POLYUNSATURATED FATTY ACIDS (PUFAs) OF *MUCOR* SP. WITH SPECIAL REFERENCE TO GAMMA LINOLENIC ACID (GLA)

THESIS

Submitted to the
UNIVERSITY OF MYSORE

For the award of Degree of
Doctor of Philosophy
In
MICROBIOLOGY

By

Mamatha S.S., M.Sc.
Food Microbiology Department
Central Food Technological Research Institute
Mysore-570020

Guide
Dr. G. Venkateswaran
Scientist (F)
Food Microbiology Department
Central Food Technological Research Institute
Mysore-570020

November- 2009

Dedicated

To

My Parents and my teachers

ACKNOWLEDGEMENT

My mentor and guide, I would extend my heartfelt gratitude to ***Dr. G. Venkateswaran,*** *Scientist &Deputy Director, Food Microbiology Department, CFTRI, Mysore-20 for his constant, invaluable and meticulous guidance, keen interest and for enabling me to grow with the freedom of thoughts and expression throughout my tenure.*

My sincere thanks goes to ***Dr. V. Prakash,*** *Director, CFTRI, Mysore for permitting me to work for my Ph.D programme at Food Microbiology Department, CFTRI, Mysore.*

I am immensely thankful to ***Dr. S. Umesh Kumar****, Deputy Director & Head, Food Microbiology Department, CFTRI, Mysore for his constant support and encouragement throughout the course.*

I am grateful to ***University of Mysore, RGNF (UGC) and CSIR, New Delhi*** *for providing me the research fellowships which rendered me to carry out this work very successfully.*

I am thankful to ***Prof. S. Shankar Bhat, Prof, K.A Raveesha****,* ***Prof, G.A. Shivamurthy,*** *the then Chairmen, Department of studies in Botany/Microbiology, Manasagangotri, Mysore* ***Dr. Ravishankar Rai****, Chairman, Department of Microbiology and all administrative staff for their kind help and co-operation that helped me in getting my financial aid from the University of Mysore.*

I immensely thank ***Dr. M.C. Varadaraj,*** *Head, HR Dept. for his kind help during my tenure.*

I am immensely thankful to ***Dr. Praksh M Halami,*** *for guidance to carry Molecular work in his lab and all his kind helpful suggestions, constructive criticism and constant support through out my tenure.*

I thank all the ***staff of Central Instrumentation Facilities & Services*** *for helping me in carrying out all analyses related to fatty acid profile studies and others.*

My sincere thanks also goes to ***Dr.S.P. MuthuKumar,*** *Scientist, Biochemistry and Nutrition, (Animal house facility), CFTRI, Mysore, for his assistance in carryout animal experiments.*

My sincere thanks also goes to ***Mr. Ravi R,*** *Sensory Science CFTRI, Mysore for his assistance in statistical analysis.*

*I would like to extend my thanks to all the **scientific & non- scientific staff** of Food Microbiology Department, CFTRI, Mysore for extending their helping hand in one or the other way which helped me a lot in completing my Ph.D work successfully.*

*I thank Dr. **(Mrs). Girija Bai R** for her help in statistical analyses, useful suggestions on various aspects and constant support pertaining to my research works.*

*I extend my sincere thanks to the staff of CFTRI, Library for their help. My sincere thanks goes to **Mr. L. Jesuraj** and My aunt **Mrs. Kamakshi A.R.,** and staff of Electrical & Mechanical Maintenance and all the departments for their constant help.*

*My heartfelt thanks goes to all my friends, Dr. (**Mrs.) Gunashree, B.S., , Ms. A. Nisha, Mrs. H.P. Mohana Kumari, Dr.(Ms.) P. Shobha Rani, Ms. M. Snigdha, Ms. D.V. Prathibha, Mrs. Divyashree, Mrs UshaRani, Dr. (Mrs) Jayalakshmi K, Mrs. Manjulatha, Mr. T. V. Logaraj, Mr. Badrinath V, Mr. Raghavendra P., Mr. Suryachandra rao, Dr. Kumaresan, Mr. Anbarasu, Mr. Deepak, Mr. Devaraju** and for all others who created a healthy and joyful environment for me to complete my Ph.D work with a great success.*

*My special thanks are due to **Mr. Khayoum**,PS, and **Mr. Gurusiddaiah**, Food Microbiology Department for their encouragement, support and various help during my tenure*

*I extend my heartful thanks to Mr. **Somanayak, S.**, for his constant support and encouragement through my tenure.*

*I express my deep sense gratitude to **Sri Pathanjali Yoga Shikshana Samithi** and its **instructors** for providing a good health fit during my tenure.*

*I extend my sincere thanks to **all my aunts, uncles and cousins,** for their constant support and encouragement through my Ph.D. tenure.*

*I extend my heartful thanks to our house owner family for their constant support and encouragement through my stay and **Mr. Krishna,** I-café Browsing centre, Saraswathipuram, Mysore, for their help to complete this thesis.*

*Last but not the least my sincere gratitude also goes to my Grand-Parents, Parents, Brother **AnanthaRamu S.S.**, Sisters, Brother in law, Sister in law and two kids for the fun, criticism, their constant encouragement and boosting up confidence in me.*

Mamatha S.S.

CERTIFICATE

I, **Mamatha S.S., certify** that this thesis is the result of the research work done by me under the supervision of **Dr. G. Venkateswaran, Scientist-F** at **Food Microbiology Department, Central Food Technological Research Institute (CFTRI), Mysore-20.** I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D.) degree in Microbiology of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree/diploma of this or any other University.

Signature of Doctoral candidate

Signed me on

Signature of Guide
Date:

Counter signed by
Date:

Signature of Chairperson/Head of Department/ Institution with name and official seal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

ABSTRACT

Lipids are one of the main classes of macronutrients required for human growth and nourishment. In order to obtain native isolate for GLA production, soil samples were collected from various habitats for screening the *Mucor* spp. in nutrient rich medium. Based on the external morphology and microscopic observation *Mucor* spp. were isolated, compared with the standard cultures and comparisons were also made with the Gilman manual of soil fungi

These isolates were screened for their oleagenicity both qualitatively and quantitatively, staining with Sudan black-B and gravimetric method respectively. All the selected *Mucor* spp. and standard cultures were cultivated on submerged fermentation to study the growth characteristics, lipid and GLA contents. The results indicated that the highest biomass, lipid and GLA content were observed in *M. rouxii* CFR-G15. The presence of GLA in the lipid of this isolate was confirmed by the mass spectrometric method.

By using gene specific primers, 18S rRNA and Δ^6 *DES* genes were amplified by PCR technique. Nucleotide sequences of 18S rRNA and Δ^6 *DES* genes exhibited 98% homology with *M. rouxii* ATCC 24905 (accession no.AF117923 and AF296076) respectively, suggesting taxonomic identity. A nucleotide sequences determined in this study was submitted to the GenBank and obtained the accession numbers EU927296 and EU526025 for 18SrRNA and Δ^6 DES genes respectively.

Cultivation conditions were selected for GLA production by optimizing appropriate selection of both physical and chemical parameters such as pH, temperature, aeration, inoculum concentration, medium composition, carbon and nitrogen sources, C:N ratio combination, different vegetable oils, and different concentration of minerals in growth media. It was concluded in this study that pH 5.5, temperature at 28±2°C, glucose, yeast extract & ammonium nitrate as carbon and nitrogen sources and C:N ratio of 60 produced maximum biomass, lipid production and GLA content in this strain. A CCR design was used for

optimizing the levels of carbon and nitrogen sources to maximize the GLA production by RSM. By using different solvent extraction, this study concluded that chloroform and methanol in 2:1 ratio gave maximum recovery of lipid.

The *M. rouxii* CFR-G15 was subjected to mutation through physical and chemical methods (UV, EMS & NTG) and after several screening, a met^- auxotroph was obtained and this amino acid marker was used for hybridization studies to obtain stable hybrids. Putative hybrids were qualitatively characterized for high lipid and GLA production. Hybrid CFR-HyG 9 showed highest GLA content of 23.34 ±1.23 % in 42.46±2.41% of lipid content which was 1.5 fold higher than the parent culture in basal medium. Thus, the various experiments carried out in this study, ultimately aimed to increase the biomass, total lipid content and maximizing the GLA production.

Plants beings the conventional sources of essential fatty acids have some limitations/disadvantages that can be over ruled by microorganisms. Hence microorganisms of GRAS status are now being exploited genetically as sources of PUFAs and tailored for maximum productivity. The potential also exists in selecting the microbial strains producing large proportions of lipid that can meet the present market demand of omega-6 fatty acids, the GLA for nutritional and pharmaceutical supplementation.

CHAPTER -1

INTRODUCTION

INTRODUCTION

Fats and oils, being essential ingredients in human diet serve as an important raw material for the industry. Presently the bulk of fats and oils are obtained mainly from plant and animal sources. The demands for fats and oils for edible and non-edible purposes are ever increasing and that from the conventional sources are unable to supply for the increasing world population. Search is going on for alternative to conventional sources. Lipid from microbes have been found to be promising to meet these requirements as some strains accumulate more than 20 to 70% lipids inside the cell, which can be extracted economically.

Microbial lipids containing high proportion of polyunsaturated fatty acids (PUFAs) of nutritional and pharmaceutical importance, which are similar in composition to those of edible and non edible oils obtained from plants and animal sources are known as single cell oil (SCO). PUFAs have diverse functions in living cells and influence membrane composition and function, eicosanoid synthesis, cellular signaling and regulation of gene expression (Gill & Valivety 1997; Horrobin 1992; Tapiero et al. 2002; Wallis 2002). Lipids rich in PUFAs, present in sufficient quantities in microorganisms, plant and animals are known as speciality lipids. Their production has been known from 100 years. For the last two decades many attempts have been made to understand the process of lipid accumulation in oleaginous species. All microorganisms don't have the capacity to accumulate lipid, in the form of triacylglycerols. A number of prokaryotic and eukaryotic microorganisms can accumulate triacylglcerol as cellular storage from 20 to 70% of the dry biomass and are known as oleaginous species (Ratledge 2003; Waltermann et al. 200). In oleaginous microorganisms, when nitrogen is exhausted in the culture medium, it triggers lipid accumulation, but glucose continues to be assimilated. Isocitrate dehydrogenase ctivity within the mitochondrion slower due to the diminution of adenosine mono phosphate (AMP) within the cells. This leads to the accumulation of citrate, which is transported into the cytosol and cleaved to acetyl-CoA by ATP: citrate lyase, an enzyme that does not occur in non-oleaginous species. In particular, oleaginous molds have the

ability to accumulate large amount of lipids, and serve as alternative source for the production of pharmaceutical grade PUFAs (Aggeslis & Sourdis 1997).

Demands for PUFAs are constantly increasing while the sources producing them are not increasing in the same pace (Wallis et al. 2002). Various alternative sources are being explored which yield these essential elements for health. These lead to the screening of new strains and better understanding of lipid production in microorganisms. It was found that

- The numbers of microorganisms that accumulate more than 20% of biomass weight were relatively small when compared to total number of species.
- Yeast, fungi, and a few bacteria are the major oil accumulating microorganisms and produce extractable oil
- The oil produced from these microorganisms are very similar to plant oils mainly composed of triacylglycerol having the same composition as that of plant fatty acids
- Algae are considered as one of the main sources for lipid production with high proportion of PUFAs, but this lipid tended to be more complex than those yeast and fungi, and some PUFAs were observed similar to those found in fish oils (Grahame et al. 2004; Van der Westhuizen et al. 1994).

The productions of microbial lipids rich in PUFAs are of current interest from an industrial point of view (Ratledge 1991; 2004)**.** PUFAs are classified into two groups, the omega-6 (n-6) or the omega-3 (n-3) fatty acids, depending on the position of the double bond (n) closest to the methyl end of the fatty acid chain. In mammals, PUFAs cannot be synthesized by *de novo* and they must be supplied through diet. They are essential fatty acids, linoleic acid (LA, 18:2n6) and alpha-linolenic acid (ALA, 18:3n3). PUFAs are produced from these fatty acids through a series of desaturation and elongation reactions catalyzed by desaturase and elongase enzymes, respectively (Certik & Shimizu 1999; Uttaro 2006).

Gamma-linolenic acid (GLA, C18:3, 6,9,12) is known as a conditional essential fatty acid in n-6 series PUFAs, because of high impacts on both its pharmaceutical and nutraceutical interest (Fan & Chapkin 1998; Harbige 2003). GLA is commercially produced from plant seed oils, which includes evening primrose (*Oenothers bennis*), borage *(Borago officinalis*) and black currant (*Ribes nigrum*). The inconsistency of the fatty acid composition and low GLA content of these plant oils, depending on seasonal and geographic variations are the major drawbacks in production these oils (Gill & Valivety 1997).

Fungal lipids are the preferred sources of pure gamma-linolenic acid (GLA) because the competing plant oils contain high amounts of other PUFAs that hinder GLA's purification (Ratledge & Wynn 2002). GLA is very important in therapeutic uses because it has selective anti-cancer properties and it also alleviates a number of diseases (Das 2004; Kenny et al. 2000). The biotechnological approach for production of SCO especially the GLA is always of importance because of the capacity of oleaginous micro-organisms to convert agro-industrial raw materials into valuable lipids, in solid-state or liquid-submerged fermentations (Certik et al. 1997; Chen & Chang 1996; Gema et al. 2002; Papanikolaou et al. 2001; 2002a, b; Papanikolaou & Aggelis 2003; Strendaska et al. 2000b). Microorganisms like *Mucor circinelloides* as a source of GLA, *Mortierella alpina* for achidonic acid (AA), and *Crypthecodinium cohnii* and *Schizochytrium* spp. for docosahexaenoic acid (DHA) production have been used commercially to produce the various SCO, being grown in stirred tank fermenters (Ratledge 2002). The first process that was developed for microbial oil with high level of GLA in industrial scale was by biotechnological means using *Mucor circinelloides* (Certik & Shimizu 1999; Ratledge 1994). The process provided an important milestone and bench mark for all future microbial oil productions. Today large scale commercial processes are operated for the production of oils rich in either arachidonic acid or docosahexaenoic acid using various microorganisms.

There are many published reports about fungal species and strains producing GLA. Sofar, only members of the lower fungi belonging to the order *Mucorales* are found to be promising GLA producers (especially *Mortierella* spp. *Mucor* spp., *Cunninghamella* spp. *Pythium* spp. and *Rhizopus* spp.) and fermentations have been developed for improving productivity of the GLA-rich oils (Jang et al. 2005; Mukherjee 1999; Stredansky et al. 2000a, b; Suzuki et al. 1981; Van der Westhuizen et al. 1994). Two large-scale processes have been developed for the commercial production of GLA from *Mortierella isabellina* and *Mucor javanicus* (Ratledge 2003). Widespread interest in this acid for dietary and pharmacological uses has inspired numerous research groups to keep on searching for particularly effective fungal strains and developing optimal conditions for their production. Since microbial production has certain advantages over plant tissue and algal cultures, efforts have been directed toward research.

Mucoraceous fungi especially the *Mucor* spp. has been reported to be an interesting organism for the production of GLA-rich oils (Aggelis & Sourdis 1997; Komaitis et al. 2001; Mamatha et al. 2008; Somashekar et al. 2002), since GLA is of great pharmaceutical interest (Das et al. 2004; Fakas et al. 2006; Horrrobin 1992). In spite of their significance, little is known about the details of biosynthesis of PUFAs in oleaginous molds, as the most studies are limited to yeast and plants (Ratledge & Wynn 2002). Furthermore the research on the details of lipid biosynthesis in the fungi mucorales and its regulation are just emerging areas (Certiik & Shimadzu 1999; Fakas et al. 2006) with special emphasis given to the role of lipid bodies in triacylglycerols (TAG) biosynthesis and storage (Aggelis & Sourdins 1997; Fakas et al. 2006; Papanikolaou et al. 2002; Waltermann et al. 2000). Some of the *Mucor* spp. are dimorphic in nature with ability to grow as yeast as well as filamentous forms depending on the environmental conditions (Orlowski 1991). M*ucor* spp. are thought to be more advantageous to produce fatty acids than algal, moss and protozoal sources (Botha et al. 1995; Ratledge 1993). Enzyme Δ^6-desaturase catalyzes, conversion of linoleic acid (C18:2 n6) and alpha linoleic acid (C18:3 n3) into GLA (C18:3 n6) and stearcodonic acid (C18:4, n3) respectively (Huang et al. 2001; Sakuradani et

al. 1999). Δ^6-desaturase enzyme is a membrane bound front-end desaturase having molecular weight of 66 kDa and it inserts the double bond inbetween 6 and 7 carbon atoms of linoleic acid forming GLA (Das et al. 2001). Identification of genes involved in synthesis of GLA helped for the characterization of organisms in relation to the taxonomy and phylogeny (Das et al. 2001; Sakuradani et al. 1999). Additionally, GLA can be used as a chemotaxonomic marker to differentiate between the genera and the species in zygomycetes (Stahl & Klug 1996).

SCOPE OF THE INVESTIGATION

Gamma linoleic acid (GLA) is an important ω-6 fatty acid and it is a precursor of long chain polyunsaturated fatty acids. Dietary supplementation with GLA is reported to be effective in treating a number of diseases like atopic eczema, diabetes, neuropathy, viral infections and cancer. It indicates that GLA has applications in both nutraceutical and pharmaceutical industries. GLA is obtained mainly from plant seed oils like *Borage* spp (*Borago officinalis* L, GLA: 10-25%), and evening primrose (*Oenothera biennis* L, GLA: 7-10%). The productions of GLA from plant sources are generally low and vary with season, climate and geographical locations. Hence, the quantity and quality of conventional sources of GLA may fall short of the market demand. Due to aforesaid drawbacks and increasing importance of GLA in pharmaceutical and nutraceutical industries, a need for an alternative source for the production of GLA which can compete in cost and quality with conventional sources was thought of from oleaginous microbial sources.

Oleaginous microorganisms have greater advantages over the other sources i.e. fast growth, easy of manipulating the organisms and also an appropriate vehicle for cloning foreign genes for the production of specific PUFAs. Zygomycetes especially mucorales are able to synthesize GLA and accumulate in large amounts within the mycelium. Microbial production of lipids that contain

unusual fatty acids like PUFAs with potential commercial application has been aimed at this study with the selection of organisms by screening and optimization. The scope of the present investigation is screening and isolation of *Mucor* spp. from local soil samples and to explore the potential strain for GLA production. *Mucor* sp. CFR-G15 strain was selected after systematic screening among the cultures and this strain was subjected to both sub-merged and solid-state fermentation studies for its biomass, total lipid and fatty profile. Taxonomic identification of *Mucor* sp. CFR-G15 was carried out through molecular method i.e. 18s rRNA sequencing. Phylogeny of Δ^6-desaturase enzymes was studied to understand its diversity. Physiology of lipid accumulation and GLA production in *M. rouxii* CFR-G15 has been studied through variations of growth conditions. Physical and chemical parameters like pH, temperature, aeration, carbon source, nitrogen source, C:N ratio, supplementation of metal ions and oils were evaluated in order to produce high biomass, lipid yield, and GLA content. Statistical optimization of medium components was also carried out by response surface methodology for increased production of GLA in mycelium of *M. rouxii* CFR-G15. A genetic study of *M. rouxii* CFR-G15 culture was carried out by mutation and protoplast fusion. This experiment was aimed to produce strains increased with lipid production and GLA content. UV radiation, chemical mutagens like EMS and NTG treatments were used to mutagenise the culture. The hybrids obtained through protoplast fusion were characterized with high lipid and GLA content in fatty acids. Thus, high GLA producing hybrids of *M. rouxii* CFR-G15 were identified.

CHAPTER -2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

LIPIDS AND FATTY ACIDS

Fats, also known as lipids are the esters of glycerol and fatty acids. They are one of the major nutrients along with protein and carbohydrates in human diet. Lipid stored in the cell membrane as oil droplets acts as energy reservoir. The major component of lipids is triacylglycerol (TG). Which consist of three fatty acids attached to a glycerol backbone as sn-1, sn-2 and sn-3 position as shown in Fig. 2.1. Triacylglycerol and fatty acid compositions can vary both within and between organisms in cell membrane. Phospholipids, glycolipids and sterols form a complex that occurs in association with the protein (Lehninger 1993; Willis et al. 1998).

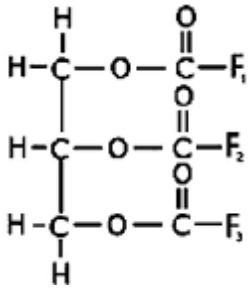


Fig.2.1 The structure of triacylglycerol. The fatty acids in the *sn*-1, *sn*-2 and *sn*-3 positions (F1, F2 and F3) will, most likely, be different.

Fatty acids are long chain of aliphatic acids (alkanoic acids) varying in chain length from, normally, C_{12} to C_{22}. In most cells like microbial, plant and animals, predominant chain lengths are 16 and 18. The chain length influences the characteristics of a fatty acid, as does the presence or absence of double bonds between carbon atoms. The structure of fatty acid represented by a simple notation system-X:Y, where X is the total number of carbon atoms and Y is the number of double bonds. Fatty acids are made up of a backbone of carbon atoms, with a methyl group (CH3) at one end [the omega (ω) or *n*-end] and a carboxyl group (COOH) at the other [the delta (Δ) end] [Fig. 2.2]. Hydrogen atoms are joined to the string of carbon atoms, forming a hydrocarbon chain. The fatty acids are classified as follows.

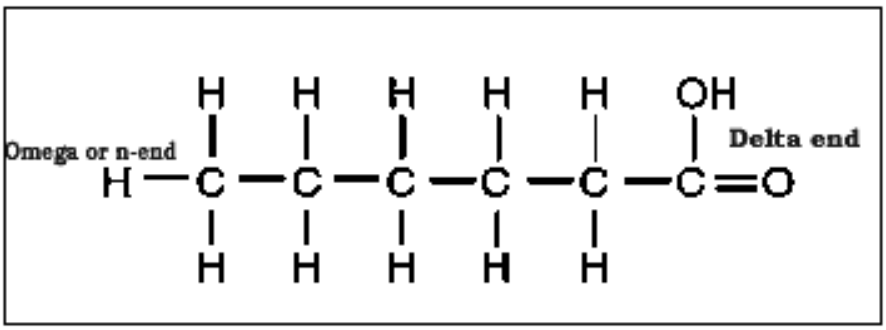


Fig. 2.2 The general structure of fatty acids

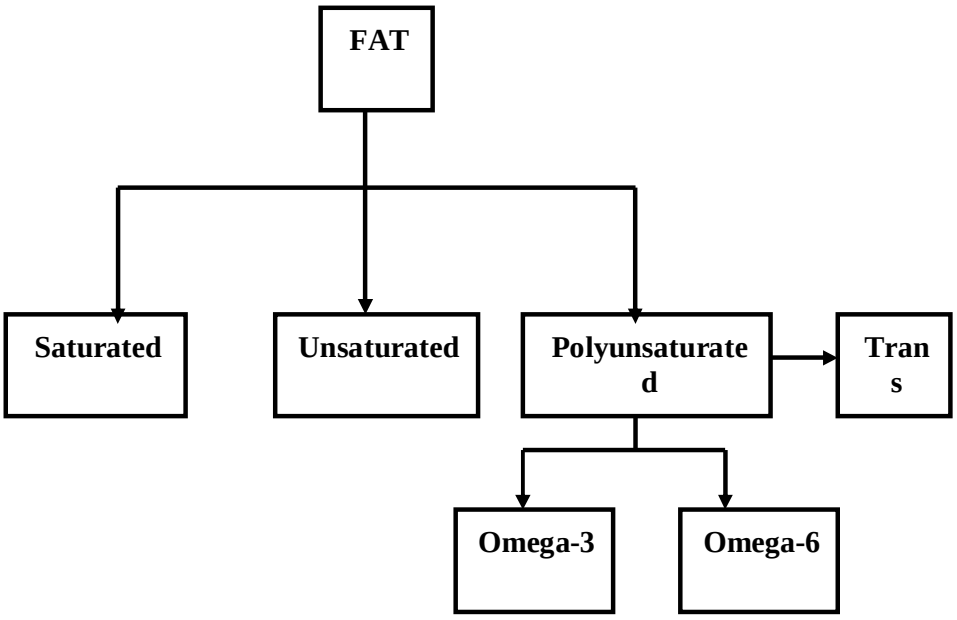


Fig. 2.3 Classification of Fats (lipids)

- If all of the carbons in the fatty acid chain are linked by single bonds is known as saturated fatty acid (SFA).
- If one or more double bonds are present in the fatty acid chain, it is considered to be an unsaturated fatty acid.
- If there is only one double bond present in an unsaturated fatty acid, it is said to be a monounsaturated fatty acid (MUFA).
- If there is more than one double bond present, the fatty acid is said to be a polyunsaturated fatty acids (PUFAs) [Fig. 2.3].

Fats and oils, being an essential constituent in human diet are also important raw materials for the industry. At present the bulk of fats and oils are obtained from plant sources. The edible and non-edible uses of fats and oils are presented in Table 2.1. Demand for oils and fats both for edible and industrial purpose is continuously increasing [Table 2.2]. The demand for oils and fats is largely met by plant sources. Animal and marine sources contribute less than 25% of total production of fat. Production of oils and fats [Table 2.3] is mainly from seven major plant crops: soybean, groundnut, cottonseed, rapeseed, palm, coconut and sunflower. In India groundnut, sunflower, cotton seed and coconut are grown as oil crops. In USA soy bean, sunflower and cotton seed are major oil producing plants. In Europe, only rapeseed is grown as an oil crop. Plants and animals, the conventional sources of edible fats and oils are unable to meet the demands of consumer and industries. Microorganisms are looked upon as an alternative or additional source of oils. As yeasts are having their long association with human diet, they, in particular appear to be the most likely source for bio oils. They produce oils similar to the composition of edible oils and are highly suitable for animal and human diet (Nigam 1999; Willis et al. 1998).

Table 2.1 Commercial application of fats and oils

(A) Oil source	Products (edible)
Soybean oil, groundnut oil,	Margarine
cottonseed oil, sunflower	Cooking fat
oil, rapeseed oil, sesame oil,	Cooking oils
palm oil, some fish oils,	Salad oils/mayonnaise/table oils
olive oil, castor oil,	Ice cream
lard and tallow	Confectionery
Coconut oil, palm kernel oil, castor oil	Pharmaceuticals
(B) Oil source	**Products (non-edible)**
Palm kernel, coconut oil	Detergents and surfactants
Palm oil	Soaps, metallic soaps, synthetic waxes
Linseed oil, tung oil, soybean oil, sunflower oil	Paints and coatings
Linseed oil, tung oil	Varnishes and lacquers
Various, mainly castor oil	Inks
Various, mainly soybean oil	Plastics and additives
Castor oil, coconut oil	Lubricants and cutting oils
Tung oil	Wood dressings, polishes
Fish oils	Leather dressing
Palm oil and tallow	Metal industry
Various, mainly soybean oil	Agrochemicals, long-chain quaternary compounds as herbicides, insecticides and fungicides
Fatty alcohols from any source	Evaporation retardants
Tallow	Fabric softeners

(Source: Nigam 1999)

Fats provide energy; indeed it is the most energy dense of all the macronutrients, it does play an important role in energy requirements and enables the absorption of fat-soluble vitamins A, D, E, and β-carotene in humans and animals (Lunn & Theoblad 2006). Lipids act as principal components of cell membranes and serve as barrier to separate from constituents and are vital for maintaining cellular integrity, shape, and flexibility. Fat is needed by all cell membranes-nerve, brain, eye, heart, adrenal and thyroid cells to function. Lipids involved in the maintenance of the vascular wall, in regulating blood pressure and in the production of eicosanoids, the family of hormone like compounds regulate

many organ systems (Ratledge et al. 1988; Tapiero et al. 2002). In recent years the public is aware of good fat that have been associated with different types of dietary fats to a number of health benefits (Lunn & Theoblad 2006).

Table 2.2 Production and consumption of edible oil during last five years in India

Oil Year (Nov.- Oct.)	Production of Oilseeds	Net availability of edible oils from all domestic sources	Consumption of Edible Oils (from domestic and import sources)
2000-2001	184.40	54.99	96.76
2001-2002	206.63	61.46	104.68
2002-2003	148.39	46.64	90.29
2003-2004	251.86	71.40	124.30
2004-2005	243.54	72.47	117.89
2005-2006	279.79	83.16	126.04
2006-2007	242.89	73.70	115.87
2007-2008(E)	282.08	84.27	121.91

(Source. Website of the Directorate of Vanaspati, Vegetable Oils & Fats, Department of Food and Public Distribution)

Table 2.3 The typical unsaturated fatty acid composition of selected fats and oils from plant and animal origin (g/100 g oil)

	Total fat (g/100 g)	MUFA			PUFA				
		14:1	16:1	18:1	18:2	18:3	20:4	20:5	22:6
Butter	82.5	0.69	1.24	15.80	0.95	0.46	0.09	0.08	-
Margarine	81.7	-	0.23	29.21	12.42	3.36	-	-	-
Cod liver oil	99.9	-	-	14.96	2.60	1.10	0.90	1.40	8.30
Olive oil	99.9	-	0.70	71.90	7.50	-	-	-	-
Linseed oil	99.9	-	-	20.20	12.70	53.30	-	-	-
Palm oil	99.9	-	Tr	37.10	10.10	0.00	-	-	-
Peanut oil	99.9	-	Tr	43.30	31.00	0.00	-	-	-
Rape oil	99.9	-	0.20	57.60	19.70	9.60	-	-	-
Soybean oil	99.9	-	0.10	20.80	51.50	7.30	-	-	-
Sunflower oil	99.9	-	0.10	20.20	63.20	0.10	-	-	-

(Source: Lunn & Theoblad 2006; Willis et al. 1998)

ESSENTIAL FATTY ACIDS

Essential fatty acids (EFAs) are those fatty acids that are required for normal physiological human health but are not synthesized by *de novo* pathway in the body; they must be obtained from dietary sources. There are two essential fatty acids, Linoleic acid (n-6, C18:2 cis LA) and alpha linolenic acid (ALA, n-3, C18:3 cis) [Fig. 2.4]. EFAs play a role in every life process of our body and life without them is unfeasible. Like vitamins EFAs are essential to human health. The major signs and symptoms associated with the deficiency EFAs are shown in Table 2.4.

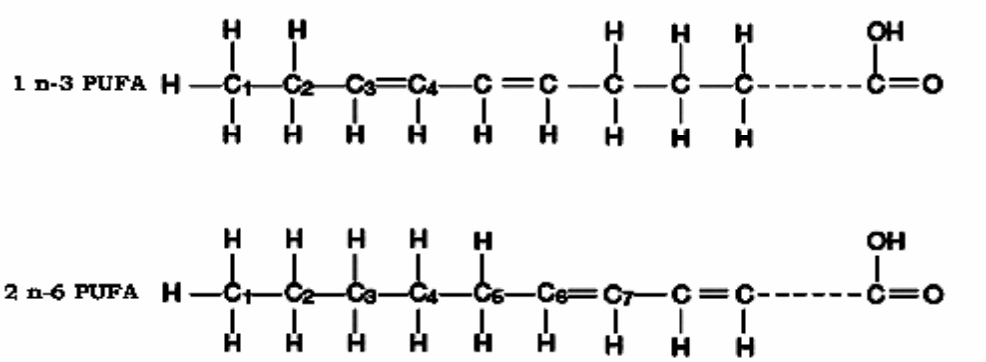


Fig. 2.4 *w*-3 and *w*-6 fatty acids (PUFAs-polyunsaturated fatty acids).

Table 2.4 The major sign and symptoms associated with the deficiency of essential fatty acids

Signs & Symptoms	Fatty acid association	Action
Emaciation, weakness, disorientation	Caloric deprivation	Add balanced of fat, protein, and CHO.
Reduced growth, renal dysplasia, reproductive deficiency, scaly skin	Classic essential fatty acid deficiency	Add good quality fats and oils
Eczema-like skin eruptions, loss of hair, liver degeneration, behavioral disturbances, kidney degeneration, increased thirst, frequent infections, poor wound healing, sterility (m) or miscarriage (f), arthralgia, cardiovascular d., Growth retardation	Linoleic acid insufficiency	Add corn or safflower oils
Growth retardation, weakness, impairment of vision, learning disability, poor coordination, tingling in arms / legs, behavioral changes, mental disturbances, low metabolic rate, high blood pressure, immune dysfunction	Alpha or gamma linolenic acid insufficiency	Add flax, primrose, borage, or black currant oils.
Depression, anxiety, slower behavioral and visual development or Cardiovascular disease risk	Long chain PUFA-dependent neuromembrane function Prostanoid balance	Add fish oils Avoid hydrogenated oils
Cancer	Low stearic to oleic ratio, Prostanoid imbalance	Add omega-3 PUFAs Use omega-6 PUFAs with caution
Rheumatoid arthritis	Low GLA & DGLA	Add primrose oil
Myelinated nerve degeneration	Increased very long chain FAs	Add high-erucate rape or mustard oils
Fatty liver	Saturated and omega-9 accumulation in liver	Restrict alcohol Add lecithin Increase Met
Accelerated aging	High PUFA intake without increased antioxidants	Add vit. E and C and Se, Mn, and Zn

(Source: www.7 007b.com fats and essential fatty acids?)

Major functions of EFAs

- It forms the membrane barrier that surrounds our cells and intracellular factories.
- Determine the fluidity and chemical reactivity of membranes.
- Increasing oxidation rate, metabolic rate and energy level
- Keeps exocrine and endocrine glands active
- Precursors for all prostaglandins series, three families of short lived hormones determines the health of our cardiovascular system, kidney function and platelet aggregation, blood pressure
- Transport cholesterol
- Strengthen the immune system to fight infection by enhancing peroxide production
- Prevents the development of allergies
    - Essential fats are necessary for normal reproduction and growth
    - ω-6 fat converts into GLA with the help of an enzyme Δ^6 -desaturase, thus manufacturing a hormone like substrate PGE1 (prostaglandins1) that plays a critical role in prevention and beneficial response to numerous health disorders.
- EFAs play a role in every life process of our body and life without them is unfeasible. Consuming EFAs poor diet, it diverts health (Lunn & Theoblad 2006; Simonopoulos 1991; Willis et al. 1998)

POLYUNSATURATED FATTY ACIDS (PUFAs)

Polyunsaturated fatty acids (PUFAs) are fatty acids with two or more double bond in cellular lipids such as triglycedies and phospholipids. PUFAs can be further classified as either ω-3, (*n*-3, omega 3) or ω-6 (*n*-6, omega 6) PUFAs, depending on the position of the first double bond in the fatty acid chain. All members of the ω-6 family of fatty acids contain their first double bond between the sixth and seventh carbon atoms from the terminal methyl group, while all

members of the ω-3 family of fatty acids have their first double bond between the third and fourth carbon atoms. They are usually named in abbreviated form as X:YnZ, where X stands for the number of carbon atoms of the chain, Y the number of double bonds, and Z the position of the first double bond counted from the methyl end (the n system of numbering). ω-6 or n-6 fatty acids are the predominating fatty acids in plants and animals where as, ω-3 or n-3 fatty acids are commonly found in marine foods, animals and phytoplanktons. The major PUFAs and their structures are given in Table 2.5 and Fig. 2.5. Linoleic acid (18:2) and alpha linolenic acid (18:3) are the essential dietary fatty acids, and are precursors for a number of PUFAs, such as, Arachidonic acid (AA, 20:4, ω-6), Eicosapentaenoic Acid (EPA, 20:5, n-3), Docosapentaenoic Acid (DPA,22:5, n-3), Docosahexaenoic Acid (DHA, 22:6, n-3).

Table 2.5 Polyunsaturated Fatty Acids and Their Chemical Name

Formula	IUPAC Name	Common Name	Abbre--viation
18:2n6	9, 12 cis, cis octadecadienoic acid	Linoleic acid	LA
18:3n6	6, 9, 12 cis, cis, cis octadecatrienoic acid	ϒ-linolenic acid	GLA
18:3n3	9, 12, 15 cis, cis, cis octadecatrienoic acid	A-linolenic acid	ALA
20:3n6	6, 9, 12 cis, cis, cis eicosatrienoic acid	Dihomo-ϒ- linoelinic acid	DGLA (DHGLA)
20:4n6	4, 7, 12, 14 cis, cis, cis, cis Eicosatetraenoic acid	Arachidonic acid	AA
20:5n3	cis, cis, cis, cis, cis Eicosapentaenoic acid	Eicosapentaenoic acid	EPA
22:6n3	cis, cis, cis, cis, cis, cis Eicosahexaenoic acid	Docosahexanoic acid	DHA

(Source : Leninhger 1993; Lopez Alonso & Garcia Maroto 2000**)**

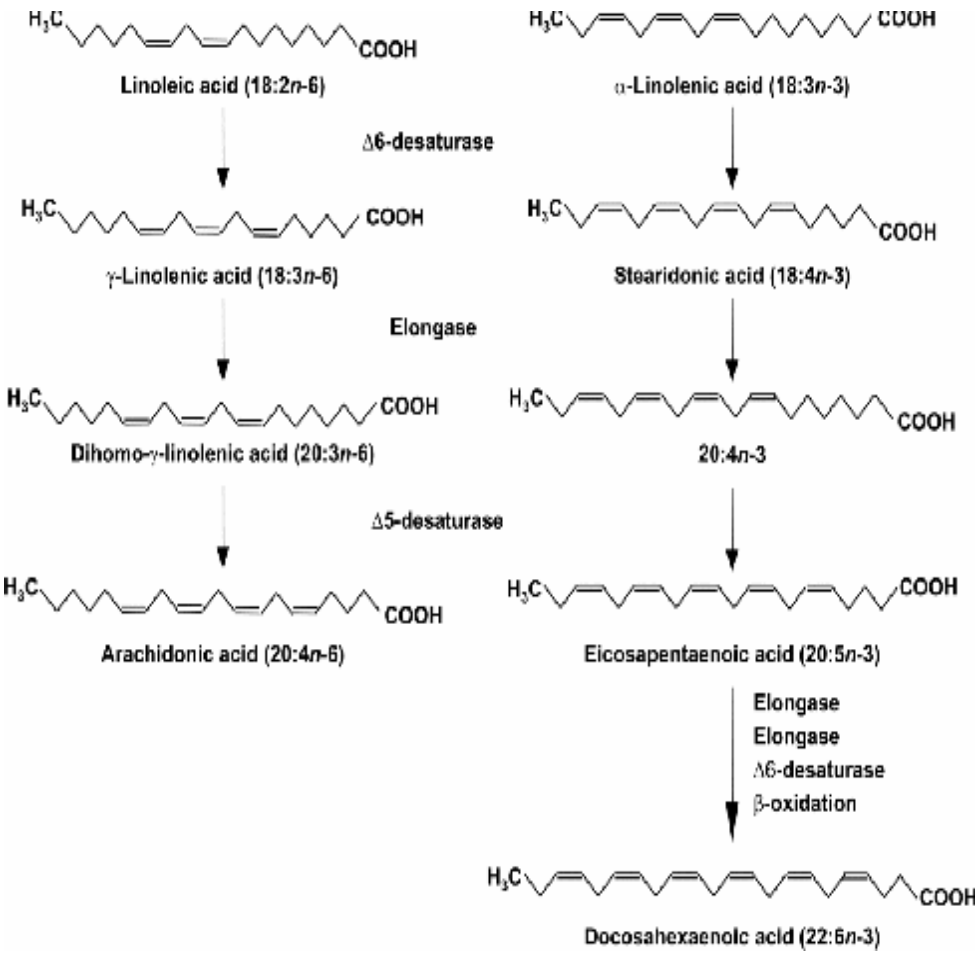


Fig. 2.5 Structure and metabolism of *w*-3 and *w*-6 PUFAs

Sources of PUFAs

Currently PUFAs are obtained from a various sources like higher plants, animal entrails, and oily fish. In recent years the demand for PUFAs in market is increasing but it has become evident that PUFAs productions from current sources are inadequate (Gill & Valivety 1997; Lopez et al. 2000; Napier et al. 1999; 2005; Wallis et al. 2002). The plant seed oils are the major commercial sources of C18 PUFAs. The plants cannot synthesise above C18 PUFAs due to a lack of the required enzymes in the cell system (Wallis et al. 2002). The long chain fatty acid like AA is mainly obtained from animal sources. EPA and DHA are commercially obtained from marine fish oils. The production of PUFAs from agricultural and

animals sources are generally low in content and vary with season, climate and geographical location. Marine fish oil, have disadvantages of objectionable taste and odours, high cholesterol and small amounts of potential toxic impurities that are difficult to remove. Therefore, the quantity and quality of conventional sources of PUFAs may come across problems meeting an increasing market demand (Napier et al. 1999; 2005; Ratledge 2003; Cohen & Ratledge 2005). Because of these drawbacks, new sources of PUFAs are needed that can compete in cost and quality with the conventional sources. A diversity of PUFAs presence has been determined in microorganisms which includes bacteria, fungi, algae and mosses (Gill & Valivet 1997; Hirano et al. 1990; Ratledge 2004; Van der Westhuizen et al. 1994). The conventional and non conventional sources of PUFAs are given in Table 2.6. These organisms are thought to be very promising and alternative producers of PUFAs because of their high growth rate on simple media and the simplicity of their manipulation (Shimizu et al. 1988; Yongmanitchai & Ward 1989; Bajpai & Bajpai 1992; Barclay et al. 1994; Somashekar et al. 2002; Papanikolaou et al. 2007). The diversity of microbial species can facilitate the selection of strains producing lipids with the target fatty acids. In recent years alternatives to conventional sources of oil production from oleaginous micro organisms have been intensively studied (Certik & Shimizu 1999). Bacteria associated with higher marine organisms like *Bacillus subtilis* have emerged as better source of highly pure PUFAs oils than fish and plant oils (Pantnayak & Sree 2005). A few bacterial species belonging to the genera *Mycobacterium, Streptomyces, Nocardia* and *Rhodococcus* are also the potential bioresources for the production of triacylglycerols (Pantnayak & Sree 2005; Waltermann et al. 2000). Fungal microorganisms like *Mortierella* and *Pythium* species are studied more intensively and found to produce high amounts of GLA, AA and EPA (Bajpai & Bajpai 1993; Certik & Shimizu 1999; Fakas et al. 2008; Shinmen et al. 1992). Single cell oil (SCO), presents a potential industrial interest for the food and pharmaceutical industries owing to their specific characteristics like pharmaceutical grade oil (Ratledge 1993; 2002; Cohen & Ratledge 2005). The most obvious target of the research in the field of microbial lipids is the

production of oil equivalent to edible oil in composition and structure i. e. lipid rich in PUFAs of medical interest (Dhyaneswar et al. 2006; du Preeze et al. 1997; Papanikolaou et al. 2001; Ratledge 1991).

Table 2.6 Sources of polyunsaturated fatty acids

PUFA	CONVENTIONAL SOURCES	MICROBIAL SOURCES
GLA	Plant seeds (Evening primrose, Borage, Black currant)	Fungi (*Mucor rouxii. M. circinelloides, M.mucedo, Mortierella isabellina, M.romanniana, Cunninghamella elegans, Rhizopus arrhizus)* Algae (*Spirulina platensis, Chlorella vulgaris*)
DGLA	Human milk, Animal tissue, Fish (*Scomber scombus),* Mosses (*Pogonatum urnigerum*	Fungi (*Mortierella* spp., *Conidiobolus nanodes, Saprolegnia freox*), mosses (*Ctenidium molluscum*)
AA	Animal tissues (Porcine liver), fish (Brevoortia, Clupea), mosses (Ctenidium molluscum)	Fungi (*Mortierella, Pythium* spp.), algae: (*Porphyridium* spp.), mosses (*Rhytidiadelphus, Brachythecium, Eurhynchium* spp)
EPA	Fish (Herring, Menhaden) Shell-fish (Blue crab, Oyster, Lobster, Mussel)	Fungi (*Mortierella, Pythium* spp.), algae: (*Chlorella, Monodus, Porphyridium, Nannochloropsis, Cryptoleura, Schizymenia, Navicula* spp.), mosses (*Brachythecium, Eurhynchium, Scleropodium* spp), bacteria (*Rhodopseudomonas, Shewanella* spp.)
DHA	Fish (Tuna, Herring, Cod, Sardine, Salmon, Menhaden), Shell-fish (Blue crab, Oyster, Lobster, Mussel)	Fungi (*Thraustochytrium aureum, T.roseum, Schizochytrium aggregatum, Entomophthora* Spp.*),* Algae (Microalgae MK8805, *Gonyaulax,Gyprodinium nelsoni, Cryptoconidium* spp*),* Bacteria (*Vibrio* sp. *Rhodopseudomonas* spp. *Shewanella* spp.)

(Source: Certik & Shimizu 1999; Gill & Valivety 1997)

Applications of PUFAs

The PUFAs play a role in phase transition and permeability of membranes and modulating the behavior of membrane bound proteins and ion channels. In addition PUFAs control the expression of certain genes, biosynthesis of cholesterol and transport in the body. These PUFAs being important as structural lipids are precursors for eicosanoids such as prostaglandins, leukotrienes and thromboxanes, collectively known as eicosanoids, which mediate vasodilation, fever, inflammations, blood pressure, clotting, pain, neurotransmission and modulate the cholesterol biosynthesis **(**Gill 2002; Johnson et al. 1997; Moreton 1988; Pullman-Mooar et al. 1990; Puri 2007; Tapiero et al. 2002**)**. PUFAs deficiencies lead to abnormalities in the spine, nervous system, immune and inflammatory systems, cardiovascular systems, endocrine systems and kidneys. PUFAs are also used for retarding the growth of tumour cells in respirosystems and reproductive systems from last decades (Das 2004; Huang et al. 200; 1Simonopoulos 2002). Another important role of PUFAs in the diet is to enable the fat-soluble vitamins A, D, E and K to be absorbed from food and for body cholesterol metabolism (Das 2004).

As the PUFAs composition of cell membranes is greatly dependent on the dietary intake, the balance of ω-6 and ω3 fatty acids can be modified by supplementing the diet with oils enriched in ω3 PUFAs Simonopulous 1991; 2002). This could compensate the unfavourable ratio of ω-3/ω-6 fatty acids of the western diet and so lead to decreases in many chronic diseases like arthritis, diabetes, inflammation, cancer and cardiovascular disease and improvement in mental health. It is probable in man's evolutionary development that there has always been the proper balance between ω-3 and ω-6 fatty acids (Das et al. 2001; Gill & Valivet 1997; Graham et al. 2004; Horrobin et al. 1992; Simonopoulos 1991; 2001).

GAMMA LINOLENIC ACID (GLA, 18:3, w-6)

There are two isomers of linolenic acid found in all organisms they are alpha linoleic acid (ALA) and gamma linoleic acid (GLA). ALA is a ω-3 essential fatty acid, comprised of 18 carbon atoms with three double bonds at 9, 12 and 15 positions. GLA is an important conditional ω-6 essential fatty acid. The fatty acid molecule is comprised of 18 carbon atoms with three double bonds at 6, 9, and 12 positions. It is also known as 18:3n-6: 6, 9, 12-octadecatrienoic acid: cis-6, cis-9, cis-12-octadecatrienoic acid: and gamolenic acid [Fig. 2.6].

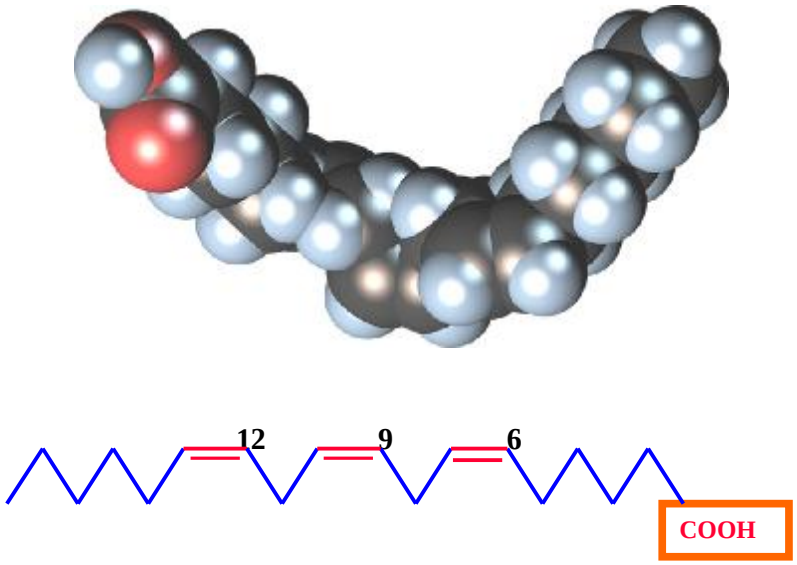


Fig. 2.6 Structure of Gamma linolenic acid (GLA)

GLA has a very long history of use, occurring as it does in the seed oil of the evening primrose (*Oenothera biennis,* EPO). EPO has been used as quasi medicinal oil for centuries; it is known as "Kings cure all" and thus has been recommended for the alleviation of a wide variety numbers of illness (Horrobin 1992; Huang & Mills 1996; Ratledge 1992a, b). In 1949, Riley re-examined the unsaturated fatty acids present in evening primrose oil and proved that GLA is other polyunsaturated fatty acid (Gunstone 1992; Horrobin 1992).

SOURCES OF GLA

GLA is found naturally in human milk and small amounts in a wide variety of common foods, notably it is found relatively high in organ meats or bird origin [Table2.7] (Horrobin 1992). GLA is present naturally in the fatty acid fractions of some plant seed oils [Table 2.8]. Most notably sources of GLA include evening primrose oil (EPO), borage oil, black currant oil, and hemp seed oil. Significant amounts of GLA have been found in the plant families *Onagraceae*, *Saxifragaceae* and *Scrophulariaceae*, but *Boraginaceae* is probably the best source. Commercial production of GLA sources [Table 2.9] are almost limited to seed oils from three plants: evening primrose, 9.6% GLA of total fatty acids (Hudson 1984; Ratledge 2003), borage, 23% GLA of total fatty acids (Guil-Guerrero et al. 2000; 2001a; 2001b) and black currant, 15±20% GLA of total fatty acids (Ratledge 2002a; Traitler et al. 1984) and some microbial sources, as *Mucor javanicus* (15±18%) and *Spirulina platensis* (21%) (Kamisaka et al. 1990; Ratledge 2004).

Table 2.7 Various food of animal or bird origin of GLA sources

Source	% lipid	% GLA in lipid	mg GLA/100g source
Beef			
Learn-raw	2.0	0.11	2.2
Fat-raw	60.4	0.10	60.4
Kidney-raw	3.1	5.0	5.0
Liver-raw	4.6	10.1	10.1
Chicken			
Learn-raw	2.9	0.13	3.8
Fat-raw	59.7	0.13	77.6
Pork			
Learn-raw	3.3	Trace	Trace
Fat-raw	69.7	0.03	20.9
Egg Yolk			
Free range hens	29.8	0.07	20.9
Commercial hens	31.5	0.06	18.9

(Source: Gunstone 1992; Horrobin 1992)

Table 2.8 GLA content of some Plant sources

Plant source	Oil content	GLA content in	
		Seeds	Oil
Oenograceae			
O. bennis	25	2.5	10.0
O. grandiflora	4	0.3	9.3
O. lamrkiana	28	2.3	8.2
O. stigosa	29	2.0	7.0
Boraginaceae			
Adelocaryum coelestinum	22	2.7	12.4
Alkanna froedini	47	4.6	9.9
A. orientalis	23	2.8	12.4
Amasinckia intermedia	28	2.3	8.2
A. leunaris	27	2.4	8.9
Brunera orientalis	27	4.2	15.4
Nonnea macrosperma	39	5.1	13/1
Pectocarya platycarpa	15	2.3	15.2
Scrophulariaceae			
S. lanceolata		2.0	8.0
S. marilandica		3.6	9.0
Saxifragraceae			
Ribes alpinum		1.7	8.9

(Source: Leman 1997; Gunastone 1992)

Table 2.9 Fatty acid profiles of various fungi and plants used for commercial production of GLA

Source	Oil content (%w/w)	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3 (n6)	C18:3 (n3)	20:1	20:2
M.cercinalloides	25	22	1	6	40	11	18	-	-	-
M.isabelliana	50	27	1	6	44	12	8	-	0.4	-
M.ramanniana	40	24		5	51	10	10	-	-	-
S.megalocarpous	22	14	-	1	12	10	62	-	-	-
Evening primrose	16	6		2	8	75	8-10	0.2	0.2	-
Borage	30	10	-	4	16	40	22	0.5	4.5	2.5
Blackcurrant	30	6	-	1	10	48	17	13		

(Source: Gunstone 1992; Ratledge 1992)

Borage and EPO are the most popular commercial sources available of the GLA oils among consumers. The growing conditions are ideal for high production of GLA in the seed oil. Borage leaves have traditionally been used in salads. Historically, herbalists have claimed that the consumption of borage leaves and flowers can treat depression and liver disorders. In addition, parts of the borage plant have diuretic characteristics which have encouraged its use for treating kidney and bladder inflammation. Traditional use of EPO has included parts of the whole plant, externally to heal wounds and to soothe the skin inflammation and internally to control coughs, as a sedative, pain killer and diuretic (Gill 2002; Gunstone 1992; Horrobin 1992; Ratledge 1992 a, b).

BIOCHEMISTRY OF GLA

The stereo specificity of GLA varies from source to source. In EPO and black currant oil, GLA is concentrated in the n-3 position, while in borage oil it is concentrated in the n-2 position. GLA is concentrated evenly in both the n-2 and n-3 positions of fungal oils [Table 2.10] (Gunstone 1992; Ratledge 2002).

Table 2.10 Stereospecific distribution (% mol/mol) of GLA in triacylglycerol oils from four sources

Position	EPO	Borage oil	Blackcurrant oil	*M. cercinelloides* oil
All	9.3	24.8	15.9	17.9
sn-1	3.6	4.0	4.1	13.3
sn-2	10.7	40.4	17.4	19.6
sn-3	13.5	30.1	25.8	19.6

(Source: o 1992; Gunstone 1992)

SCREENING FOR THE BEST GLA PRODUCING FUNGUS

The high biological activity of γ-linoleic acid and increasing need for essential lipids containing this acid led to search for alternative new natural sources. The presence of GLA in phycomyces fungi has been known since 1940 (Ratledge 1992a). It was subsequently found to be common fatty acids in fungi

classified as lower fungi, which includes the genus of phycomyces [Table 2.11] (Shaw 1965; 1966; 1966). The lower fungi are sometimes known collectively as the phycomycetes order but are more correctly classified into two subgroups; Mastigomycotina and zygomycotina, with members of both groups producing GLA in their lipids. Interestingly in recent years it has been reported that organisms like protozoa, marine and fresh water algae, bacteria, and higher fungi also produce GLA (Aggelis 1996; Barcaly et al. 1994; Dyal & Narine 2005; Gosselin 1989; Hirano et al. 1990; Patnayak & Sree 2005; Ratledge 2004).

Table 2.11 Phycomycets screened for GLA production

Absidia	Mortirella
Basiodiomycees	Mucor
Chaoenephora	Phlyctochytrium
Cunninghamella	Phycomyces
Delacroixia	Pythium
Entomopthora	Rhizopus zygorrinchus

(Source: Ratledge 1992a; 2004)

With the reports on occurrence of GLA in fungal microorganisms, it was attractive to consider a biotechnological route for its production (Ratledge 1993; 2003).

- The criteria used to evaluate GLA producing organisms in screening process are as follows;
- The organisms should grow readily in submerged fermentation. It should not create any problems for extensive filamentous growth or pellet formation.
- It should have extractable oil not less than 20% of the biomass.
- It should have a GLA content of the total fatty acids near to 20% and above.
- The oil should be over 90% triacylglycerol.
- The organisms should not have any toxic substances or no records of any causing allergic reaction or toxicity.
- The organisms should grow at room temperature or above since the low temperature is cost effective for large scale production.

There are many reports on fungal species and strains producing lipid and a few important GLA producers. These are shown in Table 2.12. Oleaginous fungi, especially zygomycetes have been recognized as candidate producers of PUFAs with high value (Certik et al. 1997; Kavadia et al. 2001; Kennedy et al. 1993; Komaitis et al. 2001). So far, only members of the lower fungi belonging to the order *Mucorales* are found to be promising GLA producers especially *Mortierella* spp. *Mucor* spp., *Rhizopus* spp. and *Cunninghamella* spp (Bandyopadhyay et al. 2001; Emelyanova 1997; Kennedy et al. 1993; Papanikolaou et al. 2004a; 2007; Torlanova et al. 1992). Two large-scale processes have been developed for the commercial production of GLA from *Mortierella isabellina* and *Mucor javanicus* (Ratledge & Wynn 2002). As fatty acids and lipids are growth-associated metabolites, many researches have attempted to search for new strain and optimized the culture conditions to improve the yield of microbial oils rich in GLA (Ahmed et al. 2006; Dyal et al. 2005; Hiruta et al. 1996; Lindberg & Hansson 1991; Somashekar et al. 2002). Since microbial production has certain advantages over plant tissue and algal cultures, most researches have been directed towards this development.

Table 2.12 Oleaginous fungus producing GLA in the dry mycelium

Fungus	**GLA (%)**
Mucor javanicus	9.00
Mortierella isabellina	9.20
Mucor cercinalooides	10.00
Cunninghamella. Echinulata	8.90
Mucor japonica	10.20
Conidiolbolus	7.70
Mortierella ramanniana	10.20
Mucor inaquiporus	9.80
Mucor hiemalis	8.80
Rhizopus oryzae	8.00
Absidia	7.90
Mucor genevensis	18.00
Mucor mucedo	15.4
Mucor racemosus	17.9
Rhizopus stolonifer	15.4
Mucor rouxii	19.4

(Source: Dyal et al. 2005; Ratledge 2003)

POTENTIAL SOURCES OF GLA

The knowledge of microbial biotechnology has advanced rapidly and this has opened up new possibilities for production of PUFAs by recombinant technology (Gill & Valivety 1997; Napier et al. 2004; 2005). Oleaginous microorganisms are attractive because of their high oil productivity. Also, well-established methods exist for commercial recovery of the oil (Dyaneswar 2006; Ratledge 1993). Thus, the genetic modification of microbes and oilseed crops to produce PUFAs is an attractive opportunity to produce GLA in high percentage in lipid biotechnology (Gill & Valivety 1997; Khoomrung et al. 2007; Ratledge 1993). Research on microbial PUFAs production was basically aimed at improving economic competitiveness of microbial lipids compared to plant and animal-derived lipids. Emphasis was placed on screening for more efficient strains, increasing the product value, using inexpensive substrates, and reducing the processing steps necessary for lipids recovered from the cells (Dyal & Narine 2005; Gema et al. 2002; Jang et al. 2000; Jangbua 2009; Jeennor et al. 2006; Papanikolaou et al. 2007).

MUCOR SPECIES

The species of *Mucor* continue to be the focus of study by a considerable number of microbiologists and mycologists with wide application in both basic and applied areas of biological research (Funtikova 2002; Orlowsky 1991). *Mucor* spp. are generally highly saprophytic and proteolytic, resulting in their ubiquitous presence in wide variety of substrates (Michinaka 2003; Orlowsky 1991).

The genus *Mucor* belongs to the order Mucorales within the class Zygomycetes (phylum Zygomycota). This order also includes the genera *Rhizopus* and *Mortierella*. Generally, fungi within this order are best known as saprophytes which favor simple sugars as opposed to more complex molecules. Organisms that implement a selected growth strategy have quick generation times that involve rapid growth, absorption of sugars, and proliferation. In addition to this

rapid cycle of growth and proliferation, the fatty acid profiles obtained from some *Mucor*, *Mortierella* and *Rhizopus* spp.have been found to have relatively high EFAs contents when compared to current industrial sources like evening primrose and borage seed oil (Carter 1988; Emelyanova 1997; Weete et al. 1998; Ward 1995; Yamada et al.1992). Furthermore, research has shown that the growth conditions of these fungi can be manipulated to give higher yields of specific EFAs.

Property of dimorphism, one characteristic which makes *Mucor* spp. distinct from other zygomycetes. Differential hyphal morphologies, mainly associated with the production of arthrospores, sporangispores or zygospores, only those that can grow in the form of spherical multipolar budding yeasts are referred to as dimorphic (Botha et al. 1997; Da Silva 2003; Orlowski 1991; Pohl 1997). Mucor sp. which show dimorphic characteristic includes *M. racemosus, M. rouxii, M. genevensis,* and *M. bacilliformis.* Other *Mucor* spp. like *M. miehei, M. pusillus, M. ramannianus M. mucedo* and *M. hiemalis* show monomorphic characteristic (Orlwosky 1991).

Metabolites produced from *Mucor* spp. have potential uses in food industry. These organisms are commonly used to produce wines fermented from rice in the orient and for the production of ethylaclohol in Europe (Orlowski 1991). Starins of *Mucor* have been used to produce sufu, tempeh (fermented soyabean food products), alpha amylase, fusidic acid and proteolytic enzyme rennin used in cheese making. *Mucor* spp. are also associated with the retting of flax and hemp, and decomposing of leathers (Botha et al. 1997; Orlowsky 1991).

Mucor spp. are most important in genetic studies on mating types (sexual reproduction) and regulatory mechanisms at the molecular level. The elucidation of zygospore formation and involvement of trisporic acid have been studied well in the *M. mucedo*. *M. racemosus* has been employed to study the relationship between overall cellular growth rate, protein synthesis, ribosome function, and

amino acid synthesis during starvation. *M. rouxii* has been used very well to study the role, regulation of chitinase synthesis in cell wall (Orlowsky 1991)

Mucor spp. in GLA production

Mucor spp. are the main organisms for the production of lipids rich in GLA and other PUFAs. Comparisons of total lipid fractions in *M. genevensis* and *M. rouxii* had much higher levels of sterols than yeast. Study of growth conditions is an approach toward better understanding of fatty acid metabolism and the role of fatty acids in cell growth and development of *M. rouxii* (Certik et al., 1997; Conti et al., 2001; Lindberg & Hansson, 1991).

M. rouxii, which is a non-pathogenic dimorphic fungus, is able to alternate its morphology between yeast-like and filamentous forms depending on its environment (Orlowski 1991; Certik et al. 1991; Michninaka et al. 2003). Most work involving fatty acid synthesis has been done on the mycelial form of *Mucor* (Botha et al. 1997; Emelyanova 1997; Hasson & Dostalek 1988; Somashekar et al. 2002). During fermentation, the high biomass of the mycelia limits oxygen and nutrient transfer. The control of cell morphology is therefore required to achieve good bioreactor performance for GLA production. With the advance of its morphogenesis, the yeast-like cells of *M. rouxii* are thought to be a candidate for developing an efficient GLA production process commercially. Since fatty acids are generally required for cell growth, development and response to environmental conditions (Botha et al. 1997; Da Silva et al. 2003; Khunyoshyeng et al. 2002), several factors which induce the formation of the yeast like cells of *M. rouxii*, may have an effect on its fatty acid composition and lipid content. It has been reported that the intracellular fatty acids of this fungus are mainly C16 and C18 acyl chains (49.0% of total fatty acids) with a substantial proportion of unsaturated fatty acids (Laoteng et al. 1999). Interestingly, the accumulation of relatively high levels of GLA has also been found in *M. rouxii* (Hansson et al. 1989). However, different fatty acid profiles have been found during growth and development of spores and mycelium (Jeennor et al. 2006; Khunyoshyeng et al.

2002; Laoteng et al. 1999; Mysyakina & Funtikova 2003). The GLA proportion was highly concentrated in sporangiospores (Khunyoshyengb et al. 2002; Mysyakina & Funtikova 2003) and at early stages of mycelial growth in *M. rouxii* (Laoteng et al. 1999). Reduced GLA content was found in association with an increase of oleic acid (C18:1, ω9) at the late logarithmic, stationary phases and also in the cells exposed to nutrient and oxygen limitations. Similar to other microorganisms, variation in culture conditions, such as medium composition, temperature and gas supply, highly influences the growth and fatty acid profiles of *M. rouxii* (Jeennor et al. 2006; Laoteng et al. 1999; Serrano et al. 2001). However, the feasibility of GLA production in large-scale fermentation should also be considered. Widespread interest in GLA for dietary and pharmacological uses has inspired numerous research groups to keep on searching for particularly effective fungal strains and developing optimal conditions for its production. In addition, GLA is the end product of PUFAs in *Mucor* sp., which differs from other Mucorales fungi, such as *Mortierella* sp. However, the production of microbial oils is so far not cost-competitive to the plant sources. Due to ability of the fungal cells to produce extracellular enzymes, such as saccharolytic and proteolytic enzymes, this fungus can use a wide range of substrates in either liquid or solid forms (Jangbua 2009). Moreover, some agricultural byproducts are being used as feed component for animals and fish. Therefore, the agricultural by-products derived from some factories, such as soybean meal, spent malt grain, orange peel, apple pomace, tomato waste hydrolysate, wheat bran, rice bran and cereals were also subjected to the fungal fermentation in order to introduce a cost-attractive process (Chen et al. 1999; Fakas et al. 2008; Gema et al. 2002; Papanikolaou et al. 2007; Stredansky et al. 2000).

GLA BIOSYNTHESIS AND D^6-DESATURASE

GLA is synthesized from linoleic acid by the action of a Δ^6-desaturase in both prokaryotes and eukaryotes. GLA represents the first product on the ω-6 PUFAs pathway [Fig. 2. 5]. Once formed, GLA is rapidly elongated to DGLA, by

the activity of a PUFAs specific elongase. DGLA can be converted to AA by the action of Δ^5 desaturase. Both DLA and AA can be metabolized to form eicosanoids (and on to prostaglandins and related compounds).

The key enzyme necessary for the synthesis of GLA is the Δ^6 desaturase. This enzyme is a subclass of microsomal membrane bound front end desaturase (Zuang et al. 2004). This catalyzes the introduction of double bonds between pre-existing double bonds and carboxyl bond (front end or Δ end) of the fatty acid molecule [Fig. 2.7] (Das et al. 2001; Michinaka et al. 2003). The desaturation of Δ^6-desaturase is aerobic reaction since it requires the oxygen for desaturation. In the recent past, the Δ^6-desaturase gene from various organisms has been identified. This development has increased the research on front end desaturase of fatty acids in biosynthesis of PUFAs (Das et al 2001; Napier et al. 2004; Utttaro 2006).

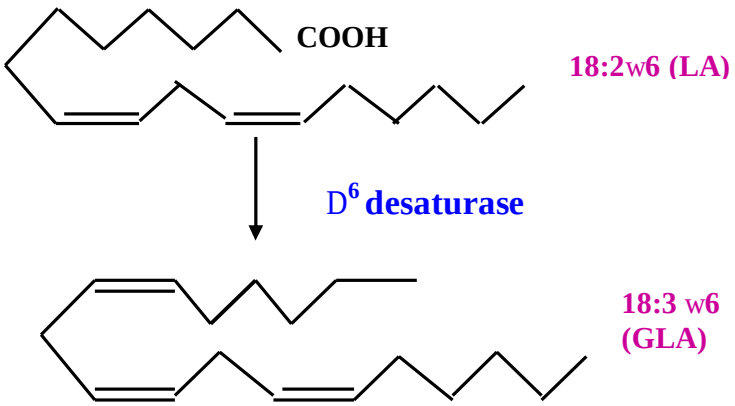


Fig. 2.7 Activity of D^6 desaturase on linoleic acid

The formation of GLA is dependent on the activity of the Δ^6-desaturase, which is hindered by numerous factors such as aping, nutrient deficiency, trans-fatty acids, use of hydrogenated oils, smoking, and excessive alcohol consumption [Fig. 2.8]. Other ω-6 fatty acid supplementation may cause an increase in AA and the undesirable pro-inflammatory, 2-series prostaglandins. A combination of GLA with EPA or DHA may antagonize the conversion of AA.

These effects will be more favourable with an increase in anti-inflammatory and antithrombotic effects (Huang & Ziboh 1997; Napier et al. 2004; 2005; 2006;).

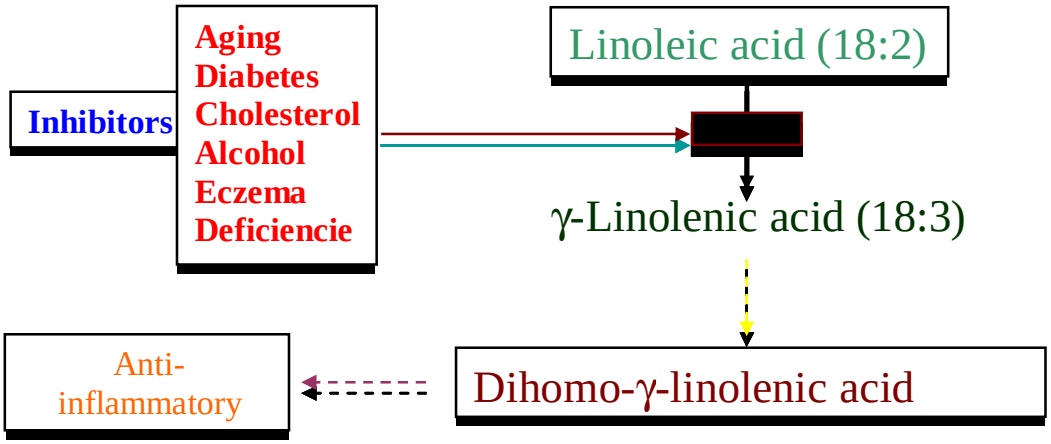


Fig 2.8 Omega-6 pathway and inhibitors of GLA synthesis

Characteristic of D^6-desaturase enzyme

Front end desaturases are fatty acids either esterified to glycerolipids or to CoA depending on the availability of the substrate in different organisms (Das et al. 2001; Na-Ranong et al. 1994; Tocher et al. 1998;). In Cyanobacteria the D^6-desaturase is believed to be esterified on C18 at Sn-1 in the thylakoids membrane and desaturation occurs to link to monogalactosyl diacylglycerol. In lower eukaryotes like fungi, the fatty acids are linked to phospholipids. In higher plants only on C18 chains on both Sn-1 and Sn-2 in the endoplasmic reticulum mainly linked to phosphatidylcholine (Ratledge & Wynn 2002). While in animals, D^6-desaturase generally utilizes the CoA linked substrates in the endoplasmic reticulum (Na-Ranong 1994; 2006; Tocher et al. 1998; Uttaro 2006). The D^6-desaturation step has been found to be the rate limiting step in the metabolic pathway of the ω-6 and ω-3 pathway i.e. LA and ALA to AA and EPA respectively (Das et al 2001; Uttaro 2006).

Identification of D^6-desaturase in fungi

Fungal D^6 -desaturase has been identified from *M. alpine* and *M. rouxii* (Huang et al. 1999; Laoteng et al. 2000). The predicted proteins showed all the characteristics of microsomal membrane bound, front end fatty acid desaturase. The *M. alpine* D^6 desaturase has been functionally expressed in yeast (*S. cereveceae*) as well as fungi *(A. oryzae*) (Huang et al. 1999; Sakuradani et al. 1999) and pnats (brassica juncea) (Huang et al. 2002). *M. rouxii* D^6 desaturase differs from that of *M. alpine* and other fungus, it is larger and more similar to plant D^6 *desaturase* than to fungal or animal D^6 desaturase. It contains a unusual histidine rich motif 'HKHHSH' down stream of cytochrome b5 domain, lying within the region of amino acid sequence that is absent in other D^6 desaturases. This region is thought to be essential for enzyme activity (Michinaka et al. 2003; Na-Ranong et al. 2006; Sakuradanai et al. 1999; Zhang et al. 2004). The reaction involves a microsomal membrane bound, cytochrome b5 reductase and fatty acid desaturase (Uttaro 2006). D^6 desaturases also studied in borage, nematode, rodents and animals (Aki et al. 1999; Napier et al. 1998; Sayanova et al. 1997). D^6 desaturase from *M. cercinelloides* showed substrate specificity, i.e it acts only on glycerolipid-linked LA (Kendrik & Ratledge 1992a).

D^6 desaturase gene from *M. alpine* has been functionally expressed in mammalian cells resulted in an increase in the endogenous levels of DGLA and AA (Das 2004). Functional expression of the *M. alpine* and *Mucor rouxii* desaturase gene showed their viability in plants, generating oilseeds rich in PUFAs. In *Brassica napus* both D^{12} and D^6 *desaturase* genes co-expressed from *M. alpine*. These genes expressed simultaneously and resulted in the accumulation of GLA in the transgenic canola oil (Lopez & Gracia 2000; Napier et al. 2006; Pereira et al. 2003). *M. alpina* is a fungus that can produce large amount of C20 PUFAs, upto 40-70% AA, depending on the strain. Intermediates in the pathway such as LA, GLA, DGLA presence indicates that *Mortierella* sp. express substantial amount of Δ^6-desaturase activity along with Δ^{12} and Δ^{15} desaturase

(Hunag et al. 1999; Sakuradani et al. 1999) However, the complete enzymology of *Mortierella* spp. has been going on to study desatursase system for biotechnological application (Certik & Shimizu 1999; Sakuradani et al. 1999).Therefore, through genetic modification it is possible to manipulate the fatty acid biosynthesis pathway *in vivo* and *ex vivo*, to enhance the production of PUFAs and their derivatives (Brown 2005; Napier et al. 2006).

Inhibitors of D^6 -desaturase in Human

- High levels of carbohydrates in the bloodstream, which slow down Δ^6-*DES* activity and decrease GLA production;
- Trans fatty acids which inhibit Δ^6-desaturase
- Viral diseases
- Elevated adrenaline (decreases Δ^6-desaturase) and cortisol (due to stress) levels which increase insulin levels and lead to excess arachidonic acid production via activation of Δ^5-desaturase enzyme. Since activation of Δ^5-desaturase depends on insulin, and insulin is balanced by glucagons (which inhibits Δ^5-desaturase), it is critical that insulin/glucogon levels are kept in balance.

FUNCTIONS OF GLA IN HUMAN

Mechanism of GLA Action in human

GLA is metabolized to the 20-carbon polyunsaturated fatty acid DGLA, which is cyclooxygenated to prostaglandin E1 (PGE1). Prostaglandin E1, exhibits anti-inflammatory, antithrombotic, antiproliferative, and lipid-lowering potential [Fig. 2.9]. It also enhances the activities by binding to surface receptors on smooth muscle cells, increasing intracellular cAMP and vasodilation. In addition, EFAs including GLA are important constituents of membrane phospholipids, including the mitochondrial membrane, where they enhance the integrity and the fluidity of the membrane (Berry 2001; Horrobin 1992; Westhuizen et al. 1994). GLA and

DGLA are not normally found in the freestate, but occur as components of phospholipids, triglycerides, neutral lipids, and cholesterol esters, mainly in cell membranes. PGE1 is metabolized to smaller prostaglandin remnants--primarily dicarboxylic acids. The majority of metabolites are excreted in the urine (GLA-Monograph).

