BIOTECHNOLOGICAL STUDIES FOR THE PRODUCTION OF ARACHIDONIC ACID FROM *MORTIERELLA ALPINA*

A THESIS

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By

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CERTIFICATE

I, **NISHA. A,** certify that this thesis is the result of research work done by me under the supervision of Dr.G.Venkateswaran at Department of Food Microbiology, Central Food Technological Research Institute, Mysore. I am submitting this thesis for possible award of Doctor of Philosophy degree in Biotechnology of 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 by me on

Signature of Guide Date
Date Counter signed by

Signature of chairperson/Head of the department/
Institution with name and office seal

DEDICATED

To my mother

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ABBREVIATIONS, SYMBOLS AND UNITS

ACL	ATP: citrate lyase
ACC	Acetyl CoA Carboxylase
ADP	Adenosine diphosphate
ALA	Alpha linolenic acid
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine mono phosphate
ANOVA	Analysis of variance
AR	Analytical reagent
AOAC	Association of Official Analytical Chemists
ARA/AA	Arachidonic acid
ARASCO	Arachidonic acid single cell oil
AST	Aspartate aminotransferase
ATCC	American type culture collection
ATP	Adenosine triphosphate
ΔA	Change in absorbance
BLAST	Basic local alignment search tool
BW	Body weight
C_f	Final concentration
C_i	Initial concentration
CBE	Cocoa butter equivalent
CBS	Central bureau voor Schimmel cultures
CCRD	Central composite rotatable design
CFU	Colony forming units
cm	Centimeter
C/N	Carbon/nitrogen
CO_2	Carbondioxide
CoA	Coenzyme A
cDNA	Complementary DNA
Corp	Corporation
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
COX	Cycloxygenase
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid

DHASCO	Docosahexaenoic acid single cell oil
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
EL	Elongase
EDTA	Ethylene diamine tetra acetic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
EMS	Ethyl methane suphonate
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FID	Flame ionization detector
FPM	Fat producing medium
FT-IR	Fourier transform infrared spectroscopy
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GLA	Gamma linolenic acid
G6PDH	Glucose 6 phosphate dehydrogenase
GRAS	Generally regarded as safe
GY	Glucose-yeast extract medium
h	Hour (s)
Hb	Haemoglobin
HCl	Hydrochloric acid
HMG CoA	3-Hydroxy 3-methyl glutaryl-CoA
HPETE	Hydroperoxy eicosatetraenoic acid
HPLC	High performance liquid chromatography
IAEC	Institutional animal ethical committee
ITS1	Internal spacer 1
ITS2	Internal spacer 2
IQ	Intelligence quotient
Kg	kilogram
Kg/h	Kilogram per hour
LC-PUFA	Long chain polyunsaturated fatty acid
LOX	lipoxygenase
LA	Linoleic acid
LDH	Lactate dehydrogenase

LT	Leukotriene
Ltd	Limited
M	Molar concentration
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
ME	Malic enzyme
MEB	Malt extract broth
mg	Milligram
mg/dL	Milligram per decilitre
min	Minutes
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
m/Z	Mass to charge ratio
N	Normal
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanogram
nm	Nanometer
nmole	Nanomole
NOAEL	No observed adverse effect level
NSAID	Non steroidal anti-inflammatory drug
NTG	N-methyl N'-nitro-N-nitrosoguanidine
OD	Optical density
OV	Ohio valley
PC	Platelet count
PCR	Polymerase chain reaction
PCV	Packed cell volume
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PE	Phosphatidyl ethanolamine
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2

PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Post menstrual age
PMS	Premenstrual syndrome
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PPG	Polypropylene glycol
PUFA	Polyunsaturated fatty acid
RAO	Refined arachidonic acid rich oil
RBC	Red blood cells
RBO	Rice bran oil
RNA	Ribonucleic acid
RP	Reverse phase
rpm	Rotation per minutes
RSM	Response surface methodology
s	Second
SCO	Single cell oil
SD	Standard deviation
SEM	Scanning electron microscopy
SESO	Sesame oil
SFE	Supercritical fluid extraction
SM	Synthetic medium
sp./sps	Species
STA	Stearidonic acid
TAE	Tris acetate ethylene diamine tetra acetic acid
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TF	Tetra hydro folate
TFA	Total fatty acid
Tris	Tris (hydroxymethyl) amino methane
TX	Thromboxane
TTC	2,3,5 Tri phenyl tetrazolium chloride
UV-VIS	Ultraviolet-visible
U/L	Units per litre
V	Volt

v/v	Volume/volume
v/v/v	Volume/volume/volume
WBC	White blood cells
w/w	Weight/weight
ω	Omega
°C	Degree celsius
%	Percent
µ	Micrometer
µg	Microgram
µL	Microlitre
µm	Micrometer
µM	Micromolar
µmole	Micromole
α	Alpha
β	Beta
γ	Gamma
δ/Δ	Delta

Abstract

Mortierella alpina CBS 528.72, an oleaginous fungus and the most promising source of arachidonic acid (ARA) was exploited for production of this nutraceutically and pharmaceutically important polyunsaturated fatty acid (PUFA). Several physical and chemical factors were investigated for their effect on biomass buildup, oleaginicity and ARA yield. Results of the study indicated that these physical and chemical parameters had a significant effect on biomass, total lipid and ARA yield in this strain. Scale up study was carried out at 15L fermentor level subsequent to optimization under shake flask conditions. One factor at a time (OFAT) and Response surface methodology (RSM) proved to be efficient strategies for maximizing ARA yield in *M. alpina*. Furthermore the use of corn solids as a cheaper alternative for yeast extract reduced the cost of the process significantly.

Lipid extraction efficacy varied considerably with the mode of extraction, solvent used and pretreatment of biomass. Mass transfer in intact cells of *M. alpina* was hindered by hard natural membrane resulting in reduced lipid extraction. HCl and enzyme pretreatment significantly increased the percentage of ruptured cells thereby improving mass transfer of solutes and lipid extraction. Use of low temperature and 2, 3, 5- Triphenyl tetrazolium chloride enabled the selection of arachidonic acid hyper producers from innumerable putative mutants. In spite of the set backs, random mutagenesis still proved effective in isolating arachidonic acid hyper-producing mutants of *M. alpina*. The safety of feeding *M. alpina* biomass was evaluated by acute and subchronic toxicity studies in Wistar rats which indicated a favourable preclinical profile of the biomass in safety evaluation. Cloning, sequencing and phylogenetic analysis of Δ^5 desaturase indicated that *M. alpina* Δ^5 desaturase was phylogenetically related to algal, slime mould and fungal desaturases with a conserved HPGG motif and three characteristic histidine boxes at the cytochrome b_5 region. These Δ^5 desaturases were phylogenetically distinct from the animal Δ^5 desaturases which formed a strong cluster in itself indicating a later divergence than the fungal and plant counterparts. In view of the above facts *Mortierella alpina* single cell oil can be accepted as a biotechnological product fulfilling the key roles in the supply of nutraceutically and pharmaceutically relevant polyunsaturated fatty acids essential for infant and geriatric nutrition.

Aim and Scope of the present Investigation

The n-3 and n-6 series of polyunsaturated fatty acids (PUFAs) have tremendous potential for use in food additives and pharmaceuticals for a multitude of chronic diseases. PUFAs with their unique structural and functional characteristics play apparent roles in every organ of human body and have uses in various fields of biomedicine and pharmaceuticals. Long chain PUFAs, ARA and DHA play particularly important roles during the development of the brain in the late foetal and early postnatal period. Although both term and pre-term infants are capable of endogenous synthesis of DHA and ARA from precursor fatty acids, this capacity appears to be sub-optimal to meet the demands of the developing tissues in pre-term infants. Infants acquire DHA and ARA initially through the maternal placenta and after birth from their diet through mother's milk. Although ARA can be obtained from conventional sources like porcine adrenal gland, liver, sardine, eggs etc., the yield of ARA is very less and hence difficult to industrialize. *Mortierella alpina,* an oleaginous zymomycete is considered as the most promising source of ARA due to the following properties.

- Active lipid synthesizing apparatus and high oleaginicity resulting in accumulation of ARA predominantly as triacylglycerols
- Extremely high growth rates on a variety of substrates without any seasonal dependence
- Ability to provide pharmaceutical grade ARA
- Existence of mutants defective in specific enzymes leading to tailor made PUFAs
- *Mortierella* is amenable to molecular genetic study and serve as useful models for elucidating biochemistry of lipid accumulation
- Rich in proteins, trace elements, vitamins, antioxidants etc., and hence could be employed as macro and micronutrients
- Simple metabolic regulation allowing growth under controlled conditions and nutritional regimes, stimulating or repressing key genes of fatty acid synthesis thereby allowing manipulation of lipid yield and profile.

The extensive research and development in ARA production carried out over the years aimed at improving the competitiveness of microbial lipids compared to plant

and animal derived oils. Owing to the importance of ARA in areas as diverse as infant and geriatric nutrition, aquaculture, cosmetics, body building etc., emphasis has to be given on increasing the product value using inexpensive substrates, screening for efficient strains and optimizing the steps necessary for oil recovery from the cells. The final cell mass, total fermentation time and the lipid content of the organism are very important for the economic feasibility, as these factors largely determine the productivity. Since lipid synthesis in *Mortierella alpina* is under strong regulatory control, mathematical modelling which determine the relationship and interactions of various nutrients have to be applied to the process for maximizing ARA yield. Submerged cultivation for ARA requires a process that consists of several operational units' viz., fermentation, cell separation and oil extraction.

Comparative success in microbial oil production has led to flourishing interest in the development of fermentation processes for ARA. Improvement of existing strains by mutagenesis is often successful in targeted PUFA production. Although it is relatively easy to produce mutants, strain improvement requires painstaking effort and ingenuity in devising screening tests. In *Mortierella,* mutation often results in suppression or activation of specific enzymes in ARA biosynthetic pathway. Such mutants are beneficial not only for the production of tailor made PUFAs but also as excellent models in elucidating fatty acid biosynthesis.

Since ARA is meant for use in infant and geriatric food formulations it is crucial that the production strains are non toxic. Although several studies aimed at safety evaluation of oil from *M. alpina,* none of these have addressed the safety of ingestion of whole organism which contains micro and macro nutrients in addition to PUFAs. Such comprehensive studies are vital since the organism after lipid extraction can be used as a source of protein for use in various fields. Such studies would project *M. alpina* as source of both single cell oils and single cell proteins.

The focus of biotechnology on value added products like ARA requires knowledge on microbial control and regulation of fatty acid biosynthesis. Elucidation of mechanisms underlying the flow and incorporation of desired fatty acids into triacylglycerols may facilitate future progress in this area. Isolation of key genes and determination of their homology with that of microbial, plant and animal counterparts is vital since it provides the necessary insight to enable the transfer of isolated genes from one organism to other thus altering the lipid profile of the latter.

Keeping in view the multifarious uses of arachidonic acid and importance of *M. alpina* as the most promising ARA producer, this oleaginous fungus was exploited and **the following objectives have been worked out.**

1. **Optimization of various fermentation conditions for biomass, total lipid and ARA content**
2. **Strain improvement of *Mortierella alpina* for higher ARA production**
   a. Mutagenesis
   b. Selection of stable mutants
   c. Selection of mutants having higher ARA content
3. **Safety evaluation studies of *M. alpina* biomass**
4. **PCR Cloning and nucleotide sequencing of Δ^5 desaturase gene**

General Introduction and Review of literature

Aim and scope of the present investigation

Chapter 1

Optimization of various fermentation conditions for biomass, total lipid and ARA production

Chapter 2

Standardization of single cell oil extraction

Chapter 3

Strain improvement of Mortierella alpina for higher ARA production

Chapter 4

Safety evaluation studies

Chapter 5

PCR Cloning, sequencing and phylogenetic analysis of Δ^5 desaturase gene

Summary and Conclusions

Future Perspectives

References

Filamentous fungi are a group of metabolically versatile organisms with a ubiquitous distribution and are widely used in biotechnology as cell factories for the production of a multitude of chemicals, pharmaceuticals and enzymes. They exist in association with other species e.g. as lichens or mycorrhiza, as pathogens of animals and plants or as free-living species and many are regarded as nature's primary degraders because they secrete a wide variety of hydrolytic enzymes that degrade waste organic materials (Archer *et al*., 2008). They influence our everyday lives in areas as diverse as medicine, agriculture and basic science and have long been applied in the traditional food fermentation industry such as the koji process (Hara *et al*., 1992).

Filamentous fungi are unique organisms rivalled only by actinomycetes and plants in producing an array of secondary metabolites with diverse structure and functions often unveiled. The ability of these fungi to grow on rather simple and inexpensive substrates as well as their capacity to produce a wide range of commercially interesting metabolites, have attracted considerable interest to exploit them as production organisms in biotechnology (Meyer, 2008). The GRAS status of many filamentous fungi makes them ideal organisms for the production of recombinant proteins and food grade polyunsaturated fatty acids (PUFAs). Features such as low cost and high productivity have attracted many research efforts in both molecular-genetic techniques and bioprocess improvements (Finkelstein and Ball, 1992; Banerjee *et al*., 2003). These fungi are employed in biotechnology as cell factories for a wide range of products ranging from simple organic acids to complex secondary metabolites (Table 1).

Fungal secondary metabolites are of intense interest to humankind due to their pharmaceutical and/or toxic properties and tremendous progress has been made in the past decade for elucidating the metabolic pathway by understanding the genes that are associated with production of various fungal secondary metabolites. Impact of molecular biology and genomics and the availability of sophisticated methods of analytical chemistry have resulted in paradigm shifts in our understanding of fungal secondary metabolism and its key role in fungal biology (Stadler and Keller, 2008). For the last two decades, molecular genetic tools have enabled us to use these organisms to express extra copies of both endogenous and exogenous genes and recent research into gene expression in filamentous fungi has explored their wealth of

genetic diversity with a view to exploiting them as expression hosts and as a source of new genes (Punt *et al*., 2002).

Fungal secondary metabolites

Fungal secondary metabolites can be broadly classified into antibiotics, immunosuppressive agents, hypocholesterolemic agents, antitumour agents, mycotoxins, pigments and polyunsaturated fatty acids (Adrio and Demain, 2003).

Antibiotics

The major antibiotics produced by filamentous fungi are Cephalosporin from *Cephalosporium*, Penicillin from *Penicillium*, Griseofulvin from *Penicillium griseofulvum*, Lentinan from *Lentinus sp*., Pleuromutilin from *Clitopilus passeckerianus* (Kilaru *et al*., 2009) and Schizophyllan from *Schizophyllum commune.* About 22% of the known 12,000 antibiotics could be produced by these fungi. Of these, pencillin and cephalosporin produced by the *Pencillium sp.* and *Cephalosporium sp*. have the highest market potential.

Immunosuppressive agents

Immunosuppressive activity of Cyclosporin A, originally produced by *Tolypocladium nivenum* has enabled its wide application with overwhelming success in organ transplants (Borel *et al*., 1976). Mycophenolic acid produced by *Penicillium brevicompactum* is widely used as an immunosuppressant (Xu and Yang, 2007; Zambonin *et al*., 2004; Bentley, 2000) during kidney and heart transplantations.

Hypocholesterolemic agents

Statins (lovastatin, pravastatin, mevastatin, monacolin J and others) produced by several fungal species viz., *Monascus ruber* (Manzoni *et al*., 1999), *Monascus purpureus* (Sayyad *et al*., 2007), *Penicillium citrinum* (Endo, 2004), *Aspergillus terreus* (Manzoni and Rollini , 2002) etc prevent the reversible inhibition of HMG-CoA reductase which catalyzes the rate limiting step of cholesterol biosynthesis thereby acting as hypocholesterolemic drugs. Compactin is a product of *Penicillium brevicompactum* and *P. citrinum* (Brown *et al*., 1976; Endo *et al*., 1976).

Antitumour agents

Few compounds produced by filamentous fungi act as antitumour agents. 3'-Amino-3'-deoxyadenosine, an antitumour agent from *Helminthosporium sp*. (Gerber and Lechevalier, 1962), glutaminase-asparaginase produced by *Aspergillus niger* (Elzainy and Ali, 2006) are examples.

Table 1 Selected examples of industrially important compounds produced by filamentous fungi

Organism	Product
Antibiotics	
Cephalosporium acremonium	Cephalosporin C
Emericellopsis sp.	Penicillin N
Penicillium chrysogenum	Penicillins G and V
Penicillium patulum, P. griseofulvum	Griseofulvin
Pleurotus mutilus, Pleurotus passeckerianus	Pleuromutilin
Strobilurus sp.	Strobilurins A and B
Enzymes	
Aspergillus sp.	Glucoamylase and pectinase
Aspergillus niger	Glucose oxidase, chymosin, xylanases
Aspergillus oryzae , A. niger	α - Amylase, lipase, proteases, phytase
Trichoderma viride,	Cellulase, cellobiohydrolase
Trichoderma reesei	Cellulose, hemicellulase, cellobiohydrolase
Rhizopus delemar, Aspergillus phoenicis	Glucoamylase
Trametes versicolor	Laccase
Trichoderma reesei	Pectin lyase
Rhizopus delemar	Proteases
Mucor miehei	Rennin
Trichoderma reesei, Trichoderma konignii	Xylanases
Mycotoxins	
Aspergillus sp.	Aflatoxins, citrinin, ochratoxin, and sterigmatocystin
Claviceps sp.	Ergot
Fusarium sp.	Trichothecenes and zearalanone
Penicillium sp.	Citrinin, ochratoxin, and patulin
Other Metabolites	
Ashbya gossypii, Eremothecium ashbyii	Riboflavin
Aspergillus niger	Citric and gluconic acid
Aspergillus oryzae	Kojic acid and biotin
Aspergillus terreus	Itaconic acid
Aureobasidium pullulans	Pullulan
Claviceps purpurea	Ergot alkaloids
Cylinrocarpum lucidum Booth	Cyclosporin A and B
Fusarium culmorum, Rhizopus japanicus	Biotin
Fusarium graminearum	Zeranol
Fusarium oxysporum	Panthothenic acid
Gibberella fujikuroi	Gibbereillic acid
Mortierella alpina	Arachidonic acid
Mortierella isabellina	Linolenic acid
Mucor circinelloides	PUFA
Phycomyces blakesleanus	β-Carotene
Schizophyllum commune	Schizophyllan
Sclerotium rolfsii	Scleroglucan
Taxomyces andrenae	Taxol
Tolypocladium inflatum	Cyclosporin A
T. reesei	Hydrophobin
Miscellaneous	
Aspergillus niger,Trichoderma reesei	Recombinant heterologous proteins including human interleukin-6 and Fab
Beauveria bassiana, Beauveria brongniartii	Entomopathogenic fungi
Fusarium graminearum	Microbial protein
Morchella sp.	Mushroom

Adapted from (Olempska-Beer *et al*., 2006; Linder *et al*., 2005; Polizeli *et al*., 2005; Adrio and Demain, 2003; Leathers, 2003; Willke and Vorlop, 2001; Archer, 2000; Ooi and Liu, 2000; Bennett, 1998; Anke, 1997; Archer and Pederby, 1997; MacKenzie *et al*., 1993; Elander and Lowe, 1992; Metz *et al*., 1979)

Taxol, a natural anticancer agent, can be produced by the fungus *Taxomyces andreanae* (Stierle *et al*., 1993). It is approved for the treatment of breast and ovarian cancer and is the only commercial antitumour drug known to act by blocking depolymerization of microtubules (Adrio and Demain, 2003).

Mycotoxins

Mycotoxins are secondary fungal metabolites with diverse structure and toxicological properties that induce a variety of toxic effects in humans and animals when foods contaminated with these compounds are ingested. The toxic effects include acute toxicity, carcinogenicity, mutagenecity, teratogenecity and estrogenic effects on animals at normal levels of exposure. The major mycotoxins produced by filamentous fungi are aflatoxins (Gunterus *et al*., 2007), ochratoxins (Mounjouenpou *et al*., 2008), fumonisins (Heinl *et al*., 2009), zearelenone (Czerwiecki *et al*., 2006), trichothecenes (Xu *et al*., 2006), citrinin (Hajjaj *et al*., 2000), citreoviridin (Frisvad *et al*., 2008) etc. Most of the known mycotoxins have been recognized as the metabolic products of the genera such as *Aspergillus, Penicillium and Fusarium*.

Pigments

Certain non toxigenic filamentous fungi of the genus *Pencillium, Aspergillus, Epicoccum* and *Monascus* are exploited to produce an array of pigments for use in food and non-food applications (Velmurugan *et al*., 2009; Sameer *et al*., 2009; Mapari *et al*., 2008; Mapari *et al*., 2005). Considering the extraordinary diversity of fungal pigments and amenability to be produced in higher yields by metabolic engineering, fungi seem to be a promising source for biotechnological production of food colourants. The fungal pigments are intended to substitute currently used synthetic colourants and/or natural colourants derived from plant materials and some of the coloured pigments produced by fungi can expand the current palette of colours used in various industrial applications. A recent survey estimated that the value of the international food colourant market is around US$ 1.15 billions (€730 million).

Polyunsaturated fatty acids (PUFAs)

Mortierella isabellina and *Mucor circinelloides* are potent producers of gammalinolenic acid (Fakas *et al*., 2009; Tauk-Tornisielo *et al*., 2009; Xian *et al*., 2003), an important PUFA of the n-6 series. Various polyunsaturated fatty acids

including arachidonic acid can be produced from *Mortierella alpina* (Sakuradani *et al*., 2009d).

Polyunsaturated fatty acids (PUFAs) are fatty acids with two or more double bonds and are the fatty acyl components of cellular lipids such as triacylglycerols and phospholipids. Long-chain polyunsaturated fatty acids (LC-PUFAs) have become high profile in the biochemical and nutraceutical areas and have recently made it to the realm of "magical molecules" based on their multiple presumably beneficial effects in biological systems and their specific therapeutic roles in certain clinical conditions. Besides pharmacological applications, public awareness of eating healthy has also brought these PUFAs to the attention of the consumers.

Classification of polyunsaturated fatty acids

PUFAs are classified based on the number of carbon atoms between the final double bond of the fatty acid chain and the terminal methyl group. Three important families of PUFAs are n-3, n-6 and n-9.

Omega 3 (n-3) fatty acids have in common a final carbon-carbon double bond in the third position from the methyl end of the fatty acid (Fig 1).

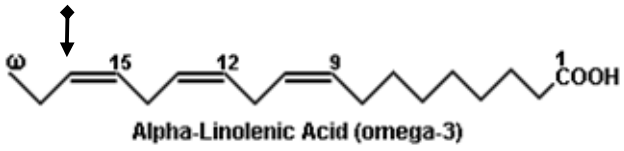


Fig 1 Structure of omega 3 fatty acid, alpha linolenic acid (ALA) showing the position of first double bond

Arrow indicates the position of the final double bond at the third position from omega end

Important nutritionally essential n-3 fatty acids are Alpha linolenic acid (ALA 18:3), Eicosapentaenoic acid (EPA 20:5), Docosapentaenoic acid (DPA 22:5) and Docosahexaenoic acid (DHA 22:6). The predominant dietary sources of n-3 fatty acids are fish, eggs, vegetable oils, green leafy vegetables such as purslane and spinach, seeds of flax, linseed, walnuts, apricot, butternuts, hempseed, etc. The major omega-3 fatty acids are given in Table 2.

Table 2 Major ω-3 fatty acids

Fatty acid	Lipid name	Chemical name
Hexadecatrienoic acid	16:3	*all-cis*-7,10,13-hexadecatrienoic acid
α-Linolenic acid	18:3	*all-cis*-9,12,15-octadecatrienoic acid
Stearidonic acid	18:4	*all-cis*-6,9,12,15-octadecatetraenoic acid
Eicosatrienoic acid	20:3	*all-cis*-11,14,17-eicosatrienoicacid
Eicosatetraenoic acid	20:4	*all-cis*-8,11,14,17-eicosatetraenoic acid
Eicosapentaenoic acid	20:5	*all-cis*-5,8,11,14,17-eicosapentaenoic acid
Docosapentaenoic acid	22:5	*all-cis*-7,10,13,16,19-docosapentaenoic acid
Docosahexaenoic acid	22:6	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid
Tetracosapentaenoic acid	24:5	*all-cis*-9,12,15,18,21-docosahexaenoic acid
Tetracosahexaenoic acid	24:6	*all-cis-6,9,12,15,18,21* tetracosenoic acid

Omega 6 (n-6) fatty acids have a final carbon-carbon double bond in the n-6 position, the sixth bond from the methyl end of the fatty acid (Fig 2).

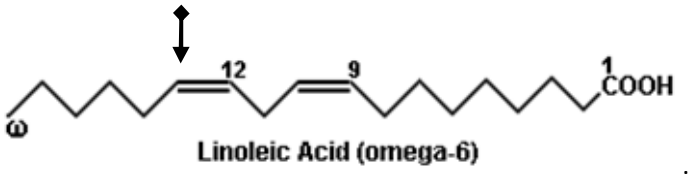


Fig 2 Structure of omega 6 fatty acid, linoleic acid (LA) showing the position of double bond

Arrow indicates the position of the final double bond at the sixth position from omega end

Linoleic acid (LA 18:2), Gamma linolenic acid (GLA 18:3), Dihomogamma linolenic acid (DGLA 20:3) and Arachidonic acid (ARA 20:4) are the important omega 6 fatty acids. The main dietary sources of n-6 fatty acids are eggs, poultry, vegetables, cereals, nuts, corn oil, safflower oil, sunflower oil, soybean oil. Plant seeds like black currant, linseed, cotton, pumpkin, avocado and evening primrose contain significant amount of n-6 fatty acids. Major omega-6 fatty acids are given in Table.3.

Table 3 Major ω-6 fatty acids

Fatty acid	Lipid name	Chemical name
Linoleic acid	18:2	9,12-octadecadienoic acid
Gamma-linolenic acid	18:3	6,9,12-octadecatrienoic acid
Eicosadienoic acid	20:2	11,14-eicosadienoic acid
Dihomo-gamma-linolenic acid	20:3	8,11,14-eicosatrienoic acid
Arachidonic acid	20:4	5,8,11,14-eicosatetraenoic acid
Docosadienoic acid	22:2	13,16-docosadienoic acid
Adrenic acid	22:4	7,10,13,16-docosatetraenoic acid
Docosapentaenoic acid	22:5	4,7,10,13,16-docosapentaenoic acid

Omega 9 (n-9) fatty acids have a final carbon-carbon double bond in the n-9 position (Fig 3). Some ω-9 fatty acids are common components of animal fat and vegetable oil. Two n-9 fatty acids important in industry are oleic acid (18:1) and erucic acid (22:1). Oleic acid is a main component of olive oil and other monounsaturated fats. Erucic acid is found in rapeseed, wallflower seed and mustard seed and is used commercially in paintings and coatings as a drying oil. Major omega-9 fatty acids are given in Table 5.

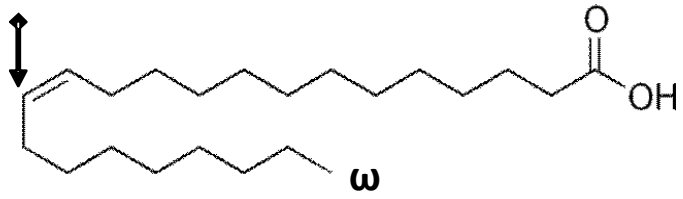


Fig 3 Structure of omega 9 fatty acid, erucic acid showing the position of double bond

Arrow indicates the position of the final double bond at the ninth position from omega end

Table 4 Major ω-9 fatty acids

Fatty acid	Lipid name	Chemical name

Oleic acid	18:1	9-octadecenoic acid
Eicosenoic acid	20:1	11-eicosenoic acid
Mead acid	20:3	5,8,11-eicosatrienoic acid
Erucic acid	22:1	13-docosenoic acid
Nervonic acid	24:1	15-tetracosenoic acid

Functions of polyunsaturated fatty acids

Being essential components of higher eukaryotes, PUFAs confer flexibility, fluidity and selective permeability to membranes (Ward and Singh, 2005). They have a strong direct influence on the molecular events governing gene expression. PUFAs mediate the activity or abundance of four families of transcription factors that play a major role in hepatic carbohydrate, fatty acid, triglyceride, cholesterol and bile acid metabolism (Jump, 2002; Clarke *et al*., 2002; Jump and Clarke, 1999). It has been demonstrated that access to essential polyunsaturated fatty acids early in life is important for intellectual development during the first years and that it can also have an impact on IQ, school abilities and academic performance up to the age of 18 (Horwood and Fergusson, 1998). Study by Field *et al*. (2000) established that in preterm babies' addition of LC-PUFAs to infant formula resulted in patterns of lymphocyte populations, phospholipid composition and cytokine production more consistent with that observed in infants fed with breast milk. Although large amounts of PUFAs may increase lipid peroxidation and oxidative stress, there is no evidence that concentrations within the range found in human milk are harmful. The most important LC-PUFAs for the brain and retina are docosahexaenoic acid (DHA) and arachidonic acid (ARA). These PUFAs not only effect membrane biophysical properties, neurotransmitter content and the corresponding electrophysiological correlates but also modulate gene expression of the developing retina and brain. Intracellular fatty acids or their metabolites activate nuclear transcription factors thereby regulating transcriptional activation of gene expression during adipocyte differentiation and retinal and nervous system development (Uauy *et al*., 2001). They find applications in areas as diverse as infant nutrition to the therapeutic treatment of chronic diseases like cancer (Table 5).

Table 5 Products/Applications of PUFAs and their sources

Product	PUFA	Source
Infant formula term/preterm	ARA, DHA	Microbial
Term infant formula	GLA	Plant
Pregnant /nursing women	DHA	Microbial
Adult diet supplement	DHA, EPA	Fish
	DHA	Microbial
	ARA	Microbial
Food Additives: cheese,yoghurt, spreads, dressings, breakfast cereals	DHA	Microbial
Eggs	DHA	Plant
Mariculture	DHA, EPA	Fish
	DHA, DPA	Fish
Pharmaceutical precursor	ARA	Microbial
Cardiovascular health	DHA, EPA	Fish
	DHA	Fish
Atherosclerosis, Hyperlipemia	EPA	Fish
Atopic eczema, rheumatoid arthritis, multiple sclerosis, schizophrenia, PMS	GLA	Plant
Schizophrenia, certain cancers	EPA	Fish

PMS-Premenstrual syndrome

Source: Ward and Singh, 2005

Biosynthesis of polyunsaturated fatty acid (PUFAs)

Biochemistry of lipid accumulation in microorganisms

Although all living organisms synthesize a minimal amount of lipids for their structural and functional roles, only a few microorganisms are capable of accumulating lipids above 20% of their cell mass as storage material. Bacteria in general produce poly–β-hydroxyl butyrates and alkanoates instead of triacylglycerols (TAG). Oil accumulation is restricted to some yeasts, fungi and algae which are termed oleaginous organisms (Ratledge, 2004).

Fatty acid biosynthesis in most oleaginous microorganisms is essentially the same as that in non-oleaginous species. However there is a crucial difference between oleaginous and non-oleaginous organisms. Lipid accumulation in an oleaginous microorganism begins when it exhausts a nutrient from the medium; usually nitrogen, but with a surfeit of carbon, usually in the form of glucose, still remaining (Ratledge, 2002). Triacylglycerols are generally best produced under limited amounts of nitrogen substrate and this is the regime of choice for main fermentation. When the organism grows, it quickly exhausts the nitrogen supplied but continues to assimilate the carbon source which is then channelled directly into lipid synthesis leading to production of TAGs as discrete oil droplets. On the other hand, non-oleaginous organisms when placed in the same nitrogen limiting growth medium, tends to cease further cell proliferation, and the excess carbon source assimilated is diverted to various polysaccharides including glycogen, glucans, mannans etc.

The oleaginicity encountered in organisms can be attributed to

1. The ability of these organisms to maintain a constant supply of acetyl CoA in cytosol as a necessary precursor of fatty acid synthase (FAS).
2. Ability to produce a sufficient supply of NADPH as the essential reductant used in fatty acid biosynthesis.

The formation of acetyl CoA in oleaginous microorganisms is due to the presence of ATP citrate lyase which does not occur in many non-oleaginous species.

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

Citric acid, the substrate for this reaction is synthesized as part of the TCA cycle within the mitochondria. In oleaginous organisms, the activity of isocitrate

dehydrogenase as a component of TCA cycle is dependent on the presence of AMP, whereas no such dependency occurs in non-oleaginous organisms. At the onset of nitrogen limitation, the activity of AMP deaminase which regulates the concentration of AMP is upregulated. The increased activity of AMP deaminase decreases the cellular content of AMP, resulting in a cessation of activity of isocitrate dehydrogenase. Since isocitrate cannot be metabolized, it is readily equilibrated and citric acid accumulates. With the aid of citrate efflux system in the mitochondrial membrane, citrate enters the cytosol and is cleaved by ATP citrate lyase (ACL) to acetyl CoA and oxaloacetate. Malate dehydrogenase converts oxaloacetate to malate which is used as the counter ion in the citrate efflux system. Acetyl-CoA thus produced is the principal building block for *de novo* biosynthesis of fatty acids (Weete, 1980). The sequence of events is given diagrammatically in Fig 4.

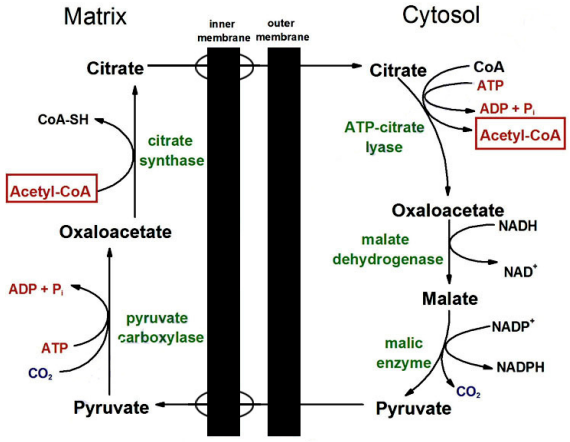
Fatty acids are highly reduced materials and to achieve their synthesis, a ready supply of reductant such as NADPH is essential. The malic enzyme (ME) generates NADPH which provides the reducing power for the synthesis of backbone of fatty acids (Boulton and Ratledge, 1981).

$$\text{Malate} + \text{NADP}^+ \longrightarrow \text{pyruvate} + CO_2 + \text{NADPH}$$

Malic enzyme activity has been found in most oleaginous microorganisms where it forms an integrated metabolon complex that combines with ACL and FAS to ensure a direct channelling of acetyl CoA into fatty acids which are finally esterified with glycerol into TAGs and incorporated into fatty acid droplets (Ratledge, 2004). However malic enzyme is not ubiquitous amongst oleaginous microorganisms and is absent in *Lipomyces* and *Candida* where an alternate NADPH generating enzyme such as a cytosolic NADPH dependent isocitrate dehydrogenase may be found (Ratledge, 2004). A correlation has been observed between the activity of the ATP citrate lyase and malic enzyme in yeast (Boulton and Ratledge, 1981) and fungi (Kendrick and Ratledge, 1992b) to accumulate NADPH in the cytoplasm of the oleaginous organisms and provides acetyl-CoA for fatty acid biosynthesis.

There is a strong correlation between the presence of ACL and the ability to accumulate lipids in yeast, filamentous fungi and other oleaginous microorganisms (Ratledge and Wynn, 2002). No organism has yet been found that accumulates more than 20% of its biomass as TAG and does not possess ACL activity. Though ACL is a

prerequisite for lipid accumulation to occur its possession does not necessarily indicate that the organism is oleaginous (Ratledge, 2002).



Source: King, 1996 The medicalbiochemistrypage.org

Fig 4 Pathway for the movement of acetyl-CoA units from mitochondrion to cytoplasm for use in fatty acid biosynthesis

Fatty acid biosynthesis

Fatty acids are synthesized from Acetyl-CoA by the two complex enzyme systems, 1. Acetyl-CoA carboxylase catalyzes the first step in the fatty acid synthesis i.e. ATP and Mn^{2+} dependent carboxylation of acetyl-CoA and malonyl-CoA (Gurr and Harwood, 1991).

2. Conversion of malonyl-CoA to fatty acid which is synthesized by fatty acid synthase and it requires acetyl-CoA and NADPH. Further synthesis involves a series of condensation - reduction - dehydration - reduction reaction that results in the lengthening i.e. addition of two carbon atoms to acyl chain and formation of even numbered fatty acids. All the carbons are supplied by malonyl-CoA except the two methyl terminal groups which are supplied by acetyl-CoA. Addition of either propionyl-CoA or isopropionyl-CoA to the acetyl-CoA results in the formation of odd chain or branched fatty acids (Bressler and Wakil, 1961).

Fatty acid biosynthesis in most organisms culminates in the formation of either C16 or C18 saturated fatty acids. These fatty acids are subsequently modified by a series of desaturases and elongases leading to the production of polyunsaturated fatty acids (PUFAs). Polyunsaturated fatty acids can be synthesized by two different pathways: an anaerobic one, by using polyketide synthase related enzymes, and an

aerobic one, which involves the action of elongases and oxygen dependent desaturases (Uttaro, 2006). In anaerobic route, the double bond is introduced to a medium-chain-length fatty acid followed by elongation. This pathway is limited to some anaerobic bacteria and marine protists. These organisms uses a polyketide synthase (PKS) like system which involves acetyl CoA and malonyl CoA as the essential building blocks but does not involve *in situ* reduction of the intermediates. Oxygen is not involved in the synthesis of double bonds and the overall requirement of NADPH needed for the reduction of the growing fatty acid acyl chain and for the operation of desaturases used in PUFA formation is reduced. This pathway has been reported in the bacteria *Shewanella* and *Moritella marina* as well as *Schizochytrium* and *Ulkenia* (Ratledge, 2004).

In aerobic route, direct desaturation of fatty acid take place in presence of molecular oxygen to produce reduced NADH or NADPH. This aerobic pathway is common to protozoa, fungi, algae, aerobic bacteria, plants and mammals (Boulton and Ratledge, 1985). Biosynthesis of PUFAs by the aerobic route involves a subclass of microsomal fatty acid desaturase enzymes, the so called front end desaturases (Napier *et al.*, 1999b). The pathway for the biosynthesis of these very long chain PUFAs involves several sequential desaturation and elongation steps (Fig 5). While higher plants contain only PUFAs with upto 18 carbon atoms and four double bonds, mosses and algae are capable of additional elongations and desaturations.

Polyunsaturated fatty acids (PUFAs) in fungi and plants are usually synthesized from oleic acid (18:1) by a series of chain elongation and desaturation reactions. Almost all fungi synthesize oleic acid (18:1) from stearic acid (18:0) by aerobic desaturation. The subsequent desaturation of oleic acid (18:1) leads to formation of alpha linolenic acid (ALA 18:3, n-3) and gamma linolenic acid (GLA 18:3 n-6) through linoleic acid (LA 18:2 n-6). In some fungi, desaturation and elongation proceed further and produce polyunsaturated fatty acid such as dihomogamma linolenic acid (DGLA 20:3 n-6), arachidonic acid (ARA 20:4 n-6), eicosapentaenoic acid (EPA 20:5 n-3), docosapentaenoic acid (DPA, 22:5, n-3) and docosahexaenoic acid (DHA 22:6 n-3). The lower fungal species particularly belonging to phycomycetes are active in the synthesis of these PUFAs. The desaturation and elongation reactions are catalyzed by respective desaturases and elongases. Chain elongation is relatively faster because it occurs in all the species

having same pathway. Desaturation reaction is slower and is considered to be the rate limiting step in PUFA synthesis.

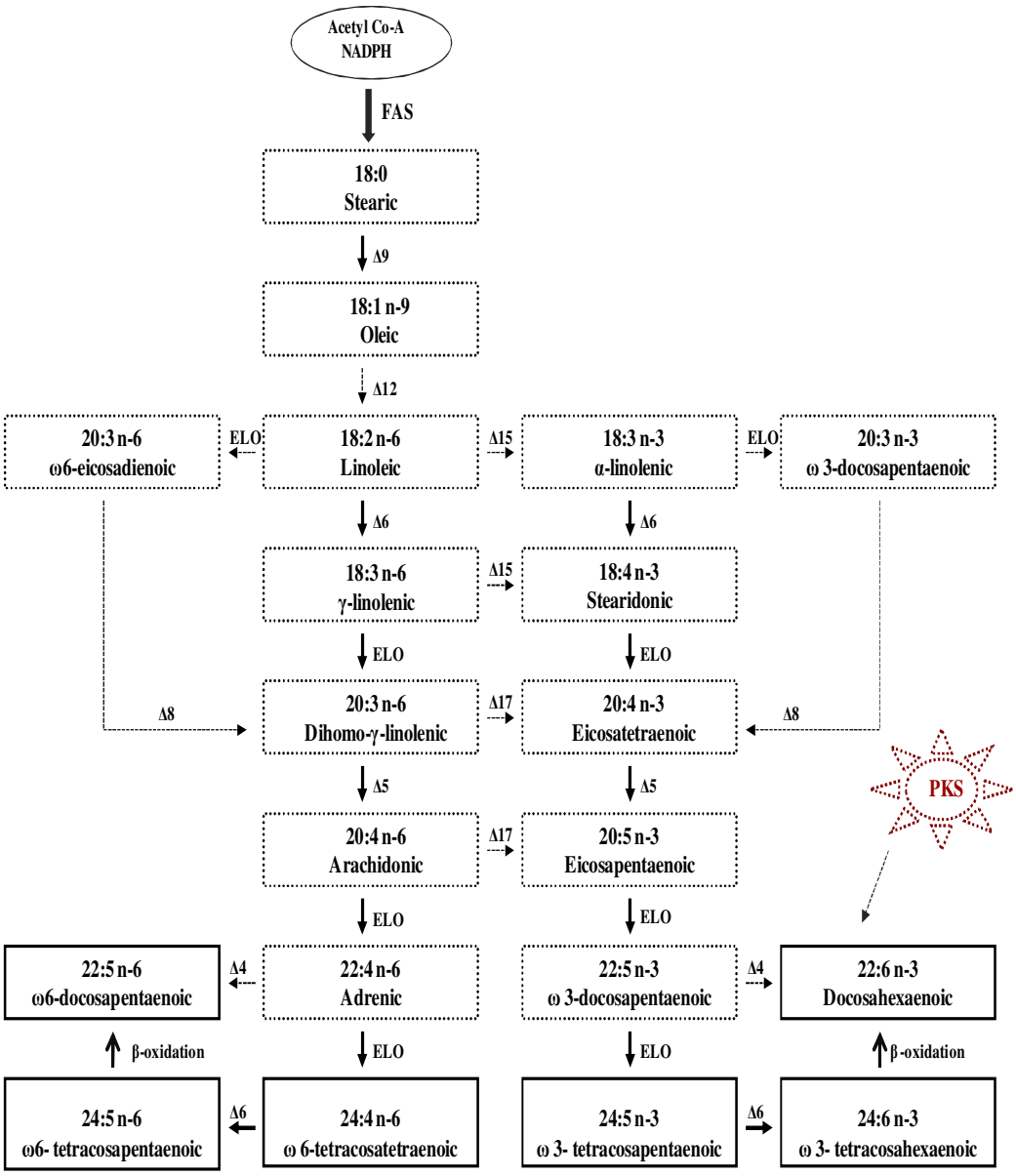


Fig. 5 Biosynthesis of long chain polyunsaturated fatty acids

Source: Pereira *et al.*, 2003

Arrows with solid line are found both in mammals and lower eukaryotes, while arrows with dotted line are exclusively for lower eukaryotes. Fatty acids in bold boxes indicate that the pathway is exclusively for mammals

Omega 3: omega 6 balance- significance

An optimum balance of omega 6-omega 3 PUFAs in the human diet is important for homeostasis of inflammatory responses (Pereira *et al*., 2004). The ratio of omega 6 to omega 3 in modern diet is approximately 15:1 or higher whereas ratio of 2:1 to 4:1 have been implicated in the prevention of several chronic diseases like breast cancer. Medical research suggests that excessive n-6 levels relative to n-3 may increase the probability of a number of diseases and depression (Okuyama *et al*., 2007; Hibbeln, 2006). Singer *et al.* (1985) have shown a defective desaturation and elongation in the n-3 and n-6 fatty acid system in patients suffering from hypertension. Imbalance in n-6 and n-3 PUFA metabolism has also been implicated in chronic diseases such rheumatoid arthritis, autoimmune disorders, Crohn's diseases and cancer (Simopoulos, 1999). The n-6 to n-3 fatty acid ratio in the diet has a profound influence on bone fatty acid composition and biosynthesis of prostaglandins which regulate bone formation and resorption (Li *et al*., 1999). An aberrant PUFA ratio has been noticed in inflammatory disease like arthritis and certain cancers which lead to over-production of prostaglandins and leukotrienes that enhance inflammation. It has been hypothesized that increased n-6 PUFA and decresed n-3 PUFA dietary intakes have contributed to the recently observed increases in asthma and other allergic diseases (Devereux, 2006). The ratio of omega 6 to omega 3 PUFAs of cells and tissues have become a biomarker to monitor the outcomes of dietary interventions and for identifying the risk factors for lipid related diseases (Harris and VonSchacky, 2004). A rational combination of ω-3 and ω-6 fatty acids is as beneficial as that of the "polypill" and may even show additional benefit in the prevention of depression, schizophrenia, Alzheimer's disease and enhance cognitive function (Das, 2008)

Major polyunsaturated fatty acids

Alpha linolenic acid (ALA 18:3 n-3)

ALA is a major source of plant omega 3 and large doses are present in dark green leaves and a variety of vegetable oils like flax, hemp, rape (canola) seed, soybean and walnut. ALA is the "parent" fatty acid to DHA and EPA and human body converts ALA rapidly into EPA, and more slowly into DHA (Gerster, 1998). Flaxseed oil is a rich natural source of ALA.

ALA, which is important for healing the body and maintaining good health is one of the least abundant of the essential fatty acids in most diet. ALA derivatives have been proven to prevent clotting and decrease the inflammatory process and are especially beneficial to individuals suffering from arthritis.

Eicosapentaenoic acid (EPA 20:5 n-3)

EPA occurs in most fish oils at about 5 to 10% by weight. In order to circumvent problems like ill-smelling EPA, difficulty in purifying EPA from fish oil and constancy of EPA supply, the microbial production of EPA by culturing of marine *Chlorella* (Feng *et al.,* 2005), *Nanochloepsis saline* (Zittelli *et al*., 1999) other algae or fungi have been attempted. It has been reported in certain bacteria (Yazawa *et al.,* 1988) belonging to the genus *Shewanella* (Hirota *et al*., 2005; Satomi *et al*., 2003), zygomcete fungus *Mortierella* (Jacobs *et al*., 2009; Shimizu *et al*., 1988b) and some more primitive fungi such as *Pythium irregulare* (O'Brien *et al.,* 1993).

EPA has numerous pharmacological effects like anti tumourigenesis (Chapkin *et al*., 2008b; Chapkin *et al*., 2007; Tsuzuki *et al*., 2007), thrombosis, lowering of plasma triglyceride level, blood pressure and blood viscosity, anti-arteriosclerosis and anti-inflammation (Serhan *et al*., 2008). EPA induces strong and selective apoptosis via lipid peroxidation (Tsuzuki *et al*., 2007; Tsuzuki *et al*., 2004; Igarashi and Mijazawa, 2000). Together with DHA, EPA has been shown to prevent platelet aggregation and reduce cholesterol thereby reducing the rate of reoccurrence of coronary heart disease. When taken in sufficient doses EPA also reduce the incidence of inflammatory diseases such as asthma and type 1 diabetes mellitus. EPA and DHA attenuate immune mediated inflammatory disease (Jolly and Fernandes, 2000; Wu and Meydani, 1998), retards progression of renal diseases (Pestka *et al*., 2002), reduces *in vitro* lymphocyte proliferation (LeBlanc *et al*., 2007; Gorjao *et al*., 2006; Jolly *et al.,* 1997), lower blood triglyceride concentration and ameliorate high fat induced insulin resistance (Giacco *et al*., 2007; Holness *et al*., 2003). Dietary long-chain n-3 PUFA act as chemotherapeutic agents which restore the normal proliferative and apoptotic pathways, have the potential for effectively treating cancers that depend on aberrations of these pathways to stay alive (Hong *et al*., 2000). Increasing evidence suggests that n-3 PUFA uniquely alter the basic properties of cell membranes (Chapkin *et al*.,

2008a). Owing to the importance of EPA, marine fish oil with slightly concentrated EPA (20-30%) has been marketed by about 60 companies.

