THE DEVELOPMENT OF AN INDUSTRIAL PROCESS TO PRODUCE γ-LINOLENIC ACID USING *CHOANEPHORA CUCURBITARUM*

by

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Abbreviations

AA	- arachidonic acid
AFA(s)	- analysed fatty acid(s)
$C_{14:0}$	- myristic acid
$C_{16:0}$	- palmitic acid
$C_{16:1}$	- palmitoleic acid
$C_{18:0}$	- stearic acid
$C_{18:1}$	- oleic acid
$C_{18:2}$	- linoleic acid
γLA	- gamma-linolenic acid
αLA	- alpha-linolenic acid
BM	- basal medium
BM-Y	- basal medium and yeast extract
C	- carbon
Cl	- cornsteep liquor
Cp	- cornsteep powder
DAP	- di-ammonium phosphate
DE	- direct extraction and esterification method
DHA	- docosahexaenoic acid
DHγLA	- dihomo-γ-linolenic acid
DM	- dry matter
EFAs	- essential fatty acids
EPA	- eicosapentaenoic acid
FA(s)	- fatty acid(s)
GBM	- glucose basal medium
GBM-Cp	- glucose basal medium and cornsteep powder
GBM-Cl	- glucose basal medium and cornsteep liquor
GBM-P	- glucose basal medium and peptone
GBM-S	- glucose basal medium and soyabean meal
GBM-Y	- glucose basal medium and yeast extract
GYE	- glucose yeast extract medium
MAP	- mono-ammonium phosphate
N	- nitrogen
NCP	- National Chemical Products
PGE_1	- prostaglandin E_1
SBM	- soyabean meal

Abstract

The objective of this work was to produce γ-linolenic acid (γLA) using a fungus in submerged fermentation. Selection work was aimed at identifying a fungal strain capable of yielding a high level of γLA in an industrial fermentation. Thirty-nine fungal strains were screened under shake flask conditions. The major criteria used in evaluating these strains were, the yield of γLA per unit volume (g/ℓ) and γLA as a percentage of fatty acids, which is important in the downstream processing of γLA. Other parameters of industrial importance such as strain handling and the fatty acid profile were also considered. Eleven fungi in the order Phycomycetes were identified after initial screening. From these fungi, a strain of *Choanephora cucurbitarum* was found to give superior γLA yields. *C. cucurbitarum* produced γLA yields of 331mg/ℓ and 674mg/ℓ in shake flask and laboratory fermenters respectively. This strain had other industrially beneficial qualities such as good sporulation, a good biomass of 22,5g/ℓ and a relatively high yield of γLA of 2,99g/100g dry matter.

Subsequently a *Zygorhynchus heterogamus* strain was found to give similar yields of γLA to *C. cucurbitarum*. *Z. heterogamus* also had a high γLA:linoleic acid ratio which aids the purification of γLA. This is the first known report of a high level of γLA in the genus *Zygorhynchus*. The industrial development of γLA production by *Zygorhynchus* is not reported.

The growth and γLA producing stages of the selected strain of *C. cucurbitarum* were further optimised by changing parameters such as the status of the inoculum, the industrial medium composition and the physical conditions of the fermentation. An improved analysis procedure, (direct extraction and esterification (DE)) allowed the measurement of more of the γLA produced by the fungus and in this way increased the apparent γLA yield. Early optimisations produced an increase in the shake flask yield of γLA from 21mg/ℓ to 396mg/ℓ. In fermentations carried out in laboratory (Chemap) fermenters, the increase was from 59mg/ℓ to 512mg/ℓ and with a subsequent fed-batch fermentation the γLA yield was 744mg/ℓ. The optimal carbon and nitrogen sources for γLA production were respectively glucose and soyabean meal. For the batch culture of *C. cucurbitarum*, optimal γLA mg/ℓ yields were obtained with a 20% inoculum grown in glucose basal medium plus soyabean (GBM-S) with a C:N ratio of 14:1. In the main fermentation the best C:N ratio in GBM-S was 22:1. No pH control was necessary in the fermentation. A dual temperature profile of 35°C for 24h followed by 30°C for 72h supported good growth and γLA production. Under these conditions there was no lag phase in fungal growth and γLA production. The optimised conditions for the production of γLA by *C. cucurbitarum* were suitable for further scale-up of the fermentation in the pilot plant.

The fermentation was scaled up by a factor of a hundred, and preliminary pilot plant trials were undertaken to evaluate the productivity of the fermentation and the recovery of γLA from the mycelial biomass of *C. cucurbitarum*. Batch fermentations at this industrial level proved to be successful in that γLA yields per unit volume were improved and the

γLA yield per gram substrate (14,7mg/g glucose) was comparable to the better reported yields.

Freeze-drying was the best means of preserving the fatty acid content of the mycelium. There was, however, a marked decrease in the unsaturated fatty acids upon storage of the dried mycelium. The batch-countercurrent system extracted most of the oil from milled dry mycelium. The overall increase in productivity from the shake flask to the pilot plant stage was 60-fold, from 0,2mg/ℓ.h to 11,9mg/ℓ.h.

CHAPTER 1

GENERAL INTRODUCTION

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1 Introduction

There has been a recent increase in research on the effects of dietary oils and fats on humans. Although there are many fats which are detrimental to health, there are those which are essential to well-being. Amongst these are the polyunsaturates $\Delta^{6,9,12}C_{18:3}$ γ-linolenic acid (γLA) (Graham, 1984 & Toplack, 1986), $\Delta^{9,12}C_{18:2}$ linoleic acid, $\Delta^{8,11,14}C_{20:3}$ dihomo-γ-linolenic acid (DHγLA), $\Delta^{5,8,11,14,17}C_{20:5}$ eicosapentaenoic acid (EPA) and $\Delta^{4,7,10,13,16,19}C_{22:6}$ docosahexaenoic acid (DHA) (Beare-Rogers, 1988). These fatty acids are usually extracted from higher plants and animals but also occur in microorganisms. Rapid advances in the field of biotechnology have led to great interest in microorganisms as alternative sources to high-priced unsaturates.

1.1 Lipid structure and nomenclature

The terms "fats", "oils" or "lipids" refer to the total material which can be extracted from a source with a solvent. The major constituent of this material is the triglyceride (a triester of glycerol with fatty acids). In this molecule the unsaturated fatty acid is usually found in position 2 of the triglyceride (s*n*-2) (Ratledge, 1982). Other minor constituents which are soluble in lipids include pigments (chlorophyll, carotenoids), sterols (phytosterol, cholesterol), phospholipids,

lipoproteins, glycolipids, hydrocarbons and vitamins (Applewhite, 1980).

Both trivial and systematic names of fatty acids (FAs) have been detailed by Weete (1974). The systematic naming of the FAs follows that used for organic compounds. The longest hydrocarbon chain is indicated by the appropriate Latin/Greek name to denote the number of carbon atoms and the suffix "oic" is used to represent the carboxyl function. For example, a fatty acid (FA) which contains 16 carbon atoms in a straight line is called palmitic acid and is systematically named hexadecanoic acid. This acid may be abbreviated by the symbols, C_{16} or $C_{16:0}$. The position of the atom along the hydrocarbon chain is indicated by the carbon number of its place of attachment beginning with the carboxyl carbon as number 1 and the subsequent carbon 2 or alpha.

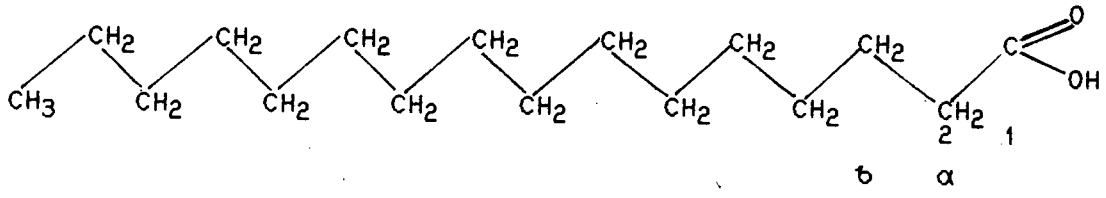

Fig.1.1 Palmitic acid ($C_{16:0}$)

Unsaturation may be designated by the number of the first carbon atom from the carboxyl end of the molecule which is involved in the double bond. For example, a C_{18} di-unsaturated FA with double bonds in the 9 and 12 positions may be designated by $\Delta^{9,12}$-octadecadienoic acid and abbreviated $\Delta^{9,12}C_{18:2}$. This FA is commonly known as linoleic acid or linoleate. The double bond position may also be defined with respect to the terminal methyl carbon atom (ω, omega). The FA mentioned above can therefore also be referred to as $\omega^{6,9}$-octadecadienoic acid. γ-Linolenic acid may be designated by $\Delta^{6,9,12}$ or

$\omega^{6,9,12}$-octadecatrienoic acid and abbreviated $\Delta^{6,9,12}C_{18:3}$.

Unsaturated FAs may occur in the *cis* or *trans* configuration. The *cis* or *trans* configuration may change the function of the FA. For example, linoleic acid, which is the main FA in the diet occurs naturally in the *cis* configuration. *Trans*-linoleic acid is not an essential FA and in fact acts as an anti-vitamin, increasing the requirements for the true essential FAs (Dry, 1985).

The distribution of the double bonds along the hydrocarbon chain may be of two types: "methylene interrupted" and "non-methylene interrupted". The methylene interrupted arrangement of the double bonds, which is the most common polyene structure of naturally occurring FAs, is a 1,4-diene non-conjugated double bond system:

$$R\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}R$$

Fig.1.2 Methylene interrupted arrangement of double bonds

The non-methylene interrupted double bond arrangement is illustrated by the conjugated double bond system or when two double bonds are separated by two or more methylene groups in the hydrocarbon chain:

$$R\text{-}CH_2\text{-}CH{=}CH\text{-}CH{=}CH\text{-}CH{=}CH\text{-}CH_2\text{-}R_1$$

Fig.1.3 Non-methylene interrupted arrangement of double bonds

Unsaturated fatty acids can also be grouped into families according to

the double bond positions relative to the terminal methyl group and the mode of biosynthesis. These families are designated ω-9, ω-6 and ω-3. γ-Linolenic acid belongs to the ω-6 family and α-linolenic acid (αLA) to the ω-3 family.

1.2 Clinical importance of lipids

The clinical significance of lipids is multifaceted and is summarised in order to substantiate the demand of certain FAs produced by microorganisms (γLA, DγLA, EPA and DHA). Lipids may serve as energy sources but also have specific roles as essential components of membrane FAs and stimulators of growth and reproduction (Weete, 1974).

Fatty acids as components of lipids are significant in the maintenance of health in animals and man: they give energy, they assist in the maintenance of body temperature, they insulate the nerves and they protect tissues (Graham, 1984). At the cellular level, FAs play a vital role in the structure of biological membranes and as immediate precursors for the formation of hormone-like prostaglandins, thromboxanes and leucotrienes and their many derivatives. These substances are collectively known as eicosanoids and are vital to human health. Eicosanoids are diverse and are short-lived molecules which regulate many body functions. There are three groups of eicosanoids.

Fatty acids which cannot be synthesised *de novo* in the body are essential fatty acids (EFAs) and include the unsaturated FAs from the ω-6 and ω-3 families, namely linoleic acid and αLA. During eicosanoid synthesis, linoleic and linolenic acids are sequentially

desaturated and elongated (Fig.1.4) (Davidson *et al.*, 1989). Eventually, the eicosanoid precursors, DHγLA, $\Delta^{15,8,11,14}C_{20:4}$ arachidonic acid (AA) and EPA are formed from linoleic acid and αLA respectively.

The FAs of the ω-6 and ω-3 families are not metabolically interconvertible but are believed to share the same desaturase enzymes and to compete for these (Horrobin, 1985). The ω-3 family has been shown to suppress the desaturation of the ω-6 family (Nassar *et al.*, 1986). For example αLA has the competitive advantage over linoleic acid for desaturation by Δ-6-desaturase (Beare-Rogers, 1988). Arachidonic acid is antagonistic to EPA (Yongmanitchai & Ward, 1989). Arachidonic acid and EPA compete for a cyclooxygenase which catalyses the initial step of conversion of these FAs to eicosanoids (Langholz *et al.*, 1989). Eicosapentaenoic acid (ω-3 family) suppresses the desaturation of DHγLA to AA (Nassar *et al.*, 1986). Rats feeding on different sources of γLA were shown by Jenkins *et al.* (1988) to produce differing FA metabolites and prostaglandins. The rats had ingested the same quantities of γLA from different oils of Evening Primrose, Borage, Black Currant and the fungus *Mortierella*. These results indicate that a novel source of γLA must not only be economic but must also perform the required metabolic role. The composition of an oil should be taken into account when formulations of γLA are prepared as the concentrations of other FAs in the oil can affect the metabolic role of γLA. For this reason subsequent chapters in this thesis include the %γLA in the analysed FAs as an important selection criterion.

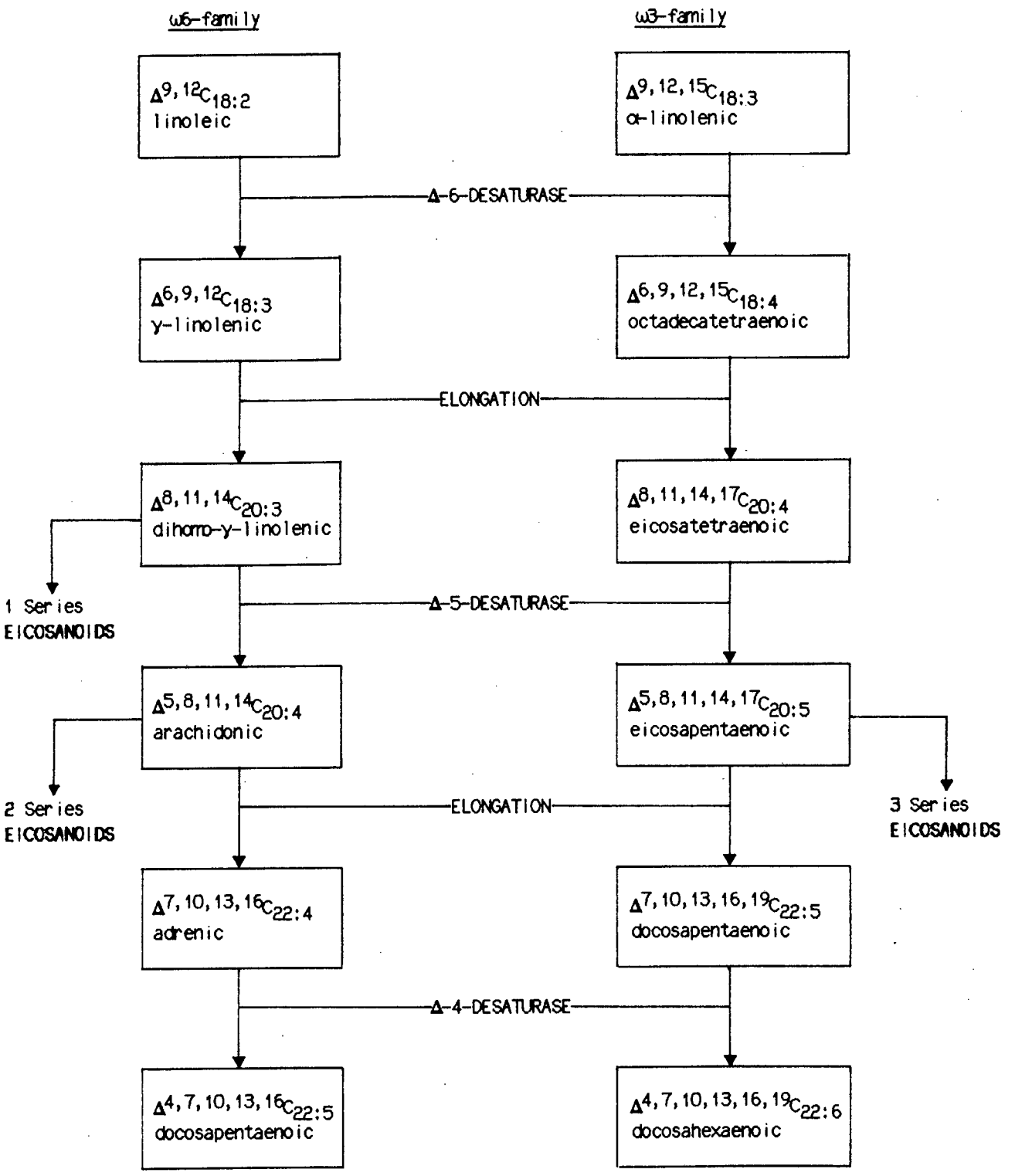

Fig.1.4 Metabolic pathways of ω-6 and ω-3 fatty acids showing the precursors of series 1, 2 and 3 eicosanoids (Davidson *et al.*, 1989).

There are factors other than αLA which influence the desaturation of linoleic acid to γLA by Δ-6-desaturase and thus assist or prevent the eventual production of the series 1 eicosanoids. *Trans* FAs (which form during the processing of vegetable oils), saturated fats, cholesterol, too little insulin, excessive alcohol, ageing, certain viruses, chemical carcinogens and ionizing radiation block the desaturation of linoleic acid whereas vitamin B_6, biotin, Zn and Mg are needed by Δ-6-desaturase (Graham, 1984). By taking γLA or DHγLA the step involving Δ-6-desaturase can be bypassed and the series 1 eicosanoids particularly prostaglandin E (PGE_1) can be successfully synthesised.

Prostaglandin PGE_1 which comes from the first eicosanoid group has the precursors, linoleic acid, γLA and DHγLA (Fig.1.4). Graham (1984) and Dry (1985) have listed some of the functions of PGE_1 as the:

- dilation of blood vessels
- lowering of arterial pressure
- increase in cardiac output
- inhibition of platelet aggregation
- inhibition of thrombosis
- inhibition of the synthesis of cholesterol
- inhibition of inflammation and types of arthritis
- healing of skin ulcers and varicose leg ulcers
- activation of defective T-lymphocytes
- inhibition of abnormal cell proliferation
- prevention of liver damage and withdrawal

symptoms in animals addicted to alcohol

- relief of angina
- dilation of the bronchia
- inhibition of gastric secretion
- stimulation of lipolysis

The absence or low concentrations of PGE_1 would certainly lead to a variety of conditions which can be altered by the intake of γLA or DHγLA. γ-Linolenic acid in particular has been shown to benefit diseases such as multiple sclerosis, cancer, benign breast disease, heart disease, vascular disorders, high blood pressure, eczema, cystic fibrosis, acne, asthma, rheumatoid arthritis (as well as other inflammatory diseases), schizophrenia, alcoholism and in conditions such as obesity, premenstrual syndrome, allergies and hyperactivity in children (Dry, 1985). In horses γLA and DHγLA have been used to treat anhydrosis (a condition lacking sweat production) (Corman & Mayhew, 1988).

The FA precursor of series 3 eicosanoids, EPA, has been reported to lower platelet aggregation and blood pressure (Beare-Rogers, 1988). Eicosapentaenoic acid was also shown to decrease the invasive and metastatic activity of cultured malignant tumour cells (Reich *et al.*, 1989). Pregnancy-induced hypertension was treated with EPA (Michael, 1988). Docosahexaenoic acid is found in the retina, cerebral cortex, spermatozoa, testes and in neural membranes of different animal species (Beare-Rogers, 1988). The physiological roles of DHA are unknown. It is known however that *in vivo* EPA and DHA are interconvertible and that intake of EPA and DHA through marine foods ensures the production of the

series 3 eicosanoids (Langholz *et al.*, 1989). It is interesting to note that in humans, mother's milk contains γLA, EPA and DHA. It is possible that these FAs can be involved in the development of brain tissue of the infant (Yongmanitchai & Ward, 1989).

1.3 The role of biotechnology in the oils and fats industry

1.3.1 Current sources of the eicosanoid precursors

The main sources of EPA and DHA are marine foods. The composition of each source varies and is affected by the species of fish, seasons and location of fishing sites. The fishing sites change according to the availability of certain types of microorganisms, the primary components of the food chain. For the above reasons and since the EPA and DHA quantities are not sufficient for global consumption, other non-conventional sources are required (Yongmanitchai & Ward, 1989).

γ-Linolenic acid is found in a number of plants such as the seeds of Black-currant, Red-currant, Gooseberry, Borage and Evening Primrose (Wolf *et al.*, 1983; Horrobin, 1985; Traitler *et al.*, 1986). The polyunsaturated FAs from the seed oils can be taken directly in the form of capsules or indirectly in produce of animals fed with these FAs. Examples of the latter include those patented by Horrobin (1985) and Maruta (1988). Horrobin (1985) fed chickens with feed containing 10% Evening Primrose oil. The γLA and cholesterol levels in the eggs were increased and decreased respectively. An increased egg size was also observed. By feeding chickens with heat-treated *Mortierella isabellina*, Maruta (1988) increased the number of eggs produced by the

chickens and the concentration of γLA in the eggs. It can be summarised that animal feed containing 0,5-5,0% ω-6 FAs not only improves animal health (prevents liver disorders, increases body mass, decreases mortality) but also improves human health by increasing the content of ω-6 FAs in egg and meat produce which are consumed by humans (Maruta, 1988).

Although oil seeds are considered natural sources of oil the climate and other agricultural aspects may influence the productivity of the oil. There is ongoing research in the development of an Evening Primrose hybrid which fits agricultural restraints, but this takes time. The plant breeder has produced reliable hybrids which yield the largest quantity of oil containing high concentrations of γLA (Graham, 1984). Matsumura *et al.* (1987) cultured the seeds of the Evening Primrose (*Oenothera biennis*) under the more controlled conditions of tissue culture and produced calluses which contained the γLA ester. The various research efforts into better γLA yields from plants and in particular *O. biennis*, have not produced a process which on economic grounds can be compared to the microbial production of γLA. The cost of a good crop of Evening Primrose or Borage oil on 30 acres in one year, can be equalled by a single 220kℓ fermenter producing oil in 4 days (4d) (Ratledge, 1987b). This output does not include the loss of oil or costs at the extraction stage. Even so, for a specialised oil such as γLA, the lower costs of the microbiological route are an added advantage.

The restraints in obtaining EPA, DHA and γLA from conventional sources together with the medical emphasis on the importance of

high-priced oils has refocused work on the production of these oils by fermentation. The increased patent application in the past few years indicates the extent of developments in industrial oil production (Suzuki & Yokochi, 1984; Agency of Industrial Sciences and Technology, 1985; Herbert & Keith, 1985; Nisshin, 1985; Suzuki & Yokochi, 1985a; Suzuki & Yokochi, 1985b; Seto, 1986; Ando *et al.*, 1987; Minoshima *et al.*, 1987; Maeda *et al.* 1988; Suzuki & Yokochi, 1988; Yazawa *et al.*, 1988b; and Yazawa *et al.*, 1989).

The reported research concerning the use of microorganisms in the fat and oil industry involves three areas. Firstly, microorganisms are potential sources of fats and oils. Secondly, microorganisms provide systems which carry out various biotransformations (Koritala *et al.*, 1987). The reactions can use the whole cell or enzymes. Thirdly microorganisms provide useful models for studying the details of biochemistry, metabolic control and function of lipids (Ratledge, 1987a). Results from these microbial studies have given invaluable leads to plant and animal lipid biochemists.

1.3.2 Microorganisms as potential sources of high priced oils

Microorganisms were first used for commercial fat production in Germany during the wars of 1914-18 and 1939-45 (Ratledge, 1984). Although this was unsuccessful, it emphasised the potential of oil production by microbes. Further developments have been reviewed by Woodbine (1959) and Ratledge (1984). Yongmanitchai & Ward (1989) have reviewed recent developments in the use of microorganisms for EPA and DHA sources. In these reviews most of the work has been carried out on yeast and moulds

in particular, as the majority of oleaginous microbes are found in the eukaryotes.

The FA compositions and profiles of prokaryotes and eukaryotes differ. In the prokaryotes only blue-green algae contain some polyunsaturated FAs. The number of carbons in the chain and the number of unsaturated bonds are usually limited to 18 and 3 respectively (Yongmanitchai & Ward, 1989). Recently, certain bacteria have been found to contain a substantial amount of EPA (Yazawa *et al.*, 1988a; Yazawa *et al.*, 1988b; Yazawa *et al.*, 1989). Among eukaryotic microorganisms, polyunsaturated FAs vary widely with respect to the chain length, the number of unsaturated bonds and the position of these on the chain. All the FAs in this group are of the *cis* configuration and come from the ω-6 and ω-3 families. Recently the fungi and algae have been extensively researched for their potential as sources of polyunsaturated FAs.

Amongst the prokaryotes few species were known to produce large amounts of extractable oils. Bacteria have only been known to synthesise monounsaturated and not polyunsaturated FAs. Recently, however, after 5 000 marine microorganisms were screened by Yazawa *et al.* (1988a), a particular Gram negative, obligate aerobe was found which produced 26mg/ℓ of EPA when cultured at 4°C for 5d. The EPA was 40% of the total FAs. Yazawa *et al.* (1988b) patented *Pseudomonas*, *Alteromonas* and *Shewanella* strains of which *P. putrefaciens* when cultured at 25°C for 24hours (24h), produced an EPA yield of 0,69g/100g dry matter (DM). Yazawa *et al.* (1989) further patented a *Pasteurella haemolytica* strain which when cultured at 25°C for 24 hours (24h) produced an EPA yield of 0,86g/100g DM. The mycobacteria, corynebacteria and nocardia are known

for their high lipid content but this is complex and is related to toxic and allergic factors (Ratledge, 1984). *Arthrobacter* has been reported to contain up to 80% of its biomass as lipid which is 90% triglycerides, 50% of which are unsaturated (Wayman *et al.*, 1984).

Other prokaryotes, the blue-green algae, resemble the bacteria in morphology but are similar to eukaryotic algae in physiology. Their FA contents and profiles are intermediate between bacteria and eukaryotic algae (Yongmanitchai & Ward, 1989). No report was found on the use of blue-green algae for the production of eicosanoid precursors.

Amongst the unicellular eukaryotes the marine phytoplankton have been shown to be promising sources of EPA and DHA (Yongmanitchai & Ward, 1989). The protozoan *Tetrahymena rostata*, produced γLA in a fed-batch fermentation. Gosselin *et al.* (1989) reported improved γLA (g/ℓ) yields under these conditions despite the sensitivity of the protozoan to shearing effects.

In yeast genera such as *Candida*, *Cryptococcus*, *Hansenula*, *Lipomyces* and *Rhodotorula* 70% of the dry matter (DM) contains FAs. The FAs of yeast are usually unsaturated and monounsaturated with carbon chain lengths of C_{16} to C_{18}. There are, however, chain lengths of C_{20} carbons or more. The polyunsaturates linoleic and αLA can also be found in yeast. Yeast are usually only used as tools for the understanding of the biochemistry and kinetics of lipid accumulation (Boulton & Ratledge, 1981; Yoon & Rhee, 1983).

The number of oleaginous moulds is greater than the number of oleaginous

yeasts. The FAs of moulds are mainly C_{16} to C_{18} with the predominant FAs (in order of their abundance) being oleic ($C_{18:1}$), palmitic ($C_{16:0}$), linoleic ($C_{18:2}$), stearic ($C_{18:0}$) and palmitoleic ($C_{16:1}$) acids. Some genera (for example in the Phycomycete family of moulds) will produce other FAs like γLA in relatively large proportions. The production of unusual FAs in the yeasts and moulds is rare.

Fungi can produce oils similar to seed oils. For example, the Agency of Industrial Sciences and Technology (1985) have patented a *Mortierella* species which produced cocoa fat-like lipids. The chance of microbial oils replacing seed oils is remote because the high technology involved in large scale fermentations is more costly than the low technology in the agriculture of low-priced oils (Ratledge & Boulton, 1985). There is however potential in the production of high value FAs (γLA, EPA, DHγLA) or FAs from wastes.

Lower fungi, the Phycomycetes in particular, have promising quantities and types of polyunsaturated FAs. Shimizu *et al.* (1988b) reported several *Mortierella* strains which produced large amounts of EPA in their mycelia when grown at 12°C (2,7g/100g DM). The production of this FA did not occur at higher temperatures. The fungus *Mortierella alpina* when cultured at low temperatures and fed with glucose, produced an EPA yield of 0,49g/ℓ and 2,9g/100g DM (Shimizu *et al.*, 1988a). A portion of the total FAs was 3,0% linoleic, 3,5% γLA, 60% AA and 13,5% EPA. Another *M. alpina* strain produced DHγLA in quantities of 10,7g/100g DM with a yield of 2,17g/ℓ (Shimizu *et al.*, 1989). The total FAs consisted of 6,6% linoleic, 4,1% γLA and 23,1% DHγLA. Sesame oil in the medium increased the concentrations of DHγLA by

repressing the conversion of DHγLA to AA. A marine fungus, *Thraustochytrium aureum* contained a high percentage of DHA (Yongmanitchai & Ward, 1989). Other strains such as *Entomophthora obscuras*, *Phytophthora infestans* and *Pythium* species are also reported to accumulate DHA. The production of γLA by various fungal strains is detailed in Chapter 2.

Other eukaryotes such as algae have lipids containing γLA, DHγLA, AA and EPA (Takagi *et al.*, 1985; Rezanka *et al.*, 1987). Hiroaki & Shinichio (1986) discuss the culturing of algae such as *Monodus subtarraneus* for the production of EPA. Ando *et al.* (1987) have patented the culturing of *Nannochloropsis* for the manufacture of a health food containing EPA. Roughan (1989) extracted oil from various commercial sources of *Spirulina* and reported γLA contents from 0,34g/100g DM to 0,6g/100g DM. Roughan (1989) states that *Spirulina* was 4 times as expensive as Evening Primrose oil as a source of γLA. In Canada, Parrish & Wangersky (1986), reported the culture of the marine diatom *Phaeodactylum tricornutum* in a continuous automated turbidostat and studied parameters affecting lipid accumulation.

1.3.3 Microbial systems involved in lipid biotransformations

Once extracted, an oil may need purification so as to diminish the influences of FAs other than the beneficial FA(s). In this way FAs such as γLA, DHγLA, EPA or DHA are concentrated. Oleaginous microorganisms contain lipase enzymes which can play a functional role in these purification/concentration procedures. Lipases catalyse the esterification, hydrolysis or exchange of FAs in esters depending on the conditions favouring the specific reaction. Lipases from different microorganisms are specific for different FAs and for the FA position on the triglyceride. If a lipase specific for a 1,3 bond is used a special FA can be removed or incorporated into the outer portion of the triglyceride without affecting the FA in the centre position. Specific lipases can therefore form triglycerides which are unobtainable by simple chemical interesterification methods (Langholz *et al.*, 1989).

The use of lipases to concentrate polyunsaturates has been recently patented in Japan. Maeda *et al.* (1988), hydrolysed triglycerides in Borage seed oil at specific positions on the triglyceride. The lipase enzyme from *Candida cylindricae* hydrolysed the glycerol-γLA ester bond less easily than in other FAs. After 18h at 30°C, the free FAs were removed and the percentage γLA in the triglycerides was increased from 20,2 to 47,4%. A lipase from an *Alcaligenes* species was used to specifically hydrolyse oil and to yield the EPA glyceride. About 38% of the product which was recovered, contained 24% EPA (Yongmanitchai & Ward, 1989).

Other conventional techniques which have been proposed to concentrate

polyunsaturated FAs include: saponification, solvent extraction, urea inclusion, molecular distillation, fractionation distillation, liquid chromatography and supercritical fluid carbon dioxide extraction (Yongmanitchai & Ward, 1989).

1.3.4 The biochemistry of microbial lipid accumulation

The types of FAs which have been detected in organisms are many. This suggests that different lipid properties are required by each organism for survival. The differences are mainly in chain length, saturation and substitution. As stated previously bacterial systems have FA chain lengths from C_{10} to C_{20}, with a predominance of those with 15 to 19 carbons. In fungi there is an abundance of C_{16} and C_{18} FAs. The same FAs are found in animals and plants as well as FAs with chain lengths of C_{20} and higher. In higher eukaryotes the FA composition is fairly constant. Microorganisms alternatively have a wide variation in FA types and in fact can be taxonomically related according to their FA profiles (Jabaji-Hare, 1988).

Lipid synthesis is better understood in bacteria, plants and animals and it is generally assumed that fungi have similar pathways to these organisms. The few aspects of lipid biosynthesis which have been investigated in fungi have been mostly in yeasts.