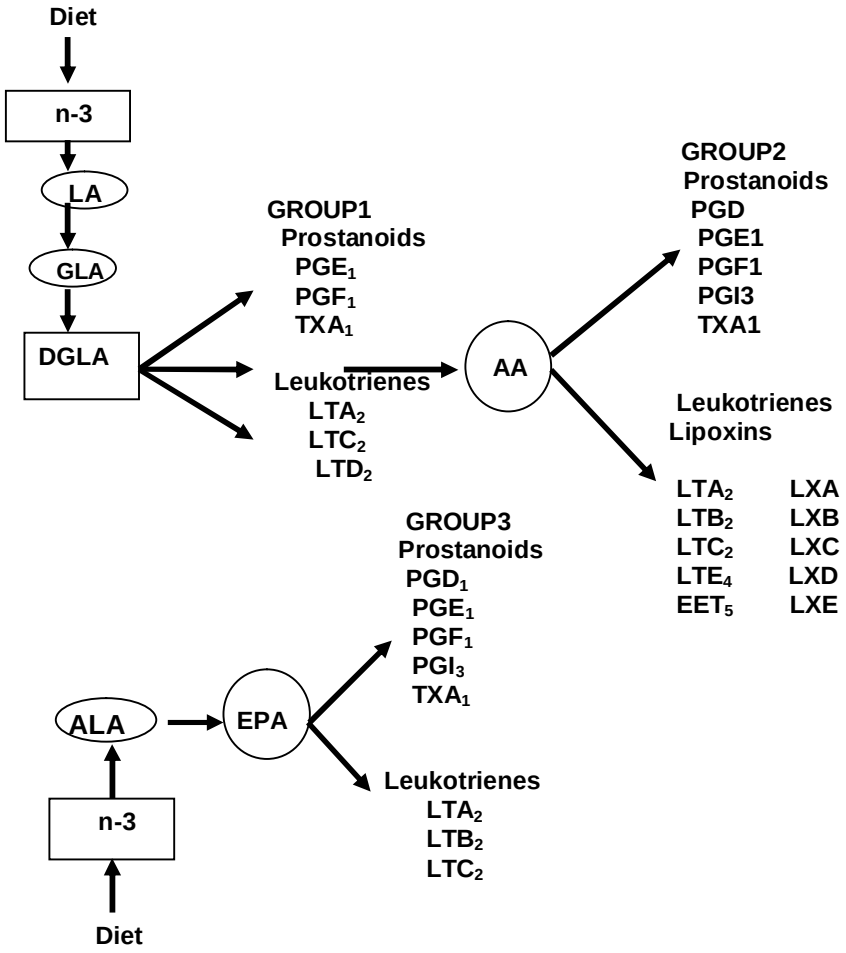


Fig. 2.9 Production of eicosanoids from PUFAs

The following are the major applications of GLA:

NUTRITIONAL USES OF GLA

The daily intake of LA can be converted into GLA and further elongated and desaturated to produce long chain-PUFAs (LC-PUFAs). If an adult should consume 5-20 g/day LA, then the conversion of GLA in endogenous will be 250-10000 mg/day. Human breast milk contains 100-400 mg/L of GLA and DGLA. If a baby consume 1L of milk then this suggests that the daily intake of GLA+DGLA in a fully breast fed infant is of the order of 20-80 mg/Kg/day (Horrobin 1992).

Infant Nutrition

A breast fed infant is presumably getting most of the nutrients, it requires during the 6 months and so from small only. The artificial formulae confirm as closely to the composition of human milk are being marketing in the recent years (Horrobin 1992). Human milk is unusually rich in EFAs, by Δ^6-desaturation body produced GLA, DGLA, AA, EPA and DHA in both the ω-6 and ω-3 series pathway (Das et al. 2001 a, b). Infants appear to lack sufficient Δ^6-desaturase activity and it is a rate limiting step in the PUFAs pathway (Fan & Chapkin 1998). Whereas breast milk is high in GLA and DGLA, but infant formula is lacking of these fatty acids. This can lead to a deficiency state in formula-fed infants, particularly skim milk-based formula (Horobin 1992). In order to assure normal development of brain, eyes, and other tissue, the human infant need to consume these EFAs through diet. Sub clinical deficiency of EFAs has been studied in pre-term and term infants. The fatty acid composition of structural membrane lipids can impact membrane function by modifying overall membrane fluidity, affecting membrane thickness, or by influencing the interaction of fatty acids with membrane proteins. Changes in neuronal membranes that affect membrane excitability have also been noted. The babies fed with artificial formulae have lower activity of Δ^6 desaturase in blood than the

breast fed babies (Fan & Chapkin 1998). This support the limiting rate of Δ^6 desaturase may be inadequate to support a babies needs if the only EFAs are available in the food are LA and ALA. Therefore there is a strong recommendation to add GLA and EPA in the preparation of infant formulae. The toxicology studies on GLA obtained from EPO showed that it is an appropriate source to add in the infant foods (Horrobin 1992; Wu et al. 1999). In Japan, Snow Brand produces baby milk to which EPO has been added to provide GLA (Horrobin 1992; Ratledge 1992a).

Ageing

In animal studies, evidence showed that the ability to Δ^6-desaturation in old animals and humans can be bypassed by supplementing GLA directly. When GLA provided in the diet it not only bypasses the GLA route but it also enhances the D^6 desaturase indirectly. D^6 desaturase also enhanced the ALA to Stearidonic acid in n-3 fatty acids inturn produce EPA and DHA (Berry 2001; Fan & Chapkin 1998; Sakurandani et al. 1999; Uttaro 2006). After 60 years the formation and activity of Δ^6 desaturase will be reduced, when nutritionally supplementation in the range of 125-500 mg/day brings up the supply of GLA available to the body to normal (Huang & Ziboh 2001).

Stress

When the human being exposed to stress, adrenal hormones inhibit the activity of Δ^6 desaturase. In animal experiment it was demonstrated that GLA can prevent stress induced blood pressure rises. Evidence showed that the formation of GLA decreases during stress and supplementation of GLA in diet can attenuate some of the consequences (Fan & Chapkin 1998; Uttaro 2006).

Poor diet

Processed foods (junk foods) may contain trans fatty acids and also isomers of LA which can inhibit the Δ^6 desaturase activity. A poor diet may also lead to elevated level of cholesterol which can also inhibit Δ^6 desaturase. Some of the junk foods may contain metallic ions like magnesium, calcium, zinc and peroxide which acts as cofactors for EFAs metabolism. Such individuals may benefit from supplementation of GLA in the diet (Horrobin 1992).

Alcoholism

Usually, high alcohol consumption depletes the formation of EFAs in human body and inhibits desaturation of LA. Therefore, the person drinking too much of alcohol, have deficiency of EFAs metabolism. So they should increase the uptake of EFAs especially GLA (omega 6) in the diet (Berry 2001; Das 2004; Horrobin 1992).

MEDICAL USES OF GLA

Some clinicians and preliminary research suggest using GLA as an unusually low risk approach to the management of several diseases and is attracting increasing interest in the pharmaceutical areas. EPO has very long history of use for various diseases like skin diseases, eczema, well being of the elderly. It contains EFAs especially GLA in substantial amount. It has been used as quasi-medicinal use for centuries. It is also known as “king of cure all” and thus recommended for use in a wide number of illness (Horrobin 1992; Moreton 1988). Traditional use of EPO has included parts of the whole plant, externally to heal wounds and to soothe skin inflammation and internally to control coughs, as a sedative, pain killer and diuretic. Historically, herbalists have claimed that the consumption of borage leaves and flowers can treat depression, liver disorders, kidney and bladder inflammation (Gill & Valivety 1997; Guil-Guerrero 2001a b; Horrobin 1992).

Skin Diseases

The first clinical use of EFAs was the curing effect of LA on skin inflammation. GLA and DGLA are important structural components of the upper skin (epidermal) layer which regulates moisture loss from the surface of the skin (Horrobin 1992; Gill 1997). Membranes surrounding the cells of skin, GLA and DGLA help in stabilizing the membrane and to reduce water loss from the surface of the skin. Several early studies suggest that EPO (rich in GLA) was more beneficial at relieving symptoms associated with skin conditions such as itching, redness and scaling (Horrobin 1993; 2000). However, blood levels of GLA, DGLA, and AA below normal indicating either reduced conversion of LA to GLA (Horrobin 1993). The eicosanoids synthesized from AA, in particular prostaglandins E2 (PGE2) and leukotrienes B4, are involved in inflammatory reactions in the skin, and are in part responsible for the redness, pain, and intense itching common in such conditions as atopic dermatitis (AD), eczema and psoriasis in both children and adults (Belch & Hill 2000; James et al. 2000; Johnson et al. 1997). Increased level of GLA in the diet enhances rate of conversion of GLA to LC-PUFAs in the system. PGE1, on the other hand, shows potent anti-inflammatory effects in the skin. PGE1 is also important for maintaining healthy skin by regulating water loss and protecting skin from injury and infection (Flieder, 2005; Horrobin 1993). In extensive research of EPO and borage oil in eczema, randomised double blind, placebo, controlled trials have been found to be of use in all features of the disease (Horrobin 1993). Finally in view of the importance of EFAs in the skin, there is a possibility that GLA may a role in the management of other skin disorders such as psoriasis and urticaria (Horrobin 1992; 2003; Uttaro 2006)

Atopic Eczema

Atopic eczema is an inherited form of dermatitis that almost develops in one year old babies. Patients with atopic eczema are more susceptible than normal to viral infections and to allergic reactions of various types. They have abnormal immune function (Horrobin 1993). Alterations in LA metabolism have been demonstrated in atopic conditions such as eczema (Horrobin 1992). Conversion

of LA to GLA is inhibited in individuals with atopic dermatitis (Horrobin 2003). Studies revealed that use of GLA obtained from EPO and borage seed oil showed improvements in eczema such as decreased inflammation and itching (Horrobin 2000; Van Gool et al. 2003).

In multicentric trials, 179 patients with atopic dermatitis were treated with 4 g of EPO daily. After 12 weeks, 62 % of patients demonstrated a significant clinical response based on a standardized clinical assessment form (Steward et al. 1991). Infants with a maternal history of atopic skin disease received a borage oil supplement (100 mg GLA) or placebo (sunflower oil) daily for the first six months of life. Outcome was based on incidence and severity of atopic dermatitis as well as total serum immunoglobulin E (IgE). Clinically, severity of atopic dermatitis was decreased favourably in the borage-oil group, although atopy was still present. Additionally, GLA had no effect on IgE levels during the first year (Leu et al. 2004).

Diabetic neuropathy

Diabetics have been shown to require higher amounts of EFAs, specifically GLA, because of impairments in Δ^6desaturase activity, which result in reductions in both GLA and DGLA levels in cell membranes (1993). DGLA is a major constituent of nerve cells and is required for normal neuronal cell membrane structure, normal regulation of nerve conduction, and for the release of eicosanoids involved in nerve microcirculation (Berry 1997; Tapiero et al. 2002). Diabetic neuropathy, a common complication of both insulin-dependent and non-insulin-dependent diabetes mellitus, is a condition where nerves degenerate and symptoms of pain and numbness follow (Berry 1997; Horrobin 1993; 2000).

There are multiple abnormalities of EFAs and eicosanoid metabolism in diabetes because Δ^6 desaturase activity is also impaired and there is block in the conversion of DGLA to PGE1. Because of this reason diabetic patients need more

EFAs than non-diabetics patients (Horrobin 1992; 1993; Pullman-Mooar 1990; Puri et al. 2007; Tapiero et al. 2002). Supplementation of GLA from EPO or borage or other sources may assist nerve function and help to prevent nerve disease in diabetic patient (called peripheral neuropathy and felt as numbness, tingling, pain, burning, or lack of sensation in the feet and/or legs). The consistency of animal experiments, human studies and biochemical analyses shows that GLA is likely to become a valuable treatment for diabetic neuropathy (Das 2006).

The effects of GLA on the prevention and treatment of diabetic neuropathy has been extensively studied. Patients receiving 360 mg GLA obtained from EPO showed statistically significant improvement in a number of parameters of neuropathy over the placebo control group (Horrobin 1993). Positive effects of GLA on diabetic neuropathy in a study in which 84 patients received GLA (480 mg/day as 12 capsules of EPO) for one year in randomized, double-blind, placebo-controlled parallel design. Sixteen parameters (neurophysical, neurological and biochemical) were evaluated quarterly. For all 16 parameters, the change over one year in response to GLA was more favorable than the change with placebo. GLA had a beneficial effect on the course of diabetic neuropathy, was not associated with important adverse events and may offer an advance in the management of diabetic neuropathy (Das et al. 2001; Fan & Chapkin 1998). Horrobin (1997) conducted a large scale trial on the beneficial effects of GLA on human diabetic neuropathy, involving 400 patients supplemented with 480 mg/ day GLA in the form of 6 g of EPO/day. It was reported that GLA supplementation improved a number of parameters of neuropathophysiology. Clinical measurements of thermal threshold also improved. These improvements increased over time from 3 to 12 months. In contrast, in the placebo treated group, all parameters were negatively affected. After treatment was continued for a second year, further improvements were noted in the GLA treated patients. The mechanism responsible for the positive effects of GLA may be due to a restoration of normal nerve conduction velocity.

Eye Disease

GLA may be beneficial in dry-eye conditions such as Sjögren's syndrome (a condition with symptoms of dry eyes, dry mouth, and, often, arthritis). Sjögren syndrome is a common autoimmune, chronic inflammatory disorder that is often associated with rheumatoid arthritis (Barham et al. 2000; Sharam et al. 2006).

Osteoporosis

Deficiency of EFAs including GLA and EPA, an ω-3 fatty acid can lead to severe bone loss and osteoporosis. Studies have shown that supplements of GLA and EPA together help maintain or increase bone mass (Horrobin 1992). EFAs may also enhance calcium absorption, increase calcium deposits in bones, diminish calcium loss in urine, improve bone strength, and enhance bone growth, (Gill & Valivety 1997; Dyaneswar 2006).

GLA in Reproductive disorders

Breast pain and premenstrual syndrome (PMS)

Breast pain and PMS are related to disorders about 0-80% of women. Breast pain and PMS are both usually relieved by the nature or induced suppression of secretion of ovarian hormones. Abnormal sensitivity of breast and other tissues in women prevents normal level of circulating hormones. Breast pain and PMS are common in women with intake of fat which is more of saturated fatty acids and less common in more unsaturated fatty acids (Horrobin 1992). Clinical studies of the use of GLA in breast pain and PMS have shown that in most cases GLA is more effective than placebo in relieving symptoms (Chenoy et al. 1994). Studies on PMS, results suggested that some women were relieved their PMS symptoms when GLA containing EPO supplement or other source like borage (Fan & Chapkin 1998; Gill & Valivety 1997). The symptoms are breast tenderness, feeling of depression, irritability and swelling, bloating from fluid retention. Breast tenderness other than PMS may also improve with use of GLA (Horrobin 1992).

Inflammation and auto immune disorders

Chronic disorder of inflammation is always associated with the immunological disorders. These disorders are associated with the activation of the AA cascade and change in production of cytokines such as interleukins, necrosis factors and the interferons. The studies showed that GLA is precursors to PGE1. Prostaglandins (PGE1) was required to normal immune response at low concentration but inhibited that immune response at high concentration (Herbige et al. 1995; Horrobin 1992). PGE1 found to be an anti inflammatory in variety of *invitro* systems and animal models by using GLA as a precursor of both PGE1 and of the other anti inflammatory metabolite 15-OH-DGLA (Fan & Chapkin 1998). This is has been demonstrated in in vivo and in vitro studies of inflammation including adjuvants arthritis, experimental allergic encephalomyelitis naturally according auto-immuno inflammatory disease, Salmonella-associated arthritis and urate induced inflammation (Barham et al. 2000; Herbige 1995). Recent studies of only GLA administration to patient and GLA along with EPA (fish source oil) has shown the same type of results in rheumatoid arthritis (Belch & Hill 2000; Zurier et al. 1996; Ziboh & Fletcher 1992). Apart from this GLA has outstanding to side effects.

Rheumatoid Arthritis

Rheumatoid arthritis is one of the more common chronic diseases. Conventional arthritis therapy treats the condition, but does not cure it. Treatments used in rheumatoid arthritis usually include anti-inflammatory drugs and corticosteroids which can lead to kidney and liver damage and gastrointestinal problems. Some preliminary information indicates that GLA, from EPO, borage oil, or black currant seed oil, may diminish joint pain, swelling, and morning stiffness. GLA may also allow for reduction in the amount of pain medication used by those with rheumatoid arthritis. Additional research would be helpful, including testing a proposed theory that using GLA and EPA (an ω-3 fatty acid from fish and fish oil) together would be helpful for rheumatoid arthritis [Table

2.13] (Belch & Hill 2000). Two clinical trials show efficacy of GLA and sources on the progression of rheumatoid arthritis. The administration of 1.1 g/day of GLA from borage oil (9 capsules/day) for 12 weeks significantly suppressed the production of the pro-inflammatory compounds PGE2, TXB2 and LTB4 in seven normal subjects and in seven patients with active rheumatoid arthritis. In six of the seven patients, improvements in sleep patterns, joint sores, morning stiffness and the patient's overall assessment of disease activity were noted. The authors postulated that the positive effects of borage oil were due to a reduction in the synthesis of AA derived eicosanoids (Johnson et al. 1997; Tapiero et al. 2002). Three trails have been conducted using EPO for rheumatoid arthritis. 40 patient took either 6 g EPO daily or placebo (olive oil) for six months (Zurier et al. 1996). The EPO group revealed a significant improvement of rheumatoid symptoms. In a six month trail, 34 patients took daily doses of 10.5 g black currant oil or placebo (soybean oil) (Leventhal et al. 1994). Patients maintained NSAIDS or corticosteroid treatment throughout the study. The study indicated significant improvement in joint tenderness scores to those on placebo no change. Borage oil appears to be the preferred oil of choice for GLA supplementation compared to EPO and black currant due to its higher concentrations of the EFA.

Table 2.13 Effect of GLA supplementation with omega 3 fatty acids in healthy and diseased subjects

Subject group	Clinical index	Response
Healthy	Leukocyte aggregation	Decrease
Raynaud phenomenon	Vasospasm duration	Decrease
Reynaud phenomenon	Vasospasm severity	Decrease
Rheumatoid arthritis	Pain severity	Decrease
Rheumatoid arthritis	Fibrinolysis	Decrease
Psoriatic arthritis	Pain severity	No change

(Source: Belch & Hill 2000)

Allergies

People who are prone to allergies may require more EFAs and often have difficulty converting LA to GLA. In fact, women and infants who are prone to

allergies appear to have lower levels of GLA in breast milk and blood. Supplements, such as GLA from EPO or other sources, have an ancient history of folk use for allergies. The supplement improves symptoms, may be vary with individuals (Horrobin 1992; 1993).

Attention Deficit/Hyperactivity Disorder (ADHD)

Studies on supplying GLA from EPO or other sources to children with ADHD, however, have been mixed and, not conclusive. Research to date has suggested that an improvement in symptoms and behaviours related to ADHD from supplements of n-3 fatty acids (Richardson 2000). In the meantime, ensuring a healthier balance of ω-3 to ω-6 fatty acids in the diet seems worthwhile for those with this behavioral condition (Carter 1988; Clader 2001). In children with borderline zinc status, EPO can improve or compensate for a mild deficiency, possibly because zinc is a cofactor in the conversion of LA to GLA (Horrobin 1992)

Cancer

Studies have being carried out about the relationship between ω6 fatty acids to cancer. While ω3 fatty acids such as LA and AA were showed to promote cancer in studies of colon, breast, and other cancers, but GLA has showed some benefit for breast cancer in certain studies (Clader & Zurier 2001; Flieder 2005; Kankaapaa et al. 2001). GLA had showed promise in the treatment of cancer, both as a cytotoxic agent and as an adjunct to chemotherapy. In the treatment of breast cancer, GLA, when used in combination with tamoxifen, was found to down-regulate estrogen receptor expression, both in an animal (Kenny et al. 2001) and clinical (Kenny et al. 2000) trials. Additionally, animal studies have showed that GLA, specifically from borage oil, can inhibit a mammary tumorigenic response by increasing the activity of ornithine decarboxylase in mammary tumors. (Bunce et al. 1990) In vitro studies demonstrate various EFAs, particularly GLA, can enhance the effect of paclitaxel, a chemotherapy drug used for breast and ovarian

cancers (Plumb et al. 1993; GLA-Monograph). In a small trial on human gliomas, a moderate improvement in patient survival was observed after 1 mg GLA was administered via cerebral reservoir for seven day (Das 2004). GLA also demonstrated a synergistic effect with gemcitabine against pancreatic adenocarcinoma cell lines (Horrobin 1993). Intravesicular GLA has been shown to be cytotoxic in superficial bladder cancer, with a response rate of 43 %, demonstrating a cytotoxic effect against transitional cell carcinoma (Das 2004). The safe and best way to reduce the cancer is to have a diet with proper balance of ω-3 to ω-6 fatty acids starting from a young age (James et al. 2000; Flieder 2005).

Weight Loss

A study suggests that if the supplement is going to work, it does so mainly for overweight individuals for whom obesity runs in the family. In addition, a few other small studies suggest that the more overweight, the more level of borage oil helps in reduction of weight in obese person (Horrobin 1992).

High Blood Pressure and Heart Disease

Four major factors involved in increasing risk of both coronary heart diseases (CHD) namely elevated level of cholesterol, elevated level of triglycerides, hypertension and enhanced platelet aggregation (plaque formation in blood vessel), and the fifth factor is diabetes. All these factors seem to be associated with the effect of each other risk factors. High cholesterol level inhibit the Δ^6 desaturase activity, supplementation of GLA bypasses the GLA synthesis and produces other LC-PUFAs which inturn produce leukotrienes, thromboxanes and prostaglandins series (Das 2007; Demaison & Moreau 2002). Dietary supplementation with GLA alone yielded variable results on circulating lipid levels (Horrobin 1992; Fan & Chapkin 1998). Animal studies suggest that GLA, either alone or in combination with two important ω-3 fatty acids such as EPA and DHA both found in fish and fish oil, may lower the plasma lipids (Laidlaw & Holub 2003; Tadaka et al. 1994; von Schacky 2000). Both animal and human

studies suggest that GLA, DGLA and EPA have been found to be effective in inhibiting platelet aggregation (Guivernau et al. 1994; Laidlaw & Holub 2003; Tadaka et al. 1994). DGLA enhances the conversion of EPA to PGE13, while EPA enhances the conversion of DGLA to PGE1(Clastro et al. 2007; Laidlaw & Holub 2003).

Atherosclerosis

Studies suggested that plaque formation in the blood vessel lead to block the flow of blood and tissue becomes functionless this can be prevented by the supplementation of GLA alone and also combination with EPA (Das 2006; 2007; Leng et al. 1998).

Another study evaluating people with peripheral artery disease blockage in the blood vessels in the legs from atherosclerosis [plaque] causing cramping pain when supplementation of EPA and GLA in combination releases the pain and it prevents the formation of plaque (Das 2007). GLA alone may not be the conferring the benefit at all but the ω-3 fatty acids, such as EPA, DPA and DHA are better known for improving blood pressure and the risks for heart disease, may be individually or combination responsible (Fan & Chapkin 1992; Demaison & Moreau 2002; Tadaka et al. 1994). Studies on 32 women of ages 36-68, assessed the effects of different combinations of EFAs on serum lipids. Received either 4 g EPA + DHA: 4 g EPA + DHA plus 1 g GLA (4:1): 4 g EPA + DHA plus 2 g GLA (4:2): or 4 g EPA + DHA plus 4 g GLA (4:4) daily for 28 days. At the end of the 28-day period, the ratio of total-to-HDL cholesterol was significantly reduced in all four groups, by 11-, 9.6-, 14-, and 14.7%, respectively. Mean group reductions in LDL:HDL ratios from days 0-28 were statistically significant in the 4:1-, 4:2-, and most dramatically in the 4:4 group, with a 19.9 % reduction. Triglyceride concentration was most predominantly lowered in the 4:2 groups. The study demonstrated a combination of GLA and marine oils may be beneficial in improving lipid profiles (GLA-Monograph).

Ulcers

Very preliminary evidence from test tube and animal studies suggest that GLA from EPO may have anti-ulcer properties (1992). Further studies are needed to treat people with GLA, stomach or intestinal ulcers or gastritis (inflammation of the stomach).

Acute Respiratory Distress Syndrome

The anti-inflammatory mechanism of GLA has successfully been evaluated in treating critically ill patients suffering with acute respiratory distress syndrome. Results of a randomized, double-blind, controlled, multicentre trial found that, nutrition supplemented with EPA, GLA, and antioxidants significantly reduced pulmonary neutrophil recruitment and inflammation compared to a control diet (Zurier et al. 1996). The EFAs and antioxidants also benefited the amount of gas exchange, requirement for mechanical ventilation, length of intensive care unit stay, and reduction of new organ failure (Uttaro 2006).

Asthma

A randomized, double-blind, placebo-controlled trial in patients with mild-to-moderate asthma examined the effectiveness of supplementing an EPA/GLA combination to stimulate leukotriene biosynthesis. No other clinically significant changes were noted in four weeks, suggesting the need for longer patient follow-up (GLA-Monograph; Ziboh & Fletcher 1992).

Antimicrobial Activity (Infections)

Immune Booster. GLA production decreases with viral infection or illness. Supplementing with GLA helps safeguard immune defenses (Horrobin 1992). Human viral infections are associated with reduced levels of LA and even further

reduced levels of LA metabolites, indicating reduced LA desaturation. Since PGs seem to be required to allow interferon's to exert its antiviral effects, a strategy of inhibiting LA desaturation may be a part of viral strategy of blocking host defenses. A reduced supply of PG precursors, DGLA and AA would reduce a cells ability to respond to interferon in defending against viral attack. When GLA along with EPA supplemented in AIDS has shown important degrees of improvement both in symptoms and in CD4 lymphocytes levels. When GLA with EPA was given to chronic fatigue sufferers, their symptoms improved dramatically (Uttaro 2006).

The studies on GLA *in vivo*, is not only the gatekeeper to our health, but to our appearance as well. It actually increases cell resilience and moistens the fatty layer beneath the skin, delivering a multitude of beautifying benefits such as:

- Producing a dewy complexion
- Aiding collagen loss
- Soothing dry, scaly skin
- Combating wrinkles
- Nourishing straw-like hair
- Strengthening brittle nails
- Helping to prevent dandruff
- It reduces the tendency of blood platelets to aggregate and thereby reduces the risk of blood clots
- It expands contract blood vessels, which may alleviate pains associated with angina pectoris (Angina)
- It expands the respiratory passages, prevents mucous formation, infections and asthma attacks
- It reduces cholesterol production
- It reinforces the effects of insulin
- It improves the activity of the immune system (primarily via its influence on the T-Lymphocytes)

- Disorders in the central nervous system: in schizophrenia and disseminated sclerosis the patient often has abnormally low PGE1 levels
- For hangovers and other withdrawal symptoms after excessive alcohol consumption
- Alcoholic liver damage
- Chest pains, aches and fluid build ups (Oedema)
- Menstrual pains caused by increased activity of harmful prostaglandins
- Premenstrual tension
- Hyperactivity in children

GLA acts as a health promoter, beautifier, and hormone balancer. Because of all this importance now days GLA is one of the giant nutrient

(Source;thttp://www.myhealthsense.com/;

http://www.fatsforhealth.com/index.php

OLEAGINOUS MICROORGANISMS AND SINGLE CELL OIL (SCO)

Lipid-producing organisms have been known for many years. Oleaginous microorganisms are defined as organisms that contain more than 25% of their dry biomass in the form of lipids (Ratledge 1988; Murphy 1991; Dyal & Narine 2005). Yeasts, molds, bacteria and algae are considered as potential interest in production of speciality lipids [Table 2.14]. Oil obtained from microorganisms

has been considered as single cell oil (SCO) because it synthesizes the oils with high purification and less expensive than agricultural and animal sources (Certik & Shimazu 2003; Ratledge 2003; Cohen & Ratledge 2005). The term single cell oil formulated by Ratledge as an obvious parallel to the term Single Cell Protein (SCP, i.e. production of edible protein in the form of microorganisms) (Moreton 1988). The term SCO was similarly meant to define edible oils being produced by unicellular organisms and allowed their use for consumption without specifically mentioning that they were derived from microorganisms (Davies 1992; Ratledge 1988). Research has shown that some moulds can store up to 80% of their biomass as lipids (Dyal & Narine 2005; Ratledge 2002). Increasing demand for commercially valuable lipids has resulted in production of PUFAs for health and dietary applications (Dyanshwesar et al. 2002; Gill & Valivety 1997; Johnson et al. 1997; Simonopoulos 1991).

Lipids obtained from moulds i.e. eukaryotic strains of microorganisms are similar to the plants lipid which contain mainly C16 and C18 fatty acids esterified in the form of TG (Ratledge 1994; 2002). The scheme obtaining for SCO from oleaginous microorganism is shown in the Fig. 2.10. Remarkable diversity of fatty acids occurs in the microbes shown in Table 2.15

Table 2.14 Oleaginous microbial sources for speciality lipid production

Microorganisms	Speciality lipids	Resemblance
Apiotrichum curvatum (yeast)	Oleic acid, palmitic acid, stearic acid and linolenic acid	Cocoa butter
Aspergillus niger (mold)	Oleic acid and unsat-urated fatty acids	Groundnut
Aspergillus sp. (mold)	Oleic acid and unsat-urated fatty acids	Seed fat of *Meduca latifolia*

A. sydowii (mold)	Oleic acid	Groundnut
Bacillus subtilis (Marine bacteria from *Aurora globostellata* marine sponge)	γ-Linolenic acid, eicosapentaenoic acid and branched chain fatty acids	-
Crypthecodinium cohnii (microalga)	Docosahexaenoic acid	-
Fusarium oxysporium (mold)	Saturated and unsaturated fatty acids	Groundnut
F. equisetti (mold)	Saturated fatty acid	Palm oil
Mucor circinelloides (mold)	γ- Linolenic acid	-
Mortierella alpine (mold)	Arachidonic acid	-
Pseudomonas sp. (Marine bacteria from *Heteronema erecta* marine-a sponge)	γ- Linolenic acid	-
Schizochytrium sp. (mold)	Docosahexaenoic acid	-

(Source: Certik & Shimizzu 1999; Leonard & Theoblad 2006)

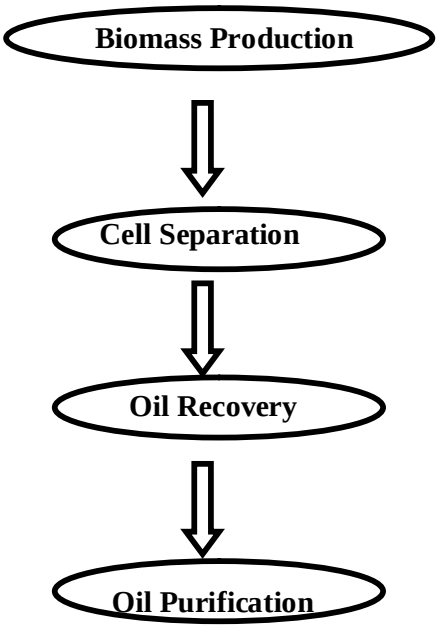


Fig. 2.10 Scheme of single cell oil process

Table 2.15 Selected Micro organisms for polyunsaturated fatty acid production

PUFA	**Strain**	**TL/DCW**	**PUFA formation**		
		(%)	**% in oil**	**% in DCW**	**g/L**
GLA	*M. ramanniana*	50	17.6	8.9	5.5
	M. isabellina	53	4.5	2.2	3.4
	M. cercinelloedes	20-25	15-18	ND	ND

	M. rouxii	20-40		2.3	
DGLA	*C. nanodes*	34	24.3	6.2	1.6
	M. alpina	45	18.3	10.7	2.6
	M. alpina	50	26.8	20.5	4.1
AA	*M. alpina*	45	42	22	13.0
	M. alpina	44	50	23	11.1
ETA	*M. alpina*	30	26	7.7	1.6
	M. alpina	26	37	9.7	2.3
EPA	*M. alpina*	55	12	6.7	1.9
	M. alpina	32	20	6.4	1.0
	M. elongata	ND	15.1	ND	0.6
	P. ultimum	18	20	3.4	0.4
DPA	*Schyzochytrium SR21*	53	7.4	4.8	1.0
DHA	*Schyzochytrium SR21*	70	37.3	26.2	15.5
	C. cohini	60	39	25.0	8.0
	T. roseum	23	48.8	11.7	2.0
18:2 n9	*M. alpina*	40	14	6.0	1.1
	M. alpina	40	16	6.3	
20:2 n9	*M. alpina*	44	25	11.0	1.7
MA	*M. alpina*	43	33	14.1	1.9

(Source: Certik & Shimazu 1999)

PATTERN OF LIPID ACCUMULATION IN OLEAGINOUS MICROORGANISM

The crucial importance for the future development of SCO process is the understanding of the mechanism of fatty acids synthesis and accumulation of higher oil content in the mycelium of microorganisms (Ratledge 2004). Oleaginous microorganisms are able to grow on fats, accumulate lipids during cellular growth, regardless of the nitrogen concentration in the medium. However, after the depletion of carbon source from the culture medium, oleaginous microorganisms degrade reserve lipids for maintenance purpose and production of new cell material (Aggelis & Sourdis 1997; Holdsworth & Ratledge 1988b). Therefore, the process of lipid synthesis-degradation is regulated by extra-cellular fat concentration: high extra-cellular fat concentration inhibits cellular oil degradation. However, the biotransformation of the storage lipid to protein also depends on the availability of some other nutrient essential for microbial growth, such as nitrogen. Since accumulation of cellular oil from fat occurred in the first steps of microbial growth, independently of the nutrient starvation, the microbial culture rapidly consume extra-cellular fat, and therefore, enter to a state favouring

storage material degradation. In general, degradation of storage lipid could be considered as phenomenon commonly met among oleaginous microorganisms, in cultures growing on fats (Aggelis & Sourdis 1997; Papanikolaou et al. 2001a; 2003a).

Biochemistry of oil-accumulation in oleaginous microorganisms

In recent years studies on metabolism of lipid synthesis has created more interest, because the wide range of lipid accumulation from 20->70% in different microorganisms. All living organisms must synthesise a minimum amount of lipid for their membranes and other structural and functional role (Evans & Ratledge 1988a; Wynn et al. 1999). Bacteria in general do not produce TG but instead produce PHB and PHA as storage polymers (Patnayak & Sree 2005; Ratledge 2003). Therefore the accumulations of oil above 20% of their cell mass only found in small number of microorganisms it includes yeasts, fungi and algae (Barclay et al. 1994; Ratledge 2004). Biosynthetic pathway of lipids in most oleaginous microorganisms is same as in non-oleaginous microorganisms. But the presence of certain key enzymes plays an important role in the production of PUFAs, where these enzymes are not found in non oleaginous microorganisms (Ratledge 1992a, b).

Several reviews have been published regarding accumulation of lipid from oleaginous microorganisms (Certik & Shimizu 1999; Moreton 1988; Ratledge 1988; 2004). The mechanism of lipid overproduction in oleaginous microorganisms appears those involving both the physiology and genetics (Ratledge 1988). Fundamental physiological requirement for lipid overproduction in these organisms is excess carbon and deficiency of nutrient, generally limited N_2 in the growth medium. During these conditions, several physiological and metabolic changes were observed (Holdsworth & Ratledge 1988).

When organisms grown in N_2 limitation medium, beyond 70% oil accumulation observed in oleaginous fungus whereas non-oleaginous fungus do

not accumulate lipid. Instead those non-oleaginous microorganisms either tend to cease further proliferation or production lipid in their cell. The physiological condition which leads to lipid accumulation is that the organisms is grown in medium such that the supply of nitrogen is quickly exhausted but carbon supply stays in excess. Various reasons have been proposed as to how oleaginous organisms may achieve this conversion of carbon substrate into lipid, such as

1. Glucose (or other substrate used) is taken up by the potential lipid accumulator regardless of the lack of supply of other important nutrients such as nitrogen source, to the cell. Thus an oleaginous organisms may continue to assimilate glucose even though it can no larger generate new cells by virtue of their being no nitrogen to synthesise proteins or nucleic acids. The glucose (substrate) is then converted into lipids whereas in other microbial cells, the glucose may be converted into polysaccharides or metabolites like citric acid, etc. by the same supplement then, the organisms which did not accumulate any such materials would have the tendency to curtail glucose transport to the cell (Botham & Ratledge 1989).

2. The rate of lipid biosynthesis is much higher in these organisms than in non-oleaginous ones in which case, recognized key enzymes of fatty acid biosynthesis (Acetyl-CoA carboxylase and fatty acid synthase complex), would be much active in oleaginous microorganisms. This does not appear to be the case of non oleaginous microorganisms (Botham & Ratledge 1989).

Sequence of Metabolic Events Leading To Initiation of Lipogenesis in Oleaginous Microorganisms

- Nitrogen becomes exhausted from medium.
- The increased activity of AMP deaminase.
- Decreases the cellular content of AMP.
- Isocitrate dehydrogenase in mitochondria slows / stops

- Isocitrate equilibrates with citrate and accumulates citrates
- Citrates exits from the mitochondrion
- Citrate enters to cytosol and cleaved by ATP citrate lyase
- Acetyl CoA generated
- Acetyl CoA is used for the fatty acid synthesis.
- Oxaloacetate converted into malate,
- Malate converted to pyruvate via malic enzyme
- The malic enzyme generates NADPH

Net reaction: Acetyl CoA + NADPH produced, which are utilized as substrate for lipid synthesis

(Ratledge 2004; Venkateswaran 1999)

This sequence of events showed in diagrammatically in Fig. 2.11 and 2.12

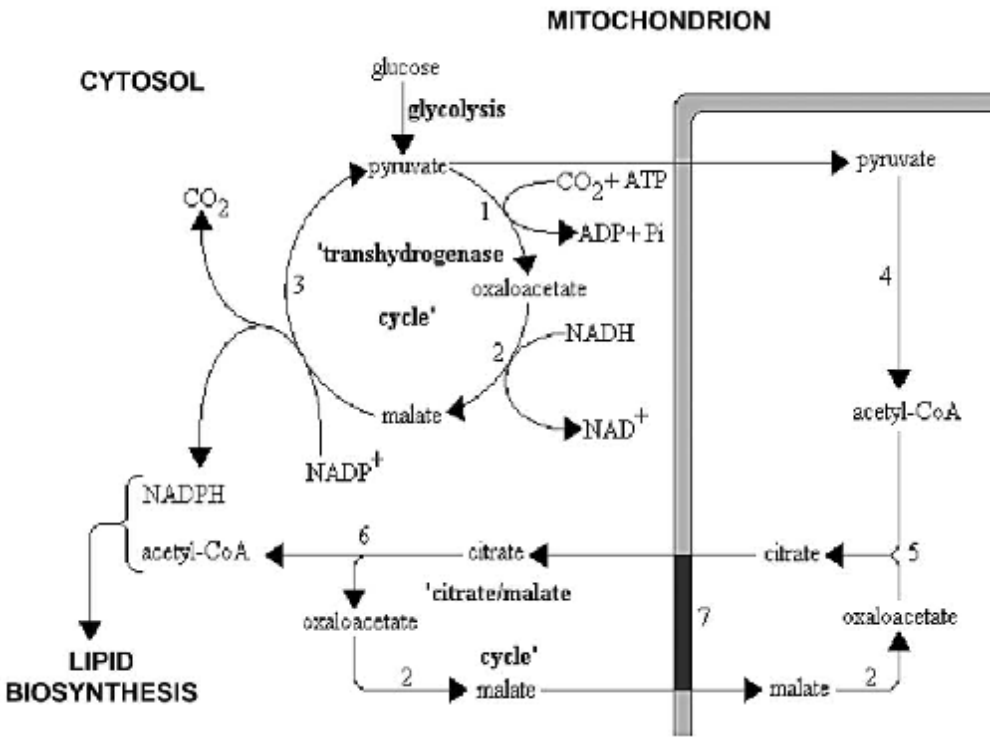


Fig. 2.11 Scheme showing how the proposed citrate/malate cycle and the cytosolic 'transhydrogenase' cycle could provide sufficient precursors (acetyl-CoA and NADPH) for lipogenesis in filamentous fungi.

Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate lyase; 7, citrate/ malate transport.

(Source: Ratledge 2004)

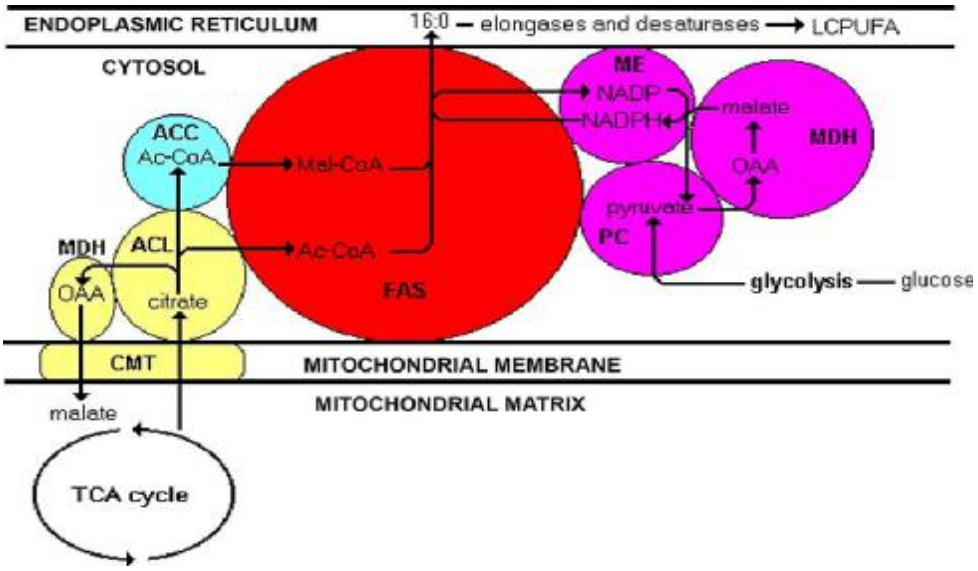


Fig. 2.12 Schematic representation of Lipid accumulation in oleaginous microorganisms

(Source: Ratledge 2004)

Biosynthesis of PUFAs production in fungi

The mechanism of lipids and fatty acids biosynthesis in plants, yeast, molds, and animals has revised by several authors (Lopez & Gracia 2000; Certik & Shimizu 1999; Finnerty & Manku 1975; Murphy 1991; Ohlrogge & Jaworsky, 1997; Ratledge & Wynn 2002; Uttaro 2006). Fungal oils have greater diversity of lipid and fatty acid in the cell. Biosynthesis is well studied in yeast, plants animals but in lower eukaryotes is still poorly understood although several pathways involved in the biosynthetic pathway and potential research has been going on this field (Certik et al. 1997; Ratledge & Wynn 2002). Generally, lipid synthesis involves three steps in the eukaryotic systems.

- Denovo synthesis of fatty acid from glucose
- The incorporation of exogenous fatty acids directly into lipid structures.
- Following desatuaration and elongation of lipid sources. In addition fatty acid biohydrogenation (saturation) and partial or total degradation (β-oxidation) also contribute to this process

Mono and poly unsaturated fatty acid are synthesized in prokaryotes and eukaryotes commonly by aerobic pathway. However, some marine prokaryotes and eukaryotes like *E.coli, Streptococcus pneumoniae* and *Thraustochytrids* respectively synthesize PUFAs via specialized polyketide synthase (PKS) (Metz et al. 2001; Napier 2002; Ratledge 2004).

In eukaryotic microorganisms the biosynthesis of PUFAs takes place by aerobic reaction. The reaction involves the introduction of double bonds (desaturation) in the aliphatic chain of fatty acids and extension (elongation) by two carbon units of the acyl chain. The enzymes currently named as desaturases and elongases (Leonard et al. 2004, Uttaro 2006). Desaturation of aerobic pathway involves three proteins (NAD(P)H-cytochrome b5 reductase, Acyl-ACP desaturase and Acyl lipid desaturases are proteins) and tree types of desturases (Acyl CoA desaturases, Acyl ACP desaturase and Acyl lipid dessaturases).

Synthesis of PUFAs starts with the condensation of acetyl-CoA with malonyl-ACP by the β-ketoacyl-ACP synthase (KAS) is followed by the reduction of the β-ketoester by a NADPH dependent β-ketoacyl-ACP reductase, the removal of water by the β-hydroxyacyl-ACP dehydrase to produce trans-2 enoyl-ACP and another reduction by the enoyl-ACP reductase to form a saturated acyl-ACP that in turn can initiate another cycle of condensation with malonyl-ACP, reductions and dehydration. The process continues up to the synthesis of C16-18 saturated acyl-ACPs. Generally stearic acid is the basic substrate for the first double bond introduction to oleic acid or oleoyl CoA or oleoyl ACP respectively. The first double bond in the aerobic pathway dominant in the eukaryotic cells and some bacteria is introduced into the Δ^9 position of saturated fatty acid. Thus plamitoleic acid (C16:1) and oleic acid (C18:1) are the major fatty acids in all microorganisms. Subsequent desaturation of oleic acid takes place in endoplasmic reticulum (ER, the formation of PUFAs is dominant in all the organisms). Oleic acid is desaturated to yield linoleic acid (LA, C18:2) by the Δ^{12} desaturase, which may be further converted into ALA (EFAs). Thus LA and ALA are precursors to ω-9, ω-6 and ω-3 fatty acid pathway [Fig. 2.13].

The next step for PUFAs production are desaturation of appropriate fatty acid precursors by Δ^6 desaturase followed by successive chain elongations and subsequent desaturation to yield respective C20 and C22 PUFAs. The ω9 family synthesized from oleic acid and sequential participation of Δ^6desaturase, elongase and Δ^5 desaturase to finally produce mead acid (MA). The ω-6 type of fatty acids is usually formed from LA via desaturation ($\Delta^{6,}$ Δ^5 and Δ^4) and elongation steps from LA through GLA, DGLA and AA. ALA is metabolized via ω-3 pathway to EPA, DPA and DHA. Once desaturation has occurred, the acyltransferation reaction then facilitates distribution of newly synthesized PUFAs to the other cellular lipids. Many microorganisms accumulate large amount of PUFAs as storage lipid in the form of triglycerides (Certik & Shimizu 1999; Ratledge 2003).

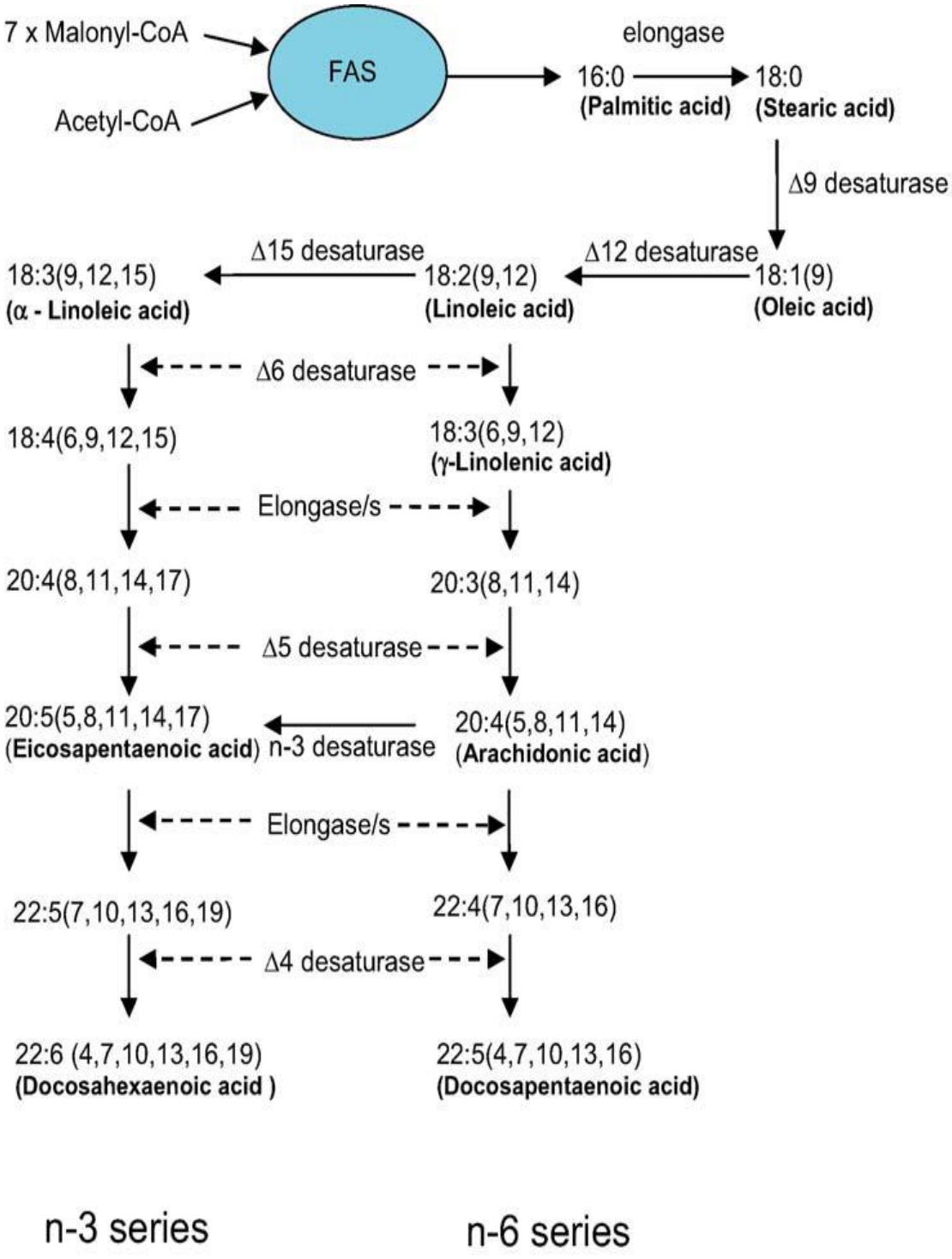


Fig. 2.13 Biosynthesis of PUFAs in eukaryotes (Fungi)

(Source: Ratledge 2004)

Advantages of microbes as SCO source

- An Active lipid synthesizing apparatus makes oleaginous micro organisms attractive oil source
- High growth rate on wide varieties of substrates including various waste substrates
- Oil production can be carried through out the year (climatic independent)
- Microbial PUFAs are the higher value of oil types moderately than commercial oils like soya oil, palm oil and sunflower oil
- Supply of high controlled (quality/purer) PUFAs with pharmaceutical grade i.e. more concentrated oil when compared to quality of control oil
- Existence of defective mutant i.e. absence of specific enzymes improve tailor made oils
- Appropriate vehicles for cloning foreign genes (plant or animals) for the production of specific PUFAs
- Microorganisms provide useful models for studying the lipid biochemistry, metabolic control and function because of fewer organelles that allows synthesis faster than complex multicellular systems of other organisms
- Microbial competence allows transformation reactions (oxidation, desaturation and dehydrogenation), enables the upgrading of PUFAs structures and simultaneous formation of other compound also
- Simplicity of metabolic regulation in microbes. They can grow under controlled condition with nutritional regimes that may stimulate or repress the key enzymes and allow for manipulation of lipid yield and fatty acid profile
- They can be employed both a source of macronutrient and as micronutrient since microbes are rich in proteins and fibre and micronutrients like vitamins, antioxidants.

(Certik et al. 1998; Gill & Valivety 1997; Venkateswaran 1999)

Disadvantages of fish and plant oils as source of PUFAs

- Seasonal and climatic variation in oil composition, which can result in inconsistencies in oil supply and quality
- Complex and expensive downstream processing
- Objectionable taste and odors of fish oil
- High cholesterol and small amounts of potential toxic impurities those are difficult to remove

(Dyaneswar 2006; Gill & Valivety 1997; Yongmanitchai & Ward 1989)

INFLUENCE OF PHYSIOLOGICAL ASPECTS ON LIPID AND PUFAs PRODUCTION

The production of PUFAs by microbial fermentation has been shown to be an ideal alternative to its amiability for the separation, purification and commercialization (Ahmed et al. 2006; Bajapai et al. 1991a, b; Ratledge 2003;). The physiology of lipid accumulation in oleaginous microorganisms specially in molds have been studied by a number of workers (Aggelis 1996; Certik & Shimizu 2002; Papanikolaou et al. 2007; Ratledge 2002; 2004), for efficient microbial strain with both high lipid and high PUFAs content. Recent research has focused mainly maximizing PUFAs yields by screening new strain, as well as medium optimization (Dyal et al. 2005; Jang et al. 2005; Lindberg & Molin 1993; Li et al. 2006). These studies have been carried out with different oleaginous mold both in batch and continuous cultures by varying different physiological parameters (Ahmed et al. 2006; Hansson & Dostalek 1988; Kavadia et al. 2001; Weinstein et al. 2000; Xian et al. 2001). This is especially vital when attempting to optimize growth conditions for a PUFAs production within a strain. The growth factors include pH, temperature, aging, carbon source, nitrogen source, metal ion types, and supplementation of the growth medium. The first detailed physiology of lipid accumulation studied in the yeast by Woodbine (1959). He observed that, a high C:N ratio is needed in the medium for lipid overproduction in the yeast.

Studies have demonstrated there is the potential to produce GLA at concentrations of 15-20% of total fatty acids by lower fungi from the order *Mucorales*, especially the species of *Mortierella*, *Mucor* and Thamnidium, *Cunninghamella* in both submerged and solid state fermentation (Certik et al. 2006; Li et al. 2006 Mamatha et al. 2008; Papanikolaou et al. 2008; Stradenska & Sajbidor 1993; Stradenska et al. 1993; Stradenska et al. 2000 a, b; Suzuki et al. 1983; Ward & Singh 2005).

pH (in the culture medium)

It has been known that in yeast, pH of the cultivation medium does not influence the biomass production but possibly in some strains pH could bring about the change in fatty acid profile of the lipid (Venkateswaran 1999). Generally, it was found that varying pH values, the amounts of saturated and monounsaturated fatty acids tended to decrease while PUFAs content tended to increase with increasing pH values. In oleaginous yeast, Ratledge et al. (1988) working with Candida 107 and Davies (1988) working with *Candida curvata* were unable to observe changes in fatty acid composition by changing the pH of the cultivation medium. Several studies on influence of pH on biomass, lipid and PUFAs production from oleaginous molds have been reported (Dyal et al. 2005; Hansoon & Dostalek 1988; Leman & Brackoniek 1996; Li et al. 2008). The study by Lindberg & Molin (1993) found that *M. alpina* did not grow at pH 8.5 and slow growth and lipid accumulation were recorded at pH 7.5. Rapid growth and a high lipid content were achieved both pH 5.5 and 6.5. AA content in total fatty acid content increased from 26% at pH 5.5 to about 31% at pH 6.5 or 7.5. The concentration of GLA was not affected. Where-as Xian et al. (2001) reported that *M. isabelliana* showed an inverse relationship between pH 5.0-9.0 and dry biomass. Dyan et al. (2005) studied pH ranging from 2.0-14.0 in different media. In *M. ramanniana var. ramanniana* showed high GLA production at pH 5.0 in dextrose yeast extract broth medium where dry biomass high at pH 3.0-4.0. This observations imply that the occurrence of interactions between media and pH which can affect the growth of *M. ramanniana var. ramanniana*. Ahmed et al.

(2006) studied the effect of pH on GLA production in *Mucor* sp. RRL001 from pH 4.0-8.0 and was found that maximum lipid production at pH 6.5. The lipid content decreased at pH 8.0 and 4.0. However, GLA production was higher at pH 5.0 and decreased in the range of pH 3.0 and 6.0. Similar observation was also made by Li et al. (2008) that total lipids, GLA and PUFAs were highest at pH 6.0 in *M. recurvus.* The study also reported, total lipid and PUFAs production increases as the pH increases from 4.0 to 6.0. However, production of PUFAs decreased sharply as the pH was raised from 7.0 to 8.0.

Growth temperature

Among physiological parameters temperature is one of the most critical factors that affect all species of living organisms and controlling the growth rate, lipid synthesis and alters the composition of fatty acids in cellular level. Earlier studies showed that temperature has pronounced effect on growth and biosynthesis of unsaturated fatty acids in certain oleaginous microorganisms (Choi et al. 1982; Kendrick & Ratledge 1992b; Linderberg & Molin 1993; Michinaka et al. 2003). Fermentation at high temperature produce more saturated than unsaturated fatty acid and vice versa when grown at low temperature. This phenomenon is a part of the adaptive response to the cold environment (Quoc & Duacq 1997; Robinson 2001; Michinaka et al. 2003).

Hansson & Dostalek (1988) studied the effect of growth temperature on biomass yield and fatty acid composition of *Mortierella ramanniana* CBS 112.08, *M. ramanniana* CBS 478.63, *M. vinacea* CBS 212.32, and *M. isabellina Oudem* at 20, 25, and 30°C. It was observed that at 25°C, there was a maximum biomass yield and was the optimal growth temperature for growth. The study also noted some variation in fatty acid composition with respect to temperature, generally, the degree of unsaturation increased as temperature decreased. This increased unsaturation was at least partially due to the fact that the GLA content of the moulds increased with a decrease in temperature. These results suggest that lower growth temperatures tend to favour the production of unsaturated fatty acids.

Effect of temperature on the fatty acid composition of *Mortierella* fungi which normally produce AA but not EPA at 28°C was studied (Shimizu et al. 1988). It was observed that the optimum temperature for AA production in *M. alpina* was 28°C Although EPA accumulation occurred up to 20°C, there was a significant decrease in EPA content when the growth temperature was above 12°C. Generally, AA content decreased with decreasing temperature whereas EPA content increased with decreasing temperature. This study suggests that ARA and EPA production are related by a metabolic pathway. Shimizu et al. (1988) hypothesize that an enzyme catalyzed reaction is activated at low temperatures which converts AA to EPA. Formation of lipid and unsaturated fatty acids tends to increase considerably and these results were also observed in certain *Mucor* spp. (Lindberg & Molin 1993; Quoc & Duacq 1997).

Nakahara et al. (1992) investigated the temperature effect on the fatty acid composition of *Mortierella* fungi in more detail by determining the fatty acid composition of both polar and neutral lipid fractions within the species of *M. isabellina.* This study found that polar lipid fractions generally contained more unsaturated fatty acids than the corresponding neutral lipid fractions. The fatty acid compositions of *M. ramanniana var. angulispora* grown at various temperatures indicated that the GLA content of the polar lipid fractions tended to increase with decreasing temperature while the GLA content of the neutral lipid components remained relatively consistent regardless of temperature. The saturated and monounsaturated fatty acids present in greater amounts at 35°C than at 25°C. Conversely, the reported PUFAs had a tendency to be present in lower amounts at 35°C than at 25°C. Michinaka et al. (2003) who found that *M. circinelloides* grown at different temperature shown that Δ^6 desaturase activity increased twice as much as that produced at room temperature thus the culture grown at low temperature shows high 18:3 fatty acids content in the lipid. Further growth in low temperature not only influences the PUFAs production but also alters the cell morphology (Carvalho et al. 1999; Higashiyama et al. 1999). However, the biomass and lipid yields obtained at lower temperatures need to be

taken into account when deciding what temperature is optimum for PUFAs production. If the biomass and/or lipid yields are too low at lower temperatures, the conditions may not be ultimately ideal for PUFAs production although (Dyal & Narine 2005; Quoc & Duacq 1997).

Shaking speed and dissolved oxygen levels

PUFAs are formed in the mycelia, through elongation and desaturation by utilizing carbon as energy source. The desaturation involves an aerobic reaction by oxygenation; therefore dissolved oxygen [DO] is an important factor for PUFAs production (Ratledge 1992). In fungal fermentation the problem of oxygen limitation generally arises because of high viscosity of the culture broth due to high biomass density and filamentous growth (Higashiyama et al. 1999; Li et al. 2006). For the commercial production of PUFAs, the achievement of a higher biomass density is essential because PUFAs are intracellular products (Ahmed et al. 2006). The O_2 requirement is higher for desaturation than for cell growth and lipid production (Ratledge 2004). In *Apiotrichum curvatum*, *Canthamoeba casrellan* and *Mortierella* spp. maximum production of fatty acid was observed when the agitation speed (RPM) increased but the concentration of fatty acid was decreased. The observation was also made that if the agitation lowers alteration in total fatty acid production was found but the individual fatty acid composition altered (Davies et al. 1990; Heighashima et al. 1999).

Lower shaking speed resulted in slower growth and lower PUFAs yields because of the lower level of dissolved oxygen (DO). However, lower PUFAs yield were also obtained in the experiment with too high shaking speed because the shear stress was increased with the increased speed. GLA and PUFAs production was high at shaking speed of 160 rpm, with the highest biomass and total lipid. PUFAs yield was reduced sharply when the shaker speed was over 180 rpm, although DBM production was not changed between 160 and 200 rpm (Li et al. 2008). There is a report that the change of DO concentration in the medium affects on morphology of fungi. Morphology of fungus changed from filamentous

to pellet when the DO concentration was maintained at 20-50 rpm. Pellet form was needed when mass transformation and cultivation of fungi in industrial scale production of PUFAs (Heighashima et al. 1999). The effects of 2-3 ppm DO on PUFAs production *E. excitalis* fungus has been reported by Kendrick & Ratledge (1992b). There have been some attempts to prevent DO limitation by monitoring and controlling the agitator speed (De Preeze et al. 1997; Emelyanova 1997; Hansoon et al. 1989; Nakahara et al. 1992; Totani et al. 1992). Hiruta et al. (1997) reported that the change of impeller design resulted in the as improvement of PUFAs production. This may be due to the fact that in general, fungi are physically weak therefore agitation rate has to be controlled within a certain range (Higashima et al. 1999). It may be due to that many fungal metabolites are extra cellular products, evaluated the distribution of productivity.

Carbon sources

Carbon sources have received much attention in attempts to find materials which are suitable and efficiently converted into lipid. The effect of carbon source on GLA production by *Mucor* spp. was investigated using different pentose, hexose sugars, mono, di and polysaccharides as well as glycerol, ethanol, acetic acid vegetable oils and cereal flours in shake flask cultures (Both et al. 1995; Certik et al. 1993; du Preeze et al. 1996; Hansoon et al. 1989; Kock & Botha 1993; Papanikolaou et al. 2002b; Tsuchivra & Sakura 1998). In the recent years, work has been carried out on numerous carbon sources including a wide variety of wastes from different areas like agricultural and industrial wastes which includes molasses, whey, organge peel, apple pomacea and other substrates like tomato waste hydrolysate (Certik et al. 2006; Chen et al. 1999; Fakas et al. 2008; Gema et al. 2001; Stredanska et al. 2000). Sajbidor et al. (1988) studied the effect of different carbon sources on growth, lipid content and fatty acid composition in four strains of mucorales namely *M. mucedo*, *M. plumbeus*, *M. ramanniana* and *R. arrhizus*. The results showed that *M. mucedo* grew very well on glycerol and produced GLA 0.58 g/100g glycerol. Kock & Botha (1993) showed that when acetic acid was used as sole carbon source *M.*

cercinalloides produces both GLA and cocoa butter equivalents consisting mainly of palmitic acid, stearic acid and oleic acid. Acetic acid is a cheap carbon source and has commercial implications (i.e. biotechnological process, the production of high value oil from low cost substrate). Tsuchivra & Sakura (1998) also found improved production of GLA when acetic acid was used as a co-substrate in glucose containing medium in *Mortierella* spp. *Mucor hiemalis* IPD 51 produced 41.1% GLA, when cultivated in media containing maltose or glucose as the sole carbon source and yeast extract as the nitrogen source (Kennedy et al. 1993). Funtikova et al (2002) performed experiment, media with various compositions using *M. lusitanicus* 306D. The results showed that glucose as the sole energy source was the most promising carbon source for obtaining lipids with a high content of GLA when compared to other carbon sources like sunflower oil, maize extract though efficiently converted to lipid. Hansoon & Dostalek (1988) found that the lipid produced on soluble starch had much higher GLA content (25.7 %) than the lipid produced on glucose (14.7%) and xylose (19.5% GLA). The GLA content in mycelia grown in xylose was somewhat higher than the mycelia grown on glycerol (Fakas et al. 2009a). *R. nigricans* shows high GLA production at 2% soluble starch when compared to other carbon sources (Bandyopadhyay et al. 2001). Lipid formation and GLA production by 48 species of *Mucorales* fungi grown in sunflower oil showed 42.7-65.8% lipids in the biomass (7.7-1.4 g/L) (Certik et al. 1997). For all the mucorales tested, lactose was the poorest carbon source for GLA production (Ahmed et al. 2006; Somashekar et al. 2002). Li et al (2008) showed that the sugar cane molasses is a good source for GLA (0.99 g/L) production by *Mucor recurvus* sp. at 15% (volume ratio). Molasses is also a good source of inorganic nitrogen and vitamins. Tauk-Tornisielo et al. (2007) studied the soluble carbohydrates and different plant oils used as carbon source. They found that biomass production was approximately 8 to 10% greater in media containing carbohydrates as the single carbon source than plant oils. They reported that no correlation between the highest yield of biomass, fatty acids and carbon source used. The lipid yield and GLA production depended on the type of carbon source and also the strains used.

There are reports on use of cereals and other waste substrates as carbon sources in solid state fermentation (Chen et al. 1999; Certik et al. 2006). Gema et al. (2002) first studied the glucose and orange peel (an agro-industrial byproduct) as a carbon source for production of lipid in semi defined nitrogen limited media with various C:N ratios in *C. echinulata*. Results revealed that the enrichment of orange peel as carbon substrate with glucose maximum GLA 80 mg/g dry biomass by fungal cultivation was obtained. Papanikolaou et al. (2007) studied the correlation of individual carbon uptake and secretion of hydrolytic enzymes for the *M .isabelliana* ATHUM 2935 and *C. echinulata* ATHUM 4411 when grown on various low cost sugar based substrates as related to the formation of biomass, and accumulation of storage lipids. Biochemical differences concerning pattern of substrate uptake, activity of intracellular key enzymes responsible for the lipid synthesis and storage of lipid breakdown process have been observed in response lipid accumulation in low cost substrate (Fakas et al. 2008; Papanikolaou et al. 2004a; 2007a, b). These discrepancies could be attributed to the difference in the assimilation of each carbon source utilized as a substrate.

Nitrogen sources

Apart from carbon sources studied various nitrogen sources have also received a lot of attention cost-effectively in PUFAs production (Certik et al. 1999; Papanokolaou et al. 2007a). Several studies indicated that use of variety of a nitrogen sources during fermentation process affect the yield of mycelial growth, lipid, PUFAs production and also mycelial morphology (Hansoon & Dostalek 1988; Park et al. 1999). In the experiment of Evans & Ratledge (1984) with 17 species of yeast in shake flask experiment with different types of nitrogen sources observed that with glutamate, arginine or urea, the lipid yields were about 50% whereas with ammonium chloride it was only 18%. Moreton (1988) was however unable to demonstrate such difference in the effects of various nitrogen sources in stirred fermenters. They concluded that the effect of different nitrogen sources on lipid yield in shake flasks experiments could have been due to the buffering capacity helping to maintain the pH at an optimal level for growth and

O_2 limitation in shake flask cultures. Hansson & Dostalek (1988) observed that potassium nitrate used as nitrogen source produced more biomass and lipid content when compared to ammonium chloride or ammonium sulphate in *M. ramanniana* CBS 112.08. However, *Mortierella* spp. produced maximum GLA at 1% yeast extract and also increased yeast extract concentration in the cultivation medium which promoted the cell growth (Bajpai & Bajpai 1992). Use of urea as nitrogen source in the cultivation medium enhanced growth and GLA production of *C. echinulata* CCRC 31840 (Chen & Chang 1996). The probable reason might be that urea has higher nitrogen content as compared to the other nitrogen sources and created an inert atmosphere that has prevented the oxidation of unsaturated fatty acids. Ammonium nitrate and urea were the better nitrogen source for growth and potassium nitrate was best for maximum lipid (35%) production in the case of *C. echinulata* CCRC 31840 (Chen & Chang 1996). Certik et al. (1999) experimented the different nitrogen sources on the activities of lipogenic enzymes in *C. echinulata,* a oleaginous fungus. He observed that increased lipid content in biomass was paralleled with an increase of acetyl Co-A carboxylase activity. They also reported that the rate of fatty acid biosynthesis is faster in *C. echinulata* when grown either on ammonium nitrate or corn steep when compared to other nitrogen sources. *M. rouxii* and *Mucor* sp.1b, both the cultures showed maximum lipid production in medium containing potassium nitrate. *M. rouxii* showed a maximum GLA (16.3%) when grown on ammonium sulfate as nitrogen source. Both the cultures grown on urea showed less biomass and poor GLA production (Somashekar et al. 2002). Dyal et al. (2005) found that 1% yeast extract as nitrogen source and 4% glucose as carbon source in the cultivation medium gave the best production of biomass, lipid and AA production in *M. ramanniana var. ramanniana*. Yeast extract as sole nitrogen source stimulated the cell growth, but not for GLA and total PUFAs production (Dyal & Narine 2005). Yeast extract enhances the growth of the organisms, since it is a complex nitrogen source but it is an expensive source for large scale production. Simple nitrogen source for microbial production of lipids have been received attentions from various workers in the recent years (Fakas et al. 2008). Ahmed et al (2006) studied the difference

of N_2 sources on GLA production by *Mucor* sp. RAL001. Yeasts extract at 1% gave higher GLA when compared to medium containing peptone. Dry biomass and GLA production was also high in corn steep liquor compared to other organic N_2 sources. In commercial production of SCO considered, corn steep liquor was the alternative to yeast extract as nitrogen source and much cheaper. In other study, urea gave the highest biomass, total lipid, GLA, and PUFAs yields followed by potassium nitrate, ammonium sulfate and ammonium chloride in *Mucor recurvus* sp. (Li et al. 2008).

C:N ratio

Oleaginous microorganisms in general are known to modulate their metabolism in response to conditions of the culture. The effects of concentration of C:N ratio in the medium have been investigated with a number of oleaginous organisms. (Fakas et al. 2008; Ratledge & Wynn 2002). The concentration of nutrients in the medium is obviously of as much importance as their relative proportion in the control of metabolic processes in fungi (Nakhara et al. 1993 ; Sattur 1989).The rate of lipid synthesis in oleaginous microorganisms depends on the high C: N ratio (Fakas et al. 2007a , b; Immelman et al. 1997; Ratledge 1987; 1989). This is attributed to induction of nitrogen-scavenging reactions. The effect of which is lowered level of AMP with consequent disruption of the citric acid cycle due to dependence of the isocitrate dehydrogenase (ICDH) reaction on AMP. The net result is said to be accumulation of citrate which is transported to the cytosol for further breakdown to produce acetyl-CoA. As the culture growth progresses a change in C:N ratio is expected with lower levels of nitrogen and the precursors for production of fatty acids might become available at the latter stage of culture growth.

Oleaginous microorganisms usually do not express their potential accumulation of lipid to any great extent in the media with C: N ratio lower than 20 and optimum for any organisms probably between a ratio of 30 and 80 (Dyal et al. 2005; Jang et al. 2005; Narine & Dyal 2005). To obtain optimum GLA production by *C. echinulata* 31840, the medium must have a 20 C:N ratio that

produces lipid accumulation. Maximum GLA content of 853-943 mg/L were obtained at C:N ratio 30-48.5. Higher GLA content in lipid was produced only in limited amount of lipid. The results show that there is a likely hood GLA was produced approximately at constant metabolic rate and extra accumulation of GLA by the cell (Chen & Chang 1996). Optimal C:N ratio of the medium was around 15-20 for AA production in culture of *M. alpina* CBS 754.68 when the C/N ratio was higher than 20 the mycelial weight and PUFAs production decreased due to N_2 limitation. *Mucor recurvus* sp. an experiment with the C:N ratio was varied at a fixed initial concentration of the carbon source. In this case the mycelial concentration was dependent on the nitrogen source concentration, when N_2 was in excess the proportion of PUFAs increased but that of saturated fatty acid decreased. However, when consumed C/N ratio was increased at a fixed initial concentration of the N_2 source, the GLA concentration depended on the initial concentration of carbon source. Although, all fungal strains utilized efficiently glucose and yeast extract as carbon and nitrogen sources respectively. This may be because of glucose enters directly into the glycolytic pathway, while yeast extract contains all the micronutrients (including metal ions) required for fungal growth, in addition to being a nitrogen source (Dyal & Narine 2005). The optimum carbon and/or nitrogen source and concentration for one fungal strain cannot be generalized to other strains within its genus or even its species.

Minerals supplements

In the recent years it is interesting to study the effect of different metal ions on lipid and PUFAs production in oleaginous microorganisms (Nakhara et al. 1992; Dyal et al. 2005; Muhid et al. 2008). Gill et al. (1977) studied the effect of N_2, P and C and Mg^{2+} limitation on lipid production by *Candida* 107 using continuous cultivation technique. Phosphate limitation alone produced a 50% reduction in biomass yield than of N_2 limitation and the lipid yield was 10>15% (w/w). Hansson & Dostalek (1988) investigated the effect of metal ions on lipid and GLA accumulation in *M. ramanniana, M. vinacea, and M. isabellina*. The results revealed that addition of Cu^{2+} and Zn^{2+} metals had a stimulatory effect on

both lipid and GLA production. However, the addition of Mg^{2+} did not influence the lipid and GLA production in *Mortierella* sp. The increased concentration of Mg^{2+} in the growth medium was also not showed any marked difference on either of lipid and GLA production. Lipid with considerably high GLA yield was possible with the fungus *Rhizopus nigricans* SSSD-8 when grown in a potato dextrose-yeast culture medium containing 0.40 % KCl (Bandyopadyay et al. 2003). Dyal et al (2005) studied effect of different metal ions with different concentration in *M. ramanniana var. ramanniana*. The study revealed that addition of Mn^{2+} at 5 mg/L induced highest yield of GLA content total lipid. Results obtained by Muhid et al. (2008) reported that the addition of Mg^{2+} to the culture medium in increased percentage it might have affected the ATP citrate lyase and Malic enzyme, thus it affected the Acetyl CoA and NADPH for lipogenesis in *Cunninghamella* sp.2A1. Metal ions are known to have important role as co-factors for enzyme activities like Malic enzyme, ATP citrate lyase and fatty acid synthase (Evans & Ratledge 1985; Wynn et al. 1999). These three enzymes play major key role in the lipid production since malic enzyme play a role in NADPH source for fatty acid synthase when ATP citrate lyase accumulate acetyl CoA as a precursors to the fatty acid biosynthesis (Wynn et al. 1999; 2001).

Supplements on lipid and PUFAs production:

Common fat or their derivatives are of special interest as substrates, since several oleaginous yeasts and molds are able to accumulate and at the same time modify the composition of the fat utilized as the carbon source (Aggelis & Sourdis 1997; Aoki et al. 1999; Papanikolaou et al. 2003a, b). Several papers have dealt with various growth media containing some supplements to examine their influence on growth, lipid and fatty acid composition in molds (Aggelis 2003; Certik et al. 1997; Papanikolaou et al. 2002a; Tornisielo et al. 2007). Kendrick & Ratledge (1996) studied supplementing triolein, sesame oil, safflower oil, linseed oil as well as oil extracted from *M. isabellina* in glucose containing media. Four fungi were studied such as *Conidiobolus nanodes, Entomophthora exitalis, M.isabellina,* and *M. cercinelloides.* It was interesting to note that *M. isabellina* did

not grow on either the linseed oil or *Mortierella* oil containing media. The medium supplemented with safflower oil showed highest cell yield where as sesame oil showed very less cell yield significantly than the glucose medium alone. With respect to GLA production, safflower oil supplemented to a glucose medium, resulted in higher cell and lipid yields than the unsupplemented glucose medium. However, the percentage of GLA that was obtained from the glucose medium without supplementation was more than two times that obtained from the supplemented medium. Lipid formation and GLA production by 48 species of *Mucorales* fungi grown in sunflower oil showed 42.7-65.8% lipids in the biomass (1.4 -7.7g/L) (Certik et al. 1997).

