THE UNIVERSITY OF HULL

The Biochemistry of Lipid Accumulation in *Mucor circinelloides* and *Mortierella alpina*

being a Thesis submitted for the Degree of Doctor of Philosophy in the University of Hull

by

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I studied for my B.Sc. in microbiology at Universiti Kebangsaan Malaysia (National University of Malaysia) between July 1989 and July 1993. The work for my doctorate was carried out firstly in the Department of Chemical and Biochemical Engineering at University College London between January 1995 and April 1995, before transferring to University of Hull for personal reasons. Most of my work at UCL focused on the study of the biosynthesis of erythromycin in *Saccharopolyspora erythraea*. Although credit has been given, no useful results were obtained and are not reported here.

My work in the University of Hull commenced on May 1995. Most of my work here concentrates on the study of lipid regulation in oleaginous fungi. I was fully sponsored by Universiti Kebangsaan Malaysia.

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Summary of Thesis Submitted for PhD Degree

by Aidil Abdul Hamid

On

The Biochemistry of Lipid Accumulation in *Mucor circinelloides* and *Mortierella alpina*

1. The profile and biochemistry of growth and lipid accumulation in *M. circinelloides* and *Mt. alpina* were investigated.

2. A nitrogen-limited condition was vital in triggering lipid accumulation in both fungi, which was in agreement with previous work reported in oleaginous yeasts (Botham and Ratledge, 1979; Boulton and Ratledge, 1984). Good growth and lipid production by *M. circinelloides* were obtained only when it was grown in fermenters. Growth in stirred bottles (whirlipots) did not result in high lipid yields. This was caused by the anaerobic nature of cultivation in the whirlipots which affected the utilization of ammonium by the cells. As a result the cultures became carbon-limited instead of nitrogen-limited. Conversly, in a fermenter culture which had an efficient aeration, the culture reached a nitrogen-limited condition at an early stage of the incubation which led to a higher lipid production of the cells.

3. The lipid production of both fungi increased in parallel with the increase in the C:N ratio of the medium but the fatty acid compositions were not affected.

4. Ten enzymes potentially linked to the regulation of lipid biosynthesis (fatty acid synthase, acetyl-CoA carboxylase, ATP:Citrate lyase, AMP:deaminase, carnitine acetyl transferase, malic enzyme, glucose-6-

phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP:isocitrate dehydrogenase and NAD:isocitrate dehydrogenase) were detected in both fungi. In both fungi, the profile of all enzymes stated above was similar with the activities increased coincidentally with the depletion of ammonia in the medium.

5. The only differences in the enzymatic profiles of the two fungi was the early depletion of ME activity in *M. circinelloides* where it disappeared after approximately 40 h of incubation, coincident with the cessation of lipid accumulation, although other key enzymes of lipid biosynthesis (FAS, ACC and ACL) together with the activities of other NADPH-generating enzymes were still active and the glucose was still present. Conversely, ME in *Mt. alpina* culture was present until the late stage of fermentation and the cell lipid continued to increase until the end of the fermentation. This suggests ME is a major provider of NADPH for lipid biosynthesis which was in agreement with previous observations in *Aspergillus nidulans* (Wynn and Ratledge, 1997) and *M. circinelloides* (Wynn et al., 1997).

6. The depletion of ME activity in *M. circinelloides* after approximately 40 h of incubation was as a result of the cessation of the protein from being synthesized, triggered by the depletion of ammonium in the culture . This was evident as malic enzyme activity returned after its initial depletion when ammonium tartrate was added into the culture. Also, the restitution of malic enzyme activity was prevented when cycloheximide, a protein synthesis inhibitor, was added simultanously with the addition of ammonium tartrate.

7. The NAD:isocitrate dehydrogenase from both fungi showed an increased affinity for its substrate, isocitrate, in the presence of AMP. However, the enzyme did not show an absolute requirement for AMP for its activity as it could still be activated in the absence of AMP at a saturating concentration of isocitrate.

8. ME was purified some 20-fold purification from both fungi. Both showed a similar K_m values for NADP (approximately 0.04 mM) but a slightly higher K_m value for malate was obtained in *Mt. alpina* compared to *M. circinelloides* (1 mM and 0.4 mM, respectively).

9. ME from both fungi showed various degrees of inhibition by tartronic acid, oxaloacetate, palmitoyl-CoA and oleoyl-CoA. At 10 mM, tartronic acid caused approximately 40 % inhibition in the activity of ME from both fungi while OAA inhibited ME from *M. circinelloides* more strongly (70 %) than that from *Mt. alpina* (45 %). At a final concentration of 1 mM, palmitoyl-CoA and oleoyl-CoA caused a 100 % inhibition on ME from *M. circinelloides* and approximately 90 % on ME from *Mt. alpina*.

10. FAS purified from both fungi showed a similar K_m values for malonyl-CoA and acetyl-CoA (approximately 0.013 and 0.016 mM, respectively) while a higher K_m value for NADPH was observed in *Mt. alpina* compared to *M. circinelloides* (0.038 and 0.01 mM, respectively).

11. Despite a range of experiment using different approaches, no direct evidence of a physical association between FAS and ME was obtained when experiments were performed to observe the formation of complexes between the two enzymes *in vitro*.

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
ACL	ATP:citrate lyase
ARA	arachidonic acid
CAT	carnitine acetyl transferase
CBE	cocoa butter equivalent
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
EPO	Evening Primrose Oil
FAS	fatty acid synthase
G-6-PDH	glucose-6-phosphate dehydrogenase
G 6-P	glucose 6-phosphate
GLA	gamma linolenic acid
6-PGDH	6-phosphogluconate dehydrogenase
MDH	malate dehydrogenase
ME	malic enzyme
NAD:ICDH	NAD:isocitrate dehydrogenase
NADP:ICDH	NADP:isocitrate dehydrogenase
OAA	oxaloacetate
PUFAs	polyunsaturated fatty acids
SCO	single cell oil

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1.0 Introduction

1.1.0 Microbial Production of Fatty Acids

1.1.1 Fatty Acids

Fatty acids are long chain carboxylic acids and are components of many lipids (Kennedy, 1993). They are rarely found naturally in the free form due to their ability to behave as detergents and thus disrupt cell membranes but are linked to a variety of molecules of which glycerol is the most common. Triacylglycerol, which is produced as a major storage product in many organisms, can constitute up to 90 % of the total lipid in some microbial cells.

Fatty acids in biological systems usually contain an even number of carbon atoms, typically 16 or 18. They may be fully saturated or contain one or more double bonds, be linear or branched, or even contain alicylic rings. Fatty acids are also an important component of biological membranes where they exist in three major kind of lipids: phospholipids, glycolipids and sterols. Decreasing the chain length and increasing the degree of unsaturation of a fatty acid enhances its fluidity and thus affects the fluidity of the membrane.

1.1.2 Single Cell Oil (SCO)

Production of oils and fats is normally achieved from plants and animals. Animal fats constitute a minor sector (approximately 22 %) of the total market and microbial oils and fats contribute nearly nothing. The

major commercial plant oils are soybean oil, palm oil and rapeseed oil with olive oil being the highest priced commodity oil (Ratledge, 1997). Animal fats (tallow and lard), however, have steadily declined in consumption over the past decade and are likely to fall even further to about 20 % of the total market by 2001 (Shukla, 1994).

Production of single cell oil (SCO), an edible oil that can be extracted from a microbial cell, has been considered as a potentially economical way to produce large quantities of fatty acids (Ratledge, 1976). Not all micro-organisms can be considered as abundant sources of oils and fats but, like all living cells, micro-organisms always contain lipids for the essential functioning of membranes and membranous structures. In terms of lipid production, micro-organisms can be divided into two groups: oleaginous and non-oleaginous. An oleaginous micro-organism can be loosely defined as one that has the potential to accumulate more than 20 % lipid but this is an arbitrary value (Boulton and Ratledge, 1981) .

Oleaginous organisms are found among yeasts, moulds and algae. There are about 600 different yeast species of which only about 25 are able to accumulate more than 20 % lipids. Of the 60 000 fungal species, fewer than 50 species accumulate more than 25 % lipid (Ratledge, 1989). Although micro-organisms contribute so little to the oils and fats industry, their biotechnological application in the production of lipids has attracted the attention of lipid production industry as they can be seen to have the following advantages: a prodigious growth rate, where 1 gram of bacteria can generate a second gram of biomass in 20

minutes. A yeast cell takes a little longer - 2 to 4 hours; they can also assimilate almost any carbon-containing-substrate to sustain growth; and they can be genetically manipulated to produce a wide range of products.

1.1.3 Historical Background

The technology of SCO consists of the production of oil-rich biomass by oleaginous micro-organisms followed by down-stream processing which includes the cell and oil recoveries (Davies, 1984; Slater, 1988). The use of micro-organisms for this purpose has attracted attention since the late 19th century. The oil contents of moulds and yeasts were established over a hundred years ago. The first publications of lipid analysis for moulds, yeast and algae were reported for *Claviceps purpurea* (Ficinus, 1873), *Saccharomyces cerevisiae* (Nageli and Loew, 1878) and *Pennales* spp. (Harder and Witsch, 1942) respectively.

Microbial lipids were originally academic curiosities and it was not until the stimulus of World War 1 in 1914, with the accompanying disruption of trade and supplies, that Germany began to develop the early work of microbial lipids into possible alternatives to plant and animal fats (Woodbine, 1959; Hesse, 1949). The stimulus of World War 2 created an even greater incentive for the Germans to develop a biotechnological route to oil and fat production. Although a large scale production was achieved, problems of scaling-up, recovering and extracting the products prevented it from being used for edible purposes.

In the early 1960s, very little interest in SCO was evident. A

multinational company, Unilever, was involved in the studies on PUFAs of moulds particularly in the production of arachidonic acid, which was mistakenly thought to be as a chicken-flavour precursor (Shaw 1966). The current interest in SCO began in the mid 1970s as a parallel to the development of single cell protein (SCP) for animal feed, which led to the development of large-scale fermentation plants in the U.K, Italy, France, Sweden, USSR and the USA. However, the large fermentation processes for the production of SCP began to close because they were unable to compete with soybean meal prices, which were too cheap for even the most productive SCP process to compete.

Because SCP production involves producing only a dried biomass, whereas SCO involves producing the biomass and then requires a further down stream-processing for oil extraction and refining, it seems that SCP would still be the preferred product rather than SCO. Therefore, the opportunities to produce triacylglycerols were limited to the highest priced materials such as cocoa butter equivalent (CBE) with yeasts and polyunsaturated fatty acids (PUFAs) with fungi.

1.1.4.0 Cocoa Butter Equivalent

Cocoa butter equivalent (CBE) is used extensively in the confectionery business: in the manufacture of chocolate and chocolate-type materials in several countries including UK, Ireland and Denmark (Ratledge, 1997). The chemical and physical properties of cocoa butter are responsible for the melting behaviour, texture and mouth-feel of chocolate which depend on the fatty acid ratios of approximately 30 % stearic acid (18:0), 30 % palmitic acid (16:0) and 30 % oleic acid

(18:1) and consists of mainly 1, 3-disaturated-2-unsaturated triacylglycerols (Ratledge, 1992).

Yeasts have been exploited for the production of a CBE, which began in the mid 1980s when the price of cocoa butter was very high (over US $ 8000 / tonne) (Ratledge, 1993). Four different approaches have been tried to produce a satisfactory yeast CBE. Strategies were designed to increase the inherently low content of stearic acid (maximally from 10 to 12 %) in oleaginous yeasts up to the required 30 % stearate or more. A short summary of the main strategies is now given below. It will then be discussed in detail in the subsequent sections.

a) Direct feeding of stearic acid or its ester to the yeasts,

b) Inhibition of stearoyl desaturase activity, which is responsible for the conversion of stearic acid to oleic acid,

c) Production of yeast mutants which lack the activity of stearoyl desaturase,

d) Metabolic manipulation, which involved the control of the amount of O_2 supplied during fermentation.

1.1.4.1 Direct Feeding of Stearic Acid.

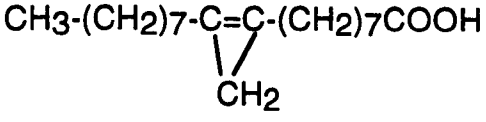
Direct feeding of stearic acid or its ester to a yeast is the simplest way to increase the content of stearic acid of a yeast oil. The fatty acid or its ester is then taken up by the yeast and esterified directly into the storage triacylglycerols in the cell, although some may be degraded. Excellent results were demonstrated by direct feeding of both stearic acid and palmitic acid to *Torulopsis* ATCC 20507, where a high content of

stearic acid were produced (43 % of the total fatty acids) (Fuji Oil Co. Ltd., 1979, 1981).

However, this approach was found to be impractical from economic point of view because of the high cost of the stearic acid or the stearate-palmitate mixture . Although stearic acid could be produced by chemical hydrogenation of oleic acid derived from cheap animal fats, the application of stearic acid from this source to produce CBE would cause a marketing problem as it will be unacceptable for vegetarians and several religious groups (Ratledge, 1997). This approach was subsequently abandoned despite a successful production of high-oleic acid sunflower oil, which could be used to produce stearic acid, at a relatively low cost.

1.1.4.2 Inhibition of Stearoyl Desaturase

Sterculic acid, which is found in the seed oil of sterculia and kapok plants, has been used as an effective inhibitor of the stearoyl desaturase (Moreton, 1985). The structure of sterculic acid is shown below :



Accumulation of up to 48 % stearic acid of the total fatty acids was achieved in several yeasts such as *Candida* sp. 107, *Rhodosporidium toruloides* and *Trichosporon cutaneum*, when as little as 100 mg sterculic acid per L of growth medium was added (Moreton, 1985). As sterculic

acid did not affect the activity of Δ 12 desaturase, which catalyzes the desaturation of oleic acid to linoleic acid (18:2), the linoleic acid proportion in the yeast oil was unaffected. In order to achieve a satisfactory CBE, the amounts of the 18:2 and 18:3 fatty acids need to be very low to maintain the melting behaviour of the CBE. *Cis*-Δ 12 analogue of sterculic acid was later found to show an inhibitory effect on the synthesis of linoleic acid (Moreton, 1988). Addition of sterculic acid and the *cis*-Δ 12 analogue of sterculic acid subsequently resulted in the production of lipids from *R. toruloides* with decreasing content of linoleic acid, with the three principal fatty acids, palmitic acid, oleic acid and stearic acid being present at a ratio of 1 : 1 : 2.

Although this approach was successful, it was found to be impractical from economic point of view in relation to the costs of the cyclopropene inhibitors which were expensive. This method was subsequently abandoned in 1986.

1.1.4.3 Mutation

Extensive work had been carried out on the yeast *Cryptococcus curvatus* (Ykema et al., 1990; Verwoert et al., 1989) to delete the stearoyl desaturase. *Cryptococcus curvatus*, which is also referred to as *Apiotrichum curvatum* and initially named *Candida curvata*, was originally isolated from dairy wastes (Moon et al., 1978). A number of auxotrophic mutants that required oleic acid for growth were isolated when the yeast was treated with chemical mutagens (Smit et al., 1992). One of these mutants, Ufa 33, contained up to 50 % stearic acid of the

total fatty acids but with a small amounts of 18:2 and 18:3 fatty acids still present (see Table 1).

A number of hybrid strains with partially functioning Δ 9 desaturase were later produced from the initial oleate-auxotroph (Ykema et al., 1990). As the Δ 9 desaturase was partially functioning, the hybrid strains could now synthesize a small amount of oleic acid. Therefore, oleic acid was no longer required to be added to the growth medium. These hybrids produced lipids with substantial amount of stearic acid and in some cases, for example the hybrid F33.10, with excellent similarities to cocoa butter (see Table 1). However, none of these mutants have been used in large scale production of CBE oils.

1.1.4.4 Metabolic Manipulation

As O_2 is a vital co-reactant in the Δ 9 desaturase reaction, an attempt to increase the stearic acid content of yeast lipids was carried out by diminishing the amount of O_2 supplied during the fermentation course (Davies et al., 1990). By this approach, up to 24 % stearic acid of the total fatty acids was obtained (see Table 1). The contents of the 18:2 and 18:3, which also required O_2 for their biosynthesis, decreased to 3 % and 1 % of the total fatty acids, respectively. Therefore, by a simple metabolic manipulation , a satisfactory production of yeast oil was achieved.

Table 1: **Cocoa butter fatty acids and the fatty acyl composition of triacylglycerols from *Cryptococcus curvatus* wild type (WT) strain, unsaturated fatty acid auxotrophic mutant (Ufa 33), a hybrid derived from Ufa 33 (F33.10) compared to the best results obtained with wild-type strain grown with limited O_2 supply (from Ratledge, 1997).**

	Major fatty acyl groups Relative % (w/w)				
	16:0	**18:0**	**18:1**	**18:2**	**18:3**
Cocoa butter	23-30	32-37	30-37	2-4	–
Yeast					
WT	17	12	55	8	2
Ufa 33[a]	20	50	6	11	4
F33.10[b]	24	31	30	6	–
WT-NZ[c]	18	24	48	3	1

WT; wild type

[a] Grown with 0.2 g L^{-1} oleic acid,

[b] Grown without oleic acid,

[c] Wild-type grown on whey lactose in a 500 L bubble column fermenter with a restricted O_2 supply.

1.1.5 The Future of CBE Production Industry

Despite all the successful attempts at optimizing the production of yeast CBE, its production could not compete with that of CBE produced from palm oil and which itself was under economic pressure as cocoa butter had become cheaper (from US $ 8600 / tonne in 1986 to about US $ 3000 / tonne in 1994) (Ratledge, 1997). Therefore, yeast CBE production will only be economically viable if either the price of cocoa butter increases, or an alternative lower cost substrates could be found. Acetic acid and whey have been successfully used as a low cost substrates in the production of a CBE. *M. circinelloides* CBS 108.16 produced up to 27 % stearic acid of the total fatty acids with up to 40 % total lipid when grown on acetic acid (Roux et al., 1994).

The use of whey as a low cost substrate in the production of a CBE was first carried out on *C. curvata* (Davies, 1988, 1992a, b; Davies and Holdsworth, 1992). The earlier discovery of the yeast that could be readily grown on lactose, which is the major carbohydrate of whey, indicated that it was the most suitable one to be used in the process. When it was grown on whey lactose in a 500 L fermenter with a restricted O_2 supply, the yeast produced up to 24 % stearic acid of the total fatty acids and with a very low content of 18:2 and 18:3 (see Table 1). It was then estimated that the yeast CBE product would sell for approximately 80 % of the price of cocoa butter (Davies and Holdsworth, 1992). However, the possiblity of commercially producing the yeast CBE still depends on the price of cocoa butter itself. Only the continuing low price of cocoa butter will prevent it from becoming an operating reality (Ratledge, 1997).

1.1.6 Polyunsaturated Fatty Acids (PUFAs)

PUFAs are fatty acids which contain two or more double bonds which are usually in the *cis* form. The double bonds are separated by one methylene group : -CH=CH-CH_2-CH=CH-. PUFAs are classified into 3 groups: n-3, n-6 and n-9, depending on the location of the terminal double bond adjacent to the terminal methyl group. As shown in Figure 1a-c, the n-3, n-6 and n-9 PUFAs have a terminal double bond 3, 6 and 9 carbon atoms counting from the terminal methyl group, respectively. Figure 2 shows the biosynthetic pathway of these PUFAs.

PUFAs of the n-3 and n-6 are known as the precursors of the various prostaglandins, leukotrienes and thromboxanes in animals (Gurr, 1992). The inclusion of the n-3 series PUFAs, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) as a dietary supplements has been suggested for the prevention of heart disease. The n-6 series, particularly GLA, have been recommended for the relief of eczema (Oxdale, 1990). The nutritional role of the n-9 series is still obscure even though eicosatrienoic acid (ETA) was first reported 42 years ago (Mead and Slaton, 1956).

1.1.6.1 Gamma-Linolenic Acid (GLA)

GLA [18:3 (6, 9,12)], was first found by Heidushka and Luft (1919) in the seed of *Oenothera biennis* (the Evening Primrose). This plant, dubbed the "King's Cure-All" in UK and Europe, has been used as the source of one of the highest priced polyunsaturated fatty acid ,

Figure 1 : Example of

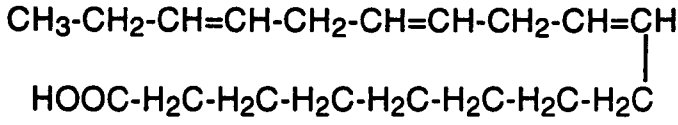
a) n-3 (α-linolenic acid)

b) n-6 (γ-linolenic acid)

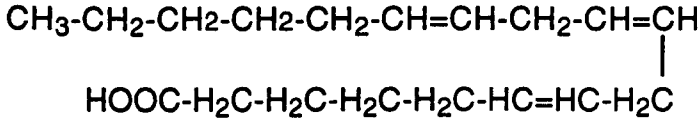
c) n-9 (octadecadienoic acid).

n-3, n-6 and n-9 PUFAs have a terminal double bond 3, 6 and 9 carbon atoms counting from the methyl group, respectively.

a) α-linolenic acid, n-3 [18:3 (9, 12, 15)].



b) γ-linolenic acid, n-6 [18:3 (6, 9, 12)]



c) Octadecadienoic acid, n-9 [18:2 (6, 9)]

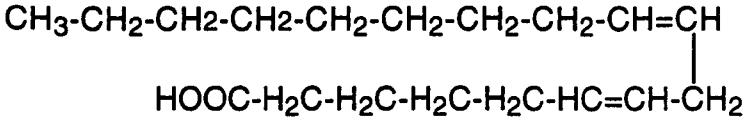
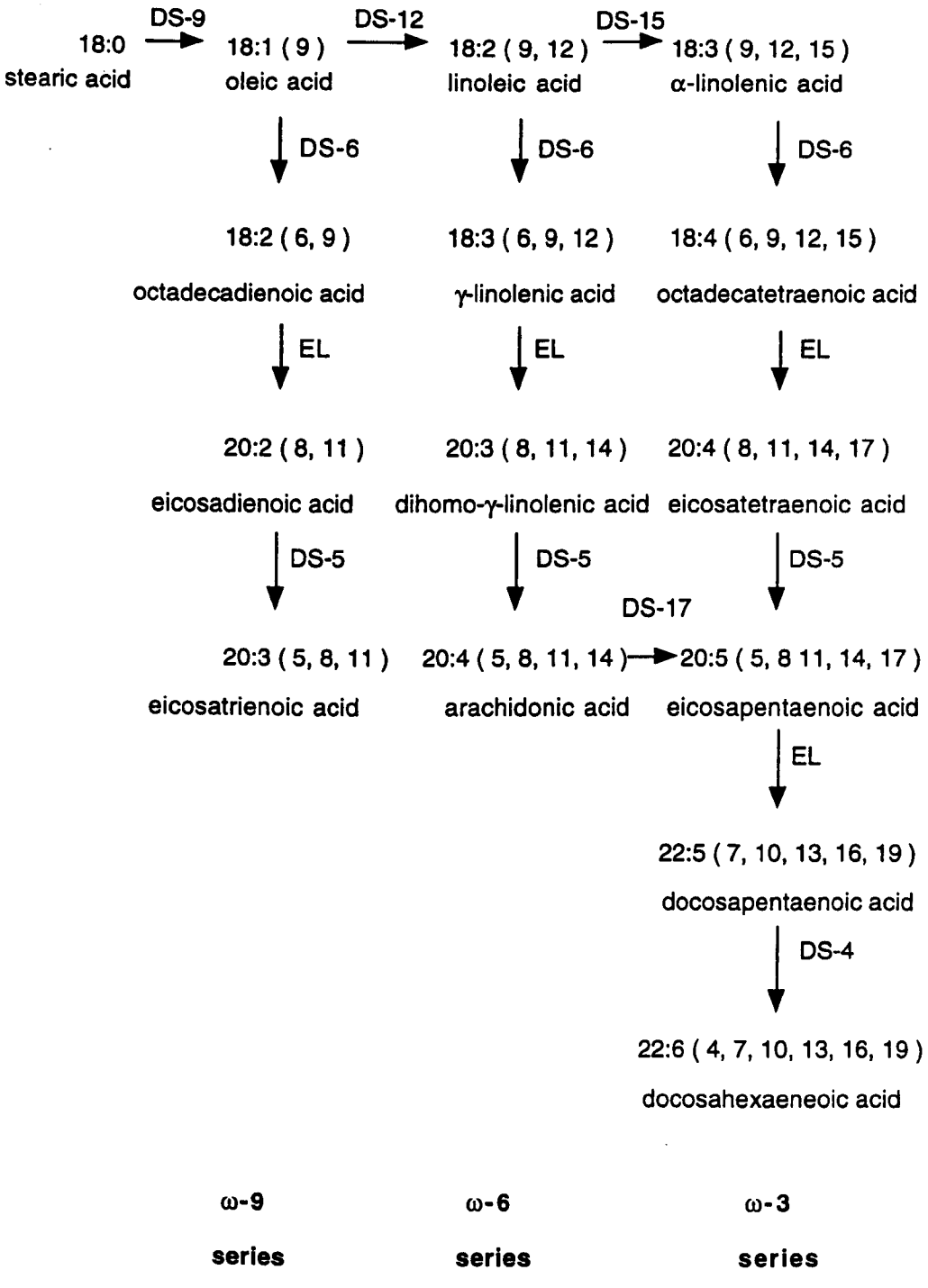


Figure 2 : **Formation of polyunsaturated fatty acids in fungi. DS-N, fatty acyl desaturase acting at Nth C atom of fatty acid; EL, elongase. (NB. DS-17 is only found in certain filamentous fungi; it is not found in animals). (from Ratledge, 1993).**


18:0
stearic acid
DS-9
18:1 (9)
oleic acid
DS-12
18:2 (9, 12)
linoleic acid
DS-15
18:3 (9, 12, 15)
α-linolenic acid
DS-6
DS-6
DS-6
18:2 (6, 9)
octadecadienoic acid
18:3 (6, 9, 12)
γ-linolenic acid
18:4 (6, 9, 12, 15)
octadecatetraenoic acid
EL
EL
EL
20:2 (8, 11)
eicosadienoic acid
20:3 (8, 11, 14)
dihomo-γ-linolenic acid
20:4 (8, 11, 14, 17)
eicosatetraenoic acid
DS-5
DS-5
DS-5
DS-17
20:3 (5, 8, 11)
eicosatrienoic acid
20:4 (5, 8, 11, 14)
arachidonic acid
20:5 (5, 8 11, 14, 17)
eicosapentaenoic acid
EL
22:5 (7, 10, 13, 16, 19)
docosapentaenoic acid
DS-4
22:6 (4, 7, 10, 13, 16, 19)
docosahexaeneoic acid
ω-9
series
ω-6
series
ω-3
series

gamma-linolenic acid (Ratledge, 1993). It has been sold in the UK for over 20 years and prescribed for the treatment of eczema (Oxdale, 1990), rheumatoid arthritis (Jantti et al., 1987), multiple sclerosis (Barber, 1988), schizophrenia (Horrobin, 1979) and premenstrual syndrome (Khoo, 1990).

The production of Evening Primrose Oil (EPO) is expensive because both a long period for growth and the huge area needed for the crop (see Fukuda and Morikawa, 1987). Therefore, much research effort was been directed towards the development of a method for the microbial production of this fatty acid. *Mucor circinelloides* was identified from a large screening programme as being a suitable organism for this purpose (Ratledge, 1993). The specifications of the fungal oil were equal or better than EPO in almost every respect including its much higher content of GLA (see Table 2) (Ratledge, 1993).

The development of the first commercial process for a microbial oil began in the UK in 1985, being run by J & E Sturge Ltd. (now Haarmann & Reimer) of Selby, Yorkshire. A similar microbial process was later launched in Japan by Idemitsu Petroleum Co. Ltd. using *Mortierella isabellina* although this mould had a much lower GLA content than *M. circinelloides* (Ratledge, 1993). Production of GLA by *M. circinelloides,* using a low cost carbon substrate, was also achieved when monocarboxylic acid (acetic acid) was used to replace glucose (Kock and Botha, 1993, 1994; Immelman et al., 1996). By using acetic acid at a subtoxic level, up to 45 mg GLA / g biomass was produced (i.e. 28 % total lipid, of which 16 % was GLA) (Preez et al., 1995). This process however has yet to be demonstrated at a pilot-scale level.

Table 2: Fatty acid profiles of a commercial fungal oil product compared with Evening Primrose Oil (from Ratledge, 1997).

	Mucor circinelloides [a]	Evening Primrose Seed Oil
Oil content [% (w/w)]	20	16
Fatty acid		
16:0	22-25	6-10
16:1	0.5-1.5	–
18:0	5-8	1.5-3.5
18:1	38-41	6-12
18:2	10-12	65-75
γ–18:3	15-18	8-12
α–18:3	0.2	0.2
20:1	–	–
22:1	–	–
24:0	–	–

[a] Production organism used by J & E Sturge Ltd., Selby, Yorkshire, UK, from 1985-1990

However, the price of GLA fell from US $ 50 - 55 kg^{-1} in 1985 to less than half this price by 1990 (Ratledge, 1993). This was caused by an increasing amount of Evening Primrose being grown by farmers as a cash crops after realizing the potential of GLA as a highly profitable oil. Competition with borage and blackcurrant oils, which were subsequently identified as an alternative sources of GLA, were also instrumental in decreasing the price of EPO. As a result, the production of GLA in the UK was subsequently suspended in 1990 following a transfer of the company to its present owner (Rhone-Poulenc Co. Ltd.).

1.1.6.2 Arachidonic Acid (ARA, 20:4 n-6)

ARA is a C_{20} PUFA that is important as a natural constituent of biological membranes and a precursor of numerous eicosanoids such as prostaglandins, thromboxanes and leukotrienes. It has been isolated from the lipids of porcine adrenal gland and liver as well as from sardines (Ahern, 1984). Formation of ARA has been reported in Phycomycetes moulds of the subdivision Mastigomycotina such as *Pythium* spp. (Gandhi and Weete, 1991), *Saprolegnia parasitica* (Gellerman and Schlenk, 1979) and *Mortierella* (Totani and Oba, 1987, 1988; Yamada et al., 1987; Chen et al., 1997; Amano et al., 1992). When grown on glucose, *Mt. alpina* can produce up to 40 % lipids with 11 % ARA of the total fatty acids(Kendrick and Ratledge, 1992).

The highest ARA contents have been demonstrated in *Mortierella alpina* which produced up to 79 % ARA of the total fatty acids which presented 26 % of the cell dry weight (Totani and Oba, 1987). When this organism was used for large scale production (300 L scale) with a long

fermentation run (up to 16 days), a slight diminution of yield was obtained (71 % ARA, where it represented 23 % of the dry cell weight).

1.1.6.3 Eicosapentanoic Acid (EPA, 20:5 n-3) and Docosahexaenoic Acid (DHA, 22:6 n-3)

EPA (20:5) and DHA (22:6) are the only n-3 PUFAs which are of significant dietetic interest (Ratledge, 1993). Currently, the major source of EPA and DHA is fish oil. EPA usually occurs in low concentrations along with DHA, which may account for over 50 % of the total fatty acids in fish oils . In fungi, EPA is formed by the activity of Δ 17 desaturase on arachidonic acid (ARA) of n-6 series. The production of EPA has been studied extensively using strains of *Mortierella* (Yamada et al., 1992; Shimizu et al., 1988a, 1989) and *Pythium* (O'Brien et al., 1993). By using *Mortierella alpina* and *Mt. hygrophila*, production of up to 29 and 41 mg EPA per g cells were achieved, respectively (Shimizu et al., 1988). EPA is produced by *Mt. alpina* when it is grown at low temperature (12 ^{o}C). This temperature-dependant formation of EPA is due to the activation of Δ 17 desaturase, which catalyzes desaturation at the n-3 position of ARA at low temperature(Shimizu et al., 1988b).Subsequently, *Mt. alpina* was demonstrated to be able to convert an exogenously added α–linolenic acid (18:3 ω-3) into EPA, increasing the yield up to 67 mg per g dry cells (Shimizu et al., 1989). However, when the same procedure was performed on *Mortierella elongata*, the EPA production only increased from 15 to 36 mg per g dry weight (Bajpai et al., 1992).

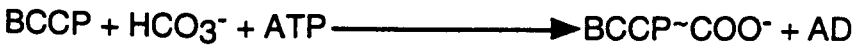
In contrast to EPA production, DHA has received relatively little research attention. Fungal species belonging to the orders of Saprolegniales and Enthomophthorales were identified as being a promising sources of DHA (Li and Ward, 1994; Singh et al., 1996). *Thraustochytrium aureum* , a marine fungi appears to be the best source of producing DHA, with up to 50 % of its fatty acids as DHA (Bajpai et al., 1991) but with relatively low oil content (10 - 15 % of biomass). An efficient production of DHA has also been demonstrated by the marine algae *Cryptheconidium cohnii*, which yielded 25 % lipid of the biomass and 39 % DHA of the total fatty acids (see Leman, 1997).

1.2.0 Biosynthesis of Fatty Acids

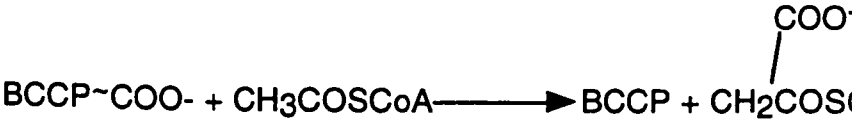
Biosynthesis of fatty acids occurs in the cytosol of microbial and animal cells. In plants, it occurs in chloroplasts. It starts with the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (reaction 1).

$$\text{Acetyl-CoA} + \text{ATP} + HCO_3^- \longrightarrow \text{malonyl-CoA} + \text{ADP} + P_i + H^+ \qquad (1)$$

Acetyl-CoA carboxylase contains biotin which is attached to a biotin carboxyl carrier protein (BCCP). Carboxylation of acetyl-CoA to malonyl-CoA proceeds in two steps: first, the carboxylation of the biotin moiety of the BCCP(reaction 2a) by biotin carboxylase , and second, the transfer of the carboxyl group to the acceptor acetyl-CoA (reaction 2b) by transcarboxylase (Wakil et al., 1983).



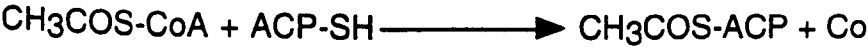
(2a)



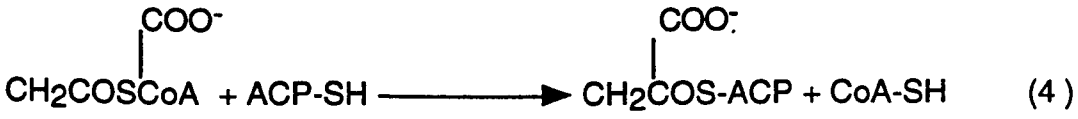
(2b)

Acetyl-CoA and malonyl-CoA are then converted to palmitate by fatty acid synthase (FAS) in the presence of NADPH. This reaction consists of several sequential reactions, each catalyzed by a specific enzyme activity. These enzymes are loosely associated with each other in bacteria and plants but in other eukaryotic cells, they occur as a tightly bound complexes (Wakil et al., 1983) .

In the first reaction, acetyl-CoA and malonyl-CoA are attached to an acyl carrier protein (ACP), forming acetyl-ACP (reaction 3) and malonyl-ACP (reaction 4) by acetyl transacylase and malonyl transacylase, respectively. The ACP consists of a 4'-phosphopanthetheine prosthetic group that binds all acyl intermediates. These reactions were reported to occur only in bacteria and plants. In yeasts, fungi and animals, the acetyl unit is transferred to a cystein sulphur in the active site of condensing enzyme by acetyl-CoA transacylase and the malonyl unit is transferred to an ACP which is covalently bound in the multienzyme complexes by malonyl-CoA transacylase. Therefore, both acetyl and malonyl units are transferred and covalently attached to the multienzyme complexes.

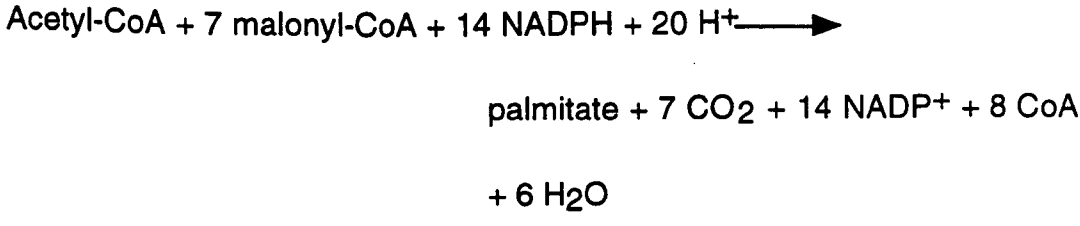


(3)



Acetyl-ACP and malonyl-ACP then react to form acetoacetyl-ACP catalyzed by acyl-malonyl-ACP condensing enzyme (reaction 5).

Acetoacetyl-ACP is then reduced to D-3-hydroxybutyryl-ACP by β-ketoacyl-ACP reductase and is then dehydrated to form crotonyl-ACP by 3-hydroxyacyl-ACP- dehydratase . In the final step of the cycle, crotonyl-ACP is reduced to butyryl-ACP. NADPH serves as the reducing agent in both reduction steps. In the second round of the biosynthesis, butyryl-ACP condenses with a new molecule of malonyl-ACP to form C_6-β-ketoacyl-ACP for further reduction to the C_6 acid. The process is then repeated up to C_{16}. The stoichiometry of the synthesis of palmitate is:



1.2.1 Sources of NADPH for fatty acid biosynthesis

The metabolism of glucose via the pentose phosphate pathway ie. the activity of glucose-6-phosphate dehydrogenase (G-6-PDH) (reaction 6) and 6-phosphogluconate dehydrogenase (6-PGDH) (reaction 7), together with NADP:isocitrate dehydrogenase (NADP:ICDH) (reaction 8) and the activity of malic enzyme (reaction 9) have all been suggested to be possible sources of NADPH which is required in the reduction steps of fatty acid biosynthesis.

glucose 6-phosphate + $NADP^+$ ⟶

6-phosphoglucono-δ-lactone + NADPH + H^+ (6)

6-phosphoglucono-δ-lactone + $NADP^+$ ⟶

ribulose 5-phosphate + CO_2 + NADPH (7)

isocitrate + $NADP^+$ ⟶ α-ketoglutarate + CO_2 + NADPH (8)

malate + $NADP^+$ ⟶ pyruvate + CO_2 + NADPH (9)

However, malic enzyme has recently been shown to act as the major provider of NADPH for fatty acid biosynthesis in two oleaginous fungi: *Aspergillus nidulans* and *Mucor circinelloides*. In *Aspergillus nidulans*, a mutant lacking this enzyme was found to produce only half the lipid (12 %, w / w of cell dry weight) that was accumulated by strains possessing this enzyme when grown in a nitrogen-limiting media,

although other NADPH generating enzymes such as G-6-PDH, 6-PGDH and NADP:ICDH were detected at high activities (Wynn and Ratledge, 1997). This difference was considered by Wynn et al. (1997) as not to be the result of impaired growth since both the wild type and the mutant strains achieved a similar culture dry weights (approximately 4.6 g / l) and cell yield (approximately 0.28 g dry weight / g glucose used).

However, when *A. nidulans* was grown in a media with excess nitrogen, the lipid content of the biomass was low (5 %, w / w of cell dry weight) and similar in all strains (wild type and mutant), regardless of their ME activity. It was presumed by Wynn et al. (1997) that under these conditions, the cell lipid produced was vital to the metabolic functioning of the cell and that the synthesis of these 'metabolically active' lipid was not limited by ME.

ME was also reported to play a vital role in lipid production of *M. circinelloides* (Wynn et al., 1997), where inhibition of ME activity in the culture caused a severe inhibition of lipid production . The inhibition of ME was achieved by inclusion of sesamol, a non-oil component of sesame seed oil, in the culture medium . Addition of approximately 6 mM sesamol in the culture medium caused a 98 % decrease in ME activity compared to ME activity of a control culture without sesamol. The inhibition of ME activity led to a parallel low total lipid production by the culture (2 %, w / w of biomass) compared to the control culture which was grown without the inclusion of sesamol, producing up to 24 % lipid of biomass. Other NADPH generating enzymes such as G-6-PDH and 6-PGDH were unaffected by sesamol except NADP:ICDH, which showed a 57 %

decrease in activity. However it was considered by Wynn et al. (1997) that the decrease in ME activity was the most important factor affecting the lipid metabolism of the fungus as the change in this activity was the most dramatic.

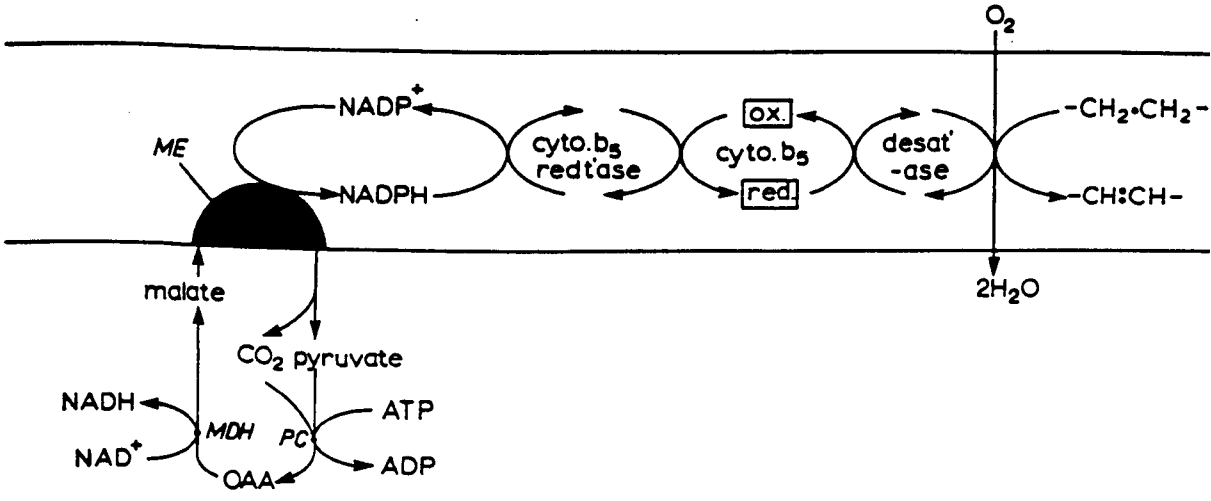
1.3.0 Desaturation of Fatty Acids

The desaturation of fatty acids beyond oleate [18:1 (cis-9)] has been reported to occur in plants and eukaryotic micro-organisms with fatty acyl groups attached to a phospholipid (Kendrick and Ratledge, 1992b). This implicates that the process is a membrane-associated activity. Desaturation of fatty acids in animals occurs in the presence of cytochrome b_5 for the transfer of electrons from NADPH (Jeffcoat et al., 1984). The same cofactor is also present in the fatty acid desaturation activity in plants (Smith et al., 1990) and yeasts (Pugh et al., 1978). Double bonds are introduced into long chain fatty acyl-CoAs by desaturases, which depend on the presence of O_2 and NADPH (Bloomfield and Bloch, 1960):

$$\text{-}CH_2\text{-}CH_2\text{-} + NADPH + O_2 \longrightarrow \text{-}CH{=}CH\text{-} + NADP^+ + 2H_2O.$$

Figure 3 illustrates a scheme for fatty-acyl group desaturation occuring in *Mucor circinelloides*, which suggests a second role for malic enzyme in the supply of NADPH for desaturation reaction (Kendrick and Ratledge, 1992). In this fungus, two distinct malic enzymes exist, one soluble and one membrane bound. Both have different roles in the production of NADPH where the former produces NADPH for the

Figure 3 : **Desaturase reactions with fatty acyl groups attached at phospholipids. Malic enzyme generates NADPH for desaturation. Malate is regenerated by the action of pyruvate carboxylase (PC) and malate dehydrogenase (MD) (from Kendrick, 1992).**


O2
NADP+
ME
NADPH
cyto.b5 redt'ase
ox.
cyto. b5
red.
desat' -ase
-CH2•CH2-
-CH:CH-
2H2O
malate
CO2 pyruvate
NADH
NAD+
MDH
PC
ATP
ADP
OAA

reduction steps in the fatty acid biosynthesis whilst the latter produces NADPH for fatty acid desaturation. Desaturation of fatty acids occurs with the fatty acyl groups attached to phospholipids. In this speculation, fatty acid desaturation in phospholipid membranes of *M. circinelloides* was found to be stimulated by malate due to the presence of the membrane bound malic enzyme. Addition of tartronic acid (Figure 4), a structural analogue of malate which acted as an inhibitor of this enzyme, resulted in the inhibition of the malate-induced stimulation of fatty acyl group desaturation in the microsomal membrane.

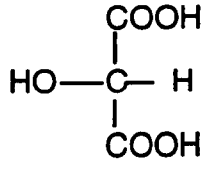


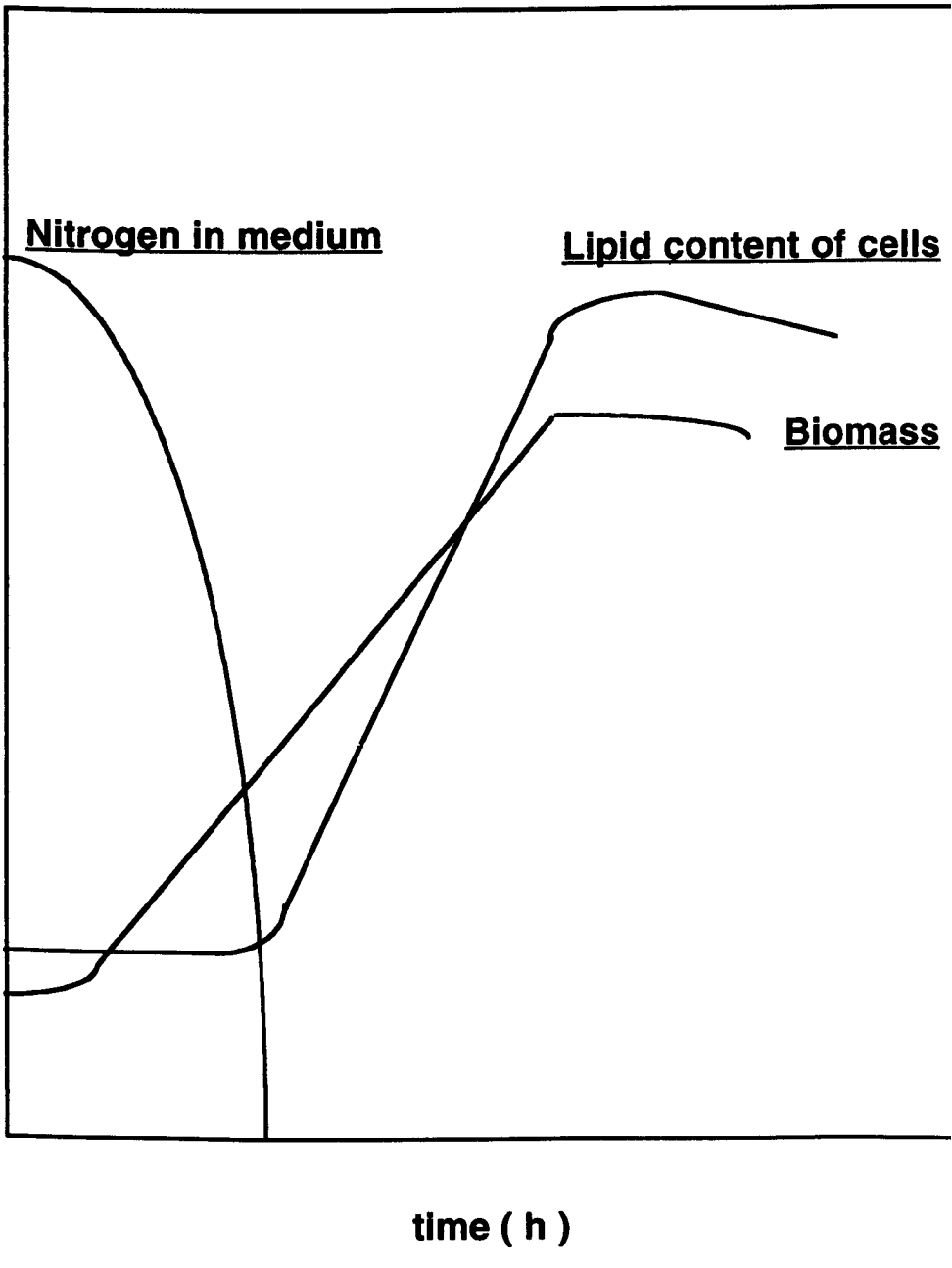
Figure 4 : Tartronic acid (hydroxymalonic acid)

This was the first report of malic enzyme being involved in the desaturation of fatty acids in any cell system.

1.4.0 Enzymatic Regulation in Lipid Accumulation of Oleaginous Yeasts

Accumulation of lipid in oleaginous yeasts is well established. It is usually a biphasic process, requiring an excess of carbon over other nutrients, particularly nitrogen (Botham and Ratledge, 1979; Boulton and Ratledge, 1984). The first phase consists of a rapid cell growth until the nitrogen becomes exhausted from the medium. This is followed by the second phase where the excess carbon is converted into lipids. A typical pattern of lipid accumulation in batch culture is shown in Figure 5.

Figure 5 : **Pattern of lipid accumulation in oleaginous organisms grown in a batch culture which involves a biphasic process : rapid cell growth in the presence of nitrogen source and lipid accumulation during nitrogen-limited conditions.**


Nitrogen in medium
Lipid content of cells
Biomass
time (h)

In general, oleaginicity is believed to be dependent on the possession of ATP:citrate lyase (ACL), and an AMP-requiring NAD^+:isocitrate dehydrogenase (NAD:ICDH) (Boulton and Ratledge, 1981). Lipid accumulation has been reported to result from a concerted action of several enzymes and metabolic events (Botham and Ratledge, 1979). A short summary of the main metabolic events is now given below. These will then be amplified in detail in the subsequent section.

a) When the nitrogen source of a culture is exhausted, AMP:deaminase is activated (Evans and Ratledge, 1985a). As a result, the concentration of AMP drops rapidly (Boulton and Ratledge, 1983) and this is then the first major trigger in the lipogenic cascade mechanism.

b) AMP is required as an activator of the enzyme NAD:ICDH operating in the mitochondrion (Evans et al., 1983). Because of the depletion of AMP, the enzyme is therefore inactivated.

c) With the cessation of NAD:ICDH activity, isocitrate cannot be further metabolized and both isocitrate and citrate begin to accumulate. As the assimilation of glucose continues unaffected by the nitrogen-limited cells and the equilibrium lies in favour of citrate, citrate becomes a major product of the cell metabolism (Boulton and Ratledge, 1983).

d) Citrate then exits from the mitochondrion in a malate-mediated citrate translocase reaction.

e) Citrate is then cleaved by ACL, producing acetyl-CoA and oxaloacetate. The acetyl-CoA then serves as a primer for fatty acid biosynthesis

1.4.1 The Role of AMP:deaminase and NAD^+:ICDH

When a culture reaches a nitrogen-limited condition, a nitrogen-scavenging process is initiated in order to maintain the synthesis of nucleic acids and proteins. This is achieved by the activation of AMP: deaminase, which hydrolyses AMP to form IMP and NH_4^+ ions (Evans and Ratledge, 1985a). As a result, the AMP concentration drops rapidly (Boulton and Ratledge, 1983). As AMP is required as an activator for the activity of NAD:ICDH, its depletion triggers the inactivation of this enzyme (Botham and Ratledge, 1979). As a result, isocitrate cannot be further metabolized and both isocitrate and citrate begin to accumulate.The isocitrate which accumulates will equillibrate to citrate via aconitase.

