The isolation of gamma-linolenic acid producing mucoralean fungi

by

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Motivation

Polyunsaturated fatty acids (PUFAs), like gamma-linolenic acid [18:3(ω6)] are considered to be precursors for human lipid hormones, which play vital regulatory roles in cellular metabolism (Thomas & Holub, 1994). Deficiencies in PUFAs, however, caused by malnutrition, stress, high sugar, cholesterol and alcohol, can lead to unbalanced concentrations of these lipid hormones. This, in turn may lead to various diseases. It is therefore necessary to include some of these fatty acids in a person's diet (Graham, 1984). The current commercial source of 18:3(ω6) is plant oils obtained from *Borago officina*, *Oenothera biennis* or *Ribes nigrum* (Ratledge, 1994). However, a possible alternative source for 18:3(ω6) is mucoralean fungi, since it was found that representatives of certain genera of Mucorales are able to produce substantial quantities of 18:3(ω6) (Lösel, 1989; Van der Westhuizen, 1994).

Studies are continually being done to improve high value fatty acid production by these fungi, thereby increasing the possibility of a commercially viable biotechnological process for the production of 18:3(ω6) (Kock & Botha, 1995). Several optimization studies have been done on the production of 18:3(ω6) by mucoralean fungi, by changing culture conditions (Hansson & Dostalek, 1988; Nakajima & Sano, 1991; Roux et al., 1994; Du Preez et al., 1995; Kock & Botha, 1995). However, an important factor to be kept in mind when developing a biotechnological process producing 18:3(ω6), is the fungal strain to be used (Aggelis et al., 1987). In most cases, fungal strains obtained from culture collections, which were originally isolated for taxonomic purposes, are screened for 18:3(ω6) production (Aggelis et al., 1987; Kock & Botha, 1995). In addition, it was found that mucoralean fungal strains differ in their ability to grow and produce fatty acids in a medium containing a specific carbon source (Sajbidor et al., 1988; Roux et al., 1994). The range of carbon sources on which mucoralean fungi are able to grow and produce 18:3(ω6), is also still mostly unknown. Only a few carbon

sources, supporting growth and 18:3(ω6) production in mucoralean fungi, were investigated (Hansson & Dostalek, 1988; Sajbidor et al., 1988; Kendrick, 1991; Linberg & Hansson, 1991; Certik et al., 1993; Roux et al., 1994).

With the above as background the aim of this study was to examine the influence of a series of 38 carbon sources on growth and 18:3(ω6) lipid content, in different mucoralean fungi. Keeping the results of this screening programme in mind, isolation media for mucoralean fungi utilizing carbon sources obtainable from industrial effluents were developed. The selectivity of these media was determined. The media were used to obtain mucoralean strains from soil using the soil plate technique (Warcup, 1950). These strains were screened for the ability to produce18:3(ω6).

1.2. Gamma-linolenic acid

1.2.1. High value fatty acids.

One of the vital components for the sustaining of life is a group of hydrophobic compounds, called lipids (Brock & Madigan, 1991). These compounds are classed as being sparingly soluble in water but readily soluble in organic solvents such as chloroform or methanol. They can be divided into two types of molecules, the first one is the terpenoid lipids which are derivatives of isoprene units. The second type include molecules which contain long-chain fatty acids (Fig. 1) which can be sub-divided into the neutral lipids, phospholipids and glycolipids (Ratledge & Wilkinson, 1988a). Neutral lipids occur as oil droplets in animal, plant and fungal cells and serve mainly as energy reserves. These oil droplets consist mainly of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids (Figs. 1 and 2). Phospholipids (Fig. 3) play a major structural role in the cellular membranes. The glycolipids (Fig. 4) contain one or more sugar residues, and are widely distributed among microorganisms, occurring mostly in the cell walls of these organisms (Ratledge & Wilkinson, 1988a).

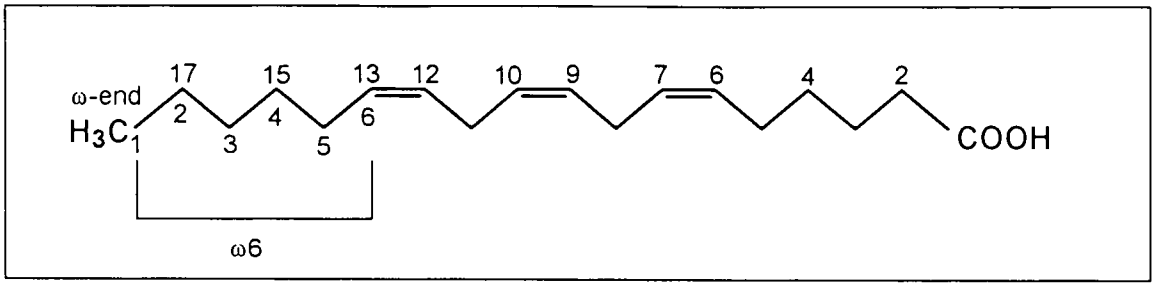


Fig. 1. The structure of gamma-linolenic acid [18:3(ω6) or 18:3(6c, 9c, 12c)]. (Cottrell 1989, Jeffery 1995).

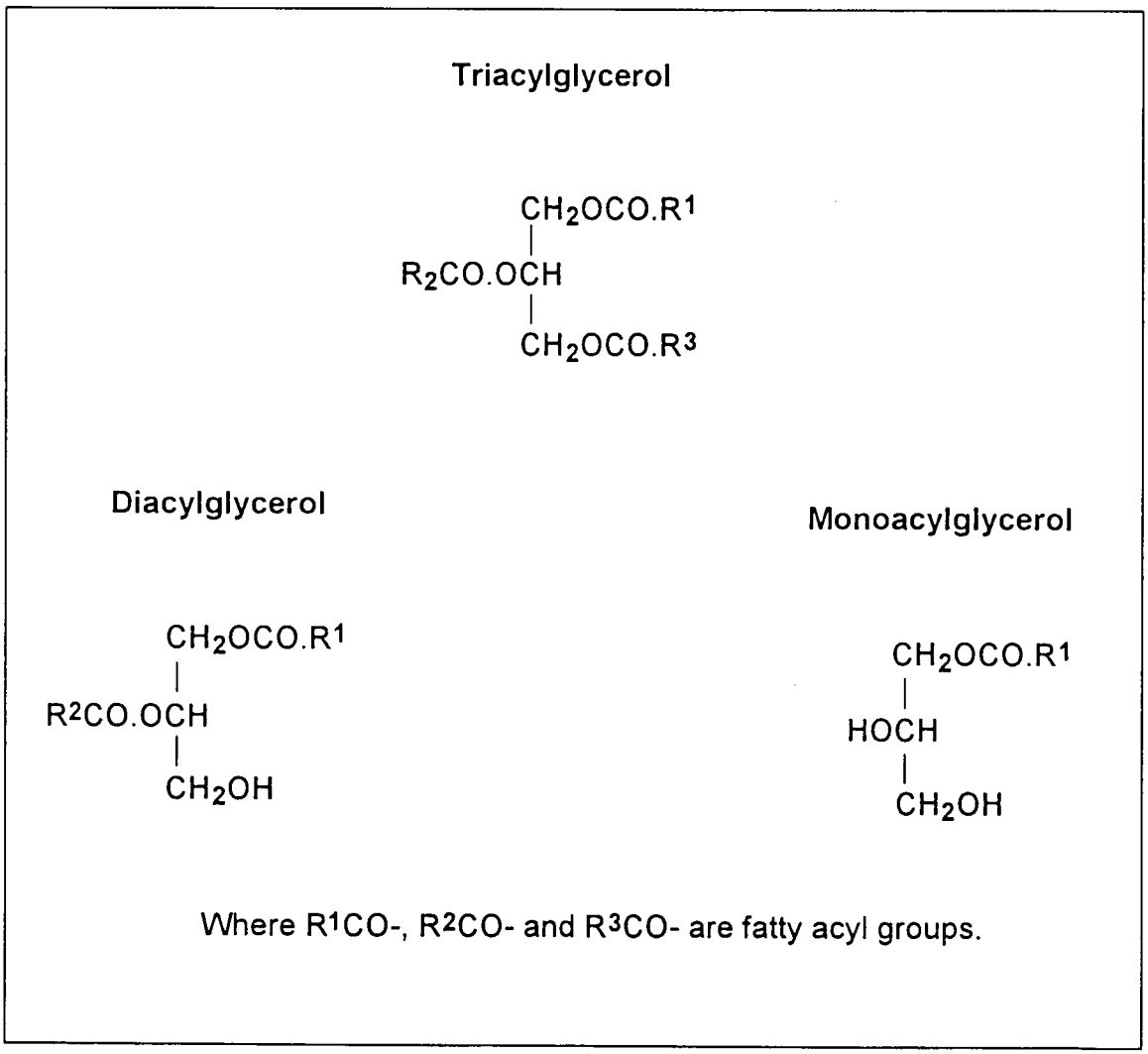


Fig. 2. Structures of the acylglycerols. (Ratledge & Wilkinson 1988b).

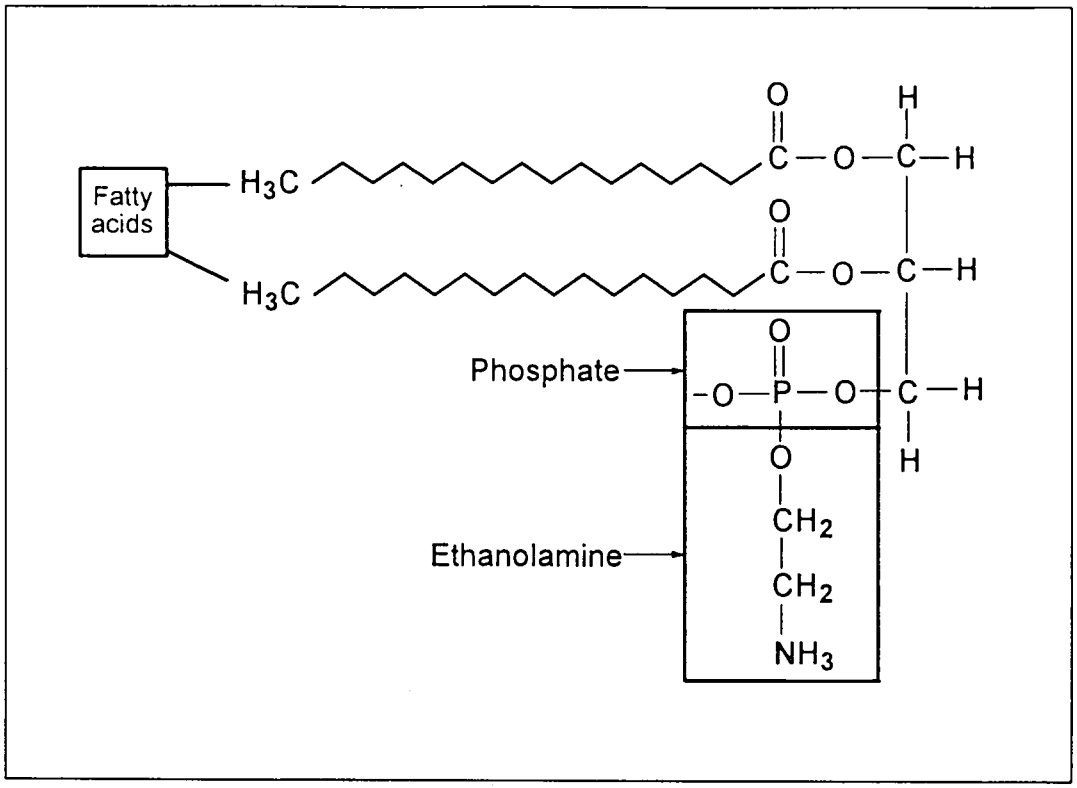


Fig. 3. The structure of phosphotidylethanolamine (a phospholipid). (Ratledge & Wilkinson 1988b).

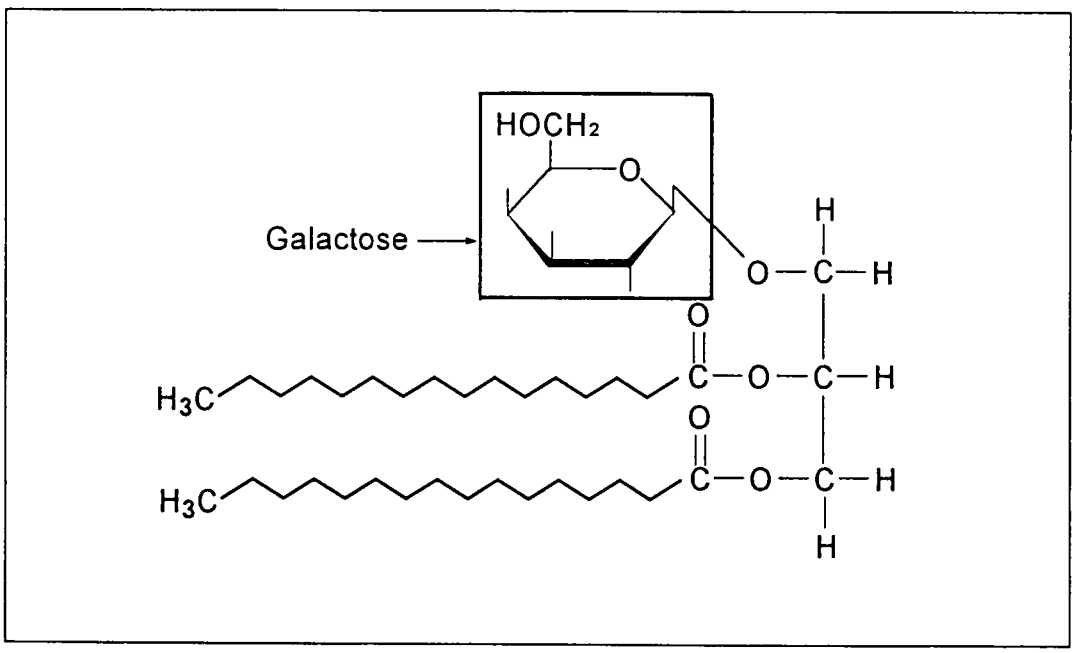


Fig. 4. The structure of monogalactosyl diglyceride (a glycolipid). (Ratledge & Wilkinson 1988b).

1.2.2. Structure and nomenclature of long-chain fatty acids.

The structure of fatty acids, which are the principle building blocks of neutral, phospho- and glycolipids is explained in Fig. 1. Two systems of nomenclature are currently widely used for naming fatty acids (Augustyn, 1991). When referring to families of fatty acids, or a specific member of a particular family, it is convenient to use the omega (ω) system. When naming a fatty acid (e.g. with the trivial name of gamma-linolenic acid) using this system (Fig. 1), the carbon atoms are counted from the omega end up to the first double bond (Jeffery, 1995). In Fig. 1 the double bond nearest to the methyl group (at the omega end) is six carbon atoms away (i.e. at carbon atom number 13). It is therefore an ω6 fatty acid. The abbreviated fatty acid notation 18:3(ω6), therefore, designates; number of carbon atoms: number of double bonds (position of last double bond nearest to the methyl group). To indicate the position of a double bond in relation to the carboxyl end of the carbon chain, or the specificity of an enzyme inserting it, the delta system of nomenclature is used (Augustyn, 1991). When a fatty acid is named according to this system, the carbon atoms are counted from the alpha end up to the various double bonds in the carbon chain. In this case the abbreviated fatty acid-notation of gamma-linolenic acid would be 18:3(6c, 9c, 12c), where the "c" indicates that the particular double bond is in the cis-formation. An enzyme responsible for the insertion of the double bond in the delta-6 position of linoleic acid [18:2(9c, 12c)], will be named a delta-6-desaturase (Ratledge & Wilkinson, 1988a). According to literature (Ratledge, 1994) fatty acids containing more than one double bond in the chain are called polyunsaturated fatty acids (PUFAs). Some of these fatty acids are known to be of high value. One of these fatty acids that can be produced by the mucoralean fungi and is considered to be of high value is 18:3(ω6) (Ratledge & Wilkinson, 1988a; Van der Westhuizen, 1994).

1.2.3. Metabolism.

To understand why 18:3(ω6) is considered to be of such high value, an overview of its metabolism in eukaryotic cells is necessary (Fig. 5). In the anabolic pathway of fatty acids, acetyl-coA acts as the precursor for the synthesis of fatty acids and is transformed to stearic acid (18:0) through the action of mainly the fatty acid synthetase complex (Schweizer, 1989). Stearic acid is then desaturased to oleic acid [18:1(ω9)] by a delta-9-desaturase. Oleic acid can either act as the precursor for the synthesis of 18:2(ω9), by the action of a delta-6-desaturase, which is then elongated and further desaturased to mead acid [20:3(ω9)] by a delta-5-desaturase. Alternatively, 18:1(ω9) can act as the precursor for the synthesis of linoleic acid [18:2(ω9)] by the action of a delta-12-desaturase. Linoleic acid can be transformed to the ω6-series of fatty acids up to 20:4(ω6) via 18:3(ω6), or it can act as the precursor for the synthesis of the ω3-series of fatty acids up to docosahexaenoic acid [22:6(ω3)] via 20:5(ω3). Arachidonic acid and 22:6(ω3) are the precursors of the lipid hormones, which play a vital regulatory role in cellular metabolism. These hormones include the prostaglandins, thromboxanes and leukotrienes (Augustyn, 1991). Any malfunction in the anabolic pathway depicted in Fig. 5 would therefore result in an imbalance in the concentration of these hormones and the concomitant detrimental effects thereof.

It is important to note that 18:2(ω6) cannot be synthesised in the body because humans and animals do not posses the delta-12-desaturase that is needed to transform 18:1(ω9) to 18:2(ω6). Linoleic acid is therefore considered to be essential because a lack of it in our diets leads to deficiency symptoms (Thomas & Holub, 1994). In addition, the delta-6-desaturase responsible for 18:3(ω6) production is inhibited by a number of factors including stress, cholesterol and alcohol (Graham, 1984). Total fatty acid deficiency (both ω3 and ω6 fatty acids) causes reduced growth, reproductive failure and dermatitis (Thomas & Holub, 1994).

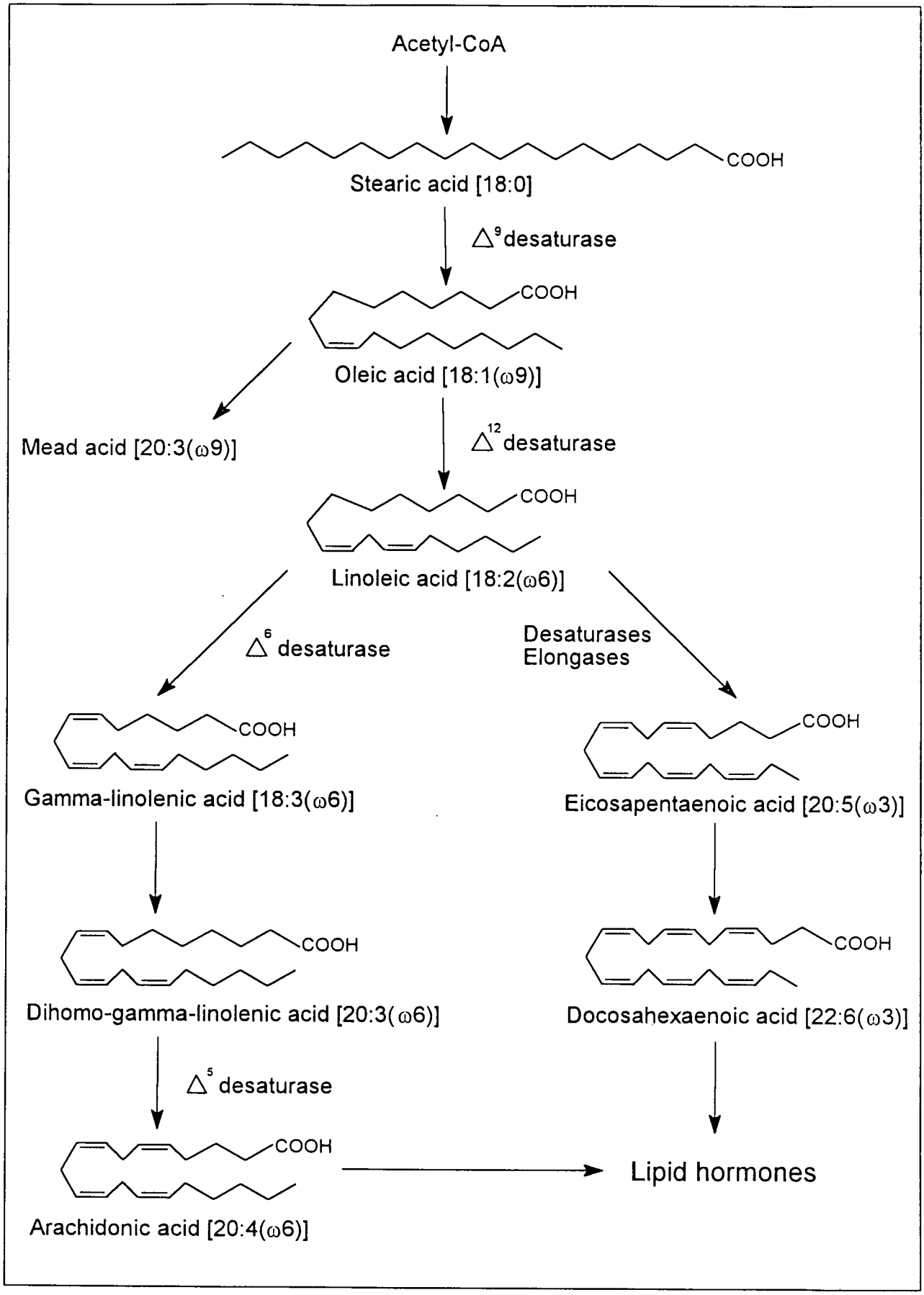


Fig. 5. The anabolic pathway for ω3 and ω6 fatty acids (Kendrick, 1991; Ratledge, 1994).

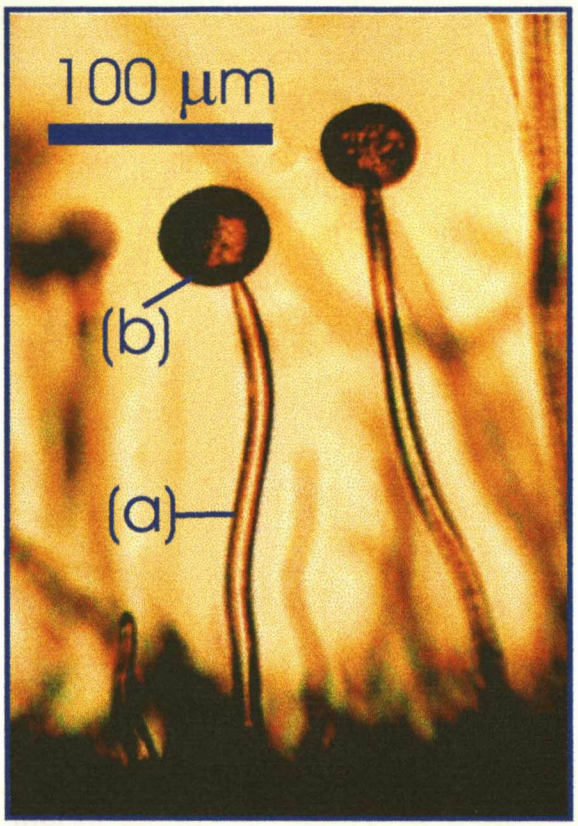
Considering the above, it is therefore not surprising that 18:3(ω6) is included in various health foods and even prescribed medicine (Graham, 1984). The current commercial sources of this fatty acid are plant oils (Thomas & Holub,1994). Gamma-linolenic acid is extracted from Evening Primrose (*Oenothera biennis*) (Gunstone et al., 1994) or Borage (*Borago officina*) (Ratledge, 1994). Borage oil contains a higher percentage w/w 18:3(ω6) (19.00-25.00%), compared to the 8.00-12.00% w/w 18:3(ω6) in Evening Primrose oil (Ratledge, 1994). However, an alternative source of these fatty acids, which has been extensively researched, is the mucoralean fungi. A percentage of 15.00-18.00% (w/w) has been obtained for 18:3(ω6) in the oil of *Mucor circinelloides* (Ratledge, 1994).