The various fungi were found to utilize the free fatty acids with varying degrees of efficiency. However, the *Mortierella* fungus effectively formed GLA, as well as, ARA and EPA from the mixture. The ability of different fungi, like *Mucor hiemalis* H-30 , utilized a fatty acid mixture to accumulate lipid added to liquid growth medium was studied (Aoki 1999). The presence of LA and ALA in the oils used for media supplementation seems to be effectively utilized by the various fungi like *M. cercinelloides*, *M. hiemalis* and *M. alpine* (Aggelis & Sourdis 1997; Auki et al. 1999; Certik et al. 1997). LA and ALA are precursors to LC-PUFAs like DGLA, AA and/or EPA. Presumably, this allows the fungi to reserve its carbon source for functions other than EFAs production instead of synthesizing them *de nova* (Dyal & Narine 2005). Complicated biological process, such as the bioconversion of industrial fats to microbial lipids studied through mathematical models (Aggelis & Sourdis 1997). It is important to quantitatively investigate bioconversions and possess a tool for predicting parameters of biochemical significance, such as the specific rates of lipid accumulation and degradation (Aggelis & Sourdis 1997; 2003; Papanikolaou et al. 2001a; 2003b).

FERMENTATION

In the past several years, research on microbial PUFAs production was basically aimed at improving economic competitiveness of microbial lipids compared to plant and animal-derived lipids. Emphasis was placed on screening for more efficient strains, increasing the product value, using inexpensive substrates, and reducing the processing steps necessary for lipid recovery from the cells. Two basic processes have been developed for microbial production of PUFAs: solid-state fermentation and submerged fermentation (Jang & Yang 2008; Conti 2001).

Solid-state fermentation for PUFAs production

Improvement of culture by cultivating on agro-industrial wastes and in the production of high lipid and PUFAs content considered to be an interesting area in the biodiesel production (Fakas et al. 2008; 2009a, b; Zhu et al. 2003). The association of fungal oil with solid state fermentation (SSF) provides another opportunity to fill marketing demands for PUFAs (Certik & Shimizu 1999). SSF is a process in which micro-organisms grow on a moist solid substrate in the absence of free water which allows utilization of cheap raw materials and residues of agro- and food-industries as fermentation substrates and convert them to useful metabolites (Certik et al. 2006; Pandey 1992; Robinson et al. 2001b). SSF used successfully in the industrial production of primary and secondary metabolites from the microbial sources such as amylase, phytase, glucoamylase, protease, cellulase, ligninase, pectinase and xylanase and various byproducts have been used successfully in SSF (Holker et al. 2004; 2005; Gunashree 2006;Pandey et al. 1999; 2000). During the past ten years, there has been a vast change in the solid-state culture with significant developments. Studies on SSF compares with submerged cultures, SSF claims higher yields, more concentrated metabolite with potential downstream recovery advantage for commercial production [Table 2.16] (Manpreet et al. 2005).

SSF might provide an alternative for PUFAs production since the association of fungal oil with solid substrate might be used as inexpensive food and feed supplement (Certik & Shimizu 1999; Jang et al. 2000; Zhu et al. 2003). Fungi play a key role in SSF, for their hyphal development allows them to effectively colonize and penetrate the solid substrate (Pandey et al. 2000; 2003). SSF cultivation was successfully used for the production of GLA and other PUFAs like AA and EPA from oleaginous microorganisms like *mucor* and *mortierella* spp. (Emelyanova 1997; Stredanska et al. 1993). Economically competitive, production of PUFAs using rice bran can be performed at the rural level. SSF can achieve this purpose by reducing the cost of growing microorganisms, high product yield and low wastewater output (Jang & Yang 2008; Pandey et al. 1999; 2003; Singhania et al. 2009). However, due to technical problems such as difficulties in contamination control, scaling-up and lipid extraction, SSF has received less attention than submerged fermentation (Conti et al. 2001; Dyal et al. 2005; Gema et al. 2002).

Submerged fermentation for PUFAs production

Submerged fermentation (SmF) is one of the industrially important techniques where the fungal products of biotechnological interest, such as primary metabolites, and secondary metabolites, are developed for use in scale up studies (Certik & Shimadzu 1999; Dyal et al. 2005). SmF cultivation for industrial use requires a process that consists of several operational units from strain cultivation to oil refining. There are three processes involved in obtaining lipids or oils the oleaginous microorganisms fermentation, cell separation and oil extraction and refining. The economic consideration is an important in PUFAs production and severely affected by the cost of raw materials and selection of growth media. It should be noted that media adequate to the production of high concentration of PUFAs in optimized conditions chosen (Certik & Shimizu 1999). Very efficient microbial strains, well adapted to SmF by genetic engineering are available for PUFAs production on an industrial scale. Oleaginous fungi, like *Mortierella* spp., *Mucor* spp., and other oleaginous microorganisms have been successfully applied

to produce good amount of PUFAs in SmF condition (Ahmed et al. 2006; Dyal et al. 2005; Somashekar et al. 2002; Papanikolaou et al. 2007). SmF offers a substantial reduction of contamination in the fermentation process and the possibility of large scale production from the different carbon and nitrogen sources, thus making the overall process more favorable to the commercial production. Success in the microbial PUFAs production has led to a flourishing interest in developing fungal fermentation processes and enabled several processes to attain commercial production levels (Ratledge & Wynn 2002; Ratledge 2003).

Table 2.16 Differences between Solid-state and Submerged liquid Fermentation

Solid state fermentation (SSF)	Submerged fermentation (SmF)
• Organisms requiring less water for growth are preferred such as filamentous fungi.	• Media concentration is very much lower as compared to water content.
• Inert support (natural or artificial), containing all components for growth in the form of solution.	• Required processed ingredients are expensive.
• Less chances of contamination because of low availability of water.	• Higher water activity becomes the major cause of contamination in SLF.
• Small size bioreactors can be used.	• Large-scale bioreactors are required because media is very much diluted.
• Less consumption of energy for aeration and gas transfer.	• High air pressure consumes more power and there is poor transfer of gas in SLF.
• Limiting factor for growth is diffusion of nutrients.	• Vigorous mixing makes diffusion easy.
• Lots of difficulties in measuring the quantity of biomass present and other online processes.	• Online sensors are available and sampling is easy for biomass measurement.
• Downstream processing is easy, cheaper and less time consuming.	• Water makes downstream process difficult and very expensive.
• Liquid waste is not produced	• High quantity of liquid waste is produced, causes difficulties in dumping

(Source: Manpreet et al. 2005)

METHODS OF LIPID EXTRACTION

The use of gas chromatography (GC) to characterize fatty acid profiles of lipids in various biological samples has became routine in laboratories of various scientific institutions and industrial organizations. The analysis of the composition of fatty acids was very important especially when developing a process for edible oil production for human consumption (Certik et al. 1996; Buurja et al. 2007). Traditionally, the procedure for fatty acid estimation consists of extraction of lipids from the biological material and subsequent preparation of fatty acid methyl esters (FAMEs) which are then used for GC analysis. The reliability of obtained results depends on efficiency of extraction procedure and efficiency of FAMEs preparation method used (Certik et al. 1996; Rodriguez et al. 1998).

Several extraction procedures may be found aiming at the improvement of lipid recovery from organisms, tissues or cell types. The extraction of lipids from intact yeast cells is connected complex membrane composition that must be taken in account; otherwise erratic results can be obtained. The lipid occurs in two forms dependent upon their ease of extraction, namely as 'readily-extractable' or 'free' lipid and 'bound' lipid, in oleaginous microorganisms (Certik et al. 1996; Rodriguez et al. 1998; Sajbidor et al. 1994). Common techniques used for lipid extraction are Folch and Bligh and Dyer, which is a quantitative extraction technique that uses chloroform and methanol (Bligh & Dyer 1959; Folch et al. 1957). The conventional methods are successful in recovering the free lipid from microorganism but poor extraction of bound lipid which is primarily associated with the cell envelope (Certik et al. 1996). The synthesis of lipids take place inside the mycelium i.e. intracellular and it should be separated for recovery and analysis. The extraction involves a pre-treatment to breakdown the tough cell wall followed by the isolation of lipid using different organic solvents (Ratledge 1992 a, b). There are three major unit operations followed during extraction of lipid from tissue, organs, plant material and in microorganisms.

- Cell disintegration
- Extraction with organic solvents

- Recovery from the solvent and refining

The efficiency of different pre-treatment methods is dependent on the rigidity of the cell wall. In case of fungi, the cell is composed of polymers of chitin, glucans and mannans (Orlawsky 1991). These polysaccharides, give high strength and rigidity to the cell wall, thus disruption process is an essential step before extraction of lipid (Burja et al. 2007; Sajbidor et al. 1994).

Cell disintegration/ disruption techniques

There are three pre-treatment methods are available to soften or break the cell wall, before the extraction of lipids using organic solvents.

a. Mechanical methods

Cell disruption by grinding action is brought about by a rapid agitation or the cell suspension with glass beads in a disintegrator (Dynomill, Braun homogenizer). Cell disruption is caused by collisions between shear force layers and also by rolling of the grinding elements (Hansoon & Dosatlek 1988). Another commonly used laboratory techniques is the French press. Liquid shear principle is applied here, where high pressure was employed to force the cell suspension through a needle valve. Pressures upto 35 Mpa are applied to samples contained in steel cylinder by means of a tight fitting piston. After the sample is bled through the needle valve at constant pressure, the cells are then subjected to sudden pressure drop results in disruption of cell wall and release of lipid globules (Gill et al. 1977; Hammond & Glatz 1988). A sonication technique has been widely employed as one of the laboratory techniques. Ultrasonic disintegrators operate at frequencies of 15 to 25 KHz, cell disruption occurs due to cavitations effect. This method can be adapted for continuous operation but does not suit for large scale processing because of difficulties in providing adequate cooling at high power input. Freezing and thawing in repeated cycles causes ice crystals to form and

melt, with some subsequent disruption of cells. But, it is a slow process with limited release of cellular materials (Hammond & Glatz 1988).

b. Biological methods

The most promising technique at laboratory scale seems to be enzyme lysis. Enzymes which have the activity include lysozyme and enzyme extracts from Leucocytes, *Streptomyces* spp. *Micromonospora* spp., *Penicillium* spp., *Trichoderma* spp., and snails. An advantage of biological method was lysis of cell wall occurs even at mild conditions. But the use of biological method is relatively expensive and the presence of enzyme in extracts complicates purification process. Autolysis is another method, in which the lytic enzymes are produced by the organism itself (Sattur 1989; Venkateswaran 1999).

c. Chemical methods

The use of chemical treatment was common to hydrolyze the cell wall of the organisms. Treatments with acid or alkali have been found to be effective in cell wall hydrolysis. Acid hydrolysis is found to be more effective compared to alkali treatment (Venkateswaran 1999; Somashekar et al. 2001). A number of detergents damage the lipoproteins of the microbial cell membrane and lead to release of intracellular components. The compounds which can be used for this purpose include quaternary ammonium compounds and sodium lauryl sulphate. Osmotic shock caused by sudden change in salt concentration is one of the gentlest methods of disruption. This method is good for fragile cell wall (Sattur 1989).

Extraction with organic solvents

After breaking the cell walls, the lipids are extracted by different solvents either individual or in mixtures like acetone, chloroform, methanol followed by folch wash (Bligh & Dyer 1959; Folch et al. 1957; Lamacka et al. 1998), acetone-methanol, ethyl ether, alcohol, hot methanol, petroleum ether, benzene, ethanol-

hexane (Certik et al. 1996; Lamacka et al. 1998; Lopez et al. 2005). Burja et al. (2007) studied using different solvent system on *Thraustochytrium* sp. ONC-T18 and observed that a drastic differences in the percentage of total lipid obtained by various methods. However, the efficiency of extraction methods differs from one organism to another and sometimes from strains to strain also (Certik et al. 1996; lewis et al. 2000; Somashekar et al. 2001).

Extraction with supercritical fluid extraction (SCF)

The traditional extraction methods used to obtain these types of products have several drawbacks; thus, they are time consuming, laborious, have low selectivity and/or low extraction yields. Moreover, these techniques employ large amounts of toxic solvents. At present, new extraction methods which are able to overcome the above mentioned drawbacks are being studied, among them, Supercritical Fluid Extraction (SFE) and Subcritical Water Extraction (SWE) are among the more promising processes (Darani & Farahani 2005; Mendes et al. 2003; Sajilata et al. 2008). These extraction techniques provide higher selectivities, shorter extraction times and avoids use of toxic organic solvents.

The need for rapid, efficient and safe methods for GLA extraction from natural sources has been emphasized (Darani & Farahani 2005). Therefore, several studies of supercritical fluid extraction of oils containing GLA and other PUFAs have been reported from fungi using CO_2, N_2O, CHF_3 and SF_6 (Certik & Horenitaky 1999; Sakaki et al. 1990), from evening primrose and borage sseeds (Favati et al. 1991; Darani & Farahani 2005) and *Spirulina platensis* and *Spirulina Maxima* (Quihui, 1999; Mendes et al. 2003; 2006) using CO_2, *Pythium irregulare* (Walker et al. 1999).

The supercritical fluid extraction (SFE) has been applied only recently to sample preparation on an analytical scale. This technique resembles Soxhlet extraction except that the solvent used is a supercritical fluid, substance above its

critical temperature and pressure. This fluid provides a broad range of useful properties. One main advantage of using SFE is the elimination of organic solvents and to avoid the degradation of thermally labile components, thus reducing the problems of their storage and disposal in the laboratory (Darani & Farahani 2005; Mendes et al. 2006; Sajilata et al. 2008).

Purification of PUFAs

The major difficulty associated with microorganisms as sources of PUFAs centres around the relatively extensive purification process to isolate and refine oils. The PUFAs concentrates can be produced by several methods, including freezing crystallization, urea complexation, molecule distillation, supercritical fluid extraction, silver ion complexation and lipase concentration. Some methods have involved a five-step process using a combination of chloroform, methanol, and water to separate lipid classes followed by transmethylation, urea fractionation, and reverse phase chromatography. Urea complexation is an effective method of PUFAs isolation because urea preferentially complexes with saturated and monounsaturated fatty acids to form solids which can be removed from the PUFAs (Bajpai & Bajpai, 1993). Ethanolic KOH used for saponification, followed by liquid chromatography to isolate the PUFAs fraction. A two-step purification process involving only urea complexation and liquid chromatography, achieved a range of 94 to 96% purity for GLA, AA, EPA and DHA (Ahmed et al. 2009).

STRAIN IMPROVEMENT IN OLEAGINOUS MICROORGANISMS

Commercial production of microbial oil has led to a flourishing interest in the development of fermentation processes and has enabled several processes to attain success (Gill & Valivety 1997; Hiruta et al 1996). When compared to health benefits obtained from microbial source (SCO), the cost of production was secondary (Certik & Shimizu 1999). Although the manipulation of microbial oil composition is rapidly growing field of lipid biotechnology, the supply of microbial lipids is still insufficient to meet pharmaceutical and nutraceutical industrial demand (Papanikolaou et al. 2007; Ratledge 2004). Therefore alternative strategies such as mutation methods, hybridization and molecular engineering techniques could be combined with classical fermentation [Fig. 2.14] (Hiruta et al. 1996; Jareokitmongkol et al. 1993 a, b; Wongsumpanchai et al. 2004).

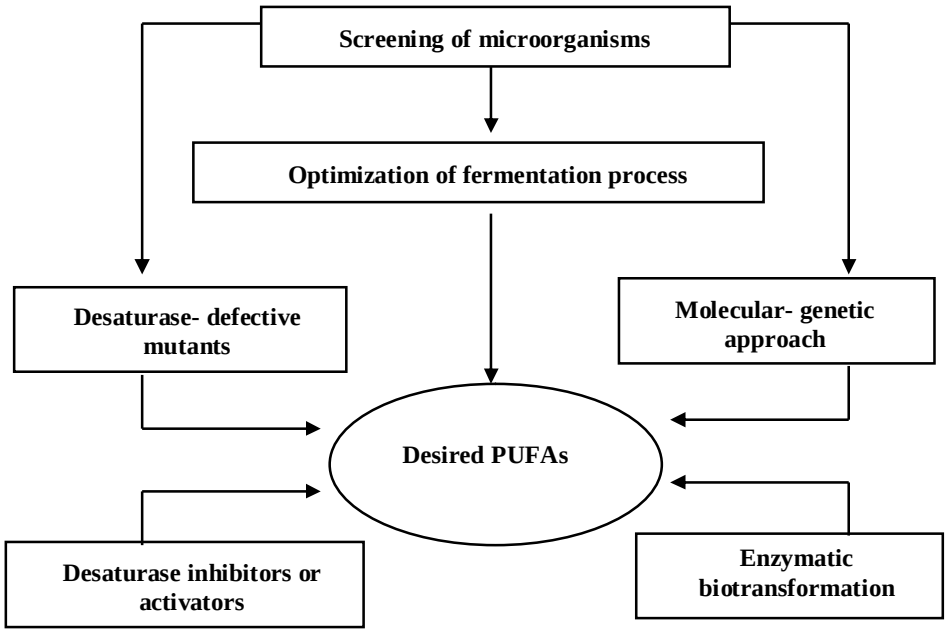


Fig 2.14 Strategy for the Modification of microbial PUFA
(Source: Certik et al. 1998)

MUTATION TECHNIQUES

Strain improvement is an essential part of process development for microbial fermentation products. It is a means of reducing costs of developing strains with increased productivity (total yield), ability to use low value added raw materials, more specific advantageous characteristic such as improved filtration properties, ability to produce under particular conditions of temperature or aeration (Certik et al. 1998; Fakas et al. 2009a, b; Jang et al. 2005; Papanikoloau et al. 2007). Inspite of the development of the newer techniques, such as rational screening and genetic engineering, the traditional method of strain improvement by mutagenesis and selection on the basis of random screening-still play central role as a reliable and cost effective procedures (Certik & Shimizu 1999).

Mutation is a method of strain improvement and is nothing but inheritable genetic change which is persists in the genome throughout the generations of the organisms. From industrial point of view mutation is the alteration in genetic level to achieve higher product yield. The change in genetic level (genotype) is manifested outside (phenotype) as a change in one or more characters, which are coded by that particular gene. Treatment with mutagens followed by selection of putative mutants at lower temperatures resulted in the identification of several new strains of microbes with higher GLA contents (Hiruta et al. 1996; Nakhara et al. 1992).

Application of mutation techniques in production of desired PUFAs

Although several wild-type oleaginous microorganisms are able to synthesize PUFAs-rich oils, these strains have a limited ability to produce new PUFAs or increase existing PUFAs formation. Mutation techniques resulting in the suppression or activation of specific desaturases and elongases are beneficial not only for the production of tailor made fatty acids, but they can also be useful for studying fatty acid biosynthetic pathway in oleaginous microorganisms (Certik et al 1998; Certik & Shimizu 2003; Gunstone (1998); Jareokitmongkol et al. 1993

a, b; Ratledge & Wynn 2002). Several mutants have been characterized and employed for PUFAs synthesis using either defective in desaturases enzymes or enhanced desaturase activities (Δ^5, Δ^6 and Δ^{12}) or their combinations (Certik et al. 1998; Jareonkitmongkol et al. 1993 a b; Laiteng et al. 2003). These mutants not only exhibit significantly improved production of naturally occurring PUFAs, but also form other PUFAs commonly not found in the wild type microorganisms (Certik & shimizu 1999; Laoteng et al. 2003; Wongsumpanchai et al. 2004). Another feature of mutants is that they can utilize exogenous fatty acids and allow the production of various PUFAs in high yield. The biotransformation is very promising from the biotechnological point of view (shinmen et al. 1992). There are various sources easily available which are natural oil containing individual fatty acid precursors. Thus, the mutants are excellent tools for regulating exogenous fatty acids flow to targeted PUFAs. Moreover, the mutants because of their simplicity of metabolism are considered to be excellent models for elucidating the reaction mechanisms involved in fatty acid biosynthesis (Certik et al. 1998; Certik & Shimizu 1999; Ratledge 2004). Among oleaginous microbial strains, the mutants of *M. alpina* with their unique fatty acid biotransforming enzyme system are probably the best studied (Certik et al. 1998; Certik & Shimizu 1999; 2000).

ii. HYBRIDISATION STUDIES

Protoplasts are osmotically fragile cells, which are completely devoid of cell wall. Protoplasts can be isolated from microbial cells by specific lytic enzymes to remove cell wall, in the presence of osmotic stabilizer. Such protoplasts can be induced to fuse in the presence of agents such as the polyethylene glycol (PEG). Protoplast fusion is a physical phenomenon, during fusion two or more protoplast come in contact and adheres with one another in the presence of fusion inducing agents. Cultivation of the protoplasts on a hypertonic growth medium induces regeneration of new cell wall material and their subsequent reversion to the normal cell form of the organism (Peberdy 1979). By

protoplast fusion it is possible to transfer some useful genes responsible for qualities such as disease resistance, nitrogen fixation, rapid growth rate, more product formation rate, quality of protein, resistant to herbicide, drought, heat and cold from one species to another. Protoplast fusion is an important tool in strain improvement for bringing genetic recombination and developing hybrids strain in filamentous fungi (Peberdy 1979). Parasexual hybrid protoplasts were obtained in two different fusion of protoplast from two genetically different cells**.** The hybrid protoplast contained heteroplasmic cytoplasm and two fused parent nuclei. Protoplast fusion has been used to combine genes from different organisms to create strains with desired properties. Fusion of protoplast is relatively a new versatile technique to induce or promote genetic recombination in a variety of prokaryotic and eukaryotic cells (Verma et al. 2007). This may be used to produce interspecific, intergeneric, intraspeicific, intrageneric, intrastrain hybrids (Peberdy et al. 1972, Prabhavathi et al. 2006). Fusion has also been found to be highly effective for obtaining heterokaryons and enabling the use of filamentous fungi for genetic studies and industrial strain improvement. Fusion technique has a great potential for genetic analysis and for strain improvement. It is a powerful technique for engineering of microbial strains with desirable industrial properties (Muralidhara & Panda 2000; Verma et al. 1999).

Protoplast isolation

Protoplast isolation is an important technique in which that cell wall of plant and microorganisms is degraded by using appropriate lytic enzymes. The isolation of protoplasts from microbial cells involves the total digestion or localized puncturing of the cell wall by enzymes allowing the cell contents enclosed by the plasma membrane to escape. To maintain the protoplast as intact structures, the protoplasts must be released into a hyper tonic or isotonic solution to provide osmotic stability (Venkateswaran 1999). The success of this method depends upon three important factors

i. Use of appropriate enzymes,

ii. Ionic conditions and

iii. Osmotic stabilizers

Various commercial enzymes have been widely used in the isolation of protoplasts from fungi. Enzymes of microbial origin are found to be highly capable of digesting the cell wall. Lytic enzymes are the most favored for the isolation of protoplast. *Trichoderma harzianum* has also been proved to be a useful lytic organism showing activity against cells of a range of species. Lysing enzymes from *T. harzianum* contains cellulase, protease and chitinase and other enzymes which act very effective in cell wall digestion. Fungal wall degraded by Novozyme-234 includes glucanase and chitinase enzymes (Peberdy 1980; Venkateswaran 1999). Bacterial cell walls are degraded efficiently by the action of lysing enzymes especially the lysozymes. In plants pectinase or macerozyme, cellulose was used efficiently for protoplast isolation.

Protoplast isolation from batch cultures of a microorganism i.e. yeasts or filamentous fungi are usually heterogeneous in nature with regard to their physiology and biochemistry (Peberdy 1979; Venkateswaran 1999). The physiological status and age of the culture is a major factor in determining protoplast yield. On the other hand, the nature of the culture medium used also has an effect. This phenomenon was observed by many workers in yeast and filamentous fungi (Peberdy 1980; Venkateswaran 1999; Prabhavathi et al. 2006). Early or exponential phase of growth cell culture are more preferable for protoplasts isolation than the culture age of lag phase (Venkateswaran 1999).

Osmotic stabilizer

Stabilizers are essential to provide osmotic support to the protoplasts following the removal of the cell wall. Many inorganic salts, sugars, and sugar alcohols have been used for this purpose. Inorganic salts have proved to be highly

effective with filamentous fungi, and sugars are found to be best suited for yeasts. (Varavallo et al. 2007; Venkateswaran 1999).

Methods of protoplast fusion

After isolation of protoplasts from their parental strains, they are mixed in equal numbers and treated with a fusogen. The treated material is then plated onto a suitable medium and the fusion products are recovered, which may later be subjected to direct or indirect selection. The protoplast fusion can be broadly classified into two categories. Spontaneous fusion and Induced fusion. During enzyme treatment the protoplasts adjoining to cells fuse through their plasmodesmata to form multinucleate protoplast and this phenomenon is called as spontaneous fusion. Fusion of free protoplast isolated from different sources with the help of fusogen is known as induced fusion. Normally isolated protoplasts do not fuse together because the surface of the isolated protoplast carries negative charges around the plasma membrane out side. Thus there is strong tendency in the protoplast to repel each other due to same charges. So this type of fusion needs inducing agents which actually reduce the electro negativity of the isolated protoplast and allow them to fuse with each other (Muralidar & Panda 2000).

The induced fusion can be brought from three ways, mechanical fusion, chemical fusion and electro fusion.

Mechanical fusion: The protoplasts are physically brought into contact by mechanically using micromanipulator or perfusion micropipette which is known as mechanical fusion**.**

Chemofusion: Chemical fusogens cause the isolated protoplast to adhere each other and leads to tight agglutination followed by fusion of protoplast. Several chemicals have been used to induce protoplasts fusion such as sodium nitrate, polyethylene glycol (PEG) and calcium ions. In *T. harzianum, T. ressei* and *A. niger* protoplast fusion has been successfully carried out using the chemofusion i.e. using PEG. Since the development of protoplast fusion in microbial systems,

PEG has been established as a universal fusogens (Prabhavathi et al. 2006). A study carried out by Anne & Peberdy (1976) confirmed the use of PEG to be highly effective in inducing fusion of protoplasts of several fungal species. Molecular weights of PEG 1000, 4000 or 6000 are equally effective in inducing the aggregation of protoplasts during fusion. The different concentration of PEG has been used to study the effect on protoplast fusion. Optimal concentration for fusion was found to be 30% PEG and below 20% its stabilizing effect was lost, in the lysis of cell wall. At higher levels i.e. above 30% the PEG was hyper tonic causing protoplasts to shrink and produced a lower frequency of fusion. PEG induced protoplast fusion of fungal protoplasts is influenced by the presence and concentration of various cations. A requirement of Ca^{2+} ion is well established. In the presence of Ca^{2+} ions, alkaline conditions stimulated the highest fusion frequency. PEG forms a molecular bridge between adjacent membranes involving hydrogen bonding in the membrane or indirectly utilizing the Ca^{2+} added to the fusion mixture.

Electrofusion: recently, mild electric stimulation is being used to fuse protoplasts. In this, two capillary microelectrodes are used to contact with the protoplast. This method is easy to control and having 100% fusion frequency but the instrument is sophisticated and expensive.

Regeneration of Protoplasts and Formation of Hybrid Cells

The induced fusion of protoplasts is clearly an artificial system, and the prospects of using this technique to generate new products unobtainable by conventional methods. In theory, protoplasts from any two organisms might be expected to fuse following the treatment with PEG. In the filamentous fungi, interspecies protoplast fusion leads to the formation of new colony forms called as fusion products or fusants. The fusants are further subjected to various selection procedures for their use in strain improvement.

Studies of the regeneration of the cell wall and its biosynthesis the protoplasts are very useful. The protoplasts of fungi, bacteria, plants, have the capacity to synthesis whole cells from which they are derived, including the ability to make a new cell wall (Peberdy 1980; Venkateswaran 1999; Verma et al. 2007). Fungal protoplast formed by various methods have been utilized in regeneration studies, particularly all washed protoplasts are capable of regeneration into new cells, their capacity being apparently determined primarily by the physical conditions in the medium. Since the regeneration process in most fungal protoplasts takes place very slowly, it is possible to follow the individual stages of biosynthesis of the new cell wall. Many workers have demonstrated the regeneration of protoplast in the fungus and yeasts (Peberdy 1980; Narayanaswamy 1994; Varavallo et al. 2007). Once protoplasts have been transferred to a growth medium with the stabilization they revert to typical normal cells. The life of growth and regeneration found vary with the osmotic strength of the medium and the cultural conditions under which they develop (Venkateswaran 1999).

The potentiality of the study of fungal protoplast helps to elucidate, many problems associated with cell structure, growth, nutrition, biosynthesis and others. The formation of protoplasts opened up new areas in the investigation of the role of the cell wall in both physiology and genetics.

Use protoplast fusion (hybridization) used in Biochemical and Genetic studies

Protoplasts contain all the intracellular organelles of cells and form a vital link in transfer of micromolecules in between cyto organelles. Currently most of the laboratories involved in fungal genetics are using gene manipulation based on protoplast fusion. Therefore to further improve genetic properties of the strain using protoplast fusion are attempts to develop methods of preparation and regeneration of protoplasts. The process involved is protoplast mutagenesis, transformation and protoplast fusion (Evans 1983; Verma et al. 2007).

Protoplast uses are as follows:

- Ø For the preparation of cell membrane and the study of cell wall structure.
- Ø For obtaining nuclei, vacuoles and tonoplasts.
- Ø Preparation of cell organelles such as mitochondria, ribosomes, endoplasmic reticulum, etc.
- Ø Preparation of cell free extracts.
- Ø The study of osmotic systems and permeability.
- Ø For growth studies.
- Ø The study of transport mechanisms of solutes.
- Ø For tests of resistance to physical factors.
- Ø Investigation of protein and enzyme synthesis effectively.
- Ø For studies of localization of enzymes in cells.
- Ø For the studies of the mode of action of antibodies and surface active agents.
- Ø For studies of the regeneration of the cell wall and its biosynthesis.
- Ø For the studies of conjugation between protoplasts.
- Ø For the studies on spore formation

(Spencer & Spencer 1981, Venkateswaran 1999)

Brume et al. (1992) showed that ten fold higher alkaloid production than the parental strains by protoplast fusion without introducing any selective auxotrophic markers. Intraspecific protoplast fusion carried out in *Streptomyces griseoflavus*, the result indicated that increased production of desferrioxamine B chelator (that absorbs additional iron from the blood of thalasemia patient) (Verma 1999). Complete set of cellulase production by the protoplast fusion in *T. reesei* and *A. niger* produced more amounts of endo and exoglucanase and other produced more β-glucosidase was the best technique used (Ahmed & Berkely 2006). Prabhavathi et al. (2006) reported that the isolated protoplast from *T. reesei* strain PTr2 showed high CMCase activity with 80% of fusants and more than two fold increased in enzyme activities with two fusants SFTr2 and SFTr3 as compared to the parental strain. Transfer of genes by protoplast fusion has been

recently demonstrated as an efficient method of improving industrially important microorganisms.

BIOTECHNOLOGY OF MICROBIAL LIPID PRODUCTION (Genetic Engineering)

A major challenge in modifying the lipid composition is to change the degree of fatty acid unsaturation and reduce or increase the chain length of fatty acids. Molecular engineering provides very powerful methodology and tools for the investigation of complex metabolic pathways involved in PUFAs formation, but also for the production of novel microbial varieties synthesising economically valuable fatty acids (Certik et al. 1998; Certik & Shimizu 1999). In lower eukaryotes i.e. in fungi, biochemical mechanism, structure and function such as positional specificity relationship between fungal desaturases, and other fatty acid modifiers, are still not well understood. The strategies for isolation and expression of the genes responsible for the desired metabolic steps have to be designed (Passorn et al. 1999). There are three independent genetic technologies to engineer fatty acid composition:

- Cloning of genes encoding protein involved in PUFAs biosynthesis,
- Transgenic expression of desaturase genes
- Modification of cloned genes in order to engineer the expressed protein (Certik et al. 1998).

Desaturases

Fatty acid desaturases play essential roles in fatty acid metabolism and the maintenance of proper structure and function of biological membranes and living organisms. There are two main types of fatty acid desaturases: the soluble and membrane bound desaturases, both of which are dioxin-oxo enzymes (Murphy 1998; Laoteng et al. 2005b). The soluble desaturases that introduce double bonds into fatty acids esterified to acyl carrier proteins are restricted to higher plants and they have two conserved histidine rich motifs (Na-Ranong et al. 2006; Zang et al. 2004). Membrane bound desaturases which distribute widely and introduce

double bonds into fatty acids esterified to acetyl -Co-A and glycerolipids are characterized by three histidine rich motifs and four transmembrane domain (Huang et al. 2001; Na-Ranong et al. 2006; Zang et al. 2004). Microsomal membrane bound desaturases, microsomal desaturases can be further divided into two groups: carboxyl directed and methyl directed desaturases such as Δ^{4-},$\Delta^{5-}$and Δ^{6-} desaturases (Laoteng et al. 2005a; Napier et al. 2004; Qiu et al. 2001; Zang et al. 2004). The desaturase introduce double bonds in between the existing double bond and carboxyl terminus of the fatty acyl chain. Methyl directed membrane bound desaturases such as Δ^{12-} desaturase introduce the double bond between the existing bond and methyl terminus of the fatty acyl chain (Passorn et al. 1999). Classification of desaturase enzymes are given in Table 2.17. Modification of the fatty acid biosynthesis pathways by genetic manipulation to produce desired oil in transgenic microorganisms and oil seed crops, as a possible alternative source, this leads to the isolation of gene encoding for desaturase enzymes from various organisms [Table 2.18] (Huang et al. 2004). The awareness of the enzymatic reactions and corresponding metabolic pathways are well studied by molecular characterization and these recombinant enzymes offer the prospect of producing a desired fatty acid in oilseed crops (Napier et al 2001).

Table 2.17 Classification of desaturases

Type	Substrate	Location	e7 donor
Soluble	Saturated acyl-ACP	Plant plastids (Δ^9)	Ferredoxin
		Mycobacterium Streptomyces	Ferredoxin
			Ferredoxin
Membrane:			
I	Saturated acyl-CoA	ER of animals and lower eukaryotes (Δ^9)	Cyt b5
		Cyanobacteria (Δ^9)	
	Saturated acyl-lipid	*Bacillus subtilis* (Δ^5)	Ferredoxin
			Ferredoxin
II (methyl-end)	Mono- or di-unsaturated acyl-lipids	Cyanobacteria (ω-6)	Ferredoxin
		Plant plastids (ω-6/ω-3)	
		ER of plants, animals and lower eukaryotes (ω-6/ω-3)	Ferredoxin
			Cyt b5
III (front-end)	PUFA acyl-CoA/acyl-lipids	ER of plants, animals and lower eukaryotes (Δ^6/Δ^5)	Cyt b5
		Euglena (Δ^8)	Cyt b5
		Lower eukaryotes (Δ^4)	Cyt b5
	PUFA acyl-lipids	Cyanobacteria (Δ^6)	Ferredoxin

[1]
Organism and subcellular location (and main regioselectivity) for desaturases.
ER, endoplasmic reticulum; Cyt b5, cytochrome b5.

(Source: Uttaro 2006)

Table 2.18 Biological sources Desaturases

Gene/enzyme	Biological source
Δ^{9-} desaturase	*Anabaena variabilis*
	Synechocystis sp.
	Rosa hybrida
	Arabidopsis thaliana
n-3 desaturase s(microsomal)	*Arabidopsis thaliana*
	Glycine max
	Brassica napus
	Limnanthes douglasii
	Nicotiana tabaccum
	Triticum aestivum
	Perilla frutescens
n-3 desaturases (plastidial)	*A. thaliana (FAD-7)*
	A. thaliana (FAD-8)
	G. max
	B. napus
	N. tabaccum
	T. aestivum
	P. frutescens
Δ^{12-} desaturase (microsomal)	*A. thaliana*
	G. max
	Borago officinalis
Δ^{12-} desaturase (plastidial)	*A. thaliana*
	A. thaliana
	Spinacia oleracea
Δ^{6-} -desaturase	*Borago officinalis*
	Physcomitrella patens
	*Synechocystis*sp.
	Helianthus annuus
	Mortierella alpina
Δ^{5-} -desaturase	*Mortierella alpina*

(Source: Lopez Garcia Alonso & Maroto 2000)

OTHER IMPORTANT w-6 & w-3PUFAs

Linoleic acid (LA, 18:2, w-6)

Linoleic acid (LA) is one of the most abundant PUFAs in all plants. It is one of the ω-6 EFAs, the human desaturase cannot insert the double bond beyond the ω-6 double bond, hence it must supply through diet. Low levels indicate dietary insufficiency that leads to a variety of symptoms. Some of these symptoms result from lack of LA in membranes where it serves a role in structural integrity (Lenhinger 1993). LA is the starting point for the production of LC-PUFAs, where it serves as precursors for production of local hormones like prostglandind and leukotirenes. Dietary sources of LA are corn oil, sun flower oil, safflower oils and soya, it needs for balanced production of hormones in human health (Lunn & Theobald 2006).

Dihomo-gamma-linolenic acid (DHGLA 20:3 ω-6)

Eicosatrienoic acid, (20:3) or dihomo-gamma-linolenic acid, is an ω-6 PUFAs which is produced from GLA by specific fatty acid elongase enzyme inserting the two carbon atoms. It is natural precursors of many C20 compounds of biological interest, including prostaglandins 1 and 3 and thromboxanes (Horrobin 1992; James et al. 2000). It is one of the many PUFAs currently undergoing evaluation by scientist as to its potential future role in medicine and also diets low in EFAs are almost universally low in DGLA (Kendrik & Ratledge 1998). In sufficiency of this acids leads to number of disorders, a wide range of cellular functions and tissue responses (Lunn & Theobald 2006). Through the amities not accumulating large quantity, but the microorganisms produce GLA. When this GLA is supplied through the diet then DGLA accumulate more by chain elongation (Huang et al. 2001; Ratledge 2004). *Mortierella* spp. are producers in low amounts since it producing high quantity of AA (Dyal & Narine 2005). There are two ways to produce DGLA by fungi (Jareokitmongkol et al. 1993a);

i. By adding inhibitors like sesamin (AA inhibitors) in the culture medium and

ii. By getting AA deficient mutants

Production of DGLA by second method is advantageous since no requirements of any inhibitors and it yields relatively high amount of DGLA (Certik & Shimizu 1999). In metabolism the conversion of AA into DGLA is slow process because desaturation is slow process, it is partly inhibited (Jareokitmongkol et al. 1993a).

Arachidonic acid (AA, 20:4, w**-6)**

The last ω-6 PUFAs is eicosatetraenoic acid (20:4, 5,8,11,14). AA serves as substrate for the cyclooxygenase and lipooxygenase enzymes, leading to the production of the 2 series prostanoids and leukotrienes (Huang & Ziboh 2001). The production of AA was carried out in industrial level from genus *Mortierella*. The highest level of AA (13 g / l, 220 mg/g mycelia) was achieved with *M. alpina* IS-4 in a 10kl fermentor (Heighashima et al. 998). The fungal process is more superior to the microalgal production among which *Prophyridium cruentum* is probably best producer (Sakuradani 2009).

Several of these products have potent, proinflammatory and thrombogenic activity. High AA also promotes gall stone formation by stimulating much production in the gall bladder mucosa. Recently AA, together with the n-3 PUFAs, DHA, has been shown to augment free radical generation in tumour cells grown in vitro (Innis 1991; Innis et al. 1994). Such research has fuelled the search for replacements for the main sources of AA from porcine liver, porcine adrenal gland and sardines. However, AA content in fish oil source is usually low and alternative to these fungal cultures like *Mortierella* spp (Yu et al. 2003). were searched for high unsaturated fatty acid especially AA(Aki et al. 2001; Buranova et al. 1990).

Eicosapentaenoic acid (EPA, 20:5, w-3)

Eicosapentaenoic acid, 20:5 (5, 8, 11, 14, 17) is an ω-3 PUFAs of great commercial potential production. Together with the ω-3 PUFAs docosahexaenoic acid (DHA), it has been shown to prevent blood platelet aggregation and reduce blood cholesterol hence reducing the rate of reoccurrence of coronary heart diseases (Das 2006; Demaison & Moreau 2002; Huang et al. 1984). EPA is the parent of the 3-series prostanoids and leukotrienes, which moderate the pro inflammatory effects of the 2-series derived from AA (Napier et al. 2003). Although EPA can be produced from the ALA, dietary intake of this fatty acid is generally poor. The conversion also requires the action of the Δ^6 desaturase enzyme that may be at a low level by virtue of inadequate Zn, Mg, or vitamins B3, B6, and C. Such enzyme impairment would be indicated if EPA levels were low and ALA levels normal or high (Graham et al. 2004; Kendrik & Ratledge 1990). High levels of saturated, monounsaturated, and trans-fatty acids and of cholesterol also limit the conversion of ALA to EPA (as well as that of GLA to DGLA) (Graham et al. 2004). Fish oils are rich sources of EPA presently (Ratledge 2003). When taken in sufficient doses, the ω-3 PUFAs also reduce the incidence of inflammatory diseases such as asthma and type I diabetes mellitus (Ruxton et al. 2004).

Docosapentaenoic Acid (DPA, 22:5, w-3) and Docosahexaenoic Acid (DHA, 22:6, w-3)

Docosapentaenoic acid, 22:5 (7, 10, 13, 16, 9) and docosahexaenoic acid, 22:6 (4, 7, 10, 13, 16, 19) are the two terminal members of the ω-3 PUFAs biosynthetic pathway. The growth and development of the central nervous system is particularly dependent upon the presence of an adequate amount of the very long chain, highly unsaturated fatty acids, DPA and DHA (Innis 1991; Kyle et al. 1992). Attention deficit hyperactivity disorder and failures in development of the visual system in EFAs deficiencies are two examples of this dependency. DHA is

an important member of the very LC-PUFAs (C22 to C26) that characteristically occur in glycosphingolipids, particularly those in brain tissue. Since this fatty acid is so important in early development, it is worth noting that the levels in breast milk are correlated with the mothers' intake of fish oils (Das 2006; Demaison & Moreau 2002), which are rich sources of DHA and DPA. Fatty acids have considerable medicinal and nutritional value for example; EPA and DHA; both ω-3 fatty acid) have physiological effects in three areas i.e. heart and circulatory, inflammatory and cancer (Kendrik & Ratledge 1988; Demaison & Moreau 2002). A characteristic of marine micro organisms is the predominance of PUFA in their cellular lipid. In the fungus *Thraustochytrium aureum* , *Schizochytrium aggregatum* and *T. roseum* are reported for the production of DPA and DHA (Kyle et al. 1992; Ratledge 2004).

Fat is more than just a source of energy PUFAs have significant role in health and disease. Inadequacy of agricultural and animal oils has necessitated the 'hunt' for suitable sources for production of biologically important fatty acids like GLA. New potential species should be screened and the characteristic should be studied. Development of new microbial technologies using fungi for this production needs lot of fundamental research on growth conditions of the fungi and the effect of incorporation of different factors into media for good yield. Mutation methods and molecular engineering steps also should be investigated. Work has been done all over the world towards these goals. This review of literature has covered the previously published literature on such researches.

This research investigation on "Polyunsaturated Fatty Acids (PUFAs) of *Mucor* sp. with Special Reference to Gamma Linolenic Acid (GLA)" has been planned based on this background material.

CHAPTER -3

MATERIALS AND METHODS

MATERIALS AND METHODS

I. MATERIALS

i. The following chemicals and reagents were procured and used in this study:

Microbiological media like potato dextrose agar, potato dextrose broth, czapek dox agar, Fine chemicals like yeast extract, malt extract, beef extract, bacteriological peptone, yeast nitrogen base without ammonium sulphate, succinic acid, glycine etc used in the study were obtained from Hi- Media Chemicals. Molecular biology reagents like acrylamide, bisacrylamide, ammonium persulphate, tris (hydroxymethyl aminomethane), ethylene diamine tetra acetic acid (EDTA), IPTG, X-gal, amino acids, sodium dodecyl sulphate (SDS), ethyl methane sulphonate (EMS), N-nitro, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), N, N, N', N'- tetramethyl ethylene diamine (TEMED), agarose, β-mercaptoethanol, tween- 80, coomassie brilliant blue G 250, bromophenol blue, xylene cyanol, bromocresol green, methyl red, ethidium bromide, fungal cell lysing enzyme from *Trichoderma harzianum*, chitinase, chloramphenicol, polyethylene glycol (PEG) 6000 and Sudan black-B, Fatty acid standards like, myristic, palmitic, plamitoleic, heptadecanoic, stearic, oleic, linoleic and gamma linoleic acids were procured from Sigma Chemicals, USA. Novozyme 234, the enzyme used for cell wall lytic purpose was from NOVO Biolabs, Denmark. PCR components like, Taq DNA polymerase, buffer, dNTPs, 1Kb, 3Kb, 10 Kb, λ-markers and restriction enzymes were procured from Genie, Bangalore Pvt. Ltd., India.

Salts of ammonium nitrate, magnesium sulphate, ferrous sulphate, manganese sulphate, potassium dihydrogen phosphate, potassium nitrate, potassium sulphate, calcium chloride, zinc chloride, copper sulphate, disodium hydrogen phosphate, ammonium sulphate, sodium hydroxide, citric acid, sorbitol,

sodium thiosulphate, sodium carbonate, formaldehyde, phosphoric acid, glacial acetic acid, hydrochloric acid, solvents like methanol, chloroform, petroleum ether, hexane, benzene, acetyl chloride, of both analytical grade and HPLC grade were obtained from Qualigens India Pvt. Ltd., E- Merck Chemicals, Glaxo India Pvt. Ltd., BDH Chemicals and Sisco Research Laboratory Chemicals. Ethanol was procured from standard distillery company. Ultra filter and RO water used in all the experiments were collected from Millipore water purification unit, USA. Whatman filter paper No.1 and No.42 were procured from Whatman Company. The different oils used in this study were of pure, refined packed ones obtained from local market of Mysore city.

All the glassware's used in this study were obtained from Borosil and Vensil Glass, India Ltd.

Standard cultures used in this study

Fungal strains *viz*, *Mucor rouxii* MTCC 386, and *Mucor hiemalis* MTCC 2877 were procured from Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh, India for a comparative purpose. These fungal strains were maintained as per the instructions given by MTCC.

ii. Culture Media Used:

i. ***Mucor*** **Screening Medium : g/L**

Glucose	30
Yeast extract	5
Potassium dihydrogen phosphate	2.4
Potassium nitrate	1.0
Magnesium sulphate	0.5
Calcium chloride	0.1
Ferrous sulphate	0.015
Zinc sulphate	0.0075
Manganese sulphate	0.01
Copper sulphate	0.0005
pH	6.0 ± 0.2

ii. Potato Dextrose Agar (PDA): g/L

Potato infusion	200.0
Dextrose	20.0
Agar-agar	20.0
pH	5.5 ± 0.2

iii. Fat Production Medium (FPM) (Enobes Medium): g/L

Glucose	40.0
Yeast extract	1.5
Ammonium nitrate	0.286
Magnesium sulphate	0.4
Calcium chloride	0.4
pH	5.8 ± 0.2

The FPM is described by the Enobes for fat production in *R. gracilis* with slight modification was used. The nitrogen limited media contained 0.286 g/l and for non-limited nitrogen medium higher concentration of 2.86 g/l of Ammonium nitrate was used (Sattur 1989).

iv. Nutrient solution (NS): g/L

Yeast extract	5.0
Peptone	4.0
Magnesium sulphate	0.75
Potassium dihydrogen phosphate	1.0

v. Luria-bertani (LB) medium: g/L

Bacto peptone	10.0
Bacto-yeast extract	05.0
Sodium chloride	10.0
pH	7.2± 0.2

vi. Potato Dextrose Broth (PDB): g/L

Potato infusion	200.0
Dextrose	20.0
pH	5.5 ± 0.2

vii. Czapek Dox Medium (CDM): g/L

Sucrose	30.0
Sodium nitrate	3.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01
Agar-agar	20.0
pH	7.3± 0.2

viii. Synthetic Medium (SM): g/L

Glucose	30.0
Yeast extract	5.0
Potassium dihydrogen phosphate	2.5
Potassium nitrate	1.0
Magnesium sulphate	0.5
Calcium chloride	0.1
Ferrous sulphate	0.02
Zinc sulphate	0.01
Manganese sulphate	0.01
Copper sulphate	0.002
pH	5.5 ± 0.2

ix. Glucose Yeast extract Medium (GY): g/L

Glucose	20.0
Yeast extract	10.0
pH	6.0± 0.2

x. Mutant Screening Medium (MYA): g/L

Maltose	4.0
Glucose	12.0
Yeast extract	4.0
Peptone	6.0
Agar-agar	20.0
pH	5.5± 0.2

(**Source: Hiruta et al. 1996**)

xi. Minimal Medium (MM): g/L

Yeast Nitrogen base w/o AA and ammonium sulphate	1.7
Ammonium sulphate	1.0
Succinic acid	10.0
Sodium hydroxide	6.0
Glucose	2.0
Agar	20.0
pH	5.8± 0.2

xi. Complete Medium (CM): g/L

Glucose	10.0
Yeast extract	2.5
Malt extract	5.0
Agar	20.0
pH	5.5 ±0.2

xiii. Protoplast Regeneration Medium (RM): g/L

Yeast Nitrogen base w/o AA and ammonium sulphate	1.7
Ammonium sulphate	1.0
Succinic acid	10.0
Sodium hydroxide	6.0
Glucose	20.0
Sorbitol	183.0
Agar	20.0
pH	5.8 ± 0.2

iii. Buffers

1.	Phosphate saline buffer	10 mM, pH 5.5
2.	Citrate buffer	50 mM, pH 5.2
3.	Sodium phosphate buffer	100 mM, pH 6-7
4.	Tris- HCl buffer	10 mM, pH 5.2
5.	TAE buffer	50 mM, pH 5.8
6.	TE buffer	10 mM, pH 8.0

Staining solution

Sudan black IV B 0.3 g in 100 ml of 70% ethanol.

METHODOLOGY

SECTION 1

ISOLATION AND SCREENING OF OLEAGINOUS *MUCOR* Spp. FOR GLA PRODUCTION

1.1 Collection of Soil Samples

Soil samples were collected from different places like river banks, humus soil, forest, agricultural land, plane land, Zoo, garden and pond soil for screening oleaginous *Mucor* species. All soil samples collected, were properly labeled and coded for further use.

1.2 Isolation of *Mucor* spp. from different soil samples

One g of soil sample was dispensed in 10 ml of sterile phosphate saline buffer (10 mM, pH 5.5), mixed thoroughly and kept undisturbed for the solid particles to settle down. 0.1 ml of the clarified supernatant was serially diluted and appropriate dilutions of the sample were plated on *Mucor* screening medium supplemented with 0.1 g/L Chloramphenicol to suppress any bacterial growth due to heterogeneity of the soil nature. The plates were incubated at 28±2ºC for 5 days to obtain mucor isolates.

1.3 Identification of *Mucor* spp.

Fungal mycelia morphologically similar to *Mucor* spp. from different agar plates were mounted on a glass slide and stained with cotton blue. Mycelial structure, sporangiophores and sporangiospores were observed under microscope (Leica Heburgg, AG, Switzerland). The morphology of the fungus was compared with standard cultures obtained from MTCC and this was further confirmed with soil fungi manual (Gilman 1999). The confirmed isolates of *Mucor* spp. were maintained on a PDA slants and stored at 4ºC. Sub-culturing was carried out in every fortnight throughout this study.

1.4 Screening of *Mucor* spp. for lipid production

Two methods were followed for screening of oleaginous *Mucor* spp. for lipid production, i) qualitative analysis by dye binding method and ii) quantitative analysis by gravimetric method

1.5 Qualitative Screening of *Mucor* spp. by staining method

The selected *Mucor* strains were stained with Sudan black B according to the method of Burnod (1964) & du Preeze et al. (1997). Qualitative screening method is given in flow chart. The presence of blue or grayish oil globules were observed under oil immersion microscope within the mycelium.

Staining Method

Fungal cell culture obtained from fermentation

↓

Filtered through Whatmann no.1 paper & washed with distilled water

↓

Resuspended in 5 ml of distilled water

↓

Add 5 ml of 1 M HCI & 0.4 ml of Sudan black B to test tube

↓

Stained cells served as the test material as against the unstained cell as control

↓

Both tubes were then placed in a boiling water bath for 30s with intermittent shaking. Centrifuged & washed with distilled water

↓

The tubes were then cooled & centrifuged at 3000 rpm for 10min

↓

Supernatant was discarded

↓

The cells were then washed with 50% alcohol 3-4 times, to remove excess stain

↓

The cells were then resuspended in distilled water

↓

Made a smear of culture solution from the cells

↓

Observe under phase contrast microscope (oil immersion)

(Source: Baroda 1964; Venkateswaran 1999)

1.6 Quantitative screening of oleaginous *Mucor* spp. by gravimetric method

The cultures selected by qualitative analysis (dye binding method) of lipid were further studied for growth characteristics, quantitative analysis of lipid and for GLA production. Nutritive medium with limited nitrogen source was used for submerged fermentation studies of oleaginous microorganisms. In the present study, fat producing medium (FPM) was used for cultivation of this fungus. It was incubated at 28±2ºC dry biomass, lipid accumulation and GLA content were observed for 6 days.

1.7 Screening of MTCC cultures for GLA production

Qualitative and quantitative screenings were also made for standard cultures (*Mucor rouxii* MTCC 386, and *Mucor hiemalis* MTCC 2877) for comparative purposes.

1.8 Cultivation of *Mucor* spp. in submerged fermentation

$1.5x10^6$/ml spores suspension of isolated culture was inoculated in 500-mL flask containing 100 mL of FPM and incubated in a rotary incubator shaker (Innova 4230, New Brunswick, USA) at 28±2ºC and 200 rpm for 6 days.

The growth and lipid accumulation of CFR-G15 was estimated for a period of 10 days. For this the flasks were removed in every 24 h intervals.

1.9 Analytical Methods

3.1.9.1 Estimation of cell dry weight

After fermentation, the mycelium was harvested from culture broth by suction filtration through Whatman No. 1 filter paper. Cell dry weight was estimated by washing the mycelia with distilled water twice and then dried at 50±2°C for 24 h and the fungal dry biomass was estimated.

1.9.2 Estimation of Lipid

Total lipid content of the *Mucor* spp. was estimated by gravimetric method of AOAC (1991). The maceration method is given in flow chart. The lipid was extracted by solvent contact method in soxhlet apparatus using analytical grade Chloroform and Methanol (2:1 V/V) for 4-6 hours (Somashekar et al. 2003). 2-3ml of solvent containing lipid fraction transferred to pre-weighed beaker and refluxed with nitrogen gas and kept in desiccators under cold condition till total lipid estimated by gravimetric method.

MACERATION PROCEDURE

1 gm of sample + 1 gm of acid washed sand &
ground with the help of pestle and mortar
⋮
The powder is transferred to Whatmann filter no.42
& tied with twine thread.
⋮
Packet, transferred to 0.5 N HCl in 500 ml beaker
⋮
Transferred to water bath, boiled for 1 hr
⋮
After boiling sample kept under running water for 30 min
⋮
Sample kept for drying in drier overnight
⋮
Lipid extraction

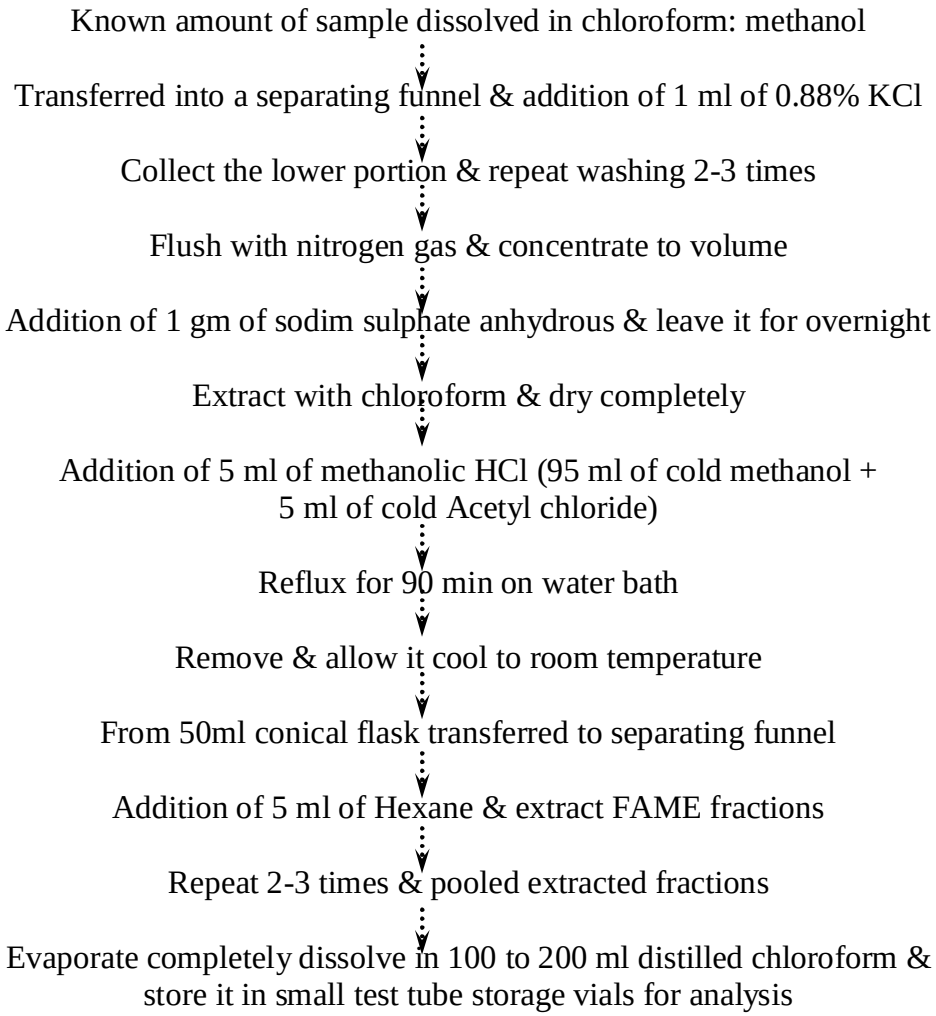
(Source: Venkateswaran 1999)

1.9.3 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid methyl esters (FAMEs) from the extracted lipid samples were prepared by Kate's method (1964) with slight modification. This method is given in flow chart below. Known amount of lipid obtained from the culture dissolved in chloroform and methanol (2:1) and saponified with 0.88% KOH. The upper

layer-containing lipid was taken and passed through an anhydrous sodium sulphate to remove the residual moisture content and solvent evaporated through nitrogen reflexing. The samples were then methylated by adding Methanolic HCl (methanol: acetyl chloride, 95:5) and kept for reflection in a water bath for 1 hr at 80ºC. After cooling to room temperature, FAMEs were extracted by hexane for two to three times and the pooled samples reflected with nitrogen gas to remove any moisture content and stored in -20°C for further use.

Preparation of FAMEs for Fatty Acid Analysis



(Source: Kate 1964; Venkateswaran 1999)

1.9.4 Fatty acid Analysis by Gas Chromatography (GC)

The samples (FAMEs) were dissolved in known amount of chloroform and analyzed by gas chromatography (Shimadzu 15A, Tokyo, Japan) instrument equipped with flame ionizing detector (FID). The separation was carried out on 3-meter column packed with 12.5% diethyl glycol succinate (DEGS) using 180°C column temperature. The temperatures of the injector and detector were maintained at 220 and 230°C respectively. Nitrogen was used as a carrier gas at a flow rate of 40ml/min. fatty acid were identified by comparing their retention time with those of known authentic fatty acid standards obtained from Sigma-Aldrich, St Louis, MO. The degree of unsaturation (Δ/mole) in the lipid fraction was calculated according to Kates & Baxter (1962). The degree of unsaturation (Δ/mole) = 1(% of monoenes) + 2(% of diene) + 3(% of triene) / 100.

1.9.5 Gas chromatography and Mass spectrometry (GC-MS)

Identification of fatty acids were carried out using GC-MS [Perkin Elmer 5906, Turbo mass Gold MS, Ausystem XL GC, Japan] at 70 eV (m/z 50–550; source at 230 ℃ and quadruple at 150 ℃) in the EI mode with an BP-21 capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness). Temperature program was set as follows: the initial temperature of the column was 120 °C (for 1 min), then raised to 220 °C at 5 °C/min, and held for 10 min. Injection and detector temperatures were maintained at 240 and 250 ℃, respectively. Helium was used as carrier gas at a flow rate of 1.0 mL/min. the injection. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic standards and the spectral data obtained from the NIST libraries.

1.9.6 Quantitative analysis of lipids using column chromatography (AOAC methods, 1984)

One gm of lipid sample dissolved in 10 ml of chloroform and transferred on to a silica gel column (80-100 mesh). Neutral lipid and polar lipid eluted with the solvents chloroform and methanol respectively.

1.9.7 Qualitative method of lipids using thin layer chromatography (AOAC methods 1984)

The extracted oil was dissolved in 1.5-2 ml of chloroform and 1.0 ml spotted on activated silica gel G coated plates. The plates were run in a solvent system containing petroleum ether: diethyl ether: acetic acid (90:10:1). After air-drying the plates, the fractions were observed in an iodine chamber. For identification of triacylglycerol (TG) and free fatty acid (FFA), triolein and oleic acid standards were used respectively.

1.9.8 Analysis of residual sugar in the medium

Residual sugar estimation was performed using dinitrosalicylic (DNS) method (Miller 1959). In brief, the broth centrifuged to remove the cells and different aliquots of the sample were taken and made upto 1 ml with distilled water. I ml of DNS reagent was added and boiled on a boiling water bath for 10 mins. The tubes were cooled to room temperature and the volume was made upto 10 ml with distilled water. OD was taken at 540 nm and the concentration of sugar in mg/ml was calculated from the calibration chart prepared with glucose as standard.

1.10 Solid- State Fermentation (SSF)

1.10.1 Media preparation

Solid- state fermentation medium was prepared as follows: 50 g fresh wheat bran was taken in clean acid- washed Erlenmeyer flask and autoclaved for 40 minutes at 121°C. After cooling, wheat bran was moistened uniformly with sterile distilled water in an aseptic condition at 60% level. This medium was inoculated with a known amount of spores ($1.5X10^6$ /ml). The flasks were incubated in an inclined position to facilitate proper aeration at ambient temperature for an optimum growth period.

1.10.2 Inoculum preparation

Inoculum preparation and the nature of inoculum play a vital role in deciding the quality and quantity of the ultimate product in solid- state fermentation. In the present study, inoculum for solid-state fermentation was prepared by cultivating the fungi in PDA slants for 7 days at 28±2ºC. To the fully sporulated fungal slant, 2-3 ml of sterile Tween- 80 was added and the spores were dislodged from the sporangiophores by gently scraping with a sterile needle. Before inoculation spore count was taken using a Haemocytometer, Feinoptik, Bad Blankenburg, Germany. An optimized level of $1.5X10^6$ ml inoculum was used for all the SSF experiment to obtain maximum fermentation efficiency.

1.10.3 Determination of moisture content in the fermented bran

For all the samples, 1 g of fermented bran was used to measure the moisture content. 1g of moldy bran was weighed exactly and dried in an oven at 55-60°C for 24 hrs until a constant weight was obtained. Final weight was then measured and difference between the initial weight and the final weight was found as the moisture content. This was converted into percentage moisture.

1.10.4 Extraction of Lipid

Fermented wheat bran (mouldy bran) dried at 55°C for 24 h and weighed. Both substrates and homogenized fermented bran lipid extracted using chloroform/methanol (volume ratio of 2:1). Total lipid content was determined gravimetrically.

1.11 Statistical analysis

Data obtained from three independent analyses was expressed as mean ± Standard deviation. Experimental data was subjected to analysis of variance and Ducan's multiple range test ($p < 0.05$) using the Statistical Analysis System (Duncans 1965).

SECTION 2

MOLECULAR CHARACTERIZATION OF *MUCOR* SP. CFR-G15 PRODUCING GLA

2.1 Genomic DNA extraction

(a) Reagents Required

- ***(i)*** **0.8 % Agarose** in 100 ml 1x TAE buffer.
- ***(ii)*** **50x TAE buffer (g/ 100 m*l*):** 24.2 g Tris base (pH 8.0), 5.7 ml of glacial acetic acid and 10 ml of 0.5M EDTA (pH 8.0) were mixed and the volume was made up to 100 ml with distilled water.
- ***(iii)*** **Lysis buffer (100 ml):** 50mM Tris HCl (pH 7.2), 50mM EDTA, 3% Sodium dodecyl sulfate (SDS), 2% β-mercaptoethnol (add just before use)
- ***(iv)*** **Tris EDTA (TE) buffer:** 10mM Tris HCl, 0.1mM EDTA.
- ***(v)*** Chloroform:phenol (1:1)
- ***(vi)*** SEVAG (chloroform:isoamyl alcohol, 24:1)
- ***(vii)*** Sodium acetate: 3M (pH 8.0)
- *(viii)* Isopropanol
- *(ix)* Ethanol (70% and 100%, ice cold)
- *(x)* **DNA Loading dye:** 0.25 g Xylene Cyanol, 0.25 g Bromophenol blue and 30 ml glycerol in 100 ml distilled water.
- ***(xi)*** **Ethidium bromide (stock):** 10 mg ethidium bromide was dissolved in 1 ml distilled water. 0.07 ml of the stock was diluted with 500 ml distilled water as working concentration, covered with aluminum foil and refrigerated.

2.1.1 Method 1

The genomic DNA extraction was performed according to method of Moller et al. (1992) and the procedure is given below.

1. 100 mg lyophilized mycelium was ground with liquid nitrogen
2. Ground lyophilized mycelium (60-100 mg dry) was taken in eppendorf tube.
3. 750 µl of lysis buffer was added, homogenized by vortexing and the mixture was incubated at 65°C for 1 hr.
4. 700 µl of chloroform:phenol (1:1 v/v) was added and vortexed briefly. Centrifuged at 12,000 g for 10 minutes.
5. 600-650 µl which is aqueous phase was transferred to a new tube.
6. 700 µl of SEVAG was added and vortexed briefly. Centrifuged at 12,000 g for 10 minutes.
7. 550-600 µl of aqueous phase was transferred to a new eppendorf tube. 20 µl of 3M sodium acetate was added and inverted gently for several times.
8. Centrifuge was done at 12,000 g for 30 seconds to pellet the DNA. Supernatant was poured out and tubes were inverted for 1 minute to drain.
9. 300 µl of TE buffer was added and placed in a water bath at 65°C for 10-15 minutes.
10. 10 µl of NaOAc was added and top off the eppendorf tube with EtOH. The tubes were inverted gently for several times.
11. Centrifuged as above for 30 to 120 sec. to pellet the DNA. Supernatant was poured off and the pellet rinsed with 70% EtOH. Tubes were inverted to drain off for 1 minute.
12. The tubes were dried in a vacuum oven at 50°C for 15 minutes.
13. DNA pellet was resuspended in 100 µl of TE buffer and stored at -20°C for further use.

2.1.2 Method 2

The genomic DNA extraction was performed according to method of Tauk-Tornisielo et al. (2007) with slight modification and the procedure given below.

1. The mycelium was harvested from a 3 days old culture and lyophilized.
2. Mycelium was ground in mortar and pestle with liquid nitrogen and transferred into 25 ml centrifuge tube.
3. Lysis buffer was added, vortexed and incubated at 65°C for 1 hour,
4. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was mixed with the solution and kept in ice for half an hour.
5. The solution was centrifuged at 14,000 rpm for 15 min. The upper phase was transferred to a new tube
6. 3 M sodium acetate and isopropanol were used to precipitate DNA.
7. The DNA pellet was washed with 70% distilled ethanol.
8. The washed pellet dried and resuspended in TE buffer. DNA was used for further study.

2.2 Analysis of DNA

Agarose gel preparation and analysis

1. o.8 % agarose was prepared and poured into the sealed boat.
2. The gel was allowed to set. The gel was placed in the electrophoresis tank to cover the surface of the gel.
3. 10 µl of each sample mixed with 2 µl of loading dye. The samples were loaded along with the marker.
4. Electrophoresis was carried out at 70 volts till the dye was reached 3/4th of the gel.
5. The gel was removed from the tank and stained by soaking in a solution of ehidium bromide for 30 min at room temperature.

6. The gel was destained in distilled water for 10 min, examined on a UV transilluminator (photodyne USA) and photographed.
7. The molecular size of genomic DNA was estimated based on the mobility of the size marker.

2.3 Polymerase Chain Reaction (PCR)

2.3.1 The PCR Reaction

Table 3.1 PCR Reaction components

Components	**Volume (ml)**	**Final concentration**
Nuclease-free water	X	
10 X reaction buffer	5.0	1X
dNTP mix (10 mM)	1.0	0.2 mM each
Taq DNA polymerase	0.6	0.02 u/ µl
*25 mM $MgCl_2$	3.0	1.5 mM
Downstream primer (R)	1.0	1.0 µM
Upstream primer (F)	1.0	1.0 µM
(The sequence and source of these primers are mentioned in Table 3.2 (Sigma Aldrich, India)		
Template DNA	Y	
50µl (Total volume)		

- The PCR reaction was carried out for 25 µl reaction by taking half of the total volume mentioned in table 3.1
- The content of the tubes was mixed properly by a brief spin of micro centrifuge
- The tubes were placed in a thermocycler (Perkin Elmer, USA) and the reaction parameters were as follows:

1. Initial Denaturation:	95°C for 5 min
2. Denaturation:	94°C for 40 sec
3. Annealing:	54°C for 40 sec
4. Extension:	72°C for 5 min
5. Final extension:	72°C for 10 min
No. of cycler (1-3)	35

The method of electrophoresis used was as same as section 2.2. Here, 1.5% of gel was used instead of 0.8% gel to estimate the PCR product. The 3Kb marker used to estimate the molecular size of PCR product. The reaction product was stored at -20°C until further use.

Table .2 PCR primers used in this study

Primers name	Sequence (5'-3')	Expected amplicon size
SSF.18S	GGTGAACCTGCGAAGG	
SSR.18S	CCTCCGCTTATTGATATGC	600bp
Des F1	GGATGAAATCCGTACCATGC	
Des R1	CAGTCAAGCCACTCTGGAC	1.2kb
SSF	CAGGCGGTGAGGCAG	
SSR	ATGACGAGGCAGTCTAGG	1.2kb
SS1N-F	AGCACGATCCCGATATCC	600bp with SSR

2.3.2 Purification of PCR product

The amplified product (100bp to 10kb), was purified by using Sigma GenElute PCR Clean up kit (Sigma Aldrich) and the protocol is given below:

Protocol

1. A GenElute Miniprep Binding column was inserted into a collection tube.
2. 1 volume of PCR reaction and 5 volumes of binding solution were added and mixed.
3. Solution was transferred into the binding column.
4. The column was centrifuged at maximum speed for 1 min.
5. Collected liquid was discarded but, retained the collection tube.

6. Binding column was replaced into the collection tube. 0.5 ml of diluted wash solution was added to the column and centrifuged at maximum speed for 1 min.
7. Collected liquid was discarded but, retained the collection tube.
8. The excess ethanol was removed by centrifuging at maximum speed for 2 min. Residual flow as well as the collection tube was discarded.
9. The column was transferred to a fresh 2 ml collection tube. 50 µl of elution solution or water was applied to the center of each column.
10. The column was incubated at room temperature for 1 min.
11. DNA was eluted by centrifuging the column at maximum speed for 1 min. The purified PCR product stored for further use.

2.3.3 A-tailing of PCR product

A-tailing of PCR product is needed to make 3' OH protruding ends on both the strands of DNA in order to make the PCR product compatible with T-tail vector.

Protocol for A-tailing using *Taq* DNA polymerase

1. 1-2 µl of purified PCR fragment generated by proof reading DNA polymerase was taken.
2. 1 µl of *Taq* DNA polymerase reaction buffer (1x) and 1 µl of 25mM $MgCl_2$ were added.
3. Final concentration of 0.2mM of dATP was added.
4. 5 Units (2 µl) of *Taq* DNA polymerase in the reaction added and reaction was make up the volume to 10 µl by demonized sterile water.
5. The reaction was incubated at 70 °C for 15 to 30 min.
6. PCR product was purified by using PCR product purification filters to remove the residual dATP present in the reaction mixture.
7. 1-2 µl of A-tailed PCR product was used in T-tail vector ligation reaction.

2.3.4 Ligation/cloning of purified PCR product to pTZ57r/ vector using Ins T/A clone PCR product cloning kit (Bangalore Genei kit, India)

Reagents required

10 x Ligation Buffer* **:** 400mM Tris-HCl, 100mM $MgCl_2$, 100mm DTT, 5.0mM ATP (pH 7.8)

PEG 4000*: 10x (50% w/v) PEG 4000 solution

T_4 DNA Ligase, 5U/ml*: Prepared in 20mM Tris-HCl (pH 7.5), 1mm DTT, 50mM KCl, 0.1mM EDTA and 50% glycerol.

* Supplied with the kit

Protocol

1. The following components were added into a 1.5 ml centrifuge tube:

Plasmid vector pTZ57/T DNA	3.0 µl
Purified PCR fragment	x µl
PEG 4000 solution	3.0 µl
T_4 DNA ligase, 5U/µl	1.0 µl
10X Ligase buffer	3.0µl
Deionized water (to make upto)	30.0 µl

   Incubated at 22°C for over night

2. The enzyme was inactivated by incubating the mixture at 65°C for 15 min.

2.4 Competent cell preparation and transformation

Reagents and bacterial strain:

***E. coli* DH5** a **HOST CELLS** (genotype: F'/endA1 hsdR17 ($r_k^- m_k^+$) glnV44thi-1recA1 gyrA (Nalr) relA1 Δ(laclZYA-argF)U169deoR(¢80dlacΔ(lacZ)M15)

Colonies of *E. coli* DH5 α strain grown on LB agar plate (1.5% agar LB medium without ampicillin).

Transformation and storage (TSS) buffer

Ingredients	**Quantity (100 ml)**
LB medium	85 (v/v)
Polyethylene glycol (PEG)	10 (W/V)
Dimethyl sulfoxide (DMSO)	5 (V/V)
Magnesium chloride ($MgCl_2$)	50mM final concentration

TSS buffer should be sterilized and kept at 4°C after cooling.

SOB (g/L)

Bacto-tryptone	20.0
Bacto-Yeast extract	5.0
Sodium chloride	0.6
Potassium chloride	0.19
Magnesium sulphate	10.0mM (added from 1.0M stock)
Magnesium chloride	10.0mM (added from 1.0M stock)

Autoclave the first four components at 121^0C for 15 min and the magnesium salt separately and then mix to constitute the SOB medium.

SOC (per 100 ml): To 1.0 ml of SOB add 7 µl of filter-sterilized glucose solution (50%w/v)

0.1M $CaCl_2$ stock solution: Dissolved 1.47 g of $CaCl_2$ in 100 ml of deionised water. The solution was sterilized by filtration and stored as 20 ml aliquots at -20°C.

Ampicillin stock solution: 100 mg of ampicillin was dissolved in 1.0 ml of deionised water, sterilized by filtration. Stored at -4^0C and working concentration of 100 µg ml^{-1} of the medium was used.

0.1M IPTG stock solution: IPTG of 0.12 g dissolved in 5.0 ml of deionised water. Filter-sterilized solution was stored as aliquots at -20°C.

X-Gal stock solution: X-gal of 100 mg dissolved in 2.0 ml of N, N'-dimethylformamide (DMF). The solution in micro centrifuge tube was wrapped in aluminium foil and stored at -20°C.