The citrate is then transported to the cytosol by citrate translocase (which will be discussed in the next section) and cleaved by ACL to form oxaloacetate and acetyl-CoA. In non-oleaginous organisms, although ACL may be present, NAD:ICDH still operates in the absence of AMP and thus limits the production of acetyl-CoA (Botham and Ratledge, 1979). NAD:ICDH has also been reported to be inhibited by ATP emphasizing that the ATP:AMP ratio is probably the major regulatory parameter controlling the oxidation of citrate in the TCA cycle (Evans and

Ratledge, 1985b).

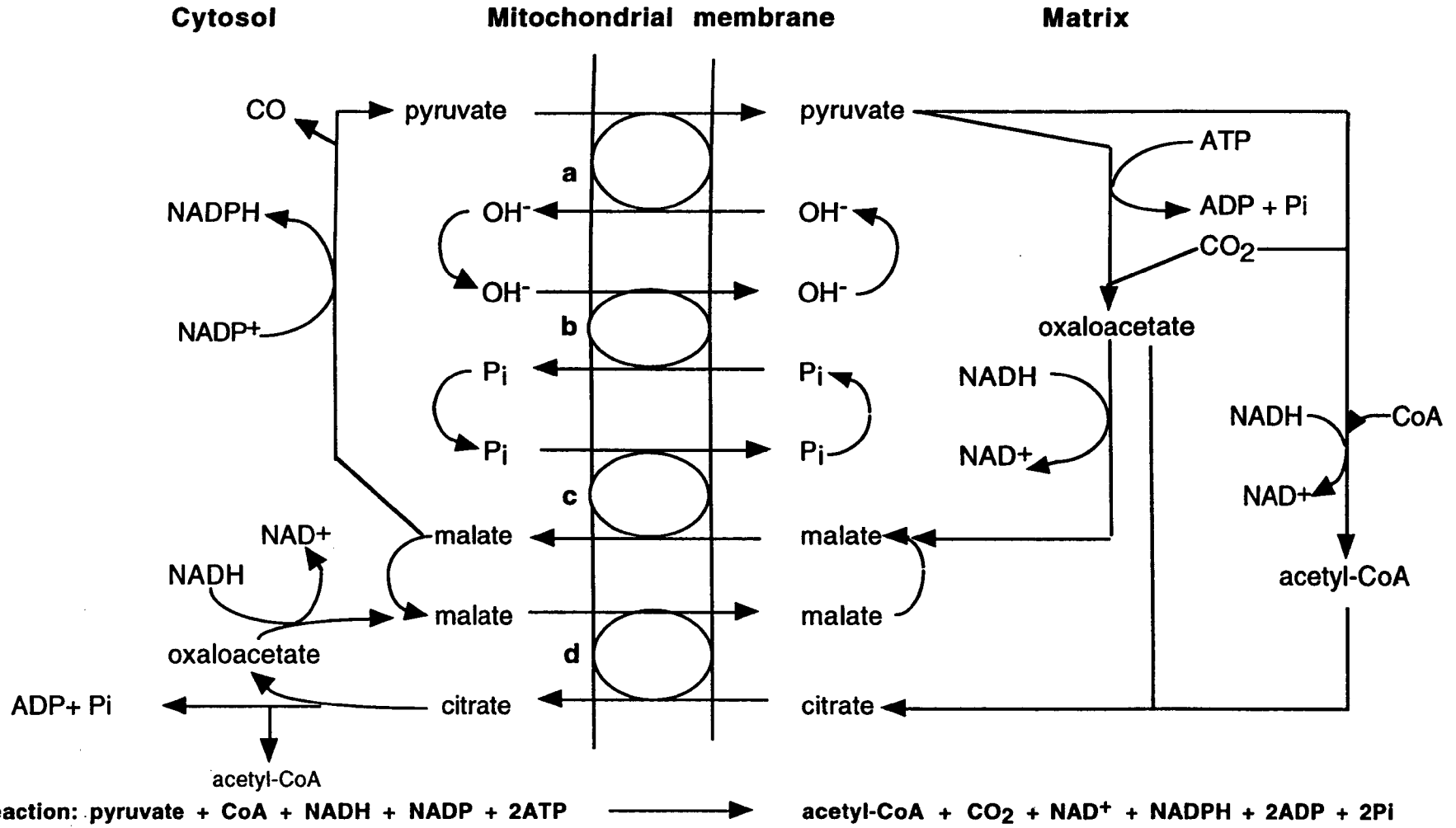
1.4.2 The Role of Malate-Citrate Shuttle and its Regulation by Fatty Acyl-CoA Esters (FACEs)

Because of the compartmentation of citrate synthesis, which occurs in the mitochondria, and fatty acid biosynthesis in the cytosol, efficient transport of citrate from mitochondria to the cytosol is important for lipid biosynthesis. The presence of malate-citrate transport system, which is carried out by citrate translocase, has been reported to occur in mitochondria of numerous oleaginous and non-oleaginous yeasts (Evans et al., 1983a).

The operation of this shuttle during lipogenesis depends on the supply of cytosolic malate. As citrate only exchanges with malate, it becomes apparent that malate should be produced in the cytosol in advance, and independently, of citrate efflux (Evans and Ratledge, 1985a).The creation of the initial pool of malate in the cytosol and its maintenance due to depletion by malic enzyme is considered to be via the coupled translocase systems of pyruvate-OH^- / OH^--phosphate / phosphate - malate (see Figure 6a) (Evans and Ratledge, 1985a). In this system, pyruvate is assumed to be able to gain entry into the mitochondria independently of malate efflux.

Pyruvate, generated from the activity of malic enzyme, would be transported back into the mitochondria via pyruvate-hydroxyl ion exchange and converted to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA then condenses with oxaloacetate to form citrate which is then

Figure 6a : **Malate-citrate shuttle and the role of malic enzyme in regulating the transport of pyruvate and citrate across the inner mitochondrial membrane. Transport process : a, pyruvate-hydroxyl ion exchange; b, hydroxyl- phosphate ion exchange; c, phosphate-malate exchange; d, malate-citrate exchange. (from Evans and Ratledge, 1985a).**



Net reaction: pyruvate + CoA + NADH + NADP + 2ATP → acetyl-CoA + CO_2 + NAD^+ + NADPH + 2ADP + 2Pi

transferred back to the cytosol with exchange of malate. Pyruvate uptake can occur independently of malate efflux but malate efflux is dependent upon pyruvate uptake (Evans and Ratledge, 1985a).

The citrate translocase in the mitochondrial membrane is known to be directly inhibited by very low concentration of long chain FACEs (Evans et al., 1983b). It was shown that citrate efflux, from a wide range of yeast mitochondria, was severely inhibited by FACEs; those with chain lengths of between 14 and 18 carbon atoms were the most potent inhibitors. Therefore the control of this system depends on the availablity of malate in the cytosol and the build up of long chain FACEs during lipid degradation.

1.4.3 The Role of ATP:Citrate Lyase and Its Regulation by FACEs

ATP:Citrate lyase (ACL) is a cytosolic enzyme and plays an important role in lipogenesis by supplying the precursor, acetyl-CoA, which is derived from mitochondrial citrate (Srere, 1975). Citrate is transported to the cytosol via citrate translocase and is cleaved by ACL to yield acetyl-CoA and oxaloacetate :

$$\text{citrate} + \text{CoA} + \text{ATP} \longrightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + P_i$$

ACL was first discovered in chicken liver (Srere and Lipmann, 1953) and has been isolated from various sources of mammalian and plant tissue (Nelson and Rinne, 1975; Hoffmann et al., 1980; and Fritsch and Beevers, 1979). In the early 1970s, ACL was isolated from microbial

sources including the yeast *Rhodotorula gracilis* (Guerritore and Hanozet, 1970) and in several species of *Mortierella* (Attwood, 1973).

The importance of ACL in lipid biosynthesis, however, was only appreciated when a correlation of lipid accumulation in yeasts with the possession of this enzyme was observed (Boulton and Ratledge, 1981; Botham and Ratledge, 1979). ACL has been shown to be present in 13 strains of yeasts, representing 6 genera of yeasts all of which are capable of accumulating more than 20 % of lipids (Table 3). This enzyme is absent in other yeasts which do not accumulate lipids (Boulton and Ratledge, 1981).

ACL is inhibited by fatty acyl-CoA esters (FACEs), ADP and glucose 6-phosphate (Evans and Ratledge, 1985a). FACEs therefore have an inhibitory effect on both citrate translocase and ACL. The action of FACEs on both ACL and malate-citrate shuttle therefore result in a very efficient regulatory control in inhibiting lipid biosynthesis from occurring if lipid degradation is stimulated.

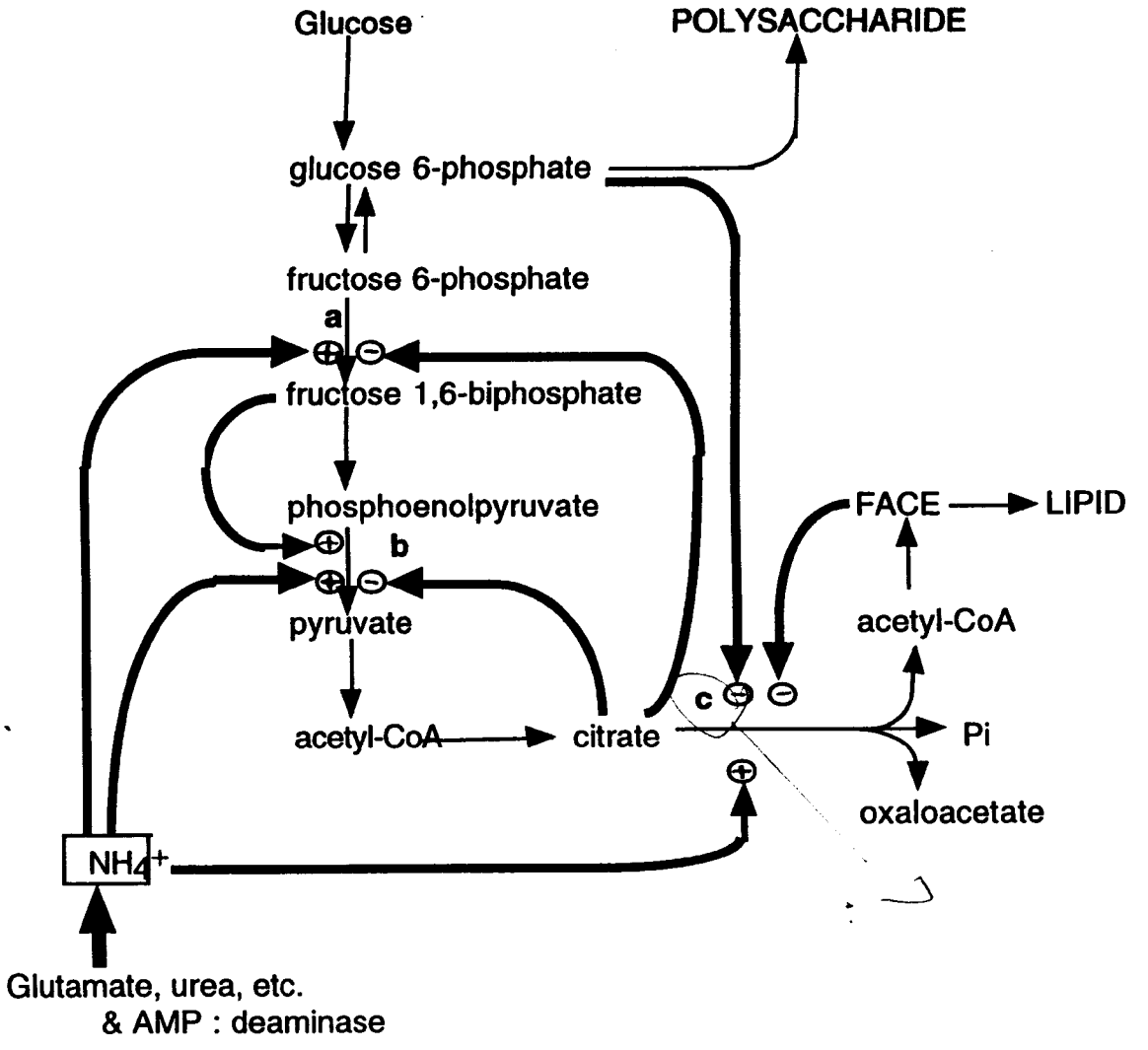
The inhibition of ACL by glucose 6-phosphate indicates that this metabolite play an important role in determining whether the carbon source flow into lipid or polysaccharide synthesis (Figure 6b). Glucose 6-phosphate decreases the affinity of ACL for its substrate, citrate which, as a result, accumulates and acts as a feedback inhibitor of phosphofructokinase (PFK), which is the crucial enzyme regulating the flux of carbon through the glycolytic pathway (Uyeda, 1979). Therefore, when glucose 6-phosphate concentration increases, polysaccharide

Table 3 : Relation of oleaginicity and the possession of ACL in yeasts (from Boulton and Ratledge, 1981).

Yeast	ATP:citrate lyase (nmol/min/mg)	lipid content (% dry wt)
Candida curvata D	7.2	33.7
Candida curvata R	5.2	33.9
Candida tropicalis NCYC 4	0	4.0
Candida utilis NCYC 359	0	3.6
Cryptococcus albidus NCYC 602	0	2.0
Hansenula ciferii CBS 111	0	7.2
Hansenula saturnus CBS 5761	11.0	24.5
Lipomyces lipofer NCYC 944	50.0	36.2
Lipomyces lipofer NCYC 692	0	1.5
Lipomyces starkeyi CBS 1809	54.0	36.5
Lipomyces starkeyi CBS 6132	0	6.4
Rhodosporidium toruloides CBS 5490	41.8	25.9
Rhodosporidium toruloides CBS 6016	0	8.0
Rhodotorula glutinis NCYC 59	12.0	23.7
Rhodotorula graminis NCYC 502	41.2	24.2
Saccharomyces cerevisieae NCYC 33	0	6.0

Figure 6b : **The role of ATP:citrate lyase in lipid biosynthesis of oleaginous yeast, its relation to the control of glycolysis and the pool of NH_4^+ which is controlled by AMP : deaminase and the use of organic nitrogen sources. a, phosphofructokinase; b, pyruvate kinase. (from Evans and Ratledge, 1985a)**

c.?


Glucose
POLYSACCHARIDE
glucose 6-phosphate
fructose 6-phosphate
a
fructose 1,6-biphosphate
phosphoenolpyruvate
b
pyruvate
FACE
LIPID
acetyl-CoA
acetyl-CoA
citrate
c
Pi
oxaloacetate
NH4+
Glutamate, urea, etc.
& AMP : deaminase

synthesis takes place .

It has been shown that lipid accumulation in *Rhodosporidium toruloides* was lower in a NH_4Cl grown culture (18 %, w / w of biomass) compared to a culture which is grown on glutamate (51 %, w / w of biomass) (Evans and Ratledge, 1983b). This is because NH_4Cl results in a lower concentration of NH_4^+ as a result of the activation of anabolic $NADP^+$:glutamate dehydrogenase. In *R. toruloides*, the affinity of ACL for its substrate, citrate, is shown to be increased in the presence of NH_4^+ . Therefore, the low concentration of NH_4^+ results in a low activity of ACL in the culture thus causing a build up of citrate which inhibits PFK and glucose-6-phosphate dehydrogenase and leads to the synthesis of polysaccharides instead of lipid. Conversely, the activity of catabolic NAD^+:glutamate dehydrogenase is derepressed in a glutamate grown culture, which then produces high concentration of NH_4^+ and activates lipogenesis. Thus we see that there can be considerable differences in the amount of lipid that oleaginous microorganisms can accumulate. In some cases this can vary in the same organism depending on its growth substrates. In other cases, the amount of lipid that is accumulated seems to be a property of the organism itself.

1.5.0 The Biochemistry of Lipid Accumulation in Oleaginous Fungi

As discussed in the previous chapter, the regulation of lipid biosynthesis in oleaginous yeasts has been well established. However, very little information has been reported in the regulation of lipid biosynthesis occurring in oleaginous fungi although the role of ME from various oleaginous fungi has been studied intensively. The first report corresponding to the lipid regulation in oleaginous fungi was published in 1979, describing the differences of ACL and NAD:ICDH characteristics between oleaginous and non-oleaginous strains of *M. circinelloides* (Botham and Ratledge, 1979). *M. circinelloides* (Strain CMI 31800), which can accumulate up to 52 % lipid under nitrogen-limitation condition, showed a similar characteristics of NAD:ICDH as observed in oleaginous yeasts. The NAD:ICDH showed a dependency for AMP for its activity where the addition of 0.2 mM AMP increased the specific activity by 9 fold compared to the activity without AMP. Conversely, in strain CMI 55452 which accumulated only 14 % lipid under the same growth conditions, the NAD:ICDH did not show a total dependency for AMP for its activity. The specific activity in the absence of AMP was three times higher than the specific activity of the same enzyme in strain CMI 31800 under the same conditions. Addition of 0.2 mM AMP increased the specific activity by two fold and did not significantly altered when up to 4 mM AMP was added. The activity of ACL in this strain was also lower than that observed in strain CMI 31800 (2 and 9 nmol / min . mg protein^{-1}, respectively) .

1.5.0 The Biochemistry of Lipid Accumulation in Oleaginous Fungi

As discussed in the previous chapter, the regulation of lipid biosynthesis in oleaginous yeasts has been well established. However, very little information has been reported in the regulation of lipid biosynthesis occurring in oleaginous fungi although the role of ME from various oleaginous fungi has been studied intensively. The first report corresponding to the lipid regulation in oleaginous fungi was published in 1979, describing the differences of ACL and NAD:ICDH characteristics between oleaginous and non-oleaginous strains of *M. circinelloides* (Botham and Ratledge, 1979). *M. circinelloides* (Strain CMI 31800), which can accumulate up to 52 % lipid under nitrogen-limitation condition, showed a similar characteristics of NAD:ICDH as observed in oleaginous yeasts. The NAD:ICDH showed a dependency for AMP for its activity where the addition of 0.2 mM AMP increased the specific activity by 9 fold compared to the activity without AMP. Conversely, in strain CMI 55452 which accumulated only 14 % lipid under the same growth conditions, the NAD:ICDH did not show a total dependency for AMP for its activity. The specific activity in the absence of AMP was three times higher than the specific activity of the same enzyme in strain CMI 31800 under the same conditions. Addition of 0.2 mM AMP increased the specific activity by two fold and did not significantly altered when up to 4 mM AMP was added. The activity of ACL in this strain was also lower than that observed in strain CMI 31800 (2 and 9 nmol / min . mg protein^{-1}, respectively) .

As previously discussed in Section 1.2.1, ME has been shown to be a major source of NADPH for the biosynthesis of fatty acids in *M. circinelloides* and *Aspergillus nidulans*. A membrane-bound form of this enzyme is also known to play an important role as NADPH provider in the desaturation of fatty acids in *M. circinelloides* (Section 1.3.0)

The possession of ACL in filamentous fungi is less well characterized. This enzyme, which is associated with those yeasts capable of accumulating substantial amount of lipids has been shown to occur in several oleaginous fungi such as *Entomorphthora exitalis*, *Conidiobolus nanodes*, and *Mt. alpina-peyron* each producing 25 %, 26 % and 40 % lipid respectively (Kendrick and Ratledge, 1992a). A widespread occurrence of this enzyme has also been reported in filamentous fungi such as *Fusarium moniliforme*, *Penicillium notatum*, *Aspergillus nidulans*, *Aspergillus parasiticus*, *M. circinelloides* and *Mt. alpina* (Wynn et al., 1998).

1.6.0 Supramolecular Organization of Fatty Acid Synthesis Regulating Enzymes

Enzymes are not independent, separate molecules in cells but they form strictly ordered structures which interact with each other and are organized in much more complex associates (Poglazof, 1996). The word 'metabolon', has been coined to describe such associations and has been defined as a supramolecular complex of metabolic enzymes and cellular structural elements within individual organelles and cell compartments (Welch, 1977; Srere, 1984). Other terminologies have also been used to describe a metabolon such as 'multienzyme

complexes', 'protein machine', 'clusters' and 'aggregates' (Srere, 1987). For the purpose of this thesis, I will use the term 'metabolon'.

A metabolon consists of not only a complex of enzymes possessing certain metabolic function but also provides an anchor site of subcellular structure such as membranes on which this complex is adsorbed. In the Kreb's cycle metabolon, a model has been proposed where the enzymes are arranged in one large aggregate with the inner mitochondria membrane serving as an anchor (Lubarev and Kurganov, 1987). It is known that the size of an enzyme is relatively big compared to its active sites. This relates to their need to have sufficient surface area to contain specific binding sites for localization in a cell and for the integration into metabolic complexes (Srere, 1984).

The formation of multienzyme complexes is believed to be influenced by the natural environment inside a cell, which consists of an extremely high concentration of proteins. The formation does not result from random association of enzymes but by associating enzymes that are related to catalyzing sequential reactions (Ovadi, 1991). Ordered aggregation of protein molecules involves the formation of non-covalent and secondary bonds. A wide range of electrostatic forces are involved in the first process of aggregation, which guide and orientate on the interacting molecules, before hydrogen bonds and finally Van Der Waals forces and the hydrophobic interactions become involved (Poglazof, 1996).

The existence of multienzyme complexes allows a series of sequential reactions to take place without having the intermediate

diffusing and equilibrating with identical molecules in the bulk phase of the same compartment of the cell (Srere, 1987). The intermediate is transferred directly to the next enzyme as its substrate, without becoming free (Ovadi, 1991). This phenomenon is referred to as channelling, which sometimes is also referred as coupling, direct transfer and vectorial transfer (Srere, 1987).

1.6.1 The Physiological Significance of Metabolic Channelling

It has been suggested that 80 % of metabolic intermediates have just one function in a cell. This implies that it would be a wasteful process if these intermediates each diffused and equilibrated with the same molecules in the bulk phase in the cell to maintain its operating concentration (Srere, 1987). The existence of multienzyme complexes leads to many catalytic advantages such as:

a) Prevention from the loss of intermediates by diffusion thus maintains a high local concentration of intermediates for the next enzyme.

b) Reduction of transit time required for an intermediate to reach the active site of the next enzyme .

c) Protection of chemically labile intermediates.

d) Segregation of intermediates from competing chemical and enzymatic reactions where the intermediates are kept in a limited microenvironment and competitions with other pathways are minimized.

e) Metabolite channeling helps conserve the solublity of the

cytoplasm, where a problem of solublity could arise from the coexistence of a multitude of molecules and electrolites. An efficient removal or recycling of one of the products of the initial reaction and the maintenance of the substrates at the active sites help keep the concentration of metabolites at a very low level thus conserve the solvent capacity of the cytoplasm.

Evidence on the existence of multienzyme complexes in metabolic pathways has been demonstrated. For example, the glycolytic pathway (Sigel et al, 1969), tricarboxylic acid cycle (Barnes et al., 1986), electron transport , and lipid metabolism (Srere, 1987).

1.6.2 Lipid Metabolism Metabolon

The FAS of eukaryotic cells (plant cells excluded) are complexes of multifunctional proteins and can be divided into two groups, exemplified by the FAS isolated from animal tissues and yeasts. The animal FAS (M_r 500 000) consists of two identical subunits (α_2) while the yeast FAS is a complex of two non-identical subunits, α (M_r 213 000) and β (M_r 203 000), with an apparent M_r of 2.4×10^6 ; it is an $\alpha_6\beta_6$ structure (Wakil et al., 1983). In bacteria and plants, the synthesis of fatty acids is carried out by seven individual enzymes (Srere, 1987).

ACL in eukaryotic cells exists as a multifunctional protein; one that contains two or more different catalytic centres on a single polypeptide

chain. In ACL activity, two distinct steps are involved: the formation of citryl-CoA and the cleavage of citryl-CoA to form acetyl-CoA and oxaloacetate. In bacteria, two separate polypeptides are involved in this process but in fungi and other eukaryotes, these two polypeptides are covalently linked. The molecular weight of ACL from yeasts, *Lipomyces starskeyi* (Boulton and Ratledge, 1983) and *Rhodosporidium toruloides* (Evans and Ratledge, 1985a) have been reported to be 510 000 and 480 000, respectively.

ACC from animal tissues has been isolated as a tightly bound multienzyme complex. The active enzyme consists of a polymer (M_r 4 - 8 million) that can be dissociated into inactive protomers (M_r 400 000). In bacteria and plants, the enzyme is dissociable into three components. ACC from *S. cerevisiae* and *Candida lypolytica* were reported to occur in a tetrameric structure (α_4) with subunit M_r of 190 000 and 230 000 respectively (Wakil et al., 1983).

Furthermore, ACL, ACC and FAS exist as a single multienzyme complex in animal tissues (Gillevet et al, 1982). These enzymes have also been shown to bind to microsomes in rat when 40 % - 90 % ACC, 30 % - 70 % ACL and 20 % - 60 % FAS were found in the microsomal fraction of rat liver. This suggests the microsome as being a major site for localization of the fatty acid synthesis metabolon. Malic enzyme, being reported as a potential provider of NADPH in fungi (Wynn et al., 1997; Wynn and Ratledge, 1997) and yeasts (Brinenberg et al., 1983) has also been shown to exist in a membrane-bound form in oleaginous fungi, *M. circinelloides* (Kendrick and Ratledge, 1992b).

Although the enzymatic regulation of fatty acid biosynthesis in yeasts has been well established, no evidence has been demonstrated so far on the possibility of the existence of fatty acid biosynthesis metabolon involving ACL, FAS, ACC and possibly ME .

1.7.0 Objectives of Research.

Apart from the earlier findings mentioned in Section 1.5.0 regarding to the biochemistry of lipid accumulation in oleaginous fungi, no further development has been reported. As oleaginous fungi have been used intensively in the commercial production of high-valued PUFAs, the understanding of the lipid biosynthesis regulation occurring in these organisms is vital for further optimization and enhancement of production. This research therefore attempts to elucidate the enzymatic regulation of lipid biosynthesis occurring in oleaginous fungi. Two filamentous fungi have been chosen for the work: *Mucor circinelloides* and *Mortierella alpina* .The work also involved the study of the role of ME in the lipid biosynthesis of both fungi as it has been suggested as a major provider of NADPH for lipid biosynthesis in several fungi (see Section 1.2.1).

CHAPTER 2

GENERAL

MATERIALS AND METHODS

2.1.0 Fungal Cultivation

2.1.1 Micro-organisms

The fungal strains examined were *Mucor circinelloides* CBS 108.16 and *Mortierella alpina* peyron CBS 696.70 .

2.1.2 Maintenance of micro-organisms

M. circinelloides and *Mt. alpina* were maintained on potato-dextrose-agar (PDA)(Difco) at 4 ^{o}C and subcultured monthly.

2.1.3 Growth media.

M. circinelloides and *Mt. alpina* were cultivated in a semi defined, nitrogen-limiting medium (Kendrick and Ratledge, 1992a) at 30 ^{o}C. The medium constituents are as stated below (g / l) :

glucose, 30 ; ammonium tartrate, 3.3 ; KH_2PO_4, 7.0 ; Na_2HPO_4, 2.0 ;$MgSO_4.7H_2O$, 1.5 ; yeast extract, 1.5 ; $CaCl_2$, 0.1 ; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 1.5; $CuSO_4.5H_2O$,0.0001; $Co(NO_3)_2.6H_2O$, 0.0001 ; $MnSO_4.5H_2O$, 0.0001.

The medium was modified to provide different carbon and nitrogen ratios in several experiments and sterilized at 121 ^{o}C for 20 minutes.

2.1.4 Growth of fungi in liquid media

M. circinelloides and *Mt. alpina* were grown either in 1 L stirred bottles (whirlipots)(Marshall et al., 1973), with final working volume of 800 ml or in 5 L fermenters with a final working volume of 4 L. A vortex was created in the stirred bottles by a magnetic bar spinning at 600 rev / min. Fermenters were stirred at 600 rev / min and the pH was maintained at 6.0 by automatic addition of 2 M NaOH and 2 M HCl. Air was supplied at 0.5 v / v / min and the temperature was controlled at 30 ^{o}C by circulating water through an internal coil in the fermenter.

Seed cultures of *M. circinelloides* were prepared by the inoculation of 1 L stirred bottles containing 500 ml of nitrogen-limiting media with a mycelia transferred from a stock culture grown on PDA. The seed cultures were grown for 16 h at 30 ^{o}C. A 5 % (v / v) inoculum was then used to inoculate the stirred bottles and fermenters. Seed cultures of *Mt. alpina* were prepared by the inoculation of 1 L Erlenmeyer flasks containing 400 ml of nitrogen-limiting media with mycelia transferred from a stock culture grown on PDA. The seed cultures were grown for 72 h at 30 ^{o}C . These were used *in toto* to inoculate the 5 L fermenter vessels.

2.2.0 Dry Weight Determination

Biomass was determined by filtering 20 ml culture under reduced pressure through a pre-weighed Whatman No 1 filter paper. The filter papers had been washed with distilled H_2O and dried at 100 ^{o}C for a

minimum of 24 hours prior to their weight being determined. Fungal biomass retained on the filter paper was washed with 2 volumes of distilled water before drying at 100 ^{o}C overnight in an oven.

2.3.0 Lipid Assay

2.3.1 Extraction of lipid from fungi

Lipid was extracted using method of Folch et. al. (1957) :
100 ml of culture was harvested by filtering through a Whatman No 1 filter paper under reduced pressure and washed with one initial volume of cold distilled H_2O. Mycelia were then freeze-dried overnight and ground to a powder in a pestle and mortar. The weight of the ground mycelia was determined and then all were added to 150 ml chloroform / methanol (2:1 v / v) and held overnight at room temperature. The material was filtered through a Whatman No 1 filter paper to remove cell debris and the organic fractions were pooled and washed with distilled H_2O (3 x 150 ml). The chloroform extract was rotary evaporated. The lipids remaining were dissolved in 10 ml diethyl ether, transferred to a pre-weighed vial and the dietyl ether removed under stream of N_2. The sample was dried in a vacuum desiccator over P_2O_2 for 24 h and reweighed.

2.3.2 Preparation of fatty acid methyl esters (FAMES) for gas chromatography using trimethylsulphonium hydroxide (TMSF)

The lipid material (20 - 30 mg) was dissolved in 400 µl chloroform

and transesterified by the addition of 400 µl of TMSH (Butte, 1983). The reaction was carried out for 5 min at room temperature after which the samples were either analysed immediately or stored at 4 ^{o}C for up to 7 days.

2.3.3 Gas chromatography of FAMES

FAMES were analysed using a Phillips PU 4500 gas chromatograph fitted with an integrator and flame ionisation detector. Separation was achieved by using a 10 % (w / w) diethylene glycol succinate column with N_2 as the carrier gas (35 ml / min). The column was maintained at 200 ^{o}C with both detector and injector port at 230 ^{o}C. Peaks were identified by comparison with authentic standards (Sigma).

2.4.0 Production of Cell Free Extracts and Microsomes

Harvested fungal mycelia were resuspended in an extraction buffer to give approximately 20 % (wet weight / v) mycelial suspensions. Unless otherwise stated, extraction buffer used was as stated below :

100 mM KH_2PO_4 / KOH pH 7.5 containing 20 % (w / v) glycerol, 1 mM each of benzamidine, mercaptoethanol, PMSF and EDTA . 0.01 % (v / v) protease inhibitor cocktail (Sigma) containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) and 1, 10-phenanthroline, was included prior before use.

The mycelial suspension was passed twice through a cold (4 ^{o}C) French pressure cell at a pressure of 35 Mpa. The mycelial suspension was placed in an ice-cold beaker between each pass to avoid excessive heating of the sample. French pressed extracts were centrifuged at 16 000 g for 20 min at 4 ^{o}C to remove cell debris. Floating layers of lipid were removed by filtration of the crude extracts through a Whatman No 1 filter paper. The filtrate was recentrifuged at 100 000 g for 1 h at 4 ^{o}C. The supernatant was termed the soluble fraction and the pellet was termed the microsomal fraction. Both the soluble fraction and the microsomes were stored at 0 ^{o}C prior to analysis.

2.5.0 Enzyme Assays

Unless otherwise stated, all enzyme assays were carried out using a single-beam Philips PU 8625 recording uv / vis spectrophotometer. All assays were carried out at 30 ^{o}C

2.5.1 ATP:Citrate lyase (EC 4.1.3.8)

The method of Srere (1962) was used. The following were added to a 1 ml cuvette:

0.1 M Tris / HCl pH 8.2, 0.1 ml ; 10 mg CoA / ml , 0.03 ml ; 0.1 M dithiothreitol, 0.1 ml ; 12 mM NADH, 0.015 ml; 100 units malate dehydrogenase / ml, 0.05 ml ; cell free extract and water, 0.44 ml; 0.1 M sodium azide, 0.015 ml. The reaction was initiated by the addition of 0.1 M ATP, 0.05 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 10 %.

2.5.2 Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

The method of Langdon (1966) was used. The following were added to a 1 ml cuvette:

1 M Tris / HCl pH 7.5, 0.5 ml ; 2 mM NADP, 0.1 ml ; cell free extract and water, 0.2 ml. Reaction was initiated by the addition of 0.1 M glucose 6-phosphate, 0.1 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 9 % .

2.5.3 6-Phosphogluconate dehydrogenase (EC 1.1.1.44)

The method of Potremoli and Grazi (1966) was used. The following were added to a 1 ml cuvette:

50 mM Tris / HCl pH 7.6, 0.5 ml ; 0.1 M $MgCl_2$, 0.2 ml ; 10 mM NADP, 0.03 ml; cell free extract and water , 0.26 ml. The reaction was initiated by the addition of 0.15 M 6 - phosphogluconate , 0.01 ml. The reaction was followed at 340 nm for approximately 5 min. Activity measurements were generally reproducible to within 10 %.

2.5.4 Malate dehydrogenase (EC 1.1.1.37)

The method of Ochoa (1955) was used. The following were added to a 1 ml cuvette ;

0.1 M KH_2PO_4 / KOH pH 7.5, 0.94 ml ; 12 mM NADH 0.017 ml ; cell free extract, 0.01 ml. Reaction was initiated by the addition of 12 mM OAA, 0.03 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 7 % .

2.5.5 Malic enzyme (EC 1.1.1.40)

The method of Hsu and Lardy (1969) was used. The following were added to a 1 ml cuvette :

0.1 M KH_2PO_4 pH 7.5, 0.82 ml ; 0.1 M $MgCl_2$, 0.03 ml ; 12 mM NADP, 0.02 ml. Reaction was initiated by the addition of 0.5 M malate, 0.07 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 6 %.

2.5.6 $NADP^+$:Isocitrate dehydrogenase (EC 1.1.1.42)

The method or Kornberg (1955) was used. The following were added to a 1 ml cuvette :

0.1 M KH_2PO_4 / KOH pH 7.0, 0.5 ml ; 5 mM $MgCl_2$, 0.05 ml ; 0.4 mM NADP, 0.1 ml ; cell free extract and water , 0.2 ml. Reaction was initiated by the addition of 0.1 M sodium isocitrate, 0.1 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 5 % .

2.5.7 NAD^+:Isocitrate dehydrogenase (EC 1.1.1.41)

The method of Kornberg (1955) was used . The following were added to a 1 ml cuvette :

0.1 M Tris / HCl pH 7.0, 0.53 ml ; 0.1 M $MgCl_2$, 0.05ml ; 10 mM AMP, 0.1 ml ; 20 mM NAD, 0.02 ml ; cell free extract and water , 0.25 ml. Reaction was initiated by the addition of 0.1 M isocitrate, 0.05 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 5 % .

2.5.8 Carnitine acetyl transferase (EC 2.3.1.7)

The method of Kawamoto et al. (1978) was used. The following were added to a 1 ml cuvette :

0.25 mM KH_2PO_4 / KOH pH 7.8, 0.1 ml ; 0.3 mM acetyl CoA in 50 mM Hepes pH 7.0, 0.3 ml ; 12 mM dithiopyridine, 0.1 ml ; 1M KCl, 0.1 ml ; cell free extract and water, 0.35 ml. The reaction was initiated by the addition of 40 mM carnitine, 0.05 ml. The reaction was followed at 324 nm for approximately 5 min . Activity measurements were generally reproducible to within 10 % .

2.5.9 Fatty acid synthase (EC 2.3.1.86)

Spectrophotometric assay :

The method of Lynen (1969) was used. The following were added to a 1 ml cuvette :

1 M KH_2PO_4 / KOH pH 6.5, 0.2 ml ; 1 mg BSA / ml, 0.3 ml ; 0.5 M cystine, 0.02 ml ; 5 mM acetyl CoA, 0.012 ; 0.017 M NADPH, 0.01 ml ; 0.01 M EDTA, 0.25 ml ; cell free extract and water, 0.187 ml. The reaction was initiated by the addition of 10 mM malonyl CoA , 0.02 ml. The reaction was followed at 340 nm for approximately 5 min . Activity measurements were generally reproducible to within 12 %.

Radioassay :

Reaction mixture containing the same final concentration of each component stated above was prepared in a test tube to a final volume of 1.5 ml. The mixture was then incubated for 3 minutes in a waterbath at 30 ^{o}C, before starting the reaction by adding 0.03 ml cold 10 mM malonyl-

CoA and 5 ul of 54 mCi / mmol [2-^{14}C] malonyl-CoA (Amersham) (0.1uCi). After 15 minutes incubation, the enzyme reaction was stopped by adding 1 ml 10 % (w / v) methanolic KOH and 2.5 micromoles each of palmitic and stearic acids as carrier materials. The solution was acidified with 1 ml 6 M H_2SO_4 then extracted 4 times with 3 ml petroleum ether. The combined petroleum ether extracts containing the fatty acids were transferred to a scintillation vial and the solvent is evaporated in a water bath . The radioactivity of the sample was then measured in a scintillation counter. Activity measurements were generally reproducible to within 12 %.

2.5.10 Acetyl-CoA carboxylase. (EC 4.1.1.9)

A modified method based on the method of Mathews et al. (1990) was used. The following were added into a scintillation vial:
200 mM Tricine buffer pH 8.0, 0.05 ml ; 0.1 M $MgCl_2$, 0.005 ml ; 0.5 M KCl, 0.02 ml ; 0.01 M DTT, 0.02 ml ; 0.05 M ATP, 0.005 ml ; cell free extract, 0.05 ml. The mixture was preincubated for 3 min at 30 ^{o}C then initiated by the addition of 10 mM (containing 0.2 µCi) $H^{14}CO_3^-$. Reaction was stopped by the addition of 4 M HCl, 0.05 ml then dried under a stream of air at 60 ^{o}C. The residue was dissolved in 0.5 ml 50 % (v / v) ethanol and scintillation fluid was added for counting. Activity measurements were generally reproducible to within 17 %.

2.5.11 AMP:deaminase (EC 3.5.4.6)

The method of Yoshino et al. (1981) was used. The following was added into a 1 ml cuvette :
40 mM cacodylate buffer pH 7.1, 0.25 ml ; 10 mg BSA / ml, 0.02 ml ; 0.4 M

KCl, 0.25 ; 0.16 M NaCl, 0.06 ml ; 0.1 M $MgCl_2$, 0.01 ml ; 0.04 M ATP, 0.025 ml ; cell free extract and water, 0.36 ml. The reaction was initiated by the addition of 0.025 ml of 0.2 M AMP and stopped after 10 minutes by placing the cuvettes immediately in cold water (4 ^{o}C). Ammonium concentration determination was then carried out for each reaction mixtures by indophenol method (see Section 2.6.0). Activity measurements were generally reproducible to within 15 %.

2.6.0 Ammonium Determination.

Ammonium was determined by the indophenol method (Chaney and Marbach, 1962).

5 ml each of reagent A (10 g phenol/ l and 50 mg sodium nitroprusside / l) and reagent B (5 g NaOH / l and 420 mg sodium hypochloride / l) were added to 1 ml of sample (diluted so that they contain less than 0.1 mg ammonium / ml).The reaction mixture was mixed by inversion and incubated at room temperature for 30 min. The A_{625nm} was determined against reagent blank. Ammonium tartrate was used as standard.

2.8.0 Glucose Determination.

Glucose concentration was measured by using Boehringer GOD-PERID test kit.

2.9.0 Protein Determination.

Protein was measured by using the method of Bradford (1976). Bovine serum albumin was used as standard.

CHAPTER 3

OPTIMIZATION OF GROWTH AND LIPID PRODUCTION IN *Mucor circinelloides.*

INTRODUCTION

This chapter discusses a series of preliminary experiments aimed at optimizing the growth and lipid production of *M. circinelloides* prior to commencing the biochemical studies on the regulation of lipid accumulation. The work involved the investigation of the growth and lipid production profiles of this organism when subjected to various culture conditions in order to determine the optimal conditions for good growth and lipid production.

3.1.0 The Effect of Inoculum Size on Growth and Lipid Accumulation in Stirred Bottles (Whirlipots)

3.1.1 Objectives

The main objective of this experiment was to investigate the effect of using different inocula sizes on the growth and lipid production of *M. circinelloides* in stirred bottles (Marshall et al., 1973). This organism is known to have the potential of producing up to 25 % (w / w of biomass) lipid with GLA content achieving 18 % of the total fatty acids when grown in a fermenter (Kendrick, 1992). This experiment therefore aimed at achieving a similar production of lipid by this organism when growing in stirred bottles, which, if successful, would significantly simplify the cultivation of the organism.

3.1.2 Materials and Methods

3.1.2.1 Cultivation of *M. circinelloides* in Stirred Bottles

M. circinelloides was grown in 1 L stirred bottles, with a final working volume of 800 ml. A vortex was created in the stirred bottles by a magnetic bar (approximately 3 cm in length) spinning at 600 rev / min. The temperature of the cultures were maintained at 30 ^{o}C by placing the stirred bottles in a water-bath.

Three separate bottles (Whirlipots 1, 2 and 3) were inoculated aseptically with different volumes of the seed culture. In this experiment, Whirlipot 1, 2 and 3 were performed with a nitrogen-limiting medium (see Section 2.1.3) with a C:N ratio at 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l). Throughout the experiment, dissolved O_2 concentration was measured continuously by using a galvanic O_2 electrode which was inserted into the bottles and connected to a chart recorder. Samples were taken at intervals throughout the experiment for the determination of culture pH, glucose and ammonium concentration, biomass and cell lipid content using methods described in Chapter 2. The fermentation was stopped at 70 h.

3.2.0 Results and Discussion

All three cultures in the Whirlipots showed a similar growth and lipid accumulation profile. In Whirlipot 1, which was inoculated at 1 % (v / v), the biomass increased rapidly from 3.8 g / l at 10 h to 6.6 g / l at 35 h.

During this period there was a decrease in culture pH (Figure 7a), ammonium concentration (Figure 7a) and dissolved O_2 (Figure 7b). The culture became anaerobic with the dissolved O_2 dropping from 95 % saturation at the beginning of the experiment to 2 % between 22 h to 28 h coincident with the decrease of glucose and a rapid increase of biomass. The total lipid increased from 3 % at 15 h to 4.8 % (w / w of biomass) at 33 h during the period of glucose utilization (graph not plotted). Ammonium was still present (0.6 g / l) at the end of the experiment .

In Whirlipot 2, which had a 5 % (v / v) inoculum, the culture showed a similar trend of growth as in Whirlipot 1 where the biomass increased rapidly from 0.5 g / l at 6 h to 7 g / l at 31 h coincident with the decrease of ammonium concentration from 3.3 g / l to 0.5 g / l (Figure 8a). As in Whirlipot 1, ammonium could still be detected (0.5 g / l) at the end of the experiment. An anaerobic phase also occurred in this culture where the dissolved O_2 in this culture dropped rapidly from 95 % at the beginning of the experiment to 2 % at 9 h and remained at this level until 24 h (Figure 8b). It later increased up to 40 % at 31 h after exhaustion of the culture glucose and the cessation of growth. The pH of the culture dropped from 6.0 to 4.6 at 24 h and remained constant until the end of the fermentation (Figure 8a). The total lipid of the culture increased from 3 % at 15 h to 4.5 % (w / w of biomass) at 31 h (graph not plotted).

The culture in Whirlipot 3, which received a 10 % (v / v) inoculum, showed a similar pattern of growth and lipid production as observed in Whirlipot 1 and 2. Again, an anaerobic condition was observed as the dissolved O_2 decreased from 93 % at the beginning of the experiment to

Figure 7: Growth profile of *M. circinelloides* grown in stirred bottles with 1 % (v / v) inoculum .

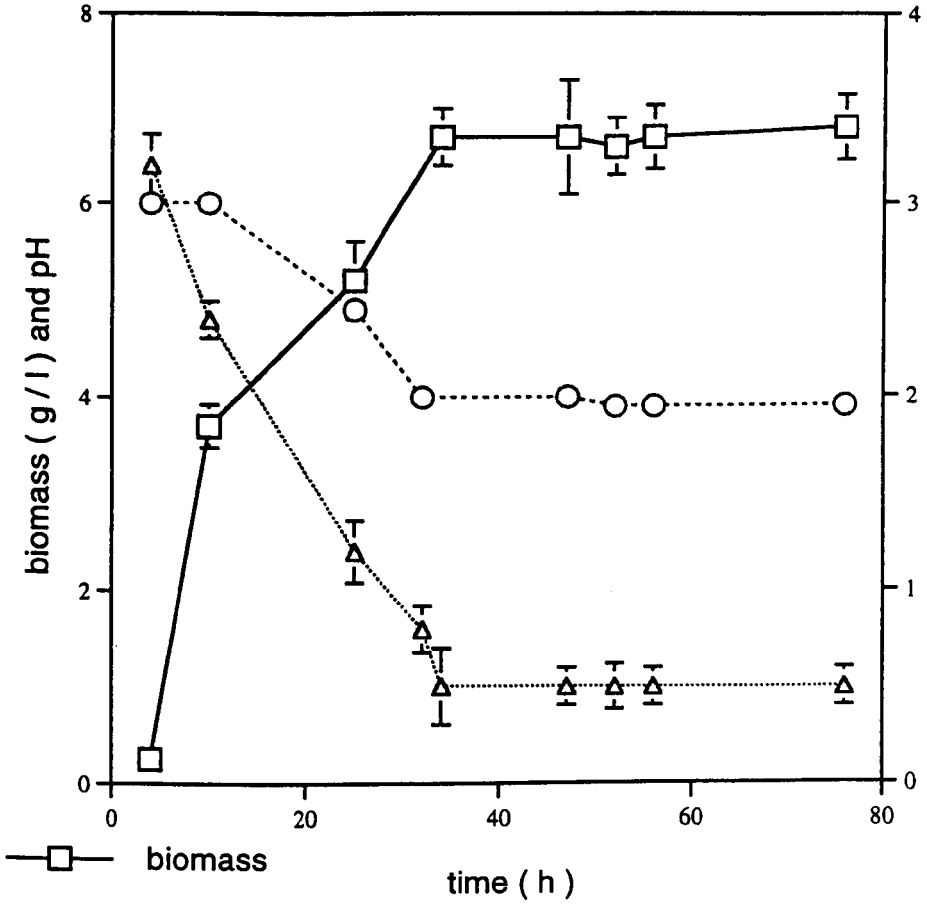
a) pH, biomass and ammonium concentration vs time.

b) glucose and dissolved O_2 vs time.

A 1 % (v / v) inoculum was added into 1 L stirred bottles containing 800 ml of nitrogen-limiting media (see Section 2.1.3) with a C:N ratio of 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The experiment was performed at 30 ^{o}C for 78 h with the culture being agitated at 600 rev / min.

Figure 7

a)


biomass (g / l) and pH
time (h)
biomass
ammonium
PH

b)

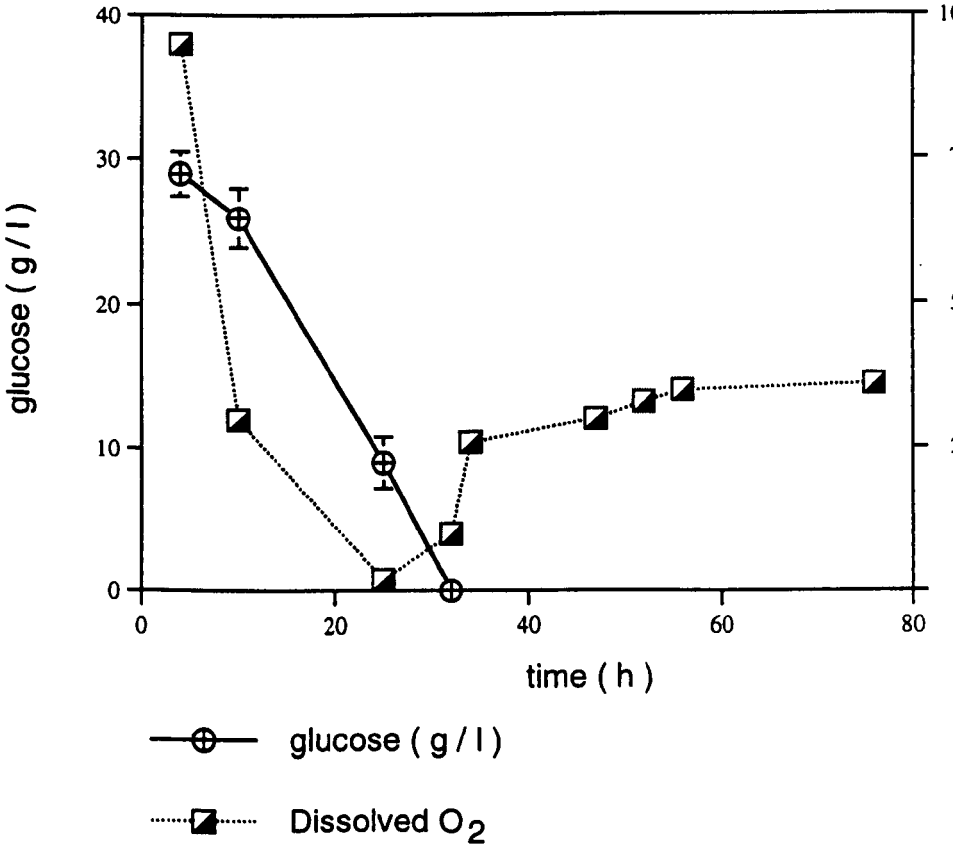

glucose (g / l)
time (h)
glucose (g / l)
Dissolved O_2

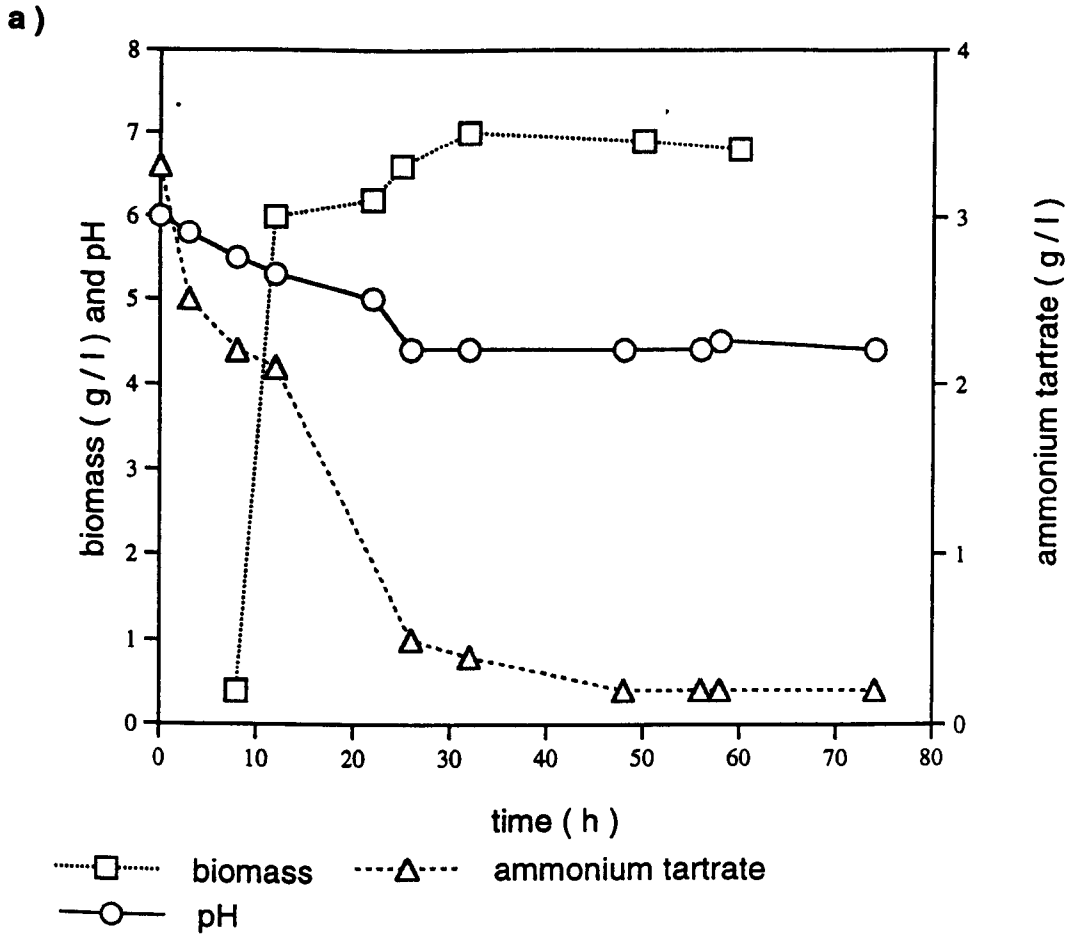
Figure 8: Growth profile of *M. circinelloides* grown in stirred bottles with 5 % (v / v) inoculum .