The pathway for the conversion of a carbohydrate to lipid in oleaginous microorganisms is illustrated by Ratledge (1986) and is shown in Fig.1.5. There is no means of directly converting pyruvate to acetyl-CoA in the cytoplasm and the acetyl-CoA formed in the

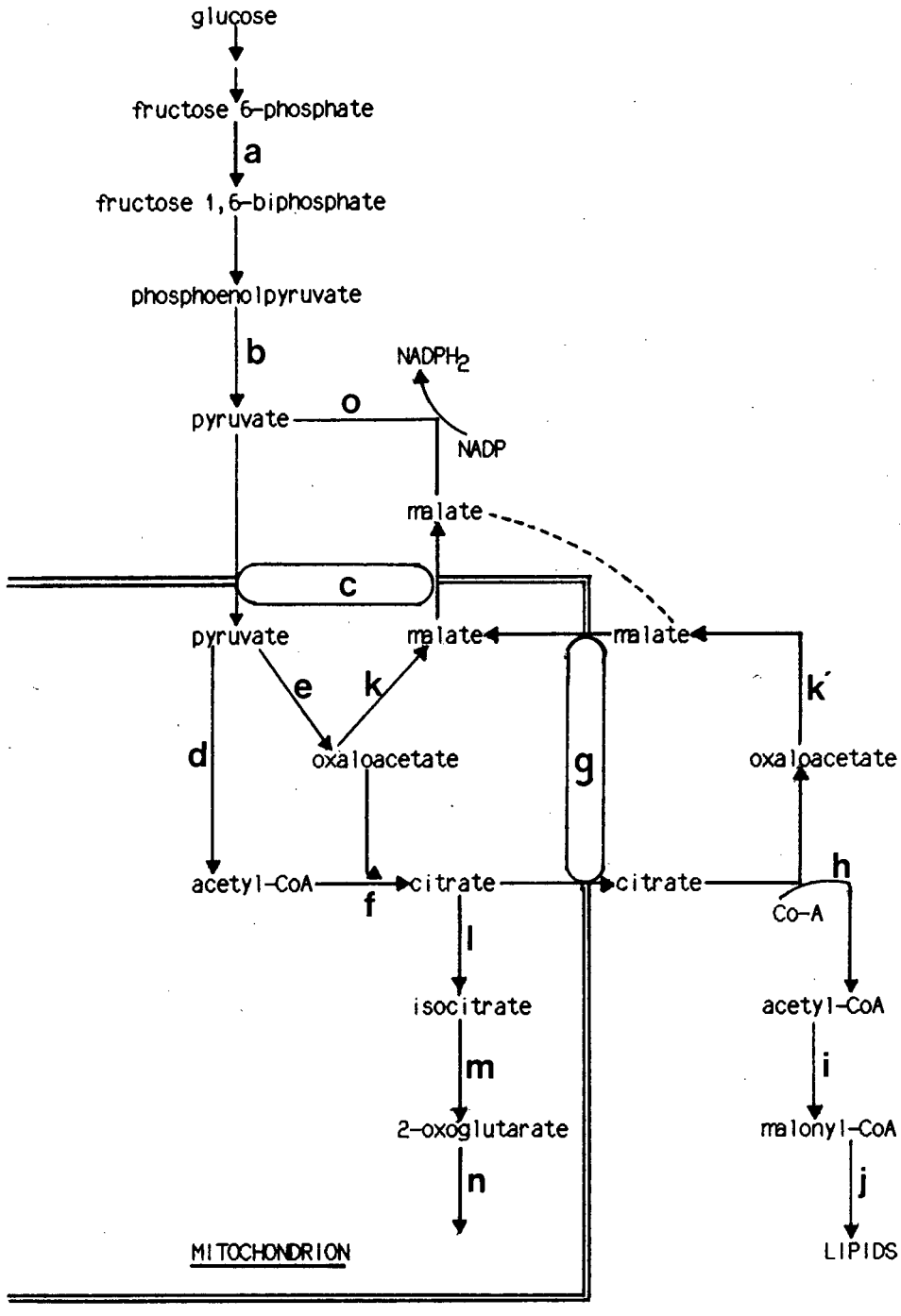

Fig.1.5 Diagrammatic representation of the pathway of conversion of carbohydrate to lipid in oleaginous microorganisms (Ratledge, 1986).

Enzymes: a-phosphofructokinase, b-pyruvate kinase, c-pyruvate-malate inter-linked translocase system, d-pyruvate dehydrogenase, e-pyruvate carboxylase, f-citrate synthase, g-citrate-malate translocase, h-ATP:citrate lyase, i-acetyl-CoA carboxylase, j-fatty acid synthetase complex, k,k'-malate dehydrogenases, l-aconitase, m-isocitrate dehydrogenase, n-2-oxoglutarate dehydrogenase, and remaining reactions of the tricarboxylic acid cycle, o-malic enzyme

mitochondrion can therefore not be transported across the mitochondrial membrane. Only acetate or acetyl-carnitine can be transported. For this reason the following sequences take place to ensure the presence of the substrate of FA synthesis (acetyl-CoA) in the cytoplasm (Fig.1.5):

1. When nitrogen is depleted from the medium, the concentration of AMP falls. The isocitrate dehydrogenase (m) within the mitochondrion, which is dependent on AMP for activity, ceases its catalytic activity. As a result of this, isocitrate is not metabolised and both isocitrate and citrate then accumulate.
2. Citrate is then transported out of the mitochondrion into the cytoplasm where it is cleaved by ATP:citrate lyase (h) to give acetyl-CoA for biosynthesis and oxaloacetate.
3. The transport of citrate out of the mitochondrion is linked to that of malate which is formed by the conversion of oxaloacetate.

An oleaginous microorganism has been defined as one which contains 20-25% of its dry matter as fat (Ratledge & Boulton, 1985). Biochemically, oleaginous yeasts (and probably moulds and eukaryotic algae) but not bacteria, can be defined by the possession of ATP:citrate lyase in their cytoplasms which catalyses the following reaction (Boulton & Ratledge, 1981):

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

In eukaryotes, acetyl-CoA cannot be produced in the cytoplasm from pyruvate as this reaction occurs in the mitochondria. Oleaginous yeasts and probably other eukaryotes accumulate citrate in the mitochondria

which is then transported into the cytoplasm where it is cleaved by ATP:citrate lyase. Non-oleaginous yeasts do not possess the citrate-cleaving enzyme and produce acetyl-CoA in the cytoplasm by other less efficient means. In prokaryotes there is no compartmentalisation of the mitochondrion and there is therefore no separation in the production of acetyl-CoA and the utilisation of acetyl-CoA for fat anabolism. For this reason the presence or absence of ATP:citrate lyase in prokaryotes has no biochemical significance as an indicator of oleaginicity.

1.3.4.1 Biosynthesis of saturated fatty acids

The synthesis of saturated FAs is catalysed by the action of two enzyme systems, acetyl-CoA carboxylase and FA synthetase (denoted (i) and (j) respectively in Fig.1.5). The properties and control of these two enzyme systems are detailed by Weete (1974), Chopra & Khuller (1984) and Dry (1985). The following information, unless otherwise quoted, has been obtained from the most recent review on lipid biosynthesis (Boulton & Ratledge, 1985).

Acetyl-CoA carboxylase (catalyses the committing step of FA synthesis)
Acetyl-CoA carboxylase is considered to catalyse the first step in the synthesis of FAs from acetyl-CoA. This enzyme complex has three functional components which have been identified, namely, biotin carboxyl-carrier protein (BCCP), biotin carboxylase and carboxyl transferase. The enzyme catalyses the carboxylation of acetyl-CoA, to give malonyl-CoA in two stages:

$$\text{biotin-BCCP} + HCO_3^- + H^+ + \text{ATP} \xrightarrow{\text{biotin carboxylase}} \text{carboxybiotin-BCCP} + \text{ADP} + P_i$$

$$\text{carboxybiotin-BCCP} + \text{acetyl-CoA} \xrightarrow{\text{carboxyl transferase}} \text{biotin-BCCP} + \text{malonyl-CoA}$$

The reaction requires biotin and ATP. The control of acetyl-CoA carboxylase consists of activation by tricarboxylic acid cycle intermediates (*e.g.* citrate), inhibition by long chain fatty acetyl-CoA esters and reversible phosphorylation. Acetyl-CoA carboxylase of *Candida lipolytica* is stabilised by glycerol and instead of being activated by citrate (as in other yeasts) is stimulated by polyethylene glycol (Chopra & Khuller, 1984). In bacteria, cellular variations of guanosine 3',3',5',5'-tetraphosphate may also regulate acetyl-CoA carboxylase.

Fatty acid synthetase (catalyses the elongation cycle in FA synthesis) Malonyl-CoA, derived from the acetyl-CoA carboxylase reaction, is used to further synthesise long chain FAs. This synthesis is catalysed by the fatty acid synthetase complex which involves a series of reactions requiring NADPH and acetyl-CoA. The series of reactions involved are similar in all organisms and consist of acetyl transacylation, malonyl transacylation, b-ketoacyl synthase condensation, b-ketoacyl reduction, hydroxyacyl dehydration and 2,3-*trans*-enoylacyl reduction. The net result of the above set of reactions is the consumption of 2NADPH and the introduction of two additional carbons to an initial acetyl group. The additional carbon atoms are also derived from acetyl-CoA, (the substrate of acetyl-CoA carboxylase). The above set of reactions are repeated until palmitate is formed.

The stoichiometry of the synthesis of palmitate ($C_{16:0}$) is:

$$\text{acetyl-CoA} + 7\ \text{malonyl-Co-A} + 14\ \text{NADPH} + 7\ H^+ \xrightarrow{\text{fatty acid synthetase}} \text{palmitate} + 7\ CO_2 + 14\ NADP^+ + 8\ \text{CoA} + 6\ H_2O \quad \langle 1 \rangle$$

The stoichiometry of the synthesis of malonyl-CoA to be used in <1> is:

$$7\ \text{acetyl-CoA} + 7\ CO_2 + 7\ \text{ATP} \xrightarrow{\text{acetyl-CoA carboxylase}} 7\ \text{malonyl-CoA}\ 7\ \text{ADP} + 7\ P_i + 7\ H^+ \quad \langle 2 \rangle$$

Hence the overall synthesis of palmitate <1> + <2> requires:

$$8\ \text{acetyl-CoA} + 7\ \text{ATP} + 14\ \text{NADPH} \longrightarrow \text{palmitate} + 14\ NADP^+ + 8\ \text{CoA} + 6\ H_2O + 7\ \text{ADP} + 7\ P_i$$

1.3.4.2 Biosynthesis of unsaturated fatty acids

Monounsaturated FAs are synthesised by either aerobic or anaerobic pathways. The aerobic pathway is the most common, and is found in yeasts, protozoa, algae, some bacteria and mammals. The double bond is introduced into the 9 position of the FA forming $\Delta^{9}C_{16:1}$ palmitoleic and $\Delta^{9}C_{18:1}$ oleic acids. The double bond is introduced into the saturated FA by a specific monooxygenase in which NADPH is the co-reductant. The reaction involves an electron transport chain usually consisting of flavoprotein or iron-sulphur protein components. Most bacteria introduce double bonds into FAs anaerobically (Yongmanitchai & Ward, 1989).

Polyunsaturated FAs are synthesised from saturated or monounsaturated precursors. The pathways involve both chain elongation and desaturation. Usually the double bonds are separated by methylene groups (*i.e.* -CH=CH-CH_2-CH=CH-) and new bonds are usually introduced between existing double bonds and the terminal methyl group of the FA. Some algae and Phycomycetes can also desaturate towards the terminal carboxyl group. The position of the first double bond from the terminal methyl group categorises the FAs into three categories namely: ω-9, ω-6 and ω-3. The ω-3 type of desaturation produces linoleic acid and αLA from oleic acid (Fig.1.6). α-Linolenic acid may accumulate in some organisms or it may undergo carboxyl desaturation and elongation to produce EPA and DHA (Fig.1.6). Wassef (1978) illustrated the formation of EPA and DHA via $\Delta^{9,12,15}C_{20:3}$ eicosatrienoic acid and not $\Delta^{6,9,12,15}C_{18:4}$ octatetraenoic acid as indicated for mammals by Davidson *et al.* (1989) and in Fig.1.4.

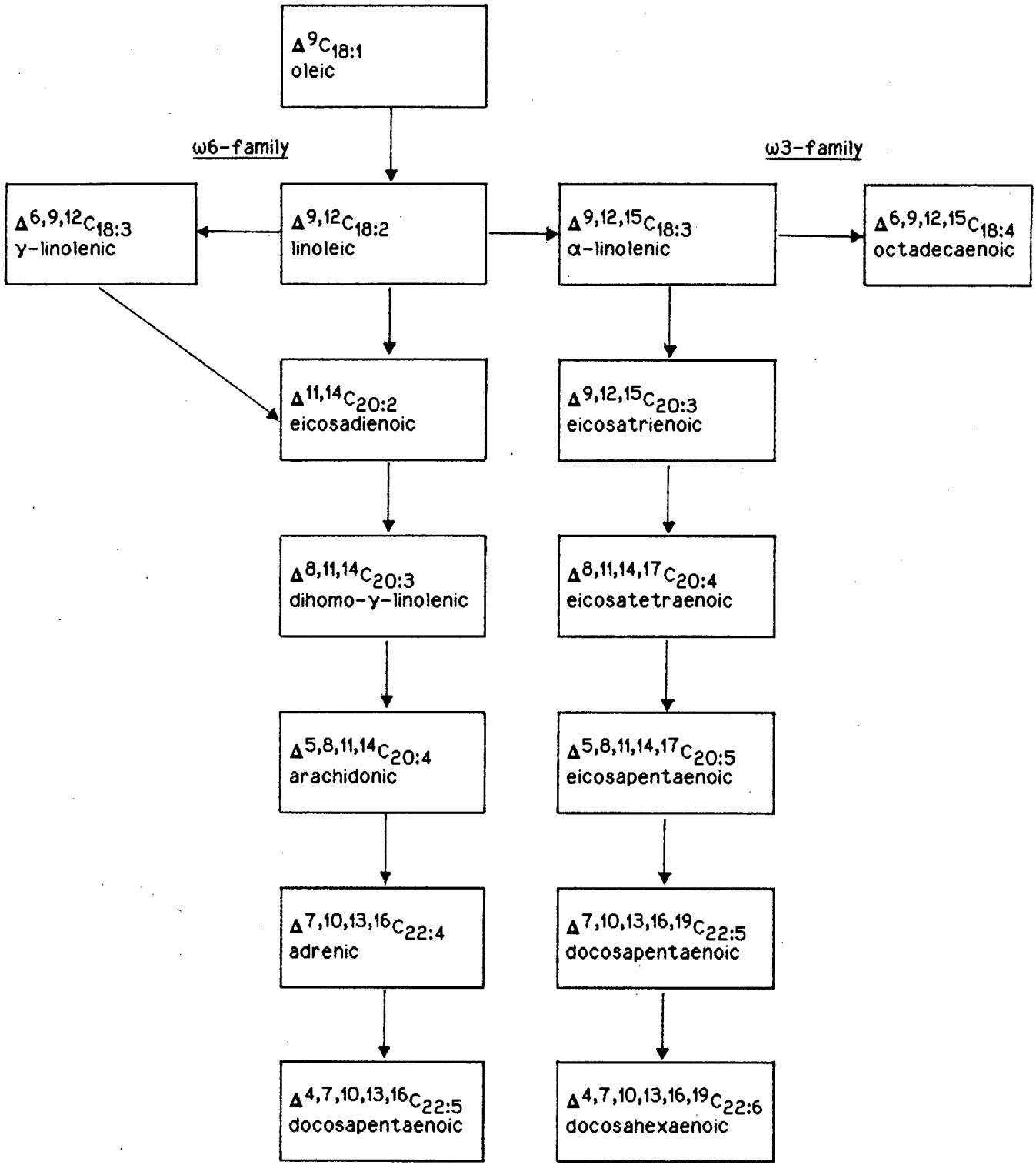

Fig.1.6 Metabolic pathways of ω-6 and ω-3 fatty acids in fungi (adapted from Wassef (1978)).

Arachidonic acid may be produced in fungi by one of two routes: desaturation of linoleic to γLA followed by chain elongation; or alternatively, chain elongation of linoleic acid to eicosadienoic acid followed by desaturation towards the carboxy terminal (Fig.1.6). The first pathway is predominant in animals and certain algae. The second route was found in the slime mould *Physarum polycephalum* and some protozoa (Wassef, 1978).

The ω9-family of polyunsaturated FAs are produced by vertebrates. Most organisms specialised in desaturating the FA from the carboxy terminal can produce linoleic acid from oleic acid. This ability is however lacking in invertebrates and therefore linoleic acid becomes an essential fatty acid.

1.4 The industrialisation of fungi

Fungi have been used on a commercial scale for many years: *Agaricus bisporus* is cultivated on a wide scale as a source of food, fungi are used as flavour enhancers in cheese, they digest protein in soyabean and produce penicillin and citric acid. Fungi therefore show applicability under industrial conditions.

The advantages of culturing fungi are the following:

1. Capable of breaking down many complex substrates
2. Few nutritional requirements
3. Tolerance to low pH values, which helps in resisting contamination
4. Ease of recovery of biomass by filtration

5. Ease of handling and drying of the biomass

Some disadvantages include:

1. Growth at lower temperatures which increases the need for cooling which affects the process economics
2. Fermentation broths are rheologically complex and difficult to aerate, especially when growth is mycelial
3. Production of undesirable metabolites (oxalic acid, mycotoxins)
4. Genetic instability

Common to all industrialised processes is the need for the highest productivity, lowest capital and running costs, high yield factor on the limiting substrate, near complete utilisation of the substrate, ease of recovery and final formulation of the material into the final product. Against this background this thesis reports the development of γLA production by a *Choanephora cucurbitarum* strain, from the selection of strain and cultural conditions, to scale-up of the optimised fermentation.

CHAPTER 2

SELECTION OF THE FUNGAL STRAIN

CHAPTER 2

SELECTION OF THE FUNGAL STRAIN

2.1 Summary

The main objective of this work was to identify a fungal strain capable of yielding a high level of γLA in industrial fermentation. Thirty-nine fungal strains were screened under shake flask conditions. The major criteria used in evaluating the strains were, the yield of γLA per unit volume (g/ℓ) and γLA as a percentage of fatty acids which is important in the downstream processing of γLA. Other parameters of industrial importance such as strain handling and the fatty acid profile were also considered. Using these criteria, the following fungi were selected for further optimisation studies: *Choanephora cucurbitarum* ATCC 12997 and its mutants, *C. cucurbitarum* NRRL 2744, *C. cucurbitarum* ATCC 46105, *C. cucurbitarum* ATCC 46106, *Rhizopus arrhizus* NRRL 1526, *R. arrhizus* NRRL 6431, *R. arrhizus* CMI 57412, *Cunninghamella blakesleeana* NRRL 1369 and *Mortierella vinacea* ATCC 20034. From these strains *C. cucurbitarum* ATCC 12997 was found to give superior γLA yields. *C. cucurbitarum* ATCC 12997 produced γLA yields of 331mg/ℓ and 674mg/ℓ in shake flask and laboratory fermenters respectively. This strain had other industrially beneficial qualities such as good sporulation, a good biomass of 22,5g/ℓ and a relatively high yield of γLA of 2,99g/100g dry matter.

Subsequently a *Zygorhynchus heterogamus* strain was discovered which gave similar yields of γLA to *C. cucurbitarum* 12997. *Z. heterogamus* NCF

47807 also had a high γLA:linoleic acid ratio which aids the purification of γLA. This is the first known report of a high level of γLA in the genus *Zygorhynchus*. The industrial development of γLA production by *Zygorhynchus* is not reported.

2.2 Introduction

Certain oleaginous fungi are capable of accumulating large quantities of oils. The commercial exploitation of this ability has been extensively reviewed by Woodbine (1959), Shaw (1966b), Weete (1974), Ratledge (1978) and Ratledge (1982). These reviews however report few studies on the production of γLA by industrial fermentation. The production of γLA by fermentation in up to 30ℓ has been reported by Herbert & Keith (1985), Nisshin (1985), Suzuki & Yokochi (1985b), Seto (1986), Fukuda & Morikawa (1987), Minoshima *et al.* (1987) and Suzuki & Yokochi (1988). Efamol Ltd. have screened over 12 000 varieties of fungi for the production of γLA, and have isolated 20 to 30 γLA-overproducers (Horrobin, 1987).

A common feature of the Phycomycetes (or lower fungi) is their ability to accumulate large amounts of lipid often seen as microscopic globules (Bergman *et al.*, 1969). There have been a few reports on the γLA-producing fungi from the genera *Pythium* and *Saprolegnia*, members of the Oomycete class (Shaw, 1965). However accumulation of γLA occurred more frequently in the Zygomycete class, within the orders of Enteromophthorales and Mucorales in particular. In the Enteromophthorales, the *Conidiobolus* and *Enteromophthora* genera produced between 15 and 20% (m/m) lipid of which only 1% was γLA (Latgé *et al.*, 1980). The main oleaginous organisms are from the Mucorales and come from the genera, *Cunninghamella*, *Mortierella*, *Mucor*, *Phycomyces*, *Choanephora*, *Thamnidia* and *Rhizopus*. The Mucorales produce lipid yields of approximately 25g/100g DM - 56g/100g DM and are the only fungi where

γLA occurs as the sole $C_{18:3}$ isomer. In other orders αLA may also occur simultaneously with, but not instead of the γ-isomer.

Strains in the Mucorales with more than 25% lipid in their biomass and/or more than 10% γLA in their lipids were selected (Tables 2.1 and 2.2). γ-Linolenic acid production by some strains involved growth under conditions which would not be considered suitable for an industrial fermentation. This was due to the fact that under stationary phase conditions mycelial mats were formed. As a result of gas/nutrient-limited transfer, stationary phase cultures could degenerate or autolyse faster than dispersed liquid cultures. Therefore, the values reported in the literature may frequently be unrepresentative for efficiency estimates of substrate to lipid conversion.

There was great variation in the quantity of γLA produced by the same strains of fungi. Reported γLA/lipid (m/m) percentage values of *C. cucurbitarum* for example were 39,41% (Kunihisa & Shimizu, 1969), 14,9% (Shaw, 1965) and 10,8% (Weete 1974), (Table 2.1). Factors that may account for this variation include the effect of culture conditions, the stage of fungal growth and the method of analysis and calculation of the γLA. From the literature it was sometimes not clear whether the %γLA value was a percentage of the lipid/oil mixture which contained many unknown FAs or a percentage of a number of FAs detected by the analysis (analysed FAs or AFAs). In the literature the γLA yields were given as mg/100g DM. This value unlike a percentage is absolute. The γLA will be expressed in this thesis as mg/ℓ medium, since it combines the biomass yield together with that of γLA produced per

Table 2.1 Production of γLA by a variety of fungal strains

Strain	Culture conditions	Biomass DM g/ℓ	FA, Lipid yield g/ℓ	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Choanephora circinans IFO 5991	5d	12,8	2,3	11,7	269	2 186	molasses/Cl medium	Kunihisa & Shimizu (1969)
Choanephora conjuncta IFO 8089	5d	5,6	1,0	34,1	338	2 186	molasses/Cl medium	Kunihisa & Shimizu (1969)
Choanephora cucurbitarum CMI 89917			2,6	14,9	362	-	γLA stable (not utilised with time)	Shaw (1965); Shaw (1966a)
Choanephora cucurbitarum				10,8				Weete (1974)
Choanephora cucurbitarum IFO 5877	5d	10,1	1,5	39,4	595	4 959	molasses/Cl medium	Kunihisa & Shimizu (1969)
Choanephora trisporum IFO 5990	5d	10,2	1,8	17,5	321	2 590	molasses/Cl medium	Kunihisa & Shimizu (1969)
Choanephora trisporum IFO 5989	5d	7,0	1,5	20,3	298	2 484	molasses/Cl medium	Kunihisa & Shimizu (1969)
Basidiobolus anarum				23,0				Weete (1974)
Basidiobolus haptosporus				22,3				Weete (1974)

Strain	Culture conditions	Biomass DM g/ℓ	FA, Lipid yield g/100gDM	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Basidiobolus meristosporus				23,2				Weete (1974)
Conidiobolus coronatus IMI 145949			52,2	2,1				Herbert & Keith (1985)
Cunninghamella blakesleeana CMI 63877	25°C 7d	4,4	3,1	17,7	24	143	stationary culture	Shaw (1966c)
	30°C 7d	9,2	4,1	13,9	52	310		
	37°C 7d	29,0	5,0	8,5	123	732		
Cunninghamella echinulata IMI 45772			28,4	15,4				Herbert & Keith (1985)
Cunninghamella elegans IMI 21199			32,2	13,3				Herbert & Keith (1985)
Cunninghamella elegans NRRL 1378	27°C 5d	2,5	16,0	24,5	98	817	peptone medium	Nisshin (1985)
Cunninghamella elegans NRRL 1378	30°C 6d	167,0	40,0	18,0			DL-methionine Ca-pantothenate	Minoshima *et al.* (1987)
Mucor ambiguus IFO 6742						17 100	immobilised system	Fukuda & Morikawa (1987)

Strain	Culture conditions	Biomass DM g/ℓ	FA, Lipid yield g/100gDM	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Mucor circinelloides HUT 1121	30°C 3d				2 074	28 800	glucose 10%m/v	Yahiro *et al.* (1988)
Mucor circinelloides IMI 55452			14,3	12,9				Herbert & Keith (1985)
Mucor genevensis NRRL 1407	30°C 30h	0,7	8,9	32,2	7	569		Gordon *et al.* (1971)
	40h	2,4	5,8	16,8	23	583		
Mucor hiemalis(+) IMI 21216	25°C 7d	1,8	18,0	18,2	57	315		Sumner *et al.* (1969)
	25°C 14d	2,7	18,1	14,8	72	214		
Mucor hiemalis(-) IMI 21217	25°C 7d	1,8	19,3	18,9	65	384		Sumner *et al.* (1969)
	25°C 14d	2,8	17,2	19,2	91	270		
Mucor hiemalis IMI 103746			28,9	6,7				Herbert & Keith (1985)
Mucor inaequisporus CBS 255.36	15°C 7d			22,3				Dexter & Cooke (1984)
Mucor javanicus CMI 25330				13,7				Shaw (1965)
Mucor miehei IMI 125824			39,2	4,0				Herbert & Keith (1985)

Strain	Culture conditions	Biomass DM g/ℓ	FA,Lipid yield g/100gDM	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Mucor mucedo IMI 26441			23,3	12,2				Herbert & Keith (1985)
Mucor mucedo	25°C 4d	0,7	12,8	22,3	36	213		Sumner
IMI 103731	25°C 7d	1,3	15,2	20,2	103	308		*et al.* (1969)
Mucor piriformis CBS 527.68	15°C 7d			26,9				Dexter & Cooke (1984)
Mucor plumbuns IMI 14781			30,3	17,1				Herbert & Keith (1985)
Mucor pusillus	25°C		26,2	6,3			thermophile	Sumner
	48°C		23,1	3,6				*et al.* (1969)
Mucor racemosus	25°C 7d	0,9	19,0	18,8	31	-		Sumner
IMI 103730	25°C 14d	1,5	9,8	19,4	29	87		*et al.* (1969)
Mucor ramannianus	25°C 7d	1,3	16,6	29,1	63	348		Sumner
IMI 35044a	25°C 14d	3,5	15,2	30,9	165	984		*et al.* (1969)
Mucor recurvus CBS 195.71	15°C 7d			20,7				Dexter & Cooke (1984)
Mucor rufescens CBS 572.70	15°C 7d			28,1				Dexter & Cooke (1984)
Mucor strictus CBS 576.66	15°C 7d			26,0				Dexter & Cooke (1984)

Strain	Culture conditions	Biomass DM g/ℓ	FA,Lipid yield g/100gDM	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Mortierella isabellina IFO 7884	30°C 7d	156	83,1	4,1	3 400	20 238	glucose 6-39%m/v	Suzuki & Yokochi (1984 & 1985a)
Mortierella vinacea IMI 147433			30,0	6,4				Herbert & Keith (1985)
Phycomyces blakesleeanus IMI 63129			49,1	17,5				Herbert & Keith (1985)
Rhizopus arrhizus CMI 57412	24°C 7d	17,6	14,2	5,7	143	851	stationary culture	Shaw (1966c)
	30°C 7d	21,5	19,5	7,8	327	1 946		
	38°C 7d	15,1	3,4	6,8	35	208		
Rhizopus arrhizus CMI 57412	25°C	15,0	45,0	20,0	1 000		controlled pH	Herbert & Keith (1985)
Rhizopus oryzae IMI 21602			32,7	13,0				Herbert & Keith (1985)
Rhizopus stolonifer IMI 17314			41,4	14,5				Herbert & Keith (1985)
Rhizopus stolonifer CMI 90609				15,6				Shaw (1965)

Strain	Culture conditions	Biomass DM g/ℓ	FA, Lipid yield g/100gDM	%γLA in FAs or Lipids	γLA mg/ℓ	γLA µg/ℓ.h	Remarks	Reference
Thamnidium elegans NRRL 1613	27°C 8d	4,0	22,5	28,1	253	1 317	shake flask	Seto (1986)
Thamnidium elegans NRRL 2468	23°C 9d	3,6	26,0	26,0	247	1 144	shake flask	Seto (1986)
Thamnidium elegans NRRL 2468	28°C 5d	45,0	31,9	27,6	2 852	23 767	30ℓ fermenter	Seto (1986)

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CI - Cornsteep liquor

FA(s) - Fatty Acid(s); part of the lipid content detected by analytical methods

DM - Dry Matter

Table 2.2 Phycomycete fungi recorded in Table 2.1, which produced lipids containing a γLA yield greater than 20g/100g lipid

Strain	%γLA in FAs or Lipids	Reference
Choanephora cucurbitarum IFO 5877	39,4	Kunihisa & Shimizu (1969)
Choanephora conjuncta IFO 8089	34,1	Kunihisa & Shimizu (1969)
Mucor genevensis NRRL 1407	32,2	Gordon *et al.* (1971)
Mucor ramannianus IMI 35044a	30,9	Sumner *et al.* (1969)
Mucor rufescens CBS 572.70	28,1	Dexter & Cooke (1984)
Thamnidium elegans NRRL 1613	28,1	Seto (1986)
Thamnidium elegans NRRL 2468	27,6	Seto (1986)
Mucor piriformis CBS 527.68	26,9	Dexter & Cooke (1984)
Mucor strictus CBS 576.66	26,0	Dexter & Cooke (1984)
Cunninghamella elegans NRRL 1378	24,5	Nisshin Oil Mills (1985)
Basidiobolus meristosporus	23,2	Weete (1974)
Basidiobolus anarum	23,0	Weete (1974)
Basidiobolus haptosporus	22,3	Weete (1974)
Mucor inaequisporus CBS 255.36	22,3	Dexter & Cooke (1984)
Mucor recurvus CBS 195.71	20,7	Dexter & Cooke (1984)
Choanephora trisporum IFO 599	20,3	Kunihisa & Shimizu (1969)
Mucor mucedo IMI 103731	20,2	Sumner *et al.* (1969)
Rhizopus arrhizus CMI 57412	20,0	Herbert & Keith (1985)

unit biomass; (the mg/ℓ value is the product of the total biomass produced per litre of medium and the amount of γLA produced per unit of biomass). The value is of importance since it enables the productivity of the fermentation to be assessed for industrial purposes.

Recent publications by Suzuki & Yokochi (1984), Herbert & Keith (1985), Nisshin (1985), Suzuki & Yokochi (1985a), Suzuki & Yokochi (1985b), Seto (1986), Fukuda & Morikawa (1987), Minoshima *et al*. (1987) and Suzuki & Yokochi (1988), indicated the increase in investigations and improvements in the production of γLA by fungi. Previous significant findings (Table 2.1) included the following strains. *R. arrhizus* NRRL 57412 at 25°C under pH controlled conditions gave a biomass yield of 15g/ℓ, with a γLA yield of 1g/ℓ which was 20% of the total lipids (Herbert & Keith, 1985). *T. elegans* NRRL 2468 after 5 days at 28°C produced DM of 45g/ℓ, a γLA yield of 4g/ℓ medium and total FAs containing 27,6% γLA (Seto, 1986). *M. isabellina* IFO 7884 grown in an unconventionally high carbohydrate concentration of glucose (390g/ℓ) for 168h produced biomass of 156g/ℓ, containing γLA of 2,18g/100g DM which was 4,1% of the total lipids (Suzuki & Yokochi, 1985a). The final γLA yield in this fermentation was 3,4gℓ. This gave a γLA productivity of 20,2mg/ℓ.h. The *Mortierella* genus was reported to produce a high-density of biomass in liquid culture, as it grew in small discrete units (pellets). This type of growth differs from the more dispersed filamentous-like growth of the other Mucorales and would be less sensitive to shearing during vigorous agitation. The effect of vigorous agitation/shear stress on mycelia is referred to in sections 3.2, 4.2.1 and 4.5.1.

In this chapter the evaluation of fungal strains for the production of γLA in shake flasks is reported. Selected strains were also grown in laboratory (Chemap) fermenters in order to verify their relative performance as indicated by the shake flask experiments or by the literature. Dry, (1985), isolated *C. cucurbitarum* ATCC 12997 mutant strains which (in the case of *C. cucurbitarum* C_1 DRY 12997) produced 57% more γLA in liquid media and 28% more γLA on solid media than the parent strain. Since this work was conducted in parallel to the strain selection work described here, the performance of these mutants was retested on a larger scale ($X10^2$). Other strains grown in Chemap fermenters included the *R. arrhizus* NRRL 57412 strain used by Herbert & Keith (1985) plus two other *Rhizopus* strains, a *Cunninghamella* strain under higher temperature conditions as recommended by Shaw (1966) and a combined *C. cucurbitarum* (+) ATCC 12997 and *C. cucurbitarum* (-) ATCC 12998 fermentation. The latter two strains were combined as the use of the same culture of the two sexual forms of a species was shown to lead to a remarkable increase in mycelial carotenoid content (Ninet & Renaut, 1979). Since carotenoid and fat production begins with the molecule acetyl-CoA, fat production could also be affected by the use of both sexual forms.

Media optimisation was done on the best γLA-producing fungal strains, *C. cucurbitarum* ATCC 12997, *C. blakesleeana* NRRL 1369 and *M. vinacea* ATCC 20034. The strains were also assessed on other desirable industrial qualities. These qualities included a short fermentation time, good sporulation, suitable growth characteristics and an appropriate FA ratio without any undesirable contaminating FAs which

would complicate the recovery process.