Docosapentaenoic acid (DPA 22:5)

The occurrence of n-6 DPA in the biosphere is universally rare except in labyrinthulids and thraustochytrids (Kumon *et al*., 2003). Most of the labyrinthulids and thraustochytrids contain DHA and a lesser amount of n-6 DPA. n-3 DPA is present at very low levels in fish oils relative to EPA or DHA, but is almost as important as either of them. Seal oil is a main source of n-3 DPA and can supply up to ten times more DPA than fish oils.

DPA levels in serum phospholipids have been inversely correlated to coronary heart disease risk (Simon *et al*., 1995). Although the physiological function of n-6 DPA has not yet been clarified, it has been reported that a relatively high level of DPA has been observed in brain and retina (Dyer and Carol, 1991; Bourre *et al.,* 1989; Homayoun *et al.*, 1988) under conditions of DHA and EPA deficiency. Deficiency of n-3 essential fatty acids during lactation resulted in an increase in n-6 DPA and a decrease in the DHA level, even in liver and serum lipids (Guesnet *et al*., 1988). DPA is beneficial to those at risk of heart disease and diabetes and is a potent inhibitor of platelet aggregation (Satoshi *et al*., 2000; Cheryk *et al*., 1999). Studies carried out in *Chaos* (amoeba) indicate a structural role ω-6 DPA in cell membrane organization (Deng *et al*., 2009).

Docosahexaenoic acid (DHA 22:6 n-3)

Current commercial sources of DHA are marine fish and its oil and their level varies from 8% to 20%. Fishes such as salmon, sardine, mackerel, menhaden, anchva and tuna which usually contain a high proportion of fat tissue are used for fish oil production. However, due to relatively low proportion of DHA in fish oil and difficulties encountered in extraction and purification, large scale production of DHA is difficult and other sources of DHA have been sought. DHA has been found in many species of phytoplankton and sea weed in levels ranging from 12% to 35% of total lipid content (Singh and Ward, 1997b). Certain psychrophilic and psychrotrophic bacteria contain a substantial amount of DHA (Hamamoto *et al.,* 1995; Yano *et al*., 1994). The DHA content of different strains of *Thrastochytrium* and *Scizochytrium sp.* range from 1.5% to 35% of total fatty acids (Singh *et al.,*

1996) and have potential for commercial exploitation. High levels of DHA have been detected in many species of microalgae and are particularly abundant in algal classes *Dinophyceae*, *Haptophyceae* and *Cryptophyceae* (Sargent *et al.*, 1987). In *Dinoflagellates*, *Crypthecodinium cohinii* (de Swaaf, 2003; Kyle, 1996; Beach and Holz, 1973), *Gonyaulax catenella* and *Gymnodinium* (Mansour *et al.*, 2003) are good producers containing more than 30% of DHA.

DHA recently attracted much attention because of its recognized beneficial effect on human health (Mendes *et al*., 2009) and various physiological functions in the human body. DHA is a unique fatty acid because it significantly alters basic properties of cell membranes, including acyl (ester-linked fatty acid) chain order and fluidity, phase behaviour, elastic compressibility, ion permeability, fusion, rapid flip-flop and resident protein function (Stillwell and Wassall, 2003). It is an essential component of cell membranes in some human tissues and account for over 60% of the total fatty acids in the rod outer segment in the retina (Giusto *et al.,* 2000). DHA is regarded to be essential for the proper visual and neurological development of infants because of its role as a structural lipid component (Das and Fams, 2003). As preterm and young infants are unable to synthesize DHA at a rate fast enough to keep up with demand of rapidly growing brain (Crawford, 1981), they should obtain these compounds from their diet.

DHA reduces or inhibits risk factor involved in cardiovascular diseases (Nordoy *et al.,* 2000) and has positive effects on diseases such as hypertension, arthritis, arterioscleriosis and thrombosis (Horrocks and Yeo, 1999). A low serum DHA level has been reported as a significant risk factor for the development of Alzeimers (Kyle *et al.,* 1999) and coronary artery disease (Paganeeli *et al*., 2001). DHA also induce strong and selective apoptosis via lipid peroxidation (Igarashi and Mijazawa, 2000) and is reported to have an anti tumour effect (Chapkin *et al*., 2008b; Chapkin *et al*., 2007; Kato *et al*., 2007). The functional significance of DHA is demonstrated by the impaired visual response, learning deficits associated with n-3 fatty acid deficiency, loss of odour discrimination and reduced spatial learning (Mitchell *et al*., 2003).

Gamma linolenic acid (GLA 18:3 n-6)

GLA has been traditionally obtained from the seeds of evening primrose and has been commercially marketed as evening primrose oil. The most important

and commercially available sources of GLA are borage (*Borago officinalis* 17-25%), black currant (*Ripes nigrum* 15-20%), evening primrose (*Oenothera biennis* 7-10%) and fungal oils from *Mucor* and *Mortierella* species (Rahmattullah *et al.,* 1994). In addition to seed oil, GLA is also found in lipids of phytoflagellates and most of the ciliated protozoa (Kyle and Ratledge, 1992). The order Mucorales of the class Zygomycetes has been reported as promising sources of GLA. It has also been reported in some thermophilic fungi (Weete, 1974). The highest level of GLA has been found in borage oil and is expected to be the best suited for the concentration of GLA. GLA production from current sources is inadequate for supplying the expanding market due to the significant properties of low productivity, complex and expensive downstream processing and unstable quality (Alonso and Maroto, 2000; Gill and Valivety, 1997). Modification of the fatty acid biosynthetic pathways by genetic manipulation to produce desired oil in transgenic microorganisms and oil seed crops as a possible alternative source has been investigated (Zhang *et al*., 2004).

GLA is used for the treatment of various diseases such as multiple sclerosis (Harbige and Sharief, 2007), schizophrenia (Irving *et al*., 2009; Vaddadi, 1992), atopic eczema and premenstrual syndrome (PMS). It is also the precursor of a number of biologically active compounds (Somshekhar *et al.,* 2002). GLA has the ability to inhibit both motility and invasiveness of human colon cancer cells (Jiang *et al*., 1995). GLA can prevent stress induced blood pressure rises in animals and attenuate surges in blood pressure due to daily events or mental stress in humans. Daily supplementation of GLA can exert anti inflammatory, anti thrombotic and anti arrhythmic activities and provide beneficial effects on glucose and lipid metabolism (Woodman *et al*., 2002; Zurier *et al.,* 1996). GLA is used for the treatment of diabetic neuropathy (Jamal, 1994; Keen *et al*., 1993; Horrobin, 1992), rheumatoid arthritis (Kast, 2001; Zurier *et al*., 1996), dry eye (Aragona *et al*., 2005) and tumour (Pham *et al*., 2006; Kokura *et al*., 1997). GLA deficiency has been associated with some skin disorders (Horrobin, 1992), viral infections as well as ageing.

Dihomo -γ-Linolenic Acid (DGLA 20:3 n-6)

Though no organism naturally accumulate large quantities of DGLA, GLA acts as the precursor for the synthesis of DGLA in human body. Shimizu *et al.*

(1989a) reported that adding sesame oil to *M. alpina* IS-4 led to a three fold increase in DGLA in the lipid which is attributed to sesamin that specifically inhibit Δ^5 desaturase. Several derivatives of anisole increased the DGLA content of *Conidiobolus nanodes* by a similar mechanism (Nakajima and Izu, 1993). DGLA containing oil was produced by a Δ^5 desaturase-defective mutant of the fungus *Mortierella alpina* IS-4 (Kawashima *et al*., 2000).

DGLA is considered a healthy omega-6 fatty acid because it promotes the body to produce a series of prostaglandins that control platelets and blood pressure. DGLA is one of the essential fatty acids and has anti-inflammatory and anti-allergic effects (Kawashima *et al*., 2009). It is widely used for preventing or treating skin diseases. Oral administration of DGLA effectively prevents the development of atopic dermatitis in NC/Nga mice and DGLA in phospholipids is a compound of key importance in the development and prevention of dermatitis (Kawashima *et al*., 2008). DGLA affects cytokine production by human PBMC independently of COX activation (Dooper *et al*., 2003). DGLA administration significantly increased the ratio of PGE_1/PGE_2 in the rat plasma and improved atopic eczema (Umeda Sawada *et al*., 2006)

Arachidonic acid (ARA 20:4 n-6)

Arachidonic acid (ARA, 20:4) is an omega-6 fatty acid and is the counter part to the saturated arachidic acid found in peanut oil. In chemical structure, arachidonic acid is a carboxylic acid with a 20-carbon chain and four *cis* double bonds (Fig. 6). It is denoted as all-*cis* 5, 8, 11, 14-eicosatetraenoic acid. It has a molecular mass of 304.5g/mol and a melting point of -49.5 °C.

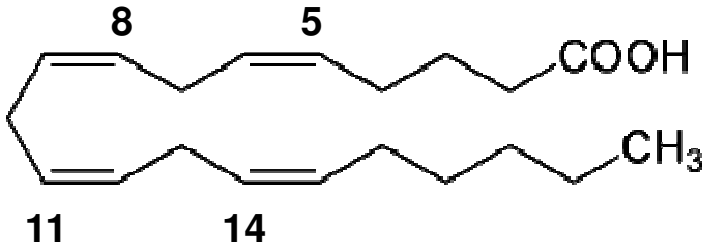


Fig 6 Structure of Arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid)

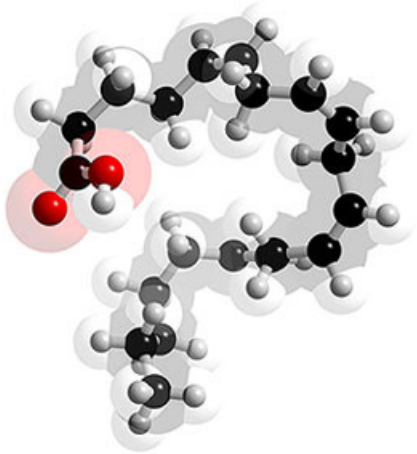


Fig 7 Three dimensional structure of arachidonic acid

Indicate oxygen atoms

Source:www.3dchem.com

Sources of ARA

Although ubiquitous in animal tissue, arachidonic acid is not available readily. ARA is isolated from the lipids extracted from pig adrenal glands, pig liver and sardines (Totani and Oba, 1987). However the arachidonic acid content is usually less than 5% and the yield per dry weight is 0.2% or lower and are difficult to commercialize. Microorganisms belonging to the genera *Pencillium, Mucor, Cladosporium, Fusarium, Hormodendram, Aspergillus and Rhodotorula* produce up to 7.5% ARA in total lipids when cultured in media containing hydrocarbons or carbohydrates (Iizuka *et al*., 1979). The moss *Physcomitrella patens* is known to contain considerable proportions of ARA (Kaewsuwan *et al*., 2006; Grimsley *et al*., 1981). Several species of algae viz., *Gracilaria spp.* (Araki *et al.,* 1990)*, Desmarestia acculeata, Phycodrys sinuosa* (Pohl and Zurheide, 1979), *Palmaria stenogona* (Khotimchenko, 2003)*, Undaria pinnatifida* (Khotimchenko, 2003), *Thalassiosira pseudonana, Amphidinium carteri* (Cobelas and Lechado, 1989), *Ochromonas danica* (Vogel and Eichenberger, 1992), *Dictyopteris membranacea* (Hofmann and Eichenberger, 1997), *Ectocarpus fasciculatus* (Makewicz *et al*., 1997), *Porphyridium creuntium* (Cohen,1999) and *Parietochloris incisa* (Solovchenko *et al*., 2008; Khozin-Goldberg *et al*., 2006; Khozin-Goldberg *et al*., 2002; Bigogno *et al*., 2002) accumulate 8-46% ARA. Some species of the genera *Delacroixia, Conidiobolus* (Miura *et al*., 1982), *Entamophthora* (Kendrick and Ratledge, 1992a), *Pythium* (Stredansky *et al*., 2000) and *Phytophthora* contain

Table 6 Fatty acid composition of algae relatively rich in arachidonic acid

Species	Major fatty acids (% TFA)									
	16:0	16:1	18:1	18:2	18:3	18:4	20:4	20:5	22:6	Reference
Thalassiosira pseudonana	10	29	-	1	-	-	14	15	-	Cobelas and Lechardo, 1989
Parietochloris incisa	10	2	16	17	1	-	43	1	-	Bigogno *et al*, 2002
Amphidinium carteri	12	1	2	1	3	19	20	-	24	Cobelas and Lechardo, 1989
Desmarestia acculeata	12	2	7	6	10	16	19	19	-	Pohl and Zurheide, 1979
Dictyopteris membranacea	20	1	14							Hofmann and Eichenberger, 1997
Ectocarpus fasciculatus	17	-	13	4	15	23	11	13	-	Makewicz *et al*., 1997
Ochromonas danica	4	-	7	26	12	7	8	-	-	Vogel and Eichenberger, 1992
Gracilaria confervoides	18	3	16	2	-	1	46	-	-	Pohl and Zurheide, 1979
Phycodrys sinuosa	22	5	5	1	1	-	44	2	-	Pohl and Zurheide, 1979
Porphyridium creuntum	34	1	12	12	-	-	40	7	-	Cohen, 1999

Source: Bigogno *et al*., 2002

relatively high levels of ARA in their lipids. Because of higher ARA contents, higher growth rates in simple media and the simplicity of manipulation, fungi are thought to be much more advantageous to produce fatty acids than the algal, moss and protozoal sources previously reported (Emelyanova, 1997; Arao *et al*., 1987; Ahern *et al*., 1983). The filamentous fungi *Mortierella alpina* is known to be the most potent producer with the arachidonic acid content ranging from 30-70% of total lipids in different strains.

Properties of Arachidonic acid (ARA)

Arachidonic acid is a slippery molecule that owes its mobility to four *cis* double bonds. These are the source of its flexibility, keeping the pure fatty acid liquid, even at subzero temperatures and helping to give mammalian cell membranes their correct fluidity at physiological temperatures. The double bonds are also the key to the propensity of arachidonic acid to react with molecular oxygen (Brash, 2001). Like other fatty acids, arachidonic acid is amphipathic, and its hydrophobic tail can remain in a lipid bilayer while its polar carboxyl group can emerge into the aqueous environment outside the membrane (Brash, 2001).

Ubiquitous among living organisms ARA primarily exists *in vivo* esterified in the sn-2 position of glycerol backbone of membrane phospholipids in and out of which it rapidly circulates (Sublette *et al.,* 2004).

Functions of ARA

ARA is found throughout the body and is essential for the proper functioning of almost every body organ, including the brain. It serves a wide variety of purposes, from being a purely structural element in phospholipids to being involved in signal transduction and being a substrate for a host of derivatives involved in second messenger function. ARA and DHA are polyunsaturated fatty acids of great physiological significance (Moore, 2001; James *et al*., 2000) and are crucial for central nervous system during development and in various pathological states (Strokin *et al*., 2003).

ARA in brain development and growth

Arachidonic acid is one of the most abundant fatty acids in the brain and together with DHA account for 20% of the brain fatty acid content (Crawford and Sinclair, 1972). It helps to maintain hippocampal cell membrane fluidity (Fukaya *et al*., 2007) and protect the brain from oxidative stress (Wang *et al*., 2006). ARA is

involved in early neurological development and activates syntaxin-3 (STX-3), a protein involved in the growth and repair of neurons (Darios and Davletov, 2006). As a component of human milk, ARA is necessary for the neurological and neurophysiological development of both term (Birch *et al*., 2000) and preterm infants (Bougle *et al*., 1999). ARA is known to be responsible for foetal cognitive development and diminished levels of ARA could lead to impaired foetal growth and nerve transmission in developing infant (Horrobin, 1997; Carlson *et al*., 1993; Birch *et al*., 1998).

Arachidonic acid is a very important compound due to its widespread role in cell signalling that leads to growth. Growth is one of the most sensitive indicators of adequate nutritional status in infants and for this reason is considered to be of key importance in all nutritional interventions. During early life, arachidonic acid may have a growth promoting effect which could be related to its role as an eicosanoid precursor or to its structural function in membrane lipids (Koletzko and Braun, 1991).

ARA for healthy skin

Arachidonic acid has been used in cosmetics as surfactant, cleansing and emulsifying agent (Eppler *et al*., 2004). It is also used to soothe rashes and in correct dosage has a positive influence on different skin problems. The use of 0.5% to 2% arachidonic acid applied under occlusion alleviated the clinical symptoms of psoriasis (Hebborn *et al*., 1988).

ARA in cell signaling and eicosanoid production

The functional role of ARA appears to be mediated either by the fatty acid itself or its bioactive derivatives -the eicosanoids. The arachidonic acid cascade which generate at least 16 multiple messenger molecules is arguably the most elaborate signalling system neurobiologists deal with. The messenger molecules of the cascade may act both within and outside the neuron, bringing into play intracellular as well as extracellular targets. ARA is thought to serve as an intercellular messenger in many parts of the central nervous system. It induces apoptotic neuronal cell death in association with intracellular Ca^{2+} rise in mitochondrial damage independently of capsase -3 (Saitoh *et al*., 2003). ARA plays a major role in synaptic transmission (Dumuis *et al*., 1988), act as an activator of protein kinase C pathway (Nishizaki *et al*., 1999) and a modulator of TRPM5 in taste signalling pathways (Oike *et al*., 2006).

Most of the effects of arachidonic acid are attributable to its conversion by oxygenases (COX, LOX, and P450) to prostaglandins, leukotrienes (Gill and Valivety, 1997) and other bioactive products (Fig 8). Arachidonic acid is the precursor of thromboxanes and prostacyclins, two most active compounds related to platelet function (Nelson *et al*., 1997a) and dietary supplementation with ARA effectively increases the levels of this fatty acid in plasma and membrane phospholipids, thereby improving platelet aggregation (Pantaleo *et al*., 2004).

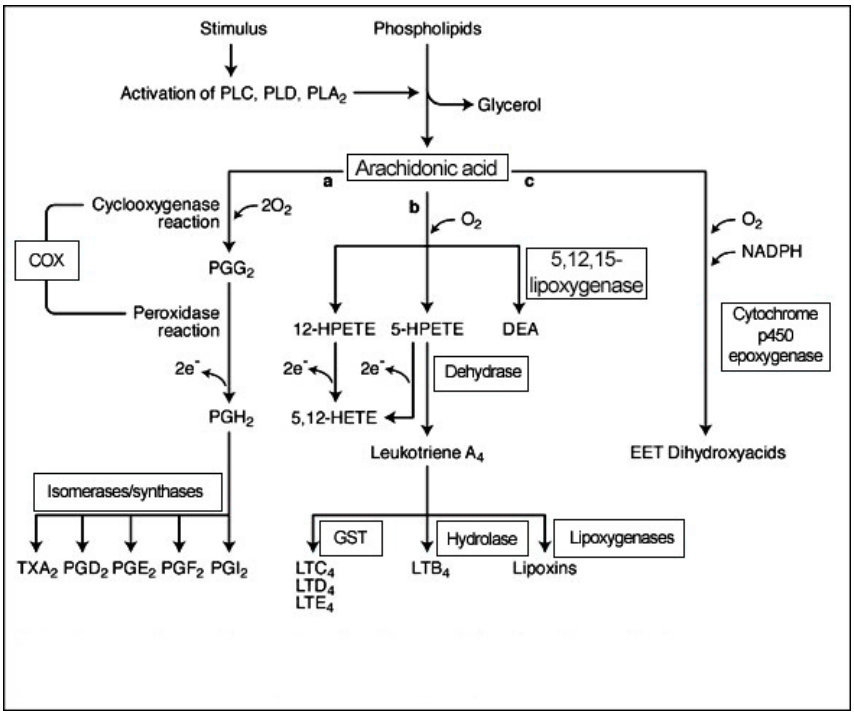


Fig 8 Release of arachidonic acid from phospholipids and eicosanoid synthesis

COX- cycloxygenase, PLC- phospholipase C, PLD- Phospholipase D, PLA_2 phospholipase A_2, PG-prostaglandin, TX- tromboxane, LT –leukotriene, HPETE- hydroperoxyeicosatetraenoic acid
Source: Expert Reviews in Molecular Medicine 2003
Cambridge University Press

ARA in anabolic muscle development

Arachidonic acid is necessary for the repair and growth of skeletal muscle tissue. Role of this nutrient in muscle anabolism and its potential for the enhancement of muscle size and strength is well documented. ARA is abundant in skeletal muscle membrane phospholipids. As a regulator of localized muscle inflammation, ARA may

be a central nutrient controlling the intensity of the anabolic/tissue-rebuilding response to weight training. Arachidonic acid has shown to improve peak muscle power, reduce systemic inflammation and produce statistically strong trends of improvements in muscle endurance and average power suggesting a definite role in muscle metabolism and performance.

ARA for the elderly

The cerebral functions of the elderly are improved by administering arachidonic acid as a supplement (Yoshinobu, 2006). ARA is also beneficial to the elderly for improving the sleep and wake rhythm as indicated by animal experiments using aged animal models. Dietary supplementation of ARA can improve cognitive function in healthy elderly men (Ishikura *et al.*, 2009). Arachidonic acid and docosahexaenoic acid supplementation increased coronary flow velocity reserve in Japanese elderly individuals which suggests beneficial effects of these PUFAs on coronary microcirculation (Oe *et al*., 2008).

ARA in apoptosis and cancer

Finstad *et al.* (1994) reported that arachidonic acid and eicosapentaenoic acids have been reported to block proliferation of the promyelocytic leukemic HL-60 cells, and that this activity, a harbinger of both apoptosis and necrosis, was unrelated to oxygenase metabolism or lipid peroxidation. The protective effect of nonsteroidal anti-inflammatory drug (NSAID) usage on the incidence of colon carcinoma was attributable to accumulation of arachidonic acid, rather than to a reduction in prostaglandin levels (Chan *et al*., 1998). A potential mechanism relates to the activation of sphingomyelin hydrolysis by free arachidonic acid and the subsequent induction of apoptosis by ceramide (Jayadev *et al*., 1994). Treatments that block the cycling of arachidonic acid from phosphatidylcholine (PC) to phosphatidyl ethanolamine (PE) inhibit proliferation and drive the cells into apoptosis (Surette *et al*., 1996). In line with this observation it was reported that addition of exogenous arachidonic acid (10–100 μM) to the medium can prevent proliferation of the HL-60 cells (Brash, 2001).

ARA in aquaculture

Like other vertebrates fish also require ARA, EPA and DHA for normal growth, development and reproduction (Rodriguez *et al*., 2004). Ogata *et al.* (2005) suggested that ARA may have greater nutritional significance in relation to egg

development and larval growth in tropical/subtropical fishes. Fish oils supplemented with ARA oil from *M. alpina* showed significant rates on hatching of larvae (Harel *et al*., 2002). Dietary ARA significantly improved the recovery of guppies from parasite infection (Khozin-Goldberg *et al.,* 2006).

ARA supplementation in humans

Arachidonic acid supplementation in daily dosages of 1,000-1,500 mg for 50 days has been well tolerated in several clinical studies, with no significant side effects reported. All common markers of health including kidney and liver function (Wilborn *et al*., 2006), serum lipids (Nelson *et al*., 1997b), immunity (Kelley *et al*., 1998) and platelet aggregation (Nelson *et al*., 1997a) appear to be unaffected with this level and duration of use. Furthermore, higher concentrations of ARA in muscle tissue may be correlated with improved insulin sensitivity (Borkman *et al*., 1993). Abnormailities in ARA metabolism is linked to brain disorders like bipolar disorder, alzheimers disease, schizophrenia and ischemia. Arachidonic acid supplementation in healthy adults appears to offer no toxicity or significant safety risk. Comprehensive clinical studies have shown that dietary supplementation with marine oil or single-cell oil sources of DHA and ARA showed a beneficial improvement in visual function in formula-fed infants matching that of human breast-fed infants (Uauy *et al*., 2001). ARA may be of particular benefit to vegetarian breast feeding mothers (Shinmen, 1989). Nelson *et al.* (1997a) studied blood clotting parameters and *in vitro* platelet aggregation and suggested that moderate intakes of foods high in ARA have few effects on blood coagulation, platelet function, or platelet fatty acid composition and adding 1500 mg/d of dietary ARA for 50 days to a typical western diet containing about 200 mg of ARA produces no observable physiological changes in blood coagulation and thrombotic tendencies in healthy, adult males compared to the un-supplemented diet. Infants fed formula supplemented with 0.32% DHA and 0.64% ARA experienced a lower incidence of bronchiolitis compared with infants fed un-supplemented formula (Pastor *et al*., 2006). The safety of arachidonic acid supplementation in patients suffering from inflammatory or other diseased states is unknown and is not recommended. Johnson *et al*. (1985) reported that deficiency of ARA and other C20 PUFA have been implicated in cirrhosis with or without alcoholism.

Kotani *et al*. (2006) assessed the effect of ARA and DHA supplementation in human amnesic patients in a 90 days study. The results were suggestive of the fact

that ARA and DHA supplementation can improve the cognitive dysfunction due to organic brain damages or ageing.

ARA production processes

ARA rich oil has been the target for biotechnologists for many years and predates the recognition of ARA in neonate nutrition. First attempt for production was undertaken by Unilever (UK) and Lion Corp (Japan). In spite of the failure of these processes, these laid the groundwork for the future development in production of ARA rich single cell oil (SCO). *Mortierella alpina* was recognized as the most promising source of ARA and three processes utilizing this fungus is being undertaken- Suntory Co Ltd, Japan, Wuhan Alking Bioengineering Co Ltd, China and DSM food specialities, Italy (sold to USA under an exclusive contract with Martek biosciences). DSM process is the most significant among these which produces over 95% of ARA rich SCO annually (Wynn and Anderson, 2001). The process involves harvesting the biomass by centrifugation, drying, pelleting and extraction with hexane. Hexane is subsequently removed from the extracted oil, refined, bleached and deodourized to obtain a yellow coloured oil. Antioxidants such as Vitamin E are added during processing to ensure complete protection against oxidation.

Products

ARA together with DHA has been fortified in a number of term and preterm infant formulae like Earth's best organic infant formula (Earth's best, USA), Neocate infant DHA and ARA (Nutricia, North America), Enfamil (Mead Jhonson Nutritionals, Canada), Similac advance early shield (Abbott nutrition, USA), Nestle good start (Nestle, USA), Kirkland Signature (Kirkland, Washington) and Baby's only essentials (Nature's one, USA).

Arachidonic acid is marketed under patent (#6,841,573) as an anabolic bodybuilding supplement in products such as X-Factor (Molecular Nutrition), Halodrol Liquigels (Gaspari Nutrition), Animal Test (Universal Nutrition), Hemodraulix (Axis Labs), Mass Caps (IDS), Max Out (iForce) and Thermaphoria (EST Nutrition). Arachidonic acid was shown to improve peak muscle power, reduce systemic inflammation and produce statistically strong trends of improvements in muscle endurance, average power, and bench press 1-rep maximum lift, suggesting a definite role in muscle metabolism and performance.

In cosmetics and personal care products, arachidonic acid has been used in the formulation of face, body, and hand skin care products, moisturizers, wrinkle smoothers, conditioners, shampoos, styling gels, facial cleansers, sunscreen lotions as well as other skin care products. It is also widely employed in certain anti aging and hair loss treatment preparations. Few ARA and DHA fortified products are given in Fig 9.

Fig 9 Products fortified with ARA and DHA available in market

Source: NASA Scientific and technical information

Market potential

It is estimated that the total worldwide market for infant formula could be as high as $80 billion. Therefore, the current estimated world sales of formula of $4 billion represent only 5% of the total potential sales. If the market grow even close to $80 billion, it is likely to spur manufacturers to find better ways to simulate breast milk. ARA and DHA would have high market potential even if only 10% of all newborn babies were fed enriched infant formulas and even if 0.1% of the weight of the formula may be ARA/DHA (Ratledge, 2005). It is highly unlikely that the demand for both fatty acids will diminish and there are indications that he demand will continue to grow until there will be infant formulae produced that will be without both ARA and DHA. The overall activities in these areas to identify new and possibly

improved sources of DHA and ARA implies considerable economic potential and the lucrative nature of markets will continue to attract further interest from established biotechnological and pharmaceutical companies. SCOs have been successful because they are products that are not obtained from other sources and fulfills the primary demand for materials essential for the development and well being of infants as well as adults.

Single cell oils-a historical perspective

The current interest in the production and use of single cell oils comes from a long history of exploitation of microorganisms as sources of oils and fats. Transition of microbial oils from mere academic curiosities to major nutraceuticals for inclusion in infant formula is an overwhelming evidence of the dietary significance of the PUFAs coupled with the realization of lack of adequate or safe plant or animal sources (Ratledge, 2005).

Exploiting microbes as an alternative source of oils and fats for human consumption started in the early years of 20^{th} century when researchers employed *Endomyces vernalis,* a species of yeast and developed a small scale process for fat production (Woodbine, 1959). The problem arose when the oils and fats produced by oleaginous species of yeast and fungi were not significantly different from those obtained from plants and was not economically feasible. Inspite of the obvious economic limitations, considerable work for the production of microbial oils took place from 1920s to 1950s which laid the foundation to unravel the process of lipid accumulation in microorganisms. Researchers in the early 1960s focussed on identifying possible sources of arachidonic acid for use as chicken flavour material with the later realization that the chicken flavour was not due to ARA, but a totally unrelated compound. In spite of the setback, this work proved invaluable for identifying microorganisms that might be useful for production of various long chain PUFAs.

In the mid 1970s, GLA- the PUFA obtained from evening primrose commended a price of about $50 per Kg when most commodity plant seed oils were fetching less than a hundredth of this. With this scenario, the prospects of commercially viable SCO were recognized and the first SCO was produced using *Mucor circinelloides* grown in large scale fermentors of $220m^3$ capacity. This oil sold under the trade name 'Oil of Javanicus' or 'GLA-Forte' achieved limited penetration

in food supplement market. The fungal oil although superior to evening primrose oil in terms of GLA content and stability to oxidation was not widely accepted.

The arrival of first SCO though not successful, encouraged several companies to explore the possibilities of utilizing microbes as a source of more expensive oils and fats. A process related to GLA-SCO was developed in Japan using *Mortierella isabellina* and *Mortierella ramanniana* (Nakahara *et al*., 1992). In the early 1980s a possible production of cocoa butter equivalent (CBE) by yeasts were investigated and mutants of a yeast *Candida curvata/Cryptococcus curvatum* were isolated which had altered activities of Δ^9 desaturase and produced the same type of CBE without using the expensive inhibitor sterculic acid (Smit *et al*., 1992). In spite of achieving a good quality CBE that could be fortified to chocolate at the permitted levels, the yeast process was abandoned as not being sufficiently cost effective. In the 1980s importance of DHA and EPA in human nutrition was recognized and the high EPA content of fish oil which interfered with the efficacy of DHA uptake, led to a search for alternative sources of this fatty acid. A major breakthrough in this search was pioneered by the launch of Martek Ltd. that focused exclusively on developing a process using *Crypthecodinium cohnnii* as the organism of choice for DHA production. The production of DHA oil by Kyle and his colleagues was feasible and had a major impact in infant nutrition market. *M. alpina* as the microbial source for production of ARA was recognized later and microbial production of oils rich in this fatty acid is one of the main SCO in current production. The growing interest in the application of arachidonic acid (ARA) in various fields of health and dietary requirements has elicited much attention on the industrial production of ARA-containing oil by the cultivation of *Mortierella* (Higashiyama *et al*., 2002).

The genus *Mortierella*

Mortierella is the zygomycete genus with the largest number of validly described species. Species of *Mortierella* can be found on almost any substrate and are often encountered in soils as saprophytes. The undulate or wavy colony is readily observed on nutrient-rich media whereas sporulation can be induced only on a variety of nutrient-poor media. First described by Coemans (1863), over 70 species of *Mortierella* have been described to date. Important members of the genus include *Mortierella alliacea, M. alpina (M. renispora), M. polycephala, M. elongata, M. spinosa, M. gamsii, M. humilis, M. hyalina, M. hygrophila, M. isabellina,*

M. ramanniana, M. reticulata and M. umbellata. Genus *Mortierella* can be classified under subgenus *Micromucor* and *Mortierella* based on their fatty acid profile. Species of the subgenus *Micromucor* include *M. ramanniana* and produce C_{18} fatty acids GLA and ALA while those belonging to *Mortierella* subgenus accumulate C_{20} fatty acids including ARA and EPA.

***Mortierella alpina* Peyronel, 1913**

The taxonomical classification of *Mortierella alpina* is given

Biota

> Fungi (Kingdom)

> Zygomycota (Division)

> Zygomycetes (Class)

> Mortierellales (Order)

> Mortierellaceae (Family)

> *Mortierella* (Genus)

>*Mortierella alpina* (species)

Distribution of *Mortierella*

Mortierella alpina is a psychrophilic soil fungi distributed through out Australia, Canada, China, Corsica, Cyprus, Gibraltar, Hungary, India, Japan, Mexico, Netherlands, New Zealand, Pakistan, Spain, Sweden, UK, former USSR and USA. Species of *Mortierella* subgenus *Mortierella* especially *M. alpina, M. elongata*, *M. parvispora* are often reported from wet alpine and tundra habitats (Onipchenko, 2004). The distribution of *M. alpina, M. elongata, M. gamsii, and M. humilis* can hardly be interpreted (McLennan and Ducker, 1957).

Isolation of *Mortierella* and screening for ARA production

The growing interest in the application of arachidonic acid (ARA) in health and dietary requirements has elicited much attention on the industrial production of ARA-containing oil by cultivation of *Mortierella* fungi. For industrial production of ARA, various studies such as isolation of a high-potential strain and optimization of culture conditions have been conducted (Higashiyama *et al*., 2002).

Carreiro and Koske (1992) found that an isolation temperature of 0°C instead of 25°C selects representatives of *Mortierella* subgenus *Mortierella.* Eroshin *et al.* (1996) reported that ARA producing *Mortierella* strains are sensitive to aspirin in the growth medium and they proposed a screening procedure, utilizing aspirin, to distinguish ARA producing *Mortierella* strains from non producers after screening 87 *Mortierella* strains. Chen *et al.* (1997) isolated a strain of *M. alpina* with a dense-lobe rosette growth pattern on malt extract agar capable of producing 504 mg/L of ARA in the screening medium. Botha *et al*. (1999) used low temperature and malt extract agar to selectively isolate representatives of the genus *Mortierella* from soil. Aki *et al.* (2001) isolated a filamentous fungus YN-15 producing significant levels of arachidonic acid from a fresh water pond sample and assigned to the species *Mortierella alliacea.* Zhu *et al.* (2004) used low temperature of 4°C coupled with triphenyltetrazolium chloride (TTC) staining to isolate strains of *M. alpina* with high arachidonic acid yield. They isolated a strain M6 which showed highest staining degree and arachidonic acid content. Ho *et al.* (2007) obtained 25 isolates of *Mortierella* species of which *M. alpina* SC9 was selected as the best PUFA producer. SC9 was identified and confirmed as a new strain of *M. alpina* after comparison analysis of the sequences of internal transcribed spacers 1 and 2 (ITS1 and ITS2) and the 5.8S rDNA region. *Mortierella alpina* I_{49}-N_{18}, a high-yielding arachidonic acid producing strain, was screened by ion implantation (Yao *et al*., 2000).

Stredanska and Sajbidor (1992) tested fifteen strains of filamentous fungi from the culture collection of fungi (Charles University, Prague) for lipid production and fatty acid accumulation. Among these fungi, the mycelium of *Cunninghamella elegans*, *Rhizopus arrhizus, Mortierella parvispora, M. elongata* and *M. alpina* contained arachidonic acid (ARA) in the range of 2.3-33.5% of the total fatty acids. Jang *et al.* (2005) investigated the effect of culture variables to improve PUFA production by *Mortierella*. Among the 11 tested microbes highest ARA yield was obtained for *M. alpina* ATCC 32222. Li *et al*. (2009) screened several *M. alpina* cultures for their capacities to produce ARA and found that a strain of *M. alpina* was found to show the highest productivity when cultivated in a soya flour supplemented medium. They further prepared a concentrate of 5% w/w arachidonic acid from the recovered mycelium.

Optimization of culture variables

In developing a biotechnology-based industrial process for single cell oil, formulating a suitable fermentation media is of crucial importance. The fermentation medium affects the product yield and volumetric productivity and hence media optimization can lead to significant increase in yield of the desired PUFA. Several studies have been carried out for optimizing the culture variables for maximizing ARA production in *M. alpina.*

Effect of carbon and nitrogen source

Many *Mortierella* species are able to convert diverse carbon sources into lipids and are thus known as oleaginous fungi (Weber and Tribe, 2003). When different compounds were tested as carbon source in glucose-yeast extract (GY) medium, growth of ATCC 16266 was found to be very poor with lactose, starch, and sucrose as carbon source, moderate with maltose, fructose and glucose and very good with linseed oil and glycerol (Bajpai *et al*., 1991b). They further concluded that of various nitrogen sources added separately at a 1% concentration to the medium containing 10% glucose, yeast extract followed by peptone resulted in the highest arachidonic acid yields. Bajpai *et al*. (1991c) reported that the highest yield of arachidonic acid per litre of culture broth in *M. alpina* ATCC 32222 was observed when glucose was used as the carbon source at 100 g/L level. Sajbidor *et al*. (1994) cultivated *Mortierella alpina* S-17 in semisynthetic liquid medium with 3% glucose as the carbon source at 28°C for 14 days to yield 2.1 g/L of intracellular lipid rich in ARA. Bao *et al*. (1997) investigated the effects of carbon and nitrogen sources on the production of arachidonic acid by *Mortierella* sp. M10. They achieved an ARA yield of 0.827 g/L when cultivated under the optimum culture conditions. Koike *et al*. (2001) investigated the influence of consumed carbon to nitrogen (C/N) ratio on arachidonic acid (ARA) production and mycelial morphology in cultures of *Mortierella alpina* using shake flasks and a fermentor. They concluded that from the viewpoint of ARA production, the optimum C/N ratio was in the range of 15 to 20 with a balance between the amounts of carbon and nitrogen sources.

Aki *et al*. (2001) used 12% glucose and 3% yeast extract in a 50L fermentor and found that the strain *Mortierella alliaceae* YN-15 is a promising fungal isolate for industrial production of ARA and other PUFAs. In order to optimize the culture conditions, Yuan *et al*. (2002) studied the effects of carbon and nitrogen sources.

Different carbon sources at mass concentration of 6.0% were tested and growth of the strain was good with glucose, moderate with starch and maltose and low with sucrose and glycerol. The nitrogen sources used were peptone, beef extract, yeast extract, peanut cake, potassium nitrate, amine sulphate, sodium nitrate and yeast powder. Results were suggestive of the fact that yeast extract was the most efficient nitrogen source, which resulted in the highest ARA content.

Zhu *et al*. (2003) employed an inexpensive medium for production of arachidonic acid by *Mortierella alpina.* Glucose derived from maize starch hydrolysate was used as the sole carbon source and defatted soybean meal and sodium nitrate were the nitrogen sources. When glucose at 100g/L level was used an optimal ARA yield of 1.47 g/L was observed. Jang *et al*. (2005) reported that in *M. alpina* ATCC 32222, soluble starch at 10% and the mixture of KNO_3 and yeast extract at 2:1 (w/w) was the best carbon and nitrogen sources for arachidonic acid and total PUFAs production. The optimal C/N ratio ranged from 5.1 to 9.0 and each gram of substrate carbon produced 110.3 mg of arachidonic acid.

Hou (2008) screened 12 *Mortierella* species for ARA and dihomo-γ-linolenic acid (DGLA) production from glycerol. Results indicated that all species tested grew on glycerol and produced ARA and DGLA except *M. nantahalensis* NRRL 5216, which could not grow on glycerol. The amount of ARA and DGLA produced by the highest ARA producers *M. alpina* and *M. zychae* were comparable with those obtained with glucose-grown mycelia.

Chen *et al.* (1997) exploited the interactions between the constituents of the medium by response surface methodology (RSM) for optimizing arachidonic acid production by the Wuji-H4 isolate. Higashiyama *et al.* (2002) combined the results derived from various studies and attained a high ARA yield in an industrial fermentor.

Effect of media pH

Bajpai *et al*. (1991c) reported that the optimum initial culture pH for arachidonic acid production in *M. alpina* ATCC 32222 was in the range 6.0-6.7. Strain ATCC 16266 grew well in the pH range of 3.8 to 8.0. The highest lipid content of biomass, degree of lipid unsaturation and arachidonic acid yield was attained at an initial pH 6.0 (Bajpai *et al*., 1991b).

Effect of incubation temperature

Shimizu *et al.* (1988a) reported that several filamentous fungi belonging to the genus *Mortierella* accumulated large amounts of 5, 8, 11, 14, 17-cis-eicosapentaenoic

acid (EPA) in their mycelia only when grown at low temperature (12°C). They postulated that this unique phenomenon is due to activation of enzymes involved in EPA formation at low temperature. Shimizu *et al.* (1989b) reported production of EPA in *M. alpina* IS-4 when grown at low temperature in a medium containing linseed oil. They suggested that this phenomenon might be due to low temperature-dependent production of EPA from arachidonic acid formed through the n-6 route coupled with conversion of α-linolenic acid in the added linseed oil to EPA through the n-3 route.

Bajpai *et al*. (1991b) reported that *Mortierella alpina* ATCC 32221 did not produce arachidonic acid at 25°C. Bajpai *et al*. (1991c) observed that *Mortierella alpina* ATCC 32222 grew well at 11°C, as well as at 25°C in a liquid medium containing glucose or linseed oil and yeast extract and demonstrated a high EPA yield at 11°C. At 25°C, EPA was not produced unless linseed oil was supplemented. When grown at 11°C for 10 days in a medium containing 2% linseed oil as carbon source, the mycelium yielded 435 mg/L EPA.

Lindberg and Molin (1993) investigated the effect of temperature on *Mortierella alpina* CBS 343.66 in order to optimize the culture conditions for rapid biomass, lipid and ARA production. They achieved high biomass, lipid and ARA production at 25°C and on decreasing the temperature to 18°C, the fraction of unsaturated fatty acids in lipids increased considerably. An increase in ARA content was observed in *M. alpina* cultures after five days of incubation, with a shift in culture temperature from 20°C to 12°C (Jang *et al*., 2000).

Effect on morphology

In *Mortierella,* studies including the investigation of morphology are important because ARA is accumulated in the mycelia and thus cultivation with high biomass concentration is essential for obtaining a high ARA yield (Higashiyama *et al*., 2002). The influence of consumed carbon to nitrogen (C/N) ratio of 5-32 on mycelial morphology was investigated in cultures of *M. alpina* using shake flasks (Park *et al*., 2001). Results indicated that the area ratio of filamentous mycelia to total mycelia was found to be independent of the consumed C/N ratio. It was clear from their studies that fungal morphology and mycelial size were affected by the ratio of carbon to nitrogen.

Park *et al*. (2002) studied the morphological parameters, such as hyphal growth rate, tip formation rate, tip extension rate and branch formation rate of *M. alpina* using a flow-through chamber under 25 different combinations of carbon and nitrogen concentrations. Their studies indicated that morphological parameters were influenced not by C/N ratio but by carbon concentration in the medium. *Mortierella alpina* was incubated aerobically for two weeks in a medium composed of urea, dextrose and various minerals including KH_2PO_4, $MgSO_4$ $7H_2O$ and $CaCl_2$ $2H_2O$ (Totani *et al*., 2002). Under mild agitation conditions the fungus grew in pulpy form; however, magnesium sulfate pelleted the fungus in the urea medium when potassium dihydrogen phosphate was present in a $KH_2PO_4/MgSO_4$ $7H_2O$ weight ratio below 1. ARA content was higher in fatty acids of the pellet, even though the overall level of fatty acids was lower.

Park *et al*. (2006) quantified morphological parameters of 11 *Mortierella sps*. and investigated the relationship between micro and macromorphology using a flow-through chamber coupled with image analysis. By observing micromorphology in a flow-through chamber, fungi were divided into 3 groups classified according to morphological parameters. Their study arrived at the conclusion that a micromorphology consisting of a high hyphal growth rate, low tip extension rate and high branch formation rate forms the suitable macromorphology for ARA production. Koizumi *et al*. (2006) investigated the effects of amino acid on morphological development and nucleus formation of *Mortierella alpina* CBS 754· 68 using flow-through chamber. Results indicated that when alanine and valine containing media were used, the hyphal growth units were 2·4-fold higher than that in the nutrient-rich medium, indicating that these amino acids stimulate the elongation of hypha.

Effect of culture period

Bajpai *et al*. (1991a) reported that in *M. alpina* ATCC 32222, production of biomass reached a plateau after 3 days incubation at 25°C, while the percentage of arachidonic acid in lipids and biomass increased dramatically from 3 to 6 days with a concurrent increase in ARA yield from 0.89 to 1.63 g/l. Yuan *et al*. (2002) studied the effect of culture period on biomass, lipid and ARA production in *M. alpina*. It was observed that the biomass continuously increased during the first six days and slightly declined there after. The highest content of the lipids in biomass was observed at the 6th day, but then it decreased intensely. The content of ARA in lipids increased in a

time-dependent manner, reaching a peak at the 11th day, however, the highest ARA content appeared at the 6th day.

Certik and Shimizu (2000) investigated kinetic analysis of ARA biosynthesis by *Mortierella alpina* 1S-4 growing under lipid-accumulating and non-lipid-accumulating conditions. Kinetic parameters of these cultivation processes demonstrated a characteristic pattern of lipogenesis in this fungus, with distinct growth, oil accumulation and ARA synthesis phases. Their work indicated that ARA was rapidly incorporated into triacylglycerols at the later cultivation phase and overall ARA yield was directly related to the total yield of fatty acid.

Eroshin *et al*. (2002) reported that *Mortierella alpina* LPM 301, a producer of arachidonic acid (ARA), possessed a unique property of intense lipid synthesis in the period of active mycelial growth. Under batch cultivation of this strain in glucose-containing media with potassium nitrate or urea, 28-35% lipids was produced at the end of the exponential growth phase and remained almost unaltered in the stationary phase. Hou (2008) who worked on 12 screened *Mortierella* species found that time course for maximum cell growth and ARA production for *M. alpina* and *M. zychae* were at 6 days of incubation.

Effect of mycelial ageing

Bajpai *et al*. (1991b) reported that arachidonic acid in biomass increased considerably when stored at 22°C for 1 week. Jin *et al*. (2009) employed improved mycelium ageing technology as a strategy for enhancing ARA production in *M. alpina* ME-1 and obtained 1.55 times higher yield than that of traditional ageing technology.

Effect of minerals

Higashiyama *et al*. (1998) evaluated the effect of mineral addition on ARA production by *M. alpina* IS-4. They concluded that 1.5% soy flour medium supplemented with 0.3% KH_2PO_4, 0.1% Na_2SO_4, 0.05% $CaCl_2{\cdot}2H_2O$ and 0.05% $MgCl_2{\cdot}6H_2O$ was found to enhance the ARA yield 1.7-fold over that of control. They further verified that the above-mentioned increase in the ARA yield was due to the minerals themselves, not a pH buffer effect.

Effect of oil supplementation

Jang *et al.* (2000) reported a hike in ARA production on supplementation with linseed and soybean oils. A similar increase in PUFA production was reported by Jang *et al*. (2005) on linseed oil supplementation at 1% level.

