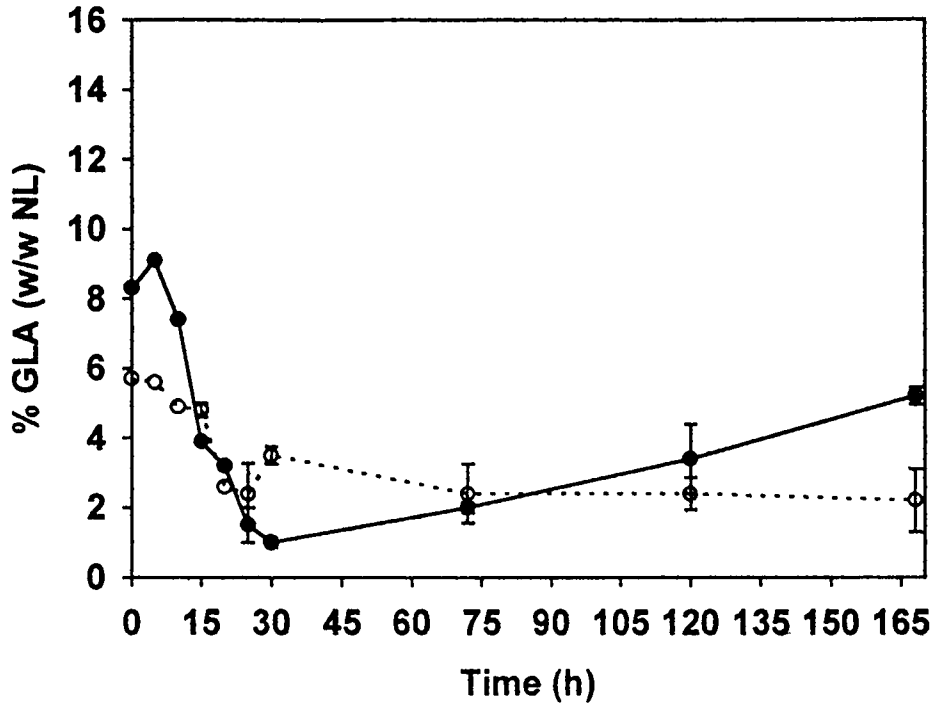

Fig. 5. Gamma-linolenic acid (GLA) content of neutral lipids (NLs) in cells grown in shake flasks on the mixed substrate (●_____●) and only sunflower oil (○---------○). Bars indicate standard deviation.

lipase activity as well as their utilisation for cell growth and production of fungal lipids.

To determine whether sodium acetate or the pH increase resulting from acetic acid assimilation was responsible for the increased sunflower oil utilisation, the above experiments were repeated in a bioreactor using the sunflower oil medium with or without sodium acetate. In a third experiment the sodium acetate was omitted from the medium but the pH was gradually increased during cultivation according to a pH profile set to mimic the natural increase in pH (due to acetic acid assimilation) observed in the above medium containing sodium acetate. In all three experiments the stationary phase was reached within 48 h. The culture parameters at this point are shown in Table 1.

The addition of sodium acetate resulted in an increased biomass production (from 9.6 g/l to 25.3 g/l), increased sunflower oil utilisation, and maximum GLA production (after 120 h from 0.03 g/l to 1.02 g/l). Comparable results were also obtained when the pH of the medium without acetate was gradually increased by titration with KOH (Table 1; experiments 1 and 2 respectively). This indicated that the pH increase alone during cultivation was responsible for most of the enhanced sunflower oil utilisation, biomass and GLA production. The greater biomass concentration in the medium with sodium acetate in comparison to the 19.0 g/l reached in the pH profile experiment could probably be largely due to the additional carbon supplied in the form of acetate. It is important to note that the sunflower oil in experiments 1 and 2

Table 1. The influence of sodium acetate and pH profiling on the performance of *Mucor circinelloides* in bioreactor cultures.