The evaluation of the strains took place throughout the duration of this study. As the process of γLA production became better understood, the evaluation tests were modified. Subsequent experiments thus involved the use of peptone or soyabean meal (SBM) as a nitrogen source and a more efficient extraction procedure (direct extraction and esterification or DE).

2.3 Materials and Methods

All media and suppliers of additives to these media are listed in Appendix A.

Initially a glucose yeast extract (GYE) medium was used (Appendix A), but was replaced by the glucose basal medium (GBM), which proved to be a more consistent medium (see yeast extract variability, Table 3.4). The direct extraction and esterification (DE) analysis method (a combined one-step fat extraction/esterification method) was developed to replace the lengthy, less efficient conventional method (a two-step fat extraction and esterification method). Since less mycelium was needed for DE, the volume of the cultures was reduced from 600mℓ to 200mℓ thereby increasing the number of experiments which could be carried out at the same time.

2.3.1 Fungal strains and their storage

The fungi which were obtained and tested are listed in Table 2.3.

The fungal spores and/or mycelia were suspended in sterile skim milk and allowed to coat dry silica gel beads (Sigma Type II S-7500) (Smith & Onions, 1983; Perkins, 1962). The silica gel beads were stored in universal bottles in a dessicator at 4°C.

Table 2.3 Fungi evaluated for γLA production

Cunninghamella elegans Lendner plus strain	UPC	11
Cunninghamella elegans Lendner minus strain	UPC	72
Cunninghamella blakesleeana Lendner	NRRL	1369
Cunninghamella echinulata (Thaxter) Thaxter	NRRL	1386
Cunninghamella elegans Lendner	NRRL	1392
Cunninghamella elegans Lendner	NRRL	1393
Mucor genevensis Lendner	NRRL	1407
Rhizopus arrhizus Went et Provisen-Gerlings	NRRL	1526
Phycomyces blakesleeanus Burgeff	NRRL	1554
Phycomyces blakesleeanus Burgeff	NRRL	1555
Conidiobolus coronatus (Constantin) Batko	NRRL	1912
Choanephora circinans Couch plus strain	NRRL	2456
Choanephora conjuncta Couch plus strain	NRRL	2560
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	NRRL	2744
Cunninghamella echinulata (Thaxter) Thaxter	NRRL	3655
Rhizopus arrhizus Went et Provisen-Gerlings	NRRL	6431
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter plus strain	ATCC	12997
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter minus strain	ATCC	12998
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	DRY	12997 mutant C_1
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	DRY	12997 mutant C_{2-9}
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	ATCC	16403
Cunninghamella elegans var chibaensis Kuwabara et Hoshino	ATCC	20230
Mortierella vinacea Dixon-Stewart	ATCC	20034
Mucor ramannianus Möller plus strain	CMI	35044a
Phycomyces blakesleeanus Burgeff	ATCC	46090
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	ATCC	46105
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	ATCC	46106
Mucor genevensis Lendner	NCF	47801
Mucor microsporus Namyslawski	NCF	47802
Mucor spinosus van Tieghem	NCF	47803
Mucor subtilissimus Oudemans	NCF	47805
Zygorhynchus exponens Burgeff strains a & b	NCF	47806
Zygorhynchus heterogamus Vuillemin strains a & b	NCF	47807
Rhizopus stonifer var stonifer (Ehrenb. :Fr.) Vuill.	NCF	47808
Absidia coerulea Bainier plus strain	NCF	47809
Absidia coerulea Bainier	NCF	47810
Absidia coerulea Bainier minus strain	NCF	47812
Rhizopus arrhizus Went et Provisen-Gerlings	CMI	57412
Choanephora cucurbitarum (Berkeley & Ravenel) Thaxter	CMI	89917

Index
ATCC - American Type Culture Collection
CMI - Commonwealth Mycological Institute
DRY - obtained from C. J. Dry (Dry, 1985)
NCF - National Collection of Fungi (Pretoria)
NRRL - Northern Utilization Research and Development Division
UPC - University of Pietermaritzburg Collection
a+b - both mating strains together

2.3.2 Fungal inocula

Silica beads were placed on GYE agar plates and the fungi were grown at room temperature for 4d. Half an agar plate of fungal mycelium and/or spores was used as the inoculum for the shake flasks. For Chemap fermenter experiments the inocula consisted of 500mℓ cultures grown from half an agar plate of mycelium. These cultures were grown for 18h in 1ℓ round-bottom flasks. The medium was the same as that used in the scaled-up Chemap fermentation. The flasks were incubated in an orbital shaker at 100r.p.m. and 30°C.

2.3.3 Fermentation conditions

Shake flask experiments were initially carried out in 2ℓ Erlenmeyer flasks containing 600mℓ of GYE medium, sealed with a 2cm thick, gauze/cottonwool/gauze sandwich type bung. The subsequent 200mℓ fermentations were carried out in 1ℓ shake flasks in GYE or GBM media. The nitrogen source in the latter was usually peptone (GBM-P) but cornsteep powder (GBM-Cp) or soybean meal (GBM-S) was also used (Appendix A.2). The carbon to nitrogen ratio (C:N ratio) in the medium unless otherwise stated was 22:1 with the C and N concentrations being 24,2g/ℓ and 1,1g/ℓ respectively. After incubation at 28-30°C in an orbital shaker (100r.p.m.) for 96h, the morphology of the cultures was observed microscopically using a Zeiss standard microscope fitted with phase contrast optics. The mycelium was harvested by filtration (Whatman No. 541) on a Buchner funnel. All shake flask experiments were done in duplicate and each duplicate was extracted individually. Results are averages of these duplicates.

For experiments in the 5ℓ Chemap fermenters, the 10% inoculum

(500mℓ added to 4,5ℓ of medium) was grown at a rotor speed of 1,6m/s, at 28°C with 1vvm of aeration for 96h. Solutions of 1:2 Bevaloid 4214 to water or 1:3 Silcolapse to water were used as antifoam. The mycelium was harvested through terylene netting. Since the Chemap fermenters produced more mycelium, two individual samples from each fermentation were extracted and analysed. The averages of these samples are reported.

2.3.4 Extraction and analysis

After harvesting the mycelium was stored at -20°C. The conventional, acid or direct esterification (DE) methods were used (Appendix B). The FAs which were finally analysed on Gas Liquid Chromatography were: myristic ($C_{14:0}$), palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), γLA and αLA.

2.4 Results

The shake flask experiments were designed to screen fungi shown in Table 2.3. The initial medium used was GYE but was replaced by the richer GBM-P medium for enhanced FA production.

The performance of selected strains was further investigated in larger scale conditions with improved physical parameter control.

Parameters assayed were :- γLA as mg/ℓ, which is an indication of efficiency/cost effectiveness of raw material to product conversion

- γLA as g/100g DM, which is an indicator of the oleaginicity of the fungus
- γLA as % of analysed FAs (AFAs), which is important in the downstream extraction and concentration of the product
- biomass as DM g/ℓ
- FA profiles which affect the downstream extraction recovery of the product

The γLA yield (mg/ℓ) is important in guiding economic calculations in the industrial process. The FA profiles indicate the quantities of FAs other than γLA present in the oil. In addition to complicating downstream concentration of γLA, the concentrations of these FAs may influence the metabolism of γLA when it is ingested for therapeutic reasons.

The results presented in this chapter were generally averages of duplicate fermentations and were therefore used as indicators of the strains giving the best γLA yields. The results were not used to show statistically acceptable differences.

2.4.1 Shake flask experiments

Strain results are grouped according to the extraction method used and in descending order of γLA mg/ℓ medium (Tables 2.4, 2.5). *C. cucurbitarum* 12997 produced markedly greater yields of γLA (mg/ℓ) than the other strains in both GYE medium and the more enriched GBM-P medium (Table 2.4). *C. echinulata* 1386, *C. echinulata* 3655 and *C. cucurbitarum* 12998 however, contained a higher content of AFAs in their mycelia. *C. echinulata* 1386 contained 30,8g AFAs per 100g DM of mycelium; this was more than double the content of *C. cucurbitarum* 12997 (AFA yield of 15,2g/100g DM). The biomass yield produced by the *C. echinulata* strains per litre of medium was much lower than that of *C. cucurbitarum* 12997 which reduced the total AFA and γLA mg/ℓ medium values. In the reviews biomass has been reported to be inversely proportional to the γLA g/100g DM content.

Microscopic examination revealed the presence of yellow spheres in *C. echinulata* 1386 and *C. echinulata* 3655. The latter strain apparently had large compartments containing these oil droplets.

C. cucurbitarum 12997, *C. cucurbitarum* 12998 and *C. blakesleeana* 1369 had a mean stearic($C_{18:0}$):oleic($C_{18:1}$):linoleic($C_{18:2}$):γLA($C_{18:3}$) ratio of 1:2:1,5:0,8 whereas the other strains had a high percentage

Table 2.4 Yields of γLA produced by various Mucorales

The following strains were grown in 600mℓ medium in 2ℓ conical flasks. The oil was extracted by the conventional method.

Strain	γLA mg/ℓ	γLA g/100g DM	AFAs g/100gDM	Biomass gDM/ℓ	Medium
C. cucurbitarum 12997	120	2,50	21,0	4,8	GYE
C. cucurbitarum 2744	60	1,09	17,3	5,5	GYE
C. cucurbitarum 16403	37	0,57	11,4	6,5	GYE
C. circinans 2456	28	0,66	10,9	4,2	GYE
C. cucurbitarum 46105	25	1,51	23,0	1,7	GYE
C. cucurbitarum 46106	27	0,68	26,0	4,0	GYE
C. conjuncta 2560	22	0,36	14,4	6,1	GYE
C. cucurbitarum 89917	17	0,40	3,0	4,3	GYE
C. cucurbitarum 12997	226	1,75	15,2	12,9	GBM-P
C. elegans 11	160	1,67	18,7	9,6	GBM-P
C. echinulata 1386	125	2,40	30,8	5,2	GBM-P
C. elegans 72	122	1,45	9,9	8,4	GBM-P
C. echinulata 3655	117	2,18	26,2	5,4	GBM-P
C. cucurbitarum 12998	91	2,16	15,8	4,2	GBM-P
C. blakesleeana 1369	49	0,57	12,9	8,6	GBM-P
C. coronatus 1912	49	0,90	10,4	5,4	GBM-P
C. elegans 1393	46	0,48	7,0	9,6	GBM-P
M. genevensis 1407	23	0,68	7,6	3,4	GBM-P
C. elegans 1392	23	0,24	7,0	9,6	GBM-P
R. arrhizus 6431	-	-	9,0	8,4	GBM-P
R. arrhizus 1526	-	-	6,4	5,5	GBM-P
Ph. blakesleeanus 1554	-	-	-	no growth	GBM-P
Ph. blakesleeanus 1555	-	-	-	no growth	GBM-P

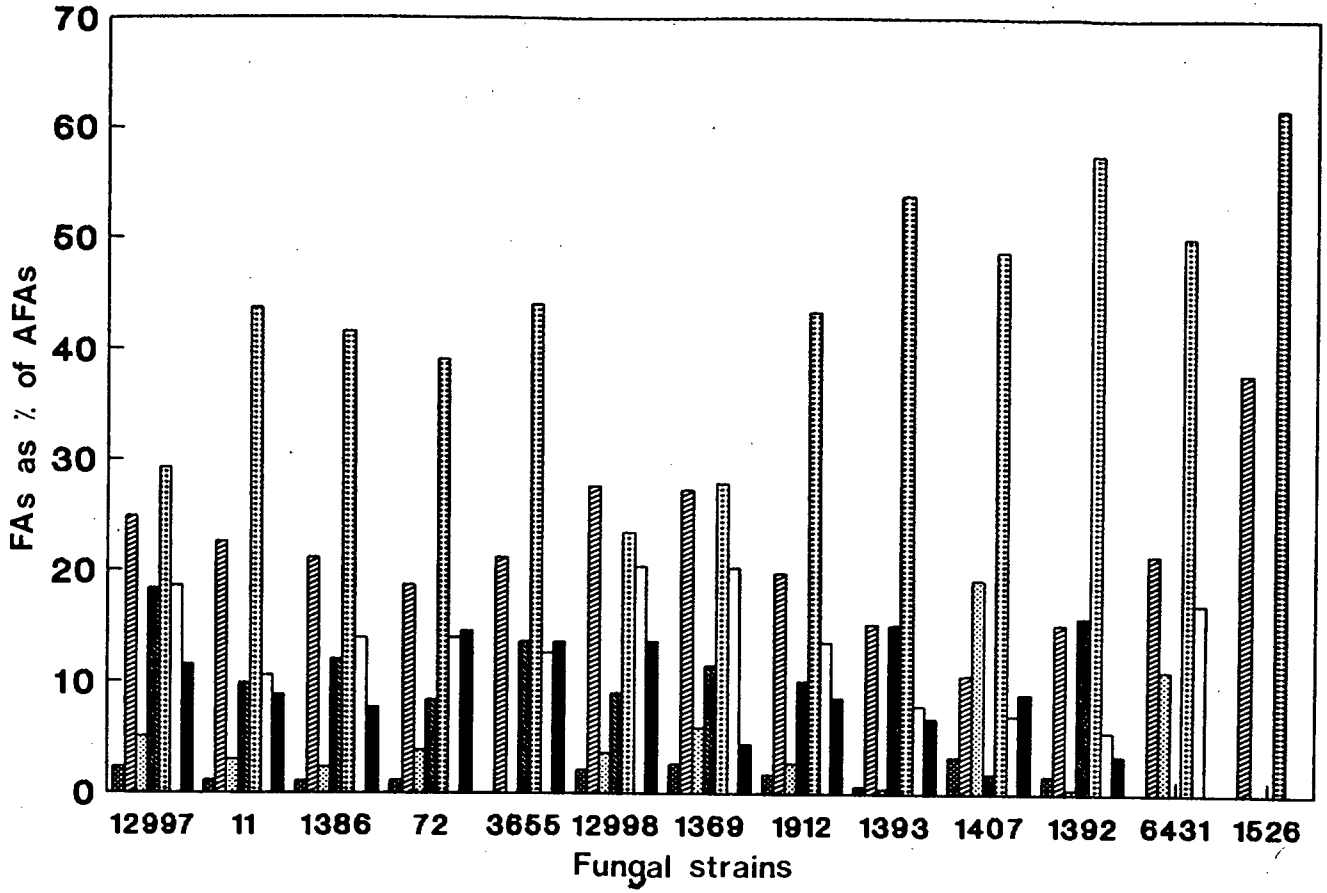

Fig.2.1 Bar graph showing the percentage distribution of analysed fatty acids in the fungal strains grown in GBM-P as shown in Table 2.4.

The fungal strains in decreasing order of γLA mg/ℓ were: *C. cucurbitarum* **12997**, *C. elegans* **11**, *C. echinulata* **1386**, *C. elegans* **72**, *C. echinulata* **3655**, *C. cucurbitarum* **12998**, *C. blakesleeanus* **1369**, *C. coronatus* **1912**, *C. elegans* **1393**, *M. genevensis* **1407**, *C. elegans* **1392**, *R. arrhizus* **6431**, *R. arrhizus* **1526**. The FAs analysed were:

C14:0 **C16:0** **C16:1** **C18:0** **C18:1** **C18:2** **γLA**

(>40%) of oleic acid (Fig.2.1), and therefore a mean FA ratio of 1:5:1:0,8. *C. cucurbitarum* 12997, *C. cucurbitarum* 12998 and *C. blakesleeana* 1369 gave good fermentation results in subsequent experiments. The γLA Chemap fermenter yields of *C. cucurbitarum* 12997 and *C. cucurbitarum* 12998 are recorded in Table 2.8 and those of *C. blakesleeana* 1369 in Table 2.10.

Z. heterogamus 47807 and *M. ramannianus* 35044a gave an 11% and 9% higher γLA yield respectively than *C. cucurbitarum* 12997 (Table 2.5). These fungi did not, however, contain more γLA in the mycelium than *C. cucurbitarum* 12997 (3,97g/100g DM). *M. vinacea* 20034 produced the highest biomass yield (19,4g/ℓ). *M. vinacea* 20034 grew as small subunits as reported by Suzuki & Yokochi (1984). *C. elegans* 20230 contained the highest percentage of γLA in the analysed FAs (Table 2.5 and Fig.2.2). This fungus together with *Z. heterogamus* 47807 and *M. genevensis* 47801 contained the ideal proportions of linoleic to γLA (Fig.2.2). A low linoleic to γLA ratio facilitates large scale extraction, purification and concentration of the γLA. Results in Fig.2.2 did not show the two different $C_{18:0}$:$C_{18:1}$:$C_{18:2}$:γLA FA ratio types recorded in (Fig.2.1), where the better γLA producers, (*C. cucurbitarum* 12997, *C. cucurbitarum* 12998 and *C. blakesleeana* 1369) had the lower linoleic ($C_{18:2}$) to oleic ($C_{18:0}$) acid ratios.

The six best γLA producers (Table 2.5), sporulated well on GYE plates.

Table 2.5 Yields of γLA produced by various Zygomycetes grown in GBM-P medium

The following strains were grown in 200mℓ of medium in 1ℓ round-bottom flasks. The FAs were extracted and esterified by the DE method.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs	Medium
Z. heterogamus 47807	332	3,02	11,0	17,8	GBM-P
M. ramannianus 35044a	326	2,49	13,1	9,1	GBM-P
C. cucurbitarum 12997	298	3,97	7,5	15,0	GBM-P
R. stonifer 47808	275	3,09	8,9	19,2	GBM-P
Z. exponens 47806	254	1,76	14,4	7,7	GBM-P
M. vinacea 20034	245	1,26	19,4	4,7	GBM-P
M. subtilissimus 47805	179	1,63	11,0	8,5	GBM-P
A .coerulea 47812	173	1,45	11,9	7,7	GBM-P
C. elegans 20230	154	2,42	6,4	24,7	GBM-P
A. coerulea 47810	136	1,41	9,6	10,3	GBM-P
A. coerulea 47809	127	1,96	6,5	19,7	GBM-P
C. blakesleeana 1369	126	2,29	5,5	10,7	GBM-P
C. cucurbitarum 2745	115	1,01	11,6	3,5	GBM-S
M. spinosus 47803	103	1,31	7,9	13,0	GBM-P
M. genevensis 47801	101	1,76	5,7	14,5	GBM-P
Ph. blakesleeanus 46090	86	1,45	5,9	16,0	GBM-P
M. microsporus 47802	41	0,86	4,8	17,1	GBM-P

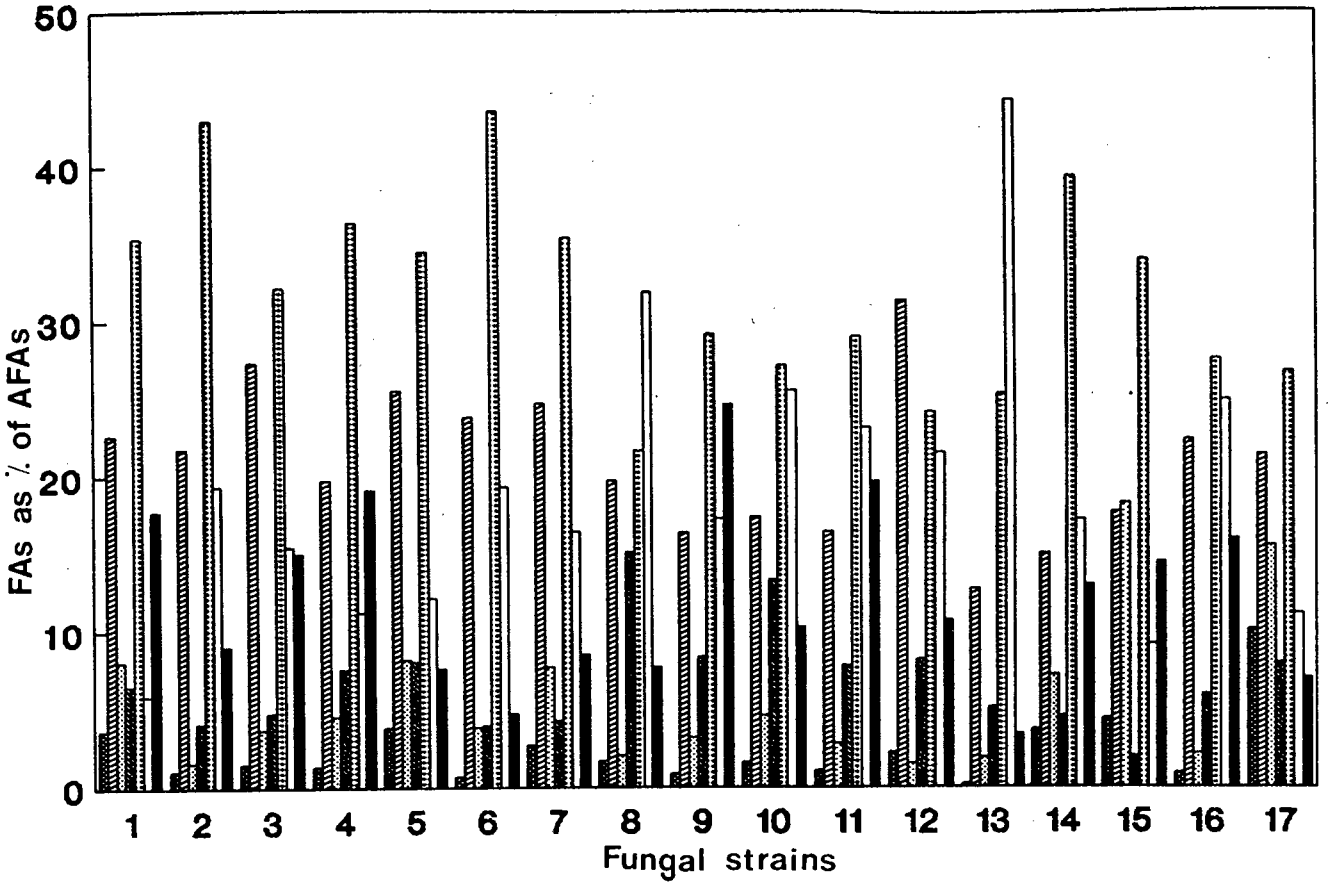

Fig.2.2 Bar graph showing the percentage distribution of DE extracted fatty acids of fungal strains grown in GBM-P as shown in Table 2.5.

Fungal strains arranged in descending order of γLA mg/ℓ were: 1 - *Z. heterogamus* 47807, 2 - *M. ramannianus* 35044a, 3 - *C. cucurbitarum* 12997, 4 - *R. stonifer* 47808, 5 - *Z. exponens* 47806, 6 - *M. vinacea* 20034, 7 - *M. subtilissimus* 47805, 8 - *A. coerulea* 47812, 9 - *C. elegans* 20230, 10 - *A. coerulea* 47810, 11 - *A. coerulea* 47809, 12 - *C. blakesleeana* 1369, 13 - *C. cucurbitarum* 2745 14 - *M. spinosus* 47803, 15 - *M. genevensis* 47801, 16 - *Ph. blakesleeanus* 46090, 17 - *M. microsporus* 47802. The FAs analysed were:

C14:0 C16:0 C16:1 C18:0 C18:1 C18:2 γLA

2.4.2 Further evaluation of selected strains in large scale fermentations

Fungal strains with fermentation characteristics of potential importance were re-evaluated under controlled Chemap fermenter conditions (*i.e.* temperature/aeration/agitation).

The strains used in these experiments were:

- four selected γLA producers (Table 2.4); *C. cucurbitarum* 12997, *C. cucurbitarum* 2744, *C. cucurbitarum* 46105 and *C. cucurbitarum* 46106 which had the best γLA or AFAs g/100g DM values
- the 2 best *C. cucurbitarum* 12997 mutants as supplied by C. J. Dry (Dry, 1985)
- *R. arrhizus* cultures; *R. arrhizus* 1526, *R. arrhizus* 6431, *R. arrhizus* 57412
- *C. blakesleeana* 1369

C. cucurbitarum 12997 produced the highest γLA yield (245mg/ℓ) (Table 2.6). *C. cucurbitarum* 2744 produced a slightly higher level of γLA per unit biomass, 2,1g/100g DM compared to 2,0g/100g DM produced by *C. cucurbitarum* 12997. However the lower biomass produced by *C. cucurbitarum* 2744, decreased the strain's overall γLA mg/ℓ value to 181mg/ℓ. The amount of γLA g/100g DM produced by *C. cucurbitarum* 12997, *C. cucurbitarum* 2744, *C. cucurbitarum* 46105 and *C. cucurbitarum* 46106 in the fermenters was relative to that produced in the shake flasks (Table 2.4), confirming the earlier results with respect to the best and the worst γLA-producers.

Table 2.6 Production of γLA by strains of *C. cucurbitarum* grown in Chemap fermenters

The strains were grown in 5ℓ GBM-Y medium.

Fatty acids were extracted by the conventional method.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997	245	2,00	12,3	15,5
C. cucurbitarum 2744	181	2,10	8,6	17,1
C. cucurbitarum 46105	145	1,49	9,7	16,8
C. cucurbitarum 46106	142	1,35	10,5	16,4

Table 2.7 Production of γLA by two mutant strains of *C. cucurbitarum* and the wild type strain, in Chemap fermenters

The strains were grown in 5ℓ GBM-P medium.

Fatty acids were extracted by the conventional method.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997 wild type	419	2,92	14,3	15,7
C. cucurbitarum 12997 mutant C_1	160	1,98	8,1	24,5
C. cucurbitarum 12997 mutant C_{2-9}	98	1,88	5,2	26,6

The γLA yield (mg/ℓ) produced by the wild type *C. cucurbitarum* strain was higher than that of the mutant strains (Table 2.7). This was a result of the low DM and the γLA yield in the DM (g/100g DM) in the mutants. However the mutants showed a higher percentage of γLA in the AFAs. The linoleic acid to γLA ratio of the mutants, 1:1,2-1,4 was lower than that of *C. cucurbitarum* 12997, 1:0,6.

The γLA yield of 396mg/ℓ produced by the two *C. cucurbitarum* sexual strains, (12997 + 12998) was probably a result of the higher biomass yield of 18,7g/ℓ, (Table 2.8).

R. arrhizus 57412 (Table 2.9) grew rapidly and large mycelial mats were prominent. It did not produce the γLA yields reported by Herbert & Keith (1985). The mat growth would inhibit efficient oxygen transfer. The Chemap fermentations of *R. arrhizus* 1526 and *R. arrhizus* 6431 improved the γLA yields (Table 2.4) from the shake flask experiments by 100%.

C. blakesleeana 1369 as reported by Weete (1974), grew well at 37°C and produced biomass of 20,9g/ℓ with a γLA yield of 351mg/ℓ, which was only slightly less than that of *C. cucurbitarum* 12997 at 30°C, (367mg/ℓ (Table 2.10)). Fermentations with *C. blakesleeana* 1369 carried out for a longer period of time and with a 20% inoculum gave a high γLA yield of 741mg/ℓ but the productivity values of 5,61mg/ℓ.h, was less than the 6,08mg/ℓ.h of *C. cucurbitarum* 12997. At a shorter fermentation time *C. blakesleeana* did not produce as much γLA as *C. cucurbitarum* 12997 (Tables 2.10, 2.12).

Table 2.8 Production of γLA by *C. cucurbitarum* 12997 and of cocultures of *C. cucurbitarum* 12997 and *C. cucurbitarum* 12998 in Chemap fermenters

Fermentations were carried out in 5ℓ of GBM-Cp and the FAs were extracted using the conventional method. A 10% and 20% inoculum was used for *C. cucurbitarum* 12997 and for the *C. cucurbitarum* cocultures respectively.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997	343	2,54	13,5	15,1
C. cucurbitarum 12997 + 12998	396	2,00	19,8	16,7

Table 2.9 Production of γLA by *C. cucurbitarum* 12997, *R. arrhizus* 1526, *R. arrhizus* 6431 and *R. arrhizus* 57412, in Chemap fermenters

Strains were grown in 5ℓ of GBM-Cp for 96h except for *R. arrhizus* 57412, which was grown for 168h. The fatty acids were extracted by the conventional method.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997	343	2,54	13,5	15,1
R. arrhizus 1526	118	1,10	10,7	6,8
R. arrhizus 6431	108	1,05	10,3	14,2
R. arrhizus 57412	217	1,09	19,9	8,3

Table 2.10 Production of γLA by *C. cucurbitarum* 12997 and the thermotolerant *C. blakesleeana* 1369 strain in Chemap fermenters

Fermentations were carried out in 5ℓ of GBM-Cp. Fatty acid results shown in part A of the table are those of the conventional extraction and those in part B are those of the DE extraction. The inoculum size was 10% and 20% in parts A and B respectively. Inocula were grown in shake flask and Chemap in parts A and B respectively.

Strain	Temperature	Time	γLA mg/ℓ	γLA mg/ℓ.h	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
Part A							
C. cucurbitarum 12997	30°C	96h	367	3,82	2,72	13,5	15,1
C. blakesleeana 1369	30°C	96h	250	2,60	1,90	13,2	12,0
C. blakesleeana 1369	37°C	96h	351	3,66	1,68	20,9	10,7
Part B							
C. cucurbitarum 12997	35°C (24h)						
	28°C (72h)	96h	584	6,08	2,99	19,5	14,9
C. blakesleeana 1369	37°C	132h	741	5,61	3,05	24,3	16,7

C. blakesleeana 1369 was the only other strain which had a similar FA distribution pattern to *C. cucurbitarum* 12997 (Fig. 2.1).

2.4.3 Media optimisation studies of *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369

C. cucurbitarum 12997 gave the best γLA yield (331mg/ℓ) in GBM-S (Table 2.11). *M. vinacea* 20034 gave a comparable yield (245mg/ℓ) in GBM-P. *M. vinacea* 20034 produced the highest biomass (19,4g/ℓ) and had the finer non-mycelial growth morphology reported by Suzuki & Yokochi (1984), Suzuki & Yokochi (1985b) and Suzuki & Yokochi (1988). However this biomass value was not comparable to 103,5g/ℓ previously reported by a 72h *M. vinacea* fermentation (Suzuki & Yokochi, 1984). No growth was obtained when *M. vinacea* 20034 was incubated in the medium used by Suzuki & Yokochi (1984) (glucose 270g/ℓ; urea 10g/ℓ; KH_2PO_4; $MgSO_4$; NaCℓ; yeast extract; peptone; minerals).

C. blakesleeana 1369 and *M. vinacea* 20034 produced the best γLA yields in a C:N ratio of 25:1 which was in the same range as the C:N ratio of 22:1 preferred by *C. cucurbitarum* 12997 (Table 2.11) and the C:N ratio used in all the fungal selection fermentations (Tables 2.4, 2.5). *M. vinacea* 20034 grew poorly in the larger volume of 500mℓ in the shake flasks (Table 2.11).

The highest γLA yields (g/100g DM) were produced in peptone media by *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369 (Table 2.11). Comparison of GBM-Cp and the GBM-P results, indicated that

Table 2.11 The production of γLA by *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369 in GBM in shake flasks

Variations of C:N ratios were achieved by altering the carbon (glucose) concentration. The γLA was extracted and analysed by the DE method.

Strain	Medium	C:N	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997	GBM-S	22:1	331	2,18	15,1	7,8
	GBM-P	22:1	298	3,97	7,5	15,0
	GBM-Cp	22:1	200	2,51	8,0	8,0
M. vinacea 20034	GBM-P	22:1	245	1,26	19,4	4,7
	GBM-Cp	22:1	121	0,91	13,3	3,7
	* GBM-Cp	15:1	8	1,06	0,7	10,8
	* GBM-Cp	7:1	8	1,00	0,8	11,3
	GBM-Cp	25:1	114	0,83	13,7	3,9
	GBM-Cp	30:1	101	0,76	13,3	3,7
C. blakesleeana 1369	GBM-P	22:1	126	2,29	5,5	10,7
	GBM-Cp	22:1	167	1,52	11,0	6,5
	GBM-Cp	15:1	172	1,74	9,9	7,8
	GBM-Cp	7:1	129	1,26	10,2	9,9
	GBM-Cp	25:1	184	1,91	9,6	9,0
	GBM-Cp	30:1	165	1,45	11,4	6,5

* Volume of medium used was 500mℓ (all other experiments had final volumes of 200mℓ) Oxygen transfer was less efficient in 500mℓ than in 200mℓ

peptone caused the decrease in the percentage of saturated FAs such as $C_{16:0}$ and $C_{18:0}$ and an increase in the unsaturates $C_{18:1}$ and γLA, (Fig. 2.3). *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369 all produced relatively more γLA in GBM-P than in GBM-Cp (Table 2.11). The total percentage of unsaturated FAs was largest in the peptone medium and least in the cornsteep powder medium (Fig. 2.3).

C. cucurbitarum 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369, grown under Chemap fermenter conditions (Table 2.12) did not produce higher γLA yields than the optimised *C. cucurbitarum* 12997 fermentation (674mg/ℓ).

The low $C_{18:2}$ and high γLA content in the FAs of *Z. heterogamus* 47807, as well as an overall good γLA yield (332mg/ℓ) (Table 2.5), justified the repeated fermentation trials with this fungus. In a 432h-long fermentation, the ratio of linoleic:γLA for *Z. heterogamus* 47807 was 1:3 whereas for *C. cucurbitarum* 12997 it was 1:0,5 (Table 2.14). *Z. heterogamus* 47807 gave the same 1:3 linoleic:γLA ratio in a shorter 96h fermentation in GBM-P. This ratio was, therefore, characteristic of the GBM-P medium and not a result of longer fermentation times such as 432h. After 432h *C. cucurbitarum* 12997 had utilised its FAs which resulted in a lower %γLA in AFAs of 9,9% as compared to 20,1% in the GBM-Cp 96h fermentation (Table 2.13). The %γLA in AFAs of *Z. heterogamus* 47807 remained unchanged in the GBM-P and GBM-Cp fermentations (Table 2.13).