Effect of glutamate supplementation

Lan *et al*. (2002) evaluated the effect of glutamate on arachidonic acid production in *Mortierella alpina.* Results suggested that biomass and arachidonic acid production were higher in the glutamate supplemented culture than those of control. Glutamate addition enhanced the activity of G6PDH thereby activating the pentose phosphate pathway leading to a higher ARA production. Yu *et al.* (2003) investigated the effect of various concentrations of glutamate on ARA production from *M. alpina* in shake flask culture. Their results indicated that glutamate supplementation promoted *Mortierella* growth, accelerated substrate metabolism, increased ARA production and supplementation of 0.8 g/L glutamate resulted in maximum ARA yield.

Effect of inhibitors

Shimizu *et al.* (1989) reported that *M. alpina* grown in liquid medium supplemented with sesame oil has reduced arachidonate and enriched dihomo-γ-linolenate contents. Sesamin (a lignan present in sesame oil) specifically inhibit Δ^5 desaturation and the formation of arachidonic acid from dihomo-γ-linolenic acid (Shimizu *et al*., 1991). Kawashima *et al*. (1996) studied the inhibitory effects of alkyl gallate and its derivatives on fatty acid desaturation in *Mortierella alpina* 1S-4. Kinetic analyses revealed that propyl gallate, a noncompetitive inhibitor of Δ^5 and Δ^6 desaturases are different from known natural inhibitors, i.e., sesamin and curcumin.

Solid state fermentation

Jang *et al.* (2000) investigated PUFA production with *Mortierella alpina* by solid substrate fermentation. Rice bran proved to be the most effective substrate for PUFA production, followed by peanut meal residue, wheat bran and sweet potato residue and each gram of substrate carbon yielded 54.5 mg of arachidonic acid after 8-12 days of incubation. Jang and Yang (2008) used a solid-state column reactor of rice bran with *Mortierella alpina* to investigate the potential production of polyunsaturated fatty acids (PUFAs). When incubated for 20°C and 12°C for 5 and 7

days respectively, each gram of substrate carbon yielded 127 mg of total PUFAs which include 6 mg ARA, 12 mg EPA, 5 mg ALA and 117 mg LA. Certik *et al*. (2008) tested *M. alpina* for their ability to utilize solid substrates during solid state fermentations and the cereal materials were effectively enriched with PUFAs.

Bioconversion

Dong and Walker (2008a) investigated canola materials including processed flake and cake as both carbon and nitrogen sources for the production of new oil containing PUFAs. They used *M. alpina* to produce the modified oil enriched with GLA, ARA and EPA. It was further noted that the mixed culture of two strains, *M. alpina* and *Pythium irregulare*, resulted in higher production of ARA and EPA than their single cultures. A method was developed to estimate the bioconversion rate from digested canola oil to fungal oil, which was nearly 50% when canola flake and cake were utilized as substrate. Dong and Walker (2008b) further used supercritical CO_2 extraction for lipid recovery from fermented canola flake and modelled the extraction kinetics of the process. The feasibility for production of PUFAs in a laboratory-scale fermentor using canola flake as a single nutrient, and for lipid extraction using supercritical CO_2 was demonstrated.

Regulation of lipogenesis

Wynn and Ratledge (2000) examined the effects of lipid based carbon sources on repression of fatty acid biosynthesis and/or fatty acid desaturation and elongation. Their results were an attestation of the fact that in *Mortierella alpina* fatty acid synthesis is regulated separately from fatty acid desaturation/elongation and that the latter reactions are not repressed by growth of the fungus on simple fatty acids. Furthermore, the data implicate the elongation of 18:3 (n-6) to 20:3 (n-6) as the rate limiting step in arachidonic acid biosynthesis by *M. alpina*.

Extraction of *M. alpina* single cell oil

The biomass, oil content and fatty acid content of oil are important parameters for the development of any successful single cell oil production process. As the fungal cultures have a tough cell wall there is a need for standardization of extraction method for total lipid recovery. A wide range of extraction procedures have been used for lipid extraction from *Mortierella.*

Bajpai *et al*. (1991a) extracted lipids from the dried *M. alpina* biomass by the method of Bligh and Dyer (1959). Tough *et al*. (2000) compared a low cost single

stage laboratory process combining fungal dehydration and lipid extraction with a traditional two stage method employing freeze drying and subsequent mechanical disruption with solvent in *Mortierella alpina*. Zhu *et al.* (2002) compared extraction of lipids from wet and dry *M. alpina* biomass and found that lipid yield of extraction from dry cells was higher than that of extraction from wet cells mainly due to non extraction of membrane lipids.

Purification studies

Sajbidor *et al*. (1994) cultivated *Mortierella alpina* S-17 in shake flasks in the semisynthetic liquid medium and obtained high pure methyl arachidonate preparation. Masahiro *et al*. (1998) purified arachidonic acid by $AgNO_3$ solution from pig liver and spleen. Giminez *et al.* (1998) purified arachidonic acid from the microalga *Porphyridium cruentum* by a three step process and obtained 81.4% pure ARA fraction. Enrichment of ARA from the fungal lipids by a urea inclusion method was investigated by Zhu *et al.* (2002) and 93.0% of the saturated and 84.6% monounsaturated fatty acids were removed by forming urea inclusion compounds.

Asao *et al*. (2005) attempted purification of ARA from *Mortierella alpina* single-cell oil by selective esterification with *Burkholderia cepacia* lipase. They screened industrially available lipases and arrived at the conclusion that lipase derived from *Burkholderia* had the highest activity on ARA. Hamam and Shahidi (2004) enzymatically modified ARA single-cell oil by incorporating capric acid with the aid of lipase PS-30 from *Pseudomonas sp.* Examination of positional distribution of fatty acid on the glycerol backbone of modified ARA single-cell oil revealed that ARA was mainly located at the sn-2 position of the modified ARA single-cell oil.

Zhang *et al*. (2006b) devised a new method to obtain high-purity arachidonic acid (ARA), from fungal single-cell oil *via* Al_2O_3-supported $CuSO_4$ column chromatography. ARA with 90.8% purity and a recovery of 46.5% was obtained by this method. Yuan *et al*. (2007) separated and purified ARA from microbial lipids by the combined method of urea inclusion reaction and reversed-phase high performance liquid chromatography. They used methanol as the solvent, free fatty acid to urea to methanol ratio of 1:2:8 (w/w), and crystallization temperature of $^{-}10°C$ to achieve ARA with 99% purity.

Mutation and Strain improvement

The history of microbial strain improvement shows that all industrially used microbial processes are run with species whose over producing properties have been known for several years. Mutation has enabled the production of tailor made PUFAs in the genus *Mortierella*. Fungal desaturase mutants with unique enzyme systems are useful not only for the regulation and over-production of valuable PUFAs, but also as excellent models for the elucidation of fungal lipogenesis (Certik *et al*., 1998).

A wide variety of mutants defective in desaturases (Δ^9, Δ^{12}, Δ^6, Δ^5 and ω3) or elongase (EL1) or ones with enhanced desaturase activities (Δ^6 and Δ^5) have been derived from *M. alpina* IS-4 by treating the parental spores with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Jareonkitmongkol *et al*., 1992b). These mutants are valuable not only as producers of novel or existing PUFAs but also for providing valuable information on PUFA biosynthesis in this fungus (Certik *et al*., 1998).

Jareonkitmongkol *et al.* (1992a) described Δ^{12} desaturase defective mutants which accumulate high levels of n-9 PUFAs such as oleic acid (18:1 n-9), octadecadienoic acid (18:2 n-9), eicosadienoic acid (20:2 n-9) and mead acid in their mycelia. Jareonkitmongkol *et al.* (1993a) described a novel Δ^5-desaturase-defective mutant derived from *Mortierella alpina* 1S-4 which produced only a trace (about 1%) amount of arachidonic acid. The ratio of dihomo-γ-linolenic acid (DGLA) to total fatty acids in each lipid class was markedly high for the mutant which produced 2.4g/L of DGLA under submerged culture conditions. Production of DGLA by this mutant is advantageous since it does not require inhibitors and the yield is relatively high.

Mutants synthesizing a high level of linoleic acid and low concentrations of GLA, DGLA and ARA are considered to be defective in Δ^6 desaturase and are characterized by accumulation of an eicosadienoic acid (20:2 n-6) and eicosatrienoic acid (20:3 n-6) (Jareonkitmongkol *et al*., 1993b). Mutants unable to synthesize n-3 PUFAs when grown at low temperature (<20°C) accumulate relatively higher content of ARA (Jareonkitmongkol *et al*., 1994). These mutants are superior to the wild strain when relatively higher content of ARA in SCO is required.

Kawashima *et al.* (1997) isolated a mutant 209-7 with an elevated Δ^6 desatuase activity from a Δ^{12} desatuarse defective mutant Mut 48. Kamada *et al.*

(1999) described production of 8, 11- *cis* -eicosadienoic acid by a Δ^5 and Δ^{12} desaturase-defective mutant derived *Mortierella alpina* 1S-4. One of the mutants, M226-9, when grown in a medium containing 4% glucose and 1% yeast extract produced 1.68 mg/mL of 20:2 n-9 per mL of culture medium and no 5, 8, 11-*cis*-eicosatrienoic acid (20:3n-9), which indicate that Δ^5 and Δ^{12} desaturases are perfectly blocked. A novel *Mortierella alpina* mutant JT-180 derived from the Δ^{12} desaturase-defective Mut48 strain produced greater amounts of 5, 8, 11-eicosatrienoic acid (mead acid) compared to its parent strain (Sakuradani *et al*., 2002). Under optimal conditions, mead acid production by JT-180 reached 1.92 g/L culture medium in a 10L jar fermentor. A mutant considered to be defective in Δ^9 desaturation of an arachidonic acid-producing fungus, *Mortierella alpina* IS-4 was isolated after treating the wild-type spores with N-methyl-N'-nitro-N-nitrosoguanidine. Steraic acid is the main fatty acid in the oil produced by these mutants (Jareonkitmongkol *et al*., 2002).

Sakuradani *et al*. (2004a) isolated five mutants Y11, Y135, Y164, Y180 and Y61 capable of accumulating higher amounts of ARA than *Mortierella alpina* 1S-4, an industrial strain. A mutant KY1 derived from *M. alpina* IS-4 which accumulated 30% diacylglycerol in the total lipid was described by Sakuradani *et al*. (2004b). This mutant may be defective in an acyltransferase involved in the conversion of diacylglycerols to triaclyglycerols. Zhang *et al*. (2006a) identified three unusual odd-chain fatty acyl residues 6, 9-heptadecadienoic acid (17:2), 8, 11 -nonadecadienoic acid (19:2) and 5, 8, 11-nonadecatrienoic acid (19:3) on cultivating the Δ^{12} defective mutant JT-180 in a media with 3% n-heptadecane. Furthermore, various mutants derived from *M. alpina* 1S-4 have led to the production of oils containing *n*-1, *n*-3, *n*-4, *n*-6, *n*-7 and *n*-9 PUFAs (Sakuradani *et al*., 2009a) (Fig.10). In another species (*M. isabellina*), Yu and Li (2009) isolated a new mutant with higher growth rate and high capability of producing arachidonic acid after protoplast ultraviolet mutation.

Abe *et al*. (2005) identified the mutation sites on Δ^5 desaturase genes in Δ^5 desaturase-defective mutants of *Mortierella alpina* 1S-4. The mutations resulted in an amino acid replacement and uncorrected transcription caused by recognition of an AG-terminal newly created on C207A gene mutation, resulting in low or no Δ^5 desaturase activity in these mutants. Sakuradani *et al*. (2009b) identified the mutation sites on ω3 desaturase genes in two ω3 desaturase-defective mutants derived from

Mortierella alpina 1S-4. They concluded that the lack of ω3 desaturase activity in these mutants is attributed to an amino acid replacement.

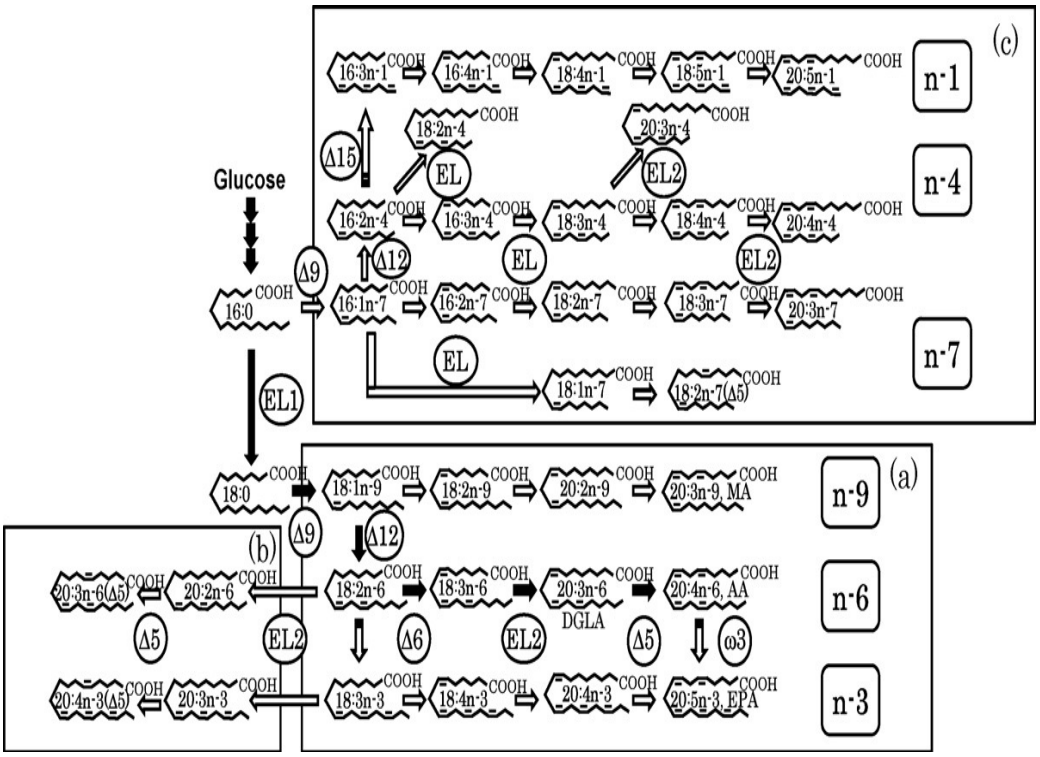


Fig 10 Pathways for the biosynthesis of PUFAs in *M. alpina* 1S-4 and its mutants

Source: Sakuradani and Shimizu (2009)

Subsequently the mutation sites on the Δ^{12} desaturase gene in *Mortierella alpina* Δ^{12} desaturase-defective mead acid accumulating mutants SR88, TM912, and Mut48 were identified (Sakuradani *et al.,* 2009a). Sakuradani *et al*. (2009c) employed methods from metabolic engineering and molecular biology for controlling *M. alpina* IS-4 cultures and breeding mutant strains. These parental and mutant strains are now used for large-scale production of a variety of PUFAs.

Toxicological studies and safety evaluation

M. alpina is a common soil fungus, to which humans are frequently exposed. Streekstra (1997) reviewed the safety of *M. alpina* for the production of food ingredients, such as arachidonic acid. They opined that the production strains, CBS 168.95 and CBS 169.95 in particular should be considered safe for the submerged

production of food ingredients since they were non pathogenic and nonallergenic. Absence of any mycotoxins produced further warranted the safety of all *Mortierella* species except *M. wolfii*.

Hempenius *et al.* (1997) carried out a preliminary safety assessment of ARA enriched oil from *M. alpina* for use in infant nutrition. HPLC analysis revealed that no mycotoxins were produced by the production strains under the conditions tested and the oil did not demonstrate any mutagenic or clastogenic activity. The sub-acute oral toxicity studies did not reveal any obvious signs of toxicity and the intake of 1000 mg ARA/kg body weight/day did not manifest any adverse effects.

Boswell *et al.* (1996) carried out the preclinical evaluation of triglyceride oils, highly enriched in ARA and DHA, by acute and subchronic gavage feedings in Sprague-Dawley rats. The results of their study indicated no significant differences in survival, clinical signs, body weight gain, food consumption, haematology, clinical chemistry and histopathological evaluations in animals administered ARASCO or DHASCO compared with that in control animals administered equal amounts of high oleic sunflower oil. The increased DHA and ARA levels in heart and liver tissues in the treated animals verified the bioavailability of these fatty acids.

ARA-oil derived from *Mortierella alpina* was further tested in a subchronic (13-week) oral toxicity study in rats, preceded by an *in utero* exposure phase (Hempenius *et al.*, 2000). There were no treatment-related effects observed up to dietary test substance concentrations of 15,000 ppm. Administration of the test substances from 4 weeks prior to mating, throughout mating, gestation, lactation of parental (F_0) animals and weaning of the F_1 pups did not affect fertility, reproductive performance, general condition of pups, viability, sex ratio or number of pups. Sub-chronic study revealed that survival, clinical signs, body weight gain and food consumption were not adversely affected by ARA oil and the minor statistically significant effects found in the high dosage groups are attributed to the intake of high-fat diet, rather than specific effects of the ARA-oil.

A developmental toxicity study was undertaken by Arterburn *et al.* (2000a) to test for potential teratogenic activity of ARA and DHA single cell oils to ensure their safe use during pregnancy. Treatment with these oils did not produce overt maternal toxicity, nor result in changes in pre or postimplantation losses, resorptions, live births or sex ratios. No foetal malformations were recorded in the treatment groups and they

concluded that these oils are not teratogenic at doses that represent a 100-fold safety factor over expected usage levels.

Microbially-derived triglycerides rich in ARA and DHA were tested for mutagenic activity in three different *in vitro* mutagenesis assays (Arturburn *et al*., 2000b). The oils were not mutagenic as per Ames reverse mutation and mouse lymphoma $TK^{+/-}$ forward mutation assays. No clastogenic activity was noticed in chromosomal aberration assays performed with Chinese hamster ovary cells and based on these assays it was concluded that the oils had no genotoxic potential.

Huang *et al*. (2002) used ARA and DHA oils in bovine- milk based formulae and fed newborn domestic piglets for a period of 30 days. They concluded that single-cell oils in formula consumed for 1 month in amounts up to 16-fold greater than that proposed for human infants did not result in any significant variation in clinical chemistry or histopathology indicative of toxic effects in neonatal pigs.

Merritt *et al*. (2003) evaluated the safety of ingredient sources of ARA and DHA for use in infant formulas in a neonatal piglet model. No test article related effects indicative of an adverse health consequence to animals was manifested in the clinical signs, body weights, food consumption, clinical chemistry, hematology, organ weights or histopathology. Their findings in this neonatal animal study supported the safety of these ingredient oil sources of DHA and ARA for use in infant formulae.

Casterton *et al.* (2009) evaluated the safety of refined arachidonic acid-rich oil (RAO) with genotoxicity studies coupled to 90-day feeding study with *in utero* exposure in Wistar rats. Their study concluded that few statistically significant effects noticed for selected histopathology, clinical chemistry and organ weight endpoints were not attributed to treatment for one or more reasons. The negative results of the genotoxicity studies further established a strong body of evidence for the safety of this RAO.

Clinical trials

A large number of clinical studies with pre-term and term infants have been undertaken with infant formula supplemented with ARA and DHA at levels approximating those found in human milk. These studies established that formula supplemented with ARA and DHA is well tolerated by human infants and is not

associated with any apparent adverse effects viz., reduced growth, changes in serological markers of spleen and liver function.

Innis *et al*. (2002) carried aout a clinical trial to determine the effect of ARA and DHA supplementation on growth and visual acuity of formula-fed premature infants. They concluded that feeding DHA+ARA from single-cell triglycerides enhances weight gain in formula-fed premature infants with no evidence of adverse effects.

Clandinin *et al*. (2005) evaluated the safety and benefits of feeding preterm infants formulas containing DHA and ARA until 92 weeks postmenstrual age (PMA), with follow-up to 118 weeks PMA. Results of their study arrived at the conclusion that infants fed the supplemented formula did not manifest any adverse events and had higher Bayley mental and psychomotor development scores at 118 weeks.

Molecular studies

Transformation

Mackenzie *et al*. (2000) isolated and used a homologous histone H4 promoter and aribosomal DNA region in a transformation vector for *M. alpina*. Qi *et al*. (2004) transformed *Arabidopsis thaliana* sequentially with genes encoding a Δ^9 specific elongating activity from *Isochrysis galbana*, a Δ^8 desaturase from *Euglena gracilis* and a Δ^5 desaturase from *Mortierella alpina* to produce substantial quantities of ARA and EPA in the higher plant. Takeno *et al*. (2004b) established a transformation system for *M.alpina* using a ura5 mutant lacking orate phosphoribosyl transferase activity. Takeno *et al*. (2004a) cloned and sequenced the ura3 and ura5 genes and isolated uracil auxotrophs in *M. alpina* IS-4. Takeno *et al.* (2005b) transformed *Mortierella alpina* IS-4 with zeocin and suggested that preincubation period and temperature had a great influence on transformation efficiency. Ando *et al*. (2009) established an agrobacterium tumefaciens-mediated transformation of *Mortierella alpina* IS-4 using the uracil auxotrophic mutant (*ura5*$^-$ strain) for its application in eicosapentaenoic acid-producer breeding. Ho and Chen (2008) sequenced and investigated a total of 44 18S-28S ribosomal gene internal transcribed spacer regions of *M. alpina* from three diverse locations. The sequences between *M. alpina* and the three closely related species (*Mortierella macrocystis, Mortierella gamsii and Mortierella humilis*) showed 74–84% sequence identity and the contructed

phylogenetic tree showed four clades of *M. alpina* isolates clearly distinct with high bootstrap values.

Cloning and heterologous expression

Knutzon *et al.* (1998) identified Δ^5 desaturase gene from *M.alpina* by heterologous expression in bakers yeast and canola. They also evaluated the effects of growth and induction conditions as well as host strain on activity of the recombinant Δ5-desaturase in *S. cerevisiae*. Expression of the *M. alpina* Δ^5-desaturase cDNA in transgenic canola seeds resulted in the production of taxoleic acid ($\Delta^{5,9}$ 18:2) and pinolenic acid ($\Delta^{5,9,12}$ 18:3). Sakuradani *et al*. (1999a) identified an NADH-Cytochrome b_5 (CbR) from *M. alpina* which had similarity to yeast, bovine, human and rat CbRs. Sakuradani *et al*. (1999b) cloned a DNA fragment from *Mortierella alpina* 1S-4 after PCR amplification with oligonucleotide primers designed based on the sequence information for Δ^6-desaturase genes from borage and *Caenorhabditis elegans*. This fragment was used as a probe to isolate a cDNA clone with an open reading frame encoding 457 amino acids from a *M. alpina* 1S-4 library, expressed under the control of the *amyB* promoter in a filamentous fungus *Aspergillus oryzae*, resulting in the accumulation of γ-linolenic acid. Parker-Barnes *et al*. (1999) identified and characterized an enzyme (GLELOp) involved in the elongation of n-6 and n-3 polyunsaturated fatty acids from *M.alpina.* Coexpression of *GLELO* cDNA with *M. alpina* Δ^5-desaturase cDNA in yeast resulted in the conversion of GLA to arachidonic acid (20:4n-6) and stearidonic acid to eicosopentaenoic acid (20:5n-3). They concluded that *GLELO* gene may play a critical role in the bio-production of both n-6 and n-3 polyunsaturated fatty acids. Wongwathanarat *et al*. (1999) isolated genes encoding to distinct Δ^9 fatty acid desaturases from *M. alpina*. Sakuradani *et al.* (1999) cloned Δ^9 desaturase gene from *M. alpina* IS-4 with heterologous expression in the fungus *Aspergillus.*

Liu *et al*. (2001) cloned Δ^6 fatty acid desaturase gene from *M. alpina* ATCC 16266 in *E. coli* shuttle vector pYES2.0 which was introduced into *Saccaromyces cerevisiae.* When linoleic acid was provided as an exogenous substrate to the yeast cultures expressing Δ^6 fatty acid desaturase activity under appropriate media and temperature conditions, the level of gamma-linolenic acid reached 31.6% of the total yeast fatty acids. Sakuradani and Shimizu (2003) demonstrated that there are two Δ^6 desaturases that are involved in desaturation of linoleic acid to γ-linolenic

acid designated as Δ^6I and Δ^6II in *M. alpina.* Chen *et al*. (2005) isolated Δ^6 fatty acid desaturase from *M. alpina* and expressed in *Lotus japonicas* and *Vigna angularis* resulting in the production of γ-linolenic acid. Sakuradani *et al*. (2005) identified a novel ω-3 desaturase with wide substrate specificity which effectively converted ARA to EPA when expressed in yeast. Chen *et al*. (2006) expressed Δ^6, Δ^5 and elongase genes from *M. alpina* for production of ARA in *Glycine max*.

1.1 Introduction

Fermentation of microorganisms aimed at the production of several value added metabolites such as single-cell oils, organic acids and lipases holds a noticeable interest in industrial microbiology and biotechnology sector. The media optimization stage is crucial when filamentous fungi are used in single cell oil (SCO) production on commercial basis. Optimization of culture medium plays a fundamental role in preventing metabolic deviations that directly affect yield of a metabolite. Optimized media formulations dramatically and positively affect media costs, process productivity and capital investments and due to these reasons even reengineering of established processes can be justified. Media optimization has potential to create higher-quality products and a more robust process for pharmaceutically and nutraceutically relevant SCOs.

Different strategies can be used for the optimization of cultivation conditions (Mao *et al*., 2007; Farid *et al*., 2000). Conventional "one-variable-at-a-time" approach is often used, but it is time-consuming and leads to confusion in understanding the process parameters (Kumar and Satyanarayana, 2007). Some statistical techniques, such as Plackett-Burman design and response surface methodology (RSM), proved to be useful for developing, improving and optimizing processes and are extensively used in formulation of culture medium for bacteria and fungi (Didier *et al*., 2007).

The efficiency of oil biosynthesis by fungi and its composition depend on the genetic properties of the strains, cultivation conditions and composition of culture medium. It is well established that media components play an imperative role in enhancing arachidonic acid accumulation and manipulation of these components is the simplest and most effective way towards increased productivity. ARA has been produced by submerged fermentation of *Mortierella alpina* in media containing various types of carbon and nitrogen sources (Zhu *et al*., 2003; Jang *et al*., 2005).

The earlier reports on ARA production by *Mortierella sp.* clearly indicated that there are considerable differences between how various growth parameters affect biomass, lipid and ARA yields. It is important to study the effect of various growth conditions on ARA as well as the gross lipid yield for each strain, as different strains of *Mortierella alpina* demonstrate varying optimization conditions. In view of the

above facts an attempt was made to formulate suitable media and culture parameters for maximal biomass, lipid and ARA production in *Mortierella alpina* CBS 528.72.

1.2 Materials and methods

1.2.1 Chemicals

Standards of fatty acid methyl esters were purchased from Sigma (St. Louis, USA). Glucose, malt extract, peptone, casein, sucrose, tween 80 and yeast extract were obtained from Hi-Media (Mumbai, India). Rhamnose and Raffinose were obtained from Ranbaxy Laboratories Ltd., India. All other chemicals were procured from Merck India Pvt. Ltd. and were used without further purifications. Solvents used in this study were of analytical reagent (AR) grade obtained from Qualigens Fine Chemicals, India. Corn solids and vegetable oils of standard companies were obtained from commercial sources. All glasswares used in this study were obtained from Borosil Glass, India Ltd.

1.2.2 Optimization of cultural conditions by one factor at a time approach

1.2.2.1 Microorganism and cultivation

Mortierella alpina CBS 528.72 procured from Dr. Donald A Mackenzie, UK was maintained on potato dextrose agar slants at 4ºC and sub-cultured every two months. The seed culture was prepared in 50mL medium containing 20 g/L glucose and 10g/L yeast extract. The pH was adjusted to 6.0 prior to autoclaving at 121ºC and 15 lbs. Cultures were incubated for 48 h at 28ºC. The production cultures were prepared in 1 litre Erlenmeyer flasks containing 300 mL medium and incubated for 7 days by shaking at 240 rpm on an orbital shaker.

1.2.2.2 Effect of culture variables

Different parameters (physical and chemical) were investigated for their effect on biomass, lipid and ARA production in *M. alpina* by the classical 'one factor at a time' approach.

1.2.2.2.1 Effect of media

Culture media used in this experiment were Malt extract broth (MEB)- g/L: Malt extract: 30; Peptone: 3, Fat producing medium (FPM)- g/L**:** Glucose: 40; Yeast extract: 1.5; NH_3NO_4: 0.286; KH_2PO_4: 0.750; $CaCl_2$: 0.4; $MgSO_4$: 0.4, Potato dextrose broth (PDB)- g/L: Infusion of 200 g peeled potato; Dextrose: 20, Synthetic medium (SM)- g/L: Glucose: 30; Yeast extract: 5; KH_2PO_4: 2.5; KNO_3: 1; $MgSO_4$.

$7H_2O$: 0.5; $CaCl_2$: 0.1; $FeSO_4.7H_2O$: 0.02; $ZnSO_4.7H_2O$: 0.01; $MnSO_4$: 0.01; $CuSO_4.5H_2O$: 0.002 and Glucose-Yeast extract medium (GY)- g/L: Glucose: 20; Yeast extract: 10.

1.2.2.2.2 Effect of media pH

In this experiment, initial pH of the media was manipulated to obtain the ideal pH range for biomass, lipid and ARA production by the organism. Media pH was adjusted prior to inoculation. The media were prepared, autoclaved and the pH was adjusted in 0.5 pH increments from 4.5 - 8.0 by adding 0.1N solutions of NaOH or HCl accordingly.

1.2.2.2.3 Effect of temperature

To investigate the effect of temperature on mycelial growth and ARA production, *M. alpina* was cultivated at different temperatures ranging from 15ºC to 40ºC. The dry biomass, lipid and ARA content were recorded as previously described.

1.2.2.2.4 Effect of carbon source

M. alpina grow to some extend over a wide range of carbon sources. Different carbon sources namely fructose, lactose, starch, raffinose, rhamnose and mannose were investigated for biomass, lipid and ARA production. To find the suitable carbon source for biomass and ARA production the carbon sources were separately provided at 20g/L level instead of glucose in the basal medium.

1.2.2.2.5 Effect of nitrogen source

For optimization of nitrogen source, individual nitrogen sources were supplemented to the medium in place of yeast extract at a concentration of 1%. The nitrogen sources used were peptone, sodium nitrate, ammonium sulphate, potassium nitrate, urea, ammonium nitrate, ammonium chloride and casein.

1.2.2.2.6 Effect of oil as carbon source

Vegetable oils were provided as the sole source of carbon to study the ability of this fungus to utilize them for growth, lipid and ARA production. Sunflower, sesame, rice bran, mustard and red palm oils were used at 2% level. After growth, the harvested cells were first washed extensively with distilled water followed by tween 80, ethanol and a rapid wash with chloroform. Cells were finally washed with distilled water before proceeding further.

1.2.2.2.7 Effect of glutamate supplementation

Glutamate at different concentrations (0.2-1.0g/L) was added to GY medium to investigate its effect of biomass, total lipid and ARA production.

1.2.2.2.8 Effect of fed batch culture

To investigate the effect of fed batch culture, *M. alpina* was grown for a period of 4 days in GY medium. After 4 days of incubation, different concentrations of glucose (1-7%) were added aseptically to replenish the spent carbon. The supplemented cultures were incubated further for 3 days and the biomass, total lipid and ARA content were determined.

1.2.2.2.9 Scale up of ARA production

ARA production process was scaled up to the level of 15L fermentor. The fermentor study was carried out with the conditions optimized by shake flask studies. pH was controlled at 6.5 and the initial dissolved oxygen was set at 100% in a stirred tank fermentor (LF 10, Murhopye Scientific company, Mysore, India). 1% PPG (polypropylene glycol) was used as the antifoam to prevent frothing. The culture was incubated for a period of 7 days.

1.2.2.3 Dry cell weight determination and lipid extraction

Biomass production was determined by harvesting the cells by suction filtration followed by drying at 55-60ºC overnight. The dried biomass was made to a fine powder by grinding with acid washed sand. These were then made into a thimble, macerated with 0.1N HCl for 20 min and washed thoroughly under running tap water to remove traces of acid. The thimble containing biomass was dried overnight and extracted by Soxhlet method with hexane as the solvent for 8-10 h. The total lipid was determined gravimetrically by standard methods (AOAC, 2000).

1.2.2.4 Methyl ester preparation and fatty acid analysis

Fatty acid methyl esters (FAMEs) were prepared using methanolic HCl as the methylating agent. The derivatized lipids were dissolved in 1 mL of benzene and any solids removed by centrifuging at 10,000 rpm for 2 min. Lipids were analyzed by gas chromatography using a Shimadzu 14B system (Shimazdu, Japan) and a 25m BP21 capillary column of 0.25µm internal diameter (Konik Tech, Barcelone, Spain). The column was operated at an initial temperature of 120ºC for 1 min before increasing the temperature to 220ºC at a rate of 10ºC /min and holding for 20 min. Carrier gas (nitrogen) was supplied at a flow rate of 1mL/min with a split ratio of 1:20. Fatty acids were identified by comparison with authentic standards procured from Sigma.

1.2.2.5 Data Analyses

The effects of different growth factors were studied for the maximum production of arachidonic acid using the traditional "one-factor-at-a-time" (OFAT) design. The experiments with the Erlenmeyer flask system were performed based on three replications. Statistical analysis was performed with SPSS software (SPSS, 1998). One way analysis of variance (ANOVA) with Duncan's multiple range test was used to assess statistical differences in this study. The values are given as mean ± SD. Levels of significance were considered at $P \leq 0.05$ unless otherwise stated.

1.2.3 Response surface optimization

1.2.3.1 Experimental design

A central composite rotatable design (CCRD) with four variables was used to study the response pattern and to determine the optimum combination of variables (Cochran and Cox, 1957). The variables were studied at five coded levels (-2,-1, 0, 1, 2), with 7 replicates at the central point (0, 0, 0, 0), 8 axial points and 16 quadrant points leading to a total of 31 experiments. The variables studied were: glucose (10-80g/L), corn solids (5-40g/L), KH_2PO_4 (1-5g/L) and KNO_3 (1-5g/L) (Table 1.7) with 45.0g/L, 22.5g/L, 3.0g/L, and 3.0g/L (Expts 24-31) respectively as their central points and their concentration ranges were fixed based on literature survey. The CCRD shown in Table 1.7 was arranged so as to fit an appropriate regression model using multiple regression program. The CCRD combines the vertices of the hypercubes whose coordinates are given by a 2^n factorial design to provide for the estimation of curvature of the model (Joglekar and May, 1987). The replicates at the centre point were used to estimate pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to superfluous factors.

1.2.3.2 Statistical analysis

Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model proposed for the response surface analysis was given as follows. The model proposed for the response Y_i was

$$Y_i = a_o + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4 \quad \text{Eqn. (1)}$$

where Y_i (i=1,2,3) is the predicted response for biomass production (Y_1), total lipid production (Y_2) and arachidonic acid production (Y_3); a_o is the value of the fitted response at the centre point of the design, a_i, a_{ii}, a_{ij} the linear, quadratic, and cross product terms, respectively. The coefficients of Eqn. 1 were obtained using MATLAB 7.0 software based on the data provided in Table 1.17 and presented in Table 1.18. The significance of each coefficient was determined by student's t-test and values are listed in Table 1.18. The ANOVA tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were analyzed statistically by computing the F-value at a probability (p) of 0.001, 0.01 and 0.05. The goodness of fit of the model was checked by the determination coefficient (R^2) values (Table 1.19).

Maximization of fitted polynomials for the responses such as biomass, total lipid and ARA was performed by a non-linear mathematical maximization procedure of the Quattro Pro software package 4.0 (Borland International Inc., USA) (Sharma *et al*., 2009; Triveni *et al*., 2001). Fitted polynomial equation was expressed as surface plot using MATLAB 7.0 in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions. Experiments were run using the optimum values for variables given by response optimization to confirm the predicted and actual values. Responses were monitored and results compared with model predictions.

1.2.3.3 Submerged fermentation

The seed culture was prepared as described in section 1.2.1.1, replacing yeast extract with corn solids at the same concentration. Various combinations of production media were prepared in 1L Erlenmeyer flasks according to the experimental design given in Table 1.18, inoculated with 5% seed culture and incubated for 7 days at 28°C. The fermentation experiments were conducted in triplicate and the average values of biomass, lipid and arachidonic acid yield were tabulated.

1.3 Results

A rosette pattern of growth was observed when *Mortierella alpina* CBS 528.72 was grown in PDA plates (Fig 1.1). Under submerged conditions in a standard medium like FPM, the fungus grew in pellet morphology. For maximizing biomass (Fig 1.2), total lipid (Fig 1.3) and ARA, various culture parameters were investigated.

Fig 1.1 Rosette growth pattern of *Mortierella alpina* CBS 528.72 in PDA

Fig 1.2 Harvested and dried biomass of *M. alpina*

A- Wet biomass, B-Dry biomass

Fig 1.3 Oil extracted from *M. alpina*

1.3.1 Optimization of culture conditions by one factor at a time approach

Physical and chemical parameters investigated had a significant effect on biomass, total lipid and ARA production in *M. alpina* CBS 528.72.

1.3.1.1 Effect of Media

Effect of culture media on biomass, lipid and ARA production is given in Table 1.1. Maximum biomass accumulation was noticed in GY medium (6.73 g/L) followed by synthetic medium (5.2 g/L). Total lipids in biomass were high for synthetic media (49.0%), GY (40.0%) and FPM (40.0%). Maximum yield of total lipids (g/L) was achieved in GY medium (2.69g/L).Though high biomass and lipid production was achieved in SM, the ARA yield was considerably lower (0.24g/L) than GY (0.92g/L) owing to reduced ARA percentage in total lipids. The fatty acid composition of lipids obtained with the different media is given in Table 1.2.

When *M. alpina* was cultured in GY medium three distinct growth phases could be noticed. i Lag phase (upto 48h)- growth is slow at this phase ii Log phase (48-120h)-Active growth and biomass production occur at this phase. iii Stationary phase with intense lipid and ARA accumulation (Fig 1.1).

Table 1.1 Effect of culture media on biomass, total lipid and arachidonic acid production by *M. alpina* CBS 528.72

Media	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
MEB	2.60±0.16[a]	0.55±0.03[a]	21.0±0.18[a]	0.07±0.00[a]
FPM	4.89±0.32[c]	1.95±0.18[b]	40.0±0.86[c]	0.29±0.03[b]
PDB	3.60±0.14[b]	0.90±0.05[a]	25.0±0.34[b]	0.03±0.00[a]
SM	5.20±0.56[c]	2.54±0.31[c]	49.0±0.42[d]	0.24±0.04[b]
GY	6.73±0.50[d]	2.69±0.25[c]	40.0±0.71[c]	0.92±0.10[c]

Culture conditions: 240 rpm; cultivation period: 7 days. MEB-Malt extract broth; FPM-Fat producing medium; PDB-Potato dextrose broth; SM-Synthetic medium; GY-Glucose yeast extract mediim.
Values are means ± SD, n =3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.2 Effect of media on fatty acid composition of *M. alpina*

Medium	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
MEB	2.02±0.12^{c}	27.61±0.28^{d}	12.07±0.04^{b}	27.28±0.18^{c}	7.23±0.04^{c}	3.00±0.10^{b}	1.89±0.08^{c}	**11.98±0.08^{c}**	1.56±0.14^{a}	1.77±0.25^{a}
FPM	1.09±0.04^{b}	22.99±0.14^{c}	17.61±0.20^{d}	22.19±0.24^{b}	7.25±0.10^{c}	3.84±0.26^{c}	1.58±0.12^{b}	**14.64±0.44^{d}**	3.48±0.30^{b}	4.18±0.16^{b}
PDB	1.9±0.10^{b}	20.73±0.22^{b}	22.9±0.16^{e}	22.14±0.09^{b}	6.39±0.16^{a}	2.78±0.14^{b}	0.67±0.09^{a}	**3.13±0.22^{a}**	3.82±0.15^{c}	4.36±0.10^{b}
SM	1.27±0.14^{c}	31.35±0.18^{e}	14.78±0.06^{c}	27.8±0.16^{d}	7.54±0.07^{d}	0.71±0.04^{a}	2.06±0.16^{c}	**9.60±0.08^{b}**	1.28±0.24^{a}	2.12±0.22^{a}
GY	0.57±0.06^{a}	13.1±0.09^{a}	10.65±0.12^{a}	18.53±0.28^{a}	6.60±0.05^{b}	0.84±0.12^{a}	3.7±0.26^{d}	**34.38±0.39^{c}**	3.29±0.20^{b}	4.04±0.24^{b}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-Total fatty acid, MEB-Malt extract broth; FPM-Fat producing medium; PDB-Potato dextrose broth; SM-Synthetic medium; GY-Glucose yeast extract medium.14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

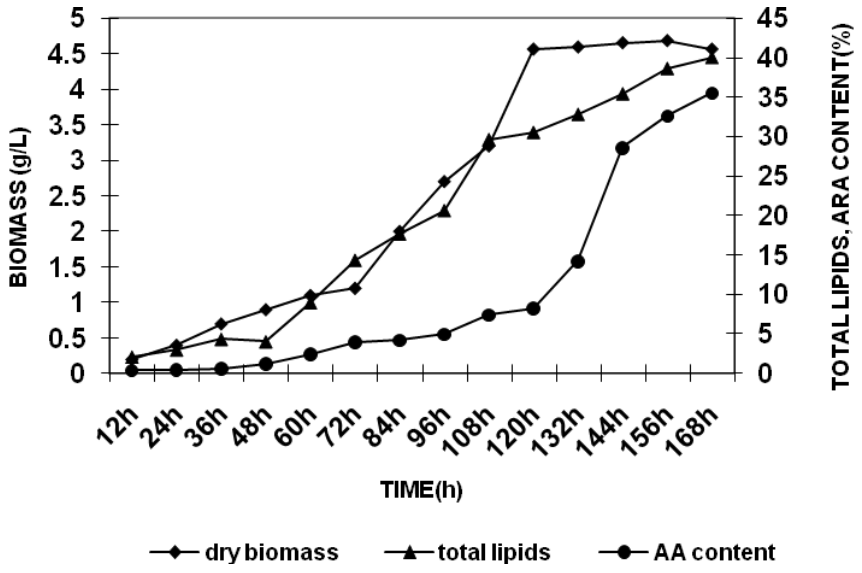


Fig 1.4 Growth curve of *M. alpina* in GY medium

1.3.1.2 Effect of pH

It was noticed that the media pH had a profound effect on biomass and ARA production in *M. alpina*. Effect of initial pH was investigated from pH 4.5 to pH 8.0 (Table 1.3). The strain showed absolute cessation of growth at pH below 4.0 and above 9.0 (data not shown). The biomass and lipid production was favoured at an acidic pH range (6.0-6.5). Initial pH in the range of 6.0-6.5 yielded no significant difference in dry biomass. The Lipid yield was maximum when the mould was cultivated at pH 6.0 (3.11g/L). Maximal ARA production was obtained at initial pH 6.5 (1.0g/L). The fatty acid composition of lipids obtained under different initial pH regimens is given in Table 1.4.

1.3.1.3 Effect of Temperature

Maximum biomass production was possible at 30ºC (6.7g/L) in GY medium (Table 1.5). A change in cultivation temperature had a profound effect on biomass production. The temperature below 25ºC or above 30ºC was found to delay mycelial growth. An absolute cessation of growth occurred at temperature at or above 35ºC. The most suitable temperature for maximum lipid accumulation in biomass (40.0 %) and ARA in total lipids (38.7 %) was 25ºC. Biomass production at this temperature was considerably less than that achieved at 30ºC leading to lower ARA yield

(0.58g/L). An intermediate temperature of 28ºC was used which proved effective and yielded 0.92g/L ARA after 7 days of incubation. Hence this temperature was employed for the subsequent experiments. The fatty acid composition of lipids obtained at different temperature is given in Table 1.6.

1.3.1.4 Effect of Carbon Source

Among the various carbon sources tested, glucose yielded the highest biomass and total lipids (6.75g/L and 2.71g/L respectively) (Table 1.7) whereas maximum ARA in total lipids was achieved in rhamnose containing medium (40.41%). In spite of a higher ARA percentage achieved with rhamnose, the dry biomass and total lipids were comparatively less (5.2 and1.77g/L respectively) than glucose. Starch was a poor growth supporter which yielded 1.6 g/L dry biomass. The important sugars for arachidonic acid yield were ranked as follows: glucose> rhamnose> fructose > mannose> lactose > raffinose > starch. The effect of carbon sources on fatty acid composition is given in Table 1.8.

1.3.1.5 Effect of Nitrogen Source

Effect of nitrogen source on biomass, lipid and ARA production was studied in GY medium wherein yeast extract was replaced by different organic and inorganic nitrogen sources. The study indicated that nitrogen source play an important role in lipid production. The influence of replacing yeast extract in the GY medium with an equal weight of different nitrogen sources on the biomass, lipid and ARA content by *M. alpina* was compared and the results are presented in Table 1.9. Of the various nitrogen sources added separately at 1% concentration to the medium containing 2% glucose, yeast extract (0.98g/L) followed by peptone (0.75g/L) gave highest ARA yield in the strain investigated. The maximum dry biomass yield was obtained with yeast extract (6.8 g/L). Total lipids in biomass were maximum with peptone as the nitrogen source (42.0%) whereas maximum lipid yield (2.79g/L) was achieved with yeast extract as the nitrogen source. Of the inorganic nitrogen sources used, maximum biomass (3.2g/L), total lipids (1.02g/L) and ARA yield (0.17g/L) was obtained when potassium nitrate was used as the source of nitrogen. It was found that inorganic nitrogen sources were inferior to organic sources in terms of biomass, lipid and ARA productivity. The effect of nitrogen sources on fatty acid composition is given in Table 1.10.