1.3. Mucorales

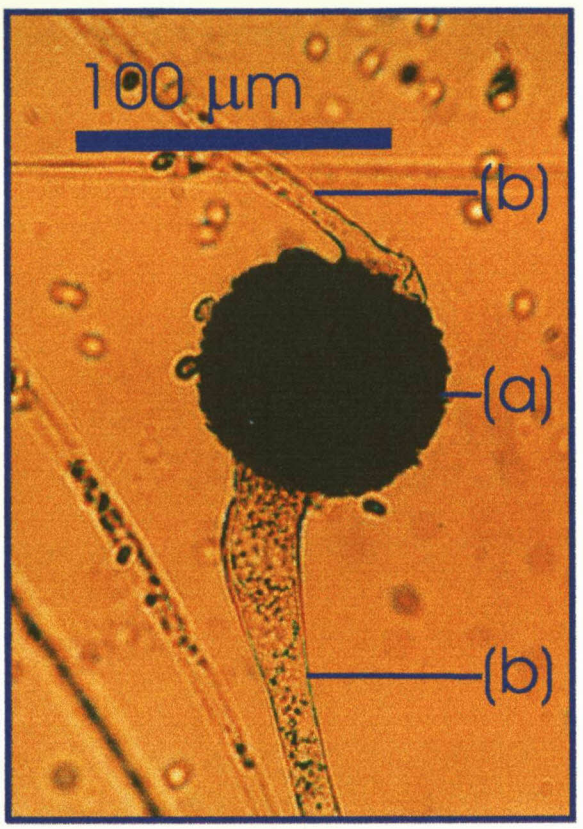
1.3.1. Families in Mucorales.

The mucoralean fungus (Fig. 6) is characterized by a thallus that is coenocytic and eucarpic with an extensive mycelium containing haploid nuclei (Benjamin, 1979). Reproduction occur asexually when one or more sporangiospores are formed in a mitosporangium. During sexual reproduction (Fig. 6) a zygospore is formed as a result of conjugation between similar gametangia. The taxa in Mucorales differ from one another with regard to the nature of their asexual means of reproduction (Hesseltine & Ellis, 1973; Benjamin, 1979).

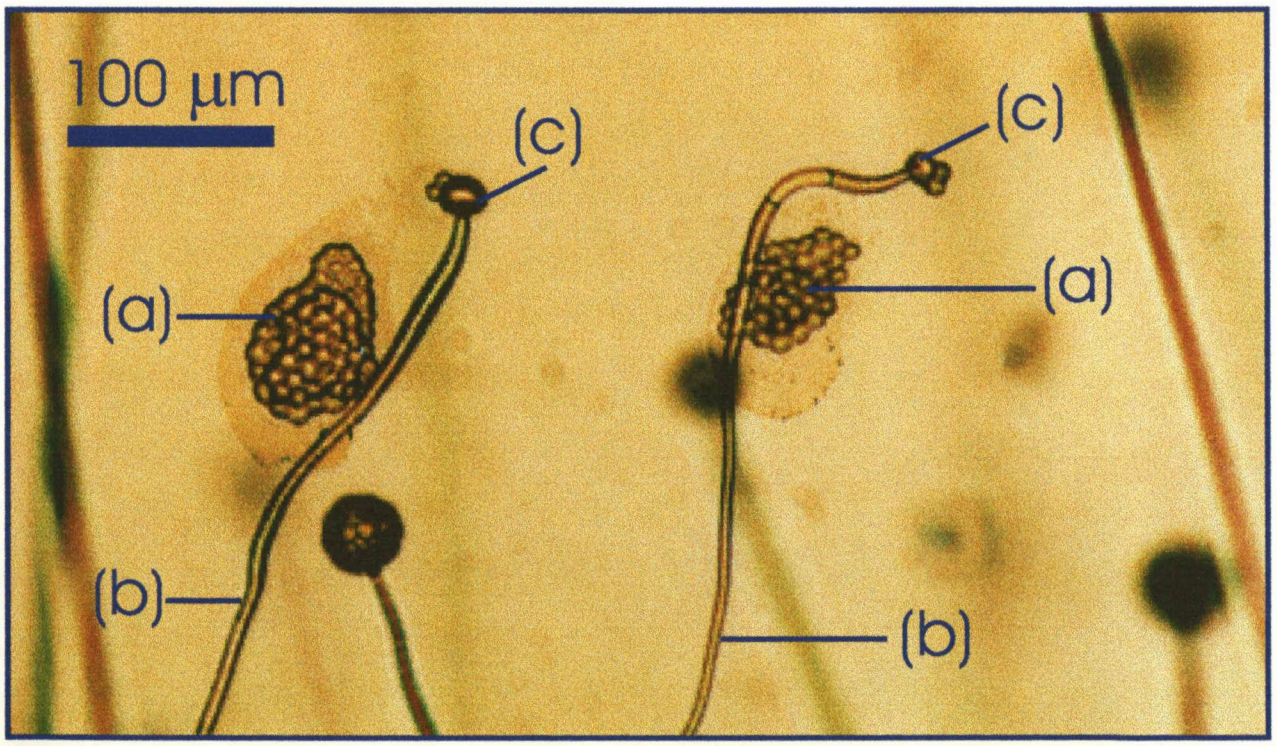
According to Benny & Benjamin (1993) Mucorales consists of sixteen families (Table 1). Members of Absidiaceae (Fig. 7 a) are characterized by sporangia with apophyses, stolons and rhizoids (Hesseltine & Ellis, 1964). Members of Chaetocladiaceae (Fig. 7 b) produce pedicellate, unispored sporangiola on small fertile vesicles, and verticillately or dichotomously branched fertile hyphae that often bear sterile spines (Benny & Benjamin, 1993). Members of Choanephoraceae (Fig. 7 c) are characterized by sporangia that are large, columellate and multispored with persistent walls which break open as two halves when releasing sporangiospores



A sporangiophore (a) with sporangium (b), belonging to *Backusella*.



A zygospore (a) suspended between two opposite aligned suspensor cells (b) in *Mucor genevensis*.



Mature sporangia in *Backusella* which had released their sporangiospores (a) by liquefaction of the sporangium wall. The sporangiophores (b) and collumellae (c) are clearly visible.

Fig. 6 Some characteristic features of fungi belonging to the order Mucorales.

Table 1. The families and genera of Mucorales (Benny & Benjamin, 1993)

Absidiaceae:	*Absidia, Apophysomyces, Chlamydoabsidia, Circinella, Gongronella, Halteromyces, Mycocladus, Rhizopodopsis, Rhizopus, Thermomucor*
Chaetocladiaceae:	*Chaetocladium, Dichotomocladium*
Choanephoraceae:	*Blakeslea, Choanephora, Poitrasia*
Cunninghamellaceae:	*Cunninghamella*
Dicranophoraceae:	*Dicranophora, Spinellus, Sporodiniella, Syzygites*
Gilbertellaceae:	*Gibertella*
Mortierellaceae:	*Aquamortierella, Dissophora, Echinosporangium, Modicella, Mortierella, Umbelopsis*
Mucoraceae:	*Actinomucor, Circinomucor, Hyphomucor, Micromucor, Mucor, Parasitella, Rhizomucor, Zygorhynchus*
Mycotyphaceae:	*Benjaminiella, Mycotypha*
Phycomycetaceae:	*Phycomyces*
Pilobolaceae:	*Pilaira, Pilobolus, Utharomyces*
Radiomycetaceae:	*Hesseltinella, Radiomyces*
Saksenaeaceae:	*Saksenaea*
Sigmoideomycetaceae:	*Reticulocephalus, Sigmoideomyces, Thamnocephalis*
Syncephalastraceae:	*Syncephalastrum*
Thamnidiaceae:	*Backusella, Cokeromyces, Ellisomyces, Fennellomyces, Helicostylum, Phascolomyces, Pirella, Thamnidium, Thamnostylum, Zychaea*

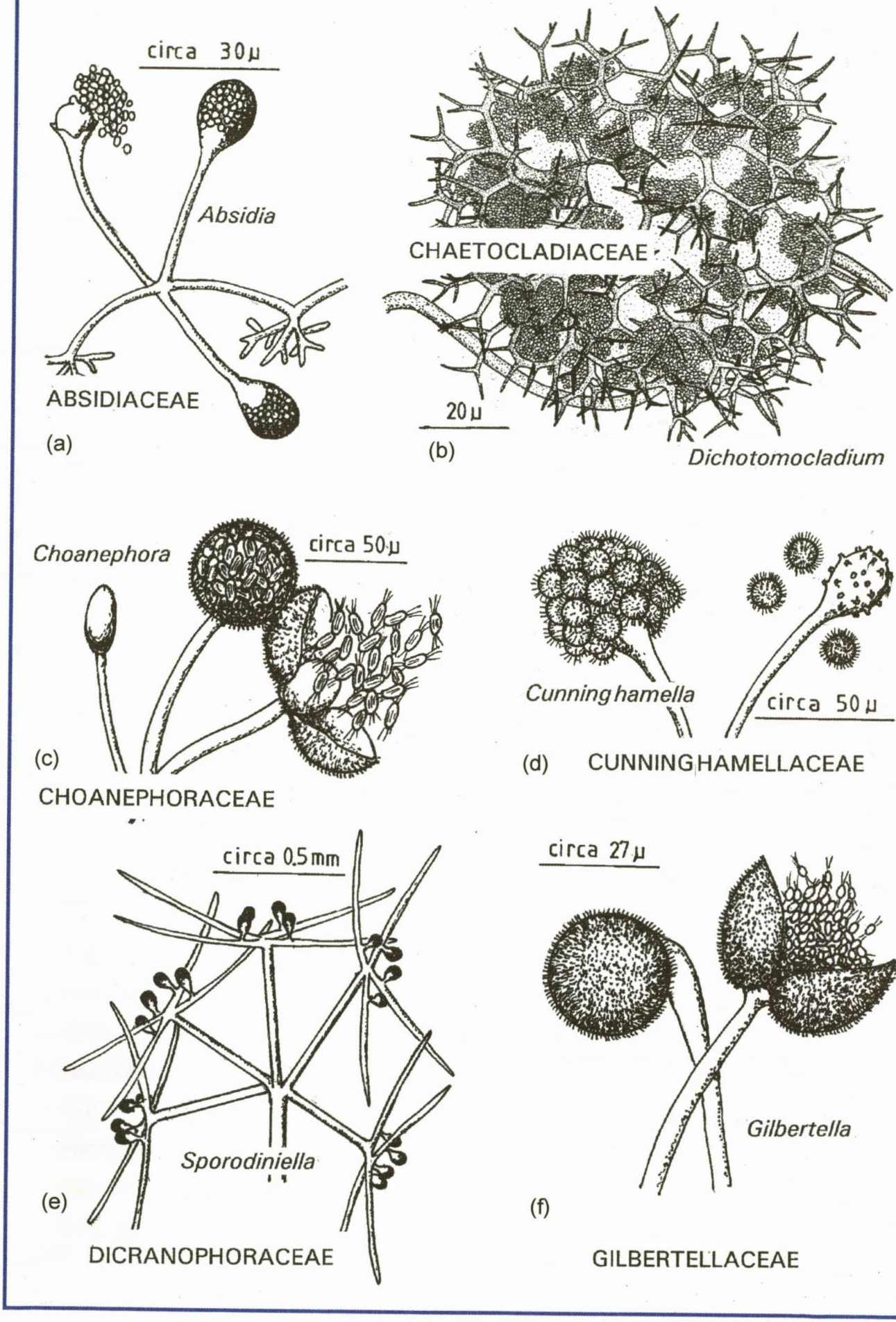


Fig.7 Families of Mucorales

that contain hairlike structures at each pole, sporangiola are also present (Hesseltine & Ellis, 1973). Cunninghamellaceae (Fig. 7 d) is characterized by single spored sporangiola borne on a swollen round vesicle at the tip of the sporangiophore (Benjamin, 1979). *Sporodiniella* is a typical member of Dicranophoraceae (Fig. 7 e) having characteristics like sporangiophores terminating in an apical verticil of sporangiophore branches and sporangia - each ultimate branch being dichotomously divided with one arm bearing a sporangium and the other arm ending in a long sterile spine (Evans & Samson, 1977). Members of Gilbertellaceae (Fig. 7 f) produce sporangia that are similar to members of Choanephoraceae, but no sporangiola are produced (Benny, 1991). In Mortierellaceae (Fig. 7 g) sporangia are not abundant, mostly chlamydospores with spiny and rough walls are produced. Zygospores are characterized by tong-like suspensor cells and the zygospore tend to get inwebbed in sterile hyphae (Hesseltine & Ellis, 1973). Members of Mucoraceae (Fig. 7 h) are characterized by columellate multispored sporangia, while the rhizoids and stolons are very much reduced or absent. When zygospores are formed they are suspended by opposite alligned suspensor cells (Hesseltine & Ellis, 1973). *Mycotypha* (Fig. 7 i), a member of Mycothyphaceae is characterized by sporangiophores ending in elongated vesicles covered with sporangiola (Alexopoulos & Mims, 1979). Members of Phycomycetaceae (Fig. 7 j) are characterized by a slender, unbranched sporangiophore with a single, dark, multispored sporangium at its tip. Zygospore formation is characterized by tong-like suspensor cells and the formation of sterile spines from one of the suspensor cells (Alexopoulos & Mims, 1979). Pilobolaceae (Fig. 7 k) is characterized by dark-coloured persistent walled sporangia containing many spores and often the sporangiophores are phototrophic (Hesseltine & Ellis, 1973; Benjamin, 1979). In Radiomycetaceae (Fig. 7 l) sporangiola are borne on secondary vesicles (Hesseltine & Ellis, 1973; Benjamin, 1979; Benny & Benjamin, 1991). Members of Saksenaeaceae (Fig. 7 m) are characterized by a sporangiophore that arizes from above short rhizoids and forms a long-necked, flask-shaped sporangium with a distinct columella in the basal venter (Hesseltine & Ellis, 1973). In Sigmoideomycetaceae (Fig. 7 n) sterile spines are produced and

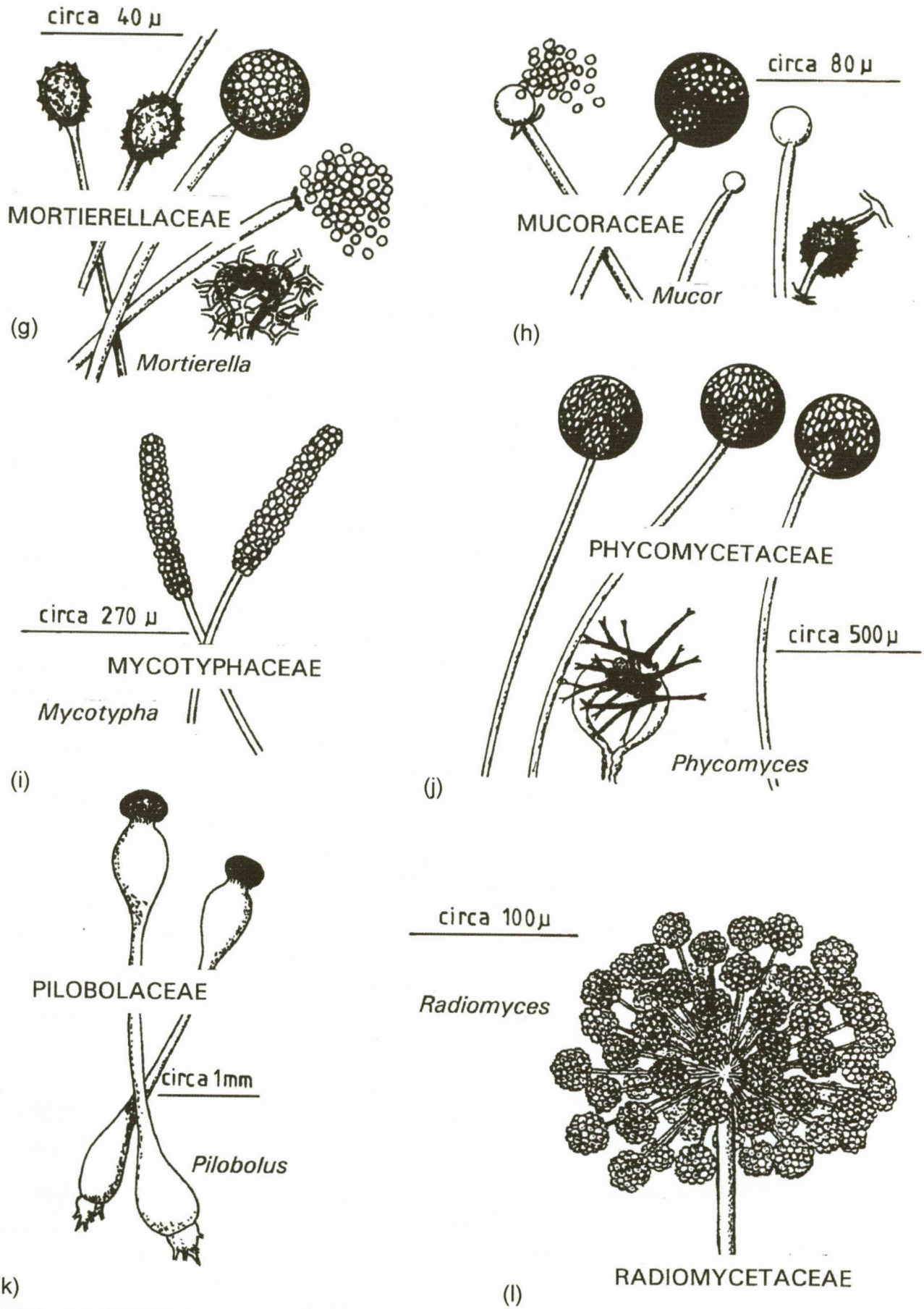


Fig. 7 Continues

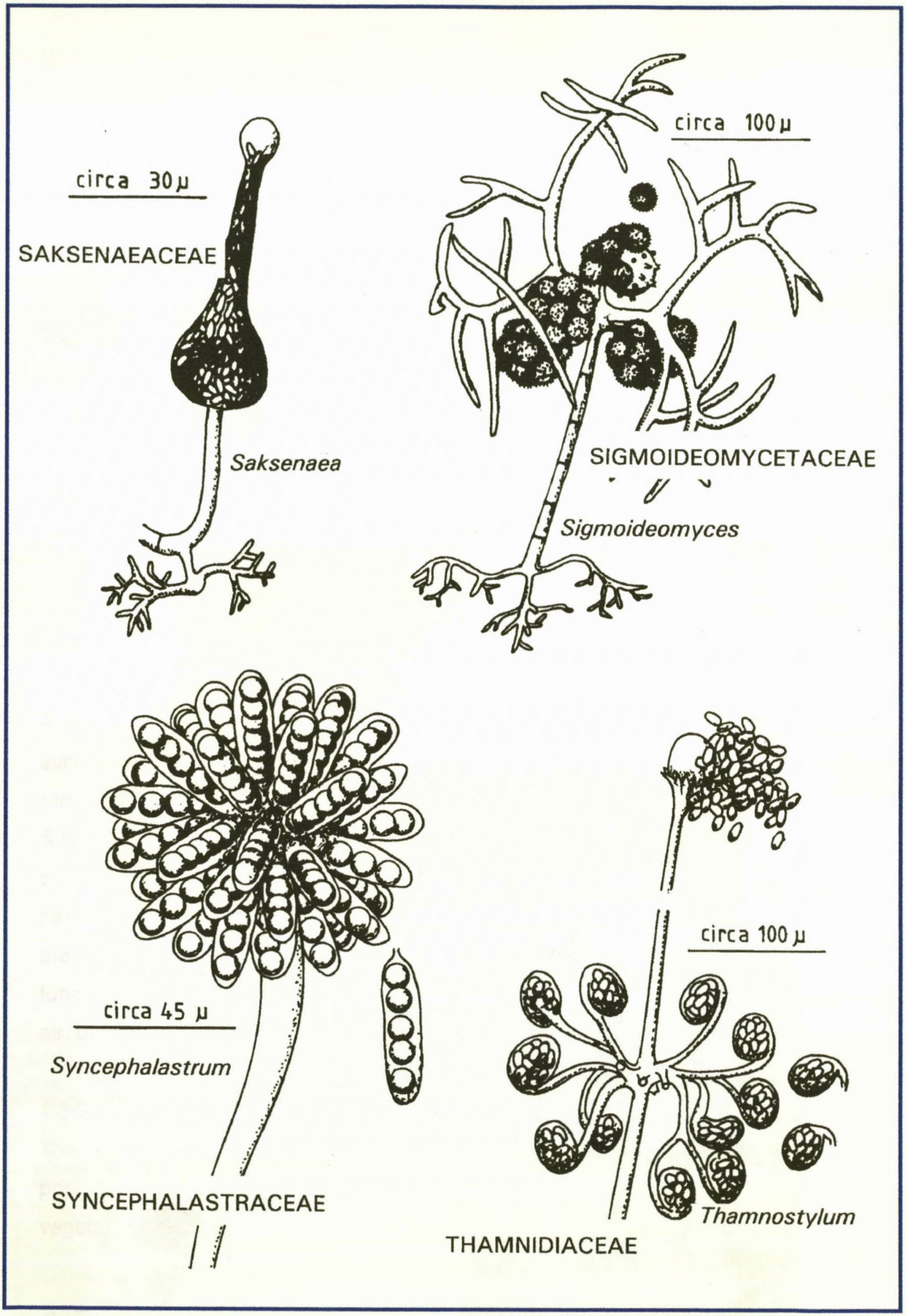


Fig. 7 Continues

the fertile vesicles containing sporangioles are stalked and arise in pairs at the branching points of the fertile hyphae. The fertile hyphae are septate (Benny et al., 1992). Members of Syncephalastraceae (Fig. 7 o) produce merosporangia borne deciduously on vesicles (Hesseltine & Ellis, 1973; Benjamin, 1979). Members of Thamnidiaceae (Fig. 7 p) produce sporangiola with persistent seperable walls and terminal sporangia with diffluent walls (Hesseltine & Ellis, 1973; Benjamin, 1979).

1.3.2. Habitat of Mucorales.

The mucoralean fungi are generally accepted to be the first saprophytic colonizers on dead or decaying plant material (Alexander, 1961). They are able to rapidly utilize the limited simple carbohydrate molecules available, before other fungi, able to utilize complex carbohydrates, like cellulose and lignin take over the decomposition of decaying plants. A typical example of such a case is the role of mucoralean fungi in fruit decay (Dennis & Blijham, 1980; Spotts & Cervantes, 1986). It was found that especially *Mucor piriformis* and some *Rhizopus* species may rapidly colonize picked pears, tomatoes and strawberries during cold storage.

A few mucoralean species have been found to be parasitic on mammals. In a survey of fungal diseases of domestic animals, *Absidia corymbifera* and *Absidia ramosa* were found to be some of the more common fungal pathogens (Hesseltine & Ellis, 1973). Another species, *Sporodiniella umbellata*, was found to be parasitic on insects (Evans & Samson, 1977), while *Parasitella parasitica* is a known parasite of fungi (Schipper, 1978). In addition, some members of the genus *Mucor* are causitive agents of spoilage of cheese (Bartschi et al., 1991). Mucoralean fungi, however, are mostly encountered when isolating microorganisms from soil, air, dung or decaying plant material (Benjamin, 1979).

1.3.3. Factors that influence the mucoralean fungal population in soil.

There are several factors that influence the composition of the mucoralean fungal population in soil. One of these factors that has a considerable influence is vegetation. In an experiment of Waid (1960) it was found that when a virgin soil

habitat such as the fore-dune of a sand-dune becomes colonized by higher plants, there is a parallel development of a fungal flora. In addition, studies were done by Thornton (1960) where the effect of changes in higher plant cover on the basic fungal flora of an oakwood soil were investigated. Some of these changes are quite remarkable, particularly the development of *Mucor ramannianus* under *Calluna* and of *Mortierella vinacea* under *Pinus* spp. Studies of Waid (1960) have shown that Mucorales is one of the most abundant fungal groups when isolating fungi from the roots of the pea plant (*Pisum sativum*) and rye grass (*Lollium perenne*), while the most abundant fungi on the roots of Scotch Pine and orchard grass belong to the Dikaryomycota.

Another important factor is the soil moisture content. Dobbs et al. (1960) found that the percentage germination of *Mucor ramannianus* is much higher in wet seasons than in dry seasons. The results of Eicker (1969) and Steiman et al. (1995), finding that forest soils are generally rich in Mucorales and that the percentage Mucorales species in desert soils is much lower, is therefore not surprising.

The soil type also plays a role. Dobbs et al. (1960) discovered that certain soil types inhibited the germination of spores of *Mucor rammanianus*. Eicker (1969) stated that forest soils are generally rich in Mucorales. Studies of Eicker (1969) revealed that deciduous forests, which are rich in bases, contained a fungal population that consisted predominantly of *Mucor flavus*, mixed forests, with acid soils contained members of *Mucor ramannianus*, while raw humus forests, with very rich soils, were characterized by the presence of *Zygorhynchus moelleri*.