2.4.1 Preparation of *E. coli* DH5 a competent cells by DMSO-PEG method (Halami 2004)

1. *E. coli* DH5 α strain was streaked on LB agar plate (1.5% agar without ampicillin) and incubated at 37° C for overnight.
2. Isolated single colony of DH5α cells from LB agar plate was picked and inoculated to 2 ml of LB media. The cells incubated for overnight at 37 ^{o}C in an incubator shaker (~200rpm).
3. 0.1ml of the overnight grown culture was inoculated into 50ml sterile LB media in 250ml Erlenmeyer flask. The cells were incubated in shaker incubator (~200rpm) at 37 ^{o}C.
4. The cell growth was monitored periodically to determine the OD_{590} reaches 0.6 to 0.8 (approximately 3-4 hrs) of the culture.
5. When the OD reaches 0.45 to 0.5, the cells transferred into polypropylene tube and culture was cooled by storing the tube on ice for 10mins.
6. Centrifuged at 4000 rpm for 5min at 4°C to recover the cells.
7. The supernatant from the cell pellet was discarded and the tubes were kept in an inverted position for 1min to allow traces of media to drain away.
8. Resuspended the cells in 1/10th volume of TSS buffer. Incubated the cells on ice for 15-20 minutes. Aliquots of 200 µl cells on ice (tubes are pre-chilled). Freeze the aliquots immediately by storing at –20^{o}C. (Note: cells can be used immediately for transformation or stored in –20^{0}C for 1-2 months durations). [Note: all micro tips, tubes were pre-chilled]

2.4.2 Transformation of *E. coli* DH5 a

1. Suspension of 200 µl competent cells was transferred into a prechilled sterile micro-centrifuge tube.
2. DNA (~50 ηg) was added into each tube, the contents of the tube mixed by swirling gently. The tubes were stored on ice for 30 minutes.

3. The following control samples were included: (a) Competent cells that receive 1 and 2µl of standard super coiled plasmid DNA, (b) competent cells that receive restricted enzyme digested DNA and (c) only competent cells as a control.
4. The tubes were transferred into water bath set at 42°C. The tubes were incubated for 90 seconds.
5. The tubes were rapidly transferred to ice and allowed the cells to chill for 1-2 minutes.
6. 800 µl of prewarmed LB medium was added to each tube. The cultures incubated for 45 minutes at 37°C in a shaker incubator set at 150 rpm. (This step allowed the bacteria to recover and express the antibiotic resistance marker encoded by plasmid).

2.4.3 Selection of transformants/recombinants

1. 100 µl of transformation mix was plated onto LB agar plates containing 100 µg ml^{-1} ampicillin, 0.5 mM IPTG and 80 µg ml^{-1} X-Gal.
2. The plates were incubated at 37°C overnight for the colonies to grow.

2.5 Analysis of transformants/recombinants

1. Plasmid isolation from the transformants.
2. Restriction digestion experiment to check for inserts release from recombinants.
3. PCR analysis for recombinants

2.5.1 Isolation of recombinants plasmid

Reagents Required

Antibiotic Stock (Ampicillin): 100 mg/ml in distilled water and sterilized by filtration. Store at -20°C and use at a working concentration of 100 µg/ml.

Solution I

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

Solution II

0.2 N NaOH (freshly prepared from 10 N NaOH)

1.0% SDS

This can be preferably prepared freshly.

Solution III

5.0M Potassium acetate	60.0ml
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

The resulting solution is 3.0M with respect to potassium and 5.0M with respect to acetate.

Protocol (Halami 2004)

1. Single colonies of appropriate strain in 2 ml of LB broth containing antibiotic was incubated.
2. The culture was incubated for overnight in a shaker incubator at 37°C and 180 rpm.
3. 1.5 ml of the overnight culture was transferred into a 1.5 ml micro centrifuge tube and the cells harvested by centrifugation at 10,000 rpm for 2 min.
4. Supernatant was discarded.
5. 100 µl of solution I was added and vortexed vigorously until no visible clumps of cells were observed. The samples were kept on ice for 5 min.
6. 200 µl of freshly prepared alkaline solution (solution II) was added and mixed gently by inverting the tubes several times. The cell suspension observed for clear. The samples were kept on ice for 5 min.

7. 150 µl of ice-cold potassium acetate solution (solution III) was added and inverted gently (the purpose of this step was to selectively renature the plasmid DNA).
8. Centrifugation of tubes at 10,000 rpm for 15 min.
9. The supernatant transferred into a fresh tube, an equal volume of phenol-chloroform was added and vortexed thoroughly. By centrifugation at 10,000 rpm for 10 min two phases formed.
10. The upper aqueous phase was transferred into fresh tube and an equal volume of chloroform added. To remove the phenol, the tubes were centrifuged at 10,000 rpm for 10 min.
11. The upper aqueous phase was transferred into a fresh tube and double volume of absolute ethanol added. The tubes were kept at -20°C for precipitation.
12. Centrifugation at 10,000 rpm for 10 min. The supernatant was discarded carefully without dislodging the pellet.
13. 300 µl of 70% ethanol was added and centrifuged to remove salts.
14. The pellet was air dried and dissolved in 20 µl of TE buffer.
15. Each sample was tested by agarose gel (0.8%) electrophoresis as per the method given in section 2.2.

Restriction digestion of plasmid DNA (Halami 2004)

1. The following reagents was added (reaction mixture was make up to a 20 µl)

Plasmid DNA	5 µl (~1 µg)
10X Restriction enzyme buffer	2 µl
Restriction enzyme	1 µl (10 U, Eco R1)
Sterile distilled water	12 µl

2. The contents were mixed gently with a pipette tip and spinned briefly.
3. The reaction mixture was incubated at 37 °C for 5- 6hrs

4. The reaction was ceased by heating at 65°C for 20 min and analyzed by agarose gel (0.8%) electrophoresis as previously mentioned.

2.6 Nucleotide sequence analysis

The nucleotide sequence was analysed by BLAST (Altschul et al. 1997) and determined sequences submitted to the GenBank with the accession numbers EU927296 and EU526025 for 18S rRNA and Δ^6 *DES* genes, respectively.

2.7 Phylogenetic analysis

The partial gene sequences obtained were aligned by Clustal X and phylogenetic tree constructed was by software MEGA 3.1 version (Kumar et al. 2004). The *DES* gene sequences obtained from NCBI, were AF296076 (*Mucor rouxii);* BOU79010 (*Borago officinalis)*; AY795076 (*Rhizopus stolonifer*); AY941161 (*Thamnidium elegans*); AF465282 (*Mortierella isabellina);* EF494667 (*Phaeodactylum tricornutum*). Neighbor joining method with bootstrap of 500 replicates generated the phylogenetic tree.

SECTION 3

OPTIMIZATION OF CULTURAL CONDITIONS FOR BIOMASS, LIPID AND GAMMA LINOLEIC ACID (GLA) CONTENT OF *MUCOR ROUXII* CFR-G15.

3.1 Fungal strain

Mucor rouxii CFR-G15 an oleaginous fungus was isolated from the soil sample. This fungus has been deposited in CFTRI, Institutional Microbial Type Culture Collection Centre. The strain has been maintained on the potato dextrose agar slants with repeated sub culturing.

3.2 Cultural condition

All experiments were performed in 500-ml Erlenmeyer flasks containing 100±2 ml of growth medium sterilized at 121°C for 20 min. The pH was adjusted by the addition of 0.1N NaOH and HCl solutions accordingly before autoclaving. Six days incubation period was chosen based on the previous experiment conducted in section 1. To ensure that the lipid yields do not include any unutilized supplemented oil, fungi which were grown on media with supplemented plant oils were first washed extensively with deionized water then with tween 80 (Kendrick & Ratledge 1996). The lab temperature was maintained at 28.0 ± 2.0°C and this has been referred in the text as room temperature (RT). All experiments were replicated in triplicate. The reported values are mean and ± Standard deviation.

3.3 Media optimization

Five different media viz., Fat producing media (FPM), synthetic media (SM), glucose yeast extract media (GY), potato dextrose broth (PDB) and czepeck dox broth (CDB) were used to determine the optimal media for growth of this organism. The composition of each media is given in material section (ii).

3.4 Physical Parameters

3.4.1 pH optimization

The initial pH of the growth media were from 3.00±0.02 to 9.00±0.02. This was adjusted by the addition of 0.1M solutions of NaOH or HCl accordingly, to determine the optimal pH range for growth of this organism. pH adjustment was done prior to autoclaving the medium.

3.4.2 Temperature optimization

To determine the optimal temperature of growth, biomass build up and lipid yield, Culture medium was incubated at different temperatures like 5, 10, 15, 20, 25, 30, 35 and 40 °C (±0.5 °C) for 6 days in the orbital shaker.

3.4.3 Aeration optimization

To study the effect of aeration on cellular lipid accumulation, batch culture was performed in 500 ml Erlenmeyer flasks containing 50, 75, 100, 125, 150 175 and 200 ml liquid medium respectively.

Ratio (R) = volume of medium/ volume of Erlenmeyer flask (500ml)

3.4.4 Inoculum size optimization

To determine the optimum concentration of inoculum and effect of the concentration on cellular lipid accumulation and GLA production, different concentration of inoculum size was experimented.

The inoculum size from 1 to 5% containing $1.5X10^6$ spores/ ml was inoculated into flasks containing liquid medium and incubated at RT for 6 days.

3.5 Chemical Parameters

3.5.1 Optimization of different carbon sources

Glucose, fructose, sucrose, maltose, galactose, lactose, and starch were used as different carbon sources, to determine their effect on biomass production, lipid accumulation and GLA content. All sugars were used at 4% level in the medium. Ammonium nitrate and yeast extract were used as the nitrogen sources. The experiment was performed at RT, which is close to the temperature where the best results have been obtained in different temperature experiment and convenient for an eventual industrial scale production.

After selecting glucose as a source of carbon, a further study was undertaken to optimize glucose concentration at different level (0-10%) supplemented in growth media. Medium without carbon source was considered as control.

3.5.2 Optimization of different nitrogen sources

The nitrogen sources in this study tested were ammonium nitrate, sodium nitrate, ammonium sulphate, ammonium chloride, potassium nitrate, urea, yeast extract, casein and peptone. Glucose at the level of 4% was used as carbon source.

The good nitrogen source was studied further at different concentrations of 0.25%, 0.50% and 1.00%. Medium containing 0% nitrogen source was used as control.

3.5.3 C:N ratio optimization

To study the effect of carbon and nitrogen ratio (C:N Ratio) on biomass, lipid and GLA production, carbon source (glucose) and nitrogen source (in the ratio of yeast extract and ammonium nitrate) were used in different ratio in the medium ranging from 20 to 120.

3.5.4 Metal ion supplementation

Magnesium (Mg^{2+}), manganese (Mn^{2+}), zinc (Zn^{2+}), and copper (Cu^{2+}) ions in the form of sulfates ($MgSO_4$, $MnSO_4.H_2O$; $ZnSO_4$ and $CuSO_4$). Iron (Fe^{2+}) and calcium (Ca^{2+}) ions in the form of iron II chloride tetrahydrate ($FeCl_2.4H_2O$, $CaCl_2$). All metal ions were added in different concentration to the media and their effect on growth, lipid yield, and GLA content were determined.

3.5.5 Plant Oil supplementation to medium

The effect of supplements such as sunflower, coconut, mustard, palm, gingelly, ground nut, niger seed oils were investigated. The control medium contained 4% glucose, whereas the oil supplemented to growth medium (FPM) was having 2% (w/v).

3.6 Medium optimization by RSM method

3.6.1 Experimental design

Based on prior trails in the laboratory and published literature (Hansson & Dostalek 1988**;** Somashekar et al. 2003) glucose, yeast extract and ammonium nitrate were found to be the most critical variables in the production of GLA by *M*ucor species. Hence these variables were selected for optimization to maximize the level of GLA production. In keeping with this objective, a RSM with CCR design proposed by Montgomery (1984 & 1997) and Myers & Montgomery (1971 & 2002) were used to maximize the level of GLA production.

Three independent variables, glucose (X_1), yeast extract (X_2) and ammonium nitrate concentrations (X_3) and the dependent response variable GLA were considered. Each independent variable was studied at five coded levels (-1.682, -1, 0, +1, +1.682). The coded and the corresponding values of the three variables are presented in Table 3.3. A series of 20 experiments including five replicates of the centre points were performed. The minimum and maximum

levels of each independent variables and the experimental design with respect to their coded and uncoded levels are presented in Table 3.4.

Table 3.3 Boundaries of experimental domain and spacing of levels expressed in coded and natural units

Variables Coded	Level of actual variables		
	Glucose (g/L) X_1	Yeast extract (g/L) X_2	Ammonium nitrate (g/L), X_3
-1.682	0	0	0
-1	20	1.01	0.10
0	50.00	2.50	0.25
1	79.27	3.99	0.40
1.682	100	5.00	0.50

Table 3.4 Design of experiments: Central composite design of variables.

	Variables Coded levels			Variables Actual level (g/L)		
Run no.	x_1	x_2	x_3	X_1	X_2	X_3
1	-1	-1	-1	20.27	1.01	0.10
2	-1	-1	1	20.27	1.01	0.40
3	-1	1	-1	20.27	3.99	0.10
4	-1	1	1	20.27	3.99	0.40
5	1	-1	-1	79.73	1.01	0.10
6	1	-1	1	79.73	1.01	0.40
7	1	1	-1	79.73	3.99	0.10
8	1	1	1	79.73	3.99	0.40
9	-1.682	0	0	0.00	2.50	0.25
10	1.682	0	0	100	2.50	0.25
11	0	-1.682	0	50.00	0.00	0.25
12	0	1.682	0	50.00	5.00	0.25
13	0	0	-1.682	50.00	2.50	0.00
14	0	0	1.682	50.00	2.50	0.50
15	0	0	0	50.00	2.50	0.25
16	0	0	0	50.00	2.50	0.25
17	0	0	0	50.00	2.50	0.25
18	0	0	0	50.00	2.50	0.25
19	0	0	0	50.00	2.50	0.25
20	0	0	0	50.00	2.50	0.25

A multiple regression analysis of the data was carried out to get empirical models that define response in terms of the independent variables. For a three factor experiment, the following second order polynomial equation was applied.

$$y_{ijk} = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j + \in_{ijk}$$

$$y_n = \beta_{n_0} + \sum_{i=1}^{3} \beta_{ni} x_i + \sum_{i=1}^{3} \beta_{nii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{nij} x_i x_j$$

$ß_0$, $ß_i$, $ß_{ii}$, and $ß_{ij}$ constant co-efficients; X_i coded independent variables by the equation, $X_i = 2[(\phi_i - \bar{\phi}_i)/ d_i]$ Where Φ_i : actual values in original units; $\bar{\phi}_i$: average of low and high levels and d_i : difference between extreme levels. The coding facilitated the computations for regression analysis and optimum search operations.

The response 3D graphs were generated from the regression equation and keeping the response function on the Z axis with X and Y axes representing the two independent variables and the third variable constant at its centre (`0', in coded level). The ANOVA was performed in coded level of variables to study the effects of independent variables. The 3D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level for maximal production of GLA.

3.6.2 RSM optimization

Optimization of variables to maximize the response was conducted by canonical method (Khuri & Cornell 1989). Accordingly, the response function was expressed in terms of the new variables (Myers & Montgomery 2002) and roots were calculated to know the nature of the response whether minimum, maximum or mini-max saddle point. The response function was considered minimum when the signs of all the roots were positive while if they were negative, then the response is maximum. If roots are mixed with positive and negative signs then response will be considered as saddle or mini-max point. The `statistica' (Stat Soft, Tulsa, OK USA) software was used to perform all the data analysis, ANOVA, regression and canonical which includes generation of 3D response surfaces.

3.7 Analytical Methods

Estimation of cell dry weight, Lipid, fatty acid analysis and DNS method has already been given in section 1.9.1, 1.9.2, 1.9.3, 1.9.4, 1.9.5 and 1.9.8.

3.8 Selection of Lipid extraction Methods with solvent systems

Estimation of fat content in *Mucor rouxii* CFR-G15 and choosing an extraction procedure was necessitated to get reliable results. Hence standardization of different extraction methods carried out. They are as follows;

3.8.1 Folch method

The method of extraction is given in below

The mycelia (about l g wet wt) homogenized with Chloroform: Methanol (2:1) in a pestle & mortar

↓

The crude extract transferred to a conical flask & addition of salt water mixed thoroughly.

↓

Mixture allowed separating into two layers at room temperature (in dark)

↓

The upper layer removed carefully without disturbing lower layer

↓

Addition of 20 ml chloroform & 20 ml water to conical flask

↓

Three layers observed, a clear upper layer of chloroform containing all the lipids, a colored aqueous layer of methanol with all water soluble material and a thick pasty interface are seen.

↓

The sample was subjected to centrifugation at 4000 rpm for 10min.

↓

Upper layer of chloroform containing lipid was collected & the methanol layer discarded

↓

Sample concentrated by liquid nitrogen

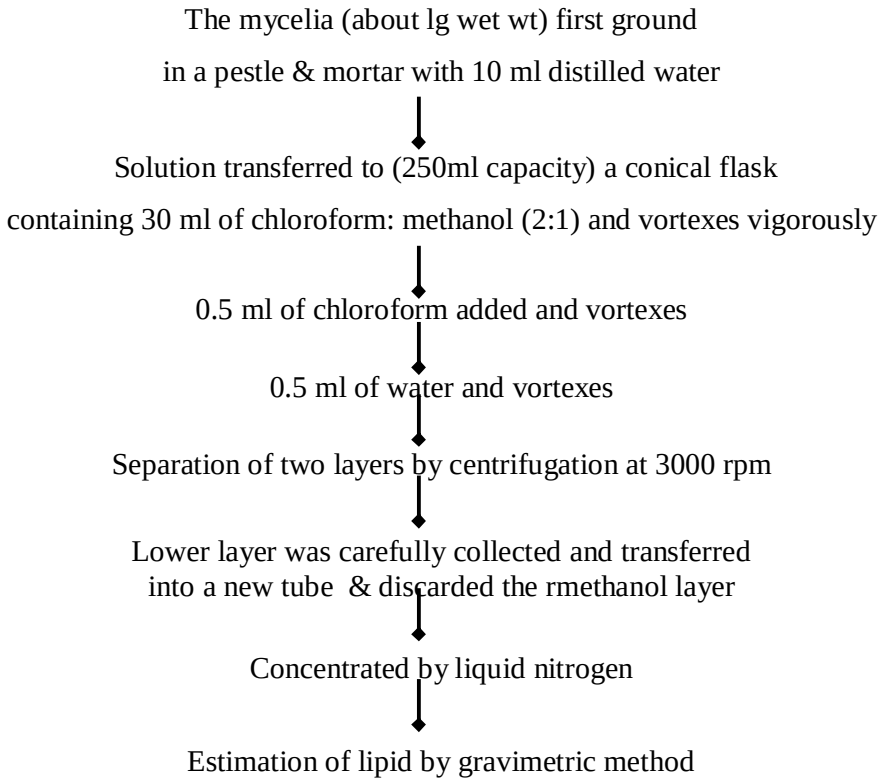
↓

Lipid estimated by gravimetric method

(Source: Folch et al. 1957)

3.8.2 Bligh and Dyer method

The method of extraction is given in flow chart



(Source: Bligh & Dyer 1959)

3.8.3 Soxhlet method

The method of extraction is as follows in section (1.9.2)

Solvent systems used in all the three methods were as follows: 1. Chloroform:Methanol (2:1), 2. Chlorofoem:Methanol(1:1), 2.Hexane:Isopropanol (3:2), 4. Hexane: Isopropanol (4:1) and 5. Hexane: Petroleum ether (1:1)

a. Statistical Analysis

The results obtained were subjected to statistical analysis as described in section 1.11

SECTION 4

GENETIC STUDIES ON *M. ROUXII* CFR-G15 WITH SPECIAL EMPHASIS ON GLA PRODUCTION

4.1 Strains used

M. rouxii CFR-G15 (parental strain) used for the mutation experiment. Methionine auxotrophic marker used for protoplast fusion experiment and the selection of mutants was carried out 15C (low temperature selection).

4.2 Reagents for protoplast fusion experiments

(i) **Citrate Phosphate buffer (50 mM, pH 5.2) g/100ml:** 9.5 g citric acid and 4.72 g Na_2HPO_4 were dissolved and the volume made up to 100 ml with distilled water.

(ii) **Protoplasting buffer (100 ml):** 20 ml of 50mM Citrate buffer, 10 ml of 60 % magnesium sulphate and 70 µl β- mercaptoethanol were mixed and the volume made up to 100 ml with distilled water.

(iii) **Fusogen (100 ml):** 30 g polyethylene glycol (PEG) 6000, 10mM calcium chloride (0.144 g), 10 mM, pH 5.2 Tris base (0.12 g) and 0.6M sorbitol (11.097 g) were dissolved and the volume made up to 100 ml with distilled water

4.3 MUTATION

4.3.1 Mutagenesis by Ultraviolet radiation (UV)

Young spores of 18 – 20 hrs old culture were harvested using 0.1 % Tween-80. The spore suspension was filtered through sterile cotton wool to remove the mycelium. Spores were pelleted by centrifuging at 5000 rpm for 5

mins. Spores pellet was washed with 10 mM potassium phosphate buffer saline pH7.2. The pellet thus obtained was resuspended in the phosphate buffer saline ($1.6X10^6$ cells/ml). 2ml cell suspension was subjected to UV irradiation for different periods of time (0, 10, 20, 30, 40,50, and 60 min) in an sterile open petridish using CAMAG UV chamber, Betrachter, Switzerland make (emitting at 254 nm radiation) at a distance of 15.0 cm height. During the course of irradiation the spores suspension was intermittently agitated. The UV treated spores suspension transferred to black bag and refrigerated at 4°C overnight prior to plating. The irradiated spore suspensions were suitably diluted with sterile saline and appropriate dilutions were plated on mutant screening medium. Killing percentage was plotted against the UV exposure in order to obtain the kill pattern. The survival colonies were picked up and incubated at low temperature (15 °C) for further selection.

4.3.2 Mutagenesis by Ethyl Methane Sulphonate (EMS)

For this experiment, the spores of *M. rouxii* CFR-G15 obtained from the previous experiment (section 1) were treated with EMS at different concentrations (2, 4, 6, 8, 10 mM) in sterile distilled water. The EMS treated spores suspensions were incubated for 1 hr at 30°C with low agitation. After incubation, the mutagen was completely removed by centrifugation at 2000 rpm for 10 minutes and this procedure was repeated twice to ensure the absence of EMS before plating on the agar medium. Spore pellet was resuspended in 0.01M sterile buffer saline (pH 7.2). Appropriately diluted spore suspension was plated on mutant screening medium. The plates were incubated at room temperature for 4-5 days and the killing effect of EMS was plotted against the concentration of EMS. As mentioned in the previous experiment the survival colonies were picked up and incubated at low temperature (15°C) for further selection.

4.3.3 Mutagenesis by N-methyl N'-nitro N-nitrosoguanidine (NTG)

NTG mutagenesis was also carried out similar to EMS method (7.1.2) with different concentrations of 5, 10, 15, 20, 25 μg ml^{-1}. The killing effect of NTG was plotted against its concentration. Here also the survival colonies were picked up and incubated at 15°C (low temperature growth selection)

4.3.4 Characterization of putative mutants for Biomass, lipid and GLA production

About 100 putative mutants with morphological, fast growing colonies at low temperature and colony variations were picked up and screened for biomass, lipid and GLA production by cultivating them in FPM medium. The biomass, lipid quantification and GLA production were estimated by the methods described in section 1.

4.3.5 Screening of auxotrophic mutants

The UV, EMS and NTG mutagenised spores grown on mutant screening medium were replicated using tooth pick on minimal medium agar plates and incubated for 24 hours. The colonies which were not grown on agar medium and grown in complete medium (master plates) were selected and individually tested for their growth factor requirements by inoculating into medium containing appropriate concentrations of individual amino acids (Venkateswaran 1999). The growth of the culture in tubes supplemented with amino acid indicated its amino acid requirements and they were marked as the respective auxotrophs. Stability of the auxotrophic mutants was confirmed by repeated experiments and they were periodically subcultured and maintained in PDA slants for further studies

4.4 Protoplast fusion techniques

Protoplast fusion was carried out among the *M. rouxii* CFR-G15-met strain (intrastrain protoplast fusion) according to the method of Peberdy (1980) and Venkateswaran (1999). Schematic representation of protoplast fusion method is given in the Fig 3.1

M. rouxii CFR-G15 was grown in FPM broth for 16-18 hrs. The fungus was found to grow as small mycelial pellets of 1-2 mm diameter. Initially, protoplasts were separated from undigested and fragmented mycelia of parental strain by being filtered through cotton wool. The protoplasts freed from small mycelial fragments by centrifugation at 1000 rpm for 10 minutes where only protoplasts sediments. The sedimental protoplasts were taken out carefully in osmotic stabilizer solution, filtered through a cotton wool and centrifuged again. Finally the pelleted protoplasts containing osmotic stabilizer solution were checked under a phase contrast microscope to ensure absence of any mycelial fragments. The number of protoplasts of each parental strain was counted with haemocytometer for fusion experiment.

4.4.1 Harvesting the fungal mycelium for protoplasting

4.4.2 Protoplast formation

The young vfungal mycelium was treated with different concentrations (100 µg - 1000 µg/ ml) of filter sterilized lytic enzyme (*Trichoderma harzianum*), Novo zyme 234 and Chitinase. The mixture was incubated for 3 hr at room temperature with mild agitation. The enzyme was removed by centrifugation at 2,000 rpm for 5 minutes. The digest was re-suspended in protoplasting buffer and the protoplast devoid of cell debris was collected by filtration using specially designed equipment shown in Fig 3.2 The protoplasts were micro photographed and the percentage of protoplasting was determined by counting them using haemocytometer (Feinoptic Bad Blankenburg, Germany). Percentage of protoplast was calculated by the following formula.

% Protoplasting = Number of protoplasts obtained/ No. of spores used for protoplasting x 100

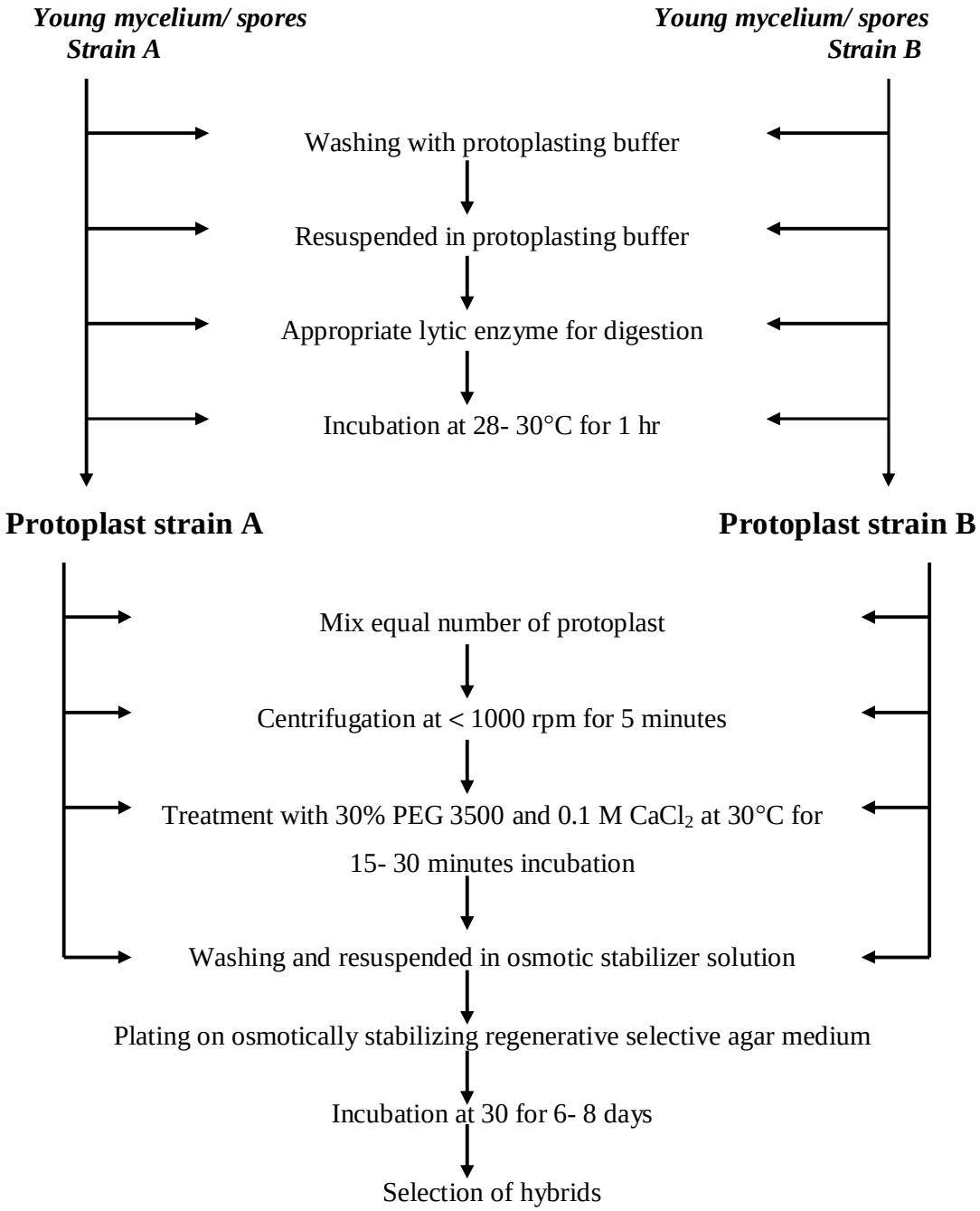


Fig. 3.1General schematic representation of protoplast formation,

fusion and regeneration

(Source: Venkateswaran, 1997)

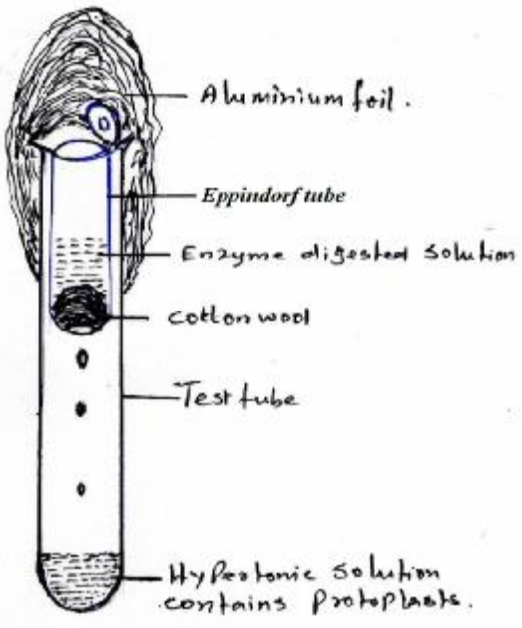


Fig. 3.2 Apparatus used for Protoplast harvest

4.4.3 Regeneration of protoplasts

Protoplasts obtained from both the parental strains were tested for their regeneration by cultivating them on regeneration medium containing 1 M sorbitol as an osmotic stabilizer. The regeneration frequency was calculated using the following formula.

Regeneration Frequency = Number of colonies on Regeneration Medium / Number of protoplasts inoculated x 100

4.4.4 Protoplast fusion

Equal numbers of protoplasts of *M. rouxii* CFR-G15-met strains were mixed with fusogen containing polyethylene glycol 6000, 10 mM Calcium chloride, 10 mM Tris- HCl and 0.6 M Sorbitol. The tubes were incubated for 30 min. to facilitate fusion between the two strains. Fusogen was removed by centrifugation at 2,000 rpm for 5 minutes under cooled condition (5°C). Fusion frequency was calculated using the formula given below after growing the fusion products on MM and CM plates.

Fusion Frequency = Number of colonies on MM/
Number of colonies on CM x 100

4.4.5 Selection of putative hybrids

Hybrids were qualitatively characterized for growth at low temperature by inoculating the hybrid strains on selection medium containing methionine amino acids (20 µg/ml, auxotroph requirement). Here also the plates were incubated for 2- 3 days at 15°C (low temperature selection). The putative hybrids thus were selected based on colony size and morphology and compared with their control *M. rouxii* CFR-G15 (parent). Hybrids were selected for further characterization. The negative hybrids attributed to either delayed growth or no morphological differences were eliminated.

4.4.6 Characterization of putative hybrids

The selected putative hybrids were cultivated in fat producing medium broth for 5 days at 30°C in an orbital shaker. After incubation, hybrids were characterized for biomass, lipid quantification and GLA production by the methods as described in section 1.9

4.4.7 Protein estimation of hybrids

Protein estimation by Kjeldhal Method

Principle: It is an oxidation of organic compounds by sulphuric acid to form CO_2 and H_2O and release of ammonia. Ammonia combines with H_2SO_4 solution to form ammonium sulphate. Ammonia is released by the reaction of ammonium sulphate with strong alkali and liberated ammonia is collected in dilute boric acid solution which in turn back titrated with dilute acid.

(a) Reagents

(i) ***Digestion mixture*****:** Powdered potassium sulphate, copper sulphate and selenium di- oxide were mixed thoroughly in 5: 2: 1 proportion respectively.

(ii) ***40 % Sodium hydroxide*****:** 40 g Sodium hydroxide pellets were dissolved in about 50 ml distilled water and the volume was made up to 100 ml in a standard volumetric flask.

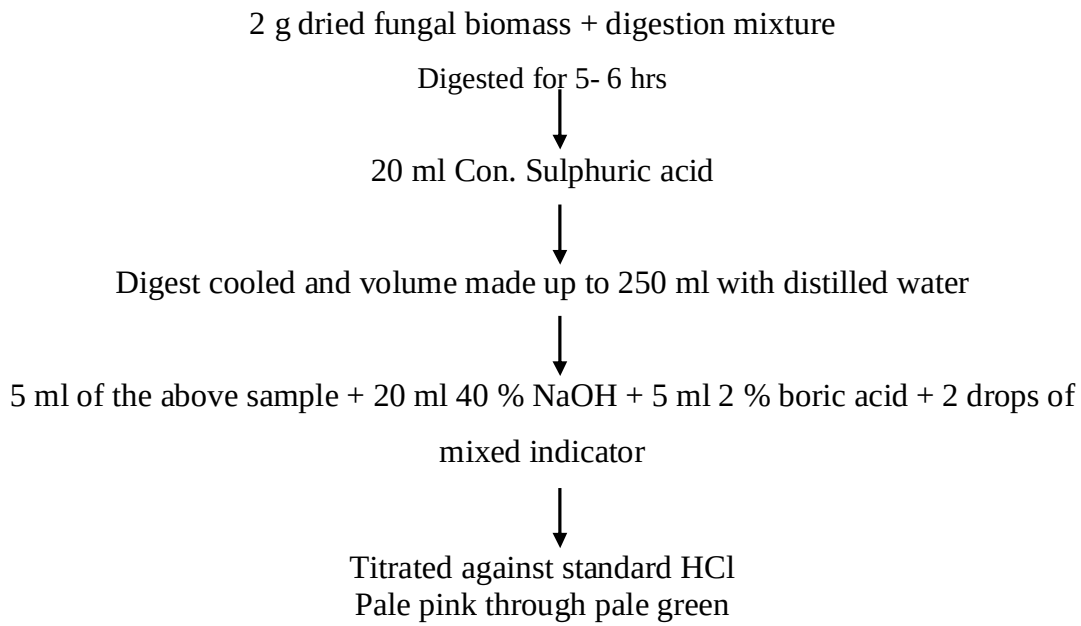
(iii) ***2% Boric acid*****:** 2 g Boric acid was dissolved in hot distilled water and the volume was made up to 100 ml in a standard volumetric flask.

(iv) ***Mixed indicator*****:** 0.1% solution each of bromocresol green and methyl red indicators were prepared in absolute alcohol and then 5 parts of bromocresol green solution was mixed with 1 part of methyl red solution.

(v) ***Standard N/70 HCl*****:** Approximately 1.3 ml of AR grade concentrated HCl was mixed with distilled water and the volume was made up to 1 L in a standard volumetric flask.

(vi) ***Standard Ammonium sulphate solution*****:** 0.942 g of Ammonium sulphate is dissolved in distilled water and the volume is 1 L in a standard volumetric flask (5 ml of this solution is equal to 1 mg of Nitrogen).

Kjeldhal method for protein content determination is given below.



Schematic chart for Kjeldhal method

Percentage protein in the biomass = Titer value - blank x 0.21 x volume of the digest made up x 6.25 x 100/ aliquot used for distillation x weight of sample taken for digestion x 1000.

4.4.8 Estimation of Total Carbohydrates of hybrids by

Determination of total carbohydrates by anthrone method (Hedge & Hofreiter, 1962)

Principle: Carbohydrates are first hyborolsed in simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloure product with an absorption maximum at 630 nm.

Materials

Anthrone Reagents: Dissolve 200 mg anthrone in 100 ml water, working standard-10ml of stock diluted to 1000ml with distilled water. Store refrigerated after adding a few drops of toluene.

Standard glucose: stock-dissolve 100mg in 100ml water. Working standard-10ml of stock diluted to 100ml with distilled water. Store refrigerated after adding a few drops of toluene.

Procedure: 100 mg of the sample was hydrolysed in boiling tube by keeping it in a boiling water bath for three hours with 5 ml of 0.25N-HCl and cool to room temperature. The reaction was neutralised by adding solid sodium carbonate until effervescence ceases. Volume was made to 100 ml and centrifuged. Supernatant the sample was collected and made into aliquots of 0.5 ml. The volume made into 1 ml in all taest tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added into all tubes and heat for 8 min in water bath. All tubes were cooled rapidly and dark green colour was measured at 630 nm. From the standard graph the amount of carbohydrate was calculated in the sample.

4.4.5 Statistical Analysis

The results obtained were subjected to statistical analysis as described in section 1.11

CHAPTER -4

RESULT & DISCUSSION

SECTION 1

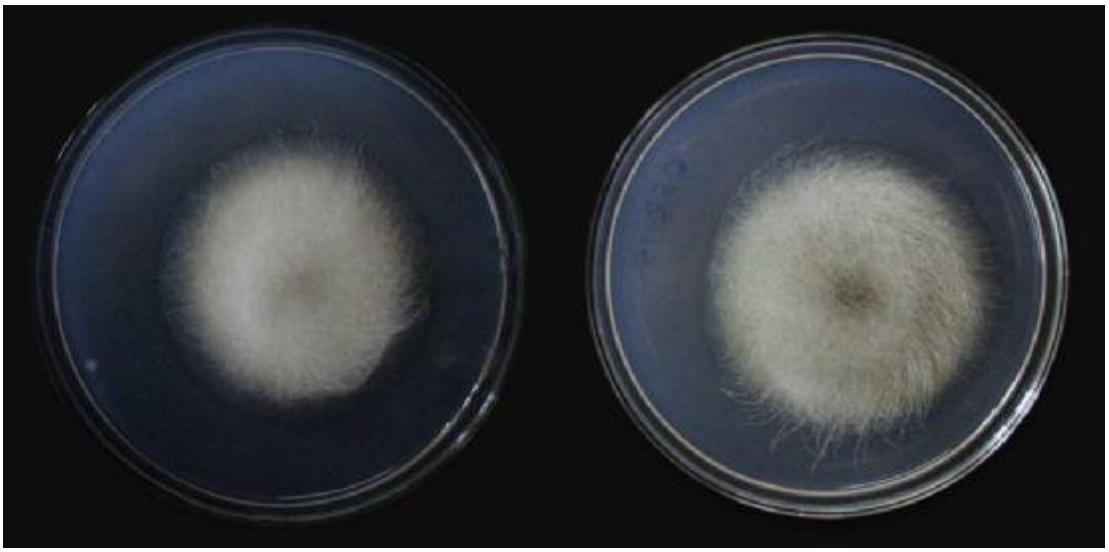
ISOLATION AND SCREENING OF OLEAGINOUS *MUCOR* Spp. FOR GLA PRODUCTION

In the past several years, research on microbial PUFAs production was basically aimed at improving economic competitiveness of microbial lipids compared to plant and animal-derived lipids. A similar pattern of distribution of fatty acids and triglycerides structure of microbial oils proved them as a feasible and cost effective source for the production of pharmaceutically important PUFAs (Certik & Shimizu 1999; Gill & Valivety 1997). Increasing interest of GLA in pharmaceutical and nutraceutical applications has lead to searches for new alternative sources of GLA. Emphasis was placed on screening for more efficient strains, increasing the product value, using inexpensive substrates, and reducing the processing steps necessary for lipid recovery from oleaginous microorganisms (Dyal & Narine 2005; Papanikolaou et al. 2008; Zhu et al. 2003). The aim of this study was to investigate the GLA contents of *Mucor* spp. isolated from local soil samples and to identify and explore a potential strain for GLA production. *Mucor* sp. CFR-G15 strain was selected among the isolates and this strain was subjected to both submerged and solid state fermentation studies for its biomass, total lipid and fatty acid profile, with special reference to GLA.

Isolation and identification of oleaginous *Mucor* spp.

In order to obtain native isolates of *Mucor* spp. for GLA production, 250 soil samples of different habitats were screened. After plating on *Mucor* screening medium, the fungal growth of *Mucor, Rhizopus, Aspergillus,* and *Penicillium* species were observed. Among these, higher percentage (40%) of *Mucor* spp. was noticed, since the medium contained more nitrogen and low percentage of minerals. All *Mucor* spp. were saprophytic and proteolytic in nature, and they grew faster on the nutrient rich medium compared to other fungi. Cottony hairy with white or gray coloured growth in morphology similar to *Mucor* spp. were tentatively identified as per fungi manual and were taken for further studies [Fig.

4.1a & b]. Non-septate coenocytic mycelium without rhizobium, swollen sporangiophores, and the similar morphological characters of *Mucor* spp. were observed in our experiment [Fig.4.2a]. All the morphological characters were additionally compared with standard culture obtained from MTCC, India and Gilman manual (1998).



a **b**

Fig. 4.1 Fungal mycelia on PDA plates
a. *Mucor rouxii* MTTC-386;
b. *Mucor* sp. CFR-G15 isolate

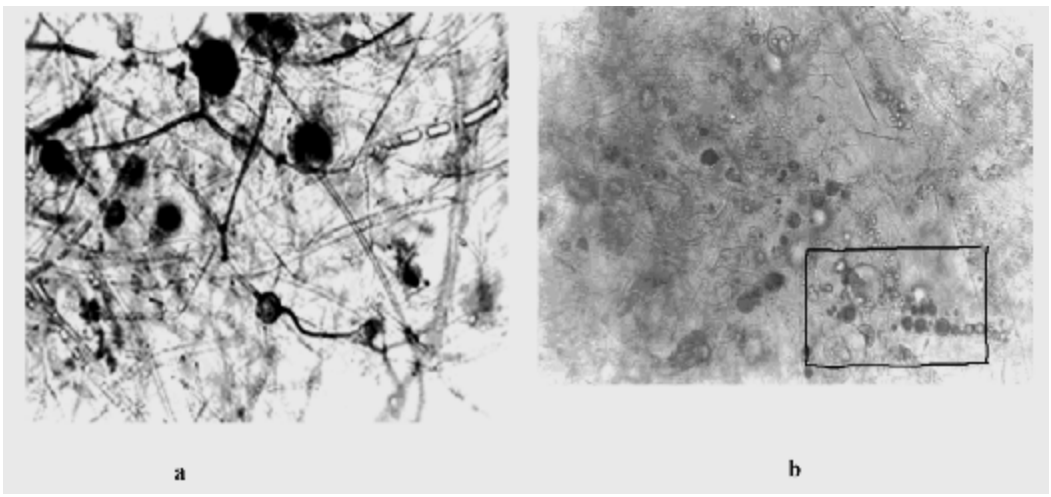


Fig. 4.2 Photomicrograph of *Mucor* sp . CFR-G15
a. Mycelia with sporangiospores and lipid globules;
b. Mycelia Stained with Sudan Black B (Box indicates lipid globules stained in dark colour)

Qualitative analysis of oleaginous *Mucor* spp.

These isolates were screened for their potentiality for producing lipid inside their mycelia both qualitatively and quantitatively by using Sudan black B staining and gravimetric method, respectively. When mycelia stained with Sudan black-B were observed under oil immersion, dark blue colour lipid globules were found [Fig.4.2b]**.** Based on the above observations, 20 species of *Mucor* were selected and named as CFR-G1 to CFR-G20 [Fig. 4.3].

Fig 4.3 Isolates of *Mucor* spp. In PDA slants

Growth characteristics of *Mucor* isolates, fatty acid content and composition

All the isolates were grown in FPM and their growth characteristics like biomass, lipid and fatty acid profiles were studied. Results indicated that, biomass (On dry weight basis) varied from 2.95±0.45 g/L to 8.80±1.22 g/ L for all the isolates. Lowest biomass was observed in CFR -G1 (2.95±0.45 g/L) and highest in CFR-G15 (8.80±1.22 g/L) cultures [Table 4.1]. Lipid production varied from 11.58±0.98 to 30.0±1.32 %. Highest total lipid was observed in CFR-G15 (30.0±1.32 %) and lowest in CFR-G4 (11.58±0.98 %) culture.

Table 4.1 Biomass, lipid and GLA content in the mycelia of selected *Mucor* isolates

Fungal isolates	DW (g/L)	Lipid (g/L)	Lipid (%)
CFR-G1	2.95±0.45	0.68±0.09	23.37±2.25
CFR-G2	3.03±0.65	0.51±0.07	17.00±1.75
CFR-G3	4.21±0.71	0.63±0.05	15.09±1.38
CFR-G4	3.08±0.21	0.35±0.08	11.58±0.98
CFR-G5	3.94±0.62	0.68±0.04	17.38±1.05
CFR-G6	6.07±0.25	1.70±0.12	26.37±2.75
CFR-G7	4.05±0.12	0.77±0.09	19.20±1.72
CFR-G8	4.58±0.51	0.54±0.12	27.43±2.35
CFR-G9	6.03±0.34	1.28±0.10	21.37±2.12
CFR-G10	5.04±0.65	1.12±0.12	22.28±2.35
CFR-G11	4.06±0.77	0.812±0.25	20.08±1.89
CFR-G12	5.50±0.45	0.95±0.18	17.29±1.69
CFR-G13	4.80±0.25	0.49±0.06	17.77±0.98
CFR-G14	2.94±0.62	0.50±0.04	17.29±1.05
CFR-G15	**8.80±1.22**	**2.97±0.35**	**30.00±1.32**
CFR-G16	3.82±0.25	0.82±0.09	14.20±0.85
CFR-G17	5.24±1.01	0.65±0.07	24.12±1.76
CFR-G18	4.57±0.01	0.56±0.12	19.14±1.23
CFR-G19	6.28±1.21	0.58±0.17	23.45±2.50
CFR-G20	5.94±0.95	0.96±0.08	16.29±2.05
M. rouxii **MTCC386**	**7.60±0.81**	**2.82±0.35**	**27.12±2.75**
M. hiemalis MTCC1277	5.2±0.85	1.26±0.18	24.35±2.22

Data are expressed as mean ±SD of three replicates.
DW: Weight of Dry Biomass (g/L)
CFR-Central Food Technological Research Institute, and
G- GLA containing isolates

Thus, CFR-G15 was considered a potent culture for the maximum production of biomass (8.80±1.22 g/L) and lipid (30.0±1.32%) among all the isolates. It was also observed that the culture CFR-G15 grew well in nitrogen limiting conditions i.e. fat producing medium (FPM).

Fatty acid composition of all isolates was analyzed by GC and was found that there were remarkable differences in fatty acid profiles among the isolates [Table 4.2]. Variations among different cultures were observed in unsaturated fatty acid index also (DBI). Oleic and palmitic acids were the major fatty acids present in all the isolates. The GC peak observed at 17.55 min indicated the presence of GLA in the isolates [Fig. 4.4a & b]. The GLA content in all isolates varied from 4.74±0.91% (CFR-G6) to 14.42±0.74% (CFR-G15). The percent GLA was found to be higher in CFR-G15 and *M. rouxii* MTCC 386. Slightly lower values were obtained CFR-G4, CFR-G7, and CFR-G12 which were 12.09±0.29, 12.51±0.82, and 11.15±0.92 as % of total fatty acids respectively. Thus, CFR-G15 isolate was selected for its rapid growth, high production of lipid and also higher GLA yield.

The presence of GLA in total lipid of CFR-G15 was additionally confirmed by mass spectrometry (MS). A molecular ion peak at m/e 292 and intense fragment ion peak at 41, 67, 79 and 93 suggested the component of GLA. Each peak was in good concurrence with the standard [Fig. 4.5a &b]. The α-isomer (ALA) was completely absent in all the isolates. Generally, phycomycetes are recognized for their ability to synthesize GLA, whereas members of ascomycetes and basidiomycetes, with few exceptions are known to produce ALA (Stahl & Klug 1996).

Table 4.2 Fatty acid composition of isolates of *Mucor* species.

Isolates	Fatty Acid Composition (%)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
CFR-G1	3.47±1.25	20.30±0.23	3.20±0.54	9.81±0.62	33.45±2.16	9.65±1.13	**10.31±0.95**	0.84±0.04
CFR-G2	3.05±0.08	16.92±2.15	1.73±0.26	12.27±0.79	44.55±3.21	15.51±1.15	**6.94±0.86**	0.95±0.07
CFR-G3	3.67±0.09	19.27±1.12	4.65±0.21	9.14±0.62	36.59±2.19	14.03±1.16	**10.61±1.11**	1.01±0.05
CFR-G4	3.60±0.46	29.86±2.65	4.25±0.15	8.90±0.49	38.94±3.11	8.28±0.89	**12.09±0.29**	0.78±0.2
CFR-G5	2.22±0.05	28.55±1.21	4.26±0.16	9.48±0.53	32.12±2.75	12.19±0.95	**5.4±0.65**	0.86±0.6
CFR-G6	3.57±0.14	26.14±1.75	4.09±0.09	14.36±0.94	37.07±2.08	5.54±1.99	**4.74±0.91**	0.66±0.03
CFR-G7	3.16±0.11	24.26±2.74	5.14±.0.11	10.12±0.53	33.04±1.19	6.24±1.57	**12.51±0.82**	0.88±0.2
CFR-G8	3.13±0.1	27.10±2.79	2.90±0.06	11.89±0.97	29.19±2.30	10.46±1.26	**10.6±1.20**	0.85±0.08
CFR-G9	5.08±0.17	25.86±3.14	4.32±0.13	12.32±0.58	33.85±2.34	9.20±0.94	**7.17±0.61**	0.78±0.05
CFR-G10	2.38±0.08	22.23±2.66	5.79±0.16	11.66±0.66	31.00±1.95	14.47±1.19	**8.02±0.97**	0.92±0.02
CFR-G11	2.11±0.06	27.21±1.44	3.85±10.09	6.94±0.94	30.65±3.16	14.10±1.12	**2.28±1.01**	1.00±0.09
CFR-G12	3.03±0.68	32.96±2.41	2.30±0.14	4.60±0.33	32.96±2.36	9.44±2.01	**11.15±0.92**	0.88±0.06
CFR-G13	3.62±0.13	18.46±1.51	5.08±0.22	9.32±0.94	35.79±1.14	14.16±0.89	**8.77±1.06**	0.96±0.03
CFR-G14	2.36±0.14	27.76±1.75	2.05±0.08	6.28±0.25	36.00±4.56	9.39±2.16	**10.56±0.89**	0.90±0.04
CFR-G15	2.21±0.08	25.78±1.58	2.46±0.61	6.42±0.58	37.40±2.26	12.26±0.91	**14.42±0.74**	1.03±0.09
CFR-G16	2.97±0.16	19.49±1.25	3.27±0.14	7.10±0.66	42.23±2.96	12.16±2.66	**10.64±0.68**	1.02±0.03
CFR-G17	3.61±0.17	22.47±2.81	4.95±0.19	9.71±1.05	42.41±4.26	11.50±1.16	**5.25±0.85**	0.86±0.05
CFR-G18	3.26±0.09	22.92±1.16	4.77±0.03	8.51±0.58	35.88±1.96	8.61±1.75	**10.45±1.11**	0.89±0.09
CFR-G19	3.65±0.15	26.51±1.59	4.94±0.46	9.49±2.01	34.19±2.68	13.20±1.01	**8.31±1.62**	0.90±0.06
CFR-G20	3.65±0.17	25.51±2.48	4.94±0.80	8.49±1.47	34.19±2.25	13.21±0.09	**8.31±0.33**	0.90±0.03
M. rouxii MTCC386	2.42±0.16	26.40±1.59	2.20±1.36	9.40±2.49	32.20±1.96	11.40±0.76	**13.80±0.85**	0.89±0.05
M.hiemalis MTCC1277	3.02±0.14	20.53±1.78	4.75±1.01	7.65±2.10	40.71±1.76	13.69±0.59	**7.39±0.46**	0.95±0.07

Data are expressed as mean ±SD of three replicates. Fatty acids are expressed as percentage of total fatty acids, DBI- Double bond index.

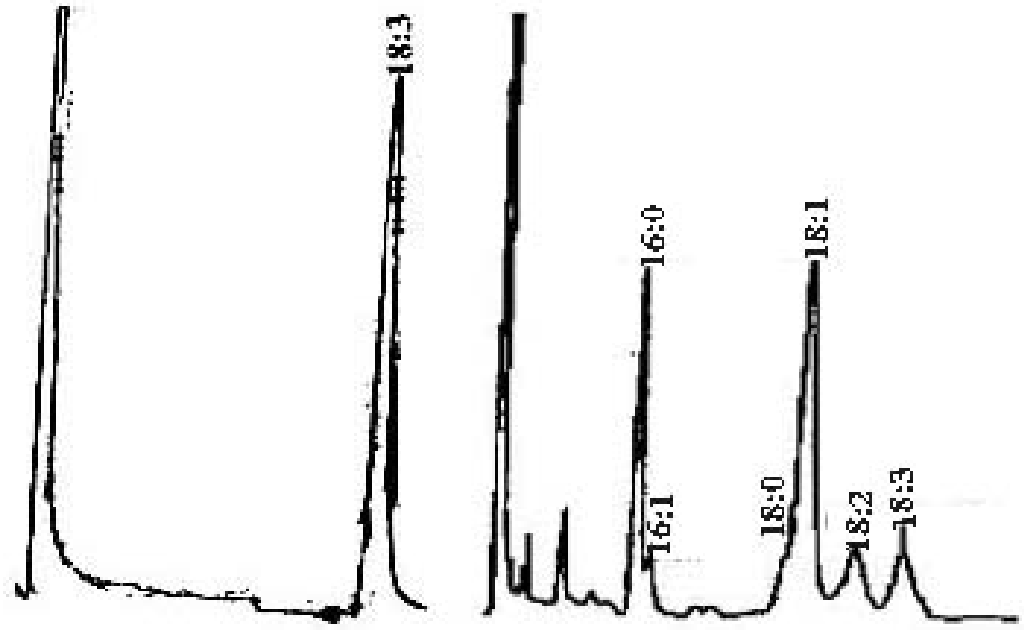


Fig. 4.4 Chromatogram showing

a. GLA standard; b. *M. rouxii* CFR-15 showing fatty acid profile with GLA

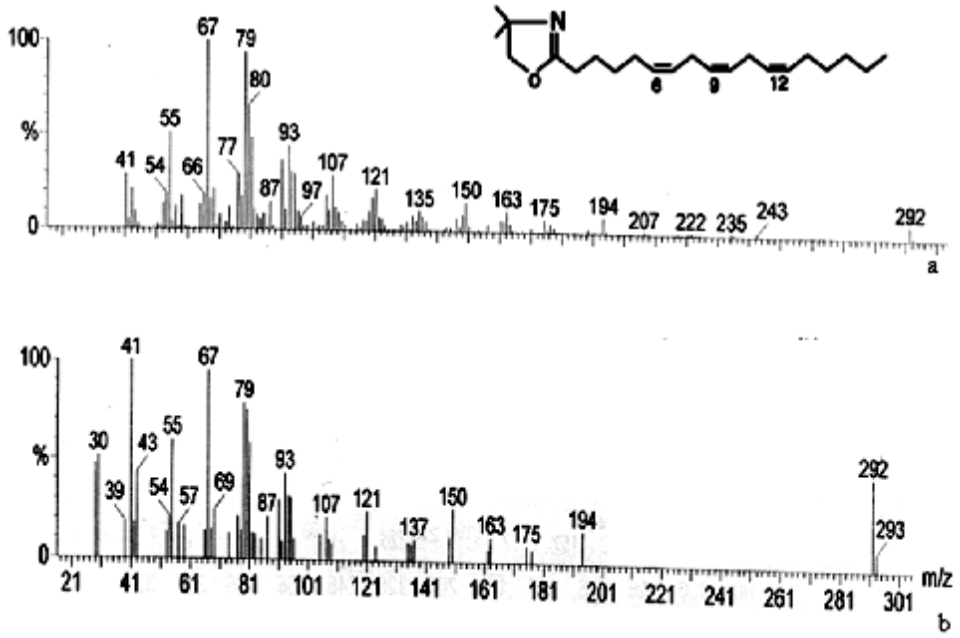


Fig. 4.5 Mass chromatogram of GLA
a. Standard; b. *M. rouxii* CFR-G15

Three variables are important for consideration when microorganisms are screened for fatty acid production. These include the cell concentration, oil content of the cell and its individual fatty acid contents. Ideally, all these variables should be very high in the selected species to achieve maximum fatty acid production. Practically, achieving high biomass, lipid, and PUFAs content is not possible at one time (Kennedy et al. 1993). While comparing with the literature, it was found that certain species of *Mucor* are known to produce GLA using different carbon sources. *M. cercinalloides* CBS 203.28 when grown at 30℃ was able to produce 15.6% GLA as a neutral lipid when acetic acid was used as carbon source (Roux et al. 1994). *Mucor* species KCTC 8405 isolated in Korea, when cultured on 3% glucose and 0.1% ammonium sulphate was able to produce 14% GLA (kang & Shin 1988). An oleaginous fungal strain isolated from the western ghat of Kerala, India when grown in a complex medium containing glucose as carbon source produced GLA at a level of 8% (by mass), (Ahmed et al. 2006). When *M. hiemalis* IPD 51 was screened for fatty acid production, maximum GLA of 15.4% in the 41.1% of total lipid was obtained (Kennedy et al. 1993). *C. echinulata* CCRC 31840 when cultivated for 5 days at shake flask fermentation, the biomass was 29.79 g dry wt/L with a total lipid of 26.94% and GLA content of 12.0% (Chen & Chang 1996). Another study in *C. echinulata* cultivated on orange pulp, enriched with glucose was able to accumulate 12.6±2.4 % GLA in 22.3±3.8 % lipid (Gema et al. 2002). Growth of *C. echinulata* on glucose and *M. isabellina* on pectin produced high GLA of 16.5 % and 6.1 % of total fatty acids respectively, as compared to starch and lactose (Papanikolaou et al. 2007). *C. echinulata* was found to produce 11.7% GLA of 47.6% lipid, upon 168 h of cultivation on tomato-waste hydrolysate media (Fakas et al. 2008). In the present investigation the selected *Mucor* sp. CFR-G15 is a promising producer of GLA, based on lipid (30±1.32%) and GLA (14.42±0.74% of total fatty acids) contents.

Growth characteristics of *Mucor* sp. CFR-G15

The culture CFR-G15 was selected for further studies, since it has shown fast growth and produced substantial amount of lipid and GLA content. Generally, to ascertain their product output fungal cultivation is carried out both by submerged fermentation (SmF) and solid state fermentation (SSF). Keeping this in view, the CFR-G15 was subjected to both SmF and SSF conditions.

Submerged fermentation

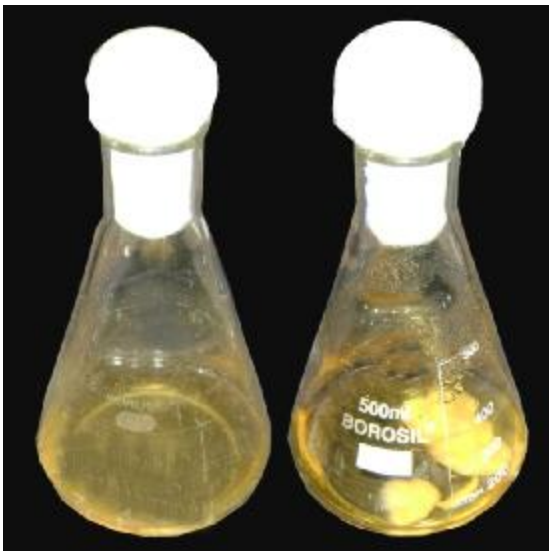
Growth characteristics of *Mucor* sp. CFR-G15 were studied in submerged fermentation. The non fermented broth, fermented broth with biomass and dry biomass (FPM) are shown in Fig. 4.6a, b, & C.

Growth and fatty acid profile of *M. rouxii* CFR-G15 is shown in Fig. 4.7a and b, respectively. Analysis of biomass, lipid and GLA production as a function of fermentation, indicated some culture variability in the lag phase, through log and stationary phases. Consequently, 10 days were chosen for comparison of data from various culture conditions. Production of biomass was increased with time and at 144 h, 8.82±0.93 g/L of dry biomass was obtained with 30±1.32% of total lipid and 14.42±0.74% of GLA content. Results revealed that *Mucor* sp. CFR-G15 showed rapid biomass formation and lipid accumulation in the reproductive phase. During stationary phase, lipid content of the dry biomass was maintained and decreased gradually. It was observed that the glucose concentration in the medium decreased rapidly in the first 48 h and then decreased a lower pace. The rapid consumption of carbon source initially exhibited faster biomass formation with apparent decrease in its lipid content. Similar patterns were also observed in lipid content of *C. echinulata* and *M. isabellina* (Papanikolaou et al. 2004b; 2007). When the nutrients get exhausted in medium other than carbon, it triggers the accumulation of lipid thus preventing cell proliferation and allows conversion of the substrate into lipid (Fakas et al. 2007b; Holdsworth et al. 1988b; Papanikolaou et al. 2004b).

Fatty acid profile of *M. rouxii* CFR-G15 revealed that GLA concentration was higher in the beginning of the fermentation that decreased on the 48 h and increased after 72 h. Maximum GLA was obtained at 96 h and was constant till 144 h [Fig. 4.7b]. Result indicated that saturated fatty acids initially decreased; however, unsaturated fatty acid index was low at 48 h and increased after 72 h. At the beginning of growth when sporangiospore development took place, saturated fatty acids like palmitic and stearic acids were found to be more. Subsequently LA and GLA were increased with the decreased saturated fatty acids, suggesting higher degree of lipid unsaturation [Fig. 4.7b]. When culture was grown for 168 h, the GLA content was found to be decreased, whereas the stearic acid increased. Our results on degree of lipid unsaturation concurred with the results on *Mortierella* sp., *Cunninghamella* sp., and *Mucor* sp. (Ahamed et al. 2006; Chen & Chang 1996; Ho et al. 2007). In general, cultures that accumulate low GLA are known to produce high quantities of lipid to perform membrane function (Papanikolaou et al. 2007; Kennedy et al. 1993).

Degradation of microbial lipid typically initiates after exhaustion of the carbon source and is referred to as 'lipid turnover' and lead to increase in biomass (Fakas et al. 2007b; Papanikolaou et al. 2004b). Lipid turnover is a phenomenon routinely observed in oleaginous microorganisms after transition from excess carbon to carbon starvation conditions that is found in molds (Fakas et al. 2007b; 2008; Papanikolaou et al. 2004b; 2007), yeasts (Holdsworth et al. 1988a; Holdsworth & Ratledge 1988b) and bacteria (Alvarez et al. 2000). In a nitrogen-limiting media when growth takes place, lipid degradation occurs to produce fatty acids. Generally fatty acid gets catabolized via β-oxidation, and acetyl CoA is produced through the Krebs cycle and anaplerotic bypass of glyoxylic acid pathway (Certik & Shimizu 1999; Fakas et al. 2008; Papanikolaou et al. 2004b). Key enzymes NAD^+ and $NADP^+$-CDH and Iso citrate lyase (ICL) play a major role in the process. Lipid turnover is an important factor in the physiology of oleaginous yeast and mold (Holdsworth & Ratledge 1988b; Papanikolaou et al. 2004b). This phenomenon

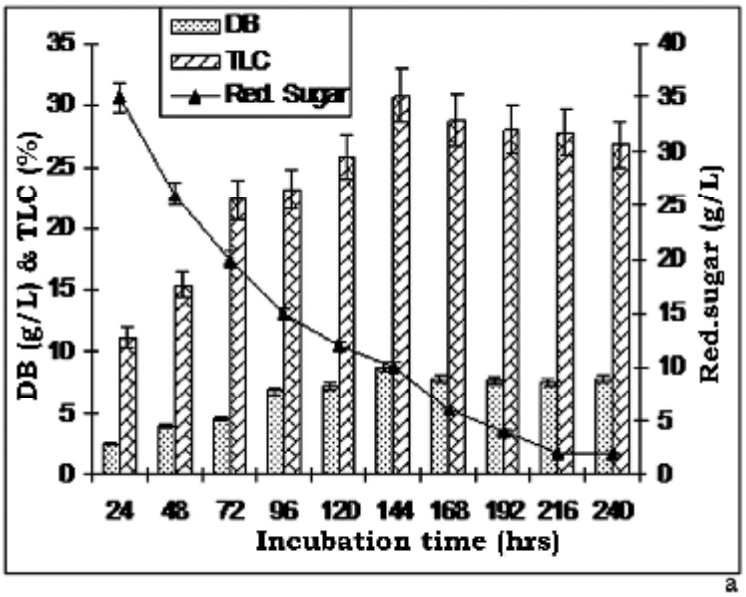
indicates lipid metabolism in oleaginous fungus is a well controlled process. Therefore key enzymes involved in the process of lipid accumulation are of prime importance when considering the potential of industrial process for microbial lipid production (Certik & Shimizu 1999; Holdsworth et al. 1988a; Papanikolaou et al. 2004b).



a **b**

c

Fig. 4.6 Submerged fermentation
a. Non-fermented broth (FPM);
b. *Mucor* sp. CFR-G15 in submerged fermentation (SmF);
c. Dry biomass of *Mucor* sp. CFR-G15



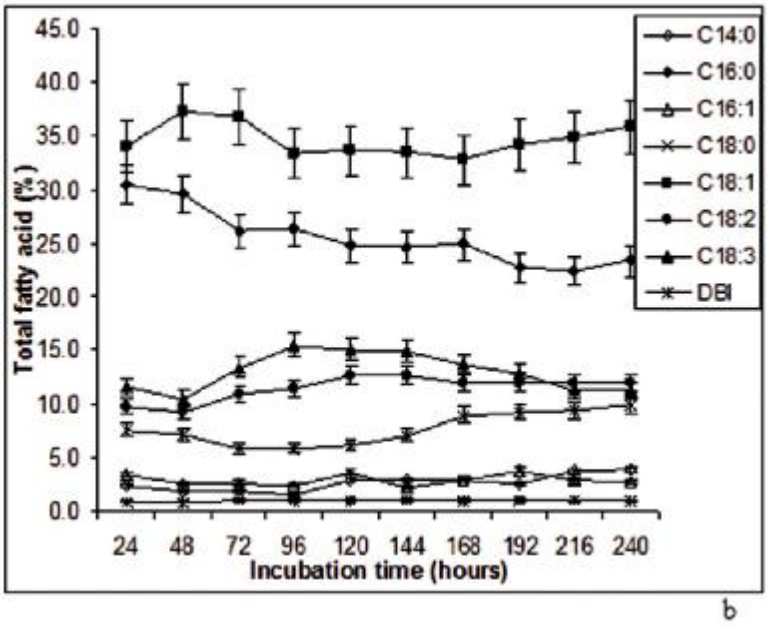


Fig. 4.7 a. Growth Characteristic of *Mucor* sp. CFR-G15 during 10 days of cultivation

b. Fatty acid profile of *Mucor* sp. CFR-G15 during 10 days of cultivation.

Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%), Reducing sugar (g/L)
Fatty acids are expressed as percentage of total fatty acids,
DBI- Double bond index.

Solid state fermentation

In order to investigate the suitability of use of wheat bran for solid state fermentation by *Mucor* sp. CFR-G15, the lipid content and fatty acid profile of the resultant substrate was studied. Fig 4.8a and b shows the wheat bran substrate before and after fermentation. Lipid composition of wheat bran (substrate as control) was analysed before it was used for growing the fungus *Mucor* sp. CFR-G15. The wheat bran, as substrate was moistened with 60% water and in other experiments with nutrient solution (NS) at the levels of 60 and 70% respectively were used for SSF studies. The results of the analyses of the moldy bran after 7 days of fermentation for its lipid and fatty acid profiles are given in Table 4.3a and b. Substrate with NS at 60 and 70 % showed good growth and lipid accumulation (12±1.01 and 14±1.03 %) which were within the optimal range for SSF process observed by other workers (Hang & Woodams 1987).

Fig. 4.8 Solid state fermentation
a. Non-fermented wheat bran
b. Fermented moldy bran of *Mucor* sp. CFR-G15

The major fatty acids found in the SSF culture were, palmitic 23.47±1.23%; stearic 12.09±0.93%; oleic 42.30±1.78%; linolenic 7.2±0.85%. Biologically important GLA was totally absent in solid substrates i.e wheat bran. After fermentation, with 60% moisture the GLA content was 8.73±0.85% of total fatty acids. When 60 and 70% nutrient solution was added to the wheat bran the GLA content was further increased to 13.64±0.84% and 12.31±0.96% respectively. Addition of a mixture of yeast extract, potassium phosphate and magnesium sulphate as a nutrient solution showed a positive effect on the total lipid (14.21±0.84%) and GLA production (13.64±0.84 %). Increase of nutrient solution from 60-70% did not show any significant change in the GLA content. Stredansky et al. (2000b) observed that, high sugar containing apple pomaceae +MSG mix, showed significant effect on the lipid yield when the substrate was supplemented with additional carbon source namely glucose, glycerol and pea nut oil. The highest GLA yield was achieved with the substrate enriched with peanut oil (not containing GLA), which serves as a direct precursor for the formation of fungal oil. Shinmen et al. (1989) also observed that Plant oils supplied to fungal cultivations showed an increased PUFAs yield.

Growth characteristics of *Mucor* sp.CFR-G15 on wheat bran with nutrient solution containing nitrogen source and metal ions was carried for 10 days. Lipid and GLA production was checked periodically after every 2 days and the results are given in Fig. 4.9. During the first 3 days of cultivation on solid substrate, the mycelial growth appeared on the surface of substrate particles. Observation under microscope revealed that fungal hyphae penetrated into the substrate in the subsequent period.

Table 4.3a Dry weight, lipid accumulation and GLA content in total fatty acids of lipid produced by *Mucor* sp. CFR-G15 cultivated on solid state substrate for 7 days.

Substrate	Dry weight (g)		Moisture content (%)		Lipid content per dry weight (%)
	Initial	AF	Initial	Final	
Wheat bran	10.0	-	0.02	0.02	1.5 ±0.09
WB+ H_20	10.0	8.98±0.22	60	45±1.13	10±0.92
WB + NS (60%)	10.0	9.34±0.32	60	39±1.49	12±0.71
WB + NS (70%)	10.0	10.21±0.34	70	40±1.78	14.21±0.84

Data are expressed as mean ±SD of three replicates. NS: Nutrient solution; AF: After fermentation.

Table 4.3b Fatty acid profile of *Mucor* sp. CFR-G15 on solid substrate

Substrate	Fatty acid composition in TFA (% w/w)							
	C14:0	C16:0	C16: 1	C18:0	C18: 1	C18: 2	C18: 3 (n6)	C18: 3 (n3)
Wheat bran	1.21±0.13	20.74±0.99	2.81±0.31	4.97±0.97	40.86±2.03	18.38±1.07	-	9.30±0.73
WB+ H_2O (60%)	2.94±0.27	23.47±1.13	2.36±0.37	12.09±0.93	42.30±1.78	7.20±0.85	8.73±0.85	7.21±0.68
WB + NS (60%)	1.53±0.16	26.61±1.03	2.24±0.25	7.51±0.60	39.74±2.17	10.6±0.93	13.64±0.84	5.34±0.37
WB + NS (70%)	2.96±0.29	25.93±1.08	2.09±0.15	6.35±1.01	30.97±1.96	9.09±0.85	12.31±0.96	5.24±0.39

Data are expressed as mean ±SD of three replicates. WB: Wheat bran; NS: Nutrient solution.
Fatty acids are expressed as percentage of total fatty acids (%).

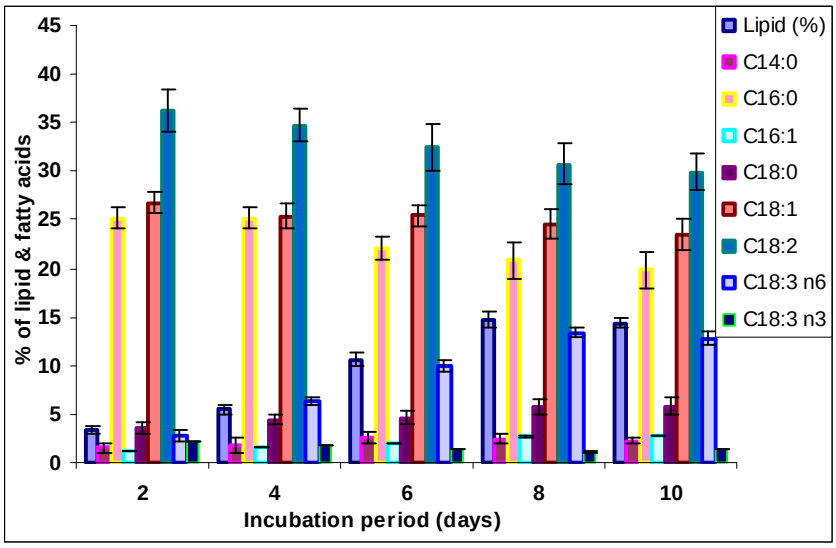


Fig. 4.9 Growth study: Lipid and Fatty acid profile of *Mucor* sp. CFR-G15 on solid-state fermentation (SSF).

Data are expressed as mean ±SD of three replicates. Fatty acids are expressed as percentage of total fatty acids. 18:3 n6-Gamma linolenic acid; 18:3 n6-Alpha linolenic acid

Fig. 4.9 shows the lipid and fatty acid profile of *Mucor* sp. CFR-G15 on solid state fermentation growth study. The lipid content after 2 days of SSF was 3.40±0.34% and steadily increased upto eight days to 14.75±0.75% and on the tenth day showed small lowering which was not significant from that of eighth day. The GLA content (as % of TFA) was 2.80±0.23 % and the increment showed the same pattern as the lipid content, the values steadily showed an increase upto the eighth day (13.94±0.56%) and was not different on the tenth day. Therefore it is inferred that GLA reached a plateau after 8 days of fermentation. Similar result was observed by Stredanska et al. (1993b) who found that, SSF process might allow to obtain GLA content near to that found in plant seed oil (evening primrose oil from 8% to 12%) after a prolonged incubation.

A considerable amount of work has been done in recent years to evaluate alternative carbon source to commercial source and to understand the physiology of fungi on SSF processing (Gema et al. 2002; Pandey 1999; Pandey et al. 2001). Certik et al (2006) studied the growth of mucorales fungi like *Thamnidium, Cunninghamella, Mucor, Mortierella* and *Rhizopus* on

different cereals. *Thamnidium*, *Cunninghamella and Mucor* showed higher capacity to synthesize GLA (4.7–6.8% of fatty acids) than strains of *Mortierella* and *Rhizopus* (1.9–3.3% of fatty acids). Cultivation of two fungi on oat flakes finally resulted in a maximum 4.8 g GLA/kg of bio-product by *T. elegans* and 4.6 g GLA/kg of bio-product by *M. mucedo*, respectively. Fungal GLA was accumulated in the by-products and its yield was affected not only by fungal strain but also by both lipid content in the byproduct and GLA concentration in fatty acids (Conti et al. 2001; Fakas et al. 2007a). High yield of GLA was also reported in *Mucorales* grown on pearled barley supplemented with additional nutrients (Certik et al. 2006).