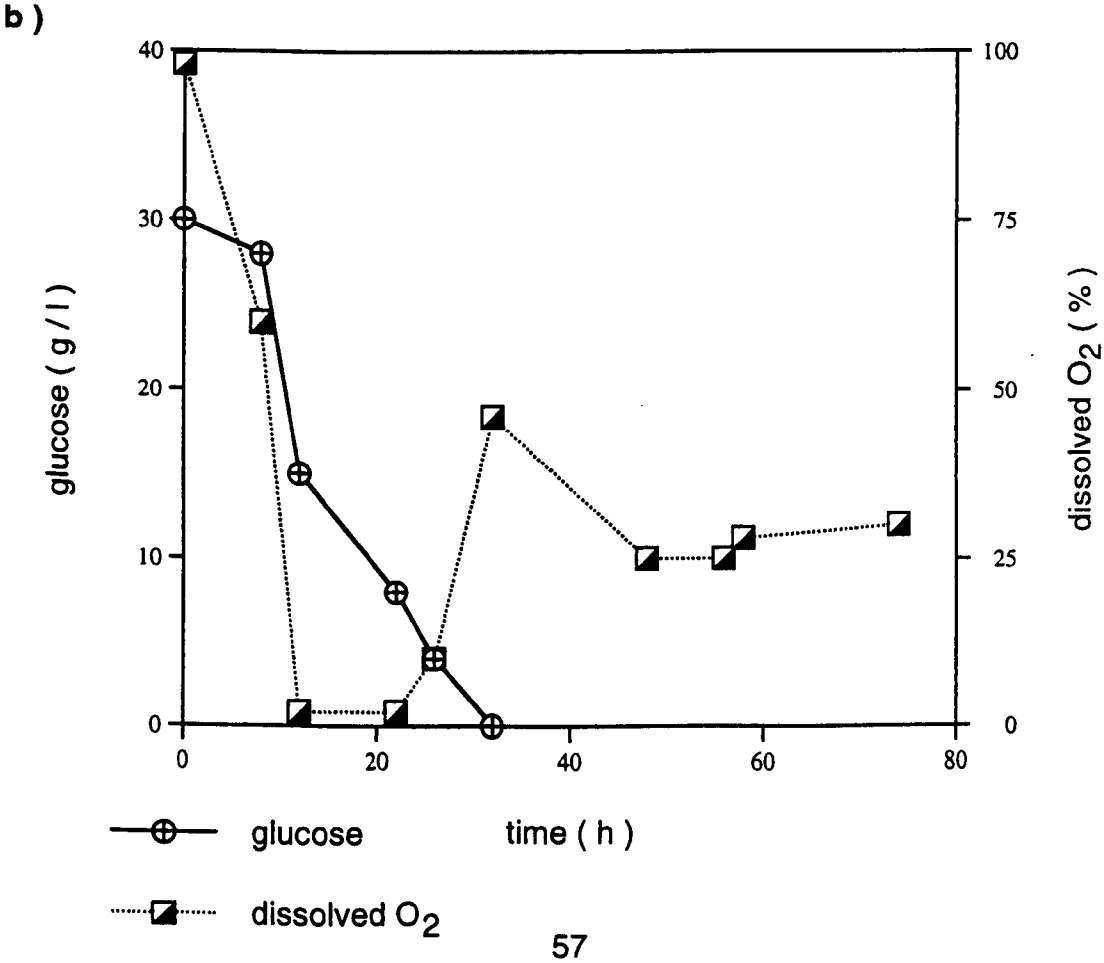
a) pH, biomass and ammonium concentration vs time.

b) glucose and dissolved O_2 vs time.

A 5 % (v / v) inoculum was added into 1 L stirred bottles containing 800 ml of nitrogen-limiting media (see Section 2.1.3) with a C:N ratio of 24:1 (30 g glucose / l and 3.3 gammonium tartrate / l). The experiment was performed at 30 ^{o}C for 78 h with the culture being agitated at 600 rev / min. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 6.


a)
biomass (g / l) and pH
ammonium tartrate (g / l)
time (h)
biomass
ammonium tartrate
pH


b)
glucose (g / l)
dissolved O_2 (%)
time (h)
glucose
dissolved O_2

1 % at 9 h (Figure 9a) and remained at that level until exhaustion of glucose after which the dissolved O_2 increased to above 20 % (Figure 9b). The total lipid of the culture increased from 3.5 % at 15 h to 4.8 % (w / w of biomass) at 27 h (graph not plotted) during the period of glucose utilization and cell growth (as measured by increasing cell dry wt) . All three cultures showed a similar fatty acid profiles where the most abundant fatty acid was 18:1, followed by 18:3, 18:2, 16:0, 18:0 and 14:0 (Table 4a).

These results show that there was no significant differences in the growth pattern and lipid production of *M. circinelloides* in stirred bottles using different sizes of inocula (Table 4b). Although a high percentage of 18:3 (26 - 30 %, of the total fatty acids) was produced (Table 4a), all three cultures produced a low cell lipid yield (approximately 5 % w /w of biomass)(Table 4b). The low lipid production of the stirred bottle cultures was due to the early exhaustion of glucose in the media while ammonium remained available until the end of the experiment. This meant that nitrogen exhaustion is vital for triggering lipid biosynthesis (Botham and Ratledge, 1979; Boulton and Ratledge, 1984; Wynn and Ratledge, 1997). Therefore, lipid accumulation was obviously not being achieved.

The decreased pH of the culture was thought to be the result of secretion of organic acids into the culture during growth. *Mucor* and many soil-inhabitating fungi such as *Fusarium* (Imshenetsky and Ulianowa, 1962), *Trichoderma*, *Aspergillus*, *Paecilomyces*, *Penicillium* are known to produce organic acids during growth (Evans and Bucke, 1998). The stabilization of pH at 4.0 was therefore due to the exhaustion of glucose which resulted in the cessation of organic acid production. These

Figure 9: Growth profile of *M. circinelloides* grown in stirred bottles with 10 % (v / v) inoculum .

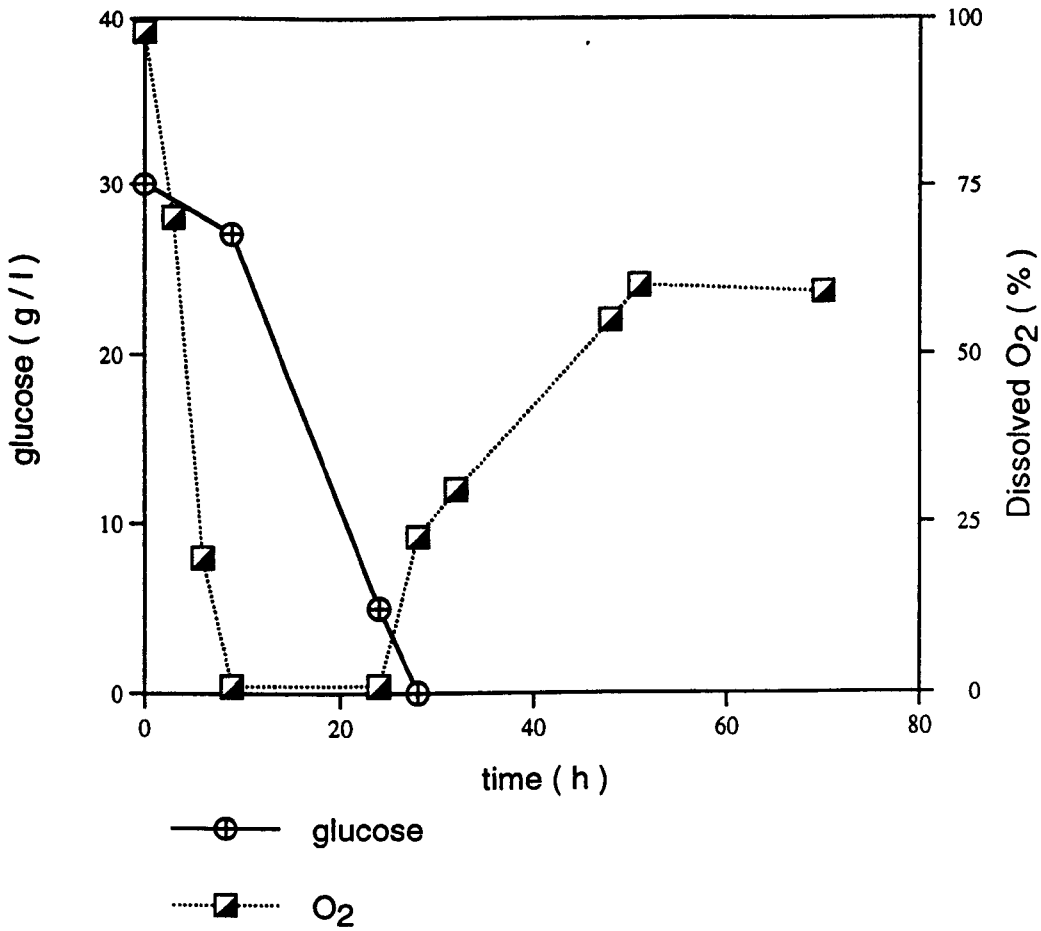
a) glucose and dissolved O_2 vs time.

b) pH, biomass and ammonium concentration vs time.

A 10 % (v / v) inoculum was added into 1 L stirred bottles containing 800 ml of nitrogen-limiting media (see Section 2.1.3) with a C:N ratio of 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The experiment was performed at 30 ^{o}C for 78 h with the culture being agitated at 600 rev / min. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 9:

a)


glucose (g / l)
Dissolved O_2 (%)
time (h)
glucose
O_2

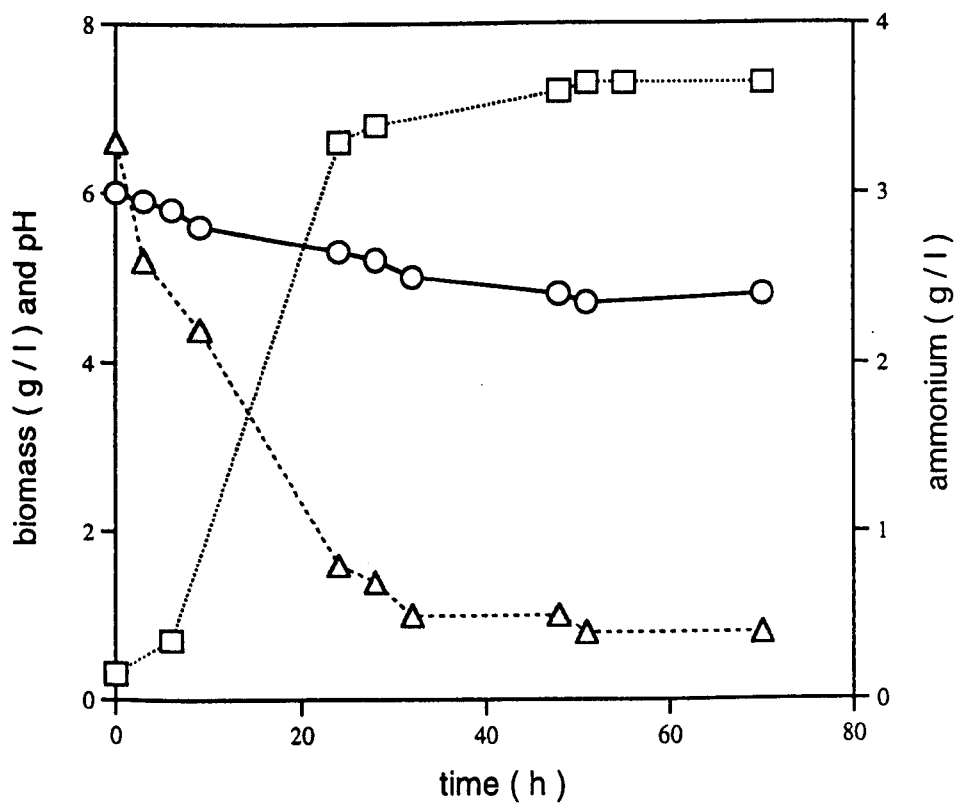

biomass (g / l) and pH
ammonium (g / l)
time (h)
biomass
ammonium
pH

Table 4a : Fatty Acid Profiles of *M. circinelloides* Grown in Stirred Bottles with Different Inocula Sizes.

Fatty Acids (%)	Inoculum Size (%, v / v)		
	1 (Whirlipot 1)	5 (Whirlipot 2)	10 (Whirlipot 3)
14:0	12-15	6-12	4-9
16:0	9-12	10-12	10-13
18:0	6-9	5-10	4-8
18:1	25-28	27-30	25-32
18:2	11-15	10-15	14-18
18:3	25-26	25-28	26-30

The fungus was grown in 1 L stirred bottles containing 800 ml of nitrogen-limiting media with a C:N ratio of 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The experiment was performed at 30 ^{o}C with the culture being agitated at 600 rev / min. A 1%, 5 % and 10 % (v / v) inoculum was used to inoculate Whirlipot 1, 2 and 3, respectively. Data presented were based on the analysis of samples from three replicates of each of the Whirlipot obtained after approximately 35 h of growth.

Table 4b: Growth profile of *M. circinelloides* Grown in Stirred Bottles with Different Inocula Sizes.

Inoculum Size (%, v / v)	Biomass of cells (g / l)	Total Lipid of Cells (% w/w, biomass)	Biomass Yield (g cells / g glucose used)	Max Lipid Yield (g lipid / g glucose used)	Ammonium at 70 h (g / l)
1 (Whirlipot 1)	5.4-6.6	4.6-4.8	0.18-0.22	0.01	0.4-0.6
5 (Whirlipot 2)	6.2-7.0	4-4.5	0.2-0.23	0.01	0.4-0.5
10 (Whirlipot 3)	6.5-7.3	4.5-4.8	0.22-0.24	0.01	0.4-0.5

The fungus was grown in 1 L stirred bottles containing 800 ml of nitrogen-limiting media with a C:N ratio of 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The experiment was performed at 30 ^{o}C with the culture being agitated at 600 rev / min. A 1%, 5 % and 10 % (v / v) inoculum was used to inoculate Whirlipot 1, 2 and 3, respectively. Data presented were based on the analysis of samples from three replicates of each of the Whirlipot approximately after 35 h of growth.

observations were similar as reported in the cultivations of *Fusarium moniliforme* (Wynn, 1994). The anaerobic phase which occurred at the early stage of fermentation may have affected the utilization of ammonium by the cultures, which caused the ammonium to be present until the end of the fermentation. The anaerobic phase was not the factor which led to the early depletion of glucose in the cultures as the rate of glucose utilization in the whirlipots slowed down during the anaerobic phase.

During cultivation of *M. circinelloides* in these stirred bottles, several operational problems were encountered. Firstly, as the agitation in stirred bottles was achieved by creating a vortex by means of a magnetic bar spinning at the bottom of the bottles, problems often occurred when the magnetic bar accidentally stopped spinning during the experiment. This occurred as a result of an uneven surface of the bottom of the stirred bottles, which obstructed the magnetic bar when it was operating. Some whirlipots possessed a convex bottom surface and this was also thought to cause the problem. Regular checking was therefore required. Experiments would be terminated if this problem occurred at night where the culture would be left unagitated overnight. The experiments shown here were carried out with duplicates for each whirlipot and repeated three times.

Another problem was the splashing of the cultures to the top of the bottles as a result of the high-speed stirring. This caused the cotton bung to become wet which affected the air flow through the cotton bung thus affecting the efficiency of aeration in the stirred bottles. The splashing of the culture also often resulted in a thick wall growth especially at the upper part of the bottles.

The small amount of media used in the stirred bottles (800 ml) also limited the frequency of sampling and also restricted the amount of culture taken for each sample. As a result, sampling for the lipid extraction assay could only be carried out twice for each whirlipot as a substantial amount of cells were required for this measurement. This problem was more pronounced when sampling was carried out at the early stage of fermentation due to a lower cell densities in the culture vessels.

3.3.0 Conclusions

1) *M. circinelloides* exhibited poor growth and lipid production when grown in whirlipots. This was due to the culture becoming carbon limited rather than nitrogen limited as was intended.

2) An anaerobic phase which occurred at the early stage of fermentation may have affected the utilization of ammonium which was still present at the end of the experiment.

3.2.0 The Effect of Increasing C:N Ratios and Improved Aeration on Growth and Lipid Production of *M. circinelloides*

3.2.1 Objectives

Previous results showed that *M. circinelloides* produced low content of lipids when grown in whirlipots. The cultures did not achieve a nitrogen-limited condition where glucose was exhausted early in the experiment and ammonia could still be detected until the late stage of incubation. An anaerobic phase which occurred at the early stage of incubation was thought to be the main factor which led to the development of this condition. Therefore, this experiment was designed to investigate the effect of improved aeration in stirred bottle cultures on the utilization of ammonium, growth and lipid production. Attempts were also made to prevent the glucose from being exhausted at the early stage of incubation by increasing its concentration in the medium.

3.2.2 Materials and Methods

3.2.2.1 Cultivation of *M. circinelloides* with Improved Aeration in Whirlipots

Improved aeration in whirlipot cultures was achieved by means of decreasing the final working volume of the medium and using a wide-necked whirlipots. Experiments were carried out in 1 L whirlipots containing a nitrogen-limiting medium (see Section 2.1.3) with a C:N ratio at 50:1 (62.5 g glucose / l and 3.3 g ammonium tartrate / l) with 4

different final working volumes: 300 ml (Whirlipot A), 400 ml (Whirlipot B), 500 ml (Whirlipot C) and 600 ml (Whirlipot D). Therefore, aeration in Whirlipot A was more efficient compared to Whirlipot B, C and D. A vortex was created in the whirlipots by using a larger magnetic bar (approximately 5.0 cm) compared to what was used in the previous experiment (3.0 cm) spinning at a lower speed (approximately 500 rev / min). This was to overcome the problem of wet cotton-bung which was caused by the splashing of the culture due to the high-speed stirring . At this speed, the vortex created reached the bottom of the stirred bottles. To overcome the faulty stirring problem observed in the previous experiments, only whirlipots which possessed a specially flattened bottom surface were used. The temperature of the cultures was maintained at 30 ^{o}C by placing the whirlipots in a water-bath.

All four whirlipots (Whirlipot A, B, C and D) were aseptically inoculated with 5 % (v / v) of seed culture. Samples were taken at intervals for determination of biomass, glucose, ammonium and lipid production. The experiment was stopped after 48 h of incubation. The experiment was repeated three times .

3.2.3 Results and Discussion

Table 5 summarizes the effect of improved aeration and an increasing C:N ratio on ammonium utilization, biomass formation and total lipid production of *M. circinelloides* cultures grown in the whirlipots. Culture in Whirlipot A produced the highest biomass concentration followed by Whirlipot B, C and D . The culture in Whirlipot A also showed a higher biomass and lipid yield compared to cultures in Whirlipot B, C and D (Table 5).

Table 5: Differences of Growth profile of *M. circinelloides* Grown in Whirlipots at Different Final Working Volumes.

Final Working Volumes (ml)	Total Lipid (%)	biomass (g / l)	Ammonium at 48 h (g / l)	Glucose at 48 h (g / l)	Biomass Yield (g/g glucose used)	Lipid Yield (g/g glucose used
300 (Whirlipot A)	2.7-3	6.6-7	0.3-0.4	5-7	0.11-0.12	0.003-0.004
400 (Whirlipot B)	2.4-2.7	5-5.8	0.5-0.6	4.6-6	0.07-0.10	0.002-0.003
500 (Whirlipot C)	2.5-2.7	4.7-5	0.8-0.9	4.6-5	0.05-0.09	0.002
600 (Whirlipot D)	1.7-2.1	4.1-4.8	1.0-1.2	5-5.2	0.07-.08	0.001-0.02

The fungus was grown in 1 L stirred bottles containing a nitrogen-limiting medium with a C:N ratio of 50:1 (62.5 g glucose / l and 3.3 g ammonium tartrate / l) with four different final working volumes. The experiment was performed at 30 ^{o}C with the culture being agitated at 500 rev / min. Data presented were based on the analysis of samples from three replicates of each of the Whirlipot taken after 48 h growth.

The glucose concentration measured at 48 h was similar in all Whirlipots. Culture in all Whirlipots produced a similar lipid content (Table 5). When the experiment was terminated at 48 h, culture of Whirlipot D contained the highest ammonium concentration followed by Whirlipot C, B and A . This indicates that growth and ammonium utilization was more efficient in Whirlipot A which was better aerated than the cultures of Whirlipot B, C and D. This result suggests that inefficient aeration in stirred bottles was the main factor which led to an inefficient utilization of ammonium by the cultures. The lower speed stirring of the Whirlipots managed to decrease the problem of wet cotton-bung where less splashing of the medium was achieved due to the slower agitation speed performed and less wall growth was observed. The use of whirlipots with a flattened bottom surface did improve the operation of the magnetic stirrer where out of twelve whirlipots used, only one was faulty.

3.2.4 Conclusions

1) Less well aerated cultures utilized ammonium more slowly than a culture which was better aerated. Therefore, the undepleted ammonium observed in whirlipot cultures in previous experiment was due to the anaerobic phase which occurred at the early stage of incubation due to the inefficient aeration of the system.

2) An optimal conditions for lipid synthesis was still unachievable although cultivation was carried out with improved aeration of the whirlipot system.

3.3.0 The Effect of Increasing C:N Ratio and Decreasing Yeast Extract Concentration on Growth and Lipid Production of *M. circinelloides* in Whirlipots

3.3.1 Objectives

As observed in previous experiments, improved aeration in whirlipots led to a better utilization of ammonium by the cultures. However, a nitrogen-limited condition was still not achieved in the cultures where ammonium was present after 48 h of incubation while glucose in the medium was nearly exhausted. Therefore, attempts were made to further optimize the culture conditions by increasing the glucose concentration in the medium to prevent it from being exhausted whilst ammonium was still present. As the yeast extract used (Difco) contains a significant amount of nitrogen (approximately 10 %, w / w of biomass), its concentration in the medium was decreased ie. from 1.5 g / l to 0.2 g / l. The concentration of ammonium tartrate used was not decreased (3.3 g / l) as this fungi already showed a low biomass formation when performed at this concentration.

3.3.2 Materials and Methods

Experiments were carried out in 1 L wide-necked whirlipots containing a nitrogen-limiting medium (see Section 2.1.3) with C:N at 56:1 (70 g glucose / l and 3.3 g ammonium tartrate / l) and at 2 different final working volumes: 300 ml (Whirlipot E) and 600 ml (Whirlipot F). The concentration of yeast extract in the medium was decreased from 1.5 g / l to 0.2 g / l. A vortex was created in the stirred bottles by a magnetic bar (5.0 cm) spinning at 500 rev / min. The temperature of the cultures

was maintained at 30 ^{o}C by placing the whirlipots in a water-bath.

The whirlipots (Whirlipot E and F, each with 3 replicates) were aseptically inoculated with 5 % (v / v) of seed culture and samples were taken at intervals for determination of biomass, glucose, ammonium and lipid production.

3.3.3 Results and Discussion

Table 6 summarizes the growth and lipid production profile observed in both cultures. Culture in Whirlipot E showed a better growth performance than that observed in Whirlipot F where the biomass of cultures in Whirlipot E reached 6 g / l compared to Whirlipot F, which was only 4.2 g / l . The culture of Whirlipot E showed a higher biomass yield (0.21 g / g glucose used) compared to the culture in Whirlipot F which was 0.16 g / g glucose used. The final glucose concentrations in both cultures were similar at 66 h, being present at approximately 40 g / l (Table 6).

Ammonium utilization in Whirlipot E was better than Whirlipot F where it finally exhausted at 66 h in Whirlipot E while in Whirlipot F, it could still be detected at high concentration (0.9-1.2 g / l). No significant differences were observed in the production of lipid by both cultures where a similar lipid production and lipid yield were achieved (Table 6).

Table 6: Differences of Growth and Lipid Production Profile of *M. circinelloides* grown at different final Working Volume and with an Increasing C:N Ratio.

	Biomass at 66 h (g / l)	Total Lipid at 66 h (% w/w, biomass)	Biomass Yield (g / g glucose used)	Lipid Yield (g / g glucose used)	Ammonium at 66 h (g / l)	Glucose at 66 h (g / l)
Whirlipot (E) (300 ml)	5.1-6	7.3-8.4	0.17-0.21	0.014-0.016	nil	39-42
Whirlipot F (600 ml)	3.5-4.2	6.5-8.0	0.11-0.16	0.01	0.9-1.2	40-43

A 5 % (v / v) seed culture was added to Whirlipots E and F (each with three replicates) containing a nitrogen-limiting medium with a C:N ratio at 56:1 (70 g glucose / l and 3.3 g ammonium tartrate / l). The experiment was performed at 30 ^{o}C with the culture being agitated at 500 rev / min. Data presented were obtained from the analysis of samples from three replicates of each of the Whirlipot at 66 h.

These results suggest that despite the modifications of the culture conditions, it was still impossible to achieve conditions favourable for lipid accumulation. A different system which facilitated efficient aeration facilities was therefore needed in order to overcome this problem.

3.3.4 Conclusions

These results suggest that whirlipots were useless for the work with *M. circinelloides* and therefore were deemed unsuitable for further work. Consequently, all work was carried out using 5 L fermenters.

3.4.0 Growth and Lipid Production of *M. circinelloides* In a Fermenter

3.4.1 Objectives

The main objective of these experiments was to investigate the growth and lipid production of *M. circinelloides* grown in a fermenter. As observed in the previous experiments, whirlipots did not provide suitable conditions for lipid accumulation to take place as nitrogen exhaustion did not occur until the late stage of fermentation. This was caused by the cells becoming anaerobic which occurred at the early stage of incubation and consequently affecting the ammonium utilization of the culture. Therefore, attempts were made to eliminate this problem by growing this fungus in a fermenter where aeration could be achieved more efficiently. In a fermenter, efficient aeration of the culture was achieved by supplying air into the fermentation vessel at a controllable rate. The fermentation vessel was also equipped with baffles which increases turbulence when the culture was agitated thus enhanced the efficiency of the aeration.

The use of a fermenter would also eliminate several technical difficulties encountered when using whirlipots. For example, the agitation system in a fermenter which was achieved by the rotation of a fixed rotor would overcome the stirring problems which often occurred in whirlipots. The presence of a sampling outlet at the bottom of the fermenter allowed sampling of the cultures to be carried out more easily. Sampling could also be performed more frequently as the larger size of the fermenter allows a higher final working volume to be used.

3.4.2 Materials and Methods

Experiments were carried out using a 5 L fermenter (Fermenter 1) containing a nitrogen-limiting medium (see Section 2.1.3) with a C:N ratio at 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l) with a final working volume of 4 L . The fermenter vessel was equipped with baffles and it was stirred at 600 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min) and pH was maintained at 6.0 by automatic addition of 2 M NaOH and 2 M KOH. Temperature was maintained at 30 ^{o}C by circulating water through an internal coil of the fermenter. The fermenter was aseptically inoculated with 5 % (v / v) of seed culture. Dissolved O_2 was measured continuously throughout the experiment using a galvanic O_2 electrode which was inserted into the fermenter vessel and connected to a chart recorder. Samples were taken at intervals for lipid assay and determination of biomass, ammonium and glucose concentration using methods described in Chapter 2.

3.4.3 Results and Discussion

Figure 10a-b show the growth and lipid accumulation profile of *M. circinelloides* grown in a fermenter. The biomass increased rapidly from 1 g / l at 3 h to 14 g / l at 32 h (Figure 10a) coincident with the depletion of ammonium at 10 h (Figure 10a) and with the decrease of glucose (Figure 10a). The dissolved O_2 decreased from 95 % at 18 h to 46 % at 30 h (Figure 10b) coincident with the decrease of glucose concentration (Figure 10a) and the increase of biomass and lipid (Figure 10b). The dissolved O_2 increased from 46 % at 32 h to 70 % at 40 h after the glucose was exhausted. Table 7a summarizes the growth and lipid production profile of this culture along with the profile of *M. circinelloides* when grown in the same growth media in whirlipots (Whirlipot 3) (data transferred from Section 3.1.2.1). The culture of Fermenter 1 showed a higher production of lipid (12 %, w / w of biomass) compared to when it was grown in Whirlipot 3 where it produced only 4.8 % lipid (w / w of biomass). This was due to the efficient aeration in the fermenter which successfully prevented an anaerobic phase from occurring in the culture thus resulted in the early exhaustion of ammonium. Nitrogen deficiency is vital in triggering lipid biosynthesis. When a culture reached a nitrogen-limited condition, the synthesis of protein and nucleic acids ceases while the excess carbon is channelled into lipids (Botham and Ratledge, 1979; Boulton and Ratledge, 1984). This suggests the possibility of *M. circinelloides* in sharing a similar lipid biosynthesis regulation system as reported in oleaginous yeasts. Glucose exhaustion in Fermenter 1 occurred between 28 and 32 h, similar to that observed in Whirlipot 3, which was depleted between 27 and 33 h (Table 7a). The fatty acid profile of the

Figure 10: Growth profile of *M. circinelloides* cultivated in a fermenter

a) biomass, glucose and ammonium concentration vs time

b) total lipid and dissolved O_2 vs time

Fermenter 1 was performed using a nitrogen-limiting medium (see Section 2.1.3) with a C:N ratio at 24:1 (30 g glucose / l and 3.3. g ammonium tartrate / l) at 30 ^{o}C for 53 h. It was stirred at 600 rpm / min and the pH maintained at 6.0. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 10:

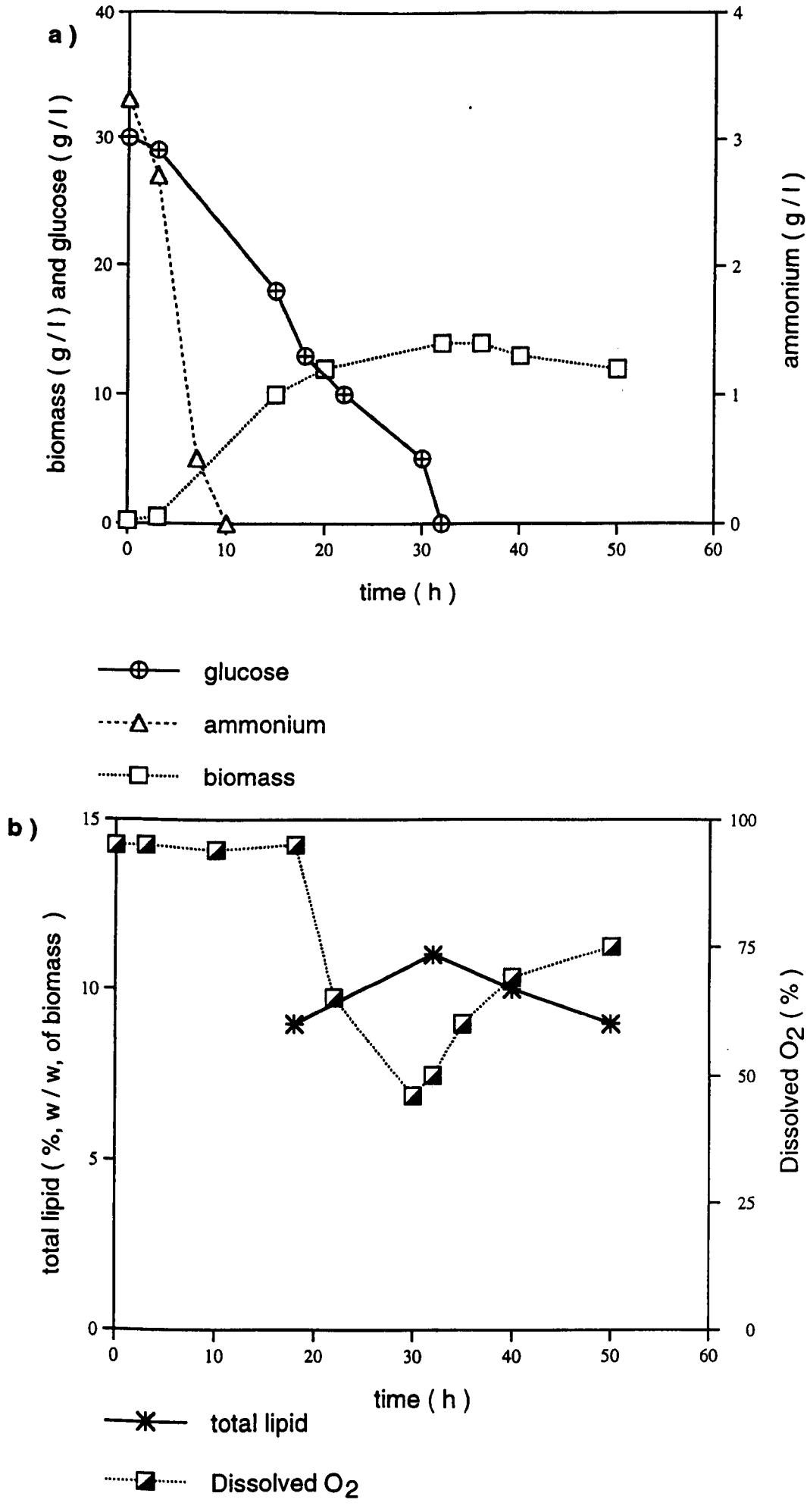

a)
biomass (g / l) and glucose (g / l)
ammonium (g / l)
time (h)
glucose
ammonium
biomass
b)
total lipid (%, w / w, of biomass)
Dissolved O_2 (%)
time (h)
total lipid
Dissolved O_2

Table 7a: Growth Profile of *M. circinelloides* Grown in a Fermenter and Stirred Bottles Performed with the same Growth Media at C:N 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l).

	Max Biomass (g / l)	Max. Total Lipid (%)	Biomass Yield g / g glucose used	Lipid Yield g / g glucose	Ammonium Depletion	Glucose Exhaustion
Fermenter 1	12-15	11-12	0.4-0.5	0.05-0.06	between 10-15 h	between 28-32 h
Whirlipot 3 (Data from Section 3.1.2.1)	6.5-7.3	4.5-4.8	0.22-0.24	0.01	undepleted at 48 h	between 27-33h

Fermenter 1 was performed with a nitrogen-limiting medium (see Section 2.1.3) with a C:N ratio at 24:1 (30 g glucose / l and 3.3. g ammonium tartrate / l) at 30 ^{o}C. Data presented were based on the analysis of samples from three replicates of Fermenter 1. Whirlipot 3 was performed in 1 L stirred bottles containing 800 ml of the same media at 30 ^{o}C.

Table7b: Differences of Fatty Acid Composition of *M.circinelloides* grown in a fermenter and stirred bottles.

Fatty Acids (%)	Fermenter 1	(Whirlipot 3)
14:0	7-13	4-9
16:0	6-10	10-13
18:0	10-12	4-8
18:1	26-30	25-32
18:2	14-16	14-18
18:3	22-25	26-30

Fermenter 1 were performed with a nitrogen-limiting medium (see Section 2.1.3) with a C:N at 24:1 (30 g glucose / l and 3.3. g ammonium tartrate / l) at 30 ^{o}C. Whirlipot 3 were performed in 1 L stirred bottles containing 800 ml of the same media at 30 ^{o}C. Data presented were based on the analysis of samples from three replicates of Fermenter 1 and Whirlipot 3 after approximately 30 h growth.

fermenter culture was similar to that observed in Whirlipot 3 (Table 7b) where the most abundant fatty acid was 18:1 followed by 18:3, 18:2, 18:0, 14:0 and 16:0. However, the content of 18:3 was lower in the fermenter culture (23 -25 % of the total fatty acids) compared to the culture of Whirlipot 3, which achieved 30 % of the total fatty acids).

Several problems have been encountered during the work with the fermenter. A thick wall growth was observed after 48 h of fermentation especially at the upper part of the fermentation vessel, around the impeller and between the baffles and the wall of the fermenter. The problem became more pronounced as the surface of the culture reached the same level as the impeller after sampling was carried out several times. This was as a result of the impeller rotating on the surface of the culture thus splashing the culture on the upper part of the vessel. The nature of the fungus, which was filamentous, also contributed to an easier adhesion of the cell to the wall of the fermenter.

Clumping of cells sometimes occurred which led to difficulties during sampling as the clumped cells often stuck in the sampling outlet at the bottom of the fermenter. However this was overcome by gently pressing the rubber tubes connected to the outlet.

3.4.4 Conclusions

Excellent aeration in a fermenter led to an improved conditions for lipid biosynthesis to occur where the nitrogen source was depleted early in the fermentation. Growth in a fermenter also resulted in a higher

biomass and lipid yield compared to when it was grown in whirlipots performed with an identical growth media.

3.5.0 The Effect of pH on Growth and lipid Production in *M. circinelloides*

3.5.1 Objectives

This experiment was designed to investigate the effect of pH on growth and lipid production of *M. circinelloides* cultivated in a fermenter. The aim was to determine whether or not the decrease of the culture pH observed during growth in whirlipots had an impact on the growth and lipid production of the fungus.

3.5.2 Materials and Methods

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 2 and 3, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). As appreciable problems with wall growth were encountered in the previous experiment, attempts were made to overcome the problem by performing the cultivation with the baffles removed from the fermenter vessel and the stirring speed increased to 800 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min).The pH of the culture in Fermenter 3 was not controlled while in Fermenter 2, the culture pH was maintained at 6.0 by automatic addition of 2 M KOH and 2 M HCl. The pH changes of the culture in Fermenter 3 were recorded manually at various

intervals throughout the experiment. Temperature was maintained at 30 ^{o}C by circulating water through an internal coil of the fermenter. Samples were taken at intervals for lipid assay and the determination of biomass, ammonium and glucose concentration and cell lipid analysis.

3.5.3 Results and Discussion

The cultures in both fermenters showed a similar growth and lipid accumulation profile (Figure 11a-b). In Fermenter 2, the biomass increased from 0.4 g / l at 3 h to 17 g / l at 28 h coincident with a period of glucose and ammonium utilization (Figure 11a). Ammonium was depleted at approximately 14 h and the total lipid increased from 5 % at 15 h to 10 % (w / w of biomass) at 28 h at the time of glucose depletion.

In Fermenter 3, the biomass increased rapidly from 0.3 g / l at 3 h to 18 g / l at 24 h (Figure 11b) coincident with the decrease of glucose and the depletion of ammonium at 12 h. The pH dropped from 6.0 initially to 3.0 at 27 h at the point of glucose exhaustion. The total lipid increased from 5 % (w / w of biomass) at 17 h to 11 % (w / w of biomass) at 27 h. Table 8a summarizes the growth and lipid production profile of both cultures. A similar fatty acid profile (Table 8b) was obtained from both cultures where the most abundant was 18:1, followed by 18:3, 18:2, 18:0, 14:0 and 16:0.

Therefore, it appears that growth and lipid production of *M. circinelloides* were unaffected by the changes of the culture pH. A similar observation has also been reported to

Figure 11: Growth and lipid accumulation profile of *M. circinelloides* in

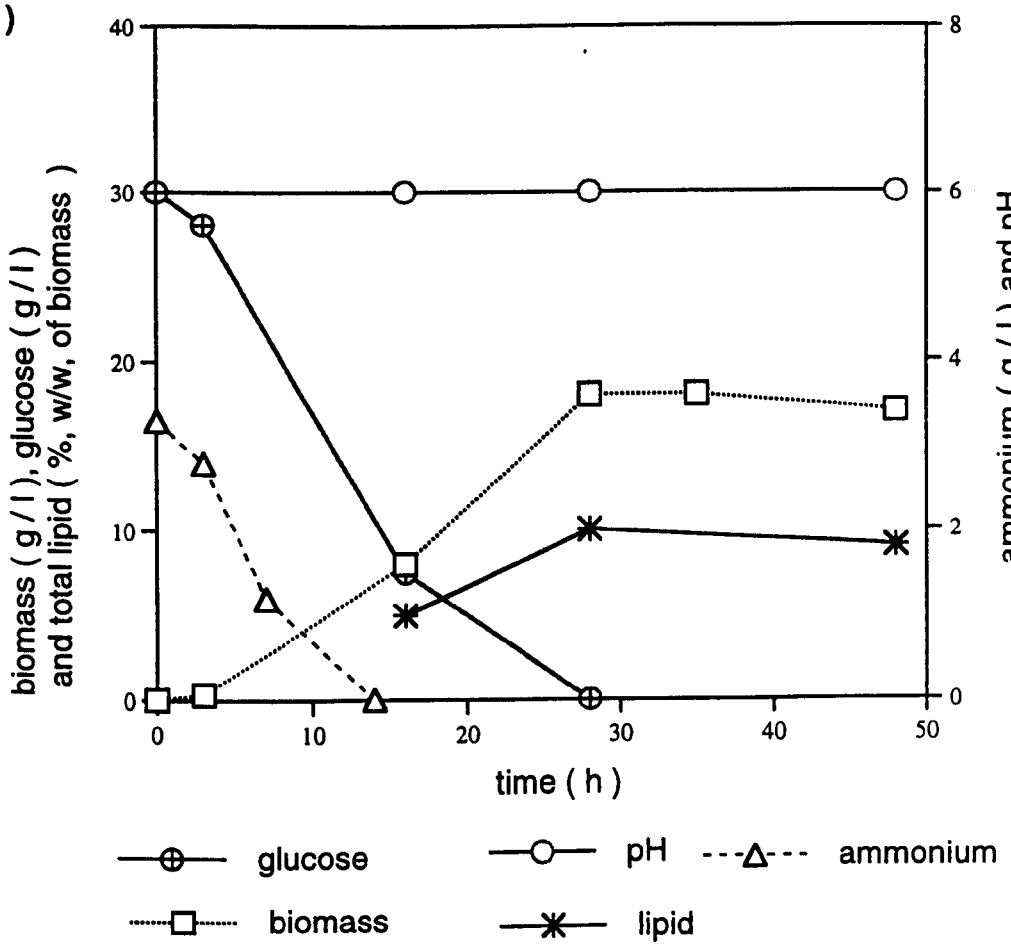
a) Fermenter 2, performed with the pH maintained at 6.0

b) Fermenter 3, performed with uncontrolled pH

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 2 and 3, containing nitrogen-limiting media (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l) . Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min).The pH of the culture in Fermenter 3 was not controlled throughout the experiment while in Fermenter 2, pH was maintained at 6.0 by automatic addition of 2 M KOH and 2 M HCl. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 11 :

a)


biomass (g / l), glucose (g / l)
and total lipid (%, w/w, of biomass)
ammonium (g / l) and pH
time (h)
0
10
20
30
40
50
0
2
4
6
8
glucose
pH
ammonium
biomass
lipid

b)

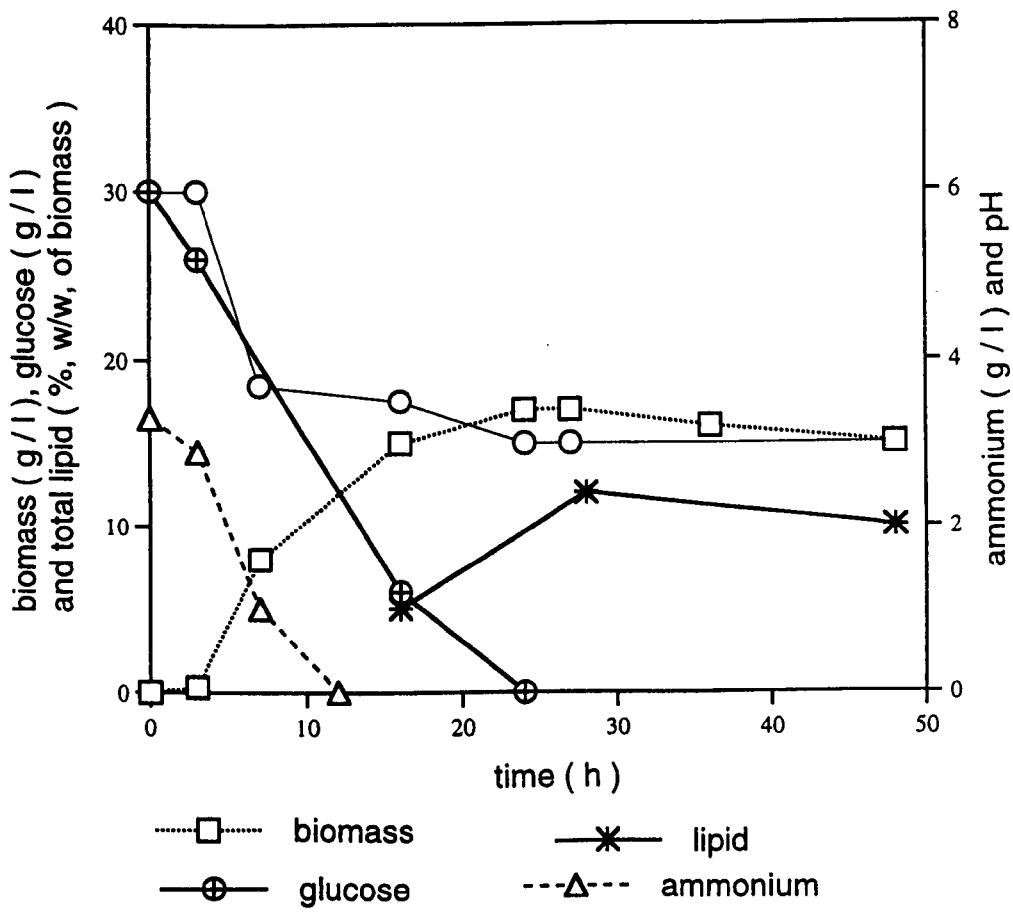

biomass (g / l), glucose (g / l)
and total lipid (%, w/w, of biomass)
ammonium (g / l) and pH
time (h)
0
10
20
30
40
50
0
2
4
6
8
biomass
lipid
glucose
ammonium

Table 8a: Differences in Growth Profile of *M. circinelloides* Grown With and Without the pH being Controlled.

	Biomass (g / l)	Total Lipid (%)	Biomass Yield g / g glucose used	Lipid Yield g / g glucose	Ammonium Depletion	Glucose Exhaustion
Fermenter 2 (controlled pH)	14-17	8-10	0.47-0.56	0.04- 0.056	13- 14 h	26-28 h
Fermenter 3 (uncontrolled pH)	16-18	8-11	0.53-0.60	0.04- 0.066	12 h	24-28 h

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 2 and 3, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The pH of the culture in Fermenter 3 was not controlled throughout the experiment while in Fermenter 2, pH was maintained at 6.0 by automatic addition of 2 M KOH and 2 M HCl. Data presented were based on the analysis of samples from two replicates of each of the fermenter taken at approximately after 30 h growth.

Table 8b : The Effect of Culture pH changes on the Fatty Acid Compositions of *M. circinelloides*

Fatty Acids (%)	Fermenter 2 (pH controlled)	Fermenter 3 (pH uncontrolled)
14:0	7-10	7-9
16:0	6-7	6-8
18:0	10-11	7-10
18:1	25-28	25-29
18:2	19-21	20-23
18:3	23-25	22-26

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 2 and 3, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The pH of the culture in Fermenter 3 was not controlled throughout the experiment while in Fermenter 2, pH was maintained at 6.0 by automatic addition of 2 M KOH and 2 M HCl. Data presented were based on the analysis of samples from two replicates of each of the fermenter after approximately 30 growth.

occur in *Saccharomonas cerevisiae* (Castelli et al., 1969), *Rhodotorula gracilis* (Kessell, 1968) and *Candida* 107 (Hall and Ratledge, 1977) where production of lipid in these organisms were unaffected when each of these organisms were cultivated in a growth media with different pH .

3.5.4 Conclusions

The growth and lipid production of *M. circinelloides* were unaffected by the changes of the culture pH. Therefore it appears that the maintenance of pH during growth of this fungus was not critical.

3.6.0 The Effect of Increasing C:N Ratio on Growth and Lipid Production of *M. circinelloides* in a Fermenter

3.6.1 Objectives

Although an optimal conditions for lipid biosynthesis (ie. a nitrogen-limited condition) have been achieved when *M. circinelloides* was cultivated in a fermenter, it still showed a significantly less lipid production (11 %, w / w of biomass) compared to what was reported in earlier work where up to 25 % (w / w of biomass) lipid was achieved (Kendrick, 1992). Therefore, attempts were made to increase the cell lipid content by growing *M. circinelloides* in a medium with an increasing C:N ratio.

3.6.2 Materials and Methods

Experiments were carried out using 5 L fermenters with a final

working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 4, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 30 :1 (37.5 g glucose / l and 3.3 g ammonium tartrate / l) and Fermenter 5 with C:N ratio at 50:1 (62.5 g glucose / l and 3.3 ammonium tartrate / l). Baffles were removed from the fermenter vessels and the fermenters were stirred at 800 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min) and the pH was maintained at 6.0 by automatic addition of 2 M NaOH and 2 M KOH. Temperature was maintained at 30 ^{o}C by circulating water through an internal coil of the fermenter. Dissolved O_2 was measured continuously using a galvanic O_2 electrode inserted to the fermentation vessels. Samples were taken at intervals for determination of biomass, ammonium and glucose concentration and lipid analysis.