Table 1.3 Effect of media pH on biomass, total lipid and arachidonic acid production by *M. alpina* CBS 528.72

Media pH	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
4.5	3.50±0.14^{c}	1.23±0.05^{b}	35.0±0.10^{bc}	0.03±0.01^{a}
5.0	2.50±0.46^{c}	0.65±0.13^{a}	26.0±0.26^{d}	0.08±0.02^{a}
5.5	4.13±0.28^{bc}	1.78±0.12^{c}	43.0±0.12^{ab}	0.32±0.03^{c}
6.0	6.77±0.62^{a}	3.11±0.29^{e}	46.0±0.06^{a}	0.95±0.09^{e}
6.5	6.90±0.45^{a}	2.87±0.23^{e}	41.6±0.51^{ab}	1.00±0.09^{e}
7.0	5.40±0.24^{b}	2.12±0.12^{d}	39.2±0.44^{b}	0.61±0.03^{d}
7.5	4.20±0.12^{bc}	1.66±0.06^{c}	39.6±0.30^{b}	0.17±0.01^{b}
8.0	2.38±0.06^{d}	0.73±0.04^{a}	30.8±0.56^{cd}	0.04±0.01^{a}

Culture conditions: Media: GY; Temperature: 30°C; Carbon source: glucose (2%); Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ±SD, n = 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.4 Effect of media pH on fatty acid composition of *M. alpina*

Initial pH	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
4.5	0.38±0.06^{ab}	35.9±0.52^{g}	17.4±0.20^{d}	29.0±0.12^{g}	7.49±0.05^{f}	0.43±0.08^{a}	1.29±0.14^{b}	**2.36±0.48^{a}**	0.68±0.09^{a}	1.24±0.05^{a}
5.0	0.88±0.14^{c}	34.36±0.26^{f}	17.35±0.26^{d}	6.17±0.10^{d}	5.26±0.16^{e}	2.18±0.24^{a}	1.10±0.30^{ab}	**12.48±0.62^{d}**	3.48±0.12^{d}	4.16±0.28^{de}
5.5	0.42±0.10^{ab}	25.51±0.12^{c}	23.8±0.18^{f}	14.64±0.14^{c}	7.74±0.20^{f}	1.02±0.15^{b}	0.88±0.06^{a}	**17.82±0.34^{e}**	1.62±0.06^{b}	1.80±0.16^{b}
6.0	0.58±0.08^{b}	15.2±0.38^{a}	12.74±0.12^{a}	13.25±0.28^{a}	4.4±0.14^{d}	2.68±0.12^{e}	3.48±0.25^{d}	**30.45±0.06^{g}**	3.04±0.26^{c}	4.20±0.10^{de}
6.5	0.50±0.06^{b}	14.8±0.22^{a}	12.64±0.09^{a}	13.13±0.32^{a}	4.06±0.22^{c}	2.80±0.16^{e}	3.44±0.10^{d}	**34.72±0.19^{h}**	3.46±0.10^{d}	4.34±0.28^{e}
7.0	0.23±0.05^{a}	16.4±0.46^{b}	13.09±0.14^{b}	14.07±0.16^{b}	3.30±0.26^{a}	3.73±0.10^{f}	5.89±0.18^{e}	**28.60±0.04^{f}**	3.14±0.19^{c}	3.86±0.15^{cd}
7.5	1.01±0.14^{c}	28.6±0.18^{d}	13.8±0.25^{c}	20.6±0.09^{f}	3.64±0.18^{b}	1.97±0.14^{d}	3.22±0.24^{d}	**10.40±0.18^{c}**	3.82±0.25^{e}	4.96±0.14^{f}
8.0	1.25±0.20^{d}	32.4±0.24^{e}	20.4±0.12^{e}	16.8±0.15^{e}	8.6±0.10^{g}	1.58±0.22^{c}	2.82±0.12^{c}	**5.40±0.50^{b}**	3.02±0.22^{c}	3.69±0.26^{c}

Values are means ± SD, n =3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test
TFA-Total fatty acid, 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid,20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

Table 1.5 Effect of temperature on biomass, total lipid and arachidonic acid production by *M.alpina* CBS 528.72

Temperature	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
15	3.20±0.14[a]	1.25±0.06[a]	39.0±0.32[b]	0.38±0.02[a]
20	3.34±0.26[a]	1.20±0.10[a]	36.0±0.06[a]	0.45±0.04[a]
25	3.80±0.22[b]	1.52±0.09[b]	40.0±0.14[c]	0.58±0.05[b]
28	6.68±0.24[c]	2.63±0.10[c]	39.4±0.05[b]	0.92±0.04[c]
30	6.70±0.08[c]	2.61±0.08[c]	39.0±0.64[b]	0.90±0.04[c]

Culture conditions: Media: GY; pH: 6.5; Carbon source: glucose (2%); Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

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Table 1.6 Effect of temperature on fatty acid composition of *M. alpina*

Temperature (°C)	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
15	0.52±0.06^{a}	10.26±0.16^{a}	10.58±0.15^{b}	21.24±0.08^{d}	4.68±0.12^{b}	2.48±0.10^{c}	3.06±0.20^{a}	**30.54±0.18^{a}**	7.24±0.09^{c}	4.54±0.18^{c}
20	0.50±0.09^{a}	10.4±0.10^{a}	10.62±0.12^{b}	16.26±0.24^{b}	4.20±0.06^{a}	2.65±0.04^{c}	3.98±0.15^{c}	**37.20±0.16^{c}**	6.20±0.12^{b}	4.28±0.06^{bc}
25	0.62±0.05^{a}	12.68±0.09^{b}	12.94±0.22^{c}	16.12±0.20^{b}	4.28±0.14^{a}	2.06±0.12^{b}	3.25±0.08^{ab}	**38.70±0.42^{d}**	3.26±0.08^{a}	3.98±0.10^{a}
28	0.59±0.04^{a}	14.85±0.20^{c}	13.6±0.12^{d}	13.45±0.15^{a}	4.29±0.15^{a}	2.60±0.19^{c}	3.48±0.16^{b}	**35.04±0.16^{b}**	3.32±0.20^{a}	4.18±0.12^{ab}
30	0.50±0.06^{a}	14.8±0.22^{c}	10.04±0.09^{a}	19.13±0.26^{c}	5.96±0.06^{c}	0.80±0.04^{a}	3.94±0.16^{c}	**34.74±0.06^{b}**	3.46±0.26^{a}	4.34±0.24^{bc}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test
TFA-Total fatty acid, 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid, 18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

Table 1.7 Effect of carbon source on biomass, total lipid and arachidonic acid production by *M. alpina* CBS 528.72

Carbon source	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
Glucose	6.75±0.25^{d}	2.71±0.12^{f}	40.2±0.24^{f}	0.96±0.05^{e}
Fructose	5.40±0.15^{c}	1.84±0.06^{e}	34.0±0.16^{d}	0.53±0.02^{c}
Starch	1.60±0.12^{a}	0.58±0.05^{b}	36.5±0.28^{e}	0.03±0.01^{a}
Lactose	4.60±0.34^{b}	0.75±0.07^{c}	16.4±0.14^{a}	0.10±0.01^{b}
Raffinose	4.60±0.24^{b}	1.13±0.08^{d}	24.6±0.36^{b}	0.09±0.01^{b}
Rhamnose	5.20±0.18^{c}	1.77±0.06^{e}	34.0±0.06^{d}	0.72±0.03^{d}
Mannose	1.40±0.08^{a}	0.40±0.03^{a}	28.9±0.10^{c}	0.12±0.01^{b}

Culture conditions: Media: GY; pH: 6.5; Temperature: 28°C; Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ±SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.8 Effect of carbon source on fatty acid composition of *M. alpina*

Carbon source	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
Glucose	0.58±0.05^{a}	14.91±0.16^{b}	13.0±0.28^{c}	13.65±0.12^{a}	4.31±0.16^{d}	2.68±0.08^{b}	3.43±0.20^{e}	**35.50±0.09^{f}**	3.29±0.12^{d}	4.04±0.10^{d}
Fructose	0.87±0.10^{ab}	19.48±0.06^{d}	3.00±0.16^{a}	31.08±0.18^{e}	3.63±0.08^{c}	4.31±0.14^{d}	0.39±0.18^{a}	**28.96±0.04^{d}**	1.57±0.06^{b}	3.06±0.05^{b}
Starch	0.92±0.08^{ab}	43.28±0.28^{g}	4.75±0.14^{b}	34.36±0.35^{f}	3.08±0.14^{b}	2.42±0.18^{ab}	0.48±0.04^{ab}	**6.11±0.42^{a}**	1.24±0.20^{a}	1.86±0.12^{a}
Lactose	2.46±0.36^{d}	18.91±0.12^{c}	3.19±0.34^{a}	28.55±0.24^{d}	3.48±0.25^{c}	3.60±0.06^{c}	1.61±0.12^{d}	**13.39±0.26^{c}**	2.09±0.14^{c}	7.13±0.28^{e}
Raffinose	1.69±0.16^{c}	28.11±0.20^{f}	2.90±0.18^{a}	44.66±0.56^{g}	1.28±0.12^{a}	2.29±0.24^{a}	1.28±0.15^{c}	**8.17±0.08^{b}**	2.10±0.18^{c}	3.38±0.14^{c}
Rhamnose	0.78±0.20^{ab}	12.21±0.26^{a}	2.93±0.15^{a}	21.48±0.20^{b}	6.56±0.22^{e}	5.24±0.20^{f}	0.68±0.03^{b}	**40.41±0.42^{g}**	1.77±0.26^{b}	3.59±0.09^{c}
Mannose	0.98±0.14^{b}	25.29±0.18^{e}	3.05±0.20^{a}	22.82±0.18^{c}	6.74±0.30^{e}	4.78±0.16^{e}	1.21±0.18^{c}	**30.23±0.28^{e}**	1.07±0.18^{a}	2.95±0.06^{b}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test
TFA-Total fatty acid, 14:0-myristic acid,16:0-palmitic acid, 18:0-stearic acid, 18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

Table 1.9 Effect of nitrogen sources on biomass, total lipid and arachidonic acid production by *M. alpina* CBS 528.72

Nitrogen source	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
Yeast extract	6.8±0.25^{d}	2.79±0.12^{f}	41.0±0.21^{f}	0.98±0.06^{e}
Peptone	6.2±0.44^{c}	2.60±0.20^{e}	42.0±0.24^{g}	0.75±0.06^{d}
Sodium nitrate	2.0±0.04^{a}	0.32±0.02^{b}	16.0±0.62^{a}	0.06±0.00^{ab}
Ammonium sulphate	3.1±0.26^{b}	0.71±0.07^{c}	23.0±0.20^{c}	0.16±0.02^{c}
Potassium nitrate	3.2±0.14^{b}	1.02±0.05^{d}	32.0±0.18^{e}	0.17±0.02^{c}
Urea	0.7±0.08^{d}	0.11±0.02^{a}	16.0±0.26^{a}	0.01±0.00^{a}
Ammonium nitrate	1.6±0.14^{a}	0.40±0.04^{b}	25.0±0.12^{d}	0.06±0.01^{ab}
Ammonium chloride	1.9±0.46^{a}	0.34±0.09^{b}	18.0±0.18^{b}	0.08±0.02^{b}
Casein	2.8±0.54^{b}	0.66±0.15^{c}	23.6±0.70^{c}	0.14±0.04^{c}

Culture conditions: Media: GY; pH: 6.5; Temperature: 28°C; Carbon source: Glucose (2 %); 240 rpm; Cultivation period: 7 days. Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.10 Effect of nitrogen source on fatty acid composition of *M. alpina*

Nitrogen source	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
Yeast extract	0.68±0.10^{a}	14.86±0.24^{a}	13.06±0.16^{b}	13.82±0.20^{a}	4.38±0.15^{ab}	2.54±0.05^{b}	3.48±0.04^{cd}	**35.28±0.29^{h}**	3.08±0.24^{c}	3.98±0.05^{e}
Peptone	1.69±0.06^{e}	16.22±0.18^{b}	14.18±0.05^{c}	15.46±0.15^{b}	6.28±0.26^{d}	3.12±0.12^{c}	3.43±0.18^{bcd}	**28.74±0.27^{g}**	3.45±0.09^{d}e	3.26±0.24^{cd}
Sodium nitrate	1.48±0.24^{de}	25.48±0.26^{g}	15.10±0.14^{f}	18.24±0.24^{d}	4.15±0.22^{a}	4.02±0.14^{d}	3.12±0.20^{b}	**18.95±0.10^{d}**	3.80±0.22^{f}	3.12±0.12^{c}
Ammonium sulphate	0.97±0.18^{ab}	19.58±0.30^{c}	12.27±0.10^{a}	21.13±0.08^{f}	7.63±0.14^{e}	4.57±0.25^{e}	2.13±0.16^{a}	**23.14±0.48^{f}**	1.38±0.16^{a}	3.08±0.09^{c}
Potassium nitrate	1.22±0.15^{bcd}	24.45±0.14^{f}	13.26±0.09^{b}	24.18±0.16^{g}	4.62±0.20^{bc}	3.25±0.26^{c}	3.56±0.14^{d}	**16.98±0.60^{c}**	1.58±0.20^{a}	3.54±0.10^{d}
Urea	1.34±0.22^{cd}	24.50±0.08^{f}	14.80±0.24^{e}	16.56±0.24^{c}	10.40±0.18^{f}	1.52±0.09^{a}	9.80±0.10^{e}	**12.06±0.52^{a}**	2.14±0.25^{b}	2.04±0.16^{d}
Ammonium nitrate	0.95±0.26^{ab}	24.80±0.25^{f}	15.62±0.18^{g}	20.82±0.18^{e}	4.36±0.26^{ab}	3.92±0.14^{d}	3.64±0.25^{d}	**14.80±0.18^{b}**	3.60±0.08^{ef}	3.18±0.32^{c}
Ammonium chloride	1.45±0.14^{de}	21.24±0.20^{d}	15.25±0.06^{f}	16.62±0.10^{c}	4.88±0.15^{c}	4.64±0.12^{ef}	3.18±0.16^{bc}	**22.60±0.44^{f}**	3.10±0.12^{c}	3.12±0.12^{c}
Casein	1.08±0.09^{bc}	23.14±0.16^{e}	14.46±0.10^{d}	18.06±0.19^{d}	4.15±0.22^{a}	4.88±0.20^{f}	3.52±0.24^{d}	**21.85±0.25^{e}**	3.24±0.26^{cd}	2.46±0.24^{b}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test
TFA-Total fatty acid, 14:0-myristic acid, 16:0-palmitic acid, 18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid,18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

1.3.1.6 Effect of Oil as Carbon Source

Glucose in the basal medium (GY) was replaced with an equal quantity of vegetable oils. The recorded total lipid content in biomass was greater or comparable with that of control except for sesame (36.4%) and mustard oil (33.78%). Of the different oils used, rice bran oil resulted in maximum total lipids in biomass (48.49%) as well as total lipid yield (2.34g/L). However ARA yield (0.01g/L) was considerably less than that of glucose supplemented media. Use of oils as carbon source led to a significant decrease in biomass and ARA production in this strain (Table 1.11). The effect of oil as the sole carbon source on fatty acid composition of *M. alpina* is given in Table 1.12.

1.3.1.7 Effect of glutamate supplementation

When the production media (GY) was supplemented with different concentrations of glutamate a significant increment in total lipid and ARA yields were achieved. Maximum biomass (8.8g/L) was obtained in a medium supplemented with 0.6g/L glutamate. Maximum lipid and ARA yield (4.37g/L and 1.87g/L respectively) were obtained when glutamate was added at 0.8g/L level. Beyond this concentration a reduction in lipid and ARA yield was evident (Table 1.13). The effect of glutamate supplementation on fatty acid composition of *M. alpina* is given in Table 1.14.

Table 1.11 Effect of oil as carbon source on biomass, total lipid and arachidonic acid production by *M. alpina* CBS 528.72

Carbon source	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
Glucose	6.50±0.38^{e}	2.60±0.17^{d}	40.00±0.30^{c}	0.92±0.07^{c}
Sunflower oil	4.32±0.16^{cd}	1.79±0.07^{c}	41.32±0.25^{d}	0.07±0.01^{b}
Sesame oil	2.60±0.06^{a}	0.95±0.02^{a}	36.40±0.22^{b}	0.03±0.00^{ab}
Ricebran oil	4.82±0.24^{d}	2.34±0.14^{d}	48.49±0.45^{e}	0.01±0.00^{a}
Mustard oil	3.68±0.52^{bc}	1.24±0.19^{ab}	33.78±0.15^{a}	0.07±0.01^{b}
Red palm oil	3.23±0.70^{ab}	1.30±0.31^{b}	40.38±0.49^{c}	0.04±0.01^{ab}

Culture conditions: Media: GY; pH: 6.5; Temperature: 28°C; Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.12 Effect of oil as carbon source on fatty acid composition of *M. alpina*

Carbon source	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
Glucose	0.62±0.12^{b}	14.12±0.24^{c}	11.8±0.20^{f}	15.25±0.25^{b}	4.26±0.10^{a}	2.14±0.12^{c}	3.29±0.22^{c}	**35.46±0.27^{e}**	3.06±0.18^{b}	4.18±0.08^{f}
SO	0.10±0.06^{a}	5.75±0.20^{b}	6.72±0.18^{e}	35.0±0.15^{d}	46.66±0.18^{f}	0.80±0.10^{a}	0.54±0.14^{a}	**4.07±0.34^{c}**	0.72±0.05^{a}	0.66±0.05^{c}
SESO	0.68±0.14^{ab}	20.12±0.16^{d}	4.35±0.15^{c}	30.24±0.12^{c}	20.52±0.09^{d}	0.86±0.15^{a}	8.68±0.12^{d}	**2.80±0.16^{b}**	0.20±0.04^{a}	0.28±0.02^{b}
RBO	0.91±0.20^{cd}	20.04±0.10^{d}	3.02±0.26^{b}	48.04±0.18^{f}	21.6±0.14^{e}	1.50±0.20^{b}	0.62±0.04^{a}	**0.27±0.32^{a}**	0.81±0.12^{a}	2.32±0.14^{e}
MO	0.82±0.12^{bcd}	3.31±0.18^{a}	1.37±0.12^{a}	11.61±0.20^{a}	12.64±0.23^{c}	4.28±0.24^{d}	0.80±0.15^{ab}	**5.41±0.10^{d}**	40.77±2.24^{c}	1.34±0.12^{d}
RO	0.95±0.08^{d}	34.60±0.25^{e}	5.71±0.22^{d}	38.95±0.25^{e}	10.10±0.17^{b}	1.23±0.16^{b}	1.07±0.26^{b}	**2.81±0.42^{b}**	0.21±0.04^{a}	0.05±0.00^{a}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-Total fatty acid, S0-sunflower oil, SESO-sesame oil, RBO-rice bran oil, MO-mustard oil, RO- redpalm oil, 14:0-myristic acid,16:0-palmitic acid, 18:0-stearic acid, 18:1-oleic acid, 18:2-linoleic acid,18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

Table 1.13 Effect of glutamate supplementation on biomass, total lipid and ARA yield in *M. alpina*

Glutamate (g/L)	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
0	6.9±0.32[a]	2.76±0.15[a]	40.0±0.34[a]	0.96±0.07[a]
0.2	7.4±0.16[b]	3.04 ±0.09[b]	41.2±0.22[b]	1.10±0.04[a]
0.4	8.2±0.12[c]	3.79± 0.07[c]	46.26±0.1[d]	1.46±0.04[b]
0.6	8.8±0.24[d]	4.31±0.15[d]	49.08±0.30[e]	1.84±0.07[c]
0.8	8.7±0.28[d]	4.37±0.17[d]	50.34±0.26[f]	1.87±0.19[c]
1.0	8.4±0.10[cd]	3.57 ±0.08[c]	42.60±0.38[c]	1.45±0.04[b]

Culture conditions: Media: GY; pH: 6.5; Temperature: 28°C; Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test

Table 1.14 Effect of glutamate supplementation on fatty acid composition of *M. alpina*

Glutamate concentration	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
Control	0.50±0.04^{a}	14.84±0.26^{c}	13.5±0.10^{d}	13.8±0.20^{c}	4.28±0.15^{b}	2.65±0.16^{ab}	3.4±0.18^{b}	**35.06±0.20^{a}**	3.2±0.18^{a}	4.10±0.15^{c}
0.2	0.56±0.08^{a}	13.20±0.26^{b}	13.64±0.15^{d}	13.6±0.18^{c}	4.20±0.11^{b}	2.68±0.12^{ab}	3.12±0.17^{ab}	**36.12±0.18^{b}**	3.34±0.14^{a}	4.25±0.10^{c}
0.4	0.52±0.12^{a}	13.36±0.10^{b}	13.8±0.26^{d}	13.48±0.26^{bc}	4.26±0.20^{b}	2.65±0.15^{ab}	3.04±0.12^{a}	**38.54±0.35^{c}**	3.14±0.26^{a}	3.68±0.26^{b}
0.6	0.58±0.05^{a}	10.68±0.22^{a}	13.04±0.14^{c}	13.25±0.10^{b}	3.82±0.18^{a}	2.8±0.26^{b}	3.06±0.24^{ab}	**42.62±0.26^{e}**	3.26±0.18^{a}	3.18±0.12^{a}
0.8	0.52±0.11^{a}	10.40±0.18^{a}	11.68±0.20^{a}	12.54±0.18^{a}	3.68±0.12^{a}	2.4±0.14^{a}	3.40±0.18^{b}	**44.80±0.50^{f}**	3.18±0.20^{a}	3.06±0.18^{a}
1.0	0.55±0.06^{a}	12.94±0.20^{b}	12.06±0.14^{b}	12.28±0.15^{a}	4.28±0.25^{b}	2.86±0.10^{b}	3.36±0.15^{ab}	**40.56±0.14^{d}**	3.15±0.14^{a}	3.98±0.20b^{c}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-Total fatty acid, 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

1.3.1.8 Effect of fed batch culture

Glucose fed batch had a significant effect on biomass, total lipid and ARA production in *M. alpina.* Maximum biomass production of 9.2g/L was achieved in a medium additionally fed 5% glucose after 4 days. Total lipids and ARA (4.49 and 1.87g/L respectively) were maximum when the spent carbon in medium was replenished with 4% glucose. Beyond 5% level a decline in total lipid and ARA was noticed (Table 1.15). Effect of fed batch culture on fatty acid profile of *M. alpina* is given in Table 1.16.

1.3.1.9 Scale up of ARA production

When ARA production was scaled up to a 15L fermentor level it was found that there was a marginal reduction in biomass production (6.8g/L to 6.2g/L). Total lipids content in biomass and fatty acid composition remained unaltered.

Table 1.15 Effect of fed batch culture on biomass, total lipid and ARA yield in *M. alpina*

Fed Batch (%)	Dry Biomass (g/L)	Total lipid		ARA (g/L)
		g/L	% w/w	
0	6.97±0.06^{b}	2.74±0.07^{b}	39.33 ±0.58^{b}	0.97±0.03^{b}
1	7.78 ±0.13^{c}	3.10± 0.08^{c}	39.9 ±0.36^{b}	1.22±0.12^{c}
2	8.60 ±0.20^{de}	3.76±0.19^{d}	43.83 ±1.04^{c}	1.50±0.10^{d}
3	8.47±0.12^{d}	4.06± 0.15^{e}	48.00 ±1.0^{d}	1.64±0.07^{d}
4	8.80 ±0.20^{e}	4.49±0.19^{f}	51.00 ±1.0^{e}	1.87±0.10^{e}
5	9.20 ±0.10^{f}	4.15±0.11^{e}	45.17 ±0.67^{c}	1.65±0.07^{d}
6	6.50 ±0.30^{a}	2.38±0.19^{a}	36.67 ±1.15^{a}	0.72±0.09^{a}
7	6.20 ±0.20^{a}	2.34±0.11^{a}	37.67 ±0.58^{a}	0.71±0.05^{a}

Culture conditions: Media: GY; pH: 6.5; Temperature: 28°C; Nitrogen source: Yeast extract (1%); 240 rpm; Cultivation period: 7 days. Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 1.16 Effect of fed batch culture on fatty acid composition of *M. alpina*

Fed batch (%)	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
0	0.56±0.04^{a}	14.82±0.22^{d}	13.15±0.16^{b}	13.70±0.26^{c}	4.26±0.18^{a}	2.60±0.10^{a}	3.50±0.13^{a}	**35.24 ±0.45^{b}**	3.22±0.15^{abc}	4.08±0.04^{a}
1	0.55±0.10^{a}	13.48±0.26^{c}	12.10±0.06^{a}	11.72±0.14^{b}	4.24±0.14^{a}	2.80±0.15^{a}	3.4±0.10^{a}	**39.43 ±2.65^{c}**	3.38±0.18^{abc}	4.14±0.10^{a}
2	0.52±0.06^{a}	13.40±0.18^{c}	12.15±0.24^{a}	11.74±0.18^{b}	4.15±0.20^{a}	2.60±0.18^{a}	3.28±0.15^{a}	**39.90 ±0.56^{c}**	3.50±0.20^{bc}	4.10±0.15^{a}
3	0.55±0.05^{a}	12.84±0.20^{b}	12.06±0.26^{a}	11.78±0.23^{b}	4.20±0.12^{a}	2.68±0.12^{a}	3.54±0.10^{a}	**40.30 ±0.36^{c}**	3.18±0.25^{ab}	4.04±0.26^{a}
4	0.58±0.06^{a}	12.15±0.16^{a}	12.05±0.15^{a}	11.14±0.26^{a}	4.22±0.11^{a}	2.58±0.20^{a}	3.45±0.22^{a}	**41.60 ±0.50^{c}**	3.15±0.22^{a}	4.06±0.18^{a}
5	0.50±0.08^{a}	13.5±0.14^{c}	12.20±0.18^{a}	11.75±0.14^{b}	4.20±0.28^{a}	2.62±0.14^{a}	3.42±0.26^{a}	**39.66 ±0.69^{c}**	3.28±0.14^{abc}	4.12±0.24^{a}
6	0.52±0.10^{a}	16.25±0.20^{e}	15.20±0.12^{c}	14.98±0.10^{d}	4.28±0.15^{a}	2.65±0.18^{a}	3.52±0.16^{a}	**30.46 ±1.02^{a}**	3.30±0.08^{abc}	4.14±0.20^{a}
7	0.55±0.08^{a}	16.28±0.18^{e}	15.16±0.22^{c}	15.16±0.22^{d}	4.30±0.24^{a}	2.68±0.15^{a}	3.48±0.18^{a}	**30.17 ±0.99^{a}**	3.54±0.16^{c}	4.15±0.15^{a}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-Total fatty acid, 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid, 18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

1.3.2 Response surface optimization

1.3.2.1 Diagnostic checking of the models

Three responses namely biomass (Y_1), total lipids (Y_2), and arachidonic acid production (Y_3) were measured. The experimental design and the responses are given in Table 1. 17. The coefficients for the actual functional relations for predicting responses (Y_i) are presented in Table 1.18. The insignificant terms were omitted based on Student's t-ratio (Khuri and Cornell, 1987). The responses under different combinations as defined in the design (Table 1.17 and 1.18) were analyzed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is given in Table 1.19.

1.3.2.2 Optimization of variables

The maximum values of second order polynomial equations (Eqn. 1) for biomass, total lipids and arachidonic acid yields (Y_1, Y_2 and Y_3, based on the coefficients provided in Table 1.18 is presented in Table 1.20. The independent variables such as glucose (X_1), corn solids (X_2), KH_2PO_4 (X_3) and KNO_3 (X_4) were 10.0 g/L (coded value -2.0), 5.0 g/L (coded value -2.0), 1.0 g/L (coded value -2.0) and 1.0 g/L (coded value -2.0) for maximum arachidonic acid production (1.39 g/L) and the corresponding biomass and total lipid were 12.49 and 5.87 g/L, respectively.

1.3.2.3 Response surface plotting

The effect of glucose, corn solids, KH_2PO_4 and KNO_3 on biomass, total lipids, and arachidonic acid production are reported by the coefficients of second order polynomials. A few response surfaces based on these coefficients for statistically significant interactions are shown in Fig. 1.5a and 1.5b. Two independent variables were kept at the condition when the arachidonic acid production was highest while the other two variables were varied within the experimental range and their effect on biomass yield, total lipids and arachidonic acid production was studied. In general, exploration of the response surfaces indicated a complex interaction between the variables. The experimental values were not statistically significant from the predicted values ($p \leq 0.05$).

Table 1.17 Central composite rotatable design and responses for biomass, total lipid and arachidonic acid production

Exp. No.	Glucose (g/L) x_1 (X_1)	Corn solids (g/L) x_2 (X_2)	KH_2PO_4 (g/L) x_3 (X_3)	KNO_3 (g/L) x_4 (X_4)	Biomass (g/L) Y_1	Lipid (g/L) Y_2	Arachidonic acid (g/L) Y_3
1	27.5(-1)	13.75(-1)	2(-1)	2(-1)	11.73	4.26	0.87
2	62.5(1)	13.75(-1)	2(-1)	2(-1)	10.30	4.16	0.73
3	27.5(-1)	31.25(1)	2(-1)	2(-1)	14.93	3.14	0.56
4	62.5(1)	31.25(1)	2(-1)	2(-1)	15.73	3.78	0.62
5	27.5(-1)	13.75(-1)	4(1)	4(-1)	10.80	3.46	0.70
6	62.5(1)	13.75(-1)	4(1)	4(-1)	9.23	3.10	0.56
7	27.5(-1)	31.25(1)	4(1)	4(-1)	14.90	3.50	0.58
8	62.5(1)	31.25(1)	4(1)	4(-1)	15.33	3.83	0.58
9	27.5(-1)	13.75(-1)	2(-1)	2(1)	10.80	3.78	0.68
10	62.5(1)	13.75(-1)	2(-1)	2(1)	8.70	2.96	0.51
11	27.5(-1)	31.25(1)	2(-1)	2(1)	16.90	4.23	0.66
12	62.5(1)	31.25(1)	2(-1)	2(1)	15.80	3.32	0.58
13	27.5(-1)	13.75(-1)	4(1)	4(1)	11.13	3.95	0.74
14	62.5(1)	13.75(-1)	4(1)	4(1)	9.70	2.90	0.54
15	27.5(-1)	31.25(1)	4(1)	4(1)	14.63	5.12	0.76
16	62.5(1)	31.25(1)	4(1)	4(1)	16.47	5.04	0.81
17	10(-2)	22.5(0)	3(0)	3(0)	10.50	2.79	0.56
18	80(2)	22.5(0)	3(0)	3(0)	10.67	2.57	0.45
19	45(0)	5(-2)	3(0)	3(0)	12.87	2.57	0.46
20	45(0)	40(2)	3(0)	3(0)	18.50	3.89	0.53
21	45(0)	22.5(0)	1(-2)	3(0)	13.50	3.62	0.58
22	45(0)	22.5(0)	5(2)	3(0)	13.10	3.95	0.66
23	45(0)	22.5(0)	3(0)	1(-2)	12.73	4.07	0.77
24	45(0)	22.5(0)	3(0)	5(2)	13.67	3.98	0.79
25	45(0)	22.5(0)	3(0)	3(0)	13.17	3.83	0.62
26	45(0)	22.5(0)	3(0)	3(0)	12.70	3.66	0.60
27	45(0)	22.5(0)	3(0)	3(0)	13.16	3.65	0.60
28	45(0)	22.5(0)	3(0)	3(0)	13.47	3.81	0.63
29	45(0)	22.5(0)	3(0)	3(0)	13.43	3.92	0.65
30	45(0)	22.5(0)	3(0)	3(0)	13.10	3.81	0.62
31	45(0)	22.5(0)	3(0)	3(0)	13.23	3.91	0.64

$X_1 = (x_1 - 45)/17.5$; $X_2 = (x_2 - 22.5)/8.75$; $X_3 = (x_3 - 3)/1$; $X_4 = (x_4 - 3)/1$
KH_2PO_4- Potassium dihydrogen phosphate; KNO_3-Potassium nitrate

Table 1.18 Estimated coefficients for the fitted second-order polynomial representing the relationship between the response and process variables

Coefficient	Biomass (g/L)	Lipid (g/L)	Arachidonic acid (g/L)
a_0	13.180 [a]	3.797 [a]	0.622 [a]
a_1	-0.176 [ns]	-0.116 [c]	-0.034 [a]
a_2	2.232 [a]	0.251 [a]	-0.002 [ns]
a_3	-0.146 [ns]	0.081 [ns]	0.010 [ns]
a_4	0.128 [ns]	0.079 [ns]	0.005 [ns]
a_{11}	-0.690 [a]	-0.220 [a]	-0.020 [c]
a_{22}	0.585 [c]	-0.084 [ns]	-0.023 [b]
a_{33}	-0.012 [ns]	0.056 [ns]	0.009 [ns]
a_{44}	-0.037 [ns]	0.116 [c]	0.048 [a]
a_{12}	0.531 [b]	0.145 [c]	0.041 [b]
a_{13}	0.194 [ns]	0.001 [ns]	0.002 [ns]
a_{14}	-0.064 [ns]	-0.211 [b]	-0.011 [ns]
a_{23}	-0.085 [ns]	0.299 [a]	0.034 [b]
a_{24}	0.290 [c]	0.303 [a]	0.054 [a]
a_{34}	0.135 [ns]	0.261 [a]	0.048 [a]

[a] Significant at 0.1%, [b] Significant at 1.0%, [c] Significant at 5.0%
[ns] Not significant even at 5% level

Table 1.19 Analysis of variance for the fitted second order polynomial model and lack of fit as per CCRD

Source of variation	df	Sum of squares		
		Biomass (g/L)	Lipid (g/L)	Arachidonic acid (g/L)
Regression				
First order terms	4	121.17[a]	2.14[a]	0.03[a]
Second order terms	10	33.11[a]	7.30[a]	0.24[a]
Total	14	154.28	9.44	0.27
Residual				
Lack of fit	11	12.20[ns]	1.15[ns]	0.03[ns]
Pure error	6	0.39	0.07	0.00
Total error	17	12.58	1.22	0.03
Grand total	31	166.86	10.66	0.30
Coefficient of determination (R^2)		0.93	0.89	0.90

[a] Significant at 0.1%, [b] Significant at 1.0%, [c] Significant at 5.0%
[ns] Not significant even at 5% level

Table 1.20 Predicted and experimental values of response at the optimum condition

Independent variables				Responses		
Glucose (g/L)(X_1)	Corn flour (g/L) (X_2)	KH_2PO_4 (g/L) (X_3)	KNO_3 (g/L) (X_4)	Biomass, g/L (Y_1)	Total lipids, g/L (Y_2)	ARA g/L (Y_3)
Conditions for maximum biomass (Y_1)						
59.53	40.00	5.00	5.00	**21.59**	8.067	1.34
(0.83)	(2.00)	(2.00)	(2.00)	(21.50±0.15)	(8.79 ± 0.10)	(1.31 ± 0.01)
Conditions for maximum total lipid (Y_2)						
35.27	40.00	5.00	5.00	20.26	**8.49**	1.31ns
(-0.56)	(2.00)	(2.00)	(2.00)	(20.02±0.25)	(8.52 ± 0.20)	(1.28 ± 0.05)
Conditions for maximum arachidonic acid (Y_3)						
10.0	5.0	1.00	1.0	12.49	5.87	**1.39**
(-2.0)	(-2.0)	(-2.0)	(-2.0)	(12.50±0.05)	(5.75 ± 0.10)	(1.41 ± 0.03)

No significant difference between the predicted and experimental values ($p \leq 0.05$).

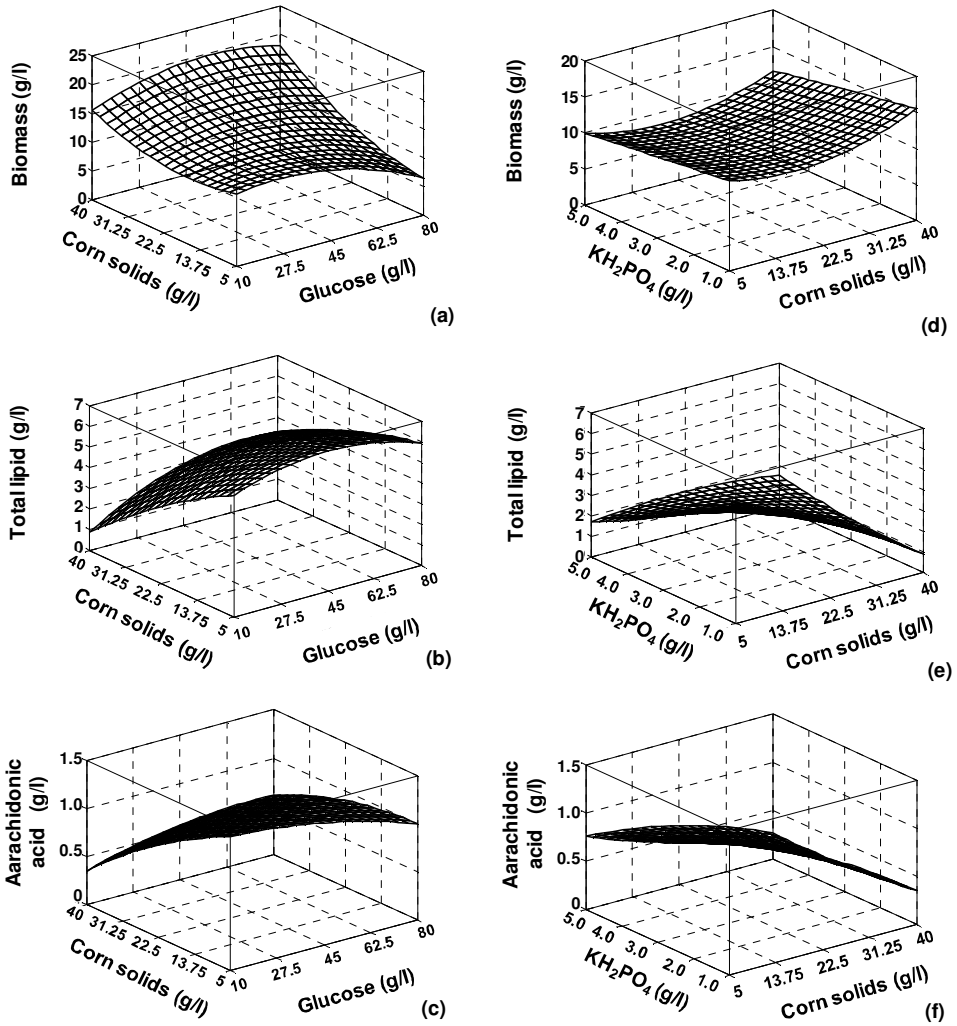

Fig. 1.5a Effect of variables on biomass, total lipid and arachidonic acid production.

Glucose and corn solids concentration (a, b, c); corn solids and KH_2PO_4 concentration (d, e, f)

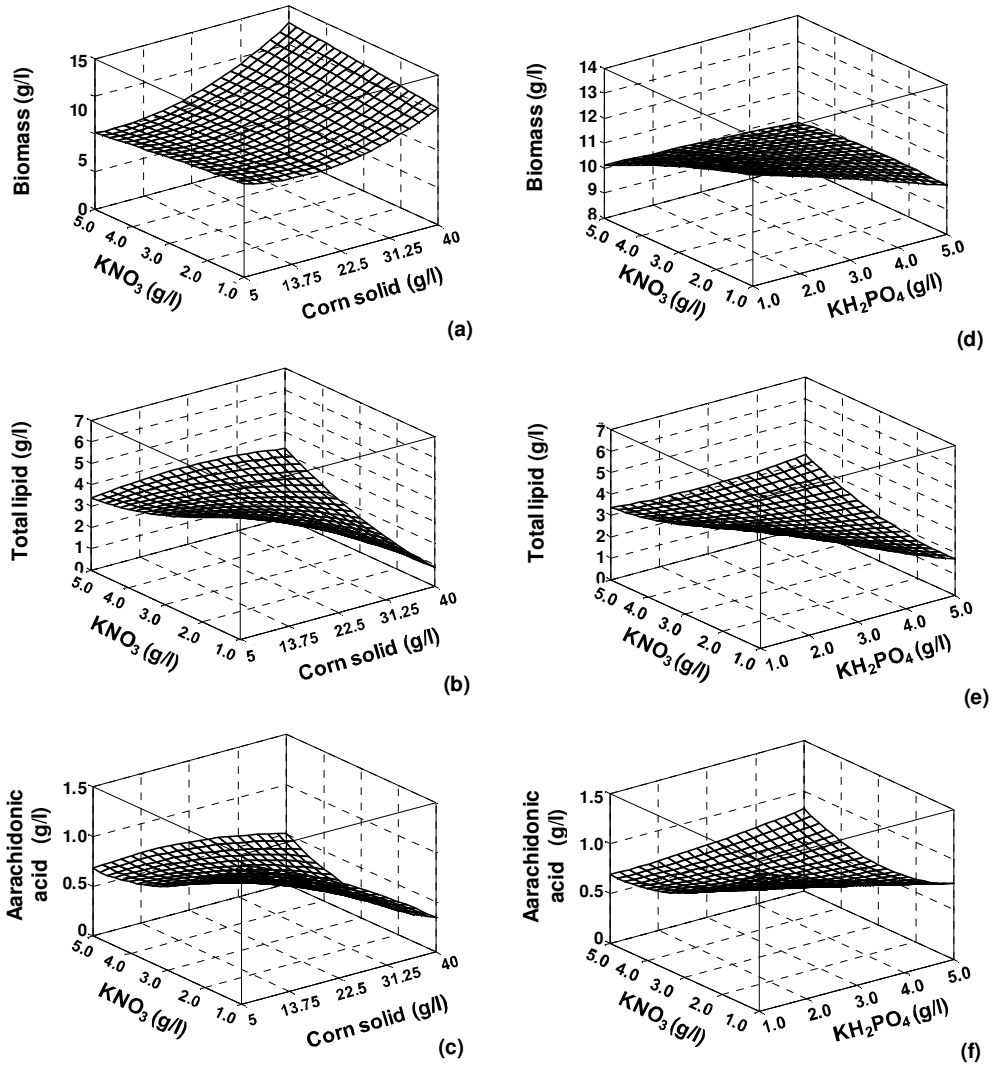

Fig. 1.5b Effect of variables on biomass, total lipid and arachidonic acid production.

KNO_3 and cornsolids concentration (a,b,c); KNO_3 and KH_2PO_4 concentration (d,e,f)

1.3.2.4 Effect of glucose and corn solids concentration

At lowest level of corn solids (5.0 g/L), the biomass and arachidonic acid were found to decrease from 12.49 to 6.5 g/L and 1.39 to 1.0 g/L, respectively with an increase in glucose concentration from 10 to 80 g/L, whereas no significant change in total lipid concentration was observed.

At the highest level of corn solids (40 g/L), biomass yield (15.5 to 18.04 g/L), total lipids (0.9 to 3.27 g/L), and arachidonic acid production (0.35 to 0.62 g/L) were found to increase with an increase in glucose concentration from 10 to 80 g/L (Fig. 1.5a a, b and c). Similarly, for all the levels of glucose concentration (10 to 80 g/L), increase in corn solids from 10 to 40 g/L resulted in an enhancement in the biomass yield, whereas total lipids and arachidonic acid were decreased (Fig. 1.5a a, b and c).

1.3.2.5 Effect of corn solids and KH_2PO_4 concentration

At lowest level of corn solids (5.0 g/L), an increase in KH_2PO_4 concentration (from 1.0 to 5.0 g/L) resulted in a decline in the biomass (12.49 to 6.5 g/L), total lipid (5.87 to 1.71 g/L) and arachidonic acid (1.39 to 1.0 g/L) production. At the highest level of corn solids (40 g/L), biomass and arachidonic acid yields were found to decrease from 15.53 to 11.64 g/L and 0.35 to 0.26 g/L, respectively with an increase KH_2PO_4 concentration from 1.0 to 5.0 g/L, whereas total lipids was found to increase from 0.9 to 1.52 g/L (Fig. 1.5a d, e and f). Similarly, for all the levels of KH_2PO_4 concentration (1.0 to 5.0 g/L), increase in corn solids from 10 to 40 g/L resulted in an increase in the biomass yield, whereas total lipid and arachidonic acid were decreased (Fig. 1.5a d, e and f).

1.3.2.6 Effect of corn solids and KNO_3 concentration

For all the levels of KNO_3 concentration (1.0 to 5.0 g/L), biomass yield was found to increase and arachidonic acid was found to decrease with an increase in corn solids from 5.0 to 40.0 g/L. Total lipid production decreased (from 5.87 to 0.90 g/L) and remained almost constant for lowest (1.0 g/L) and highest (5.0 g/L) concentrations of KNO_3, respectively (Fig. 1.5b a, b and c).

At lowest level of corn solids (5.0 g/L), the biomass, total lipid and arachidonic acid were found to decrease from 12.49 to 10.11 g/L, 5.87 to 3.36 g/L and 1.39 to 1.0 g/L, respectively with an increase in KNO_3 concentration from 1.0 to 5.0 g/L. At the highest level of corn solids (40 g/L), biomass yield (15.53 to 17.79 g/L),

total lipids (0.9 to 3.24 g/L), and arachidonic acid production (0.35 to 0.50 g/L) were found to increase with an increase in KNO_3 concentration from 1.0 to 5.0 g/L (Fig. 1.5b a, b and c).

1.3.2.7 Effect of KH_2PO_4 and KNO_3 concentration

At lowest level of KH_2PO_4 concentration (1.0 g/L), the biomass yield, total lipid and arachidonic acid were found to decrease from 12.49 to 10.11 g/l, 5.87 to 3.36 g/L and 1.39 to 0.68 g/L, respectively with an increase in KNO_3 concentration from 1.0 to 5.0 g/L. KH_2PO_4 proved to be a better nitrogen source than KNO_3 and was better assimilated by the fungus. At the highest level of KH_2PO_4 concentration (5.0 g/L), biomass yield was found to decrease from 9.96 to 9.74 g/L, however, total lipids and arachidonic acid production were found to increase from 1.71 to 3.37 g/L and 0.76 to 0.82 g/L, respectively with an increase in KNO_3 concentration from 1.0 to 5.0 g/L (Fig. 1.5b d, e and f).

Similarly, at lowest level of KNO_3 concentration (1.0 g/L), the biomass yield, total lipid and arachidonic acid were found to decrease from 12.49 to 9.96 g/L, 5.87 to 1.71 g/L and 1.39 to 0.76 g/L, respectively with an increase in KH_2PO_4 concentration from 1.0 to 5.0 g/L. At the highest level of KNO_3 concentration (5.0 g/L), biomass yield was found to decrease from 10.11 to 9.74 g/L, whereas, arachidonic acid production was found to increase (from 0.68 to 0.82 g/L) and no significant change in total lipid with an increase in KH_2PO_4 concentration from 1.0 to 5.0 g/L (Fig.1.5b d, e and f).

1.3.2.8 Verification of results

Suitability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions was determined to be optimum, which was also used to validate experimentally and predict the value of the responses using model equations. The experimental value was found to be in agreement with the predicted ones ($P \leq 0.05$ Table 1.20).

1.4 Discussion

The effects of various factors such as media composition, initial pH of the cultivation media, temperature, carbon sources, nitrogen sources, oil as sole carbon source, glutamate supplementation and fed batch culture on biomass, total lipid, and arachidonic acid production by *Mortierella alpina* was investigated by the traditional "one-factor-at-a-time" (OFAT) design, where the experimental design involved the

step-by-step improvement of each factor in an individual and non simultaneous manner, while maintaining the other factors at constant values (Moen *et al*., 1999).

Media composition is a major contributor for microbial biomass and metabolite formation. Cultivation media had a statistically significant influence on biomass and arachidonic acid production in this fungus. Reduced efficacy of potatao dextrose broth (PDB) in biomass and total lipid production is attributed to the presence of dextrose in addition to its potato starch and absence of an efficient nitrogen source like yeast extract. Results indicated that glucose when used at 2-4% level proved to be a better carbon source than malt extract as indicated by the lower lipid and ARA in malt extract broth. Results of the present investigation corroborated well with that of other studies in *Mortierella* species (Bajpai *et al*., 1991c; Shinmen *et al*., 1989), where GY medium proved to be the most effective for ARA production.

The pH of the medium was very crucial for growth and metabolite production in *Mortierella*. Our results are in agreement with that of Lindberg and Molin (1993) who previously recorded that *M. alpina* did not grow at pH 8.5 and that the dry biomass was lower at pH 7.5 compared to that recorded at pH 6.5. This can be possibly due to some media-pH interactions which reduce the efficiency of the strain to utilize the carbon source provided. Many investigators claimed that diverse morphology of fungal mycelia under a different initial pH value was a critical factor in biomass accumulation and metabolite formation (Shu and Lung, 2004; Wang and Mc Neil, 1995). Most fungi require more acidic pH optima for growth in submerged culture and that an increase in initial pH was found to stimulate the accumulation of metabolites (Shu and Lung., 2004; Wang and Mc Neil, 1995).

Temperature is one of the most important environmental factors affecting the growth of microorganisms, and causes changes in many biosynthetic pathways. In the present study, profuse growth of *M. alpina* was observed at temperatures ranging from 25° to 30°C. The highest arachidonic acid % in total lipids occurred at 25°C where as the maximum ARA yield was obtained at 28°C. At lower temperature, the fungus produced more eicosapentaenoic acid (EPA). Chen *et al*. (1997) reported maximum ARA production for Wuji H-4 isolate (*M. alpina* strain) at 24ºC. This can be attributed to the proposed adaptive role of PUFAs in membrane stabilization under stress conditions of low temperatures. PUFAs including ARA and EPA may play an important role in the regulation of membrane fluidity in this organism, thereby

compensating for the decreased functionality of the biomembranes under cold stress conditions. The regulation of fatty acid saturation by desaturase enzymes is known as homeoviscous adaptation wherein the organism adjusts the membrane fluidity to maintain the optimal function of biological membranes. Another possible explanation is that at lower temperature more dissolved oxygen is available in the culture medium for desaturase enzymes that are oxygen dependent (Cohen *et al*., 1987) thereby resulting in production of more unsaturated fatty acids. Colla *et al*. (2008) have shown temperature to be the most important variable for biomass concentration in *Rhodotorula dairenensis.*

In the present study, the most effective carbon source for ARA production was glucose. It was evident that *Mortierella alpina* effectively utilized simple sugars as reported by Kendrick (2001). This may be due to the fact that generally fungi belonging to *Mortierellales* are saprophytes which prefer to grow rapidly and proliferate extensively on simple sugars compared to complex molecules. This is in contradiction to the result obtained in several fungi where supplementing with a complex carbon source promoted mycelial growth and highly enhanced the production of fungal metabolites. Our results are in agreement with that of Dyal *et al*. (2005) in *M. ramanniana* that both fructose and glucose containing media yielded much higher biomass. A higher concentration of glucose was not conducive for lipid production. This may be due to the intolerance of the cells to high concentrations of glucose since it increased the osmotic potential of the medium. Lower glucose concentrations were also not effective. It is possible that different carbon sources have different effect of catabolic repression on the cellular secondary metabolism. Although rhamnose yielded higher ARA in total lipids, biomass and lipid production was comparatively lower. In addition, rhamnose is an expensive sugar and hence it is not feasible to produce ARA on a commercial basis using this sugar as the carbon source.