PUFAs production in fungi was significantly influenced by the moisture content of substrate in solid substrate fermentation. Preliminary trials with wheat bran, and mixture of nutrient solution at 60% and 70% suggested that 60-70 water content was favourable for both lipid and GLA production. Similar moisture level between 60–75% of water in the substrate was reported as optimal for growth, substrate utilization and GLA formation in fungi (Conti et al. 2001; Stredansky 2000a, b). Solid substrate should be having well-balanced sources of carbon with adequate levels of organic nitrogen and other nutrients necessary for fungal proliferation (Pandey 2003). Jang et al. (2000) reported that initial moisture content of solid substrate ranging from 60% to 65% was good for PUFAs production. However, suitable moisture of the substrate was also required for satisfied fungal growth during SSF (Pandey 2000; 2002). The low moisture levels reduced the solubility of nutrients and the swelling of substrate and increased the water tension (Pandey et al. 2001). Correspondingly, high moisture content decreased the porosity and the gas exchange induced the loss of particle structure and the production of stickiness, reduced the gas volume, and enhanced the aerial mycelium formation (Pandey et al. 2001; Singhania et al. 2009).

The main drawback of this type of cultivation is in scale-up studies. This is largely due to several problems encountered in the heat transfer and

homogeneity of the cultures. Separation of mycelia mass from the substrate was difficult in SSF cultivation and recovery of lipid has to be done along with the substrate. So accuracy of lipid content obtained cannot be comparable with submerged fermentation in different fungi.

As SmF is more suitable for scale up studies, our further detailed studies using *Mucor sp.* CFR-G15 was confined to SmF only.

Lipid composition of *Mucor* sp. CFR-G15 in submerged fermentation

The lipid composition of *Mucor* sp. CFR-G15 was analyzed by thin layer chromatography. In the culture cultivated in basal medium (FPM) upto the stationary phase, the ratio of neutral and polar lipid was about 4:1. Neutral lipid, when separated by thin layer chromatography, showed that, triacylglycerols, diacylglycerols, and monoacylglycerols being the predominant lipid classes. Neutral and polar lipid fractions were analysed by GC and it was found that GLA present in both the lipid classes with high concentration in neutral lipid (15.43±1.08%) when compared to polar lipid (10.88±0.97%) [Table 4.4]. Since the ratio of neutral to polar lipid was high in *Mucor* sp. CFR-G15, it has more potential for commercial production of GLA. *M. cercinalloides* CBS 203.28 produced GLA content of 15.6% **as** a neutral lipid when acetic acid was used as carbon source and grown at 30 °C (Roux et al. 1994). Based on above result *Mucor* sp. CFR-G15 was selected for further studies like optimization of cultural conditions in submerged fermentation.

Table 4.4 Lipid composition of *Mucor* sp. CFR-G15 in submerged fermentation

Data are expressed as mean ±SD of three replicates. Fatty acids are expressed as percentage of total fatty acids.

Types of lipid	Lipid Content (%)	Fatty acid composition (%)							
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	DBI
Neutral lipid	72.00 ±2.49	2.66±0.19	19.8±0.91	3.45±0.24	5.87±0.19	39.64±2.18	12.28±0.67	**15.43±1.08[b]**	**1.14±0.05[b]**
Polar lipid	28.23 ±1.47	3.93±0.04	29.1±1.63	2.61±0.21	7.72±0.96	34.31±2.09	9.53±0.92	**10.88±0.97[a]**	**0.89±0.03[a]**

SECTION 2

MOLECULAR CHARACTERIZATION OF OLEAGINOUS *MUCOR* SP. CFR-G15 WITH SPECIAL REFERENCE TO GLA

Since *Mucor* sp. CFR-G15, was proliferating fast and accumulated high lipid (28±3.32%) in its mycelium, it was selected for further studies. In order to taxonomically identify the culture through molecular method i.e. 18S rRNA gene sequencing and to study the diversity of this fungal culture, Δ^6 *DES* gene sequencing was carried out and used for characterization.

Multiple sequence alignment of 18S rRNA and D^6 *DES* genes

To amplify target DNA by PCR, multiple sequence alignment was carried out to design primers. Major *Mucor* species, strains: *Mucor rouxii* ATCC (AF296076), *Amylomyces rouxii* strain CBS416.77 (EF203695.1), *Mucor* sp. KJ-2007a strain KJ1119 18S (EF203698.1), *Mucoraceae* sp. CGMCC-0817 (AF545631.1), *Mucor circinelloides* (AJ878535.1), *Mucor plumbeus* strain A220 (EF203696.1), *Mucor plumbeus* strain A162 (EF203697.1) were used for designing primers. The more conserved region present in the sequences was used to construct forward and reverse primers [Fig 4.10a]. The primers were named as SS-18S F and SS-18S R according to convenient. The PCR of 18S rRNA was expected to 600 bp in *Mucor* sp CFR-G15.

For the cloning of Δ^6 *DES* gene by PCR, two sets of primers were designed. Selected molds which produce GLA, *Mucor rouxii* (AF296076, AF296073) and *Mucor circinelloides* (AB090360, AB052086) were used for multiple sequence alignment. All the sequences were multaligned using the Multalin version 5.4.1. The more conserved region present in the alignment sequences were identified and selected to design both forward and reverse primers [Fig 4.10b]. The primers were named SS-F, SS-R, DES-F and DES-R conveniently. The expected PCR size of Δ^6 *DES* gene was approximate 1.2 kb.

```
                    18S Forward primer
DQ118998.1| TCCGTAGGTGAACCTGCGGAAGGATCATTAAATAATCAATAA
DQ119007.1| TCCGTAGGTGAACCTGCGGAAGGATCATTAAATAATCAATAA
AM745433.1| TCCGTAGGTGAACCTGCGGAAGGATCATTAAATAATCAATAA
AF412288.1|                                TTAAATAATCAATAA
AY625074.1| AAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGA
CONSENSUS   .................TCCGTAGGTGAACCTGCGGAAGGA
                                ***************
                                        18S Reverse primer
DQ118998.1| CAGGCGGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
DQ119007.1| CAGGCGGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
AM745433.1| CAGGCGGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
AF412288.1| CAGGCGGGATTACCCGCTGAACTT
AY625074.1| CAGGCGGGATTACCCGCTGAACTTAA
CONSENSUS   CAGGCGGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
          *********************************
```

(A)

```
                    DES Forward primer
AF296076.1M. AGATGTCACGGATGAAATCCGTACCATGCATCCACCACAGGTATATGA
AF290983.1M. AGATGTCACGGATGAAATCCGTACCATGCATCCACCACAGGTATATGA
AB090360.1M. AGATGTCACGGATGAAATCCGTACCATGCATCCACCACAGGTATATGA
AB052086.1M. AGACGCTACAGATGTCTTTCATGAAATGCATCCTccctctgcttacga

                    DES Reverse primer
AF296076.1M. ATGTTGCGTACTACAATGGATGTTGATTGTCCAGAGTGGCTTGACTGG
AF290983.1M. ATGTTGCGTACTACAATGGATGTTGATTGTCCAGAGTGGCTTGACTGG
AB090360.1M. ATGTTGCGTACTACAATGGATGTTGATTGTCCAGAGTGGCTTGACTGG
AB052086.1M. ----------------------------TTGATTGTTCATTGACTGG
```

(B)

Fig. 4.10 Multiple sequence alignment sequence a. 18S rRNA b. 6 Δ^6*DES* gene

(DQ118998.1 *Amylomyces rouxii* strain CBS 416.77; DQ119007.1 *Rhizomucor variabilis var. regularior* strain CBS 384.95; AM745433.1 *Mucor circinelloides*; AF412288.1 *Mucor circinelloides* strain CBS203.28
AY625074.1 *Mucor racemosus* strain NRRL 1428
AF296076.1M. *Mucor rouxii* delta-6 desaturase mRNA, complete cds; AF290983.1M.*Mucor rouxii* delta-6 desaturase gene, complete cds; AB090360.1M.*Mucor circinelloides* mcD6-2 mRNA for delta-6 fatty acid desaturase, complete cds; AB052086.1M. *Mucor circinelloides)*

Optimization of extraction procedure for genomic DNA

In order to obtain good quality of DNA from *M. rouxii* CFR-G15, two methods were followed as mentioned in the materials and methods. The extracted genomic DNA by these methods all dissolved in 20 µL TE buffer and 5 µL of each sample loaded to the agarose gel. Intensity of fungal DNA recovered with the two extraction methods is shown in Fig. 4.11a and 4.11b. a band of less intensity was observed in agarose gel in first method, it indicates low yield of DNA, may be due to the poor lysis of cell wall of culture. In the method 1, the little shearing was observed in agarose gel. Results indicated that good quality of DNA was obtained by second method. The quantity and quality of DNA obtained by this method was analyzed by gel and used for further studies.

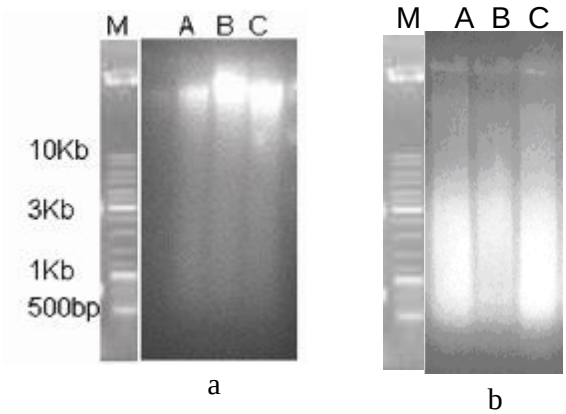


Fig.4.11 a. Gel analysis of genomic DNA from *Mucor rouxii* CFR-G15
b. Restriction digestion (M ; 10 Kb marker)

Since fungi have cell walls that hinder cell lysis and influence the recovery of DNA using conventional extraction methods (Fredricks et al. 2005; Maaroufi et al. 2004). Several methods are available for the extraction of genomic DNA from various filamentous molds (Schwarz 2006; Iwen et al. 2005; 2007). The simple lysis procedures, such as use of sequential freeze-thaw cycles or incubation with hot detergent and proteases, have not produced high yields of DNA from many fungal species. Alternative approaches for the lysis of fungal cells include the agitation of tissue samples with microspheres or

particulates within a sealed tube for physical disruption and the enzymatic digestion of cell wall polysaccharides to form spheroplasts followed by membrane lysis procedures (Goodwin & Lee 1993; Haugland et al. 1999). Other DNA extraction methods for fungi, such as grinding cells frozen with liquid nitrogen using a mortar and pestle and disrupting cell walls with a probe sonicator were also reported (Graham et al. 1994; Muller et al. 1998; Van Burik et al. 1998). Some factors during the recovery of DNA must be considered when selecting a DNA extraction method. These include processing time, sample volume, additional reagents, and equipment for each DNA extraction method (Fredricks et al. 2005). In DNA extraction methods different lysis strategies that are suitable for use on different samples are followed. The extraction of genomic DNA from fungal cultures is a critical step in the process of cloning and sequencing (Van Burik et al. 1998). Results presented here revealed that different DNA extraction methods may produce considerably different yields of fungal DNA.

PCR cloning

i. Amplification of 18S rRNA gene

The optimized annealing temperature of 54°C for 18S rRNA, clear band was observed. The amplification of 18S rRNA gene by PCR was analyzed after electrophoresis of agarose gel and approximate product of 600 bp was obtained and non specific bands were completely absent [Fig. 4.12a]. This suggests primers used in this study were shown strong specificity for fungal rDNA sequences. The PCR product was purified and used directly for nucleotide analysis using SS-18S F and SS-18S R primers. In literature the identification of Mucorales which are medically important fungi, the rDNA was amplified at 52°C for 1 min (Michinaka et al. 2003). Amplification of rRNA of *M. cercinelloides* was done at 50°C for 30 s (Iwen et al. 2007). Oleaginous fungus *Mortierella alpina* which is isolated from soil identified through ITS regions amplification using 57 °C for 1 min as annealing temperature (Yuen et al.

2007). *M. hiemalis* strains were evaluated and probable genetic differences between these strains were determined using 55 ºC for 1 min as annealing temperature (Tauk-Tornisielo et al. 2007). PCR amplification of 18S rRNA and ITS region is known to be used for rapid and specific identification of species both industrially and medically important fungi.

ii. Amplification of D^6 Desaturase gene

The amplification of Δ^6 *DES* gene by PCR using genomic DNA as a template was carried out. The annealing temperature for Δ^6 *DES* gene was optimized in this study to get the specific band. When the primers set 1 were used, the non specific bands were present in PCR products in all the annealing temperature tried. But when DES-F & R used non specific bands were completely absent and only the specific band was obtained. PCR at 54 °C was shown to be optimum for Δ^6 *DES* genes, the expected approximate size of 1.2 Kb was obtained [Fig. 4.12b] and this was compared with the marker. The second set DES-F & R primers showed strong specificity for *Mucor rouxii* CFR-G15 Δ^6 *DES* sequences. The nested primers showed the approximate size of 600 bp for Δ^6-DES at 54 °C. Further the specific band eluted using the commercial kit and purified PCR product was used for further cloning experiment. PCR amplification of Δ^6- *DES* gene was carried out in most of the *Mucor* spp. *Mortierella* spp. *Rhizopus* spp. and *Thamindium elegans* by using mRNA as template and construction of cDNA library to obtain the full length sequence were also reported (Michinaka et al. 2003; Sakuradanai et al. 1999; Wang et al. 2007; Zhang et al. 2004).

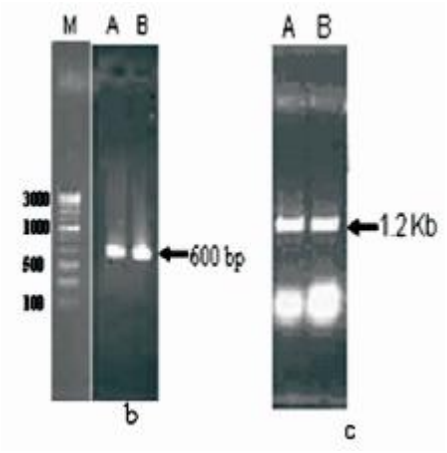


Fig. 4.12 PCR amplification of 18S rRNA and D^6 *DES* gene of *M. rouxii* CFR-G15.

a. PCR product of 18S rRNA gene and

b. PCR product of Δ^6 *DES* gene

(a), M is a 3 Kb Marker (b), A and B PCR products in duplicates, Arrow indicates expected size of amplicon.

Cloning of D^6 DES gene in *E coli* DH5a and recombinant analysis

E-coli DH5α competent cells were prepared by PEG-DMSO method, since this method showed more competitiveness in transformation. The PCR product of Δ^6 *DES* was ligated into T-tail cloning vector and transformed into PEG-DMSO treated *E-coli* DH5α cells. The controls were vector self ligated positive and negative control used. Among transformants putative clones were selected based on the blue/white colonies. When plates were observed, the numbers of white colonies observed were higher than the blue colonies, indicating more efficiency of cloning. About 18 recombinants were obtained in blue/white screening method among 22 colonies analysed.

Recombinants were further confirmed by plasmid isolation, restriction digestion and as well as by PCR. Plasmids were isolated from all 18 recombinants and analysed by gel. The size of the plasmid DNA was compared to that of the parent vector. Plasmid DNA appeared larger due to presence of

insert [Fig. 4.13a]. Additionally, recombinants were subjected to restriction digestion showed the release of inserts in the plasmid. Comparisons were made on the sizes of the restriction digests product with those that are expected based on the desired clone [Fig. 4.13b]. The recombinants further analyzed by PCR, using DES-F and R primers. The agarose gel analyses after electrophoresis showed an expected approximate size of 1.2 Kb correspond with the marker [Fig. 4.13c].

To study the function of a particulars DNA, sequencing and manipulation of those sequences is a basic part in molecular biology. There two ways followed to get this, i). the PCR and ii) use of restriction enzymes and modifying enzymes to "cut and paste" the desired DNA fragments from genome into cloning vectors, then which were replicated using live cells. PCR products were ligated into a suitable vector, which later transformed into suitable host like *E. coli*. Several methods were used to screen transformants colonies such as Blue/white selection, restriction digestion and PCR. Blue/white selection was the easiest screening method, utilized with large class of cloning vectors (Sambrook & Russel 2001).

In this study, the pTZ57R/T vector system was used. This vector system provides the following information for cloning of PCR product. It is an efficient system for cloning of PCR products with addition of 3'A by *Taq* DNA polymerase. The vector pTZ57R/T is prepared by cutting vector pUC57 with E*co*RV and adding a 3'– terminal thymidine (T) to both ends. DNA polymerases that lack 3'→5' exonuclease activity (*i.e.* proof reading activity) possess deoxynucleotidyl terminal transferase activity in addition to primer extension activity (Sambrook & Russel 2001). This results in the addition of extra adenine at 3' ends of amplified products. This terminal transferase activity of some DNA polymerases is independent of the template.

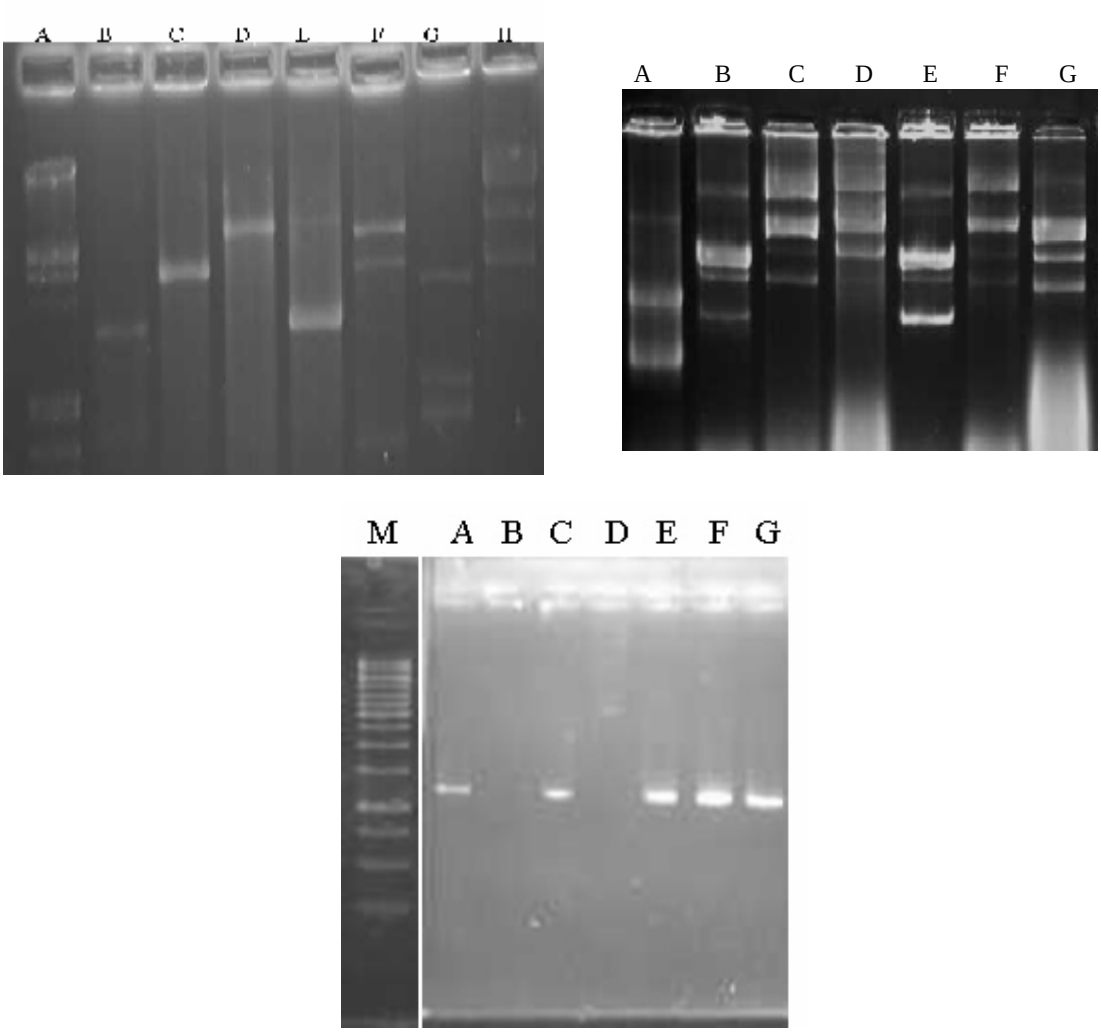


Fig. 4.13 Analysis of Recombinants

a. Plasmid isolation (A Marker B= Control plasmid, C, D, F, H = Recombinant clones and E & G Non recombinants clones)

b. Restriction digestion of plasmid (A= Negative control, B= Positive Control, C, D, F= Recombinants

c. PCR amplification of Δ^6 *DES* gene in recombinants (A=10Kb Marker, B= Control plasmid, C, D, F, H Clones)

Nucleotide sequencing and Phylogenetic analysis

i. Analysis of 18S rRNA gene

For sequencing of the PCR product of rRNA gene was SS-18S F and SS-18S R primers were used. The resultant sequence of rDNA gene obtained was around 600 bp. This rDNA sequence analyzed using BLAST analysis (NCBI), it contains, ITS1, 5.8S rRNA, ITS2 and 28S rRNA regions. The *M. rouxii* CFR-G15 was identified to be 98% homology with *M. rouxii* ATCC 24905 (AF117923.1), 96% *A. rouxii* (DQ118998.1), 95 and 92% similarity with the *Mucor sp*. KJ-2007a strain KJ1119 18S (EF203698.1) *M. cercinelloides* (AJ878535.1) respectively.

Molecular phylogenetic analysis of *M. rouxii* CFR-G15 was confirmed as a new strain of *M. rouxii*. The phylogenetic relationship of these fungal strains is presented in a distance-based NJ tree [Fig. 4.14]. Stains of the same species that had identical sequences are merged into one cluster, but strains of different species that shared the same sequence were kept as separate clusters on the NJ tree. Literature suggested that molecular characterization was a useful tool to phylogenetically related fungi besides their characteristic morphological features as well as physiological and functional aspects (Fliegerova et al. 2004; Sharma et al. 2008).

Molecular identification has been evaluated for several groups of microorganisms which are industrially and medically important (Abe et al. 2006; Sharma et al. 2008; Woll et al. 2003; Wu et al. 2003). Different molecular targets have been used, including conserved ribosomal DNA genes and the more variable internal transcribed spacer (ITS) regions between those genes which allow identification to the species level (Schwarz et al. 2006).

In recent past, molecular tools are routinely used for taxonomic identification. Molecular techniques using genomic targets within the rRNA complex have been shown to be reliable for the species identification of the zygomycetes, including *Mucor* species (Abe et al. 2006; Iwen et al. 2007;

Komaitis et al. 2001; Schwarz et al. 2006). The results of these studies indicated that ITS sequences shared a high level of identity between isolates within a species used, contrasting with a low level of identity between species. This indicates that ITS sequencing is a reliable molecular tool for precise identification of zygomycetes to the genus and species level and could used for "DNA bar coding" of this group of fungi (Iwen et al. 2007; Schwarz et al. 2006). Similar to this study, three alleles namely, 18S rDNA, ITS region and D1 / D2 hyper variable region were considered for phylogenetic studies. Combination of two alleles, i.e. either 18S rRNA and ITS or ITS and 26S rRNA have also been used to determine medically important yeast and zygomycetes, respectively (Abe et al. 2006; Chen et al. 2001; Morakotkarn et al. 2007; Voigt et al. 1999). The ITS1/2 rDNA region proved to provide appropriate markers for genetic studies in the orders *Mortierellales* and *Mucorales*. It also showed that the ITS1/2 rDNA region may be used for phylogenetic studies of *Mucorales* at the population and species levels (Ho & Chen 2008; Kilo et al. 2009; Kwasna et al. 2006). Comparative studies of the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (rDNA) have become a useful tool in fungal taxonomy as these regions evolve sufficiently rapidly to distinguish different species within a genus (Iwen et al. 2002; kilo et al. 2009).

The molecular characterization can also be a useful tool to study phylogenetically relate the fungi on the basis of their characteristic morphological features (Fliegerova et al. 2004) as well as physiological and biochemical aspects. Similarly, two zygomycete fungi *M. indicus* and *R. pusillus*, that expectedly formed as out groups and classified as mucorales. These have been considered to have an early phylogenetic origin as compared with ascomycetes and were found to be good sources of phytases (Chadha et al. 2004).

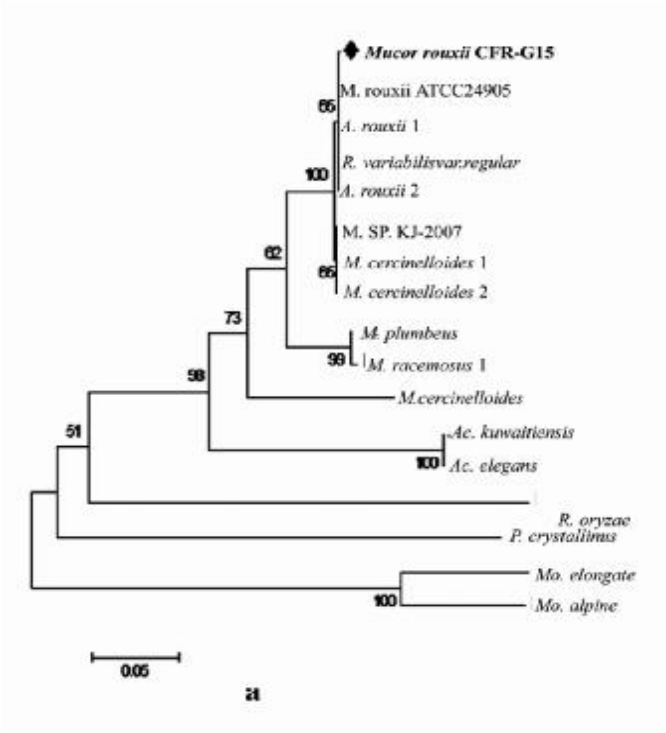


Figure 4.14 Phylogenetic analysis of 18S rRNA taxonomy (Native isolate is highlighted in bold letters)

ii. Analysis of D^6-*DES* gene

The recombinant clone was sequenced in both direction using MB3 primers and the sequence obtained was around 1200 bp. Sequencing results showed that it contain regions that encode the Δ^6-desaturase enzyme involved in the GLA biosynthesis pathway. Using BLAST (NCBI), the percentages of identical matches of with other sequences in the GenBank database (NCBI) were determined; it showed 98% similarity with *Mucor rouxii* ATCC (AF296076).

In this study, phylogenetic tree of Δ^6 *DES* generated by MEGA 3 version showed *M. rouxii* CFR-G15 was clustered with the *Mucor* spp. and this indicates that, they are more related [Fig. 4.15]. Translated Δ^6 desaturase of *M. rouxii* CFR-G15 predicted protein showed all the characteristic of membrane bound front-end fatty acid desaturases. It contained an usual histidine rich

motif 'HKHHSX' down stream of cytochrome b5 domain, lying within the region of amino acid sequence; this region is essential for enzyme activity (Sakuradanai et al. 1999; Michinaka et al. 2003; Zhang et al. 2004). Homology and deduced amino acid sequence, suggested a novel sequence coding a putative Δ^6 fatty acid desaturase that was involved in the synthesis of GLA in *M. rouxii* CFR-G15. *Mucor* spp. are considered to be the lipid-producing organisms, and has attracted the attention for the biosynthesis of PUFAs and genes encoding Δ^6, Δ^9, and Δ^{12} fatty acid desaturases have also been cloned. Cloning of desatuarse enabled to understand the regulation of the production of PUFAs *in vivo* or *in vitro* (Brown 2005).

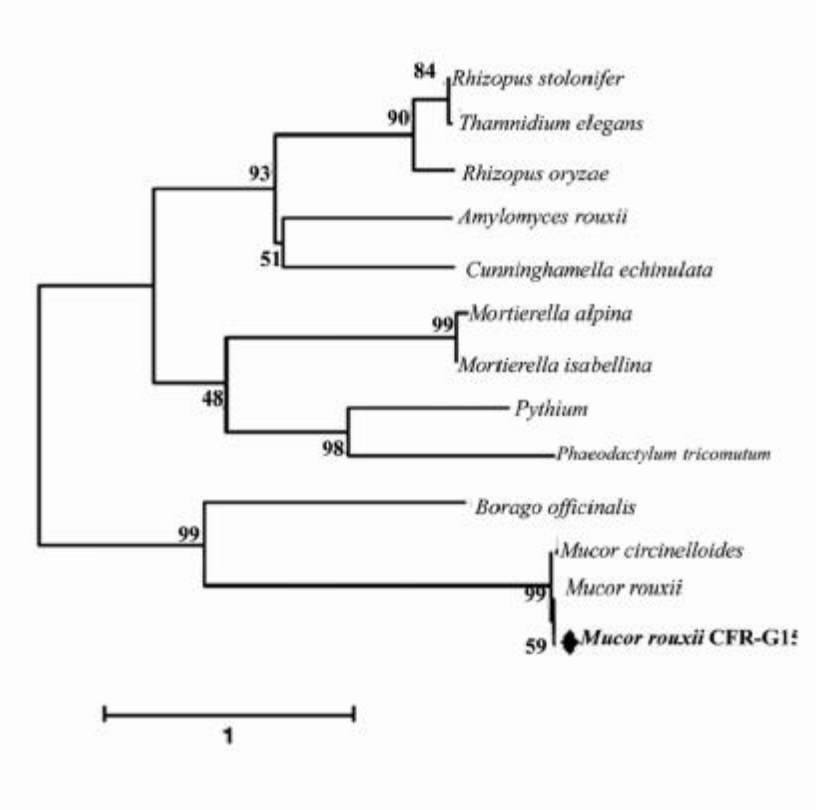


Figure 4.15 Phylogenetic analysis of D^6 *DES* gene diversity between the GLA producers. Native isolate is highlighted in bold letters.

Δ^6-desaturase enzyme is a membrane bound front-end desaturases with molecular weight 600kDa. It catalyzes, conversion of linoleic acid (C18:2 n6) and Alpha linoleic acid (C18:3 n3) into GLA (C18:3 n6) and stearcodonic acid (C18:4, n3) respectively (Michinaka et al. 2003). GLA is an omega-6 (ω-6) polyunsaturated fatty acid and has wide number of application in medicinal and nutritional area (Horrobino 1992; Gill & Valivety 1997). To study the diversity of D^6 desaturase enzyme in GLA producers, several studies on cloning and expression has been under taken (Gill & Valivety 1997; Sakuradanai et al. 1999; Wang et al 2007). The Δ^6 desatuarse enzyme has been diversified from various organisms like plants (Sayanova et al.1997; 2003), animals (Aki et al.1999; Das et al. 2001), insects (Napier et al. 1998), algae (Domergue et al. 2002; Hirano et al. 1990) and fungi (Michinaka et al. 2003; Sakuradani et al. 1999; Wang et al. 2007). Δ^6-desaturases in higher plants; *Borage*, *Echium*, *Primula* and *Anemone* appear to be distantly related to the fungal and lower-plant Δ^6-desaturases. This diverged from those in lower plants at an early stage of evolution. The fungal *Mucor rouxii* Δ^6-desaturase is more related to plant species Borage than the animals, algae and fungi like *Mortirella* spp. (Michinaka et al. 003; Zhang et al. 2004).

Gene sequencing of 18S rRNA is suggesting taxonomic identity of the native isolate *M. rouxii* CFR-G15. In fungal taxonomy, comparative studies of the 18S rRNA and ITS regions of the ribosomal RNA genes has become a useful tool for rapid and accurate identification. The genes and genes products involved in the biosynthesis of PUFAs is being identified and characterized for higher production of PUFAs. Additionally, gene encoding Δ^6-desaturase enzyme involved in GLA biosynthesis in *M. rouxii* CFR-G15 was also identified. This study provides useful information for further work on PUFA metabolic pathways and gene engineering about GLA production from microbial sources. A potential strain was reported that could be used for the development of an economical process in industrial GLA production.

SECTION 3

OPTIMIZATION OF CULTURAL CONDITIONS FOR BIOMASS, LIPID AND GLA CONTENT OF *MUCOR ROUXII* CFR-G15.

Several growth conditions are known to influence lipid production in oleaginous fungi. The physiology of lipid production in different oleaginous microorganisms has been studied by a number of workers through various growth conditions (Ahmed et al. 2006; Dyal et al. 2005; Fakas et al. 2007a, b; Kavadia et al. 2001; Leman 1997; Li et al. 2008; Nakahara et al. 1992; Papanikolaou et al. 2004a; Somashekar et al. 2002; Stredanska et al. 1993). Some microorganisms grown under particular environmental conditions were able to accumulate significant quantities of storage material, lipid or polysaccharides (Aggelis et al. 1996; Fakas et al. 2008; Ratledge 1987; 1993). A high C:N ratio in the medium was initially found essential for lipid overproduction in oleaginous microrganisms (Holdsworth & Ratledge 1988a; Ratledge 2004). However, lipid over production can also result from limitation of certain other essential nutrients and environmental parameters. Microbial production of lipids that contains speciality lipids (PUFAs) with potential commercial value has been aimed at organism's selection and optimization (Ahmed et al. 2006; Fakas et al. 2007b; Kendick & Ratledge 1992). In commercial production of GLA by fermentation, usually optimization of media was carried out for biomass and product maximization. Optimization is directly proportional to the effect of nutrients of cost effective substrate and also factors like pH, temperature, aeration or agitation on the yield of products (Hansson & Dostalek 1988; Kendrick & Ratledge 1996; Mamatha et al. 2008; Xian et al. 2006).

In this study, the influence of different physiological parameters was carried out for *M. rouxii* CFR–G15, a locally isolated strain in a lab scale level using 500 ml shake flask cultivation with 100 ml working volume. Thus, this study was aimed to optimize the pH, temperature, aeration, carbon source,

nitrogen source, C:N ratio, supplementation of metal ions and oils in order to produce high biomass, lipid yield, and GLA content. As this experiment was focused for the development of a scale-up and mass production of GLA from *M. rouxii* CFR-G15 in submerged fermentation, these parameters were optimized.

Effect of Different Media Composition on Lipid Accumulation

In this experiment, different media compositions were tried for their suitability for biomass buildup, total lipid productions and GLA content. The media used were fat producing medium (FPM), synthetic medium (SM), glucose yeast extract medium (GY), potato dextrose broth medium (PDB) and Czepack Dox medium (CDB) based on the nutrient conditions and results of pilot experimental studies. The results of the experiment using *M. rouxii* CFR-G15 strain, is presented in Fig. 4.16 The results indicated that biomass yield was more in SM medium (8.80±1.21 g/L) followed by FPM and GY. Biomass production in PDB media was lower than SM. The biomass build up (4.21±0.61 g/L) was found to be least in CDB. The lipid percentage calculated on the basis of dry biomass is given in Fig 4.3.1. The lipid percent varied from 16.91±1.12% to 30.27±1.12%. The media CDB and PDB showed the least lipid percent in the dry mycelia. The media FPM and GY were similar in the production of lipid content (%) by the culture *M. rouxii* CFR-G15. The culture grown on SM contained a lipid % intermediate between these two sets (23.34±1.98%). The lipid content of dry biomass was calculated for all the experiments. Though the percentage lipid content varied between media used, because of the variation in the quantity of biomass produced (consequently dry biomass content) the absolute quantity of lipid produced varied. The total lipid yield thus calculated was expected to give basis the about choice of the media for future studies using *M. rouxii* CFR-G15. The production of total lipid in CDB media was the least 0.72±0.15 g/L which was not different from PDB media namely 1.28±0.27 g/L. The other media studied namely SM GY and FPM produced 2.06±0.41, 2.33±0.45 and 2.50±0.54 g/L respectively. The

production of lipid (g/L) in these groups was not different. In FPM media almost all sugar was utilized for growth and lipid accumulation.

The final content of reducing sugar per litre was used as an indicator of sugar utilization by *M. rouxii* CFR-G15, in the media studied [Fig. 4.16]. This residual sugar content was lowest in FPM, 2.87±0.64 g/L and in SM 3.21±0.51 g/L which were not different. The residual sugar was highest in CDB viz., 5.56±0.45 g/L. A value of 5.30±0.43 g/L found in GY was not different from that. In media PDB, the value was intermediate between these two groups (4.14±0.24 g/L). Result showed that among the media studied FPM and SM were utilized more by *M. rouxii* CFR-G15.

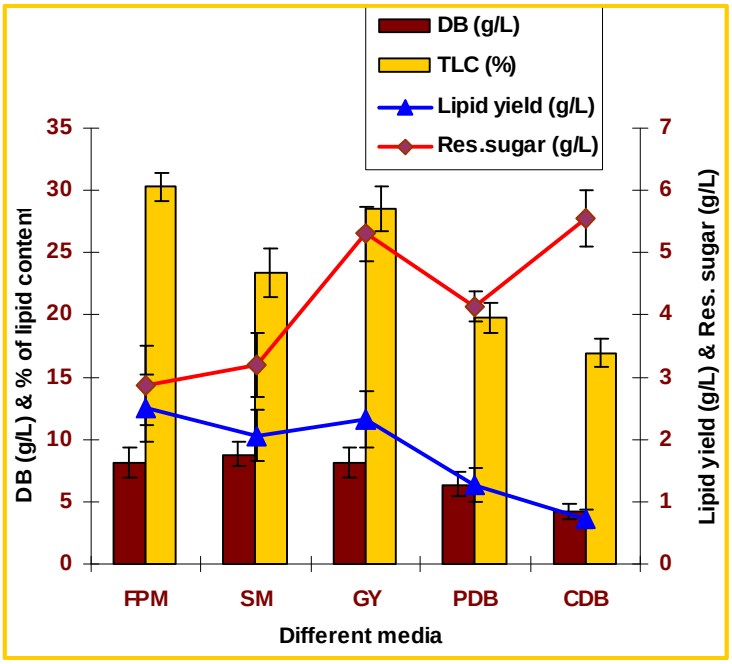


Fig. 4.16 Effect of Different Media on biomass, lipid production and residual sugar in *M. rouxii* CFR-G15

Data are expressed as mean ±SD of three replicates. DB- dry biomass (g/L), TLC-Total lipid content (%), Residual sugar (g/L).

Effect of different media on GLA production and other fatty acids is shown in Table 4.5. The highest GLA content of 14.98±1.00% was noticed when FPM was used. Lowest value was found when CDB media used (7.15±0.58%). Use of SM resulted in a GLA content of 13.55±0.97% which is not different from FPM media. Media PDB and GY produced 11.15±1.02%

and 13.12±1.05% GLA respectively which were intermediate in their efficiency in GLA production in *M. rouxii* CFR-G15. For all further experiments FPM was selected to optimize the cultural conditions, since it showed highest lipid and GLA content when compared to other media

Oleaginous microorganisms accumulate high lipid content when the nitrogen source exhausted from the medium and excess carbon diverted into lipid synthesis (Fakas et al. 2008; Holdsworth & Ratledge 1988b; Papanikolaou et al. 2004b). FPM was commonly used since the oleaginous microorganisms grow well in N_2 limiting media and produce high lipid content. It provides favorable conditions necessary for the accumulation of lipid inside the mycelium of fungi. The result concurred with these found in oleaginous microorganisms like zygomycetes fungus which shows good growth and more lipid accumulation in N_2 limited medium (Buranova et al. 1990; Chen & Chang 1996; Kendrik & Ratledge 1992a; Komaitis et al. 2001). *Mortierella* spp. produces more biomass, lipid and PUFAs in GY medium, since it is nutrient rich medium (Buranova et al. 1990; Dyal et al. 2005; Hansson & Dostalek 1988; Kavadia et al. 2001; Leman 1997). PDB has components such as metal ions and/or other micronutrients which provide meticulous conditions to enhance growth of the organisms but at the same time it was found that it was not suitable for lipid and PUFAs production (Dyal et al. 2005; Somashekar et al. 2002). Similar observation was also made in our study. CDB medium was nutritionally intermediate, having poor growth and less lipid accumulation. It may be due to the occurrence of variation in pH (dropped to acidic) during growth phase. pH of the medium was maintained in the FPM, SM and GY throughout the growth period.

Table 4.5 Effect of different media composition on fatty acid profile of *M. rouxii* CFR-G15

Different Media	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
FPM	2.98±0.31	23.92±1.08	3.27±0.50	6.49±0.58	32.37±1.23	13.83±0.83	**14.98±1.00^{d}**	**1.08±0.04d**
SM	1.42±0.27	27.97±1.15	2.28±0.42	7.90±0.45	35.69±1.42	11.02±0.98	**13.55±0.97^{cd}**	**1.01±0.04cd**
GY	3.26±0.35	20.12±1.08	4.58±0.51	8.02±0.53	36.63±1.61	12.42±1.06	**13.12±1.05^{c}**	**1.05±0.06cd**
PDB	3.03±0.22	29.96±1.27	2.34±0.46	8.34±0.63	34.84±1.12	9.44±0.90	**11.15±1.02^{b}**	**0.90±0.04b**
CDB	5.05±0.36	25.86±1.05	4.32±0.55	12.32±0.76	33.45±1.21	9.20±0.70	**7.15±0.58^{a}**	**0.79±0.01a**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

Influence of Culture Conditions on Lipid and GLA Production

Physical parameters

Growth and lipid accumulation in oleaginous microorganisms are highly influenced by a number of environmental or physical parameters like pH, temperature, light and aeration. Among these, temperature and aeration are the most critical factors that affect the overall metabolism in the microbes. These physical parameters also controlling the growth rate, morphology, lipid synthesis and their composition in cellular level (Morenson 1988; Ratlegde & Wynn 2002).

pH optimization

In cultivation medium, pH is one of the most important environmental factors affecting cell growth and product formation (which can be controlled) in microbial fermentations (Hong et al. 2006). To know the optimal pH for the maximum production of biomass, lipid and GLA in *M. rouxii* CFR-G15 this study was carried out. Cultivation medium was prepared (FPM) and pH was adjusted from pH-3.0 to pH-9.0 (pH of the medium was adjusted before autoclaving using 0.1N HCl and/or NaOH accordingly). The results revealed that original pH of the medium yielded maximum biomass. At pH 5.5 it was 8.10±0.45 g/L. The dry biomass content was maximum at pH values of 4.5 to 5.5. Below a pH of 4.5 down to 3 the values were same and biomass was dipping down to a low of 6.60±0.53 g/L. Above the pH of 7 there was a steep fall in the biomass to a low value of 3.27±0.34 g/L at pH 9. This confirms that a pH of 5.5 is the most suitable for maximum biomass production.

The lipid percentage of the dry biomass at different pH levels gave a clear picture for the choice of optimum pH in the range of 5.5 to 6. The lipid contents were 32.58±1.35% and 30.50±1.24% at the above said levels respectively. Very low lipid percent were found at pH values at neutral range and above as well as at 3.0 and 3.5 pH values. There was a trend of increase in the lipid content from pH 4.0 onwards which reached a peak at 5.5. At 6.5 the lipid content became considerably lower and was 25.9±1.51% only. The total

lipid yield (g/L) was highest at pH 5.5 and was 2.64±0.26 g/L and at pH 5.0 the value was 2.43±0.32 g/L which were not different from pH pH 6.0 it was 2.20±0.19 g/L. This is due to both high lipid percent and higher biomass contents. The total lipid content showed a deep continuous lowering from pH 7.0 onwards (1.00±0.18 g/L) to pH 9.0 (0.44±0.03 g/L). A gradual slow increase was found from pH 3.0 (1.16±0.15 g/L) upto pH 4.5 (1.99±0.23 g/L). The residual sugar content (g/L) did not show any definite trends. Above pH 4.5 upto the 7.5 utilization was almost same and was between 3.30±0.46 to 4.56±0.51 g/L. the least utilization was at pH 3.0 namely 6.00±0.57 g/L at neutral and alkaline pH also the utilization was low.

The GLA content was high at pH 5.5 (16.45±0.93%) and pH 6.00 (16.69±1.01%). Low values for GLA content was noticed at pH values 3.0, 3.5, 4.0 and at 8.0, 8.5 and 9.0. GLA content from 14.09±1.04% to 15.59±1.17% from pH 4.5 to pH 6.5 with the exception of 5.5 and 6.0 gave similar values. This again confirms that the pH 5.5 chosen as the proper pH for the growth of *M. rouxii* CFR-G15 as far as GLA production is concerned. The low GLA content was found at both pH 3.0 and 9.0 indicated that acidic and alkali ranges didn't favours the GLA production though a small amount of growth was observed in the cultivation medium. The major fatty acids found in lipid were oleic and palmitic acids. The level of unsaturated fatty acids were increasing till 6.0, after that a decline was observed. The results are given in Fig. 4.17 and Table 4.6.

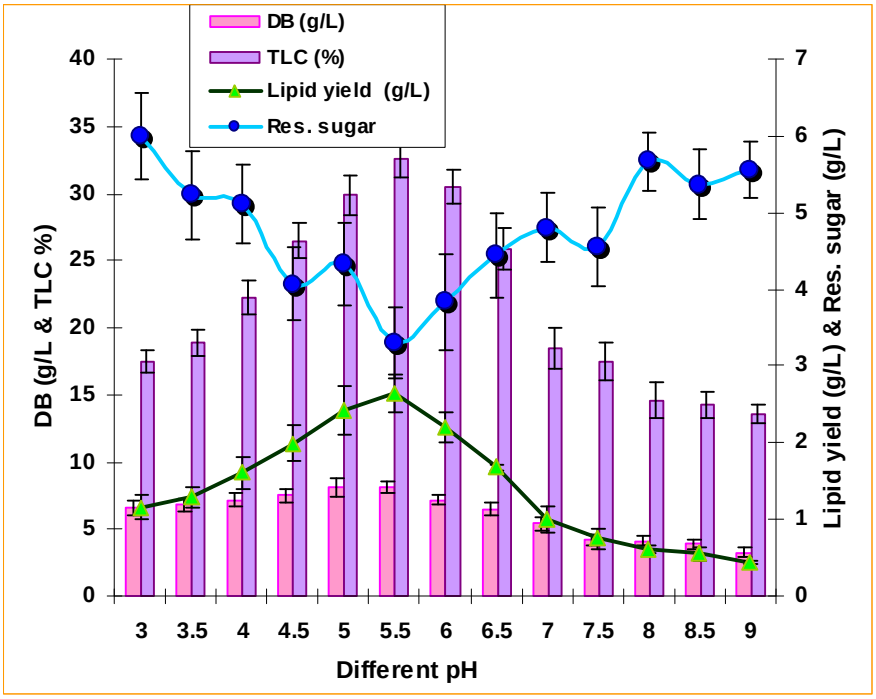


Fig. 4.17 Effect of initial pH on biomass, lipid production and residual sugar in *M. rouxii* CFR-G15.

Data are expressed as mean ±SD of three replicates. DB- dry biomass (g/L), TLC-Total lipid content (%), residual sugar (g/L).

The GLA content was high at pH 5.5 (16.45±0.93%) and pH 6.00 (16.69±1.01%). Low values for GLA content was noticed at pH values 3.0, 3.5, 4.0 and at 8.0, 8.5 and 9.0. GLA content from 14.09±1.04% to 15.59±1.17% from pH 4.5 to pH 6.5 with the exception of 5.5 and 6.0 gave similar values. This again confirms that the pH 5.5 chosen as the proper pH for the growth of *M. rouxii* CFR-G15 as far as GLA concerned. The low GLA content was found at both pH 3.0 and 9.0 indicated that acidic and alkali ranges didn't favours the GLA production though a small quantity growth was observed in the cultivation medium. The major fatty acids found in lipid were oleic and palmitic acids. The level of unsaturated fatty acids were increasing till 6.0, after that a decline was observed. The results are given in Fig. 4.17 and Table 4.6.

This trend was very much similar to those found with the *Mortierella* sp. and *Mucor* sp. RRL001 where lipid production was high when the mould was cultivated at the pH 6.5 (Ahamed et al. 2006; Dyal et al. 2005; Leman &

Brackoniek 1996; Lindberg & Molin 1993). Result obtained in this study also agreed with the results of studies with fungus *Rhizopus nigricans* SSSD-8, where maximum GLA yield from total lipid content was observed at pH 5.5 (Bandyopadhyay et al. 2001). The results of the study was different from studies on *Mucor cercinelloides* I.M.I. 307741 and *Rhizopus arrhizus* where maximum GLA and lipid content were obtained at pH 4.0 and 7.0 respectively (Kristofikova et al. 1991). It is inferred that the pH of the cultivation medium does not influence the biomass production. In general, it was found that with respect to varying pH values, the amount of saturated fatty acid and monounsaturated fatty acid decreased with increasing pH values, while the PUFAs content tend to increase with increasing pH values (Dyal et al. 2005). Thus our result revealed that GLA production in *M. rouxii* CFR-G15 was maximum at pH 5.5 and 6.0 whereas the biomass and lipid content obtained were maximum at pH 5.5. Hence in all our future experiments pH 5.5 was fixed as optimal pH.

Table 4.6 Influence of pH on fatty acid profile of *M. rouxii* CFR-G15

Different pH	Fatty acid composition (as % of total fatty acids)							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	DBI
3.0	2.98±0.34	25.04±1.32	3.24±0.23	7.24±0.65	37.68±2.02	10.95±0.85	**10.90±0.78^{a}**	**1.04±0.07^{a}**
3.5	2.57±0.45	26.14±1.45	3.09±0.36	7.39±0.78	37.07±1.91	10.54±0.94	**10.75±0.79^{a}**	**1.27±0.08**
4.0	2.80±0.58	27.86±1.52	3.50±0.48	6.84±0.92	36.61±1.86	10.61±0.95	**10.77±0.85^{a}**	**1.31±0.06**
4.5	2.59±0.44	24.19±1.17	2.98±0.44	5.93±0.87	35.24±2.09	12.51±0.93	**14.09±1.04^{cd}**	**1.06±0.08**
5.0	2.50±0.37	24.96±1.12	3.21±0.41	6.12±0.30	34.36±1.83	12.78±0.97	**15.43±1.12d^{ef}**	**1.09±0.11**
5.5	2.22±0.56	23.55±1.10	4.26±0.39	5.48±0.52	33.12±1.67	13.19±0.79	**16.45±0.93^{ef}**	**1.13±0.21**
6.0	2.66±0.52	20.86±1.57	4.25±0.26	6.19±0.39	34.94±1.89	13.28±0.65	**16.69±1.01^{e}**	**1.16±0.18**
6.5	2.68±0.60	23.41±1.34	3.12±0.31	8.56±0.37	34.62±2.09	10.75±0.95	**15.59±1.17^{def}**	**1.06±0.14**
7.0	4.53±0.33	21.44±1.52	3.13±0.38	7.12±0.55	35.72±2.15	11.25±0.98	**15.04±1.08^{def}**	**1.06±0.21**
7.5	3.46±0.43	23.53±1.33	3.49±0.29	8.47±0.41	35.57±2.12	9.47±0.84	**14.79±1.11^{def}**	**1.02±0.09**
8.0	1.86±0.38	25.61±1.08	2.13±0.57	9.15±0.72	36.67±2.05	11.51±0.88	**12.11±0.95^{ab}**	**0.98±0.08**
8.5	2.23±0.36	27.70±1.17	2.06±0.41	7.78±0.54	36.71±2.15	10.71±0.81	**12.69±0.93^{bc}**	**0.98±0.07**
9.0	3.23±0.41	27.72±1.25	2.16±0.56	9.78±0.69	35.71±1.94	10.25±0.93	**10.69±0.87^{a}**	**1.09±0.08^{a}**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index

Optimization of Temperature

PUFAs are the primary metabolites produced in *M. rouxii* CFR- G. Their yield was associated with the growth of the fungus, lipid accumulation and growth temperature under cultivation. Our focus in this study was to optimize the cultivation temperature for *M. rouxii* CFR-G15 an oleaginous fungi at different levels ranging from low temperature (5-15°C) to the high temperature (40 to 45°C) for enhanced production of biomass, lipid accumulation and their fatty acid profiles with special reference to GLA. Thus the culture was grown in association of two varied temperatures and supply of additional glucose in the culture medium.

Results indicated that this strain did not show any significant ($p>0.05$) growth (dry biomass) (4.3±0.3 g/L and 2.42±0.22 g/L) and lipid yield (22±1.2% and 14.2±1.2 %) both in low temperature (5ºC) and high temperature (40ºC) when compared to room temperature (7.8±0.80 g/L and 30.20±1.23 %). Optimal growth with reference to biomass (7.8±0.80 g/L), and total lipid content (30.20±1.23 %) was observed at 30°C under normal culture conditions [Fig. 4.18]. Further, to optimize the temperature for biomass build up, lipid accumulation and pattern of lipid profile, temperatures of 14ºC, 21ºC, 28ºC and 35ºC were selected. Optimal growth with reference to biomass (8.20±1.8 g/L) and total lipid yield (30.12±1.20%) was observed at 28ºC when compared to low temperature on the 6th day. Maximum GLA production, was obtained when the fungus was grown at 14ºC for 6 days. This resulted 21.97±1.09% of the total fatty acids [Table 4.7]. This clearly indicated that low temperature favors PUFAs formation particularly GLA in the cell of *M. rpuxii* CFR-G15.

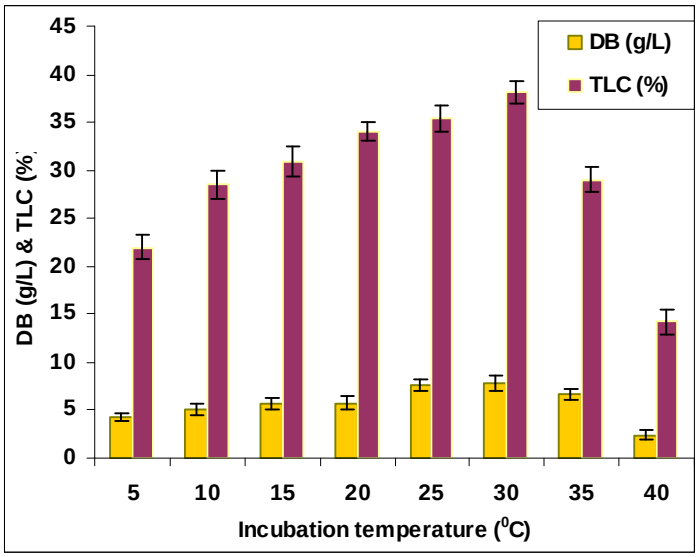


Fig. 4.18 Effect of incubation temperature on biomass and lipid content in *M. rouxii* CFR-G15.

All parameters are expressed as Mean ± SD values of triplicate. DB-Dry Biomass (g/L) TLC-Total lipid content (%)

It was observed that, this culture when grown in low temperature, the biomass and lipid production showed variations. While altering the temperature to low level from the optimal, the highest yield of GLA and other PUFAs were recorded. Another interesting observation was that during the exponential growth condition, the lipid profiles were showing more of the saturated form than unsaturated whereas in stationary phase, the culture was more of an unsaturated form. Hence it was concluded that the degree of unsaturation in the fatty acid composition was influenced by low temperature. This observation was very much in accordance with the studies of Jang et al. (2005). Further, growth in low temperature not only influenced the PUFAs production but also altered the cell morphology (Higashiyama et al. 1999). In this study, culture medium, during the first 24 hours of the growth at 35ºC large fluffy pellets (3-5mm dia) were formed and gradually these pellets transformed into mycelial form whereas in low temperature (14°C) smaller pellets were formed and they were stable.

Table 4.7 Effect of incubation temperature on Biomass, Lipid and GLA production of *M. rouxii* CFR-G15 on 6th day of cultivation.

Temp ° C	Biomass (g/L)	Lipid (%, DB w/w)	GLA/ Total Fatty acids (% w/w)
14	5.6±0.6 [a]	15.75 ±0.61[a]	**21.97±1.09 [c]**
21	7.218±1.1[b]	19.1±0.85[b]	**15.27±0.10 [b]**
28	8.20±1.8[c]	30.12±1.20 [c]	**14.42±0.52[b]**
35	5.9±0.23[a]	27.8±0.24 [b]	**11.17±0.56 [a]**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DB; Dry biomass Lipid; Total lipid in the dry biomass

Effect of temperature on fatty acid profiles of total lipid of *M. rouxii* CFR- G15 on 6th day of cultivation is given in Fig 4.19. Oleic acid (C18:1) was found to be a major fatty acid in all the temperatures tested, followed by palmitic acid (C16:0), GLA (C18:3, n-6), linoleic acid (C18:2) and stearic acid (C18:0). The remaining fatty acids were only fractional. An important observation was made that the increase in GLA and linoleic acid contents inversely proportional to the availability of palmitic acid and oleic acid contents at low temperature level (14°C).

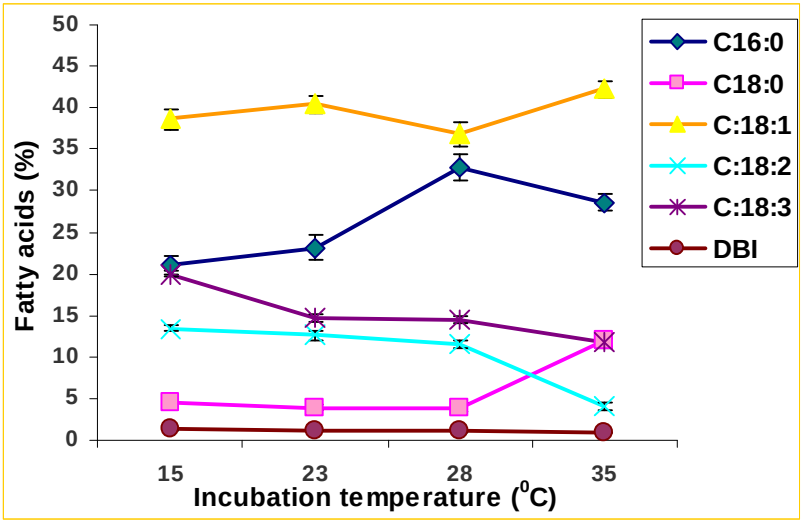


Fig. 4.19 Effect of incubation temperature on Fatty Acid Profiles of *M. rouxii* CFR-G15 (on 6th day of incubation period).
All parameters are expressed as Mean ± values of triplicate.
The fatty acid contents were expressed in relative percentage of total fatty acids.

The increased level of GLA at low temperature could be due to presence of desaturase genes that received signals from a specific sector in the cytoplasmic membrane. Influence of carbon in the cystol led to accumulation of acetyl CoA which further led to unsaturation of long chain fatty acids from fatty acid synthase (Jiang & Gao 2004). This study also revealed that low temperature brought about higher contents of GLA per unit mass. The degree of unsaturation of membrane lipid increased with the lowering of growth temperature.

The degree of unsaturation was also found to be increaseing steadily with lowering of temperature in *M. rouxii* CFR-G15 tested here. This modification could be due to the membrane fluid stability of the organisms as an adaptive mechanism to cold environment. This observation correlates very much with results observed by Robinson (2001) and Sumner et al. (1969b). Multiple interlocking controls and regulatory mechanisms operate within the cell system necessitate or adjust the cell lipid to alter the lipid profile. Certain key enzymes responsible for fatty acid formation become inactivated due to sudden temperature shock. It was observed that temperature shock also alters the structural and functional moiety of the lipid in the cell level (Devem & Manocha 1976; Higashiyama et al. 1999; Shimizu et al. 1988). At lower temperature the synthesis of unsaturated fatty acids was less retarded than their degradation, so that under these conditions, more linoleic acid (C18:2) accumulates in the lipids, with consequent increase in the degree of lipid unsaturation. Varying the temperature for growth of the organism modified its biosynthetic metabolites resulting in an increased or decreased level of unsaturation (Δ/mole) (Quoc & Duacq 1997; Suutari & Laakso 1994). Our results indicated that, the degree of unsaturation was primarily dependant on the concentration of GLA and oleic acid content i.e. higher the concentration of GLA the higher the degree of unsaturation (Δ/mole) was observed in the *M. rouxii* CFR- G15 [Fig. 4.19].

The conversion of saturated into unsaturated fatty acid is a well known phenomenon regulated by desaturase enzymes which require oxygen as a cofactor along with acetyl coenzyme A (acetyl Co-A), acyl carrier protein, reduced nicotinamide adenine nucleotide ($NADH_2$) and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$). Therefore, when the oxygen concentration of the medium falls to a low level, it becomes rate limiting for the desaturation reaction (Choi et al. 1982; Devem & Manocha 1976; Robinson 2001a).

The results of the present study indicated that, *M. rouxii* CFR-G15 produced 30.20±1.20% lipid in their dry cell weight, in which 14.2±0.52% accounted for GLA in an ambient cultivation condition. When the culture was grown at low temperature, the lipid content was 15.75±0.61% the GLA content increased significantly ($p<0.05$) i.e. 21.97±1.09% of the total fatty acid content. Therefore further optimization of temperature as fermentation parameter was needed to produce high percentage of lipid and higher GLA production. Kates & Baxter (1962) have reported that the rate of synthesis and degradation of unsaturated fatty acids were temperature dependent and these rates had different coefficients.

Lower temperatures in the medium brought about higher contents of GLA and PUFAs per unit mass, but fungal growth rate, and lipid yield are decreased. Therefore, the highest overall production of PUFAs and GLA could not be achieved at low temperature. In order to combine the beneficial effect of rapid biomass production at its optimal temperature and then shifting it to a lower temperature generated higher yield of lipid, GLA and other PUFAs in *M. rouxii* CFR-G15. It could be concluded through our results that, maximum biomass and lipid production were obtained at 28ºC and maximum GLA production at 14ºC. Our results also revealed that when culture was transferred from room temperature to low temperature, the lipid content of the mycelium decreased. This indicated that the culture utilized the accumulated lipid in the mycelium as a carbon source when the glucose becomes exhausted in the

medium. In order to adapt to cold environment, the fungus utilized lipids produced by it and protein in the culture medium for mycelial growth. This observation was also made by Sumner & Morgan (1969a); Shimizu et al. (1988). Hence, the combination of two temperatures and also addition of carbon source (glucose) to the culture medium were performed. Fig. 4.20 shows that the biomass, lipid and GLA content were significantly increased when the fungus was grown at 28ºC for four days of incubation and later the temperature was altered to low temperature (14ºC). Addition of glucose (5%) to medium, and the culture was transferred to low temperature; there was significant ($p<0.05$) increase in lipid and GLA content viz, 23.56±0.56% and 19.5±0.52% of the total fatty acids respectively. Formation of lipid and unsaturated fatty acids tends to increase considerably and these results were in agreement with the observation of Lindberg & Molin (1993) and Quoc & Duacq (1997).

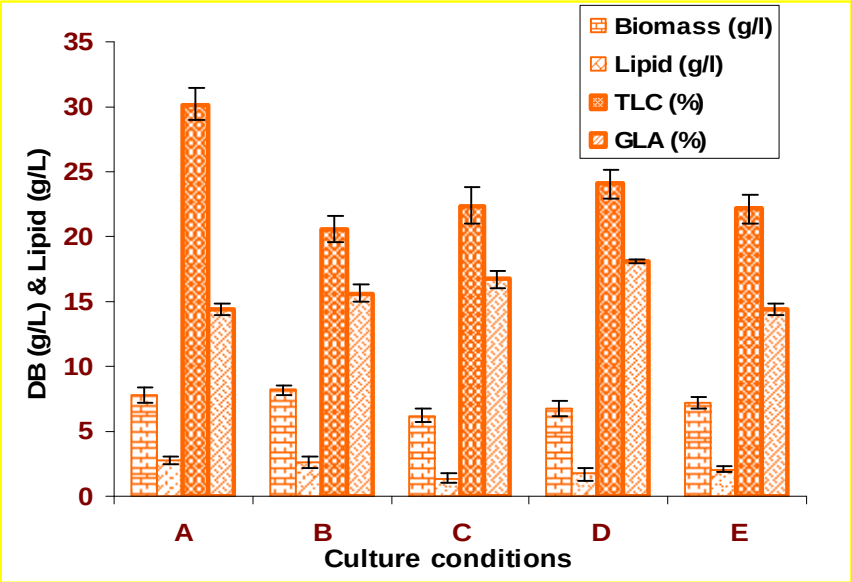


Fig. 4.20 Effect of growth temperature and addition of glucose on biomass, lipid and GLA production by *M. rouxii* CFR-G15

Culture Conditions are as follows: [A] 5 days at 28°C; [B] 5 days at 28°C, 3 days at 12 °C; [C] 5 days at 28°C; 3 days at 12 °C + 3% Glucose; [D] 5 days at 28°C 3 days at 12 °C + 5% Glucose, and [E] 5 days at 28°C, 3 days at 12 °C + 10% Glucose

All parameters are expressed as Mean ± values of triplicate.

Thus our studies concluded that temperature is the principal regulatory factor in the degree of unsaturation in the lipid profile of the organisms. In general, organisms growing under low temperature possess relatively a high degree of unsaturation in their lipid profile and it is a part of the adaptive response to the cold environment. Temperature is also regulating most of the other factors, like pH, water activity, aeration and nutrients inside the cell in relation to other cellular functions and environments. Therefore microbes have to adapt to their cellular composition in accord with the demand. The changes in fatty acid composition of a cell by change in the ambient temperature have also altered the growth rate and dissolved oxygen in the growth medium. In this study, it was possible to enhance the lipid and GLA production of *M. rouxii* CFR-G15 from the baseline significantly ($p<0.05$) 15.75±0.10% to 23.56±0.56% and 14.2±0.52% to 19.5±0.52% respectively. The results presented in this study showed the significant influence of growth temperature and supply of glucose enhanced the production of biomass, lipid and GLA. These finding suggest that *M. rouxii* CFR-G15 may have a potential for commercial exploitation for the production of GLA by fermentation. Upon optimization, for all further studies, room temperature (28±2ºC) was used for culturing this fungus.

Optimization of aeration

To study the effect of aeration on cellular lipid accumulation in *M. rouxii* CFR-G15, batch culture was performed by changing the ratio (R) of volume of the medium to the total volume of the flask (500 ml). The ratio used to optimize the aeration was 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, and 0.4. In this study it was found that, there was an increase of biomass when the ratio of media to volume of flask ratio 0.1 (6.40±0.67 g/L) to 0.2 (7.80±0.67 g/L). Other ratios (0.15, 0.25, 0.3, 0.35 and 0.4) didn't promote biomass production in *M. rouxii* CFR-G15. In this experiment a ratio of 0.2 promoted maximum percentage of lipid on the basis of dry biomass in the cultivation media (33.01±1.14%). The

least lipid percentage (23.35±0.76) was observed at the ratio was 0.4. Other ratios did not greatly vary in their lipid percentage. Lipid g/L, the values were 1.60±0.21 for a ratio of 0.4 and 2.52±0.38 for a ratio of 0.2. None of the values were different. Therefore it was inferred that the ratio of volume of media to volume of flask didn't make a difference in this criteria between 0.2 and 0.4 [Fig.4.21]. It was also noticed that at 0.4, biomass, lipid production and sugar utilization (4.14±0.31 g/L) were considerably reduced. Rests of them were not different. These results suggested that oleaginous microorganisms require considerable aeration for energy, biosynthesis and metabolism. Similar observation was made by Hong et al. (2006).

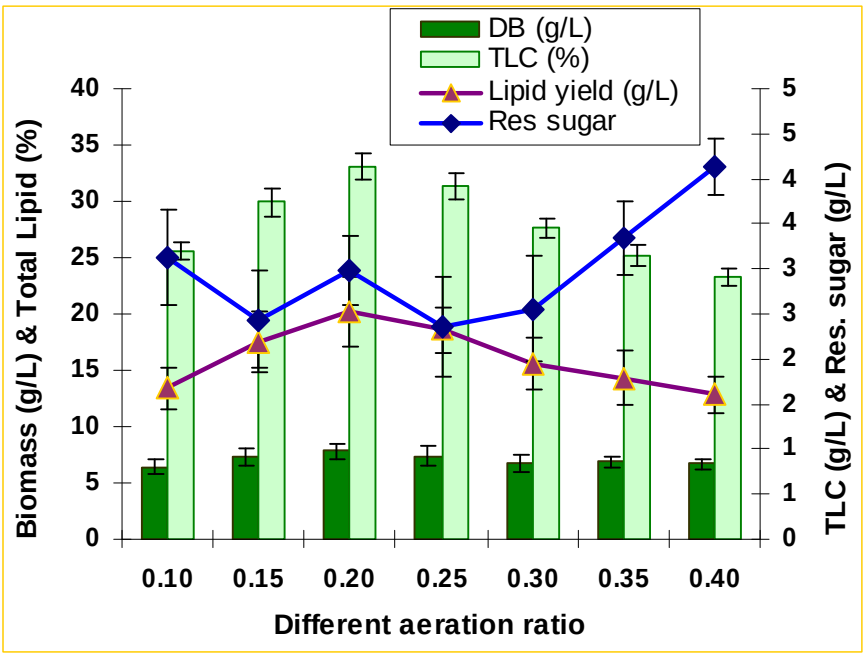


Fig. 4.21 Effect of aeration on biomass, lipid production and residual sugar in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%), Residual sugar (g/L).

The effect of aeration on fatty acid profile of *M. rouxii* CFR-G15 presented in Table 4.8. An analysis of the data showed that, the GLA content expressed as percentage of fatty acids were 12.20±0.64% and 14.74±0.92% at 0.40 and 0.20 ratios respectively. A ratio of 0.2 gave the highest GLA content

(14.74±0.92%). A ratio of 0.25 also produced a high GLA content of 13.50±0.78% and was not different from 0.2 ratio, the GLA ratio value at rest of the aeration ratios were similar. The result also revealed that there was a tendency of increase in the unsaturation index with the increase in aeration but the mean values were not significantly different. At 0.2 ratio unsaturation index was 1.02±0.04 (Δ/mol). These results indicated that aeration was needed for the conversion of monounsaturation status into polyunsaturation. PUFAs are formed through desaturation and elongation from a carbon source in any of the organisms in presence of oxygen i.e. aerobic reaction (Ratledge 1992a). The conversion of saturated into unsaturated fatty acid is known to be regulated by desaturase enzymes which require oxygen as a cofactor along with acetyl coenzyme A (acetyl Co-A), acyl carrier protein, reduced $NADH_2$ and reduced $NADPH_2$. These are greatly necessary for the citrate formation from acetyl CoA in the presence of citrate lyase. If the oxygen concentration of the medium falls to a low level, it becomes rate limiting for the desaturation reaction (Choi et al. 1982; Devem & Manocha 1976; Robinson 2001a).

Table 4.8 Effect of aeration on fatty acid profile in *M. rouxii* CFR-G15

Aeration ratio	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
0.10	3.50±0.19	22.24±1.14	3.07±0.32	9.52±0.84	35.47±1.91	10.28±1.02	**12.68±0.93[a]**	**0.96±0.02**
0.15	2.28±0.39	23.64±1.42	4.20±0.24	6.09±0.82	36.64±2.04	11.88±0.96	**13.17±0.78[a]**	**1.02±0.0.2**
0.20	3.17±0.32	24.05±1.27	3.73±0.35	6.70±0.79	34.05±1.78	12.05±0.74	**14.74±0.92[b]**	**1.02±0.04**
0.25	3.28±0.35	23.64±1.35	4.20±0.49	6.09±0.72	34.64±2.15	12.22±0.96	**13.50±0.78[b]**	**1.02±0.06**
0.30	2.84±0.23	23.83±1.42	2.82±0.51	6.23±0.65	36.99±2.26	12.60±0.81	**12.73±0.86[a]**	**1.00±0.06**
0.35	4.04±0.25	22.23±1.35	3.82±0.38	7.23±0.52	35.99±2.17	12.60±0.95	**12.64±0.78[a]**	**0.99±0.08**
0.40	3.07±0.46	24.55±1.21	2.73±0.34	9.70±0.83	35.05±2.05	12.05±0.91	**12.20±0.64[a]**	**0.97±0.09**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

Optimization of Inoculum Size

Size of the inoculum in the culture medium plays an important role on fungal metabolites like antibiotics (*Penicillium)*, enzymes (*Aspergillus)* and lipid (*Cunninghamella*, *M. rouxii)* (Chen & Liu 1997; Jangbua 2009; Pandey 2003). Inoculum size/density (number of spores per unit volume of fermentation broth) has been attributed as one of the major factors influencing mycelial morphology. This in turn affects mycelial growth and product formation by determining the rheology of the culture and causing diffusion limitation of nutrients within mycelial floes (Chen & Liu 1997; Metz 1977). In this experiment, to study the effect of inoculum concentration on the production of biomass buildup, lipid accumulation and GLA content, lower concentration to higher concentration of inoculum size (0.5 ml, 1 ml, 2ml, 3ml, 4ml and 5ml containing $1.5x10^6$ per ml) were used. Normal inoculum size through out the experiment had been fixed as $1.5x10^6$ spores/ ml. The result obtained in this study is presented in Fig 4.22. Results indicated that 0.5 ml containing $1.5x10^6$ spores /ml produced biomass of 6.40±0.41 g/L and 1 ml produced 7.36±0.55 g/L biomass. Inoculum size 5ml containing $1.5x10^6$ spores /ml had maximum content of biomass (9.12±0.45 g/L). Inoculum size of 4 ml also gave similar results of biomass 8.34±0.65 g/L. Other quantity of inoculum also produced similar results. At 5.0 ml of inoculum size the lipid content (%) was 24.02±1.78, though the biomass production was high the lipid percent was lower. At 0.5 ml 26.20±1.92 % was found. Lipid percentage was highest at 3 ml (32.34± 2.11 %) this value was not different from value obtained from 4.ml (31.34± 2.12 %) or 2.0 ml also it was 29.60±2.48 they were not different. The level of lipid percentage was decreased after inoculum size 3, may be due to lack of aeration (dissolved oxygen) in the culture. An inoculum size of 0.5 ml resulted in a total lipid grams per litre 1.68±0.02.g/L. An inoculum size of 1 ml did not result in great increases in the total lipid content (2.08±0.16 g/L). There was an increase in the total lipid content when the inoculum size was increased to 2, 3, 4, or 5 ml, which were not different. Highest total lipid content

observed with 4 ml of inoculum i.e. 2.61±0.34 g/L. Residual sugar content is given in Fig 4.22. At an inoculum size of 0.5 ml the residual sugar was 5.12±0.46 g/L, this was higher than all the other groups which showed residual sugar content from 2.98±0.90 g/L (1 ml) to 3.56±0.34 g/L (3 ml). Residual sugar content of inoculum sizes 1.0 ml to 5.0 ml was not different.

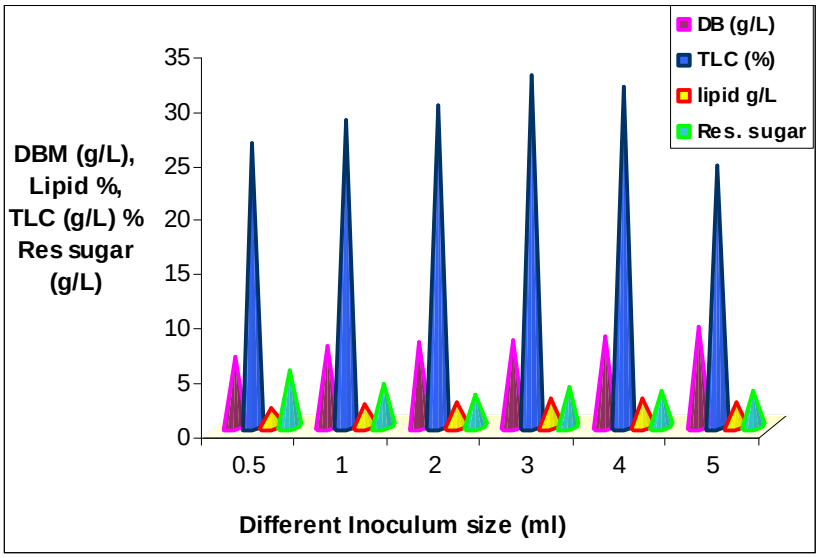


Fig. 4.22 Effect of different inoculum size on biomass, lipid production and residual sugar in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%), Residual sugar (g/L).