Table 2.12 Production of γLA by *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369 in Chemap fermenters

The fermentation volumes were 5ℓ. Fatty acid results are averages of duplicate samples of mycelium from each fermentation. The DE method was used.

Strain	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997 (a)	674	2,99	22,5	8,7
M. vinacea 20034 (b)	334	1,56	21,5	4,9
C. blakesleeana 1369 (c)	326	1,47	22,2	ND
C. blakesleeana 1369 *	741	3,05	24,3	16,7

* - 132h fermentation, 20% inoculum (see Table 2.10)
ND - not determined

Conditions for the fermentations were as follows:-

Index term	Stage of fermentation	Medium	C:N ratio	Temperature
(a)	Inoculum	GBM-S	22:1	30°C
(a)	Fermentation	GBM-S	22:1	30°C
(b)	Inoculum	GBM-Cp	25:1	30°C
(b)	Fermentation	GBM-Cp	22:1	30°C
(c)	Inoculum	GBM-Cp	25:1	35°C
(c)	Fermentation	GBM-Cp	22:1	35°C

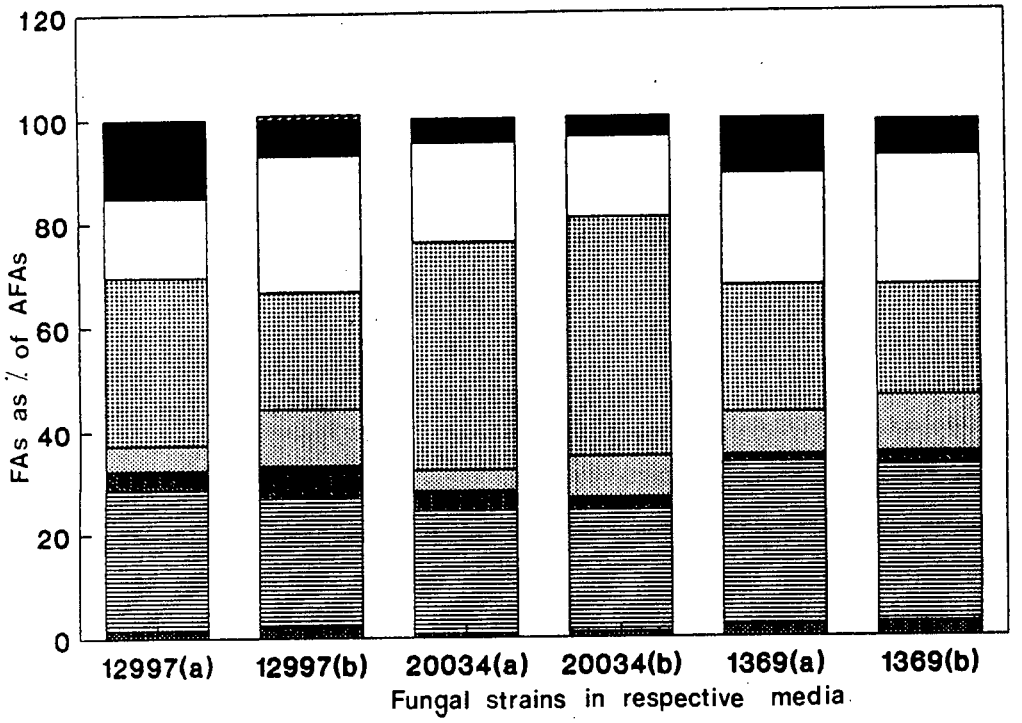

Fig.2.3 Comparison of percentages of AFAs of *C. cucurbitarum* 12997, *M. vinacea* 20034 and *C. blakesleeana* 1369, grown in GBM-P or GBM-Cp in shake flasks.

The fungi *C. cucurbitarum* **12997**, *M. vinacea* **20034** and *C. blakesleeanus* **1369**, were grown in **(a)** GBM-P or **(b)** GBM-Cp. The fatty acids analysed were:

C14:0 **C16:0** **C16:1** **C18:0**
C18:1 **C18:2** **γLA** **αLA**

Table 2.13 Production of γLA by *C. cucurbitarum* 12997 and *Z. heterogamus* 47807 grown in shake flasks, in various media and for different time intervals

The fermentation volumes were 200mℓ. The DE method was used for oil extraction and analysis.

Strain	Media	Time h	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
C. cucurbitarum 12997	GBM-P	432	234	1,61	14,5	9,9
Z. heterogamus 47807	GBM-P	432	308	2,80	11,0	17,3
C. cucurbitarum 12997 *	GBM-S	168	202	1,45	14,0	5,2
Z. heterogamus 47807	GBM-S	168	236	1,44	16,4	6,5
C. cucurbitarum 12997	GBM-Cp	168	248	2,94	8,4	20,1
Z. heterogamus 47807	GBM-Cp	168	224	1,84	12,2	17,2

* Results are low as fungus in this case grew in a mat form which limits oxygen transfer. Growth in GBM-S is usually in pelleted form.

Table 2.14 The percentage distribution of AFAs in the fatty acid content of *C. cucurbitarum* 12997 and *Z. heterogamus* 47807 after the 432h fermentation in GBM-P (Table 2.13)

		Fatty Acid given as % of AFAs	
Fatty Acid		*C. cucurbitarum* 12997	*Z. heterogamus* 47807
Myristic	$C_{14:0}$	3,14	3,67
Palmitic	$C_{16:0}$	28,73	26,11
Palmitoleic	$C_{16:1}$	6,77	5,58
Stearic	$C_{18:0}$	7,61	5,66
Oleic	$C_{18:1}$	23,83	35,59
Linoleic	$C_{18:2}$	20,05	6,09
γLA	$C_{18:3}$	9,89	17,29

2.5 Discussion

For industrial purposes and in comparison with the other strains, *C. cucurbitarum* 12997 combined a relatively good biomass yield with a good concentration of γLA in the biomass. This fungus had further advantages of fine pelleted growth in the fermenter and good sporulation. *C. cucurbitarum* 12997 consistently gave the highest γLA mg/ℓ yields and in its most optimum medium GBM-S, still surpassed all the strains studied.

The high γLA yields obtained by *C. blakesleeana* 1369 were only achieved by a change in temperature. This highlights the problem in strain selection in that each fungus has its optimal requirements for maximum lipid production. This is why some optimisation work was done with each of the selected strains (Tables 2.11, 2.12, 2.13). When optimal fermentation conditions of the strains were known they were repeated, but often the results did not correspond with those reported in the literature. *R. arrhizus* 57412 and *M. vinacea* 20034 were two examples. The latter was not the same strain as that used by Chesters & Peperdy (1965) or by Suzuki & Yokochi (1984) but the medium used by the latter authors should not have inhibited total growth of *M. vinacea* 20034. No growth with *M. vinacea* 20034 was obtained in the medium used by Suzuki & Yokochi (1984) (reported in results section 2.4.). Suzuki & Yokochi (1984) claimed that at the higher glucose concentrations of 390g/ℓ the fungal growth rate was slower resulting in a longer final fermentation time. The high glucose concentration used by Suzuki & Yokochi (1984 & 1985b) may have not been the only growth inhibitor as Weete (1974) referred to a study where 40% glucose in a medium gave the

highest fat yields in certain fungi. Suzuki & Yokochi (1984) have patented "special culture conditions" giving "advantageous fungal growth of small discrete units" and a "high velocity of multiplication". These conditions were not elaborated and there was no glucose feed.

The poor results with *M. vinacea* 20034 grown under limited oxygen transfer conditions in 500mℓ of GBM-Cp (Table 2.11), indicated the limitation in oxygen transfer in shake-flask cultures. This may be the reason why the *R. arrhizus* and *Ph. blakesleeanus* strains did neither grow nor produce γLA (Table 2.4).

The relative order of γLA producers in Chemap fermenters (Table 2.6) followed that determined by shake flask experiments (Table 2.4). This showed that although the physical conditions in shake flasks were not optimal the experiments served their purpose in the screening for the better fungal γLA producers.

The FA profiles of the various strains were considered important as they could affect future extraction and concentration work of γLA. If the concentrations of closely related FAs are high they cannot be separated by downstream processes. The FA profiles were also investigated as possible indicators of superior γLA producers in future screening experiments. This was because the fungi which had the low oleic ($C_{18:1}$) to γLA ratio (Figs.2.1), (*C. cucurbitarum* 12997, *C. cucurbitarum* 12998 and *C. blakesleeana* 1369) were the only fungi which gave good γLA yields in further scale-up fermentations (Tables 2.8, 2.10). There were however no prominent differences in the FA ratios of fungi in Fig.2.2. The improved extraction procedure (DE) in

this case could have released FAs from all parts of the mycelium thereby changing the ratios.

The six best γLA-producers sporulated well on GYE plates. The inocula of these fungi therefore contained mycelium and spores. This gave the fungi an added advantage over strains which did not sporulate on GYE. The mycelial/spore inoculum would have increased the number of growing points in the fermenter thus decreasing the lag phase in growth and therefore allowing more time within the fermentation for fat accumulation. This indicates the importance of the spore inoculum (see also Tables 3.14, 3.15 and 3.16, for effects of inoculum size and age).

The *C. cucurbitarum* 12997 C_1 and C_{2-9} mutants did not produce the reported (Dry, 1985) increase in γLA as a result of having reverted to a deficient state. Work done in shake flasks, after the reported Chemap fermenter runs (Table 2.7) showed the instability of these mutant strains (Dry, 1985). The low biomass of the mutant strains (Table 2.7) could indicate a growth factor requirement caused by a multiple mutation. The lower linoleic acid to γLA ratio (1:1,2-1,4) in the mutant strains would benefit the concentration and purification of γLA in the final product. However, the observed increase in the percentage of uncharacterised FAs (*i.e.* FAs other than those analysed for) could complicate the oil purification and concentration of γLA.

Ratledge (1982) reported that fungi often lost their lipid accumulating ability and that oleaginicity was often not stable. Examples of this were *M. ramannianus* 35044a and *C. blakesleeana* 1369 where production of high γLA yields deteriorated in repeated fermentations.

Z. heterogamus which was the last strain screened, produced similar yields to *C. cucurbitarum* 12997 and had an advantageous lower linoleic acid:γLA ratio. It however contained many uncharacterised FAs which would make the extraction/concentration procedure difficult. Further experimentation is suggested with this strain to verify and possibly exploit its potential.

The fungi in GBM-P produced more unsaturated FAs. It would be of value if the components in peptone were identified so that those stimulating FA desaturation could be used in the optimised medium. Peptone itself is very expensive for use on an industrial scale. GBM-P did not have contaminating FAs and had enough nutrients to support each fungus in γLA production. GBM-P was therefore the best medium for the shake flask screening test.

CHAPTER 3

OPTIMISATION OF THE CULTURE CONDITIONS

CHAPTER 3

OPTIMISATION OF THE CULTURE CONDITIONS

3.1 Summary

The growth and γLA producing stages of the selected strain *C. cucurbitarum* 12997 were optimised. Optimised parameters were the industrial medium composition, the status of the inoculum and physical conditions such as temperature, pH and oxygen. By optimising these conditions the composition and quantity of the fat produced by *C. cucurbitarum* 12997 was changed considerably. The improved analysis procedure (DE) allowed measurement of more γLA and thus increased the apparent γLA yield. Early optimisations produced an increase in the shake flask γLA yield from 21mg/ℓ to 396mg/ℓ. In the Chemap fermentations the increase was from 59mg/ℓ to 512mg/ℓ and with a subsequent fed-batch fermentation the γLA yield was 744mg/ℓ. The optimal carbon and nitrogen sources for γLA production were respectively glucose and soyabean meal (SBM). For the batch culture of *C. cucurbitarum*, optimal γLA mg/ℓ yields were obtained with a 20% inoculum grown in glucose basal medium plus soyabean (GBM-S) with a C:N ratio of 14:1. In the main fermentation the best C:N ratio in GBM-S was 22:1. No pH control was necessary in the fermentation. A dual temperature profile of 35°C for 24h followed by 30°C for 72h supported good growth and γLA production. Under these conditions there was no lag phase in fungal growth and γLA production. The optimised conditions for the production of γLA by

C. cucurbitarum were suitable for further scale-up of the fermentation in the pilot plant.

3.2 Introduction

Major improvements in the productivity of fermentation processes are often ascribed to the development of superior strains through mutation. However, other parameters such as the nutritional and physical environment to which an organism is exposed are also known to greatly alter product yield.

3.2.1 Industrial fermentation media

The development of an industrial medium requires the selection of economical carbon, nitrogen, phosphorous, sulphur, potassium and trace element sources. Other important factors are the raw material variability, availability and price stability. The price may be affected by the substrate's cost of transportation, handling, storage and pretreatment (hydrolysis and special sterilisation costs). The medium composition must not only support good microbial growth but it must also maximise product yield and reduce the synthesis of compounds which are difficult to separate and therefore contaminate the product.

Fermentation substrates are classified according to their predominant function within a nutrient medium as carbon-containing and nitrogenous substances, minerals or vitamins. Carbohydrates for example serve as carbon-sources and energy sources. Complex raw materials such as molasses or whey additionally contain nitrogen, minerals and vitamins, (molasses contains a high content of biotin, (0,8-1,0mg/kg molasses) Sugar Milling Research Institute (SMRI), personal communication). Proteins are important sources of nitrogen but can also supply carbon and minerals; *e.g.* soyabean meal is a source of certain FAs and lipids.

Carbon sources range from monosaccharides such as glucose and xylose or disaccharides such as maltose, lactose and cane molasses (molasses also contains a few polysaccharides). Other polysaccharides are starch, dextrose, inulin and cellulose. Other carbon sources include alcohol, carboxylic acids, fats, hydrocarbons as well as gases.

Urea is a suitable organic nitrogen source with the added advantage of a high buffering capacity. Urea is however not stable during thermal sterilisation and the use of filter sterilisation for industrial media would be impractical. Meals such as soyabean, cottonseed, rape seed and fish are inexpensive sources of amino acids, mineral salts and vitamins. Soyabean meal is available in three different grades according to the fat content; full-fat meal (SBM) with >18% fat, partly defatted meal with 4,5-9% fat and fully defatted meal with a maximum of 2% fat. Fully defatted SBM contains all essential amino acids, glutamic acid being the main component with little methionine and cysteine. Protease inhibitors present in the meal are inactivated by thermal treatment at 100^{o}C.

Cornsteep liquor (CI), a by-product of cornstarch extraction is used frequently as a nitrogen source but its composition fluctuates depending on the grade and germinating characteristics of the corn. It is, however, a good source of phosphorous and the dried product is more consistent in quality. Yeast autolysates and dried yeasts are rich in protein and amino nitrogen as well as important vitamins. Additives to the medium can include phosphorous, sulphur and macroelements (K, Mg, Ca, Zn, Fe and Cℓ). Microelements (Co, B, Cd, Cr, Cu, Mo and Ni) are usually supplied by water and the raw materials. Biotin and thiamine, the most common essential vitamins may not be present in

sufficient quantities in the raw materials and are therefore added in the form of pure vitamins or as yeast extract. Other additives include certain amino acids, enzyme preparations, precursors or inducers of a biochemical pathway, detergents as emulsifiers, antifoam agents and antiseptics which increase the viscosity of the medium. Strongly foaming substrates are soyabean meal, cornsteep liquor, yeast extracts and sulphite waste liquors.

A variety of substrates have been considered for the growth of oleaginous microorganisms. These include cellulose, rice hull hydrolysate, starch waste liquor, molasses, beet molasses, sweet potato hydrolysate, date extract, domestic waste water, whey, potato processing waste, peat hydrolysates/peat oxidates and alcohol (Ratledge, 1982; Farag *et al.*, 1983; Abraham & Srinivasan, 1984; Suzuki *et al.*, 1984; Tahoun *et al.*, 1987). Oleaginous yeasts have been grown in olive oil, animal fat (Tan & Gill, 1985) and mixtures of soapstock (Picataggio & Smittie, 1979). The FA content of *C. elegans* and other Mucorales grown on alkanes has been reported by Ratledge (1980).

3.2.2 Conditions influencing microbial lipid synthesis

Woodbine (1959), Wassef (1978), Weete (1979) and Ratledge (1982) have reviewed the effects of culture conditions on fat production. Unless otherwise cited the information below has been extracted from these references.

3.2.2.1 Mode of fermentation

Fat accumulation in most microorganisms growing in batch culture follows a two-stage pattern. The first stage is a cell proliferation stage in which cells continue growing at their maximum rate until nutrient(s) other than carbon (*i.e.* nitrogen, phosphate) become limiting. In the second stage, as the mycelium ages, the excess carbon is consumed and converted to lipids. A few organisms like *Ph. blakesleeanus*, *M. vinacea* and *R. arrhizus* produce fat constitutively or can accumulate fat under non-limiting growth conditions. The fats are utilised during the stationary stage of growth.

Little work has been done on the application of continuous culture methods to FA production. This is because the observed lipid accumulation patterns in batch culture were thought to need a two-stage continuous system which was unrealistic if scale-up were to be considered. There have been reports of yeast producing lipids in single-stage continuous culture (Yoon & Rhee, 1983).

The recently reported production of γLA by a fungus has been in dispersed form in a shake flask or in a stirred tank reactor. Fukuda & Morikawa (1987), however, have reported early work on the immobilisation

of *Mucor ambiguus* in reticulated foam biomass support particles. After seven consecutive batch fermentations, *M. ambiguus* IFO 6742 was reported to have a relatively good coefficient yield of γLA per gram Glucose (14,2mg/gG) and a γLA productivity of 17,1mg/ℓ.h (Table 2.1). The batch immobilisation system for γLA production has potential but was not investigated as *C. cucurbitarum* 12997 did not grow in a fine yeast-like form. The production of γLA by *C. cucurbitarum* 12997 in a submerged batch fermentation was more practical. Dry (1985) reported much higher γLA g/100g DM with *C. cucurbitarum* 12997 on solid media. This system, however, posed several additional difficulties including poor control of contamination, temperature and water availability.

3.2.2.2 Carbon and nitrogen sources in the fermentation medium

Strains belonging to the Mucorales produced γLA from glycerol, maltose, soluble starch and sodium acetate (Sajbidor *et al.*, 1988). Lactose was not converted efficiently to γLA. Glucose however, remained the best carbon source for γLA production. Chesters & Peberdy (1965) reported that *M. vinacea* grown in glucose or maltose produced the best γLA yield. Yeast assimilated and converted xylose to lipids (Fall *et al.*, 1984) but there has been no report of other fungi converting xylose to γLA. Although fungi vary in their ability to convert different carbon substrates into fat, the three best and most common carbohydrates for lipid production were in descending order: glucose > sucrose > fructose. All patented processes used glucose as the carbon source for γLA production.

Apart from the carbohydrate source, nitrogen has the most pronounced effect on lipid production, especially at low concentrations as it

initiates lipid accumulation (Ratledge, 1982). In some species ammonium was best for fat accumulation and organic nitrogen was best for mycelial growth. Chu (1959) reported *C. cucurbitarum* growth on nitrogen sources of glutamic acid, yeast extract, casein hydrolysate, asparagine, proline, leucine or arginine. Patented processes of Phycomycete fungi producing γLA used NH_4SO_4 (Seto, 1986), $NH_4C\ell$ (Herbert & Keith, 1985), any ammonium source or urea, cornsteep liquor, yeast extract and peptone (Suzuki & Yokochi, 1984). Soyabean meal has been known to have a stimulatory effect on fermentations and was therefore included for screening as a nitrogen source for γLA production. The cornsteep powder (Cp) nitrogen source, although more expensive than cornsteep liquor, was included in media optimisation to alleviate the problem of quality inconsistency.

The C:N ratio is of importance in lipid production. Depending on the fungus a C:N ratio of 80:1 (Chesters & Peberdy, 1965) or 22:1 (Suzuki & Yokochi, 1984) was found to be suitable for fat production. The actual carbon and nitrogen concentrations are also vital. Increasing the carbon and nitrogen content of the medium while keeping the C:N ratio constant results only in increased mycelial production. Suzuki & Yokochi, (1984) increased the glucose concentration from 60-400g/ℓ in the production of γLA by *M. vinacea*. At the higher glucose concentrations the efficiency of conversion of substrate to γLA and the productivity (rate of production) were lower.

3.2.2.3 Additives and supplements to the fermentation medium

Suzuki & Yokochi (1985b) improved the γLA production efficiency of *Mortierella* spp. by adding acetic acid or an alkali metal acetate.

Fukuda & Morikawa (1987), increased the secretion of γLA into the culture broth by the use of nonionic surfactants such as polyoxyethylene sorbitan FA ester and polyethylene glycol FA ester. Various additives to the medium may be precursors of the FA pathway such as acetate and citrate or they may have no obvious biological role like α-DL-Tocopherol. This natural anti-oxidant of lipids increased the degree of unsaturation in the FAs of the fungus *Glomera cingulata* (Ratledge, 1982).

Nutromix, a commercial yeast food, was found to stimulate fermentations at the local breweries. It is not known what Nutromix contained but typical commercial yeast foods can contain a nitrogen content of 7g/100g, a phosphate content of 50g/100g, as well as calcium, inositol, niacin, pyridoxin, pantothenic acid, thiamine, biotin, zinc and manganese. Commercial yeast foods stimulate biomass production and decrease the rate of fermentation (Axcell *et al.*, 1988). Nutromix was used to supplement GBM-Cp so as to increase γLA yields.

Some additives like antifoams and exogeneous FAs may have inhibitory effects on lipid production. Shifrin (1984) reported copper tolerant clones of *Chlorella vulgaris* which had 50% more lipid. Shifrin (1984) suggested that lipid production in these clones could aid in excluding the inhibitory effect of the copper ion by some exclusion mechanism or by sequestering the ion within the cell.

Other elements used as supplements for fat production are sodium, potassium, magnesium, sulphate and phosphate ions. Zinc sulphate, manganese chloride and potassium phosphate in large amounts induced cell proliferation in yeast and in small concentrations induced lipid

accumulation (Naganuma, 1985).

The relationship between lipid production and vitamin deficiency in fungi has not been established. In some cases vitamin deficiencies cause a reduction in lipid production. Minoshima *et al.*, (1987) added pantothenic acid and thiamine to enhance γLA production by *C. elegans*. Pyridoxin, and biotin deficiency also reduce lipid production. Biotin is needed as a cofactor in the synthesis of acetyl-CoA carboxylase the FA chain lengthening enzyme. Inositol and nicotinic acid deficiencies result in an increase in the lipid content of cells. Lilly & Barnett (1951) have listed *C. cucurbitarum* as a thiamine deficient fungus.

3.2.2.4 Physical factors affecting lipid synthesis

Illumination has not been reported to play a role in γLA production. The duration of light, however, affects fungal spore formation. The optimum pH values for growth of most fungi are between pH 6,0 and 7,0. During fungal growth in unbuffered media the pH may rise or fall slightly depending on the medium. *R. arrhizus* grown in a buffered medium contained a higher content of fat and showed a more efficient utilisation of glucose (Herbert & Keith, 1985). However, according to Weete (1974) and Wassef (1978) there were several reports that indicated that between pH 4 and 8 there was very little correlation between lipid production by fungi and the pH of the medium.

Aeration allows fungi to grow more rapidly and to utilise most of the available carbon. Oxygen is necessary for desaturation of FAs. Umeki & Nozawa (1984) reported that under oxygen stress there were decreases in

the rate of radioactive palmitic acid incorporation into the unsaturates; palmitoleic ($C_{16:1}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and γLA in *Tetrahymena pyriformis*. Dry (1985) reported increased γLA yields when the oxygen to medium ratio in shake flasks was increased. Ratledge (1982) however, stated that large scale aeration should be decreased to low levels (*i.e.* with an oxygen partial pressure of 1mmHg) to visibly affect growth and the FA profile. Efficient agitation could aid efficient gas transfer in the fermenter but it could also increase the shear stress of the mycelium (Section 4.2.1 & 4.5.1).

The growth morphology of the fungus affects nutrient and gas transfer in submerged culture. Fungi can grow in submerged culture in single-cell, pelleted and filamentous forms. Factors which affect growth morphology include the fungal species, the size and type of inoculum, medium shear and medium composition (Byrne & Ward, 1989a; Byrne & Ward, 1989b). Filamentous growth increases the viscosity of the medium, and therefore a higher power input is required to maintain adequate mixing and aeration. Less power is required to mix pelleted growth. In pelleted growth the centre of the pellets can, however, become anaerobic and therefore decrease the overall specific growth rate of the fungus. It is thus important to be able to control the fungal morphology in the fermenter. The growth morphology of *R. arrhizus* changed from filamentous and pelleted to dispersed with anionic polymers, maize particles and various concentrations of glucose and soyabean meal (Byrne & Ward, 1989a).

Sumner *et al.* (1969), reported that increasing temperatures increased lipid levels. This was true, however, within certain temperature ranges which differed depending on the species. The most pronounced effect of

temperature on fungal lipids was in the relative increase of unsaturation of FAs grown under lower temperature conditions (Sumner *et al.*, 1969; Dexter & Cooke, 1984). Kates & Baxter (In Sumner *et al.*, 1969) proposed that the rates of synthesis and degradation of unsaturated FAs were both temperature dependent. They suggested that at lower temperatures the synthesis of unsaturated FAs was less retarded than their degradation which led to an increased concentration of unsaturates. Brown & Rose (1969) observed increases in the unsaturated FAs of *Candida utilis* at lower temperatures and at increased dissolved-oxygen tensions. The increased desaturation at lower temperatures may therefore be associated with more oxygen going into solution at these temperatures. Since the desaturation of saturated FAs requires oxygen, the abundant availability of this molecule would be important.

Even though controls on microbial fat/γLA synthesis may be understood the purpose for γLA production by the Mucorales has not been clearly identified. Dexter & Cooke (1984) suggested that the production of unsaturated FAs is an adaptive mechanism for the organism to survive at lower temperatures by keeping its membrane fluid. There is no evidence, however, which shows that the control of the degree of saturation is a result of γLA biosynthesis. Nevertheless, with the available information experiments were designed to identify and understand conditions which enhanced lipid/γLA production in *C. cucurbitarum* 12997, bearing in mind the final use of such conditions in large-scale submerged fermentations.

3.3 Materials and Methods

3.3.1 Fungal inocula

Initially the inoculum was prepared as in 2.3.2. For sporulation optimisation malt extract agar (Oxoid), nutrient agar (Oxoid), potato carrot agar and GYE agar were used (Appendix A). These agars were also supplemented with lactose, raffinose, maltose and thiamine (Barnett & Lilly, 1956).

The inoculum into shake flasks was 1 X 10^7 spores/200mℓ. A control fermentation was always included in groups of experiments so that the extra parameter change from mycelial to spore inoculum would not influence the experimental observations. Spores were prepared from 4d old sporulating mycelia grown on GYE agar plates. The spores were loosened from the plate with 10mℓ of sterile distilled water using a glass rod. The spore suspension was then transferred into a McConkey bottle and a portion of this was quantified microscopically using a haemocytometer.

For pilot plant inoculum build-up, the fungus was transferred from an agar plate to a shake flask to the Chemap fermenter and in some cases to the 180ℓ pilot plant prefermenter. The inoculum size at each stage (unless indicated) was 10%. Conditions for shake flask inocula were described in 2.3.2. For Chemap fermenter and pilot plant prefermenter inocula the mycelium was grown at 35°C for 16h. In the Chemap, agitation and aeration were 1,6m/s and 1vvm respectively whereas in the pilot plant the same were 5,5m/s and 0,35vvm. The growth medium for the inoculum was the same as that of the fermentation.

3.3.2 Fermentation conditions

Initially the molasses/cornsteep liquor medium (Kunihisa & Shimizu, 1969), was used. However, it became apparent through media trials that the basal medium (BM) with various carbon and nitrogen supplements was a superior medium under the conditions of these experiments. The basal medium was GBM without glucose (Appendix A.2). The GBM with Cl, Cp, peptone, SBM or yeast extract is referred to as GBM-Cl, GBM-Cp, GBM-P, GBM-S or GBM-Y respectively. The C:N ratio of the medium was (unless indicated) kept at 22:1 with carbon and nitrogen concentrations of 24,2g/ℓ and 1,1g/ℓ respectively. The nitrogen content of complex sources was determined by the Kjeldahl method (Florence & Milner, 1979). The total invert sugar of the molasses was measured by Gas Liquid Chromatography (Appendix B.3).

Fermentation conditions described in 2.3.3 (for shake flask and Chemap fermenters) and in 4.3.1 (for pilot plant) were used.

3.3.3 Monitoring of fermentations

The pH of shake flask media was determined using an Orion Research Model 701A/digital Ionanalyser. The pH in Chemap fermenters was measured *in situ* by an Ingold pH probe.

Total nitrogen in solution in the medium was determined by the Kjeldahl method. The glucose concentration was determined by the Merck GOD-PAP 3393 assay method.

3.3.4 Extraction and analysis

The conventional or DE methods were used for final oil analysis. Since the DE method had not been developed when the profile fermentations were run, the HP method (Dry, 1985) was used to analyse the oil changes during the fermentations.

3.4 Results

Shake flask experiments were used to screen various media for optimum production of γLA by *C. cucurbitarum* 12997. Certain media were further investigated under larger scale (Chemap) conditions with improved physical parameter control. The Chemap fermenters were also used for identifying the physical conditions needed for the optimum production of γLA by the fungus.

Parameters assayed were as in 2.4

- γLA as g/100g DM
- γLA as % of AFAs,
- biomass as DM g/ℓ
- γLA as mg/ℓ
- γLA as mg/g substrate also accounts for efficiency/cost effectiveness of raw material to product conversion. It is mentioned in experiments where the substrate concentration per litre medium varies considerably
- γLA mg/ℓ.h is the rate of γLA production (productivity)
- FA profiles which affect the downstream extraction and concentration of the γLA

The γLA mg/ℓ, γLA mg/g substrate and γLA mg/ℓ.h are the more important variables and guide economic calculations in the industrial process. The FA profiles indicate the quantities of FAs other than γLA present in the oil. In addition to complicating downstream concentration of γLA, the concentrations of these FAs may influence the metabolism of γLA when it is ingested for therapeutic reasons.

The results presented in this chapter were averages of duplicate fermentations and were therefore used as indicators of the conditions giving the best γLA yields. The results were not used to show statistically acceptable differences.

3.4.1 The fermentation medium composition

3.4.1.1 The carbon and nitrogen composition of the medium

The molasses/cornsteep liquor medium did not promote γLA production by *C. cucurbitarum* 12997. The fungus produced the most γLA in the GBM-Y, 77mg γLA/ℓ in shake flasks and 205mg γLA/ℓ in Chemap fermenters (Table 3.1). The (clarified molasses)BM-Y produced only 20mg γLA/ℓ medium. *C. cucurbitarum* 12997 also produced low γLA mg/ℓ in other complex carbon sources in BM-Y (Table 3.2). The final pH of the (sucrose)BM-Y was unusually high (Table 3.2). The increased %γLA in the biomass and in the AFAs was the main effect of glucose in the medium.

The fermentations in Chemap fermenters produced more γLA than fermentations in the same media in shake flasks (Table 3.1, Section B;Section A).

The highest γLA (mg/ℓ) and a good %γLA in the biomass was produced by *C. cucurbitarum* 12997 in GBM and mono-ammonium phosphate (MAP) (Table 3.3, Section A). In this fermentation the biomass (4g/ℓ) and the pH (2,85) were low. MAP and DAP media produced a higher %γLA in AFAs and less linoleic acid ($C_{18:2}$) lowering the

Table 3.1 Production of γLA by *C. cucurbitarum* 12997 in shake flasks and Chemap fermenters in industrial media (molasses/cornsteep liquor)

In Section A *C. cucurbitarum* 12997 was grown in 600mℓ of medium in 2ℓ conical flasks whereas in Section B the *C. cucurbitarum* 12997 grown in 5ℓ of medium in Chemap fermenters. The conventional extraction method was used to extract and analyse the oil in all experiments.

Medium		γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
Section A					
High test molasses Cornsteep liquor	(6,0% m/v total invert sugar) (2,5% v/v)	22	0,43	5,1	6,2
Clarified molasses Cornsteep liquor	(6,0% m/v total invert sugar) (2,5% v/v)	21	0,48	4,3	8,3
Unclarified molasses Cornsteep liquor	(6,0% m/v total invert sugar) (2,5% v/v)	19	0,38	4,9	6,9
Crop syrup Cornsteep liquor	(6,0% m/v total invert sugar) (2,5% v/v)	10	0,20	5,1	7,8
(Clarified molasses)BM-Y	(C:N 22:1)	20	0,52	3,9	8,3
GBM-Y	(C:N 22:1)	77	1,48	5,2	17,5
Section B					
Clarified molasses Cornsteep liquor	(5,0% m/v total invert sugar) (2,5% v/v)	59	1,00	5,9	15,5
GBM-Y	(C:N 22:1)	205	1,78	11,5	16,1

Table 3.2 The production of γLA by *C. cucurbitarum* 12997 in shake flasks in BM-Y with glucose, malt extract, sucrose or molasses

The fungus was grown in 2ℓ conical flasks in 600mℓ of BM-Y. The type of carbon source was varied. The C:N ratio of the medium was 22:1. The oil was extracted by the conventional method.