3.6.3 Results and Discussion

In Fermenter 4 the dissolved O_2 concentration dropped continuously between 20 h and 32 h (Figure 12b) but then increased after glucose exhaustion at 35 h (Figure 12a). The initial decrease in O_2 was due to the growth of the organism as seen by the increase in biomass (Figure 12a), cellular lipid (Figure 12b) and with the utilization of the carbon source. The total lipid content of the biomass (Figure 12b) increased from 7 % to 14 % (w/w of biomass) between 18 h to 50 h (no data available before 18 h due to insufficient cell density).

After glucose depletion (Figure 12a) the biomass gradually decreased until the end of the fermentation period. It was thought that the

Figure 12: Growth and lipid production profile of *M. circinelloides* cultivated in nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 30:1 (37.5 g glucose / l and 3.3 g ammonium tartrate / l)

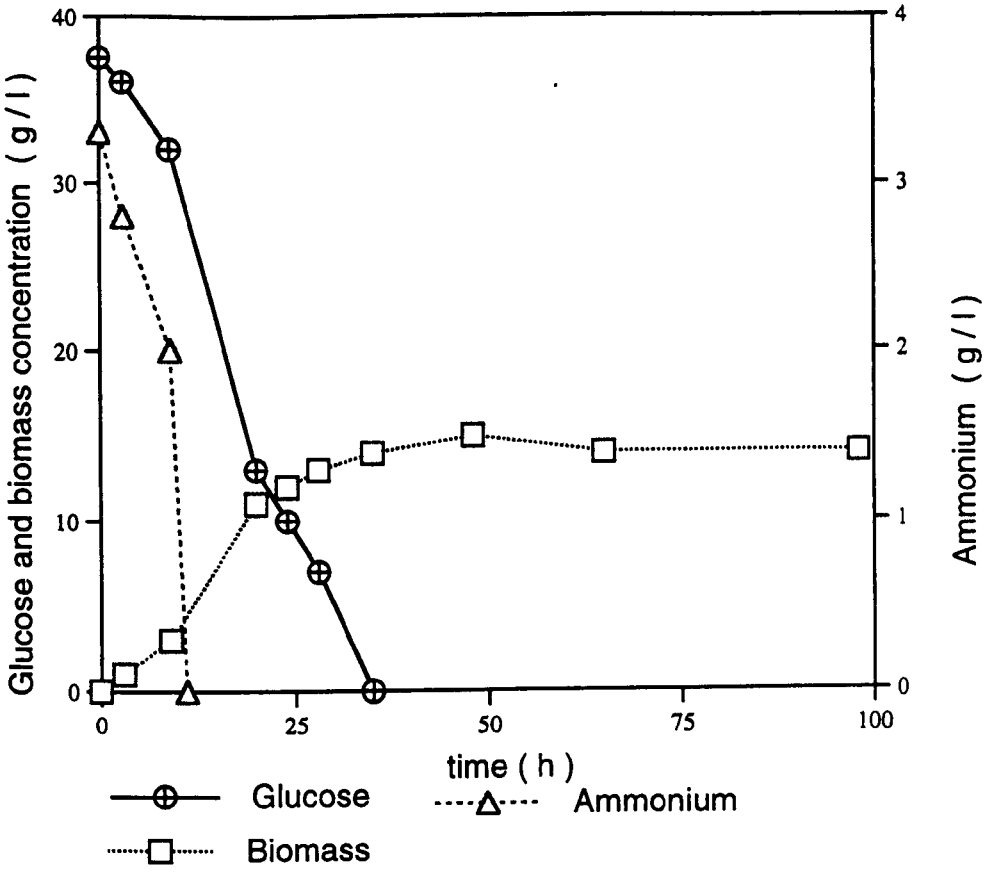
a) glucose, biomass and ammonium concentration vs time

b) dissolved O_2 and total lipid vs time

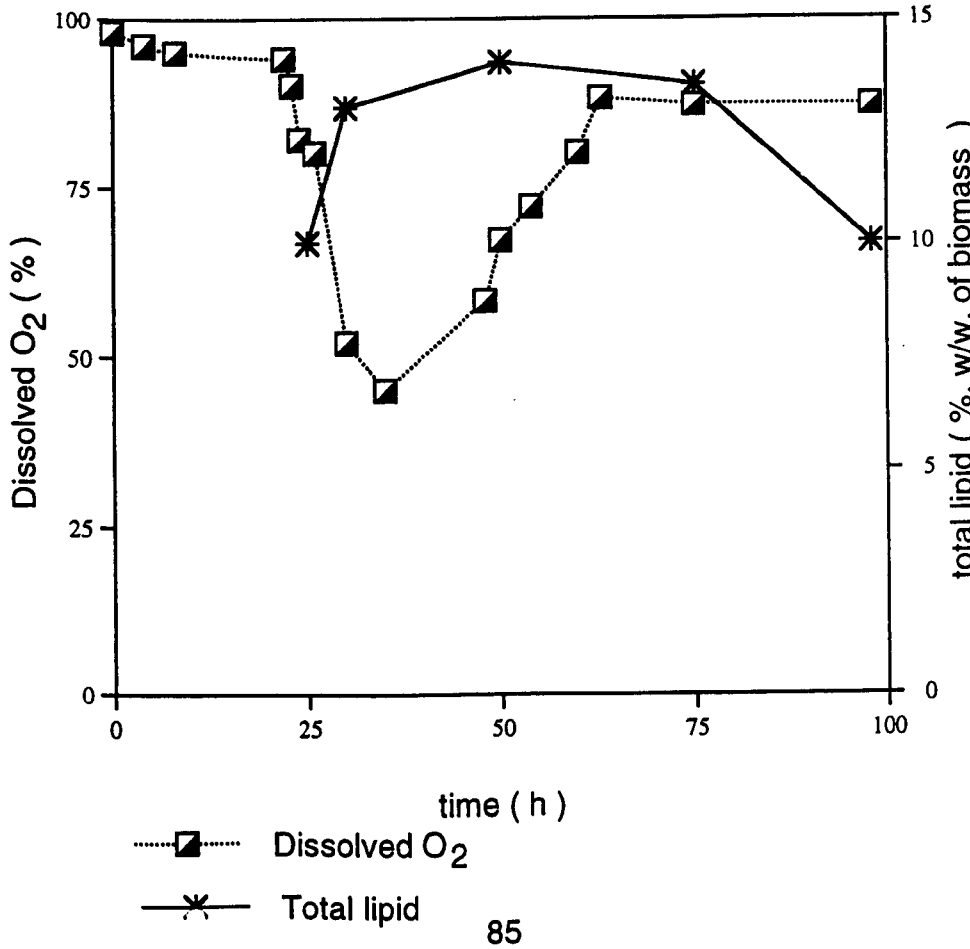
Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 4 and it was stirred at 800 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min).The pH of the culture was maintained at 6.0 by automatic addition of 2 M KOH and 2 M HCl. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 12:

a)


Glucose and biomass concentration (g / l)
Ammonium (g / l)
time (h)
Glucose
Ammonium
Biomass

b)


Dissolved O_2 (%)
total lipid (%, w/w, of biomass)
time (h)
Dissolved O_2
Total lipid

decrease in biomass was due to the fungus mobilizing and utilizing its stored lipid as a carbon and energy source. Evidence of this is seen as the total lipid decreased from 14 % to 9 % (w / w of biomass) between 40 h and 98 h (Figure 12b). It has been shown that intracellular lipids synthesized during active growth of *Mortierella ramanniana* were depleted when cells were transferred to a medium lacking glucose (Peberdy and Toomer, 1975).

In Fermenter 5, performed with C:N ratio at 50:1 (62.5 g glucose / l and 3.3 g ammonium tartrate / l), a similar pattern of growth was observed (figure 13a-b). The dissolved O_2 decreased during active growth until 20 h (Figure 13b) and the total lipid increased from 12 % to 25 % (w / w of biomass) between 20 h to 50 h (Figure 13b), some 2-fold higher than what was produced in Fermenter 4 (14 %, w / w of biomass).The cell lipid content stopped increasing at approximately 50 h although glucose was still present. The increased biomass was presumably due to an increased lipid as the initial concentration of nitrogen (the growth limiting nutrient) was the same in both Fermenter 4 and 5 . The biomass yield produced in Fermenter 4 and 5 was similar (0.32 and 0.4 g / g glucose used, respectively) (Table 9a). However, the lipid yield in Fermenter 5 was higher (0.08 g / g glucose used) compared to Fermenter 4 which was 0.056 g / glucose used. It is known that lipid production is favoured by a high C:N ratio (Ratledge, 1986; Weete, 1980). Lipid production in *Mortierella ramanniana* (Hansson and Dostalek, 1988), *Mortierella vinacea* (Chesters and Peberdy, 1965), *Candida utilis* (Babij et al.,1969) and *Mortierella* sp. S-17 (Sajbidor et al., 1990) were demonstrated to produce higher lipid content when cultivated in a high C:N ratio. However, although both fermenters contained media with a different C:N ratio, both

Figure 13: Growth and lipid production profile of *M. circinelloides* cultivated in nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 50:1 (62.5 g glucose / l and 3.3 g ammonium tartrate / l)

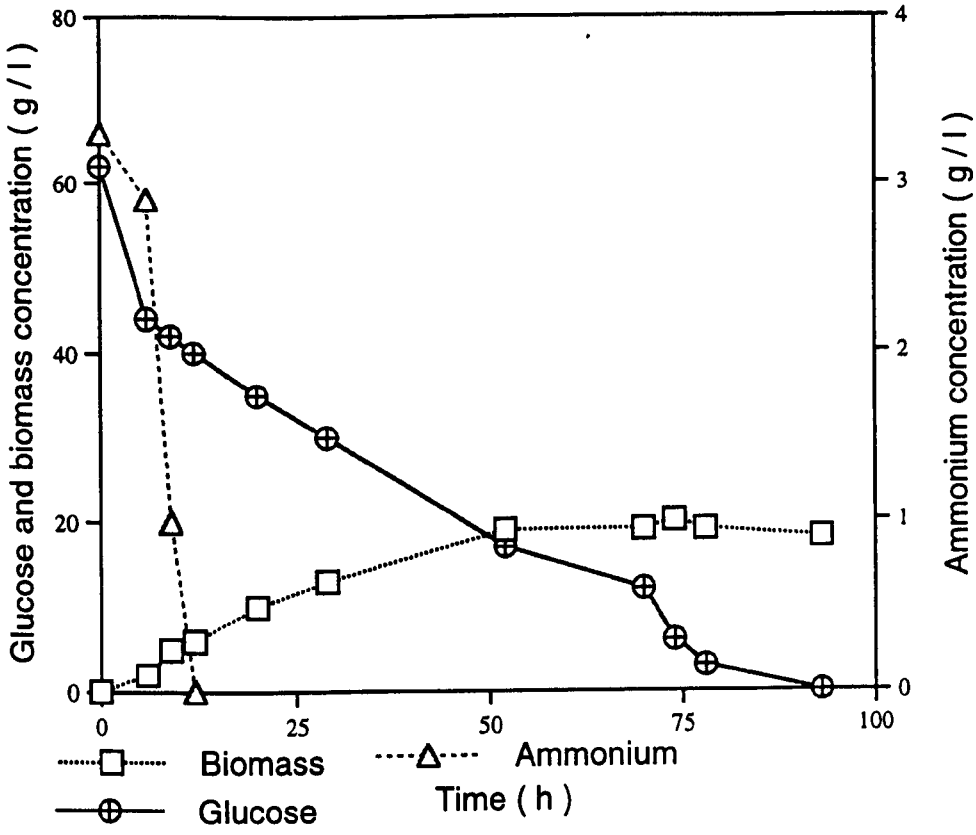
a) glucose, biomass and ammonium concentration vs time

b) dissolved O_2 and total lipid vs time

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 4 and it was stirred at 800 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min).The pH of the culture was maintained at 6.0 by automatic dripping of 2 M KOH and 2 M HCl. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 13:

a)


Glucose and biomass concentration (g / l)
Ammonium concentration (g / l)
Time (h)
Biomass
Ammonium
Glucose

b)

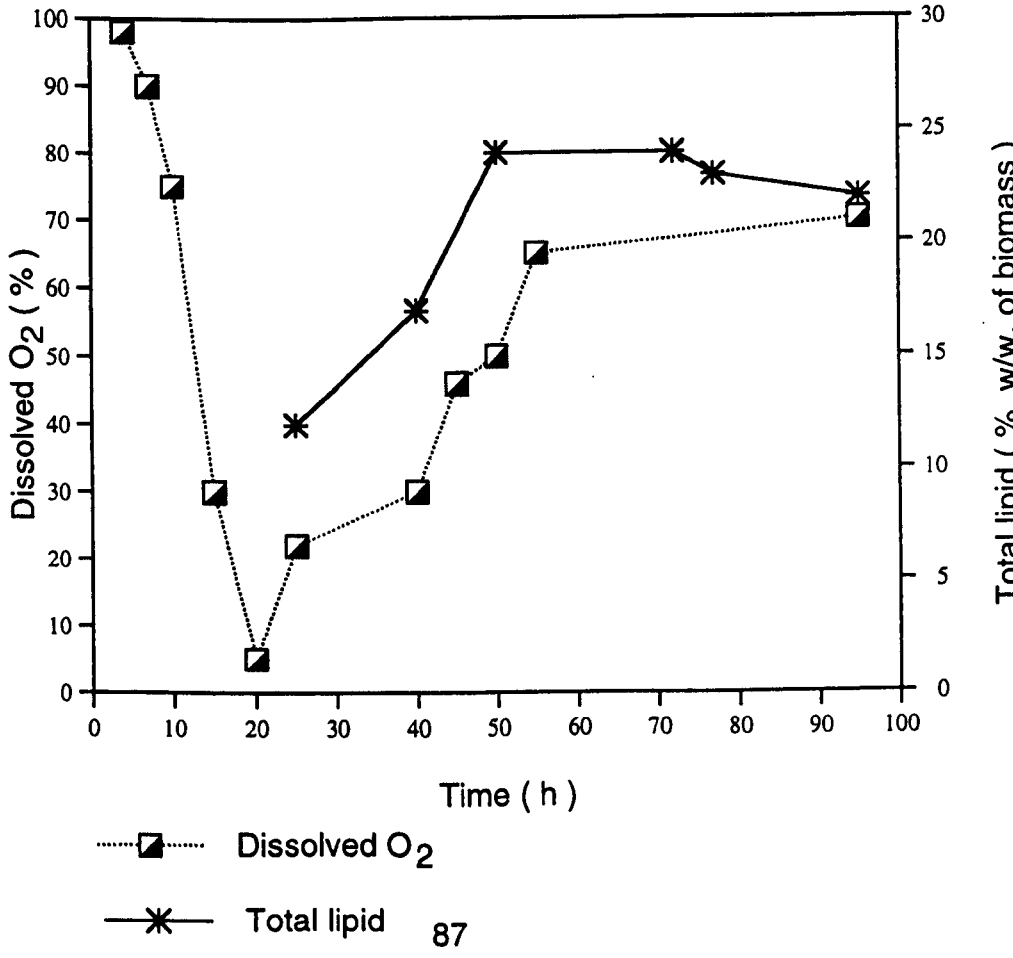

Dissolved O2 (%)
Total lipid (%, w/w, of biomass)
Time (h)
Dissolved O2
Total lipid

Table 9a : The biomass and total lipid produced by *M. circinelloides* in fermenters performed at different C : N ratios.

	total lipid (%)	Biomass (g/l)	Biomass Yield g / g glucose used	Lipid Yield g / g glucose used
Fermenter 4 C : N (30 : 1) 37.5 g glucose / l and 3.3 g ammonium tartrate / l	12-14	15-16	0.4-0.42	0.051-0.056
Fermenter 5 C : N 50 : 1 62.5 g glucose / l and 3.3 g ammonium tartrate / l	22-25	18-20	0.29-0.32	0.06-0.08
Fermenter 6 C : N 40 : 1 30 g glucose / l and 2 g ammonium tartrate / l	13-14	11-12	0.36-4	0.051
Fermenter 7 C : N 66 : 1 50 g glucose / l and 2 g ammonium tartrate / l	23-25	13-15	0.26-0.3	0.06-0.075

Experiments were carried out using 5 L fermenters as described in Section 3.62. Data presented were based on the analysis of samples from two replicates of each of the fermenter after approximately 50 h growth.

Table 9b : Fatty Acid Compositions of *M. circinelloides* grown at a different C:N ratios.

Fatty Acids (%)	Fermenter 4 C:N 30:1	Fermenter 5 C:N 50:1	Fermenter 6 C:N 40:1	Fermenter 7 C:N 66:1
14:0	6-8	6	7-9	6-8
16:0	9-11	17-18	10-12	10-12
18:0	11-13	7-10	9-11	9-14
18:1	28-31	24-34	29-31	29-33
18:2	13-15	12-15	14-17	13-16
18:3	22-26	19-23	21-22	21-23

Experiments were carried out using 5 L fermenters with a final working volume of 4 L. A 5 % (v / v) inoculum was added to the fermenters, containing a nitrogen-limiting medium (see Section 2.1.3) with different C:N ratios. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min) and the pH of the culture was maintained at 6.0. Data presented were based on the analysis of samples from two replicates of each of the fermenter.

cultures showed a similar fatty acid profiles except a higher content of 16:0 was observed in Fermenter 5 (Table 9b).

A similar experiment was then repeated but with decreased concentration of ammonium tartrate incorporated into the media (ie. from 3.3 g / l to 2 g / l). Fermenter 6 was run with 30 g glucose / l and 2 g ammonium tartrate / l (C:N 40:1) and Fermenter 7 was performed with 50 g glucose / l and 2 g ammonium tartrate / l (C:N 66:1). Table 9a summarizes the differences in the lipid yield, biomass yield, total lipid and biomass produced in Fermenter 4, 5, 6 and 7, each performed with two replicates .

Fermenter 7 showed a higher production of lipid, achieving 25 % total lipid (similar to that produced in Fermenter 5) compared to Fermenter 6 which produced only 14 % total lipid (graph not plotted). Again, the fatty acid profiles of lipid from both cultures were similar despite being performed at a different C:N ratio with the most common fatty acid residues being 18:1, 18:3, 18:2, 16:0, 18:0 and 14:0, respectively (Table 9b). It was therefore concluded that a media with 50 g glucose / l and 2 g ammonium tartrate / l (C:N 66:1) was favourable for cultivating *M. circinelloides* as it showed a high lipid production (25 %, w / w of biomass) and lipid yield with a lower concentration of glucose used compared to the media with C:N at 50:1 (62.5 g glucose / l and 3.3 g glucose / l).

3.6.4 Conclusions

1) *M. circinelloides* produced a higher lipid content when cultivated in a media with an increased C:N ratio. However, no significant differences

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4.1.2 Materials and Methods

The experiment was carried out using a 5 L fermenter (Fermenter 8) containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24:1 (30 g glucose / l and 3.3 g ammonium tartrate / l) and a final working volume of 4 L. The baffle was removed from the fermenter vessel to prevent wall growth and it was stirred at 800 rev / min. Aeration was achieved by supplying air at a flow rate of 0.5 (v / v / min) and pH was maintained at 6.0 by automatic addition of 2 M NaOH and 2 M KOH. Temperature was maintained at 30 ^{o}C by circulating water through an internal coil of the fermenter. The fermenter was aseptically inoculated with 5 % (v / v) of seed culture. Samples were taken at intervals for determination of biomass, glucose, ammonium concentration, lipid production and fatty acid analysis using methods described in Chapter 2.

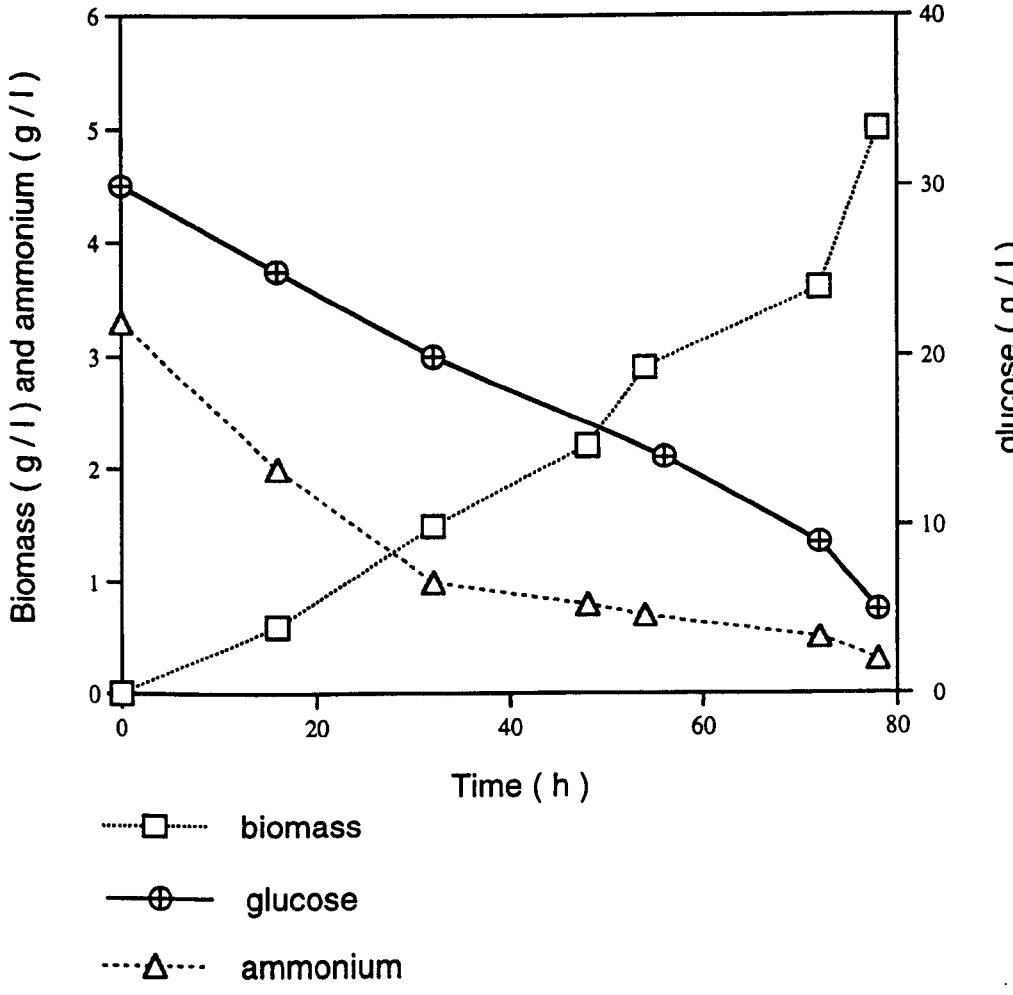
4.1.3 Results and Discussion

A very slow growth and lipid production rate were observed when *Mt. alpina* was grown in a fermenter (Figure 14a). The biomass increased from 0.6 g / l at 16 h to 5.0 g / l at 78 h coincident with the decrease of glucose from 30 g / l at the beginning of the experiment to 5 g / l at 78 h. Ammonium was not exhausted until the end of the experiment. A low biomass and lipid yield (0.2 and 0.02 g /g glucose used, respectively) were observed. The total lipid increased from 6 % (w / w of biomass) at 54 h to 10 % (w / w , of biomass) at 78 h (graph not plotted). Earlier work on this fungus showed a significantly higher production of lipid (achieving 38 %, w / w of biomass) when it was grown in an identical growth media in whirlipots (Kendrick and Ratledge, 1992a).

Figure 14a: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with 5 % (v / v) inoculum.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 8, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 14a


Biomass (g / l) and ammonium (g / l)
glucose (g / l)
Time (h)
0
20
40
60
80
0
1
2
3
4
5
6
0
10
20
30
40
biomass
glucose
ammonium

The low lipid production was due to the unfavourable conditions for lipid biosynthesis which occurred as the nitrogen source was not depleted until the late stage of the experiment. It was thought that the poor performance of this fungus may be due to an insufficient amount of seed culture used for the inoculation (5 % , v / v). Problems associated with poor growth exhibited by this fungus were first encountered during the preparation of the seed culture prior to the experiment. Inoculation of mycelia from a stock culture grown on PDA into whirlipots containing 500 ml of nitrogen-limiting media (see Section 2.1.3) resulted in no growth after 48 h. This was done in duplicate and repeated twice with the same results. The seed culture was subsequently prepared by transferring mycelia from a stock culture (PDA) into shake flasks containing 500 ml of the same nitrogen-limiting media where a substantial amount of biomass was formed after 72 h of incubation.

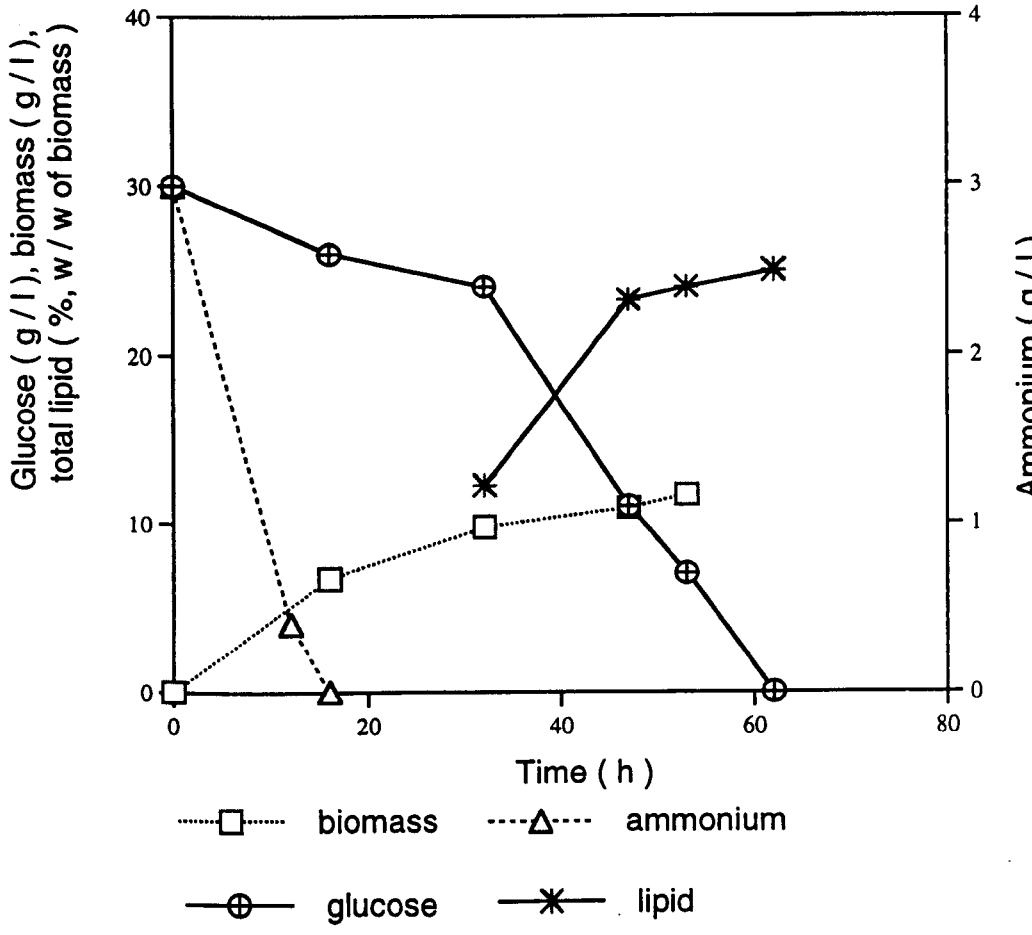
When the same experiment was repeated but by adding a larger inoculum size (10 %, v / v) into a fermenter (Fermenter 9), an improved growth and lipid accumulation were observed (Figure 14b). The biomass increased rapidly from 8 g / l at 18 h to 11 g / l at 53 h coincident with the decrease of glucose from 26 g / l to 7 g / l. In contrast to the previous run in Fermenter 8 , the ammonium concentration was now depleted at an early stage of fermentation (16 h) which triggered the biosynthesis of lipid as seen by the increase of total lipid from 12 % (w / w of biomass) at 31 h to 24 % (w / w of biomass) at 62 h .

This experiment showed that by increasing the amount of seed culture from 5 % to 10 % (v / v), improved growth and lipid production were achieved. The culture showed a high biomass and lipid yield (0.37 and

Figure 14b: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with 10 % (v / v) inoculum.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 14b


Glucose (g / l), biomass (g / l), total lipid (%, w / w of biomass)
Ammonium (g / l)
Time (h)
0
10
20
30
40
0
1
2
3
4
0
20
40
60
80
biomass
ammonium
glucose
lipid

0.088 g / g glucose used, respectively). However, the lipid production was still not as high as previously reported (Kendrick and Ratledge, 1992a). The fatty acid composition of the lipid is shown in Table 10.

Another problem encountered when growing this fungus was the occurrence of foam on the surface of the media after approximately 72 h of incubation in Fermenter 8. A more severe problem of foam formation was observed in Fermenter 9 where a stable formation of foam occurred after 16 h of incubation. The problem was so severe that a substantial amount of the liquid culture was transported by the foam into the air outlets causing the air filter connected to the outlet to become wet. This problem was subsequently overcome by adding approximately 0.3 ml of anti-foam to the culture at the beginning of the fermentation. These experiments were repeated twice and typical data are presented.

As the fermentation was performed without baffles, less wall growth was observed. However, adhesion of cells still occurred particularly around the impeller of the fermenter due to the filamentous morphology of the fungus, which caused it to be easily entangled to the rotating impeller during agitation.

Table 10: Growth, Lipid Accumulation and Fatty Acid Profiles of *Mt. alpina* Grown in a Fermenter Using 10 % (v / v) Seed Culture.

Max. Biomass (g / l)	10-11
Max. Total Lipid (%, w / w of biomass)	24-27
Biomass Yield (g / g glucose used)	0.33-0.37
Lipid Yield (g / g glucose used)	0.088-0.09
Fatty acids (Relative %, w/w)	
14:0	9-12
16:0	17-18
18:0	12-15
18:1	28-33
18:2	13-17
18:3	4-6
20:4	4-7

The organism was grown in 5 L fermenters (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9 (two replicates), containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). Samples analysed were taken after approximately 60 h growth. The values given represent the range of estimates from duplicate experiments.

4.1.4 Conclusions

1) Cultivation of *Mt. alpina* using 5 % (v / v) inoculum in a fermenter resulted in poor growth and lipid production. Optimal growth and lipid production were achieved when *Mt. alpina* was cultivated in a fermenter using 10 % (v / v) of seed culture .

3) Further optimization of the growth condition was required as the lipid production achieved (24 %, w / w of biomass) was lower than previously reported where up to 38 % (w / w of biomass) was produced.

4.2.0 The Effect of Increasing C:N Ratio on Growth and Lipid Production of *Mt. alpina* in a Fermenter

4.2.1 Objectives

As previously observed, *Mt. alpina* showed a better growth and lipid production when cultivated in a fermenter using 10 % (v / v) inoculum compared to when inoculation was carried out using a 5 % (v / v) inoculum. Although an improved performance was achieved, the lipid production was still low (24 %, w / w of biomass) compared to what was reported in an earlier work on this fungus where up to 38 % (w / w of biomass) was produced (Kendrick and Ratledge, 1992a). Therefore, attempts were made to further improve the lipid production performance of this fungus by increasing the C:N ratio of the media.

4.2.2 Materials and Methods

Experiment was carried out using a 5 L fermenter (Fermenter 10) as described in Section 4.1.2, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40:1 (30 g glucose / l and 2.0 g ammonium tartrate / l). Samples were taken at intervals for determination of biomass, glucose, ammonium concentration, cell lipid content and fatty acid analysis using methods described in Chapter 2.

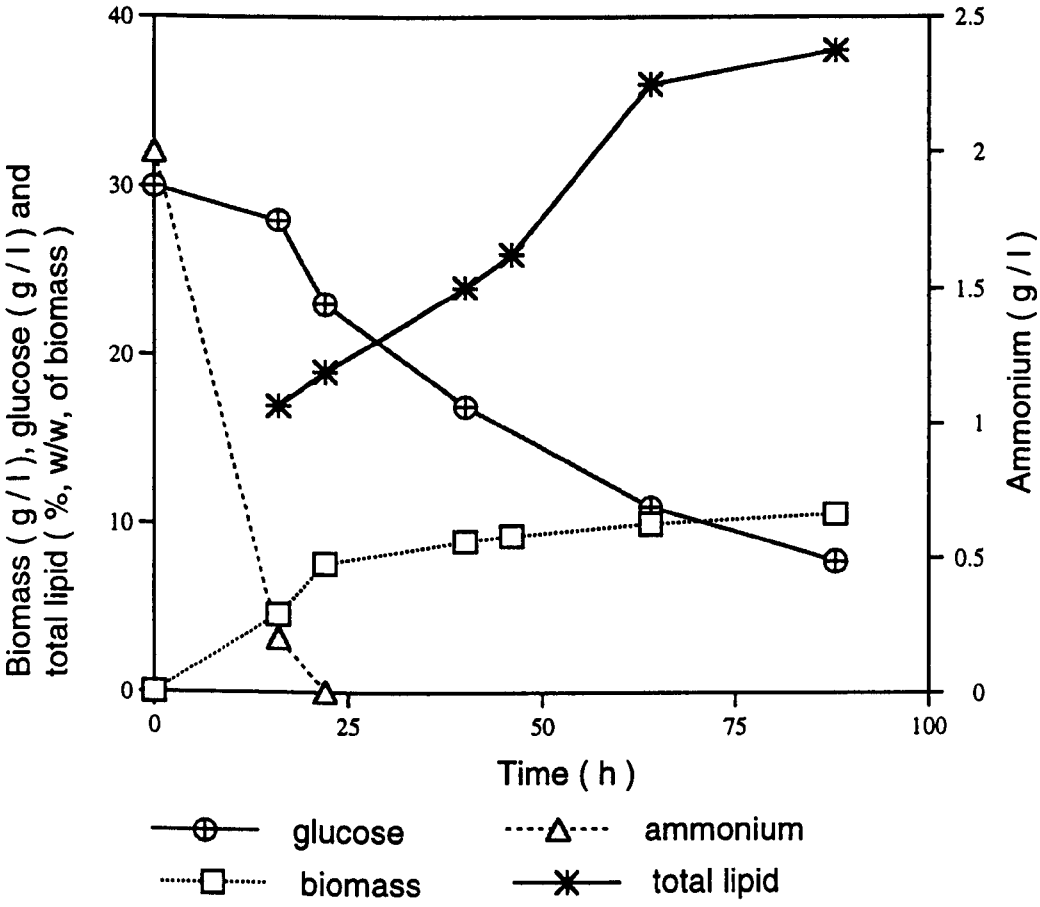
4.2.3 Results and Discussion

Figure 15 shows the growth and lipid accumulation profile of *Mt. alpina* in Fermenter 10. The biomass increased rapidly from 5 g / l at 16 h

Figure 15: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with an increased C:N ratio.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40 :1 (30 g glucose / l and 2.0 g ammonium tartrate / l). 10 % (v / v) inoculum. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 15


Biomass (g / l), glucose (g / l) and total lipid (%, w/w, of biomass)
Ammonium (g / l)
Time (h)
0
10
20
30
40
0
0.5
1
1.5
2
2.5
0
25
50
75
100
glucose
ammonium
biomass
total lipid

to 9 g / l at 40 h associated with the decrease of glucose and the depletion of ammonium. The lipid content of the culture increased from 19 % (w / w of biomass) at 22 h to 38 % (w / w of biomass) at 64 h coincident with the rapid decrease of glucose concentration from 23 g / l to 8 g / l. This result suggests that by increasing the C:N ratio of the growth media, a better growth and lipid accumulation performance could be achieved. Optimal fungal growth is known to occur under optimal nutritional and environmental conditions, and is the result of various metabolic activities in a certain balance.The C:N ratio is known as one of the most important nutritional parameter for lipid production (Weete, 1980). High production of lipid is associated with low rates of protein synthesis. A high C:N ratios reduces protein synthesis resulting in more carbon substrate being available for conversion to lipid (Ratledge and Wilkinson, 1988).

The biomass and lipid yields of the culture were higher (0.5 and 0.19 g / g glucose used, respectively) than that observed in Fermenter 9 (Section 4.2.0) which were 0.37 and 0.088 g / g glucose used, respectively. Therefore, with glucose concentration still kept at 30 g / l, but by decreasing the ammonium tartrate concentration from 3.3 g / l to 2 g / l , higher lipid production was achieved. However the fatty acid profiles obtained were similar as observed in Fermenter 9 (Section 4.2.0) where the most abundant fatty acid was 18:1 followed by 18:2, 18:0, 16:0,14:0, 20:4 and 18:3 (Table 11).

During the fermentation run, no difficulties were encountered. The problem of excessive foam formation was overcome by the addition of anti-foam agent (0.3 ml) at the beginning of the experiment. The problem of wall growth has been decreased by performing the fermenter

Table 11: Fatty acid Profiles of *Mt. alpina* Grown in a fermenter with an Increased C:N ratio

Fatty Acids	Relative % (w / w)
14:0	5-9
16:0	16-20
18:0	13-16
18:1	29-31
18:2	15-21
18:3	3-5
20:4	4-6

Mt. alpina was cultivated in a fermenter as described in Section 4.1.2 . A 10 % (v / v) seed culture was added to a fermenter containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40:1 (30 g glucose / l and 2 g ammonium tartrate / l).Data presented were based on the analysis of samples from three replicates of Fermenter 10 obtained after 90 h of growth .

without baffles where less cells adhered to the fermenter walls.

4.2.4 Conclusions

Mt. alpina showed improved growth and lipid accumulation when cultivated in a medium with an increased C:N ratio. This was thought to be the result of the decreased ammonium tartrate concentration used in the medium which allowed more carbon to be channelled into lipids rather than being used for protein synthesis. No significant changes in the fatty acid profiles were observed.

CHAPTER 5

THE BIOCHEMISTRY OF LIPID ACCUMULATION IN *M. circinelloides and Mt. alpina*

INTRODUCTION

This chapter discusses on the work carried out in the attempt to elucidate the enzymatic regulation of lipid accumulation in *M. circinelloides* and *Mt. alpina*. The activity profiles of several enzymes, which are related to the regulation of fatty acid biosynthesis as previously established in oleaginous yeasts (Evans and Ratledge, 1985a; Boulton and Ratledge, 1981; Botham and Ratledge, 1979), were studied. The discussion will also include the description of preliminary work (ie. optimization of cell extracts preparation) carried out prior to the biochemical studies being performed.

5.1.0 Disruption of Fungal Biomass

5.1.1 Objectives

This experiment was carried out to develop an efficient method of cell disruption for the production of cell extracts from these two fungi. As the French press technique has been regularly used in this laboratory and proved to be efficient in the applications toward several organisms (Kendrick, 1992; Wynn, 1994), this method was adopted and its efficiency on the disruption of cells of *M. circinelloides* and *Mt. alpina* was investigated.

5.1.2 Materials and Methods

5.1.2.1 Methods of Cultivation

M. circinelloides and Mt. alpina were cultivated in 5 L fermenters (as described in Section 4.1.2) containing nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l) and 40:1 (30 g glucose / l and 2 g ammonium tartrate / l), respectively. For the cultivation of *M. circinelloides,* a 5 % (v / v) seed culture was used while 10 % (v / v) inoculum was used for the growth of *Mt. alpina.* Both cultures were harvested after 24 h of inoculation.

5.1.2.2 Disruption Technique

Harvested fungal mycelia were resuspended in an extraction buffer (see Section 2.4.0) to give approximately 20 % (wet wt / v) mycelial suspensions. The mycelial suspension was passed four times through a cold (4 ^{o}C) French pressure cell at a pressure of 35 MPa. After each passage, approximately 20 ml samples were taken from the resulting homogenate and placed in a separate ice-cold beaker. French pressed extracts were centrifuged at 16 000 g for 20 min at 4 ^{o}C to remove cell debris. Floating layers of lipid were removed by filtrating the crude extracts through a Whatman No 1 filter paper. The protein concentration of each resulting supernatant was then determined using method described in Section 2.9.0.

5.1.3 Results and Discussion

Table 12 summarizes the effect of number of passes through the French press on the disruption of *M. circinelloides* and *Mt. alpina*. In *M. circinelloides*, only 36 % (1.2 mg / ml) of the protein in the final extract after 4 passes, was released after a single pass through the French press. After the second pass, the protein concentration increased to 73 % of the final protein concentration. In *Mt. alpina*, 41 % (1.4 mg / ml) of the total protein was released after the first pass and 71 % (2.4 mg / ml) after two passes.

A yellowish cell extract was obtained from *M. circinelloides* while a slightly milky extract was observed in the cell extract produced from *Mt. alpina*. In the subsequent work, both fungi were disrupted by only two passes through the French press as a substantial amount of protein was liberated from the mycelia with the minimum physical stress. Vigorous methods of cell disruption have been reported to affect the activity and the structure of certain enzymes (Dutton, 1988). In this method, cells were forced through a small orifice at a very high pressure; shear forces disrupted the cells.

Several difficulties were encountered when using this method. Firstly, the temperature of the French press increased very quickly from 4 ^{o}C to between 8 to 13 ^{o}C. So, after several passes, it had to be held in ice in order to restore the low temperature. This caused difficulties when processing a large amount of samples. Secondly, the rubber seal which was attached to the plunger of the French press wore out very quickly and sometimes the seal was cut during the process causing the plunger to

Table 12: The Effect of Number of Passes through a French Press on the disruption of *M. circinelloides and Mt. alpina*

	Protein concentration in extract (mg / ml)	
No. of Passes	*M. circinelloides*	*Mt. alpina*
1	1.2	1.4
2	2.4	2.4
3	2.8	2.6
4	3.3	3.4

The fungi were cultivated in 5 L fermenters and cells were harvested after 24 h of incubation. Harvested fungal mycelia was resuspended in an extraction buffer (see Section 2.4.0) to give approximately 20 % (wet wt / v) mycelial suspension. The mycelial suspension was passed four times through a cold (4 ^{o}C) French pressure cell at a pressure of 35 MPa where after each passes, approximately 20 ml of the resulting cell extracts were taken and placed in a separate ice-cold beaker. French pressed extracts were centrifuged at 16 000 g for 20 min at 4 ^{o}C to remove cell debris. Floating layers of lipid were removed by filtration of the crude extracts through a Whatman No 1 filter paper. The protein concentration of each resulting supernatants was then determined.

stick and difficult to pull out. This problem was more pronounced when processing a large volume of sample where it could cause severe delays in order to mend the unit. Therefore, regular checking of the seal was required between each passes.

5.1.4 Conclusions

1) Two passes of cell extracts through the French press were sufficient in the disruption of cells from both fungi. Therefore, this technique was adopted for the preparation of cell extracts from both fungi for the rest of the work.

2) It was important to maintain the French pressure cell at a low temperature by holding it in ice after performing several passes in order not to over-heat the samples. In this experiment, the temperature of the final extracts varied between 8 to 13 ^{o}C.

5.2.0 The Investigation of Enzymatic Regulation Involved In the Lipid Biosynthesis of *M. circinelloides* and *Mt. alpina*

5.2.1 Objectives

In the previous chapter, the growth and lipid accumulation profiles of both fungi were elucidated. Both fungi showed a similar profile of lipid accumulation as reported in oleaginous yeasts (Botham and Ratledge, 1979; Evans and Ratledge, 1985a) where depletion of nitrogen source in the presence of excess glucose triggered lipid accumulation. This experiment attempts to investigate the profile of several enzymes which

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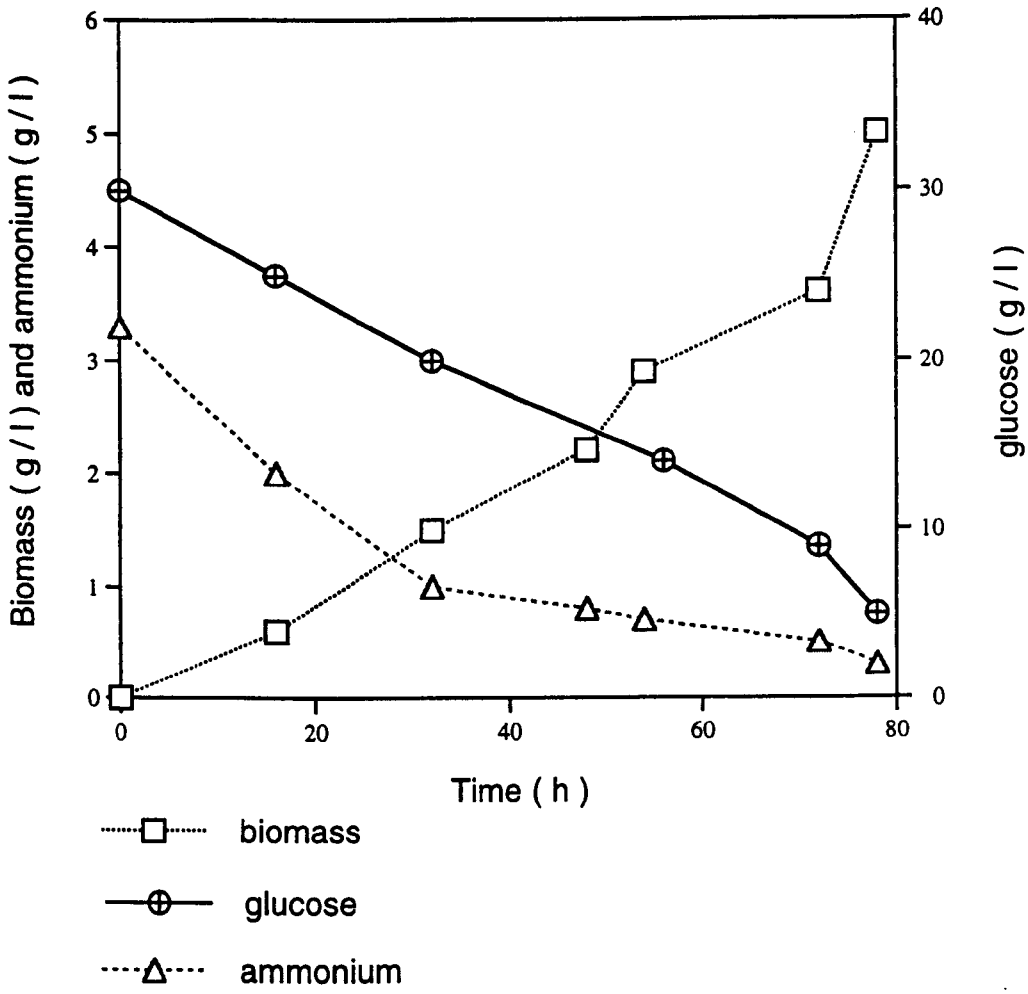
Figure 14a: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with 5 % (v / v) inoculum.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 5 % (v / v) inoculum was added to Fermenter 8, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 14a


Biomass (g / l) and ammonium (g / l)
glucose (g / l)
Time (h)
0
20
40
60
80
0
1
2
3
4
5
6
0
10
20
30
40
biomass
glucose
ammonium

The low lipid production was due to the unfavourable conditions for lipid biosynthesis which occurred as the nitrogen source was not depleted until the late stage of the experiment. It was thought that the poor performance of this fungus may be due to an insufficient amount of seed culture used for the inoculation (5 % , v / v). Problems associated with poor growth exhibited by this fungus were first encountered during the preparation of the seed culture prior to the experiment. Inoculation of mycelia from a stock culture grown on PDA into whirlipots containing 500 ml of nitrogen-limiting media (see Section 2.1.3) resulted in no growth after 48 h. This was done in duplicate and repeated twice with the same results. The seed culture was subsequently prepared by transferring mycelia from a stock culture (PDA) into shake flasks containing 500 ml of the same nitrogen-limiting media where a substantial amount of biomass was formed after 72 h of incubation.

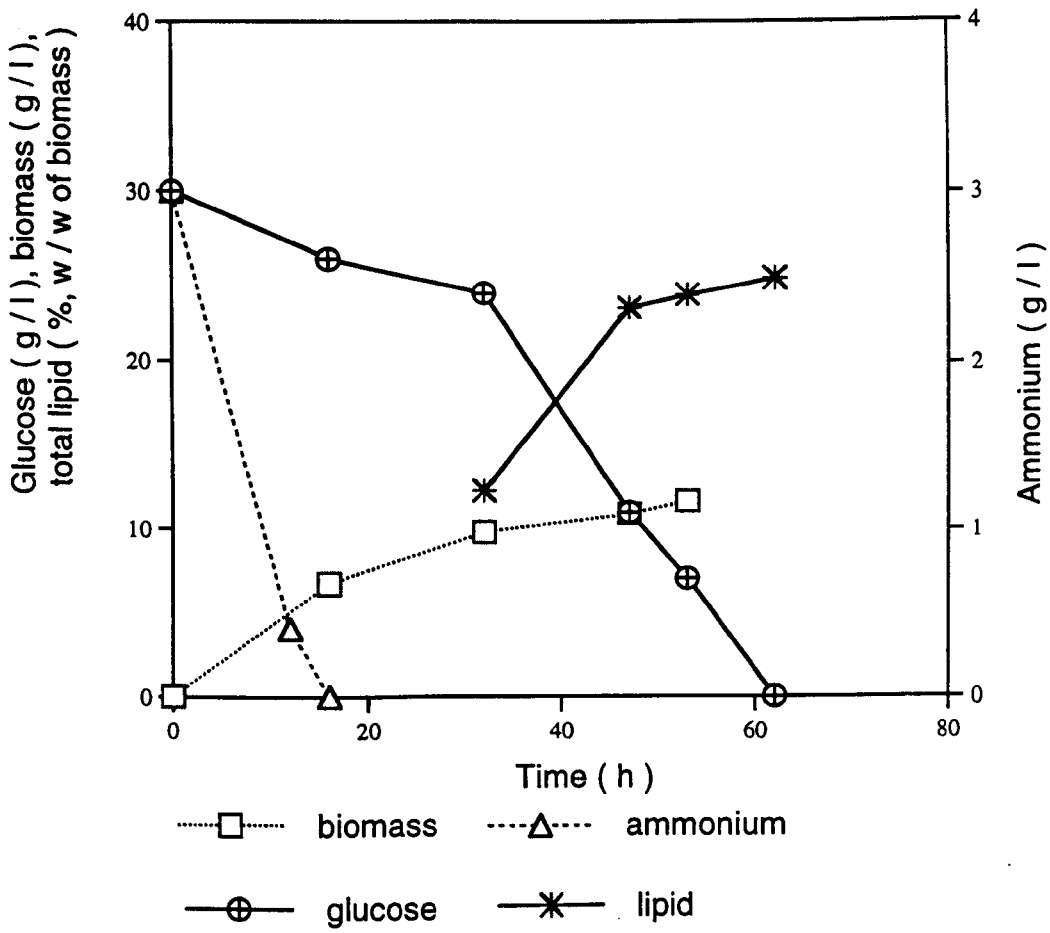
When the same experiment was repeated but by adding a larger inoculum size (10 %, v / v) into a fermenter (Fermenter 9), an improved growth and lipid accumulation were observed (Figure 14b). The biomass increased rapidly from 8 g / l at 18 h to 11 g / l at 53 h coincident with the decrease of glucose from 26 g / l to 7 g / l. In contrast to the previous run in Fermenter 8 , the ammonium concentration was now depleted at an early stage of fermentation (16 h) which triggered the biosynthesis of lipid as seen by the increase of total lipid from 12 % (w / w of biomass) at 31 h to 24 % (w / w of biomass) at 62 h .