Different size of inoculum altered fatty acid profiles in *M. rouxii* CFR-G15 is shown in Table 4.9. Inoculum size 2 ml, 3ml, 4ml produced the same quantities of GLA viz., 15.29±0.95, 14.33±1.04 and 14.23±0.97% respectively. Five ml of inoculum produced a biomass with 11.80±0.81% GLA for the total fatty acid content. Inoculum size of 0.5 ml and 1.0 ml resulted in similar lower values 12.24±0.90% and 13.24±1.10% respectively. The influence of inoculum size on the production of unsaturated fatty acids was studied through double bond index. Though the values varied from 0.96±0.04 (for 5 ml inoculum size) to 1.08±0.08 (for 2 ml of inoculum size) the results were not significantly different. It is evident from the result that 2 or 3 ml of inoculum could be chosen for further studies based on percentage of GLA content. The result

obtained in this study is differing to Chen & Liu (1997) in C. *echinulutu* CCRC 31840, where an increase in mycelial weight apparently resulted in an increased yield of biomass, lipid, and GLA. Generally in fungal fermentation, when the inoculum size increases, the growth of the organisms tends to be more, but the problem of O_2 limitation arises because of the high viscosity of the culture broth due to high biomass and filamentous growth (Shimizu et al. 1988; Sumner & Morgan 1969a). At high inoculum size, stearic acid was present in greater concentration, oleic acid and other unsaturated fatty acid in less concentration. The reason might be that, conversion of stearic acid to oleic acid by desaturase enzyme, when oxygen depletes, possibly the activity of the enzyme also declines since it is an aerobic reaction (Uttaro 2006).

Table 4.9 Optimization of inoculum size on fatty acid profile of *M. rouxii* CFR-G15

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

Different inoculum size (ml)	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
0.5	3.71±0.41	24.86±1.22	2.83±0.34	7.59±0.79	35.58±2.12	11.25±0.61	**12.24±0.90^{b}**	**0.97±0.05**
1.0	2.41±0.51	23.86±1.38	2.83±0.31	7.19±0.67	36.58±2.11	12.35±0.72	**13.24±1.10^{ab}**	**1.01±0.06**
2.0	2.20±0.42	24.57±1.28	3.71±0.41	7.09±0.72	32.07±1.80	13.33±0.88	**15.29±0.95^{c}**	**1.08±0.08**
3.0	2.10±0.25	25.67±1.11	2.43±0.54	7.13±0.47	33.43±2.08	13.25±0.91	**14.33±1.04b^{c}**	**1.05±0.06**
4.0	2.10±0.31	25.79±1.12	2.43±0.57	8.93±0.71	33.43±1.52	12.55±0.89	**14.23±0.97b^{c}**	**1.04±0.07**
5.0	3.37±0.34	26.09±1.61	2.91±0.45	9.82±0.63	30.91±1.25	13.18±1.05	**11.80±0.81^{a}**	**0.96±0.04**

Optimization of chemical parameters

Effect of Different carbon sources

During microbial fermentation, the carbon source acts as a major nutrient for building cellular material and also as energy source (Aggelis et al. 1997; Dyal & Narine 2005; Papanikolaou et al. 2007). Biomass buildup, lipid accumulation, fatty acid profile and its constitution have been influenced by different type of sugars during fermentation (Dyal & Narine 2005; Hansoon & Dostalek 1988; Jang et al. 2005; Papanokolaou et al. 2007; Sajbidor 1988; Somashekar et al. 2002). The carbon sources tested were glucose, fructose, galactose, maltose, lactose, sucrose and starch in *M. rouxii* CFR-G15. The biomass produced during the growth of *M. rouxii* CFR-G15 in media containing different carbon sources is given in Fig.4.23. The disaccharides lactose gave the lowest biomass viz., 2.50±0.54 g/L. The highest biomass production was exhibited in media with starch, glucose and fructose as the source of carbon viz., 8.44±0.34, 8.12±0.61 and 7.51±0.72 g/L respectively. Where as sucrose, galactose and maltose as source of carbon produced biomass between these values 4.20±0.35, 6.40±0.45 and 7.39±0.31 g/L respectively. These results were used as the basis for selection of glucose as carbon source for further studies. Similar results also found in *M. rouxii* and *Mucor* sp. 1b, where the biomass production was in high in both starch and glucose were used as carbon source individually in the media (Somashekar et al. 2002). Findings in this study is in agreement with the results reported by Hansoon & Dostalek (1998) in which biomass production in *M. ramanniana* grown in media containing fructose as the carbon source was only slightly higher than the dextrose. Weete (1980) also observed that both glucose and fructose produce much higher biomass than the sucrose in the media. Result obtained in this study also corresponds to other *Mucor* spp. where the lactose was the poorest carbon source for biomass production (Ahmed et al. 2006; Somashekar et al. 2002).

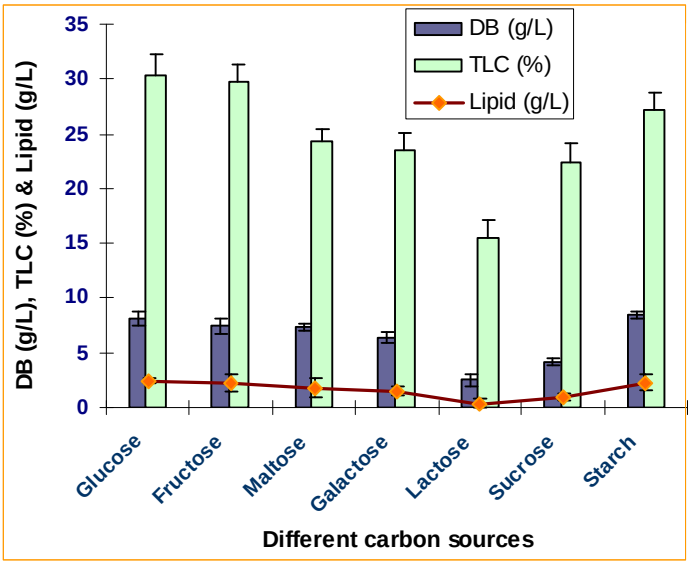


Fig. 4.23 Effect of different carbon sources on biomass and lipid production in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%).

The lipid production (%) in *M. rouxii* CFR-G15 by different carbon sources is given in Fig.4.23. Least percentage of lipid produced by lactose 15.56±1.60 and highest production was by glucose and fructose namely 30.34±1.91% and 29.67±1.73% respectively. These two values were not different. The other sugars galactose, lactose, maltose and starch gave values intermediate between these two levels. It is proved from these studies and similar studies in literature that glucose and fructose are best suited as carbon sources for the media as judged by this criterion. The production of total lipids by *M. rouxii* CFR-G15 showed a different picture when glucose, starch, fructose, maltose and galactose were used as carbon source. They were similar in their total lipid production capacities (g/L) (1.51±0.41 to 2.46±0.23 g/L). When lactose and sucrose were the carbon sources, the total lipid production was as low as 0.39±0.34 g/L and 0.94±0.31 g/L respectively. The variations in the total lipid were neutralised by the difference in the biomass contents when the yield as g/L was calculated.

The GLA content as % of total fatty acids produced from different sources showed the following picture. Glucose produced the highest GLA

content (15.20±0.85%). Lowest GLA content was found with lactose, sucrose and galactose containing media, viz., 9.89±0.57%, 10.39±0.91% and 11.02±0.62% respectively. Little lower values were exhibited by maltose (12.02±0.89%), starch (12.74±0.87%) and fructose (13.31±1.03%) when they were provided as carbon sources [Table 4.10]. Therefore it is inferred that glucose was the best suited carbon source for GLA production when *M. rouxii* CFR-G15 was used. The degree of unsaturation of the lipids from mycelia from lactose containing media was the lowest (0.87±0.06). Rest of the carbon sources gave unsaturation index (DBI) between 0.92±0.07 to 1.08±0.08 (Δ/mol) [Table 4.10]. The result obtained in this experiment was in concurrence with *Mucor* sp. *RRL* 001 where maximum GLA (8.0% of total fatty acids) was obtained when glucose was used as carbon source (Ahmed et al. 2006). Kennedy et al. (2001) also observed that growth of *M. cercinelloides* on glucose showed maximum GLA production (15.0 %) in the total PUFAs. For all the mucorales observed by others, lactose was the poorest carbon sources for GLA production (Ahmed et al. 2006; Somashekar et al. 2002). Furthermore, starch has been considered as an additional competent substrate for biomass production compared with glucose for strains of *C. echinulata* and *M. alpina* (Chen & Liu 1997; Jang et al. 2005; Papanikolou et al 2004). But starch was not suitable for *M. ramanniana* and *Mucor* sp. (Hansoon & Dostalek 1988; Ahmed et al. 2006). Soluble starch was the best carbon source for GLA and total PUFAs production, glucose and glycerol were the next, and galactose and lactose were the poorest in the formation of lipid and GLA in *M. ramanniana var.ramannian* (Dyal et al. 2005). Furthermore, in accordance with the results of this study, growth of *C. echinulata* CCRS 31840 on soluble starch resulted in the synthesis of storage lipid that contained slightly lower GLA quantities compared with the glucose (Chen & Chang 1996). Increased saturated fatty acids of the cellular lipid was observed when the *M. isabellina* and *C. echinulata* grown on apple pectin than glucose, starch or lactose (Papanikolaou et al. 2007).

Table 4.10 Effect of different carbon source on fatty acid profile in *M. rouxii* CFR-G15

Different carbon sources	Fatty acid composition (as % of composition)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
Glucose	2.17±0.37	24.05±1.21	3.73±0.47	5.70±0.65	35.05±2.03	12.05±0.94	**15.20±0.85^{d}**	**1.08±0.08^{c}**
Fructose	1.42±0.31	25.63±1.36	2.21±0.28	6.37±0.54	38.20±1.83	11.24±1.03	**13.31±1.03^{c}**	**1.03±0.05^{bc}**
Maltose	3.16±0.27	24.14±1.28	2.12±0.38	9.28±0.41	33.76±1.14	13.72±0.69	**12.02±0.89^{bc}**	**1.00±0.04^{bc}**
Galactose	3.14±0.53	25.14±1.33	3.12±0.65	8.28±0.81	35.70±1.61	11.72±0.73	**11.02±0.62^{ab}**	**0.92±0.07^{ab}**
Lactose	3.19±0.45	28.80±1.29	1.20±0.58	11.20±1.01	33.37±1.89	10.32±0.84	**9.89±0.57^{a}**	**0.87±0.06^{a}**
Sucrose	3.51±0.51	23.97±1.39	1.72±0.39	8.94±0.90	39.76±1.78	11.11±0.96	**10.39±0.91^{a}**	**1.01±0.07^{bc}**
Starch	1.41±0.49	22.80±1.15	2.89±0.55	8.52±0.83	38.80±1.45	11.27±1.03	**12.74±0.87^{c}**	**1.07±0.08^{c}**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

In microbial fermentations, the simple carbohydrates, especially the glucose assimilated most efficiently and rapidly for the formation of biomass and lipid. In the recent past, for economical production of single cell oil (SCO) a variety of low cost substrates like dairy, distilleries and agricultural wastes have found to be utilized as a major carbon source (Chen et al. 1999; Certik et al. 2006; Conti et al. 2001; Fakas et al. 2008; Gema et al. 2002; Li et al. 2008; Papanikolaou et al. 2007; Waltermann et al. 2000). Several oleaginous microorganism like *Cunninghamella* spp. and *Rhizopus* spp. use starch as a carbon source but they need more incubation period to degrade the substrates and convert into lipid (Chen & Chang 1996). In this study, mucorales which are saprophytic, grew very rapidly and proliferate extensively on simple sugars compared to complex substrates. Similar observation have been reported earlier by Ahmed et al. (2006); Jang et al. (2005); Kennedy et al. (2001); Somashekar et al. (2002). Based on the above observation, all the further experiments have been carried out in FPM medium containing glucose as carbon source.

Optimization of glucose concentration in fermentation medium

Optimization of glucose concentration for maximum biomass, lipid and GLA production was carried out by keeping the concentration of nitrogen source as constant in FPM medium. When glucose levels of 2-10% were tested as the carbon source in the FPM medium, the biomass content was 6.72±0.37 g/L at 2.0% level which was the lowest quantity. There was an increased level (mean values) in the biomass content with increase in the glucose level. At 4, 6, 8 and 10 the values were 7.70±0.58 g/L, 8.53±0.68, 8.27±0.90 and 7.83±0.63 g/L, but the values were not different. The result obtained in this study is presented in Fig 4.24. At a glucose level of 2% the lipid (% of biomass) was 25.60±0.97 %. The lipid level increased from 4 % onward (37.80±1.21%) and was similar at 6, 8, and 10% also. At 8% it was 39.50±1.24% and was the maximum lipid produced. Total lipid (g/L) was 1.72±0.16 at glucose level of 2%. It showed steep rise at 4% level to 2.92±0.31 g/L. The total lipid content 6, 8, 10 % was not different and was 3.19±0.40 g/L, 3.28±0.46 g/L and 3.09±0.30

g/L respectively. The glucose utilized by *M. rouxii* CFR-G15 at 2, 4, 6,8 and 10% was as follows 19.2±0.36, 36.94±0.45, 53.5±0.58, 69.77±0.52 and 83.51±0.70 g/L. With every increment in the glucose levels supplied an increment in the utilization was noticed.

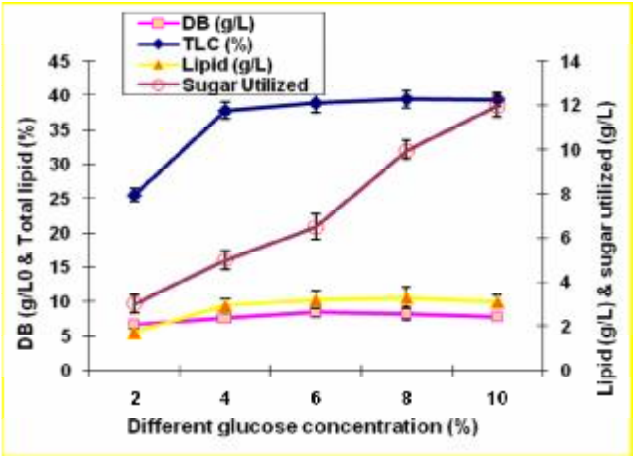


Fig. 4.24 Effect of different concentration of glucose on biomass, lipid production and sugar utilized in *M. rouxii* CFR-G15.

Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%),
Residual sugar (g/L).

Effet of different glucose concentration on fatty acid profile in *M. rouxii* CFR-G15 is shown in Table 11 GLA content at 2 % it was 12.91±0.74%, at 10% level 11.96±0.65% and at 8 % 13.51±0.86 %. Highest GLA content was found at 6% 15.67±0.97 %, at 4% also the GLA content was similar (14.79±0.84%). There is no difference at double bond index in all the levels tested. It ranged from 1.02±0.13 (10%) to 1.12±0.09 (Δ/mol) (6%).

The results obtained in this study agreed with the results of Chen & Chang (1996). When *Cunninghmella* was cultivated for GLA production at higher percentage of carbon source (glucose), didn't yield good amount of biomass and lipid. This effect was also observed in various fungi including *Mortierella* spp. *Mucor* spp. and *Cunninghamella* spp. (Mamatha et al. 2008; Nakhara et al. 1993; Papanikalaou et al. 2004a). It was also reported that fatty acids content increased with the concentration of glucose from 2.0 to 12.0% in *M. alpina* CBS 754.68, while AA content was reversed (Bajpai et al. 1991c; Koike et al. 2001).

Table 4.11 Effect of initial glucose concentration on fatty acid profile in *M. rouxii* CFR-G15

Different conc. of glucose	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
2	2.35±0.53	21.41±1.23	3.35±0.53	7.44±0.64	38.22±1.56	12.65±0.96	**12.91±0.74^{a}**	**1.06±0.08**
4	2.42±0.34	23.53±1.87	3.49±0.64	8.47±0.79	33.41±1.21	13.14±0.98	**14.79±0.84^{bc}**	**1.08±0.07**
6	2.56±0.45	21.78±1.11	3.34±0.45	6.75±0.34	32.59±1.35	14.55±1.02	**15.67±0.97^{c}**	**1.12±0.09**
8	3.41±0.54	21.70±1.15	3.59±0.54	7.91±0.62	34.95±1.85	13.5±1.01	**13.51±0.86^{ab}**	**1.06±0.10**
10	3.40±0.58	21.67±1.26	4.02±0.76	7.65±0.73	37.93±1.87	11.94±1.19	**11.96±0.65^{a}**	**1.02±0.13**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

It was also observed in this study that, further increase in glucose concentration from 6 to 10%, yielded decreased biomass, which may be due to intolerance of the cells to the higher concentration of glucose as the osmotic potential increased in the cultivation medium. This result is concurred with the Ahmed et al. (2006) where glucose concentration increased from initial concentration 2.0 to 12.5%, dry biomass production drastically decreased.

Optimization of nitrogen source

Oleaginous organisms, generally, accumulate lipid as storage compounds when the nitrogen gets exhausted in the cultivation medium by preventing cell growth and the excess carbon gets diverted to lipid storage in the cells as TG (Lindberg & Molin 1993; Papanikalou et al. 2004; Weete 1988). Thus, nitrogen plays an important role in lipid accumulation in all oleaginous microorganisms. In this experiment, various nitrogen sources were used in the cultivation medium to optimize the maximum biomass buildup, lipid accumulation and GLA production. Ammonium sulphate, ammonium nitrate, sodium nitrate, potassium nitrate and ammonium chloride were used as inorganic nitrogen sources and yeast extract, peptone, casein and urea as organic sources. Among the nine sources of nitrogen chosen for the study, ammonium chloride gave lowest biomass of 7.10±0.45 g/L and that shown by the medium containing sodium nitrate was not different and was 7.20±0.34 g/L. Urea gave highest biomass of 8.97±0.35 g/L, yeast extract 8.90±0.48 g/L and ammonium sulphate 8.80±0.31 g/L. Other nitrogen sources were intermediate in the production of biomass in *M. rouxii* CFR-G15. Result is shown in [Fig. 4.25].

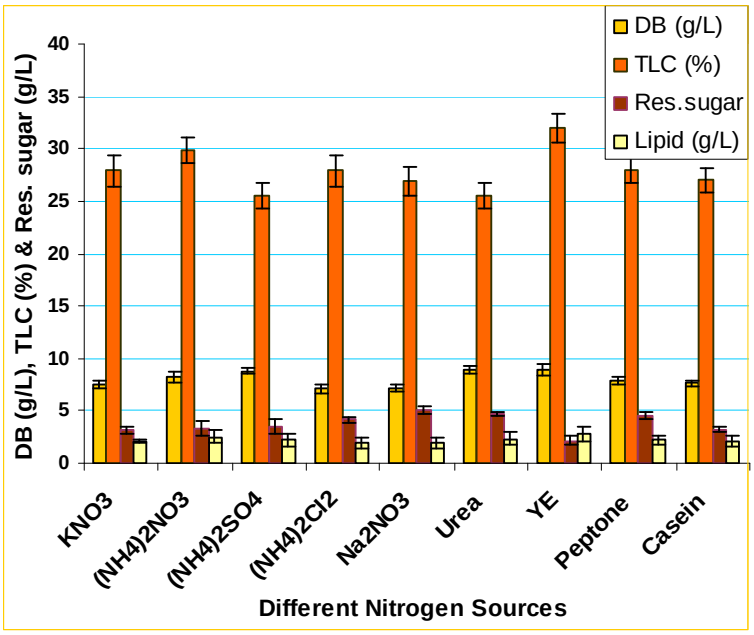


Fig. 4.25 Effect of different nitrogen sources on biomass, lipid Production and residual sugar in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates.
DB- dry biomass (g/L), TLC-Total lipid content (%),
Residual sugar (g/L). YE-Yeast extract

The values of lipid percentage of dry biomass of *M. rouxii* CFR-G15 showed that all the nitrogen sources promoted similar lipid accumulation ranging from 25.49±1.23% (ammonium sulphate) to 28.00±1.31% (peptone). Ammonium nitrate (29.86±1.30%) and yeast extract (32.0±1.37%) showed higher percentage of lipid accumulation. When the total lipid content as g/L, *M. rouxii* CFR-G15 showed that all the nitrogen sources yielded 1.94±0.20 g/L (sodium nitrate) to 2.29±0.17 g/L (urea). Yeast extract (2.85±0.24 g/L) showed high lipid yield in *M. rouxii* CFR-G15. Ammonium nitrate showed little higher value 2.45±0.27 g/L than the other sources but was less than yeast extract [Fig. 25]. The residual sugar content in yeast extract was the lowest i.e. 2.12± 0.42 g/L, this was expected as yeast extract showed very good biomass production and lipid accumulation. The medium containing sodium nitrate as nitrogen source showed the highest amount of residual sugar namely 5.12±0.34 g/L, the biomass content also the lowest in the group. Rest of them values were in between for e.g., potassium nitarate (3.12±0.38 g/L) to urea (4.70±0.21 g/L). Ammonium sulphate and urea though gave more biomass, the lipid content was

found to be very less. Many studies have shown the effect of nitrogen source on the biomass and lipid yield in yeast and fungal species (Evans & Ratledge 1984; Fakas et al 2007a; Park et al. 1999; Somashekar et al. 2002; Totani et al. 2000). Hansson & Dostalek (1988) observed that when potassium nitrate used as nitrogen source gave more biomass, lipid content when compared to ammonium chloride or ammonium sulphate in *M. ramanniana* CBS 112.08. Dyal et al. (2005) showed that 1% yeast extract and 4% glucose as nitrogen source in the cultivation medium gave the best production of biomass, lipid and AA production in *M. ramanniana var ramanniana*. Our result is also very much in agreement with this observation.

The effect of different nitrogen supplement on GLA and other fatty acids production is illustrated in Table 4.12. The GLA content as percent of total fatty acids was lowest in yeast extract medium with the value of 11.02±0.87%. Casein containing medium also resulted a low GLA containing 11.11±0.71%. Though the biomass and lipid content were high, with these two nitrogen sources (casein and yeast extract) the total lipid content was high in yeast extract and a slight variation observed in casein. But these two nitrogen sources when graded on GLA content were as lowest performers in. Ammonium nitrate, peptone, ammonium chloride and potassium nitrate as nitrogen sources produced lipid with GLA contents of 16.64±1.13, 15.77±1.08, 14.94±1.04 and 14.92±0.85% respectively. The unsaturated fatty acid content as measured with the double bond index gave the following results. Media containing casein as source of nitrogen had the lowest double bond index namely 0.82±0.09. The highest values were obtained with media with ammonium nitrate (1.11±0.09) and peptone (1.08±0.07). The other had values in between these two nitrogen sources.

The result obtained in our study is not concomitant with the results obtained by Lindberg & Molin (1993), in *Mortierella alpine* CBS 343.66 where ammonium nitrate was replaced with KNO_3 enhanced the cell growth, total PUFAs and AA production. Yeast extract as sole nitrogen source

stimulated the cell growth, but not for GLA and total PUFAs production (Dyal et al. 2005; Dyal & Narine 2005). Ammonium nitrate was the best nitrogen source for GLA and total PUFAs production. However, *Mortierella* spp. produce maximum GLA at 1% yeast extract and also increase of yeast extract concentration in the cultivation medium promoted the cell growth (Bajpai & Bajpai 1992). Chen & Chang (1996) in their study reported that use of urea as nitrogen source in the cultivation medium enhanced growth and GLA production of *C. echinulata* CCRC 31840. The probable reason that urea has higher nitrogen content as compared to the other nitrogen sources and created an inert atmosphere that has prevented the oxidation of unsaturated fatty acids. Several studies indicated that use of variety of nitrogen sources during fermentation process affect the yield of mycelial growth in different ways (Park et al. 1999).

Table 4.12 Effect of different nitrogen sources on fatty acid profile of *M. rouxii* CFR-G15

Different nitrogen sources	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
KNO_3	3.55±0.43	21.63±1.04	3.45±0.9	6.21±1.02	36.38±1.53	13.64±0.98	**14.92±0.85^{cd}**	**0.93±0.07^{ab}**
$(NH_4)_2NO_3$	3.07±0.38	19.64±1.13	3.36±0.93	6.11±0.98	36.01±1.98	13.56±0.76	**16.64±1.13^{d}**	**1.11±0.09^{c}**
$(NH_4)_2SO_4$	2.89±0.24	24.44±1.75	3.17±0.98	4.10±0.87	39.53±2.02	11.04±0.65	**13.24±0.77^{b}**	**1.01±0.07^{bc}**
$(NH_4)_2Cl_2$	2.14±0.25	24.92±1.59	3.92±0.76	9.47±1.23	29.46±1.98	14.23±1.04	**14.94±1.04^{cd}**	**1.03±0.06^{bc}**
Na_2NO_3	3.22±0.23	21.43±1.45	4.06±0.78	5.62±0.34	37.49±2.32	12.64±0.78	**14.35±1.09^{bc}**	**1.04±0.07^{bc}**
Urea	1.98±0.30	23.32±1.65	1.01±0.83	8.74±1.04	36.15±2.14	13.77±0.92	**14.14±1.05bc**	**0.93±0.08^{ab}**
YE	3.21±0.54	23.20±1.74	4.51±0.93	6.45±0.67	36.21±2.04	13.9±0.87	**11.02±0.87^{a}**	**1.07±0.09^{bc}**
Peptone	1.46±0.26	25.99±1.45	2.34±0.56	5.78±0.57	36.46±1.98	12.12±1.12	**15.77±1.08^{cd}**	**1.08±0.07^{c}**
Casein	2.19±0.12	34.92±2.57	4.19±0.99	8.89±1.03	26.32±1.46	11.21±1.15	**11.11±0.71^{a}**	**0.82±0.09^{a}**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index; YE-Yeast extract.

Effect of C:N ratio

The carbon to nitrogen (C:N) ratio in the growth medium plays a major factor for achieving the high lipid accumulation in oleaginous microorganisms (Fakas et al. 2008; Moreton 1988). The media used for lipid production have to be high C:N ratio so that sufficient carbon left in medium will be utilized for lipid accumulation during culture growth (Ratledge & Wynn 2002). The effect of C/N ratio on lipid production was well studied by most of the researcher (Chen & Chang 1996; Immelman et al. 1997; Koike et al. 2001; Nakhara et al. 1992; Papnikolaou et al. 2008). In this experiment, optimization of C:N ratio for biomass, lipid and GLA production of *M. rouxii* CFR-G15 was carried out. The result obtained in this study is shown in Fig. 4.26. The C:N ratio produced a biomass of 8.60±0.53 g/L, C:N ratio of 20 and 140 resulted in biomass content of 9.28±0.74 g/L and 9.80±0.87 g/L respectively. The highest biomass content was observed when C:N ratio 80 which was 11.73±0.9 g/L. The other C: N ratios values were in between. Percentage of lipid production was lowest in C: N ratio 160 namely 15.34±0.93 %, again the lipid % was highest with the C:N ratio of 80 i.e. 39.45±1.54. No other trends could be influenced from the data. At C:N ratio 160 had a lowest total lipid yield (1.32±0.14 g/L), the highest lipid yield was obtained at C:N ratio of 80 i.e. 4.64±0.61 g/L. A C:N ratio 20 also showed a trend similar to 160 C:N ratio. The residual sugar content increased from C:N 20 to 140. There is a slight lowering in the residual sugar in 160 C:N ratio. Residual sugar in 20 C:N ratio was 1.15±0.23 g/L and at 140 it was 13.78±0.57 g/L, at 160 it was 12.17±0.53 g/L.

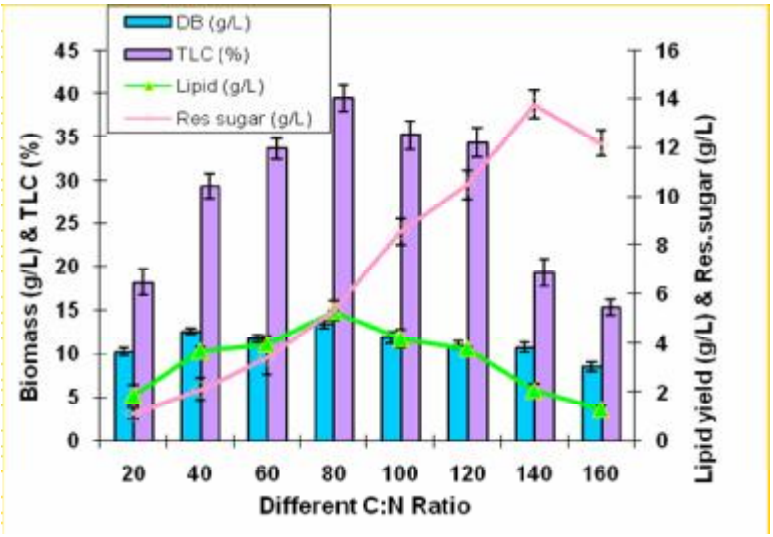


Fig. 4.26 Effect of different C:N ratio on biomass, lipid production and residual sugar in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates. DB- dry biomass (g/L), TLC-Total lipid content (%), Residual sugar (g/L).

The GLA content as a percent of lipid content was lowest at a C:N ratio 160 and 140 and were 8.44±0.66 and 9.33±0.59% respectively. The GLA content was highest at 60 and 40 C:N ratio and were 15.17±1.15 and 13.78±1.04% respectively. The double bond indices of C:N ratio of 20 to 160 was the same[Table 4.13]. Optimum C:N ratio for maximum biomass, lipid and GLA production in this organism was found between C:N ratio 40 and 80 .

In general, when the C:N ratio was tested, the lipid accumulation accelerated after depletion of nitrogen source in the medium and high lipid accumulation observed at high C:N ratio. When high C:N ratio was used in cultivation medium, the rate of glucose uptake could be higher that lead to increased ATP production. Consecutively the ATP production increases cell energy charge that would inhibit isocitrate dehydrogenase (ICDH) activity (Ratledge & Wynn 2002). However in oleaginous fungi the inhibition of ICDH, triggers the lipid accumulation in the mycelium (Papanikolou et al. 2004; Wynn et al. 2001). Our study also revealed that during lipogenic phase, GLA content was found to be decreased. Furthermore, the result obtained in the experiment is supported with the report of Kennedy et al. (1993). They used

two strains to study lipid and GLA production potentially and observed that lipid accumulation increased at high C:N ratio but GLA content was very low. Kavadia et al. (2001) indicated in his study, the change of GLA content in the lipogenic phase was observed in oleaginous zygomycetes fungi. It could be the commencement of growth i.e. spores development, GLA synthesized in more amount and utilized for proper functioning of membrane while in lipogenic phase GLA synthesis decreases. Similar observation was made on *M. ramannian var. angulispora,* when organisms enter the lipogenic (stationary) phase, the conversion of LA to GLA decreases (kamashika et al. 1990). All the above findings lipid accumulation and GLA synthesis and our results are similar with the.

Fakas et al. (2008) & Koike et al. (2001) studied the control of C:N exerts on lipogenesis in media comprising agro-industrial waste that contain organic nitrogen was investigated. The studies showed that rate of carbon uptake and nitrogen availability were major factors affecting lipid accumulation process in oleaginous microorganisms. When the carbon uptake was more, the lipid accumulation occurred even in the presence of high amount of nitrogen in the growth medium. Therefore, when the waste residues were evaluating, the medium C:N ratio should not be considered as an adequate criterion during their fermentation time. When the C:N ratio was higher than 20, the mycelial weight and AA decreased due to N_2 limitation. The GLA production and total PUFAs in *Mucor recurvus* sp increased significantly to the maximal level. C:N ratio of 35 was found to be optimal for maximum GLA production (Li et al. 2008). GLA content decreased sharply when the C:N ratio was lower than 30 or higher than 40 in *Mucor* sp. RRL 001(Ahamed et al. 2006). Jang et al. (2005) studied the effect of C:N ratio on PUFAs production in *M. alpina*. Results obtained indicated that there was significant differences among C:N ratio, final pH, cell dry weight and PUFAs production. Thus, it could be concluded through our studies that, the relative proportion of C:N ratio play major role in biomass production, lipid and GLA accumulation in *M. rouxii* CFR-G15.

Table 4.13 Optimization of C:N ratio on fatty acid profile of *M. rouxii* CFR-G15

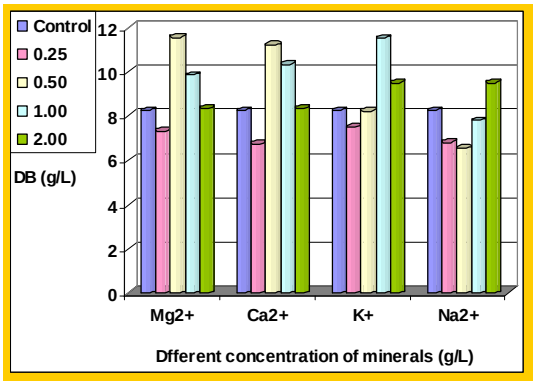
Different C:N ratio	Fatty acid composition (as % of total fatty acids)							
	14:00	**16:00**	**16:01**	**18:00**	**18:01**	**18:02**	**18:03**	**DBI**
20	2.98±0.56	23.39±1.34	4.45±0.26	10.91±1.23	35.57±2.13	10.61±1.01	**11.56±0.90^{b}**	**0.90±0.07**
40	2.57±0.47	22.26±1.23	4.10±0.34	10.04±1.34	32.70±2.04	12.72±1.36	**13.78±1.04^{cd}**	**1.04±0.09**
60	2.80±0.78	22.81±1.52	4.22±0.63	9.10±1.56	32.00±1.98	12.70±1.81	**15.17±1.15^{d}**	**1.07±0.12**
80	1.59±0.82	22.12±1.31	4.33±0.56	10.30±1.03	33.88±2.32	12.12±1.45	**12.34±0.85bc**	**0.99±0.05**
100	1.51±0.45	23.92±1.45	3.67±0.34	12.06±1.56	34.11±2.56	11.27±1.31	**12.17±0.87^{bc}**	**0.97±0.10**
120	2.22±0.39	22.24±1.63	3.91±0.78	12.50±1.34	35.51±2.69	10.23±1.09	**11.48±0.85^{b}**	**0.94±0.09**
140	2.66±0.43	25.26±1.37	3.84±0.78	13.50±1.78	34.30±2.64	9.61±1.12	**9.33±0.59^{a}**	**0.85±0.06**
160	2.68±0.71	27.13±1.56	2.10±0.56	13.50±1.65	36.30±2.87	8.06±0.98	**8.44±0.66^{a}**	**0.80±0.04**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

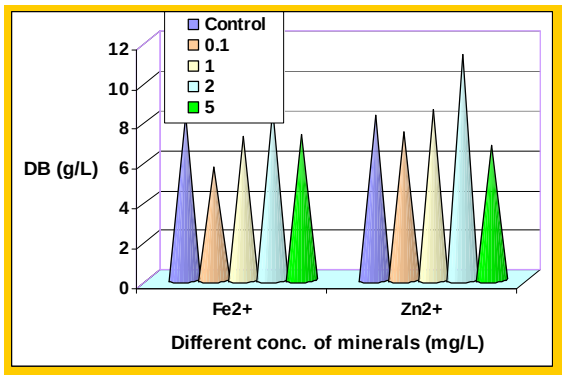
Supplementation of Minerals to enhance the GLA production

Several studies indicated that addition of minerals like Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+} ions with different concentration influence the growth, lipid and PUFAs production, in *M.ramanniana var ramanniana, M. ramanniana, M. isabeeliana, Rhizopus nigricans* SSS 0088 and *Cunninghamella* sp.2A1 (Bandyopadhyay et al. 2003; Dyal et al. 2005; Higashiyama et al. 1999; Muhid et al. 2008). A study was carried out for optimization of Mg^{2+}, Ca^{2+}, K^{+} and Na^{2+}, (at levels of 0.25, 0.5, 1.0 and 2.0 g/L), Zn^{2+}, Fe^{2+} (at levels of 0.1, 1, 2, 5 mg/L) and Cu^{2+} and Mn^{2+} (0.1, 0.25, 0.5 1 mg) supplemented to basal medium (FPM) to know their influence on lipid and GLA content in *M. rouxii* CFR-G15. The results indicated that when this organism cultivated in basal medium without any addition of minerals, the production of biomass (8.24±0.43 g/L), lipid (30±1.32%) and GLA (14.2±0.53 %) was observed. Supplementation of Mg^{2+} in the culture medium and biomass build up at 0.25 & 2 g/L resulted in the lowest biomass content were 7.36±0.97 g/L and 8.35±1.01 respectively. At 0.5 g/L gave highest biomass of 11.56±0.25 g/L which was higher than in control. Addition of Mg^{2+} at a level of 0.5 g/L resulted in a significant increase in the biomass production by the fungus *M. rouxii* CFR G15. Further increment of Mg^{2+} level at 1 g/L and 2 g/L resulted in a biomass level lesser than 0.5 g/L. Ca^{2+} supplementation to media at 0.25 g/L resulted in the lowest biomass 6.82±0.36 g/L, which was lower than the control. Raising the Ca^{2+} level in media to 0.5 g/L resulted in the biomass production in the group 11.20±0.34 g/L, which was higher than that found in basal medium. There was a lowering biomass when Ca^{2+} level raise to 1 and 2 g/L. Addition of K^{+} at 0.25 and 0.5 g/L did not result in the increment in the biomass content. Supplementation of K^{+} at 1 g/L resulted in the considerable increment in the biomass content to 11.43±0.50 g/L. Addition of Na^{2+} at 2 g/L to 9.45±0.51 g/L resulted in a small increases in the biomass content when compared to control. Since the source of Na^{2+} was sodium sulphate salt, it was noticed that apart from contributing to the medium, it resulted in a change in

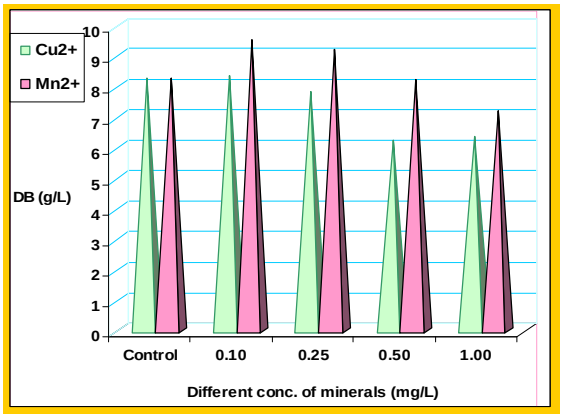
pH of the medium, which might have impact on biomass production. Addition of Fe^{2+} at the level chosen namely at 0.1, 1, 2 and 5 mg/L did not result any improvement in the production of biomass content. Addition of Zn^{2+} to medium resulted in an increase in the biomass 11.25±0.47 g/L at 2 mg/L. Addition of Cu^{2+} at the levels used in this study did not show any increase in the biomass content (rather it brought down the biomass level considerably). Addition of Mn^{2+} to the medium resulted in increased biomass at 1 mg/L. Further increments in Mn^{2+} addition brought down the biomass production in the levels studied. The results are given in Fig. 4.27a, b, & c.



a



b



c

Fig. 4.27a,b,c Effect of different Minerals on biomass production in *M. rouxii* CFR-G15.

Data pressed as mean ±SD of three replicates. DB- dry biomass (g/L).

Addition of different mineral at different concentration to the medium on percentage of lipid content shown in Fig. 4.28a, b, & c. Addition of Mg^{2+} to medium at 0.5 g/L resulted in an increase in the lipid content (36.19±0.86%). Supplementation of Ca^{2+} at 0.5 g/L resulted in an increase of lipid 35.38±0.92 %. K^{+} supplementation at 0.5 g/L increased lipid level to 34.67±1.25. Levels used in the Na^{2+} supplement experiment didn't result any increase the lipid percent. 0.1 mg/L of Fe^{2+} resultant lipid 33.46±1.25 %. Further increase in levels lowered the lipid percent level. Addition of 1 mg/L of Zn^{2+} slight increase in the lipid percentage 35.33±1.11, further addition resulted in decrease in the lipid percent. Addition of Cu^{2+} at the levels of used in this experiment didn't show any raise in the lipid content. Addition of Mn^{2+} in the level used in the experiment didn't result in any increase in the lipid percent level. Total lipid yield (g/L) of *M. rouxii* CFR-G15 in basal medium was 2.48± 0.18 g/L. Addition of Mg^{2+} in the medium resulted in a value of 4.19±0.16 g/L, which is above the control value. Addition of 0.5 g/L Ca^{2+} to the medium resulted in 3.96±0.22 g/L. Supplementation of 1 g/L K^{+} resulted in total lipid values of 3.63±0.0.15 g/L. Na^{2+} level didn't increase total lipid yield at any level of the supplement studied. Addition of iron at the levels studied didn't improve the total lipid yield in the *M. rouxii* CFR-G15, rather the total lipid yield was considerably lower at all the four level studied. Addition of the Zn^{2+} didn't result in an increase in yield of total lipid. Supplementation of Cu^{2+} to the medium at the levels studied didn't result an increment the total lipid yield. Supplementation of Mn^{2+} at the levels studied didn't result in increase of lipid yield.

Minerals influenced significantly the production of GLA in *M. rouxii* CFR-G15 [Fig. 4.29a, b & c]. GLA content of the group supplemented with Mg^{2+} at 0.5 g/L resulted in a GLA content of 24.56±0.72 % as total fatty acids. When compare to the unsupplemented basal medium which was 14.25±0.53 %. Ca^{2+} content at 0.5 g/L resulted in an increase in the GLA content to 23.52±0.96%. K^{+} supplementation at 0.5 g/L resulted in an increment in GLA content over the basal medium (20.83±1.23 %). 1 g/L resulted in a value of

22.91±1.27 % which was not significantly different. Na^{2+} at 1.0 g/L supplementation resulted in a higher GLA content of 21.05±1.21% which similar values of 20.68±1.25 and 21.54±0.82 at 0.5 g/L and 2.0 g/L respectively.

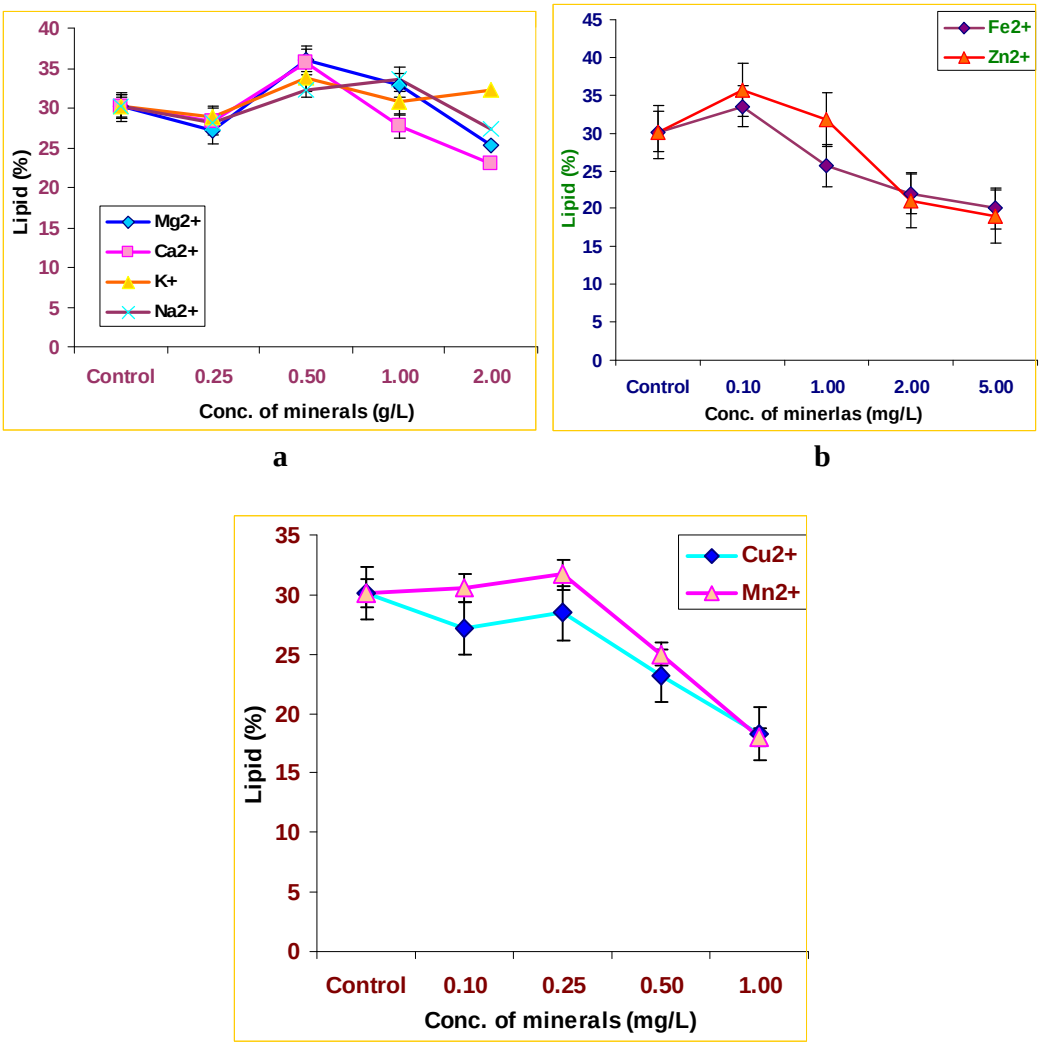


Fig. 4.28 Effect of different Minerals on total lipid content in *M. rouxii* CFR-G15.

Data are expressed as mean ±SD of three replicates.
TLC-Total lipid content (%),

Supplementation of Fe^{2+} resulted in a GLA of 19.47±0.84% at 0.1 mg/L and was higher than the content of the basal media in *M. rouxii* CFR-G15. Supplementation of Zn^{2+} at 0.1 mg/L resulted in a slight increase in GLA content to 16.79± 0.73%. Cu^{2+} didn't result any increment in the GLA content of the lipid. Supplementation of 1 mg/L Mn^{2+} resulted in a value similar to that of basal medium and was 15.06±0.84%. The GLA production of the fungus for the individual minerals was the best (among the several concentrations of the minerals) at the mineral concentration of Mg^{2+} and Ca^{2+} at 0.5 g/L. Thus comparing the results obtained by the addition of minerals at their various concentrations it was observed that Mg^{2+} and Ca^{2+} as an additive to the basal medium was most effective for enhancing GLA yield. The degree of GLA production was almost 1.5 fold in comparison to the basal medium alone and this enhancement can be considered to be significant.

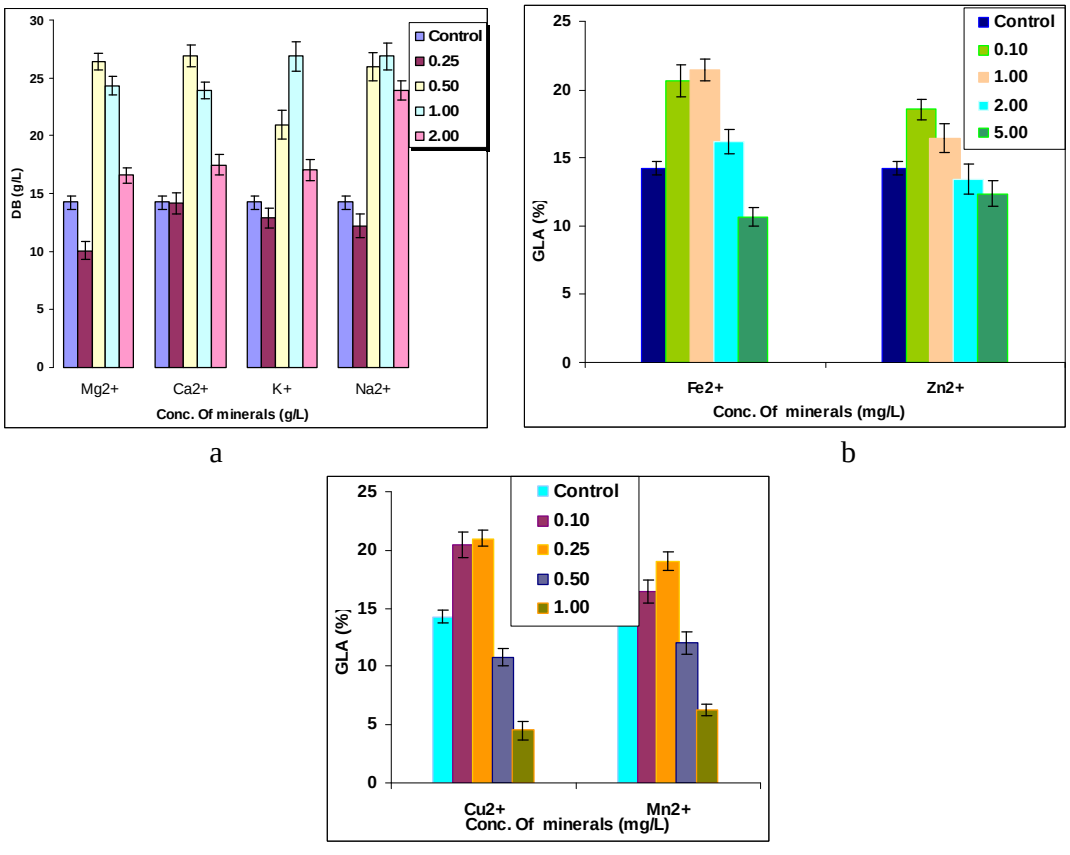


Fig. 4.29 Effect of different Minerals on GLA content in *M. rouxii* CFR-G15.
Data are expressed as mean ±SD of three replicates.
GLA expressed as relative % of total fatty acids

Furthermore, results obtained in this study concurred with the *M. ramanniana var. ramanniana,* that media supplemented with Mg^{2+}, Cu^{2+} and Mn^{2+} yielded more lipid when compare to medium without any mineral supplements (Dyal et al 2005). Maximum lipid production was attained in *M. ramanninana* when addition of Cu^{2+} (0.005 g/L) and Zn^{2+} (0.0075 g/L) to the growth media (Nakhara et al. 1992). This study also revealed that lower concentration of Fe^{2+} and Zn^{2+} ions increased lipid production from 30 to 36% in *M. rouxii* CFR-G15. The probable reason may be due to low activity of enzyme concentration caused implicating in lipogenesis. Metal ions are known to have important role as co-factors for enzyme activities like Malic enzyme, ATP citrate lyase and fatty acid synthase. These three enzymes play major key role in the lipid production since malic enzyme plays a role in NADPH source for fatty acid synthase when ATP citrate lyase accumulate acetyl CoA as a precursors to the fatty acid biosynthesis (Evans & Ratledge 1985). Similar observation was made by Muhid et al. (2008) when Mg was added to the culture medium it showed an increased percentage of lipid in *Cunninghamella* sp.2A1, it had influence the ATP citrate lyase and Malic enzyme, thus inturn influence the Acetyl CoA and NADPH formation lipogenesis (Muhid et al. 2008; Wynn et al. 1999; 2001) and also in *Mortierella alpine* CBS (Hansoon & Dostalek 1988). Shasi et al. (1989) also observed that, ATP citrate lyase dependent on Mg^{2+} for lipogenesis activity in oleaginous yeast like *Rhodotorula gracili.* Metal ions like Mg^{2+}, Mn^{2+} and other bivalent ion have also found to be an important co factor for malic enzyme activity (Totani et al. 2000). Higashiyana et al (1998) reported that influence of addition of minerals, such as Na^{2+}, K^{2+}, Ca^{2+}, and Mg^{2+} on AA production by *Mortierella alpine* IS-4. He observed that 1.5% soy flour medium with the addition of 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2$ and 0.05% $MgCl_2$ enhanced the AA yield 1.7 fold over that of unsupplementation (without any minerals) medium. He also observed that variations in the morphology of the oraganism, when minerals were added to media. The morphology of the fungus was filamentous when KH_2PO_4 supplemented to the media. Where as the morphology of fungus was

large pellet form (with diameters of 2-3 mm) with the addition of Na_2SO_4, $CaCl_2$ and $MgCl_2$ minerals. The possible explanation may be that an excess electrolyte concentration, when added in the form of minerals mixture inhibited the microbial growth and consequent lipid production and GLA yield. Small amount of these ions are usually needed for cell growth and enzyme activity. The lipid yield per unit biomass and the fatty acid profile are both influenced by the type and concentrations of the added metal ion our observation is in with other observation (Hansoon & Dostalek 1988; Sajbidor et al. 1992).

Effect of oils supplements on GLA production

Several reports had indicated that, oleaginous yeasts and molds as well as non oleaginous microorganisms grow and accumulate significant amounts of lipids, when vegetable oils and certain animal fats were used in the growth medium as a sole carbon source. (Aggelis 1996; Aggelis & Sourdins 1997; kamasika et al. 1990). The idea behind the supplementation of various vegetable oils in growth media was that, unlike conventional carbon sources, they would provide precursors for the formation of long chain fatty acid in the cell system (Aggelis et al. 2003; Dyal et al. 2005; Papanikolaou et al. 2002a). When microorganisms grow on plant oils, the organisms either cease the lipid synthesis or it continues to produce longer chain length fatty acids with higher degree of unsaturation. The objective of this study is to incorporate certain oils in the growth medium as carbon source to increase the biomass, lipid and GLA content in *M. rouxii* CFR-G15. The exogenous oils like coconut, sunflower, gingelly, mustard, palm, ground nut and niger seed oils as an alternative to carbon source were supplemented to media at 2% level by replacing glucose. Basal medium which contain 4% glucose was used as the control. The selected oils were rich in myristic, palmitic and linoleic acids. The effect of different oils supplements on biomass, lipid percent and lipid yield is given in Fig. 4.30 The biomass produced with control was 8.27±0.41 g/L. The quantity of biomass obtained when mustard and palm oil were used where 4.60±0.89 g/L

and 6.27±0.71 g/L respectively. All the other oils produced higher biomass than the control. The production of biomass when different oils were supplemented as follows: gingelly 10.44±0.72 g/L, Sunflower 12.13±0.63 g/L, niger seed 12.49±0.69 g/L, coconut 14.20±0.87 and ground nut 16.01±0.74 g/L. All these oils were capable of generating higher biomass production by the organism *M. rouxii* CFR-G15 when compared to control.

The lipid percent produced by the *M. rouxii* CFR-G15 in basal medium was 30.67±1.53%. The lipid production was higher in mustard and sunflower oil and were 41.53±1.15% and 43.79±1.57% respectively. These two values were not different. Lower lipid production was shown when other oils were supplemented studied they were coconut (14.47±1.53%), palm oil 15.91±1.31% gingelly oil 21.74± 1.22%, groundnut oil 22. 53±1.00 and niger seed oil 27.73±1.31. Total lipid yield (g/L) accumulated was 2.54±0.23 in the control. *M. rouxii* CFR-G15 grown in niger seed oil, ground nut oil and sunflower oil had yielded higher lipid values, they were 3.41 ±0.26, 3.60±0.19 and 5.32±0.65 g/L respectively. Palm oil and mustard oil containing media could generate only 0.99±0.09 g/L and 1.90±0.31 g/L respectively.

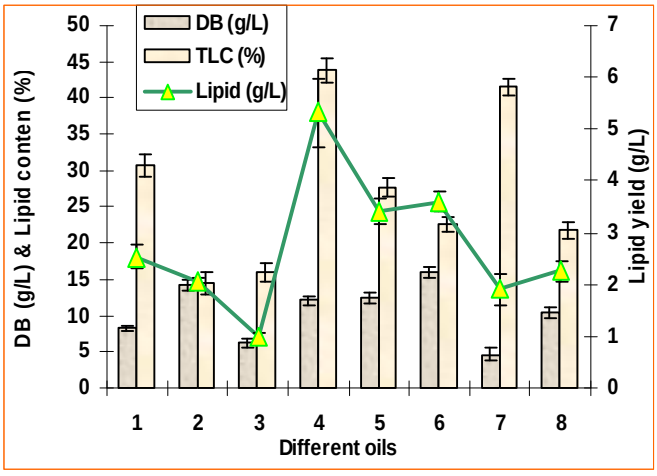


Fig. 4.30 Effect of Different plant oils on biomass, and lipid production in *M. rouxii* CFR-G15.

Data are expressed as mean ±SD of three replicates.

DB: Dry Biomass (g/L); TLC: Total lipid content (%).

1. Glucose 2. Coconut oil 3. Palm oil 4. Sunflower oil 5. Niger seed oil 6. Groundnut oil 7. Mustard oil 8. Gingelly oil

The lipid percent produced by the *M. rouxii* CFR-G15 in basal medium was 30.67±1.53%. The lipid production was higher in mustard and sunflower oil and was 41.53±1.15% and 43.79±1.57% respectively. These two values were not different. Lower lipid production shown by the other oils studied they were coconut (14.47±1.53%), palm oil 15.91±1.31% gingelly oil 21.74± 1.22%, groundnut oil 22. 53±1.00 and niger seed oil 27.73±1.31. Total lipid yield (g/L) accumulated was 2.54±0.23 in the control. *M. rouxii* CFR-G15 grown in niger seed oil, ground nut oil and sunflower oil had yielded higher lipid values, they were 3.41 ±0.26, 3.60±0.19 and 5.32±0.65 g/L respectively. Palm oil and mustard oil containing media could generate only 0.99±0.09 g/L and 1.90±0.31 g/L respectively.

Fatty acid profile of the *M. rouxii* CFR-G15 grown in media with supplementation of different plant oil is shown in Table 4.14. GLA content of 14.42±1.16% was observed in control medium. Medium with niger seed oil resulted in lipid accumulation with higher GLA content of 16.30±1.19%. Ground nut oil and mustard oil promoted lipid accumulation of 14.90±1.03 % and 13.30±1.12 % which were similar to that observed in the control. Palm oil, coconut oil, gingelly oil and sunflower oil promoted lipid accumulation with a GLA content of 5.94±0.58, 6.46±0.79, 9.97±0.58 and 12.40±1.04% respectively. LA content was found to be more in most of the plant oils and it made a precursor for formation of fatty acids (GLA). In our results, we could also find that the GLA was very less when medium was supplemented with palm oil and coconut oil as there was little LA content in these oils, this may be due to poor incorporation of saturated fatty acids into mycelium to be converted to LA and GLA (PUFAs). It is confirmed through our experiment that LA is the precursor for the formation of PUFAs in oleaginous microorganisms.

Table 4.14 Effect of different Plant oils on fatty acid profile of *M. rouxii* CFR-G15

Different Plant Oils	Fatty acid composition (as % of total fatty acids)							
	14:0	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**DBI**
Glucose	1.5±0.14	20.6±2.15	3.4±0.01	8.4±0.98	37.4±1.85	13.5.±1.10	**14.42±1.16^{cd}**	**1.11±0.9^{c}**
Coconut	8.09±0.13	32.4±2.67	1.6±0.0.34	8.16±0.96	28.54±1.56	13±1.07	**5.94±0.58^{a}**	**0.76±0.09^{a}**
Palm	5.14±0.15	39.6±2.56	2.2±0.03	12.2±1.04	26.8±1.98	6±1.36	**6.46±0.79^{a}**	**0.80±0.08^{a}**
Sunflower	1.55±0.143	18.5±1.43	2.6±0.68	7.67±1.23	35.67±2.13	18.00±1.31	**12.30±1.12^{c}**	**1.04±0.11^{b}**
Niger	2.5±0.13	12.2±1.01	2.6±0.42	4.95±1.02	29.30±1.90	31±2.18	**16.30±1.19^{d}**	**1.12±0.19^{cd}**
Ground nut	4.35±0.19	20.6±2.16	1.2±0.23	12.9±1.67	28.77±1.78	16.3±0.98	**14.90±1.03^{cd}**	**1.03±0.09^{bc}**
Mustard	1.37±0.0.12	18.6±1.04	4.9±0.85	5.96±0.98	24.47±1.45	32.05±2.29	**13.30±1.12^{c}**	**0.9±0.09^{b}**
Gingelly	3.26.37±0.14	19.94±1.23	2.8±0.71	8.26±1.03	23.47±1.25	27±1.78	**9.97±0.58^{b}**	**1.19±0.12^{d}**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.

Optimization of supplementation of niger seed oil

Niger oil is a low cost carbon source when compared to other oils like sunflower oil, ground nut oil, mustard oil and gingelly oil. In yet another experiment, we observed that supplementation of niger seed oil (2% level) in the cultivation medium yielded 16.30±1.19% GLA. To optimize the niger seed oil concentration in the growth medium, the niger oil was incorporated in different concentration (1, 2, 3, 4 and 5% level). Two types of comparison were made for studies with niger seed oil, one with 4% glucose control and other one with 2% glucose control. 1, 2, 3, 4 and 5 % of niger seed oil was added to a 2% glucose containing media. The biomass production when compared with the 4% basal control which produces a biomass 8.27± 0.41 g/L, 1% and 2% didn't make any difference in the biomass content. A level of 3% niger seed oil gave the highest content of biomass of 14.20 ±1.04 g/L. 4 and 5 % respectively generated a biomass content of 12.00±0.68 and 11.34±0.71 g/L. The result is given in Fig 4.31a.

The Fatty acids as percentage of fatty acids of *M. rouxii* CFR-G15 grown in media with 1-5% niger seed oil supplementation was as follows: The GLA as percentage of fatty acids of *M. rouxii* CFR-G15 grown in media with 1-5% niger seed oil supplementation was as follows: GLA content of the control (unsupplemented media) was 14.42±1.16%, higher value at 4 and 3% supplementation was noticed and the values were 16.42±1.01% and 19.32±0.95% respectively. Supplementation of niger seed oil at 1 and 2% resulted in similar values of GLA and 5% lowered the GLA content. DBI of the control was 1.16±0.09%, 1 and 2% supplementation didn't increase the DBI. Niger seed oil supplementation with 3% gave slightly higher DBI viz., 1.37±0.06. 4 and 5% didn't result any change in DBI. The results is given is shown in Fig. 4.31b

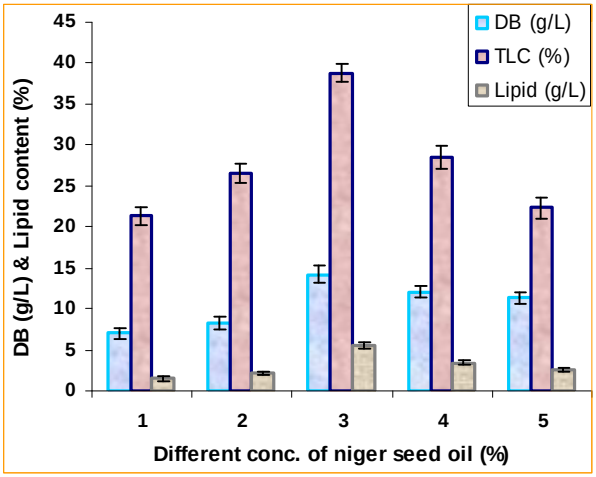


Fig. 4.31a Effect of different concentration of niger seed oil on biomass and lipid production in *M. rouxii* CFR-G15

Data are expressed as mean ±SD of three replicates. DB: Dry Biomass (g/L);TLC: Total lipid content (%).

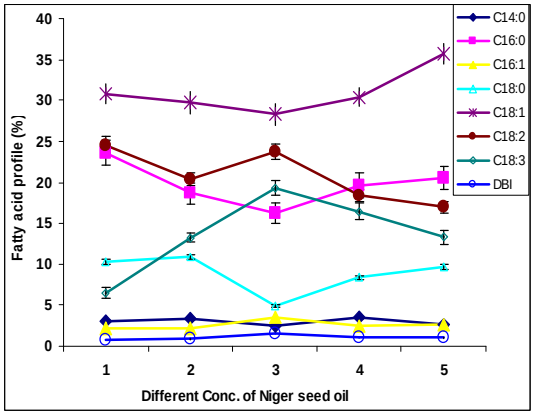


Fig. 4.31b Effect of different concentration of niger seed oil on fatty acid composition of *M. rouxii* CFR-G15.

Data are expressed as mean ±SD of three replicates. Fatty acids are expressed as relative percentage of total fatty acids, DBI- Double bond index.

Comparison of the same experimental results with 2% basal medium (Control) showed the following result. Addition of niger seed oil at all the levels used, resulted in the increase of biomass. The control showed the dry biomass of 6.72± 0.37 g/L. there is no difference in the 1 and 2% level of addition. There is an increase to 14.20±1.04 g/L at 3% addition. At 4 and 5% addition the values were 12.00±0.68 and 11.34±0.71 g/L respectively. The biomass content doubled at 3% level supplementation *M. rouxii* CFR-15. The lipid content at 2% control was 24.93±0.58%. At 3 % level the lipid content

was 38.80±1.10 %, at 5% level there was a lowering in the lipid content. At level 2 % niger seed oil supplementation it was 22.34±1.15 %. The lipid yield at 2% control was 1.67±0.10 g/L. Addition of niger seed oil at 1, 2, and 5% were not different but slightly higher values than the control. At 3 % level a lipid yield of 5.51 ±0.51 g/L was noticed. At 4% it was 3.42±0.31 g/L. The GLA content of the 2% control was 11.24±0.63%. There is an increase in GLA content with 1, 2, 3, and 4% but the higher mean values at 5% level it was similar to control. At 3%, supplementation gave GLA content of 19.32±0.96%. The DBI of 2% control was 1.01±0.04. At 3% supplementation level an increase in DBI to 1.37±0.06 was noticed.

Similar results observed in *M. hiemalis* HA-30, when free fatty acids like different PUFAs were supplemented in the medium showed more absorption of unsaturated fatty acid in TG compared to the saturated fatty acids (Aoki et al. 1999). The results obtained in this study are also similar to the experiments by Aggelis et al. (1995) in *M. cercinelloides* CBS 172-27. Certain fatty acids in plant oils could be selectively absorbed in the cellular lipid where as other fatty acids were used for beta oxidation degradation to provide energy and intermediate cell metabolism (Aggilis 1997; Auki et al. 1999; Dyal and Narine 2005). The incorporated fatty acids will either assimilated for growth or become a substrate for endo-cellular biotransformation (Papanikolaou et al. 2001; 2002a). The phenomenon of controlling the fatty acid composition was specific process of substrate and the endo-cellular changes of fatty acid by the enzymatic capabilities of the microorganisms (Papanikolaou et al. 2001). In bacteria like *Vibrio* sp., *E. coli*, *Bacillus* fatty acids were mainly accumulated in the phospholipids (Ando et al. 1992; Patnayak & Sree 2005) but in fungi such as *M. remanniana var. angulispora, M. alpina* IS-4 and *M.cercinalloides* exogenous fatty acid accumulated in triacylglycerol (Aggelis & Sourdins 1997; Kamishaka et al. 1990; Shinmen et al. 1992). Mutants or by genetic manipulation could be the between choice for carrying out biotransformation of PUFAs from plant sources into higher value oils (Certik et al. 1998; Wang et al. 2009).

OPTIMIZATION OF MEDIUM COMPONENTS THROUGH RESPONSE SURFACE METHODOLOGY (RSM)

Microbial production of lipids that contains unusual fatty acids with potential commercial application has been aimed at organisms selection and optimization. In commercial production of GLA by fermentation, optimization of media components are carried out for biomass and product maximization. Optimization is directly proportional to the effect of nutrients of cost effective substrate and also factors like pH, temperature, aeration or agitation on the yield of products (Ahmed et al. 2006; Hansoon & Dostalek 1988; Kendrick & Ratledge 1996). The conventional method of optimization involves varying one parameter at a time and keeping the others constant, but this method does not give the interaction of various parameters as compared to factorial design. To overcome these problems, Response Surface Methodology (RSM) technique was employed to study the effect of several factors influencing the responses by altering the multiple variables simultaneously and carrying out a limited number of experiments (Ravi & Susheelamma 2005). RSM is a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses. This technique was used in the various areas to optimize the conditions such as enhanced of lipid production and enzyme optimization (Linder et al. 2005), cultural medium optimization (Chang et al. 2006), biosurfactants in probiotics (Rodrigues et al. 2006) and other fields. The optimization of the medium in any fermentation process plays a fundamental role in preventing metabolic deviation that directly affects the yield. RSM is the common approach to the optimization of growth medium. In this study we aimed to design an optimal medium for an efficient GLA production using RSM. Variables consisting of glucose as the main carbon and the energy source, yeast extract and ammonium nitrate as nitrogen sources were selected for optimizing proportion for maximum GLA accumulation. This experiment was conducted based on the optimization of carbon and nitrogen sources previously explained.

Result indicated that glucose, yeast extract and ammonium nitrate were the major sources of nutrients for the enhancement of GLA. The optimizations of above parameters were already carried out in one at factor method. The data obtained were subjected to regression analysis by applying the RSM. The coefficients of the regression equation were calculated using the Statistica software and the results are given in Table 4.15. The GLA production is expressed in terms of the following regression equation.

Regression equation

"GLA = 5.134+0.367x_1 - 0.003*x_1^2 + 0.036*x_2 + 0.046*x_2^2 - 14.236*x_3 + 41.056*x_3^2 + 0.008*x_1*x_2 - 0.011*x_1*x_3 - 1.421*x_2*x_3"

Where X_1, X_2 and X_3 were the glucose, yeast extract and ammonium nitrate respectively. The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given in Table. 4. A good fit of the regression model was checked by the coefficient of determination (R^2), correlation coefficient (R) values and total regression F test. The R^2 value was 0.852 indicates 85% of the variance could be explained by this model successfully. The ANOVA results [Table 4.16] indicated that the independent variable glucose is influencing the GLA production significantly ($P<0.05$).

Response surface plots

The 3D response surface curves were then generated to explain the interaction of variables and the optimum levels of each variable required for the maximum production of GLA. Response surface plots as a function of two factors varied at a time by maintaining all other factors at fixed level (zero level, for instance), which was helpful in understanding both the main and interaction effects of these two factors. These plots were easily obtained by calculating from the model values taken by one factor where the second varies with constraint of a given Y value. The yield values for different concentrations of the variables were also predicted from the respective response surface plots

[Fig 4.32a, b, c]. The maximum predicted yield was indicated by the surface confined in the response surface diagram.

Table 4.15 Model coefficients estimated by multiples linear regression

Factor	Regression Co -efficient	Std. Error	t(10)	p
Mean/Interc.	5.134	4.127	1.244	0.2418
Glucose (X_1)	0.367	0.079	4.626	0.0009
Glucose ($X_1^* X_1$)	-0.003	0.001	-5.418	0.0002
Yeast extract (X_2)	0.036	1.586	0.023	0.9823
Yeast extract ($X_2^* X_2$)	0.046	0.225	0.206	0.8413
Ammonium nitrate (X_3)	-14.262	15.817	-0.902	0.3884
Ammonium nitrate ($X_3^* X_3$)	41.056	22.466	1.827	0.0975
X_1*X_2	0.008	0.015	0.523	0.6122
X_1*X_3	-0.011	0.150	-0.071	0.9447
X_2*X_3	-1.421	2.993	-0.475	0.6452

Table 4.16 ANOVA of the variables

	SS	df	MS	F	p
Glucose (x_1)	75.31	1.00	75.31	20.94	0.00
Glucose (x_1*x_1)	104.78	1.00	104.78	29.14	0.00
Yeast extract (x_2)	2.86	1.00	2.86	0.80	0.39
Yeast extract (x_2*x_2)	0.15	1.00	0.15	0.04	0.84
Ammonium nitrate (x_3)	1.46	1.00	1.46	0.41	0.54
Ammonium nitrate (x_3*x_3)	11.79	1.00	11.79	3.28	0.10
x_1*x_2	0.98	1.00	0.98	0.27	0.61
x_1*x_3	0.02	1.00	0.02	0.01	0.94
x_2*x_3	0.81	1.00	0.81	0.22	0.65
Error	35.96	10.00	3.60		
Total SS	243.21	19.00			

R^2=0.8521; Adj: 0.7190

Fig. 4.32a, shows the response surface plot obtained as a function of glucose concentration vs. yeast extract concentration, while third variable ammonium nitrate was maintained at zero level (coded). An increase in GLA

yield with increase concentration of glucose versus yeast extract was observed. Among the two independent variables glucose plays a major role in the production of GLA. Its level is very critical as evidenced from the 3D surface graph. The optimum value was near the centre point of glucose level and the interaction of glucose and yeast extract did not result in any further beneficial effect on the system.

Fig. 4.32b, shows the response surface plot obtained as a function of concentration of glucose vs. ammonium nitrate, while the third variable yeast extract was kept at its mid level. An increased GLA with increase concentration of glucose vs. ammonium nitrate were also observed. As indicated in Fig. 4.32a, the same trend was observed here also. The role of glucose was very critical and significant when compared with ammonium nitrate. The interaction effect of both independent variables resulted in decrease in the production of GLA after the mid level.

Fig. 4.32c, shows the effect of concentration of yeast extract vs. ammonium nitrate, while the third variable glucose was maintained at zero level. Increase in GLA yield with increased concentration of yeast extract was observed. From the response surface it is clear that the increase in concentration of ammonium nitrate decreases the GLA content and yeast extract increases the GLA production. But the extent of decrease of ammonium nitrate or increase of yeast extract is only marginal for GLA increase in the total lipid of the mycelium.