Parameter	Cultivation Time (h)	Experiment no. 1	2	3
Biomass (g/l)	48	25.3	19.0	9.6
Lipid content (% w/w)	48	62.4	59.3	45.5
Residual lipids (g/l)	48	1.7	1.5	6.8
pH	48	8.54	8.43	3.33
Acetic acid (g/l)	48	0	-	-
Neutral lipids (% w/w)	48	95.6	94.6	88.7
γ-Linolenic acid (% w/w of NL)	120	5.9	7.0	1.3

Experiment 1: Sunflower oil with sodium acetate; Experiment 2: Sunflower oil without sodium acetate, but with an increasing pH profile; Experiment 3: Sunflower oil without sodium acetate

(Table 1) was completely emulsified within 16 h of cultivation whereas no emulsification was observed in experiment 3. Consequently, it was impossible to obtain a representative sample containing residual oil during experiment 3, especially since most of the residual oil remained on the surface of the medium during cultivation and subsequent sampling. The value of 6.8 g/l residual oil reported for experiment 3 was probably a significant under-estimation.

From these results it is clear that it is not sodium acetate *per se* that was responsible for the emulsification and consequent enhanced utilisation of sunflower oil, but rather an effect of the rise in pH. Our findings may give new insight into the utilisation of plant oils as a possible source for the production of high-value fatty acids such as GLA.

4.4 ACKNOWLEDGEMENTS

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SUMMARY

Surveys launched across South Africa indicate that many frying establishments abuse their frying oils and fats during the frying process, resulting in degradation and concomitant production of potentially toxic oxidation products. Some of these compounds have been shown to be toxic to animals and in human *in vitro* studies. Consequently, strict regulations under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972) were published on 16 August 1996. It is now an offense to use or sell used cooking oil or fat for human consumption containing high levels of these degradation products. Since frying establishments are not allowed to discard their used oils and fats by selling to the public for consumption or dumping into municipal drainage systems, it is important that these oils and fats are collected for re-use in another form. Consequently, the aim of this study was the biotransformation of used oil wastes (containing no toxic substances) to high value lipids containing gamma-linolenic acid (GLA). This polyunsaturated fatty acid is prescribed for the treatment of eczema. In order to achieve this, *Mucor circinelloides* f. *circinelloides* CBS 108.16 was first grown on 40 g/l unused sunflower oil and, as expected, produced neutral lipids (NL) similar in fatty acyl composition to the original oil. The apparent repression of the Δ^6 fatty acid desaturation was partially reversed when cells were grown on oil (30 g/l) and sodium acetate (10 g/l) as mixed substrates resulting in an increase in GLA content. Furthermore, a three-fold increase in oil substrate utilisation and doubling of biomass production to 19.1 g/l occurred when sodium acetate was added to the oil substrate. When sodium acetate (10 g/l) was added to a growth medium containing used cooking oil (UCO) similar results were obtained. This experimental procedure was

repeated for seven additional *Mucor* strains and again the stimulatory effect of sodium acetate in combination with UCO was obvious. Next, the effect of different UCO concentrations in the presence of 10 g sodium acetate/l on biomass and lipid production was investigated in *Mucor circinelloides* CBS 108.16. According to our results, a maximum biomass concentration of 48 g/l consisting of 82 % oil yielding about 35 g NL/l and up to 900 mg GLA/l was achieved. The addition of 30 g UCO/l in combination with 10 g sodium acetate/l proved to be the optimum UCO concentration in order to obtain maximum GLA yield. Similar results with this strain were obtained when UCO was replaced with fresh unused cooking oil. When these experiments were repeated with linseed oil and sodium acetate as sole carbon sources, much less GLA was produced (351 mg GLA/l). According to bioreactor studies, the effects of sodium acetate addition can be attributed to the change in pH of the medium during cell growth in the presence and absence of acetate. In the absence of sodium acetate the pH decreased to 2.2, whereas in its presence it increased to about pH 8.0. During metabolism of sunflower oil in the presence of sodium acetate, the percentage of saturated fatty acids in the extracellular lipids increased, suggesting a higher specificity of the fungal lipase for unsaturated fatty acids. When the sodium acetate was omitted from the medium and the pH gradually increased according to a pH profile mimicking the natural increase in pH found in the medium containing sodium acetate, similar results as in the presence of sodium acetate were obtained. This observation indicated that the pH increase alone during cultivation was responsible for the increased sunflower oil utilisation, biomass and GLA production.