The pH of soil also influence the composition of the mucoralean fungal population. Griffin (1972) found *Mortierella isabellina* and *Mucor rammanianus* to be the most common mucoralean fungi in acid soils, while *Absidia glauca* and *Mortierella alpina* are the most common mucoralean fungi in alkaline soils. Carreiro & Koske (1992) found that lower isolation temperatures enhanced the number of *Mortierella* and *Mucor* species obtained from soil, while the number of dikaryomycotan fungi

decreases at the lower temperatures.

1.3.4. Isolation methods.

The soil plate technique, originally developed by Warcup (1950) to estimate fungal populations in soil, is commonly used to isolate mucoralean fungi (Eicker, 1969; Eicker, 1974; Vardavakis, 1990; Steiman et al., 1995). The method encompasses the aseptic transfer of 0.005 - 0.015 g soil into a sterile Petri dish. The soil is then thoroughly mixed with eight to ten millilitres of molten agar medium at *circa* 45°C. The soil inoculated medium is then incubated and the developing fungal colonies counted and isolated.

It was found that this method of isolation is more selective for mucoralean fungi than the dilution-plate method (Menzies, 1957). The latter method selects for fungi sporulating abundantly (e.g. *Penicillium* species), while the soil plate method is more selective for fungi present as hyphae or chlamydospores in the soil. Interestingly, it is known that the viable cells of *Mucor ramannianus* in soil, are mostly chlamydospores, not sporangiospores (Parkinson & Waid, 1960).

1.3.5. Isolation media.

The media and carbon sources commonly used to isolate mucoralean fungi are listed in Table 2. It is important to note that, except for Czapek-Dox agar, most mucoralean fungi were isolated using complex media, with carbohydrates as carbon sources (Table 2). These media are non-selective for differrent fungal groups and are used to isolate fungi in general from different habitats. However, several screening programmes for growth and 18:3(ω6) production by mucoralean fungi have shown that these fungi can grow and produce 18:3(ω6) on other carbon sources than maltose, sucrose, starch and glucose, which are usually included in isolation media (Hansson & Dostalek, 1988; Roux et al., 1994; Kock & Botha, 1995).

Table 2. A list of isolation media and carbon sources commonly used to isolate mucoralean fungi. Eicker (1969), Michailides et al. (1992), Botha et al. (1996).

Species	Medium	Carbon source
Actinomucor elegans	Malt extract agar	Maltose
Absidia cylindrospora	Czapek-Dox agar	Sucrose
Circinella simplex	Czapek-Dox agar	Sucrose
Cunninghamella elegans	Czapek-Dox agar	Sucrose
Gongronella butleri	Czapek-Dox agar	Sucrose
Merosporangiferous Mucorales	Corn meal agar	Starch/Glucose
	Potato dextrose agar	Glucose
	Yeast extract-soluble starch agar	Starch/Glucose
	Malt extract-Yeast extract agar	Maltose/Glucose
Mortierella alpina	Malt extract agar	Maltose
Mortierella vesiculose	Potato dextrose agar	Glucose
Mortierella isabellina	Czapek-Dox agar	Sucrose
	Malt extract agar	Maltose
Mucor circinelloides	Malt extract agar	Maltose
Mucor flavus	Czapek-Dox agar	Sucrose
Mucor fragilis	Czapek-Dox agar	Sucrose
Mucor piriformis	Acidified potato dextrose agar	Glucose
Mucor silvaticus	Czapek-Dox agar	Sucrose
Rhizopus oryzae	Czapek-Dox agar	Sucrose
Zygorhynchus moelleri	Czapek-Dox agar	Sucrose

A selective medium was developed to detect low numbers of *Mucor* among air-borne fungi in cheese factories, since *Mucor* species are the causative agents of cheese spoilage (Bartshi et al., 1991). The authors determined the selectivity of the medium (Table 3) by testing 29 fungal strains, representing different unrelated fungal groups, for growth on the medium. This included 12 hyphomycetous fungi, three blastomycetous and five hemiascomycetous fungi. In addition, nine mucoralean fungi, representing the genera *Actinomucor*, *Mucor*, *Rhizomucor* and *Rhizopus* were tested. It was found that only strains representing species within the genera *Actinomucor*, *Mucor* and *Rhizomucor* showed significant growth on the medium.

The selective agent in this medium was ketoconazole, a compound structurally related to the benzimidazole fungicides (Fig. 8). These compounds selectively inhibited mitosis in mainly ascomycetous and hyphomycetous fungi by binding to certain amino acid sequences on the tubulin sub-units, thereby preventing spindle formation (Fig. 9) (Lyr, 1989).

Benzimidazole was included in another selective medium, developed for the isolation of mucoralean fungi able to produce 18:3(ω6) from acetate as carbon source (Botha et al., 1995). The composition of this medium is given in Table 3. These authors tested the selectivity of the medium among the mucoralean fungi by inoculating 105 strains representing the genera *Actinomucor*, *Mucor*, *Mortierella* and *Rhizopus* onto the isolation medium. After two weeks of incubation the inoculated cultures were observed for growth, it was found that only strains representing species in the genus *Mucor* could grow on the medium. However, in subsequent isolations from leaf litter from different geographical areas in South Africa, isolates representing the genera *Actinomucor* and *Thamnostyllum* were also found to grow on the medium.

Table 3. Selective media for the isolation of mucoralean fungi

Medium of Bartschi *et al.* (1991)

Malt extract	20.00 g/l
Yeast extract	2.00 g/l
Chloramphenicol	500.00 mg/l
Ketoconazole	50.00 mg/l
Agar	15.00 g/l
pH	5.6

Medium of Botha *et al.* (1995)

Sodium acetate	20.00 g/l
NH_4Cl	1.00 g/l
KH_2PO_4	0.50 g/l
$MgSO_4.7H_2O$	0.25 g/l
Yeast extract	0.50 g/l
$CaCl_2$	0.05 g/l
$FeSO_4.7H_2O$	10.00 mg/l
$ZnSO_4.7H_2O$	10.00 mg/l
$MnSO_4.H_2O$	0.80 mg/l
$CuSO_4.5H_2O$	0.05mg/l
Agar	16.00 g/l
Benzimidazole	0.02 g/l
pH	5.5

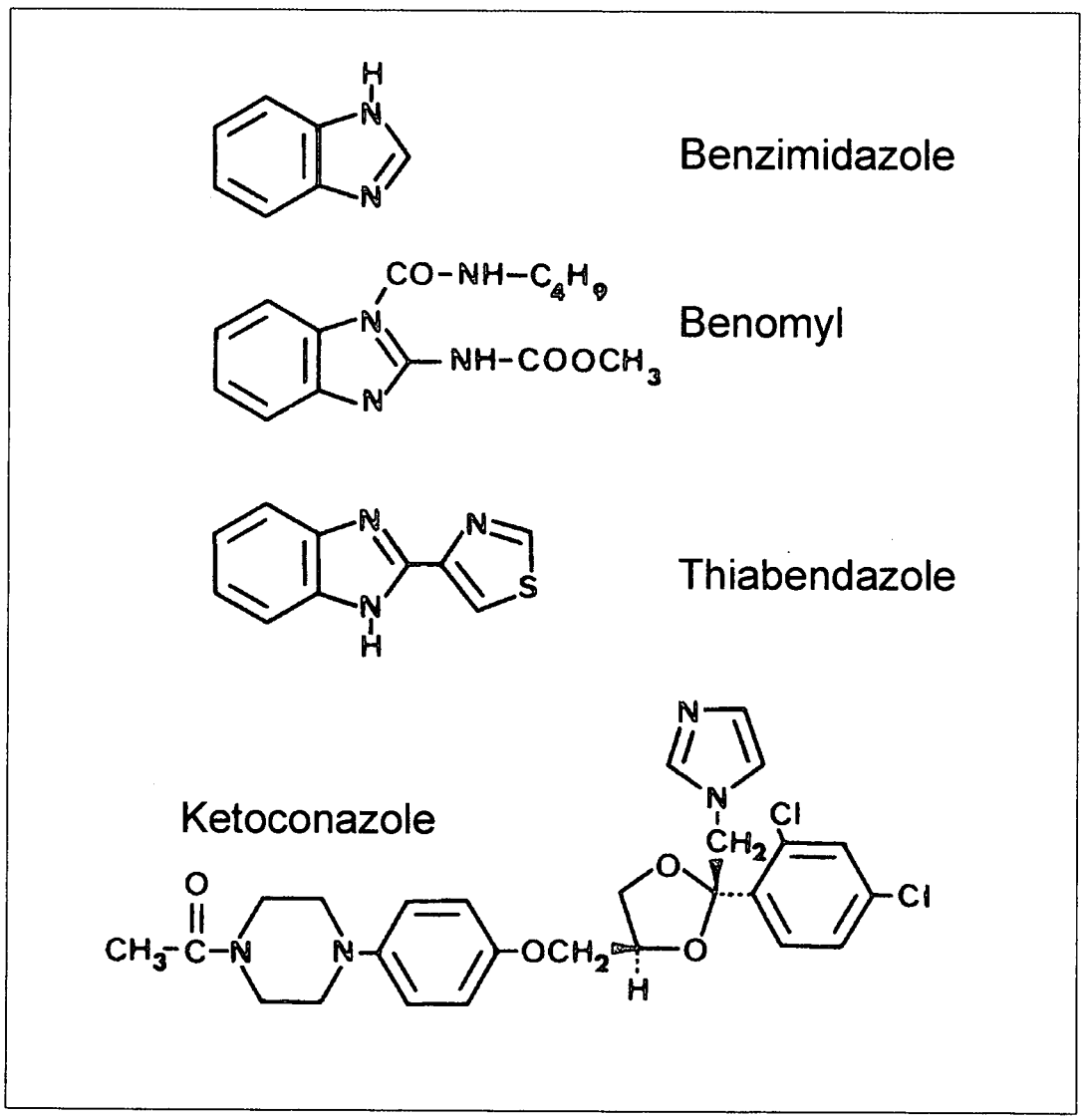


Fig. 8. Some benzimidazole fungicides and related compounds (Lyr, 1989).

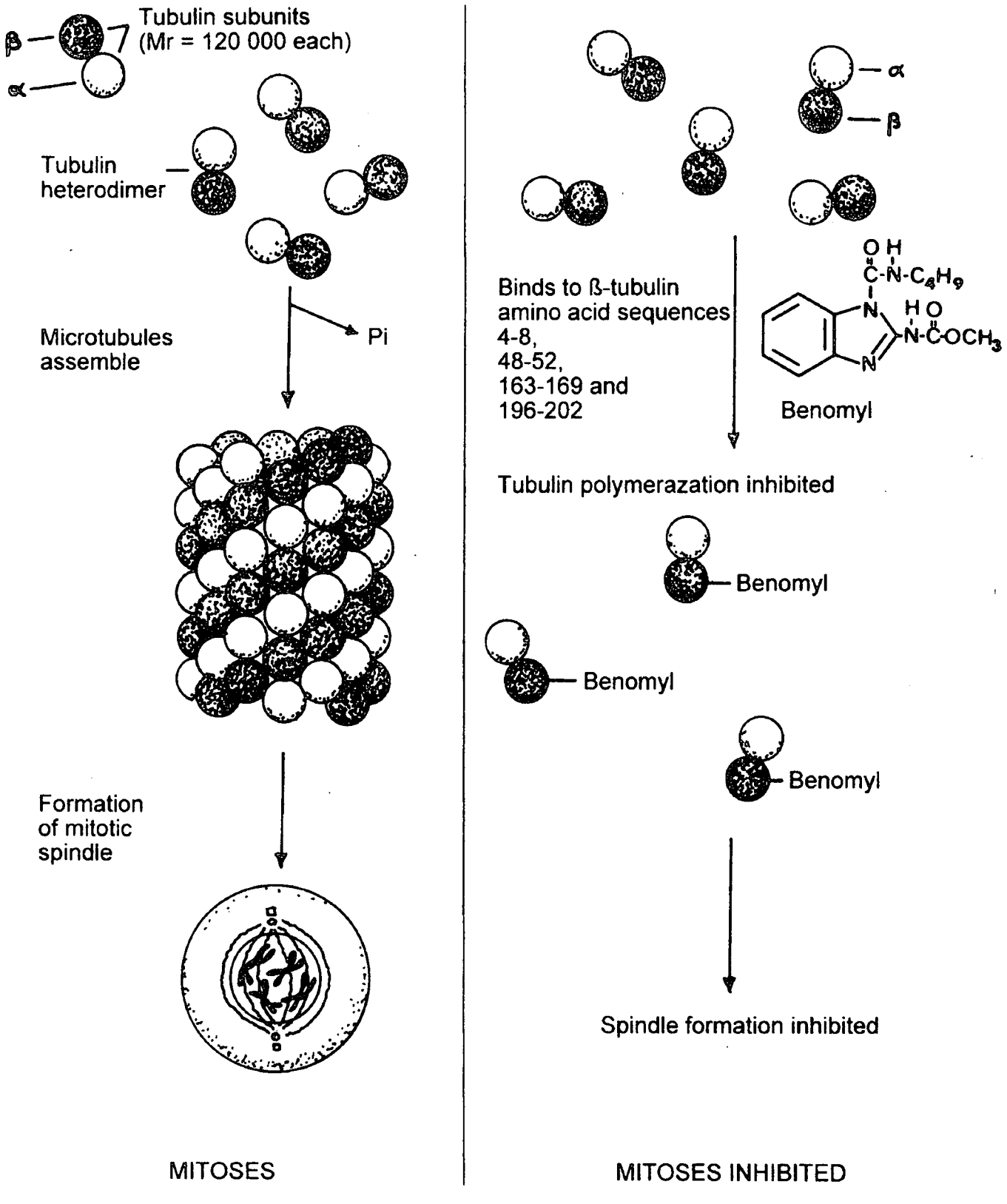


Fig. 9 An illustration of the mechanism of action of benzimidazole fungicides (Lyr, 1989).

1.4. Carbon source utilization and gamma-linolenic acid production by mucoralean fungi.

An important factor in the production of gamma-linolenic acid [18:3(ω6)] by Mucorales is the carbon source on which the organism grow (Israilides et al., 1994; Kock & Botha, 1995). This is important since the carbon source can determine the economic viability of the 18:3(ω6) production process by these fungi. Secondly, the carbon source is a key element in the isolation media of fungi (Botha et al., 1995). The question therefore arises what carbon sources can be utilized by these fungi and do 18:3(ω6) production occur on all these carbon sources, because it is known that carbon sources do influence fatty acid production in the fungal domain (Pohl, 1996). Several studies have been conducted on members of *Mortierella*, *Mucor* and *Rhizopus* to examine the influence of different carbon sources on high value fatty acid production (Hansson & Dostalek, 1988; Sajbidor et al., 1988; Kendrick, 1991; Lindberg & Hansson, 1991; Certik et al., 1993; Roux et al., 1994). The results of these studies are summarized in Table 4.

Mortierella ramanniana 1022 was examined by Sajbidor et al. (1988) on glucose, glycerol, lactose, maltose, sodium acetate and starch as sole carbon sources at a concentration of 30.00 g/l in a complex medium at 28°C in shake flasks. The most biomass was obtained on glucose (13.83 g/l) and starch (11.17 g/l) as sole carbon sources (Table 4). Likewise, the highest volumetric concentrations of 18:3(ω6) were obtained on glucose (182.00 mg/l) and starch (229.21 mg/l) as sole carbon sources (Table 4).

Another strain of *Mo. ramanniana* was examined by Hansson & Dostalek (1988) unfortunately under other culture conditions than the strain examined by Sajbidor et al. (1988). In this case the strain was grown on fructose, glucose, lactose, maltose, starch, sucrose and xylose as sole carbon sources at a concentration of 1 mole carbon/l in a complex medium at 25°C in shake flasks. The most biomass

Table 4. The influence of carbon sources on gamma-linolenic acid production by the mucoralean fungi. (Partly taken from Pohl, 1996).

Name	Carbon source	Biomass (g/l)	Total lipid %(w/w)	Percentage 18:3(ω6) #	18:3(ω6) content of biomass (mg/g)	18:3(ω6) concentration (mg/l)
Mortierella ramanniana 1022 *	Glucose	13.83	14.00	9.40	13.60	182.00
	Glycerol	6.69	13.20	10.00	13.20	88.31
	Lactose	8.53	11.70	11.70	13.69	116.77
	Maltose	9.67	7.10	13.20	9.37	90.63
	Sodium acetate	0.06	17.40	6.10	Trace	Trace
	Starch	11.17	12.00	17.10	20.52	229.21
Mortierella ramanniana CBS 112.08 •	Fructose	12.00	24.90	15.10	37.60	451.19
	Glucose	11.20	23.40	14.70	34.40	385.26
	Lactose	8.70	13.20	19.40	25.61	222.79
	Maltose	6.90	19.10	19.30	36.86	254.35
	Starch	10.70	12.50	25.70	32.13	343.74
	Sucrose	8.70	13.30	21.00	27.93	242.99
	Xylose	8.00	15.40	19.50	30.03	240.24
Mucor circinelloides CBS 108.16 Δ	Glucose	n.d.	15.50	23.90	32.30	n.d.
	Sodium acetate	4.62	40.30	5.00	20.15	93.09
Mucor circinelloides CBS 203.28 Δ	Glucose	n.d.	17.00	11.30	14.50	n.d.
	Sodium acetate	3.64	26.30	11.30	29.72	108.18
Mucor circinelloides UOFS 100 Δ	Glucose	n.d.	47.70	9.20	38.30	n.d.
	Sodium acetate	4.18	31.10	8.20	25.52	106.60
Mucor mucedo 1384 *	Glucose	6.30	23.70	8.00	18.96	119.45
	Glycerol	7.48	18.60	14.30	26.60	198.62
	Lactose	1.23	23.60	25.80	60.68	74.89
	Maltose	8.17	22.80	11.70	26.68	217.94
	Sodium acetate	1.56	27.10	24.50	66.40	103.58
	Starch	4.02	16.20	22.30	36.13	145.23

Abbreviations: 18:3(ω6): Gamma-linolenic acid. n.d.: not determined.
References: *: Sajbidor et al. (1988). •: Hansson & Dostalek (1988). Δ: Roux et al. (1994).
#: Percentage 18:3(ω6) in the lipids, relative to other long chain fatty acids i.e. palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)].

Table 4. (Continues)

Name	Carbon source	Biomass (g/l)	Total lipid %(w/w)	Percentage 18:3(ω6) #	18:3(ω6) content of biomass (mg/g)	18:3(ω6) concentration (mg/l)
Mucor mucedo F-1384 ∫	Fructose	n.d.	n.d	23.90	n.d	33.92
	Galactose	n.d	n.d	27.10	n.d	21.40
	Glucose	n.d	n.d	15.20	n.d	71.44
	Glycerol	n.d	n.d	18.70	n.d	82.27
	Lactose	n.d	n.d	24.60	n.d	31.22
	Maltose	n.d	n.d	11.00	n.d	82.51
	Starch	n.d	n.d	20.00	n.d	68.80
	Starch hydrolysate	n.d	n.d	11.90	n.d	84.23
	Sucrose	n.d	n.d	22.60	n.d	49.18
	Xylose	n.d	n.d	17.20	n.d	70.65
Mucor plumbeus CCM 474 *	Glucose	6.96	22.10	11.40	25.19	175.35
	Glycerol	2.79	9.40	15.80	14.85	41.44
	Lactose	1.53	9.40	28.10	26.41	40.41
	Maltose	5.65	23.50	12.70	29.84	168.62
	Sodium acetate	1.44	19.30	17.00	32.81	47.25
	Starch	1.47	13.10	21.70	28.43	41.79
Mucor rouxii CBS 416.77 Δ	Glucose	n.d.	22.60	9.00	17.00	n.d.
	Sodium acetate	3.67	40.00	8.30	33.20	121.84
Mucor rouxii CBS 416.77 □	Glucose	8.10	7.10	25.00	17.75	143.78
	Molasses	3.50	8.50	37.00	31.45	110.08
	Starch	9.80	11.00	19.00	20.90	204.82
	Starch hydrolysate	12.00	10.00	17.00	17.00	204.00

Abbreviations: 18:3 (ω6): Gamma-linolenic acid. n.d.: not determined.
References: ∫: Certik et al. (1993). *: Sajbidor et al. (1988). Δ: Roux et al. (1994). □: Lindberg & Hansson (1991).
#: Percentage 18:3(ω6) in the lipids, relative to other long chain fatty acids i.e. palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)].

Table 4. (Continues)

Name	Carbon source	Biomass (g/l)	Total lipid %(w/w)	Percentage 18:3(ω6) #	18:3(ω6) content of biomass (mg/g)	18:3(ω6) concentration (mg/l)
Rhizopus arrhizus VUPL 23 *	Glucose	4.53	18.40	10.80	19.87	90.02
	Glycerol	9.01	12.50	9.20	11.50	103.62
	Lactose	1.59	8.20	13.90	11.40	18.12
	Maltose	4.95	21.20	9.40	19.93	98.64
	Sodium acetate	3.33	17.20	2.30	3.95	13.17
	Starch	7.81	16.20	8.90	14.42	112.60

Abbreviations: 18:3 (ω6): Gamma-linolenic acid.
References: *: Sajbidor et al. (1988).
#: Percentage 18:3(ω6) in the lipids, relative to other long chain fatty acids i.e. palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)].

was obtained on fructose (12.00 g/l), glucose (11.20 g/l) and starch (10.70 g/l) as sole carbon sources (Table 4). The highest volumetric concentrations of 18:3(ω6), were obtained on fructose (451.19 mg/l), glucose (385.26 mg/l) and on starch (343.74 mg/l).

Roux et al. (1994) examined different strains of *Mucor circinelloides* on glucose and sodium acetate as sole carbon sources (Table 4). The concentration of the glucose in the culture media was 50.00 g/l and the final concentration of sodium acetate amounted to between 15.00 and 18.00 g/l, fed on demand to the organism. Both carbon sources were fed to the organisms in a complex medium at 28°C. The highest percentage 18:3(ω6) in the oil, was obtained in *Mucor circinelloides f. circinelloides* CBS 108.16 grown on glucose as carbon source (Table 4).

Mucor mucedo 1384 was examined by Sajbidor et al. (1988) on glucose, glycerol, lactose, maltose, sodium acetate and starch as sole carbon sources, at a concentration of 30.00 g/l in a complex medium at 28°C in shake flasks. The most biomass was obtained on glucose 6.30 g/l), glycerol (7.48 g/l) and maltose (8.17 g/l) as sole carbon sources (Table 4). The highest volumetric concentrations of 18:3(ω6), were obtained on glycerol (198.95 mg/l), maltose (217.94 mg/l) and again on starch (145.23 mg/l), as in the case of the experiments on *Mortierella ramanniana* (Hansson & Dostalek, 1988; Sajbidor et al., 1988). It must be kept in mind, however, that the same culture conditions were not used for all three organisms.

Mucor mucedo F-1384 was examined by Certik et al. (1993) on fructose, galactose, glucose, glycerol, lactose, maltose, starch, starch hydrolysate, sucrose and xylose as sole carbon sources at a concentration of 1 mole carbon/l in a complex medium at 28°C in shake flasks (Table 4). The highest volumetric concentrations of 18:3(ω6), were obtained on glycerol (82.27 mg/l), maltose (82.51 mg/l) and starch hydrolysate (84.23 mg/l) (Table 4).

Mucor plumbeus CCM 474 was examined by Sajbidor et al. (1988) on glucose, glycerol, lactose, maltose, sodium acetate and starch as sole carbon sources at a concentration of 30.00 g/l in a complex medium at 28°C in shake flasks. The most biomass was obtained on glucose (6.96 g/l) and maltose (5.65 g/l) as sole carbon sources (Table 4). Similarly, the highest volumetric concentrations of 18:3(ω6), were obtained on glucose (175.35 mg/l) and maltose (168.62 mg/l) as sole carbon sources (Table 4). It is interesting to note that in the experiments with *M. mucedo* 1384, *M. mucedo* F-1384 and *M. plumbeus* CCM 474, maltose also looks promising for obtaining a high concentration of 18:3(ω6) in the oil from representatives of the genus *Mucor*. However, this substrate was not tested with *Mucor rouxii*, a species known for substantial amounts of 18:3(ω6) production (Aggelis et al., 1988).