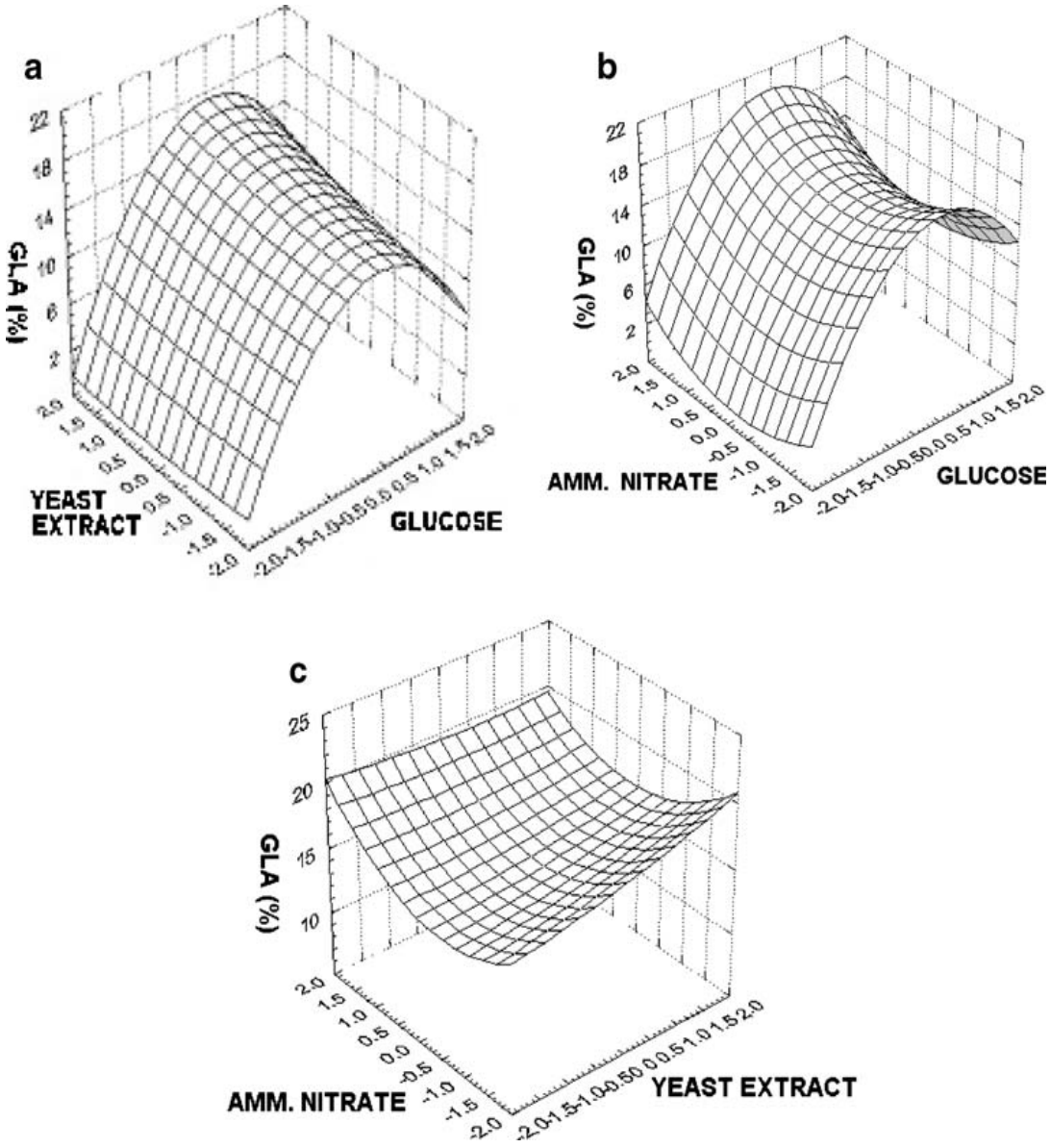


Fig. 4.32

a. **Effect of glucose and yeast extract concentration on the production of GLA by *M.rouxii* CFR-G15.**

   Other variable was held at zero level (coded)

b. **Effect of glucose and ammonium nitrate concentration on the production of GLA by *M. rouxii* CFR-G15.**

   Other variable was held at zero level (coded);

c. **Effect of yeast extract and ammonium nitrate concentration on the production of GLA by *M. rouxii* CFR-G15.**

   Other variable was held at zero level coded)

Canonical analysis

The signs of the roots, the independent variables (Eigen values) indicated the nature of the response surface. The calculated roots of the variables had mixed signs, meaning that the response function is a saddle point or mini-max response. Verification experiment was accomplished by using the optimized conditions and obtained 18.55 % GLA of total fatty acids [Table 4. 17]. This result therefore corroborated the predicted values and the effectiveness of the model. A 4.77 % increase in production of GLA was achieved when compared to that of basal medium. The results of the conditions for maximizing the GLA production within the experimental region were predicted and given as Table 4. 18. The maximum production of GLA (18.55 %) could be obtained when the levels of independent variables glucose, yeast extract and ammonium nitrate were at 65.0 (g/L), 3.5 (g/L) and 0.5 (g/L), respectively. The results were validated by doing the experiments with the predicted levels by the model. The results indicated that the predicted and observed values for GLA did not differ significantly ($P \leq 0.05$). This indicated that the optimized medium components favoured the production of GLA in the mycelium.

Table 4.17 Optimized values - Estimated

Optimized Values				
Independent Variables		**Dependent Variables**		
			Predicted Value	Actual value
Glucose (g/L)	65.00	Biomass (g/L)	12.620	10.67±0.22
Yeast extract (g/L)	3.50	Lipid (g/g)	0.399	0.35±0.81
Ammonium nitrate (g/L)	0.50	GLA (% of total fatty acids)	18.90	18.55±0.46

Optimization experiments are designed to provide in depth information on a few variables identified during fatty acid enhancement. In this study, the nutritional medium components for maximum GLA production from an oleaginous fungus *M.rouxii* CFR-G15 were optimized by the method of CCRD and RSM. It was observed that, biomass and GLA production were predominantly influenced by carbon and nitrogen concentrations. They also played key nutrient materials, which controlled the biosynthesis of the lipid production in the mycelium (Hansson & Dostalek 1988). Similar observations were also made by Chang et al. (2006), Lin et al 2007 on the production of biomass and triterpenoid and alkaline B-mannanase respectively. In this experiment, low concentration of nutrients had caused low production of GLA. At higher concentrations, both nutrients caused inhibition of lipid and PUFAs formation especially GLA synthesis. Similar observations were also made by Certik & Shimizu (1999) and Kendrick & Ratledge (1992). The optimal calculated values of tested variables for maximum production of GLA were glucose (65.0 g/L), yeast extract (3.5 g/L) and ammonium nitrate (0.5 g/L) with a predicted percentage of GLA 19.97%. These predicted optimal parameters were tested and final GLA obtained was 18.55% of total fatty acids [Table 4.17]. The 3-D response surface curves were plotted to understand the interaction of medium components and their effect on mycelium growth. In Fig. 4.32a, the high concentration of yeast extract repressed the growth and GLA production in *M. rouxii* CFR-G15 at all levels (+1.685, 0, -1.685). Variations in concentration of ammonium nitrate didn't significantly affect the growth and GLA production [Fig 4.32c]. These results therefore corroborate the predicted values, and the effectiveness of the model. Yeast extract was used in all the fermentation studies as supplement, because this contains complex nutrients such as vitamin, nucleic acid, lipid and other substances which could be necessary for growth of microbes, but the optimum concentration of yeast extract was necessary for growth and secondary metabolites production. In our earlier experiments, we observed that combination of yeast extract and ammonium nitrate were the significant nitrogen sources for biomass, lipid and

GLA production. These observations were also made by Hansson et al. (1989) and Kendrick & Ratledge (1992). It was also observed in this study that the main effects of carbon and nitrogen levels as well as the interactions were highly significant for growth and GLA accumulation. However, controlled fed batch cultivation and chemostat cultures have so far been used successfully for improving metabolites production in a number of microorganisms (Hansson et al. 1989).

The use of a multifactorial statistically approach for determining the conditions that lead to the optimum yield of secondary metabolites production (especially the PUFAs production) was the major noteworthy observation in this study. This observation is very much concurrence with the result of Chang et al 2006. CCRD maximizes the amount of information that can be obtained, while considering the interaction of independent variables and limiting the number of individual experiments required. The response surface methodology, a smaller and less time consuming experimental design, could generally satisfy the optimization of many microbial processes (Chang et al. 2006; Linder et al. 2005). The optimum culture medium obtained in this experiment has given a basis for further study with batch or fed batch cultivation in bioreactor for large scale production of secondary metabolites i.e. GLA from *M. rouxii* CFR-G15.

Thus, we conclude this experiment that CCR design with RSM was found to be useful in determining the conditions leading to the maximum yield of GLA. The use of an experimental design allowed the rapid screening of a large experimental domain in search of the best condition and levels for the GLA production. The R^2 value showed a good fit of the model with an experimental data. With the best of our knowledge, there were no much reports available on the GLA production by media engineering. Thus our experiment mainly focused on an attempt to demonstrate the application of statistical design and RSM to maximize GLA production.

SELECTION OF EXTRACTION METHODS WITH SOLVENT SYSTEMS

This research work was aimed on microbial production of lipids that contain unusual fatty acids. Lipid extraction from biomass is an important step in the quantification of lipid from microorganisms. Rapid and efficient method for extraction of lipid forms the basis for the optimization of culture conditions for lipid production in oleaginous microorganisms. The extract ants may also have a function in preventing enzymatic hydrolysis (oxidation of fatty acids). At the same time, attention was given to the potential toxicity of the solvents. Finally, the extractability of cells or tissues was found to be variable and depended on both the nature of the cells and of the lipids. This study was carried out to compare the efficacy of three extraction methods along with different solvent system for lipid yield and fatty acid composition of *M. rouxii* CFR-G15.

In most of the oleaginous microorganisms, lipids are present in cell membranes and in the cytosol also. Since the lipid contains heterogeneous compounds with different structures and properties, quantity of lipid produced in the cells depends on the extraction method having different concentration of solvents in oleaginous microorganisms. Hence, different methods were chosen for lipid extraction such as Folch method, Bligh and Dyer method and soxhelt method. The data obtained in this study i.e. lipid content extracted using various solvent system is shown in Fig. 33a, b, and C.

Results indicated that among the three methods used viz., Folch, Bligh & Dyer and soxhlet apparatus methods with five different solvent system they were: chloroform: methanol (2:1), chloroform: methanol (1:1), hexane: isopropanol (3:2), hexane: isopropanol (4:1) and hexane: petroleum ether (2:1). The highest extraction gave a lipid content of 39.00±1.34 % in folch method when chloroform: methanol used in 2:1 ratio. Hexane and sopranos (3:2) gave similar to that (38±1.18 %) and chloroform and methanol (1:1) gave slightly lower extraction of total lipid content 35±1.21%. The GLA content of the lipid

was also in the same order viz., 13.80±1.11%, 13.03±0.61% and 11.34±0.64 % respectively.

Maintaining the folch method as control chloroform:methanol (2:1) as a comparison the extractability by bligh and dyer method was assessed in *M. rouxii* CFR-G15. The lipid content extracted by any of the solvent used in the bligh and dyer method was lower than that of folch control. Chloroform and methanol in the ratio of 2:1 and 1:1 gave lipid content extraction of 35.02± 1.21 and 34.21±1.01% respectively. These values were not different and were higher than the other systems used in this method. The GLA obtained by this extraction method using chloroform: methanol in the ratio of 2:1 and 1:1 were 12.87±0.92 % and 11.82±0.72%. The GLA content in the lipid extracted using chloroform: methanol (2:1) by this system was similar to that of folch control.

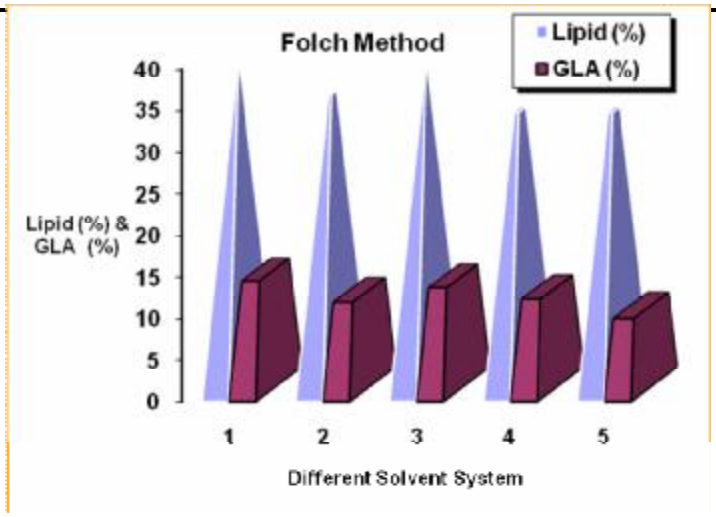
Soxhlet apparatus method extracted was slightly lower than the folch control. The highest extraction obtained when chloroform: methanol (2:1) was used viz., 36.73±1.09%. GLA content of the lipid extracted by soxhlet method was highest with chloroform: methanol (2:1) in the solvent system i.e. 14.47±0.66%. It was similar to that of control. Chloroform: methanol in the ratio 1:1 also gave values comparable to control viz., 12.70±1.12% in *M. rouxii* CFR-G15.

The results obtained in this study also supported by Certik et al (1996) and Somashekar et al (2001). Since solvent system contained polar and non - polar solvents. Chloroform: methanol (2:1) showed efficacy of extraction of lipid yield very high from this. The ideal solvent system for extracting lipids from cells should be sufficiently polar to extract bound lipids from cell membranes. At the same time, the solvent should not be so polar since the non polar lipids do not dissolve but should not react chemically with these lipids. Generally, non-polar solvents are usually needed for lipid extraction their combinations with polar solvents are advantageous, mainly because of dehydration, protein denaturation and degradation of hydrogen bonds between

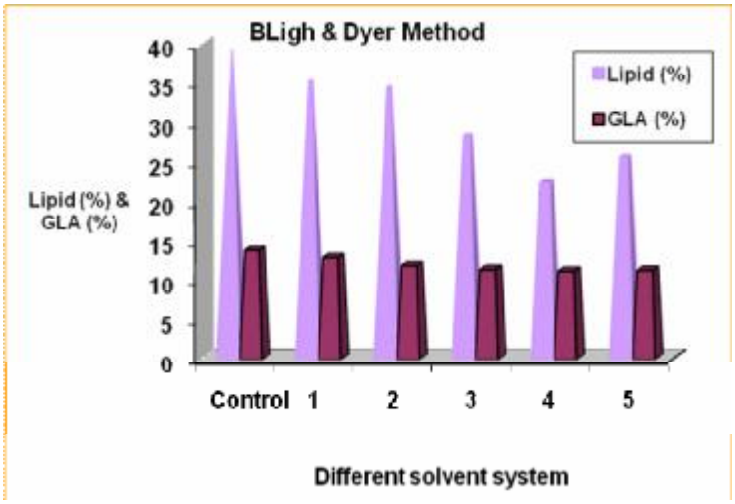
the complex lipid and proteins. The combination of polar and non polar solvents is thus advantageous for the extraction of lipid from microorganisms.

When compare to all the methods used in this study, soxhlet method was advantageous over the other two methods since the separation of the cell debris and lipid didn't arise. Unlike other two methods in which the cells were ground with the sand along with solvent system, which causes the cells to lyse and release oil, while in soxhlet extraction the cell to solvent contact was very less. The oil extraction from soxhlet method can be used for various applications in industries as the solvent is considered to be safe (Somashekar et al. 2001). Various literatures have reported that the members of zygomycetes produce 10-20% of GLA under different cultural conditions and observed maximum GLA production when chloroform: methanol was used as solvent system (Emelyanova 1997, Weete et al 1998, Certik and Shimizu 1999).

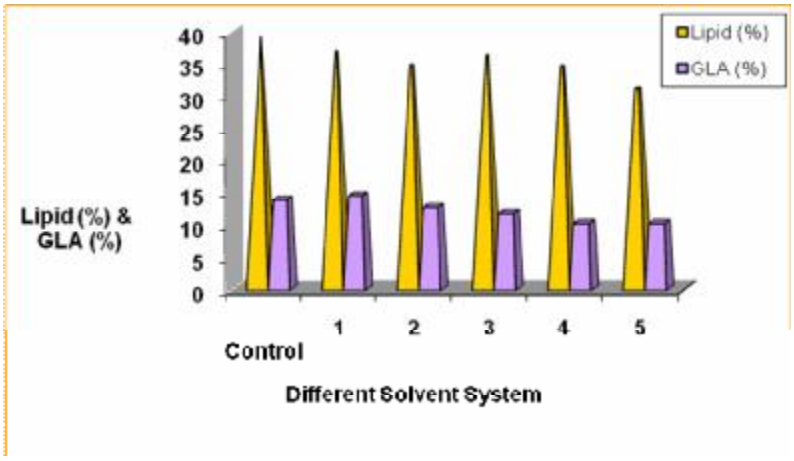
This study suggest that the chloroform: methanol was found to be the best solvent system in soxhlet method for the extraction of lipid both bound fatty acids and free fatty acids. As the fungal cell wall is tough, there was a need of maceration or disruption of cell wall by acid or alkali hydrolysis before extraction of lipid from biomass in soxhlet method. Thus there is a lot of scope to improve the rapid and reliable extraction methods and better recovery of intracellular lipid (purification) from biomass for further development of microbial lipid biotechnology.



(A)



(B)



(C)

Fig. 33. Selection of extraction methods with solvent systems
A. Folch method; B. Bligh and dyer Method; C. Soxhlet method

SECTION 4

GENETIC STUDIES ON *M.ROUXII* CFR-G15 WITH SPECIAL EMPHASIS ON GLA PRODUCTION

MUTATION

Strain improvement is considered as one of the major factors involved in the achievement of higher titres of industrial metabolites (Certik et al. 1998; Peberdy 1980). On the other hand, strain improvement has been achieved through mutation, selection, or genetic recombination. In many cases, mutations are harmful, but occasionally may lead to a better adapted organism to its environment with improved biocatalytic performance. Mutagenesis in microorganisms is an important property conferred by DNA, since it creates new variations in the gene pool. The challenge is to isolate those strains which are true mutants that carry beneficial characters (Parekh et al. 2000; Wang et al. 2009). Random mutagenesis and fermentation have been reported as an effective way to improve the productivity of industrial microbial cultures (Parekh et al. 2000; Wang et al. 2009). The most commonly used mutagens are ethylmethane sulfonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), methyl methane sulphonate (MMS) and ultraviolet (UV) irradiation (S).

The objective of this study has been to introduce auxotrophic marker in *M. rouxii* CFR-G15, which could be used for marker for hybrids selection derived from protoplast fusion. UV radiation (physical), chemical mutagens like EMS and NTG were used to mutagenise the culture. The variants obtained were also examined for modification in fat content and fatty acid profile from the biomass and screening of high GLA producing hybrids of *M. rouxii* CFR-G15 was also performed in this study.

i. UV-radiation

UV radiation is a common mutagen used to induce mutations for strain improvement. UV at 254 nm wavelength is absorbed by DNA and cause damage in cell level and is therefore mutagenic. The pyrimidines (thymine and cytosine) are especially sensitive to modifications by UV rays absorption. This may result in the production of thymine dimers that alter the DNA helix and block future replications; this causes permanent mutation (Lenhinger 1993). This method is commonly applied in the field of microbiology for improving biotechnologically important microbial strains in large scale product development.

In the present work, mutants of *M. rouxii* CFR-G15 were developed by exposing the young spores to UV irradiation at different time intervals. The killing pattern of this culture under UV is shown in Fig. 4.34. Dose dependent inactivation of the culture was also observed in this study. The results indicated that with 10 mins exposure, the killing rate was 9.56 %, 50 min 80.27% and with 1 h 97.5% were observed. It was noticed that the killing rate increased with the increase in the exposure time and an inhibitory effect on growth was observed in mutant screening medium.

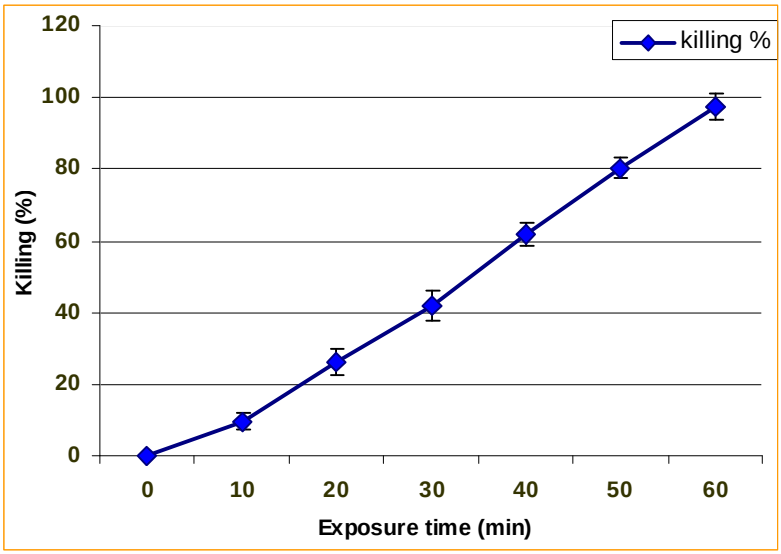


Fig. 4.34 Effect of UV on *M. rouxii* CFR-G15

ii. Treatment with EMS

In this experiment, the culture was treated with different concentrations of EMS ranging from 2-10mM for 1 h. Killing pattern with different concentration of EMS on this culture is shown in Fig 4. 35. The results indicated that, 10mM concentration of EMS was found to be optimum in giving 65% killing rate. A lower killing rate of 32% was noticed when treated with 2 mM concentration. The survival rate decreased with the increase in concentration of EMS. Unlike UV, results obtained by EMS treatment of *M. rouxii* CFR-G15, the killing rate was found to be less than 65%.

EMS, an alkylating agent and highly mutagenic in lower organisms but only moderately carcinogenic in mice and rats. The main effect of EMS on DNA *in vitro* and *in vivo* without activation by cellular components is alkylation. However, the main product, 7- alkylguanine is not necessarily the major cause of mutagenesis and the major mutagenic effects of EMS must be examined by biological means. The use of EMS as mutagen produces almost all kinds of genetic effects in all the organisms tested (Gunashree 2006). EMS is not hazards to human and till now no reports on epidemiological studies are known.

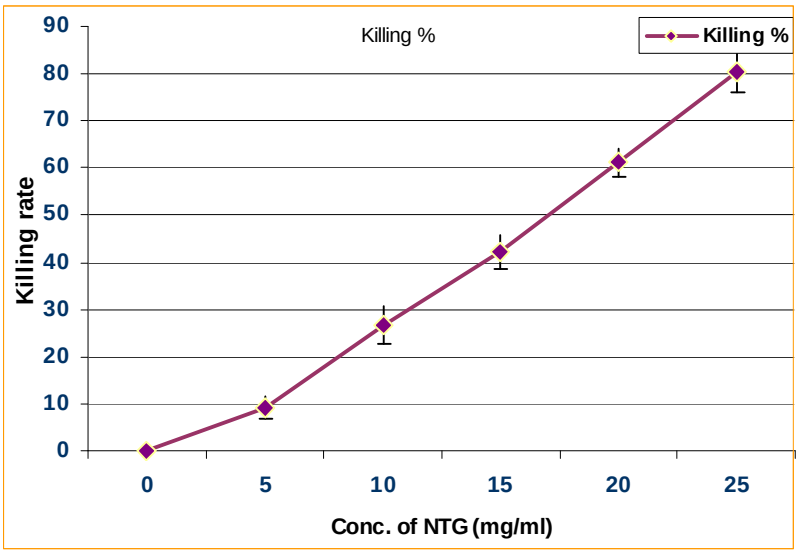


Fig. 4.35 Effect of EMS on *M. rouxii* CFR-G15

Treatment with NTG

In NTG treatment, a maximum killing rate of 80% was observed when the spores were subjected to 25µg/ml which was higher than the reported value. A killing rate of 100% was observed when the concentration of NTG increased more than 25µg/ml. The data obtained in this study revealed that minimal killing rate could be observed when spores were treated with 5µgml^{-1} of NTG. Unlike in EMS, results obtained through NTG treatment, the killing rate was found to be more. The results are indicated in Fig. 4.36.

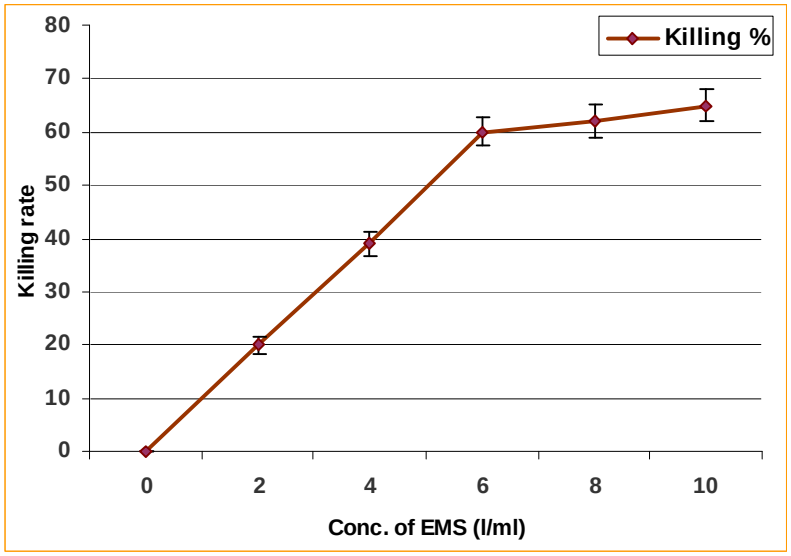


Fig. 4. 36 Effect of NTG on *M. rouxii* CFR-G15

NTG reacts with the sulfhydryl groups for conversion of very reactive carbonium ions or alkyl diazonium ions. This is a widely used laboratory mutagen and carcinogen known by a number of abbreviations that includes most commonly MNNG. It has been applied to a wide array of organisms starting from prokaryotes through mammals and has been clearly demonstrated to produce a spectrum of genetic alterations in nearly all test systems (Moore 1969).

Studies of specific-locus NTG mutability have been conducted at a number of loci in *Neurospora*. The vast majority of induced mutations were found to behave like point mutations of the base pair substitution type and a few base pair deletion mutations were also obtained. NTG was found to induce both forward and reverse mutation in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* with a linear dose response relationship and the lowest effective dose tested was 10 $\mu g ml^{-1}$(Venkateswaran 1999). In addition to point mutation, MNNG was found to induce both mitotic crossing over and gene conversion in *S. cerevisiae* (Venkateswaran 1999).

Screening of Auxotroph mutants

The spores of *M. rouxii* CFR-G15 exposed to different mutagens like UV, NTG and EMS had shown a respective killing pattern during mutagenesis. The survivors from this treatment were screened for auxotrophic mutants by standard chemical methods (Holiday 1956; Venkateswaran 1999). The result revealed that the colony which grew on complete medium (CM) but did not grow on minimal medium (MM) was considered as auxotrophic mutants with the requirement of one or more growth factors especially the amino acids.

About 4000±200 colonies were screened for auxotroph mutant, of which 5 auxotrophic mutants having the requirement of lysine, alanine, isoleusine and methionine were detected. Among 5 auxotroph mutants 2 were found to be lysine requirement, and one each of alanine, isoleusine and methionine requirements respectively. Some of these auxotroph mutants while sub culturing on agar slants reverted back during experiments. Only the methionine auxotroph (met^-) was found to be very stable even after 3-4 subculturing. This methionine auxotroph was used for protoplast fusion studies as selection criterion. The mutagen induced cultures were also examined for its morphological characters and pigmentation on the growth medium. The results are given in Fig. 4.37a and 4.37b.

(A)

(B) (C)

Fig 4.37a Microphotographs showing various colony morphology after mutagenesis and low temperature selection

A. UV treatment; B. EMS treatment; C. NTG treatment

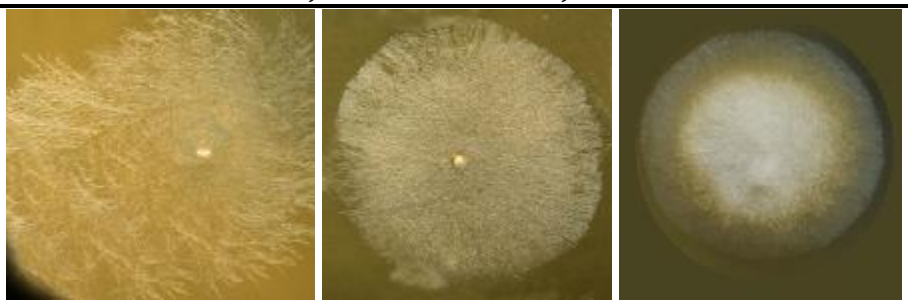


Fig 4.37b Auxotrophic mutants of *M. rouxii* CFR-G15 showing colony variation on selective media

Screening and selection of low temperature resistant mutants

When *M. rouxii* CFR-G15 cultivated at low temperature, the GLA content of the mycelia increased, but the cell growth (biomass formation) and total lipid production were found to be very low. It is presumed that the cultures which grow faster at low temperature would also contain more GLA in their cellular lipid or vise versa. The ability to grow well at low temperature was considered as an index for cell improvement (Hansoon & Dostalek 1988; Hiruta et al. 1996).

About 250 mutagenised colonies from complete medium were picked up, plated out separately and these colonies were grown under low temperature (at 15 °C) for selection. The results indicated that nearly 30 mutants were selected on the basis of their fast growth and larger in colony size when compared to parent culture. The selected mutants were cultivated in Erlenmeyer flasks containing nitrogen limiting medium (FPM) for 5 days at 28 °C to evaluate the biomass and GLA production. Fatty acid methyl esters of mutants were prepared by direct transesterification by the method of Rodriguez et al. (1998) and Lepage & Roy (1984)

Biomass production in parent was 7.98±0.56 g/L. The lowest biomass content was 4.6±0.32 g/L (M23) and highest biomass production was in 10.6 ±0.85 g/L (M27). Among the thirty mutants selected five showed lower biomass production and five showed higher biomass when compared with the parent. Rests of the mutants were similar to the parent. The results are presented in Table 4.18.

GLA content of the parent was 14.42±1.02 %. Mutant no. 26 didn't show any GLA content in its lipid. Mutant no. 6 had the lowest GLA value 0.52±0.05% and highest GLA content observed in mutant 21 (18.37±0.83 %). Among the mutants selected twenty three mutants produced GLA content lower than the parent and seven were higher [Table 4.18]. The mutants which had more GLA content did not show any variations in repeated experiments.

Thus, this experiment represented that the mutants that could able to accumulate lipid with high GLA content were successfully obtained by using low temperature growth as a selection index.

Table 4.18 Biomass and GLA Content of Mutants (selected at lower temperature, 15°C) of *M. rouxii* CFR-G15 isolated after mutagen treatment

Mutants	Biomass (g/L)	GLA (%)
Parent	7.98±0.45	14.42±1.02
M1	7.35±0.45	15.84±0.45
M2	8.2±0.53	9.28±0.53
M3	7.8±0.42	7.36±0.42
M4	6.2±0.39	10.28±0.39
M5	9.0±0.46	10.24±0.46
M6	8.00±0.51	**0.52±0.05**
M7	7.6±0.62	5.08±0.27
M8	8.0±0.60	6.87±0.35
M9	6.8±0.38	1.55±0.07
M10	8.6±0.42	15.84±0.42
M11	9.2±0.53	15.65±0.53
M12	10.2±0.72	10.95±0.72
M13	8.4±0.47	16.70±0.78
M14	8.6±0.53	16.70±0.47
M15	9.04±0.76	6.87±0.60
M16	8.86±0.80	3.43±0.21
M17	8.0±0.74	1.7±0.09
M18	7.20±0.68	10.01±0.68
M19	7.60±0.50	11.85±0.96
M20	8.4±0.49	11.20±0.83
M21	8.8±0.53	**18.37±0.83**
M22	9.21±0.63	7.30±0.32
M23	**4.60±0.32**	6.68±0.64
M24	9.20±0.64	17.26±0.84
M25	8.40±0.84	1.46±0.08
M26	7.20±0.42	0.00±0.00
M27	**10.6±0.85**	1.27±0.06
M28	7.7±0.63	0.68±0.03
M29	6.8±0.45	11.46±0.36
M30	5.4±0.36	7.30±0.32

Data are expressed as mean ±SD of three replicates.
GLA expressed as relative percentage of total fatty acids,

PROTOPLAST FUSION

Protoplasts fusion has always been considered as one of the acceptable tools in microbial genetics. This technique is considered for the development of microbial strain by way of nuclear exchanges between two parental strains. Gene transfers during protoplast fusion and chromosomal aberrations have also been reported (Venkateswaran 1999). The aim of the study is to isolate the hybrid with high amount of GLA production in total lipid content in *M. rouxii* CFR-G15.

Protoplast formation/isolation

In this experiment, strains of *M. rouxii* CFR-G15 mycelia with auxotrophic marker (met^-) was subjected to lytic activity with different fungal lysing enzymes such as Lyticase, Novozyme 234 and Chitinase for 2 to 3h period with intermittent agitation. The enzymes effectively acted on mycelial mass and produced spheroplasts and protoplasts. The enzyme reacted with the cell wall of the mycelial mass became swelled up and round hyaline globules were formed by the action of these lysing enzymes. The action of these lytic enzymes on the mycelial mass depended upon the type of lytic activity of the enzyme, concentration of the enzymes, the incubation period and age of mycelial mass.

The result of the formation of spheroplasts and protoplasts from the parental strain is given in Fig. 4.38a&b. Our experiment concluded that complete digestion of mycelia and the formation/release of protoplast have occurred in 1 h of the incubation with Novozyme 234 lytic enzyme. Hence, an optimal enzyme concentration and the incubation period were selected to get sufficient protoplast/spheroplast from the strain for the fusion purpose.

Three lytic enzymes with various concentration used in this experiment had different lytic nature during the incubation period (1 h). Among the lytic enzymes tested 800 µg/ml Novozym 234in an osmotic stabilizer was found to be optimal for lysis of cell wall and release of protoplast [Table 4.19].

Peer and Chete (1990) obtained highest protoplast yield from *T. harzianum* using Novozym 234 at 10 mg/ml with 0.6 M KCl, where as Tschen & Li (1994) used 15 mg/ml of Novozyme 234 with 0.6 M sucrose to obtain maximum number of protoplast from *T. harzianum* and *T. koningii.* The results obtained by Prabhavathi et al. (2006) maximum protoplasts from *T. reesei* obtained when 8 mg/ml lysing enzyme at 0.6 M KCl as osmotic stabilizers. Hence, the study suggests that, lytic enzyme with various concentration play vital role in digesting the cell wall of fungal mycelium and also depends upon the nature of cell wall composition of the particular fungal strain. Venkateswaran (1999) reported that preparation of protoplasts from various yeast cells, Novozym 234 enzyme (100 µg/ml) was found to be the most effective lytic enzyme among the various lytic enzymes tested.

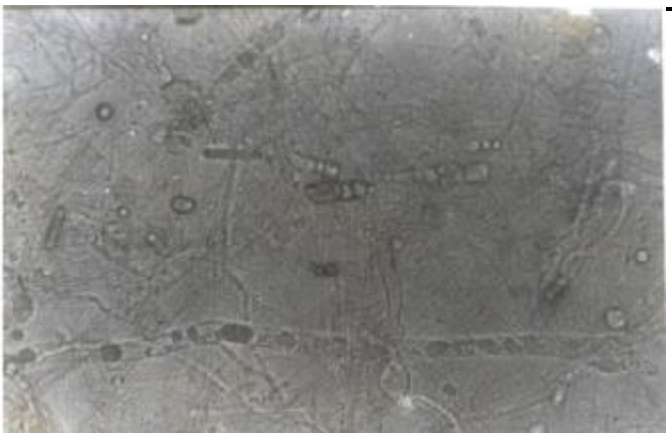
Table 4.19 Influence of different lytic enzymes on the production of protoplast of *M. rouxii* CFR-G15

Conc. of enzymes mg/ml	Protoplasts counts / ml		
	Novozyme 234	Lysing	Chitinase
100	ND	ND	ND
200	$0.81x10^5$	$1.81x10^3$	ND
300	$1.25x10^5$	$3.25x10^3$	$1.25x10^2$
400	$2.43x10^5$	$4.43x10^3$	$2.43x10^2$
500	$3.25x10^6$	$5.25x10^3$	$3.25x10^3$
600	$6.45x10^6$	$3.45x10^4$	$4.45x10^3$
700	$1.94x10^7$	$1.14x10^5$	$1.14x10^4$
800	$2.25x10^7$	$2.25x10^5$	$2.25x10^4$
900	$4.46x10^6$	$3.46x10^5$	$2.46x10^4$
1000	$6.25x10^6$	$4.25x10^5$	$3.25x10^4$

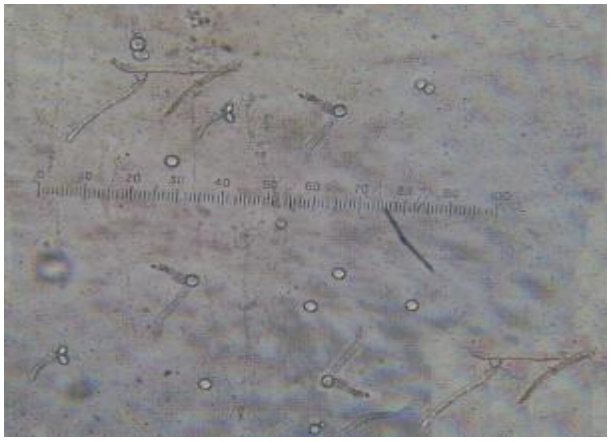
The stages of protoplast formation were observed by using different types of lytic enzymes with different concentration. Swelling and agglutination of cells were observed initially and subsequently the *M. rouxii* CFR-G15 mycelium started lysing. Almost complete digestion of mycelia and release of protoplast occurred prominently after 1h of incubation when 800 µg/ml Novozyme 234 was used. The protoplasts released soon after the lytic activity from the mycelial structure were found to be smaller in size and later slowly

enlarged to a hyaline spherical structure. It was also observed that the protoplast yield significantly affected by the concentration of lysing enzymes. The lysis of fungal mycelium was confirmed when the tip portion of the mycelia released protoplast at low concentration of enzyme [Fig. 4.38]. On the other hand, at appropriate concentrations of enzymes, the mycelium become lysed very effectively yielded large numbers of protoplasts. The high concentration enzymes totally digested the mycelial structure and did not yield any protoplast. It is probably due to the imbalance between the concentration of enzyme, the crowd of the protoplast and the hypotonic solution used. Thus optimization of enzyme concentration for the production of viable protoplast became highly important during this experiment.

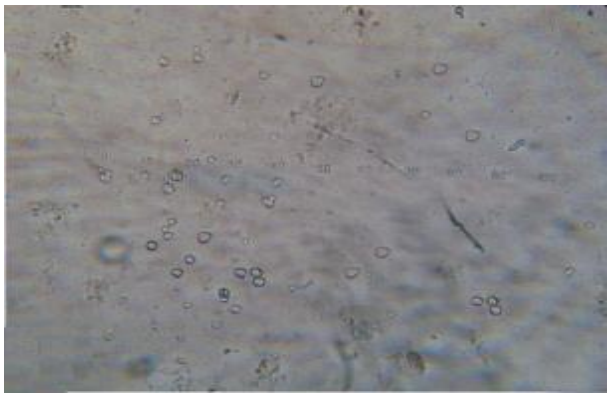
Thus this experiment concludes that the amount of protoplast formation depended on the effectiveness of the lytic enzyme that made release of protoplast from mycelia for the formation of osmotically fragile bodies called protoplasts. The frequency of protoplast was calculated by using the standard formula. Our experiment thus revealed that the total protoplast frequency of *M. rouxii* CFR G-15 having methionine auxotrophic marker was found to be 35 %.



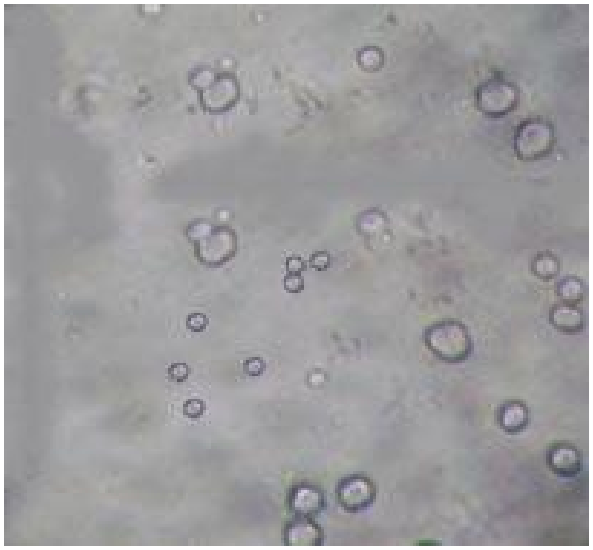
(A)



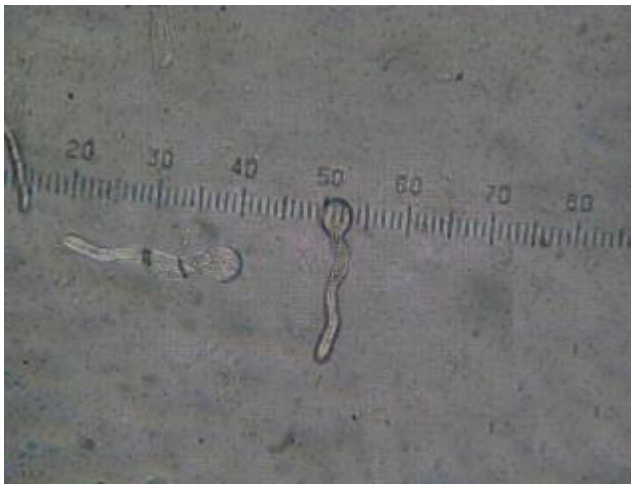
(B)



(C)



(D)



(E)

A) Microphotograph of *M. rouxii* CFR- G15 (met^-) before enzyme treatment showing mycelial structure

B) Microphotograph showing ell degradation and protoplast release in *M. rouxii* CFR-G15 (met^-)

C) Microphotograph showing protoplast formation in *M. rouxii* CFR-G15 (met^-)

D) Microphotograph showing protoplast formation in *M. rouxii* CFR-G15 (met^-)

E) Microphotograph showing protoplast germination after fusion in selective media containing osmotic stabilizer (Sorbitol)

Regeneration Protoplast

Protoplast regeneration is an important event in protoplast fusion experiment and a study on the effect of different osmotic stabilizers used in the regeneration medium showed that 1M sorbitol was the best for this experiment [Fig. 4.39]. Kirimura et al. (1988) also reported similar levels of regeneration of protoplast grown on a medium stabilized with 0.7 M KCl. The poor regeneration of protoplast was observed in our experiment, when MgSO4 at 0.5M concentration used in the regeneration media. Venkateswaran (1999) reported that among the osmotic stabilizer used 1M sorbitol facilitated maximum of protoplast in regeneration selective medium. The results obtained in this study also in concominent with the above results. In our experiment 1M sorbitol was used in regeneration medium for regeneration of protoplast obtained from *M. rouxii* CFR-G15 auxotroph culture. Our results indicated that the regeneration frequency was found to be 0.46%.

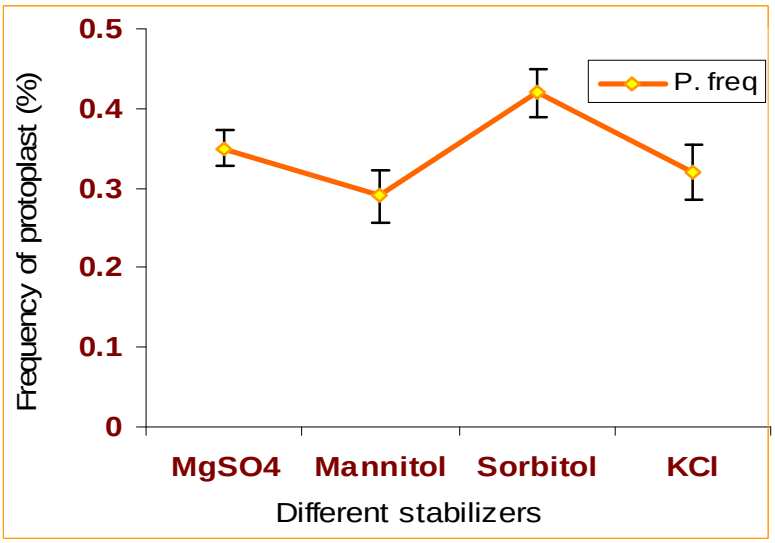


Fig. 4.39 Effect of different stabilizers on frequency of protoplast fusion

Protoplast Fusion

The intrastrain protoplasts obtained from *M. rouxii* CFR-G15 having auxotrophic marker were fused in presence of 30% PEG 6000 and after 1 h incubation at 30 °C the fused protoplasts were plated on protoplast regeneration medium for its appearance. Our results indicated that the fusion frequency was found to be 0.92 % which was considered to be very optimal. Fusion frequency between the protoplasts from *M. rouxii* CFR-G15 was calculated based on the ratio between the numbers of colonies on MM to the number of colonies on CM. Several reports indicated that the fusion frequency ranged between 0.2% and 0.9% (Gunashree 2006; Venkateswaran 1999). Our experiment also revealed that during fusion of protoplast clumping of protoplasts were formed [Fig. 4.38]. Similar concentration of PEG used was reported as optimum for inter-specific and intra strain fusion of protoplast between *T. harzianum, T. reesei* and *A. niger* by EL Bondkly 2006; El Bondkly & Talkhan 2007; Prabhavathi et al. 2006.

The colonies which showed very fast growth on transfer to selective complete medium [Fig. 4.40] and grown at 15 °C (low temperature selection) were picked up and transferred to a complete medium. Based on the mycelial growth and morphology, 15 fast growing colonies of intra strain fusants of *M. rouxii* CFR-G15 were selected and designated as CFR-HyG1 to CFR-HyG15 [Fig.4.41]. Later these selected putative hybrids were cultivated in a normal room temperature. The characteristics of putative hybrids were performed on basis of their biomass buildup, fat production and GLA content and these putative hybrids were compared with the parental cultures. Difference in growth of parental and hybrid culture grown at both room temperature and low temperature (15°C) is shown in Fig. 4.42.

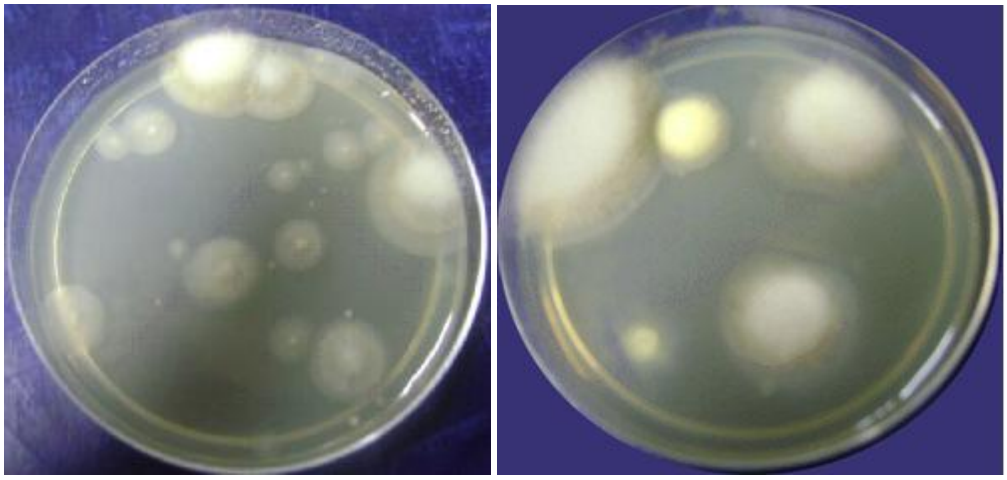


Fig 4.40 Fusant colonies of *M. rouxii* CFR-G15 on selective regeneration plates

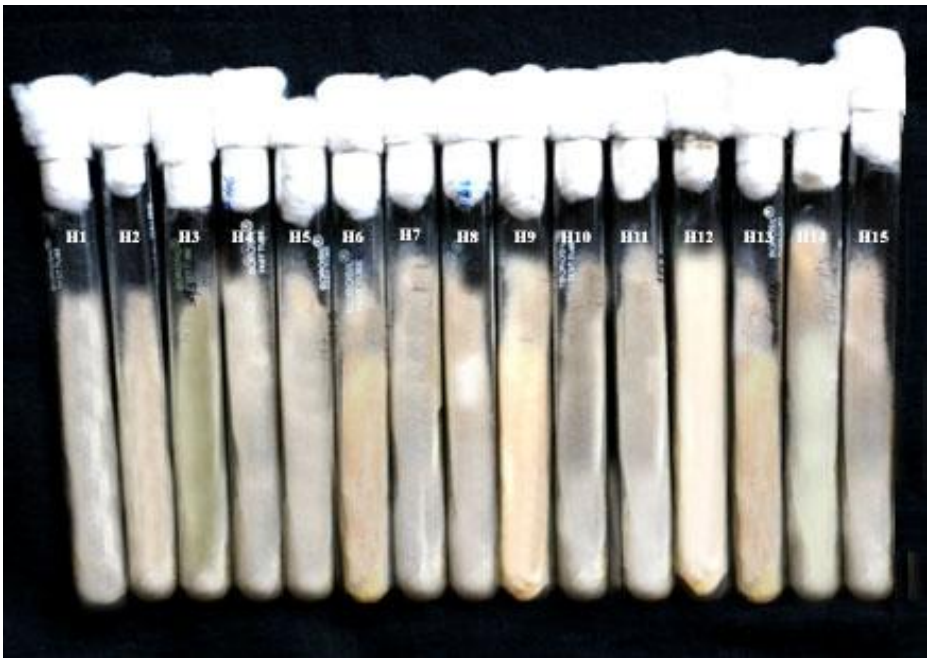
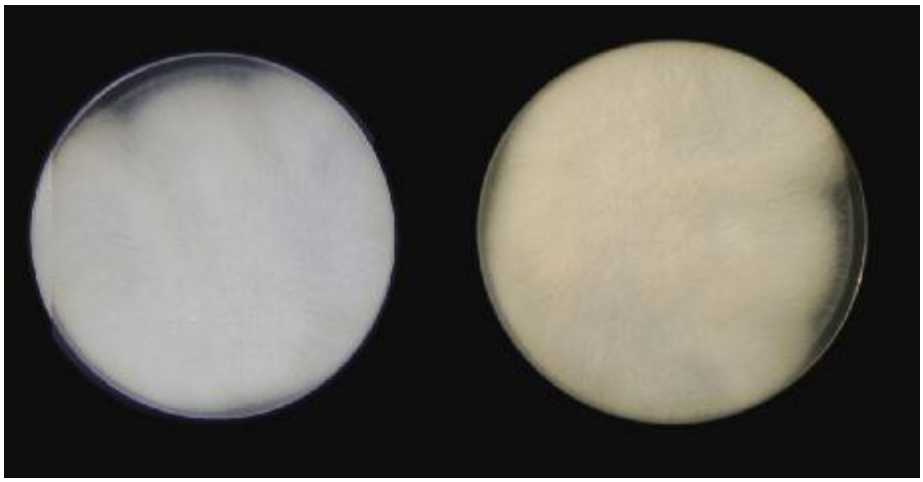


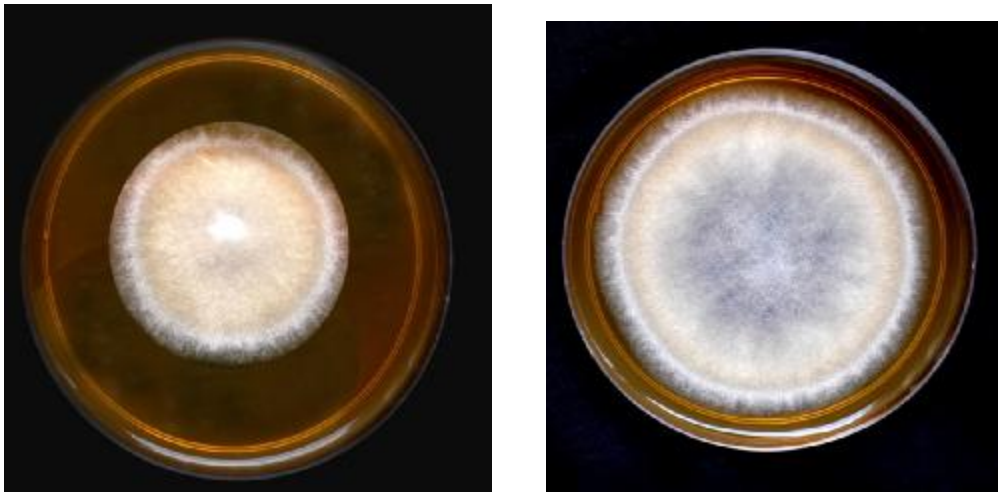
Fig 4.41 Selected Hybrid cultures

Plate-1



(A) (B)

Plate-2



(A) (B)

Fig. 4.42 Plate 1: A. Parent colony grown in 28°C (RT);
B. Hybrid colony grown in 28°C (RT)

Plate 2: A. Parent colony grown in 15°C (LT);
B. Hybrid colony grown in 15°C (LT)

(RT-Room temperature; LT-Low temperature)

Characterization of putative hybrids strain for biomass and lipid content

All the putative hybrids, parent strain and auxotroph mutant were grown on FPM and their growth characteristic like biomass production, lipid and fatty acid profiles were analysed. Table 4.20 shows the content of biomass of parent, auxutrophic mutant and selected hybrids. The biomass content of parent and auxotroph mutant was 7.98±0.45 g/L and 8.64±9.38 g/L respectively. The dry biomass of selected putative hybrids was ranging from 9.94±0.39 g/L (CFR-HyG2) to 17.80±1.15 g/L (CFR-HyG12). Four hybrids contained almost double the quantity of dry biomass.

Lipid content of parent and auxotrophic mutant were 28.32±1.23 % and 30.78±1.41% respectively. The range of lipid content was 20.52±1.63% (CFR-HyG8) to 42.46 ±2.41% (CFR-HyG9). Two hybrids had low lipid content than the parent and auxotroph mutants. Among the 10 putative hybrids four had lipid percent above 36 %. Three putative hybrids were similar to the parent.

Thus, CFR-HyG12 and CFR-HyG9 was considered a potent culture for the maximum production of biomass of 17.80±1.04 g/L and 15.80±0.76 g/L and lipid production of 38.22±1.14% and 42.46±2.32% respectively, among all the hybrids selected. The lipid production in parental strain and auxotroph mutant were found to be 28.32 and 30.78 % respectively. The biomass production in hybrids increased 2 fold than the parental culture but the lipid production increased only 10 % in the hybrids (42.46±2.32%). The total yield per g/L was 2.26±0.11 in the parent and 2.66±0.23 in the auxotroph mutant. Lowest lipid yield was in CFR-HyG8 which was 2.33±0.21 g/L, highest was 6.81 ±0.37 (CFR-HyG12). Among the putative hybrids four of them were similar in their lipid yield and eleven of them were higher than parent. When a medium containing 4 % glucose was used, the residual sugar content of the media parent was cultured was 3.25±0.03 g/L and auxotroph mutant was 3.13 ±0.09 g/L. No difference in the residual sugar content was noticed in all the media in which the putative hybrids were cultured

Table 4.20 Biomass, and lipid characteristics of hybrids obtained by intraspecific protoplast fusion of *M. rouxii* CFR-G15 (Fat Producing Medium, Room temperature, pH-6.0, 180rpm)

Parent/ Aux. mutants/ Hybrids	Dry Biomass (g/L)	Total Lipid content (%)	Lipid yield (g/L)	Residual content (g/L)
Parent	7.98±0.45	28.32±1.23	2.26±0.11	3.25±0.03
Aux.mut (met$^-$)	8.64±0.38	30.78±1.41	2.66±0.12	3.13±0.09
CFR-HyG1	12.40±1.02	34.76±1.76	4.30±0.46	3.60±0.26
CFR-HyG2	**9.94±0.39**	35.25±1.78	3.51±0.36	2.88±0.06
CFR-HyG3	10.38±0.58	32.78±1.86	3.41±0.32	3.47±0.08
CFR-HyG4	11.84±0.81	20.97±1.15	2.49±0.35	3.87±0.05
CFR-HyG5	13.76±1.20	30.79±1.59	4.24±0.28	3.87±0.03
CFR-HyG6	11.20±1.07	26.59±1.26	2.97±0.31	3.47±0.06
CFR-HyG7	15.80±1.06	40.63±2.13	6.43±0.48	3.53±0.07
CFR-HyG8	11.40±1.04	**20.52±1.63**	2.33±0.21	4.27±0.07
CFR-HyG9	15.80±0.76	**42.46±2.41**	**6.72±0.32**	3.53±0.07
CFR-HyG10	10.40±0.83	28.85±2.12	3.01±0.24	4.27±0.04
CFR-HyG11	14.00±1.02	32.31±2.10	4.53±0.46	4.00±0.17
CFR-HyG12	**17.80±1.15**	40.22±1.14	**6.81±.037**	4.20±0.07
CFR-HyG13	15.20±1.01	34.32±2.43	5.23±0.46	4.13±0.06
CFR-HyG14	13.21±0.87	31.75±2.18	4.18±0.54	4.13±0.05
CFR-HyG15	13.62±0.76	40.36±2.48	5.50±0.38	3.40±0.08

Data are expressed as mean ±SD of three replicates.
CFR-Central Food Technological Research Institute,
Hy-Hybrid strains and G1-GLA containing isolates

Characterization of putative Hybrids for its Fatty Acid Profiles

Fatty acid composition of all hybrids, parental strains and auxotroph mutant were analyzed by GC. The results revealed that there were significant differences in fatty acid profiles among the hybrids [Table 4.21]. Parental strains and auxtrophic mutants did not have significant amount of GLA when compared to CFR-HyG9 and CFR-HyG12 hybrids. The GC chromatogram of

all the hybrids indicated that the presence of GLA in considerable amount when compare to parent and auxotrophic mutant [Fig. 4.44 a and b. c]. The presence of GLA in selected hybrids was further confirmed by MS. However, the GLA production varied among the hybrids and also the composition of unsaturated fatty acid index. Oleic and palmitic acids were the major fatty acids present in all the hybrids, parent and mutants. Palmitic acid and oleic acid content were showed drastic reduction in the hybrids when compared to parental strains and auxotroph mutant. In CFR-HyG9 and CFR-HyG10 the palmitic acid present at 15.9 % (less) and 22.8 % (high) respectively. It was also observed that the LA (C18:2) which is considered to be one of the essential fatty acids was found to be more quantity in all the hybrids when compared to parental strain and auxotrophic mutant. e.g. CFR-HyG4 and CFR-HyG10 the LA content was found to be more than the GLA content. Whereas in all other hybrids LA content was less than the GLA. The GLA as a percentage of total fatty acid in the lipid in parent was 15.42 ±00.59 % and the auxotrophic mutant was 10.90±0.57 %. All the putative hybrids had greater percentage of GLA than auxotrphic mutant. Among the putative hybrids eight of them had similar GLA content as the parent and seven putative hybrids had higher GLA content. The range of GLA percent of the putative hybrids was from 14.74±1.23% (CFR-HyG3) to 23.52±1.78% (CFR-HyG9). The unsaturated fatty acid content as indicated by the double index of the parent was 1.03±0.06 and in auxotrophic mutant it was 0.98±0.04. There is no significant difference in the DBI of all the putative hybrids. Thus, CFR-HyG9 and CFR-HyG12 were selected for production of GLA since, these two hybrids were producing high growth rate of biomass, lipid accumulation and GLA content in their mycelia compared to remaining hybrids. This indicates the activity of D^6-desaturase was more in the CFR-HyG9 and CFR-Hy12 when compared to other hybrids. The results obtained in this study supported by others in oleaginous microorganisms (Nakhara et al. 1992; Hiruta et al 1996). The parent, mutant (auxotroph) and hybrid cultures in selective medium is shown in Fig. 4.44

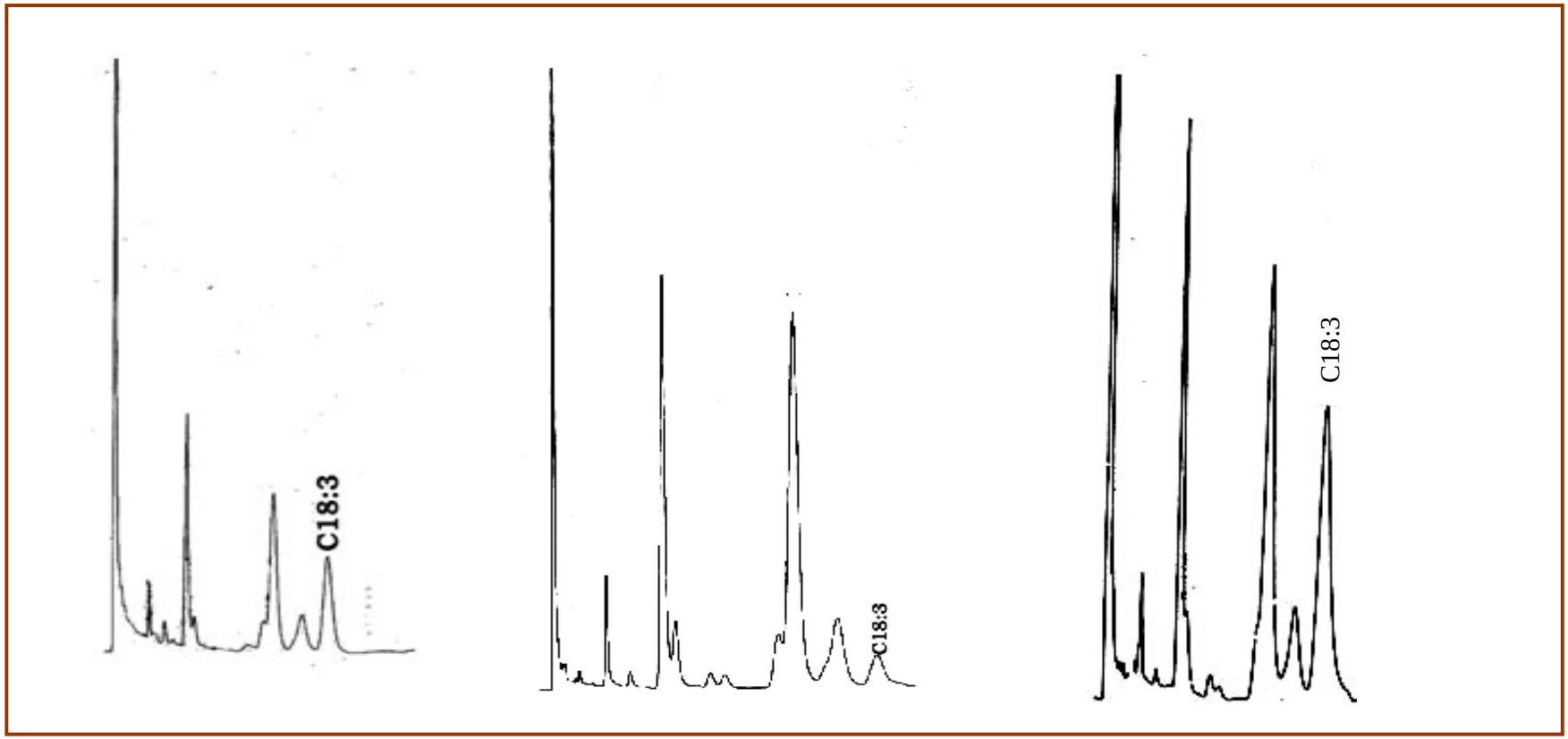


Fig 4.43 Gas chromatogram showing the increased GLA
a. Parent; b. Auxotroph mutant; c. Hybrid culture

Table 4.21 Fatty acid profile of hybrids obtained by intrastrain protoplast fusion of *M. rouxii* CFR-G15

Parent/ Aux.mutant/ Hybrids	Fatty acid composition (as % of total fatty acids)							
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	DBI
Parent	2.2±0.35	18.8±1.23	1.6±0.07	9.2±0.86	30.8±1.67	13.8±1.12	**14.42±1.02^{b}**	**1.03±0.06**
Aux.mut (met^{-})	2.9±0.41	25.3±1.26	2.7±0.09	6.1±0.50	38.8±2.18	11.7±1.02	**10.9±0.99^{a}**	**0.98±0.04**
CFR-HyG1	3.6±0.21	21±1.32	2.1±0.13	10.5±0.78	31.45±1.97	14.3±1.23	**15.9±1.23^{c}**	**1.10±0.7**
CFR-HyG2	2.1±0.15	19.7±1.37	1.4±0.06	11.5±0.96	32.87±1.65	14.6±1.42	**16.7±1.43^{cd}**	**1.14±0.4**
CFR-HyG3	4.0±0.43	21.4±1.57	2.1±0.15	9.5±0.73	33.3±1.87	13.6±1.13	**14.74±1.23^{b}**	**1.07±0.3**
CFR-HyG4	2.1±0.17	15.6±1.21	2.7±0.17	10.5±0.51	33.1±1.92	18.7±1.56	**15.96±1.26^{bc}**	**1.21±0.5**
CFR-HyG5	4.0±0.35	17.2±1.06	3.0±0.21	9.6±0.38	31.3±1.46	14.76±1.37	**17.97±0.84^{cd}**	**1.17±0.07**
CFR-HyG6	4.4±0.34	19.8±1.09	4.7±0.32	5.2±0.23	28.6±1.79	16.5±1.35	**17.63±1.46^{cd}**	**1.23±0.05**
CFR-HyG7	5.5±0.54	17.7±1.11	3.8±0.42	8.1±0.42	27.4±1.86	17.3±1.36	**20.13±0.90^{e}**	**1.23±0.04**
CFR-HyG1	4.0±0.43	18.2±1.24	3.1±0.42	6.3±0.46	25.3±1.65	22.4±1.67	**18.5±1.85^{cde}**	**1.29±0.06**
CFR-HyG9	**2.5**±0.21	15.9±1.01	3.6±0.29	5.4±0.34	26.4±1.25	21.5±1.45	**23.52±1.78^{e}**	**1.44±0.07**
CFR-HyG10	1.6±0.16	22.8±1.56	2.4±0.32	8.8±0.76	31.93±1.32	15.6±1.21	**15.4±1.34^{bc}**	**1.18±0.0**
CFR-HyG11	4.4±0.41	17.5±1.21	2.2±0.21	8.5±0.69	32.9±1.53	15.9±1.53	**17.8±1.62^{cde}**	**1.20±0.05**
CFR-HyG12	4.9±0.32	17.5±1.23	2.6±0.31	5.2±0.37	30.3±1.29	17.9±1.45	**19.9±1.56^{e}**	**1.28±0.4**
CFR-HyG13	6.2±0.41	18.6±1.14	3.7±0.43	7.6±0.39	29.3±1.31	16.8±1.39	**16.5±1.53^{bcd}**	**1.16±0.03**
CFR-HyG14	6.6±0.53	19.2±1.24	2.8±0.38	8.8±0.42	28.7±1.54	15.8±1.61	**16.8±1.53^{bcd}**	**1.14±0.4**
CFR-HyG15	3.7±0.32	17.6±1.17	2.1±0.28	10.4±0.32	34.2±1.78	14.7±1.59	**15.9±1.34^{bcd}**	**1.13±0.06**

All parameters are expressed as Mean ± SD values in the same column with different letters are significant different at p< 0.05 according to Duncan's multiple range tests. DBI- Double bond index.CFR-Central Food Technological Research Institute, Hy-Hybrid strains and G1-GLA containing isolates

Fig. 4.44 Photomicrograph of Parent, Mutant and Hybrid cultures of *M rouxii* CFR-G15 in FPM agar slants

Characterization of putative Hybrids for the protein, total soluble sugar, lipid and ash content

The selected hybrids were also subjected to proximate analysis of total protein, sugars and ash content on the dry biomass basis. The results are given in Table 4.22. The crude protein was estimated by Kjeldhal method and the results revealed that, protein content of the parent was 38.81±1.76% and that of auxotrophic mutant 40.1±2.01%. The range of protein in the putative hybrids was from 31.30±1.78% (CFR-HyG9) to 50.80±2.23% (CFR-HyG4). Six putative hybrids were higher than parent and two were lower. Rests of them were similar in protein percentage in their dry biomass. The total carbohydrates content in hybrids and parental strains of the biomass were estimated by phenol-sulphuric acid method. The results showed that the total crude carbohydrate in parent was 32.86±1.45% and the auxotroph mutant 30.10±1.52%. The values for carbohydrate content of the dry biomass of the putative hybrids varied from 20.12±1.05% (CFR-HyG3) to 32.55±1.96% (CFR-HyG10). The ash content of the parent was 0.16±0.03% and of that of auxotroph mutant was 0.18±0.05%. The ash content ranged from 0.14±0.03 to 0.35±0.09 and was not greatly different.

Thus, the various experiments carried out in this study, ultimately aimed to increase the biomass, total lipid content and maximizing the GLA production through study of various techniques. Based on the above results, hybrids culture namely CFR-HyG9 and CFR-HyG12 were selected for maximum biomass, lipid and GLA production. Future study may be undertaken for scale up studies using fermenters/reactors for the production of biomass and lipid extraction under optimized controlled conditions for food and pharmaceutical applications.

Table 4.22 The lipid, protein, carbohydrate and ash content of the parent/aux. mutant/ putative hybrids.

Parent/ Aux. mutant/Hybrid	Lipid (%)	Protein (%)	Carbohydrate (%)	Ash (%)
Parent	28.32±1.23	38.81±1.76	32.86±1.23	0.16±0.03
Aux.mut (met^-)	30.78±1.41	40.10±2.01	28.94±1.52	0.18±0.05
CFR-HyG1	34.76±1.76	36.91±1.89	28.07±1.61	0.27±0.07
CFR-HyG2	35.25±1.78	46.65±2.12	17.83±1.78	0.35±0.09
CFR-HyG3	32.78±1.86	45.22±2.17	20.12±1.05	0.24±0.08
CFR-HyG4	20.97±1.15	50.80±2.23	26.33±1.73	0.14±0.03
CFR-HyG5	30.79±1.59	36.31±1.78	31.09±1.69	0.23±0.05
CFR-HyG6	26.59±1.26	40.63±1.34	31.49±2.07	0.32±0.09
CFR-HyG7	40.63±2.13	30.45±1.51	28.74±1.67	0.18±0.02
CFR-HyG8	20.52±1.63	46.90±1.96	32.43±1.52	0.15±0.02
CFR-HyG9	42.46±2.41	31.30±1.78	25.97±1.37	0.27±0.04
CFR-GyG10	28.85±2.12	38.31±1.63	32.55±1.96	0.29±0.07
CFR-HyG11	32.31±2.10	42.60±1.87	24.83±1.31	0.26±0.08
CFR-HyG12	40.22±1.14	38.40±1.93	21.25±1.98	0.18±0.05
CFR-HyG13	34.32±2.53	40.24±1.97	25.23±1.73	0.21±0.06
CFR-HyG14	31.75±2.08	45.91±1.98	22.13±1.26	0.22±0.08
CFR-HyG15	40.36±2.78	35.36±1.37	24.01±1.43	0.27±0.06

Data are expressed as mean ±SD of three replicates.
CFR-Central Food Technological Research Institute;
Hy-Hybrid strains; G1-GLA containing isolates

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

Single cell oil (SCO) in the recent past, has become an accepted biotechnologically important product, fulfilling major roles in human health. They are also become very essential for nutrition of infants as well as geriatrics for overall maintenance. SCO are the edible oils extracted from microorganisms. As the single celled entities they are all at the bottom of the food chain (Zui and Ratledge 2005). Yeasts, fungi and several algae are able to produce high levels of nutritionally and pharmaceutically important SCO, rich in PUFAs (Cohen & Ratledge 2005; Waltermann et al. 2009).

In this study, *M. rouxii* CFR-G15, a zygomycetous fungi producing high amount of gamma linolenic acid was isolated from soil and has been subjected various experimental conditions to produce maximum lipid and GLA in its biomass.

In this section an overall conclusions from each study have been described:

In order to obtain native isolate for GLA production, 250 soil samples from various habitats (river banks, humus soil, forest agricultural land, Zoo zone, garden and pond) were collected and screened for oleaginous fungi with special reference to *Mucor* spp. Normally, *Mucor, Rhizopus, Aspergiluus and penicillium species were* observed from the above soil samples. Higher percentage (40%) of *Mucor* species were noticed among the fungal population. All *Mucor* spp. were saprophytic and proteolytic in nature and they grew faster on the nutrient rich medium when compared to other fungi.

Cottony hairy with white or grey coloured growth was the morphological character of *Mucor* sp. Later these *Mucor* spp. were identified with standard culture obtained from MTCC Bank, confirmation was also performed using Gilman manual of soil fungi and further confirmed by molecular methods (Fig. 4.1.1) using rRNA analysis and Δ^6-desaturase gene cloning and sequencing analyses.

All *Mucor* species isolated through screening methods were observed for their oleagenecity by qualitative method using Sudan B black stain and quantitatively by gravimetric method, respectively.

Mycelia with highly stained fat globules inside were selected for further study. About 20 *Mucor* isolates along with the standard cultures were cultivated in fat production medium by submerged fermentation to find out the growth characteristics, total lipid production and GLA content. Among all the 20 isolates, CFR-G15 showed higher biomass production (8.82±0.93 g/L) on dry basis, lipid percentage (30±1.32%) and GLA (14.42±0.74as % of fatty acids) content.

Optimization of cultural conditions for maximum biomass, lipid and GLA content of *Mucor rouxii* CFR-G15 (screened and selected as potent culture for GLA production) was carried out. The cultivation conditions for GLA production were optimized by appropriate selection of pH, temperature, aeration, effect of inoculum size/concentration, media composition, various carbon and nitrogen sources in the cultivation media, C:N ratio, incorporation of certain vegetable oils in the growth media and certain minerals with different concentrations. From the above experiments on optimization parameters a pH of 5.5-6.0, temperature at 28 ±2°C for good biomass buildup and 15°C for GLA formation in mycelial structure were found to be optimum.

Effect of aeration on the culture with different volumes of medium in the cultivation flask was tested. Our result concluded that 100 ml working volume in 500 ml capacity culture flasks gave maximum biomass, lipid and GLA production in *M. rouxii* CFR-G15.

Evaluation of various carbon sources for maximizing the GLA production was carried out. Carbon sources like glucose, fructose, sucrose, starch, galactose, maltose, and lactose were tested for the above purpose. The result indicated that, when glucose and soluble starch were used as carbon source individually, a good biomass build up (8.44±0.34 and 8.12±0.81 g/L) was found. Sucrose and lactose didn't give any significant growth of *M. rouxii*

CFR-G15. Slight variation was observed among the other carbon sources in growth of *M. rouxii* CFR-G15.

The effect of nitrogen on the production of GLA in *M. rouxii* CFR-G15 was studied by using different nitrogen compounds. They are yeast extract, peptone, ammonium sulphate, ammonium nitrate, potassium nitrate and urea. Our results concluded that the medium containing yeast extract and ammonium nitrate gave the highest cell biomass and total lipid yield and GLA content. Urea in the cultivation medium gave more biomass but very low lipid content.

Studies on C:N ratio by varying the carbon and nitrogen sources were carried out for optimal production of GLA in the fungus. Through this study, it was concluded that C:N ratio 40-80 gave maximum lipid and GLA production with *M. rouxii* CFR-G15.

Several studies indicated that addition of certain minerals such as Ca^{2+}, Mg^{2+}, Na^{2+}, K^{+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} ions at different concentrations had an effect on growth, lipid and GLA production. Our results indicated that Mg^{2+}, and Ca^{2+} when added at 0.5 g/L in growth medium increased the concentration of GLA more than 10% in *M. rouxii* CFR-G15. Other minerals like Na^{2+}, K^{+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and Mn^{2+} didn't show any significant increases on these parameters.

Similarly inclusion of certain plant oils also had an effect on biomass, lipid and GLA production. Coconut, palm, sunflower, ground nut, niger, gingelly and mustard oils were added to cultivation medium. The results concluded that niger seed oil showed increase in biomass, lipid content and GLA content in the *M. rouxii* CFR-G15. When growth medium was supplemented with coconut oil and palm oil, the GLA content was found to be very low when compared to basal medium, because these oils very low linoleic acid content in their lipid. Hence we conclude that oil sources containing saturated fatty acids didn't favour the production of GLA. It is confirmed again from our experiment that linoleic acid is the precursors for the formation of GLA and other PUFAs. Niger seed oil contains good amount of linoleic acid

thus incorporation of this oil in the growth medium favoured the GLA production in *M. rouxii* CFR-G15.

Response surface methodology (RSM) was used with the aim of optimizing the levels of carbon and nitrogen source to maximize the GLA production in *M. rouxii* CFR-G15. Various trial experiments revealed that glucose, yeast extract and ammonium nitrate played a major role in GLA production in this fungus. The optimization study was carried out with CCR design (CCRD) with these variables. 20 experiments were carried out and central point was experimented 6 times. The results indicated that the maximum yield of biomass (12. 2 g/L), lipid (39.9 %) and GLA (18.89%) observed with glucose 65 g/L, yeast extract 3.5 g/L and ammonium nitrate 0.5 g/L were used.