This experiment showed that by increasing the amount of seed culture from 5 % to 10 % (v / v), improved growth and lipid production were achieved. The culture showed a high biomass and lipid yield (0.37 and

Figure 14b: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with 10 % (v / v) inoculum.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 14b


Glucose (g / l), biomass (g / l), total lipid (%, w / w of biomass)
Ammonium (g / l)
Time (h)
0
20
40
60
80
0
10
20
30
40
0
1
2
3
4
biomass
ammonium
glucose
lipid

0.088 g / g glucose used, respectively). However, the lipid production was still not as high as previously reported (Kendrick and Ratledge, 1992a). The fatty acid composition of the lipid is shown in Table 10.

Another problem encountered when growing this fungus was the occurrence of foam on the surface of the media after approximately 72 h of incubation in Fermenter 8. A more severe problem of foam formation was observed in Fermenter 9 where a stable formation of foam occurred after 16 h of incubation. The problem was so severe that a substantial amount of the liquid culture was transported by the foam into the air outlets causing the air filter connected to the outlet to become wet. This problem was subsequently overcome by adding approximately 0.3 ml of anti-foam to the culture at the beginning of the fermentation. These experiments were repeated twice and typical data are presented.

As the fermentation was performed without baffles, less wall growth was observed. However, adhesion of cells still occurred particularly around the impeller of the fermenter due to the filamentous morphology of the fungus, which caused it to be easily entangled to the rotating impeller during agitation.

Table 10: Growth, Lipid Accumulation and Fatty Acid Profiles of *Mt. alpina* Grown in a Fermenter Using 10 % (v / v) Seed Culture.

Max. Biomass (g / l)	10-11
Max. Total Lipid (%, w / w of biomass)	24-27
Biomass Yield (g / g glucose used)	0.33-0.37
Lipid Yield (g / g glucose used)	0.088-0.09
Fatty acids (Relative %, w/w)	
14:0	9-12
16:0	17-18
18:0	12-15
18:1	28-33
18:2	13-17
18:3	4-6
20:4	4-7

The organism was grown in 5 L fermenters (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9 (two replicates), containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 24 :1 (30 g glucose / l and 3.3 g ammonium tartrate / l). Samples analysed were taken after approximately 60 h growth. The values given represent the range of estimates from duplicate experiments.

4.1.4 Conclusions

1) Cultivation of *Mt. alpina* using 5 % (v / v) inoculum in a fermenter resulted in poor growth and lipid production. Optimal growth and lipid production were achieved when *Mt. alpina* was cultivated in a fermenter using 10 % (v / v) of seed culture .

3) Further optimization of the growth condition was required as the lipid production achieved (24 %, w / w of biomass) was lower than previously reported where up to 38 % (w / w of biomass) was produced.

4.2.0 The Effect of Increasing C:N Ratio on Growth and Lipid Production of *Mt. alpina* in a Fermenter

4.2.1 Objectives

As previously observed, *Mt. alpina* showed a better growth and lipid production when cultivated in a fermenter using 10 % (v / v) inoculum compared to when inoculation was carried out using a 5 % (v / v) inoculum. Although an improved performance was achieved, the lipid production was still low (24 %, w / w of biomass) compared to what was reported in an earlier work on this fungus where up to 38 % (w / w of biomass) was produced (Kendrick and Ratledge, 1992a). Therefore, attempts were made to further improve the lipid production performance of this fungus by increasing the C:N ratio of the media.

4.2.2 Materials and Methods

Experiment was carried out using a 5 L fermenter (Fermenter 10) as described in Section 4.1.2, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40:1 (30 g glucose / l and 2.0 g ammonium tartrate / l). Samples were taken at intervals for determination of biomass, glucose, ammonium concentration, cell lipid content and fatty acid analysis using methods described in Chapter 2.

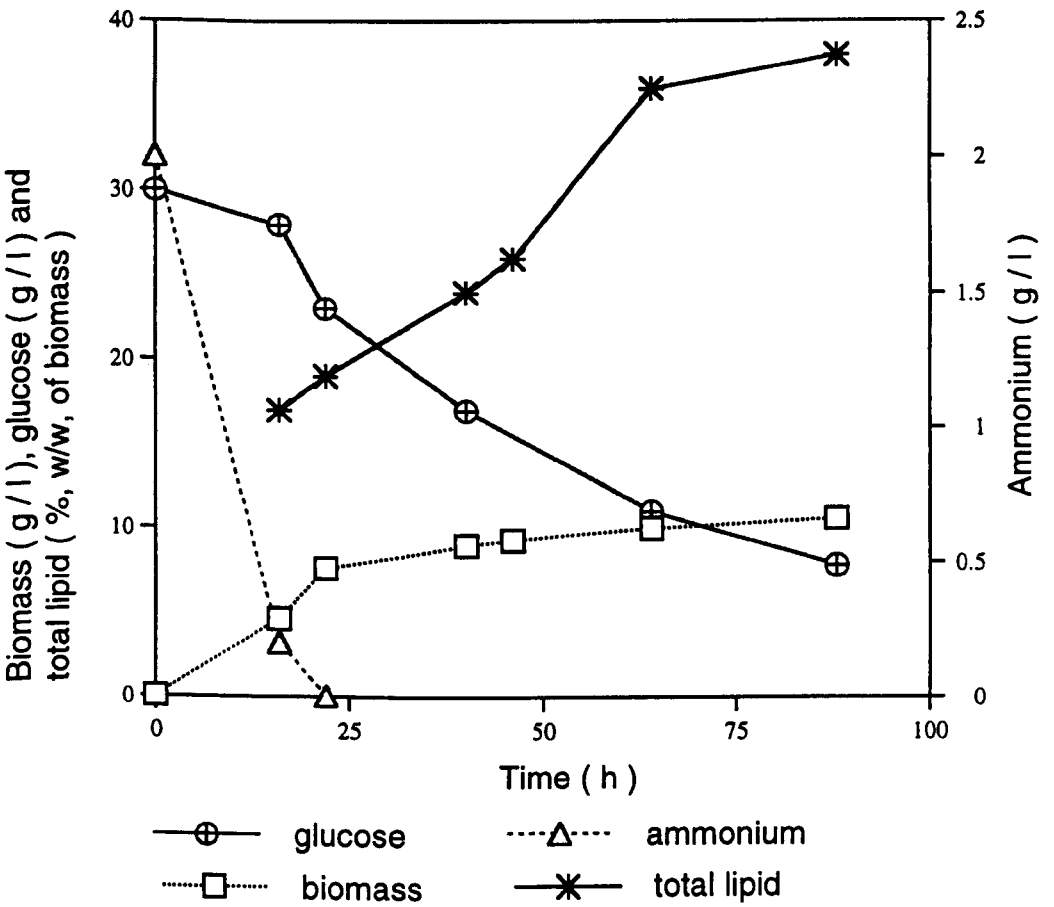
4.2.3 Results and Discussion

Figure 15 shows the growth and lipid accumulation profile of *Mt. alpina* in Fermenter 10. The biomass increased rapidly from 5 g / l at 16 h

Figure 15: Growth and lipid production profile of *Mt. alpina* grown in a fermenter with an increased C:N ratio.

Experiment was carried out using a 5 L fermenter (as described in Section 4.1.2) with a final working volume of 4 L. A 10 % (v / v) inoculum was added to Fermenter 9, containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40 :1 (30 g glucose / l and 2.0 g ammonium tartrate / l). 10 % (v / v) inoculum. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.

Figure 15


Biomass (g / l), glucose (g / l) and total lipid (%, w/w, of biomass)
Ammonium (g / l)
Time (h)
0
10
20
30
40
0
0.5
1
1.5
2
2.5
0
25
50
75
100
glucose
ammonium
biomass
total lipid

to 9 g / l at 40 h associated with the decrease of glucose and the depletion of ammonium. The lipid content of the culture increased from 19 % (w / w of biomass) at 22 h to 38 % (w / w of biomass) at 64 h coincident with the rapid decrease of glucose concentration from 23 g / l to 8 g / l. This result suggests that by increasing the C:N ratio of the growth media, a better growth and lipid accumulation performance could be achieved. Optimal fungal growth is known to occur under optimal nutritional and environmental conditions, and is the result of various metabolic activities in a certain balance.The C:N ratio is known as one of the most important nutritional parameter for lipid production (Weete, 1980). High production of lipid is associated with low rates of protein synthesis. A high C:N ratios reduces protein synthesis resulting in more carbon substrate being available for conversion to lipid (Ratledge and Wilkinson, 1988).

The biomass and lipid yields of the culture were higher (0.5 and 0.19 g / g glucose used, respectively) than that observed in Fermenter 9 (Section 4.2.0) which were 0.37 and 0.088 g / g glucose used, respectively. Therefore, with glucose concentration still kept at 30 g / l, but by decreasing the ammonium tartrate concentration from 3.3 g / l to 2 g / l , higher lipid production was achieved. However the fatty acid profiles obtained were similar as observed in Fermenter 9 (Section 4.2.0) where the most abundant fatty acid was 18:1 followed by 18:2, 18:0, 16:0,14:0, 20:4 and 18:3 (Table 11).

During the fermentation run, no difficulties were encountered. The problem of excessive foam formation was overcome by the addition of anti-foam agent (0.3 ml) at the beginning of the experiment. The problem of wall growth has been decreased by performing the fermenter

Table 11: Fatty acid Profiles of *Mt. alpina* Grown in a fermenter with an Increased C:N ratio

Fatty Acids	Relative % (w / w)
14:0	5-9
16:0	16-20
18:0	13-16
18:1	29-31
18:2	15-21
18:3	3-5
20:4	4-6

Mt. alpina was cultivated in a fermenter as described in Section 4.1.2 . A 10 % (v / v) seed culture was added to a fermenter containing a nitrogen-limiting medium (see Section 2.1.3) with C:N ratio at 40:1 (30 g glucose / l and 2 g ammonium tartrate / l).Data presented were based on the analysis of samples from three replicates of Fermenter 10 obtained after 90 h of growth .

without baffles where less cells adhered to the fermenter walls.

4.2.4 Conclusions

Mt. alpina showed improved growth and lipid accumulation when cultivated in a medium with an increased C:N ratio. This was thought to be the result of the decreased ammonium tartrate concentration used in the medium which allowed more carbon to be channelled into lipids rather than being used for protein synthesis. No significant changes in the fatty acid profiles were observed.

CHAPTER 5

THE BIOCHEMISTRY OF LIPID ACCUMULATION IN *M. circinelloides and Mt. alpina*

INTRODUCTION

This chapter discusses on the work carried out in the attempt to elucidate the enzymatic regulation of lipid accumulation in *M. circinelloides* and *Mt. alpina*. The activity profiles of several enzymes, which are related to the regulation of fatty acid biosynthesis as previously established in oleaginous yeasts (Evans and Ratledge, 1985a; Boulton and Ratledge, 1981; Botham and Ratledge, 1979), were studied. The discussion will also include the description of preliminary work (ie. optimization of cell extracts preparation) carried out prior to the biochemical studies being performed.

5.1.0 Disruption of Fungal Biomass

5.1.1 Objectives

This experiment was carried out to develop an efficient method of cell disruption for the production of cell extracts from these two fungi. As the French press technique has been regularly used in this laboratory and proved to be efficient in the applications toward several organisms (Kendrick, 1992; Wynn, 1994), this method was adopted and its efficiency on the disruption of cells of *M. circinelloides* and *Mt. alpina* was investigated.

5.1.2 Materials and Methods

5.1.2.1 Methods of Cultivation

M. circinelloides and Mt. alpina were cultivated in 5 L fermenters (as described in Section 4.1.2) containing nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l) and 40:1 (30 g glucose / l and 2 g ammonium tartrate / l), respectively. For the cultivation of *M. circinelloides,* a 5 % (v / v) seed culture was used while 10 % (v / v) inoculum was used for the growth of *Mt. alpina.* Both cultures were harvested after 24 h of inoculation.

5.1.2.2 Disruption Technique

Harvested fungal mycelia were resuspended in an extraction buffer (see Section 2.4.0) to give approximately 20 % (wet wt / v) mycelial suspensions. The mycelial suspension was passed four times through a cold (4 ^{o}C) French pressure cell at a pressure of 35 MPa. After each passage, approximately 20 ml samples were taken from the resulting homogenate and placed in a separate ice-cold beaker. French pressed extracts were centrifuged at 16 000 g for 20 min at 4 ^{o}C to remove cell debris. Floating layers of lipid were removed by filtrating the crude extracts through a Whatman No 1 filter paper. The protein concentration of each resulting supernatant was then determined using method described in Section 2.9.0.

5.1.3 Results and Discussion

Table 12 summarizes the effect of number of passes through the French press on the disruption of *M. circinelloides* and *Mt. alpina*. In *M. circinelloides*, only 36 % (1.2 mg / ml) of the protein in the final extract after 4 passes, was released after a single pass through the French press. After the second pass, the protein concentration increased to 73 % of the final protein concentration. In *Mt. alpina*, 41 % (1.4 mg / ml) of the total protein was released after the first pass and 71 % (2.4 mg / ml) after two passes.

A yellowish cell extract was obtained from *M. circinelloides* while a slightly milky extract was observed in the cell extract produced from *Mt. alpina*. In the subsequent work, both fungi were disrupted by only two passes through the French press as a substantial amount of protein was liberated from the mycelia with the minimum physical stress. Vigorous methods of cell disruption have been reported to affect the activity and the structure of certain enzymes (Dutton, 1988). In this method, cells were forced through a small orifice at a very high pressure; shear forces disrupted the cells.

Several difficulties were encountered when using this method. Firstly, the temperature of the French press increased very quickly from 4 ^{o}C to between 8 to 13 ^{o}C. So, after several passes, it had to be held in ice in order to restore the low temperature. This caused difficulties when processing a large amount of samples. Secondly, the rubber seal which was attached to the plunger of the French press wore out very quickly and sometimes the seal was cut during the process causing the plunger to

Table 12: The Effect of Number of Passes through a French Press on the disruption of *M. circinelloides* and *Mt. alpina*

	Protein concentration in extract (mg / ml)	
No. of Passes	*M. circinelloides*	*Mt. alpina*
1	1.2	1.4
2	2.4	2.4
3	2.8	2.6
4	3.3	3.4

The fungi were cultivated in 5 L fermenters and cells were harvested after 24 h of incubation. Harvested fungal mycelia was resuspended in an extraction buffer (see Section 2.4.0) to give approximately 20 % (wet wt / v) mycelial suspension. The mycelial suspension was passed four times through a cold (4 ^{o}C) French pressure cell at a pressure of 35 MPa where after each passes, approximately 20 ml of the resulting cell extracts were taken and placed in a separate ice-cold beaker. French pressed extracts were centrifuged at 16 000 g for 20 min at 4 ^{o}C to remove cell debris. Floating layers of lipid were removed by filtration of the crude extracts through a Whatman No 1 filter paper. The protein concentration of each resulting supernatants was then determined.

stick and difficult to pull out. This problem was more pronounced when processing a large volume of sample where it could cause severe delays in order to mend the unit. Therefore, regular checking of the seal was required between each passes.

5.1.4 Conclusions

1) Two passes of cell extracts through the French press were sufficient in the disruption of cells from both fungi. Therefore, this technique was adopted for the preparation of cell extracts from both fungi for the rest of the work.

2) It was important to maintain the French pressure cell at a low temperature by holding it in ice after performing several passes in order not to over-heat the samples. In this experiment, the temperature of the final extracts varied between 8 to 13 ^{o}C.

5.2.0 The Investigation of Enzymatic Regulation Involved In the Lipid Biosynthesis of *M. circinelloides* and *Mt. alpina*

5.2.1 Objectives

In the previous chapter, the growth and lipid accumulation profiles of both fungi were elucidated. Both fungi showed a similar profile of lipid accumulation as reported in oleaginous yeasts (Botham and Ratledge, 1979; Evans and Ratledge, 1985a) where depletion of nitrogen source in the presence of excess glucose triggered lipid accumulation. This experiment attempts to investigate the profile of several enzymes which

5.2.3 Results and Discussion

5.2.3.1 FAS

FAS catalyzes the following reaction:

Acetyl-CoA + 7 malonyl-CoA

+ 14 NADPH + 20 H^+ ⟶ palmitoyl-CoA + 7 CO_2

+14 $NADP^+$ + 7 CoA + 6H_2O

Verification of Assay. Table 13 summarizes the procedure carried out in the verification of FAS assay using the spectrophotometric method described in Section 2.5.9. In Reaction 1, performed without acetyl-CoA included in the reaction mixture, the activity obtained after initiation with malonyl-CoA (7 nmol / min.mg protein^{-1}) was similar to the endogenous rate observed (before initiation). When reaction was performed with malonyl-CoA excluded from the assay mixture (Reaction mixture 2), a similar result was obtained where the activities observed before and after initiation with acetyl-CoA were the same. When the reaction was carried out with the inclusion of acetyl-CoA, a higher activity compared to the endogenous rate was achieved when the reaction was initiated by the addition of malonyl-CoA. These experiments showed that the reaction was dependent on the presence of both substrates thus confirming the specificity of the reaction. The initial velocity of the reaction was also shown to be proportional to the concentration of the enzyme used when doubling the amount of the extract used in the assay gave a 2 fold increase in activity (data not shown).

Table 13: Verification of FAS assay

Cell-Free Extract (µl)	Reaction No.	Acetyl-CoA	Malonyl-CoA	Activity Before initiation**	Activity After initiation**	Initial Activity**
20	1	-	+*	7	7	0
	2	+*	-	7	7	0
	3	+	+*	7	27	20

+, included in reaction mixture; -, omitted in reaction mixture; +*, used for initiation.

**, nmol / min.mg protein^{-1}

Data presented were based on analysis of sample taken after 16 h of growth from *M. circinelloides* culture. Reaction was carried out using standard protocol described in Section 2.5.9. Activity before initiation refers to the endogenous rate observed before initiation was carried out. Initial activity was obtained by subtracting the activity observed after initiation with the endogenous rate. The protein concentration of the cell extract was 1.8 mg / ml.

FAS Activity Profile. A similar profile of FAS activity was observed during growth of both fungi. As ammonium was depleted at 16 h and 22 h in the culture of *M. circinelloides* and *Mt. alpina*, respectively (Figure 16a and b) the total lipid in both culture increased coincidentally with the increase of FAS activities in both fungi (Figure 17a). Both cultures showed a subsequent decrease in FAS activity, approximately after 40 h in *M. circinelloides* and after 50 h in *Mt. alpina.*

The importance of FAS in lipogenesis is obvious and this enzyme has been reported to play an important role in controlling biosynthesis of fatty acids in non-oleaginous yeasts where addition of fatty acids into the growth medium resulted in repression of FAS activity (Meyer and Schweizer, 1976). To date, there have been very few studies on the FAS in oleaginous fungi and so its possible regulatory role remains uncertain.

5.2.3.2 ACC

ACC catalyzes the following reaction:

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \longrightarrow \text{malonyl-CoA} + \text{ADP} + P_i + H^+$$

Verification of Assay. Table 15 summarizes the procedure carried out in the verification of the enzyme assay using method described in Section 2.5.10. When the reaction was performed with the inclusion of 50 µl cell-free extract in the reaction mixture, a mean value of 1.4 nmol / min.mg protein^{-1} was obtained when the reaction was stopped after 10 min (Reaction 1). Linearity of the reaction was determined by

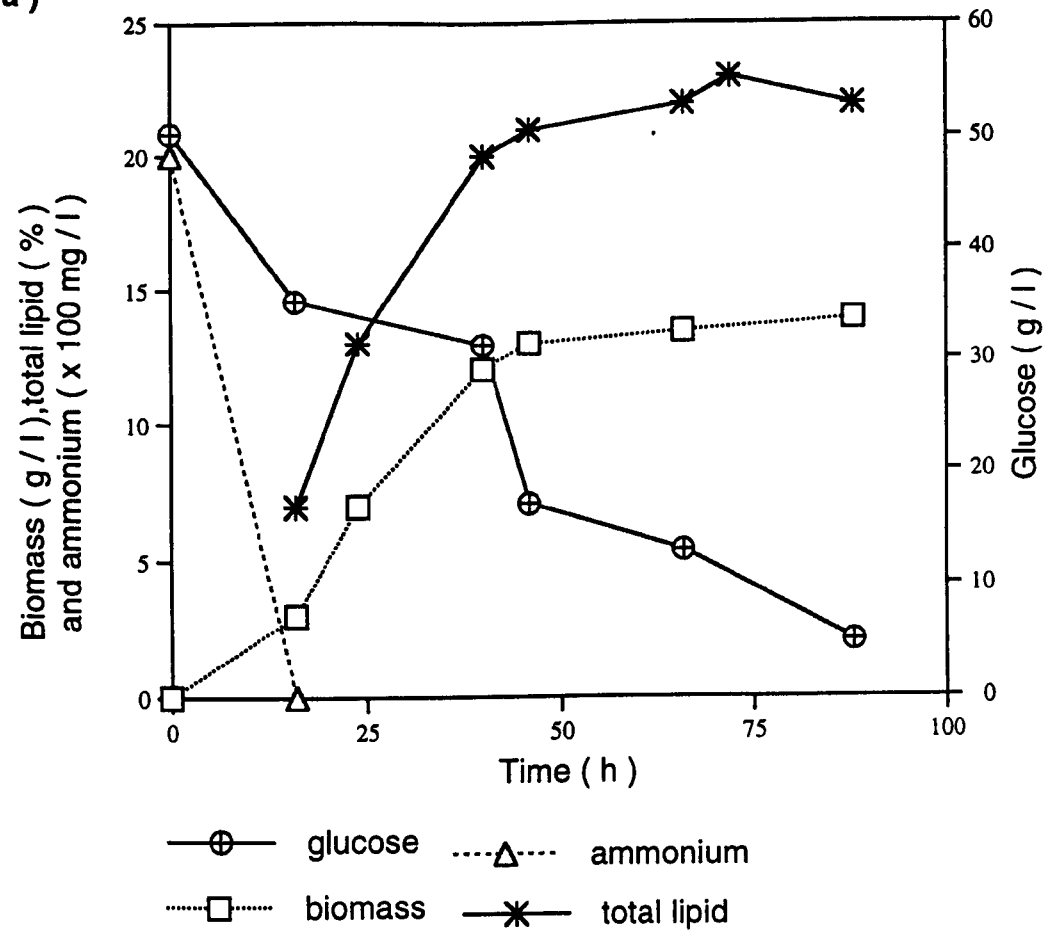
Figure 16 : Biomass, glucose, ammonium concentration and total lipid content of:

a) *M. circinelloides* culture in a fermenter with C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l).

b) *Mt. alpina* culture in a fermenter with C:N ratio at 40:1 (30 g glucose / l and 2 g ammonium tartrate / l).

***M. circinelloides* and *Mt. alpina* were cultivated in nitrogen-limiting media (as described in Section 2.1.3) using 5 % and 10 % (v / v) inoculum, respectively. The variance for biomass, ammonium and glucose assays were generally similar to those shown in Figure 7a and b.**

a)


Biomass (g / l),total lipid (%) and ammonium (x 100 mg / l)
Glucose (g / l)
Time (h)
0
5
10
15
20
25
0
10
20
30
40
50
60
0
25
50
75
100
glucose
ammonium
biomass
total lipid

b)

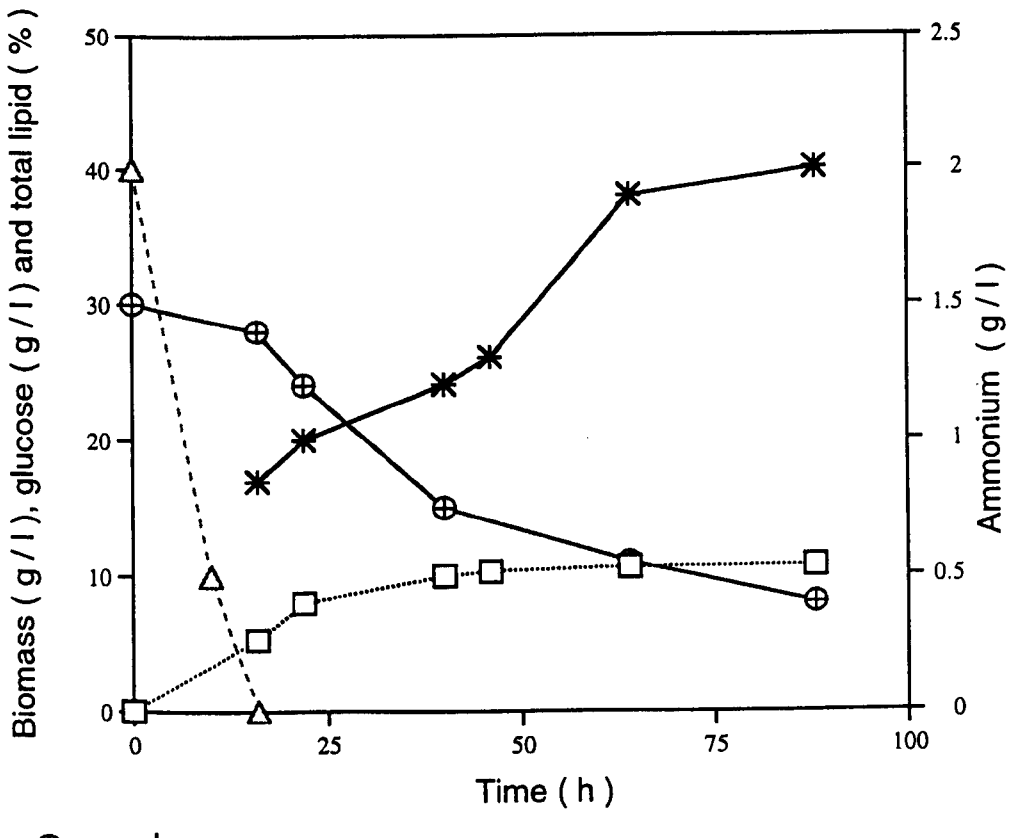
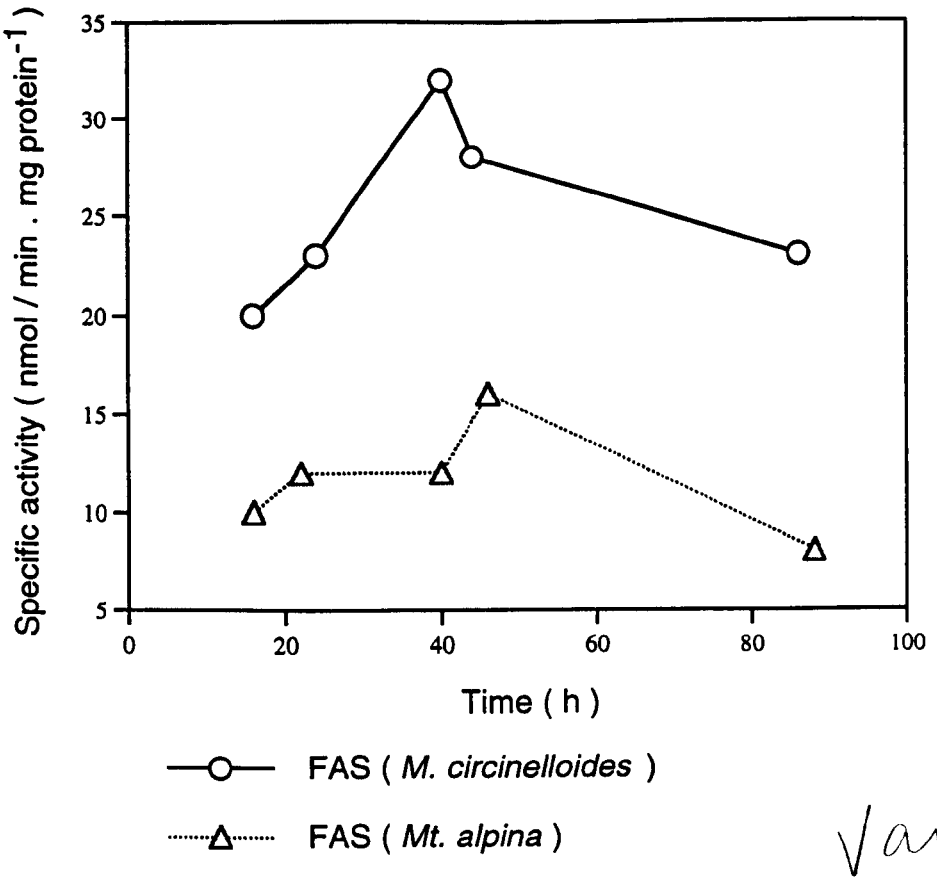

Biomass (g / l), glucose (g / l) and total lipid (%)
Ammonium (g / l)
Time (h)
0
10
20
30
40
50
0
0.5
1
1.5
2
2.5
0
25
50
75
100
glucose
ammonium
biomass
total lipid

Figure 17a: FAS Activity Profile of *M. circinelloides* and *Mt. alpina*



Activities were determined using method described in Section 2.5.9

Table 14: Verification of ACC assay

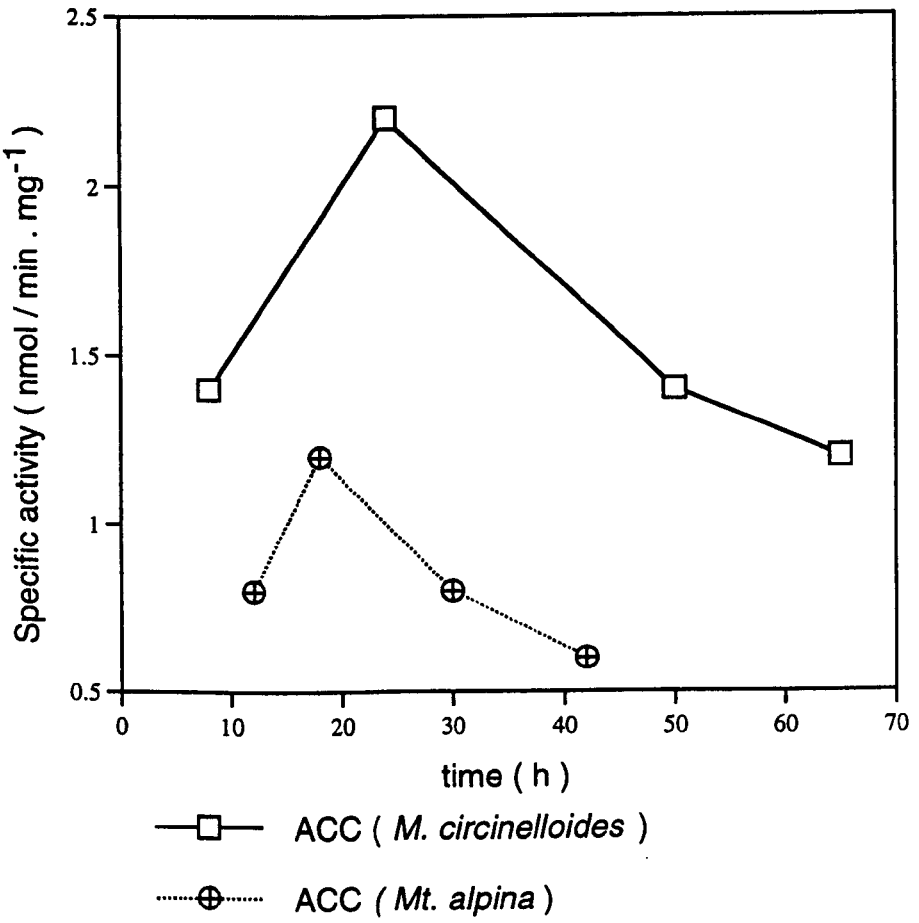
Cell-free Extract (µl)	Reaction No.	Reaction Period (min)	Specific activity nmol /min.mg protein^{-1}		Mean
			R1	R2	
50	1	10	1.4	1.3	1.4
	2	30	1.5	1.1	1.3
25	3	10	1.3	1.5	1.4

Data presented were based on analysis of sample from *M. circinelloides* taken after 6 h of inoculation. All assays were carried out in duplicates and the reaction rates were calculated based on the initial cpm obtained from each assay which had been subtracted with the cpm obtained from a blank assay mixture. Blank assays were carried out by using the same procedure but the cell extracts used for the reaction were boiled for approximately 3 minutes. The protein concentration of the extract was approximately 1.3 mg / ml.

performing the reaction for 30 minutes (Reaction 2). A similar activity was observed (1.3 nmol / min. mg protein^{-1}) as in Reaction 1 (1.4 nmol / min.mg protein^{-1}). This showed that the activity measured was the steady-state rate of the reaction. When experiment was repeated using half the amount of cell-free extract (Reaction 3), the total activity measured was half of that observed in Reaction 1 (data not presented). This showed that the activities were proportional to the amounts of protein included. Dependency of the reaction on acetyl-CoA and ATP was determined by measuring the activities with the exclusion of one of the cofactors while the other one was included in the reaction mixture. No activity was observed in both cases, showing that the reaction was dependent on the presence of both cofactors (data not presented). The reaction rates were calculated based on the initial counts per min (cpm) obtained from each assay which have been subtracted with the cpm obtained from a blank assay. The blank assay was performed using the same procedure but the cell extracts used for the reaction were boiled for approximately 3 minutes.

ACC Activity profile. ACC activities varied from 0.6 nmol / min.mg protein^{-1} to 2.2 nmol / min.mg protein^{-1} in both *M. circinelloides* and *Mt. alpina* (Figure 17b). The activities increased at approximately 22 h immediately after the depletion of ammonium in both cultures. A low activity of ACC has also been reported in *Candida* 107 and *Candida utilis* where it occurred between 1 to 2.7 nmol / min.mg protein^{-1}(Botham and Ratledge, 1979). However, in both fungi, the rates of ACC reaction were lower than the rates of lipid synthesis observed indicated by the following calculations.

Figure 17b: ACC activity Profile of *M. circinelloides* and *Mt. alpina*



Assays were performed using method described in Section 2.5.10

The lipid synthesis rate of *M. circinelloides* and *Mt. alpina* between 16 h to 25 h was 0.18 and 0.28 nmol / min.mg protein^{-1}, respectively, assuming a protein concentration of 40 % in the biomass and the molecular weight of triacylglycerol as 890. As ACC must provide 24 mol malonyl-CoA to produce 1 mol triacylglycerol, the enzyme in *M. circinelloides* and *Mt. alpina* must be operating at a minimum rate of 0.18 x 24 = 4.3 nmol / min.mg protein^{-1} and 0.28 x 24 = 6.72 nmol / min.mg protein^{-1}, respectively. Therefore, the rate of ACC observed in both fungi was insufficient to provide the required amounts of malonyl-CoA for the synthesis of triacylglycerol. The low activities observed in both fungi may probably due to the inactivation of the enzyme during cell disruption.

ACC is an important enzyme in lipid biosynthesis as it catalyzes the conversion of acetyl-CoA to malonyl-CoA to initiate fatty acid biosynthesis (Wakil et al., 1983). Indeed the key position occupied by this enzyme in fatty acid biosynthesis has led to suggestion that it is a key enzyme in the regulation of lipid biosynthesis. Growth of *Saccharomyces cerevisiae* (Kamiryo and Numa, 1973) and *Yarrowia (Candida) lipolytica* (Mishina et al., 1976) in a medium containing saturated, monounsaturated and polyunsaturated fatty acids was shown to result in 80 % decrease in the activity of acetyl-CoA carboxylase. This was caused by the reduction of mRNA coding for this enzyme (Horikawa et al., 1983).

5.2.3.3 ACL

ACL catalyzes the following reaction:

Citrate + ATP +CoA ⟶ acetyl-CoA+oxaloacetate+ADP+P_i

Verification of Assay. Verification of ACL assay was carried out using method described in Section 2.5.1 by determining the dependency of activity for ATP, CoA and citrate. The reaction was totally dependent on the presence of the three cofactors (Table 15) where no activity was detected when one of the substrates was excluded from the reaction mixture (ie. the rate measured after initiation was similar to the endogenous rate). The activity was also proportional to the amount of proteins added where a two fold increase of total activity was observed when the amount of cell-free extracts included was doubled (data not presented). In this assay, the ATP used was prepared fresh daily and the pH was adjusted at 7.0 as failure to do so will cause it to deteriorate very quickly. Addition of 15 µl of 1 M sodium azide into the reaction mixture (1 ml) was also necessary in order to inhibit the high activity of NADH oxidase observed before initiation. Addition of glycerol (20 % w / v) and thiol reagent (mercaptoethanol) in the buffer used for preparation of cell extracts was necessary (Boulton and Ratledge, 1983; Shashi et al., 1990; Wynn et al., 1998) as this enzyme was reported to be deactivated when cell extracts were prepared using buffers lacking these compounds.

ACL Activity Profile. The activity of ACL in *M. circinelloides* increased between 16 h to 48 h (Figure 18a), after the depletion of ammonium (Figure 16a) which was exhausted at 16 h and with an increasing total lipid content of the cells (Figure 16a). Figure 18a shows a similar profile of ACL activity in *Mt. alpina*, which increased from 22 h as ammonium was depleted, coincident with the increase of

Table 15: Verification of ACL Assay

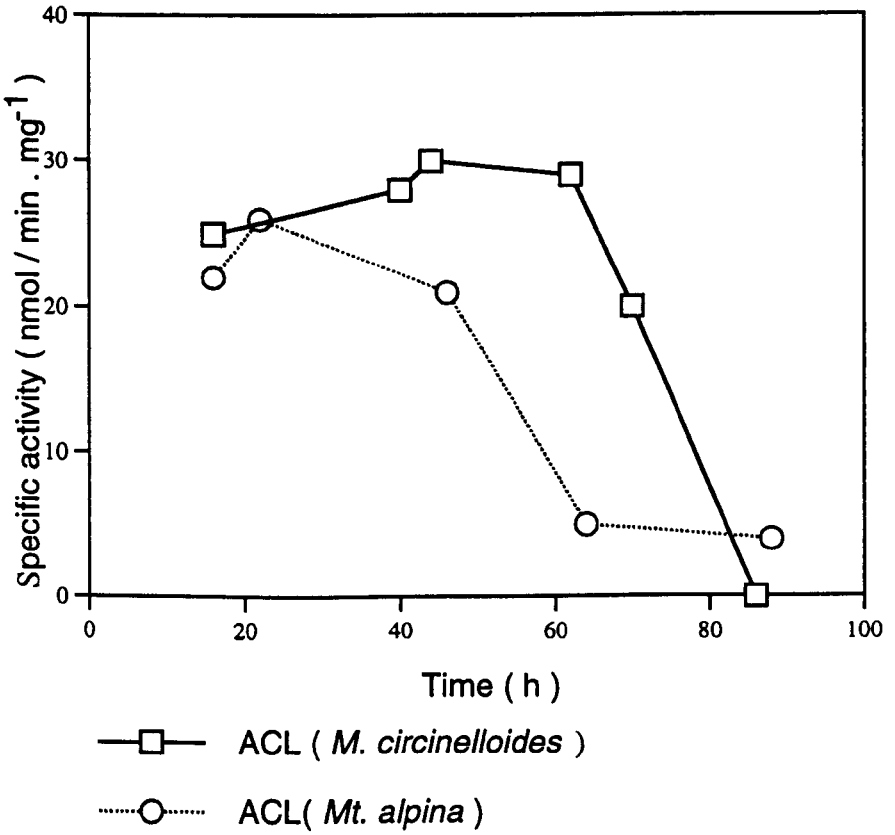
Cell-free Extract (µl)	ATP	CoA	Citrate	Activity Before Initiation**	Activity After Initiation**	Initial Rate**
50	+*	+	-	19.0	19.0	0
	+*	-	+	18.1	18.0	0
	-	+*	-	20.1	19.2	0
	+*	+	+	20.0	45.0	25.0

+, included in reaction mixture; -, omitted in reaction mixture; +*, used for initiation.

**, (nmol/min.mg protein^{-1})

Data presented were based on the analysis of sample from *M. circinelloides* taken after 16 h of growth. Assays were carried out using standard procedure described in Section 2.5.1. The protein concentration of the cell extract was 2.1 mg / ml. Activity before initiation refers to the endogenous rate observed before initiation was carried out. Initial activity was obtained by subtracting the activity observed after initiation with the endogenous rate.

Figure 18a: ACL Activity Profile of *M. circinelloides* and *Mt. alpina*



total lipid content of the cells (Figure 16b).

It has been reported that in oleaginous yeast *Candida curvata*, the activity of ACL increased 5-fold as the culture reached a nitrogen-limited phase (Evans and Ratledge, 1983). A similar observation has also been reported to occur in *Trichosporon cutaneum*, *Rhodosporidium toruloides* (Holdsworth et al., 1988) and *Lipomyces starkeyi* (Naganuma et al., 1987). ACL constitutes the only means of cytosolic citrate metabolism in eukaryotic cells. The cleavage of citrate transported from mitochondria yields acetyl-CoA which then serves as a primer for fatty acid biosynthesis. The occurrence of this enzyme has been correlated to the ability of micro-organisms capable of accumulating more than 20 % lipid (Boulton and Ratledge, 1981). It is also known to occur in a wide range of filamentous fungi (Wynn et al., 1998).

5.2.3.4 CAT

CAT catalyzes the following reaction:

Acetyl-CoA + carnitine ⟶ acetylcarnitine + CoA-SH

Verification of Assay. Verification of CAT assay was carried out using method described in Section 2.5.8 by determining the dependency of the reaction towards acetyl-CoA and carnitine and also by testing the proportionality of the activity to the amounts of protein added (Table 16a). The reaction showed a total dependency on the presence of acetyl-CoA and carnitine where omission of either one of the cofactors resulted in no stimulation of activities after the initiation. The reaction also showed a

Table 16a: Verification of CAT Assay

Cell-free Extract (μl)	Acetyl-CoA	Carnitine	Activity Before Initiation**	Activity After Initiation**	Initial Activity**
50	-	+*	10.8	11	0
	+*	-	13.1	13	0
	+	+*	12.1	22.2	10.1

+, included in reaction mixture; -, omitted in reaction mixture; +*, used for initiation.

**, (nmol/min.mg protein^{-1})

Results were based on the analysis of sample of *M. circinelloides* taken after 16 h of growth. Assays were carried out using standard protocol described in Section 2.5.8.The protein concentration of the cell extract was 1.7 mg / ml. Activity before initiation refers to the endogenous rate observed before initiation. Initial activity was obtained by subtracting the activity observed after initiation with the endogenous rate.

proportional increase in total activity to the amounts of protein added (data not presented).

CAT Activity Profile. The activity of CAT in *M. circinelloides* and *Mt. alpina* (Figure 18b) increased after nitrogen depletion, coincident with the increase of cell lipid in both cultures. This could be explained as CAT serves as a minor supplementary pathway to the citrate efflux system coupled with ACL for the provision of acetyl-CoA for lipid synthesis (Ratledge and Gilbert, 1985). CAT transfers acetyl units both into and out of mitochondria (Kohlaw and Tan-Wilson, 1977) for respectively, either degradation via tricarboxylic acid cycle or fatty acid biosynthesis. This enzyme also occurs in peroxisomes (Ueda et al., 1982) for the efflux of acetyl units derived from the oxidation of fatty acids. This enzyme has been reported to occur in a wide variety of filamentous fungi (Wynn et al., 1998).

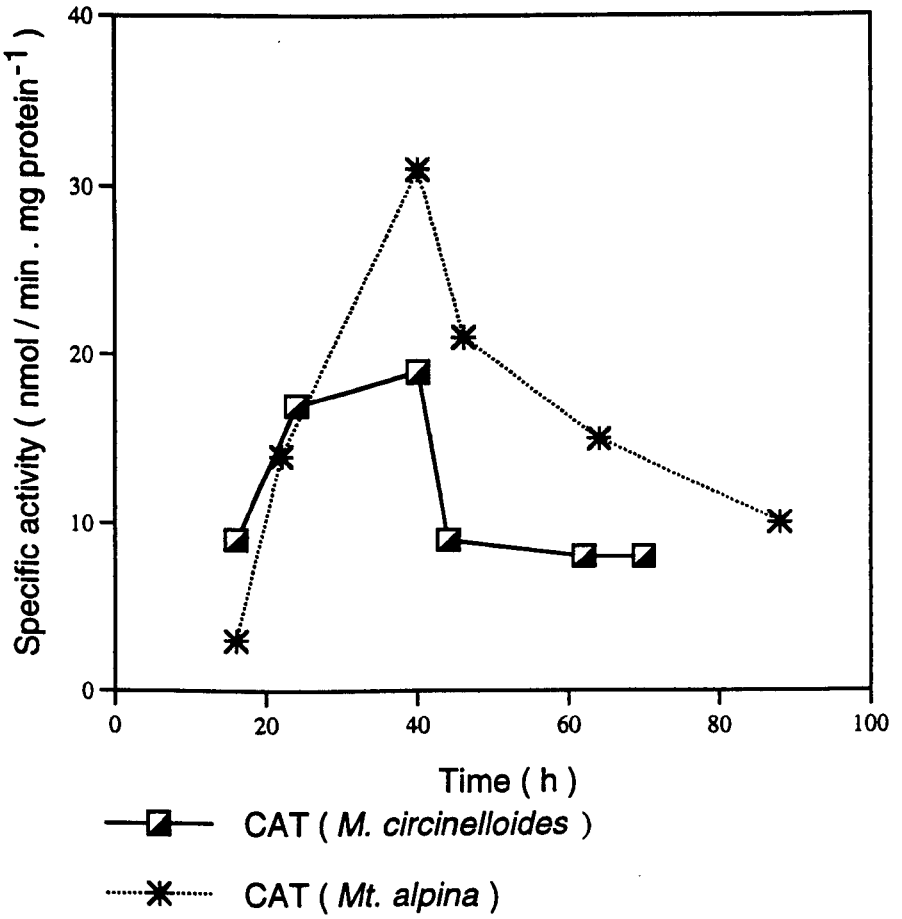
5.2.3.5 AMP:deaminase

AMP:deaminase catalyzes the following reaction:

$$AMP + H_2O \longrightarrow IMP + NH_4^+$$

Verification of assay. Verification of AMP:deaminase assay was carried out using method described in Section 2.5.11 by determining the proportionality of the reaction to the amounts of protein added into the reaction mixture. As a discontinuous method of assay was used, the linearity of the reaction was also checked every time the assay was carried out. Calculations of the activity for each assay were based

Figure 18b: CAT activity Profile of *M. circinelloides* and *Mt. alpina*



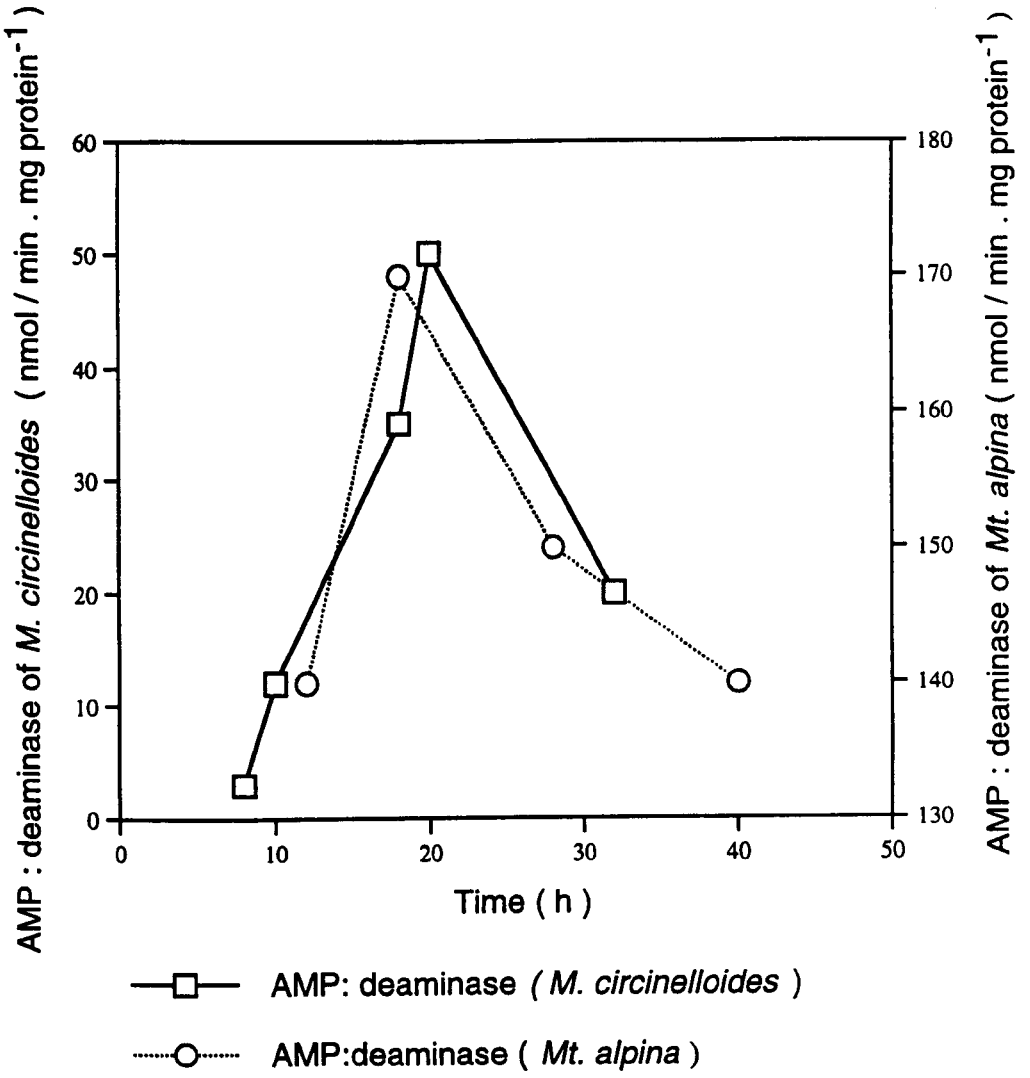
Assays were carried out using method described in Section 2.5.8

on the amounts of ammonium produced in each complete reaction mixture subtracted with the amount of ammonium detected in a control assay mixture containing the same amounts of cell extract but was not initiated with AMP.

The reaction was found to be linear when assays were carried out for 10 and 30 min (data not presented). A proportional increase in the total activity was also observed when the amounts of protein added was increased. Freshly prepared indophenol reagents were prerequisite in obtaining reproducible results as these reagents failed to work if stored for more than 2 months (at 25 ^{o}C). As ammonium tartrate was used in the growth media, thorough washing of mycelia when harvesting the fungi was necessary to prevent excessive ammonium being present in the extract as it will affect the accuracy of the assay.

AMP:deaminase Activity Profile. As shown in Figure 19a, AMP:deaminase activity in *M. circinelloides* increased from 4 nmol / min . mg protein^{-1} at 8 h to 50 nmol / min . mg protein^{-1} at 20 h after ammonium was exhausted before decreasing slowly to 20 nmol / min . mg protein^{-1} at 32 h. In *Mt. alpina*, the activity increased, though not as dramatically as in *M. circinelloides* , from 140 nmol / min . mg protein^{-1} to 170 nmol / min. mg protein^{-1} after the depletion of ammonium and remained high until 38 h. The activation of this enzyme which leads to the depletion of AMP has been reported to occur in oleaginous yeasts for example in *Rhodosporidium toruloides* where the activity of AMP:deaminase increased after exhaustion of nitrogen source (Evans and Ratledge, 1985a).