Roux et al. (1994) examined *Mucor rouxii* CBS 416.77 on glucose and sodium acetate as sole carbon sources, with a glucose concentration of 50.00 g/l and a final sodium acetate concentration which amounted to 18.00 g/l, fed on demand to the organism. The carbon sources were fed to the strain in a complex medium at 30°C. In this case, the highest percentage of 18:3(ω6) in the oil was obtained with glucose (9.00%) as sole carbon source (Table 4).

Mucor rouxii CBS 416.77 was also examined by Lindberg & Hansson (1991), but under different conditions than used by Roux et al. (1994). In these experiments the strain was grown on glucose, molasses, starch and starch hydrolysate as sole carbon sources. The concentration of the glucose, starch and starch hydrolysate was 60.00 g/l and the molasses 154.00 g/l in a complex medium, however, in contrast to the experiments of Roux et al. (1994), the strain was grown at a lower temperature of 25°C. The most biomass was obtained on starch hydrolysate (12.00 g/l) and starch (9.80 g/l) as sole carbon source (Table 4). The highest percentage of 18:3(ω6) in the oil was obtained with molasses (37.00%) as carbon source, but when comparing the volumetric concentrations of 18:3(ω6) obtained,

the highest concentration was obtained on starch (204.82 mg/l) as sole carbon source (Table 4). Interestingly, when you compare the percentage of 18:3(ω6) obtained in the oil of *M. rouxii* grown on glucose, you will notice that in the experiment of Lindberg & Hansson (1991) the percentage 18:3(ω6) obtained is much higher than in the experiments of Roux et al. (1994). A possible explanation for this may be the influence of temperature. When the strain was grown at a lower temperature, a higher percentage 18:3(ω6) in the oil was obtained - it is known that lower temperatures can promote the production of PUFAs like 18:3(ω6) (Rattray, 1988). The reason for this phenomenon was given by Rattray (1988), who stated that more PUFAs are included in fungal membranes at lower temperatures, in order to keep up the membrane fluidity, which is essential for various enzymatic processes in the cells.

Rhizopus arrhizus VUPL 23 was examined by Sajbidor et al. (1988) on glucose, glycerol, lactose, maltose, sodium acetate and starch as sole carbon sources, at a concentration of 30.00 g/l in a complex medium at 28°C in shake flasks. The most biomass was obtained on glycerol (9.01 g/l), maltose (4.95 g/l) and starch (7.81 g/l) as sole carbon sources (Table 4). The highest volumetric concentrations of 18:3(ω6), were obtained on glycerol (103.62 mg/l), maltose (98.64 mg/l) and starch (112.60 mg/l) as sole carbon sources (Table 4).

In addition to the experiments discussed here, the influence of different oils as carbon sources on high value fatty acid production by mucoralean fungi was examined by Kendrick (1991) (Table 5). It is interesting to note that 18:3(ω6) production in both *M. circinelloides* and *Mortierella isabellina* were lower in the experiments where the oils were used as carbon sources, than when glucose was used as carbon source (Table 5). According to Kendrick (1991) this may be the result of an inhibition of the cytosolic malic enzyme activity by the presence of an excess of free fatty acids originating from the oils fed to the fungi. The role of malic enzyme in lipogenesis is to provide NADPH for fatty acid biosynthesis and fatty acid

Table 5. The influence of different oils as carbon sources on high value fatty acid production by the mucoralean fungi.

Name	Carbon source	Percentage 18:3(ω6) #	18:3(ω6) concen= tration (mg/l)
Mucor circinelloides No. 1 *	Glucose	8.30	173.88
	Safflower oil	1.10	24.53
	Sesame oil	2.60	45.15
	Triolein	7.80	140.43
Mortierella isabellina No. 2 *	Glucose	3.90	63.90
	Safflower oil	1.40	62.34
	Sesame oil	0.90	28.36
	Triolein	0.50	18.11
Mucor circinelloides CBS108.16 Δ	Sunflower oil	0.60	27.90
	Sunflower oil plus Sodium acetate	4.60	446.32

Abbreviations: 18:3(ω6): Gamma-linolenic acid.
References: * Kendrick (1991), Δ Jeffery et al. (1997).
#: Percentage 18:3(ω6) in the lipids, relative to other long chain fatty acids i.e. palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)]

desaturation. Under conditions of NADPH limitation, induced by growth on oils, the fungi no longer have the ability to synthesise fatty acids or desaturase them further. The fungi therefore incorporate these fatty acids directly into cellular lipids without modification.

Interestingly, recent results obtained on the utilization of sunflower oil by *M. circinelloides* suggested that the delta-6-desaturation reaction is less repressed when sodium acetate is added together with sunflower into the medium (Jeffery et al., 1997). When 30 g/l sunflower oil and 10 g/l sodium acetate were added to a complex medium, *M. circinelloides* CBS108.16 was therefore able to produce up to 446.32 mg/l 18:3(ω6) in its neutral lipid fraction (Table 5). In contrast, only 27.90 mg/l 18:3(ω6) was produced when 40 g/l sunflower oil was used as sole carbon source.

Although a considerable amount of work has been conducted on enhancing high value fatty acid production by mucoralean fungi, the influence of carbon sources on growth and 18:3(ω6) production has been largely overlooked. It seems that with a few exceptions, starch and perhaps maltose, may be potential carbon sources for the production of substantial amounts of 18:3(ω6) by mucoralean fungi. Unfortunately, most authors have examined only a few carbon sources on a limited number of fungal strains. In addition, no standardized culture conditions were used. This is unfortunate, since it is known that factors such as oxygen, pH, temperature, C:N ratio and the type and concentration of the carbon source can influence fungal lipid production including fatty acid composition (Rattray,1988). No direct comparison between the results obtained by the various authors can therefore be drawn.

1.5. Aim

The ultimate aim of this study was to develop media capable of isolating mucoralean fungi, that can grow and produce 18:3(ω6), on carbon sources present in industrial effluents. In order to achieve this, it was first necessary to examine the influence of 38 carbon sources on the 18:3(ω6) content of the lipids present in different mucoralean fungi (Chapter 2). Thereafter, carbon sources obtainable from industrial effluents, which support growth and the accumulation of lipids containing high percentages 18:3(ω6) had to be selected. Isolation media utilizing these carbon sources had to be developed (Chapter 3). In order to achieve this, the selectivity of these media among members of Mucorales, as well as the ability of the media to select mucoralean fungi from soil, had to be determined. The mucoralean fungal isolates that were obtained from the soil were subsequently evaluated for growth and 18:3(ω6) production in media containing the carbon sources present in industrial effluents (Chapters 4 and 5).

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CHAPTER 2

CARBON SOURCE UTILIZATION AND GAMMA-LINOLENIC ACID PRODUCTION BY MUCORALEAN FUNGI

(Published in Systematic and Applied Microbiology 20, 165 -170)

2.1. Introduction

Gamma-linolenic acid [18:3(ω6)] is a long-chain, polyunsaturated fatty acid (Ratledge, 1994). In mammals, including man, 18:3(ω6) has important nutritional value, since it is a precursor for the synthesis of lipid hormones (prostaglandins, thromboxanes or leukotrienes), which play vital regulatory roles in cellular metabolism (Graham, 1984; Thomas & Holub, 1994). The current commercial source for 18:3(ω6) in the diet, is the oil extracted from plants such as Evening Primrose (*Oenothera biennis*) or Borage (*Borago officina*) (Graham, 1984; Gunstone et al., 1994). Borage oil contains a higher percentage w/w 18:3(ω6) (19.00 - 25.00 %), compared to 8.00 - 12.00 % w/w 18:3(ω6) in Evening Primrose oil (Ratledge, 1994).

However, an alternative source that has been extensively researched, is the fungal domain, especially the zygomycotan fungi. According to Kendrick (1992) the primitive protoctistan fungi gave rise to the terrestrial dikaryomycotan fungi and Zygomycota. A well-known order of the Zygomycota is Mucorales, known for the production of significant quantities of 18:3(ω6) (Ratledge, 1994). Percentages of 15.00 to 18.00 % (w/w) have been obtained for this fatty acid in the oil of *Mucor circinelloides,* a well-known member of Mucorales (Ratledge, 1994; Du Preez et al., 1995). Interestingly, although 18:3(ω6) has also been found in the lipids of the protoctistan fungi, evidence in literature suggests that the Dikaryomycota has lost the ability to produce this fatty acid (Van der Westhuizen, 1994).

Several optimization studies have been done on the production of 18:3(ω6) by mucoralean fungi, by changing the culture conditions (Hansson & Dostalek, 1988; Nakajima & Sano, 1991; Roux et al., 1994; Du Preez et al., 1995; Kock & Botha, 1995). However, only a few studies were conducted where the influence of different carbon sources on 18:3(ω6) production by the mucoralean fungi were examined. Also in most cases, no standardized culture conditions were used and only a few carbon sources were investigated (Hansson &

Dostalek, 1988; Sajbidor et al., 1988; Kendrick, 1991; Lindberg & Hansson, 1991; Certik et al., 1993; Roux et al., 1994). A more elaborate study was reported by Botha et al. (1996) on the ability of a strain representing *Mucor circinelloides* to utilize 40 different carbon sources and produce polyunsaturated fatty acids including 18:3(ω6). They found that the strain could germinate and grow on a wide variety of carbon sources in synthetic liquid media. The highest percentages polyunsaturated fatty acids were produced when acetic acid, glucose, mannitol, soluble starch or trehalose was used as sole carbon sources. Therefore, the aim of this study was to examine the influence of 38 carbon sources on the 18:3(ω6) content of the lipids present in four mucoralean strains.

2.2. Materials and methods

2.2.1. Fungal strains.

Mucor circinelloides f. *circinelloides* CBS 119.08, *Mucor flavus* CBS 234.35, *Thamnostylum piriforme* PPRI 5534 and *Mortierella alpina* ATCC 3221 were used in this study. These fungal strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), the American Type Culture Collection (ATCC) and the Plant Protection Research Institute, South Africa (PPRI).

2.2.2. Preparation of inoculum.

A culture of each strain was incubated at 21°C in the dark for seven to ten days on 2 % w/w malt extract agar (Biolab). A spore suspension of *circa* 2.5 x 10^6 spores/ml was obtained by transferring the sporangiospores from each culture with an inoculation loop to 10.00 ml sterile distilled water. This spore suspension was then used as inoculum.

2.2.3. Culture conditions.

The spore suspension obtained for each fungal strain was used to inoculate thirty eight sets of four test tubes (150 mm x 12 mm). Each set of four tubes contained a synthetic medium with a particular carbon source (Tables 1-7). Each tube, which contained 5.00 ml of a synthetic medium consisting of 7.60 g/l

Yeast Nitrogen Base, Difco (YNB) and 2.00 g/l carbon, received 40 µl of the spore suspension as inoculum (Van der Walt & Yarrow, 1984). The inoculated tubes were incubated at 18°C on a rollordrum rotating at 100 rpm. From the start of growth, as determined visually, the biomass was harvested every second day by filtration (Whatman, GF/A), until the stationary growth phase was reached. The biomass was freeze dried and weighed.

2.2.4. Fatty acid analyses.

The lipids were extracted from the freeze dried biomass using chloroform/methanol (2:1, v/v) (Kendrick & Ratledge, 1992). The extracted lipids were dried under nitrogen gas and then methylated by the addition of trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The methylated fatty acids were analysed with a Varian 3300 gas chromatograph and a Supelcowax 10 glass capillary column (0.75 mm x 30.00 m) with nitrogen (5.00 ml/min) as carrier gas (Kock, 1988). Peaks were identified by reference to authentic standards and the percentage 18:3(ω6) in the lipid was calculated relative to the other long-chain fatty acids present. These fatty acids include palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)] and linoleic acid [18:2(ω6)].

2.3. Results and discussion

The results obtained on biomass and 18:3(ω6) production by the four mucoralean strains are depicted in Tables 1 to 7. In general, an increase in biomass occurred during incubation. In some cases, however, a decrease in biomass was apparent at the end of the incubation period. This may be due to lyses of cells during the stationary phase. Also apparent in some cases, is the decrease in the percentage 18:3(ω6), as the cultures were incubated into the stationary phase. This may be ascribed to the utilization of this fatty acid for energy purposes.

Table 1. Percentage 18:3(ω6) and biomass produced by *Mucor circinelloides* f. *circinelloides* CBS 119.08 during growth on different carbohydrates as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		2 days	4 days	6 days	8 days
Pentoses					
L-Arabinose	G	+++	+	++	+
	B	+	+	++++	+++++
D-Xylose	G	++	++	++	+
	B	+	++	+++	+++++
D-Ribose	G	++++	+	++	++
	B	+	+	++++	++++
Hexoses					
D-Galactose	G	+	+	+	+
	B	++	+++	+++++	++++
D-Glucose	G	++	++	+	+
	B	+++	++++	+++++	+++++
L-Rhamnose	G	-	-	+++	++
	B	-	-	+	+
Disaccharides					
Cellobiose	G	++	+++	n.d.	+++++
	B	++	++	++	++
Maltose	G	+++	++	n.d.	++
	B	+	+++	+++	+++
Melibiose	G	-	-	+++	++
	B	-	-	+	+
Sucrose	G	-	-	+++++	++
	B	-	-	+	+
Trehalose	G	++++	++	+++	++
	B	++	+++	+++	++++
Trisaccharides					
Melezitose	G	+++	++	+++	++++
	B	+	++	+	++
Polysaccharides					
Inulin	G	++	++++	+++	+++
	B	+++	+++	+++	+++++
Starch	G	-	++	+++	++
	B	+	+++	+++++	+++++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

n.d. = not determined

Carbon sources not utilized: D-Arabinose, L-Sorbose, Lactose, Raffinose

Table 2. Percentage 18:3(ω6) acid and biomass produced by *Mucor circinelloides* f. *circinelloides* CBS 119.08 during growth on different glucosides, alcohols or organic acids as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		2 days	4 days	6 days	8 days
Glucosides					
Salicin	G	++	++	++	+++
	B	+	++	++	++
Alcohols					
Adonitol	G	-	-	++	++
	B	-	-	+++++	+++++
Dulcitol	G	-	-	++	+++++
	B	-	-	+	++
Ethanol	G	-	+	++	++
	B	-	+	++	+
Mannitol	G	-	-	+++	++
	B	-	-	+++++	+++++
Sorbitol	G	++	n.d.	+++	+++
	B	+	++	++++	++++
Organic acids					
Acetic acid	G	+	+	+	+
	B	+	+	++++	+++
Butyric acid	G	-	-	+++	+++
	B	-	-	++	++
D-Gluconate	G	++	+++	++	+++
	B	+	+	+++	+++
Lactate	G	++	++	++	++
	B	+	+	++++	+++
Succinate	G	+++	+++	+++++	+++
	B	+	++	+++	++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

n.d. = not determined

Carbon sources not utilized: Butane-2,3-diol, Erythritol, Glycerol, Inositol, Methanol, Propane-1,2-diol, Citrate, Formic acid, Propanoic acid

Table 3. Percentage 18:3(ω6) and biomass produced by *Mucor flavus* CBS 234.35 during growth on different carbohydrates as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		2 days	4 days	6 days	8 days
Pentoses					
L-Arabinose	G	++	+++	+++	+++
	B	+	+	++	+
D-Xylose	G	-	++	+++	+++
	B	-	+	+	++
Hexoses					
D-Galactose	G	++++	+++++	+++++	+++++
	B	+++	+++++	+++++	+++++
D-Glucose	G	++++	n.d.	+++++	+++++
	B	+++++	n.d.	+++++	+++++
L-Rhamnose	G	-	+++	+++++	++
	B	-	+	++	++
Disaccharides					
Cellobiose	G	+++++	++++	+++++	++
	B	++	+++	++	++
Maltose	G	++++	+++++	+++++	++++
	B	++	++	++	+
Trehalose	G	++++	+++++	+++++	+++++
	B	++	+++	+++	++
Trisaccharides					
Melezitose	G	+++	++	+++	+
	B	+	++	++	+
Raffinose	G	+	++	++++	++++
	B	+	+	+	+
Polysaccharides					
Inulin	G	+++	++	+++	++++
	B	+	+	+	+
Starch	G	++++	+++++	+++++	+++++
	B	+++	++++	+++++	++++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

n.d. = not determined

Carbon sources not utilized: D-Arabinose, D-Ribose, L-Sorbose, Lactose, Melibiose, Sucrose

Table 4. Percentage 18:3(ω6) and biomass produced by *Mucor flavus* CBS 234.35 during growth on different glucosides, alcohols or organic acids as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		2 days	4 days	6 days	8 days
Glucosides					
Salicin	G	+++	++++	++++	++++
	B	+	+++	+++	++
Alcohols					
Erythritol	G	+	+++	++++	+++
	B	+	+	++	++
Ethanol	G	+++	++	+++++	++++
	B	+	+	++	+++
Glycerol	G	+	+++++	+++++	+
	B	+	+	+++	++
Inositol	G	-	-	+++++	+++
	B	-	-	++	+
Mannitol	G	+	++	++	++++
	B	+	+	++	+
Sorbitol	G	-	+++	++	+
	B	-	+	++	+
Organic acids					
Acetic acid	G	+++	+++++	+++++	+++
	B	++	+++	+++	+++
D-Gluconate	G	++	+++++	n.d.	+++++
	B	+	++	n.d.	++
Lactate	G	-	+++++	n.d.	+++++
	B	-	+	n.d.	+++
Succinate	G	+++	+++++	+++++	+
	B	+	++	+++++	+++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

n.d. = not determined

Carbon sources not utilized: Adonitol, Butane-2,3-diol, Dulcitol, Methanol, Propane-1,2-diol, Butyric acid, Citrate, Formic acid, Propanoic acid

Table 5. Percentage 18:3(ω6) and biomass produced by *Thamnostylum piriforme* PPRI 5534 during growth on different carbohydrates as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		4 days	6 days	8 days	10 days
Pentoses					
L-Arabinose	G	-	+	+	+
	B	-	+	+	++
D-Xylose	G	-	+	++	++
	B	-	+	+	+++
Hexoses					
D-Galactose	G	+	+	+	+
	B	+	++	+++	++
D-Glucose	G	+	++	+	++
	B	+	++	+++	++
Disaccharides					
Cellobiose	G	+	+	+	++
	B	+	+	+	++
Maltose	G	++	+++	++	+
	B	+	++	++	+++
Melibiose	G	-	-	+	+
	B	-	-	++	++
Trehalose	G	-	++	n.d.	+
	B	-	++	n.d.	++
Trisaccharides					
Melezitose	G	+	+	++	n.d.
	B	+	++	+	n.d.
Polysaccharides					
Inulin	G	-	+	+	+
	B	-	+	++	+
Starch	G	+	+	+	+
	B	+	++	++	+++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

n.d. = not determined

Carbon sources not utilized: D-Arabinose, D-Ribose, L-Rhamnose, L-Sorbose, Lactose, Sucrose, Raffinose

Table 6. Percentage 18:3(ω6) and biomass produced by *Thamnostylum piriforme* PPRI 5534 during growth on different glucosides, alcohols or organic acids as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		4 days	6 days	8 days	10 days
Glucosides					
Salicin	G	+	++	++	+
	B	+	+	++	++
Alcohols					
Sorbitol	G	-	-	+	+
	B	-	-	++	+
Organic acids					
Acetic acid	G	++	++	+	+
	B	+++	++++	++++	+++++
Butyric acid	G	+++	++	+	+
	B	++	++	++	++
Lactate	G	-	-	++	+
	B	-	-	++	++
Succinate	G	+	+	+	+
	B	+	++	+	+

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

Carbon sources not utilized: Adonitol, Butane-2,3-diol, Dulcitol, Erythritol, Ethanol, Glycerol, Inositol, Mannitol, Methanol, Propane-1,2-diol, Citrate, Formic acid, D-Gluconate, Propanoic acid

Table 7. Percentage 18:3(ω6) and biomass produced by *Mortierella alpina* ATCC 3221 during growth on different carbohydrates, glucosides, alcohols or organic acids as sole carbon sources.

Carbon sources		Percentage gamma-linolenic acid and biomass produced			
		6 days	8 days	10 days	12 days
Pentoses					
D-Ribose	G	-	+	+	+
	B	-	++	++	++
Hexoses					
D-Glucose	G	+	+	+	+
	B	++++	+++	++++	++++
Disaccharides					
Maltose	G	+	+	+	+
	B	+++	++	+++	+++
Trehalose	G	+	+	+	+
	B	++++	+++	++++	++++
Polysaccharides					
Inulin	G	+	+	+	+
	B	++	++	++	++
Starch	G	+	++	+	+
	B	+++	+++	++++	++++
Alcohols					
Glycerol	G	+	+	+	+
	B	++	++	+++	+++

G = % Gamma-linolenic acid calculated relative to the other long-chain fatty acids in the oil (Palmitic acid[16:0]; Palmitoleic acid[16:1(ω7)]; Stearic acid[18:0]; Oleic acid[18:1(ω9)]; Linoleic acid[18:2(ω6)]): 0.00% = -; 0.01-5.00% = +; 5.01-10.00% = ++; 10.01-15.00% = +++; 15.01-20.00% = ++++; > 20.00% = +++++

B = Biomass: 0.000 g/l = -; 0.001-0.300 g/l = +; 0.301-0.600 g/l = ++; 0.601-0.900 g/l = +++; 0.901-1.200 g/l = ++++; > 1.200 g/l = +++++

Carbon sources not utilized: D-Arabinose, L-Arabinose, D-Xylose, D-Galactose, L-Rhamnose, L-Sorbose, Cellobiose, Lactose, Melibiose, Sucrose, Melezitose, Raffinose, Salicin, Adonitol, Butane-2,3-diol, Dulcitol, Erythritol, Ethanol, Inositol, Mannitol, Methanol, Propane-1,2-diol, Sorbitol, Acetic acid, Butyric acid, Citrate, Formic acid, D-Gluconate, Lactate, Propanoic acid, Succinate

The results obtained with the first *Mucor* strain that was tested, *Mucor circinelloides* f. *circinelloides* CBS 119.08, are depicted in Tables 1 and 2. This strain assimilated 65 % of the carbon source series presented to it. The highest percentage 18:3(ω6) obtained with this strain was 27.17 %, from cellobiose as carbon source after eight days of incubation. In general, with this strain lower percentages 18:3(ω6) were obtained from hexoses as carbon sources compared to the pentoses as carbon sources. Regarding the percentage 18:3(ω6) obtained with carbon sources such as glucosides, alcohols and organic acids (Table 2), the highest percentage 18:3(ω6) was obtained with dulcitol as carbon source (24.04 %), after eight days of incubation. In contrast to the results reported here, another strain of this species was found by Botha et al. (1996) to produce the highest percentage 18:3(ω6) on starch as carbon source (28.30 %). These contrasting results are not surprising since it is known that 18:3(ω6) production from a specific carbon source may vary within *Mucor circinelloides* (Roux et al., 1994; Botha et al., 1995).

The results obtained with *Mucor flavus* CBS 234.35 are depicted in Tables 3 and 4. This strain assimilated 60 % of the carbon source series presented to it. From all the strains tested, *M. flavus* CBS 234.35 produced the highest percentage 18:3(ω6). After eight days of incubation a value of 36.40 % 18:3(ω6) was reached with starch as carbon source.

In general, for *M. flavus* CBS 234.35 where growth occurred, the percentages 18:3(ω6) obtained with hexoses and disaccharides as carbon sources were higher than the percentages 18:3(ω6) obtained with the pentoses and trisaccharides as carbon sources (Table 3). Regarding the percentage 18:3(ω6) obtained with carbon sources like glucosides, alcohols and organic acids (Table 4), the highest percentages 18:3(ω6) were obtained with ethanol and acetic acid after six days of incubation, reaching values of 28.95 % and 25.82 % respectively.

The results obtained with *Thamnostylum piriforme* PPRI 5534 are depicted in Tables 5 and 6. This strain assimilated less carbon sources i.e. 44 % compared

to the two strains representing *Mucor flavus* and *Mucor circinelloides.* The highest percentage 18:3(ω6) obtained in this strain, with a carbohydrate as carbon source, was 10.07 %, obtained on maltose after six days of incubation. However, 12.84 % 18:3(ω6) was obtained on butyric acid as carbon source after four days of incubation (Table 6). In general, lower percentages 18:3(ω6) were obtained with this strain than in the two *Mucor* strains tested.