Carbon Source	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs	Final pH
Glucose	77	1,48	5,2	17,5	5,04
Malt extract	34	0,50	6,9	9,1	4,98
Sucrose	34	1,05	3,2	14,0	7,18
Clarified molasses	20	0,52	3,9	8,3	5,88

Table 3.3 Production of γLA by *C. cucurbitarum* 12997 in shake flasks in media containing inorganic nitrogen sources and urea combined with carbon sources of molasses or glucose

In Section A *C. cucurbitarum* 12997 was grown in 600mℓ of medium in 2ℓ conical flasks. The BM composition was used with various combinations of carbon and nitrogen sources to give a C:N ratio of 22:1. In Section B results are from 5ℓ scale-up fermentations with the best inorganic nitrogen source from Section A. The conventional method of extraction was used in all cases.

Carbon Source	Nitrogen Source	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs	Final pH
Section A						
Glucose	MAP*	104	2,59	4,0	13,2	2,85
Glucose	Yeast Extract	77	1,50	5,2	17,5	5,04
Glucose	DAP*	73	1,25	5,9	8,4	2,88
Glucose	Urea*	51	1,00	5,0	12,1	5,77
Glucose	Urea(acid ethyl ester complex)	-	-	-	-	-
Molasses	DAP	56	1,04	5,4	23,4	5,75
Molasses	Yeast Extract	48	1,15	4,2	18,2	4,41
Molasses	Urea	45	0,95	4,8	15,1	7,99
Molasses	MAP	20	0,33	5,9	6,3	4,41
Section B						
Glucose	Yeast Extract*	227	2,21	10,3	13,6	4,95
Sucrose	MAP*	113	1,06	10,7	29,4	4,00
Glucose	MAP*	107	1,94	5,5	17,3	3,04

Index terms

* - 0,1% yeast extract added as vitamin source

DAP - di-ammonium phosphate

MAP - mono-ammonium phosphate

Urea(acid ethyl ester complex) - residual urea from fungal lipid purification process; urea contained fatty acid ethyl esters 97% of which was the linoleic fatty acid ethyl ester

linoleic:γLA ratio. The high γLA yield with MAP was not produced in the scale-up fermentation (Table 3.3, Section B); the fungus produced more γLA in the yeast extract and biotin medium.

The addition of biotin to GBM-Y (Table 3.4) mainly improved the γLA content in the mycelium although there was also an increase in the biomass yield. The addition of biotin to GBM-Cp did not improve the overall γLA mg/ℓ yield, (S. P. Hanley, personal communication).

C. cucurbitarum 12997 in GBM-P produced the highest γLA (mg/ℓ) yield (Table 3.4). The dual cornsteep liquor/yeast extract medium did not improve the overall γLA yield. The %γLA in the extracted oil varied considerably in fermentations with different yeast extracts. This variation was different and not so marked in the %γLA in AFAs values.

C. cucurbitarum 12997 in shake flask experiments (Table 3.5) produced almost twice as much γLA in GBM-S (349mg/ℓ) than in GBM-Cp (187mg/ℓ). This yield improvement was in the range of that obtained in GBM-P. The γLA yield reached 674mg/ℓ in GBM-S in Chemap fermentations (Table 2.12). The FA αLA was present in GBM-S and absent from GBM-Cp (Fig.3.1). In GBM-S there was a higher linoleic:γLA ratio than in GBM-Cp. There was a resultant decrease in the %γLA in AFAs from 10,8% to 6,7% (Table 3.5). Combinations of SBM/Cp did not change the linoleic:γLA ratio (Fig.3.1) or produce better γLA yields than 349mg/ℓ (Table 3.5).

C. cucurbitarum 12997 grown in GBM and fully defatted SBM contained very little αLA in its AFAs (Fig.3.2). The %γLA in AFAs increased

Table 3.4 The effect of various organic nitrogen sources and biotin supplementation on the production of γLA by *C. cucurbitarum* 12997 in GBM in Chemap fermenters

C. cucurbitarum 12997 was grown in an average volume of 5ℓ medium in Chemap fermentations. Peptone (-P), cornsteep powder (-Cp), cornsteep liquor (-Cl) or yeast extract (-Y) were the nitrogen sources in GBM. Biotin in concentrations of 5µg/ℓ was added to fermentations with the lower γLA yields. The C:N ratio of the medium was 22:1. The conventional extraction method was used to extract and analyse the oil.

Media	Yeast Extract Type	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in oil	%γLA in AFAs
GBM-P	-	397	2,73	14,6	11,1	15,5
GBM-Cp	-	347	2,49	13,9	11,3	14,7
GBM-Cl	-	319	3,29	9,7	9,4	14,4
GBM-Y	Oxoid	246	2,00	12,3	12,0	15,5
GBM-Y	Bovril paste	215	1,69	12,7	6,5	13,7
*GBM-Y/Cl	Bovril paste	209	2,53	8,2	10,4	18,5
GBM-Y	Beechams paste	177	1,37	12,9	7,7	13,0
GBM-Y	Beechams powder	205	1,78	11,5	9,2	16,1
GBM-Y + biotin	Beechams powder	227	2,21	10,3	13,6	14,9
GBM-Y	Lab M	84	0,67	12,6	6,4	13,4
GBM-Y + biotin	Lab M	251	1,61	15,5	10,7	20,8

*GBM-Y,Cl - Bovril yeast paste contributed half the required nitrogen and cornsteep liquor the remaining half of the nitrogen content of GBM

Table 3.5 The production of γLA by *C. cucurbitarum* 12997 in GBM with various soyabean meal, cornsteep powder, yeast extract and ammonium sulphate combinations

C. cucurbitarum 12997 was grown in 200mℓ GBM in 1ℓ round flasks. The nitrogen sources to GBM (SBM, Cp, yeast extract and ammonium sulphate $(NH_4)_2SO_4$) were combined to give a final nitrogen of 1,1g/ℓ medium. The ratio of nitrogen source combination indicates the fraction of nitrogen supplied by each source. The resulting C:N ratio of the medium was 22:1. The oil was extracted and analysed by DE.

Nitrogen Source	Ratio of Nitrogen Source combination	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
SBM		349	2,27	15,4	6,7
SBM/Cp	3:1	195	1,44	13,5	5,3
SBM/Cp	2:1	252	1,76	14,4	5,7
SBM/Cp	1:1	261	1,84	14,2	6,2
SBM/Cp	1:2	185	1,39	13,3	5,5
Cp		187	1,57	12,0	10,8
SBM/Cp/yeast extract	3:2:1	235	1,78	13,2	7,0
SBM/Cp/yeast extract	2:3:1	209	1,64	12,7	6,9
SBM/Cp/yeast extract/$(NH_4)_2SO_4$	3:2:1:1	115	1,10	10,4	5,7
SBM/Cp/yeast extract/$(NH_4)_2SO_4$	2:3:1:1	114	1,02	11,2	4,5

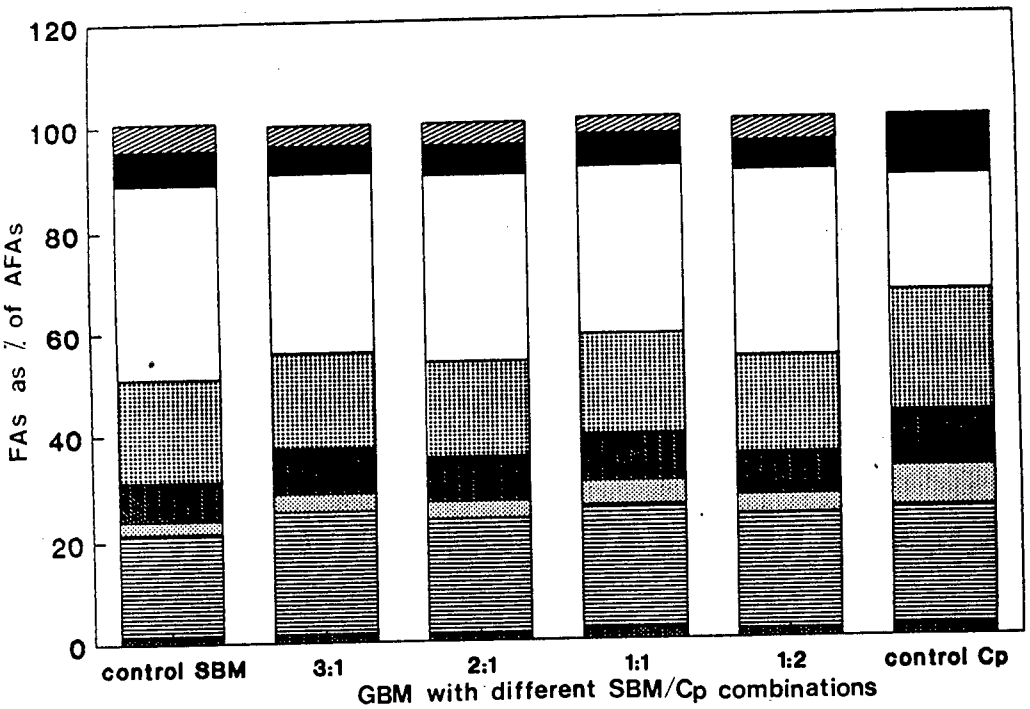

Fig.3.1 The effect of the nitrogen sources SBM, Cp and their combinations in GBM on the AFA proportions in *C. cucurbitarum* 12997.

The nitrogen sources were combined in the ratios (SBM:Cp) 3:1, 2:1, 1:1 and 1:2. The control experiments contained either SBM or Cp. The fermentations were in shake flasks (Table 3.5). The FAs as % of AFAs were: C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γLA, αLA

from 7,3 to 21% (Table 3.6) but the overall γLA yield of 205mg/ℓ was less than the 325mg/ℓ obtained from fermentations in GBM and ordinary SBM.

The fungus grown in GBM with cottonseed meal (Epic) or groundnut meal had no αLA and relatively good γLA (mg/ℓ) yields (Fig.3.2, Fig.3.3, Table 3.6). More γLA (mg/ℓ) was produced in the dual SBM/cottonseed meal or SBM/groundnut meal media than in the single cottonseed or groundnut media (Table 3.6). There was more %γLA in the AFAs in the media with the dual nitrogen sources than in the medium with SBM (Fig.3.3). Note that the media with the dual nitrogen sources also had less linoleic acid than the medium with SBM. The 280mg/ℓ of γLA in the SBM/cottonseed medium was, however, lower than the 325mg/ℓ of γLA in the SBM medium (Table 3.6).

The high fungal biomass obtained in fermentations with mustardseed meal and linseed meal contained seed fibre which contributed to the biomass (g/ℓ) values. The biomass yields of 32,5 and 23,9g/ℓ (Table 3.6) were therefore not true reflections of fungal growth. Olive waste had a low nitrogen content and therefore large quantities of the waste were included in the medium giving a biomass value of 200mg/ℓ which was not included in Table 3.6.

The best C:N ratio was between 20:1 and 25:1 in GBM-Y, GBM-C and GBM-S (Tables 3.7, 3.8). The more efficient conversion of glucose in GBM-S was at the C:N ratio of 18:1 and 25:1. As the carbon concentration was increased in GBM-Y (Table 3.7) the overall γLA yield increased from 190 - 212mg/ℓ. This was, however, at the expense of the glucose substrate (note the decrease of γLA mg/gGlucose from 4,52 to 2,36).

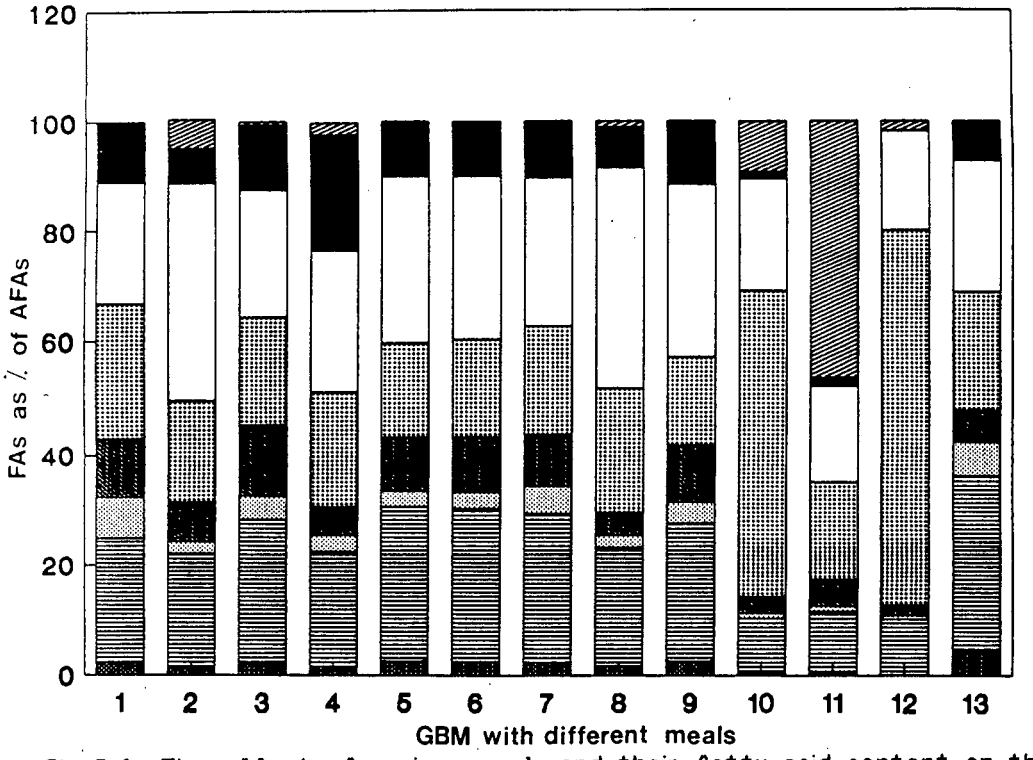

Fig.3.2 The effect of various meals and their fatty acid content on the AFA proportions in *C. cucurbitarum* 12997.

The nitrogen sources in GBM were 1 control Cp, 2 control SBM, 3 mildly defatted SBM, 4 fully defatted SBM, 5 cottonseed (Epic), 6 cottonseed (Nola), 7 groundnut, 8 maize, 9 sunflower, 10 mustardseed, 11 linseed, 12 olive waste and 13 fishmeal. The fermentations were in shake flasks (Table 3.6). The FAs as % of AFAs were: C14:0, C16:0, C16:1, C18:1, C18:2, γLA, αLA, C18:0

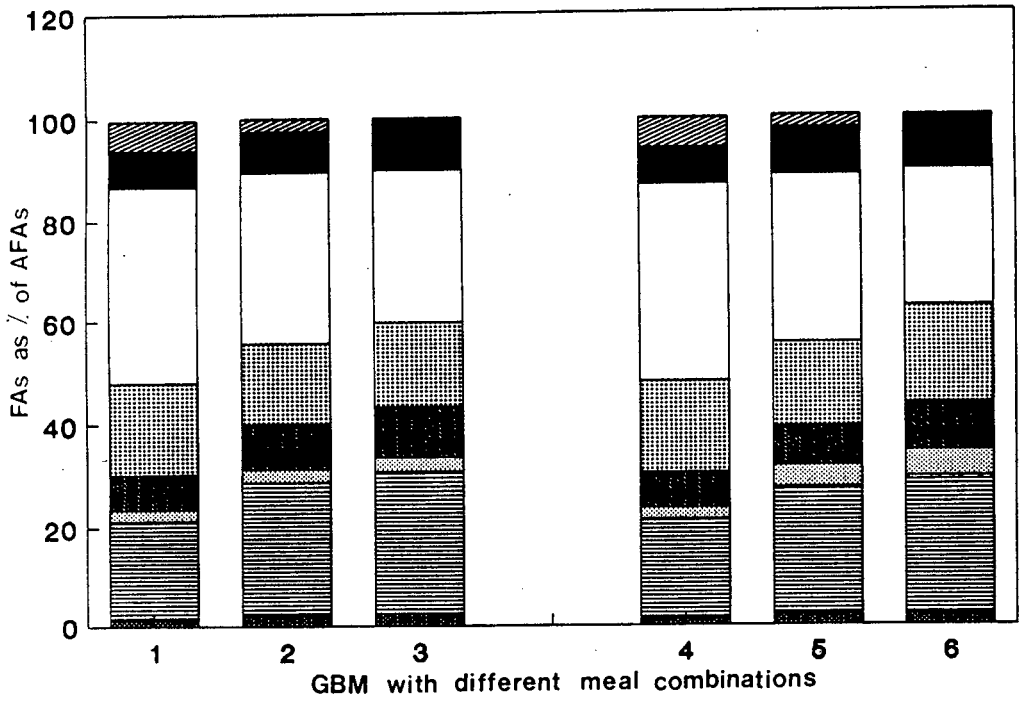

Fig.3.3 The effect of combinations of cottonseed, groundnut and SBM in GBM on the AFA proportions in *C. cucurbitarum* 12997.

The nitrogen sources were 1 SBM, 2 SBM/cottonseed (Epic), 3 cottonseed (Epic), 4 SBM, 5 SBM/groundnut and 6 groundnut. The fermentations were in shake flasks (Table 3.6). The FAs as % AFAs were: C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γLA, αLA

Table 3.6 The production of γLA by *C. cucurbitarum* 12997 in shake flasks in GBM with meals of soyabean, linseed, mustardseed, cottonseed, groundnut, maize, fish, and olive waste

C. cucurbitarum 12997 was grown in 200mℓ GBM in 1ℓ round flasks. The meal or meal combinations were added so as to give a final nitrogen concentration of 1,1g/ℓ medium and a resulting C:N ratio of 22:1. The results with SBM as a nitrogen source are averages of 7 fermentations, all other results are averages of duplicate fermentations and extractions. The oil was extracted and analysed by DE.

Nitrogen Source	Ratio of meal combination	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
SBM		325	2,10	15,5	7,3
SBM (mildly defatted)		292	2,21	13,2	12,1
SBM (fully defatted)		205	1,55	13,2	21,0
SBM/cottonseed (Epic)	1:1	280	1,71	16,4	8,1
cottonseed (Epic)		278	1,45	19,2	10,0
cottonseed (Nola)		230	1,04	22,1	10,0
SBM/groundnut	1:1	285	2,28	12,5	9,0
groundnut		252	1,92	13,1	10,3
maize		218	0,80	27,0	7,5
sunflower		182	1,55	11,8	11,5
mustardseed		*180	0,55	32,5	1,4
linseed		*168	0,70	23,9	1,7
olive waste		40	0,02	N/A	0,3
fishmeal		31	1,13	2,7	6,9

N/A - not applicable as fungal biomass could not be separated from olive waste

* - slightly overestimated as fungal biomass could not be separated from mustardseed/linseed particles

Table 3.7 The effect of the C:N ratio on the production of γLA by *C. cucurbitarum* 12997 in GBM-Y and GBM-Cp in Chemap fermenters

C. cucurbitarum 12997 was grown in 5ℓ GBM in Chemap fermenters. To alter the C:N ratio of the medium, the carbon concentration (glucose) was changed in the GBM-Y whereas the nitrogen concentration (Cp) was changed in the GBM-Cp. The conventional extraction method was used to extract and analyse the oil.

Medium	C:N	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	γLA mg/gGlucose
variation of [carbon]					
GBM-Y	14:1	190	1,62	11,8	4,52
GBM-Y	22:1	205	1,78	11,5	3,11
GBM-Y	30:1	212	1,45	14,7	2,36
variation of [nitrogen]					
GBM-Cp	22:1	343	2,54	13,5	5,20
GBM-Cp	39:1	280	2,35	11,9	4,24
GBM-Cp	55:1	225	2,01	11,2	3,41

Table 3.8 The production of γLA by *C. cucurbitarum* 12997 in GBM-S with different glucose and SBM concentrations, in shake flask fermentations

C. cucurbitarum 12997 was grown in 200mℓ GBM-S in 1ℓ round shake flasks. The oil was extracted and analysed by DE.

Medium	C:N	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	γLA mg/gGlucose
variation of [carbon]					
GBM-S	18:1	234	1,65	15,7	4,93
GBM-S	25:1	319	1,95	16,4	4,83
GBM-S	40:1	184	1,32	15,3	1,74
GBM-S	80:1	225	1,57	16,1	1,07
2 X [carbon]; 2 X [nitrogen]					
GBM-S	25:1	181	0,73	24,8	1,37

When the nitrogen concentration was decreased in GBM-Cp the γLA yield per litre and per gram glucose decreased. Note the higher biomass yield (24,8g/ℓ) in the medium with the double concentrations of carbon and nitrogen (Table 3.8).

3.4.1.2 Additives and supplements to glucose basal medium

The addition of calcium to the GBM-Cp increased the biomass to an average of 20g/ℓ (Table 3.9). The γLA (g/100g DM) was however, halved. The addition of Nutromix at 0,05g/ℓ improved the γLA yield from 161mg/ℓ to 182mg/ℓ but this was not as high as the GBM-S fermentation in Table 3.10. Sodium citrate did not increase the production of γLA by *C. cucurbitarum* 12997 (Table 3.9).

There was no difference between γLA production in GBM-S with or without the trace salt solution (Table 3.10). The addition of Gibberellic acid to GBM-S slightly lowered the γLA yield from 396mg/ℓ to 379mg/ℓ. Sodium acetate and $CuSO_4$ decreased the γLA yield to 218mg/ℓ and 291mg/ℓ respectively.

Inorganic Bevaloid 4241 and Silcolapse did not affect the γLA production by *C. cucurbitarum* 12997 (Table 3.11). None of the antifoams changed the %γLA in the AFAs.

Table 3.9 The effect of the additives Nutromix, calcium, and sodium citrate on the production of γLA by *C. cucurbitarum* 12997 in GBM-Cp

In Section A *C. cucurbitarum* 12997 was grown in 200mℓ GBM-Cp in 1ℓ round flasks. The oil was extracted and analysed by DE.
In Section B *C. cucurbitarum* 12997 was grown in 5ℓ GBM-Cp in Chemap fermenters. The oil was extracted and analysed by the conventional method.

Additive	Concentration g/ℓ	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
Section A					
No additive	-	161	1,20	13,4	6,0
Nutromix	0,05	182	1,31	13,9	5,8
Nutromix	1,00	173	1,17	14,8	5,6
Calcium carbonate ($CaCO_3$)	10,00	139	0,71	19,5	5,8
Calcium hydroxide ($Ca(OH)_2$)	10,00	126	0,62	20,4	5,4
Section B					
No additive	-	394	2,92	13,5	14,9
Sodium citrate	0,50	298	1,99	15,0	14,3

Table 3.10 The effect of the additives sodium acetate, copper sulphate and trace elements on the production of γLA by *C. cucurbitarum* 12997 in GBM-S in shake flasks

C. cucurbitarum 12997 was grown in 200mℓ GBM-S in 1ℓ round flasks. The medium marked GBM-S(no trace salts) is the GBM-S without the trace element solution. The oil was extracted and analysed by DE.

Medium	Additive	Concentration	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
GBM-S	-	-	396	2,74	14,4	8,3
GBM-S(no trace salts)	-	-	406	2,62	15,5	7,9
GBM-S	Gibberellic acid	4ppm	379	2,73	13,9	8,1
GBM-S	$CuSO_4$	50ppm	291	1,83	15,9	6,0
GBM-S	Na acetate	10g/ℓ	218	1,27	17,1	5,6

Table 3.11 The effect of various antifoams on γLA production by *C. cucurbitarum* 12997 in GBM-S

C. cucurbitarum 12997 was grown in 200mℓ of GBM-S with the various antifoams in 1ℓ round flasks. Experiments with Silcolapse antifoam have their own control as the spore inoculum differed. The oil was extracted and analysed by DE.

Antifoam	Quantity added g/ℓ medium	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
Control	(No Antifoam)	363	2,52	14,4	7,7
Chemspec	1,0	216	1,56	13,9	4,6
Chemspec	5,0	224	1,32	17,0	4,4
Struktol	1,0	245	1,93	12,7	5,7
Struktol	5,0	191	1,07	17,9	3,9
Bevaloid 4214 Organic	1,0	282	2,02	14,0	6,0
Bevaloid 4214 Organic	5,0	268	1,80	14,9	5,5
Bevaloid 4241 Inorganic	1,0	344	2,34	14,7	6,6
Bevaloid 4241 Inorganic	5,0	313	1,92	16,3	6,2
Control	(No Antifoam)	292	1,82	16,1	6,5
Silcolapse	0,5	282	1,82	15,5	6,9
Silcolapse	2,5	256	1,7	15,1	6,1

3.4.2 Physical parameters affecting γLA production

The pH tended to drop in the GBM-S medium (Table 3.12) but tended to rise half a unit in the GBM-Cp medium (Fig.3.5a,b). When the pH was controlled at pH 5,5 throughout the fermentation the γLA yield (346mg/ℓ) was less than that of the control fermentation (Table 3.12).

The biomass was increased from 6,8g/ℓ to 17,5g/ℓ as the fermentation temperature was increased from 25°C to 35°C (Table 3.13). The %γLA in the oil decreased from 10,2% at 25°C to approximately 8,0% at 30°C and 35°C. The yield of γLA in the DM also decreased from 1,99g/100g DM to 0,86g/100g DM at 25°C and 35°C respectively. The fermentation at 28°C produced the highest γLA yield of 205mg/ℓ. This yield was increased further to 236mg/ℓ in the dual temperature fermentation.

The combined effect of a 20% inoculum and a dual fermentation temperature can be seen if growth profiles Figs.3.4a and 3.4b are compared.

Table 3.12 The effect of pH on the production of γLA by *C. cucurbitarum* 12997 in GBM-S

In Section A *C. cucurbitarum* 12997 was grown in 200mℓ GBM-S in 1ℓ round flasks. The pH was adjusted with sulphuric acid (H_2SO_4) or sodium hydroxide (NaOH) prior to medium sterilisation.

In Section B *C. cucurbitarum* 12997 was grown in 10ℓ GBM-S in Chemap fermenters. The pH was kept constant at pH 5,5 with NaOH. In the control the pH was not adjusted.

The oil extraction and analysis was done by DE.

pH Initial	pH After sterilisation	pH After fermentation	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs
Section A						
4,0	4,1	4,1	173	1,02	17,0	3,4
5,0	5,2	4,6	203	1,32	15,4	4,9
5,5 (control)	6,0	4,6	322	2,07	15,6	7,1
6,0	5,9	5,5	249	1,49	16,7	5,8
7,0	6,3	6,1	254	1,53	16,6	5,9
Section B						
5,5 (control)	5,3	4,0	512	2,80	18,3	11,1
5,5	5,5	5,5,	346	1,16	29,8	5,5

Table 3.13 The production of γLA by *C. cucurbitarum* 12997 in GBM-Cp at different temperatures

C. cucurbitarum 12997 was grown in an average of 7ℓ GBM-Y in Chemap fermenters. In the profile experiment the temperature was 30°C for 48h and then 25°C for 25h. The oil was extracted and analysed by a modified conventional method. The only modification to the method was a single and not a double extraction step.

Temperature	Time h	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in Oil
25°C	96	135	1,99	6,8	10,2
28°C	96	205	1,78	11,5	9,2
30°C	96	167	1,05	16,0	7,9
35°C	96	151	0,86	17,5	8,0
Profile	96	236	1,91	13,9	12,3

3.4.3 The inoculum preparation and its affect on γLA production

The agar media which supported fungal growth were, from best to worse, malt extract, glucose yeast extract, nutrient and potato carrot. The agar media which supported sporulation were, from best to worse, glucose yeast extract, potato carrot, nutrient and malt extract. The addition of lactose, raffinose, maltose and thiamine to these media made no observable difference to the sporulation of *C. cucurbitarum* 12997. Room temperatures between 22°C - 25°C and a high humidity produced copious spores.

The aged mycelium which had been transferred 3 times had lower values of %γLA in AFAs (3,84%) and γLA in the biomass (0,72mg/100g DM) than the control (Table 3.14). The final γLA yield was greater in the control.

In shake flasks, Chemap fermenters and the pilot plant, an increase in the inoculum from 1% to 10% resulted in a 12% increase in productivity (Table 3.15). The higher initial fermentation temperature in *20% (Section B) would have increased the rate of production of biomass which, in addition to the 20% inoculum, would have increased the γLA productivity (*i.e.* less time was would be needed to produce a certain γLA yield as was the case in Fig.3.4b).

There was an increase in biomass, γLA mg/ℓ and the γLA mg/gGlucose when the fungal inoculum was grown in the Chemap fermenter (Table 3.16). These values were increased if the inoculum was grown in GBM-Cp with C:N 15:1. The use of the pilot plant prefermenter increased the biomass yield from 14,4mg/ℓ to 18,3mg/ℓ and the γLA

Table 3.14 The effect of the age of the inoculum on the production of γLA by *C. cucurbitarum* 12997 in GBM-Cp in shake flasks

The control 500mℓ GBM-Cp was inoculated with fungal spores and after 48h the mycelium (as 20%) was inoculated into 500mℓ fresh medium where the fungus was allowed to grow under normal fermentation conditions for 96h.

In the aged mycelium cultures, fungal spores of *C. cucurbitarum* 12997 were grown in 500mℓ GBM-Cp. After 48h 100 - 300mℓ of the mycelium/broth were transferred into 500mℓ fresh medium. This transfer was repeated after 60h and again after 48h. After the third transfer the fungus was allowed to grow under normal fermentation conditions for 96h. The oil was extracted and analysed by DE.

Type of mycelium	γLA mg/ℓ	γLA g/100gDM	AFAs g/100gDM	Biomass g/ℓ	%γLA in AFAs
Control	183	1,17	15,7	15,7	7,43
*Aged mycelium	139	0,72	19,1	19,3	3,84

* due to difficulty of mycelial inoculum handling on this small (500mℓ) scale the inoculum size was 30% and not 20% as for the control

Table 3.15 The effect of inoculum size on the γLA produced by *C. cucurbitarum* 12997 in GBM-Y and GBM-Cp at shake flask, Chemap or pilot plant level

In Section A, *C. cucurbitarum* 12997 was grown in 200mℓ in 1ℓ round flasks. The inoculum was in spore form. The oil was extracted and analysed by DE.

In Section B, *C. cucurbitarum* 12997 was grown in Chemap fermenters. The inoculum was grown in shake flasks. The oil was extracted and analysed by the conventional method.

In Section C, the fungus was grown in 1 800ℓ medium in the pilot plant. The 1% inoculum was grown in 18ℓ GBM-Cp in the Chemap fermenter whereas the 10% inoculum was grown in 180ℓ in the pilot plant prefermenter. The oil was extracted and analysed by DE.

The fermentation indicated *20% was grown at 35°C for 24h and at 30°C for 72h. All other fermentations in Sections A, B, C were at 30°C for 96h.

Inoculum size	Medium	γLA as mg/ℓ.h
Section A		
1 X 10^7 spores	GBM-Cp	0,65
1 X 10^8 spores	GBM-Cp	0,73
1 X 10^9 spores	GBM-Cp	0,83
Section B		
10%	GBM-Cp	2,57
*20%	GBM-Cp	2,91
Section C		
1%	GBM-Y	4,18
10%	GBM-Y	4,71
1%	GBM-Cp	7,41
10%	GBM-Cp	7,77

Table 3.16 Biomass and γLA yields of *C. cucurbitarum* 12997 after the inoculum was grown under different conditions

C. cucurbitarum 12997 was grown in GBM-P in the experiment marked with an asterisk(*). All other fermentations were in GBM-Cp. The fermentation volumes were: Chemap - 5ℓ, prefermenter - 180ℓ, pilot plant - 1 800ℓ. The biomass is given as DM. The oil was extracted and analysed by DE.

CONDITIONS OF CULTIVATION						BIOMASS AND γLA YIELDS			
Inoculum			Fermentation			Biomass		γ-Linolenic	
Grown in	C:N	Size	Grown in	C:N	Feed	(g/ℓ)	(mg/gGlucose)	(mg/ℓ)	(mg/gGlucose)
*Shake flask	22:1	10%	*Chemap	22:1	-	14,9	226	520	7,9
Chemap	22:1	10%	Chemap	22:1	-	19,5	295	584	8,9
Chemap	22:1	20%	Chemap	22:1	+	25,8	195	639	4,8
Chemap	14:1	20%	Chemap	22:1	+	30,1	228	744	5,5
Chemap	22:1	1%	Pilot plant	22:1	-	14,4	218	830	12,5
Prefermenter	22:1	10%	Pilot plant	22:1	-	18,3	277	886	13,5

yield very slightly (830mg/ℓ to 886mg/ℓ). The pilot plant prefermenter caused mycelial breakage and damage. The GBM-Cp feed increased the γLA yield from 584mg/ℓ to 639mg/ℓ. In fermentations where glucose was fed, the substrate to γLA conversion was not efficient (Table 3.16).

A decreased lag phase in the *C. cucurbitarum* fermentation profile (Fig.3.4b) was the result of a higher inoculum and an increased initial fermentation temperature. In Fig.3.4a the increase in biomass (DM g/ℓ) began after a 50h lag phase and was rapid up to 76,5h after which there was only a slight increase in biomass (stationary phase). At the onset of the stationary phase of growth at ±74h, the glucose and nitrogen concentrations were 50g/ℓ and 0,76g/ℓ respectively (Fig.3.5a). The amount of γLA (g/100g DM) increased until 92,5h after which it remained stable (Fig.3.4a). At 92,5h the glucose and nitrogen were at non-limiting concentrations of 34g/ℓ and 0,76g/ℓ respectively (Fig.3.5a). Linoleic acid ($C_{18:2}$) an immediate precursor of γLA was produced at the same growth stages as γLA (Fig.3.6a). Both oleic ($C_{18:1}$) and palmitic ($C_{16:0}$) levels continued to increase after 93,5h and leveled off at 110h. The levels of palmitoleic ($C_{16:1}$), myristic ($C_{14:0}$) and stearic ($C_{18:0}$) were less than 1g/100g DM during the entire fermentation. The highest γLA yield (440mg/ℓ) was produced at 131,5h and was equivalent to a productivity of 3,35mg/ℓ.h. A higher productivity of 4,2mg/ℓ.h was obtained at 92,5h.