The type of nitrogen source in the cultivation media had a profound effect on ARA production. Exclusion of nitrogen greatly affects fungal growth and metabolite production, but in most oleaginous organisms, depletion of nitrogen after growth is a prerequisite for lipid accumulation (Wynn *et al*., 2001; Venkateswaran *et al*., 1992). Biomass production diminished in medium comprising ammonium nitrate, sodium nitrate or ammonium sulphate as the nitrogen source suggesting that *M. alpina* hardly

assimilate inorganic nitrogen and require amino acid or protein for high biomass buildup. According to Weete (1980), organic nitrogen sources are the best for growth of many fungi. A number of researchers have studied the effect of nitrogen source on biomass and lipid yield of various *Mortierella* fungi. In all the cases yeast extract proved to be the best nitrogen source for maximizing biomass, lipid and ARA yields. These investigations used 2% dextrose as the carbon source and indicated that 1% yeast extract was the optimum. Yeast extract is an excellent nitrogen source for profuse growth of fungi owing to the presence of metal ions and required micronutrients which are vital for the growth of fungi. The total lipid content in biomass was higher when peptone was used as the nitrogen source. This is comparable to several previous reports which show that peptone reduces the lag phase of growth and promote metabolite production in submerged culture. The nature and concentration of nitrogen source used in the medium is an essential factor regulating lipogenesis (Certik *et al*., 1999). Rise in lipid accumulation closely parallel to the use of organic nitrogen is possibly due to increase in acetyl CoA carboxylase (ACC) activity. A high degree of oleaginicity depends on the rate of ACC by which acetyl CoA is efficiently converted to malonyl CoA providing the backbone for lipid synthesis. The rapidly increased malic enzyme (ME) activity in the presence of organic nitrogen and the close relationship among ACC, ME, and lipid accumulation imply that ME is closely bound with lipid synthesis and channels the reducing power into condensation reaction of acetyl CoA to yield fatty acids (Certik *et al*., 1999). Potassium nitrate was better than the ammonium salts for biomass lipid and ARA production. Chen *et al*. (1997) who worked on Wuji-H4 isolate (*M. alpina* strain) concluded potassium nitrate to be superior to the ammonium salts. In the present study, ARA yield was drastically reduced when urea was used as the nitrogen source. This may be due to the fact that urea acts as an inhibitor for Δ^5 desaturase, a key enzyme which catalyzes the conversion of Dihomo-γ-Linolenic acid to ARA (Obukowicz *et al*., 1998). Natural nitrogen sources change markedly the morphology of *Mortierella alpina* in submerged culture. It was noticed that yeast extract when used as the nitrogen source induced the formation of fluffy circular pellets whereas other nitrogen sources induced filamentous mycelial growth.

Biomass production was considerably reduced when vegetable oils were used as the sole carbon source in place of glucose in the cultivation media. This can be

attributed to the reduced efficacy of the fungus to assimilate oils when compared to simple carbon sources like glucose. Carbohydrates are usually metabolized via the Embden –Meyerhoff pathway to generate pyruvate or acetyl –CoA. Utilization of oils by microorganisms is accompanied with production of extracellular lipases which cleave fatty acid residues from glycerol and the fatty acids produced can either be incorporated to lipid structures or degraded to basic skeletons serving the biomass synthesis (Akhtar, 1983; Esfhani *et al*., 1981). Amendment of oil resulted in a substantial increase in the percentage of total lipids in biomass when compared to glucose supplemented media. Growth of fungi in a carbohydrate –containing medium after optimization of cultural conditions results in a constant lipid yield with the demanded fatty acid profile. Oil addition to the medium usually not only increases lipid accumulation in the fungal cell but the composition of the accumulated lipid reflects the length of carbon source and structure of oil source (Koritala *et al*., 1987). In all the oils tested in this study there was a significant decrease in the ARA content. Several studies have clearly indicated that Δ^5 desaturase, the enzyme which catalyze the conversion of DGLA to ARA could be inhibited by the presence of certain oils in the growth media. Inhibition of PUFA production by oils has earlier been reported. When microorganisms are grown on oils there is generally little change in the fatty acid profile of added oil and the organism appears to cease desaturation and elongation of the presented fatty acids (Ratledge, 1989). Thus growing microorganisms on the precursors of PUFA does not enhance PUFA formation but leads to cessation of PUFA production (Kendrick and Ratledge, 1996). The nature of oil added to the medium plays a very important role in its utilization by microorganisms. Kendrick and Ratledge (1996) reported that fungi grown on extracellular oils have a much decreased capacity for PUFA biosynthesis due to strong repression of the desaturases and elongases. This study indicated that sesame oil when used in place of glucose resulted in a markedly lower cell and ARA yield but lipid yield was not significantly reduced. Sesamin contained in sesame oil was found to be a potent inhibitor of Δ^5 desaturase thereby preventing the conversion of DGLA-ARA (Shimizu *et al*., 1999; Shimizu *et al*., 1991).

Glutamate supplementation significantly improved ARA production in the strain investigated. Glutamate is involved in nitrogen metabolism and is required as an essential precursor of protein and nucleotide synthesis as well as a substrate for

energy metabolism (Wice *et al*., 1981). Glutamate also stimulates aerobic glycolysis thereby influencing cell growth. Furthermore it play a role in activating acetyl CoA carboxylase (ACC) leading to formation of malonyl CoA, an essential substrate for fatty acid synthase and fatty acid elongation systems (Kowluru *et al*., 2001). However supplementation of greater than 0.8g/L glutamate led to a decline in total lipids and ARA as reported by Yu *et al*. (2003). This can be ascribed to the fact that at higher concentrations, glutamate will be converted to proline which is accompanied by NADPH consumption (Andarwulan and Shetty, 1999) leading to a decline in ARA.

A higher ARA yield was obtained with fed batch culture in *M. alpina.* This has been used as an important strategy for improving ARA in *Mortierella.* Since excess carbon in a nitrogen limiting media is converted to lipids, carbon concentration is very crucial for maximized production of total lipids and ARA. On the contrary, low initial glucose concentration is beneficial for fungal growth and shortens the lag phase after inoculation. When glucose concentration is high, osmotic potential is increased which prevents the growth of fungi. To circumvent this, glucose was replenished after the initial growth period thereby achieving higher total lipid and ARA yield.

The one-factor-at-a-time approach though widely used for optimizing process variables, often leads to misinterpretation of results when interactions between factors are prevalent. Hence Response surface methodology (RSM), a powerful tool and efficient mathematical approach (Xin *et al*., 2005; Murthy *et al.,* 2000), based on the fundamental principles of statistics, was employed for further improvement in ARA production. Attempts were made to replace yeast extract in the production medium with a cheaper alternative ie., corn solids. Since phosphate and nitrate ions were important for ARA production in *Mortierella,* KH_2PO_4 and KNO_3 were incorporated in the media and interaction of these variables with glucose and corn solids were studied in a 2^4 factorial experiment.

From the results it is evident that the first and second (quadratic and cross product) order terms are significant while the lack of fit was not significant for the biomass, total lipids and arachidonic acid yields. The lack of fit measures the failure of the model to represent data in experimental domain at points, which are not included in the regression. The high values of coefficient of determination (R^2) also

suggested that the model is a good fit. The R^2 is proportion of variability in response values explained or accounted for by the model (Montgomery, 1984; Myers, 1971).

At lowest level of corn solids, biomass and ARA decreased with an increase in glucose concentration. With sufficient nitrogen in the medium, biomass production was a function of the amount of carbon source provided and lipid accumulation commences at the onset of nitrogen depletion. It has been reported that low initial concentration of glucose was conducive for fungal growth (Zhu *et al*., 2006). This may be due to repression of growth at higher glucose concentrations due to increased osmotic potential.

There was an increment in biomass yield for all glucose concentrations when the level of corn solids was increased. However under these conditions total lipid and ARA were decreased. It is evident that a combination of corn solids and glucose had complementary effects in promoting cell growth in *Mortierella alpina*. A combination of corn flour and glucose contributed to the highest mycelia yield by *Hericium erinaceus* was previously reported (Huang *et al*., 2007). This is an attestation of the fact that corn solids serves as an efficient carbon source (Liu *et al*., 2009; Shi *et al*., 2009; Altaf *et al*., 2007) in addition to its role as nitrogen source. Corn flour carbon in addition to the carbon supplied as glucose probably aided in biomass buildup. It was noted that similar to most oleaginous organisms, lipid synthesis in *Mortierella alpina* was not upregulated by a surfeit of nitrogen. PUFAs are accumulated due to a metabolic shift in Kreb's cycle caused by activation of adenosine triphosphate (ATP) dependent citrate lyase. Therefore a very important prerequisite for lipid over production is the metabolic shift caused by nitrogen depletion (Hall and Ratledge, 1977). It is apparent from our studies that lipid biosynthesis was unregulated by surplus nitrogen provided as corn solids.

At lowest level of corn solids, an increase in KH_2PO_4 concentration resulted in a decrease in biomass, total lipid and ARA. At high carbon/ nitrogen ratios, the biomass production decreased with increasing KH_2PO_4. A similar result was obtained by Park *et al.* (2005) in *Rhodotorula glutinis*. KH_2PO_4 possibly had an inhibitory effect on lipid and ARA production when used at high levels.

At low to moderate C/N ratios, increases in KH_2PO_4 led to decreased production of cell biomass and ARA whereas total lipid production was increased.

Carbon utilization was switched from cellular growth to lipid biosynthesis under these conditions.

For all the levels of KH_2PO_4, increase in corn solids resulted in an increase in biomass whereas total lipid and arachidonic acid was decreased. Biomass yield tends to increase when provided with sufficient nitrogen. Corn solids and KH_2PO_4 provided the nitrogen flux essential for a high biomass buildup but could not accelerate the production of total lipids which occur in a nitrogen depleted media. A decrease in total fatty acid content is attributed to a differential increment in cell division and subsequent reduction in the rate of lipid synthesis.

Addition of excess nitrogen in the form of KNO_3 did not accelerate cell growth when corn solid concentration was minimal. *M. alpina* being a saprophyte hardly assimilates inorganic nitrogen source and requires amino acids and proteins for high biomass buildup (Nisha and Venkateswaran, 2009). Growth rate of mycelia in N-free media stopped because of nutrition limitation resulting in reduced biomass yield. The degree of oleaginicity is dependent on the rate at which acetyl CoA carboxylase converts acetyl CoA to malonyl COA, thus providing the backbone of lipid synthesis (Certik *et al*., 1999). Nitrogen starvation imposed a reduction in cellular soluble protein content, variation in fatty acid composition and reduction in the *in vitro* activity of the enzyme acetyl CoA carboxylase (Livne and Sukenik, 1992) thereby reducing lipid yield.

Conclusions

From the above study it can be concluded that the biomass buildup, oleaginicity and arachidonic acid production in *M. alpina* strain CBS 528.72 was influenced by several cultural parameters viz., media composition, carbon and nitrogen source, cultivation temperature, pH etc. The results indicated that under the conditions mentioned in this study, highest arachidonic acid production was achieved in GY medium (2% glucose and 1% yeast extract) at a pH of 6.5 and temperature of 28°C. Glutamate supplementation and fed batch culture had a significant positive effect on lipid and ARA yield. A drastic reduction was noticed in ARA and biomass yield when vegetable oils were used instead of glucose as the sole carbon source. The ideal conditions when employed triggered the enzymes in ARA biosynthetic pathway leading to higher production.

For improving ARA production further, statistical method of optimization was employed and interaction between different factors was investigated. Experimental results illustrated that RSM was an efficient method to obtain optimized parameters for maximum production of arachidonic acid. Exploration of the response surfaces indicated a complex interaction between the variables such as glucose, corn solids, KH_2PO_4 and KNO_3 concentrations. The optimum conditions when employed resulted in maximum production of arachidonic acid (1.39 g/L) and the corresponding biomass and total lipid were 12.49 and 5.87 g/L, respectively. The CCRD together with response surface methodology provided insight into the interaction and identified the optimum combination of variables (within a specified range) for maximized arachidonic acid production with the help of a relatively small number of experiments, thus reducing the time and cost of the study. The use of corn solids as a cheaper alternative for yeast extract also tremendously reduced the cost of the optimized process.

2.1 Introduction

Lipids play a vital role in the cells, where they occur as heterogeneous compounds with diverse structures and properties. Complete isolation of lipids from cells is difficult and is wholly dependent on the method of extraction employed. As the fungal cultures have a rigid cell wall, standardization of extraction method is pivotal for total lipid recovery. A wide range of extraction procedures have been used for lipid extraction from filamentous fungi.

The biomass, total lipid and fatty acid profile of oil are important parameters for the development of a successful single cell oil production process for ARA. Owing to the increased demand of ARA single cell oil for inclusion in infant and geriatric food formulations, attempts are being made over the past two decades for rapid and efficient extraction of ARA rich oil. Being an intracellular product, a suitable extraction method is required for maximum lipid and ARA yield.

Although pure lipids are soluble in a wide variety of organic solvents, many of these are not suitable for lipid extraction from fungal cells. Since PUFA rich *M. alpina* oil is intended for use in pharmacological, medical and food applications, solvents should be selected that are acceptable in terms of toxicity, handling, safety and cost. Most widely used solvent system for lipid extraction from tissues is a combination of chloroform and methanol. Bligh and Dyer (1959) as well as Folch (1957) employed this solvent system for extraction from wet and dry tissues respectively. Jin *et al*. (2009), Zhu *et al.* (2002), Singh and Ward (1997a), Bajpai *et al*. (1991c) and Totani and Oba (1987) used chloroform: methanol as the solvent for extracting *M. alpina* lipids.

In most of the commercial oil extraction processes hexane is used as the solvent. Hexane has been preferred over other solvents due to its high solubility for fats and oils and its chemical stability. Solvent extraction with hexane is a widely used approach for obtaining edible oils because of its low cost and efficiency in terms of oil and solvent recovery (Gaur *et al*., 2007). The search for alternative solvents was prompted by the 1990 Amendments to the Clean Air Act, which listed n-hexane as a hazardous air pollutant. Isopropanol is one of the most favourable alternatives to hexane for oil extraction. Compared to hexane and other solvents like ethanol, it is less flammable and less toxic and is free of restrictive governmental regulations.

Although organic solvent extraction has been widely used for production of single cell oils, a major problem persists due to the oxidative susceptibility of highly unsaturated fatty acids. PUFAs degrade at high-temperature, oxygen-rich conditions typical of conventional hot solvent-extraction and distillation methods (Walker *et al*., 1999). Furthermore there is an increasing global concern to minimize the use of organic solvents, particularly the chlorinated ones because of their suspected human toxicity. Supercritical fluid extraction (SFE) has received increased attention as an important alternative to conventional separation methods because it is simpler, faster, efficient and avoids consumption of large amounts of organic solvents which are often expensive and potentially harmful (Gouveia *et al*., 2007). SFE can eliminate costly downstream processing such as caustic refining and bleaching that are necessary when using liquid solvents. When the solute becomes dissolved in supercritical fluids, they exhibit a higher diffusability than they do in lipids, facilitating rapid mass transfer of the solutes from the matrix. For these reasons they become alternative solvents for lipid extraction. Most of the supercritical extraction processes utilize CO_2 as the extraction fluid since this is the most economical and compatible fluid. Moreover when CO_2 is used, the extraction process is relatively benign permitting the removal of heat and oxygen sensitive compounds thereby avoiding molecular alteration. Carbon dioxide is an ideal supercritical fluid because of its environmentally benign, non-toxic, non-flammable, non-polluting, recoverable characteristics and its ability to solubilize lipophilic substances (Sahena *et al*., 2009). Super critical CO_2 extraction is suitable for extraction of non polar compounds with molecular weights less than 400 (Saykhedkar and Singhal, 2008) and would be appropriate in the perspective of 'green technology'.

SFE has been employed for extraction of several PUFAs particularly γ-linolenic acid from *Cunninghamella echinulata* (Certik and Horenitzky, 1999), *Spirulina platensis* (Sajilata *et al*., 2008) and seeds (Guil-Guerrero *et al*., 2008). CO_2 was employed for extraction of oil from fungi *Mortierella ramanniana* var. *angulispora* under supercritical conditions (Sakaki *et al*., 1990). Cygnarowicz-Provost *et al.* (1994) effectively extracted fungal lipids by supercritical CO_2. Extraction of lipids from fermented LC-PUFA rich microorganisms thus holds great promise in SCO industry. In the present investigation *M. alpina* SCO was extracted by diverse methods employing organic solvents and CO_2 and their differential ability for lipid

recovery from the intact and treated biomass was assessed. The effect of Solvent/solvent systems on lipid extraction efficacy extraction was also investigated.

2.2 Materials and methods

2.2.1 Chemicals

Methyl esters of fatty acids, PPG and lytic enzyme were purchased from Sigma chemicals, USA. Glucose and yeast extract were procured from High Media Pvt. Ltd., Mumbai, India. Solvents used were of high purity obtained from Qualigens Fine Chemicals, India. Food grade supercritical carbon dioxide was obtained from Kiran Corp, Mysore, India.

2.2.2 Microorganism and culture maintenance

Mortierella alpina CBS 528.72 was cultivated in GY medium for a period of seven days as described in section 1.2.2.1. In all extractions except Bligh and Dyer method the harvested mycelia was freeze dried and made to a fine powder by grinding.

2.2.3 Effect of different extraction methods

To investigate the effect of extraction methods on lipid recovery, *M. alpina* biomass was subjected to lipid extraction by diverse methods.

2.2.3.1 Organic solvent extraction

Bligh and Dyer, Folch, Soxhlet and Soxtec methods of extractions employing organic solvents were carried out with *M. alpina* biomass.

2.2.3.1.1 Bligh and Dyer method

In Bligh and Dyer method (Bligh and Dyer, 1959), the solvent used was chloroform: methanol (1:2). 2 g of biomass was homogenized with 1mL of water and 3.75 mL chloroform: methanol and vortexed for 10-15min. 1.25 mL chloroform and 1.25 mL water were subsequently added and mixed, followed by centrifugation at 2000rpm. After centrifugation, the lipids were obtained in the organic lower phase. The organic phase was taken and evaporated under vacuum in a rotary evaporator (Buchi Rotavapour R250/V, Germany) or under a stream of nitrogen.

2.2.3.1.2 Folch method

In Folch method (Folch, 1957), 2g of 0.1N HCl treated biomass was homogenized with 40 mL chloroform: methanol (2:1), agitated in an orbital shaker for 15-20 min and then filtered to remove the liquid phase. After washing with 0.9%

NaCl solution and centrifugation at 2000rpm, the lipids were obtained in the chloroform phase.

2.2.3.1.3 Soxhlet method

Powdered biomass (2g) was taken in porous cellulose thimble. The thimble containing biomass was placed in the extraction chamber of Soxhlet apparatus and extracted with hexane for 8-10 h.

2.2.3.1.4 Soxtec method

5 g of 0.1N HCl treated biomass was used for soxtec extraction (Soxtec system HT-2, 1045 Extraction unit, FOSS TECATOR). Extraction was done according to AOAC official method (AOAC, 2005). The samples were packed in thimbles and extracted with sufficient amount of hexane by boiling for 20 min and extraction for 2h.

Subsequent to extraction by all the above methods, solvent was evaporated under vacuum in a rotary evaporator (R -210 Buchi, Germany) or under a stream of nitrogen (N-EVAP ^{TM} 112 nitrogen evaporater, Organomation Associates, USA) and the percentage of total lipids was calculated as follows.

% of lipid= (F-T)/S x 100

F=Weight of the cup+ Lipid, T=Weight of the cup, S=Weight of the sample

2.2.3.2 Supercritical method

A Nova Swiss, Werke AG, Ex1000, Switzerland system in the supercritical mode was used for extraction. The powdered sample was packed in an extraction vessel. The extraction chamber was a stainless steel cell. For each extraction, 50g of ground biomass was charged in to the extraction cell. Extraction was carried out at 250 bars and 50°C. Polypropylene bottles were used for collecting the fractions. The extracted lipids were collected at regular time intervals and weight determined gravimetrically. On completion of the experiment, lipids remaining in the tubing were recovered by organic solvent washing and were considered when the final yield was calculated. Total volume of CO_2 used was also quantified by the flow meter system. After quantification, the extracted lipids were stored under nitrogen at $^{-}$20°C.

2.2.4 Effect of biomass pretreatment

To investigate the effect of pretreatment on lipid recovery, control, HCl treated and enzyme treated biomass were extracted by organic solvent as well as supercritical method.

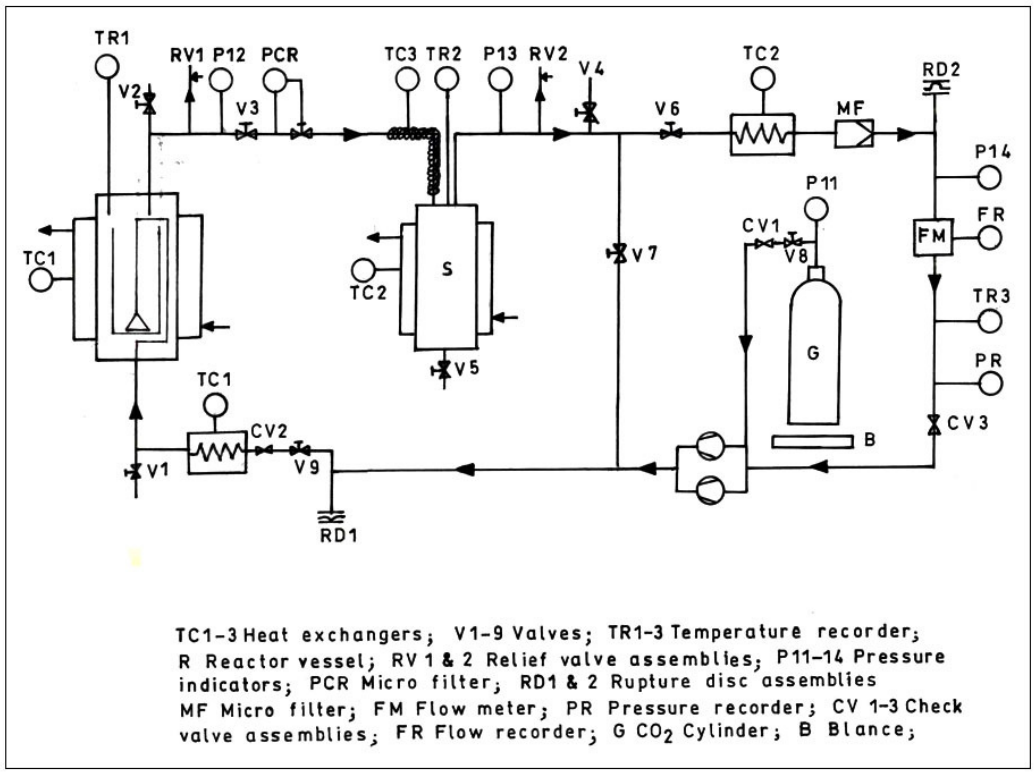

Fig 2.1 Schematic diagram of Supercritical CO_2 extraction system

2.2.4.1 Disruption of fungal cell wall and lipid extraction

Disruption of the fungal cell wall was done by a) treating with 0.1N HCl for 20 min b) treating with lytic enzyme from *Trichoderma harzianum* for a period of 12h. Subsequent to treatment, the biomass was rinsed thoroughly with distilled water and freeze dried.

The intact and treated cells were extracted using supercritical and organic solvent methods. The procedure followed for extraction is described in sections 2.2.3.2 and 2.2.3.1.4. In supercritical method, the extracted lipids were collected at regular time intervals and weight determined gravimetrically.

2.2.4.2 Modeling of mass transfer in untreated and treated cells

The extraction of lipids from the biomass differs depending on the fact that the biomass is treated or not. The treated cells behave differently to the untreated cells.

The mass transfer, essentially a diffusion controlled process is based on Fick's second law of diffusion (Udaya Sankar *et al*., 1986). Liquid diffusion in a solid can be expressed as

$$\frac{\partial C}{\partial t} = D\left[\frac{\partial C}{\partial t} + \frac{2}{r}\frac{\partial C}{\partial r}\right] \qquad \textit{Eqn (1)}$$

where C is the solute concentration, r is the radial distance of the cell, D is the diffusion coefficient and t is the time. The equation can be solved for the present case with the following secondary boundary and initial conditions.

$\partial C/\partial t = 0$ at $r = 0$ for $t>0$; $C=C_f$ at $r=r_0$ for $t>0$; $C=C_i$ at $0<r<r_0$ for $t=0$

The first boundary equation specifies a finite concentration at the centre of the sphere. The second boundary equation means that the surface is at the final concentration (C_f). According to the initial condition, the initial concentration (C_i) is uniform throughout the object. Using separation of variables, equation (1) can be solved to obtain the following relationships for lipid extraction rate as a function of time.

$$\frac{C-C_f}{C_i-C_f} = \frac{6}{\pi^2}\sum_{n=1}^{n=\infty}\frac{1}{n^2}\exp\left[\frac{-D\,n^2\pi^2 t}{r_0^2}\right] \qquad \textit{Eqn (2)}$$

When diffusion of lipids has taken over a long period of time, the second and the higher terms of series can be neglected, thus eq (3) can be solved for time of extraction

$$\frac{C-C_f}{C_i-C_f} = \frac{6}{\pi^2}\exp\left[\frac{-D\,n^2\pi^2 t}{r_0^2}\right] \qquad \textit{Eqn (3)}$$

$$\ln\frac{6}{\pi^2}\left[\frac{C-C_f}{C_i-C_f}\right] = -t/\gamma \qquad \textit{Eqn (4)}$$

where

$$\gamma = \frac{r_0^2}{\pi^2 D}$$

From a plot of the equation (4) which gives a straight line, the diffusion coefficient can be calculated.

2.2.4.3 Colour measuring L, a, b values

Labscan XE Hunter *L*, *a* and *b* colour measuring system (Hunter, USA) was used to measure the colour attributes and to illustrate the colour differences between the extracts. The sample cup was placed on the instrument measurement port so that the extract will be read through the flat bottom of the cup.

2.2.4.4 Phase contrast microscopy

The untreated and treated cells were observed under a phase contrast microscope (Olympus BX 40, USA) and the percentage of lysed cells in each sample was calculated by considering the average ratio of ruptured to intact cells in different microscopic fields taken an average of 10 fields with at least colony of mean 30 cells.

2.2.4.5 Scanning electron microscopy

The control, treated and extracted biomass were examined by scanning electron microscopy (SEM) to investigate the morphological changes in fungal cells during the breaking process and extraction. For SEM analysis, the control, HCl treated, enzyme treated and extracted biomass were fixed for 50 min by incubating in a solution containing 2.5% gluteraldehyde in 0.1 M phosphate buffer. The samples were recovered and post-fixed using 1% OsO_4 in 0.1 M phosphate buffer. To improve the surface architecture, post-fixed cells were rinsed thoroughly using 0.1 M phosphate buffer, and treated with 6% thiocarbohydrazide. The cells were finally washed with double-distilled water and dehydrated through a graded series of ethanol. The moisture free samples were mounted on aluminium stubs, sputter-coated with a gold layer and used for scanning. Samples were scanned with a Leo 435 VP scanning electron microscope (Cambridge, UK) at an accelerating voltage of 15 kV.

2.2.4.6 FT-IR spectral analysis

The absorption spectra of the supercritical and organic solvent extracted oil were recorded by FT-IR spectroscopy using a high resolution FT-IR spectrometer Nicolet 5700 (Nicolet, Madison, WI, USA). Typical apodized resolution was between 500 and 4000 cm^{-1}. The spectra were recorded by placing the samples in a KBr plate.

2.2.5 Effect of solvents on lipid extraction

Frequently used solvent systems were compared to evaluate their effects on lipid yield and fatty acid composition of *M. alpina*. Lipid extraction was carried out from the macerated biomass using diverse solvent systems, which are polar, non polar or combinations of different polarity levels. Recovery of oil from the disrupted mycelia was used as a measure of extraction efficiency of the different solvents.

The extractions were carried out according to the procedure adopted by Certik *et al*. (1996) with modifications. The freeze dried biomass was made to a fine powder by grinding with neutral sand. These were then made in to a thimble, macerated with 0.1N HCl for 30 min. The macerated mycelium (2 g) was taken for each extraction. Lipid extraction was done by the following methods.

1. Fungal biomass was extracted twice by 100 mL chloroform:methanol (2:1, v/v) for 3 h at room temperature with occasional stirring. The mixture was subsequently filtered and 1.2 fold volume of 0.9% KC1 added, stirred vigorously for 1 min and centrifuged at 1500 rpm for 10 min to effect phase separation. The chloroform/lipid containing layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum.
2. Method is the same as (1), but chloroform:methanol (1:1, v/v) was used.
3. The same as (1), but chloroform: methanol (1:2, v/v) was used.
4. Method is the same as (1), but chloroform was used as a sole solvent.
5. Fungal biomass was extracted twice by 100 mL methanol for 3 h at room temperature with occasional stirring. The mixture was filtered subsequently and the extracts collected, evaporated and extracted twice with hexane (ratio 1: 1, v/v). The hexane fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum.
6. Fungal biomass was extracted two times by 100 mL hexane: isopropanol (3:2, v/v) for 3 h at room temperature with occasional stirring. After extraction, the mixture was filtered to collect extracts. KCI (0.9%) (1.2-fold total extract volume) was added, stirred vigorously for 1 min, and the mixture was centrifuged to effect phase separation. The hexane layer was filtered through anhydrous Na_2SO_4 and evaporated under vacuum.
7. Method is the same as (6), but hexane: isopropanol (4:1, v/v) was used.
8. Method is the same as (6), but hexane as a sole solvent was used.

9. Method is the same as (5), but isopropanol was used as a sole solvent.
10. Fungal biomass was extracted by 100 mL ethanol for 3 h with occasional stirring. After filtration, the biomass was again extracted with 100 mL hexane for 3 h with occasional stirring and then filtered. The ethanol fraction was evaporated, 100 mL hexane and 120 mL 0.9 % KCI were added, vortexed for 1 min, and the hexane/lipid containing fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum. The hexane fraction after the second extraction was mixed with 0.9% KCI (1.2-fold), stirred vigorously for 1 min, and the hexane/lipid-containing fraction was filtered through anhydrous Na_2SO_4 and evaporated under vacuum.
11. Method is the same as (5), but acetone was used as a sole solvent.
12. Method is the same as (5), but acetone: benzene: isopropanol (1: 1: 1, v/v/v) was used.
13. Method is the same as (5), but benzene: methanol (1: 1, v/v) was used.
14. Method is the same as (5), but ethyl acetate was used as a sole solvent.
15. Method is the same as (5), but diethyl ether was used as a sole solvent.
16. Method is the same as (5), but acetonitrile was used a sole solvent.
17. Method is the same as (5), but petroleum ether was used a sole solvent.
18. Method is the same as (5), but benzene was used a sole solvent.

2.2.6 Methyl ester preparation

Saponification of the total lipids were carried out using methanolic potassium hydroxide, and fatty acid methyl esters were prepared using boron trifluoride in methanol for gas chromatographic analysis (Morrison and Smith, 1964). The derivatized lipids were dissolved in 1 mL of benzene and any solids removed by centrifuging at 10,000 rpm for 2 min.

2.2.7 GC and GC-MS analysis

Fatty acid analyses were carried out by gas chromatography (Fisons GC 8000 series) fitted with FID, using a fused-silica capillary OV-1 bonded column 30 m and 0.25 mm (Ohio Valley speciality, USA). The column was operated at an initial temperature of 120°C for 1 min before increasing the temperature to 220°C at a rate of 10°C/min and holding for 20 min. Carrier gas (nitrogen) was supplied at a flow rate of 1 mL/min with a split ratio of 1:20. Methyl heptadecanoate was used as the internal standard.

GC-MS analysis was done using a GC-17A (Shimadzu, Japan) fitted with a QP-5000 mass spectrometer. The ionization energy was 70eV with a scan time of 1sec and mass range of 40 to 400. Samples were prepared in hexane with a dilution of 0.1%. Fatty acids were identified by matching their mass spectra and retention time with those of authentic standards (Sigma). NIST mass spectra library was also used as reference

2.2.8 Statistical analysis

Statistical analysis was performed with SPSS software (SPSS, 1998). The extractions were done in triplicate and the mean values are reported. Comparison of results were carried out by one - way analysis of variance (ANOVA) and post hoc analysis was performed by Duncan's multiple range test. The values are given as mean ± SD. Levels of significance were considered at $P \leq 0.05$

2.3 Results

M. alpina was cultured in GY medium (Fig 2.2) for period of 7 days. Biomass was harvested, freeze dried (Fig 2.3) were used for all extractions except Bligh and Dyer method.

2.3.1 Effect of different extraction methods

The extraction methods employed differed considerably in their lipid extraction efficacy. Maximum lipids were extracted by soxtec method (43.15%) followed by Soxhlet (40.5%) and Folch method (36.2%). Bligh and Dyer method of extraction proved to be inefficient for complete lipid recovery as indicated by the reduced extraction (24.6%). The supercritical method of extraction yielded 26.78% lipids which was comparatively less when compared to all organic solvent extraction methods except Bligh and Dyer method.

Fatty acid composition of *M. alpina* oil extracted by different methods is given in Table 2.1. It is evident that the extraction method employed had a significant effect on fatty acid composition in *M. alpina*. The use of Chloroform-methanol based methods resulted in an increment in the percentage of DHA when compared to hexane or CO_2 based methods. Maximum ARA as %TFA (35.6%) was obtained when soxhlet method of extraction was used. Bligh and Dyer as well as Folch method were not effective in terms of ARA yield.

Fig 2.2 Growth of *M. alpina* under submerged cultivation in GY medium

Fig 2.3 Harvested and lyophilized biomass of *M. alpina* used for extraction

A-Wet biomass, B-Lyophilized biomass

Table 2.1 Fatty acid composition of lipids extracted from *M. alpina* by different methods

Major fatty acids	Extraction method				
	Bligh and Dyer	**Folch**	**Soxhlet**	**Soxtec**	**Supercritical**
14:0	1.28±0.32^{b}	1.31±0.28^{b}	0.62±0.08^{a}	0.64±0.05^{a}	0.90±0.05^{a}
16:0	16.98±0.54^{c}	17.28 ± 0.42^{c}	14.08±0.56^{a}	15.4±0.01^{b}	19.92±0.32^{d}
18:0	6.40±0.12^{b}	6.88 ±0.18^{b}	13.30±0.32^{c}	13.82±0.89^{c}	2.15±0.15^{a}
18:1	25.60±0.68^{b}	25.01±1.2^{b}	13.35±0.26^{a}	13.49±1.07^{a}	27.74±0.36^{c}
18:2	4.92±0.36^{b}	4.88±0.24^{b}	4.01±0.34^{a}	6.69±0.11^{c}	7.81±0.21^{d}
18:3	4.74±0.22^{d}	4.69±0.18^{d}	2.60±0.04^{c}	2.24±0.03^{b}	0.52±0.01^{a}
20:3	3.12±0.20^{c}	3.06±0.32^{c}	1.50±0.02^{b}	1.46±0.01^{b}	0.25±0.03^{a}
20:4	**26.48±2.4^{a}**	**25.97±1.6^{a}**	**35.60±0.88^{b}**	**35.58±0.38^{b}**	**33.28±0.32^{b}**
20:5	3.10±0.08^{bc}	3.03±0.12^{b}	3.26±0.12^{c}	3.47±0.02^{d}	0.87±0.09^{a}
22:6	6.38±0.12^{d}	6.45±0.14^{d}	4.09±0.18^{c}	3.57±0.15^{b}	1.24±0.22^{a}

Values are means ± SD, n – 3 expressed as % total fatty acids. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid.18:2-linoleic acid,18:3-gamma linolenic acid,20:3-dihomogamma linolenic acid,20:4-arachidonic acid,20:5-eicosapentaenoic acid,22:6-docosahexaenoic acid

2.3.2 Effect of biomass pretreatment

2.3.2.1 Supercritical fluid extraction

In control, extraction commenced after 6h and the extraction curve reached a plateau subsequent to 30 h of extraction after which no significant increment in the yield was observed. The maximum extraction that could be achieved with the untreated biomass was significantly lower than that of the treated biomass. In HCl treated biomass, the lipid recovery was 26.78% after 42h. With enzyme treatment, the extraction process was relatively faster and a near complete lipid extraction was achieved in 18h. The results indicated that biomass pretreatment by enzymatic means considerably accelerated the mass transfer of solutes thereby extracting 38.73% lipids after 42 h. This was significantly greater than the control where the maximum lipid recovery was 7.85% (Fig 2.4). The flow rates of CO_2 were 1.5-2.0 Kg/h during the experiments. The initial fractions extracted were solid like while the latter fractions were semi-liquid.

Oil is embedded to systemic membrane of the cells and the extraction is mostly controlled by diffusion of the oil through the treated or untreated cell walls. The logarithmic fractional extraction of the biomass with time for the enzyme treated cells is depicted in Fig 2.5. The slope of the straight line gives the diffusion coefficient as per equation (4). The average diameter as extrapolated from the microscopic observation of 40-50 cells was found to be $3.14X\ 10^{-4}$ m. The diffusion coefficients calculated as per Eqn. 4 were 4.995E-14, 7.492E-14 and 1.998E-13 m^2/s respectively for the control, HCl treated and enzyme treated cells.

2.3.2.2 Organic solvent extraction

The extraction of lipids by organic solvent method for the control, HCl treated and enzyme treated biomass was similar to supercritical extraction; pretreatment of biomass resulted in a substantial increase in lipid recovery. Lipid recovery from the untreated, HCl and enzyme treated biomass were 10.53%, 43.0% and 51.47% respectively increasing in proportion with the percentage of ruptured cells (Fig 2.4).

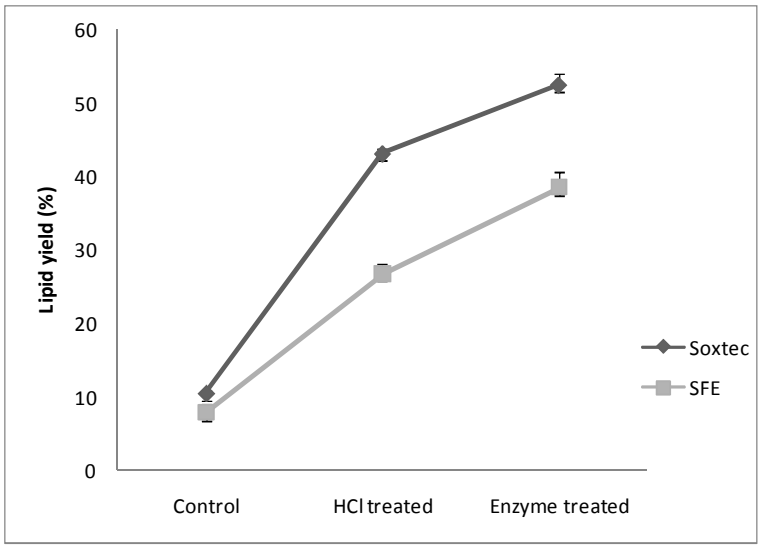

Fig 2.4 Total lipids extracted by supercritical and soxtec method in the control and treated biomass of *M. alpina*

The values are mean± SD of 3 replicates. The different alphabets for the control, HCl treated and Enzyme treated cells indicate that the percentage of ruptured cells and the corresponding lipid yield are significantly different at 5% levels according to Duncan's multiple range test.

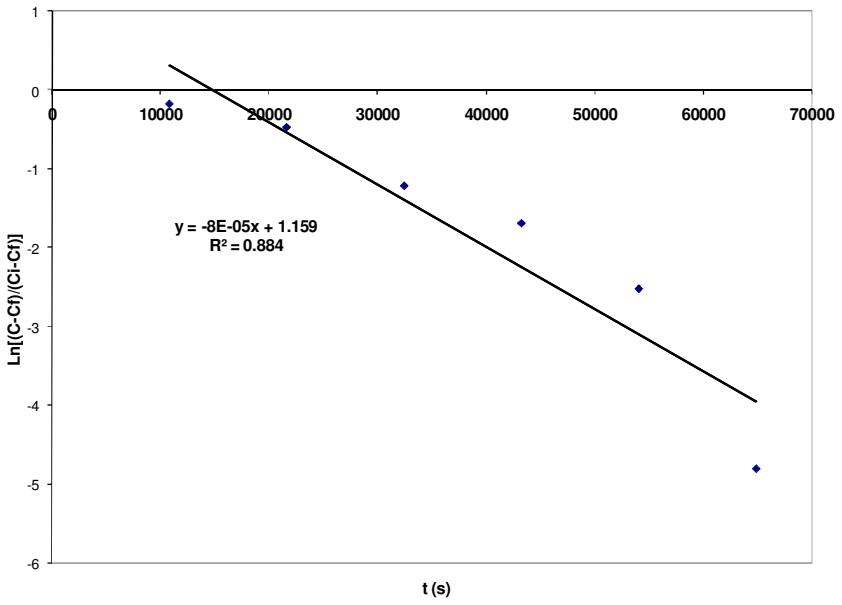

Fig 2.5 Graph depicting the log of fractional biomass extracted versus time for enzyme treated biomass using supercritical carbon dioxide

2.3.2.3 Colour measuring

The oil obtained from the treated and untreated biomass showed a significant difference in colour attributes as indicated by the l, a, b values. In case of supercritical extraction, the HCl and enzyme treated samples showed a reduction in the lightness (l) values when compared to that of the control (Fig 2.6). The maximum 'a' value was given by the control extract which signify a greater 'redness' than that of the treated samples. The negative 'a' value for the enzyme treated sample indicate that this extract was greener than the control or HCl treated samples. Positive 'b' values obtained for all the samples are indicative of a trend towards 'yellowness' with the maximum value shown by the HCl treated sample.

Solvent extracted samples had higher 'l' values than the supercritical extracts which reveal that the former were lighter in colour (Fig 2.6). The negative 'a' values indicate a trend towards 'greenness' which is contrary to that of supercritical extracts. Maximum 'yellowness' in the soxtec extracts was given by the untreated sample. The colour attributes of the control and treated samples were not considerably different in the case of soxtec extracted samples.

2.3.2.4 Phase contrast microscopy

The percentage of ruptured cells quantified in control, HCl treated and enzyme treated biomass were 20.52, 62.92 and 86.5% respectively.

2.3.2.5 Scanning electron microscopy (SEM)

Scanning electron microscopic images of intact, treated and extracted biomass are presented in Fig 2.7. A comparison of SEM images revealed a greater rupture of cell wall in the treated biomass compared to that of intact cells. Maximum disruption of cell wall was observed in the extracted cells.

2.3.2.6 FT-IR analysis

The spectrum showed no discrepancies between the supercritical and solvent extracts (Fig 2.8a, 2.8b). The CH=CH stretching vibrations at 721.9 was prominent whereas the CH=CH trans stretching vibration (at 960-970) was not evident which is an attestation of the fact that in the extracted oil most of the fatty acids are in the cis – configuration. CH_2 stretching vibrations at 2924.3 and 2853.8, CH_2 bending vibrations at 1465.9 and C=O stretching vibrations (conjugated) at 1744.3 were evident. The broad band at 3468.0 indicted the presence of intermolecular hydrogen bonds.