The results obtained with *Mortierella alpina* ATCC 3221 are depicted in Table 7. This strain could only utilize 18 % of the carbon source series presented to it. The highest percentage 18:3(ω6) obtained with *M. alpina* ATCC 3221 was 5.61 %, obtained on starch as carbon source after eight days of incubation. Interestingly, Hansson & Dostalek (1988) and Sajbidor et al. (1988), also obtained higher percentages 18:3(ω6) in the oil of another *Mortierella* species (i.e. *M. ramanniana*), using starch as carbon source, compared to other carbon sources. Of all the glucosides, alcohols and organic acids presented to *M. alpina* ATCC 3221, only glycerol could be utilized (Table 7). On this carbon source, the highest percentage 18:3(ω6) obtained was 4.20 %, after eight days of incubation.

2.4. Conclusions

When comparing carbon source utilization and 18:3(ω6) production with morphology of the fungal strains an interesting pattern emerges (Fig. 1). The two representatives of *Mucor* had a greater ability to utilize the series of carbon sources and produced higher percentages 18:3(ω6) than the other two strains representing *Mortierella* and *Thamnostylum.* Interestingly, a similar pattern was found within the dikaryomycotan genus *Kluyveromyces* when morphology and carbon source utilization were compared, and this corresponded with phylogenetic development (Kock et al., 1988). Whether the emerging pattern

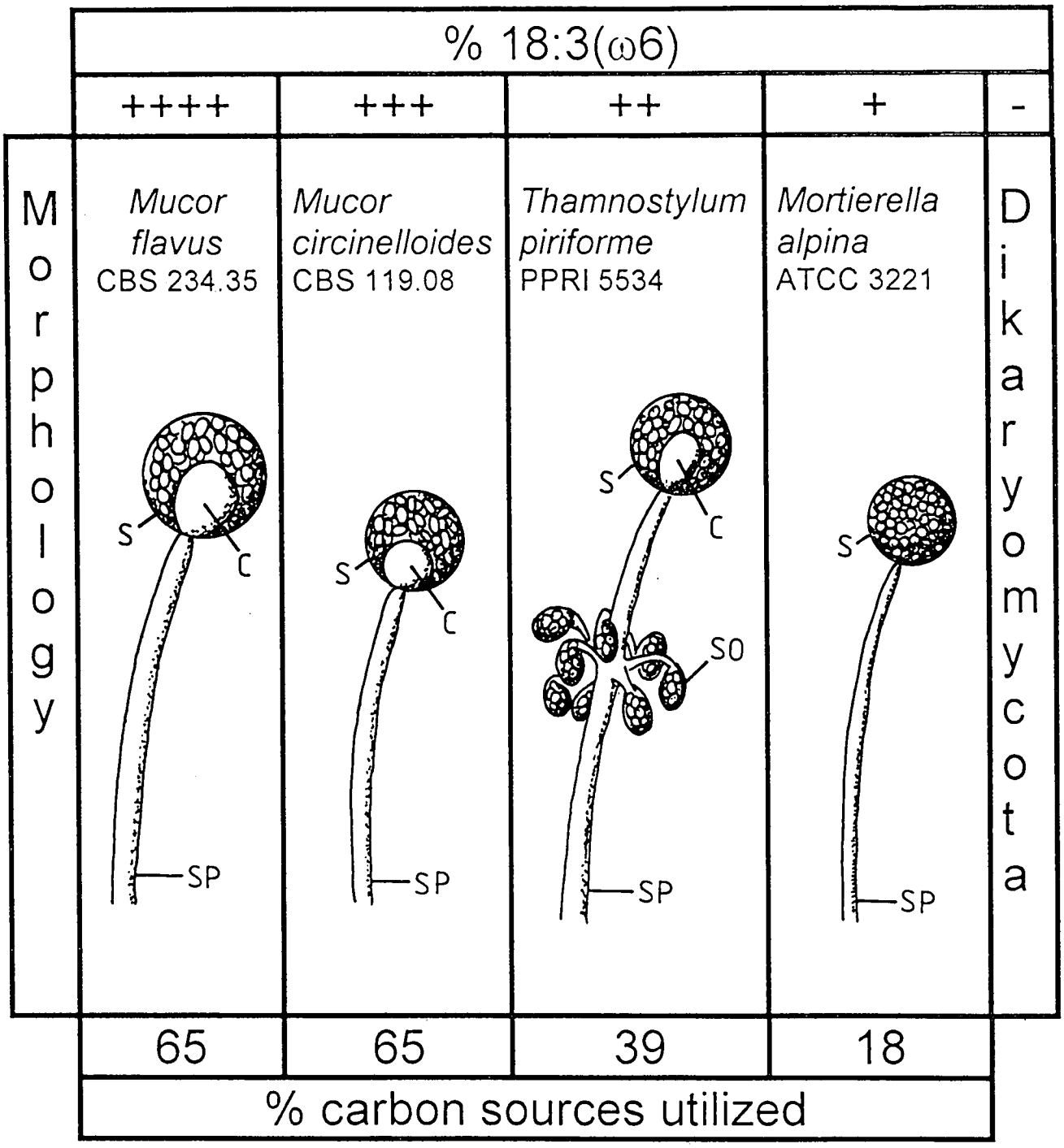

Fig. 1. Comparison of carbon source utilization with morphology and gamma-linolenic acid [18:3(ω6)] production.

Horizontal scale - Top: General ability to produce 18:3(ω6), highest values = +++++, lowest values = -.
Horisontal scale - Bottom: The percentage of the carbon source series that could be utilized by the fungal strains in this study.
Vertical scale - Left: Distinguishing morphological features are illustrated.
C = columella, S = sporangium, SO = sporangiolum, SP = sporangiophore.

(Fig. 1) is a result of phylogenetic development within Mucorales, remains to be seen.

With this study, the foundations have been laid for a database from which combinations of mucoralean fungi and their carbon sources can be selected to grow and produce lipids rich in 18:3(ω6). It has been found that mucoralean fungi can grow and produce 18:3(ω6) on a wide variety of carbon sources, including pentoses, hexoses, disaccharides, trisaccharides, polysaccharides, glucosides, alcohols and organic acids.

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CHAPTER 3

DEVELOPMENT AND TESTING OF SELECTIVE MEDIA FOR MUCORALEAN FUNGI

(Submitted for publication in Antonie van Leeuwenhoek)

3.1. Introduction

In the screening programme conducted in Chapter 2, it was found that mucoralean fungal strains representing the genera *Mortierella*, *Mucor* and *Thamnostylum* can grow and produce gamma-linolenic acid [18:3(ω6)] on a wide diversity of carbon sources, including starch, sucrose and acetic acid. Starch, and sucrose as a component of molasses, are important since they are agro-industrial wastes (Israilides et al., 1994). The utilization of these cheap substrates in biotechnological processes would therefore be economically advantageous. Not surprisingly, studies have been conducted on the ability of mucoralean fungi to grow and produce 18:3(ω6) on these carbon sources (Hansson & Dostalek, 1988; Sajbidor et al., 1988; Certik et al., 1993). Similarly, the ability of mucoralean fungi to grow and produce 18:3(ω6) from acetic acid, a component of effluents from the petro-chemical industry, have been well studied (Kock & Botha, 1995).

However, in order to improve 18:3(ω6) production, continuous searches are being conducted to obtain more mucoralean fungi from soil able to grow on specific carbon sources (Botha et al., 1995). Unfortunately, it was found that other soil fungi, like *Trichoderma* species, produce substances that inhibit growth of certain mucoralean fungi (Tronsmo & Dennis, 1978). This may result in less mucoralean fungi being recovered from the isolation plates because of the presence of antagonistic fungi.

Isolation media specific for mucoralean fungi have been constructed (Bartshi et al., 1991; Botha et al., 1995). However, these authors either used a few strains to determine the selectivity of the media within Mucorales, or did not quantitatively determine the ability of the media to select mucoralean fungi amongst a natural occuring fungal population.

The aim of this study was therefore to construct isolation media for mucoralean fungi with starch, glucose or acetate as carbon sources. In order to achieve this,

selectivity of these media among members of Mucorales, as well as the ability of the media to select mucoralean fungi from soil, had to be determined.

3.2. Materials and methods

3.2.1. Strains used.

Some of the strains used in this study were obtained from the Centraalbureau voor Schimmelcultures (CBS), The Netherlands, and from the Mucoralean Fungal Culture Collection of the Free State University (MUFS), South Africa (Table 1). The other fungal strains were isolated from soil of the Dry *Cymbopogon-Themeda* Veld (Acocks, 1988) at the Soetdoring Nature Reserve in the central Free State, South Africa (Table 3).

3.2.2. Selectivity of isolation media.

Three media were evaluated for their selective properties. The media were tested for their ability to support growth of 134 mucoralean fungal strains representing 66 species and seven genera (Table 1). The composition of the media were as follows: Isolation media A contained (g/l); starch, 10.00; NH_4Cl, 1.00; KH_2PO_4, 1.00; $MgSO_4.7H_2O$, 0.50; yeast extract (Difco), 0.50 and chloramphenicol, 0.20. It also contained the following mineral salts (mg/l): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium which had a pH of 5.5 and was solidified with 16 g/l agar, also contained 0.02 g/l benlate (Aldrich). Isolation medium B had an identical composition than isolation medium A, except it contained 10 g/l sucrose as carbon source instead of starch.

Isolation medium C contained (g/l): sodium acetate, 10.00; NH_4Cl, 1.00; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.25; yeast extract (Difco), 0.5 and $CaCl_2$, 0.05. It also contained (mg/l): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium had a pH of 5.5, was solidified with 16 g/l agar and also contained 0.02 g/l benlate.

A loopfull of one week old hyphal growth on malt extract agar (Difco), from each of the fungal strains listed in Table 1, was used to inoculate each of the three selective media contained in Petri dishes (diameter, 80 mm). In each case, an area not more than 25 mm^2, in the centre of the Petri dish containing the medium, was inoculated. The cultures were incubated at 20°C in the dark and the diameter of the colonies were measured after 14 days of incubation.

In order to determine the effect of the antifungal agent, benlate, on the strains, isolation media without benlate, as well as malt extract agar (Difco) without and with 0.02 g/l benlate, were also included in the experiment (Table 1).

3.2.3. Testing of isolation media.

The above mentioned isolation media were evaluated for their ability to selectively isolate mucoralean fungi from soil.The sampling site comprising an area of 10 m^2, is located in the Soetdoring Nature Reserve, on the Dry *Cymbopogon-Themeda* Veld (Acocks, 1988) within a summer rainfall region in the central Free State, South Africa. The mean annual rainfall is 564 mm, while the mean annual temperature is 16.1°C. The characteristics of the soil at the sampling site is summarized in Table 2.

The sampling took place in mid-summer. At the sampling site, the surface litter was first scraped away to reduce contamination from this habitat. A soil sample of 2900 g, comprising of 9 subsamples, was taken at random over the area of the site, each to a depth of 100 mm. The subsamples were thoroughly mixed in the laboratory to produce the sample which was then further processed.

By using the soil plate technique of Warcup (1950) the ability of isolation media A, B and C (each containing 0.02 g/l benlate) to isolate mucoralean fungi, was compared. For each medium, soil plates were prepared by transferring 0.005 g of soil from the sample to each of 10 sterile Petri dishes. Eight millilitres of cooled

able 1. The ability of mucoralean fungi to grow on malt extract agar, Isolation medium A, Isolation medium B and Isolation medium C, in the absence or in the presence of 0.02 g/l benlate.

	MEA -ben	MEA +ben	A -ben	A +ben	B -ben	B +ben	C -ben	C +ben
amily: ABSIDIACEAE								
enus: *Absidia*								
bsidia sp.	+++	+++	+++	+++	+++	+++	+++	+++
enus: *Rhizopus*								
. microsporus var. *chinensis* (Saito) Schippers & Stalpers CBS631.82	+++	+++	+++	+++	+++	+++	+++	+++
. microsporus Tiegh. var. *microsporus* PPRI5560	+++	+++	+++	+++	+++	+++	+++	+++
. oryzae Went & Prins. Geerl. CBS112.07T	+++	+++	+++	+++	+++	+++	+++	+++
. stolonifer (Ehrenb.: Fr.) Vuill. var. *stolonifer* CBS609.82, PPRI5167	+++	+++	+++	+++	+++	+++	+++	+++
. stolonifer var. *reflexus* (Bainier) Schipper CBS319.35	+++	+++	+++	+++	+++	+++	+++	+++
amily: MORTIERELLACEAE								
enus: *Mortierella*								
. acrotona W. Gams CBS386.71T	+++	-	+++	-	+++	-	-	-
. alpina Peyronel CBS219.35	+++	++	+++	++	+++	-	-	-
. alpina Peyronel CBS527.72, CBS529.27, ATCC3221	+++	+	+++	+	++	-	-	-
. alpina Peyronel CBS224.37	+++	-	+++	-	+++	-	-	-
. amoeboidea W. Gams CBS889.72T	+++	-	+++	-	+++	-	-	-
. angusta (Linnem.) Linnem. CBS293.61	+++	-	+++	-	+++	-	-	-
. antarctica Linnem. CBS609.70	+++	++	+++	+	+++	+	+	+
. bainieri Cost. CBS442.68	+++	-	+++	-	+++	-	-	-
. basiparvispora W. Gams & Grinbergs CBS518.72T	+++	+	++	+	+++	-	-	-
. chlamydospora (Chesters) Plaats-Nit. CBS120.34	+++	-	+++	-	+++	-	-	-
. claussenii Linnem. CBS294.59	+++	-	+++	-	+++	-	-	-
. clonocystis W. Gams CBS357.76T	+++	-	+++	-	+++	-	-	-

EA -ben = Malt extract agar (Difco); MEA +ben = Malt extract agar (Difco) containing benlate; A -ben = olation medium A containing starch as carbon source; A +ben = Isolation medium A containing starch as arbon source and benlate; B -ben = Isolation medium B containing sucrose as carbon source; B +ben = olation medium B containing sucrose as carbon source and benlate. C -ben = Isolation medium C containing odium acetate as carbon source. C +ben = Isolation medium C containing sodium acetate as carbon source nd benlate.

= no growth; + = growth: 5 - 40 mm diameter; ++ = growth: 40 - 75 mm diameter; +++ = growth: 75 - 80 mm iameter.

able 1. Continues.

	MEA -ben	MEA +ben	A -ben	A +ben	B -ben	B +ben	C -ben	C +ben
. decipiens (Thaxt.) Björl. CBS263.61	+++	-	+++	-	+++	-	-	-
. dichotoma Linnem. ex W. Gams BS221.35	+++	-	+++	-	+++	-	-	-
. gamsii Milko CBS 749.68T	+++	-	+++	-	+++	-	-	-
. globalpina W. Gams & Veenb.-Rijks BS718.88	+++	+	+++	+	+++	-	-	-
. indohii C.Y. Chien CBS720.71	+++	+	+++	+	+++	+	+++	+++
. isabellina Oudem. CBS208.32	++	++	++	++	++	+	+	+
. kuhlmanii W. Gams CBS157.71T	+++	-	+++	-	+++	-	-	-
. longicollis Dixon-Stew. CBS209.32	+++	+++	+++	+++	++	++	-	-
. minutissima Tiegh. CBS307.52	+++	-	+++	-	+++	-	-	-
. multidivaricata R. K. Benj. BS227.78T	+++	-	+++	-	+++	-	-	-
. mutabilis Linnem. CBS308.52	+++	-	+++	-	+++	-	-	-
. oligospora Björl. CBS650.68	+++	+	+++	+	+++	+	-	-
. parvispora Linnem. CBS304.52	+++	+	+++	+	+++	+	-	-
. polygonia W. Gams & Veenb.-Rijks BS685.71T	+++	-	+++	-	+++	-	-	-
. ramanniana (A. Möller) Linnem. ar. *ramanniana CBS*112.08	+++	+++	++	++	+	+	+	+
. ramanniana var. *autotrophica* . H. Evans CBS212.72	+++	+++	++	++	+	+	+	+
. reticulata Tiegh. & Le Monn. BS523.70	+++	-	+++	-	+++	-	-	-
. rishikesha B. S. & B. R. Mehrotra BS652.68T	+++	-	+++	-	+++	-	-	-
. schmuckeri Linnem. CBS295.59	+++	-	+++	-	+++	-	-	-
. sclerotiella Milko CBS529.68	+++	-	+++	-	+++	-	-	-
. sossauensis Wolf CBS898.68	+++	-	+++	-	+++	-	+	-
. turficola Y. Ling-Yong CBS430.76	+++	+	+++	+	+++	-	-	-
. umbellata C.Y. Chien CBS124.71	+++	+	+++	+	+++	-	-	-
. vinacea Dixon-Stew. CBS212.32	+++	++	++	++	+	+	+	+
. zychae Linnem. CBS316.52T	+++	+	+++	+	+++	-	-	-

IEA -ben = Malt extract agar (Difco); MEA +ben = Malt extract agar (Difco) containing benlate; A -ben = olation medium A containing starch as carbon source; A +ben = Isolation medium A containing starch as arbon source and benlate; B -ben = Isolation medium B containing sucrose as carbon source; B +ben = olation medium B containing sucrose as carbon source and benlate. C -ben = Isolation medium C containing odium acetate as carbon source. C +ben = Isolation medium C containing sodium acetate as carbon source nd benlate.

no growth; + = growth: 5 - 40 mm diameter; ++ = growth: 40 - 75 mm diameter; +++ = growth: 75 - 80 mm iameter.

able 1. Continues.

	MEA -ben	MEA +ben	A -ben	A +ben	B -ben	B +ben	C -ben	C +ben
amily: MUCORACEAE								
enus: ***Actinomucor***								
. *elegans* (Eidam) C. R. Benj. & Hesselt. CBS100.09, MUFS018, MUFS 193, MUFS194	+++	+++	+++	+++	++	++	+++	+++
. *elegans* (Eidam) C. R. Benj. & Hesselt. MUFS022, MUFS089, MUFS090	+++	+++	+++	+++	+++	+++	+++	+++
enus: ***Mucor***								
M. aligarensis B. S. & B. R. Mehrotra CBS993.70T	+++	+++	+++	+++	+++	+++	+++	+++
M. ardhlaengiktus B. S. Mehrotra & B. M. Mehrotra CBS210.80T	+++	+++	+++	+++	+++	+++	++	++
M. azygosporus R. K. Benj. CBS292.63T	+++	+++	+++	+++	+++	+++	+++	+++
M. bacilliformis (Hesselt.) CBS251.53	+++	+++	++	++	+++	+++	++	++
M. bainieri Mehrotra & Baijal CBS293.63T	+++	+++	++	++	+++	+++	+++	+++
M. circinelloides Tiegh. f. *circinelloides* CBS119.08, MUFS028, MUFS088	+++	+++	+++	++	+++	+++	+++	+++
M. circinelloides Tiegh. f. *circinelloides* MUFS027	+++	+++	++	+++	+++	+++	+++	+++
M. circinelloides Tiegh. f. *circinelloides* MUFS045, MUFS051	+++	+++	++	++	+++	+++	+++	+++
M. circinelloides Tiegh. f. *circinelloides* MUFS055	+++	+++	+++	+++	+++	+++	+++	+++
M. circinelloides f. *griseocyanus* (Hagem) Schipper CBS116.08, MUFS006, MUFS007, MUFS010, MUFS021, MUFS029, MUFS030, MUFS084	+++	+++	++	++	+++	+++	+++	+++
M. circinelloides f. *griseocyanus* (Hagem) Schipper MUFS001, MUFS004, MUFS005	+++	+++	+++	++	+++	+++	+++	+++
M. circinelloides f. *griseocyanus* (Hagem) Schipper MUFS040	+++	++	+++	+++	+++	+++	+++	+++
M. circinelloides f. *griseocyanus* (Hagem) Schipper MUFS052, MUFS067, MUFS103, MUFS104	+++	+++	+++	+++	+++	+++	+++	+++

MEA -ben = Malt extract agar (Difco); MEA +ben = Malt extract agar (Difco) containing benlate; A -ben = solation medium A containing starch as carbon source; A +ben = Isolation medium A containing starch as arbon source and benlate; B -ben = Isolation medium B containing sucrose as carbon source; B +ben = solation medium B containing sucrose as carbon source and benlate. C -ben = Isolation medium C containing odium acetate as carbon source. C +ben = Isolation medium C containing sodium acetate as carbon source nd benlate.

= no growth; + = growth: 5 - 40 mm diameter; ++ = growth: 40 - 75 mm diameter; +++ = growth: 75 - 80 mm iameter.

Table 1. Continues.

	MEA -ben	MEA +ben	A -ben	A +ben	B -ben	B +ben	C -ben	C +ben
M. circinelloides f. *griseocyanus* (Hagem) Schipper MUFS094	+++	+++	+++	+++	++	++	+++	+++
M. circinelloides f. *janssenii* (Lendn.) Schipper CBS232.29, MUFS108, MUFS116	+++	+++	++	++	+++	+++	+++	+++
M. circinelloides f. *janssenii* (Lendn.) Schipper MUFS013, MUFS024, MUFS056, MUFS102	+++	+++	+++	+++	+++	+++	+++	+++
M. circinelloides f. *janssenii* (Lendn.) Schipper MUFS017, MUFS046	+++	+++	+++	++	+++	+++	+++	+++
M. circinelloides f. *janssenii* (Lendn.) Schipper MUFS086	+++	+++	++	++	+++	++	+++	+++
M. circinelloides f. *lusitanicus* (Bruderlein) Schipper CBS108.17, MUFS043	+++	+++	++	++	+++	+++	+++	+++
M. circinelloides f. *lusitanicus* (Bruderlein) Schipper MUFS026	+++	+++	+++	+++	+++	+++	+++	+++
M. circinelloides f. *lusitanicus* (Bruderlein) Schipper MUFS031	+++	+++	+++	++	+++	+++	+++	+++
M. flavus Bainier CBS234.3587	+++	+++	++	+++	+++	+++	+++	+++
M. fragilis Bainier sensu Zycha CBS236.35	+++	+++	+++	+++	+++	+++	+++	+++
M. fuscus Bainier CBS132.22T	++	++	+	+	+++	+++	+++	+++
M. genevensis Lendner MUFS038	+++	+++	+++	+++	+++	+++	+++	+++
M. hiemalis f. *luteus* (Linnem.) Schipper CBS243.35	+++	+++	++	++	+++	+++	+++	+++
M. hiemalis f. silvaticus (Hagem) Schipper CBS249.35	+++	+++	+++	+++	+++	+++	++	++
M. indicus Lendn. CBS120.08	+++	+++	++	++	+++	+++	-	-
M. minutus (Baijal & B. S. Mehrotra) Schipper CBS586.67T	+++	+++	+++	+++	+++	+++	+++	+++
M. oblongiellipticus H. Nagan., Hirahara & Seshita ex Pidopl. & Milko CBS568.70T	+++	+++	+++	+++	+++	+++	+++	+++
M. plumbeus Bonord. CBS111.07, MUFS035 MUFS183	+++	+++	+++	+++	+++	+++	+++	+++
M. plumbeus Bonord. MUFS162, MUFS186	+++	+++	++	++	+++	+++	+++	+++
M. prayagensis B. S. Mehrotra & Nand ex Schipper CBS816.70	+++	+++	++	++	+++	+++	++	++
M. racemosus f. *chibinensis* MUFS009	+++	+++	++	++	+++	+++	+++	+++

MEA -ben = Malt extract agar (Difco); MEA +ben = Malt extract agar (Difco) containing benlate; A -ben = Isolation medium A containing starch as carbon source; A +ben = Isolation medium A containing starch as carbon source and benlate; B -ben = Isolation medium B containing sucrose as carbon source; B +ben = Isolation medium B containing sucrose as carbon source and benlate. C -ben = Isolation medium C containing sodium acetate as carbon source. C +ben = Isolation medium C containing sodium acetate as carbon source and benlate.

- = no growth; + = growth: 5 - 40 mm diameter; ++ = growth: 40 - 75 mm diameter; +++ = growth: 75 - 80 mm diameter.

able 1. Continues.