Figure 19a: Specific Activity Profile of AMP:deaminase of *M. circinelloides* and *Mt. alpina*



Assays were performed using method described in Section 2.5.11

5.2.3.6 NAD:ICDH

NAD:ICDH catalyzes the following reaction:

Isocitrate + NAD^+ ⟶ α-ketoglutarate + NADH

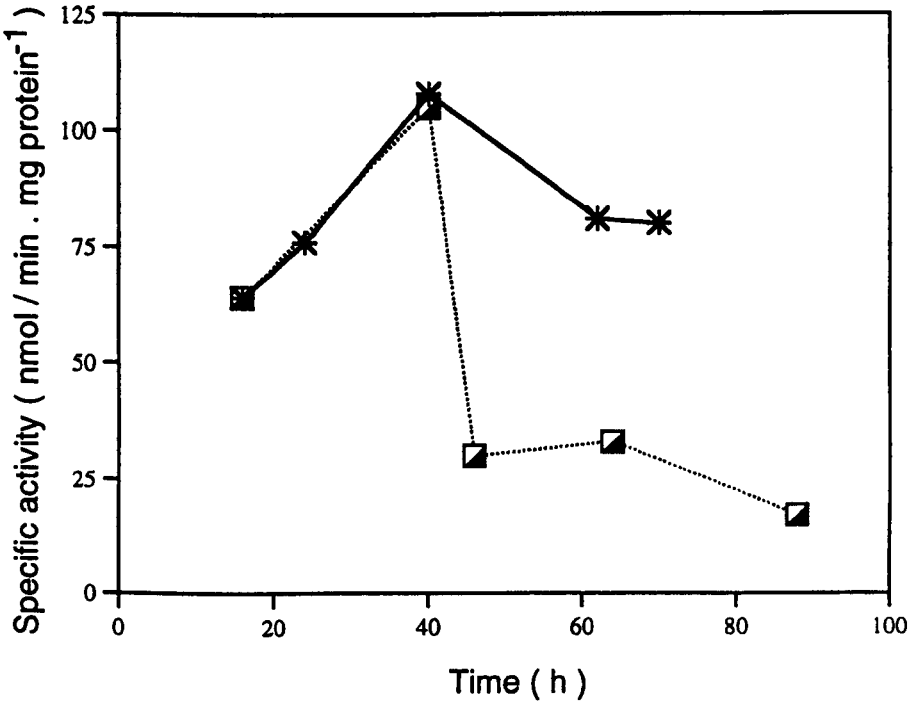
Verification of Assay. The activity of the enzyme was found to be proportional to the amounts of protein added where a 2-fold increase of total activity was observed when the amount of protein included into the reaction mixture was doubled (data not presented). Verification of this assay was carried out using assay method described in Section 2.5.7.

NAD:ICDH Activity Profile. The activity of NAD:ICDH observed in both cultures (Figure 19b) increased at an early stage of experiment but then decreased in the late stages. This enzyme in oleaginous yeasts has been reported to have an absolute requirement for AMP (Evans and Ratledge, 1985b; Botham and Ratledge, 1979). Depletion of AMP during transition to nitrogen-limited condition leads to the inactivation of this enzyme and hence a build-up of mitochondrial citrate which is then transported to the cytosol .

5.2.3.7 ME, G-6-PDH, 6-PGDH and NADP:ICDH

Verification of Assays. Verification of these enzymes were carried out using method described in Chapter 2. The total activities of all enzymes stated above were found to increase proportionally to the amounts of protein used. No difficulties were encountered when performing these assays.

Figure 19b: NAD:ICDH Activity Profile of *M.circinelloides* and *Mt. alpina*



Assays were performed using method described in Section 2.5.7

The ME, G-6-PDH, 6-PGDH and NADP:ICDH Activity Profiles. These enzymes, which generate NADPH, were present in both fungi. The activities of G-6-PDH (Figure 20b), 6-PGDH (Figure 20c) and NADP:ICDH (Figure 20d) from both fungi were high throughout the fermentation.

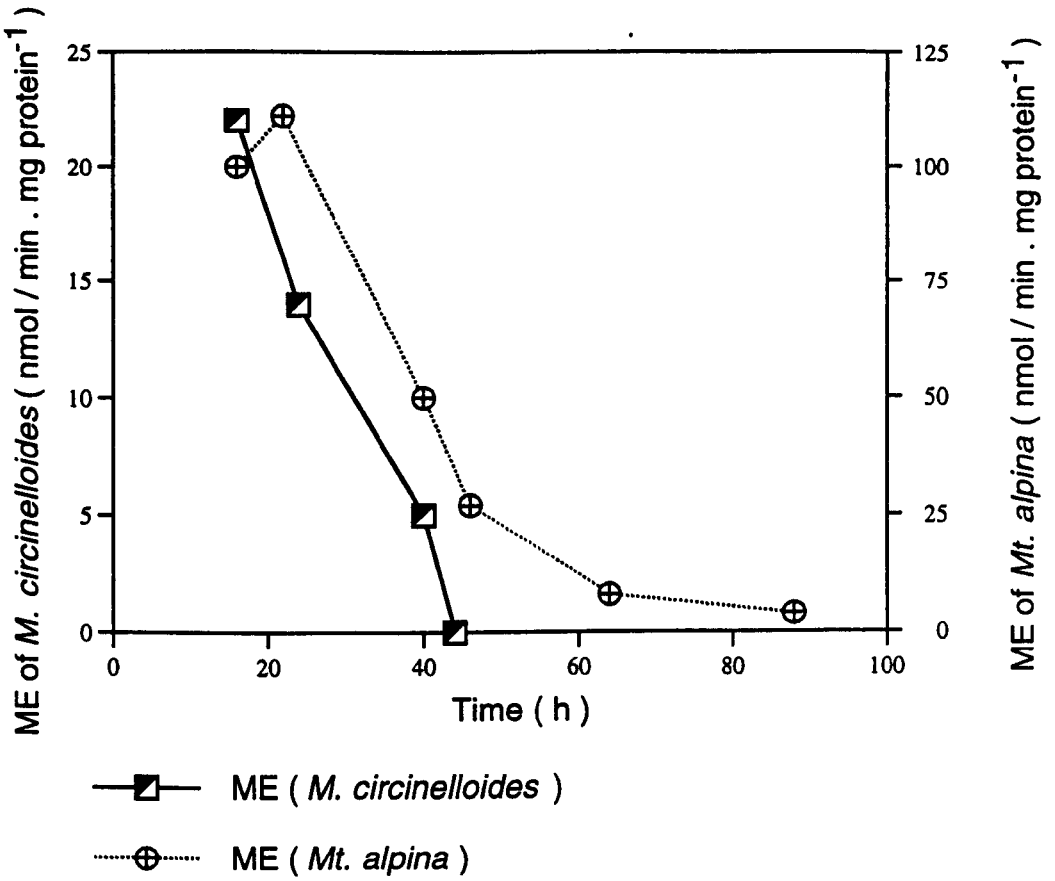
In *M. circinelloides*, the activity of ME diminished after nitrogen exhaustion and disappeared at 40 h (Figure 20a), coincident with the cessation of lipid accumulation (Figure 16a) while ME activity in *Mt. alpina* was still present until 88 h. Although the activity of key enzymes for fatty acid biosynthesis ie. FAS, ACL and ACC, together with G-6-PDH, 6-PGDH and NADP:ICDH which could be a possible alternative sources of NADPH for fatty acid biosynthesis still were active after ME activity had disappeared in *M. circinelloides*, no accumulation of lipid was observed after 40 h despite glucose still being present.

When experiments were repeated and cell extracts containing ME activity obtained at early hours of experiment were mixed with extracts obtained after ME has disappeared, no inhibition was observed thus indicating that the depletion of ME activity at 40 h was not due to the action of intermediary inhibition (see Table 16b).

The correlation between the depletion of ME activity and the diminishing accumulation of lipid in *M. circinelloides*, suggests the possibility of ME having a role as a major provider of NADPH for FAS activity. A possible explanation for this observation is the channelling of NADPH between ME and FAS, perhaps, as a result of a physical association between these two enzymes. ME has been shown to be a major source for lipid biosynthesis in *Aspergillus nidulans* where a mutant

Figure 20:

a)


ME of M. circinelloides (nmol / min . mg protein-1)
ME of Mt. alpina (nmol / min . mg protein-1)
Time (h)
ME (M. circinelloides)
ME (Mt. alpina)

b)

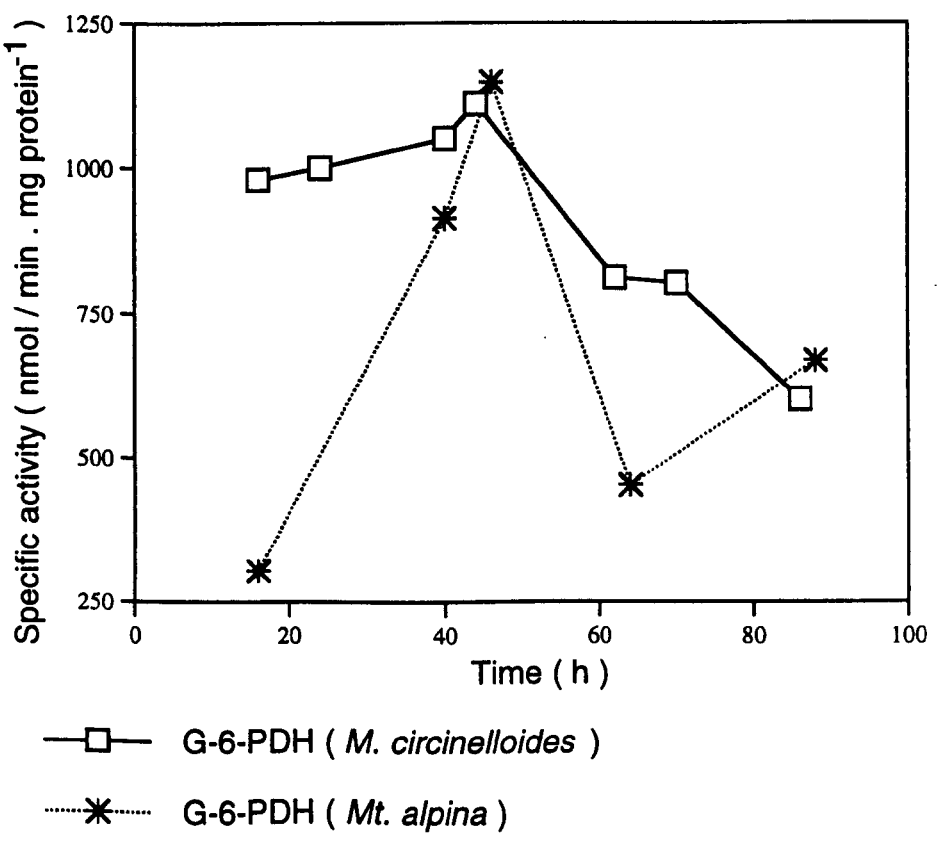

Specific activity (nmol / min . mg protein-1)
Time (h)
G-6-PDH (M. circinelloides)
G-6-PDH (Mt. alpina)

Figure 20: Specific activity profile of

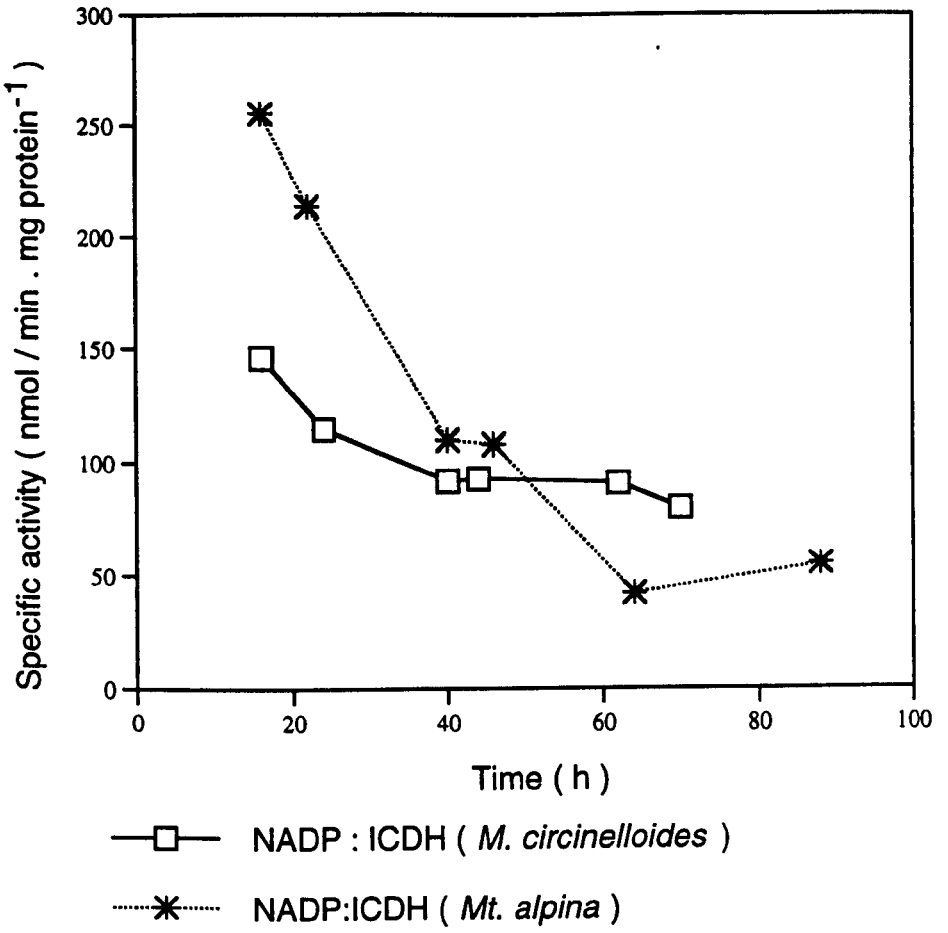
c) NADP:ICDH

d) 6-PGDH

of *M. circinelloides* and *Mt. alpina* cultures grown in a fermenter, as described in Section 5.2.3.

Figure 20:

c)


Specific activity (nmol / min . mg protein^{-1})
300
250
200
150
100
50
0
0
20
40
60
80
100
Time (h)
NADP : ICDH (M. circinelloides)
NADP:ICDH (Mt. alpina)

d)

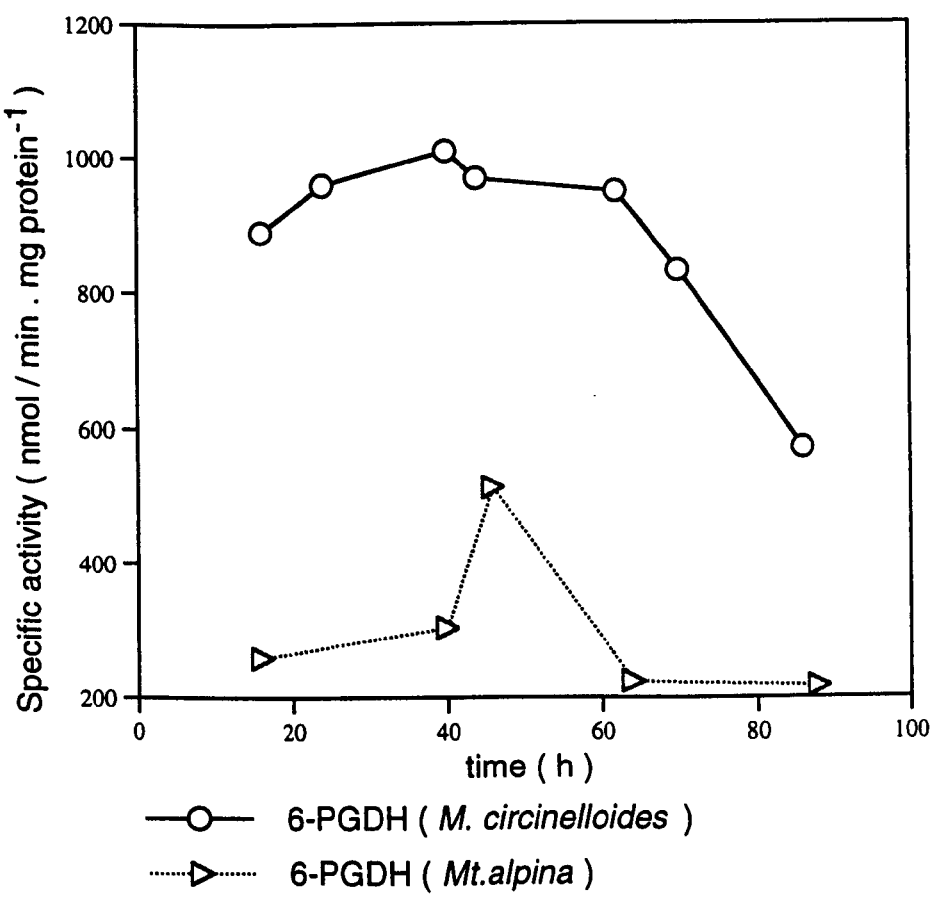

Specific activity (nmol / min . mg protein^{-1})
1200
1000
800
600
400
200
0
20
40
60
80
100
time (h)
6-PGDH (M. circinelloides)
6-PGDH (Mt.alpina)

Table 16b: The Mixing of Crude Extracts of *M. circinelloides* Containing ME with Extracts Obtained After ME Depletion.

Extract Containing ME* (µl)	Extract Lacking ME** (µl)	Specific Activity***
60	0	25
60	53	12.4

Approximate protein concentration: * 1.5 mg / ml

** 1.7 mg / ml

***, (nmol / min.mg protein^{-1})

Assays were carried out using assay method described in Section 2.5.5. Cell extracts containing ME activity were prepared using mycelia obtained after 24 h growth while cell extracts lacking ME were prepared using cells obtained after 45 h growth. When 60 µl (containing 0.09 mg protein) of extract containing ME activity was mixed with 53 µl (also containing 0.09 mg protein) extract lacking ME, ME activity was found to be approximately half of the sum of the individual activities (12.5 nmol /min.mg protein^{-1}). Therefore, no inhibition of ME activity was evident.

lacking ME activity was shown to accumulate only half the lipid (12 %, w / w, of biomass) accumulated by strains of *A. nidulans* possessing ME (Wynn and Ratledge, 1997). ME from *M. circinelloides* has also been demonstrated to play an important role in lipid biosynthesis where the addition of sesamol, a non-oil component of sesame seed which act as an inhibitor of ME activity, severely affected the accumulation of lipid *in vivo* (Wynn et al., 1997).

5.3.0 The Dependency of NAD:ICDH Activity From *M. circinelloides* and *Mt. alpina* on AMP

5.3.1 Objectives

The regulatory role of NAD:ICDH in lipid accumulation o f oleaginous yeasts has been well characterized (Botham and Ratledge, 1979; Evans and Ratledge, 1985b). The absolute dependency of its activity on AMP plays a vital part in causing the accumulation of citrate which serves as the source of acetyl-CoA for fatty acid biosynthesis. When a culture reaches a nitrogen-limited condition, AMP:deaminase is activated which leads to the depletion of AMP. This results in the cessation of NAD:ICDH activity which leads to the accumulation of both citrate and isocitrate. As the equilibrium lies in favour of citrate and the assimilation of glucose continues unabated by the nitrogen-limited cells (Botham and Ratledge, 1979), citrate becomes a major product of its metabolism. Citrate is then transported to the cytosol and cleaved by ACL to generate acetyl-CoA which serves as the primer for fatty acid biosynthesis.

As the properties of NAD:ICDH have not been investigated in oleaginous fungi, this experiment attempted to investigate the dependency of NAD:ICDH from both fungi on AMP for its activity.

5.3.2 Materials and methods

5.3.2.1 ***Cultivation of fungi.*** *M. circinelloides* and *Mt. alpina* were cultivated in 5 L fermenters as described in Section 4.1.2. Cells were harvested after 24 h of inoculation. Cell free extracts were then prepared as described in Section 2.4.0.

5.3.2.2 ***Dialysis of cell extracts.*** Cell extracts were dialyzed against 300 volumes of the same extraction buffer for 5 h with a constant stirring by a magnetic bar at 4 ^{o}C. After 5 h, the buffer was replaced with the same volume of extraction buffer and the cell extracts were dialyzed for a further 16 h. The cell extracts were then recovered and stored at 0 ^{o}C prior to analysis.

5.3.2.3 ***Determination of NAD:ICDH activity.*** NAD:ICDH activity from both fungi was analysed using the assay method described in Section 2.5.7. The activity was tested with the inclusion of various concentrations of AMP and isocitrate in the assay mixture.

5.3.3 Results and Discussion

The activity of NAD:ICDH from both fungi varied over a range of isocitrate concentrations in the presence of varying concentrations of AMP (Figure 21a and 22a). In *M. circinelloides*, the activity increased approximately 70-fold when AMP was included at 0.1 mM in the presence of 0.5 mM isocitrate compared to the activity without AMP. However, at a saturating concentration of isocitrate which was 3.5 mM and 7.5 mM in *M. circinelloides* and *Mt. alpina*, respectively (based on figure 21b and 22 b, the K_m value of NAD:ICDH for isocitrate was 0.16 mM and 1.1 mM for *M. circinelloides* and *Mt. alpina*, respectively), the activities achieved were the same and were unaffected by the concentration of AMP used.These results suggest that AMP increases the affinity of NAD:ICDH towards isocitrate. ME activities from both fungi did not show an absolute dependency on AMP as the activities could still be activated (Figure 21a and 22a) in the absence of AMP and a maximum activity was achieved when a final concentration of 3.5 mM and 7.5 mM of isocitrate were used, respectively. As the reaction of aconitase lies in favour of citrate (an equilibrium mixture consists of 90 % citrate and 4 % isocitrate), the concentration of isocitrate in the mitochondria is very low (Zubay, 1983). Therefore, the depletion of AMP will regulate the activity of NAD:ICDH in both fungi as the intramitochondrial isocitrate concentration will be below NAD:ICDH K_m value for isocitrate.

A similar observation has also been made in *Rhodosporidium toruloides* where this enzyme also showed a sigmoidal activation curves with respect to isocitrate concentration at various concentration of AMP where AMP acted by increasing the affinity of NAD:ICDH towards isocitrate (Evans and Ratledge, 1985b).

Figure 21 :

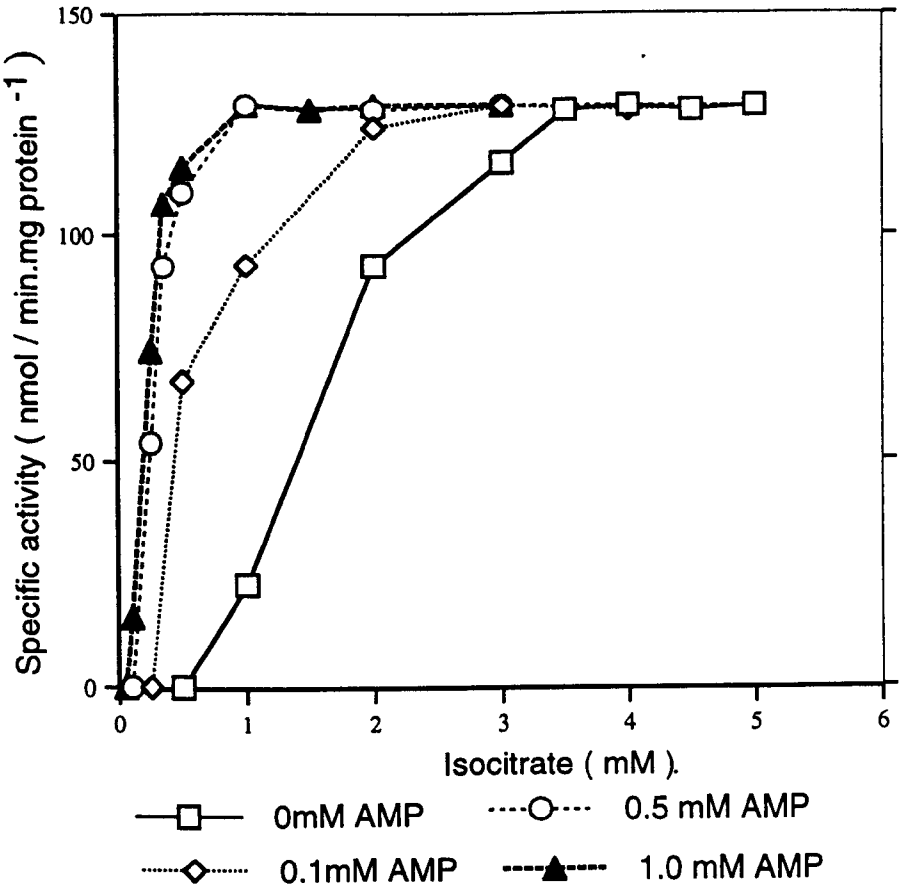
a) Activity of NAD:ICDH from *M. circinelloides* with various concentrations of isocitrate and AMP

b) Lineweaver-Burk plot for the determination of the *Km* value for isocitrate of NAD:ICDH from *M. circinelloides* at 1 mM AMP

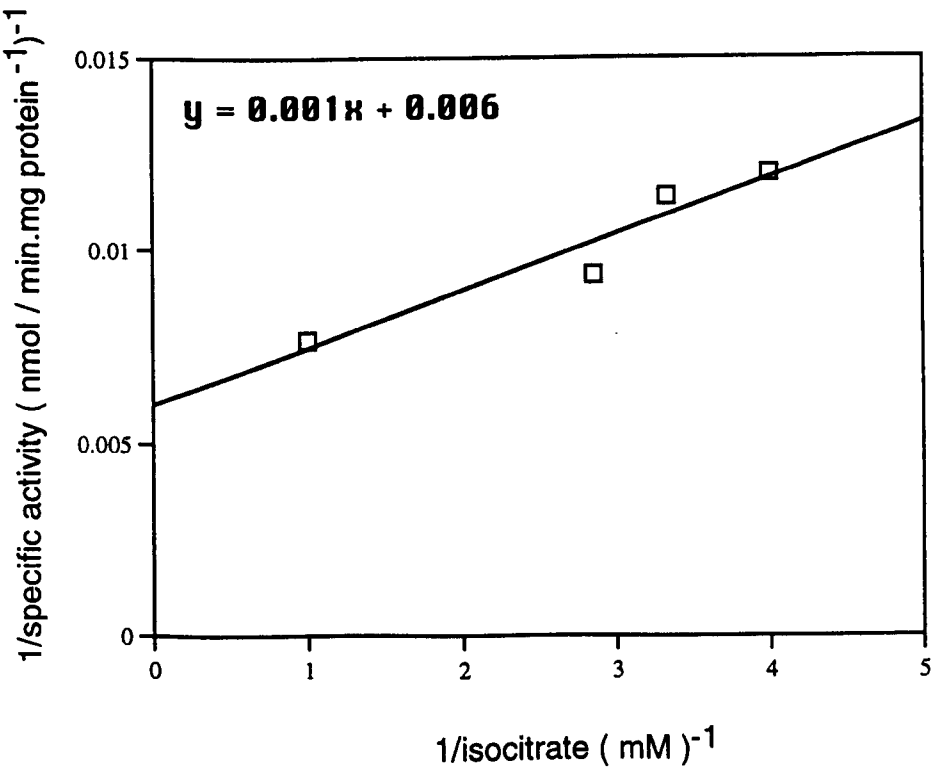
Crude extracts were prepared from a fermenter culture after 16 h of inoculation as described in Section 2.4.0.

Figure 21:

a)


150
100
50
0
Specific activity (nmol / min.mg protein -1)
0
1
2
3
4
5
6
Isocitrate (mM).
0mM AMP
0.5 mM AMP
0.1mM AMP
1.0 mM AMP

b)


y = 0.001x + 0.006
0.015
0.01
0.005
0
1/specific activity (nmol / min.mg protein -1)-1
0
1
2
3
4
5
1/isocitrate (mM)-1

5.3.4 Conclusions

NAD:ICDH from *Mt. alpina* and *M. circinelloides* did not show an absolute dependency for AMP as reported in oleaginous yeasts where in the absence of AMP and at a saturating concentration of isocitrate, its activity achieved a maximum value.

5.4.0 Investigation on the Cessation of ME activity in *M. circinelloides* Culture

5.4.1 Objectives

Attempts were made to investigate the cause of the cessation of ME activity in *M. circinelloides* which occurred approximately 20 h after ammonium exhaustion. As discussed in Section 5.2.3, the culture of *M. circinelloides* lost the activity of ME at approximately 40 h after inoculation which led to the cessation of lipid accumulation although glucose was still present and all other key enzymes involved in lipid biosynthesis were still active. As seen in the growth profile (see Figure 16a), ME activity decreased drastically (Figure 20a) after the depletion of ammonium in the culture while other parameters such as the pH (maintained at 6.0) and glucose concentration remained unchanged. It was thought that the ammonium depletion in the culture medium may be responsible in the diminishing activity of ME. Therefore, this experiment was designed to determine whether or not adding ammonium tartrate into the culture media could restore the activity of ME.

5.4.2 Materials and Methods

Experiments were carried out in 5 L fermenters (as described in Section 4.1.2) containing nitrogen-limiting media (see Section 2.1.3) with C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l). A 5 % inoculum was used for inoculating Fermenter 11, 12 and 13. The activity of ME in each culture was followed from the beginning of the experiment and additional ammonium tartrate at a final concentration of 2 g / l was added to Fermenter 11, 1 hour after the activity of ME of the culture depleted and to Fermenter 12, 24 hours after ME activity depleted. Culture of Fermenter 13 served as a negative control where no additional ammonium tartrate was added to the culture. The activity of ME was observed at intervals throughout the experiment using assay method described in Section 2.5.5.

5.4.3 Results and Discussion

Figure 23a and b show the ME activity of *M. circinelloides* after the addition of ammonium tartrate at 39 h (1 hour after ME depletion) and 64 h (24 h after ME depletion), respectively. In Fermenter 10 (Figure 23a), ME activity increased from zero at 39 h to 22 nmol / min. mg protein^{-1} at 40 h, 1 h after ammonium tartrate was added, and the activity subsequently reached a similar level as observed at 15 h approximately 4 h after the addition of ammonium tartrate.

When addition of ammonium tartrate was carried out 24 h after the depletion of ME (Figure 23b), a similar profile of restitution of ME activity was observed. Addition of ammonium tartrate at 64 h resulted in

Figure 23 : Restitution of ME activity in the culture of *M. circinelloides* by the addition of ammonium tartrate;

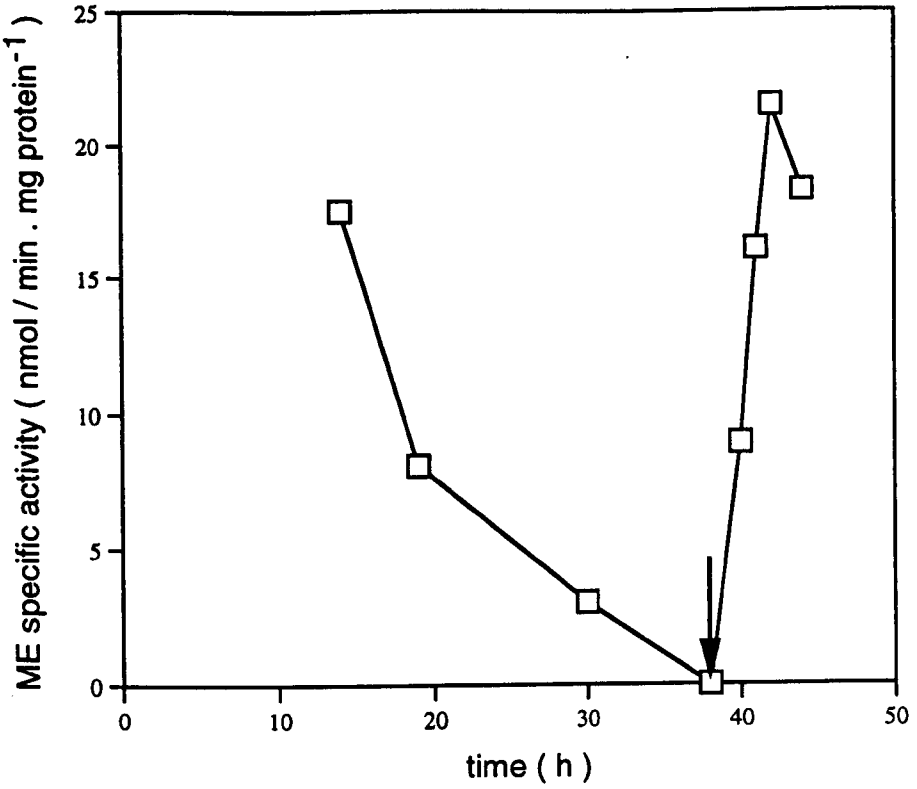
a) 1 hour after ME depletion

b) 24 hours after ME depletion.

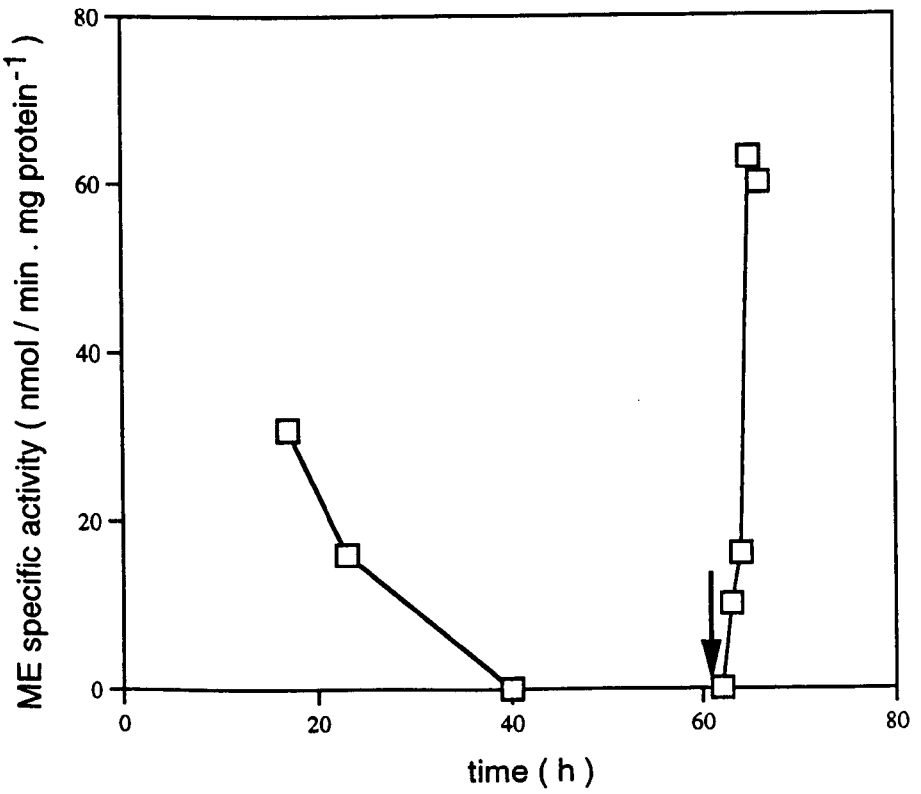
Additional ammonium tartrate (indicated by arrows) was aseptically added into each culture to a final concentration of 2 g / l .

Figure 23 :

a)


ME specific activity (nmol / min . mg protein^{-1})
25
20
15
10
5
0
0
10
20
30
40
50
time (h)

b)


ME specific activity (nmol / min . mg protein^{-1})
80
60
40
20
0
0
20
40
60
80
time (h)

the increase of ME activity from zero at 64 h to 60 nmol / min . mg protein^{-1} at 67 h (3 hours after ammonium tartrate addition). No activity of ME was detected in Fermenter 13 (where no ammonium tartrate was added) after its depletion at 41 h.

These results proved that ammonia plays an important role in determining the presence of ME activity in *M. circinelloides* culture and restitution of activity could be achieved even after 24 h after the initial activities had gone.

The exact cause of ME depletion was as yet unclear but the possibility is that it may be related to the cessation of the synthesis of the protein when the nitrogen source in the culture was exhausted and that the addition of ammonium tartrate then triggered the synthesis of this enzyme to take place. A long-term control in the regulation of a certain pathway could occur by the changes of the rate of synthesis and degradation of enzymes involved in the pathway (Wakil et al., 1983). For example, the growth of *Saccharomyces cerevisiae* (Kamiryo and Numa, 1973) and *Candida lypolytica* (Mishina and Kimiryo, 1976) in media containing saturated, monounsatured or polyunsaturated fatty acids was reported to result in the 80 % decrease of ACC activity. This decrease was caused by the reduction of mRNA coding for the enzyme (Horikawa et al., 1980) and thus repression of DNA transcription was evidently occurring.

5.5.0 The Effect of Cycloheximide Inclusion on the Restitution of ME Activity by Ammonium Tartrate in *M. circinelloides.*

5.5.1 Objectives

As previously observed, the addition of ammonium tartrate into *M. circinelloides* culture resulted in the restitution of ME activity. Experiments were carried out to determine whether the restitution of ME activity could be prevented by inhibiting the protein biosynthesis of the fungus by inclusion of a potent protein-synthesis inhibitor, cycloheximide. This compound acts by inhibiting the activity of peptidyl transferase which is responsible in the formation of peptide bonds during protein synthesis.

5.5.2 Materials and Methods

Three fermenters (Fermenter 14, 15 and 16) (performed as described in Section 4.1.2) containing nitrogen-limiting media (see Section 2.1.3) with C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l) were inoculated with 5 % inoculum. A final concentration of 100 mg cycloheximide / l and 2 g ammonium tartrate / l were added simultaneously to Fermenter 14 and 15, 1 h and 24 h after the depletion of ME, respectively. In Fermenter 16 (a negative control), only ammonium tartrate was added 1 h after ME depletion. ME activity was measured at intervals throughout the experiment in all three cultures using method described in Section 2.5.5. The activity of G-6-PDH, 6-PGDH and MDH were also determined in the culture of Fermenter 14 using methods described in Section 2.5.2, 2.5.3 and 2.5.4, respectively. This was to investigate whether cycloheximide affected the activity of any

other existing enzymes in the culture.

5.5.3 Results and Discussion

Figure 24a shows the restitution of ME activity 1 h after its depletion with and without the inclusion of cycloheximide. In Fermenter 14 (with the inclusion of cycloheximide), no ME activity was detected after ammonium tartrate addition was carried out at 40 h. Conversely, when ammonium tartrate was added without the inclusion of cycloheximide (Fermenter 16), ME activity increased rapidly starting 1 h after the addition increasing from zero at 41 h to 37 nmol / min. mg protein^{-1} at 43 h. When ammonium tartrate was added with the inclusion of cycloheximide (Fermenter 15) 24 h after ME depletion, no ME activity was observed. The activities of G-6-PDH, 6-PGDH and MDH observed in Fermenter 14 (Figure 24b) remained high and stable throughout the experiment. When cycloheximide was added into the culture at 40 h, the activity of all three enzymes stopped increasing and slightly decreased at the late stage of experiment. The decrease in the activities was probably due to the effect of cycloheximide which caused the cessation of the synthesis of these enzymes.

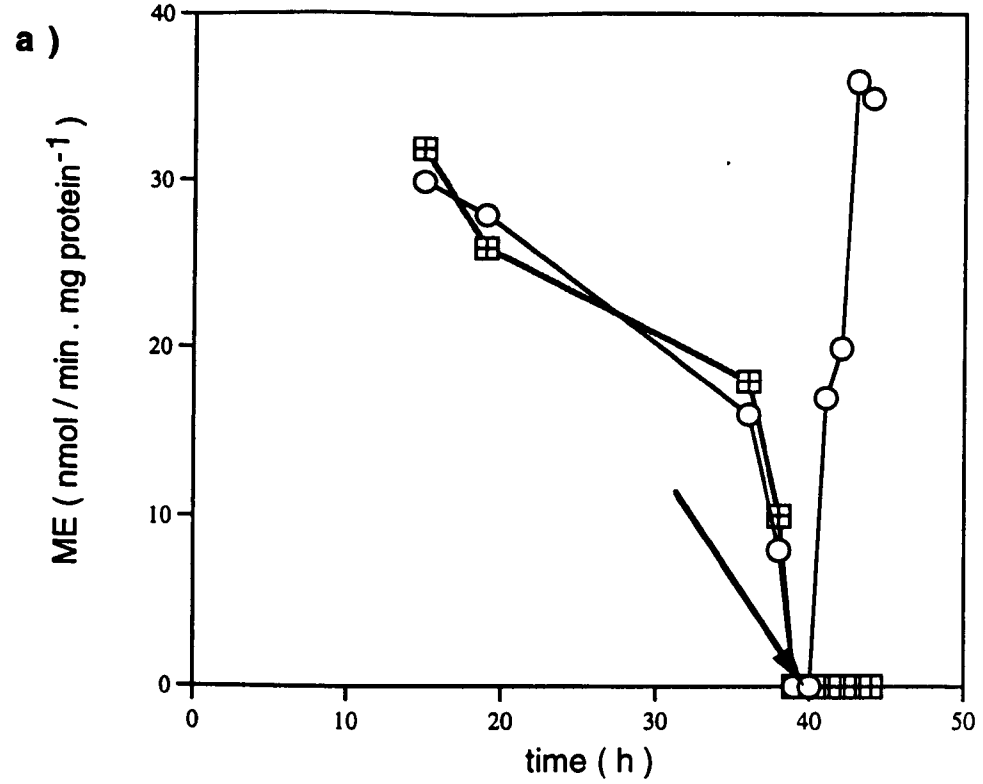
This experiment showed that the inclusion of cycloheximide to the culture, did not cause inactivation of enzyme systems in the cell. This confirmed that the inhibition of the restitution of ME activity was due to the inhibition of protein synthesis. Furthermore, no inhibitory effect of cycloheximide towards the activity of ME, G-6-PDH, 6-PGDH and MDH were observed *in vitro* when up to 200 mg / l of cycloheximide was

Figure 24 :

a) The restitution of ME activity in the culture of *M. circinelloides* 1 hour after ME depletion (indicated by arrow) with and without the inclusion of cycloheximide

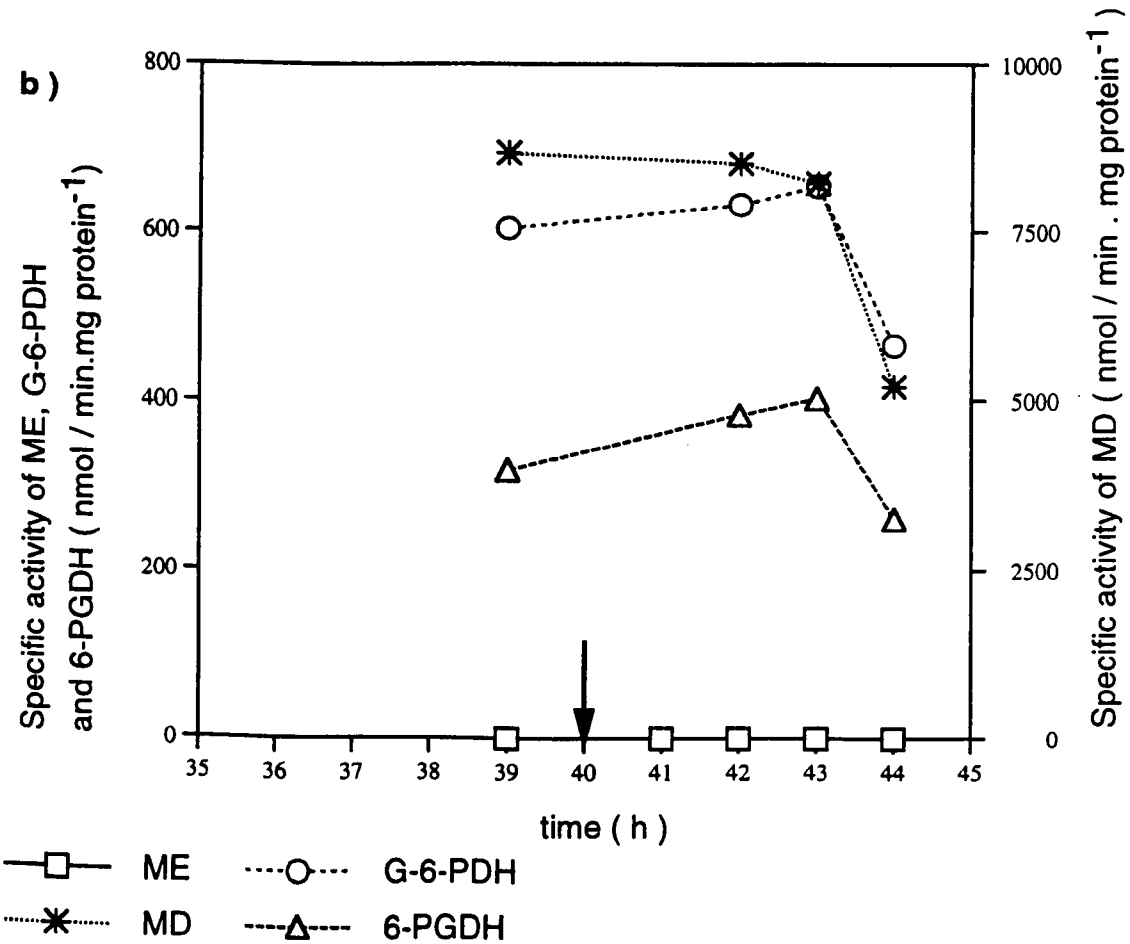
b) The activity of ME, G-6-PDH, 6-PGDH and MD in the culture of Fermenter 14 before and after the addition of ammonium tartrate and cycloheximide 1 hour after ME depletion (indicated by arrow).

Cycloheximide was added simultaneously with the addition of ammonium tartrate.



—○— ME specific activity of *M. circinelloides* culture without cycloheximide

—⊞— ME specific activity of *M. circinelloides* culture with cycloheximide



included into the reaction mixture of each assays.

This experiment showed that by adding cycloheximide (therefore inhibiting protein synthesis), restitution of ME activity was prevented. Thus the depletion of ME activity observed in cultures of *M. circinelloides* was probably due to the cessation of protein synthesis.

These results suggest that the depletion of ME activity in *M. circinelloides* which occurred at an early stage of fermentation was due to the cell stopped synthesizing this enzyme when ammonium tartrate was depleted and was not a result of intermediary inhibition (see Section 5.2.3).

5.5.4 Conclusions

The restitution of ME activity was prevented by the addition of cycloheximide in the culture. This suggests that the depletion of the activity was as a result of the cessation of the protein synthesis and that ammonium tartrate addition in the culture triggered the synthesis of the protein.

5.6.0 Partial Purification and Characterization of ME from *M. circinelloides* and *Mt. alpina*.

5.6.1 Objectives

This experiment attempted to partially purify and characterize ME from both fungi. The purified enzymes were to be used in the further investigations on the existence of complex formation between this enzyme and FAS. The work also involved the comparison of the kinetic properties of the enzyme from the two fungi.

5.6.2 Materials and Methods

5.6.2.1 Cultivation of Fungi

M. circinelloides and *Mt. alpina* were cultivated in 5 L fermenters as described in Section 4.1.2 in nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 66:1 (50 g glucose / l and 2.0 g ammonium tartrate / l) and 40:1 (30 g glucose / l and 2.0 g ammonium tartrate / l), respectively. Cells were harvested after 24 h (during active growth) of incubation and used for the preparation of cell-free extracts.

5.6.2.2 Preparation of Cell Extracts

Cell extracts were prepared using a French press (see Section 2.4.0). Buffer used was 10 mM KH_2PO_4 / KOH pH 7.5 containing all supplements as described in Section 2.4.0.

5.6.2.3 Ammonium Sulphate Precipitation.

Solid ammonium sulphate was slowly added to a constantly stirred crude extracts to 50 % saturation. Once the saturation level was achieved, the crude extracts were left stirring for a further 15 minutes. The extracts were then centrifuged at 16 000 g for 15 minutes. The supernatant was recovered and brought to 60 % saturation, stirred for a further 15 minutes, before recentrifuging at 16 000 g for 15 minutes. Care was taken to prevent excessive foaming and accumulation of solid ammonium sulphate in the beaker during the stirring process. The pellet collected at 60 % saturation, containing ME, was resuspended in a minimal volume of buffer and dialysed overnight against 300 volumes of the same extraction buffer before being used for the next step.

5.6.2.4 Affinity Chromatograpy

Samples were loaded onto a Mimetic Green 1A6XL column (2 x 10 cm, Affinity Chromatography Ltd. Ballasalla, Isle of Man, UK) which had been equilibrated with 3 column volumes of the same extraction buffer. Elution of the enzyme was accomplished by first washing with 2 column volumes of extraction buffer, and then using a 0 to 250 mM NaCl gradient (500 ml) in the same buffer. Fractions with high activity of ME were collected, pooled and stored at 0 ^{o}C prior to analysis.

5.6.3 Results and Discussion

In the ammonium sulphate precipitation step, a good recovery of ME was achieved (60 and 65 % in *M. circinelloides* and *Mt. alpina*,

respectively) (Table 17a and b). No problem was encountered during the experiment. In the second stage which involved the application of Mimetic Green column, no ME activity was eluted when the column was washed with 2 column volumes of extraction buffer, which indicates binding of ME to the column. When the column was washed with a gradient of 0 to 250 mM NaCl (500ml) in the same buffer, the enzyme activity was eluted as a single peak (Figure 25a and 25b). This method produced ME from *M. circinelloides* and *Mt. alpina* which achieved 24 and 22-fold purification, respectively (Table 17a and 17b). ME produced from both fungi was free from the activity of G-6-PDH, 6-PGDH, NADP:ICDH and FAS. However, the recovery of the enzyme was poor, which was only 2 % in both cases. There were three possiblities which may have caused this observation; Firstly, 98 % of the enzyme was denatured; Secondly, its activity may have been affected due to some structural modification during the purification; Thirdly, the enzyme isolated may represent a minor form of ME, not representative of the main ME activity of the cell. However, as the K_m values of ME from *M. circinelloides* were similar to those previously reported (see Section 5.7.0), I think it most likely that the latter two possiblities can be ignored.

Although buffer used included all necessary components for stabilizing the enzyme, such as glycerol, PMSF, mercaptoethanol and benzamidine, the attempts to stabilize the enzyme were unsuccessful. It is known that addition of PMSF (Mcelhaney-Feser and Cihlar, 1994), a thiol group reagent (Boulton and Ratledge, 1983) and glycerol (Fritsch and Beevers, 1979) were prerequisites in obtaining maximal recoveries in various enzyme purification procedures. Although ME has been detected in a number of fungi such as *Mortierella elongata*, *Conidiobolus nanodes* and *Aspergillus nidulans* (Savitha et al., 1997) its metabolic

Table 17: Partial purification of ME from

a) *M. circinelloides*

b) *Mt. alpina*

Purifications were carried out using methods described in Section 5.6.2. ME obtained was free from the activity of FAS, G-6-PDH, 6-PGDH and NADP:ICDH.