In Fig.3.4b at the higher initial inoculum and temperature the lag phase was eliminated and at 67,5h the most γLA was produced. This increased the γLA productivity from 4,2mg/ℓ.h to 6,2mg/ℓ.h.

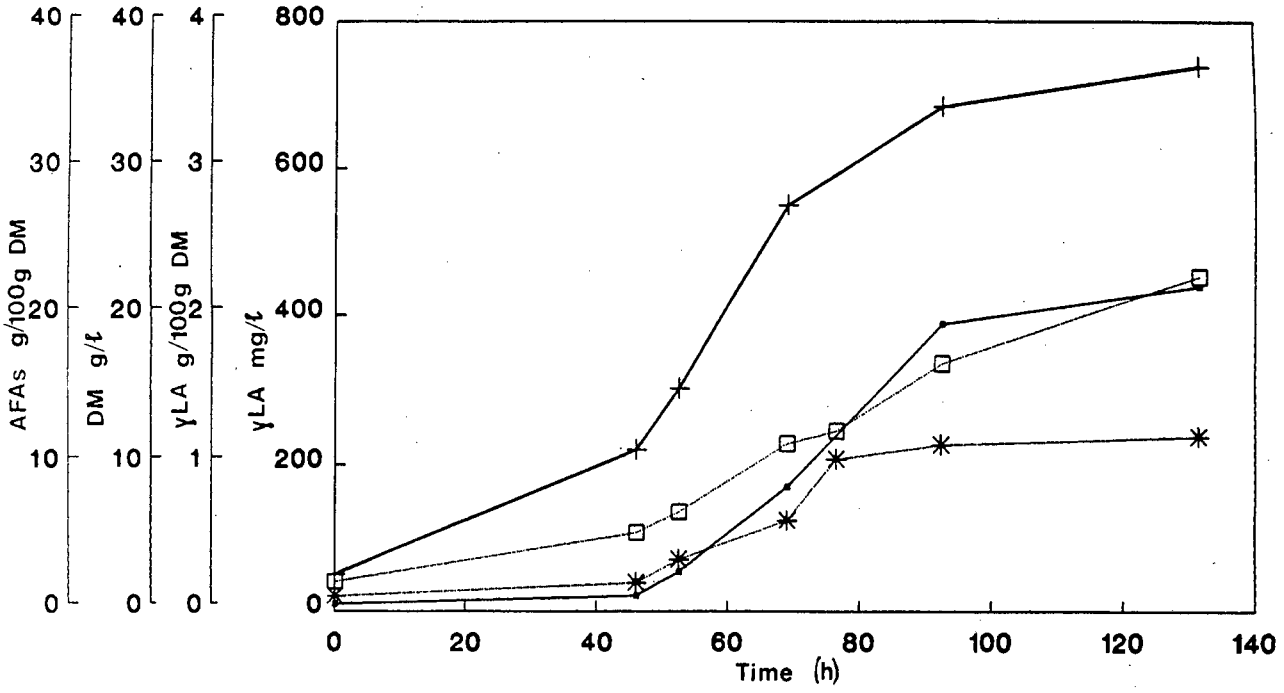

Fig.3.4a Profiles of growth and fat production by *C. cucurbitarum* 12997 in a 15ℓ Chemap fermentation inoculated with a 10% inoculum.

The fungus was grown in GBM-Cp at 30°C for 132h. Extraction and analysis of oil was by the HP method. The γLA, biomass and AFAs are indicated as:

γLA mg/ℓ —■— γLA g/100g DM —+— DM g/ℓ —✳— AFAs g/100g DM —□—

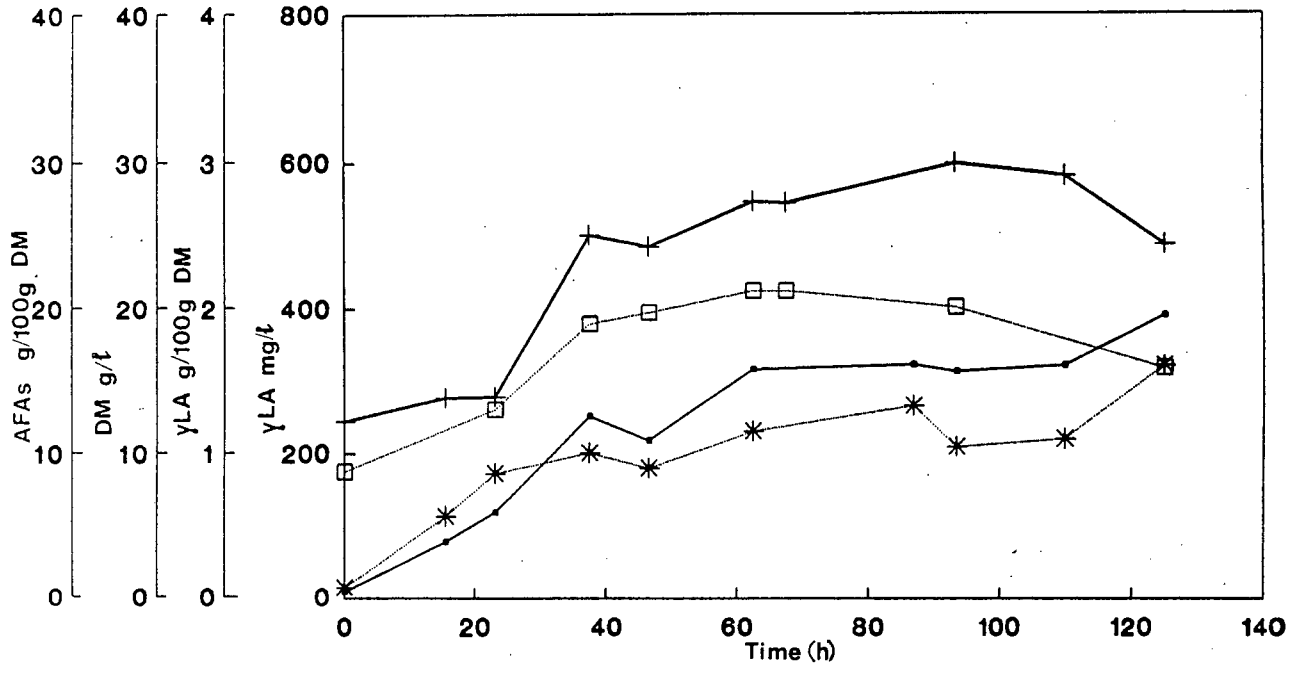

Fig.3.4b Profiles of growth and fat production by *C. cucurbitarum* 12997 in a 15ℓ Chemap fermentation inoculated with a 20% inoculum.

The fungus was grown in GBM-Cp at 35°C for 24h and at 30°C for 100h. Extraction and analysis of oil was by the HP method. The γLA, biomass and AFAs are indicated as:

γLA mg/ℓ —■— γLA g/100g DM —+— DM g/ℓ —✳— AFAs g/100g DM —□—

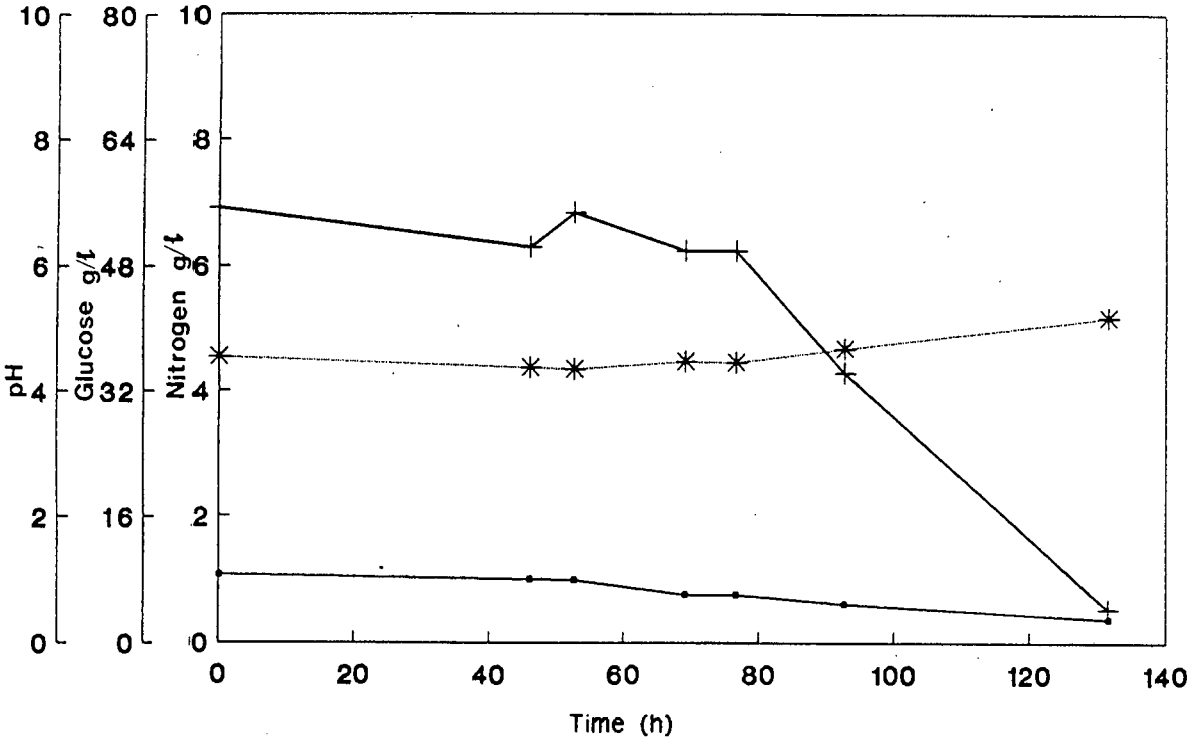

Fig.3.5a Glucose, nitrogen and pH levels during the production of γLA by *C. cucurbitarum* 12997 in a 15ℓ Chemap fermentation inoculated with a 10% inoculum.

The fungus was grown in GBM-Cp at 30°C for 132h. Nutrient and pH levels are indicated as: Glucose —+— Nitrogen —•— pH ···✳···

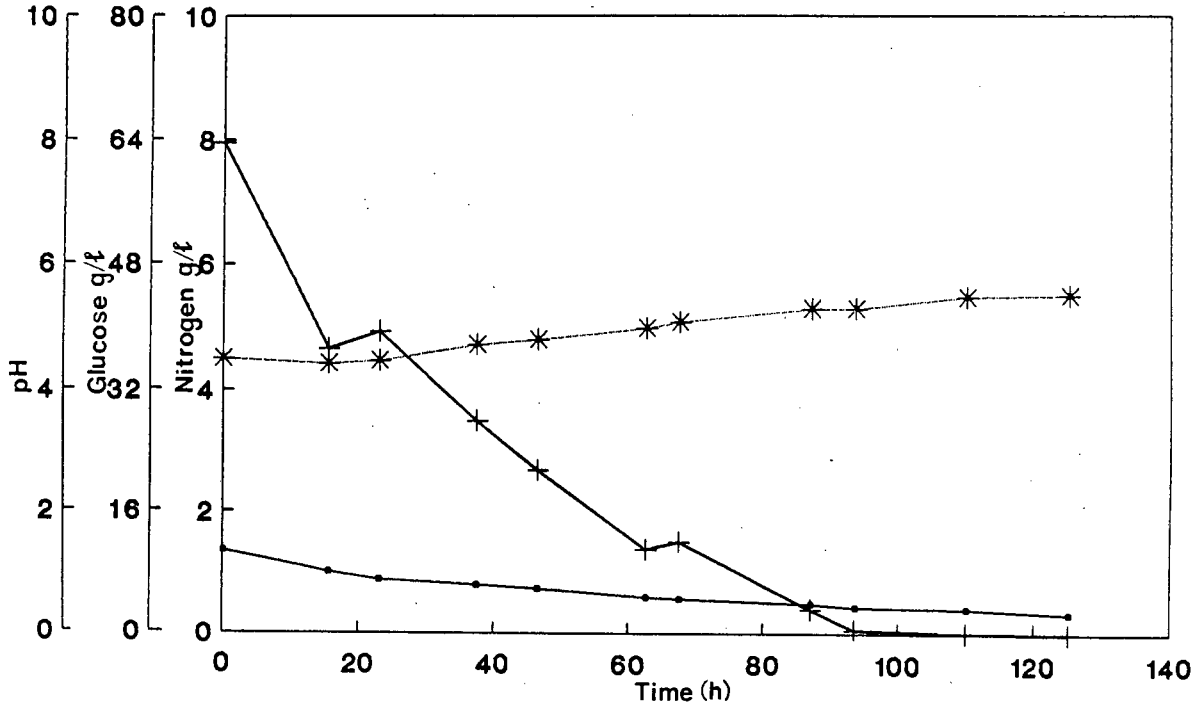

Fig.3.5b Glucose, nitrogen and pH levels during the production of γLA by *C. cucurbitarum* 12997 in a 15ℓ Chemap fermentation inoculated with a 20% inoculum.

The fungus was grown in GBM-Cp at 35°C for 24h and at 30°C for 100h. Nutrient and pH levels are indicated as: Glucose —+— Nitrogen —•— pH ···✳···

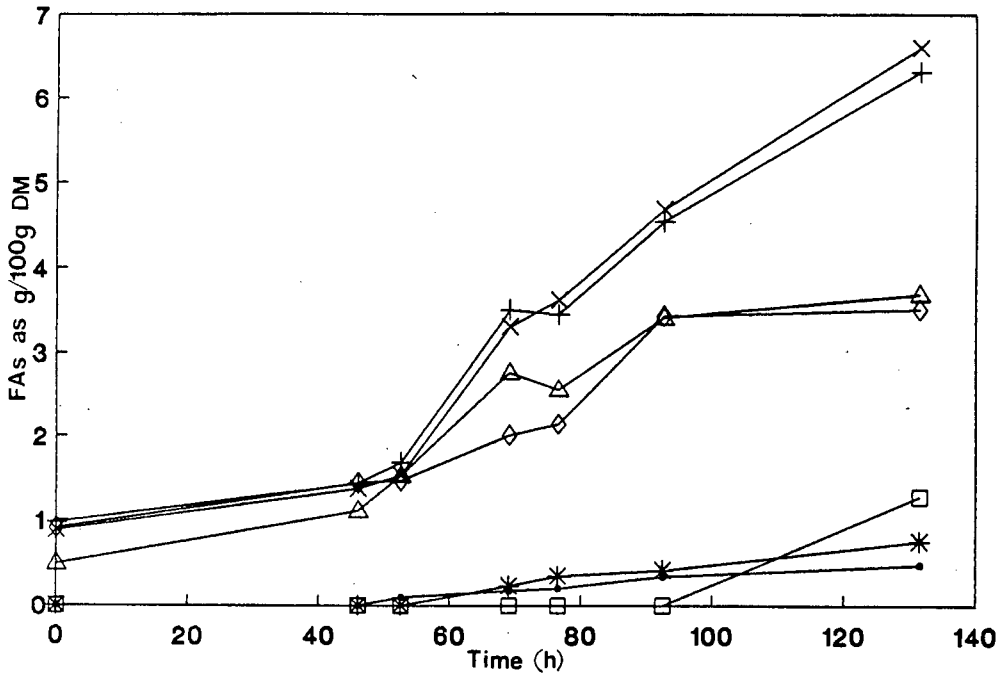

Fig.3.6a The concentration of fatty acids in *C. cucurbitarum* 12997 during a 15ℓ Chemap fermentation inoculated with a 10% inoculum.

The fungus was grown in GBM-Cp at 30°C for 132h. Extraction and analysis of the oil was by the HP method. The FAs analysed were:

C14:0 C16:0 C16:1 C18:0
C18:1 C18:2 γLA

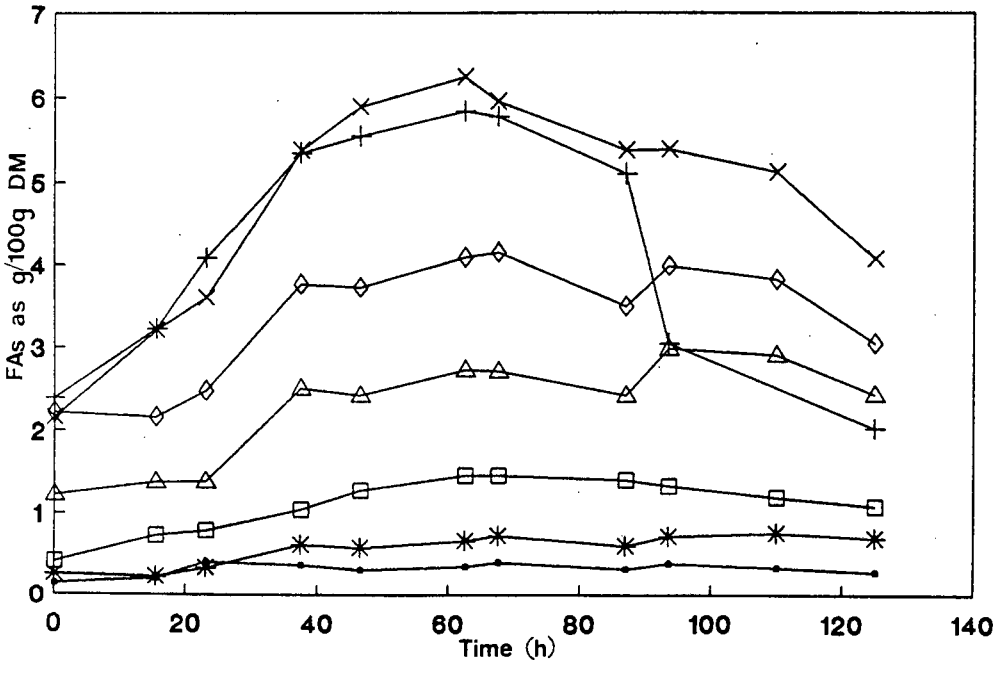

Fig.3.6b The concentration of fatty acids in *C. cucurbitarum* 12997 during a 15ℓ Chemap fermentation inoculated with a 20% inoculum.

The fungus was grown in GBM-Cp at 35°C for 24h and at 30°C for 100h. Extraction and analysis of oil was by the HP method. The FAs analysed were:

C14:0 C16:0 C16:1 C18:0
C18:1 C18:2 γLA

The productivity per g glucose was also higher in this fermentation even though the initial glucose concentrations in the two fermentations differed from 80g/ℓ to 55g/ℓ (Fig3.5b and Fig3.5a respectively). The amount of biomass increased for the first 30h after which the rate of increase became gradual. The glucose was used completely by 125h (Fig.3.5b). The nitrogen in the medium decreased from 1,35g/ℓ at the start to 0,48g/ℓ at 87h after which it remained at ±0,45g/ℓ. The pH of the medium decreased from 4,5 at 0h to 4,4 at 10h and then gradually increased to pH 5,5 at 125h (Fig.3.5b). The total amount of FAs produced reached a peak of 3,26g/100g DM at 67,5h. This corresponded to the peak production of oleic ($C_{18:1}$), palmitic ($C_{16:0}$), linoleic ($C_{18:2}$) and stearic ($C_{18:0}$) acids (Fig.3.6b). The concentrations of these FAs started to decrease from 67,5h onwards, when the nitrogen and glucose available for fungal assimilation was almost depleted or low. The greatest reduction of FA content was in the oleic ($C_{18:1}$) and palmitic ($C_{16:0}$) acids (Fig.3.6b). At that point the glucose concentration was 13,7g/ℓ.

At the end of both profile fermentations the biomass harvested through terylene netting was much higher than the biomass yields (g/ℓ) calculated from the 500mℓ sample. During the fermentation the ratio of fungal pellet to mat growth varied. The biomass values in Figs.3.4a and 3.4b were therefore not absolute but sufficient to give an indication of the production of γLA by the fungal biomass in suspension.

3.5 Discussion

Both the quantity and the composition of the microbial fat were changed considerably by culture conditions and by the composition of the medium. The improved analysis procedure (DE) also improved the γLA yield from the biomass. Experiments showed an increase in the shake flask γLA yield from 21mg/ℓ (Table 3.1) to 396mg/ℓ (Table 3.10). In the Chemap fermentations the increase was from 59mg/ℓ (Table 3.1) to 512mg/ℓ (Table 3.12) and with a fed-batch fermentation the γLA yield was 744mg/ℓ (Table 3.16). In the latter fermentation, however, the carbon source was not utilised efficiently. The conditions of growth giving the highest and economical γLA yields were GBM-S at a C:N ratio of 22:1, with no pH control. A dual fermentation profile system of 35°C for 24h followed by 30°C for 72h supported good growth and γLA production. In addition to the high yields the above conditions gave a growth morphology of 1mm diameter pellets which improved mass transfer in the fermentation, aided downstream processing of the mycelium and made extraction more effective (Section 4.5.2).

The molasses/cornsteep liquor medium as used by Kunihisa & Shimizu, (1969) did not produce good γLA yields because *C. cucurbitarum* 12997 was inhibited even by clarified molasses. Molasses contains glucose, fructose and sucrose which in that order are preferentially used by most fungi. The fungal preference for glucose was shown by the higher γLA yields in GBM-Y and not in (molasses)BM-Y (Table 3.1).

As reported by Weete (1974) the inorganic nitrogen sources (MAP and DAP) gave good γLA and FA yields with low biomass. These γLA

yields were not, however, obtained on a larger scale where the organic nitrogen sources supported better growth and overall better γLA yields (mg/ℓ).

The order of best to worst organic nitrogen sources were SBM, peptone, Cp and yeast extract. The cost and availability of SBM were also more reasonable than those of the other organic nitrogen sources. The active ingredients in SBM for increased γLA yields may have been nutritional and/or physical. In the SBM and peptone media the pH dropped half a unit whereas with Cp and yeast extract the pH rose half a unit. The different nutrients in these media may have caused different metabolites to be produced which lowered or increased final pH. The physical effect on γLA production may also have been a result of the different gas transfer qualities of the media. The GBM-S and GBM-Cp had large and fine insoluble particles respectively whereas GBM-P and GBM-Y formed clear solutions. Axcell *et al.* (1988) suggested that commercial yeast nutrients and SBM, because of their irregular particulate nature, provide nucleating sites for CO_2 bubble formation. As a result of this the inhibitory effect of CO_2 is lessened. The GBM-S was particulate at the start of the fermentation when O_2 was required for growth. After 18h when CO_2 as well as O_2 were needed for increased fat formation and desaturation (Fig.3.6b) the medium contained no particles.

Use of different yeast extracts resulted in different γLA yields. The γLA yields with the poorer yeast extracts were improved by supplementation with biotin. Even though Beechams yeast extract did not produce the best γLA yield it was used in all further experiments as it was the cheapest of all the yeast extracts. The

increased γLA production in GBM-Y supplemented with biotin was not repeated in GBM-Cp supplemented with the same vitamin (S. P. Hanley, personal communication). Hanley found that vitamins such as thiamine, calcium pantothenate and inositol also had no effect on γLA yields in GBM-Cp. Trace elements such as vanadium, nickel, tin, chromium, chloride and sodium had no significant effect on γLA production. Iron, zinc, copper and manganous sulphates had a detrimental effect on the final γLA yield (S. P. Hanley, personal communication).

Various changes to GBM-Cp (*i.e.* C:N ratio, Cp/SBM combinations, supplementation) were done in an effort to increase the *C. cucurbitarum* γLA levels in the GBM-Cp to levels obtained in GBM-S. The preference for GBM-Cp was because Cp did not introduce contaminating FAs to the fungal oil whereas SBM in GBM-S introduced a high percentage of linoleic and αLA in the AFAs (making γLA purification and concentration difficult). Although calcium in various forms increased biomass production and the commercial yeast food Nutromix increased the overall γLA yield from 161mg/ℓ to 182mg/ℓ, these increases never reached levels obtained in GBM-S (Table 3.9). The positive yields of γLA in GBM-S merited further work in this medium, even though SBM introduced problems with contaminating FAs.

The FA composition of the meals exerted substantial influence over the composition of the fungal oil (Fig 3.2). In most cases the proportions of the FAs in the fungus were a reflection of the actual proportions of these FAs in the meal. For example SBM and linseed in their AFAs have high contents of 55,5% linoleic acid ($C_{18:2}$) and 56% αLA respectively (Epic Oil Mills (PTY) LTD., personal communication). These FAs remained in the fungus and were present in the oil extracted

from the fungus. Purification of the oil for the concentration of γLA would be necessary as certain concentrations of FAs of the ω-3 family (αLA) affect the human metabolism of FAs from the ω-6 family (Beare-Rogers, 1988). The purification of the oil can be complicated by the chemical similarity of the FAs such as αLA and γLA. Exogeneous FAs not only complicate the purification and concentration of the oil, they also inhibit γLA production by the fungus. When the percentage αLA in AFAs was high the percentage linoleic acid ($C_{18:2}$) or oleic acid ($C_{18:1}$) was high (Fig.3.2 compare SBM, mustardseed to fully defatted SBM). This could have been due to inhibition of desaturation of linoleic acid ($C_{18:2}$) to γLA. Delta-6-desaturase, the enzyme catalysing this reaction, recognises both linoleic acid ($C_{18:2}$) and αLA ($C_{18:3}$) as substrates for further desaturation. Beare-Rogers (1988) reports that αLA has the competitive advantage over linoleic acid for desaturation.

Fatty acids not in the triglyceride form (the form normally found in meals) inhibited the fermentation. Dry (1985) reported that when linoleic acid was added to the medium it had a negative effect on growth and γLA production. The imiscibility of linoleic acid in the liquid medium would have affected efficient gas transfer. The result with urea(acid ethyl ester) complex (Table 3.3), may also have been due to the imiscibility of the free FAs. The complex could also have been toxic. The complex was a by-product of FA purification and concentration by urea complexing. The presence of free FAs as esters should not have been toxic to the fungus as they have been found to be incorporated into a wide variety of yeasts with considerable increase in the lipid content of the cells (Ratledge, 1982). Heat sterilisation could, however, have degraded urea into toxic forms.

The mildly defatted SBM did not affect the γLA production as badly as the fully defatted SBM. The fully defatted SBM had a powder consistency whereas SBM and mildly defatted SBM were more particulate. The similar γLA yields of SBM and mildly defatted SBM may have been a result of their particulate similarity (allowing for nucleation sites for CO_2). Most other meals had particles which differed from SBM and which, in the case of olive waste, linseed and mustardseed, could not be separated from the fungal biomass giving erroneous biomass results (Table 3.6). The lower γLA yields in fully defatted SBM may have also been a result of essential nutrient loss during steaming in the oil extraction process.

Some of the antifoams would have decreased oxygen transfer in the medium which affects FA desaturation and results in lower γLA yields (g/100g DM) (Table 3.11). Inorganic Bevaloid 4241 or Silcolapse should be used for foam control as they did not affect γLA yields.

It was found that in the Chemap profiles (Fig.3.6a,b) the proportion of fatty acids in AFAs varied during the fermentation. The reason for this may have been the changing gas transfer rate in the medium due to fungal growth. Fatty acid composition, however, has also been known to be affected by changes in the growth rate of the organism and by the concentration of substrate in the medium. In batch culture the fungal growth rate and the substrate concentration would be continually changing. It therefore follows that the FAs would change in their relative amounts. The use of batch culture as a biochemical tool for understanding various controls on γLA production is limited as any effects on growth rate will change the lipid composition. One needs

the constant growth rate of continuous culture for such an exercise. The purpose of this thesis was, however, the development of industrial conditions for improved γLA production and therefore less emphasis was placed on the understanding of lipid anabolism.

The main physical parameter which affected the production of γLA by *C. cucurbitarum* 12997 was temperature. This could have been a dual effect of temperature and dissolved O_2 availability. At higher temperatures there was a decrease in the amount of soluble O_2. This would happen especially towards the end of the fermentation when the mycelial biomass had increased the viscosity of the broth. The higher temperatures therefore increased growth but inhibited FA desaturation especially at the end of the fermentation (there was a lower unsaturate:saturate ratio in the final fungal oil). For *C. cucurbitarum* 12997 inoculum preparation and growth production, a temperature of 35°C should be used. For the fungus to produce a higher γLA yield in the biomass, the temperature should be decreased to between 25°C and 30°C.

The control of the pH at 5,5 with NaOH decreased γLA yields (Table 3.12). Herbert & Keith (1985) controlled the pH of a *Rhizopus* fermentation at pH 6 ± 0,2. In shake flasks the highest γLA yield was obtained at pH 5,5 which was the normal pH of GBM-S. When the pH was kept constant at pH 5,5 γLA production was not ameliorated.

The physiological state and size of the inoculum were important factors in ensuring good growth and γLA production. Initially the inoculum from an agar plate was a mixture of mycelia and spores and varied depending on the growth of the fungus. When the conditions for

sporulation were optimised using GYE medium the inoculum for GBM-S was a spore suspension. The larger the inoculum (in mycelial or spore form) the higher the γLA yield obtained (Table 3.15). This was not, however, the case with the aged mycelium (Table 3.14) where the inoculum was larger than that for the control but the γLA yield (139mg/ℓ) was less than the control (183mg/ℓ). The number of generations needed in the fermentation were therefore important. In the larger spore or mycelial inoculum it would take fewer cell divisions/generations to reach an important fermentation stage whereas with the lower inoculum more cell divisions/generations would be needed to reach that stage. The growth of the inoculum in a medium supporting growth (*i.e.* C:N of 14:1; temperature 35^{0}C; good aeration in Chemap) also gave better yields (Table 3.16). Good growth together with the age of the culture would influence the nutrient balance in the fermentation medium which in turn would control fat synthesis. In the exponential phase, growth and fat accumulation in *C. cucurbitarum* 12997 occurred simultaneously (Figs.3.4a,b). In the exponential phase, however, the accumulation of γLA (g/100g DM) was not concurrent with the increase in biomass (Fig.3.4a,b). The lag phase in the production of γLA may be decreased by a young (24h), high (20%) inoculum.

The effect of inoculum size on γLA production was again observed in the pilot plant fermentation where a 10% inoculum gave better yields than a 1% inoculum (Table 3.16). There are however engineering limitations on the inoculum size in a scale-up greater than the 1 800ℓ pilot plant. In further scale-up, a 20% mycelial inoculum would be the maximum size allowed. Under such limitations a concentrated spore inoculum could be used as an alternative to the

mycelial inoculum. The reasons for mycelial breakage and damage in the prefermenter are discussed in Chapter 4.

The profiles of the yLA fermentation (Figs.3.4, 3.5 and 3.6) not only showed the dramatic increase in yLA productivity with temperature and inoculum changes but also gave an understanding of the glucose and nitrogen utilisation patterns. These are important for the design of feeding patterns in a fed-batch or continuous culture. In Fig3.6b the decrease in oleic acid ($C^{18:1}$) and linoleic acid ($C^{18:2}$) concentrations began when the glucose concentration was 13,7g/ℓ. For a fed-batch fermentation one needs to determine whether the utilisation of oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:0}$) could be prevented by levels of glucose kept above 13,7g/ℓ. If so the further desaturation of these FAs could result in increased yLA yields. At the end of the fermentation (Fig.3.5b) glucose was depleted whereas nitrogen remained at 0,45g/ℓ.

The manipulation of culture conditions and the improved oil analysis gave an overall 10-fold increase in the yLA yield. Future improvements should concentrate on modifications of the batch fermentation. Controlled glucose feeding should be optimised and a form of continuous culture should also be investigated.

CHAPTER 4

PRELIMINARY PRODUCTION AND EXTRACTION OF γ-LINOLENIC ACID ON PILOT SCALE

CHAPTER 4

PRELIMINARY PRODUCTION AND EXTRACTION OF γ-LINOLENIC ACID ON PILOT SCALE

4.1 Summary

Preliminary pilot plant trials were undertaken to evaluate the fermentation and product recovery of γLA using *C. cucurbitarum* 12997. The overall increase in the γLA yield from the shake flask to the pilot plant stage was 60-fold from 21mg/ℓ (0,2mg/ℓ.h) to 966mg/ℓ (11,9mg/ℓ.h). The yield of γLA per gram substrate (14,7mg/gGlucose) was comparable to published data. Freeze-drying was the best means of preserving the fatty acid content of the mycelium. There was, however, a marked decrease in the unsaturated fatty acids upon storage of the dried mycelium. The batch-countercurrent system extracted the most oil from milled dry mycelium.

4.2 Introduction

Scale-up is the transfer of a process to a larger capacity. It is difficult to simulate laboratory conditions at industrial scale so further experimental work is usually required on an intermediate pilot plant level. This work is also needed so that process data and economics can be evaluated in detail. These include power consumption, mixing requirements, heat transfer, unit cost of product and its output rate. These factors in turn govern economic decisions. For this reason and because the industrial environment is competitive, time is always a critical factor and governs decisions regarding experimental design.

4.2.1 Fermentation on pilot scale

In the recently reported production of γLA by a fungus, the organism has been in dispersed form in a shake flask or stirred tank reactor. Fukuda & Morikawa (1987), however, have reported early work on the immobilisation of *Mucor ambiguus* in reticulated foam biomass support particles. *M. ambiguus* IFO 6742 was reported to have a relatively good coefficient yield of γLA per gram Glucose (14,2mg/gG) and a γLA productivity of 17,1mg/ℓ.h (Table 2.1). Fukuda & Morikawa (1987) also claim lipid secretion by the cells into the broth and/or onto the surface of the cell wall without any damage to the fungus. This assists the purification of γLA. The batch immobilisation system for γLA production has potential but was not investigated. The available pilot scale facility for work in this thesis, was a 2kℓ stirred tank reactor. Parameters which affect the effective use of such a vessel are introduced in this section.