	MEA -ben	MEA +ben	A -ben	A +ben	B -ben	B +ben	C -ben	C +ben
M. racemosus f. *chibinensis* MUFS039	+++	+++	+++	+++	+++	+++	+++	+++
M. racemosus Fres. f. *racemosus* CBS113.08	+++	+++	+++	+++	+++	+++	+++	+++
M. racemosus f. *sphaerosporus* (Hagem) Schipper CBS115.08, MUFS014, MUFS015, MUFS020	+++	+++	+++	+++	+++	+++	+++	+++
M. racemosus f. *sphaerosporus* (Hagem) Schipper MUFS057	+++	+++	++	++	+++	+++	+++	+++
M. racemosus f. *sphaerosporus* (Hagem) Schipper MUFS111	+++	+++	+++	+++	++	++	+++	+++
M. recurvus E. E. Butler var. *recurvus* CBS317.52	+++	+++	++	++	+++	+++	+++	+++
M. recurvus var. *indicus* Baijal & Mehrotra CBS786.70	+++	+++	++	+++	++	++	+++	+++
M. rouxii (Calmette) Wehmer sensu Bartnicki-Garcìa CBS416.77	+++	+++	+++	+++	+++	+++	+++	+++
M. saturninus Hagem CBS137.40	+++	+++	+++	+++	+++	+++	+++	+++
M. sinensis Milko & Beljakova CBS204.74	+++	+++	++	++	+++	+++	+++	+++
M. subtilissimus Oudem. CBS735.70	+++	+++	+++	+++	+++	+++	+++	++
M. tuberculisporus Schipper CBS562.66T	+++	+++	++	++	+++	+++	+++	+++
M. ucrainicus Milko MUFS096	+++	+++	+++	++	+++	+++	+++	+++
Family: THAMNIDIACEAE								
Genus: *Backusella*								
Backusella lamprospora (Lendner) Benny & R.K. Benjamin MUFS002, MUFS008, MUFS012	+++	+++	+++	++	+++	+++	+++	+++
B. lamprospora (Lendner) Benny & R.K. Benjamin MUFS011	+++	++	+++	+++	+++	+++	+++	+++
Genus: *Thamnostylum*								
Thamnostyum piriforme (Bainier) von Arx & Upadhyay MUFS025	+++	+++	+++	+++	+++	+++	+++	+++
T. piriforme (Bainier) von Arx & Upadhyay MUFS053	+++	+++	++	++	++	++	+++	+++

MEA -ben = Malt extract agar (Difco); MEA +ben = Malt extract agar (Difco) containing benlate; A -ben = solation medium A containing starch as carbon source; A +ben = Isolation medium A containing starch as carbon source and benlate; B -ben = Isolation medium B containing sucrose as carbon source; B +ben = solation medium B containing sucrose as carbon source and benlate. C -ben = Isolation medium C containing sodium acetate as carbon source. C +ben = Isolation medium C containing sodium acetate as carbon source and benlate.

= no growth; + = growth: 5 - 40 mm diameter; ++ = growth: 40 - 75 mm diameter; +++ = growth: 75 - 80 mm diameter.

Table 2. Characteristics of soil at the sampling site.

Loamy soil	
Soil temperature at sampling depth	20.5°C
pH of soil *	6.38
Soil moisture content •	13.10% ± 0.17%
Organic matter content Δ	2.92% ± 0.09%

• Determined according to the method of Spotts and Cervantes (1986).
Δ Determined according to the method of Eicker (1970). • Determined according to the method of Eicker (1970).

molten isolation medium was then added to each Petri dish. The cultures were incubated at 25°C in the dark and observed for growth. In order to determine the selectivity of the isolation media, soil plates prepared with a relatively non-selective medium among members of the fungal domain (Carreiro & Koske, 1992), were also included in the experiments. This medium (MYPps), contained (g/l): Malt extract (Difco), 7.00; Peptone (Oxoid), 1.00; Yeast extract (Difco), 0.50; Penicillin G, 0.50; Streptomycin sulphate, 0.50 and agar, 16.00.

In order to confirm the superiority of the soil plate technique above that of the dilution plate method (Warcup, 1960) in isolating mucoralean fungi, the latter method was also employed in this study. A dilution series (1:10; 1:100; 1:1000 and 1:5000) was prepared in sterile physiological salt solution. The 1:5000 dilution was then used as inoculum for plates containing MYPps medium. The cultures were incubated at 25°C in the dark and observed for growth.

In all of the above cases, growth from each developing colony on the isolation plates, was transferred to fresh malt extract agar, Difco (MEA) and incubated at 25°C. Further purification was obtained by successive inoculation and incubation of each isolate on MEA.

3.2.4. Identification.

The fungal isolates belonging to the Dikaryomycota were identified up to genus level using the keys of Ellis (1976), Kreger-van Rij (1984) and Baxter et al. (1994). The mucoralean fungi were identified using the key and description given by Hesseltine & Ellis (1964), Ellis & Hesseltine (1965), Gams (1977), Schipper (1973, 1975, 1976, 1978 and 1984) and Lunn & Shipton (1983).

3.3. Results and discussion

3.3.1. Selectivity of isolation media.

The results on the determination of the selectivity of the three isolation media within Mucorales, are summarized in Table 1. It can be seen that the three isolation media supported growth of the representatives of the genera *Absidia*, *Actinomucor*, *Backusella, Mucor*, *Rhizopus* and *Thamnostylum*, regardless of the presence of 0.02 g/l benlate in the medium. Interestingly, growth of most representatives of the genus *Mortierella*, was inhibited by the presence of 0.02 g/l benlate in the medium. This is clear from the results obtained when the strains were inoculated onto MEA, Isolation medium A and Isolation medium B, and observed for growth (Table 1).

In accordance with the results of Botha et al. (1995), who found that an isolation medium containing 20 g/l acetate as carbon source could not support growth of *Mortierella* species, few of the strains representing this genus could grow on Isolation medium C, which contained 10 g/l acetate.

The results therefore indicate that all three isolation media containing 0.02 g/l benlate, are selective for mucoralean fungi belonging to the genera *Absidia*, *Actinomucor*, *Backusella, Mucor*, *Rhizopus* and *Thamnostylum*.

3.3.2. Testing of isolation media.

The ability of the isolation media and techniques used to isolate mucoralean fungi from soil, are summarized in Table 3. Included is the results obtained when a relatively non-selective medium (MYPps) is employed to isolate fungi using the soil plate technique. It can be seen that 20 % of the total colony forming units per gram (CFU/g) soil obtained in this manner represented Mucorales. In contrast, when this same medium was used utilizing the dilution plate technique, less than three percent of the total CFU/g soil, was found to be mucoralean fungi. This is in accordance with the view of Warcup (1960), who found that the dilution plate

Table 3. Fungi isolated from the soil of the Dry *Cymbopogon-Themeda* Veld.

Fungi	MYPps (CFU/g soil) dilution plate	MYPps (CFU/g soil) soil plate	Med. A (CFU/g soil)	Med. B (CFU/g soil)	Med. C (CFU/g soil)
Dikaryomycota					
Acladium	600	1400	-	-	-
Acremonium	1200	2200	-	-	-
Alternaria	-	-	-	200	-
Aspergillus	100	600	-	-	-
Candida	-	-	-	400	-
Cladosporium	600	-	-	200	-
Culcitalna	200	-	-	-	-
Curvularia	-	-	1200	2200	-
Cylindrocarpon	-	-	-	200	-
Drechslera	-	-	400	200	-
Epicoccum	-	200	-	-	-
Fusarium	200	-	-	-	-
Gonatobotrys	-	200	-	-	-
Humicola	600	1400	-	-	-
Paecilomyces	2200	1000	600	1000	-
Penicillium	24000	9400	-	-	-
Periconia	3400	600	-	-	-
Rhodotorula	-	200	-	400	-
Scytalidium	400	600	-	200	-
Sporothrix	800	800	-	-	-
Trichoderma	2000	16800	-	-	-
Trichosporon	-	-	-	200	-
Basidiomycetous black yeast	-	-	-	200	-
TOTAL	37200	35400	2200	5400	-
Zygomycota					
Absidia	-	-	-	200	-
Cunninghamella	-	-	2000	-	-
Gongronella	-	-	-	400	-
Mortierella	600	8600	-	-	-
Mucor	-	200	-	-	200
Rhizopus	200	-	1200	1400	-
TOTAL	800	8800	3200	2000	200

CFU: Colony forming units; MYPps: Relative non-selective medium (Carreiro & Koske, 1992); Med. A: Isolation medium A containing starch as carbon source and 0.02 g/l benlate; Med. B: Isolation medium B containing sucrose as carbon source and 0.02 g/l benlate; Med. C: Isolation medium C containing sodium acetate as carbon source and 0.02 g/l benlate.

technique is more selective for fungi sporulating abundantly (e.g. *Penicillium* species). The soil plate method is more selective for fungi present as hyphae or chlamydospores in soil. Interestingly, it is known that the viable cells of *Mucor rammanianus* in soil, are mostly chlamydospores, not sporangiospores (Parkinson & Waid, 1960).

By using the soil plate technique, Isolation medium C was found to be the most selective for mucoralean fungi (Table 3). All the fungi which were able to grow on the medium, were representatives of the mucoralean genus *Mucor*. The second most selective medium for mucoralean fungi was found to be Isolation medium A. Sixty percent of the total CFU/g soil was found to be representatives of *Cunninghamella* and *Rhizopus*. The rest were dikaryomycotan fungi. Of the three selective media tested, the least selective was Isolation medium B. Only 27 % of the total CFU/g was found to be representatives of Mucorales. However, the mucoralean isolates obtained on this medium represented more genera (i.e. *Absidia*, *Gongronella* and *Rhizopus*) than on the other two isolation media. The species which the isolates obtained on media A, B and C represent, are listed in Table 4.

In accordance with results obtained during the determination of the selectivity of the three isolation media (Table 1), no isolates representing *Mortierella* grew on the isolation media (Table 3). However, 19 % of the total CFU/g soil obtained with the MYPps-medium using the soil plate technique was found to be representatives of *Mortierella*.

3.3.3. Ecological observations.

In order to asses the validity of the results obtained with the dilution plate method and the soil plate method utilizing the MYPps medium, the results were compared to the data generated with similar ecological studies done in southern Africa. Comparing the fungal genera identified in this study to the genera identified in other veld types in southern Africa, an interesting pattern emerges (Fig. 1).

Table 4. Mucoralean species obtained from the soil sample using the selective isolation media.

Species	A (CFU/g soil)	B (CFU/g soil)	C (CFU/g soil)
Absidia californica	-	200	-
Cunninghamella echinulata	2000	-	-
Gongronella butleri	-	400	-
Mucor circinelloides	-	-	200
Rhizopus microsporus	1200	1400	-

A: Isolation medium A containing starch as carbon source and 0.02 g/l benlate; B: Isolation medium B containing sucrose as carbon source and 0.02 g/l benlate; C: Isolation medium C containing sodium acetate as carbon source and 0.02 g/l benlate; CFU: colony forming units.

Sourish Open Savannah Veld (Eicker, 1976)

Type of vegetation: Grass, shrubs and trees

Dikaryomycotan genera detected:
1, 3, 5, 6, 7, 8, 9, 15, 16, 17, 18, 19, 20, 21, 24, 26, 29, 32, 35, 36, 37, 38, 41, 43, 45, 46, 47, 49, 50, 51, 52, 53, 55, 58, 64, 68, 70, 71, 72, 73,74, 76, 77

Oomycotan genera detected:
78

Zygomycotan genera detected:
79, 80, 81, 82, 83, 84,86

Percentage similarity with the results of the present study regarding the presence or absence of the genera listed in the legend:

43 %

Springbok Flats (Opperman & Wehner, 1994)

Type of vegetation: Grass and thorny trees

Dikaryomycotan genera detected:
4, 10, 17, 23, 25, 26, 27, 29, 30, 32, 33, 38, 39,40, 42, 44, 48, 49, 51, 52, 54, 55, 56, 57, 59, 61, 67, 70, 71, 76

Oomycotan genera detected:
-

Zygomycotan genera detected:
82

Percentage similarity with the results of the present study regarding the presence or absence of the genera listed in the legend:

61 %

Coastal forest and thornveld (Eicker, 1969)

Type of vegetation: Grass, herbs, shrubs and trees

Dikaryomycotan genera detected:
5, 6, 11, 12, 13, 14, 15, 17, 26, 28, 29, 38, 43, 46, 49, 51, 53, 54, 63, 64, 66, 74, 75, 76

Oomycotan genera detected:
-

Zygomycotan genera detected:
81, 82, 83, 84, 85, 86

Percentage similarity with the results of the present study regarding the presence or absence of the genera listed in the legend:

69 %

Grassland of the Giribes Plains (Eicker et al., 1982)

Type of vegetation: Grass only

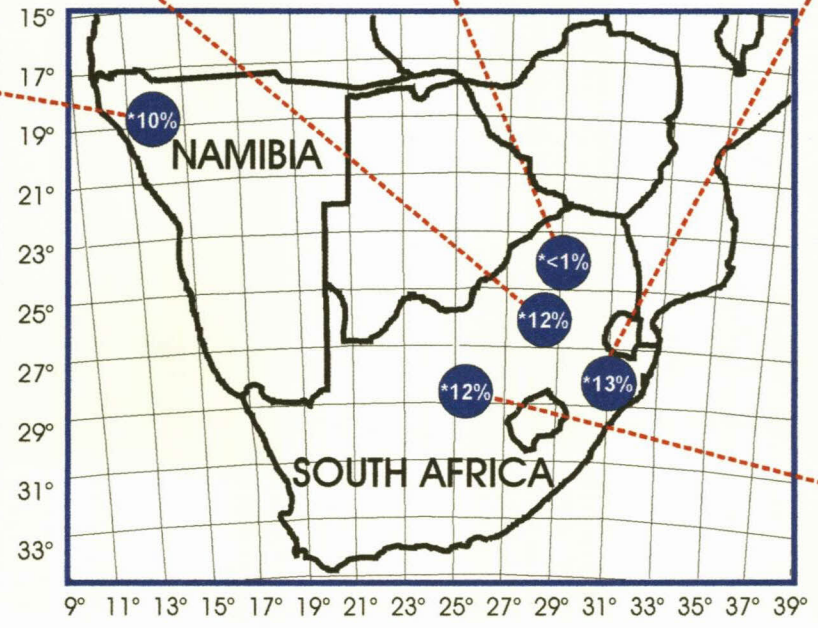
Dikaryomycotan genera detected:
4, 5, 6, 7, 17, 25, 26, 29, 31, 32, 38, 49, 50, 51, 52, 55, 58, 59, 70, 74

Oomycotan genera detected:
-

Zygomycotan genera detected:
79, 81, 85

Percentage similarity with the results of the present study regarding the presence or absence of the genera listed in the legend:

77 %



Dry *Cymbopogon-Themeda* Veld (This study)

Type of vegetation: Grass only

Dikaryomycotan genera detected:
2, 4, 6, 17, 22, 26, 29, 34, 38, 49, 51,52, 62, 65, 69, 74

Oomycotan genera detected:
-

Zygomycotan genera detected:
83, 84, 85

Percentage similarity with the results of the present study regarding the presence or absence of the genera listed in the legend:

100 %

Fig. 1

Fig. 1. A comparison of the fungal genera identified in five ecological surveys of soils from different veld types (Acocks, 1988) in southern Africa.

Dikaryomycotan genera:
1: *Achaetomiella*, 2: *Acladium*, 3: *Acremoniella*, 4: *Acremonium*, 5: *Alternaria*, 6: *Aspergillus*, 7: *Aureobasidium*, 8: *Auxarthron*, 9: *Beauveria*, 10: *Bipolaris*, 11: *Bispora*, 12: *Botryotrichum*, 13: *Candida*, 14: *Cephalosporium*, 15: *Chaetomium*, 16: *Chaetomidium*, 17: *Cladosporium*, 18: *Cochliobolus*, 19: *Coniella*, 20: *Coniothyrium*, 21: *Corticium*, 22: *Culcitalna*, 23: *Cylindrocarpon*, 24: *Doratomyces*, 25: *Dreschlera*, 26: *Epicoccum*, 27: *Exserohilum*, 28: *Fumago*, 29: *Fusarium*, 30: *Fusicoccum*, 31: *Geotrichum*, 32: *Gliocladium*, 33: *Gliomastix*, 34: *Gonatobotrys*, 35: *Gonytrichum*, 36: *Graphium*, 37: *Helminthosporium*, 38: *Humicola*, 39: *Idriella*, 40: *Macrophomina*, 41: *Mammaria*, 42: *Microsphaeropsis*, 43: *Monilia*, 44: *Myrothecium*, 45: *Nectria*, 46: *Neocosmospora*, 47: *Neurospora*, 48: *Nigrospora*, 49: *Paecilomyces*, 50: *Papulaspora*, 51: *Penicillium*, 52: *Periconia*, 53: *Pestalotia*, 54: *Phialophora*, 55: *Phoma*, 56: *Phomopsis*, 57: *Pithomyces*, 58: *Pleospora*, 59: *Pyrenochaeta*, 60: *Rhinocladiella*, 61: *Rhizoctonia*, 62: *Rhodotorula*, 63: *Sclerophoma*, 64: *Scopulariopsis*, 65: *Scytalidium*, 66: *Septonema*, 67: *Spegazzinia*, 68: *Spicaria*, 69: *Sporothrix*, 70: *Stachybotrys*, 71: *Stagonospora*, 72: *Thielavia*, 73: *Trichocladium*, 74: *Trichoderma*, 75: *Trichosporon*, 76: *Verticillium*, 77: *Volutella*

Oomycotan genera:
78: *Saprolegnia*

Zygomycotan genera:
79: *Absidia*, 80: *Actinomucor*, 81: *Cunninghamella*, 82: *Gongronella*, 83: *Mortierella*, 84: *Mucor*, 85: *Rhizopus*, 86: *Zygorhynchus*

*Percentage of total isolates on non-selective media representing Mucorales.

Regarding the presence or absence of the fungal genera listed, the highest similarity was obtained when comparing the results of this study with that of a survey conducted on another type of grassveld by Eicker (1982). A 78 % similarity was obtained with results of these authors on the genera present in soil of grassveld between bare patches on the Giribes plains in Namibia. Interestingly, the percentage similarity decreased when the results of this study were compared with data obtained in ecological surveys on soil from veld types which consist of grass and other types of vegetation, such as shrubs and trees (Fig. 1). However, representatives of the genera *Cladosporium*, *Epicoccum*, *Fusarium*, *Humicola*, *Paecilomyces* and *Penicillium* were found during all five ecological surveys depicted in Figure 1. This is not surprising, since it is known that members of these genera occur commonly in soils (Gilman, 1959; Waid, 1960; Eicker, 1970, Eicker, 1974; Carreiro & Koske, 1992).

In all surveys except one (Fig. 1), the percentage isolates representing Mucorales compared to the total number of isolates, ranged from 10 % to 13 %. The exception being the survey of Opperman & Wehner (1994) who sampled the soil and root fungi of vegetation on the Springbok flats and found that mucoralean fungi comprises less than one percent of the total isolates. This discrepancy may be ascribed to the fact that unlike in other surveys where the soil plate and dilution plate techniques were used, in the study of Opperman & Wehner (1994), fungi present on the roots of vegetation were used to inoculate isolation plates. According to Waid (1960), mucoralean fungi are abundant on the roots of *Lolium perenne*, however this grass type was not examined by Opperman & Wehner (1994).

It is important to note that by using selective media to study the fungal population in the soil samples of this study, the percentage mucoralean fungi in relation to the total number of fungal isolates increased from 12 %, obtained on the MYPps-

medium, to 16%, obtained on MYPps-medium as well as on the three selective media (Table 3).

3.4. Conclusions

Selective media, with starch, sucrose or acetate as carbon source were used to isolate mucoralean fungi representing the genera *Absidia*, *Cunninghamella*, *Gongronella*, *Mucor* and *Rhizopus*.

When using only a relative non-selective medium for fungal isolation, the fungal genera obtained in this study corresponded with the genera obtained by others in southern Africa while surveying similar habitats. It was found that in surveys of different veld types, the percentage of mucoralean isolates, compared to total number of isolates, ranged from 10 % to 13 %, when isolation techniques such as the soil plate method and the dilution plate method were imployed.

No representatives of the genera *Absidia*, *Cunninghamella* or *Gongronella* were detected in the soil samples when only non-selective media were used as isolation media. However, isolates representing these genera were obtained on the selective media with starch or sucrose as carbon source.

Members of the genus *Mortierella* were found to be sensitive towards 0.02 g/l benlate in the isolation medium. No isolates representing this genus, therefore, were obtained when the selective media were used as isolation media.

However, the results of this study indicate that by using selective media for mucoralean fungi, more isolates representing this fungal group, as well as more taxa can be obtained from soil. In Chapter 4, the mucoralean isolates obtained in this study, were screened for growth and 18:3(ω6) production on glucose, sucrose and starch carbon sources.

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CHAPTER 4

EVALUATION OF MUCORALEAN ISOLATES FOR GROWTH AND GAMMA-LINOLENIC ACID PRODUCTION

4.1. Introduction

Mucoralean fungi are known producers of the high value polyunsaturated fatty acid (PUFA), gamma-linolenic acid or 18:3(ω6) (Lösel, 1989; Van der Westhuizen et al., 1994). Recently, isolation media were developed to isolate these fungi from among a natural occurring fungal population in soil (Chapter 3). Four media were used for this purpose, containing either acetic acid, maltose, sucrose or starch as carbon source.

Some of these carbon sources were identified as industrially significant since they are associated with the effluents of various industries, ranging from agricultural to petrochemical industries (Israilides et al., 1994; Kock & Botha, 1995). Utilization of these carbon sources in the biotechnological production of 18:3(ω6), may therefore be advantageous for the economy of such a process. However, it has been shown that different carbon sources may influence 18:3(ω6) accumulation in Mucorales and other fungi (Chapter 2; Sajbidor et al., 1988; Lindberg & Hansson, 1991; Kendrick & Ratledge, 1992; Roux et al., 1994; Du Preez et al., 1995; Pohl, 1996).

Consequently, the purpose of this study, was to examine a number of the above mentioned isolates representing different families in Mucorales that were isolated on different media, for growth and 18:3(ω6) production in liquid media containing glucose, sucrose or starch as carbon source. This was done to confirm that representative isolates of all the families can produce of 18:3(ω6) in liquid media containing the different carbon sources. In addition, lipid metabolism regarding 18:3(ω6), would also be examined in representatives of various taxa within Mucorales.

4.2. Materials and methods

4.2.1. Isolates used.

Mucoralean fungal isolates, isolated from the Dry *Cymbopogon-Themeda* Veld (Acocks, 1988) as explained in Chapter 3, were used in this study (Table 1). The isolates are held in the Mucoralean Fungal Culture Collection (MUFS) at the University of the Orange Free State.

4.2.2. Changes in biomass and lipid content during growth.

In order to determine an appropriate incubation period before harvesting and lipid analyses, it was first necessary to determine the biomass, the lipid content and volumetric 18:3(ω6) concentration during growth in isolates representing each family listed in Table 1. Three isolates, *Cunninghamella echinulata* MUFSCu002 (Cunninghamellaceae), *Mucor circinelloides* MUFS244 (Mucoraceae) and *Rhizopus microsporus* MUFSR009 (Absidiaceae), were subsequently each investigated for biomass and lipid content and composition during growth in three different media.

4.2.2.1. Medium preparation. To stimulate lipid accumulation (Ratledge, 1989), including 18:3(ω6), an amount of carbon source was included in each medium which resulted in a final C:N ratio of *circa* 50:1 (w/w). Each of the three media used in this study, contained either glucose, sucrose or starch as carbon source. Medium A contained (g/l): glucose, 50.00; NH_4Cl, 1.80; yeast extract, 0.10; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.00; $CaCl_2.2H_2O$, 0.05. Medium B contained (g/l): sucrose, 50.00; NH_4Cl, 1.87; yeast extract, 0.10; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.00; $CaCl_2.2H_2O$, 0.05. Medium C contained (g/l): starch, 50.00; NH_4Cl, 1.98; yeast extract, 0.10; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.00; $CaCl_2.2H_2O$, 0.05. The three media each had a pH of 5.8.

4.2.2.2. Culture conditions. For each of the above mentioned three isolates, the following inoculation and cultivation procedure was performed: The fungal isolate was inoculated into all three of the above mentioned media, contained in 100 ml quantities in 1000 ml conical flasks. Two loopfulls of one week old

Table 1. The mucoralean isolates screened for growth and 18:3(ω6) production in this study. The families to which these isolates belong, as well as the isolation media and carbon sources on which these isolates were originally isolated on, are also included.

Isolates	Families	Isolation media and carbon sources	
Absidia californica (MUFS200)	Absidiaceae	Sucrose	*
Gongronella butleri (MUFSGo002)	Absidiaceae	Sucrose	*
Rhizopus microsporus (MUFSR012)	Absidiaceae	Starch	Δ
R. microsporus (MUFSR009)	Absidiaceae	Starch	Δ
R. microsporus (MUFSR010)	Absidiaceae	Sucrose	*
R. stolonifer (MUFSR012)	Absidiaceae	Maltose	•
Cunninghamella echinulata (MUFSCu003)	Cunninghamellaceae	Starch	Δ
C. echinulata (MUFSCu002)	Cunninghamellaceae	Starch	Δ
C. echinulata (MUFSCu001)	Cunninghamellaceae	Starch	Δ
Mucor circinelloides (MUFS244)	Mucoraceae	Maltose	•
M. circinelloides (MUFS243)	Mucoraceae	Acetate	□

* Composition of isolation medium (g/l): sucrose, 10.00; NH_4Cl, 1.00; KH_2PO_4, 1.00; $MgSO_4.7H_2O$, 0.50; yeast extract (Difco), 0.50 and chloramphenicol, 0.20; mineral salts (mg/l): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium which had a pH of 5.5 and was solidified with 16 g/l agar, also contained 0.02 g/l benlate (Aldrich).