1. Supercritical extracted **2. Solvent extracted**

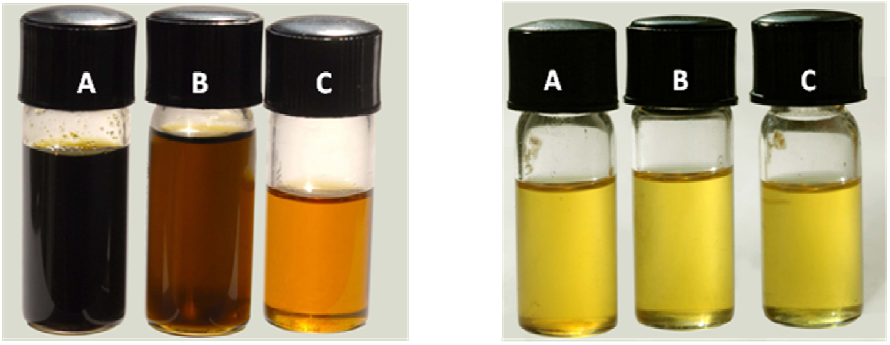

Fig 2.6 Comparison of untreated and treated extracts of *M. alpina*

A-Control, B-HCl treated, C-Enzyme treated

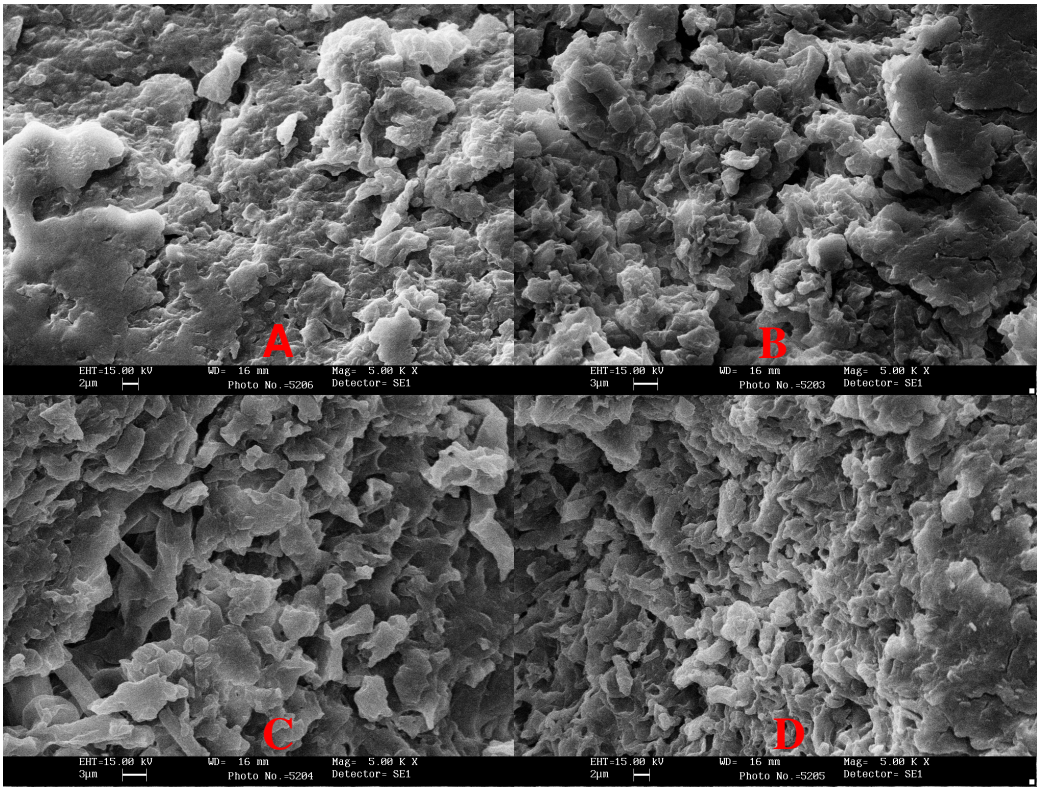

Fig 2.7 Scanning Electron Microscopic images of control, treated and extracted cells of *M. alpina*

A-Control, B- HCl treated, C-Enzyme treated, D- extracted

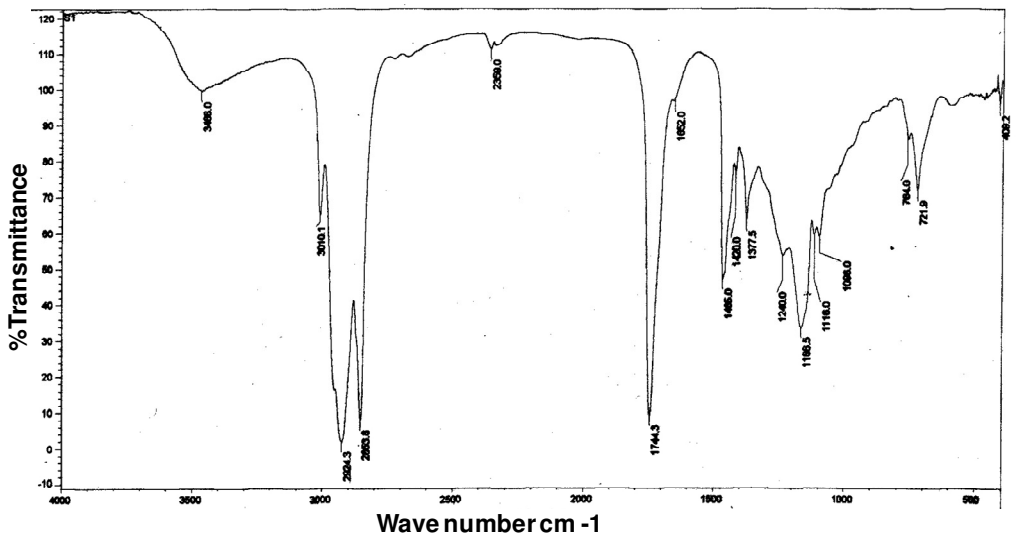

Fig 2.8a FT-IR spectrum of *M. alpina* single cell oil extracted by supercritical CO_2

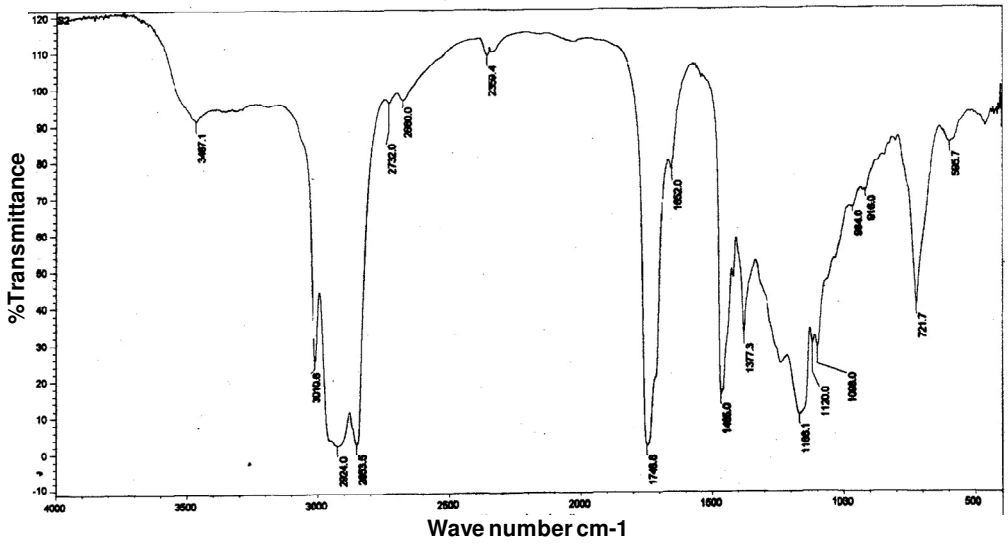

Fig 2.8b FT-IR spectrum of *M. alpina* single cell oil extracted by organic solvent

2.3.2.7 Fatty acid analysis

Fatty acid composition of the lipids obtained by supercritical and organic solvent extraction is given in Table 2.2 and 2.3. It is evident that palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4) are the major fatty acids in *M. alpina* single cell oil. In supercritical and soxtec extraction, maximum ARA (% TFA) was obtained with HCl treated biomass. Purity of ARA obtained with enzyme treated biomass was considerably lower than that of HCl treated biomass.

The significant variations observed in the percentage of fatty acids in different extracts is indicative of the fact that both biomass pretreatment and mode of extraction had an influence on ARA recovery from *M. alpina* biomass. Although enzymatic pretreatment had a significant positive effect on lipid recovery, ARA (%TFA) was reduced considerably probably due to degradation of ARA with the enzyme. Hence enzymatic method was not feasible for biomass pretreatment in *M. alpina*

On GC-MS analysis the spectrum of arachidonic acid in supercritical extract (Fig 2.8) showed the molecular ion M at m/z 318 and other characteristic ions at m/z 289(318-29), 264(318-54), 247(318- 71), 233(318-85), 220(318-98), 203(318-115), 175(318-143), 161(318-157), 150(318-168), 133(318-185), 119(318-199), 105(318-213), 91(318-227), 79(318-239), 67(318-227) and 41(318-277). The molecular ion at *m/z* = 318 though not very abundant, the diagnostic ion for the *n*-6 moiety at *m/z* = 150 does stand out. The alpha ion for the $\Delta^{5,8}$ double bonds at *m/z* = 180 is small but distinctive (Fig. 2.8). The fragmentation pattern corroborates well with that of authentic standard (Fig 2.9). A similar mass spectrum was obtained for the peak corresponding to ARA in solvent extracted samples. The major fatty acids and the fragmentations ions used for identification is given in Table 2.4.

Table 2.2 Fatty acid composition of lipids extracted from *M. alpina* biomass by super critical extraction

Fatty acid	Control	HCl treated	Enzyme treated
14:0	0.77±0.01^{b}	0.90±0.05^{c}	0.56±0.03^{a}
16:0	16.24±2.01^{a}	19.92±0.32^{b}	15.49±2.19^{a}
18:0	3.74±0.87^{a}	2.15±0.15^{a}	2.54±1.38^{a}
18:1	28.86±3.09^{ab}	27.74±0.36^{a}	32.70±1.46^{b}
18:2	7.93±0.11^{b}	7.81±0.21^{b}	7.21±0.13^{a}
18:3	0.39±0.16^{ab}	0.52±0.01^{b}	0.22±0.02^{a}
20:3	0.44±0.18^{ab}	0.25±0.03^{a}	0.58±0.01^{c}
20:4	**24.56±0.23^{a}**	**33.28±0.32^{c}**	**27.07±0.15^{b}**
20:5	3.91±2.67^{a}	0.87±0.09^{a}	1.48±0.04^{a}
22:6	7.89±1.46^{b}	1.24±0.22^{a}	6.41±1.78^{b}

Values are means ± SD, n – 3 expressed as % total fatty acids. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. 14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

Table 2.3 Fatty acid composition of lipids extracted from *M. alpina* biomass by solvent extraction

Fatty acid	Control	HCl treated	Enzyme treated
14:0	0.58±0.23^{a}	0.64±0.05^{a}	0.49±0.16^{a}
16:0	16.94±0.72^{b}	15.4±0.01^{a}	18.15±0.06^{c}
18:0	14.20±0.42^{a}	3.82±0.89^{a}	4.14±0.56^{a}
18:1	14.15±1.77^{a}	23.49±4.07^{a}	32.15±0.20^{a}
18:2	7.19±0.18^{b}	6.69±0.11^{a}	7.57±0.12^{c}
18:3	2.50±0.09^{b}	0.24±0.03^{a}	0.19±0.03^{a}
20:3	1.36±0.12^{a}	1.46±0.01^{a}	1.41±0.12^{a}
20:4	**28.74±0.34^{a}**	**35.58±0.38^{c}**	**32.89±1.14^{b}**
20:5	3.16±0.08^{a}	3.47±0.02^{a}	3.10±0.39^{a}
22:6	5.78±1.70^{a}	3.57±0.15^{a}	4.37±0.92^{a}

Values are means ± SD, n – 3 expressed as % total fatty acids. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. 14:0-myristic acid, 16:0-palmitic acid, 18:0-stearic acid,18:1-oleic acid, 18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

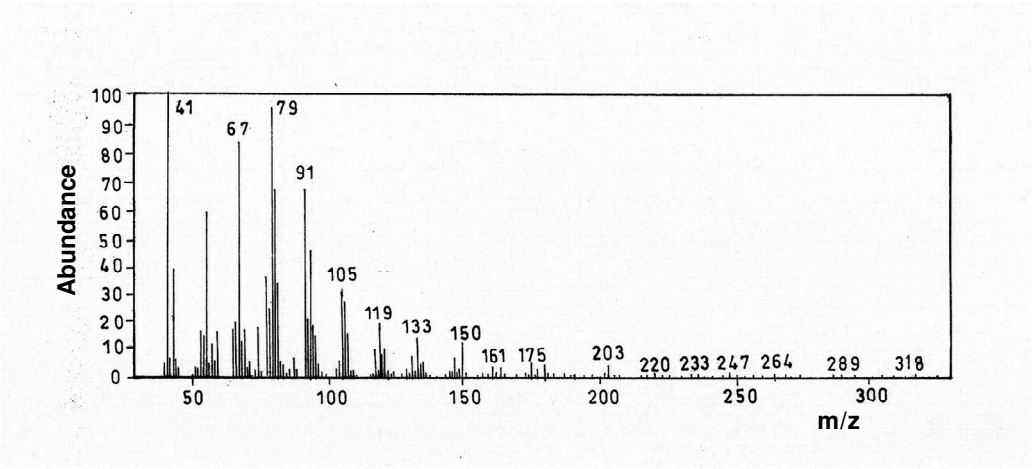

Fig 2.9 Mass spectrum of peak corresponding to arachidonic acid in HCl treated supercritical extract

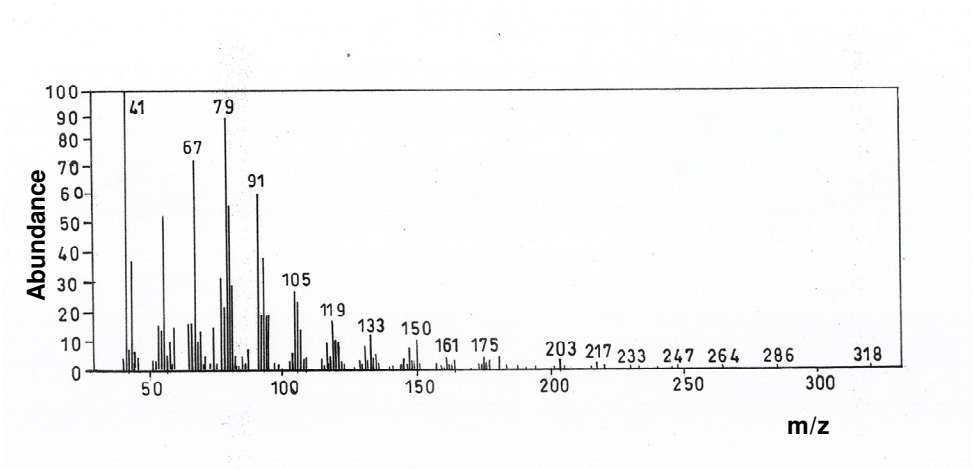

Fig 2.10 Mass spectrum of standard arachidonic acid

Table 2.4 Fragmentation ions used in the identification of fatty acids in *M. alpina* single cell oil

Fatty acid	(M^+)*	Other fragmentation ions
Palmitic acid	270	43, 55, 74, 87, 101, 115, 129, 143, 157, 171, 185, 199, 213, 227, 239, 241
Gamma linoleic acid	292	41, 55, 67, 79, 93, 107, 121, 135, 150, 163, 175, 189, 194, 208, 235, 243, 263, 271
Linoleic acid	294	41, 55, 67, 81, 95, 110, 123, 136, 150, 164, 178, 192, 205, 220, 234, 250, 263
Oleic acid	296	41, 55, 69, 74, 83, 96, 110, 124,141, 152, 66, 180, 194, 222, 236, 253, 264
Stearic acid	298	43, 55, 74, 87, 97, 111, 129, 143,158 177, 185, 199, 213, 227, 255, 267
Eicosapentaenoic acid	316	41, 55, 67, 79, 91,105, 108, 119, 131, 147 161, 180, 187, 201. 215, 247, 273, 287
Arachidonic acid	318	41, 67, 79, 91, 105, 119, 133, 150, 161 203, 233, 247, 264, 289
Dihomo -gamma-linolenic acid	320	41, 55, 67, 79, 93, 107, 121, 135, 150, 163, 177, 191, 203, 222, 249, 265, 290
Docosahexaenoic acid	342	41, 55, 67, 79, 91, 105, 108, 119, 131, 145, 166, 187, 201, 215, 241, 255, 273, 299, 313

*M^+ indicate the molecular ion

2.3.3 Effect of solvents/solvent systems

To investigate the effect of various solvents/solvent systems, Folch method of extraction was kept as standard and lipids extracted by this method was considered as 100% or the maximum extractable lipids. The different solvents/ solvent systems were compared in terms of lipid recovery (Fig 2.11). The results indicated that solvents with lower polarity extracted a significantly higher amount of total lipids. For individual solvents, acetonitrile (highest polarity) extraction resulted in the lowest lipid yield (18.52%), while chloroform extracted the highest amount (92.59%). Since chloroform is a good solvent for non polar compounds, it can be assumed that *M. alpina* strain contains higher amounts of non polar lipid structures than polar lipids. Methanol which extracted 29.6% of the total lipids proved to be ineffective for lipid extraction from *M. alpina*.

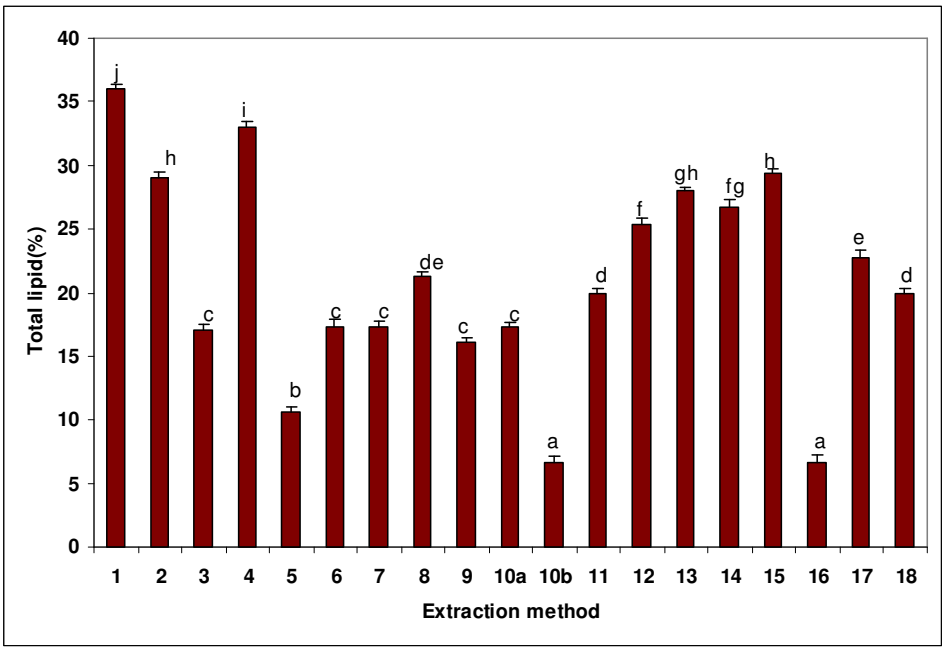

Fig 2.11 Effect of solvents/solvent system on lipid yield in *Mortierella alpina*

Values are means ± SD, n - 3. The different alphabets for the extractions indicate that they are significantly different at 5% levels according to Duncan's multiple range test 1.Chloroform :Methanol 2:1, 2.Chloroform :Methanol 1:1,3.Chloroform :Methanol 1:2, 4.Chloroform,5. Methanol, 6.Hexane:isopropanol 3:2,7.Hexane:isopropanol 4:1, 8.Hexane, 9. Isopropanol, 10a Ethanol,10b Hexane ,11. Acetone12.Acetone:Benzene:Isopropanol (1:1:1), 13Benzene:Methanol (1:1), 14.Ethyl acetate, 15 Diethyl ether, 16.Acetonitrile17.Petroleum ether, 18.Benzene

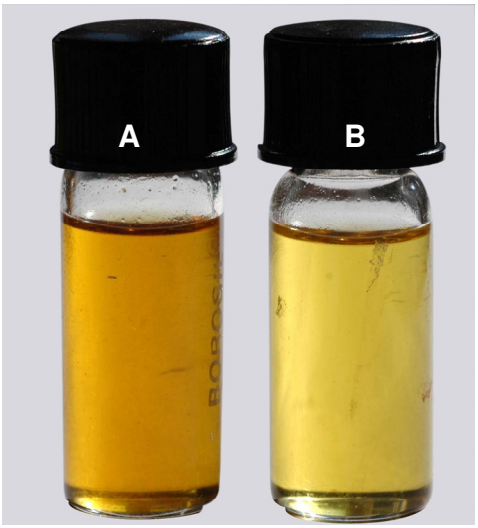

Fig 2.12 Comparison of chloroform:methanol and hexane extracted oil

a.Chloroform : methanol; b. Hexane

Of the non polar solvents used, diethyl ether (81.48%) extracted higher amount of lipids than hexane (59.26 %) or petroleum ether (62.96).

Among the different solvent systems examined, the most efficient extraction method in terms of g of oil extracted /100g of biomass was chloroform: methanol system. This system was able to extract more of the minor oil components than hexane extraction system. In contrast to chloroform: methanol experiments, lipid yields from *M. alpina* biomass were lower for hexane: isopropanol system (hexane: isopropanol 3:2, 48.15%; hexane: isopropanol, 4:1, 51.85%) increasing in proportion with the ratio of hexane. Although chloroform proved superior to hexane in terms of lipid recovery, hexane extraction gave a greater clarity of oil (Fig 2.12). The effect of different solvent systems on fatty acid composition of *M.alpina* lipid is given in Table 2.5a and 2.5b. The chromatographic analysis of the oil samples revealed an array of fatty acids both saturated and unsaturated. Arachidonic acid content varied from 30.19 to 20.96% with the use of diverse solvent systems. Maximum ARA in total lipids was observed when extracted using choloroform:methanol 1:2. Oleic acid constituted the next highest proportion to ARA and the content ranged from 28.27 to 23.13%.

Table 2.5a Effect of solvents/solvent systems on fatty acid composition in *M. alpina*

No	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
1	1.31±0.28^{cd}	17.28 ± 0.42^{defg}	6.88 ±0.18^{efgh}	25.01±1.2^{cde}	4.88±0.24^{def}	4.69±0.18^{cde}	3.06±0.32^{b}	**25.97±1.6^{bc}**	3.03±0.12^{bcd}	6.45±0.14^{e}
2	1.86±.05^{e}	16.68 ±0.36^{cd}	6.99 ±0.42^{fghi}	23.13±1.1^{a}	3.24±0.18^{a}	5.16±0.32^{defg}	3.34±0.24^{b}	**28.8±1.2^{ef}**	3.48±0.22^{de}	5.12±0.20^{d}
3	1.16±.14^{abc}	15.52±0.54^{a}	6.12±0.26^{cd}	24.29±0.86^{abcd}	4.87±0.28^{def}	5.02±0.40^{defg}	3.17±0.16^{b}	**30.19±0.98^{f}**	2.16±0.08^{a}	5.20±0.15^{d}
4	1.45±.18^{cde}	17.24±0.38^{defg}	6.36±0.30^{cde}	24.53±0.92^{abcde}	5.26±0.34^{fgh}	4.77±0.28^{cde}	1.02±0.42^{a}	**29.13±1.3^{ef}**	3.77±0.18^{e}	3.34±0.82^{ab}
5	1.63±.25de	17.62±0.26^{fgh}	6.80±0.15^{efg}	24.63±0.80^{abcde}	4.47±0.36^{bcd}	3.87±0.16^{b}	1.09±0.44^{a}	**28.19±0.94^{de}**	3.16±0.26^{bcd}	5.19±0.12^{d}
6	1.05±.32^{abc}	16.83±0.48^{cde}	6.99±0.24^{fghi}	24.81±0.94^{bcde}	4.82±0.22^{def}	5.24±0.25^{efgh}	3.20±0.35^{b}	**28.72±0.88^{ef}**	3.04±0.24^{bcd}	3.66±0.64^{b}
7	1.34±.28^{cd}	17.73±0.30^{gh}	7.50±0.48^{i}	26.63±1.0^{efg}	4.65 ±0.20^{cde}	4.61±0.34^{cd}	2.98±0.28^{b}	**24.61±0.94^{b}**	3.35±0.30^{de}	4.87±0.38^{cd}
8	1.01±.20^{abc}	16.30±0.52^{bc}	6.85±0.32^{efg}	24.40±0.82^{abcd}	4.05±0.32^{b}	5.15±0.28^{defg}	3.06±0.20^{b}	**29.25±1.0^{ef}**	2.84±0.26^{bc}	5.02±0.24^{cd}
9	0.82±.18^{ab}	16.85±0.45^{cdef}	6.82±0.16^{efg}	24.88±0.68^{bcde}	4.66±0.44^{cde}	5.01±0.22^{def}	3.19±0.36^{b}	**28.83±0.84^{ef}**	3.14±0.22^{bcd}	4.60±0.14^{cd}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-total fatty acids,14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid.18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid, 1,2,3… indicate the method of extraction. 1.Chloroform :Methanol 2:1, 2.Chloroform :Methanol 1:1,3.Chloroform :Methanol 1:2, 4.Chloroform,5. Methanol, 6.Hexane:isopropanol 3:2,7.Hexane:isopropanol 4:1, 8.Hexane, 9. Isopropanol

Cont…

Table 2.5b Effect of solvents/solvent systems on fatty acid composition in *M. alpina*

No	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
10a	1.31±.14^{cd}	16.92±0.40^{cdef}	5.88±0.20^{c}	25.79±0.74^{def}	4.77±0.35^{def}	5.46±0.18^{fgh}	3.18±0.28^{b}	**29.03±0.95^{ef}**	2.69±0.18^{b}	3.18±0.12^{ab}
10 b	1.20±.26^{abcd}	15.36±0.32^{a}	6.85±0.25^{efg}	23.44±0.78^{abc}	4.20±0.28^{bc}	4.38±0.44^{c}	3.33±0.56^{b}	**26.03±0.90^{bc}**	3.75±0.24^{e}	9.35±0.40^{g}
11	1.04±.18^{abc}	17.58±0.58^{efgh}	7.41±0.14^{hi}	26.12±0.64^{efg}	4.86±0.22^{def}	5.06±0.20^{defg}	3.05±0.25^{b}	**26.66±1.1^{cd}**	3.04±0.40^{bcd}	3.56±0.28^{b}
12	0.78±.06^{a}	16.81±0.34^{cde}	6.51±0.26^{def}	25.83±0.86^{def}	5.13±0.16^{efg}	5.04±0.34^{defg}	3.23±0.20^{b}	**28.24±0.85^{def}**	3.12±0.36^{bcd}	3.74±0.35^{b}
13	1.19±.34^{abcd}	19.01±0.35^{j}	7.32±0.20^{ghi}	27.71±0.94^{gh}	4.82±0.24^{def}	2.79±0.26^{a}	3.19±0.18^{b}	**20.96±0.82^{a}**	3.41±0.28^{de}	7.60±0.26^{f}
14	1.37±.16^{cd}	17.98±0.42^{gh}	6.52±0.50^{def}	26.74±0.98^{fgh}	4.87±0.28^{def}	4.69±0.34^{cde}	2.86±0.85^{b}	**25.70±0.96^{bc}**	3.06±0.22^{bcd}	4.45±0.14^{c}
15	1.22±.28^{abcd}	16.28±0.28^{bc}	6.58±0.18^{def}	24.10±0.85^{abc}	4.80±.40^{def}	4.87±0.25^{cde}	3.01±0.38^{b}	**28.76±1.0^{ef}**	2.73±0.15^{b}	5.92±0.18^{e}
16	1.25±.35^{bcd}	15.68±0.35^{ab}	6.62±0.35^{cde}	23.26±0.64^{ab}	4.04±.32^{b}	4.80±0.32^{cde}	2.96±0.15^{b}	**29.43±0.92^{ef}**	2.24±0.20^{a}	7.51±0.22^{f}
17	1.25±.35^{cd}	18.26±0.60^{i}	2.21±0.36^{a}	26.88±0.68^{efg}	5.45±.26^{gh}	5.56±0.18^{gh}	3.46±0.26^{b}	**27.83±0.86^{de}**	3.31±0.36^{cde}	3.39±0.24^{b}
18	1.05±.12^{abc}	18.25±0.38^{i}	3.44±0.26^{b}	28.27±0.85^{h}	5.69±.32^{h}	5.70±0.24^{h}	3.24±0.15^{b}	**28.21±0.88^{de}**	3.06±0.18^{bcd}	2.76±0.46^{a}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. 10a,10b.11,… indicate the method of extraction TFA-total fatty acids,14:0-myristic acid,16:0-palmitic acid,18:0-stearic acid,18:1-oleic acid.18:2-linoleic acid,18:3-gamma linolenic acid,20:3-dihomogamma linolenic acid,20:4-arachidonic acid,20:5-eicosapentaenoic acid,22:6-docosahexaenoic acid. 10a Ethanol, 10b Hexane, 11. Acetone 12.Acetone:Benzene:Isopropanol (1:1:1), 13Benzene:Methanol (1:1), 14.Ethyl acetate, 15 Diethyl ether, 16 Acetonitrile, 17. Petroleum ether, 18.Benzene

2.4 Discussion

Lipid extraction is a fundamental process in food science and technology and the methods employed must be versatile, relatively simple and safe for both consumers and operating personnel. In spite of the focus given for organism screening and optimization, less attention has been given on oil extraction and most of the extraction methods have been originally described for animal and plant materials. Reliable processes of easy recovery of microbial oils have to be devised and the choice of the process depends on the nature of microbial cells and the type of extract desired. The extraction and fractionation of polyunsaturated fatty acids to produce concentrates has had limited commercial success, despite the extensive research that has been carried out in this area (Catchpole *et al*., 2009).

In the present investigation, the two methods employed, supercritical and organic solvent extraction exhibited significant variation in lipid extraction efficacy. SFE was inferior to all organic solvent extraction methods except Bligh and Dyer method in terms of total lipid yield. This is attributed to the inability of supercritical CO_2 to extract phospholipids. CO_2 can only extract neutral lipids from lipid mixtures, and a co-solvent like ethanol must also be used to extract phospholipids (Catchpole *et al*., 2009).

The lipid yield was significantly lower when extracted by Bligh and Dyer method when compared to other methods of extraction. Iverson *et al*. (2001) previously reported that the Bligh and Dyer method produced significantly lower estimates of lipid yield when the lipid content of a sample is greater than 2% and this underestimation increased significantly with increasing lipid content of the sample.

The significant difference in fatty acid composition observed with the different extraction methods may be attributed to the differential solubility of individual fatty acids in these solvents ie., Chloroform: methanol, hexane or CO_2. In extractions using Chloroform: methanol based systems, increase in % of DHA was evident possibly due to enhanced phospholipid extraction than hexane or CO_2 based systems.

In both supercritical and soxtec methods lipid yield was dependent on whether the biomass was pretreated or not. The fungal wall is a complex structure composed typically of chitin, 1,3-β- and 1,6-β -glucan, mannan and proteins, although wall composition varies markedly between species of fungi (Adams, 2004). Fungal cells proved to be particularly resistant towards some disintegration methods commonly

used for yeasts and bacteria (Taubert *et al.,* 2000). The rigidity of fungal cell wall is higher than that of plant cells and hence resistance of the fungal cell wall becomes the rate limiting step for mass transfer of oils from the cells. The mass transfer is diffusion controlled as the oil is embedded in the cells and extraction involves breaking or lysing the cells. It was observed that the oil yield was very low in control due to low diffusion through the hard wall. After lysing the cells with HCl the rate of extraction was almost doubled as the diffusion coefficient is improved. In enzymatic treatment the diffusion coefficient appeared to be 40 times higher than HCl treatment.

After the complete maceration of cells, the lipids surround the biomass and the solvent (CO_2 or hexane) penetrates the core of biomass leading to higher extraction as observed in the treated biomass. On the contrary, the rate of extraction in the untreated cells (control) was hindered by the hard natural membrane. Gouveia et al. (2007) reported that degree of crushing significantly influenced extraction of pigments in *Chlorella vulgaris.* Results imply that the rate of mass transfer was significantly lower in the unlysed cells leading to a dwindled total lipid yield.

Although supercritical method proved inferior to soxhlet and soxtec methods in terms of lipid recovery, the use of carbon dioxide offers certain advantages concerning the quality of the extracted oil and the efficiency of the process. The complete removal of organic solvents from the solvent extracted oil is a time- and energy-consuming process in present-day oil technology (Aleksovski *et al*., 1998). Since the oil obtained by SFE is solvent free, the processing time is several times shorter than that needed for the same yield by solvent extraction. Moreover cis-isomers were much more soluble in supercritical CO_2 than trans-isomers which reduce the possibility of extraction of trans fat in small amounts. Mendes *et al.* (2003) have reported that the cis/trans ratio, in the supercritical extracts, increased significantly, relatively to the original one in the microalga *Dunaliella salina.*

A low lipid yield was observed when methanol was used as the solvent. This is ascribed to the reduced ability of methanol to extract non polar lipid classes. Methanol extraction is suitable for polar compounds and can be accepted for selective extraction of phospholipids from microbial biomass (Certik *et al.,* 1996). It was also observed that isopropanol alone is not a convenient extractant for quantitative lipid isolation from *M. alpina* biomass.

The non polar solvents hexane and petroleum ether proved to be less efficient than diethyl ether in lipid recovery from *M. alpina* biomass. Petroleum ether may not be able to "break apart some of the more polar shells surrounding some lipids and give a near complete lipid extraction and diethyl ether is usually used despite its higher flammability and cost. Lower extraction with hexane and petroleum ether could be attributed to their reduced efficacy for extraction in cold extraction conditions when compared to soxhlet method of extraction.

Among the different solvent systems examined, the most efficient extraction method was chloroform:methanol system. The reduced efficacy of hexane: isopropanol system is possibly due to reduced isolation of less polar lipids (Somshekhar *et al*., 2001). Generally, non polar solvents are needed for lipid extraction, where most lipids are adequately dissolved. Nevertheless, their combinations with polar solvents are advantageous, mainly because of dehydration, protein denaturation and degradation of hydrogen bonds between the lipid complex and proteins (Certik *et al.*, 1996). Present study also indicated a higher rate of oil recovery with Chloroform: methanol compared to hexane when extracted by Folch method. Efficiency of Chloroform: methanol method over hexane was also reported by Miraliakbari and Shahidi (2008). Study by Phillips *et al.* (2008) also projected Chloroform: methanol method to be the best for thorough total lipid extraction. On the other hand hexane extraction yielded a higher clarity of oil implying that hexane extraction provided more refined oil when compared to the chloroform:methanol system.

2.5 Conclusion

The results imply that method of extraction, solvent system used and pretreatment of biomass had a significant effect on lipid recovery in *Mortierella alpina* cells. Organic solvent methods Soxhlet and soxtec proved superior to supercritical extraction in terms of lipid yield. In spite of this fact *M. alpina* oil extracted using supercritical carbon dioxide could be competitive with the conventional process, since it simplifies considerably the oil refinement stages and completely eliminates the solvent distillation stages, which are the most costly processing stages in terms of energy consumption.

3.1 Introduction

Filamentous fungi play important roles in biotechnology as producers of low molecular weight compounds and enzymes and genetic manipulations of these microbes are often used in industry to obtain hyper producing strains. Recent developments in industrial biotechnology have resulted in the exploitation of new and undiscovered microorganisms by devising improved methodologies, which have led to better yields, thus making the industrial process feasible. Industrial strain improvement plays a central role in the commercial development of microbial fermentation processes (Zhao *et al.*, 2009). Exponential growth in fungal metabolic engineering has resulted in over-production of rate limiting enzymes of important biosynthetic pathways, consequently increasing yield of the final products.

An organism isolated from the wild, often produce low quantities of any detected desirable products and while process design lead to significant improvements in performance, genetic methods based on the use of largely empirical whole cell mutagenesis coupled with high throughput screening methods, tend to bring about the most significant one step improvements. Although most of the strain improvement strategies are focussed on product yield, it can also be effectively utilized to alter the spectrum of metabolites and growth characteristics thereby improving the performance of microorganisms. The progress in strain improvement over the years has increased fermentation productivity and decreased costs tremendously (Demain and Adrio, 2008; Bapiraju *et al.*, 2004) and the spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Abdelghani *et al.*, 2005). These genetic programs also serve other goals such as the elimination of undesirable products or analogs, discovery of new metabolites and deciphering of biosynthetic pathways (Demain and Adrio, 2008). Induced mutagenesis presents substantial advantage compared to genetic engineering as it does not require the wealth of biochemical and genetic information in the organism at stake (Queener and Lively, 1986).

Over the past few decades microbes have been recognized as potent sources of polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series. Strain improvement by mutation is a traditional method used with great success for isolating mutants producing enhanced levels of desired PUFAs. Increasing demand for biologically

active PUFAs has focussed commercial attention on PUFA based nutraceuticals and therapeutics and on the provision of a suitable biosynthetic framework for their production (Certik *et al*., 1998). Industrial biotechnology employs the controlled use of microorganisms for the production of PUFAs that can further be used in a diverse array of applications that span the pharmaceutical, chemical and nutraceutical industries In recent years, industrial systems employing microbes have been developed to produce PUFAs, recovery of which is otherwise unprofitable from higher plants and animals. Examples include γ-linolenic acid production by the genera *Mortierella* and *Mucor* (Suzuki *et al*., 1996), arachidonic acid and eicosapentaenoic acid by the genus *Mortierella* (Shimizu and Yamada, 1989; Aki *et al*., 2001).

The filamentous fungus *M. alpina* is a potent producer of arachidonic acid (ARA), an important PUFA of the n-6 series. The ARA production process is dependent on a suitable strain with high titre and ideal production profile. The PUFA content of *Mortierella* can be modulated by culture conditions and several studies aimed at optimization of culture variables in diverse strains (Nisha and Venkateswaran, 2009; Zhu *et al*., 2003; Jang *et al*., 2005).

The exponential increase in the application of ARA in various fields over the last few decades demands extension in both qualitative and quantitative improvement. Quantitative enhancement requires strain improvement and medium optimization for over-production of the desired PUFAs. To obtain such strains we use genetic manipulation including traditional methods such as random mutagenesis, followed by screening and selection. In *Mortierella,* mutation techniques often results in suppression or activation of regioselective fatty acid desatuarses leading to the production of tailor made PUFAs. A wide variety of mutants defective in desaturases (Δ^9, Δ^{12}, Δ^6, Δ^5, ω3) have been derived from *M. alpina* by chemical mutagenesis. In these mutants one or more enzymes of the ARA biosynthetic pathway is blocked leading to the over-production of fatty acids other than ARA. To facilitate the isolation of a mutant strain with higher ARA from innumerable negative mutants, a versatile screening strategy has to be used which can elude the elaborate process of biomass drying, lipid extraction and fatty acid analysis. In order to accomplish a high production of ARA, screening of mutants derived from *M. alpina* CBS 528.72 was carried out. Selection of arachidonic acid hyper-producers was done using a simple

staining technique. Mutagenesis was carried out by employing both physical and chemical mutagens.

3.2 Materials and methods

3.2.1 Chemicals

Standards of fatty acid methyl esters, N-methyl-N'-nitro-N'nitrosoguanidine (NTG) and ethyl methane sulphonate (EMS) were purchased from Sigma (St. Louis, USA). Glucose, 2, 3, 5 tri phenyl tetrazolium chloride (TTC) and yeast extract were procured from Hi-Media (Mumbai, India). All other chemicals were of AR grade procured from sD Fine chemicals, India. Solvents used were obtained from Qualigens Fine Chemicals, India

3.2.2 Microorganism and Culture maintenance

Mortierella alpina strain CBS 528.72, used as the parent for mutagenesis was maintained on potato dextrose agar, PDA slants at 4ºC and sub-cultured every two months.

3.2.3 Mutagenesis

The mutation programme used UV as the physical mutagen and NTG and EMS as the chemical mutagens.

3.2.3.1 UV treatment

Spores from two week old slants were suspended in 0.9% (w/v) NaCl solution containing 0.1% (v/v) Tween 80 for UV-treatments. 1 mL spore suspension ($2x10^6$cells/ml) was transferred into sterile petri dish and irradiated for different time intervals using a CAMAG UV chamber, which emits radiations of 254 nm at a distance of 15 cm. The spore suspension was agitated during irradiation. Sampling was done at regular intervals and maintained at 4°C in darkness overnight to avoid photo reactivation. Suspensions were suitably diluted and appropriate dilutions were plated on glucose-yeast extract (GY) medium and incubated for 4-5 days at 4°C. Spore-killing percentage was plotted against duration of UV exposure. Preliminary analyses of survival curve indicated that UV irradiation for 24 min resulted in 99% killing and was considered for further experiments. Spores unexposed to radiation were taken as control and percentage survivors were calculated by considering the colonies developed on control plates as 100%.

3.2.3.2 EMS and NTG treatment

Aliquots of 1 mL of the spore suspensions harvested as described in section 3.2.3.1 were treated with different concentrations of mutagen EMS/NTG (5, 10, 15, 20 and 25µg/mL). The individual suspensions were incubated at 30°C for different time intervals (30, 60, 90 and 120 min) with vigorous agitation in an orbital shaker. Subsequent to incubation, the samples were centrifuged for 5 min at 5000rpm and the supernatant was decanted. Cells were washed thoroughly with sterile water and resuspended in 0.01 M sterile buffered saline (pH 7.0). The treated cells were refrigerated overnight in the dark before plating for any DNA repair to take place. The samples were serially diluted and appropriate dilutions plated on GY medium and incubated at 4°C. The survival percentage was calculated from the number of colonies obtained for each treatment.

3.2.4 Screening of mutants

Two selection strategies were employed: 1. incubation at low temperature 2. Staining with 2, 3, 5 tri phenyl tetrazolium chloride.

3.2.4.1 Selection at low temperature

The plates incubated at 4°C were observed and fast growing colonies were selected, subcultured in 250 mL Erlenmeyer flasks and incubated for a period of seven days in Glucose - Yeast extract (GY) medium.

3.2.4.2 TTC staining and quantification of staining degree

TTC staining was carried out according to the method described by Zhu *et al.* (2004). Fresh mycelia was harvested and washed twice with sterile distilled water. 1 mL of 0.8% TTC solution in 0.2 mol/L borate buffer (pH 8.0) was added to 0.1 g of fresh mycelia in a screw cap tube and incubated in the dark for one hour at 25°C. Mycelia were subsequently rinsed twice with sterile distilled water and homogenized by grinding. The red tetra hydro folate (TF) was extracted twice with 2 ml of ethyl acetate. Quantification of staining degree was done by measuring absorbance of TF in the ethyl acetate solvent at 485nm. The hyper stained putative mutants were selected and cultured in 1 L Erlenmeyer flasks.

3.2.5 Analytical Methods

3.2.5.1 Determination of dry cell weight

Fungal growth was measured by determining the mycelial weight after harvesting the cells by suction filtration followed by drying at 55ºC. The dried biomass was made to a fine powder by grinding with neutral sand. These were then made in to a thimble, macerated with 0.1N HCl for 20 min and extracted by Soxhlet method with hexane as solvent for 8-10 h. The total lipid was determined gravimetrically.

3.2.5.2 Methyl ester preparation and fatty acid analysis

Saponification of the total lipids were carried out using methanolic potassium hydroxide, and fatty acid methyl esters were prepared with slight modification using boron trifluoride in methanol for gas chromatographic analysis (Morrison and Smith, 1964). The derivatized lipids were dissolved in 1 mL of benzene and any solids removed by centrifuging at 10,000 rpm for 2 min. Fatty acid analyses were carried out by gas chromatography (Fisons GC 8000 series fitted with FID) using a fused-silica capillary OV-1 bonded column 30 m and 0.25 mm (Ohio Valley speciality, USA). The column was operated at an initial temperature of 120°C for 1 min before increasing the temperature to 220°C at a rate of 10°C/min and holding for 20 min. Carrier gas (nitrogen) was supplied at a flow rate of 1 mL/min with a split ratio of 1:20.

3.2.5.3 GC-MS analysis

GC-MS analysis was done using a GC-17A (Shimadzu, Japan) fitted with a QP-5000 mass spectrometer. The ionization energy was 70eV with a scan time of 1sec and mass range of 40 to 400. Samples were prepared in hexane with a dilution of 0.1%. Fatty acids were identified by matching their mass spectra and retention time with those of authentic standards purchased from Sigma, USA. NIST mass spectra library was also used as reference.

3.2.6 Statistical Analyses

Statistical analysis was performed with SPSS software version 8.0 (SPSS, 1998). One way analysis of variance (ANOVA) with Duncan's multiple range test was used to assess statistical differences in this study. The values are given as mean ± SD. Levels of significance were considered at $P \leq 0.05$.

3.3 Results

3.3.1 Survival of *M. alpina* exposed to mutagens

Survival curve of *M. alpina* exposed to UV irradiation indicated that 24 min exposure resulted in a killing rate of 99% (Fig 3.1). NTG treatment for 90 min at 25 µg/mL concentration resulted in 98% killing and was used for the subsequent experiments. The survival curve of *M. alpina* exposed to different concentrations of NTG as a function of time is given in Fig 3.2. With EMS, a maximum killing rate of 98% was observed when used at 20 µg/mL level for 120 min (Fig 3.3). Beyond this concentration, 100% killing was noticed.

3.3.2 Screening for arachidonic acid hyper- producing mutants

Low temperature screening coupled with TTC staining was used for selection of hyper arachidonic acid producing mutants. At the restrictive temperature of 4°C, the mutants grew faster than the parent and this was employed as the primary selection strategy. A total of 540 colonies selected after low temperature screening were grown in GY medium and were subjected to TTC staining technique. The results indicated a direct positive correlation between TTC staining and ARA in total lipids and had no relation to the amount of total lipids present in the mycelium. It was further observed that only arachidonic acid producing *M. alpina* was stained red with TTC whereas oleaginous *Mucor sp.* failed to show positive staining with TTC (Fig 3.4).

Mutagenesis gave ten prospective mutant strains after TTC staining (Table 3.1). These strains M18, M24 (UV mutants), M110, M118, M130, M237 (EMS mutants), M240, M241, M242 and M386 (NTG mutants) were selected based on the higher staining degree with TTC. At ambient temperature, there were no morphological discrepancies between the parent and mutants which indicate the absence of any morphological variations attributable to the treatment. On the contrary, the mutants exhibited a faster growth rate at 4°C. Disparity in growth of parent and the UV mutant M18 at the restrictive temperature is given in Fig 3.5. In submerged culture all the mutants grew in GY medium with pellet morphology but statistically significant variations were observed in their biomass yield (Table 3.1). Maximum biomass was obtained with the UV mutant M18 which yielded 10.33g/L dry biomass

and 3.44g/L total lipid after 7 days of incubation. Mutant M386 yielded 9.54g/L dry biomass 4.2g/L total lipids and 1.95g/L ARA, which was significantly higher than that of the parent.

3.3.3 Gas chromatographic analysis

Fatty acid analysis of the mutants revealed a higher ARA% in total lipids than control as indicated by their higher staining with TTC (Table 3.2). Though mutant M110 gave higher ARA percentage in total lipids, the yield of ARA/L was considerably lower owing to the drastic reduction in biomass and total lipid production. Maximum ARA yield was obtained with the NTG mutant M386 which produced 1.95 g/L ARA after seven days of culture in GY medium. The mutant M240 did not give any significant improvement in terms of ARA yield inspite of the higher percentage of ARA in total lipids. It was noticed that ARA being an intracellular product, sufficient biomass buildup was mandatory for better ARA yield. Results indicated that the mutants M386, M18, M237 and M242 gave a statistically significant increment in ARA yield. The best mutant M386 showed 203.13% higher yield of ARA over the parent, which was found to be statistically significant. Fatty acid profiles of the mutants were significantly different from that of parent. In most mutants with higher ARA productivity a concomitant decrease in the percentage of EPA and DHA was noticed. In mutants M242 and M130 a significant reduction in the percentage of stearic (18:0) acid was evident.

3.3.4 GC-MS analysis

On GC-MS analysis the spectrum of arachidonic acid in mutant M386 showed the molecular ion M at m/z 318 and other characteristic ions at m/z 262(318-56), 247(318-71), 234(318-84), 220(318-98), 203(318-115), 175 (318-143), 161(318-157), 150(318-168), 133(318-185), 119(318-199), 105(318-213), 91(318-227), 79(318-239), 67(318-227), 41(318-277). The molecular ion at *m/z* = 318 though not very abundant, the diagnostic ion for the *n*-6 moiety at *m/z* = 150 does stand out. The alpha ion for the $\Delta^{5,8}$ double bonds at *m/z* = 180 is small but distinctive (Fig 3.6 and 3.7). The fragmentation pattern corroborated well with that of authentic standard. A similar mass spectrum was obtained for the peak corresponding to ARA in other mutants.

3.3.5 Stability of mutants

Stability of mutants was assessed by repeated subculture. In the present study the selected mutant strain (M386) maintained the morphological characteristics in terms of growth, lipid yield and ARA production efficiency for 6 generations showing that the mutation was stable.