	Total activity (nmol / min)	Specific activity (nmol / min / mg)	purification (fold)	Recovery (%)
Crude extracts	10 271	26	1	100
$NH_4(SO_4)_2$ precipitation 50 %-60 %	6163	441	17	60
Mimetic Green	205	620	24	2

	Total activity (nmol / min)	Specific activity (nmol/min.mg $^{-1}$)	Purification (fold)	Recovery (%)
Crude extracts	15 780	93	1	100
$NH_4(SO_4)_2$ precipitation 50 %-60 %	10 257	551	6	65
Mimetic Green	316	2009	22	2

Figure 25: Elution of ME of

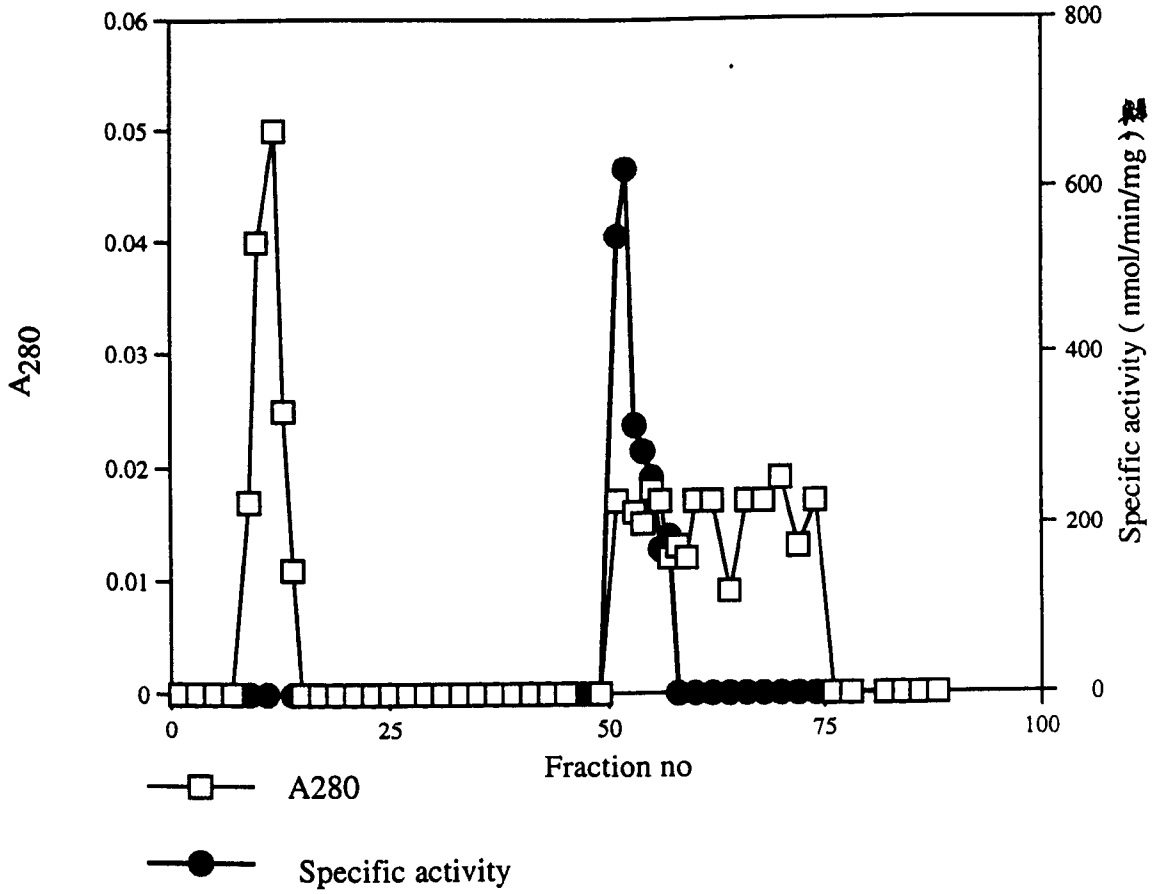
a) *M. circinelloides*

b) *Mt. alpina*

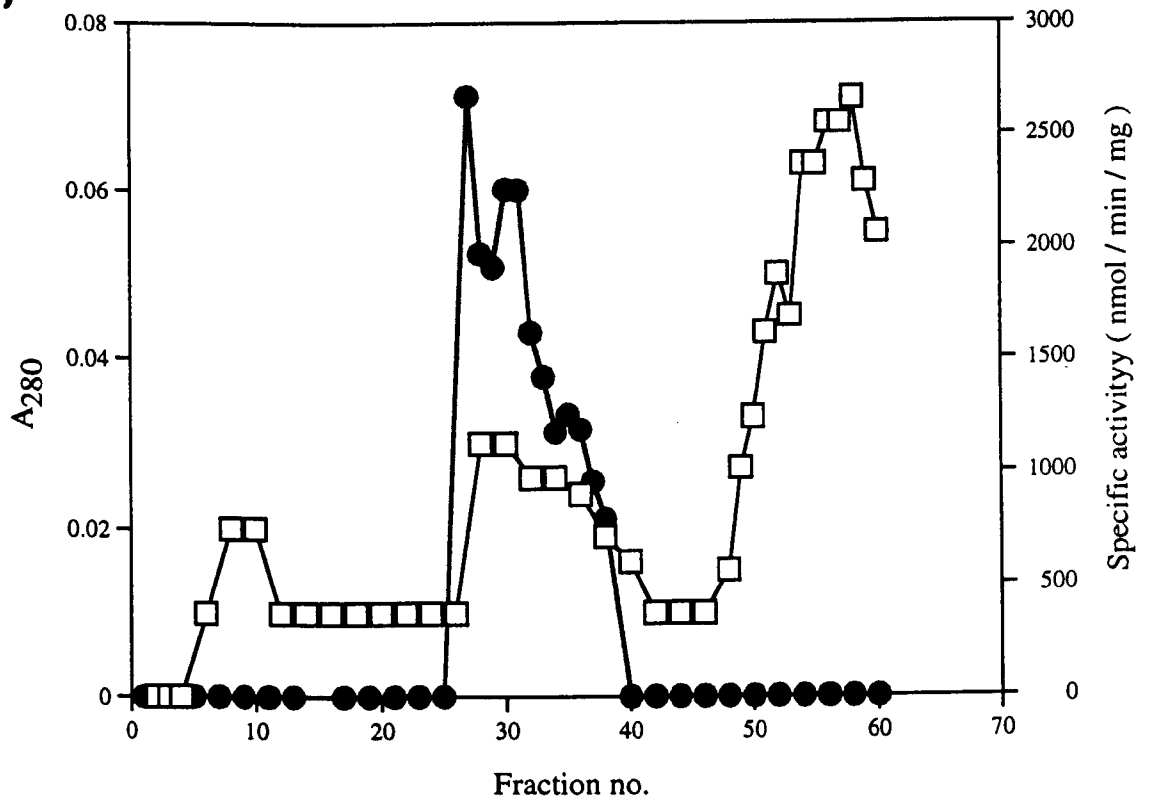
from a Mimetic Green column. Elution of the enzyme was achieved by washing with a 0 to 250 mM NaCl gradient (500 ml) in extraction buffer.

Figure 25 :

a)


0.06
0.05
0.04
0.03
0.02
0.01
0
A280
800
600
400
200
0
Specific activity (nmol/min/mg)
0
25
50
75
100
Fraction no
A280
Specific activity

b)


0.08
0.06
0.04
0.02
0
A280
3000
2500
2000
1500
1000
500
0
Specific activityy (nmol / min / mg)
0
10
20
30
40
50
60
70
Fraction no.
A280
Specific activity

has been suggested primarily to be a generator of pyruvate whereas in others it has also been implicated in the provision of NADPH for lipid biosynthesis and fatty acid desaturation.

5.7.0 Determination of K_m Values of Purified ME from *M. circinelloides* and *Mt. alpina* for NADP and Malate

5.7.1 Objectives

These experiments attempt to determine the K_m values of ME from both fungi for malate and NADP. This was to establish the differences of the values between these two fungi and to compare with values obtained from the study of ME isolated from other sources.

5.7.2 Materials and Methods

Specific activities of ME purified from both fungi were measured using method described in Section 2.5.5. Activities were determined with the inclusion of various concentrations of each of the substrates, where the other was kept at a saturating concentration in the reaction mixture. Lineweaver-Burk plots were obtained and the K_m values for each substrates were calculated.

5.7.3 Results and Discussion

Figure 26a-d show a straight line obtained for each plots. The apparent K_m values for NADP of ME from *M. circinelloides* and *Mt. alpina*

Figure 26 : Lineweaver-Burk plots for determining the K_m value of ME for NADP from

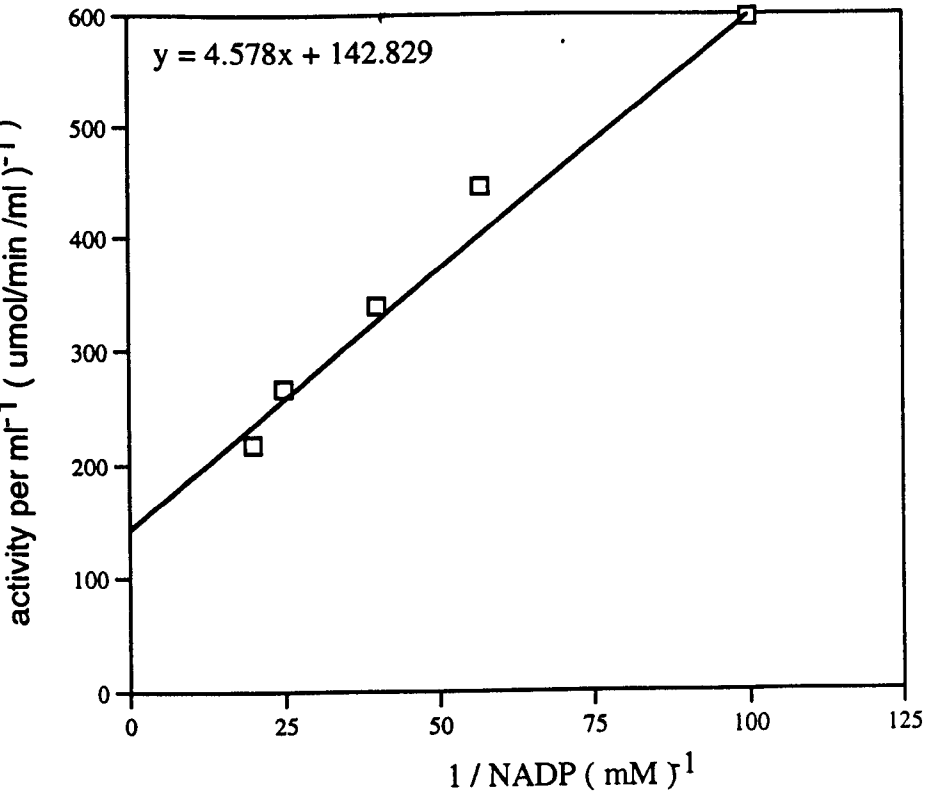
a) *M. circinelloides*

b) *Mt. alpina*.

ME activities were determined using method described in Section 2.5.5 with the inclusion of various concentrations of NADP with the concentration of malate being kept constant.

Figure 26 :

a)


y = 4.578x + 142.829
activity per ml-1 (umol/min /ml)-1)
600
500
400
300
200
100
0
0
25
50
75
100
125
1 / NADP (mM)1

b)

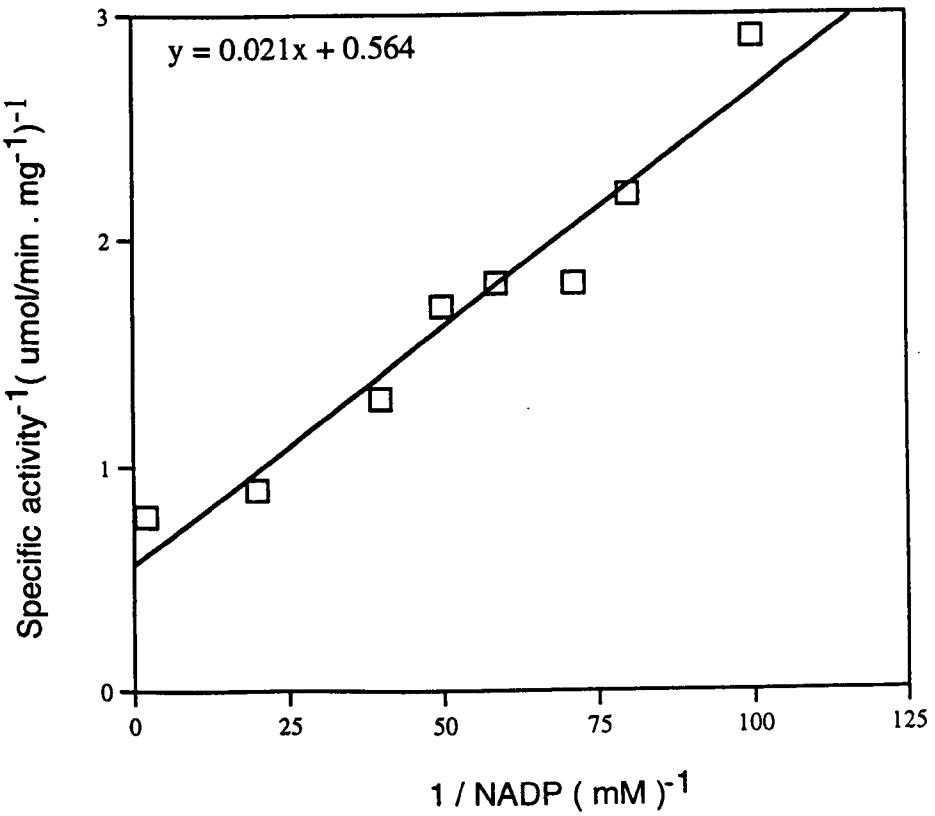

y = 0.021x + 0.564
Specific activity-1(umol/min . mg-1)-1
3
2
1
0
0
25
50
75
100
125
1 / NADP (mM)-1

Figure 26 : Lineweaver-Burk plots for determining the K_m for malate of ME purified from

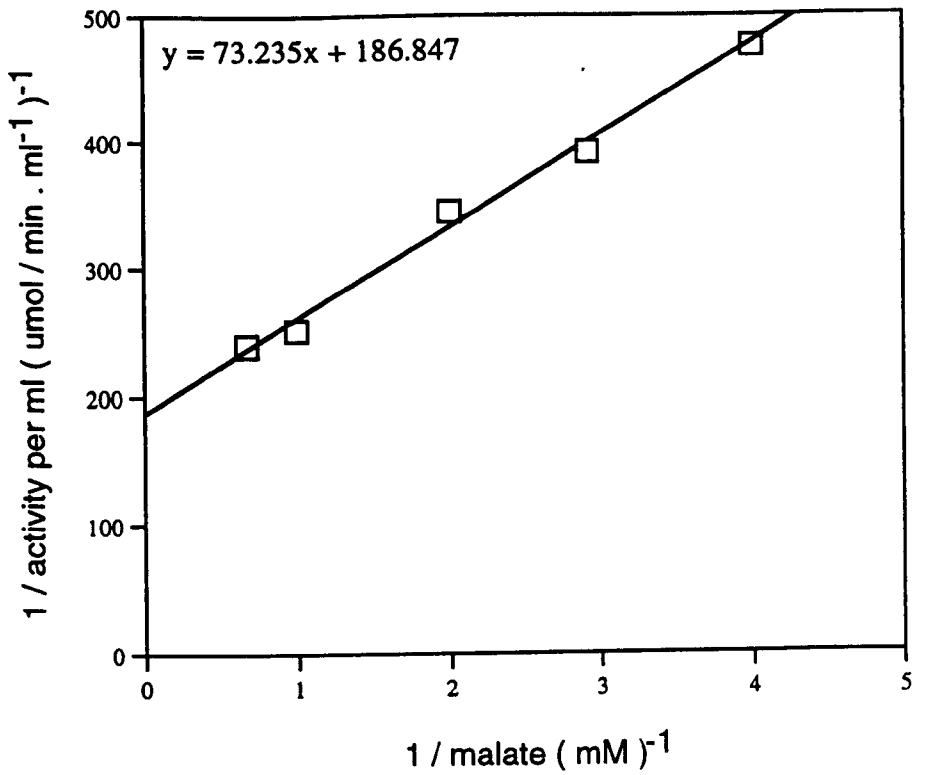
c) *M. circinelloides*

d) *Mt. alpina.*

ME activities were determined using method described in Section 2.5.5 with the inclusion of various concentrations of malate into the reaction mixture and with the concentration of NADP being kept constant.

Figure 26:

c)


y = 73.235x + 186.847
500
400
300
200
100
0
0
1
2
3
4
5
1 / activity per ml (umol / min . ml-1)-1
1 / malate (mM)-1

d)

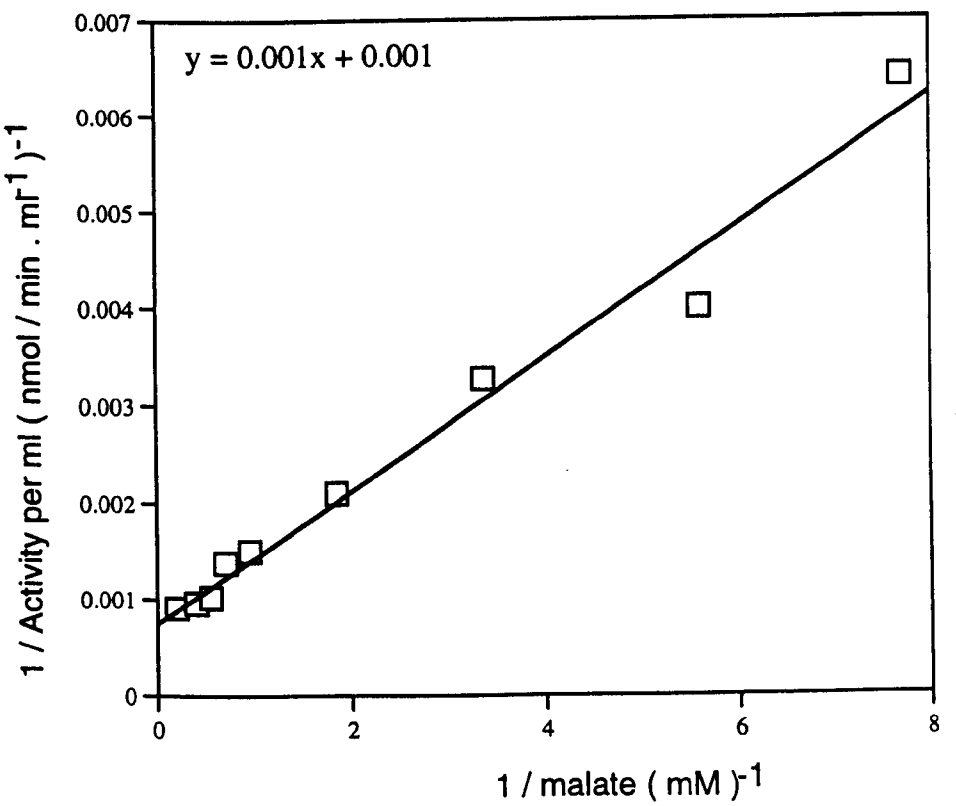

y = 0.001x + 0.001
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0
2
4
6
8
1 / Activity per ml (nmol / min . ml-1)-1
1 / malate (mM)-1

Table 18: Apparent K_m Values of ME from *M. circinelloides* and *Mt. alpina* for NADP and malate.

	M. circinelloides	*Mt. alpina*
K_m NADP (mM)	0.032	0.038
K_m malate (mM)	0.4	1

Specific activities of ME purified from both fungi were measured using method described in Section 2.5.5. Activities were determined with the inclusion of various concentrations of each of the substrates (NADP and malate), where the other one was kept constant in the reaction mixture. Lineweaver-Burk plots were obtained and the K_m values for each substrates were calculated.

were similar: 0.032 mM and 0.038 mM, respectively but ME from *M. circinelloides* had a lower K_m value for malate (0.4 mM) than ME from *Mt. alpina* (1 mM). Table 18 summarizes the differences of K_m values of ME from both fungi for both substrates.

The K_m values of ME from both fungi for NADP were similar to those reported of ME isolated from stems and roots of wheat (Casati et al., 1997) and also from *M. circinelloides* (Savitha et al., 1997). ME has also been purified from various sources such as from developing fruits of tomato (Knee et al., 1996), *Streptococcus bovis* (Kawai et al., 1996) and wheat (Casati et al., 1997).

5.8.0 The Effect of Four Potential Inhibitors on ME Activity from *M. circinelloides* and *Mt. alpina*.

5.8.1 Objectives

The effect of four potential inhibitors on ME activity isolated from *M. circinelloides* and *Mt. alpina* was investigated. Compounds selected were malate analogues (tartronic acid and oxaloacetate, OAA) and fatty acyl-CoA esters (palmitoyl-CoA and oleoyl-CoA) . As the effect of these compounds on the activity of ME from *M. circinelloides* has been investigated by previous researchers (Savitha et al., 1997), this experiment therefore attempts to compare the inhibitory effect of the compounds on ME from *Mt. alpina* to that observed in *M. circinelloides*.

5.8.2 Materials and Methods

The activities of purified ME from both fungi were determined at various final concentrations of each of the potential inhibitors in the reaction mixture using method described in Section 2.5.5. The degree of inhibition was determined by comparing the activities obtained with the activities of ME performed without the inclusion of any of the inhibitors. Stock solutions of the compounds were prepared by dissolving each of the compounds in distilled H_2O prior to the experiment.

5.8.3 Results and Discussion

All four compounds showed various degrees of inhibition of ME activity from both fungi (Table 19). Tartronic acid demonstrated a similar degree of inhibition on ME from both fungi where at a final concentration of 10 mM, approximately 40 % inhibition was achieved . At a final concentration of 10 mM, OAA showed a 70 % inhibition on ME from *M. circinelloides* but a slightly lower degree of inhibition (45 %) on ME from *Mt. alpina*. At a final concentration of 1 mM, oleoyl-CoA and palmitoyl-CoA caused a 100 % inhibition on ME from *M. circinelloides* and approximately 90 % on ME from *Mt. alpina*.

The inhibition of ME activities by oleoyl-CoA and palmitoyl-CoA was more likely due to these compounds acting as detergents as the concentrations of each of the compounds included in the reaction mixtures were well over the critical micellar concentration (the critical micellar concentration of oleoyl-CoA and palmitoyl-CoA was reported to occur at approximately 3 μM and 4 μM, respectively, Hsu and Powell

Table 19: The Effect of Four Potential Inhibitors on the Activity of ME from *M. circinelloides* and *Mt. alpina*

Inhibitors	Final conc. (mM)	% of Inhibition	
		M. circinelloides	*Mt. alpina*
Tartronic acid	2.5	4-6	0
	5.0	16-20	11-15
	10.0	36-42	35-40
OAA	2.5	16-20	0
	5	38-40	5-10
	10	65-70	41-45
Oleoyl-CoA	0.1	59-65	66-70
	0.5	71-75	72-75
	1	100	83-88
Palmitoyl-CoA	0.1	57-60	55-60
	0.5	71-75	70-75
	1	100	81-85

The activities of ME from both fungi were determined with the inclusion of various final concentrations of each of the compounds in the reaction mixture. The degree of inhibition was determined by comparing the activities obtained with the activities of ME performed without the inclusion of any of the inhibitors. 100 % ME activity of *M. circinelloides* and *Mt. alpina* was 60 and 90 nmol / min.ml^{-1}, respectively.

1975). It is known that fatty acyl-CoA esters are powerful detergents and may, therefore, exert their effects by non-specific binding or by disruption of the quarternary structure of enzymes. The inhibition of various enzymes by FACEs has been shown to be due to an irreversible disaggregation of the enzymes (Bloch and Vance, 1977; Dorsey and Porter, 1968).

FACEs play an important role in regulating lipid biosynthesis in oleaginous yeasts by affecting the efficiency of mitochondrial citrate translocase at 1 μM (Evans, Scragg and Ratledge, 1983). Oleoyl-CoA and palmitoyl-CoA were also demonstrated to cause approximately 90 % inhibition on ACL activity from *Lipomyces starkeyi* at a final concentration of 10 μM and both also produced significant inhibition at concentrations below their critical micellar concentration values (Boulton and Ratledge, 1983).

5.8.4 Conclusions

Tartronic acid showed a similar degree of inhibition towards ME activity from both fungi (approximately 40 %) while OAA exhibited a slightly higher inhibition on ME from *M. circinelloides*. The inhibition by palmitoyl-CoA and oleoyl-CoA was probably due to the detergent-like action of both FACEs toward the enzyme as the concentrations used were well over the critical micellar concentration of each of the compounds.

5.9.0 Partial purification of FAS from *M. circinelloides* and *Mt. alpina*

5.9.1 Objectives

This experiment attempted to partially purify FAS from both fungi. This was to establish the differences of the kinetic properties of the enzyme between the two organisms and for use in the further study of the hypothesised lipid biosynthesis metabolon involving FAS and ME.

5.9.2 Materials and Methods

5.9.2.1 Cultivation of Fungi

M. circinelloides and *Mt. alpina* were cultivated in 5 L fermenters as described in Section 4.1.2 in nitrogen-limiting media (see Section 2.1.3) with a C:N ratio at 66:1 (50 g glucose / l and 2.0 g ammonium tartrate / l) and 40:1 (30 g glucose / l and 2.0 g ammonium tartrate / l), respectively. Cells were harvested after 24 h of incubation and used for the preparation of cell-free extracts.

5.9.2.2 Preparation of Cell Extracts

Cell extracts were prepared using French press (see Section 2.4.0) in 10 mM KH_2PO_4 / KOH pH 7.5 containing all supplements as described in Section 2.4.0.

5.9.2.3 Partial Purification of FAS from *M. circinelloides.*

Ammonium sulphate precipitation. FAS was obtained from crude extracts by recovering the pellet sedimented between 35 to 55 % saturation after centrifugation at 16 000 g for 15 minutes. The pellet was recovered in a minimal volume of the same extraction buffer and used for the next step.

Gel filtration. 20 ml of extract was loaded on to a Sephacryl S 300 gel filtration column (3 x 72 cm, Pharmacia Biotech) which had been equilibrated with 2 column volumes of the same extraction buffer. The column was washed with the same buffer and fractions with high activity of FAS were collected, pooled and used for analysis.

5.9.2.4 Partial Purification of FAS from *Mt. alpina.*

Ultracentrifugation. Crude extracts were centrifuged at 100 000 g for 6 h at 4 ^{o}C. The supernatant was discarded and the yellowish brown pellet was resuspended in a minimum volume of extraction buffer. The resulting suspension was centrifuged for 20 min at 37 000 g. The clear yellow supernatant containing the enzyme was recovered and diluted 10 times with the same extraction buffer and recentrifuged at 100 000 g for 6 h. The pellet containing the enzyme was dissolved in a minimum volume of extraction buffer and used for the next stage.

Affinity chromatography. Sample was loaded onto a Red Sepharose column (2 x 15 cm, Pharmacia Biotech)which was

equilibrated with 10 mM KH_2PO_4 / KOH pH 7.5 containing all supplements as described in Section 2.4.0. Elution of the enzyme was achieved by washing first with two column volumes of the same extraction buffer, and then using a gradient of 0 to 250 mM NaCl (500 ml) in the same buffer. Fractions with high activity of FAS were pooled and used for analysis.

5.9.4 Results and Discussion

A summary of the purification procedure for *M. circinelloides* and *Mt. alpina* is given in Table 20a and 20b, respectively. Both procedures resulted in a successful purification of FAS from both fungi although the recovery obtained at the last stage were low: 3 % and 6 % for *M. circinelloides* and *Mt. alpina,* respectively. The ammonium sulphate precipitation technique used in the purification of FAS from *M. circinelloides* proved to be very efficient, producing FAS with 3 fold purification and 95 % recovery. However, only 3 % recovery was achieved after elution from the Sephacryl S 300 gel filtration column although it yielded an overall 15-fold purification of FAS.

A similar result was also obtained for FAS from *Mt. alpina* . After the ultrcentrifugation of the crude extract, FAS was purified 5-fold with 90 % recovery. The second stage involving the Red Sepharose affinity column produced FAS with a 17 purification fold but with a low recovery (6 %). FAS purified from both fungi was free from the activity of G-6-PDH, 6-PGDH, NADP:ICDH and ME.

In both procedures, the lost of activities may be due to the

Table 20a: The Purification of FAS from *M. circinelloides*

	Volume (ml)	Total activity (nmol / min)	Specific activity (nmol / min.mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	200	35 200	21	100	1
$(NH_4)_2SO_4$ precipitation (35 %-55 %)	13	33 400	64	95	3
Gel filtration (Sephacryl S 300)	4	885	316	3	15

The purification was carried out using methods described in Section 5.9.2.3.

Table 20b: The Purification of FAS from *Mt. alpina*

	Volume (ml)	Total activity (nmol / min)	Specific activity (nmol / min.mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	90	1710	14	100	1
Ultracentrifugation	1.6	1543	67	90	5
Affinity column (Red Sepharose)	4	94	234	6	17

The purification was carried out using methods described in Section 5.9.2.4.

inactivation of the enzyme during the purification process although the buffer used for the enzyme preparation was included with PMSF, mercaptoethanol, glycerol and protease inhibitor cocktail in the extraction buffer as described in Section 2.4.0. Addition of these components was demonstrated to be prerequisites in obtaining maximum recoveries in the purification procedures of FAS from *Candida albicans* (Mcelhaney-Feser and Cihlar, 1994) and other enzymes (Wynn, 1994; Boulton and Ratledge, 1983). It was also possible that its activity may have been affected due to some form of structural modification during the purification process. FAS has been isolated from chicken liver (Arslanian et al., 1976), rabbit mammary gland (Paskin and Mayer, 1976) and other micro-organisms such as from *Saccharomyces cerevisiae* (Lynen, 1969 ; Stoops et al., 1978) and *Escherichia coli* (Valope and Vagelose, 1976; Wakil, 1970).

The application of ultracentrifugation method in the purification of FAS from *Mt. alpina* proved easier and efficient as it gave high recoveries and better purification compared to ammonium sulphate precipitation method used in the purification of FAS from *M. circinelloides*. The only disadvantage of the procedure was the need to work at odd hours in order to recover the pellet on time as the pellet would dissolve in the supernatant if left for several hours. This method has been applied in the purification of FAS from *Saccharomyces cerevisiae* (Lynen, 1969). The application of gel filtration column (Sephacryl S-300) was also easier compared to the Red Sepharose column as the latter required careful regeneration of the matrix every time before use. Failure to do so resulted in an inefficient performance of the column where traces of all NADPH-generating enzymes, excluding ME, were present in FAS fractions eluted from the column. The gel filtration column only required

washing and equilibrating with the same buffer prior to the experiment and no problem was encountered during the experiments. The only disadvantage of the procedure was the longer time required (approximately 24h) for the experiment to complete as the flow rate of the column was very slow. This prevented regular checking of the fraction collector from being carried out overnight, which raised the possibility of losing the enzyme due to the malfunction of the machine.

5.10.0 Determination of K_m Values of FAS Isolated from *M. circinelloides* and *Mt. alpina* for Malonyl-CoA, Acetyl-CoA and NADPH.

5.10.1 Objectives

This experiment attempts to determine the K_m values of FAS from *M. circinelloides* and *Mt. alpina* for its substrates: malonyl-CoA, acetyl-CoA and NADPH. This was to compare the properties of the two enzymes from the different organisms before commencing the work involving the association between FAS and ME.

5.10.2 Materials and Methods

Specific activities of FAS purified from both fungi were measured using methods described in Section 2.5.9. Activities were determined with the inclusion of various concentrations of each of the substrates (NADPH, malonyl-CoA and acetyl-CoA), where the other two were kept constant in

the reaction mixture. Lineweaver-Burk plots were obtained and the K_m values for each substrate was calculated.

5.10.3 Results and Discussion

Figure 27a-f show the Lineweaver-Burk plots obtained in the determination of K_m values of FAS from both fungi for each of the substrates. FAS from *M. circinelloides* and *Mt. alpina* showed a similar K_m values for malonyl-CoA (0.013 mM and 0.012 mM respectively). Similar K_m values for acetyl-CoA were also obtained: 0.017 mM and 0.015 mM for *M. circinelloides* and *Mt. alpina,* respectively but a higher K_m value for NADPH was observed in FAS from *Mt. alpina* (0.038 mM) compared to FAS from *M. circinelloides* (0.010 mM). Table 21 summarizes the K_m values of FAS from both fungi for each substrates.

Similar K_m values for malonyl-CoA and acetyl-CoA have been reported to occur in *Saccharomyces cerevisiae* (Lynen, 1969) and *Candida albicans* (Mcelhaney and Cihlar, 1994). However, the K_m values for NADPH of *M. circinelloides* and *Mt. alpina* (0.010 mM and 0.038 mM respectively) were much lower than that observed in *Saccharomyces cerevisiae* (0.067 mM) (Lynen, 1969).

Figure 27: Lineweaver-Burk plots for determining the K_m values for NADPH of FAS purified from

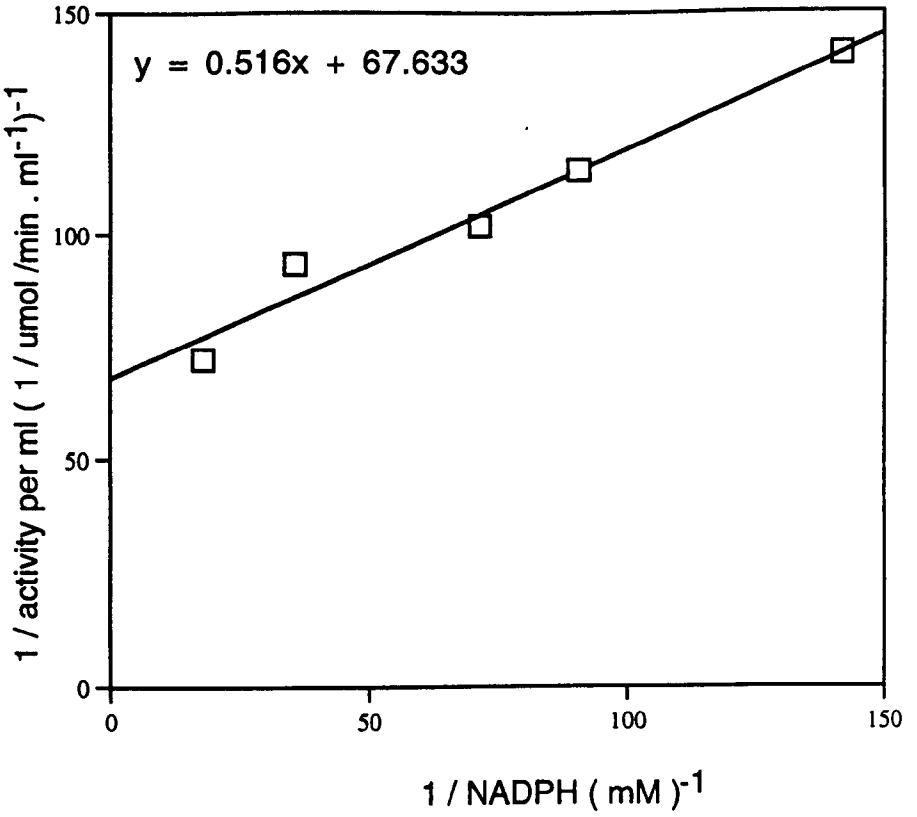
a) *M. circinelloides*

b) *Mt. alpina.*

FAS activities were determined using method described in Section 2.5.9 with the inclusion of various concentrations of NADPH into the raction mixture and with the concentration of acetyl-CoA and malonyl-CoA being kept constant.

Figure 27:

a)


y = 0.516x + 67.633
1 / activity per ml (1 / umol /min . ml-1)-1
1 / NADPH (mM)-1
0
50
100
150

b)

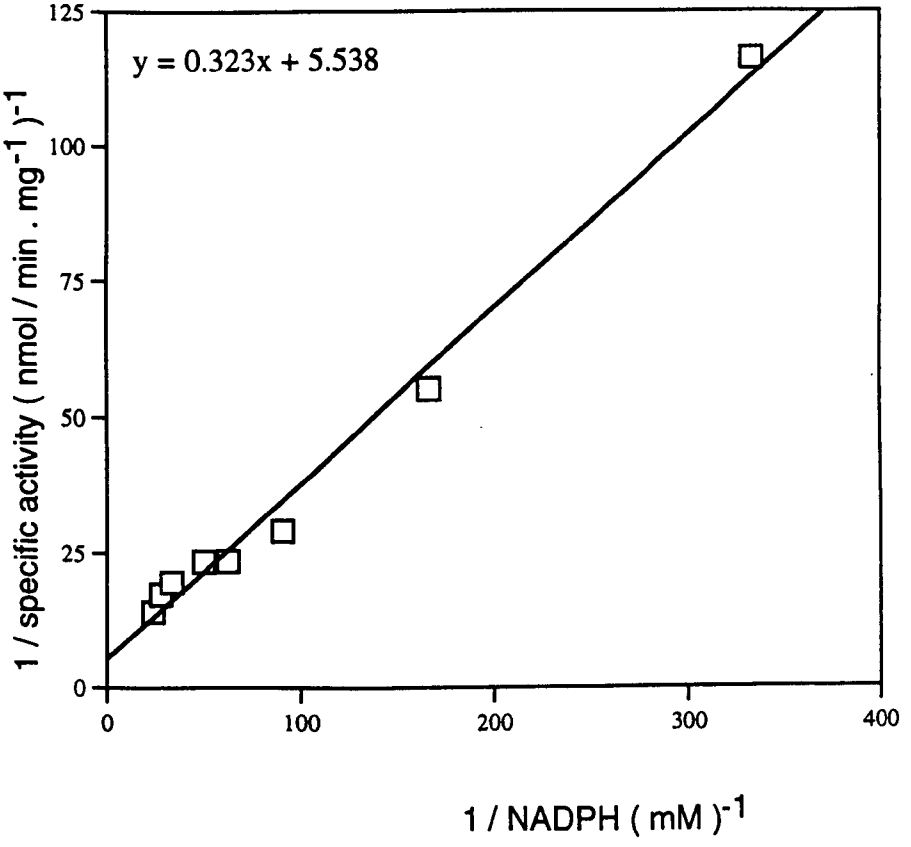

y = 0.323x + 5.538
1 / specific activity (nmol / min . mg-1)-1
1 / NADPH (mM)-1
0
25
50
75
100
125
200
300
400

Figure 27: Lineweaver-Burk plots for determining the K_m values for acetyl-CoA of FAS purified from

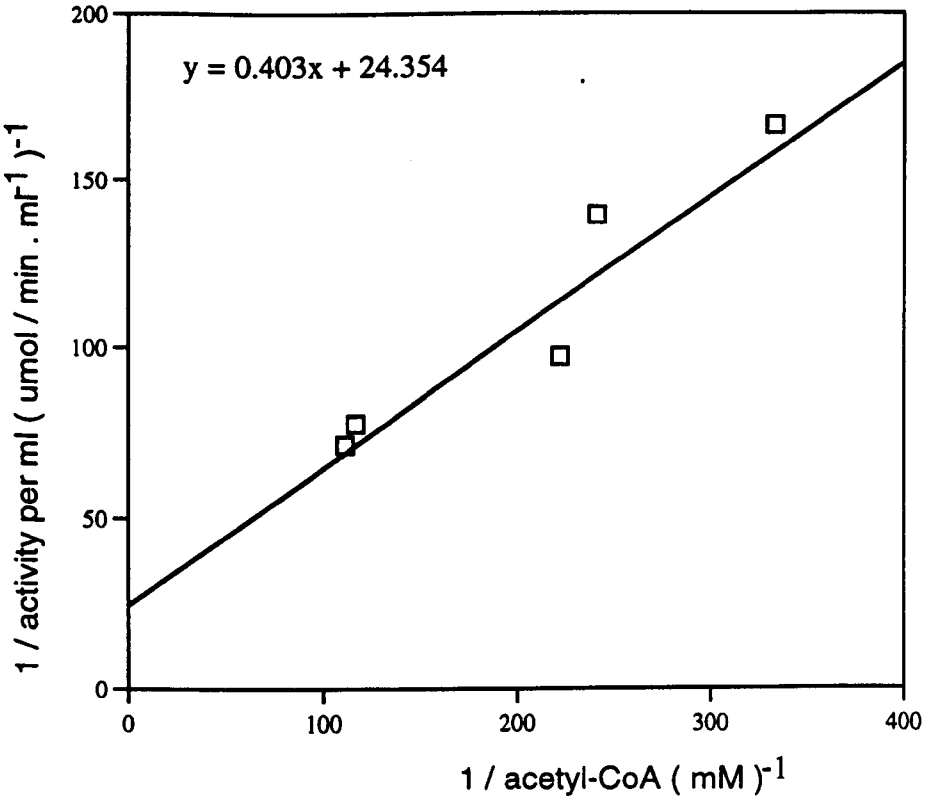
c) *M. circinelloides*

d) *Mt. alpina.*

FAS activities were determined using method described in Section 2.5.9 with the inclusion of various concentrations of acetyl-CoA into the reaction mixture and with the concentration of NADPH and malonyl-CoA being kept constant.

Figure 27:

c)


y = 0.403x + 24.354
1 / activity per ml (umol / min . ml^{-1})$^{-1}$
1 / acetyl-CoA (mM)$^{-1}$
0
50
100
150
200
0
100
200
300
400

d)

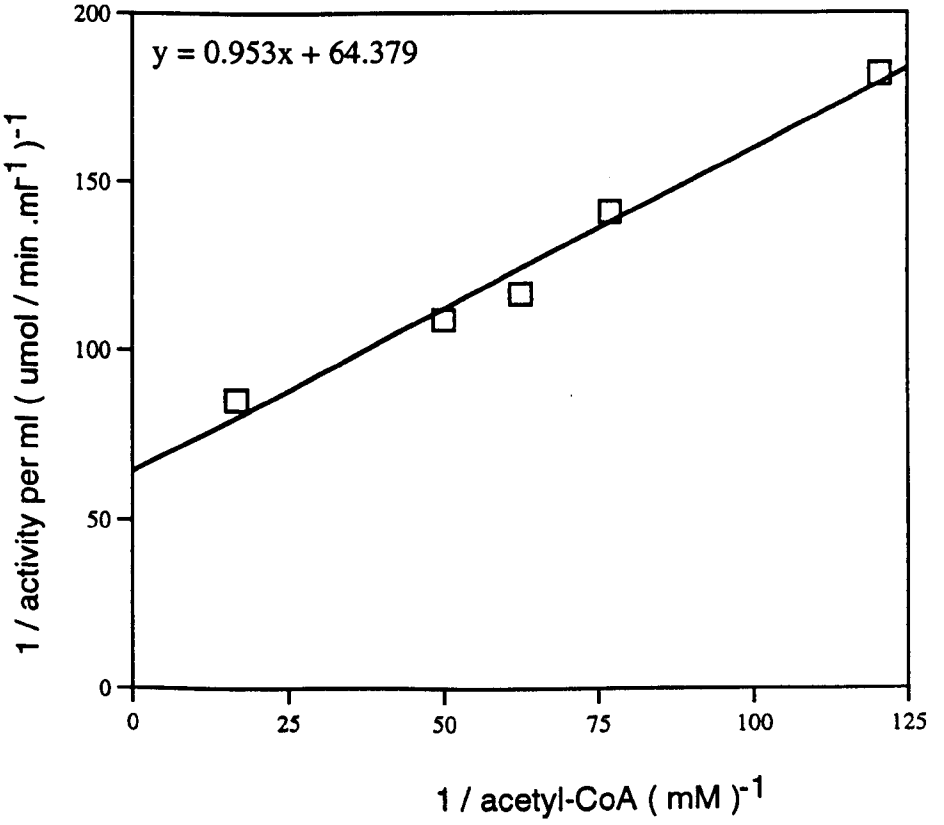

y = 0.953x + 64.379
1 / activity per ml (umol / min .ml^{-1})$^{-1}$
1 / acetyl-CoA (mM)$^{-1}$
0
50
100
150
200
0
25
50
75
100
125

Figure 27: Lineweaver-Burk plots for determining the K_m values for malonyl-CoA of FAS purified from

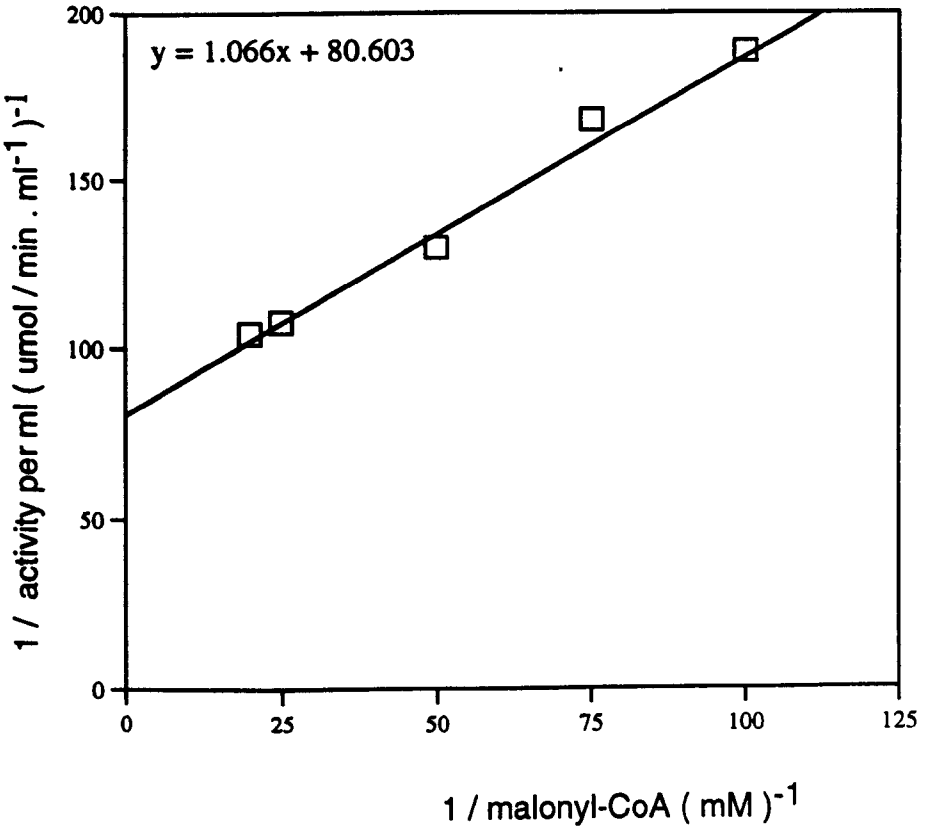
e) *M. circinelloides*

f) *Mt. alpina.*

FAS activities were determined using method described in Section 2.5.9 with the inclusion of various concentrations of malonyl-CoA into the reaction mixture and with the concentration of NADPH and acetyl-CoA being kept constant.

Figure 27.

e)


y = 1.066x + 80.603
1 / activity per ml (umol / min . ml-1)-1
1 / malonyl-CoA (mM)-1
0
50
100
150
200
0
25
50
75
100
125

f)

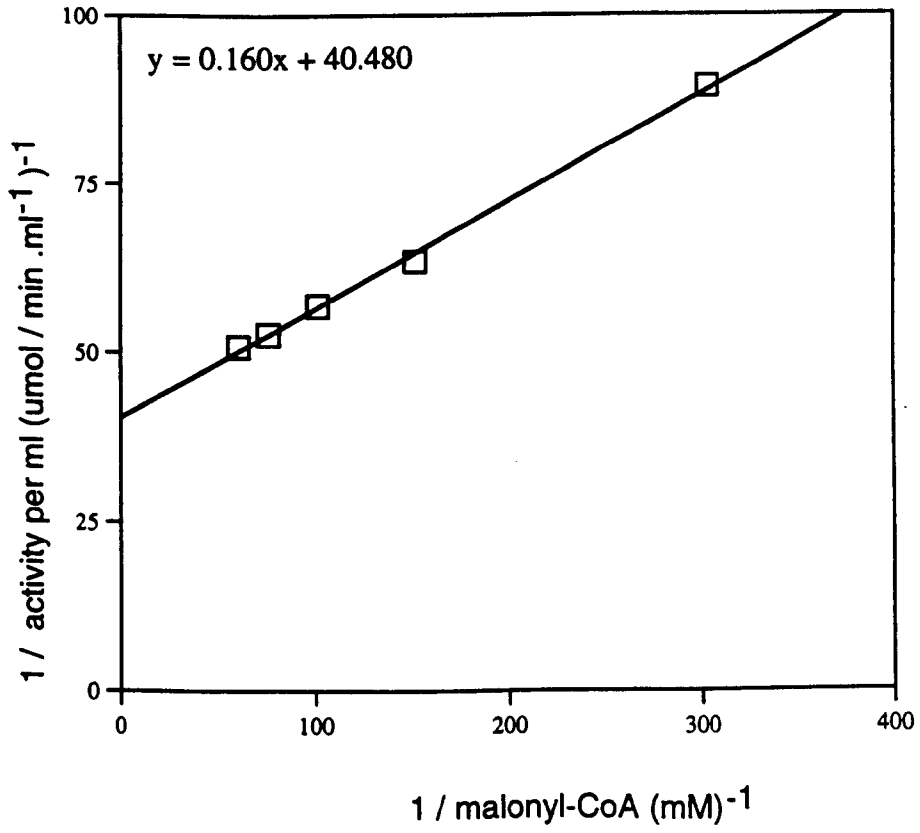

y = 0.160x + 40.480
1 / activity per ml (umol / min .ml-1)-1
1 / malonyl-CoA (mM)-1
0
25
50
75
100
0
100
200
300
400

Table 21: K_m Values of FAS from *M. circinelloides* and *Mt. alpina* for Its Substrates

K_m (mM)	*M. circinelloides*	*Mt. alpina*
Malonyl-CoA	0.013	0.012
Acetyl-CoA	0.017	0.015
NADPH	0.01	0.038

Specific activities of FAS purified from both fungi were measured using method described in Section 2.5.9. Activities were determined with the inclusion of various concentrations of each of the substrates (NADPH, malonyl-CoA and acetyl-CoA), where the other two were kept constant in the reaction mixture. Lineweaver-Burk plots were obtained and the K_m values for each substrates were calculated.

CHAPTER 6

THE INVESTIGATION ON THE OCCURRENCE OF A LIPID BIOSYNTHESIS METABOLON INVOLVING ME AND FAS IN *M. circinelloides* and *Mt. alpina.*

INTRODUCTION

As previously discussed in Chapter 5, the lipid accumulation of *M. circinelloides* culture in a fermenter was shown to stop coincident with the depletion of ME activity when other key enzymes such as FAS, ACC, ACL and other NADPH generating enzymes, G-6-PDH, 6-PGDH and NADP:ICDH were still active and the glucose was still present. Conversely, in *Mt. alpina* culture, ME activity could be detected throughout the experiment and lipid accumulation occurred until the late stage of fermentation. These observations suggest ME as a major provider of NADPH for lipid biosynthesis which was in agreement with earlier observations reported in *Aspergillus nidulans* (Wynn and Ratledge, 1997) and *M. circinelloides* (Wynn et al., 1997). This phenomenon could only be explained by direct channelling of NADPH generated by ME to FAS, which may suggest a physical association between the two enzymes. This chapter describes a series of experiments carried out to determine the possibility of the occurrence of this phenomenon in both fungi.

6.1.0 The Investigation of the Channelling of NADPH from ME to FAS by Determining the Apparent K_m Value of FAS for NADPH when Coupled with ME.