Δ Isolation medium had an identical composition than isolation medium *, except it contained 10 g/l starch as carbon source instead of sucrose.

• Medium contained (g/l): Malt extract (Difco), 7.00; Peptone (Oxoid), 1.00; Yeast extract (Difco), 0.50; Penicillin G, 0.50; Streptomycin sulphate, 0.50 and agar, 16.00.

□ Isolation medium contained (g/l): sodium acetate, 10.00; NH_4Cl, 1.00; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.25; yeast extract (Difco), 0.5 and $CaCl_2$, 0.05. It also contained (mg/l): $FeSO_4.7H_2O$, 10.00; $ZnSO_4.7H_2O$, 10.00; $MnSO_4.H_2O$, 0.80 and $CuSO_4.5H_2O$, 0.05. The medium had a pH of 5.5, was solidified with 16 g/l agar and also contained 0.02 g/l benlate.

hyphal growth on malt extract agar (Difco), was used to inoculate each 100 ml of medium. The cultures were incubated at 30°C on a rotary shaker (160 r.p.m.; throw = 50 mm). The biomass was periodically harvested by filtration (Whatman No. 1), washed with distilled water, freeze-dried and weighed. All experiments were performed at least in triplicate.

4.2.2.3. Lipid extraction and fatty acid analyses. Lipids were extracted from the freeze-dried biomass as described by Kendrick & Ratledge (1992). This included 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. The lipids were dissolved in diethyl ether and transferred to preweighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 55°C. The extracted lipids were dissolved in chloroform and applied to a collumn (140 mm x 20 mm) of activated silicic acid. Neutral, glyco- and phospholipids were eluted by the successive addition of 1-,1-,1- trichloroethane, acetone and methanol (Kendrick & Ratledge, 1992). The different lipid fractions were dissolved in chloroform and methylated by the addition of trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph and a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 ml / min) as carrier gas (Kock, 1988). Peaks representing methylated fatty acids were identified using authentic standards.

4.2.3. Screening for 18:3(ω6) production.

The isolates, listed in Table 1, were screened for biomass production, lipid content and volumetric concentration of 18:3(ω6) reached, after seven days of incubation in three different media containing glucose, sucrose or starch as carbon source.

4.2.3.1. Medium preparation and culture conditions. The same three media containing glucose, sucrose or starch as carbon sources were prepared as

described under "Medium preparation" (4.2.2.1.). The inoculation of these media with the fungal strains and the subsequent cultivation of the cultures, were identical to the procedure explained under "Culture conditions" (4.2.2.2.). However, the cultures were only harvested once, after seven days of incubation. Harvesting was accomplished by filtration of the biomass (Whatman No. 1). This was followed by washing the biomass with distilled water, freeze-drying and weighing it. All experiments were performed at least in triplicate.

4.2.3.2. Lipid analyses. Lipids were extracted and analysed as explained under "Lipid extraction and fatty acid analyses" (4.2.2.3.).

4.3. Results and discussion

4.3.1. Changes in biomass and lipid content during growth.

Figures 1, 2 and 3 depicts the results obtained when biomass, total lipids (% w/w) and volumetric 18:3(ω6) concentration, in the neutral lipid fraction, were monitored during growth of *C. echinulata* MUFSCu002, *M. circinelloides* MUFS244 and *R. microsporus* MUFSR009 in the three media containing glucose, sucrose or starch as carbon source. From these results (Figures 1, 2 and 3) it can be seen for each isolate, the biomass, total lipids and volumetric 18:3(ω6) concentrations differed significantly during growth in media containing different carbon sources. Nevertheless, stationary phase has been reached by all three isolates in all three media after six to eight days of incubation.

Interestingly, by this time of incubation, the percentage total lipids in the biomass (w/w), have already peaked in *M. circinelloides* MUFS244 in media containing glucose or sucrose. With *C. echinulata* MUFSCu002 grown on glucose, *M. circinelloides* MUFS244 grown on starch and with *R. microsporus* MUFSR009 grown on starch as carbon source, a plateau was reached in lipid accumulation after about four days of incubation. The percentage lipids in the other cultures, increased towards the end of incubation. No significant changes in volumetric 18:3(ω6) concentration occurred after six days of incubation. The only

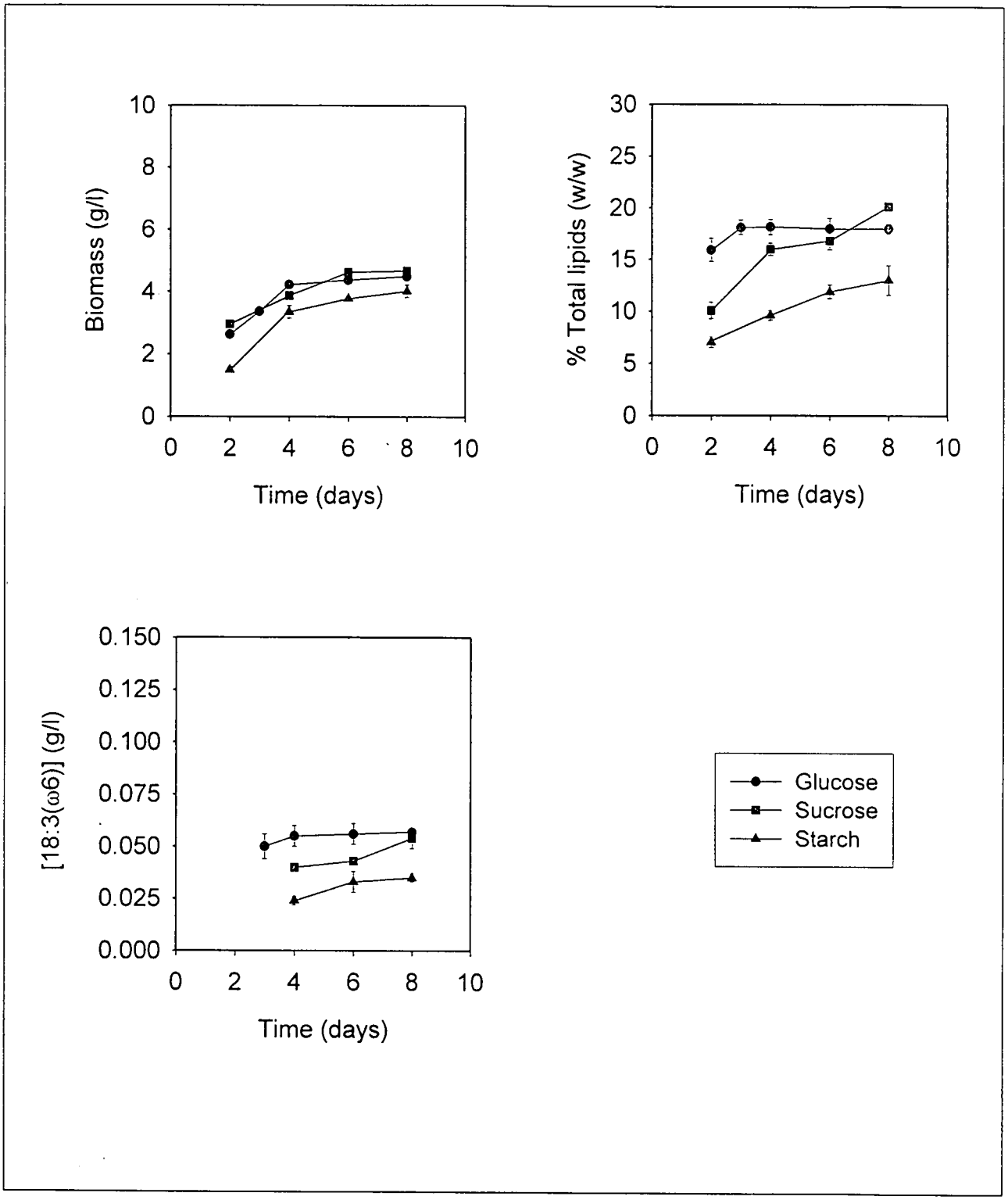


Fig. 1. Biomass, total lipids and gamma-linolenic acid concentration obtained during growth of *Cunninghamella echinulata* MUFSCu002 in media containing glucose, sucrose or starch as carbon source.

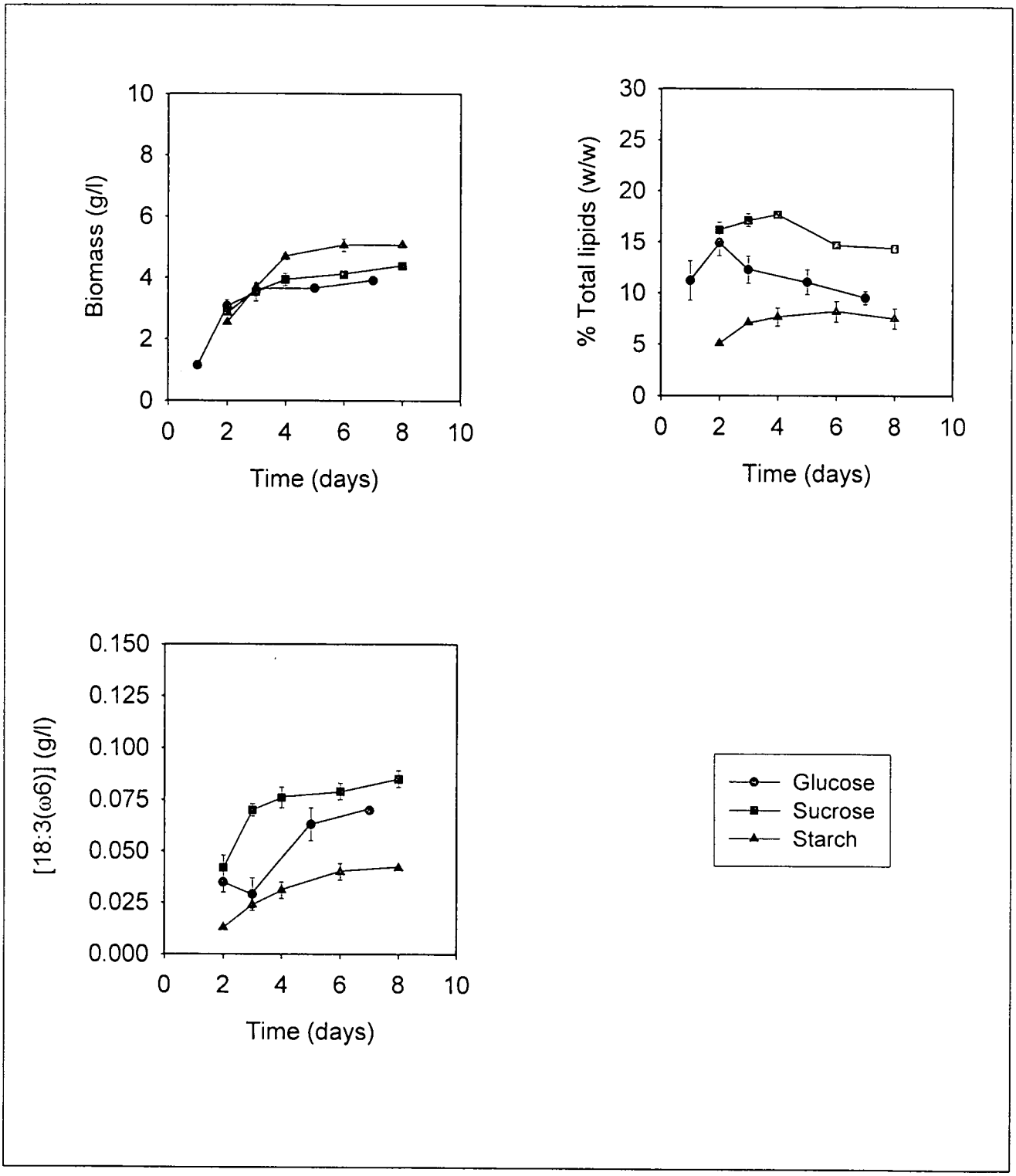


Fig. 2. Biomass, total lipids and gamma-linolenic acid concentration obtained during growth of *Mucor circinelloides* MUFS244 in media containing glucose, sucrose or starch as carbon source.

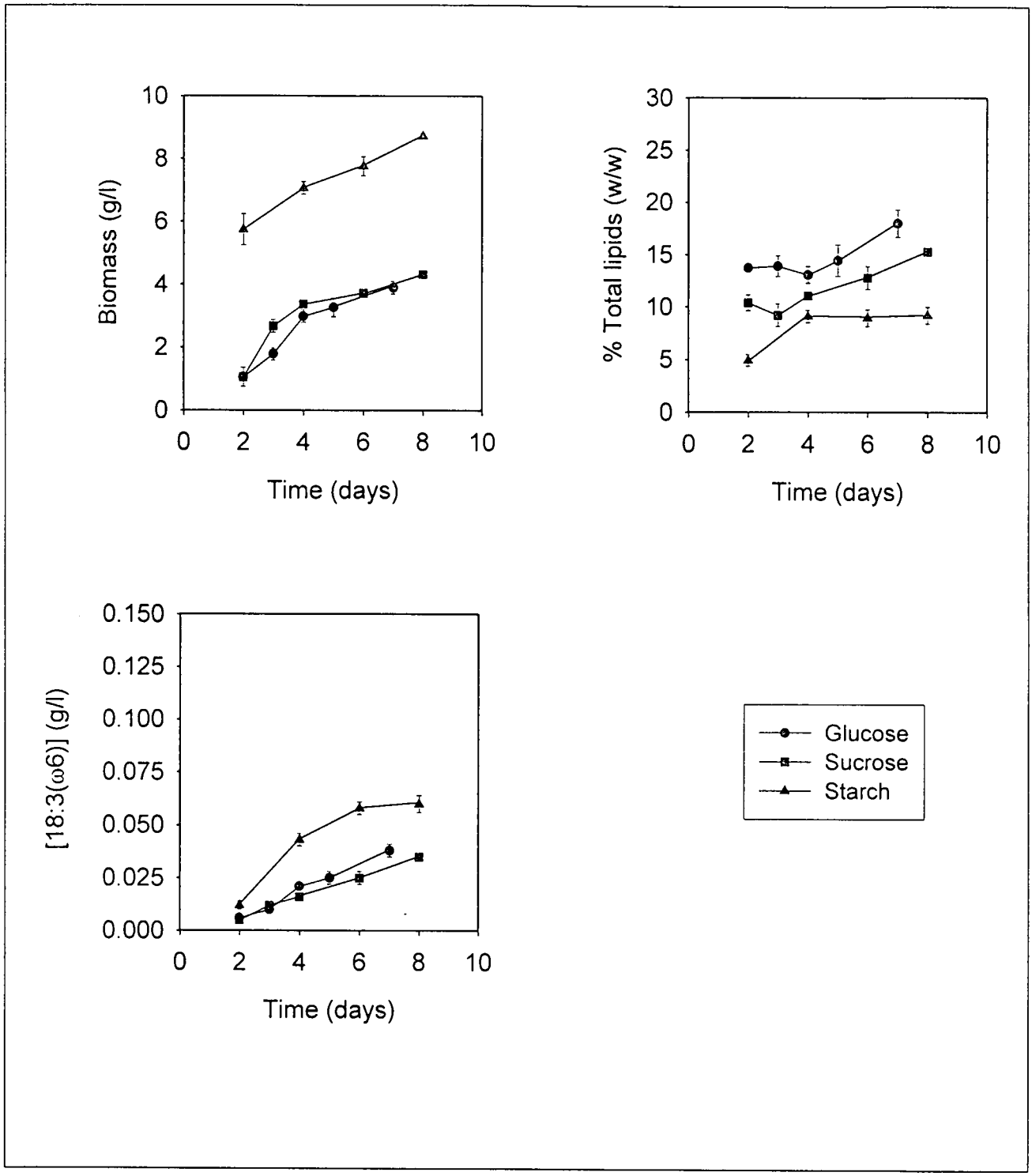


Fig. 3. Biomass, total lipids and gamma-linolenic acid concentration obtained during growth of *Rhizopus microsporus* MUFSR009 in media containing glucose, sucrose or starch as carbon source.

exceptions were *C. echinulata* MUFSCu002 grown on sucrose, and cultures of *R. microsporus* MUFSR009 grown in media containing glucose or sucrose as carbon source. In these cultures the volumetric concentration of 18:3(ω6) increased up until the end of the incubation period. Considering all of the above results, it was decided to harvest the cultures during the screening process, in the stationary phase after seven days of incubation.

4.3.2. Screening for 18:3(ω6) production.

The results obtained when the isolates listed in Table 1 were screened for 18:3(ω6) production, in the neutral lipid fraction, on glucose, sucrose or starch as carbon source after seven days of incubation, are depicted in Figure 4. Also included in this figure, is the biomass and the percentage total lipid (w/w) in the biomass obtained. In addition, the volumetric concentration of neutral lipid (g/l) reached, was also calculated for each isolate and carbon source investigated.

With the exception of *M. circinelloides* MUFS243 and *R. stolonifer* MUFSR008, all the isolates tested showed strong growth on all three carbon sources tested (Figure 4a). However, for each isolate, the biomass obtained on starch was significant higher than that obtained on glucose or sucrose. The exception in this case was the results obtained with *C. echinulata* MUFSCu002. The most biomass (12.50 g/l) was obtained with *C. echinulata* MUFSCu003 on starch as carbon source, while the least biomass (0.18 g/l) was obtained with *R. stolonifer* MUFSR008 on sucrose as carbon source.

When comparing the percentage total lipid (w/w) reached on the three different carbon sources for each isolate (Figure 4b), it is clear for all the tested isolates, the least lipid per cell was produced on starch as carbon source. While more lipid per cell was accumulated with glucose and/or sucrose as carbon source. The higher percentages lipid (32.84 and 42.44, w/w) were obtained with *G. butleri* MUFSGo002 and *R. stolonifer* MUFSR008 on respectively glucose and sucrose as carbon sources. However, it must be kept in mind that less than 0.2 g/l biomass was obtained with *R. stolonifer* on sucrose as carbon source.

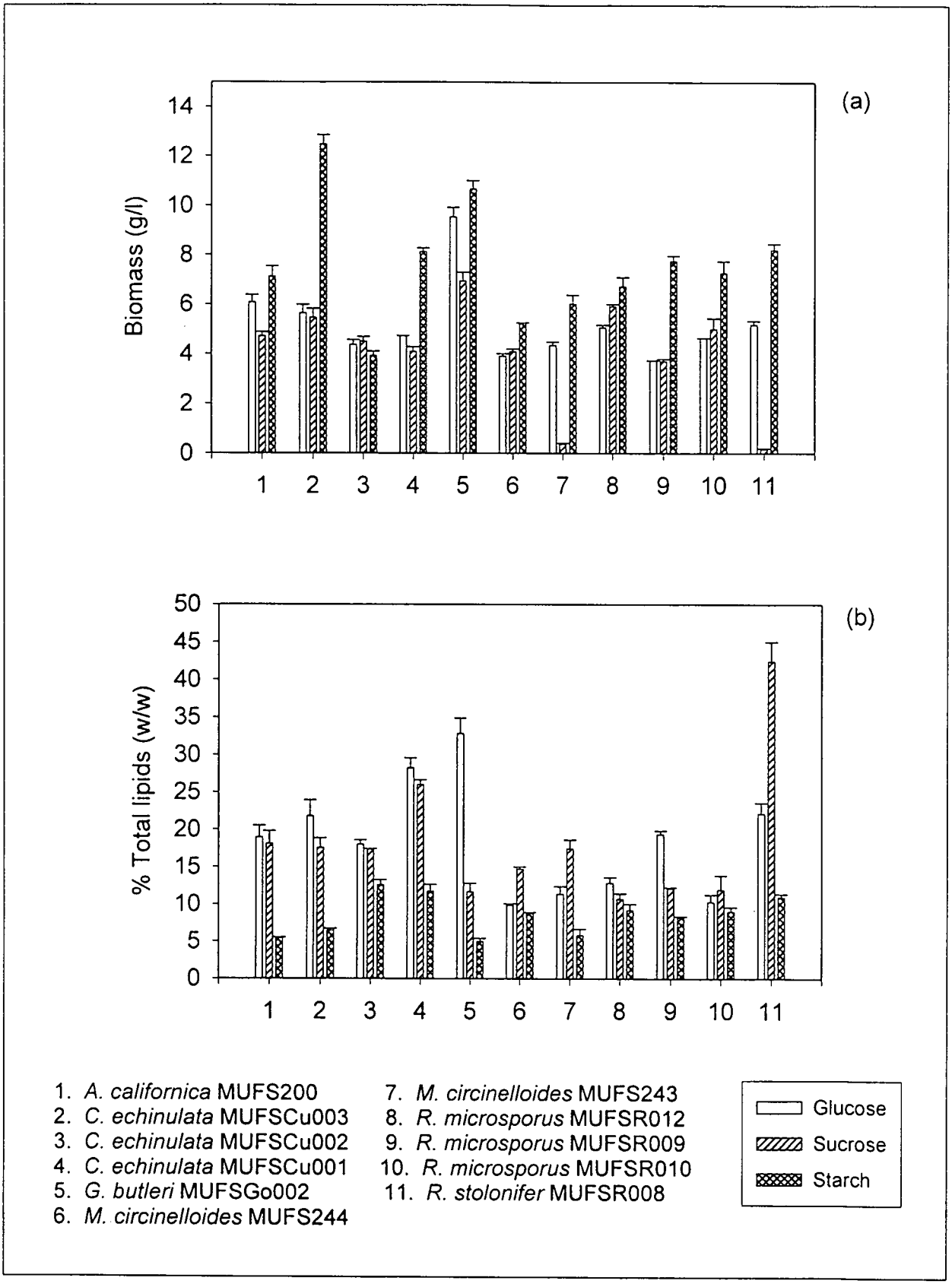
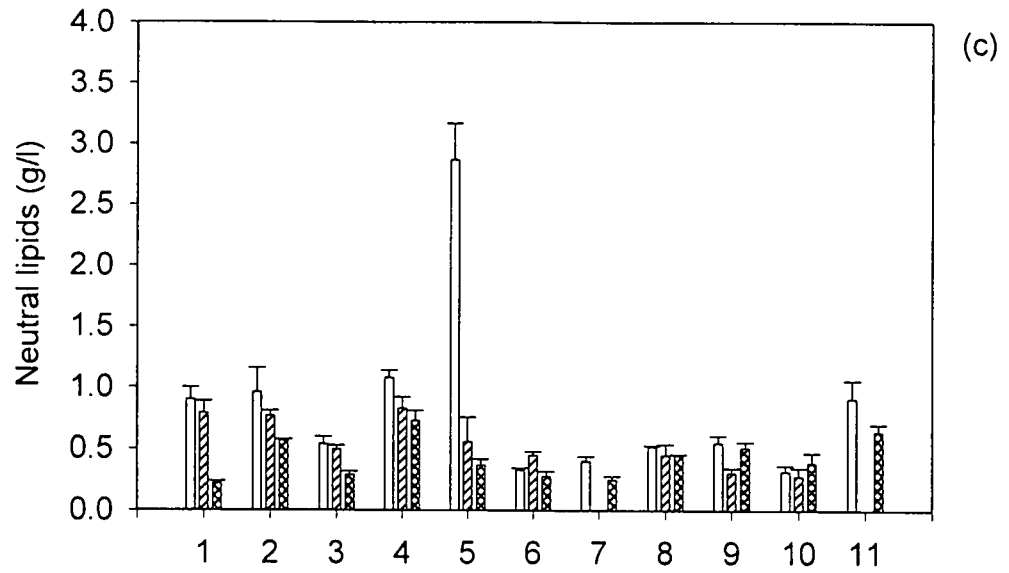
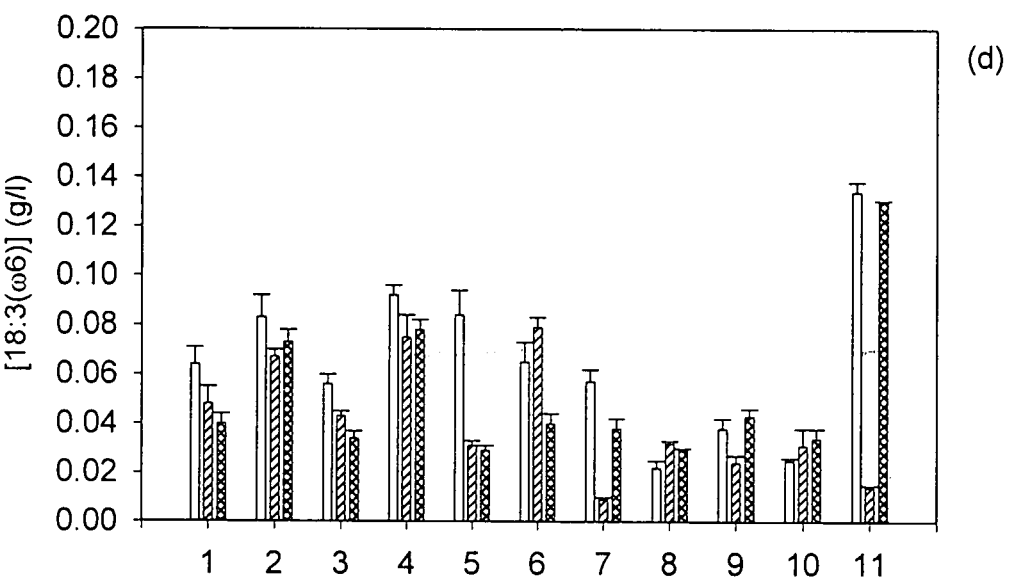


Fig. 4. Results obtained on biomass production (a), percentage total lipids reached (b), as well as volumetric concentration of neutral lipids (c) and 18:3(ω6) (d) reached, during screening of mucoralean isolates.