Some of the factors which govern the successful design of a fermenter are maintenance of sterility, oxygen transfer to the cells and heat removal from the culture broth. Oxygen and heat transfer are interrelated in that a high rate of oxygen consumption results in increased heat production which necessitates more rapid heat removal.

Fermentations have been predominantly carried out in the batch mode in a stirred vessel. Stirring transfers energy, disperses gas bubbles, separates and mixes particles. Microbial cultures can be agitated mechanically, pneumatically and hydraulically. In each case different forces are generated which can affect the microorganism in various ways. The amount of agitation can influence fungal pellet formation and therefore the pellet size. The pellet can either break-up or have a smaller diameter and will be affected differently depending on the culture age (Märkl & Bronnenmeier, 1985). Some mycelial hyphae can adapt by becoming shorter and tougher when the stirring speed is increased. The extraction of the oil would be less efficient from tougher cells. Air agitation can be used in solutions with low viscosity to avoid the complexities introduced by mechanical agitation and to diminish any shear stress on the microorganism. This has been recommended by Solomons (1985) for certain *Mucor* spp which are sensitive to mechanical shear. Suzuki & Yokochi (1988) used air agitation in the production of γLA by *M. vinacea*.

With the above and laboratory scale knowledge the production of γLA by *C. cucurbitarum* 12997, was scaled-up a hundred times so as to understand the requirements for its successful production industrially.

4.2.2 Oil extraction on pilot scale

Extraction of oil in general has two main pitfalls: 1)incomplete extraction and 2)the formation of free FAs or transesterified FAs during the extraction process. Both these FAs can be toxic to certain forms of life.

The extraction rate is affected by the rate at which equilibrium can be attained between the lean solvent/oil mixture (miscella) on the outside of the oil containing particles and the inside of these particles. The viscosities of the miscella and the particles, the size and shape of the particles, the ease of penetration of the solvent and the solubility of various FAs in the solvent influence the rate at which equilibrium will be attained in the system.

The solvent for extraction must be safe from a fire or explosion standpoint and non-toxic in handling. The solvent choice is naturally governed by the polarity and location of the triglycerides which are to be extracted. If the FA is found in the polar triglycerides a more polar solvent such as chloroform will extract this FA successfully. Dry (1985) found most of the γLA in the neutral FAs and very little in the more polar phospholipids and glycolipids. A chloroform:methanol mixture in the ratio of 1:1 was found to extract the most γLA from the mycelium of *C. cucurbitarum* 12997 (National Chemical Products (NCP), personal communication). The less polar acetone was, however, found to be as effective as chloroform:methanol once the mycelium had been dehydrated by acetone initially. Since acetone is a safer solvent it was used in the semi-countercurrent extraction experiments.

For the large scale extraction of oil, the quantity of solvent used must be kept to a minimum to avoid energy costs for eventual recovery of the solvent (Norris, 1982). The solvent is usually reused until equilibrium is established between the oil content of the solid material and that of the solvent. Extraction is therefore carried out in a batch countercurrent or continuous countercurrent system. In this system the solvent flows in an opposite direction to the oil containing phase. At the end of the extraction the solvent containing oil (miscella) is brought out of the system at a uniform high oil content.

Extraction of lipid from a microbial cell is known to require some disruption of the cell. Methods include acid or alkali treatment, passing through the French press or ultrasonic treatment and freeze-drying or oven-drying (Ratledge, 1982). Various methods of cell disruption had been tried at NCP to improve solvent contact with the oil. The acid pretreatment of the mycelium increased the γLA extraction 1,6 times (NCP, personal communication). Acid, however, hydrolyses triglycerides forming the more toxic free FAs and it therefore can only be used for analysis purposes (Ratledge, 1982). Maceration and sonication of wet mycelium were also shown to increase the extractability of γLA but large scale facilities were not available (NCP, personal communication). Enzyme digestion of the cell wall was shown to be lengthy and expensive.

The γLA had to this stage been extracted from the mycelium on a laboratory scale for analysis purposes only. Pilot scale extractions were therefore necessary to direct future developments in the downstream processing of the γLA fermentation.

4.3 Materials and Methods

4.3.1 Fermentation on pilot scale

The initial stages of the inoculum were prepared as in 2.3.2. The 18ℓ Chemap stage was grown under conditions described in 2.3.3 for 48h and was transferred aseptically to two 10ℓ containers which were used to inoculate the pilot plant prefermenter or the main fermenter. The media composition of the seed stages was the same as the final stage. There was no pH adjustment or control in the fermentation. Foaming was controlled by a sensor relay which activated a pump to add a 1:2 Bevaloid 4214:water solution.

The pilot plant facilities used are diagramatically represented in Fig.4.1. During the growth cycle in the prefermenter and the fermenter the culture was continuously stirred with the impeller tip speed being 5,7m/s and 5,5m/s respectively. The culture was aerated with sterile air at ± 0,35vvm.

4.3.2 Extraction of oil on pilot scale

After filtering through bags made of terylene netting, the water content of the biomass was ±30%. This mycelium was then spread on a double layer of terylene netting and air-dried in fumehoods or with fans keeping the air in good circulation. A 1952 Secfroide model with six plates was used for freeze-drying 12ℓ quantities of moist mycelium. The dried mycelium was either left in 1-2cm lumps or milled using a hammer mill.

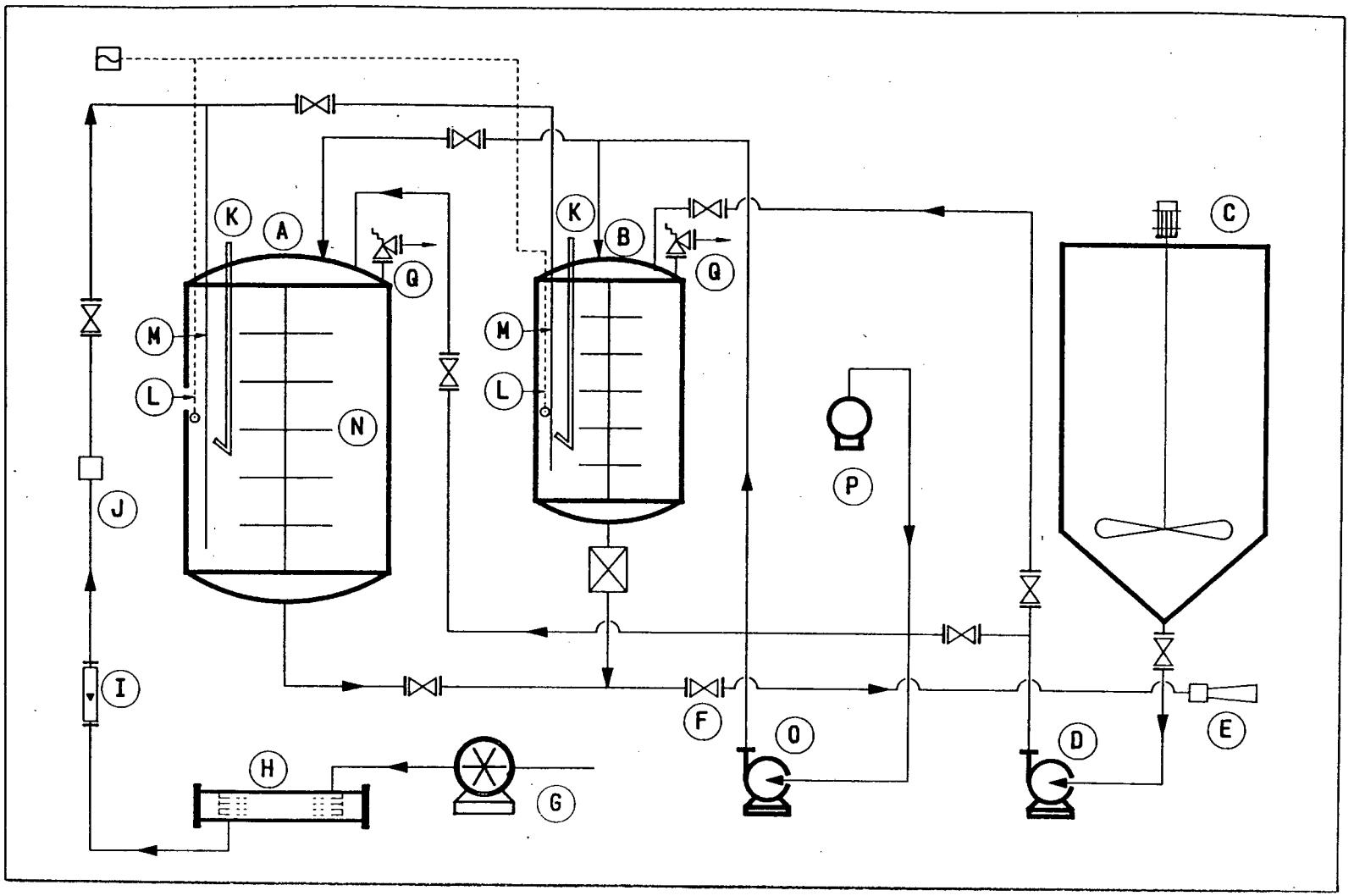

Fig.4.1 Diagramatic representation of fermentation pilot plant.

Parts indicated as: **A** - fermenter, **B** - prefermenter, **C** - mixing tank **D** - transfer tank, **E** - ejector, **F** - valve, **G** - compressor, **H** - condenser, **I** - rotameter, **J** - air filter, **K** -inoculation port, **L** - antifoam sensor, **M** - air sparger, **N** - rotating impellers, **O** - antifoam pump, **P** - antifoam container, **Q** - pressure release valve

4.3.2.1 Semi-countercurrent extraction

The column (Fig.4.2) (10ℓ) was packed with 8kg air dried mycelium. Acetone was allowed to flow by gravity at approximately 6ℓ/h through the bottom of the column. The oil rich solvent was collected from the top of the column. The extraction was considered complete when the solvent had no residue on evaporation.

4.3.3 Sampling and analysis of oil

One litre samples were taken at various intervals during the fermentation and analysed in duplicate by the conventional method for Runs 1, 2 and 3 and by DE for Runs 4, 5, 6 and 7.

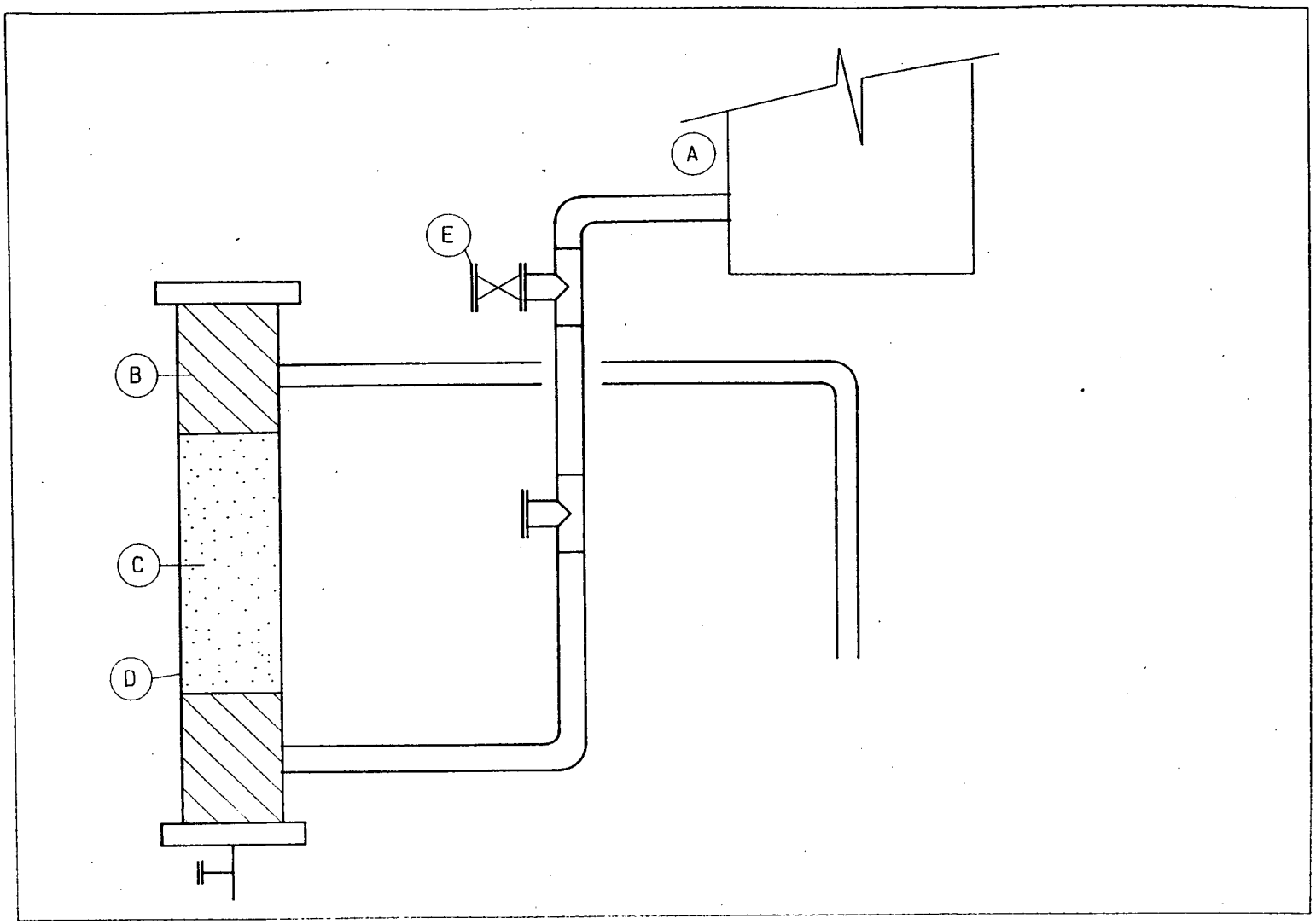

Fig.4.2 Column used in the semi-counter current extraction of oil from dry mycelium.

Parts indicated as: **A** - solvent holding tank, **B** - glass wool, **C** - mycelium, **D** - extraction column, **E** - valve

4.4 Results

Parameters assayed were as in 3.4

- γLA as g/100g DM
- γLA as % of analysed fatty acids (AFAs),
- biomass as DM g/ℓ
- γLA as mg/ℓ
- γLA as mg/g substrate
- γLA as mg/ℓ.h

Emphasis in this chapter was placed on γLA mg/g substrate and mg/ℓ.h.

4.4.1 Fermentations on pilot plant scale

C. cucurbitarum 12997 produced the most γLA mg/ℓ.h in Run 7 in GBM-S (Table 4.1). The increase in the γLA curve (mg/ℓ) was sharper in Runs 6 and 7 than in Runs 4 and 5 (Fig.4.3). There was also a marked decrease in the lag phase of the profiles of γLA (g/100g DM) and DM (g/ℓ) in Runs 6 and 7. In Runs 6 and 7 γLA (g/100g DM) concentration began decreasing after 90h. This decrease was also observed in the concentration of all the AFAs (Fig.4.4). There was a difference in the FA quantities produced in GBM-Cp (Runs 4 and 5) and in GBM-S (Runs 6 and 7) (Fig.4.4). The concentration of linoleic acid ($C_{18:2}$) and αLA was higher in GBM-S than in GBM-Cp (Figs.3.2, 4.2). The concentration of both these fatty acids decreased during the fermentations in GBM-S. The total AFA concentration decreased with increasing temperature (Fig.4.5). The γLA concentration reached an exceptional level at 72h in Run 5 (Fig.4.4). There, however, followed an

Table 4.1 Comparison of the γLA yields by *C. cucurbitarum* 12997 under pilot plant fermentation conditions

In 10% inocula the pilot plant prefermenter was used

* - with agitation in the prefermenter stage

** - with intermittent agitation in the prefermenter stage

Run no.	Fermentation medium	Inoculum size	Oil analysis method	γLA mg/ℓ	γLA g/100gDM	Biomass g/ℓ	%γLA in AFAs	γLA mg/g glucose	γLA mg/ℓ.h
Run 1	GBM-Y	1%	Conventional	254	2,27	11,2	22,2	3,8	2,4
Run 2	GBM-Cp	1%	Conventional	467	2,78	16,8	18,0	7,1	2,6
Run 3	GBM-Cp	10%	Conventional	583	3,27	17,8	24,5	8,8	4,7
Run 4	GBM-Cp	1%	DE	830	5,76	14,4	23,3	12,8	7,4
Run 5	GBM-Cp	10%	DE	886	4,83	18,3	22,1	10,5	7,8
*Run 6	GBM-S	10%	DE	805	4,13	19,5	12,76	13,1	8,8
**Run 7	GBM-S	10%	DE	966	4,04	23,9	13,7	14,7	11,9

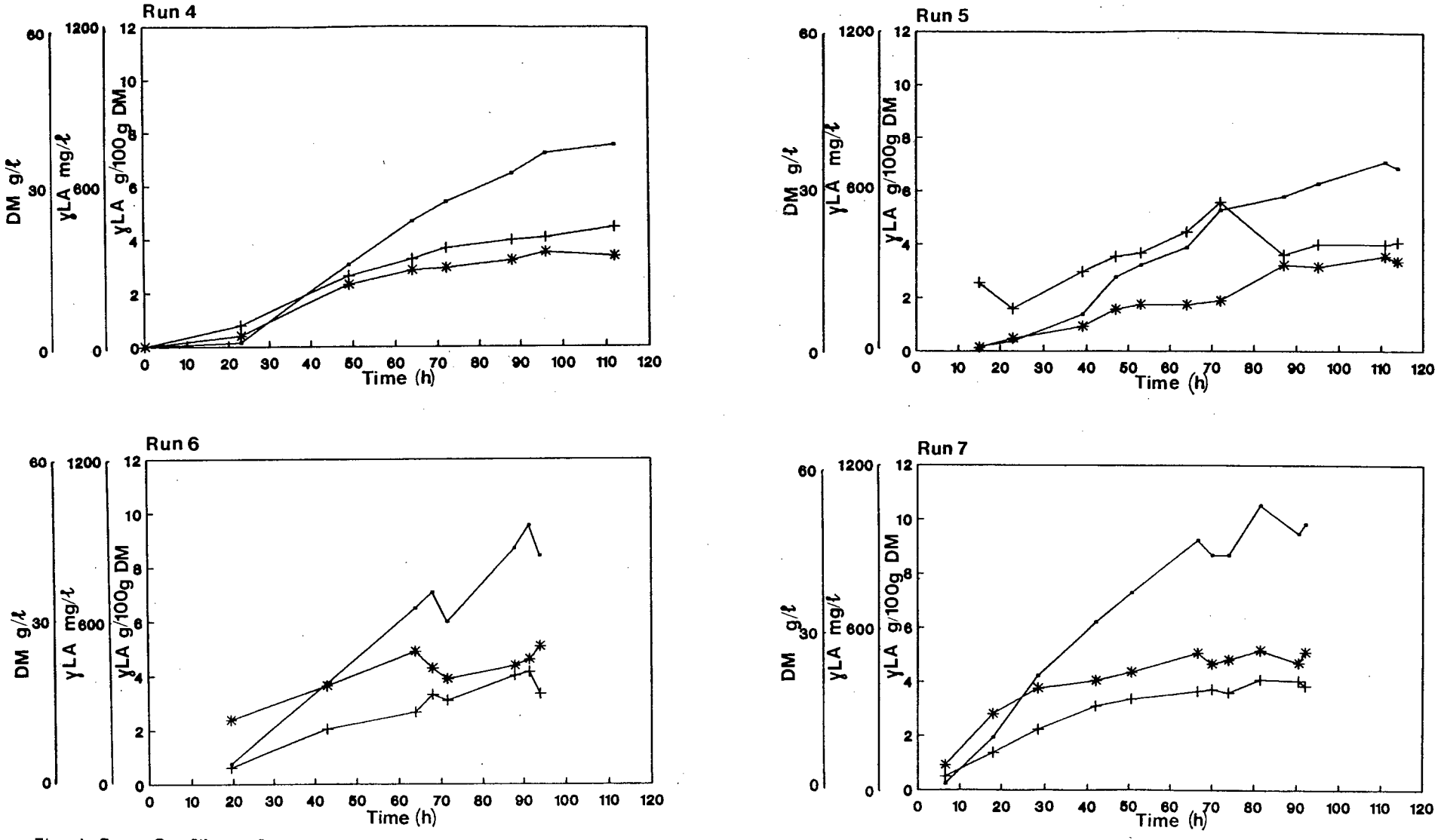

Fig.4.3 Profiles of growth and fat production by *C. cucurbitarum* 12997 in pilot plant fermentations; Runs 4, 5, 6 and 7 (Table 4.1).

The fungus was grown in 1 800ℓ in the pilot plant. The fermentation medium and seed stage conditions for each run are described in Table 4.1. The oil was extracted and analysed by DE. Biomass and yLA are indicated as:
yLA mg/ℓ —●— yLA g/100g DM —+— DM g/ℓ —✳—

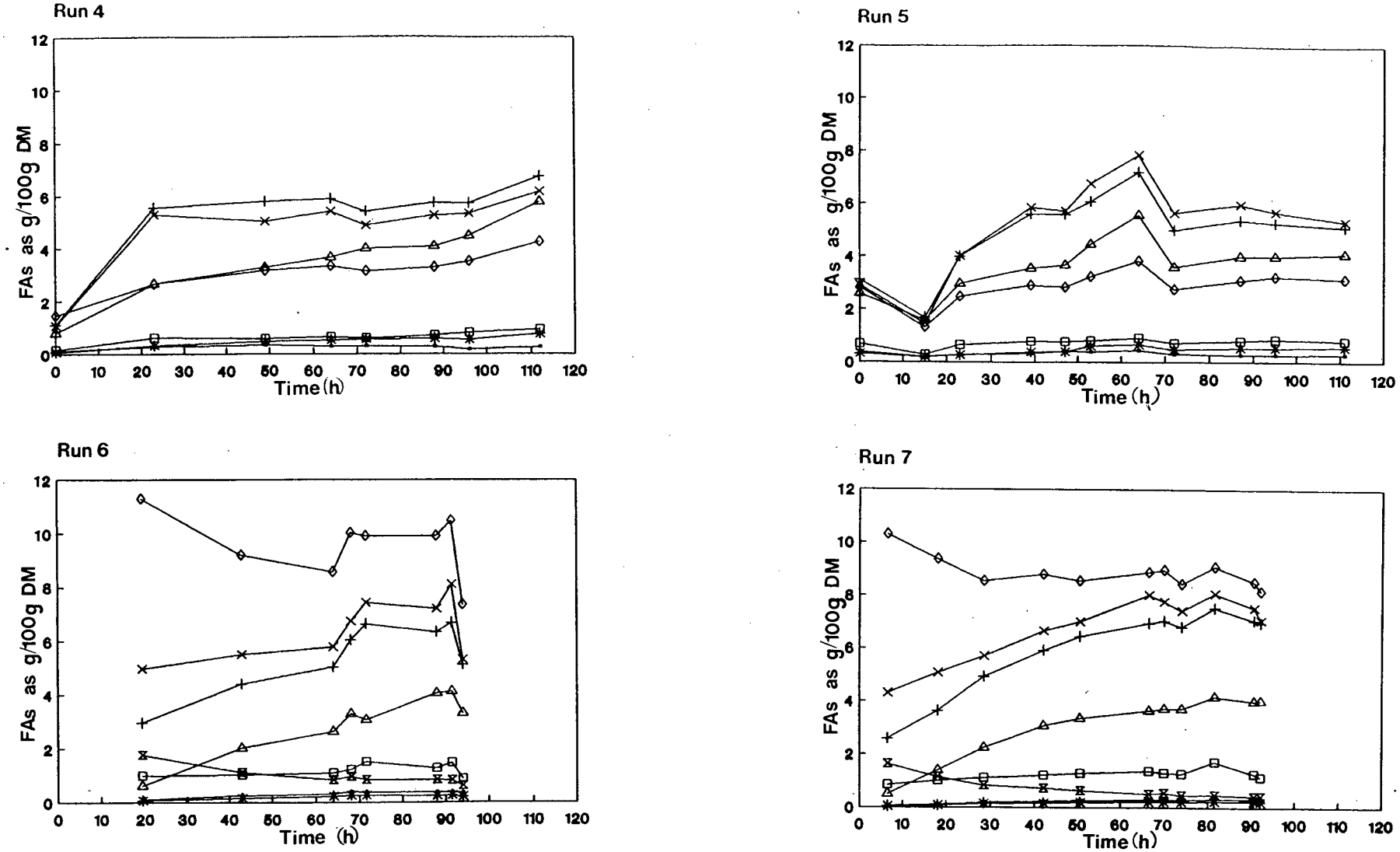

Fig.4.4 The concentration of fatty acids in *C. cucurbitarum* 12997 in pilot plant fermentations; Runs 4, 5, 6 and 7 (Table 4.1).

The fungus was grown in 1 800ℓ in the pilot plant. The fermentation medium and seed stage conditions for each run are described in Table 4.1. The oil was extracted and analysed by DE. The fatty acids analysed were: C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γLA

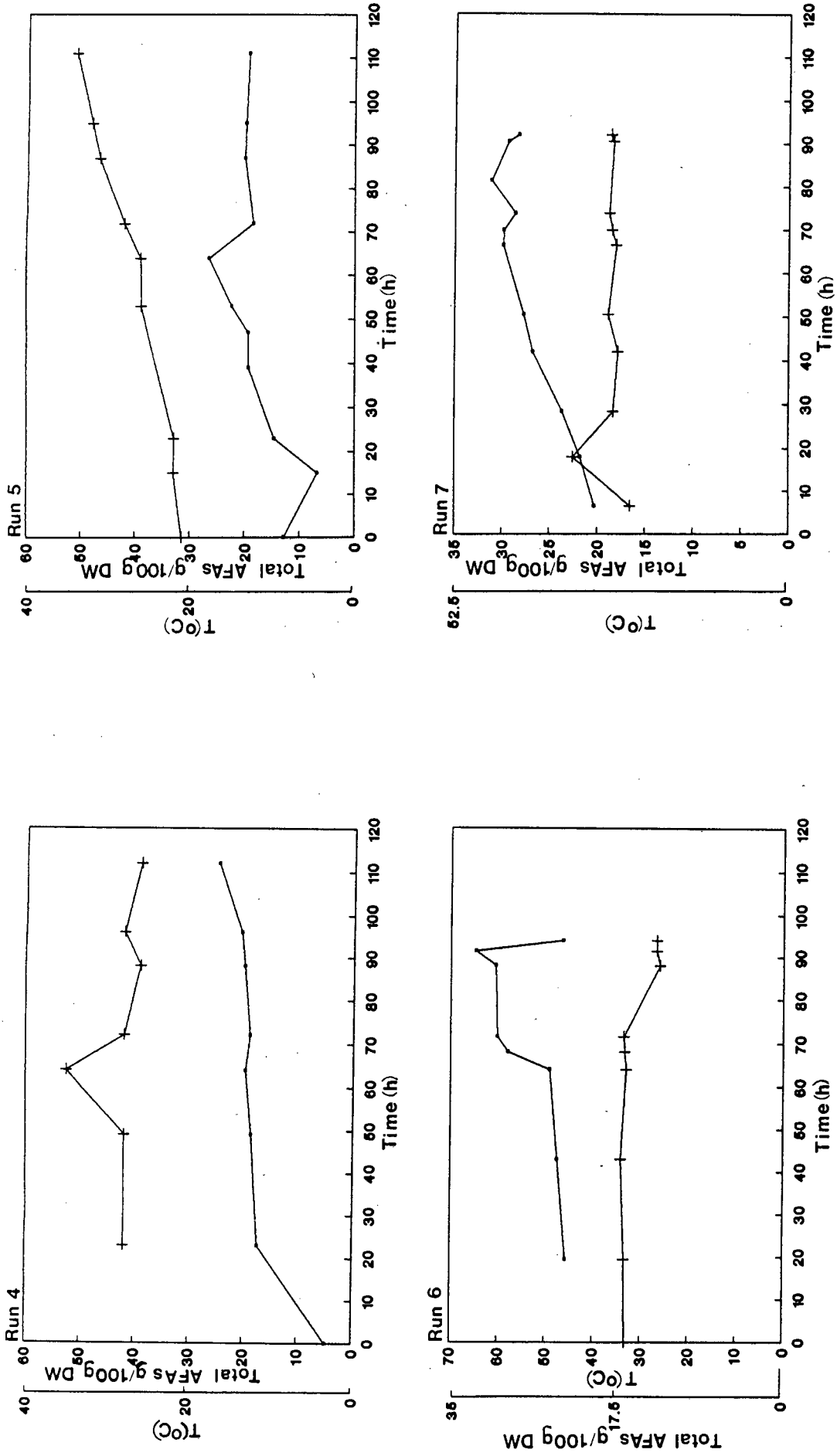

Fig. 4.5 The variation in the concentration of total AFAs with changes in the fermentation temperature.

Fermentations took place in 1 800ℓ in the pilot plant. The oil was extracted and analysed by DE.
Total AFAs —■— and Temperature as T(°C) —+— were compared.

increase in temperature (Fig.4.5, Run 5) and a decline in γ_{LA} Fig.4.3, Run 5).

The total N decreased rapidly and stabilised at a constant value (Fig.4.6). The C content of glucose is 37,5%. Using this value the C:N ratio can be calculated from Fig.4.6. The C:N ratio increased rapidly at the start while N was consumed for biomass production but decreased later as the C was consumed and the N stabilised. The glucose concentration decreased more sharply in Run 7. The pH increased in GBM-Cp (Runs 4 and 5) and decreased in GBM-S (Runs 6 and 7). This was also observed on the smaller laboratory scale (Table 3.12 and Fig.3.5a,b).

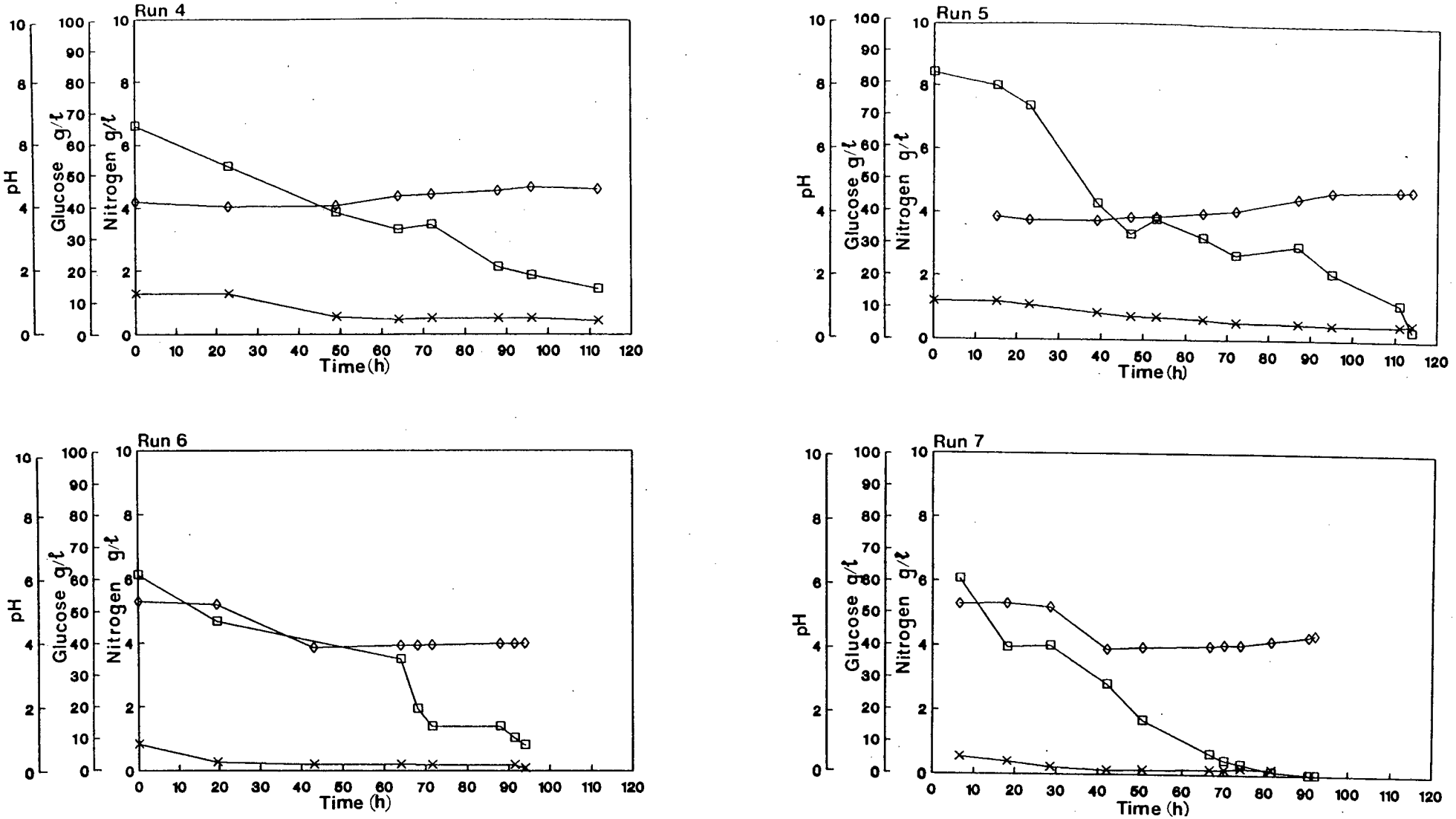

Fig. 4.6 Glucose, nitrogen and pH levels during the production of YLA by *C. cucurbitarum* 12997 in pilot plant fermentations; Runs 4, 5, 6, and 7 (Table 4.1).