6.1.1 Objectives

This experiment attempts to investigate the possibility of the occurrence of NADPH channelling from ME to FAS in *Mt. alpina* by determining the change in the K_m value of FAS for NADPH when it was coupled with ME activity which may serve as the NADPH provider. The occurrence of the channelling of NADPH from ME to FAS would result in a decreased K_m value of FAS for NADPH in the coupled system in comparison to the K_m value obtained in the previous experiment using 'free' NADPH (Section 5.10.0).

6.1.2 Materials and Methods

6.1.2.1 Experimental Design

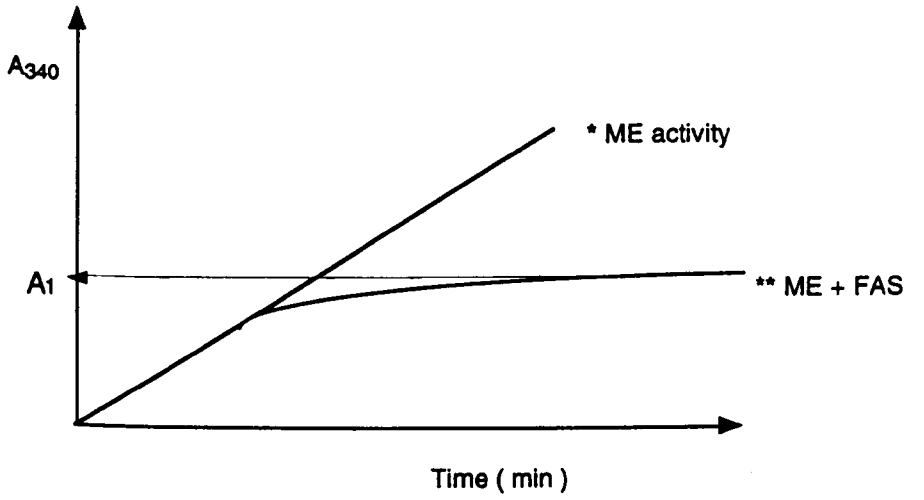
Reaction Assay Mixture. As this experiment involved the coupling of FAS and ME activity, the reaction mixture consisted of the same final concentration of all components of FAS spectrophotometric assay mixture (see Section 2.5.9) excluding NADPH and with the inclusion of the same final concentration of ME cofactors and substrate (NADP, $MgCl_2$ and Malate) as described in Section 2.5.5 at a total volume of 1.5 ml. The reaction was performed at 25 ^{o}C and initiation was carried out by the addition of 0.24 mM NADP. Both enzymes were stable

at this temperature up to 1 h when tested prior to this experiment. FAS and ME included in each of the reaction mixtures were at a total activity of 9.0 nmol NADPH oxidized / min and 4.5 nmol NADP reduced / min, respectively. In this experiment, 4 different reactions were carried out:

1) **Cuvette A:** This assay was to determine the K_m value of FAS for NADPH when it was coupled with ME which served as the NADPH provider for its activity. The assay mixture was as described in Table 22a. The reaction was initiated by the addition of NADP into the assay mixture and followed for 30 min. The increase of adsorbance was recorded continuously by a chart recorder connected to the spectrophotometer. In this system, the adsorbance would stop increasing when the final concentration of NADPH generated from ME in the assay mixture reached the K_m value of FAS for NADPH as FAS would be operating at 4.5 nmol / min (half the activity included in the mixture ie. $1/2\ V_{max}$) and therefore would utilize all NADPH generated by ME (which operated at 4.5 nmol / min) (see Figure 28). The value obtained would be compared to the K_m value observed in the previous experiment (Section 5.10.0).

2) **Cuvette B:** This assay was as a control to confirm the linearity of ME activity throughout the experiment. ME activity was measured using a complete reaction mixture but without the inclusion of FAS (see Table 22a). The activity was initiated with malate and followed for 30 minutes.

Figure 28a: The Determination of K_m Value of FAS for NADPH in a Coupled System with ME Activity.



In this experiment, ME and FAS total activity included in the reaction mixture was 4.5 nmol / min and 9 nmol / min, respectively. Therefore, in a system where ME activity was performed without the inclusion of FAS (*), a steady increase of adsorbance would be observed where ME would be generating 4.5 nmol NADPH / min. In a system where ME activity was coupled with FAS activity (**), the adsorbance would cease to increase when the NADPH generated by ME reached the K_m value of FAS for NADPH (K_m = A1 x 6220^{-1} M) as FAS would be performing at half its maximum activity therefore utilizing 4.5 nmol NADPH / min, which was equal to the rate of NADPH generated by ME.

Table 22a : Summary of Assay Mixture Contents

Reaction Mixture	FAS*	ME *	Malonyl-CoA (0.2 mM)	NADPH (0.04 mM)	NADP (0.24 mM)	Malate (35 mM)
Cuvette A	+	+	+	-	+*	+
Cuvette B	-	+	+	-	+	+*
Cuvette C	+	-	+*	+	-	+
Cuvette D	+	-	+*	+	+	-

* FAS and ME included in the reaction mixtures were at a total activity of 9.0 nmol / min and 4.5 nmol / min, respectively.

+, included in the reaction mixture

-, excluded from the reaction mixture

+*, used for initiation

Assays were performed using modified FAS standard assay mixture (see Section 2.5.9) in a final volume of 1.5 ml. The reaction was carried out at 25 ^{o}C as both enzymes showed to be stable at this temperature up to 1 h when tested prior to the experiment.

3) **Cuvette C:** This assay was to determine the effect of malate on the activity of FAS by measuring the activity of FAS in the presence of malate in the reaction mixture (see Table 22a). The reaction was initiated by the addition of malonyl-CoA and the activity of FAS was determined and compared to a control performed with the same assay mixture but without the inclusion of malate.

4) **Cuvette D:** This assay was to determine the effect of NADP on the activity of FAS. FAS activity was measured with the inclusion of NADP in the reaction mixture (see Table 22a). The reaction was initiated by the addition of malonyl-CoA and the activity of FAS was determined and compared to a control performed with the same assay mixture but without the inclusion of NADP.

6.1.3 Results and Discussion

Results obtained from the reaction observed in Cuvette A showed that the attempts to achieve a channelling of NADPH from ME to FAS were unsuccessful. After the initiation of the reaction, the initial ME activity observed was 4.5 nmol / min and it slightly decreased after 6 minutes to 2.8 nmol / min (see Figure 28b and Table 22b). It was presumed that the decrease in the activity was due to the utilization of NADPH by FAS as ME activity observed in Cuvette B was linear until 30 minutes thus suggesting that the decreased rate in Cuvette A was not due to any possibility of a decrease of ME activity.

Figure 28b: The increase of adsorbance vs time of a reaction in Cuvette A. The trace was obtained by following the reaction using a chart recorder connected to the spectrophotometer.

Table 22b: Observations of Total Activity changes of ME and FAS and the Changes of NADPH concentration in Cuvette A

* As the activity of ME in Cuvette B (see Table 22a) was linear within 30 minutes (total activity of 4.5 nmol / min), the decrease in activity observed at the various intervals in Cuvette A were presumably caused by the activity of FAS. Therefore, the total activity of FAS at the various intervals were obtained by subtracting the initial activity of ME (4.5 nmol / min) with the total activity of ME observed at each intervals.

** Concentration of NADPH was calculated based on the changes of adsorbance (ΔA) observed using the equation stated below:

$\Delta A = C\Delta M$ where **C** = Molar Coefficient Correllation (6220 for NADPH)

ΔM = Changes of Molarity

therefore, $\Delta M = \frac{\Delta A}{6220}$

Figure 28b

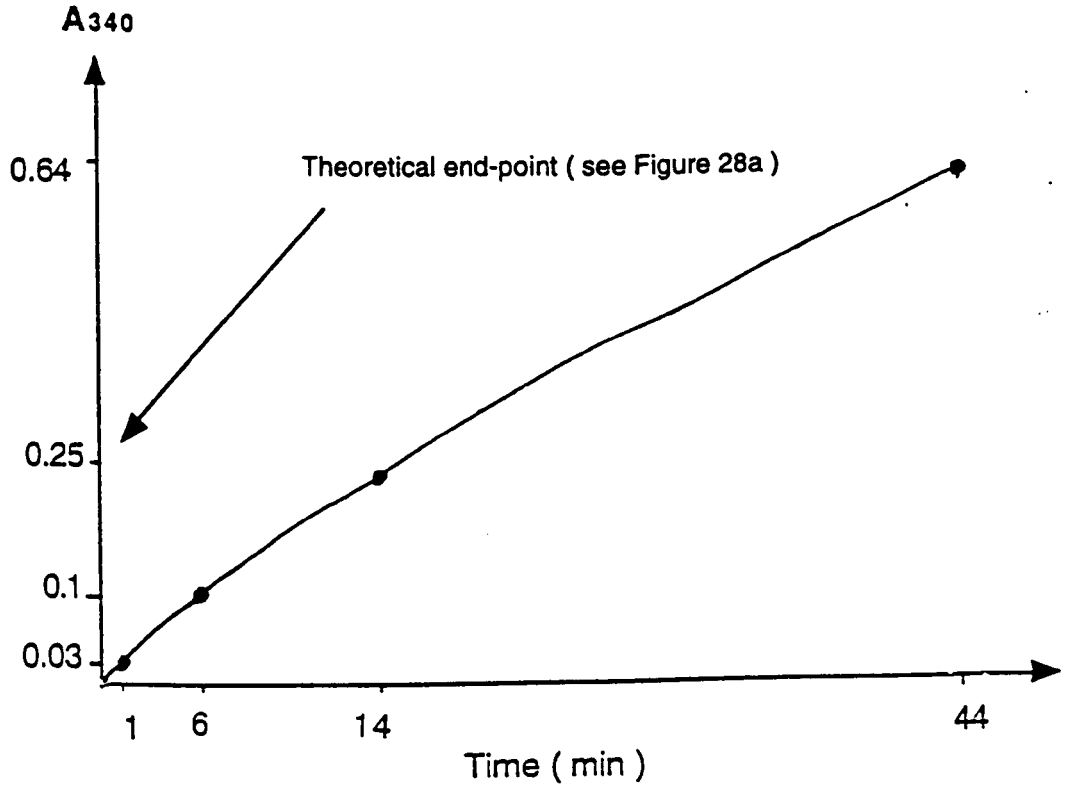


Table 22b

		Time (min)			
		1	6	14	44
Total Activity (nmol / min)	ME	4.5	2.8	2.4	1.7
	FAS*	0	1.7	2.1	2.8
NADPH** (mM)		0.005 (ΔA=0.03)	0.016 (ΔA=0.10)	0.04 (ΔA=0.25)	0.10 (ΔA=0.64)

No evidence of a decrease of the K_m value of FAS for NADPH was observed in this coupled assay as the adsorbance increased continuously until the concentration of NADPH in the system reached 0.04 mM (K_m value of FAS for NADPH) as seen by the change of adsorbance of 0.25 (see Figure 28b and Table 22b). The reason was that if the channelling occurred, the K_m value of FAS for NADPH would be decreased (lower than 0.04) and therefore the adsorbance would stop increasing before the NADPH concentration in the assay mixture reached 0.04 mM.

As there was no evidence of NADPH channelling between the two enzymes, it was expected that the adsorbance would stop increasing when the NADPH concentration in the assay mixture reached 0.04 mM. However, the adsorbance continued to increase even after reaching 0.04 mM where after 44 minutes, the NADPH concentration reached 0.1 mM (as seen by the changes of adsorbance of 0.64) where FAS total activity was 2.8 nmol / min.

Results from reaction of Cuvette D subsequently showed that these observations were due to the inhibitory effect of NADP towards FAS as a 66 % inhibition in FAS activity was observed when 0.24 mM NADP was present in the reaction mixture compared to a control where FAS activity was measured without the inclusion of NADP. No inhibitory effect of malate towards FAS was observed as a similar FAS activity was observed in Cuvette C compared to a control assay, which was performed using the same procedure but without the inclusion of malate. Therefore, the unsuccessful attempts were caused by the inhibition of

FAS activity by NADP in the assay mixture which prevented it from operating at its full activity.

6.2.0 The Investigation of the Channelling of NADPH from ME to FAS by Determination of FAS Activity when Coupled with either ME or G-6-PDH

6.2.1 Objectives

As observed in the previous experiment, the attempts to demonstrate the existence of NADPH channelling from ME to FAS by determining the K_m value of FAS for NADPH in a system when its activity was coupled with ME was unsuccessful due to the inhibitory effect of NADP which was present in the reaction mixture towards the activity of FAS. Therefore, experiments were designed to investigate the possiblity of the occurrence of NADPH channelling from ME to FAS by measuring the activity of FAS when it was coupled with ME as NADPH generator in comparison to its activity when coupled with G-6-PDH. It was presumed that if the channelling occurred, a higher activity of FAS would be observed when FAS was coupled with ME than when it was coupled with G-6-PDH. G-6-PDH was chosen to be coupled with FAS as a negative control of this experiment as no evidence of NADPH channelling between the two enzymes was observed (Section 5.2.0). In this attempt, NADP was not included into the coupling assay mixture due to its inhibitory effect on FAS but the reaction was initiated by the inclusion of a very low concentration of NADPH. Therefore, the radioassay method was applied for adequate accuracy and sensitivity.

6.2.2 Materials and Methods

6.2.2.1 Reaction mixture

As the reaction involved the coupling of FAS with either ME or G-6-PDH, the reaction mixture consisted of the same final concentration of all components of FAS standard reaction mixture (see Section 2.5.9) with the inclusion of the same final concentration of substrate and cofactors (excluding NADP) of either ME or G-6-PDH as described in Section 2.5.5 and 2.5.2, respectively. ME (purified from *Mt. alpina*) and G-6-PDH (Sigma) each with a total activity of 6.8 nmol / min were added into separate test tubes containing the same total activity of FAS (3.2 nmol / min). Table 23 summarizes the reaction mixture of both coupling systems. Prior to the experiment, both malate and glucose 6-phosphate were shown not to have any inhibitory effect towards the activity of FAS. The activity of ME and G-6-PDH were also shown not to be affected by the components of FAS reaction mixture.

All test tubes were incubated for 3 minutes in a water bath at 25°C before the reaction was initiated by the addition of NADPH at a final concentration of 0.0038 mM (1 / 10 of FAS K_m value for NADPH). The reaction was stopped after 15 minutes. To ensure linearity of the activity, the same experiment was then repeated for each coupling mixtures but with the reaction being stopped after 30 minutes and 1 hour after initiation. All three enzymes were demonstrated to be stabile when each were incubated for 1 h at 25°C, prior to the experiment being performed. Blank assays were performed without the inclusion of both enzymes into an identical reaction mixture.

Table 23 : The Summary of FAS Assay Mixture when Coupled with either ME or G-6-PDH

Reaction Mixture	FAS*	ME *	G-6-PDH	Malonyl-CoA (0.2 mM)	Malate (35 mM)	G 6-P (mM)
Cuvette A	+	+	-	+	+	-
Cuvette B	+	-	+	+	-	+

* FAS, G-6-PDH and ME included in the reaction mixtures were at a total activity of 3.2 nmol / min and 6.8 nmol / min, respectively.

+, included in the reaction mixture

-, excluded from the reaction mixture

The assays were performed at 25 ^{o}C with the reaction being initiated with the addition of NADPH (3.8 μM). Linearity of the reaction was checked by performing each of the reactions for 15, 30 and 60 min.

6.2.3 Results and Discussion

No evidence of the channelling of NADPH from ME to FAS was observed as the activity of FAS when coupled with either ME or G-6-PDH was similar (0.13 nmol / min . ml^{-1} and 0.16 nmol / min . ml^{-1} , respectively). It was thought that the unsuccessful attempts were caused by the conditions of the coupling mixture, which was probably still not optimal to initiate the association between ME and FAS.

Many factors have been reported to deter association between enzymes from taking place *in vitro* . For example, the pH and molarity of coupling buffer have been demonstrated to affect the association of the TCA cycle 'metabolon' (Barnes and Weitzman, 1986) . The multienzyme complexes showed a decrease of cluster formation up to 50 % when a shift of pH away from 7.5 to 6.5 or 8.9 occurred and a high salt buffer (1 M KCl) almost eliminated the cluster formation (Barnes and Weitzman, 1986). A similar effect of salt concentration on the formation of complexes involving enzymes and structural elements has also been reported where the binding of ACL from rat liver towards the microsomal fraction was inhibited up to 60 % when the concentration of KCl in the assay buffer was increased from 20 mM to 80 mM (Linn and Srere, 1984). Therefore, it was thought that the buffer used in the experiment, 133 mM KH_2PO_4 at pH 6.5, may have contributed a similar effect, preventing on the formation of clusters between FAS and ME.

Another possibility which may have prevented the association of FAS and ME was the lack of a probable anchor site in the assay mixture

for the enzymes to attach to. It has been reported that a metabolon not only consists of enzyme complexes but also an anchoring site of subcellular structure such as membrane, on which these complexes are adsorbed (Poglazof, 1996). ACL, ACC and FAS from rat liver have been shown to bind to the microsomal fraction where 40-90 % ACC, 30-70 % ACL and 20-60 % FAS were found to exist in the microsomal fraction (Linn and Srere, 1985). A membrane-bound ME has also been reported to occur in *M. circinelloides* although its role was only related to the generation of NADPH for fatty acid desaturation (Kendrick and Ratledge, 1992b). Therefore, as the microsomal fraction of the cells may be a major locus for lipid biosynthesis metabolon, its presence in the reaction mixture may be crucial in order for the association between ME and FAS to occur.

6.2.4 Conclusions

No evidence of the channelling of NADPH from ME to FAS was observed. This was possibly as a result of unsuitable conditions in the reaction mixture used which prevented the association between the two enzymes from occurring.

6.3.0 Determination of Physical Association between FAS and ME from *M. circinelloides* by Ultracentrifugation

6.3.1 Objectives

As the pH and molarity of the coupling buffer used in the previous experiments were probably still not optimal for the association between ME and FAS to occur, attempts were made to investigate the possibility of an association between the two enzymes from *M. circinelloides* using an improved buffer system. The effect of the addition of microsomal fractions into the coupling mixture was also investigated as such membranes were probably a prerequisite for association to take place. The effect of including malate into the coupling mixture was also investigated as its presence was possibly necessary for the formation of complementary binding sites between ME with FAS. In these experiments, ME and FAS were mixed together with the presence of various concentrations of microsomal fraction and malate. The mixture was then centrifuged at 100 000 g (detailed explanation in Section 6.3.2.1) and the activity of both enzymes in the pellet and supernatant determined. A decreased activity of each enzymes in the supernatant followed by the recovery of the activity in the pellet would indicate the association of the enzymes with the pellet fraction.

6.3.2 Materials and Methods

6.3.2.1 Coupling Assay Mixture

Buffer used was 2 mM Hepes pH 7.3 containing 1 mM each of EDTA, PMSF, mercaptoethanol, benzamidine and 220 mM mannitol. This

buffer has been used in previous work involving the investigation of numerous enzyme-membrane association systems (Linn and Srere, 1984; Brent and Srere, 1987; D'Souza and Srere, 1983). All manipulations were carried out at 4 ^{o}C. Into separate 4 ml cuvettes each containing 500 µl of purified ME, increasing volumes of purified FAS were added (see Table 24a). Each of the reaction mixtures varied in the presence and the amounts of microsome and malate included. The coupling mixtures were then incubated at 25 ^{o}C for 10 minutes. 500 µl of each of the complete coupling mixtures were removed from each of the cuvettes for ultracentrifugation while the remaining 1000 µl was kept at 4 ^{o}C in a cold room. The reaction mixtures which included microsomal fractions were centrifuged at 100 000 g for 1 h while the coupling mixtures which did not contain microsomal fractions were centrifuged for 6 h at 100 000 g. The activity of ME and FAS in each of the resulting supernatants after the centrifugation was determined and compared to the activity of ME and FAS in each of the corresponding reaction mixtures which were not centrifuged (kept at 4 ^{o}C) using methods described in Section 2.5.5 and 2.5.9, respectively.

The initiation of ME activity was carried out by the inclusion of NADP into the reaction mixture. A decrease in the total activity of ME in the supernatant followed by the recovery of the decreased activity in the pellet would indicate the binding of ME to the pellet fraction. Prior to the experiment, both ME and FAS were dialyzed against 500 volumes of the coupling buffer overnight. Microsomal fractions were prepared using methods described in Section 2.4.0 but were resuspended in the coupling buffer. It was free from the activity of ME and FAS. No endogenous

Table 24a: The Summary of the Coupling Assay Mixtures Between FAS and ME

Reaction Mixture	ME (µl)	FAS (µl)	* Microsome (µl)	0.5 M Malate (µl)	Buffer (µl)
1a	500	-	-	-	1000
1b	-	60	-	-	1440
2a	500	60	-	-	940
2b	500	60	20	-	920
2c	500	60	20	70	850
3a	500	120	-	-	880
3b	500	120	60	-	820
3c	500	120	60	70	750
4a	500	300	-	-	700
4b	500	300	130	-	570
4c	500	300	130	70	500

* Protein concentration was 3.5 mg / ml.

Purified ME and FAS used were dialyzed overnight against 500 volumes of the coupling buffer (see Section 6.3.2.1). Both enzymes were free from the activity of 6-PGDH and G-6-PDH and no endogenous activity was observed when both extracts were tested for the activity of ME prior to the experiment. The final volume of each assay mixtures was 1.5 ml.

Each assay mixtures (except 1b) contained ME at a total activity of 20 nmol / min. Assay mixture 1b and 2a-c contained FAS at a total activity of 15 nmol / min while assay mixture 3a-c and 4a-c contained FAS at a total activity of 30 and 75 nmol / min, respectively.

activity was observed in all ME, FAS and microsomal fractions when each were tested for the activity of ME prior to the experiment.

6.3.3 Results and Discussion

No evidence of physical association between ME with either FAS or the microsomal fractions was observed. When FAS was centrifuged at 100 000 g for 6 h (Mixture 1b), all of FAS activity was recovered in the pellet and none in the supernatant indicating that the ultracentrifugation was sufficient to sediment FAS (Table 24b). The lack of FAS activity in the supernatant was not due to the inactivation of the enzyme as all activity was recovered in the pellet. However, when ME was mixed together with FAS (Mixture 2a, 3a and 4a) and then centrifuged for 6 h at 100 000 g, approximately 98 % of ME activity were still recovered in the supernatants while approximately 100 % of FAS activity was detected in the pellet. This indicated that ME was not associating with FAS as, if it did, ME would have been recovered with FAS in the pellet.

When FAS and ME were mixed with microsomes (Mixture 2b, 3b and 4b) and with both malate and microsomes in the mixture (Mixture 2c, 3c and 4c), similar results were obtained where no evidence of association between ME with FAS and the microsomal fractions were observed after centrifugation at 100 000 g for 1 h (see Table 24b). These results also indicated that FAS did not associate with the microsomal fractions as after the ultracentrifugation, approximately 100 % FAS activity was still in the supernatant while the microsomes were sedimented as a pellet. Therefore, despite the modifications of the buffer system and the inclusion of microsomal fractions into the coupling

Table 24b: The Recoveries of ME and FAS Activity in the Supernatant and Pellet After Centrifugation

		Recovery (%)			
		ME		FAS	
Reaction Mixture	Centrifugation	Supernatant	Pellet	Supernatant	Pellet
1a	**	100	0	ND.	ND.
1b	**	ND.	ND.	0	100
2a	**	100	0	0	100
2b	*	100	0	100	0
2c	*	100	0	100	0
3a	**	100	0	0	100
3b	*	100	0	100	0
3c	*	100	0	100	0
4a	**	100	0	0	100
4b	*	100	0	100	0
4c	*	100	0	100	0

ND., not detected

Each assay mixtures (except 1b) contained ME at a total activity of 20 nmol / min. Assay mixture 1b and 2a-c contained FAS at a total activity of 15 nmol / min while assay mixture 3a-c and 4a-c contained FAS at a total activity of 30 and 75 nmol / min, respectively.

500 µl of each of the coupling mixtures (see Table 20a for the content of each mixtures) were obtained for centrifugation while the other remaining 1 ml were not centrifuged and kept at 4 ^{o}C.

Reaction mixtures which were included with microsomal fractions were centrifuged at 100 000 g for 1 h (*) while the coupling mixtures which did not contain microsomal fractions were centrifuged at 100 000 g for 6 h (**).

mixture, a physical association between FAS and ME was not established. Although ME from *M. circinelloides* has been reported to exist as both a cytosolic and as a membrane-bound form (Kendrick and Ratledge, 1992b), the effort to associate these two components were unsuccessful.

There were two possibilities which may have prevented the associations from taking place. Firstly, the vigorous method used (French press) for cell disruption of the fungus during cell extract preparation may have affected the structural elements of the enzyme and damaged the binding sites required for the association. It has been demonstrated that the formation of multienzyme complexes in the TCA metabolon of *Escherichia coli* was eliminated when French pressing was used in the cell disruption procedure (Barnes and Weitzman, 1986). The metabolon was also shown to be permanently dissociated when it was subjected to sonication.

Secondly, the use of purified enzymes in this experiment may have affected the ability of both enzymes to associate if there were some other yet unidentified components of the cell which were required for the association which may have been separated from the enzymes after its purification.

6.3.4 Conclusions

No evidence of physical association between ME with either FAS or the microsomal fractions was observed despite the application of modified buffer system and the inclusion of microsomal fractions into the

coupling mixture. The vigorous cell disruption technique used may have affected the structure of the enzymes which as a result prevented association from taking place. The use of purified enzymes in this experiment also may have excluded certain components of the cell whose presence may have been necessary for the association to occur.

6.4.0 Determination of Association between ME and FAS from *Mt. alpina* Using Whole-Cell Extracts Produced by Gentle Grinding

6.4.1 Objectives

As observed in previous experiments, attempts to associate ME and FAS by the mixing of purified enzymes in the presence of microsomal fractions were unsuccessful despite the application of a modified coupling buffer. It was thought that the vigorous cell disruption method (French press) may have affected the structural elements of the enzymes and so prevented the association from taking place. The use of purified enzymes in the experiments also may have excluded some other components of the cell the presence of which was possibly necessary for association. These experiments therefore attempt to isolate an aggregate of FAS and ME from a whole-cell extracts of *Mt. alpina* produced by gentle grinding in a modified buffer system.

6.4.2 Materials and Methods

6.4.2.1 Production of Cell Extracts

Mt. alpina was grown in a fermenter as described in Section 4.1.2 and harvested after 24 h of incubation. Cells were then resuspended in 50 ml of an ice-cold buffer (as described in Section 6.3.2.1 but with the inclusion of 10 mg BSA / ml) to form a slurry. It was then ground in a cold (approximately 4 ^{o}C) pestle and mortar for approximately 2 minutes. The temperature of the pestle and mortar was maintained at approximately 4 ^{o}C by keeping it in an ice-bath during the grinding process.The slurry was then used for the next step.

6.4.2.2 Isolation of Enzyme Clusters by Ultracentrifugation and Gel Filtration.

The slurry produced was then centrifuged at 3000 g for 15 minutes at 4 ^{o}C to remove undisrupted cells and large cell particles. The supernatant was recovered and half of the volume (25 ml) loaded onto a Sephacryl S 300 gel filtration column (3 x 72 cm, Pharmacia Biotech) which had been equilibrated with the same buffer (see Section 6.4.2.1). The column was washed with the same buffer and fractions collected were then analysed for the activity of FAS, ME, ACL, G-6-PDH and 6-PGDH (Approximate molecular weight for each of the enzymes is 2 x 10^{6}, 62 000, 4 x 10^{5}, 121 000 and 100 000, respectively). If a single peak of high-molecular mass eluted from the gel filtration and contained both FAS

and ME, this would indicate association of these enzymes, as ME and FAS should be well seperated by such chromatography.

The 25 ml remaining supernatant was recentrifuged at 16 000 g for 15 minutes at 4 ^{o}C. The supernatant was obtained and recentrifuged at 100 000 g for 1 hour . The activities of FAS, ME, ACL, G-6-PDH and 6-PGDH in the pellet and both supernatants were measured after each centrifugation. A decrease in the total activity of each enzyme in the supernatant after each centrifugation followed by the recovery of the activity in the pellet, would indicate a positive association of the enzymes with the pellet fraction.

6.4.3 Results and Discussion

Table 25 summarizes the activity of each of the enzymes detected in the supernatant and the pellet after each centrifugation of the crude extract. After centrifugation at 16 000 g for 15 min, no significant decrease in the total activity of each of the enzymes was observed, approximately 97 % recovery was obtained for each enzymes in the supernatant. When the supernatant was recentrifuged at 100 000 g for 1 h, only 43 % of FAS total activity after centrifugation at 3000 g were recovered in the supernatant while G-6-PDH showed an 18 % decrease in the total activity in the supernatant. Approximately 100 % of the decreased activities of both enzymes were detected in the microsomal fraction. This indicated that FAS and G-6-PDH were associated with the microsomal fraction. No significant changes of the total activity in the supernatant was observed for 6-PGDH, ME and ACL where a 97 %, 96 % and 97 % recovery were

Table 25: The recoveries of ME, FAS, ACL, G-6-PDH and 6-PGDH Activity in the Supernatant and Pellet Fractions after Three stages of centrifugation.

	Recoveries (%)									
	ME		FAS		ACL		G-6-PDH		6-PGDH	
Centrifugation	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant	Pellet
3000 g, 15 min	100	nd.	100	nd.	100	nd.	100	nd.	100	nd.
16 000 g, 15 min	97	tr.	98	tr.	97	tr.	98	tr.	98	tr.
100 000 g, 15 min	96	tr.	43	54	97	nil	82	16	97	tr.

nd; not detected, tr; trace.

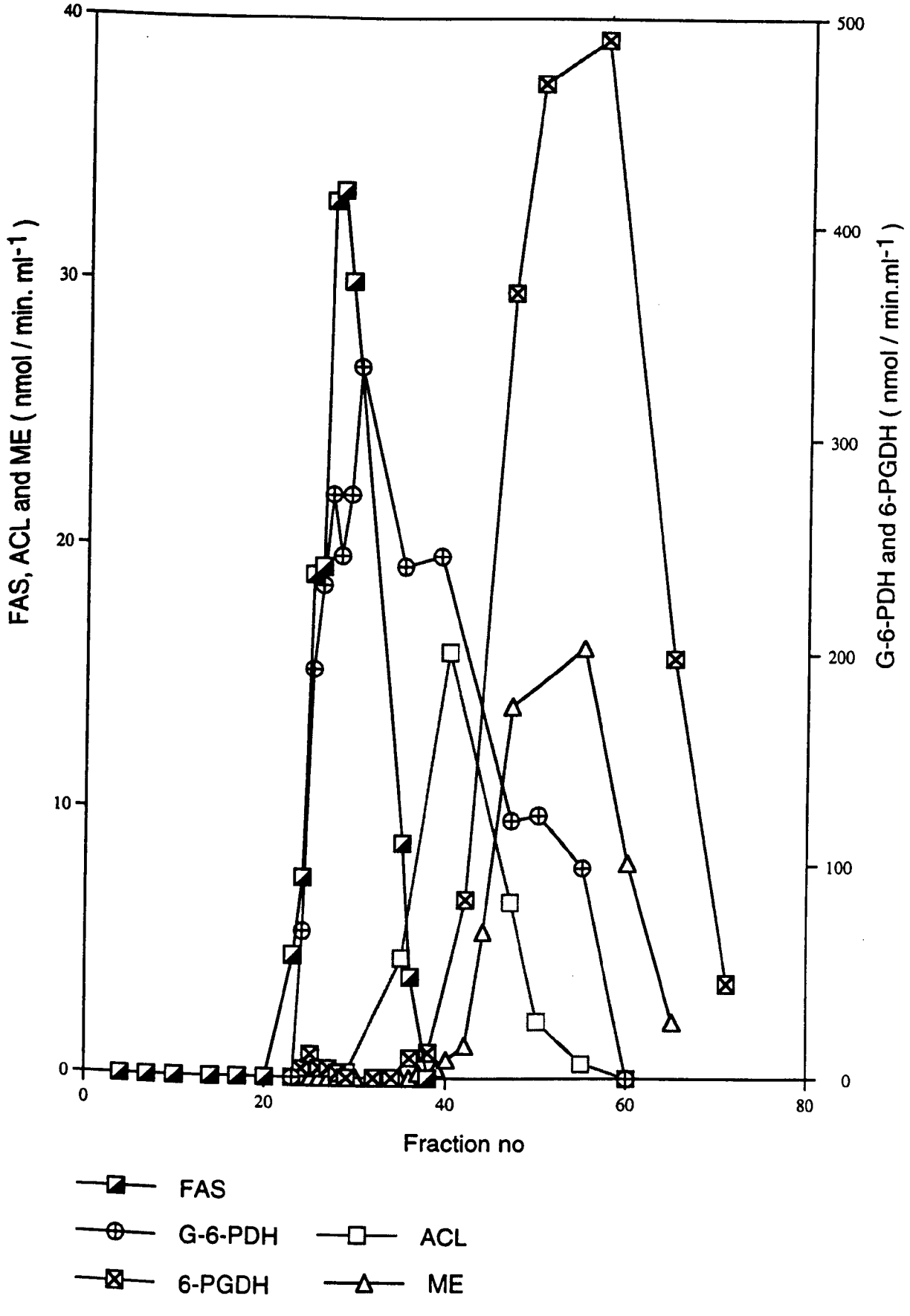
The recoveries of each enzymes were calculated by measuring the total activity of the enzymes in the supernatant and pellet after each step of centrifugations compared to the total activity of the enzymes in the supernatant after centrifugation at 3000 g for 15 min. 100 % activity of FAS, ME, ACL, G-6-PDH and 6-PGDH were 965, 4014, 4260, 2067 and 15 755 nmol / min, respectively.

obtained after the centrifugation, respectively.

The gel filtration of the supernatant obtained after the 3000 g centrifugation gave the elution profile shown in Figure 29 . Both FAS and G-6-PDH were eluted as a single peak but the other enzymes were eluted at distinct positions corresponding to their individual molecular masses. This result suggests that FAS and G-6-PDH have the tendency to aggregate together. This was in agreement with the observations in the ultracentrifugation step where a significant decrease in the total activity of both enzymes in the supernatant were observed after centrifugation at 100 000 g for 1 h, indicating positive association with the microsomal fraction. Therefore, the attempts to isolate clusters of FAS and ME were unsuccessful.

These results do not support the idea of a physical association between FAS and ME as discussed in Section 5.2.0. There were several possibilities which may have led to these observations. Firstly, as the enzyme profile obtained in the previous experiments (see Section 5.2.0) indicated that an association between G-6-PDH and FAS were unlikely to occur, the elution of G-6-PDH and FAS as a single peak from the gel filtration column (see Figure 29) was possibly due to a non-specific association between these two enzymes. This may possibly be caused by the formation of a non-specific binding sites that could occur during cell disruption (Robinson and Srere, 1985) which subsequently resulted in the binding of both enzymes. It was also possible that the active site of G-6-PDH for NADP binding was complementary to the active site of FAS for NADPH which resulted in the binding of both enzymes when the cells were disrupted.

Figure 29: The Elution of FAS, ME, ACL, G-6-PDH and 6-PGDH from Sephacyl S 300 gel filtration column of a whole-cell extracts of *Mt. alpina* . The extracts were prepared using method described in Section 6.4.2.1. The column was washed using the same buffer and the activitiy of the enzymes were measured using methods described in Chapter 2.


FAS, ACL and ME (nmol / min. ml-1)
G-6-PDH and 6-PGDH (nmol / min.ml-1)
Fraction no
0
10
20
30
40
0
100
200
300
400
500
0
20
40
60
80
FAS
G-6-PDH
ACL
6-PGDH
ME

Secondly, the coupling buffer used was possibly still not optimal to promote binding between ME and FAS despite the inclusion of 10 mg BSA / ml in the buffer, which was to simulate the intracellular milieu which is known to consist of high concentration of proteins. It was demonstrated that the yield of cluster formation of the TCA metabolon in *E. coli* increased up to 3-fold in the presence of a similar concentration of BSA (Barnes and Weitzman, 1986).

6.4.4 Conclusions

1) Despite the improved buffer system that included 10 mg BSA / ml and a gentler method of cell disruption that was employed, the attempts to observe a physical association between ME and FAS were still unsuccessful. It was thought that a further modification of the buffer system was required in order to achieve an optimal conditions for the association to take place.

2) The observed apparent binding of G-6-PDH and FAS was probably as a result of a non-specific binding as no evidence of the possibility of NADPH channelling from this enzyme to FAS was observed in previous experiments (see Section 5.2.0).

6.5.0 Determination of Association between ME and FAS of *M. circinelloides* Using Whole-Cell Extracts Produced by Gentle Grinding

6.5.1 Objectives

As demonstrated in previous experiments, the attempts to observe

an association between ME and FAS by using a whole-cell extract obtained from *Mt. alpina* prepared by gentle grinding method were unsuccessful despite the inclusion of high concentration of BSA in the coupling buffer. It was thought that an optimal conditions for the association was probably still not achieved. Therefore, these experiments attempt to isolate a cluster of FAS and ME by using a whole-cell extract from *M. circinelloides* produced by gentle grinding in a modified buffer system where the cofactors and substrate of ME were included in the buffer. It was thought that the inclusion of these components may be necessary for the formation of specific binding sites between the two enzymes.

6.5.2 Materials and Methods

M. circinelloides was cultivated in a 5 L fermenter using method described in Section 3.6.0. Cells were harvested after 24 h growth and disrupted using method described in Section 6.4.2.1. The buffer used was as described in Section 6.4.2.1 but with the inclusion of 5 mM malate, 0.04 mM NADP and 3 mM $MgCl_2$. The isolation of enzyme clusters was carried out using gel filtration method (see Section 6.4.2.2).

6.4.3 Results and Discussion

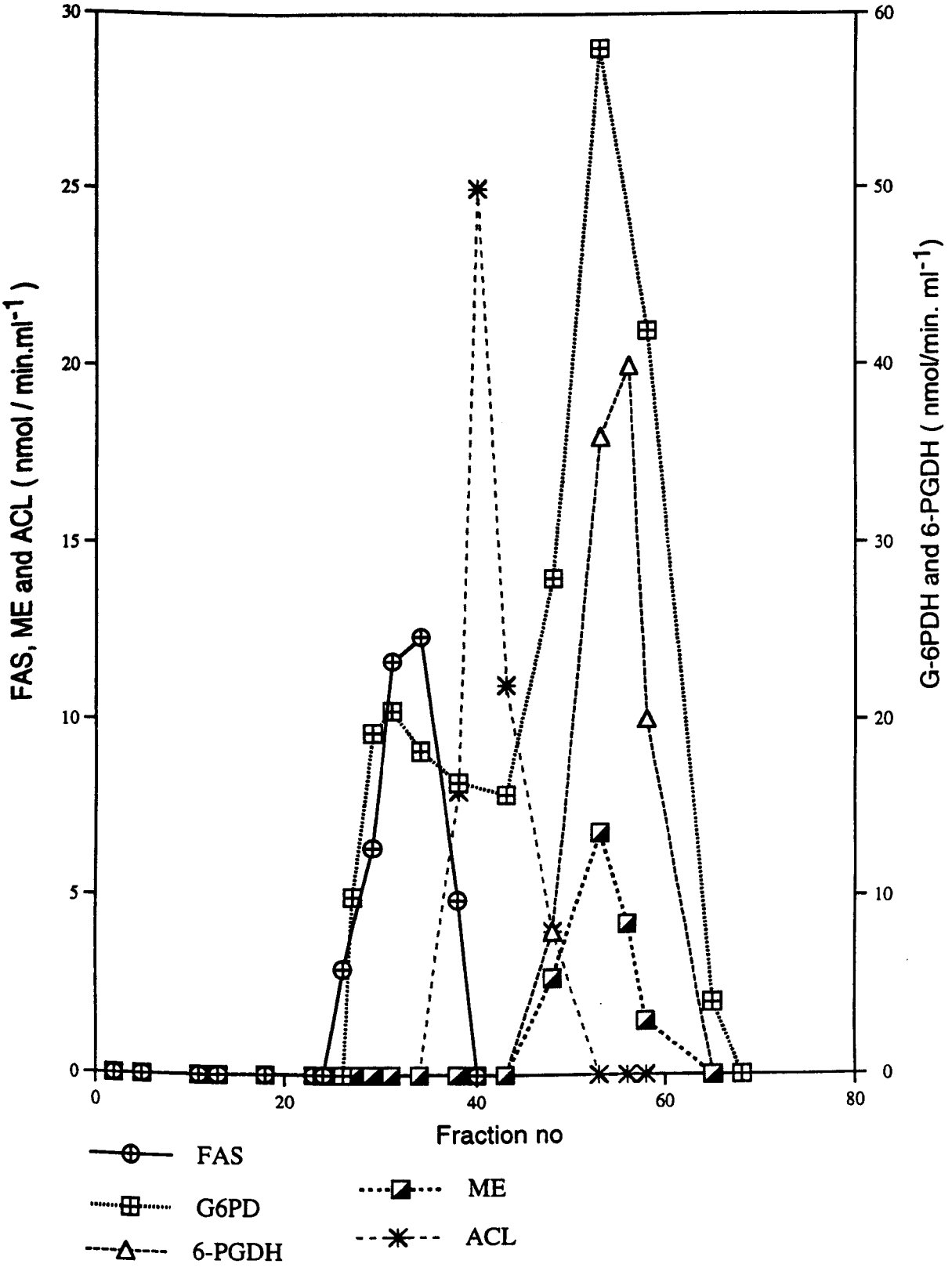
Similar results as observed in the previous experiments were demonstrated where no evidence of physical association between ME and FAS was obtained. The gel filtration of the supernatant obtained after the 3000 g centrifugation gave a similar elution profiles as observed in the previous experiment where FAS and a small proportion of G-6-PDH

activity were eluted as a single peak while the other enzymes and a major proportions of G-6-PDH were eluted at distinct positions corresponding to their individual molecular masses (Figure 29). This result indicated that FAS and G-6-PDH have the tendency to aggregate together and despite the inclusions of NADP and malate in the coupling buffer, the attempts to observe an association between ME and FAS were again unsuccessful.

It was thought that the unsuccessful attempts to observe the association between the two enzymes *in vitro* could be due to using conditions that were still far from physiological. It is known that the composition of the cytosol, obtained by methods of disruption and fractionation, bears almost no resemblance to those in the aqueous cytoplasm of intact cells (Clegg, 1984). Therefore, it was difficult to assess the exact combinations of the parameters in order to achieve the right conditions for the association to occur. The majority of previous work involved in the characterization of metabolons (Barnes and Weitzmann, 1986; Robinson et al., 1987; Robinson and Srere, 1985 ; Brent and Srere, 1987) achieved association by the application of gentle cell disruption either by osmotic disruption or gentle sonication. The method of osmotic disruption was not employed in these study as according to previous work on the same fungi, a long time (up to 6 h) would be required in order to achieve a spheroplast formation of the fungi (J.P. Wynn, personal communication). It was thought that this could lead to a change of the biochemical activities within the fungi, as the cells would be transferred and subjected to a totally differrent environment for 6 h which may subsequently affect the overall experiment.

The occurrence of FAS and G-6-PDH as a cluster was similar to that

Figure 30: The Elution of FAS, ME, ACL, G-6-PDH and 6-PGDH from Sephacyl S 300 gel filtration column of whole-cell extracts of *M. circinelloides*. The extracts were prepared using method described in Section 6.4.2.1. Buffer used was as described in Section 6.5.2. The column was washed using the same buffer and the activitiy of the enzymes were measured using methods described in Chapter 2.


FAS, ME and ACL (nmol / min.ml-1)
G-6PDH and 6-PGDH (nmol/min. ml-1)
Fraction no
0
5
10
15
20
25
30
0
10
20
30
40
50
60
0
20
40
60
80
FAS
G6PD
6-PGDH
ME
ACL

observed in the previous experiment when cell extracts from *Mt. alpina* were used. As there is no evidence which suggests that G-6-PDH functions as a major provider of NADPH for FAS activity (see Section 5.2.0), the association between these two enzymes seen in this experiment was probably due to a non-specific binding. This could occur if the disruption of the cells resulted in the formation of a non-specific binding sites on both enzymes.

6.5.4 Conclusions

All attempts to observe a physical association between ME and FAS from *M. circinelloides* were unsuccessful although further modification of the coupling buffer was carried out. It was thought that suitable conditions for coupling were still not being achieved.

CHAPTER 7

GENERAL CONCLUSIONS

7.1 The Optimization of Growth and Lipid Accumulation of *M. circinelloides* and *Mt. alpina*

1) Both fungi showed good growth and lipid production when cultivated in fermenters but not when grown in whirlipots. This was due to unfavourable conditions for lipid biosynthesis occurring in whirlipot cultures as a result of inefficient aeration. In whirlipot cultures, an anaerobic phase occurred at the early stage of incubation which affected the utilization of ammonium by the cells. As a result, the ammonium remained present in the culture until the late stage of fermentation while glucose was depleted thus resulting in low lipid production by the cells. Conversely, in a fermenter culture which had an efficient aeration, the culture reached a nitrogen-limited condition at the early stage of incubation which led to a higher lipid production of the cells. Therefore, a nitrogen-limited condition was vital in triggering the lipid biosynthesis of both fungi, which was in agreement with previous work reported in oleaginous yeasts. It is well established that lipid accumulation requires an excess of carbon over other nutrients, particularly nitrogen (Botham and Ratledge, 1979; Boulton and Ratledge, 1984). When the nitrogen is exhausted, synthesis of nitrogenous cellular components (protein, DNA, RNA etc.) ceases while the remaining carbon continues to be channelled into lipid.

2) The lipid production of both fungi increased in parallel with the increase in the C:N ratio of the medium. For example, *M. circinelloides* produced up to 25 % lipid (w / w of biomass) when cultivated in a medium with C:N ratio at 66:1 (50 g glucose / l and 2 g ammonium tartrate / l), however when it was grown in a medium with C:N ratio at 40:1 (30 g glucose / l and 2 g ammonium tartrate / l), it produced only 14 % lipid (w/w of biomass). *Mt. alpina* produced 40 % lipid (w / w of biomass) when grown in a medium with C:N ratio at 40:1 (30 g glucose / l and 2 g ammonium tartrate / l) whereas when it was cultivated in a medium with C:N ratio at 24:1 (30 g glucose / l and 3.3. g ammonium tartrate / l), it produced only 24 % lipid (w/w of biomass). However, the fatty acid compositions of both fungi were not affected by the change of the C:N ratio .

7.2 The Investigation on the Enzymatic Profile of *M. circinelloides* and *Mt. alpina* during lipid Accumulation

1) Ten enzymes possibly involved in the regulation of lipid biosynthesis (fatty acid synthase, acetyl-CoA carboxylase, ATP:citrate lyase, AMP:deaminase, carnitine acetyl transferase, malic enzyme, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP:isocitrate dehydrogenase and NAD:isocitrate dehydrogenase) were detected in both fungi. The profile of all enzymes examined were similar in both fungi. Generally the activities increased after the depletion of ammonia in the culture. The only difference observed between the two fungi was the early depletion of ME activity in *M. circinelloides*, where it disappeared after approximately 40 h of incubation coincident with the cessation of lipid accumulation although other key enzymes of lipid

biosynthesis (FAS, ACC and ACL) together with the activities of other NADPH-generating enzymes were still active and the glucose was still present. Conversely, ME in *Mt. alpina* culture was present throughout the incubation period and the cell lipid continued to increase until the late stage of fermentation.

Together these observations suggested that ME is a major provider of NADPH for lipid biosynthesis which was in agreement with previous observations in other oleaginous fungi (Wynn et al., 1997; Wynn and Ratledge, 1997). The depletion of ME activity in *M. circinelloides* was demonstrated not to be caused by the action of intermediary inhibition as the mixing of cell extracts containing ME with extracts obtained after ME had disappeared resulted in no inhibition of the activity.

2) The depletion of ME activity was demonstrated to be a result of the cessation of the synthesis of the protein, triggered by the depletion of ammonium in the culture. This was evident as the restitution of the activity was achieved when ammonium tartrate was added into the culture after the initial depletion of ME activity at approximately 40 h after inoculation and that the restitution was prevented when cycloheximide, a potent protein synthesis inhibitor, was added simultanously with the addition of ammonium tartrate.

3) The acitivity of NAD:isocitrate dehydrogenase of both fungi did not show an absolute requirement for AMP for its activity as it could still be activated in the absence of AMP at a saturating concentration of isocitrate.

4) ME was purified some 20-fold purification from both fungi. Both

showed a similar K_m values for NADP (approximately 0.04 mM) but a slightly higher K_m value for malate was obtained in *Mt. alpina* compared to *M. circinelloides* (1 mM and 0.4 mM, respectively).

5) ME from both fungi showed various degrees of inhibition by tartronic acid, oxaloacetate, palmitoyl-CoA and oleoyl-CoA. At 10 mM, tartronic acid caused approximately 40 % inhibition in the activity of ME from both fungi while OAA showed a higher inhibition in ME from *M. circinelloides* (70 %) compared to *Mt. alpina* (45 %). Oleoyl-CoA and palmitoyl-CoA showed a potent inhibitory effect on ME from both fungi only when the concentrations of both compounds were well over the critical micellar concentration and at unphysiological concentrations. Therefore, the inhibition was more likely due to these compounds acting as detergents which probably exert their effects by disruption of the structure of the enzyme.

6) FAS was purified from both fungi but with a low recovery (approximately 5 %). Both showed similar K_m values for malonyl-CoA and acetyl-CoA (approximately 0.013 and 0.016 mM, respectively) while a higher K_m value for NADPH was observed in *Mt. alpina* compared to *M. circinelloides* (0.038 and 0.01 mM, respectively).

7) Attempts to demonstrate the occurrence of physical association between FAS and ME were not successful despite the application of various approaches in the attempt to associate both enzymes *in vitro*. Two methods of cell disruption were used during the preparation of cell extracts from the fungi: French pressing and gentle grinding in a pestle

and mortar. The former was subsequently abandoned as earlier work showed that vigorous method of cell disruption resulted in the elimination of the association of enzymes in the TCA cycle metabolon (Barnes and Weitzmann, 1986).

In the first attempts, experiments were performed to establish the existence of NADPH channelling from ME to FAS by determination of the K_m value of FAS for NADPH when coupled with ME. However, the determination of the value was not achieved due to the inhibitory effect of NADP in the reaction mixture towards the activity of FAS where it was demonstrated that approximately 60 % of FAS activity was inhibited in the presence of 0.24 mM NADP. In the second experiment, the determination of the channelling of NADPH from ME to FAS was carried out by comparing the activity of FAS when coupled with either ME or G-6-PDH (a negative control) using a very low concentration of NADPH in the reaction mixture. No evidence was obtained as both coupling assays resulted in a similar activity of FAS.

As no positive results were obtained in the earlier stages, where experiments were performed using purified ME and FAS, the later experimentations were performed using whole-cell extracts (produced by gentle grinding) as there may be some other yet unidentified proteins that were essential for the association being separated during the purification process. However, despite the application of this method, the association between the two enzymes was not observed. This was demonstrated when whole-cell extracts from both fungi were placed on a gel filtration column (Sephacryl S 300) resulting in the elution of both enzymes as two distinctive peaks. Various steps had also been taken in order to

achieve an optimal conditions for the association to take place by altering the contents of the coupling buffer. This included the use of high concentration of BSA (up to 10 mg / ml), which was to imitate the intracellular environment, and also the inclusion of the substrate and cofactors of ME: NADP, malate and $MgCl_2$ into the buffer. It was thought that a suitable conditions for the association remain elusive.

CHAPTER 8

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