1. *A. californica* MUFS200
2. *C. echinulata* MUFSCu003
3. *C. echinulata* MUFSCu002
4. *C. echinulata* MUFSCu001
5. *G. butleri* MUFSGo002
6. *M. circinelloides* MUFS244
7. *M. circinelloides* MUFS243
8. *R. microsporus* MUFSR012
9. *R. microsporus* MUFSR009
10. *R. microsporus* MUFSR010
11. *R. stolonifer* MUFSR008

Glucose
Sucrose
Starch

Fig. 4. Continues

Significant differences in the volumetric concentration of neutral lipid (g/l) reached on at least two of the three different carbon sources, were also obtained for each isolate investigated (Figure 4c). Except for *M. circinelloides* MUFS244 and *R. microsporus* MUFSR010, the highest volumetric neutral lipid concentration for each isolate was reached on glucose as carbon source. The overall highest neutral lipid concentration (2.87 g/l) was obtained with *G. butleri* MUFSGo002 on glucose as carbon source.

All the isolates were able to produce 18:3(ω6) on all three carbon sources tested (Figure 4d). However, at least one significant difference in volumetric 18:3(ω6) concentrations reached on the different carbon sources, were obtained for each isolate investigated. Where significant differences existed, the volumetric concentration of 18:3(ω6) reached within each isolate was found to be higher for glucose than for sucrose and/or starch as carbon source. The exceptions being *M. circinelloides* MUFS244 and *R. microsporus* MUFSR012, which produced more 18:3(ω6) on sucrose, *R. microsporus* MUFSR010, which produced more 18:3(ω6) on starch, and *R. microsporus* MUFSR009 which produced a similar quantity of 18:3(ω6) on glucose and starch. The highest volumetric concentrations of 18:3(ω6) were obtained with *R. stolonifer* MUFSR008, on starch (0.130 g 18:3(ω6)/l) and glucose (0.134 g 18:3(ω6)/l).

4.4. Conclusions

The results of this study has shown that isolates obtained with the isolation media in Chapter 3, are able to produce volumetric concentrations of 18:3(ω6) comparable to the results of some authors (Sajbidor et al., 1988; Lindberg & Hansson, 1991; Certik et al., 1993; Roux et al., 1994). The volumetric concentrations of 18:3(ω6) obtained in this study was notably lower than the 4.40 g 18:3(ω6)/l obtained by Jeffery et al. (1997) with *Mucor circinelloides* grown on sunflower oil and acetate. However, the purpose of this study was

only to confirm that the isolates obtained in Chapter 3 can produce 18:3(ω6) in liquid media with glucose, starch or sucrose as carbon source.

Although it is known that representatives of *Mucor* and *Rhizopus* are able to produce significant volumetric concentrations of 18:3(ω6) (Sajbidor et al., 1988; Lindberg & Hansson, 1991; Certik et al., 1993; Roux et al., 1994; Du Preez et al., 1995; Kock & Botha, 1995; Jeffery et al., 1997), the results of this study showed that representatives of *Absidia* and *Gongronella* may also produce comparable quantities of18:3(ω6).

In most cases, the lipid metabolism of each isolate, measured as the percentage total lipids (w/w) produced, as well as the volumetric concentration of neutral lipids and 18:3(ω6) obtained, differed significantly in media containing different carbon sources. This is in accordance with the results of others obtained with representatives of *Mucor* and *Rhizopus* (Chapter 1).

From the results of this study, it is clear that 18:3(ω6) production may differ within a mucoralean species, since isolates representing a particular species may produce different volumetric concentrations of this PUFA, in a medium containing a specific carbon source (Figure 4d). This is especially true for the isolates representing *Cunninghamella echinulata*, *Mucor circinelloides* and *Rhizopus microsporus*.

The isolates were obtained on isolation media containing different carbon sources (Table 1). However, during the screening process, all the isolates except *C. echinulata* MUFSCu002, produced significantly more biomass on starch as carbon source, than on glucose or sucrose as carbon source (Figure 4a). Although many more studies should be done, results indicate that no carbon source used in the isolation media (Table 1), would consistently select for superior producers of 18:3(ω6) (Figure 4d).

It should be noted, however, that some isolates obtained on an isolation medium utilizing a particular carbon source, may not produce significant volumetric

concentrations of 18:3(ω6) on another carbon source. Two examples of such isolates are *M. circinelloides* MUFS243 and *R. stolonifer* MUFSR008, which were obtained on isolation media containing respectively acetate and maltose as carbon sources (Table 1). These two isolates failed to produce significant biomass on sucrose (Figure 4a) and consequently could not produce significant volumetric concentrations of 18:3(ω6) during the screening process (Figure 4d). Nevertheless, where significant growth on a carbon source occurred, all the isolates produced substantial volumetric concentrations of 18:3(ω6).

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CHAPTER 5

EVALUATION OF MUCORALEAN ISOLATES FOR GROWTH AND GAMMA-LINOLENIC ACID PRODUCTION IN AN INDUSTRIAL EFFLUENT

(Sponsored by African Products Pty. Ltd.)

5.1. Introduction

In a recent study (Chapter 2) it was found that members of Mucorales can grow and produce 18:3(ω6) on a variety of carbon sources, including carbon sources present in industrial effluents (e.g. acetic acid, starch and sucrose) (Israilides et al., 1994; Kock & Botha, 1995). Media containing either acetic acid, maltose, starch or sucrose as carbon source were developed to isolate mucoralean fungi from soil (Chapter 3). Some of these mucoralean isolates were screened for growth and 18:3(ω6) production in liquid media containing either glucose, starch or sucrose as carbon source (Chapter 4). The results indicated that these carbon sources do influence growth and 18:3(ω6) production in mucoralean fungi. The quantities of 18:3(ω6) obtained (Chapter 4), were also comparable with the results obtained by others (Sajbidor et al., 1988; Lindberg & Hansson, 1991; Certik et al., 1993).

In addition, it has been found that the biomass obtained from fungi grown in industrial effluents, can also be utilized for other purposes. An example is yeasts belonging to the genus *Geotrichum* which were used to produce bioprotein from effluents of petrochemical industries (Botha & Kock, 1993). The bioprotein obtained during this process was used as a supplement in animal feed.

The purpose of this study was therefore to screen some of the mucoralean isolates obtained in Chapter 3 for growth and 18:3(ω6) production in an industrial effluent. The effluent which consisted mainly of dextrins, galactans and starch, was obtained from African Products (Pty. Ltd.), an industry producing glucose-syrups through the enzymatic hydrolyses of starch. The isolates were screened for the ability to grow in a medium containing the effluent as carbon source, as well as the ability to reduce the chemical oxygen demand (COD) of the effluent. The isolates that showed the most reduction in COD of the effluent, were then analysed for 18:3(ω6) production.

5.2. Materials and methods

5.2.1. Isolates used.

Mucoralean fungal isolates, isolated from the Dry *Cymbopogon-Themeda* Veld (Acocks, 1988) (Chapter 3), were used in this study (Table 1). The isolates are held in the Mucoralean Fungal Culture Collection (MUFS) at the University of the Orange Free State.

5.2.2. Medium preparation.

The industrial effluent of African Products (Pty. Ltd.) was used as carbon source. This effluent (COD = 9.58 g/l) contained arabinose (0.03 g/l), galactose (0.01 g/l), glucose (0.02 g/l), galactans (14.39 g/l) and dextrins/starch (12.22 g/l) as well as phosphate (0.01 g/l), nitrogen (0.24 g/l) and sodium chloride (40.00 g/l). The medium that was prepared from the effluent contained (g/l): yeast extract, 0.10; $MgSO_4.7H_2O$, 0.25; K_2HPO_4, 10.00; $(NH_4)_2SO_4$, 5.00 and 1.00 l effluent. The medium had a pH of 5.8 and a COD of 20.26 g/l.

5.2.3. Preparation of inoculum.

A culture of each strain was incubated at 25°C in the dark for seven days on malt extract agar (Difco). A spore suspension of *circa* 2.5 x 10^6 spores / ml was obtained by transferring the sporangiospores from each culture with an inoculation loop to 10.00 ml sterile distilled water. This spore suspension was then used as inoculum.

5.2.4. Culture conditions.

The spore suspension obtained for each fungal isolate was used to inoculate the above mentioned medium, contained in 100 ml quantities in 1000 ml conical flasks. The cultures were incubated at 30°C on a rotary shaker (160 r.p.m.; throw = 50 mm). After six days of incubation, the biomass was harvested by filtration (Whatman No.1), washed with distilled water, freeze-dried and weighed. All experiments were performed at least in triplicate.

Table 1. Biomass produced and COD obtained when the mucoralean isolates were screened for the ability to grow and reduce COD of a medium which contained the industrial effluent (COD = 9.58 g/l) as carbon source.

Isolates	Biomass (g/l)	Standard deviation	COD (g/l)	Standard deviation
Absidia californica (MUFS200)	2.42	0.16	7.06	1.15
Gongronella butleri (MUFSGo002)	5.81	0.18	5.48	0.41
Rhizopus microsporus (MUFSR012)	2.99	0.08	12.36	0.48
R. microsporus (MUFSR009)	4.66	0.15	4.78	0.40
R. microsporus (MUFSR010)	3.25	0.07	5.84	0.70
R. stolonifer (MUFSR008)	2.70	0.22	9.48	1.70
Cunninghamella echinulata (MUFSCu003)	2.32	0.10	3.87	0.63
C. echinulata (MUFSCu002)	2.29	0.13	6.51	0.63
C. echinulata (MUFSCu001)	2.37	0.09	5.55	1.29
Mucor circinelloides (MUFS244)	2.43	0.07	5.01	0.57
M. circinelloides (MUFS243)	2.50	0.04	6.12	0.78

5.2.5. Determination of chemical oxygen demand.

The chemical oxygen demand (COD) was determined according to the method of Jirka & Carter (1975), using a HACH DR/2000 spectrophotometer.

5.2.6. Lipid extraction and fatty acid analyses.

Lipids were extracted from the freeze-dried biomass as described by Kendrick & Ratledge (1992). This included 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. The lipids were dissolved in diethyl ether and transferred to preweighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 55°C. The extracted lipids were dissolved in chloroform and applied to a collumn (140 mm x 20 mm) of activated silicic acid. Neutral, glyco- and phospholipids were eluted by the successive addition of 1-,1-,1- trichloroethane, acetone and methanol (Kendrick & Ratledge, 1992). The different lipid fractions were dissolved in chloroform and methylated by the addition of trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed using a Varian 3300 gas chromatograph and a polar Supelcowax 10 glass capillary column (0.75 mm x 30 m) with N_2 (5 ml/min) as carrier gas (Kock, 1988). Peaks representing methylated fatty acids were identified using authentic standards.

5.3. Results and discussion

Table 1 lists the biomass that was obtained with the mucoralean isolates when grown in the medium containing the industrial effluent. It also lists the COD values that were obtained for each isolate. From these results it can be seen that the mucoralean isolates were able to grow in this medium. The most biomass (5.81 g/l) was obtained with *Gongronella butleri* MUFSGo002.

When comparing the COD values obtained with these isolates (Table 1) it is clear that all the isolates, except *Rhizopus microsporus* MUFSR012 and

R. stolonifer MUFSR008, were able to significantly reduce the COD of the original effluent (COD = 9.58 g/l). *Cunninghamella echinulata* MUFSCu003 were able to reduce this COD value to 3.87 g/l and *R. microsporus* MUFSR009 reduced the value to 4.78 g/l. Since the lowest COD values were obtained for these two isolates, they were consequently analysed for 18:3(ω6) production.

The results of the testing of *C. echinulata* MUFSCu003 and *R. microsporus* MUFSR009 for 18:3(ω6) production, are depicted in Table 2. *Cunninghamella echinulata* MUFSCu003 produced a volumetric 18:3(ω6) concentration, in the neutral lipids, of 0.04 g/l and *R. microsporus* MUFSR009 produced 0.03 g/l 18:3(ω6).

5.4. Conclusions

From the results obtained in this study it is clear that the mucoralean isolates could grow on the medium containing the industrial effluent as carbon source. In addition, these isolates were able to significantly reduce the COD value of the effluent, the exceptions being *R. microsporus* MUFSR012 and *R. stolonifer* MUFSR008.

Two of these isolates which showed the most reduction in COD values (*C. echinulata* MUFSCu003 and *R. microsporus* MUFSR009), were tested for the ability to produce 18:3(ω6) in the medium containing the industrial effluent. The results indicated that the volumetric 18:3(ω6) concentrations obtained were comparable with the 18:3(ω6) quantities obtained when the mucoralean isolates were grown in media specifically designed for lipid accumulation (Chapter 4). The results of this study has therefore shown that isolates obtained with the isolation media in Chapter 3, are able to produce 18:3(ω6) in the industrial effluent. Many more studies on the optimization of 18:3(ω6) production however, should be done before the commercial potential of such a process could be evaluated. The results of this study indicate that certain members of

Table 2. Results on the lipid analyses of the isolates, grown in the medium prepared from the industrial effluent.

	C. echinulata MUFSCu003	Standard deviation	*R. microsporus* MUFSR009	Standard deviation
Biomass (g/l)	2.32	0.10	4.66	0.16
% Total lipids (w/w)	17.57	0.91	7.83	0.19
Neutral lipids (g/l)	0.27	0.01	0.27	0.01
[18:3(ω6)] (g/l)	0.04	0.004	0.03	0.002

Mucorales can not only be used for 18:3(ω6) production, but can also be used to reduce the COD value of industrial effluents. In addition, the possibility exists to use the biomass obtained during cultivation as a supplement in animal feed, since mucoralean fungi are already used in the preparation of many Eastern foods (Hesseltine, 1965). However, as in the case with the 18:3(ω6) production, many more studies on the optimization of biomass production and COD reduction should be done before the commercial potential of such a process could be evaluated.

5.5. References

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SUMMARY

Members of Mucorales are known to produce the high value fatty acid gamma-linolenic acid [18:3(ω6)]. Although few studies have been conducted, it is known that the type of carbon source included in the medium, influences the production of 18:3(ω6) by these fungi. The range of carbon sources on which mucoralean fungi are able to grow and produce 18:3(ω6), is still mostly unknown. Another factor that influences the quantities of 18:3(ω6) that are being produced by these fungi, is the specific fungal strain that is used in the process. Consequently, in this study it was decided first to investigate the ability of different mucoralean fungi to grow and produce 18:3(ω6) on a wide range of carbon sources. Isolation media for obtaining new strains from nature, which utilize carbon sources obtainable from industrial effluents, would subsequently be developed.

The influence of 38 different carbon sources on growth and consequent 18:3(ω6) content of the lipids produced by four mucoralean fungal strains were therefore investigated. The strains represented the species *Mortierella alpina*, *Mucor circinelloides*, *Mucor flavus* and *Thamnostylum piriforme*. The representatives of *M. circinelloides* and *M. flavus* respectively utilized 25 and 23 of the 38 carbon sources in the series. The highest percentages 18:3(ω6) obtained with the representatives of *M. circinelloides* and *M. flavus* were 27.17 % and 36.40 % respectively. In contrast, the highest percentages 18:3(ω6) obtained with the representatives of *Mo. alpina* and *T. piriforme* were only 5.61 % and 12.84 % respectively. These two strains could respectively utilize only seven and 17 of the carbon sources. This study indicated that mucoralean fungi can grow and produce 18:3(ω6) on a variety of carbon sources, including carbon sources present in industrial effluents (e.g. starch, sucrose and acetic acid).

Three selective media were subsequently developed in order to isolate mucoralean fungi from soil, using the soil plate technique. The media, which were complex, respectively contained starch, sucrose and sodium acetate as carbon sources, as well as 0.02 g/l of the anti-fungal agent, benlate. The selectivity of the media for members of Mucorales was first determined by testing the media for the ability to support growth of 134 mucoralean fungal strains representing 66 species and seven genera. The three isolation media supported growth of strains representing *Absidia*,

Actinomucor, *Backusella*, *Mucor*, *Rhizopus* and *Thamnostylum*. The ability of the isolation media to select mucoralean fungi from a natural fungal population in soil, was then determined and representatives of the genera *Absidia*, *Cunninghamella*, *Gongronella*, *Mucor* and *Rhizopus* were obtained. The results further showed that by using selective media in combination with a relatively non-selective medium, instead of the non-selective medium alone, more mucoralean taxa could be isolated from a particular soil sample.

Mucoralean fungal isolates that were obtained from the soil sample, were subsequently evaluated for growth and 18:3(ω6) production in media containing starch, sucrose or glucose as sole carbon sources. Isolates representing the families Absidiaceae, Cunninghamellaceae and Mucoraceae were inoculated in complex media containing the above mentioned carbon sources. It was found that all the isolates were able to produce 18:3(ω6) on all three carbon sources. However, significant differences in volumetric 18:3(ω6) concentrations reached on different carbon sources were noted for each isolate investigated. The highest volumetric concentrations of 18:3(ω6) were obtained with an isolate representing *R. stolonifer* on starch (0.130 g/l) and glucose (0.134 g/l) as carbon sources.

In order to prove that the isolates obtained using the above-mentioned isolation media, are able to grow in an industrial effluent, some of the isolates representing different families, were grown in a medium prepared from an industrial effluent containing dextrins, galactans and starch as carbon sources. The lipids of the isolates which reduced the COD value of the effluent the most, were analysed. It was found that these isolates were able to produce 18:3(ω6).

This study has therefore shown that it is possible to construct isolation media to isolate 18:3(ω6) producing mucoralean fungi from a natural fungal population. It was also found that such isolates can be used to produce biomass and 18:3(ω6) from carbon sources present in industrial effluents.

OPSOMMING

Lede van Mucorales is bekend vir hul vermoë om die hoë waarde vetsuur, gamma-linoleensuur [18:3(ω6)], te produseer. Hoewel daar slegs enkele studies gedoen is, is dit bekend dat die tipe koolstofbron in die medium die produksie van 18:3(ω6) deur die fungi beïnvloed. Die spektrum koolstofbronne waarop mucoraliese fungi kan groei en 18:3(ω6) produseer, is egter nog grootliks onbekend. Nog 'n faktor wat die hoeveelheid 18:3(ω6) wat deur die fungi geproduseer word beïnvloed, is die spesifieke fungusstam wat in die proses gebruik word. Daar is gevolglik besluit om in hierdie studie eers ondersoek in te stel na die vermoë van die mucoraliese fungi om op 'n reeks koolstofbronne te groei en 18:3(ω6) te produseer. Daarna sal isolasiemedia ontwerp word waarmee nuwe fungusstamme wat koolstofbronne teenwoordig in industriële aflope kan benut, uit die natuur geïsoleer kan word.

Die invloed van 38 verskillende koolstofbronne op groei en gevolglike 18:3(ω6) inhoud van die lipiede geproduseer deur vier mucoraliese fungusstamme, is ondersoek. Die stamme het die spesies *Mortierella alpina*, *Mucor circinelloides*, *Mucor flavus* en *Thamnostylum piriforme* verteenwoordig. Die verteenwoordigers van *M. circinelloides* en *M. flavus* kon onderskeidelik 25 en 23 van die 38 koolstofbronne benut. Die hoogste persentasies 18:3(ω6) wat verkry is met die verteenwoordigers van *M. circinelloides* en *M. flavus,* was 27.17 % en 36.40 % onderskeidelik. In teenstelling hiermee, was die hoogste persentasies 18:3(ω6) wat verkry is met die verteenwoordigers van *Mo. alpina* en *T. piriforme* slegs 5.61 % en 12.84 % onderskeidelik. Hierdie twee stamme kon slegs onderskeidelik sewe en 17 van die koolstofbronne benut. Hierdie studie is dus 'n aanduiding dat mucoraliese fungi kan groei en 18:3(ω6) produseer op 'n wye verskeidenheid van koolstofbronne, insluitende koolstofbronne teenwoordig in industriële aflope (bv. stysel, sukrose en asynsuur).

Drie selektiewe media is hierna ontwikkel om mucoraliese fungi vanuit grond te isoleer d.m.v. die grondplaattegniek. Die media, wat kompleks is, het onderskeidelik stysel, sukrose en natrium asetaat as koolstofbronne bevat, asook 0.02 g/l van die anti-fungus middel, benlate. Die selektiwiteit van die media vir Mucorales is bepaal deur die media se vermoë te toets om groei te ondersteun van

134 mucoraliese fungus stamme, wat 66 spesies en sewe genera verteenwoordig. Die drie isolasiemedia het groei van die stamme van *Absidia*, *Actinomucor*, *Backusella*, *Mucor*, *Rhizopus* en *Thamnostylum* ondersteun. Die vermoë van die isolasiemedia om vir mucoraliese fungi in 'n natuurlike fungus populasie in grond te selekteer, is bepaal. Verteenwoordigers van die genera *Absidia*, *Cunninghamella*, *Gongronella*, *Mucor* en *Rhizopus* is verkry. Die resultate het verder bewys dat deur selektiewe media in kombinasie met 'n relatiewe nie-selektiewe medium te gebruik, i.p.v. die nie-selektiewe medium alleen, meer mucoraliese taksa geïsoleer kan word vanaf 'n spesifieke grondmonster.

Mucoraliese fungusisolate wat uit die grondmonster verkry is, is gevolglik geëvalueer vir groei en 18:3(ω6) produksie in media wat stysel, sukrose of glukose as koolstofbronne bevat. Isolate wat die families Absidiaceae, Cunninghamellaceae en Mucoraceae verteenwoordig is geïnokuleer in komplekse media wat die bogenoemde koolstofbronne bevat. Daar is gevind dat al die isolate 18:3(ω6) kon produseer op al drie koolstofbronne, hoewel betekenisvolle verskille in die volumetriese 18:3(ω6) konsentrasies verkry is op die verskillende koolstofbronne vir elke isolaat wat getoets is. Die hoogste volumetriese konsentrasies van 18:3(ω6) is verkry van die isolaat wat *R. stolonifer* verteenwoordig. Stysel (0.130 g/l) en glukose (0.134 g/l) is as koolstofbronne gebruik.

Om te bewys dat die isolate wat verkry is van die bogenoemde isolasiemedia in 'n industriële afloop kan groei, is sommige van die isolate wat verskillende families verteenwoordig, opgegroei in 'n medium wat voorberei is van 'n industriële afloop wat dekstriene, galaktane en stysel as koolstofbron bevat. Die lipiede van die isolate wat die CSB waarde van die afloop die meeste verminder het, is geanaliseer. Dit is gevind dat die isolate 18:3(ω6) kon produseer.

Die studie het dus bewys dat dit moontlik is om isolasiemedia te ontwerp om 18:3(ω6) produserende mucoraliese fungi uit 'n natuurlike fungus populasie te isoleer. Dit is ook gevind dat die isolate gebruik kan word om biomassa en 18:3(ω6) te produseer van koolstofbronne wat in industriële aflope teenwoordig is.