The fungus was grown in 1 800ℓ in the pilot plant. The fermentation medium and seed stage conditions for each run are described in Table 4.1. The nutrient conditions are indicated as:
—X— Nitrogen g/ℓ —□— Glucose g/ℓ —◇— pH

4.4.2 Oil extractions on pilot plant scale

The concentration of all FAs decreased after the mycelium was air-dried (Fig.4.7). Freeze-drying did not affect the γLA concentration in the mycelium (D5 and D6). The FAs decreased the most when the mycelium was air-dried in the sun (D4). The %γLA in the AFAs of the mycelium did not change after drying but remained at ±22% (D1, D2, D3, D4 & D6). In D5, however, the %γLA in the AFAs increased to 36%.

On storage, the concentration of the unsaturated FAs (linoleic acid and γLA) decreased markedly in both air-dried and freeze-dried mycelium (Figs.4.8a, 4.8b). The concentration of palmitic ($C_{18:0}$) and oleic ($C_{18:1}$) acids only decreased in the 3d period of air-drying (Fig.4.8a). The concentration of the same FAs remained constant after freeze-drying (Fig.4.8b).

4.4.2.1 Semi-countercurrent extraction

The extraction of unmilled mycelium utilised 150ℓ of acetone to extract only 66g of oil and left unextracted 59,3% of the total oil in the mycelium (Table 4.4). This amounted to 1 871g of oil and 302,4g γLA from the total biomass of Run 1. The extraction of milled mycelium left unextracted 21,7% of the total oil in the mycelium (Table 4.4). In this extraction the rate at which the oil was extracted decreased rapidly from 94,72g/ℓ at 0h to 2,00g/ℓ at 5,5h (Table 4.2). The %γLA in the oil also decreased with time from 14,8% at 0h to 7,5% at 42,5h.

The chloroform:methanol solvent ($CHC\ell_3$:MeOH) extracted more oil than

hexane or acetone (Table 4.4). The %γLA in the oil was, however, low in the $CHCl_3$:MeOH extract.

4.4.2.2 Batch countercurrent extraction

Only 0,6% of the total oil remained in the mycelium after the batch countercurrent extraction (Table 4.4). The %γLA in the oil decreased with each extraction from 15,4% in A_1 to 6,9% in A_7.

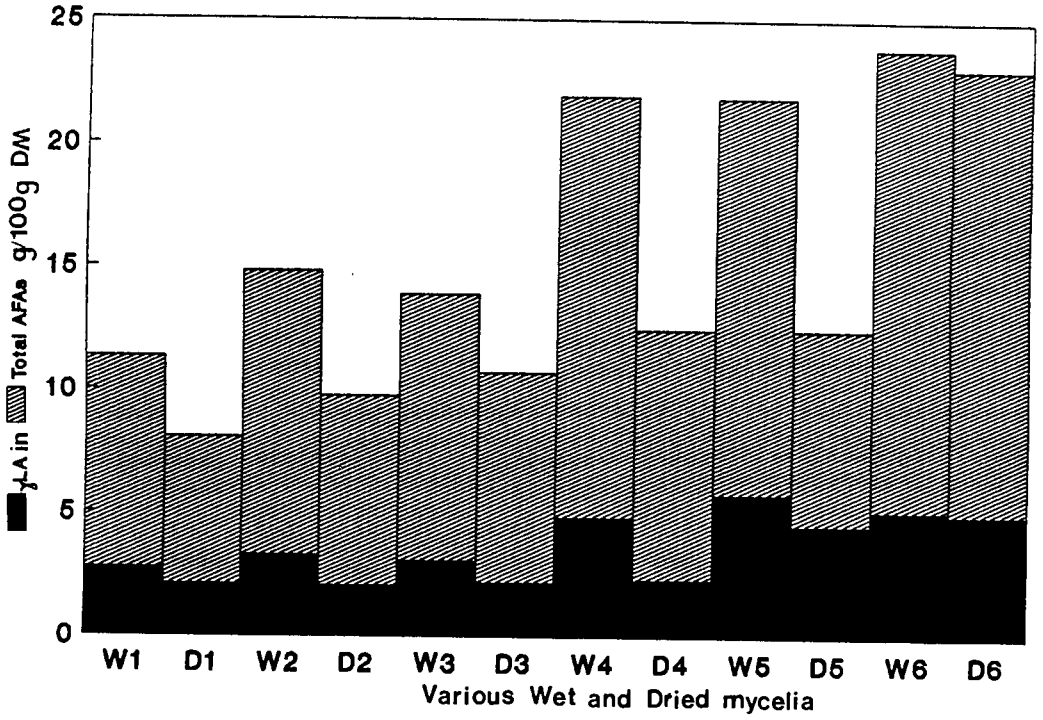

Fig.4.7 The concentration decrease of γLA and total AFAs after wet mycelium was air-dried or freeze-dried.

Wet mycelium of *C. cucurbitarum* 12997 from 6 pilot plant fermentations (W1-W6), was air-Dried (D1-D3), air-Dried in the sun (D4) and freeze-Dried (D5-D6) over a period of 3d. All mycelia except D5 were dried immediately after they had been harvested. Mycelium D5 was stored at -20°C for 30d before being freeze-Dried. The conventional method was used for extraction and analysis of FAs in W1-W4 and D1-D4, whilst DE was used for the analysis of FAs W5-W6 and D5-D6.

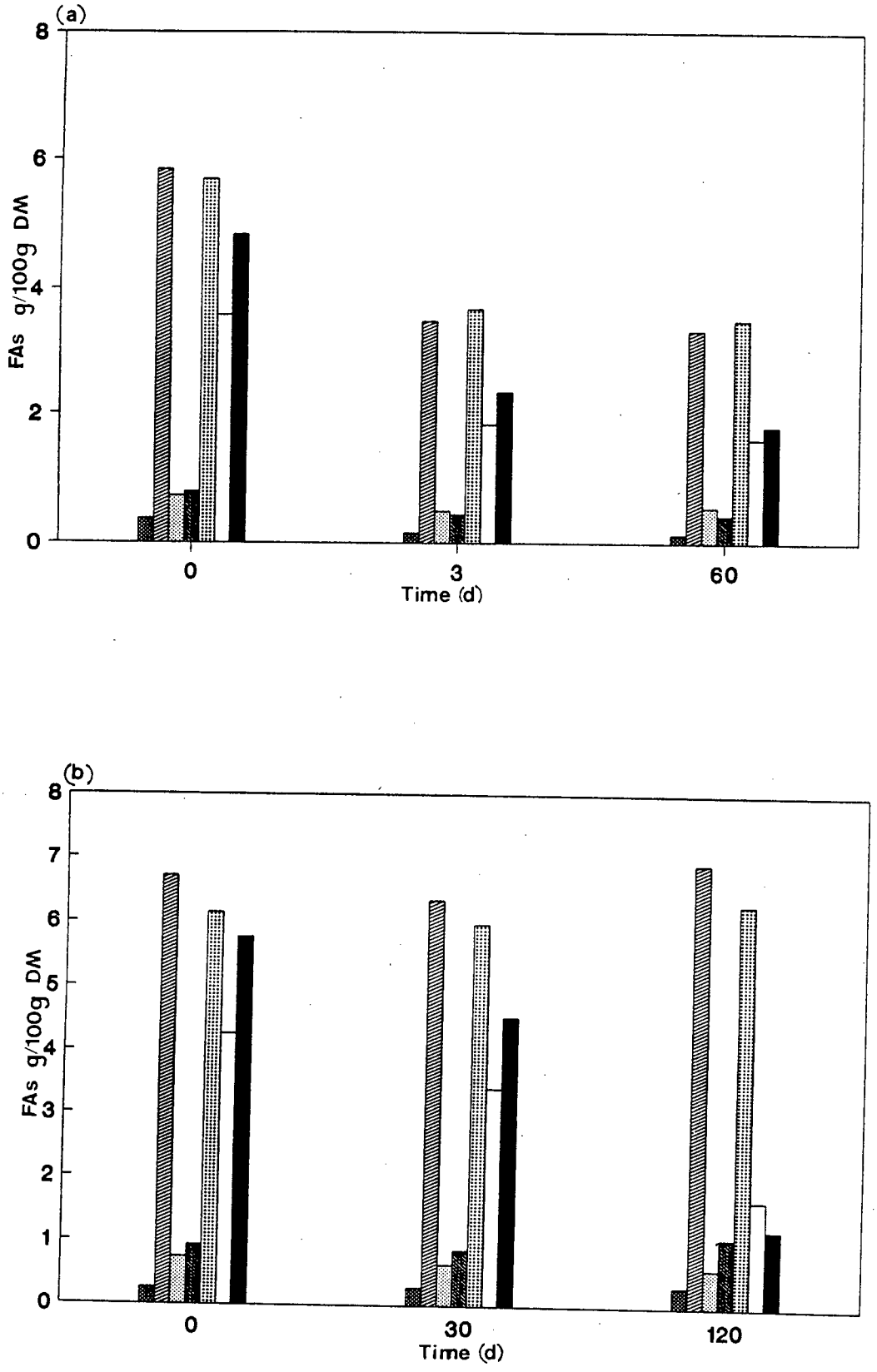

Fig.4.8 The decrease in the concentration of AFAs in (a) air-dried and (b) freeze-dried mycelium over a period of storage.

The mycelium from Run 4 was (a) air-dried immediately or (b) frozen immediately. The frozen mycelium (b) was further freeze-dried after a period of 30d. The FAs which were extracted and analysed by DE were:

C14:0 C16:0 C16:1 C18:0
C18:1 C18:2 γLA

Table 4.2 The amount of oil in 1ℓ samples taken from semi-countercurrent extraction of milled mycelium

The milled air-dried mycelium was slurried with acetone and poured into the column. The excess acetone was drained off at the bottom and reused to slurry more mycelium and pack the column. The miscella obtained from the packing is labelled "Pre" in the Table. Fresh acetone was allowed to flow through the column at approximately 6ℓ/h. One litre samples were collected at intervals and the γLA content determined. The mycelium stood in solvent for (a) 6h and (b) 18h at the end of the extraction.

Time (h)	Flow rate (ℓ/h)	Oil in miscella g/ℓ	%γLA in oil
Pre	0	88,80	15,69
0	6,4	94,72	14,83
5,5	7,8	2,00	12,48
17,5	4,3	0,67	10,93
20,5	2,2	0,67	10,61
31,5	5,3	0,54	8,72
42,5	5,9	0,26	7,49
48,5	(a)	0,21	9,94
66,5	(b)	3,80	15,86
84,5	(b)	0,68	11,96

Table 4.3 The amount of oil extracted on a batch countercurrent system

The extraction was carried out in two 20ℓ bell jars (A & B) each containing 4kg of air-dried, milled mycelium. The mycelium was the product of Run 2 (Table 4.1). Solvent was added into A and the resulting miscella was reused to extract oil from the mycelium of B. Acetone was used for the initial extractions and $CHC\ell_3$:MeOH for the later extractions. The mycelium/solvent slurry was stirred with an overhead stirrer for 0,5h. The oil was analysed as in the conventional method. The oil of B_2 was not determined (ND).

Step	Solvent	γLA g/ℓ solvent	Oil g/ℓ solvent	% γ L A in oil
A_1	acetone	4,15	27,0	15,4
A_2	acetone	1,36	9,0	15,1
A_3	acetone	0,54	4,0	13,5
A_4	acetone	0,19	1,7	10,9
A_5	$CHC\ell_3$:MeOH	0,25	2,6	9,4
A_6	$CHC\ell_3$:MeOH	0,17	2,4	7,2
A_7	$CHC\ell_3$:MeOH	0,10	1,5	6,9
B_1	acetone (A_1)	11,95	69,7	17,2
B_2	acetone (A_2)	ND	ND	ND
B_3	acetone (fresh)	2,40	14,7	16,3
B_4	acetone (A_4)	0,54	4,0	13,6
B_5	$CHC\ell_3$:MeOH (A_5)	0,48	4,0	11,9
B_6	$CHC\ell_3$:MeOH (A_6)	0,54	8,0	6,8
B_7	$CHC\ell_3$:MeOH (fresh)	0,36	6,0	6,1

Table 4.4 Oil remaining in mycelia after semi-countercurrent and batch extractions

Section A (semi-countercurrent extraction)

Unmilled mycelium was extracted under conditions described in the introduction to Table 4.2. When the miscella contained no oil the extraction was terminated and the mycelium was refluxed in 200mℓ $CHC\ell_3$:MeOH (1:1) for 1h. The milled mycelium was air-dried at the end of the extraction period (84,5h, Table 4.2), to remove any remaining acetone. Representative portions of this extracted mycelium were stirred for 16h in 200mℓ acetone, hexane or $CHC\ell_3$:MeOH (1:1).

Section B (batch countercurrent extraction)

The oil from batches A and B was analysed and the total (g/100g DM) was subtracted from the oil content of the unextracted control.

In all cases the oil was dried and analysed as in the conventional method. The total unextracted oil was calculated using the pilot plant mycelial yield of Run 1 (20,16kg DM) for Section A and of Run 2 (30,24kg DM) for section B (Table 4.1).

Solvent	Oil g/100g DM	%γLA in oil	Total unextracted oil (g)	Unextracted oil % of total oil
Section A (semi-countercurrent extraction)				
Unmilled mycelium (Run 1)				
$CHC\ell_3$:MeOH	9,28	16,16	1 871	59,3
Milled mycelium (Run 1)				
Hexane	0,48	8,61	98	3,1
Acetone	0,39	12,45	79	2,5
$CHC\ell_3$:MeOH	3,40	5,70	692	21,7
Section B (batch countercurrent)				
Milled mycelium (Run 2)				
$CHC\ell_3$:MeOH	0,14	5,26	42	0,6

4.5 Discussion

4.5.1 Pilot scale fermentations

C. cucurbitarum 12997 produced the most yLA mg/ℓ.h in Run 7 in GBM-S (Table 4.1). In this run, the inoculum was higher (10%) and was grown without agitation in the prefermenter. Under the above pilot plant conditions and with the improved DE analysis there was a 5-fold increase in the rate of yLA production from 2,4mg/ℓ.h to 11,9 mg/ℓ.h. The overall increase in productivity from the shake flask to the pilot plant stage was 60-fold from 0,2mg/ℓh (21mg/ℓ) to 11,9mg/ℓh (966mg/ℓ) (Tables 3.1, 4.1). The increased yLA yield and its productivity (*i.e.* reduction of the lag phase and the fermentation time) is economic and helps avoid contamination. The decrease in the pH of the GBM-S fermentation also decreases the chances of contamination.

The final yLA yield of *C. cucurbitarum* 12997 per unit volume (966mg/ℓ) is not in the same range as the three best reported yields; 3 400mg/ℓ produced by *M. isabellina* IFO 7884, 2 852mg/ℓ produced by *T. elegans* NRRL 2468 and 2 074mg/ℓ produced by *M. circinelloides* HUT 1121 (Table 2.1). However, *C. cucurbitarum* 12997 under pilot plant conditions produced a comparable coefficient yield of yLA mg/gGlucose (mg/gG): In decreasing order of yLA mg/gG *M. circinelloides* 1121, *C. cucurbitarum* 12997, *T. elegans* 2468 and *M. isabellina* 7884 produced 20,73mg/gG, 14,7mg/gG, 14,3mg/gG and 8,7mg/gG respectively. *M. ambiguus* IFO 6742 has also been reported to have a relatively good coefficient yield of yLA (14,2mg/gG). The best

reported rate of γLA production was by *M. circinelloides* 1121 (28,8mg/ℓ.h), *T. elegans* 2468 (23,7mg/ℓ.h) and *M. isabellina* 7884 (20,2mg/ℓ.h) (Table 2.1). The productivity of *C. cucurbitarum* 12997 in a pilot plant batch fermentation was 11,9mg/ℓ.h.

The best yield of γLA/gGlucose (14,7mg/gG) produced by C. *cucurbitarum* 12997 suggests that a ton of substrate produces 14,7kg of γLA. The economics of the process hinge on the cost of the substrate, the sale price of the product and the cost of the conversion of substrate to product (fermentation). Since the cost factors are constantly changing, a fermentation process has always to be improved to produce economically acceptable yields.

The increased linoleic and αLA levels in the mycelium grown in GBM-S was characteristic of that medium (Fig.3.2). The concentrations of these FAs decreased by the end of the fermentation. The linoleic and αLA final concentrations in the biomass were respectively 70% and 30% of the original concentrations. The decreased concentrations are important as αLA should not be present in the final oil product. α-Linolenic acid competes for the enzyme Δ-6-desaturase which forms γLA from linoleic acid. The ingestion of a mixture of αLA and γLA would therefore affect the body's natural desaturation of linoleic acid to γLA increasing the need of γLA from an external source. To minimise this effect αLA should be removed from the oil or be present in very small quantities only. The chemical separation or purification of γLA from αLA is, however, difficult as these FAs have similar physical properties. The decrease in the concentration of αLA by biological means (*i.e.* by *C. cucurbitarum* 12997) therefore aids the purification of γLA.

The AFAs (g/100g DM) which decreased at the end of Runs 6 and 7 may have been utilised by the fungus (Fig.4.4). Dry (1985) reported lipid depletion in *C. cucurbitarum* 12997 at 14d and 4d in shake flask and agar cultures respectively. The pilot plant conditions were such that lipid depletion began at 90h in Run 6. The glucose levels in the medium were noticeably low at the end of the runs (Fig.4.6) and may have favoured lipolysis and subsequent lipid oxidation in the fungus. The total nitrogen decreased rapidly and stabilised at a constant value (Fig.4.6). This indicates that the residual nitrogen is only partly available or unavailable to the fungus. Organic nitrogen is best for biomass production while inorganic nitrogen sources may be better for lipid synthesis. The fed-batch fermentation in Chemap fermenters increased the yLA yield (Table 3.16). Research should be continued into the feeding of glucose and an inorganic nitrogen source to increase yLA yields further.

Microscopic examination of mycelia from fermentations involving agitation in the prefermenter indicated that the mycelia were damaged. The impeller tip speed of the fermenter and prefermenter were 5,5m/s and 5,7m/s respectively. The shear forces in the prefermenter would have been greater as this vessel had a smaller diameter and a higher impeller tip speed than the fermenter vessel. Since the agitation in the prefermenter could not be varied, it was disengaged to minimise high shear of the mycelium. The use of air agitation with intermittent mixing was shown to improve the growth of the inoculum. In the fermenter vessel no obvious shear problems were observed. A prefermenter stirrer with variable speed control would allow for stirring at low speeds which would decrease shear stress and optimise

aeration. These low speeds may better the already favourable conditions created by the absence of agitation in the prefermenter and improve the γLA productivity in the main vessel. To avoid unfavourable effects on γLA production, it is important that rheological properties in the available pilot plant facilities are understood.

The importance of heat removal in the γLA fermentation was illustrated by the decrease in total AFA concentration with the uncontrolled increase in temperature (Fig.4.5). Lower temperatures produced higher FA yields. The temperature control in the fermentation is therefore critical.

Future pilot plant work should investigate biochemical engineering parameters which would affect the successful production of γLA. These would include rheological data, oxygen and heat transfer as well as information on the shear stress caused under pilot plant conditions. The culture conditions of the fermentation could be further optimised especially if a fed-batch culture system is to be introduced.

4.5.2 Pilot scale oil extraction

The drying of the mycelium has to be rapid in order to avoid the degradation of γLA (Figs.4.7, 4.8a, 4.8b). Lipases and phospholipases can be active in mycelium which is not actively growing. These are even active in the presence of organic solvents and can be inactivated if harvested cells are treated with 80% ethanol at 80°C for 15min (Ratledge, 1982). Herbert & Keith (1985), however, kept the mycelium in refrigeration at a 10% water content until it was required for extraction. Fatty acid degradation was not reported in this case. Fatty acids can also be converted by chemical means to hydroperoxides, epoxides or acid polymers. Chemical oxidation can be accelerated by irradiation. This was evident in the decreased total AFA and γLA concentrations in mycelium which was air-dried in the sun (Fig.4.7).

C. cucurbitarum 12997 grew in 1mm diameter pellets in GBM-S under pilot plant conditions which made the oil easier to extract than the thick, mat growth usually found under different culture conditions or in different species like *R. arrhizus* (Byrne & Ward, 1989a; Byrne & Ward 1989b). When the biomass was harvested, the mycelium was compacted and mycelial clumps (diameter 3-20mm) were formed. The initial semi-countercurrent extraction with unmilled mycelium was inefficient (59,3% of total oil was unextracted) (Table 4.4). This was a result of poor penetration of solvent to the core of mycelial clumps. The second extraction using milled mycelium was more efficient (21,7% of total oil was unextracted). Uneven compacting, however, produced channels hampering sufficient contact of solvent with mycelium. When the mycelium was left in acetone for 18h, solvent penetration was better and more oil was recovered from the resulting miscella. The suspension

should be well mixed to allow good contact between the solvent and mycelial oil. Extractions in the batch countercurrent system were most effective mainly due to constant mixing. This system also decreased the quantity of solvent used to extract the oil.

The %yLA in the oil decreased with time in both types of countercurrent extractions. This could be because yLA and other FAs are found in neutral or polar lipid fractions in differing quantities. Since the solvent initially removes a certain amount of moisture from the mycelium the milieu becomes more hydrophobic later on in the extraction. The yLA and FAs would therefore be extracted at differing rates depending on how hydrophobic their lipid environment is.

The solvent mixture $CHCl_3$:MeOH extracted more yLA than acetone (Table 4.4) but it also extracted many unknown FAs which would later complicate purification and concentration of yLA. Dense gases especially carbon dioxide have been successfully used to extract oils from seeds (supercritical extraction) and may be viable alternatives for yLA extraction from fungal biomass (Friedrich *et al.*, 1982).

CHAPTER 5

GENERAL CONCLUSION

5 General Conclusion

Preliminary pilot plant trials in Chapter 4 showed that yLA was produced on a larger scale in 1 800 litres using the selected strain *C. cucurbitarum* 12997 (Chapter 2) and conditions developed in Chapter 3. At this level the yLA yield per g substrate is of the same range as the reported high yields of *C. circinelloides* HUT 1121 and *T. elegans* NRRL 2468.

The results reported and discussed in this thesis indicate that there has been a successful translation of the industrial production of yLA from the strain selection stage through the stage of optimisation of the culture conditions to the final scale-up of the fermentation. This was achieved with a 60-fold increase in the productivity from 0,2mg/ℓ.h (initial shake flask results) to 11,9mg/ℓ.h (final pilot plant results).

APPENDICES

APPENDIX A

A Media composition

All media were sterilised in the autoclave for 15min at 121°C and 105kPa.

A.1 Glucose Yeast Extract (GYE) medium

Ingredients		Supplier
Glucose	11g	African Products (PTY) LTD.
Yeast extract	3g	Beechams SA (PTY) LTD.
H_2O	1ℓ	

To make agar plates 15g of agar and 250mg of Novobiocin (for sterility purposes) were added to 1ℓ of the above medium.

A.2 Glucose Basal Medium (GBM)

Ingredients		Supplier
glucose	66g	African Products (PTY) LTD.
potassium dihydrogen orthophosphate	5g	NT Supplies (PTY) LTD.
magnesium sulphate	1g	NT Supplies (PTY) LTD.
trace element solution	1mℓ	
H_2O (tap water)	1ℓ	

Trace element solution

Ingredients		Supplier
ferric sulphate	5g	BDH Chemicals LTD.
zinc sulphate	1g	Carlo Erba

copper sulphate	0,5g	NT Supplies (PTY) LTD.
ammonium molybdate	0,1g	BDH Chemicals LTD.
manganese sulphate	0,1g	Jones & Adams CO.
boric acid	0,1g	NT Supplies (PTY) LTD.
calcium hydroxide	0,1g	NT Supplies (PTY) LTD.
H_2O (distilled)	1ℓ	

The trace element solution was made up in two solutions, A and B, so as to prevent precipitation. Whenever needed the solutions were taken out of storage at $4^{o}C$. Solution A contained calcium hydroxide, boric acid and ammonium molybdate. Solution B contained ferric sulphate, zinc sulphate, manganese sulphate and copper sulphate.

The GBM medium without glucose named basal medium (BM), was used as the base for carbon and nitrogen variation. The carbon source was always added to give a final carbon concentration in the medium of 24,2g/ℓ. The various nitrogen sources were added to give a final nitrogen concentration of 1,1g/ℓ. Unless indicated the C:N ratio was 22:1 and the pH was not adjusted and varied between pH 4,5-5,5 depending on the medium composition. The yeast extract unless otherwise indicated was obtained from Beechams.

A.3 Potato Carrot Agar

Grated potato and carrot of mass 20g and 2g respectively was boiled for 1h in 1ℓ of tap water. The cooked material was drained through a fine sieve and 20g of agar was added. The medium was sterilised at $121^{o}C$ for 20min.

A.4 Suppliers of additives to media

All additives were obtained from local (South African) sources except for Struktol which was obtained from Hamburg, Germany.

Nitrogen sources	Supplier
ammonium sulphate	PAL chemicals
Bovril paste	Beechams SA (PTY) LTD.
cornsteep liquor	African Products (PTY) LTD.
cornsteep powder	National Fermentation Products (PTY) LTD.
cottonseed meal (Epic)	Epic Oil Mills (PTY) LTD.
cottonseed meal (NOLA)	NOLA Industries (PTY) LTD.
di-ammonium phosphate	NT Supplies (PTY) LTD.
fishmeal	EPOL (PTY) LTD.
groundnut meal (NOLA)	NOLA Industries (PTY) LTD.
linseed	S. Schmidt (feeds and seeds) (PTY) LTD.
maize	S. Schmidt (feeds and seeds) (PTY) LTD.
mono-ammonium phosphate	NT Supplies (PTY) LTD.
mustardseed	S. Schmidt (feeds and seeds) (PTY) LTD.
Nutromix	South African Breweries LTD.
olive waste	Costa & Son, "NERVI", Huguenot, Cape
SBM	Jabula Foods LTD.
SBM (mildly defatted)	Epic Oil Mills (PTY) LTD.
SBM (fully defatted)	Epic Oil Mills (PTY) LTD.
sunflower meal	NOLA Industries (PTY) LTD.
urea	Riedel-de Haën
yeast extract	Beechams SA (PTY) LTD.

Antifoams	Supplier
Bevaloid (4214 & 4241)	Bevaloid SA (PTY) LTD.
Chemspec	Chemical Specialties (Durban) (PTY) LTD.
Silcolapse	ICI
Struktol	Schill & Seilacher

Miscellaneous	Supplier
biotin	Sigma
calcium carbonate	Riedel-de Haën
calcium hydroxide	NT Supplies (PTY) LTD.
crop syrup	Mount Edgecombe Sugar Mill (Natal)
high test molasses	Mount Edgecombe Sugar Mill (Natal)
molasses	Mount Edgecombe Sugar Mill (Natal)
sodium acetate	NT Supplies (PTY) LTD.
sodium citrate	Protea lab services (PTY) LTD.

APPENDIX B

B Analytical methods

B.1 Conventional method of fatty acid extraction and analysis

B.1.1 Extraction of the oil

Mycelium (±12g) was weighed and macerated in 200mℓ of a (1:1) volume/volume $CHC\ell_3$:MeOH mixture for 0,5-1min using an Ultraturrex homogeniser. The homogenate was refluxed for 1h and then the mycelium was filtered from the lipid extract (miscella) through Whatman No. 541 under vacuum. The filtrate was kept aside whilst the mycelium was resuspended in 200mℓ of solvent and refluxed for another hour. The miscella from the second reflux was added to the first and the solvent removed using a rotary evaporator in a 70°C waterbath. The crude oil was purified by resuspending it in 200mℓ of hexane for 1h. This miscella was dehydrated with sodium sulphate and then filtered through cottonwool. The hexane was evaporated off with a rotary evaporator in a 70°C water bath. The lipid was then esterified and analysed. If needed the lipid was stored under nitrogen at 4°C. The %DM of the mycelium was determined after being dried in an 100oC oven for 18h.

B.1.2 Esterification

Samples containing about 250mg of the lipid extract were transferred into 25mℓ volumetric flasks and approximately 50mg of pentadecanoic acid was added to each flask as an internal standard. The solvent was evaporated off in a water bath in the fume hood and 4mℓ of 0,5N KOH in methanol

(MeOH) was added to each sample. This was heated for 5-10min to ensure that all the lipid had been saponified. The flasks were allowed to stand in a water bath until cool (it is important that the saponified material is properly cooled). A 5mℓ quantity of boron trifluoride (BF_3) in MeOH (Merck) was added and the samples were heated for 2-3min. A saturated salt solution was added to float the methyl esters into the neck of the flask.

B.1.3 Analysis by Gas Liquid Chromatography (GLC)

The esters solution was diluted in hexane to a 10% concentration and was used for analysis by gas liquid chromatography. A Hewlett-Packard 20 M carbowax capillary column was used. The sample took 15min to elute from the Hewlett-Packard 5880 chromatograph. The calibration mixture for verifying the accuracy of the column consisted of the following :

Pentadecanoic acid	± 250mg
Myristic acid	± 100mg
Palmitic acid	± 300mg
Palmitoleic acid	± 100mg
Stearic acid	± 100mg
Oleic acid	± 200mg
Linoleic acid	± 200mg
γ-Linolenic acid	± 100mg
α-Linolenic acid	± 100mg

The calibration mixture was made up in 25mℓ hexane and stored under nitrogen at 4°C, in the dark. A 5mℓ portion of this solution was esterified in conjunction with mycelial oil samples.

The calibration mixture was used to calibrate the gas chromatograph. The preparation and analysis of the fatty acid methyl esters (FAME) was considered satisfactory if the relative response factor (RRf) was close to unity ($\pm$1,0000).

RRf was calculated as follows:

Abbreviations

Rf - Response factor

RRf - Relative Response factor

[FAME] - Fatty Acid Methyl Ester concentration (myristic - αLA)

[Is] - Internal standard concentration (pentadecanoic acid $C_{15:0}$)

$$Rf_{Is} = \frac{\text{area on chromatogram}}{[Is]}$$

$$Rf_{FAME} = \frac{\text{area on chromatogram}}{[FAME]}$$

$$RRf = \frac{Rf_{sample}}{Rf_{Is}}$$

B.2 Direct Extraction and Esterification, analysis method (DE)

This method used ten times more mycelium than the HP method (Dry, 1985) and was therefore more suitable for analysing a more representative sample of mycelium obtained from the larger scale fermentations.

Approximately 1g samples of the mycelium were transferred into 25mℓ volumetric flasks and 25µℓ of a 1% solution of pentadecanoic acid in hexane was added to each flask as an internal standard. The solvent was evaporated off in a water bath in the fume hood and 4mℓ of 0,5N KOH in methanol (MeOH) was added to each sample. This was heated for 5-10min to ensure that all the lipid had been saponified. The flasks were allowed to stand in a water bath until cool (it is important that the saponified material is properly cooled). A 5mℓ quantity of BF_3 in MeOH (Merck) was added and the samples were heated for 2-3min. A saturated salt solution was added to float the methyl esters into the neck of the flask.

The fatty acids were analysed by GLC as in B.1.3.

B.3 Total invert sugar analysis in molasses

Reagents

Sodium benzoate (BDH) - 0,3% aqueous solution with pH adjusted to 6,9 ± 0,1. Solution prepared freshly every 2 weeks.

Hydroxylamine hydrochloride (BDH) - 2,5g dissolved in 100mℓ pyridine (Merck). Solution prepared freshly every 2 weeks.

Oximation reagent (Riedel-de Haën) - 270µℓ of dimethyl-aminoethanol added to 5mℓ of hydroxylamine hydrochloride reagent just before use.

Hexamethyldisilazane (HMDS) (Merck)

Trifluoracetic acid (TFA) (Merck)

The standard and sample mixtures were made up in 25mℓ Erlenmeyer flasks with ground necks. The sugars which had been stored in a dessicator were weighed accurately.

Sugars were : Standard (calibration) mixture

Fructose (AR) dried	160mg
Glucose (AR) dried	120mg
Sucrose (AR) dried	600mg
Trehalose (AR) dried	660mg
Xylose (AR) dried	150mg
Sample mixture	
Molasses	2g
Trehalose (AR) dried	660mg
Xylose (AR) dried	150mg

The sodium benzoate solution was added in 2,7mℓ and 2mℓ quantities

to the standard and sample solutions respectively. The flasks were stoppered and shaken to dissolve the sugars and the molasses. After this point all procedures were performed under anhydrous conditions. The oximation was performed in 5mℓ glass vials fitted with teflon lined caps and magnetic stirrers. 10µℓ of the standard and sample mixtures was placed into separate reacti-vials. The oximation reagent was added in a 1mℓ quantity to both and the sealed vials were placed on a reacti-therm (Pierce-heating and stirring module) at 80°C for 10min. The samples were cooled to room temperatures and 900µℓ of HMDS and 100µℓ TFA were injected through the seal into each vial. The contents were gently swirled and the lid was opened to release the pressure. The contents were heated in the reacti-therm at 80°C for 10min. The contents were allowed to cool and the precipitate to settle. The supernatant was transferred to a GLC vial and was analysed on a stainless steel column (24 6'X$^{1}/_{8}$" 3% OV-17 on W/AW DMCS).

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