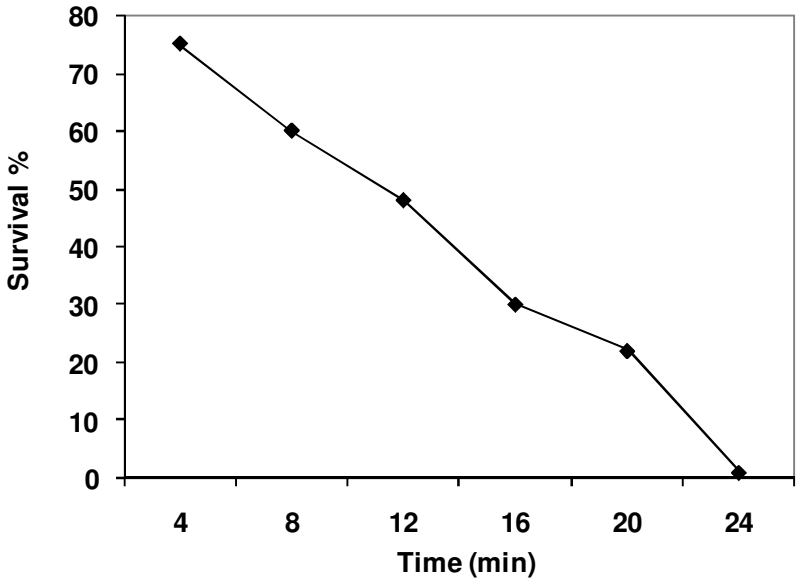

Fig 3.1 Survival curve of *M. alpina* exposed to UV

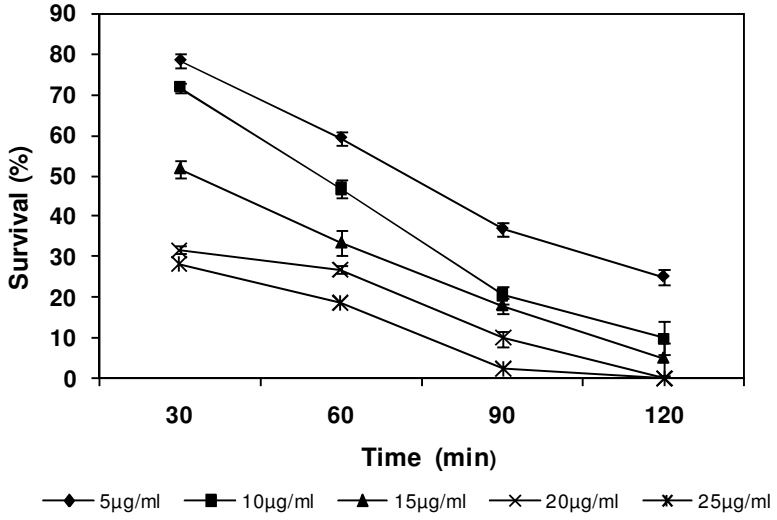

Fig 3.2 Survival curve of *M. alpina* exposed to NTG

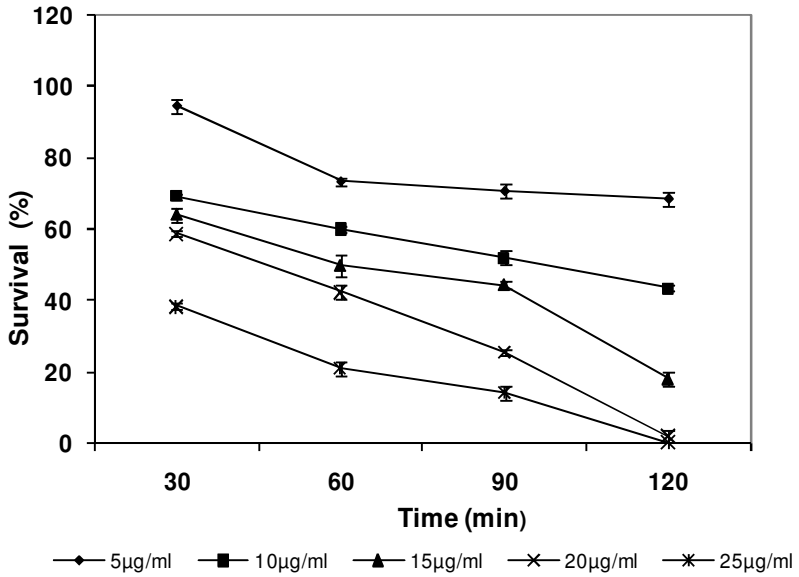

Fig 3.3 Survival curve of *M. alpina* exposed to EMS

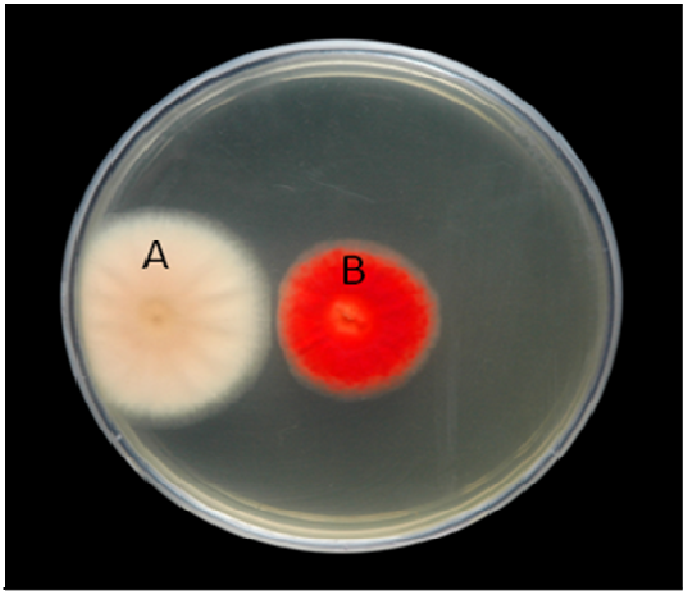

Fig 3.4 TTC staining of *M. alpina* and *Mucor sp.*
A-Mucor *sp.* showing negative staining, B- *M. alpina* showing positive staining

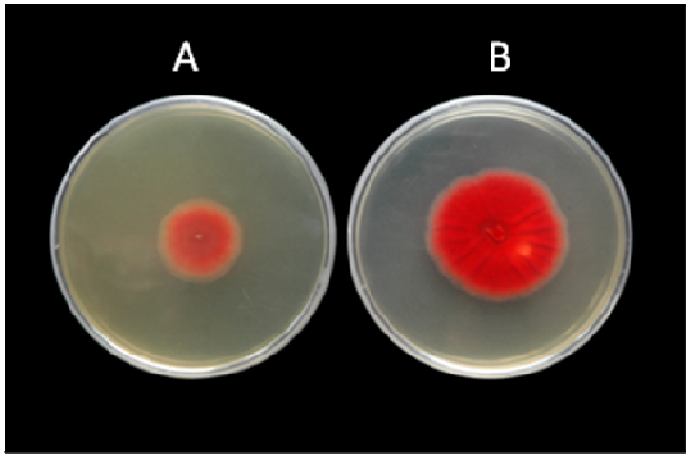

Fig 3.5 Comparison of growth rate and staining degree of parent and mutant M18 at 4°C
A-parent, B-Mutant M18

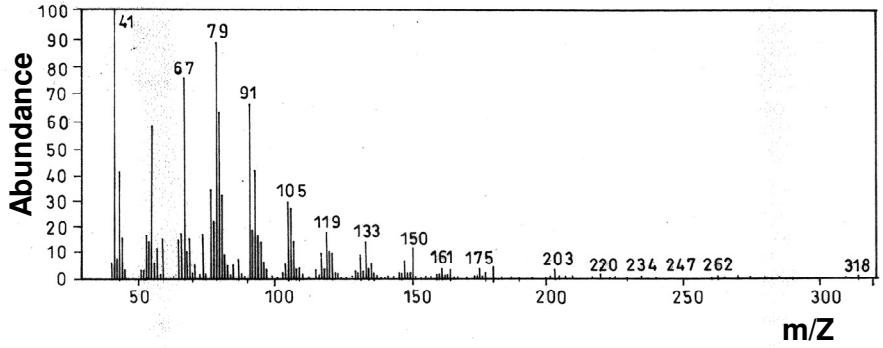

Fig 3.6 Mass spectrum of the peak corresponding to ARA in mutant M386

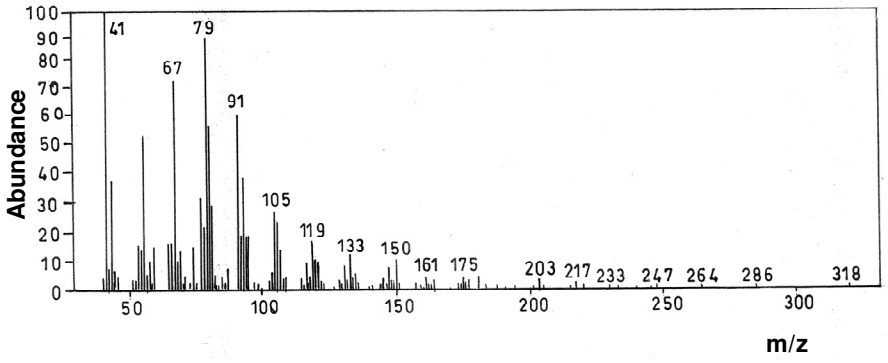

Fig 3.7 Mass spectrum of standard ARA

Table 3.1 Biomass, total lipid and ARA yield of selected *M. alpina* mutants

Strain	Dry biomass (g/L)	Total lipid (g/L)	ARA(g/L)
Parent	6.81±0.07^{bcd}	2.74±0.08^{bc}	0.96±0.03^{b}
M18	10.33±0.55^{f}	3.44±0.30^{f}	1.35±0.13^{e}
M24	7.2±0.30^{de}	3.01±0.18^{de}	1.16±0.07^{cd}
M110	5.8±0.21^{a}	1.78±0.17^{a}	0.69±0.06^{a}
M118	6.49±0.12^{b}	2.66±0.09^{bc}	1.02±0.04^{b}
M130	7.20±0.09^{de}	2.67±0.09^{bc}	1.06±0.05^{bc}
M237	9.60±0.19	3.65±0.10	1.55±0.05
M240	5.83±0.21^{a}	2.49±0.10^{b}	0.96±0.05^{b}
M241	7.2±0.18d^{e}	2.79±0.10^{cd}	1.14±0.05^{cd}
M242	6.72±0.28^{bc}	2.76±0.17^{bc}	1.18±0.08^{d}
M386	9.54±0.13^{f}	4.27±0.07^{g}	1.95±0.04^{e}

All values are mean± SD of 3 determinations. Values in the same column followed by different alphabetical superscripts are significantly different at 5% level according to Duncan's multiple range test
ARA-arachidonic acid

Table 3.2 Fatty acid composition of parent and mutants

Strain	Major fatty acids (%TFA)									
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**20:3**	**20:4**	**20:5**	**22:6**
Parent	0.62±0.14^{cd}	14.91±0.20^{e}	13.0±0.25^{f}	13.65±0.15^{a}	4.31±0.12e	2.68±0.14e	1.38±0.20^{d}	**35.12±0.14^{a}**	3.29±0.18^{d}	4.07±0.18^{f}
M18	0.36±0.10^{ab}	9.42±0.28^{a}	10.46±0.20^{d}	16.28±0.22^{d}	6.28±0.18f	5.64±0.15g	2.54±0.18^{e}	**39.38±0.12^{c}**	3.15±0.24^{d}	2.46±0.18^{d}
M24	0.85±0.08^{e}	19.20±0.64^{g}	10.4±0.18^{d}	20.84±0.10^{f}	1.86±0.25^{b}	1.44±0.11^{b}	0.62±0.06^{b}	**38.51±0.11^{b}**	1.10±0.12^{ab}	1.92±0.14^{c}
M110	0.45±0.05^{abc}	12.62±0.26^{d}	10.84±0.24^{e}	25.18±0.32^{h}	4.32±0.40^{e}	2.65±0.19^{e}	1.28±0.12^{cd}	**38.55±0.11^{b}**	0.85±0.18^{ab}	0.96±0.10^{a}
M118	0.92±0.16^{e}	10.40±0.33^{b}	10.82±0.19^{e}	25.12±0.38^{h}	4.28±0.16^{e}	1.52±0.20^{bc}	0.55±0.04^{ab}	**38.35±0.25^{b}**	1.18±0.22^{bc}	2.56±0.26^{de}
M130	0.87±0.14^{e}	14.81±0.18^{e}	2.52±0.24^{a}	28.57±0.40^{j}	3.79±0.28^{d}	2.87±0.16^{e}	1.14±0.26^{cd}	**39.84±0.20^{d}**	1.50±0.25^{c}	2.77±0.23^{e}
M237	0.40±0.06^{ab}	10.22±0.38^{b}	2.56±0.12^{a}	26.90±0.18^{i}	2.48±0.14^{c}	1.82±0.14^{d}	0.64±0.10^{b}	**42.43±0.38^{f}**	3.20±0.36^{d}	3.98±0.14^{f}
M240	0.26±0.08^{a}	20.06±0.50^{h}	8.63±0.18^{b}	15.41±0.24^{c}	2.60±0.22^{c}	1.46±0.08^{bc}	0.98±0.15^{c}	**38.45±0.48^{b}**	1.20±0.12^{bc}	2.55±0.18^{de}
M241	0.48±0.10^{bc}	18.27±0.35^{f}	10.03±0.26^{c}	14.40±0.08^{b}	7.19±0.50^{g}	5.31±0.24^{f}	1.02±0.18^{c}	**41.0±0.18^{e}**	1.07±0.25^{ab}	1.93±0.10^{c}
M242	0.80±0.12^{de}	18.47±0.14^{f}	2.46±0.10^{a}	19.08±0.12^{e}	1.36±0.26^{a}	1.72±0.12^{cd}	2.89±0.34^{f}	**42.77±0.21^{f}**	0.71±0.14^{a}	0.99±0.11^{a}
M386	0.76±0.15^{de}	11.42±0.22^{c}	8.85±0.17^{b}	24.68±0.15^{g}	2.46±0.14^{c}	1.02±0.05^{a}	0.28±0.08^{a}	**45.70±0.09^{g}**	0.95±0.10^{ab}	1.28±0.09^{b}

Values are means ± SD, n - 3. Values in the same column that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. TFA-Total Fatty acids, 14:0-myristic acid,16:0-palmitic acid, 18:0-stearic acid,18:1-oleic acid,18:2-linoleic acid, 18:3-gamma linolenic acid, 20:3-dihomogamma linolenic acid, 20:4-arachidonic acid, 20:5-eicosapentaenoic acid, 22:6-docosahexaenoic acid

3.4 Discussion

Several approaches have been employed to improve the economy of biologically-based industrial processes by "improving" the organism involved and the efficacy of this technique is dependent on the type of organism and the nature of the end-product. Strain improvement is considered to be one of the key factors involved in the achievement of higher titers of industrial metabolites (Podojil *et al*., 1984). The classical strain improvement is robust although it is a time-consuming and resource intensive practice (John and Nampoothiri, 2008). Random mutagenesis and screening have been reported as an effective way to improve the productivity of industrial microbial cultures (Parekh *et al*., 2000). The most extensively used mutagens for strain improvement are N-methyl-N'-nitro-N nitrosoguanidine (NTG), methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS) and ultraviolet (UV) irradiation (Baltz, 1999). Over-production of primary or secondary metabolites is a complex process and successful development of improved strains requires knowledge of physiology, pathway regulation and control and the design of creative screening procedures (Parekh *et al*., 2000). Strain improvement is vital for enhanced PUFA production by industrial microorganisms.

For improved production of ARA, *M. alpina* was subjected to random mutagenesis by physical and chemical methods. UV light was chosen as a physical mutagen because it is considered as one of the simplest ways to obtain a wide variety of mutant strains from different organisms. Non-ionizing radiations such as UV at wavelength 254 nm is absorbed by DNA leading to thymine dimers that cause mispairing during DNA replication, and subsequent permanent mutation if not repaired (Shivanna and Govindarajulu, 2009). In *M. alpina* young spores were not sensitive to UV irradiation when given for very short time intervals. The mutagenic agent UV though practical, is not an effective way to induce mutagenesis and hence it is preferable to use long exposures and concomitant high killing rates to increase the probability of mutation. It was found that UV irradiation had a higher spore killing efficiency than NTG and EMS as indicated by the respective survival curves. In *Mortierella,* mutation possibly altered the essential functions of the cells beyond repair and in doing so killed them.

The success of any strain improvement program is directly influenced by the selection strategy employed for screening mutants. In the present study, low

temperature and TTC staining were used as the selection methods for isolating high ARA producing mutants. It is well documented that organisms capable of thriving at low temperature have higher membrane fluidity due to the increased levels of PUFAs including ARA. Hiruta *et al.* (1996) used low temperature growth as an index, to isolate *Mortierella ramanniana* mutant MM 15-1, which produced lipids with high γ-linolenic acid (GLA) content. We employed this strategy and the putative mutants with higher growth rate at lower temperature were selected and sub-cultured for further evaluation by TTC staining. The principle of TTC staining relies on the oxidation of TTC by intact mitochondrial dehydrogenase, which yields the carmin red product, tri phenyl formazan. One of the earliest uses of the tetrazolium salts was in the rapid testing of the viability of germinating seeds (Lakon, 1953). It is also used extensively to visualize the infract size in brain cells (Cheng *et al*., 2006; Joshi *et al*., 2004). A positive correlation between TTC staining degree and ARA content in lipids was observed in the present study as reported by Zhu *et al*. (2004). The staining action of TTC in *M. alpina* is based on the presence of a Δ^5 desatuarse, a dehydrogenase enzyme which catalyzes the formation of ARA from dihomo-γ-linolenic acid (DGLA). Mutants with high levels of the enzyme are hyper stained, whereas negative mutants remain less stained or unstained owing to reduced or eliminated Δ^5 desaturase enzyme activity. This forms the basis of the selection scheme, for rapid screening of mutant strains of *M. alpina* with enhanced arachidonic acid production.

In *Mortierella,* mutation often resulted in the reduced functionality of one or more enzymes in the ARA biosynthetic pathway resulting in a total or partial cessation of ARA production as assumed by the large number of negative mutants obtained after TTC staining. Mutants derived from *M. alpina* 1S-4, defective in Δ^5 and Δ^6 desaturases have been previously described (Sakuradani *et al*., 2009d).

In spite of the higher spore killing efficiency of UV over chemical mutagens, the latter yielded more prospective mutants after TTC staining technique. EMS is an alkylating agent that induces point mutagenesis by A-T to G-C transition (French *et al*., 2006). This mutagenic agent has been used to increase glucose oxidase activity and citric acid production in *Aspergillus niger* (Lotfy *et al*., 2007; Khattab and Bazaraa, 2005), to improve recombinant strains (Lussier, 1997) and to isolate free fatty acid secreting mutants of *S. cerevisiae* (Mobini-Dehkordi *et al*., 2008). In

addition, mutant strains of *S. cerevisiae* that have resistance to antifungal drugs have been isolated using EMS (Hapala *et al*., 2005).

Mutagenesis and selection effectively enhanced arachidonic acid yield in *Mortierella*. It is possible that the mutagens gave the cell an oxidative stress that had an inductive effect which ultimately led to increased ARA production. The best mutant M386 was isolated from screened strains which showed maximum ARA production after repeated subculture.

The enhanced total lipid and ARA production by the mutant strain M386 confirmed the efficiency of NTG as a chemical mutagen. NTG has been widely used to induce mutations in microorganisms. It has been successfully employed for the production of carotenoids from *Rhodotorula rubra* (Frengova *et al*., 2004), lipase from *Aspergillus niger* (Elliah *et al*., 2002), phosphate accumulating mutants of *E. Coli* (Morohoshi *et al*., 2003), kojic acid by *Aspergillus oryzae* (Futamura *et al*., 2001) and lipase from *Pseudomonas* (Caob and Zhanga, 2000). Efficacy of NTG may be attributed to the multiple mutations at the replication point through its effect on DNA polymerase III.

From the results it is evident that the mutagenic agents employed in this study were effective for strain improvement of *Mortierella alpina*. A superior mutant (M386) with an ARA productivity of 203.13% (0.98 to 1.95g/L) higher than that of the parent strain was obtained. Furthermore the use of TTC made the effective screening of large number of putative mutants possible.

3.5 Conclusions

The results of the present study illustrated that conventional random mutagenesis may still be a useful and effective technique for the development of fungal strains capable of increased arachidonic acid production. Effectiveness of physical and chemical mutagens in strain improvement and TTC staining technique for rapid screening of mutants for enhanced ARA productivity was established in the present investigation. It is crucial that the high yielding fungal mutant strain can be subjected to statistical optimization for further improvement in ARA yield. The enhancement of arachidonic acid production in mutants could be ascribed to structural change of the Δ^5 desaturase enzyme, elevated enzyme levels, increased level of modulator proteins or cofactors promoting the enzyme activity or decreased level of inhibitory proteins.

4.1 Introduction

Health and safety are priorities for food and drug manufacturers, particularly when microbes are involved in the process. Numerous microorganisms have a long history of safe use in food production that predates scientific assessment. Few microorganisms have been assessed scientifically in a manner that would fully characterize all potential risks associated with the food they are used to produce including in some instances, the consumption of viable microorganisms. The safety assessments that have been conducted have focussed primarily on the absence of pathogenicity and adverse effects attributed to ingestion of these microorganisms, rather than evaluating the results of prescribed studies.

The market for functional products such as single cell oils, is one of the fastest growing areas of food science and product development which introduces a new paradigm for food safety professionals. These aim at providing favourable physiological benefits beyond those of good nutrition without any undesirable effects. In case of fortified foods, the objective is to formulate products that are at least as safe as conventional counter parts regardless of potential health benefits. In case of existing strains, the history of safe use criterion is very much associated with detection, enumeration and characterization of strains to ensure the safety of consumers ingesting the same organism throughout the claimed exposure period. A crucial step in ensuring the safety of a source organism is to perform a battery of toxicity tests in an appropriate animal model and in many instances animal data from toxicity studies is the only information available in supporting human overdose effects. Although acute toxicity test has been widely criticized as a parameter for assessing toxicity (Timbrel, 2002; Klassen, 2001; Lorke, 1983), there are still some occasions when some useful information could be obtained from such studies. Apart from giving an idea on the range of doses that could be used for subsequent toxicity testing, it could equally divulge the potential harmful effects elicited by the substance under investigation.

Subchronic studies give valuable information on the probable health hazards expected to arise from continuous exposure over a period of rapid growth into young adulthood. It also predicts the vulnerability of a long term, low dose exposure to particular compound. These studies give insights into the major toxic effects, target organs of toxicity and possibility of accretion of the test material and can provide an

estimate of the no observed adverse effect levels (NOAEL) of exposure which can be used for selecting dose levels for chronic study and for establishing safety criteria for human exposure.

While most filamentous fungi are not known to have any adverse effects on human health, some can produce highly toxic metabolites and initiate allergic responses. A mass influx of polyunsaturated fatty acid (PUFA) fortified functional foods in world market has prompted research into possible fungal sources and an oleaginous mould *M. alpina* has been recognized as a robust candidate for the production of arachidonic acid - a pharmaceutically and nutraceutically important PUFA. Although there is no indication that species of *Mortierella* have a strong toxigenic potential, compared to other fungi commonly used for food products, this must be shown experimentally for the actual strains used in production and hence great emphasis has been placed on safety testing of the strains used. Several safety studies of ARA enriched triglyceride obtained from *Mortierella alpina* have been reported (Sakuradani and Shimizu, 2003; Hempenius *et al.*, 1997, 2000; Streekstra, 1997). Most of the studies have focussed on the safety of ARA oil from *M. alpina* and not the organism *per se*. The present safety assessment looks not only at the safety of the intended dietary ingredient (ARA) but also at the safety of ingestion of the whole organism, which includes other nutritional compounds and metabolites.

4.2 Materials and methods

4.2.1 Chemicals

Glucose and Yeast extract were procured from Hi media Pvt. Ltd, India; EDTA and fatty acid standards from Sigma Chemicals, USA. All other chemicals used were of analytical grade obtained from E Merck, Pvt. Ltd., India. Solvents used were also of analytical reagent grade procured from Qualigens Pvt. Ltd., India.

4.2.2 Organism and culture maintenance

Mortierella alpina strain CBS 528.72 was grown in glucose yeast extract (GY) medium for 7 days at 28±1ºC by shaking at 240 rpm in an orbital shaker as reported (Nisha and Venkateswaran, 2009). After growth, biomass was harvested by suction filtration and freeze dried. This was subsequently powdered and used for experimental diet preparation.

4.2.3 Proximate analysis of *M. alpina* biomass

Prior to feeding, proximate composition of *M. alpina* biomass was determined by standard methods.

4.2.3.1 Determination of moisture content

Moisture content of the biomass was determined according to AOAC method (2005). Sample (2g) was weighed in an aluminium dish and placed in a hot air oven maintained at 130±1°C for 4 h. The sample was subsequently cooled to room temperature in a dessicator and the loss of weight in percentage was reported as the moisture content.

4.2.3.2 Determination of total ash content

Ash content was determined according to the procedure described by AOAC (2005). Sample was accurately weighed in a silica crucible. The contents of the crucible were charred on a hot plate (100-200°C) and after the sample stopped emitting smoke, the crucible was kept in a muffle furnace. The temperature of the muffle furnace was slowly raised to 550°C and ashing was done for 5 h. The weight of the crucible with its ash content was recorded. The ash content was calculated and expressed as percentage of original sample.

4.2.3.3 Determination of fat

Total fat content was determined according to the AOAC method (2005) with slight modification. Powdered biomass (10g) was macerated with 0.1N HCl for 20 min. After treatment, the sample was packed in a filter paper and washed thoroughly under running tap water to remove traces of acid. This was dried and accurately weighed into a thimble and extracted using diethyl ether as solvent for 12 h. The extract was collected in a previously weighed flat bottom flask and the solvent was evaporated over hot water bath. The flask was dried in an oven at 100°C, cooled and weight was taken. The fat content was expressed as g/100g of the sample.

4.2.3.4 Determination of the crude fibre content

Crude fibre content was determined according to AOAC method (2005). Sample (2g) was extracted thrice with petroleum ether and dried in a hot air oven for 30 min. The dried sample was taken in a digestion flask. 200 mL of preheated sulphuric acid (1.25%) was added, connected to a condenser and boiled for 30 min. The digested material was immediately filtered through a linen cloth and washed with near boiling water until free of acid. To the residue, 200 mL of preheated NaOH

solution (1.25%) was added, connected to the condenser and boiled for 30 min. The digested material was immediately filtered through a sintered crucible and washed thoroughly with near boiling water to remove the alkali. Finally the residue was washed with alcohol, diethyl ether and acetone, dried at 100°C for 3 h and weight was taken. The sintered crucible was transferred to muffle furnace and the material was made to ash and weight was determined. The crude fiber in percentage of original sample was calculated.

4.2.3.5 Determination of protein content

Total protein content was determined by micro Kjeldahl's method according to AOAC (2005). 2g biomass and 1g digestion mixture were taken in a Kjeldahl's flask. 20 mL sulphuric acid was added to this and digested until the organic matter was oxidized. The digest was cooled and the volume made up to 100 mL with distilled water.

An aliquot of 25 mL was taken for steam distillation with 20 ml of 40% NaOH solution. The liberated ammonia was absorbed in 10 ml of 2% boric acid containing a few drops of mixed indicator. This was titrated against N/70 HCl. Simultaneously standard (Ammonium sulphate) was titrated to estimate the amount of nitrogen content of the sample. The protein content of the sample was calculated in percentage using the factor 6.5.

4.2.3.6 Analysis of mineral constituents

Analysis of the mineral constituents was done according to the procedure described by Mani *et al.* (2007).

4.2.3.6.1 Preparation of standard solutions

4.2.3.6.1.1 Zinc

Zinc sulphate (0.439g) was dissolved in 200 ml of distilled water to which 5 mL of 1:5 H_2SO_4 was added. The contents were transferred to a volumetric flask and the volume was made up to one litre. From this 100 µg/mL zinc stock solution, working standards of 0, 0.1, 0.2, 0.4 and 0.6µg/mL solutions were prepared.

4.2.3.6.1.2 Iron

Ferrous ammonium sulphate (0.702 g) was dissolved with 5 mL of 1:5 H_2SO_4 and made up to one litre to give 100 µg/mL Fe^{2+} stock solution. Working standards 0, 0.1, 0.2, 0.4 and 0.6µg/mL concentrations were prepared from this stock solution.

4.2.3.6.1.3 Manganese

Potassium permanganate (0.288 g) was dissolved in 300 mL of distilled water, 20 mL concentrated H_2SO_4 was added and warmed to 60°C. Oxalic acid was added drop by drop to make the solution colourless. The contents were cooled and made up to one litre to get 100 µg/mL stock solution. From this stock solution, working standards of 0, 0.1, 0.2, 0.4 and 0.6µg/mL were prepared.

4.2.3.6.1.4 Copper

Copper sulphate (0.392 g) was dissolved in distilled water and made up to one litre to give 100 µg/ml of copper. From this, working standards of 0, 0.1, 0.2, 0.4 and 0.6µg/mL were prepared.

4.2.3.6.2 Preparation of triple acid extract

Powdered biomass (0.5 g) was transferred into a 100 mL conical flask and 15 ml of triple acid mixture (nitric: sulphuric: perchloric acid 9:2:1) was added. The contents of the flask were digested over a sand bath until clear, filtered through a Whatman No. 42 filter paper and collected in a 100 mL volumetric flask. Contents of the flask and residue were washed with small increments of hot water. The volumetric flask was subsequently cooled and the volume was made up to 100 mL with cold distilled water. The triple acid extract so prepared was stored for the analysis of mineral constituents.

The reading of the filtrate was taken in an atomic absorption spectrophotometer (Shimadzu AA-6701, Japan) and the concentration of each micronutrient in the sample was estimated by using the standard curve.

4.2.3.6.3 Determination of phosphorus

Reagent A

a. Ammonium molybdate (12 g) in 250 mL of distilled water.

b. Antimony potassium tartarate (0.291 g) in 100 mL distilled water.

c. 100 mL of 5N H_2SO_4 was prepared

All the three reagents were mixed well and made upto 2 litres.

Reagent B

Ascorbic acid (1.056g) was dissolved in 200 mL of reagent A. Biomass (1g) was weighed accurately and transferred to a polypropylene shaking bottle. 50mL of 0.5M $NaHCO_3$ (pH 8.5) and a pinch of activated charcoal powder (High media, India) was added. The contents were mixed well in a mechanical shaker

for 30 min and filtered through Whatman No. 42 filter paper. From this, 5 mL of the filtrate was pipetted out into 25mL volumetric flask, 4 mL of freshly prepared reagent B was added and made up to 25mL. The contents were shaken well and allowed to stand for few minutes. The intensity of blue colour developed was measured in a spectrophotometer at 660nm. Standard curve was plotted and concentration of phosphorus was calculated.

4.2.4 Animals and Treatment Design

Animal care and handling conformed to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and the protocols were approved by the Institutional Animal Ethical Committee (IAEC). The animals were housed in individual stainless steel cages and were provided food and water *ad libitum.* During the experimental period, there were no environmental changes that affected the outcome of the study. The animals were maintained under specifically controlled environmental conditions viz., temperature 26±2ºC under a light and dark cycle (12-12 h) and relative humidity of 60-70%. These values were within acceptable ranges with no significant deviations.

4.2.5 Diet Preparation

Standard rat feed was procured from M/s Sai Durga Feeds and Foods, Bangalore. Freeze dried *M. alpina* biomass was powdered and added at different levels to prepare the experimental diet. There were no known contaminants in the feed or water which would interfere with the results. For subchronic study, diet was prepared once every 10 days. The stability of the test substance in the experimental diet was ensured by storage at 4°C and confirmed by routine fatty acid analysis. The composition of standard rat feed was 22.11% crude protein, 61% carbohydrate, 4.1% crude fat, 3.16% crude fiber, 5.13% ash and 4.5% moisture.

4.2.6 Viability test

Viability of fungal biomass and presence of live fungus in the faecal matter was determined by standard spread plate method. One gram of dried biomass or faecal pellet was taken under aseptic conditions and serial dilutions plated on potato dextrose agar and incubated at room temperature for 96 h. The number of colonies formed were counted and expressed as colony forming units per gram (CFU/g).

4.2.7 Acute oral exposure in rats

In acute study, a total of thirty healthy adult male albino Wistar rats (260±10g) were randomly assigned to five feeding regimens with six rats in each group, and were given a single dose of biomass at different levels (0, 0.5, 1.0, 2.5, 5.0 g/kg BW) mixed with 2g feed and monitored for a period of 14 days. Unintentional predisposition was minimized during allocation of animals to their respective groups. Only males were used for acute study. Daily feed and water intake, faecal and urine output was recorded throughout the experimental period. Body weights were obtained on the day of test administration and on 2^{nd}, 7^{th} and 14^{th} day. On completion of the experiment, the animals were weighed, sacrificed by ether anesthesia, and blood samples were collected for haematological and serum biochemical investigations. The vital organs were weighed and relative organ weights (g/100g body weight) were calculated. Complete histological analysis was done for any treatment related anomalies. The protocols followed are detailed in the following section.

4.2.8 Subchronic oral exposure study

Three week old male and female (36-38g) Wistar rats bred in our animal house facility were randomly assigned to six groups, each comprising of six males and six females and were fed *M. alpina* biomass at 0, 2.5, 5.0, 10.0, 20.0 and 30.0 g/kg of the diet, for groups 1 through 6, respectively, for 13 weeks. Since the biomass was intended for use in infant formulae, the test diet administration was commenced at the weanling stage. The animals were observed twice daily for any mortality and clinical signs. Weekly body weight was recorded throughout the treatment period. The amount of supplied and residual diet was weighed daily and the mean daily feed intake for each week was calculated. Test substance intake was calculated from the feed intake and concentration of the test substance in the diet admixture. Test diet was available *ad libitum* for 13 weeks except for one-night fasting prior to scheduled sacrifice.

On completion of the experiment, the rats were anesthetized with diethyl ether and euthanized via cervical decapitation. A portion of the blood was allowed to clot at room temperature for 20 min. Serum was separated by centrifugation (R8c Remi, India) at 2000 rpm for 10 min and stored at $^{-}40°C$ until analysis.

4.2.8.1 Haematology

Haematological examination was performed using a K-4500 automated haematology analyzer (Sysmex Corp, Japan). Aliquots of whole blood samples were mixed with a 4 fold volume of suppliers buffer containing 0.5% ethylene diamine tetra acetic acid (EDTA.2K) and applied to the analyzer for the following parameters: haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet count (PC). For measuring differential leukocyte count, blood samples were mixed with ¼ volume of 5% EDTA.2K and analyzed with a Microx HEG-120A (Omron Tateishi Electronics Co., Ltd, Tokyo, Japan).

4.2.8.2 Relative organ weights

At necropsy all the organs/tissues were carefully observed macroscopically for any lesions. The fresh organ weights were noted for liver, lungs, kidneys, heart, spleen, testis/ovary, brain and adrenals. The organ weights were recorded as absolute values and their relative values were calculated on the basis of the final body weights of the rats.

4.2.8.3 Histopathology

At autopsy, the vital organs were surgically removed from the rats, washed with normal saline, fixed and preserved in 10% neutral buffered formalin. All major tissues were further processed and trimmed. The samples were dehydrated in an alcohol series, cleared in xylene and embedded in paraffin. Blocks were cut at a microtome setting of 4-6µm, mounted on glass slides and stained with hematoxylin and eosin for complete histopathologic evaluations. Liver, lungs, kidneys, heart, spleen, testis/ovary, brain and adrenals were examined microscopically for any histological deviations. The stained sections were examined and photographed using a microscope (Olympus BX 40, USA).

4.2.8.4 Serum biochemical indices

The serum levels of glucose, triglycerides, cholesterol, urea, creatinine and activities of key marker enzymes viz., alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST) and lactate dehydrogenase (LDH) were assayed using standard kits.

4.2.8.4.1 Glucose

Glucose estimation was done using standard kit procured from AccuDx technologies Pvt. Ltd, India. GOD/POD method (Basak, 2007; Ambade *et al*., 1998) was used for estimation. The sample and the reagent were allowed to attain room temperature prior to use. To 1mL of the reagent, 10µl sample /standard was added and incubated at 37 °C for 10 min. After incubation, the solution was mixed well and the absorbance at 505nm was taken against the reagent blank. Concentration of glucose in sample (mg/dl) =Abs of test/Abs of Standard x 100

4.2.8.4.2 Triglyceride

Determination of Triglycerides was done using GPO-POD method (Bucolo and David, 1973) according to the instructions of the kit manufacturer (Aspen Laboratories Pvt Ltd, India). To 1 mL of the reagent, 10µl of distilled water, standard or sample was added for the blank, standard and test respectively, mixed and incubated for 10 min at 37°C and the absorbance was read within 60 min against reagent blank.

Triglyceride (mg/dl) = Abs of sample/Abs of Std x Conc. of Std (mg/dl)

4.2.8.4.3 Cholesterol

Reagent kit procured from Span diagnostics Ltd, India was used for cholesterol estimation. One step method by Wybenga *et al*. (1970) was used, based on reaction of cholesterol hot solution of ferric perchlorate, ethyl acetate and sulphuric acid, yielding a lavender color complex. To 3 mL of the cholesterol reagent, 15 µl of standard /sample was added, mixed well and kept in a boiling water bath for 90 seconds. The tubes were immediately cooled to room temperature under running tap water. The absorbance of the standard and test against blank were taken on a spectrophotometer at 560nm.

Total cholesterol (mg/dl) = Absorbance of the sample/absorbance of Standard x 200

4.2.8.4.4 Urea

Diacetylmonoxime (DAM) method was used for urea estimation. The assay kit was procured from Span diagnostics Ltd, India. The reagents were allowed to attain room temperature prior to use. To 2.5 mL of the urea reagent, 10 µl of sample /standard were added for test and standard respectively. After mixing well, 250 µl DAM was added. The tubes were mixed well and kept in boiling water bath for 10

min and subsequently cooled immediately for 5 min, mixed gently by inversion and the absorbance at 525nm was measured within 10 min.

Urea in mg/100ml =absorbance of test/absorbance of standard x 30

4.2.8.4.5 Creatinine

Standard kit for creatinine estimation was obtained from Span diagnostics Ltd, India. Modified Jaffe's reaction was used for the *in vitro* quantitative determination of creatinine in serum. To 1 mL of working reagent prepared by mixing equal volume of picrate reagent and sodium hydroxide, 100 µl of serum or standard was added for test and standard respectively. Initial absorbance of the standard AS1 was measured after 30 sec and final absorbance AS2 after an interval of 120 sec. Reading for the test AT1 and AT2 were similarly recorded.

Creatinine mg/dl = (AT2- AT1) / (AS2-AS1) x 2

4.2.8.4.6 Alkaline phosphatase (ALP)

Alkaline phosphatase activity was determined by modified IFCC method (Tietz *et al*., 1983) using standard kit obtained from Aspen Laboratories Pvt Ltd, India. To 1 mL of working reagent, 20µl of the sample was added, mixed well and incubated for 1 min at 37°C. Absorbance at 405nm was read immediately and measured again after 1, 2 and 3 min. The change in absorbance (ΔA) per min was calculated.

ALP activity (U/L) = ΔA/min x 2757(factor)

4.2.8.4.7 Alanine aminotransferase (ALT)

The kit for determining ALT activity was procured from Aspen Laboratories Pvt Ltd, India. Modified IFCC method (Bergermeyer *et al*., 1986) was used for ALT determination. To 1mL of the working reagent, 100µl of the sample was added, mixed well and the absorbance at 340 nm was read after 1min. The absorbance was again read after 1, 2 and 3 min at 37°C. The change in absorbance (ΔA) per min was calculated.

ALT activity (U/L) = ΔA/min x 1745(factor)

4.2.8.4.8 Aspartate amino transferase (AST)

AST estimation was done by using standard kit procured from Aspen Laboratories Pvt Ltd, India. Modified IFCC method was used for estimation. To 1mL of the working reagent, 100µl of the sample was added, mixed well and the absorbance at 340 nm was read after 1min. The absorbance was again read after 1, 2 and 3 min at 37 ℃. The change in absorbance (ΔA) per min was calculated.

AST activity (U/L) = ΔA /min x 1745(factor)

4.2.8.4.9 Lactate dehydrogenase (LDH-L)

Reagent kit procured from Teco diagnostics, USA was used for LDH estimation. To 1mL of the reagent, 25µl of the sample was added, mixed well and incubated at 37°C for 60 sec. The absorbance at 340 nm was measured exactly at one min interval for the next two minutes. Absorbance change per minute (ΔA /min) was calculated.

LDH activity (U/L) = ΔA /min x 6592(factor)

4.2.9 Statistical analysis

Statistical analysis was performed with SPSS software version 8.0 (SPSS, 1998). Comparison of results from control and treatment groups were carried out by one - way analysis of variance (ANOVA) and post hoc analysis of group differences were performed by Duncan's multiple range test. The values are given as mean ± SEM. Level of significance was considered at $P \leq 0.05$ unless otherwise stated.

4.3 Results

4.3.1 Proximate analysis

On analysis, *M. alpina* biomass yielded 10.75% crude fiber, 40.95% protein, 3.98% total ash, 3.88% moisture, 3.57% carbohydrates and 36.87% total lipids (Fig 4.1). Micronutrient analysis indicated that the biomass contained 0.613µg/g Zinc, 1.410µg/g Iron, 1.370µg/g Copper and 1.090µg/g phosphorous in addition to the macronutrients. Manganese was not detected in biomass.

4.3.2 Viability test

The viability of *M. alpina* biomass was assessed prior to feeding. No viable spores were found either in the biomass or faecal matter of rats in any of the treatment groups.

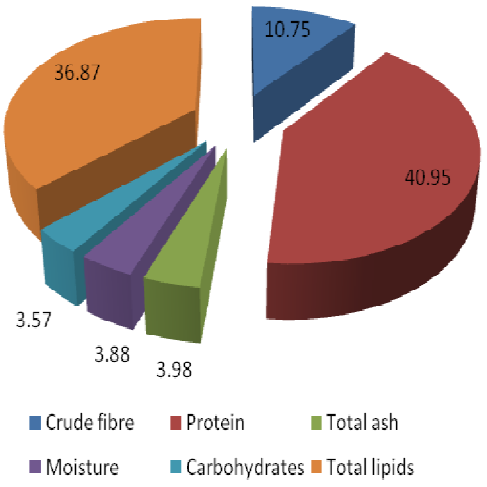

Fig 4.1 Composition of *M. alpina* biomass

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4.3.3 Acute oral exposure

The 14 days acute study of *M. alpina* biomass on male Wistar rats caused no deaths or treatment-related signs. No incidence of clinical signs of toxicity ascribed to biomass ingestion viz., ill health, abnormal behaviour, changes in locomotor activity, ataxia and gastrointestinal intolerance were noted either immediately or during the post treatment period even at the highest dose of 5g/kg BW. Thus, the LD_{50} is considered to be greater than 5 g/kg BW, which indicate that the biomass was not toxic according to the criteria for acute toxic classifications. No abnormal clinical signs or gross pathological abnormalities were manifested in any organ or tissue in the external, cephalic, thoracic or abdominal regions of the animals.

4.3.3.1 Body weight

The trend in body weights in control and biomass fed rats during the experimental period is given in Table 4.1. Augmentation of *M. alpina* biomass at 1and 2 g/kg BW levels had a stimulatory effect on growth and body weight gain of rats when compared to that of control. This marginal increase was not statistically significant. On the contrary, when given at 5g level, there was an initial weight loss and body weight of these rats at the completion of experiment were lower than that of control rats, though not statistically significant.

4.3.3.2 Food and water intake

The effect of biomass supplementation on food consumption pattern is given in Table 4.2. No significant alteration was noticed in food consumption of animals between control and treatment groups. There was no dose dependent decline in food intake except in the highest dosage group which showed a reduction in food intake on the second day. This reduction in food intake was not permanent and the rats continued to take food at normal levels for the rest of the experimental period. Water intake also failed to show any significant variation from that of the control (Table 4.3).

Table 4.1 Body weight of male Wistar rats given an acute dose of *Mortierella alpina* biomass

Dose (g/kg BW)	Body weight (g)			
	Day 0	Day 2	Day 7	Day14
Control	276.8±12.9	272.7±19.2	279.7±17.9	287.3±17.3
0.5	275.7±11.1	278.5±11.8	286.8±14.5	297.3±18.4
1.0	275.8±14.6	278.3±16.0	284.6±16.2	292.8±19.2
2.0	275.0±9.3	276.9±8.4	280.6±11.5	280.9±20.0
5.0	276.3±13.2	278.8±11.2	278.5±18.2	283.8±33.3

BW-body weight; Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$).

Table 4.2 Feed intake of male Wistar rats given an acute dose of *Mortierella alpina* biomass

	Feed intake(g/day)				
Day	**Control**	**0.5g/Kg BW**	**1g/Kg BW**	**2g/Kg BW**	**5g/Kg BW**
1	15.37±1.3	15.17±1.6	14.30±1.7	14.16±1.9	13.46±2.2
2	14.75±1.0	13.71±1.7	13.85±1.4	14.81±1.4	12.85±2.8
3	15.95±1.2	15.58±1.5	15.08±1.8	15.29±1.3	14.68±2.3
4	15.17±0.7	15.18±2.0	14.63±1.1	15.19±0.8	15.09±1.2
5	14.22±1.9	14.09±2.0	15.47±1.1	14.75±1.4	14.08±0.9
6	14.79±1.4	15.22±1.3	15.23±1.1	15.89±1.2	15.12±1.5
7	16.73±2.4	15.39±1.4	15.67±0.9	15.63±1.5	15.76±1.4
8	16.29±1.2	15.40±2.3	15.69±2.4	15.73±1.2	15.60±1.2
9	14.68±1.5	15.14±1.4	16.75±1.0	16.83±0.6	16.52±1.6
10	14.43±2.2	14.79±2.0	15.65±0.4	15.07±1.8	15.67±1.6
11	15.58±2.2	15.86±2.2	15.65±1.0	16.24±1.3	15.83±1.8
12	15.19±2.1	15.51±1.3	15.38±1.7	16.04±1.9	16.12±1.8
13	14.80±2.3	15.16±1.1	15.10±2.9	15.83±3.1	16.41±2.3
14	15.44±2.7	15.30±2.4	16.11±1.2	17.30±1.5	16.25±1.5

BW-Body weight; Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$).

Table 4.3 Water intake of male Wistar rats given an acute dose of *Mortierella alpina* biomass

Day	Water intake(ml/day)				
	Control	0.5g/Kg BW	1g/Kg BW	2g/Kg BW	5g/Kg BW
1	22.21±2.5	23.07±5.0	22.91±3.8	22.61±3.6	22.64±4.8
2	21.41±2.4	22.25±1.4	20.88±2.3	21.07±3.3	21.61±3.2
3	21.79±2.2	20.64±2.9	21.12±3.1	21.64±4.1	21.37±1.9
4	23.29±4.4	23.81±3.7	23.42±2.3	22.78±2.2	22.56±2.3
5	24.09±4.6	23.05±1.9	23.34±1.9	22.88±3.1	22.58±4.4
6	23.32±5.3	21.83±3.6	21.75±3.6	24.13±3.4	23.24±4.4
7	24.5±2.8	23.71±2.0	22.20±4.7	23.97±2.2	24.34±2.5
8	24.01±3.8	22.58±3.6	22.90±4.0	25.29±2.6	23.05±1.3
9	24.46±2.6	23.36±1.9	25.31±4.2	25.56±2.5	24.57±2.7
10	23.71±3.5	23.00±3.9	23.02±3.0	23.36±1.7	24.20±4.5
11	21.35±2.2	20.96±2.2	21.13±4.2	22.64±1.4	22.90±2.9
12	22.65±2.1	21.49±2.0	21.51±1.8	24.86±3.8	24.35±3.0
13	24.19±2.9	24.48±3.7	23.14±3.4	24.38±3.7	23.85±4.1
14	22.87±1.9	22.80±1.7	22.10±1.0	23.39±2.0	23.24±2.4

BW-Body weight; Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$

4.3.3.3 Faecal and urine output

An insignificant increment in faecal and urine output of rats fed *M. alpina* biomass when compared to that of control was noticed (Fig 4.2). This increase was not dose related.

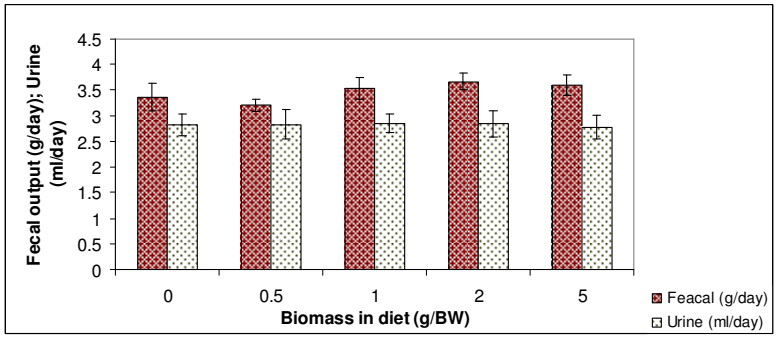


Fig 4.2 Faecal and urine output of male Wistar rats given an acute dose of *Mortierella alpina* biomass

BW-Body weight; Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$).

4.3.3.4 Relative organ weights and histopathology

Relative organ weights did not reveal any statistically significant difference between the control and treatment groups (Table 4.4). Histopathological studies of the vital organs did not exhibit any significant difference between the control and biomass fed animals.

4.3.3.5 Hematology and serum biochemistry

There were no significant changes in haematological profile and clinical biochemistry of rats fed *M. alpina* biomass (Table 4.5 and 4.6). Haematological investigations revealed a marginal reduction in haemoglobin concentration, packed cell volume and RBC count at the highest dose (5g/kgBW), but these were not statistically significant. The treatment did not reveal any significant increase in the serum levels of diagnostic marker enzymes of myocardial infarction LDH, ALT and AST and hence no leakage of these enzymes from heart (Table 4.6).