OPSOMMING

Opnames deur Suid-Afrika het getoon dat menige diepbraai-ondernemings hulle kookolies en vette misbruik, wat lei tot die afbraak daarvan asook gelyktydige produksie van potensiële toksiese oksidasie produkte. Daar is bewys dat van hierdie verbindings toksies is vir diere en in menslike *in vitro* studies. Gevolglik is streng regulasies gepubliseer op 16 Augustus 1996 onder die 1972 Akte van Voedings-, Skoonheids- en Ontsmettingsmiddele (Akte 54 van 1972). Dit is nou teen die wet om gebruikte olies en vette wat groot hoeveelhede van die afbraakprodukte bevat, te gebruik of te verkoop vir menslike gebruik. Aangesien diepbraai ondernemings nie ontslae mag raak van hul gebruikte olies en vette deur dit aan die publiek te verkoop vir gebruik of om dit in die munisipale dreinsisteme te gooi nie, is dit belangrik dat die olies en vette versamel word vir hergebruik in 'n ander vorm. Die doel van die studie was derhalwe om die gebruikte olie-afval (wat geen toksiese verbindings bevat nie) te biotransformeer na hoë waarde lipiede wat gamma-linoleensuur (GLA) bevat. Laasgenoemde polionversadigde vetsuur word voorgeskryf vir die behandeling van ekseem. Om die doel te bereik, is *Mucor circinelloides* f. *circinelloides* CBS 108.16 eerstens gegroei op 40 g ongebruikte sonneblomolie/l, en soos verwag, het die neutrale lipiede (NL) geproduseer 'n soortgelyke vetsuursamestelling gehad as die oorspronklike olie. Die oënskynlike onderdrukking van die Δ^6 vetsuuronversadiging, is gedeeltelik opgehef toe die selle gegroei is op olie (30 g/l) en natrium-asetaat (10 g/l) as gemengde substraat en het gelei tot 'n toename in die GLA inhoud. Daar was verder 'n drie-voudige toename in die benutting van die oliesubstraat en 'n verdubbeling in biomassa produksie tot 19.1 g/l toe natrium-asetaat by die oliesubstraat gevoeg is. Soortgelyke resultate is

verkry toe natrium-asetaat (10 g/l) by die groeimedium bevattende gebruikte kookolie (GKO) gevoeg is. Die eksperimentele prosedure is herhaal vir 'n verdere sewe stamme van *Mucor* en die stimulerende effek van natrium-asetaat in kombinasie met GKO was weereens duidelik. Die effek van verskillende GKO konsentrasies, in die teenwoordigheid van 10 g natrium-asetaat/l, op biomassa en lipiedproduksie is vervolgens ondersoek in *Mucor circinelloides* CBS 108.16. Volgens ons resultate, is 'n maksimum biomassa konsentrasie van 48 g/l bestaande uit 82 % olie wat ongeveer 35 g NL/l en tot 900 mg GLA/l opgelewer het, bereik. Die byvoeging van 30 g GKO/l in kombinasie met 10 g natrium-asetaat/l, was die optimum GKO konsentrasie om maksimum GLA te lewer. Soortgelyke resultate is verkry toe die GKO vervang is met ongebruikte kookolie. Toe die eksperimente herhaal is met lynsaadolie en natrium-asetaat as enigste koolstofbronne, is baie minder GLA geproduseer (351 mg GLA/l). Na aanleiding van bioreaktorstudies, kon die effek van natrium-asetaat byvoeging toegeskryf word aan die verandering in pH van die medium gedurende groei in die teenwoordigheid sowel as afwesigheid van asetaat. In die afwesigheid van natrium-asetaat, het die pH na 2.2 gedaal, terwyl dit tot ongeveer pH 8.0 in die teenwoordigheid van asetaat gestyg het. Gedurende die metabolisme van sonneblomolie in die teenwoordigheid van natrium-asetaat, het die persentasie versadigde vetsure in die ekstrasellulêre lipiede toegeneem, wat 'n aanduiding is dat die fungale lipases 'n hoër spesifisiteit vir onversadigde vetsure het. Toe geen natrium-asetaat by die medium gevoeg is nie en die pH geleidelik verhoog is volgens 'n pH profiel wat die natuurlike styging in pH naboots soos in 'n natrium-asetaat-bevattende medium, is soortgelyke resultate verkry as in die

teenwoordigheid van natrium-asetaat. Hierdie waarneming het aangetoon dat die verhoging in pH gedurende kweking alleen verantwoordelik was vir die verhoogde sonneblomolie-benutting, biomassa- en GLA-produksie.