Strain improvement is an important criterion for microorganisms to improve the product performance. This fungus, *M. rouxii* CFR-G15, was subjected to mutation (UV, EMS and NTG) and the mutants were selected based on the auxotrophic growth requirements. Lysine (lys^-), alanine (ala^-), isoleusine ($isoleu^-$) and methionine (met^-) auxotrophs were selected as markers for protoplast fusion studies. Though we could produce a few auxotrophic markers, many of them have reverted back to original genetic status due to DNA autorepair mechanism. A double mutation on this fungus, have resulted in getting met^- auxotroph permanently. This marker was used as selection criterion for hybrid selection.

Intrastrain/intraspecific protoplast fusion (hybridization) experiment was carried out to obtain hybrids with high lipid and GLA content. PEG 6000 at 30% level was used as fusogenic agent. By using standard protocol a few hybrids were selected. Quantitative analysis revealed that, out of 15 hybrids, CFR-HyG9 and CFR-HyG12 had more GLA content when compared to parent and met^- auxotroph mutant cultures of *M. rouxii* CFR-G15.

This study on GLA production using *Musor* sp. (*Mucor rouxii* CFR-G15) indicates a promising alternative/ additional source for industrial scale production from conventional sources like evening primrose, borage seed oil and other potential microbial sources. Screening studies are useful identifying organisms for PUFAs production. Further study can be carried out to identify potential PUFAs carried out to identify potential PUFAs producers especially GLA. Exploring the native isolate for the production of the speciality lipid GLA using biotechnological approaches is an important step. The present work enabled the identification of new fungal strains for their potential GLA production through manipulation of growth conditions, mutation and hybridization.

The genes and genes products involved in the biosynthesis of PUFAs is being identified and characterized all over the world for higher production of PUFAs from microbial sources. Additionally, gene encoding Δ^6-desaturase enzyme involved in GLA biosynthesis in *M. rouxii* CFR-G15 was also identified. The present research work provides useful information for further work on PUFA metabolic pathways and gene engineering about GLA production from microbial sources. A potential strain was reported that could be used for the development of an economical process in industrial GLA production.

OUTCOME OF THIS RESEARCH WORK

PAPERS

Mamatha, S.S., Ravi, R.and Venkateswaran, G. "Medium optimization of Gamma Linolenic Acid production in *Mucor rouxii* CFR-G15 by RSM". Food and bioprocess technology An International journal (Published)

Mamatha, S. S. and Venkateswaran, G**.** "Differential temperature effect on Gamma linolenic acid prodcuton in *Mucor rouxii* CFR-G15. Indian journal of Microbiology (Accepted)

Mamatha S S, Prakash M Halami and Venkateswaran G. "Identification and characterization of omega 6 fatty acids producing *Mucor rouxii* native isolate CFR-G15" .European Journal of lipid science and technology (Accepted)

Mamatha S S, Muthukumar S P and Venkateswaran G. **"**Safety evaluation of M*ucor rouxii CFR*-G15 biomass containing ω -6 fatty acids in rats". Regulatory toxicology and pharmacology (communicated)

Papers presented in National/International Conferences/Symposia

Mamatha S.S., Gayathri N and G. Venkateswaran (2004). Intraspecific protoplast fusion for altered lipid profile in *Mucor rouxii*. 45th Annual Conference of AMI held between 23rd and 25th Nov 2004 at National Dairy Research Institute, Karnal, Haryana.

Mamatha S. S and G.Venkateswaran (2005) Differential temperature effect on growth, lipid and lipid profiles of *Mucor rouxii*: a study. 46th Annual Conference of AMI held between 8th and 10th Dec 2005 at Osmania University, Hyderabad.

Mamatha S. S. and G.Venkateswaran (2005) Studies on Gamma Linolenic Acid (GLA) of *Mucor* sps: Isolation and screening from soil sources. 17th Indian Convention of Food Scientists and Technologists organized by AFSTI (I), CFTRI and DFRL held between 9th and 10th December 2005 at NIMHANS, Bangalore. **Best poster Award in the area of Microbiology and Biotechnology**

Mamatha, S. S. and Venkateswaran, G. "Effect of extraction methods on lipid yield with special reference to γ-linolenic acid from *Mucor rouxii* CFR-G15" at the 17th ICFOST jointly organized by CFTRI, and

AFST (I) held between 16th and 17th of Nov-2006 at Agricultural university, Hyderabad Also awarded **Best poster award in the area of Biotechnology and Microbiology**

Mamatha, S. S. and Venkateswaran, G**.** "Influence of C:N ratio on the production of lipid and γ-linolenic acid by *Mucor rouxii* CFR-G15" 47th Annual Conference of AMI held at Biotechnology and bioinformatics department, Barkatulla University, in the month of Dec-2006

Mamatha, S.S., Ravi, R.and Venkateswaran, G**.**. "Response Surface Optimization Of Media Components For Gamma Linolenic Acid Production By *Mucor Rouxii* CFR- G15" at the 75th Annual Conference of SBC(I) held at life sciences department, Jawaharlal Nehru University, in the month of Dec-2006

Mamatha S. S and G.Venkateswaran (2007) "Enhancing γ-linolenic acid production by M*ucor rouxii* CFR-G15 using oils". "New horizons in biotechnology (NHBT)" organized by NIIST, Trivandrum held between 26th and 29th November 2007

Mamatha S. S and G.Venkateswaran (2007) "Influence of Mineral components for the enhanced production of γ-linolenic acid by M*ucor rouxii* CFR-G15" 1st International Conference of Applied Bioengineering held at Sathyabhama University, Chennai.

Mamatha S S, Prakash M Halami and Venkateswaran G (2008) "Molecular characterization of omega 6 fatty acids producing *Mucor rouxii* native isolate CFR-G15" IFCON-2008 organized by AFST(I) held at CFTRI, Mysore

Mamatha S S, MuthuKumar S P† and Venkateswaran G (2008) "Safety evaluation of omega 6 fatty acids containing *Mucor rouxii* CFR-G15 biomass in albino rats for food formulation" IFCON-2008 organized by AFST(I) held at CFTRI, Mysore

BIBLIOGRAPHY

Abe A, Oda Y, Asano K & Sone T (2006**)** The molecular phylogeny of the Genus *Rhizopus* based on rDNA sequences. *Biosci Biotechnol Biochem*. 70, 2387-2393.

Aggelis G (1996**)** Two alternative pathways for substrate assimilation by *Mucor circinelloides*. *Folia Micro*biol, 41, 254–256.

Aggelis G & Sourdis J (1997**)** Prediction of lipid accumulation-degradation in oleaginous micro-organisms growing on vegetable oils. *Antonie Leeuwenhoek*, 72, 159–165.

Aggelis G (2003**)** Selective uptake of fatty acids by the yeast *Yarrowia lipolytica*. *Eur J Lipid Sci Technol*, 105, 651–655.

Ahmed S U, Singh S K, Pandey A, Kanjilal S & Prasad R B N (2006) Effects of Various Process Parameters on the Production of γ-Linolenic Acid in Submerged Fermentation. *Food Technol Biotechnol*, 44, 283–287.

Ahmed S U, Reddy K K, Swathy L S, Singh S K, Kanjilal S, Prasad R B N & Pandey A (2009**)** Enrichment of γ-Linolenic Acidin the extracted from *Mucor zychae* MTCC 5420. *Food Res Internat*, 42, 449-453.

Aki T, Shimida Y, Inagaki K, Higashimoto H, Kawamoto S, Shigete S, Ono K & Suzuki O (1999) Molecular cloning and functional characterization of rat delta-6 fatty acid desaturase. *Biochem Biophys Res Commun*, 255, 575-579.

Altschul S F, Maddan T L, Schaffer A A, Zhang J, Zhang Z, Miller W & Lipman D J (1997**)** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids Res*, 25, 3389-3402.

Alvarez H, Kalscheuer R & Steinbul A (2000**)** Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. *Appl Microbiol Biotechnol*, 54, 218–223.

Ando S, Nakajima K & Hatano M (1992**)** Incorporation of n-3Polyunsaturated fatty acids into phospholipids of a marine bacterium *Vibrio* sp. cultivated with the sardine oil. *J Ferment Bioeng*, 73, 169-171.

Anne J & Peberdy J F (1976**)** induced fusion of fungal protoplasts following treatment with polyethylene glycol. *J Gen Microbiol*, 92, 413-417.

AOAC (1991**)** Official methods of Analysis, 15th edn, Vol 1and 2. Assoc. Offici. Anal. Chem. Arlington.

AOAC Official methods of Analysis (1984a), 13 Edition, Association of official Analytical Chemists, Washington 529

AOAC Official methods of Analysis (1984b), 13 Edition, Association of official Analytical Chemists, Washington 447

Aoki H, Nishioka K, Mankura M, Endo Y & Fujimoto K (1999**)**The incorporation of Accumulation of Supplemented Fatty acids in *Mcor hiemalis HA-30*. *Biosci Biotechnol biochem*, 63, 1032-1036.

Bajpai P, Bajpai P K & Ward O P (1991b**)** Eicosapentaenoic acid (EPA) formation: Comparative studies with *Mortierella* strains and production by *Mortierella elongata*. *Mycological Res*, 95, 1294–1298.

Bajpai P K, Bajpai P & Ward O P (1991d**)** Optimization of production of docosahexaenoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. *J Am Oil Chem Soc,* 68, 509–514.

Bajpai P K & Bajpai P (1992) Review: arachidonic acid production by microorganisms. Biotechnol. *App. Biochem,* 15, 1–10.

Bajpai P & Bajpai P K (1993) Eicosapentaenoic acid (EPA) production from microorganisms: a review. *J Biotechnol*, 30, 161–183.

Bandyopadhyay S, Ghosh S, Chaudari S & Bhattacharyya D K (2001) production of γ-linolenic acid bt *Rhizopus nigricans* SSD-8. *J Oleo Sci.* 50, 641-647.

Bandyopadyay K, Bandyopadhyay S, Bhattacharyya A K & Ghosh G (2003) Mineral addition on production of γ-Linolenic acid by *Rhizopus nigricans. J Oleo Sci*, 52, 215-219.

Barclay W R, Meager K M & Abril J R (1994) Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae like microorganisms. *J Appl Phycol*, 6, 123–129.

Barham, J.B, Edens M B, Fonteh A N, Johnson M M, Easter L & Chilton F H (2000) Addition of eicosapentaenoic acid to gamma-linolenic acid-supplemented diets prevents serum arachidonic acid accumulation in humans. *J Nutr,* 130, 1925-1931.

Belch J J F & Hill A (2000). "Evening primrose oil and Borage oil in rheumatologic conditions", *Ame J Clin Nut*r. 71, 352S-356S.

Berry E M (1997) Dietary fatty acids in the management of diabetes mellitus. *Am J Clin Nutr*, 66 (Suppl), 991S–7S.

Berry E M (2001) Are diets high in omega-6 polyunsaturated fatty acids unhealthy? *Europ Heart J Suppl*, 3, D37–D41.

Bligh E C & Dyer W J (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*, 37, 911–917.

Botha A, Kock J L F, Roux C, Coetzee D J & Botes P J (1995) An isolation medium for γ-linolenic acid producing mucoralean fungi. *Syst Appl Microbiol*, 18, 448-453.

Both A, Kock J L F Coetzee D J & Botes P J (1997) Physiological properties and fatty acid composition in *Mucor cercinelloides f circinelloides. Antonie van leeuvenhoek* 71, 201-207.

Botham P A & Ratledge C (1979) A biochemical explanation for lipid accumulation in Candida 107 and other oleaginous micro-organisms. *J Gen Microbiol*, 114, 361-375.

Brown J E (2005) A critical review of methods used to estimate γ-linoleic acid-delta-6 desaturase, *exo vivo* and *in vivo*. *Eur J Lipid Sci Technol*, 107, 119-134.

Brume M D, Parker V G, Alacemi M & Socici H (1992) Strain improvement of *Claviceps purpurea* by protoplast fusion without introducing auxotrophic markers. *Appl Microbiol Biotechnol* , 38-42.

Burdon K L (1964) Fatty material I bacteria and fungi revealed by staining dried, fixed slide preparation. *J Bacteriol*, 52, 665-578.

Burja A M, Aementa R E, Radianingtyas H Barrow C J (2007) Evaluation of fatty acid extraction methods for *Thraustochytrium* sp. ONC-T18. *J Agric Food Chem*, 55, 4795-4801.

Calder P C (2001) Polyunsaturated fatty acids, inflammation, and immunity. *Lipids*, 36, 1007–1024.

Carter J P (1988) ϒ-Linolenic acid as a nutrient. *Food Technol*, 6, 72–82.

Carvalho P O, Oliveira J G & Pastore G M (1999) Enhancement of Gamma-Linolenic Acid production by the fungus *Mucor* sp LB-54 by growth temperature. *Rev Microbiol*, 30, 170-175.

Certik M, Sereke Berhan S & Sajbidor J (1993) Lipid production and fatty acid composition of selected strains belonging to Mucorales. *Acta Biotechnol*, 13, 193–196.

Certík M, Andrasi P & Sajbidor J (1996) Effect of extraction methods on lipid yield and fatty acid composition of lipid classes containing gamma-linolenic acid extracted from fungi. *J Ame Oil Chem Soc*, 73, 357-365.

Certik M, Balteszova L & Sajbidor J (1997) Lipid formation and gamma-linolenic acid production by Mucorales fungi grown on sunflower oil. *Lett Appl Microbiol*, 25, 101–105.

Certik M, Sakuradani E & Shimizu S (1998) Desaturase-defective fungal mutants:useful tools for the regulation and overproduction of polyunsaturated fatty acids. *Trends Biotechnol*, 16, 500–505.

Certik M & Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J Biosci Bioeng*, 87, 1–14.

Certik M, Megova J & Horenitzky R (1999) Effect of nitrogen sources on the activities of lipogenic enzymes in oleaginous fungus *Cunninghamella echinulata*. *J Gen Appl Microbiol*, 45, 289–293.

Certik M & Shimizu S (2000) Kinetic analysis of oil biosynthesis by an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. *Appl Microbiol Biotechnol*, 54, 224–230.

Certik M, Slavikova L, Masrnova S & Sajbidor J (2006) Enhancement of Nutritional Value of Cereals with γ-Linolenic Acid by Fungal Solid-State Fermentations. *Food Technol Biotechnol*, 44, 75–82.

Chadha B S, Gulati H K, Minhas M, Saini H S & Singh N (2004) Phytase production by thermophilic fungus *Rhizomucor pusillus*. *World J Microbiol Biotechnol*, 20, 105–109.

Chang C Y, Lue M Y & Pan T M (2006) Statistical optimization of medium components for the production of *Antrodia cinnamomea* AC0623. *Appl Microbiol Biotech*, 72, 654-661.

Chen H C & Chang C C (1996) Production of γ-linolenic acid by the fungus *Cunninghamella echinulata* CCRC31840. *Biotechnol Prog*, 12, 338-341.

Chen H C, Chang C C, & Chen C X (1997) Optimization of arachidonic acid production by *Mortierella alpine* Wuji-H14 isolate. *J Am Oil Chem Soc*, 74, 569-578.

Chen H C & Liu T M (1997) Inoculum effects on the production of γ-linolenic acid by the shake culture of *Cunninghamella echinulata* CCRC 31840. *Enzyme Microb Technol*, 21, 137–142.

Chen Y, Eisner T D, Kattar M M, Barrett S L R, Lafe K, Bui U, Limaye A P & Cookson B T (2001) Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. *J Clin Microbiol*, 39, 4042–4051.

Cheng H M, Walker T H, Hulbert J G & Raman R D (1999) Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oil. *Biores Technol,* 67, 101-110.

Choi S Y, Ryu D D W & Rhee J S (1982) Production of microbial lipid: Effects of growth rate and oxygen on lipid synthesis and fatty acid composition of *Rhodotorula gracik. Biotechnol Boeing*, 24, 1165-1172.

Cohen Z & Ratledge C in: Single Cell Oils, American Oil Chemists' Society, Champaign, IL, USA, 2005

Conti E, Stredansky M, Stredansky S & Zanetti F (2001) Gamma-linolenic acid production by solid-state culture of Mucorales strains on cereals. *Biores Technol*, 76, 283–286.

Das T, Huang Y S & Mukerji P. δ6-desaturase and γ-linoleic acid biosynthesis: A biotechnology perspective *In: gamma linolenic acid: recent advances in biotechnology and clinical application* Eds. Y.S. Huang, V. A. Ziboh. AOCS press, 2001c, 6-23.

Das U N (2004) From bench to the clinic: [gamma]-linolenic acid therapy of human gliomas. *Prostaglandins Leukot Essent Fatty Acids,* 70, 539–552.

Das U N (2006) Essential fatty acids: biochemistry, physiology and pathology. *Biotechnol J,* 1, 420–439.

Das UN (2007**)** A defect in the activity of D6 and D5 desaturases may be a factor in the initiation and progression of atherosclerosis. *Prostaglandins Leukot Essent Fatty Acids,* 76, 251-268.

Da Silva T L, Pinheiro H M & Roseiro J C (2003) Stress-induced morphological and physiological changes in γ-linolenic acid production by *Mucor fragilis* in batch and continuous cultures. *Enzyme Microb Technol,* 32, 880–888.

Davies R J, Scale up of yeast technology; In *Industrial Application of Single Cell Oils*, kyle D J & Ratledge C. Eds. American oil Chemists Society, Champaign, IL, 1992 pp. 196-218.

Davies R J, Holdswoth J E & Reader S L (1990) The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast *Apiotrichum curvatum. Appl Microbiol Biotechnol*, 33, 569–573.

Demaison L & Moreau D (2002) Dietary ω-3 polyunsaturated fatty acids and coronary heart disease-related mortality: a possible mechanism of action. *Cell Mol Life Sci,* 59, 463–477.

Devem J M &. Manocha M S (1976) Effect of various cultural conditions on the fatty acid and lipid composition of *Choanephora cucurbitarum. Can J Microbiol*, 22, 443-449.

Domergue F, Abbadi A, Zahringer U, Moreau H, Heinz E (2005) *In vivo*-characterisation of the first acyl-CoAvDelta6-desaturase from the plant kingdom, the microalga *Ostreococcus tauri*. *Biochem J,* 389, 483–490.

Dyal S D, Bouzidi L & Narine S (2005) Maximizing the production of γ-linolenic acid in *Mortierella ramanniana var. ramanniana* as a function of pH, temperature and carbon source, nitrogen source, metal ions and oil supplementation. *Food Res Inter,* 38, 815-829.

Dyal S D & Narine S (2005) Implications for the use of *Mortierella* fungi in the industrial production of essential fatty acids. *Food Res Inter,* 38, 445–467.

Dyaneshwar W, Kalpana J & Abhay H (2006) Polyunsaturated fatty acids: Biotechnology. *Crit Rev Biotech,* 26, 83-93.

Duncan DB (1955) Multiple range and multiple F- tests. *Biometrics* 11, 1-42.

du Preez J C, Immelman M & Kilian S G (1996) The utilization of short-chain monocarboxylic acids as carbon sources for the production of gamma-linolenic acid by *Mucor* strains in fed-batch culture. *World J Microbiol Biotechol,* 12, 68.

du Preez J C, Immelman M, Kock J L K & Kilian S G (1997) The effect of acetic acid concentration on the growth and production of gamma-linolenic acid by *Mucor circinelloides* CBS 203.28 in fed-batch culture. *World J Microbiol Biotechol,* 13, 81-86.

EI-Bondkly A M (2006) Gene transfer between different *Trichoderma* species and *Aspergillus niger* through intergeneric protoplast fusion to convert ground rice straw to citric acid and cellulases. *Appl Biochem Biotechol,* 135, 117-132.

El Bondkly A M & Talkhan F N (2007) Intra-strain crossing in *Trichoderma harzianum via* protoplast fusion to enhance chitinase productivity and bio control activity. *Arab J Biotech,* 10, 233-240.

Emelyanova E V (1996) γ-Linolenic acid production by *Cunninghamella japonica* in solid state fermentation. *Process Biochem,* 31, 431-434.

Emelyanova E V (1997) Lipid and γ-linolenic acid production by *Mucor inaquisporus*. *Process Biochem,* 32, 173- 177

Evans D A (1983) Agricultural application of protoplast fusion. *Nature Biotechnol,* 1, 253-261.

Evans C T, Scragg A H & Ratledge C (1983a). A comparative study of citrate efflux from mitochondria of oleaginous and non oleaginous yeasts. *Eur J Biochem,* 130, 195-204.

Evans C T & Ratledge C (1983b) Biochemical activities during lipid accumulation in *Candida curvata*. *Lipids,* 18, 630-635.

Evans & Ratledge (1984) Effect of nitrogen source on lipid accumulation in oleaginous yeasts. *J Gen Microbiol,* 47, 39-43.

Evans C T & Ratledge C (1985) Possible regulatory roles of ATP: citrate lyase, malic enzyme, and AMP deaminase in lipid accumulation by *Rhodosporidium toruloides* CBS 14. *Can J Microbiol,* 31, 1000-1005.

Fakas S, Papanikolaou S, Galiotou-Panayotou M, Komaitis M & Aggelis G (2006) Lipids of *Cunninghamella echinulata* with emphasis to γ-linolenic acid distribution among lipid classes. *Appl Microbiol Biotech,* 73, 676–683.

Fakas S C, Certik M, Papanikolaou S, Aggelis G, Komaitis M & Galiotou-Panayotou M (2007a) γ-linolenic acid production by *Cunninghamella echinulata* growing on complex organic nitrogen sources. *Bioresour Technol,* 99, 5986–5990.

Fakas S, Galiotou-Panayotou M, Papanikolaou S, Komaitis M & Aggelis G (2007b) Compositional shifts in lipid fractions during lipid turnover in *Cunninghamella echinulata. Enzyme Microb Technol,* 40, 1321–1327.

Fakas S, Papanikolaou S, Galiotou-Panayotou M, Komaitis M & Aggelis G (2008) Organic nitrogen of tomato waste hydrolysate enhances glucose uptake and lipid accumulation in *Cunninghamella echinulata. J Appl Microbiol,* 105, 1062–1070.

Fakas S, Papanikolaou S, Batsosa A, Panayotoua M G, Mallouchosa A, Aggelis G (2009a) Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabellina. Biomass & Bioenergy,* 33, 573-580.

Fakas S, Makri A, Mavromati M, Tselepi M & Aggelis G (2009b) Fatty acid composition in lipid fractions length wise the mycelium of *Mortierella isabellina* and lipid production by solid state fermentation. *Bioresource Technol,* 100, 6118-6120.

Fan Y Y & Chapkin R S (1998) Importance of dietary g-linolenic acid in human health and nutrition. *J Nutr,* 128, 1411–1414.

Favati F, King J W & Mazzanti M (1991) Supercritical carbon dioxide extraction of Primrose oil. *J Am Oil Chem Soc,* 68, 422–427.

Finnerty W R & Makula P A (1975) Microbial lipid metabolism. *Crit Rev Microbiol,* 4, 1–40.

Fliegerova K, Hodrova B & Voigt K (2004) Classical and molecular approaches as a powerful tool for characterization of rumen polycentric fungi. *Folia Microbiol,* 49, 157–164.

Flider F J (2005) GLA: Uses and new sources. *Inform,* 16, 279-282.

Folch J, Lees M & Sloane-Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem, 199,* 833–841.

Fredricks D N, Smith C & Meier A (2005) Comparison of Six DNA Extraction Methods for Recovery of Fungal DNA as Assessed by Quantitative PCR. *J Clin Microbiol,* 43, 5122–5128.

Funtikova N S & Mysyakina I S (1997) Synthesis of γ-linonenic acid by mucaraceous fungi utilizing exogenous fatty acids. *Microbiol,* 66, 76–79.

Funtikova N S, Mysyakina I S & Konova I V (2002) Synthesis of biologically Active Lipids by the Fungus *Mucor lusitanicus* 306D Grown on Media with Various Composition. *Appl Biochem Microbiol,* 38, 553–557.

Gamma Linolenic acid-GLA-Mongraph. *Alternative Med Rev*, March 2004. Health Care Industry.

Gema H, Kavadia A, Dimou D, Tsagou V, Komaitis M & Aggelis G (2002) Production of γ-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. *Appl Microbiol Biotechnol*, 58, 303–307.

Gil A (2002) Polyunsaturated fatty acids and inflammatory diseases. *Biomed Pharmacother,* 56, 388–396.

Gill C O, Hall M J & ratledge C (1977) Lipid accumulation in an oleaginous yeast (*Candida* 107), growing on glucose in single cell -stage continuous culture. *Appl Environ Microbiol,* 32, 231-239.

Gill I & Valivety R (1997) Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. *Trends Biotechnol,* 15, 401-409.

Gilman C J: Manual Soil Fungi. *Biotech Books,* Delhi (India). 1998

Goodwin D C & Lee S B (1993) Rapid, microwave mini-prep of total genomic DNA from fungi, plants, protists and animals for PCR. *Biotechniques,* 15, 438–444.

Gosselin Y, Lognay G, & Thonart P (1989) Improvement of fed-batch mass culture for gamma-linolenic biosynthesis by *Tefrahyenu rostrufa* (protozoa). *Biotechnol Lett,* 11, 423-426.

Graham G C, Mayers P & Henry R J (1994) A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Biotechniques,* 16, 48–50.

Graham I A, Cirpus P, Rein D & Napier J A (2004) The use of very long chain polyunsaturated fatty acids to ameliorate metabolic syndrome: transgenic plants as an alternative sustainable source to fish oils. *Nutr Bulletin,* 29, 228–233.

Guil-Guerrero J L, Go´mez-Mercado F, Garcı´a-Maroto F & Campra-Madrid P (2000) Occurrence and characterization of oils rich in γ-linolenic acid (I): *Echium* seeds from *Macaronesia*. *Phytochem*, 53, 451-456.

Guil-Guerrero J L, Go´mez-Mercado F, Rodrı´guez-Garcı´a I, Campra-Madrid P & Garcı´a-Maroto F (2001a) Occurrence and characterization of oils rich in γ-linolenic acid (III): the taxonomical value of the fatty acids in Echium (*Boraginaceae*). *Phytochem*, 58, 117-120.

Guil-Guerrero J L, Garcı´a-Maroto F F & Gime´nez-Gime´nez A (2001b) Fatty acids profiles in organs from forty-nine plant species that are potential new sources of γ-linolenic acid. *J Am Oil Chem Soc,* 78, 677- 684.

Gunashree B S (2006) Ph.D Thesis, University of Mysore, Mysore, India.

Gunstone F D (1992) Gamma linolenic acid. Occurrence and physical and chemical properties. *Prog Lipid Res,* 31, 145- 161.

Gunstone F D (1998) Movements towards tailor-made fats. *Prog Lipid Res*, 37, 277–305.

Hammond & Glatz (1988) Extraction and analysis of single cell oils. *In Single Cell Oil* ed. Moreton R S. pp. 147–165. Harlow: Longmans Scientific & Technical. New York

Hang Y D & Woodams E E (1987) Effect of substrate moisture content on fungal production of citric acid in a solid state fermentation system. *Biotechnol Lett,* 9, 183-186.

Hansson L & Dostalek M (1988) Effect of culture conditions on mycelial growth and production of γ- linolenic acid by the fungus *Mortierella ramanniana*. *Appl Microbiol Biotechnol,* 28, 240–246.

Hansson L, Dostalek M & Sorenby B (1989) Production of γ-linolenic acid by the fungus *Mucor rouxii* in fed-batch and continuous culture. *Appl Microbiol Biotechnol,* 31, 223–227

Harbige L S (2003) Fatty acid, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids*, 38, 323–341

Hastings N, Agaba M, Tocher D R, Leaver M J, Dick J R, Sargent J R, Teale A J (2001) A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc Nat Acad Sci*, 98, 14304–14309.

Haugland R A, Heckman J L & Wymer L J (1999) Evaluation of different methods for the extraction of DNA from fungal conidia by quantitative competitive PCR analysis. *J Microbiol Methods,* 37, 165–176.

Hedge J E & Hofreiter B T (1962) In: Carbohydrate Chemistry 17 Ed: Whiltler R L & Be Miller J N, Academic Press New York.

Higashiyama K, Yaguchi T, Akimoto K, Fujikawa S & Shimizu S (1998) Effects of mineral addition on the growth morphology and arachidonic acid production by *Mortierella alpina* 1S-4. *J Am Oil Chem Soc*, 75, 1815-1819

Higashiyama K, Murakami K, Tsujimura H, Matsumoto N & Fujikawa S (1999) Effect of dissolved oxygen on the morphology of an arachidonic acid production by *Mortirella alpina* 1S-4. *Biotechnol bioeng*, 63, 442-448.

Hirano M, Mori H, Miura Y, Matsunaga N, Nakamura N & Matsunaga T (1990) Gamma-linolenic acid production by microalgae. *Appl Biochem Biotechnol*, 24-25, l83- 191

Hiruta O, Futamura T, Takebe H, Satoh A, Kamisaka Y, Yokochi T, Nakahara T & Suzuki O (1996) Optimization and scale-up of γ-linolenic acid production by *Mortierella ramanniana* MM15-1, a high γ-linolenic acid producing mutant. *J Ferment Bioeng*, 82, 366–370.

Hiruta O, Yamamura K, Takebe H, Futamura T, Linum K & Tanka H (1997) Application of Macbled fermentor for microbial processes. *J Ferment Bioeng*, 83, 79-86.

Ho S Y, Jiang Y & Chen S (2007) PUFAs Contents of the fungus *M. alpina* isolated from soil. *J Agric Food Chem*, 55, 3960-3966.

Ho S Y & Chen F (2008) Genetic characterization of *Mortierella alpina* by sequencing the 18S-28S ribosomal gene internal transcribed spacer region. *Lett Appl Microbiol,* 47, 250-255.

Holdsworth J E, Veenhuis M & Ratledge C (1988a) Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids. *J Gen Microbiol,* 134, 2907–2915.

Holdsworth J E & Ratledge C (1988b) Lipid turnover in oleaginous yeasts. *J Gen Microbiol,* 134, 339–346.

Holiday, R (1956) *Nature* , 178, 987.

Holker U, Hofer M & Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl Microbiol Biotechnol,* 64, 175-186.

Holker U & Lenz J (2005) Solid-state fermentation-are there any biotechnological. *Curr Opin Microbiol*, 8, 301-306.

Hong H, Datla N, Reed D W, Covello P S, Mackenzie S L & Qiu X (2002) High-level production of gamma-linolenic acid in *Brassica juncea* using a Delta-6 desaturase from *Pythium irregulare*. *Plant Physiol*, 129, 354-362.

Hong L Y, Bo L, Bao Z Z & Wu B F (2006) Optimization of Culture Conditions for Lipid Production by *Rhodosporidium toruloides. Chinese J Biotechnol,* 4, 650–656.

Horrobin D F (1992) Nutritional and medical importance of g-linolenic acid. *Prog Lipid Res,* 31, 163-194.

Horrobin D F (1993) Fatty acid metabolism in health and disease: the role of Δ-6 desaturase. *Am J Clin Nutr,* 57, 732S-737S.

Horrobin D F (2000) Essential fatty acids metabolism and its modification in atopic eczema. *Am J Clin Nutr,* 71, 367S-372S

Hudson B J F (1984) Evening primrose (*Oenothera* spp.) oil and seed. *J Am Oil Chem Soc,* **61**, 540–543.

Huang Y S, Manku M S & Horrobin D F (1984) The effect of dietary cholesterol on blood and liver polyunsaturated and on plasma cholesterol in rats fed various types of fatty acid diets. *Lipids*. 19, 664-672.

Huang Y S & Milles D E (1996) Gamma-Linolenic Acid:Metabolism and Its Roles in Nutrition and Medicine. Champaign IL: AOCS.

Huang Y S, Chaudhary J M & Thumond J M, Bobik E G, Jr. Yuan L, Chan G M & Kircher (1999) Cloning of Δ^{12} and Δ^{6} desaturases from *Mortierella alpine* and recombinant production of gamma linolenic acid in *Saccharomyces cerevisiae. Lipids*, 34, 649-659.

Huang Y S & Ziboh A (2001) Gamma-Linolenic Acid:Recent Advances in Biotechnology and Clinical Applications. Champaign IL: AOCS.

Huang S, Pereira S L & Leonard L E (2004) Enzymes for transgenic biosynthesis of long-chain polyunsaturated fatty acids. *Biochimie*, 86, 793–798.

Innis S M (1991) Essential fatty acids in growth and development. *Prog Lipid Res,* 30, 39–103.

Innis S M, Nelson C M, Rioux M F & King D J (1994) Development of visual acuity in relation to plasma and erythrocyte w-6 and w-3 fatty acids in healthy term gestation infants. *Am J Clin Nutr,* 60, 347–352.

Immelman M, Du Preez J C & Kilian S G (1997) Effect of C:N ratio on gamma-linolenic acid production by *Mucor circinelloides* grown on acetic acid. *System Appl Microbiol,* 20, 158–164.

Iwen P C, Hinrichs S H & Rupp M E (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. *Med Mycol*, 40, 87–109.

Iwen P C, Freifeld A G, Sigler L & Tarantolo S R (2005) Molecular Identification of *Rhizomucor pusillus* as a Cause of Sinus-Orbital Zygomycosis in a Patient with Acute Myelogenous Leukemia. *J Clin Microbiol,* 43, 5819–5821.

Iwen P C, Sigler L, Noel R K & Freifeld A G (2007) *Mucor circinelloides* Was Identified by Molecular Methods as a Cause of Primary Cutaneous Zygomycosis. *J Clin Microbiol,* 45, 636–640.

James M J, Gibson R A & Cleland L G (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production, *Ame J Clin Nutr,* 71, 343S–348S.

Jang H D, Lin Y Y, Yang S S (2000) Polyunsaturated fatty acid production with *Mortierella alpina* by solid substrate fermentation. *Bot Bull Acad Sinica,* 41, 41-48.

Jang H D, Lin Y Y & Yang S S (2005) Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortirella alpina*. *Bioresour Technol,* 96, 1633-1644.

Jang H D & Yang S S (2008) Polyunsaturated fatty acids production with a solid-state column reactor. *Biores Technol,* 6181-6189

Jangbua P, Laoteng K, Kitsubun P, Nopharatana M & Tongta A (2009) Gamma-linolenic acid production of *Mucor rouxii* by solid-state fermentation using agricultural by-products. *Lett Appl Microbiol,* 49, 91-97.

Jareokitmongkol S, Sakuradani E & Yamada H (1993a) A novel desaturase mutants of *Mortierella alpina* IS-4 and its dihomo- -linolenic acid productivity. *Appl Environ Microbiol,* 59, 4300-4303.

Jareokitmongkol S, Shimizu S & Yamada H (1993b) Production of an eicosapentaenoic acid-containing oil by a Δ^{12} desaturase defective mutant of *Mortierella alpina* 1S-4. *J Am Oil Chem Soc,* 70, 119–123.

Jeennor S, Laoteng K, Jeennor S, Laoteng K, Tanticharoen M & Cheevadhanarak S (2008) Evaluation of inoculum performance for enhancing gamma-linolenic acid production from *Mucor rouxii*. *Lett Appl Microbiol,* 46, 421–427.

Jiang H & Gao K (2004) Effect of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum trieornutum* (*Bacillariophyceae*). *J Phycol,* 40, 651-654.

Johnson M M, Swan D D, Surette M E, Stegner J, Chilton T, Fonteh A N & Chilton F H (1997) Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. *J. Nutr,* 127, 1435-1444.

Kamisaka Y, Yokochi T, Nakahara T & Suzuki O (1990) Incorporation of linoleic acid and its conversion to γ-linolenic acid in fungi. *Lipids,* 25, 54–60.

Kang H S & Shin H D (1989) Influence of medium composition on the production of γ-linolenic acid by *Mucor* sp KCTC 8405P. *Kor J Appl Microbiol Bioeng,* 17, 568-573.

Kates M & Baxter M (1962) Lipid composition of mesophilic and psychrophilic yeasts (*candid* species) as influenced by environmental temperatures. *Can J Biochem Physiol,* 40, 1213-1227.

Kates M (1964) Bacterial lipids. *Advan Lipid Res*, 2, 17-90.

Kavadia A, Komaitis M, Chevalot I, Blanchard F, Marc I & Aggelis G (2001) Lipid and γ-linolenic acid accumulation in strains of Zygomycetes growing on glucose. *J Ame Oil Chem Soc*, 78, 341–346.

Kendrick A & Ratledge C (1992a) Lipids of selected moulds grown for production of n-3 and n-6 polyunsaturated acids. *Lipids*, 27, 15–20.

Kendrick A & Ratledge C (1992b) Lipid formation in the oleaginous mould *Entomophthora exitalis* grown in continuous culture: effects of growth rate, temperature, and dissolved oxygen tension on polyunsaturated fatty acids. *Appl Microbiol Biotechnol*, 37, 18-22.

Kendrick A & Ratledge C (1992c) Desaturation of polyunsaturated fatty acids in *Mucor cercinelloides* and the involvement of a novel membrane bound malic enzyme. *Eur J Biochem*, 209, 667-673.

Kendrick A & Ratledge C (1996) Cessation of polyunsaturated fatty acid formation in four selected filamentous fungi when grown on plant oils. *J Am Oil Chem Soc*, 473, 431–435.

Kenny F, Pinder S, Ellis I, Gee J, Nicholson R, Bryce R & Robertson J. (2000) Gamma linolenic acid with tamoxifen as primary therapy in breast cancer. *Int J Cancer*, 85, 643–648.

Kennedy M J, Reader S L & Davies R J (1993) Production, characterization of fungi with particular emphasis on gamma linolenic acid production. *Biotech Bioeng*, 42, 625-634.

Kilo H, Abe A, Sujaya I N, Oda Y, Asano K & Sone T (2009) Molecular characterization of the relationships among *Amylomyces rouxii, Rhizopus oryzae*, and *Rhizopus delemar*. *Biosci Biotechnol Biochem*, 73, 861-864

Kirimura K, Nakajima J, Lee S P, Kawabe S, Usami S (1988) Citric acid production by the diploid strains of *Aspergillus niger* obtained by protoplast fusion. *Appl Microbiol Biotechnol*, 27, 504-506.

Khoomrung S, Laoteng K, Jitsue S & Cheevadhanarak S (2008) Significance of fatty acid supplementation on profiles of cell growth, fatty acid and gene expression of three desaturases in *Mucor rouxii*. *Appl Microbiol Biotechnol*, 80, 499–506.

Khunyoshyeng S, Cheevadhanarak S, Rachdawong S & Tanticharoen M (2002) Differential expression of desaturases and changes in fatty acid composition during sporangiospore germination and development in *Mucor rouxii*. *Fungal Genet Biol*, 37, 13–21.

Khuri A I & Cornell J A (1989) Response surfaces: Designs and analysis. Marcel Dekker, NY.

Kock J L F & Botha A (1993) Acetic acid – a novel source for the production of gamma-linolenic acid and cocoa butter equivalents. *South Afr J Sci*, 89, 465-471.

Koike Y, Jie Cai H, Higashiyama K, Fujikawa S & Park E Y (2001) Effect of consumed carbon to nitrogen ratio of mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. *J Biosci Bioeng*, 91, 382–389.

Komaitis A, Chevalot M, Blanchard I, Marc F & Aggelis G (2001) Lipid and gamma-linolenic acid accumulation in strains of Zygomycetes growing on glucose. *J Am Oil Chem Soc*, 78, 341–346.

Kristofikova L, Rosenberg M, Vlnova A, Sajbidor J & Certik M (1991) Selection of *Rhizopus* strains for L(+)-lactic acid and gamma linolenic acid production. *Folia Microbiol,* 36, 451–455.

Kumar S, Tamur K & Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetic analysis and sequence alignment. *Brief Bioinform,* 5, 150-163.

Kwasna H, Ward E & Bateman G L (2006) Phylogenetic relationships among Zygomycetes from soil based on ITS1/2 rDNA sequences. *Mycological Res,* 110, 501–510.

Kyle D J, Sicotte V J, Singer J J & Reeb S E (1992) Bioproduction of docosahexaenoic acid (DHA) by microalgae. In: Industrial Applications of Single Cell Oils. Kyle D J & Ratledge C (Eds.), AOCS Press, IL, pp. 287–300.

Lamacka M, Sajbidor J & Bohov P (1998) Lipid isolation and fatty acid analysis in *Saccharomyces cerevisiae*. Comparison of different methods. *Biotechnol Techniq*, 12, 621–625.

Laoteng K, Anjard C, Rachadawong S, Tanticharoen M, Maresca B & Cheevadhanarak S (1999) *Mucor rouxii* D9-desaturase gene is transcriptionally regulated during cell growth and by low temperature. *Mol Cell Biol Res Commun,* 1, 36–43.

Laoteng K, Mannontarat R, Tanticharoen M & Cheevadhanarak S (2000) Δ6 -desaturase gene of *Mucor rouxii* with high similarity to plant Δ6-desaturase and its heterologous expression in *Saccharomyces cerevisiae. Biochem Biophys Res Commun,* 279, 17-22.

Laoteng K, Pongchuachidthai R, Rueksomtawin K, Dandusitapunth Y, Tanticharoen M & Cheevadhanarak S (2003) A *Mucor rouxii* mutant with high accumulation of an unusual trans-linoleic acid (9c,12t-C18:2). *FEMS Microbiol Let,t* 223, 159–165.

Laoteng K, Ruenwai R, Tanticharoen M & Cheevadhanarak S (2005a) Genetic modification of essential fatty acids biosythesis in *Hansenula polymorpha*. *FEMS Microbiol Lett,* 245, 169-178.

Laoteng, K, **Cheevadhanarak S, Tanticharoen M & Maresca B** (2005b) Promoter analysis of *Mucor rouxii* ? Δ9 -desaturase: Its implication for transcriptional regulation in *Saccharomyces cerevisiae. Biochem Biophys Res Commun*, 335, 400-405

Lehninger Priniciple of Biochemistry, Ed Nelson D L & Cox M M Fouth edition (1993).

Leman J & Brakoniecka-Sikorska A (1996) Effect of growth conditions on biosynthesis of oil by *Mortierella ramanniana*. *Polish J Food Nutr Sci,* 5, 111–120.

Leman J (1997) Oleaginous Microorganisms: An assessment of the potential, *Adv Appl Microbiol,* 43, 195-244.

Leng G C, Lee A J, Fowkes F G, Jepson R G, Lowe G D, Skinner E R, Mowat B F (1998) Randomized controlled trial of gamma-linolenic acid and eicosapentaenoic acid in peripheral arterial disease. *Clin Nutr*, 17, 265-71.

Leonard A E, Pereira S L, Sprecher H & Huang Y S (2004) Elongation of long-chain fatty acids. *Prog Lipid Res,* 43, 36–54.

Lepage G & Roy C C (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J Lipid Res,* 25, 1391–1396.

Leventhal L J, Boyce E G & Zurier R B (1994) Treatment of rheumatoid arthritis with blackcurrant seed oil. *Br J Rheumatol.* 33, 847–852.

Lewis T, Nichols P D & Mc Meekin T A (2000) Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *J Microbiol Meth,* 43, 107–116.

Li N, Deng Z N, Qin Y L, Chen C L & Liang Z Q (2008) Production of Polyunsaturated fatty acids by *Mucor recurvus* sp. with Sugarcane Molasses as the carbon source. *Food Technol Biotechnol,* 46, 73-79.

Li Y H, Liu B, Zhao Z B & Bai F W (2006) Optimized culture medium and fermentation conditions for lipid production by *Rhodosporidium toruloides. Chinese J Biotechnol,* 22, 650–656.

Lin S S, Dou W F, Xu H Y, Li H Z, Xu Z H & Ma Y H (2007) Optimization of medium composition for the production of alkaline β-mannanase by alkaliphilic *Bacillus* sp. N16-5 using response surface methodology. *Appl Microbiol Biotechnol,* 75, 1015-1022.

Lindberg A M & Hansson L (1991) Production of gamma linolenic acid by the fungus *Mucor rouxii* on cheap nitrogen and carbon sources. *Appl Microbiol Biotechnol,* 36, 26–28.

Lindberg A M & Molin G (1993) Effect of temperature and glucose supply on the production of polyunsaturated fatty acids by the fungus *Mortierella alpina* CBS343.66 in fermentor culture. *Appl Microbiol Biotechnol,* 39, 450-455.

Linder M, Kochanowski N, Fanni J & Parmentier M (2005) Response surface optimization of lipase catalyzed esterification of glycerol and n-3 polyunsaturated fatty acids from salmon oil. *Process Biochem,* 40, 273-279.

Liu J W, De Michelle S J, Palombo J, Chuang LT, Hastilow C, Bobik E J & Huang YS (2004) Effect of long-term dietary supplementation of high- g -linolenic canola oil versus borage oil on growth, hematology, serum biochemistry, and n-6 fatty acid metabolism in rats. *J Agric Food Chem.* 52, 3960–3966.

Lopez A D & Garcia M G (2000) Plants as 'chemical factories' for the production of polyunsaturated fatty acids. *Biotechnol Adv,* 18, 481–497.

Lopez-Martínez J C, Campra-Madrid P, Rincon-Cervera M A & Guil-Guerrero J L (2005) Ecological and simultaneous seed oil extraction/saponification/γ-linolenic acid concentration. *Eur J Lipid Sci Technol,* 107, 180–186.

Lunn J & Theobald H E (2006) The health effects of dietary unsaturated fatty acids. *Nutr Bulletin,* 31, 178–224

Maaroufi Y, Ahariz M, Husson M & Crokaert F (2004) Comparison of different methods of isolation of DNA of commonly encountered *Candida* species and its quantitation by using a real-time PCR-based assay. *J Clin Microbiol,* 42, 3159–3163.

Mamatha S S, Ravi R & Venkateswaran G (2008) Medium Optimization of Gamma Linolenic Acid Production in *Mucor rouxii* CFR -G15 using RSM. *Food Bioprocess Techno,l* 1, 405–409.

Manpreet S, Sawraj S, Sachin D, Pankaj S & Banerjee U C (2005) Influence of Process Parameters on the Production of Metabolites in Solid-State Fermentation. *Mal J Microbiol,* 1, 1-9.

Mendes R L, Nobre B P, Cardoso M T, Pereira A P & Palavra A F (2003) Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. *Inorganica Chimica Acta,* 356, 328-334

Mendes R L, Reis A D & Palavra A N F (2006) Supercritical CO_2 extraction of γ-linolenic acid and other lipids from *Arthrospira* (*Spirulina*) *maxima*: Comparison with organic solvent extraction. *Food Chem,* 99, 57–63.

Metz B & Kossen N W F (1977) The growth of molds in the form of pellets. *Biotechol Bioeng,* 19, 781-799.

Metz J G, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, & Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science*, 293, 290–293.

Michinika Y, Aki T, Shimachi-Nakajima T, Kawamoto S, Shigeta S O, Suzuki & Ono K (2003) Differential response to low temperature of two Δ^6 desaturase from *M. cercinelloides*. *Appl Microbiol Biotechnol,* 62, 362-368.

Miller G L (1959) Use of dinitro salicylic acid reagent for determination of reducing sugar. *Anal Chem,* 31, 426-428.

Moller, Bahnweg, Sandermann & Geiger (1992) DNA extraction method in fungi. *Nucleic Acids Res,* 20, 6115-16.

Montgomery D C (1984) Response surface methodology. In: Design and analysis of experiments (2nd edn), John Wiley, NY.

Montgomery D C (1997) Response surface methods and other approaches to process optimization. In: Design and analysis of experiments, John Wiley, NY, 427-510.

Morakotkarn D, Kawasaki H & Seki T (2007) Molecular diversity of bamboo-associated fungi isolated from Japan. *FEMS Microbiol Lett,* 266, 10–19.

Moore D (1969) The mutagenic action of N-methyl-N'-nitro-Nnitrosoguanidine on *Coprinus lagopus*. *J Gen Microbiol,* 55, 121-125.

Moreton R S (1988) Physiology of lipid accumulation. In Single Cell Oil ed. Moreton R S. pp. 11–27. Harlow: Longmans Scientific & Technical. New York.

Muhid F, Navi W N W N, Kader A J A, Yosyff W M W & Haamid A A (2008) Effects of metal ion concentration on lipid and gamma linolenic acid production by cunninghamella sp. 2A1. *OnLine J Biological Sci,* 8, 2008.

Mukherjee K D (1999) Production and use of microbial oils. *Inform,* 10, 308–313.

Muller F M, Werner K E, Kasai M, Francesconi A, Chanock S J & Walsh T J (1998) Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *J Clin Microbiol,* 36, 1625–1629.

Murlidhar R V & Panda T (2000) Fungal protoplast fusion : A revisit. *Bioprocess Biosyst Engg,* 22, 429-431.

Murphy D (1991) Storage lipid bodies in plants and other organisms. *Prog Lipid Res,* 29, 299–324.

Myers R H (1971) Response Surface Methodology. Allyn and Bacon, Boston M A.

Myers R H & Montgomery D C (2002) Response surface methodology: process and product optimization using designed experiments. John Wiley, NY.

Mysyakina I S & Funtikova N S (2003) Changes in the lipid composition of *Mucor hiemalis* sporangiospores related to the age of the spore-forming culture. *Microbiol*, 72, 461–465.

Nakahara T, Yokochi T, Kamisaka Y, & Suzuki O. Gamma-linolenic acid from genus *Mortierella*. In: Industrial applications of single cell oils. Champaign, IL, American Oil Chemists Society Press, 2002, 61–97.

Nakahara T, Yokochi T, Kamisaka Y & Suzuki O (1993) Physiological effects of gamma-linolenic acid and its production by fungal cultivation. *J Jp Oil Chem Soc,* 42, 242-253.

Napier J A, Sayanova O , Stobart A K & Shewry P R (1997) A new class of cytochrome b5 fusion proteins. *Biochem J,* 328, 717–718.

Napier J A, Hey S J, Lacey D J & Shewry S R (1998) Identification of *Caenorhabditis elegans* D6-fatty-acid desaturase by heterologous expression in *Saccharomyces cerevisiae.* Biochem J, 330, 611–614.

Napier J A, Michaelson L V & Stobart A K (1999) Plant desaturases: harvesting the fat of the land. *Curr Opin Plant Biol,* 2, 123–7.

Napier J A (2002) Plumbing the depths of PUFA biosynthesis: A novel polyketide synthase-like pathway from marine organisms. *Trends Plant Sci, 7*, 51-54.

Napier J A, Michaelson L V & Sayanova O (2003) The role of cytochrome b5 fusion desaturases in the synthesis of polyunsaturated fatty acids. *Prostaglandins Leukot Essent Fatty Acids,* 68, 135–143.

Napier J A, Beaudoin F, Michaelson L V & Sayanova O (2004) The production of long chain polyunsaturated fatty acids in transgenic plants by reverse-engineering. *Biochimie*, 86, 785–792.

Napier J A, Beaudoin F & Sayanova O (2005) Reverse engineering of long-chain polyunsaturated fatty acid biosynthesis into transgenic plants. *Eur J Lipid Sci Technol,* 107, 249–255.

Na-Ranong S, Laoteng K**, Kittakoop P, Tanticharoen M & Cheevadhanarak S** (2005) Substrate specificity and preference of ? 6 -desaturase of *Mucor rouxii*. *FEBS Lett,* 579, 2744-2748.

Na-Ranong S, Laoteng K**, Kittakoop P, Tanticharoen M & Cheevadhanarak S** (2006) Targeted mutagenesis of a fatty acid delta-6 desaturase from *Mucor rouxii*: Role of amino acid residues adjacent to histidine-rich motif II. *Biochem Biophys Res Commun,* 339, 1029—1034.

Narayanswamy S (1994) Plant cells and tissue cultures. Plant Protoplast:Isolation,Culture and Fusion. 391-469. TATA MCGraw Hill Publishing Company ,New Delhi,India.

Nigam P (1999) Fermentation (industrial); Production of Oils and Fatty Acids. Academic Press.

Ohlrogge J B & Jaworski J G (1997) Regulation of fatty acid biosynthesis. *Annu Rev Plant Physiol Plant Mol Biol,* 48, 109–36.

Orlowski M (1991) *Mucor* Dimorphism. *Microbiol Rev,* 5, 234–258.

Pandey A (1992) Recent process developments in solid state fermentations. *Proc Biochem,* 27, 109-117.

Pandey A, Selvakumar P, Nigam P & Soccol C R (1999) Solid-state fermentation for the production of industrial enzymes. *Curr Sci,* 77, 149–162.

Pandey A, Soccol C R & Mitchell D A (2000) New developments in solid-state fermentation I—bioprocesses and products. *Proc Biochem,* 35, 1153- 1169.

Pandey A, Soccol C R, Rodriguez-Leon J A & Nigam P (2001). Solid-state Fermentation in Biotechnology. Asiatech Publishers Inc., New Delhi, p. 221.

Pandey A (2003) Solid-state fermentation. *Biochem eng,* 13, 81-84.

Papanikolaou S, Chevalot I, Komaitis M, Aggelis G & Marc I (2001) Kinetic profile of the cellular lipid composition in an oleaginous *Yarrowia lipolytica* capable of producing a cocoa-butter substitute from industrial fats. *Antonie Van Leeuwenhoek,* 80, 215–224.

Papanikolaou S, Chevalot I, Komaitis M, Marc I & Aggelis G (2002a) Single cell oil production (SCO) production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. *Appl Microbiol Biotechnol,* 58, 308–312.

Papanikolaou S & Aggelis G (2002b) Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. *Bioresour Technol,* 82, 43–49.

Papanikolaou S & Aggelis G (2003a) Modeling lipid accumulation and degradation in *Yarrowia lipolytica* cultivated on industrial fats. *Curr Microbiol,* 46, 398–402.

Papanikolaou S & Aggelis G (2003b) Selective uptake of fatty acids by the yeast *Yarrowia lipolytica*. *Eur J Lipid Sci Technol,* 105, 651–655.

Papanikolaou S, Komaitis N & Aggelis G (2004a) Single cell oil (SCO) production by *Mortierella isabellina* grown on high sugar content media. *Bioresour Technol,* 95, 287–291.

Papanikolaou S, Sarantou S, Komaitis M & Aggelis G (2004b) Repression of reserve lipid turnover in *Cunninghamella echinulata* and *Mortierella isabellina* cultivated in multiple limited media. *J Appl Microbiol,* 97, 867–874.

Papanikolaou S, Galiotou-Panayotou M, Fakas S, Komaitis M & Aggelis G (2007) Lipid production by oleaginous Mucorales cultivated on renewable carbon sources. *Eur J Lipid Sci Technol,* 109, 1060–1070.

Papanikolaou S, Fakas S, Fick M, Chevalot I, Galiotou-Panayotou M, Komaitis M, Marc I & Aggelis G (2008) Biotechnological valorisation of raw glycerol discharged after

bio-diesel (fatty acid methyl-esters) manufacturing process: production of 1,3-propanediol, citric acid and single cell oil. *Biomass & Bioenerg,* 32, 60–71.

Parekh S, Vinci V A & Strobel R J (2000) Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol,* 54, 287–301.

Park E Y, Koike Y, Higashiyama K, Fujikawa S & Okabe M (1999) Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of *Mortierella alpina*. *J Biosci Bioeng,* 88, 61–67.

Passorn S, Laoteng K, Rachadawong S, Tanticharoen M & Cheevadhanarak S (1999) Heterologous expression of *Mucor rouxii* Δ12-desaturase gene in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun,* 263, 47-51.

Patnayak S & Sree A (2005) Screening of bacterial associates of marine sponges for single cell oil and PUFA. *Let Appl Microbiol,* 40, 358–363.

Peberdy J F (1979) Fungal protoplasts: Isolation, reversion and fusion. *Ann Rev Microbiol,* 33, 21-39.

Peberdy J F (1980) Protoplast fusion- a tool for genetic manipulation and breeding in industrial microorganisms. *Enz Microbial Technol,* 2, 23-29.

Pereira S L, Leonard A E & Mukerji P (2003) Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins Leuk Essent Fatty Acids,* 68, 97-106.

Prabavarthy V R,mathiavanan N, Sagadevan E, Murugesan K, Lalithakumari D (2006) Intrastrain protoplast fusion enhances carboxymethylcellulase activity in *Trichodermma reesei*. *Enzyme Microbial Technol,* 38, 719-723.

Prakash M Halami (2004) Ph.D Thesis, University of Mysore, Mysore, India.

Pohl C H, Botha A, Kock J L F, Coetzee D J & Botes P J (1997) The production of γ-linolenic acid by selected members of the Dikaryomycota grown on different carbon sources. *Antonie van Leeuwenhoek,* 72, 191-199.

Pullman-Mooar S, Laposata M, Leto D, Holman R, Leventhal LJ, DeMarco D & Zurier R B (1990) Alteration of the Cellular Fatty Acid Profile and the Production of Eicosanoids in Human Monocytes by Gamma-linolenic Acid'. *Arthritis Rheum.* 33, 1526-1531.

Puri B K (2007) The safety of evening primrose oil in epilepsy. *Prostaglandins Leukot Essent Fatty Acids,* 77, 101-103.

Qiu X, Hong H P, MacKenzie S L (2001) Identification of a Δ4 fatty acid desaturase from *Thraustochytrium* sp. Involved in biosynthesis of docosahexaenoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J Biol Chem,* 276, 31561–31566.

Quihui H (1999) Supercritical carbon dioxide extraction of *Spirulina platensis* component and removing the stench. *J Agri Food Chem,* 47, 2705–2706.

Quoc P K & Duacq P J (1997) Effect of growth temperature on the biosynthesis of eukaryotic lipid molecular species by the cyanobacterium *Spirulina platensis*. *Biochemica et Biophysica Acta,* 1346, 237-246.

Ratledge C (1987) Lipid biotechnology, a wonderland for the microbial physiologist. *J Am Oil Chem Soc,* 64,1647–1656.

Ratledge C (1988) Biochemistry, stoichiometry, substrates and economics. In Single Cell Oil ed. Moreton R S pp 33–70 Harlow: Longmans.

Ratledge C (1989) Microbial routes to lipids. *Biochem Soc Trans.* 17, 1139–41.

Ratledge C (1991) Microorganisms for lipids. *Acta Biotechnol* 11, 429–438.

Ratledge C (1992a) Microbial lipids: Commercial realities or academic curiosities. pp.1-15. In: Industrial Applications of Single Cell Oils, D J Kyle, & Ratledge C (eds.), AOCS Press, IL, USA.

Ratledge C (1992b) Tailor made oils and fats—possibilities for microbial oils. *Rev Franaise des Corps Gras*, 39, 325–332.

Ratledge C (2002) Regulation of lipid accumulation in oleaginous micro-organisms. *Biochem Soc Trans*, 30, 1047–1050.

Ratledge C & Wynn J P (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv Appl Microbiol*, 51, 1–51.

Ratledge C (1993) Single cell oils-have they a biotechnological future?. *Trends Biotechnol,* 11, 278-284.

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, 86, 807-815.

Ravi R & Susheelamma N S (2005) Simultaneous optimization of a multi-response system by desirability function analysis of 'Boondi' making - a case study. *J Food Sci*, 70, S539-547.

Robinson H C (2001a) Cold adaptation in Arctic and Antarctic fungi. New *Phytologist*, 151, 341-353.

Robinson T, Singh D & Nigam P (2001b) Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Appl Microbiol Biotechnol,* 55, 284-289.

Rodriguez R J, Belarbi E H, Sanchez J L G & Alonso D L (1998) Rapid simultaneous lipid extraction and transesterification for fatty acid analyses. *Biotechnol Tech*, 12, 689–691.

Rodrigues L, Teixeira J, Oliveira R & Van der Mai H C (2006) Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. *Proc Biochem*, 41, 1-10.

Roux M P, Kock J L F, Botha A, Du Preez J C, Wells G V & Botes P J (1994) *Mucor* a source of cocoa butter and gamma-linolenic acid. *World J Microbiol Biotechol,* 10, 417-422.

Ruxton C H S, Reed S C, Sumpson M J A & Millington K J (2004) The health benefits of omega-3 Polyunsaturated fatty acids: a review of the evidence. *J Hum Nutr Dietet,* 17, 449-459.

Sajbidor J, Certik M & Dobronova S (1988) Influence of different carbon sources on growth, lipid content, and fatty acid composition in four strains belonging to Mucorales. *Biotechnol Let,* 10, 347–350.

Sajbidor J, Dobronova S & Certik M (1990) Arachidonic acid production by *Mortierella* SP. S-17: influence of C/N ratio. *Biotechnol Lett,* 12, 455-456.

Sajbidor J, Kozelouiiova D & Certik M (1992) Influence of some metal ions on the lipid content and arachidonic acid production by *Mortierella* sp. *Folia Microbiol,* 37, 404–406.

Sajbidor J, Certik M & Grego J (1994) Lipid Analysis of Baker's Yeasts. *J Chromatogr,* 665, 191-195.

Sajilata M G, Singhal R S & Kamat M Y (2008) Supercritical CO_2 extraction of γ-linolenic acid (GLA) from *Spirulina platensis* ARM 740 using response surface methodology. *J Food Engineer,* 84, 321–326.

Sakaki K, Yokochi T, Suzuki O & Hakuta T (1990). Supercritical fluid extraction of fungal oil using CO_2, N_2O, CHF_3 and SF_6. J Ame Oil Chem Soc, 67, 553–557.

Sakuradani E, Kobayashi M & Shimizu S (1999) δ6-Fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus. Gene cloning and its heterologous expression in a fungus, *Aspergillus*. *Gene*, 238, 445–453.

Sakuradani E & Shimizu S (2009) Single cell oil production by *Mortierella alpine. J Biotechnol,* 44, 31-36

Sambrook J & Russell D W (2001) Molecular cloning. A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sattur A (1989) Ph.D Thesis, University of Mysore, Mysore, India.

Sayanova O, Mark A S, Peter L, Stobart A K, Dobson G, William W C, Peter R S & Johnathan A N (1997) Expression of a Borage Desaturase cDNA Containing an N-Terminal Cytochrome *b*5 Domain Results in the Accumulation of High Levels of Δ6-Desaturated Fatty Acids in Transgenic Tobacco. *Proc Natl Acad Sci, USA,* 94, 4211–4216.

Sayanova O V, Beaudoin F, Michaelson L V, Shewry P R, Napier J A (2003) Identification of Primula fatty acid delta 6-desaturases with n-3 substrate preferences. *FEBS Lett,* 542, 100–104.

Schwarz P, Bretagne S, Gantier J C, Garcia-Hermoso D, Lortholary O, Dromer F & Dannaoui E (2006) Molecular identification of zygomycetes from culture and experimentally infected tissues. *J Clin Microbiol,* 44, 340–349.

Serrano I, Lopes da S T & Carlos R J (2001) Ethanol-induced dimorphism and lipid composition changes in *Mucor fragilus* CCMI 142. *Lett Appl Microbiol,* 33, 89–93.

Sharma A K, H Raj, A Pandita, V R Tandon & A Mahajan (2006) Gamma Linolenic Acid in Dry Eye. *J K science,* 8, 24-27.

Sharma M, Chadha B S, Kaur M, Ghatora S K & Saini H S (2008) Molecular characterization of multiple xylanase producing thermophilic /thermotolerant fungi isolated from composting materials. *Lett Appl Microbiol,* 46, 526–535.

Shasi K **Menan** (1989) Ph.D Thesis, University of Mysore, Mysore, India.

Shaw R (1965) The occurrence of gamma linolenic acid in fungi. *Biochim Biophys Acta,* 98, 230-237.

Shaw R (1966a) The polyunsaturated fatty acids of microorganisms. *Adv Lipid Res,* 4, 107-174.

Shaw R (1966b) The fatty acids of phycomycete fungi, and the significance of the γ−linolenic acid component. *Comp Biochem Physiol,* 18, 325–331.

Shimizu S, Shinmen Y, Kawashima H, Akimoto K & Yamada H (1988) Fungal mycelia as a novel source of eicosapentaenoic acid: activation of enzyme(s) involved in eicosapentaenoic acid production at low temperature. *Biochem Biophys Res Commun,* 150, 335-341.

Shimizu S, Akimoto K, Shinmen Y, Kawashima H, Sugano M & Yamada H (1991) Sesamin is a potent and specific inhibitor of Δ5-desaturase in polyunsaturated fatty acid biosynthesis. *Lipids*, 26, 512–516.

Simpoulos A P (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother,* 56, 365–379.

Simonopoulos A P (1991) Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr* , 54, 438–63.

Shinmen Y, Shimizu S, Akimoto K, Kawashima H & Yamada H (1989) Production of arachidonic acid by *Mortierella* fungi. *Appl Microbiol Biotechnol,* 31, 11–16.

Shinmen Y, Kawashima H , Shimizu S & Yamada H (1992) Concentration of eicosapentaenoic acid in arachidonic acid producing fungus. *Mortierella alpine* IS-4, grown with fish oil. *Appl Microbiol Biotechnol,* 38, 301-304.

Somashekar D, Venkateswaran G, Sambaiah K, Lokesh B R (2002) Effect of culture conditions on lipid and gamma linolenic acid production in mucoraceous fungi. *Process Biochem* , 38, 1719-1724.

Somashekar D, Venkateswaran G, Srividya C, Krishnanand, Sambaiah K & Lokesh B R (2003) Efficacy of lipid extraction methods for lipid and fatty acid composition from fungal cultures. *World J Microbiol Biotechnol,* 17, 317-320.

Spencer J F T & Spencer D M (1982) Hybridization studies in Yeast. *Current Genetics,* 8, 49-53.

Stahl P D & Klug M J (1996) Characterization and differentiation of filamentous fungi based on fatty acid composition. *Appl Environ Microbiol, 62,* 4136-4146.

Stredanska S & Sajbidor J (1993a) Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by *Mortierella alpina*. *Acta Biotechnologica,* 13, 185–191.

Stredanska S, Slugen D, Stredansky M & Grego J (1993b) Arachidonic acid production by *Mortierella alpina* grown on solid substrates. *World J Microbiol Biotechnol,* 9, 511–513.

Stredansky M, Conti E & Salaris A (2000a) Production of polyunsaturated fatty acids by *Pythium ultimum* in solid-state cultivation. *Enz Microbial Technol,* 26, 304-307.

Stredansky M, Conti E, Stredanska S & Zanetti F (2000b) γ-linolenic acid production with *Thamnidium elegans* by solid-state fermentation on apple pomace. *Biores Technol,* 73, 41-45.

Sumner J L & Morgan E D (1969a) The fatty acid composition of sporangiospores and vegetative mycelium of temperature-adapted fungi in the order Mucorales. *J Gen Microbiol,* 5, 215-221.

Sumner J L, Morgan E D & Evans H C (1969b) The effect of growth temperature on the fatty acid composition of fungi in the order mucorales. *Can J Microbiol,* 15, 515-520.

Suutari M & Laakso S (1994) Microbial fatty-acids and thermal adaptation. *Crit Rev Microbiol,* 20, 285-328.

Suzuki O, Yokochi T & Yamashina T (1981) Studies on production of lipids in fungi (II). Lipid compositions of six species of Mucorales in Zygomycetes. *J Jpn Oil Chem Soc,* 30, 863-868.

Tadaka R, Saito M & Mori T (1994) Dietary γ-linolenic acid enriched oil reduces body fat content and induces liver enzymes activities relating to fatty acid β-oxidation in rats. *J Nutr.* 124, 469-474.

Tapiero H, Ba G N, Couvreur P & Tew K D (2002) Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Alternative Med Rev,* 56, 215-222.

Tauk-Torniselo S M, Viera J M, Ceilia M, Carneiro V S, Govone J S (2007) Fatty acid production by four strains of *M. hiemalis* grown in plant oil and soluble carbohydrate. *Afric J Biotechnol,* 6, 1840-1847.

Tocher D R, Leaver M J & Hodgson P A (1998) Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Prog Lipid Res*, 37, 73–117.

Torlanova B O, Konova I V, Funtikova N S, Babanova N K, Katomina A A, Mysyakina I S (1992) Influence of the conditions of culturing of a *Mucor* fungus, treatment of biomass, and extraction ability on the production of lipids containing gamma-linolenic acid. *Appl Biochem Microbiol,* 28, 461–468.

Totani N, Someya K & Oba K (1992) Industrial production of arachidonic acid by *Mortierella*: *In Industrial applications of single cell oils*. pp. 52–60. Champaign, IL: Ame Oil Chem Soc.

Totani N, Hyodo K & Ueda T (2000) A study on new nitrogen source for cultivation of genus *Mortierella*. *J Jpn Oil Chem Soc,* 49, 479–485.

Traitler H, Wille H J & Struder A (1988) Fractionation of blackcurrant seed oil. *J Am Oil Chem Soc,* 65, 755–760.

Uttaro A D (2006) Biosynthesis of Polyunsaturated fatty acids in Lower Eukaryotes. *IUBMB Life*, 58, 563 – 571.

Van Burik J A, Schreckhise R W, White T C, Bowden R C & Myerson D (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Med Mycol,* 36, 299–303.

Van der Westhuizen J P J, Kock J L F, Botha A & Bores P J (1994) The distribution of the omega 3 and 6-series of cellular long-chain fatty acids in fungi. *Sys Appl Microbiol,* 17, 327-345.

van Gool W J, Thijs C, Henquet CJM, van Houwelingen AC, Dagnelie PC, Schrander J, Menheere P P & van den Brandt P A (2003) γ-Linolenic acid supplementation for prophylaxis of atopic dermatitis—a randomized controlled trial in infants at high familial risk1–3. *Am J Clin Nutr,* 77, 943–51.

Vanheeswijck R (1984) The formation of protoplasts from *Mucor* species. *Carlsberg Res Commun,* 49, 597-609.

Varavallo A M, Queiroz M V, Lana T G, de Brito A T R, Gonacalves D B & de Araujo E F (2007) Isoaltion of recombinant strains with enhanced pectinase production by protoplast fusion between *Penicillium expansum* and *Penicillium griseoroseum. Brazillian J Microbiol,* 38, 52-57.

Vega E Z, Glatz B A & Hammond E G (1988) Optimization of bana juiece fermentation for the production of microbial oil. *Appl Env Microbiol,* 54, 748-752.

Venkateswaran G (1997) Ph. D Thesis, University of Mysore, Mysore, India.

Verma N, Bansal M C & Kumar V (2007) Protoplast Fusion Technology and Its Biotechnological Applications .

Voigt K, Cigelnik E, & O'donnell K (1999) Phylogeny and PCR Identification of Clinically Important Zygomycetes Based on Nuclear Ribosomal-DNA Sequence Data. *J Clin Microbio,* 37, 3957–3964.

Wallis J G, Watts J L & Browse J (2002) Polyunsaturated fatty acid synthesis: What will they think of next? *Trends Biochem Sci,* 27, 467–470.

Walker T H, Cochran H D & Hulbert G J (1999) Supercritical Carbon Dioxide Extraction of Lipids from Pythium irregulare. *J Ame Oil Chem Soc,* 76, 597-602.

Waltermann M, Luftmann H, Baumeister D, Kalscheuer R, Steinbüchel A (2000) *Rhodococcus opacus* strain PD630 as a new source of high-value single-cell oil? Isolation and characterization of triacylglycerols and other storage lipids. *Microbiol,* 146, 1143–1149.

Wang D, Le M, Wei D, Cai Y, Zhang Y & Xing L (2007) Identification and Functional Characterization of the Delta-6 Fatty Acid Desaturase Gene from *Thamnidium elegans. J Eukaryot Microbiol,* 54, 110-117.

Wang J, Li R, Lu D, Ma S, Yan Y & Li A (2009) quick isolation method for mutants with high lipid yield in oleaginous yeast. *World J Microbiol Biotechnol,* 25, 921–925.

Ward O P (1995) Microbial production of long-chain PUFAs. *INFORM,* 6, 683-688.

Ward O P & Singh A (2005) Omega-3/6 fatty acids: Alternative sources of production. *Proc Biochem,* 40, 3627–3652.

Weete J D (1980) Lipid biochemistry of fungi and other organisms. New York, NY: Plenum Press, 97–111.

Weete J D, Shewmaker F & Gandhi S R (1998) g-Linolenic acid in zygomycetous fungi *Synzygites megalocarpus. J Am Oil Chem Soc,* 75, 1367–1372.

Westhuizen J P, Kock J L F, Botha A, Botes P J (1994) The distribution of the ω3 and ω6 of cellular long chain fatty acids in fungi. *Syst Appl Microbiol,* 17, 327–345.

Weinstein R N, Montierl P O & Johnstone K (2000) Influence of growth temperature on lipid and soluble carbohydrate synthesis by fungi isolated from fell field soil in the maritime Antarctic. *Mycologia,* 92, 222–229.

Willis W M, Lencki R W & Marangoni A G (1998) Lipid Modification Strategies in the Production of Nutritionally Functional Fats and Oils. *Crit Rev Food Sci Nutr,* 38, 639–674.

Wongsumpanchai W, Anamnart S, Laoteng K & **Petsom A** (2004) Elongation of C16:0 to C18:0 fatty acids in methylotrophic yeast *Hansenula polymorpha* CBS1976 and fatty acid auxotrophic mutants. *FEMS Microbiol Lett,* 237, 213-218.

Woll A M, Appel K F, Petersen J B, Poulsen U & Arnau J (2002) Identification and analysis of genes involved in the control of dimorphism in *Mucor circinelloides* (syn. *racemosus*). *FEMS Yeast Res,* 2, 203-213.

Woodbine M (1959) Microbial fat: Microorganisms as potential fat producers. Prog Ind Microbiol, 1, 179-245.

Wu Z, Tsumura Y, Blomquist G & Wang X R (2003) 18S rRNA gene variation among common airborne fungi, and development of specific oligonucleotide probes for the detection of fungal isolates. *Appl Environ Microbiol,* 69, 5389–5397.

Wynn J P, Hamid A A & Ratledge C (1999) The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. *Microbiol,* 145, 1911-1917.

Wynn J P, Hamid A A, Li Y & Ratledge C (2001) Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi *Mucor circinelloides* and *Mortierella alpina*. *Microbiol,* 147, 2857–2864

Xian M, Yan J, Kang Y, Liu J, Bi Y & Zhen K (2001) Production of γ-linolenic acid by *Mortierella isabellina* grown on hexadecanol. Lett Appl Microbiol, 33, 367–370.

Yamada H, Shimizu S, Cmen Y, Akimoto K, Kawashima H & Jareonkitmongkol S (1992) Production of dihomo-gammalinolenic acid, arachidonic acid, and eicosapentaenoic acid by filamentous fungi. Industrial applications of single cell oils. Champaign, IL: Ame Oil Chem Soc, pp.118–138.

Yongmanitchai W & Ward O P (1989) Omega-3 fatty acids: alternative sources of production. *Proc Biochem,* 24, 117-125.

Yu L J, Qin W M, Lan W Z, Zhou P P & Zhu M (2003) Improved arachidonic acids production from the fungus *Mortierella alpina* by glutamate supplementation. *Bioresource Technol,* 88, 265–268.

Zhang Q, Li M, Ma H, Sun Y & Xing L (2004) Identification and characterization of a novel δ6-fatty acid desaturases gene from *Rhizopus arrizus*. *FEBS Letters,* 556, 81-85.

Zhu M, Yu L J & Wu Y X (2003) An inexpensive medium for production of arachidonic acid by *Mortierella alpina. J Ind Microbiol Biotechnol,* 30, 75–79.

Zhu L Y, Zong M H & Wu H (2003) Efficient lipid production with *Trichosporon fermentans* and its use for biodiesel preparation. *Bioresource Technol,* 99, 75–79.

Ziboh, V A & Fletcher M P (1992) Dose-response effects of dietary gamma-linolenic acid-enriched oils on human polymorphonuclear-neutrophil biosynthesis of leukotriene B4. *Am J Clin Nutr*, 55, 39-45.

Zurier R B, Rossetti R G, Jacobson E W, DeMarco D M, Liu N Y, Temming J E, White B M, Lapsota M (1996) Gamma-linolenic acid treatment of Rheumatoid Arthritis. A randomized, placebo-controlled trial. *Arthritis Rheum*, 39, 1808-17.