Table 4.4 Relative organ weights of male Wistar rats given an acute dose of *Mortierella alpina* biomass

Organ	*M.alpina* biomass in the diet (g/kg BW)				
	0	**0.5**	**1.0**	**2.0**	**5.0**
Liver	2.77±0.21	2.70±0.23	2.76±0.26	2.62±0.23	2.70±0.22
Lungs	0.49±0.07	0.46±0.09	0.46±0.09	0.54±0.09	0.42±0.07
Kidney	0.60±0.06	0.56±0.02	0.57±0.05	0.56±0.04	0.56±0.04
Heart	0.27±0.02	0.27±0.02	0.26±0.01	0.28±0.03	0.26±0.02
Brain	0.56±0.09	0.57±0.08	0.58±0.10	0.48±0.04	0.53±0.06
Spleen	0.18±0.02	0.20±0.02	0.19±0.03	0.19±0.02	0.19±0.03
Adrenals	0.05±0.01	0.04±0.01	0.05±0.00	0.04±0.00	0.04±0.01
Testis	0.98±0.06	0.96±0.09	0.92±0.10	0.96±0.04	0.96±0.05

Values are mean ± SEM (Standard error mean) of six animals and are expressed in g%. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$).

Table 4.5 Haematological profile of male Wistar rats given an acute dose of *Mortierella alpina* biomass

Parameter	*M. alpina* biomass in the diet (g/kg BW)				
	0	**0.5**	**1.0**	**2.0**	**5.0**
Hb(g/dl)	16.12±0.6	16.22±0.3	16.07±0.7	16.58±0.4	15.60±0.5
RBC(10^6/µl)	9.29±0.5	9.21±0.6	9.31±0.4	9.48±0.1	8.98±0.2
WBC(10^3/µl)	14.3±2.6	15.2±1.0	14.4±2.0	12.7±1.3	15.7±2.9
PCV(%)	51.6±2.9	51.5±1.1	51.2±2.9	52.8±1.9	49.8±1.5
MCV(fl)	55.6±2.9	56.2±4.4	54.9±1.4	55.7±1.7	55.4±1.9
MCH (pg)	17.37±0.7	17.70±1.2	17.23±0.4	17.48±0.3	17.35±0.5
MCHC(%)	31.23±0.7	31.54±0.3	31.40±0.4	31.42±0.5	31.35±0.4
PLC(10^5/mm^3)	8.58±0.99	8.69±0.82	8.02±1.30	8.43±1.16	8.55±0.77
Differential count (%)					
Neutrophils	20.00±3.4	17.60±5.9	20.50±8.3	16.50±3.7	17.67±4.7
Lymphocytes	70.5±4.7	74.0±6.4	71.2±8.8	75.3±4.7	73.3±4.4
Eosinophils	1.50±0.6	1.20±0.4	1.17±0.4	1.17±0.4	1.17±0.0
Monocytes	8.0±1.5	7.2±0.7	7.2±0.4	7.0±2.0	8.0±0.6
Basophils	0	0	0	0	0

Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$). BW-body weight, Hb - haemoglobin, RBC - red blood cells, WBC - white blood cells, PCV - packed cell volume, MCH - mean corpuscular haemoglobin, MCV - mean corpuscular volume, MCHC - mean corpuscular haemoglobin concentration, PLC - platelet count.

Table 4.6 Effect of dietary *Mortierella alpina* biomass on serum biochemistry of male Wistar rats

Parameter	*M. alpina* biomass in the diet (g/kg BW)				
	0	0.5	1.0	2.0	3.0
Glucose (mg/dl)	107.97±5.5	100.13±7.9	103.36±9.6	97.15±13.9	99.95±12.7
LDH (U/L)	1267.18±111.64	1095.42±152.92	1099.21±135.24	1203.04±84.33	1075.33±173.90
ALP (U/L)	102.7±11.2	104.2±10.8	95.12±9.60	98.16±14.3	98.95±16.3
AST (U/L)	102.9±23.96	78.68±15.01	89.54±18.58	96.70±17.09	90.34±12.42
ALT (U/L)	12.22±5.03	13.48±3.4	13.67±3.33	9.88±2.41	14.37±2.64
TG (mg/dl)	127.3±19.68	128.5±24.28	123.5±14.08	146.0±6.21	142.0±5.39
Cholesterol (mg/dl)	50.96±5.42	45.02±6.31	44.27±6.39	48.75±10.38	46.35±7.28

Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$). LDH, lactate dehydogenase (U/L); ALP, alkaline phosphatase (U/L); AST, asparate animo trasferase (U/L); ALT, alanine amino transferase (U/L); TG, triglycerides.

4.3.4 Sub chronic oral exposure study

4.3.4.1 Clinical signs

All the animals survived the experimental period. During the administration period, animals in their cages appeared normal with no overt clinical signs of toxicity or allergic reactions. No incidence of diarrhoea, constipation or other gastrointestinal disorders was observed. General condition and behaviour were not adversely affected by the test substance in any of the treatment groups. On autopsy, macroscopic observation of the animals revealed no alterations in the external surface and all the orifices of the cranial, thoracic and abdominal cavities were normal.

4.3.4.2 Feed intake and body weight

In general, compliance with the experimental diet was excellent, and all animals tolerated the dietary changes well, as assessed by 24-h feed intake (Table 4.7 and 4.8). Biomass fortification did not show any adverse effect on the palatability of food. A statistically significant increase was observed in the feed intake of animals in the *M. alpina* fed groups during the second week. The average daily biomass intake in the 2.5, 5.0, 10.0, 20.0 and 30.0 g/kg groups were 44.62, 86.15, 176.92, 356.92 and 536.92 mg/rat/day for males and 33.85, 64.62, 131.54, 270.77 and 424.62 mg/rat/day for females respectively, increasing in proportion with the concentration of the test compound in the diet (Table 4.9 and 4.10).

No treatment related body weight loss was manifested in *M.alpina* fed groups throughout the study (Table 4.11 and 4.12). A statistically significant increase in the body weight of *M .alpina* fed males was noted during the 2^{nd}, 3^{rd} and 4^{th} week. Group 2 males attained the highest body weight on completion of the experiment. In females, a statistically significant increment was observed in the body weight during the 1^{st} week.

4.3.4.3 Relative organ weights and histopathology

The relative organ weights did not show any significant deviation in males (Table 4.13). The marginal increase in relative brain weights observed in males (Groups 3, 4 and 5) was not statistically significant. In females, adrenals, lungs, kidney and heart did not show any significant deviation from that of control in any of the treated animals (Table 4.14). Variation documented in relative spleen weight in females failed to show any significance at the highest dose. A significant increase in relative ovary, liver and brain weights was noticed in females in *M. alpina* fed groups when

Table 4.7 Daily feed intake of male Wistar rats fed *Mortierella alpina* biomass for 13 weeks

Week	Daily feed intake(g)					
	Control	2.5g/kg	5.0g/kg	10.0g/kg	20.0g/kg	30.0g/kg
1	8.60±0.84[a]	9.32±2.33[a]	8.67±1.21[a]	8.69±0.49[a]	8.83±1.48[a]	9.02±0.24[a]
2	9.20±0.26[a]	10.29±0.18[b]	10.80±0.24[bc]	10.86±0.14[bc]	11.22±0.84[cd]	11.62±0.18[d]
3	9.88±1.24[a]	10.16±1.28[a]	11.28±1.54[a]	11.46±1.28[a]	12.06±1.22[a]	12.42±1.24[a]
4	10.69±0.84[a]	11.02±0.60[a]	11.84±0.62[a]	12.01±0.70[a]	12.48±0.89[a]	12.86±0.79[a]
5	11.61±0.46[a]	12.51±2.13[a]	13.01±1.12[a]	12.95±0.99[a]	13.68±0.01[a]	13.98±0.61[a]
6	13.09±0.73[a]	14.40±0.98[a]	13.90±0.85[a]	13.63±0.52[a]	13.73±1.50[a]	14.28±0.59[a]
7	12.44±0.79[a]	13.36±1.02[a]	13.08±0.66[a]	13.14±0.82[a]	13.36±0.72[a]	14.66±1.20[a]
8	12.18±0.50[a]	12.26±0.71[a]	12.90±0.42[a]	12.94±0.25[a]	14.08±1.23[a]	14.98±1.17[a]
9	14.01±0.59[a]	14.28±0.61[a]	14.84±0.97[a]	14.11±1.32[a]	14.44±0.68[a]	15.28±1.09[a]
10	14.87±0.55[a]	14.88±0.60[a]	14.97±0.60[a]	15.04±1.26[a]	14.98±0.61[a]	15.62±1.42[a]
11	14.82±0.65[a]	14.99±0.73[a]	14.93±2.08[a]	15.02±0.96[a]	15.28±0.01[a]	15.18±0.72[a]
12	14.64±1.40[a]	14.68±1.09[a]	14.74±1.66[a]	15.02±0.68[a]	16.24±1.62[a]	16.46±1.09[a]
13	15.43±0.66[a]	15.88±0.82[a]	15.98±1.42[a]	16.40±0.73[a]	16.45±1.99[a]	17.28±1.07[a]

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 4.8 Daily feed intake of female Wistar rats fed *Mortierella alpina* biomass for 13 weeks

Week	Daily feed intake (g)					
	Control	**2.5g/kg**	**5.0g/kg**	**10.0g/kg**	**20.0g/kg**	**30.0g/kg**
1	6.91±0.38[a]	7.30±0.73[a]	7.66±0.66[a]	7.32±0.85[a]	7.83±0.69[a]	8.01±0.46[a]
2	11.24±0.52[a]	11.98±0.08[ab]	11.80±0.10[ab]	11.81±0.16[ab]	11.77±0.14[ab]	12.34±0.15[b]
3	14.52±0.63[a]	15.72±0.88[a]	15.22±0.87[a]	14.82±0.87[a]	16.26±0.62[a]	16.78±1.34[a]
4	15.88±1.36[a]	17.06±0.68[a]	17.56±0.54[a]	17.76±0.83[a]	17.92±1.27[a]	18.36±1.26[a]
5	16.89±1.17[a]	17.34±0.57[a]	16.90±0.83[a]	16.94±0.64[a]	17.78±0.37[a]	17.91±0.51[a]
6	17.28±0.72[a]	18.55±0.81[a]	17.90±0.64[a]	17.54±1.49[a]	18.15±0.95[a]	18.18±0.68[a]
7	17.64±0.70[a]	18.96±0.68[a]	18.13±0.94[a]	19.56±1.72[a]	19.34±1.31[a]	20.46±0.96[a]
8	17.58±0.68[a]	19.81±0.96[a]	17.92±1.43[a]	19.44±1.20[a]	19.01±1.21[a]	19.36±1.31[a]
9	16.53±1.19[a]	19.21±1.05[a]	18.24±1.11[a]	19.36±0.75[a]	18.41±1.21[a]	18.68±1.87[a]
10	17.87±0.67[a]	19.86±1.15[a]	18.87±0.73[a]	19.84±0.98[a]	19.47±0.76[a]	20.56±1.44[a]
11	18.99±0.96[a]	20.12±0.87[a]	19.59±0.41[a]	21.04±1.07[a]	21.51±0.51[a]	19.48±1.83[a]
12	21.68±0.75[a]	22.29±0.82[a]	21.82±0.68[a]	21.83±0.61[a]	22.34±0.69[a]	21.92±0.47[a]
13	20.50±0.28[a]	21.28±1.78[a]	20.6±0.72[a]	21.48±1.06[a]	21.24±0.76[a]	20.68±0.53[a]

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 4.9 Daily biomass intake of male Wistar rats fed dietary *Mortierella alpina* biomass for 13 weeks

Week	Daily Biomass intake(mg)					
	Control	**2.5g/kg**	**5.0g/kg**	**10.0g/kg**	**20.0g/kg**	**30.0g/kg**
1	-	18.25±1.8^{a}	38.3±3.3^{b}	73.20±8.5^{c}	156.6±13.8^{d}	240.3±13.8^{e}
2	-	29.95±0.2^{a}	59.0±0.5^{b}	118.1±1.6^{c}	235.4±2.80^{d}	370.2±4.50^{e}
3	-	39.30±2.2^{a}	76.1±4.4^{b}	148.2±8.7^{c}	325.2±12.4^{d}	503.4±40.2^{e}
4	-	42.65±1.7^{a}	87.8±2.7^{b}	177.6±8.3^{c}	358.4±25.4^{d}	550.8±37.8^{e}
5	-	43.35±1.4^{a}	84.5±4.2^{b}	169.4±6.4^{c}	355.6±7.40^{d}	537.3±15.3^{e}
6	-	46.38±2.1^{a}	89.5±3.2^{b}	175.4±14.9^{c}	363.0±19.0^{d}	545.4±20.4^{e}
7	-	47.4± 1.7^{a}	90.7±4.7^{b}	195.6±17.2^{c}	386.8±26.2^{d}	613.8±28.8^{e}
8	-	49.53± 2.4^{a}	89.6±7.2^{b}	194.4±12.0^{c}	380.2±24.2^{d}	580.8±39.3^{e}
9	-	48.03± 2.6^{a}	91.2±5.6^{a}	193.6±7.5^{b}	368.2±24.2^{c}	560.4±56.1^{d}
10	-	49.65± 2.9^{a}	94.4±3.7^{b}	198.4±9.8^{c}	389.4±15.2^{d}	616.8±43.2^{e}
11	-	50.30± 2.2^{a}	97.9±2.1^{b}	210.4±10.7^{c}	430.2±10.20^{d}	584.4±54.9^{e}
12	-	55.73± 2.2^{a}	109.1±3.4^{b}	218.3±6.1^{c}	446.8±13.8^{d}	657.6±14.1^{e}
13	-	53.2± 4.5^{a}	103.0±3.6^{b}	214.8±10.6^{c}	424.8±15.2^{d}	620.4±15.9^{e}

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 4.10 Daily biomass intake of female Wistar rats fed *Mortierella alpina* biomass for 13 weeks

Week	Daily Biomass intake(mg)					
	Control	2.5g/kg	5.0g/kg	10.0g/kg	20.0g/kg	30.0g/kg
1	-	23.3±5.83^{a}	43.4±6.1^{a}	86.9±4.9^{b}	176.6±29.6^{c}	270.8±7.2^{d}
2	-	25.7±0.45^{a}	54.0±1.2^{b}	108.6±1.4^{c}	224.4±16.8^{d}	348.6±5.4^{e}
3	-	25.4±3.2^{a}	56.4±7.7^{a}	114.6±24.2^{b}	241.2±24.4^{c}	372.6±37.2^{d}
4	-	27.6±1.5^{a}	59.2±3.1^{b}	120.1±7.0^{c}	249.6±17.8^{d}	385.8±23.7^{e}
5	-	31.3±5.3^{a}	65.1±5.6^{b}	129.5±9.9^{c}	273.6±0.20^{d}	419.4±18.3^{e}
6	-	36.0±2.5^{a}	69.5±4.25^{b}	136.3±5.2^{c}	274.6±30.0^{d}	428.4±17.7^{e}
7	-	33.4±2.6^{a}	65.4±3.3^{b}	131.4±8.2^{c}	267.2±14.4^{d}	439.8±36.0^{e}
8	-	30.7±1.8^{a}	64.5±0.0^{a}	129.4±2.5^{b}	281.6±24.6^{c}	449.4±35.1^{d}
9	-	35.7±1.5^{a}	74.2±4.9^{b}	141.1±13.2^{c}	288.8±13.6^{d}	458.4±32.7^{e}
10	-	37.2±1.5^{a}	74.9±3.0^{a}	150.4±12.6^{b}	299.6±12.2^{c}	468.6±42.6^{d}
11	-	37.5±1.8^{a}	74.7±10.4^{b}	150.2±9.6^{c}	305.6±0.2^{d}	455.4±21.6^{e}
12	-	36.7±2.7^{a}	73.7±8.3^{a}	150.2±6.8^{b}	324.8±32.4^{c}	493.8±32.7^{d}
13	-	39.7±2.1^{a}	79.9±7.1^{a}	164.0±7.3^{b}	329.0±39.8^{c}	518.4±32.1^{d}

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 4.11 Weekly body weight of male wistar rats fed *M.alpina* biomass for 13 weeks

Week	Body weight (g)					
	Control	2.5g/kg	5.0g/kg	10.0g/kg	20.0g/kg	30.0g/kg
Initial	36.17±0.66[a]	36.17±0.54[a]	36.03±0.68[a]	36.28±0.73[a]	36.20±0.45[a]	36.78±0.27[a]
1	56.20±1.27[a]	62.38±1.08[a]	60.22±0.58[a]	60.13±1.52[a]	60.53±1.16[a]	63.50±0.77[a]
2	89.18±5.45[a]	101.07±2.41[b]	95.47±1.99[ab]	96.07±2.34[ab]	96.72±2.21[ab]	98.43±3.10[ab]
3	119.17±5.81[a]	136.33±3.46[b]	126.00±4.22[ab]	122.67±4.87[ab]	126.67±2.86[ab]	128.17±4.58[ab]
4	135.00±7.92[a]	159.17±3.91[b]	147.20±5.90[ab]	142.50±5.06[ab]	148.83±3.66[ab]	145.50±3.15[ab]
5	169.83±9.84[a]	190.50±5.25[a]	176.80±6.59[a]	171.67±8.51[a]	178.33±6.49[a]	192.33±9.28[a]
6	200.67±10.96[a]	219.67±10.18[a]	210.40±7.77[a]	202.17±11.71[a]	206.00±9.69[a]	228.33±10.58[a]
7	234.83±10.85[a]	249.00±15.62[a]	240.60±9.35[a]	236.66±11.97[a]	237.67±10.13[a]	258.16±11.34[a]
8	249.17±10.78[a]	268.17±14.10[a]	250.60±11.69[a]	257.00±8.38[a]	253.00±8.74[a]	271.33±10.12[a]
9	268.00±11.03[a]	288.33±13.07[a]	275.00±8.98[a]	270.17±14.37[a]	269.50±12.94[a]	284.17±10.44[a]
10	274.33±11.55[a]	298.17±12.85[a]	282.60±9.78[a]	278.50±14.5[a]	283.5±12.22[a]	293.33±9.59[a]
11	282.6±8.04[a]	309.50±14.68[a]	286.50±10.41[a]	296.33±12.6[a]	302.50±11.43[a]	302.00±11.77[a]
12	287.40±7.33[a]	318.00±13.61[a]	289.17±10.63[a]	302.17±11.65[a]	305.67±11.02[a]	307.33±11.46[a]
13	296.33±11.37[a]	324.67±14.89[a]	298.17±14.59[a]	310.83±10.56[a]	314.00±11.37[a]	311.33±11.40[a]

Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups ($P<0.05$).

Table 4.12 Weekly body weight of female wistar rats fed *M.alpina* biomass for 13 weeks

Week	Body weight (g)					
	Control	2.5g/kg	5.0g/kg	10.0g/kg	20.0g/kg	30.0g/kg
Initial	36.95±0.67[a]	36.77±0.42[a]	36.03±0.82[a]	36.60±1.12[a]	36.02±0.69	36.92±0.90[a]
1	54.67±1.46[a]	57.57±1.03[ab]	58.93±0.98[ab]	58.80±0.65[ab]	56.95±2.20[ab]	59.82±1.16[b]
2	84.58±3.07[a]	90.42±2.11[a]	89.17±1.67[a]	87.92±1.21[a]	85.92±2.93[a]	84.58±3.46[a]
3	106.17±2.96[a]	110.50±2.70[a]	106.67±2.03[a]	109.17±3.16[a]	106.67±1.89[a]	108.67±1.54[a]
4	109.00±4.91[a]	116.17±2.20[a]	113.33±1.15[a]	112.67±3.45[a]	114.17±1.30[a]	119.00±4.02[a]
5	124.67±4.31[a]	129.33±2.80[a]	126.67±1.89[a]	125.33±3.56[a]	128.00±2.49[a]	132.67±3.70[a]
6	137.50±3.49[a]	140.83±2.54[a]	137.67±1.84[a]	144.00±3.91[a]	141.17±2.94[a]	139.67±3.94[a]
7	145.33±2.40[a]	151.33±4.12[a]	148.83±3.68[a]	156.67±3.65[a]	150.83±3.05[a]	155.17±4.76[a]
8	151.83±3.13[a]	158.83±3.62[a]	152.33±2.99[a]	161.50±3.58[a]	156.67±2.85[a]	162.50±5.35[a]
9	159.67±4.70[a]	167.33±4.10[a]	162.00±4.30[a]	160.00±3.79[a]	164.17±2.89[a]	168.33±5.00[a]
10	163.83±3.79[a]	173.00±5.29[a]	168.67±5.00[a]	167.50±4.35[a]	168.83±3.09[a]	176.83±5.79[a]
11	166.67±3.33[a]	171.67±4.66[a]	172.00±4.40[a]	177.67±5.90[a]	173.17±2.41[a]	179.50±6.45[a]
12	169.83±3.48[a]	175.17±4.59[a]	181.5±5.91[a]	176.33±4.69[a]	176.67±2.68[a]	184.33±6.86[a]
13	175.18±4.62[a]	180.42±3.46[a]	184.32±4.80[a]	178.64±5.34[a]	180.44±3.68[a]	186.28±6.48[a]

Values are mean ± SEM (Standard error mean) of six animals. No significant difference between control and *Mortierella alpina* biomass fed groups (P<0.05).

compared to that of the control. The changes observed in relative organ weights were not accompanied by relevant changes in haematology or treatment related histopathological findings.

There were no adverse histopathology deviations in spleens, livers or kidneys of animals of any test group compared to controls. Livers of treated animals showed no shrunken hepatocytes or congestions in portal tracts and sinusoids. Sections revealed well-formed structure of parenchyma and portal triads. No evidence of centrilobular degenerative changes, sleatosis or necrosis was observed in the treatment groups. Lungs showed organized alveolar spaces and no thickening of inter alveolar septa or cellular infiltrations were observed. Kidneys of treated animals were also normal and did not present any glomerular or vascular congestion. No swelling of epithelium and occlusion of lumen were noticed even in the highest dose group. Other vital organs like heart and brain also showed normal structure. Histological examination of ovaries in the treatment groups revealed no abnormalities in germinal epithelium, stages of follicular development, maturation and corpus luteum. There were no microscopic or macroscopic lesions in any organs that could be attributed to the treatments. Histopathological investigations failed to reveal any incidence of organ toxicity in this study.

4.3.4.4 Haematological and serum biochemical indices

Haematological investigations revealed a dose dependent significant decrease in haemoglobin concentration in group 3, group 4, group 5 and group 6 males which was not manifested in females. WBC counts showed a significant increase in group 6 animals. Significant increase in lymphocyte count was found in males in groups 5 and 6 males whereas no such alteration was found for eosinophils, monocytes or basophils. No significant differences were found in RBC counts, PCV, MCV, MCH, MCHC and platelets in both the sexes (Table 4.15 and 4.16).

On serum biochemical analysis, statistically significant dose-dependent reduction was detected for triglyceride in both males and females. No significant alteration was seen in glucose, cholesterol, urea, and ALT values in both the sexes (Table 4.17 and 4.18). AST and LDH levels showed a significant reduction in group 4, group 5 and group 6 males whereas no such alteration was noticed for females. Alkaline phosphatase activity showed an increment in *M. alpina* fed males. AST concentrations were consistently higher than ALT levels.

Table 4.13 Relative organ weights of male Wistar rats fed *Mortierella alpina* biomass for 13 weeks

Organ	*M.alpina* biomass in the diet (g/kg)					
	0	**2.5**	**5.0**	**10.0**	**20.0**	**30.0**
Liver	3.09±0.05[a]	2.92±0.03[a]	2.90±0.63[a]	3.03±0.29[a]	3.37±0.29[a]	2.92±0.53[a]
Lungs	0.48±0.02[a]	0.45±0.02[a]	0.46±0.05[a]	0.46±0.03[a]	0.46±0.02[a]	0.47±0.02[a]
Kidney	0.77±0.02[a]	0.71±0.04[a]	0.75±0.05[a]	0.74±0.05[a]	0.82±0.07[a]	0.77±0.07[a]
Heart	0.33±0.01[a]	0.30±0.01[a]	0.29±0.02[a]	0.30±0.02[a]	0.34±0.03[a]	0.28±0.02[a]
Brain	0.54±0.05[a]	0.59±0.01[a]	0.69±0.09[a]	0.60±0.02[a]	0.61±0.03[a]	0.56±0.02[a]
Spleen	0.23±0.01[a]	0.22±0.02[a]	0.21±0.02[a]	0.25±0.01[a]	0.24±0.02[a]	0.21±0.02[a]
Adrenals	0.05±0.01[a]	0.05±0.00[a]	0.05±0.01[a]	0.05±0.02[a]	0.05±0.01[a]	0.05±0.02[a]
Testis	1.12±0.04[a]	1.04±0.06[a]	0.96±0.19[a]	1.04±0.03[a]	1.09±0.05[a]	0.95±0.04[a]

Values are means ± SEM, n = 6 and are expressed as g%. No significant difference between control and *Mortierella alpina* fed groups ($P \leq 0.05$)

Table 4.14 Relative organ weights of female Wistar rats fed *Mortierella alpina* biomass for 13 weeks

Organ	*M.alpina* biomass in the diet (g/kg)					
	0	**2.5**	**5.0**	**10.0**	**20.0**	**30.0**
Liver	2.45±0.23[a]	2.91±0.04[b]	2.91±0.04[b]	2.82±0.06[b]	2.93±0.10[b]	2.75±0.11[ab]
Lungs	0.56±0.06[a]	0.57±0.02[a]	0.55±0.02[a]	0.54±0.03[a]	0.54±0.02[a]	0.58±0.02[a]
Kidney	0.74±0.06[a]	0.78±0.02[a]	0.76±0.02[a]	0.77±0.02[a]	0.78±0.03[a]	0.74±0.03[a]
Heart	0.34±0.04[a]	0.36±.01[a]	0.34±0.02[a]	0.35±0.00[a]	0.35±0.01[a]	0.33±0.01[a]
Brain	0.66±0.09[a]	0.91±0.05[b]	0.89±0.04[b]	0.91±0.04[b]	0.92±0.02[b]	0.84±0.02[b]
Spleen	0.22±0.02[a]	0.28±0.01[ab]	0.30±0.02[b]	0.30±0.02[b]	0.27±0.02[ab]	0.23±0.01[a]
Adrenals	0.03±0.01[a]	0.03±0.01[a]	0.03±0.01[a]	0.03±0.00[a]	0.03±0.05[a]	0.03±0.03[a]
Ovary	0.08±0.01[a]	0.12±0.02[b]	0.11±0.01[b]	0.13±0.02[b]	0.11±0.01[b]	0.11±0.01[b]

Values are means ± SEM, n = 6 and are expressed as g%. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test.

Table 4.15 Haematological profile of male Wistar rats fed *M.alpina* biomass for 13 weeks

Parameter	*M.alpina* biomass in the diet (g/kg)					
	0	2.5	5.0	10.0	20.0	30.0
Hb(g/dl)	16.90±0.24^{c}	16.88±0.32^{c}	16.57±0.33^{bc}	15.85±0.22^{ab}	15.86±0.13^{ab}	15.62±0.26^{a}
RBC(10^6/µl)	9.99±0.15^{a}	9.90±0.16^{a}	9.70±0.28^{a}	9.5±0.16^{a}	9.55±0.11^{a}	10.23±0.62^{a}
WBC(10^3/µl)	18.73±1.30^{a}	18.93±0.83^{a}	19.05±2.82^{a}	19.48±2.77^{a}	19.87±1.67^{a}	23.01±2.71^{b}
PCV(%)	57.52±1.36^{a}	59.68±1.21^{a}	60.17±1.00^{a}	58.83±1.45^{a}	60.72±0.20^{a}	59.70±0.86^{a}
MCV(fl)	57.32±1.15^{a}	55.30±5.87^{a}	62.18±1.09^{a}	61.90±0.89^{a}	63.67±1.93^{a}	62.45±0.58^{a}
MCH(pg)	16.78±0.21^{a}	17.08±0.36^{a}	17.10±0.31^{a}	16.68±0.20^{a}	16.63±0.37^{a}	16.33±0.17^{a}
MCHC(%)	26.95±1.58^{a}	28.35±0.25^{a}	27.52±0.23^{a}	26.97±0.33^{a}	26.13±0.23^{a}	26.15±0.18^{a}
PLC(10^5/mm^3)	8.96±0.55^{a}	10.07±0.19^{a}	8.92±1.07^{a}	9.08±0.30^{a}	9.25±0.36^{a}	9.45±0.35^{a}
Differential count (%)						
N	19.67±0.42^{a}	20.17±0.41^{a}	18.83±2.80^{a}	19.89±0.24^{a}	18.88±1.06^{a}	19.92±0.48^{a}
L	75.83±2.94^{a}	75.98±1.25^{a}	76.00±1.42^{a}	76.17±1.94^{a}	77.83±1.54^{b}	80.83±0.65^{b}
E	1.00±0.01^{a}	1.33±0.21^{a}	1.17±0.17^{a}	1.17±0.17^{a}	1.17±0.17^{a}	1.33±0.21^{a}
M	2.50±0.34^{a}	2.64±0.42^{a}	2.00±0.52^{a}	2.33±0.42^{a}	2.00±0.89^{a}	2.17±0.40^{a}
B	-	-	-	-	-	-

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. Hb - haemoglobin, RBC - red blood cells, WBC - white blood cells, PCV - packed cell volume, MCH - mean corpuscular haemoglobin, MCV - mean corpuscular volume, MCHC - mean corpuscular haemoglobin concentration, PLC - platelet count, N- Neutrophils, L- Lymphocytes, E- Eosinophils, M-Monocytes, B-Basophils

Table 4.16 Haematological data for female wistar rats fed *M. alpina* biomass for 13 weeks

Parameter	*M.alpina* biomass in the diet (g/kg)					
	0	**2.5**	**5.0**	**10.0**	**20.0**	**30.0**
Hb(g/dl)	15.57±0.32[a]	15.03±0.27[a]	15.12±0.15[a]	15.28±0.22[a]	15.17±0.17[a]	15.27±0.13[a]
RBC(10^6/µl)	9.03±0.13[a]	8.96±0.19[a]	8.85±0.10[a]	8.90±0.4[a]	8.87±0.15[a]	8.86±0.09[a]
WBC(10^3/µl)	14.88±1.84[a]	13.95±1.65[a]	14.10±2.82[a]	14.31±0.27[a]	14.90±1.04[a]	17.28±1.34[b]
PCV(%)	60.65±1.26[a]	58.42±1.28[a]	58.80±0.69[a]	60.00±1.15[a]	59.58±1.28[a]	60.07±0.99[a]
MCV(fl)	67.15±0.57[a]	66.20±0.65[a]	66.47±0.48[a]	67.40±1.21[a]	67.25±0.83[a]	67.32±0.49[a]
MCH(pg)	17.22±0.19[a]	16.80±0.25[a]	17.10±0.20[a]	17.17±0.21[a]	17.17±0.21[a]	17.28±0.09[a]
MCHC(%)	25.65±0.22[a]	25.73±0.20[a]	25.73±0.20[a]	25.48±0.21[a]	25.67±0.32[a]	25.60±0.26[a]
PLC(10^5/mm^3)	9.68±0.50[a]	9.72±0.32[a]	9.02±0.39[a]	9.50±0.43[a]	8.86±0.28[a]	9.12±0.39[a]
Differential count (%)						
N	13.33±1.78[a]	12.50±1.06[a]	12.33±0.88[a]	12.67±1.65[a]	16.00±1.48[a]	11.83±0.17[a]
L	83.00±1.75[a]	84.5±1.38[a]	84.17±1.08[a]	83.83±1.81[a]	81.17±1.96[a]	85.00±0.45[a]
E	1.17±0.17[a]	1.17±0.17[a]	1.17±0.17[a]	1.17±0.17[a]	1.17±0.17[a]	1.17±0.17[a]
M	2.50±0.22[a]	1.83±0.31[a]	2.33±0.33[a]	2.33±0.42[a]	1.87±0.49[a]	2.00±0.37[a]
B	-	-	-	-	-	-

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. Hb - haemoglobin, RBC - red blood cells, WBC - white blood cells, PCV - packed cell volume, MCH - mean corpuscular haemoglobin, MCV - mean corpuscular volume, MCHC - mean corpuscular haemoglobin concentration, PLC - platelet count, N- Neutrophils, L- Lymphocytes, E- Eosinophils, M-Monocytes, B-Basophils

Table 4.17 Effect of dietary *Mortierella alpina* biomass on serum biochemistry of male Wistar rats

Parameter	*M. alpina* biomass in the diet (g/kg)					
	0	**2.5**	**5.0**	**10.0**	**20.0**	**30.0**
Cholesterol(mg/dl)	85.35±9.60^{a}	68.92±6.95^{a}	84.34±3.78^{a}	82.42±3.11^{a}	75.53±4.92^{a}	87.42±3.65^{a}
TG (mg/dl)	124.83±4.5^{b}	96.12±4.95^{a}	93.71±6.52^{a}	90.23±5.98^{a}	88.56±5.74^{a}	88.23±6.52^{a}
AST(U/L)	142.66±7.09^{b}	143.38±3.28^{b}	143.53±2.98^{b}	115.32±2.09^{a}	107.32±4.87^{a}	104.41±6.42^{a}
ALT(U/L)	42.46±2.63^{a}	36.65±1.66^{a}	42.02±1.91^{a}	41.30±3.88^{a}	40.57±2.31^{a}	37.37±2.45^{a}
ALP(U/L)	91.61±6.14^{a}	107.75±10.67^{a}	124.99±7.01^{a}	132.33±20.59^{ab}	171.16±19.54^{bc}	194.14±6.08^{c}
LDH(U/L)	1219.52±19.03^{b}	1211.28±15.78^{b}	1219.53±58.65^{b}	865.20±15.78^{a}	898.16±08.24^{a}	889.92±26.91^{a}
Glucose(mg/dl)	76.88±2.19^{a}	76.19±2.61^{a}	76.78±1.54^{a}	88.67±8.15^{a}	85.04±8.65^{a}	84.24±7.31^{a}
Urea(mg/dl)	17.19±1.09^{a}	16.47±2.16^{a}	19.25±2.21^{a}	18.74±1.20^{a}	21.18±3.17^{a}	15.58±2.84^{a}
Creatinine(mg/dl)	0.84±0.03^{a}	0.88±0.05^{a}	0.88±0.05^{a}	0.83±0.07^{a}	0.80±0.04^{a}	0.80±0.03^{a}

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. LDH, lactate dehydogenase; ALP, alkaline phosphatase; AST, aspartate animo trasferase; ALT, alanine amino transferase;TG,Triglycerides

Table 4.18 Effect of dietary *Mortierella alpina* biomass on serum biochemistry of female Wistar rats

Parameter	*M.alpina* biomass in the diet (g/kg)					
	0	**2.5**	**5.0**	**10.0**	**20.0**	**30.0**
Cholesterol (mg/dl)	75.02±2.06[a]	73.17±2.07[a]	71.21±2.42[a]	70.25±4.90[a]	72.26±1.05[a]	72.26±1.48[a]
TG (mg/dl)	126.52±3.82[c]	123.37±4.84[bc]	118.98±2.60[b]	106.55±2.48[a]	108.06±2.85[a]	104.67±4.23[a]
AST(U/L)	102.29±3.92[a]	106.88±2.58[a]	102.53±2.95[a]	106.70±2.58[a]	103.70±2.45[a]	105.56±3.34[a]
ALT(U/L)	29.37±1.98[a]	27.37±1.45[a]	28.79±1.45[a]	30.98±1.91[a]	27.20±2.15[a]	26.39±1.69[a]
ALP(U/L)	78.18±2.67[a]	77.63±3.27[a]	79.57±1.98[a]	78.11±2.46[a]	76.79±2.89[a]	77.21±1.64[a]
LDH(U/L)	1211.28±56.09[a]	1227.76±72.93[a]	1213.92±44.30[a]	1211.28±36.54[a]	1236.00±67.95[a]	1326.56±36.5[a]
Glucose(mg/dl)	101.06±3.81[a]	107.85±4.92[a]	94.88±11.73[a]	88.50±6.87[a]	105.03±11.78[a]	76.98±8.83[a]
Urea(mg/dl)	27.95±2.67[a]	25.42±1.82[a]	24.56±1.61[a]	23.60±2.54[a]	24.84±1.69[a]	24.68±1.57[a]
Creatinine(mg/dl)	0.54±0.03[a]	0.53±0.02[a]	0.52±0.01[a]	0.53±0.02[a]	0.52±0.01[a]	0.48±0.01[a]

Values are means ± SEM, n = 6. Values in the same row that do not share the same alphabetic superscripts are significantly different at 5% levels according to Duncan's multiple range test. LDH, lactate dehydogenase; ALP, alkaline phosphatase; AST, asparate animo trasferase; ALT, alanine amino transferase; TG,Triglycerides

4.4 Discussion

A considerable interest has developed in recent years for production of ARA since it is well documented that ARA is important for both infants and geriatrics. ARA supplementation improves spatial cognition in aged rats (Okaichi *et al*., 2005) and also ameliorates age related endothelial dysfunction that leads to various cardiovascular diseases (Nakano *et al*., 2007). Since the activity of Δ^5 desaturase enzyme which catalyzes the conversion of dihomogamma linolenic acid to arachidonic acid decline with age (De Gomez *et al*., 1983; Blond and Bezard, 1991), dietary supplementation of long chain PUFAs including ARA for the elderly is crucial. Extensive application of ARA in nutraceutical and pharmaceutical fields coupled with their significance in health and dietary requirements has encouraged "hunting" for more suitable sources and great emphasis has been placed on safety evaluation of the actual strains used in production.

The present study is the first to evaluate the safety of the *M. alpina* biomass in rats as such as all previous studies were done with the extracted oil. The fortification of biomass as such could evade the solvent extraction of oil which made the PUFAs prone to oxidative damage. Further more in addition to PUFAs, *M. alpina* biomass proved to be a good source of protein, dietary fibre and micronutrients.

Acute studies did not reveal any overt signs of toxicity which indicated that the biomass was safe at the levels given in this study. Absence of mortality or morbidity in the experimental groups further confirmed the safety of *M. alpina* biomass.

The test diets were well accepted by the treated rats suggesting that *M.alpina* biomass did not result in any change in carbohydrate, protein or fat metabolism in these experimental animals. Further more it is an attestation of the fact that the diet did not adversely impede with the nutritional benefits viz., weight gain and stability of appetite expected of animals that are continually supplied with biomass fortified food and water *ad libitum.*

In the present study a stimulatory effect of biomass fortification on body weight gain during the 2^{nd}, 3^{rd} and 4^{th} week in males and first week in females was observed. It was reported earlier that five day-old piglets supplemented for 2 weeks with ARA exhibit a 27% increase in body weight with no changes in body length

(Merritt *et al*., 2003). ARA is positively correlated to growth in preterm infants (Weiler, 2000). Postulation of a specific role of ARA in growth and body weight gain is complex because of its multiple and diverse functions. It appears that supplementation of biomass containing ARA facilitates growth, consistent with the known role of fatty acids in growth (Xiang and Zetterstrom., 1999; Carlson *et al*., 1993; Koletzko and Braun, 1991). The stimulatory effect found in the present study was not evident in the succeeding weeks pointing to the fact that ARA effect was more prominent in the early phase of growth.

Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs and in toxicological experiments, comparison of organ weights between control and treated groups have conventionally been used to predict toxic effect of a test article (Pfeiffer, 1968; Peters and Boyd, 1966). Significant increase in liver and spleen weight seen in the present study was manifested only in females and did not result in any adverse health consequences. This was not coupled with morphological changes and no evidence of toxicity was found. Increased spleen weight without histopathological correlation indicative of toxicity is generally considered to be a physiological adaptation to high dietary levels of unsaturated fatty acids and not a manifestation of toxicity (Lina *et al*., 2006). Burns *et al.* (1999) also reported an increase in spleen and liver weight of rats fed high levels of ARA/DHA. Increased relative brain weights encountered in the *M. alpina* fed groups can be attributed to the stimulatory effect of ARA on brain development (Weiler, 2000; Carlson *et al*., 1993). Though noticed in males, the effect of ARA was more pronounced in females. Increased ovarian weight in treatment groups cannot be considered as a manifestation of toxicity due to the variability attributable to its small size and physiological factors unrelated to treatment like estrus cycle and relative infrequency of these organs as target organs of toxicity. Further more arachidonic acid has a stimulatory effect on ovarian steroidogenesis and production of luteinizing hormone- releasing hormone (LHRH) (Wang and Leung, 1989), which in turn leads to a spur in ovarian follicular growth. This possibly might have resulted in increased relative ovarian weight in biomass supplemented groups. The absence of significant changes in other vital organs in the present study points to the fact that ingestion of *M. alpina* biomass did not induce any anomalous growth or inflammation of these

organs which would otherwise have resulted in higher relative organ weights in the treatment groups.

Feed supplementation of *M.alpina* biomass did not induce any statistically significant alteration in the haematological profile of rats except for a reduced haemoglobin concentration in males and elevated WBC counts at the highest dose. In the present study, a dose dependent reduction in haemoglobin concentration was found in males. This is in agreement to the result obtained by Lina *et al*. (2006) who opined that accumulation of PUFAs in the red blood cell membrane increase lipid peroxidation and subsequently RBC senescence. In humans it has been reported that n-6 rich safflower oil increased the susceptibility of erythrocytes to oxidative damage by free radical generation (Mills *et al*., 1995). High dietary levels of *M. alpina* biomass rich in PUFAs possibly modified the fatty acid composition of the erythrocyte membrane and affected the survival time of erythrocytes in the circulation. Enhanced leucocytes count in the highest dosage group may be correlated with the increased immuno protective condition upon supplementation of PUFA rather than a toxic effect (Roy *et al*., 2008). This could be an evidence of the fact that *M. alpina* biomass may contain biologically active principles that have the ability to increase the population of defensive WBC thereby triggering the immune system. Other haematological parameters had no significant differences between the control and treatment groups which indicated that haematological values were not significantly affected by fortification of *M. alpina* biomass. These findings also suggest that the biomass of *M. alpina* may not be toxic as they do not significantly affect the circulating red blood cells nor the haematopoiesis or leucopoiesis that could otherwise have caused a megaloblastic anemia, or significant changes in packed cell volume (PCV) and eosinophils. These also indicate that the normal metabolism of the animals was not affected.

The observed diminution in triglycerides found in the present study can be attributed to the lowering effects of PUFAs on blood lipids (Meritt *et al*., 2003; Harris, 1989; Herzberg, 1989). Although unsaturated fatty acids of the n-3 series are more effective than n-6 PUFAs, it was reported by Hempenius *et al.* (2000) that both ARA and DHA oils were equally effective in lowering blood lipids. Similar reduction of serum triglycerides has been reported in humans following administration of fish oil (Saynor and Gillot, 1992). This reduction in triglycerides may be due to increased

fatty acid oxidation or reduced lipogenesis (Rodriguez Cruz *et al*., 2005) as well as reduced activity and possibly the expression of all lipogenic enzymes (Delzenne and Kok, 1999; Kok *et al*., 1996). The normal levels of urea and creatinine attained in the present study indicated that *M. alpina* biomass did not interfere with the renal capacity of metabolite excretion and is an evidence of the conserved renal integrity of treated rats (Kaneko, 1989).

The results of the present study reveal no elevated activity of any of the marker enzymes (LDH, ALT and AST). Elevated levels of lactate dehydrogenase are found in pathologic situations like myocardial infraction, liver diseases, renal disease, and certain forms of anemia, malignant diseases and progressive muscular dystrophy. Leakage of liver enzyme lactate dehydrogenase is very commonly used for measuring cytotoxicity of test reagents. ALT localized primarily in cytosol of hepatocytes is considered as a sensitive marker of hepatocellular damage and within limits can provide a quantitative assessment of the degree of damage sustained by the liver (Al Mamary *et al*., 2002). Absence of elevated activities of these diagnostic marker enzymes suggest the safety of *M. alpina* augmentation at levels used in the present study. The increase in alkaline phosphatase activity in *M. alpina* fed males is attributed to the administration of extra fat in the diet. Similar increases in alkaline phosphatase activity have been reported previously with high fat diets (Kroes *et al*., 2003; Hempenius *et al*., 2000; Burns *et al*., 1999)

Absence of any significant histological findings viz., cellular infiltrations, inflammation and lesions in the vital organs emphasizes the safety aspect of *M. alpina* biomass at levels given in this experiment. The fundamental issue in this study was not the safety of ARA *per se* but rather the safety of *M. alpina* biomass as a source of arachidonic acid. NOAEL of *M.alpina* biomass in the model studied was identified as 5.0 g/kg diet. No test article related health outcomes, histopathologic changes of concern or other evidence of altered organ structure or function were found. Few statistically significant changes observed were manifested either in one sex and/or were not concomitant with relevant histopathological abnormalities. These variations are attributable to the incorporation of extra fat in the diet. Our findings indicate that *M. alpina* biomass was nontoxic and well tolerated in rats. The absence of any apparent negative effects combined with the positive effect on growth, biomass gain

and triglyceride diminution should encourage feed usage of ARA rich *M. alpina* biomass.

Despite the limitations for predicting human safety based on experiments in rodents, the results of the study point out that *M. alpina* biomass has a positive preclinical profile in safety evaluation. However evaluation should be done in a suitable non-rodent model before extrapolating the results in humans. The present investigation could be regarded as preliminary probes necessitating further studies to establish the safety of *M. alpina* biomass.

4.5 Conclusions

The present safety evaluation study did not reveal any overt toxic effects either immediately or during the course of feeding. A preliminary acute toxicity study revealed that the biomass was safe at acute doses and that the LD50 exceeded 5000 mg/kg BW, the highest dose used in the study. *M. alpina* biomass in diet was well accepted with a positive influence on growth and no evident toxic effects on the survival, food consumption and body weight gain throughout the treatment interlude. The statistically significant changes in relative organ weights, serum biochemical and hematological indices in *M. alpina* fed groups' viz., higher relative weights of spleen, liver, brain and ovary in females, reduced hemoglobin concentration in males, elevated WBC counts at highest dose, reduction in serum triglycerides and increased alkaline phosphatase activity were not concomitant with pertinent histopathological changes and hence toxicologically inconsequential. No microscopic or macroscopic lesions attributable to the treatment were manifested in the experimental groups. The results of the present study strongly advocate the safety of *M. alpina* biomass in rats at levels used in the study.

5.1 Introduction

Polyunsaturated fatty acids (PUFAs) are important components of cellular structure and function and serve as precursors to eicosanoids, including prostaglandins and leukotrienes. They are sophisticated signalling molecules that can mediate a myriad of processes involved in cellular communication, differentiation, and cell death (Palmer *et al*., 1998). LC-PUFA are not only required for the development of the foetal neuronal system but also contribute via a multiplicity of beneficial roles to the maintenance of health with increasing development and age, particularly by reducing the incidence of cardiovascular diseases (Demaison and Moreau, 2002).

Biosynthesis of pharmacologically and nutraceutically important PUFAs in organisms involves a series of desaturation and elongation steps. In long chain PUFA biosynthesis, the desaturases, along with elongases introduce consecutive series of desaturations and elongations to generate biologically potent fatty acids including arachidonic acid (Periera *et al*., 2003). The most ubiquitous and wide spread modification of fatty acids is the insertion of double bonds which dramatically increase the membrane fluidity due to decrease in electrostatic interaction between lipid molecules (Hashimoto *et al*., 2006). Fatty acid desatuarses (EC 1.14.99), a family of enzymes omnipresent in most living cells catalyze the addition of regioselective double bonds in a fatty acyl chain and play a key role in regulating the fluidity of membrane lipids. The desaturation reaction essentially an aerobic process, utilizes molecular oxygen and reducing equivalents from electron transport chain (Pereira *et al*., 2003).

A surfeit of information exists in the role of dietary PUFAs in the prevention and mitigation of chronic diseases and current research is focussed mostly on understanding the mechanisms regulating desaturase gene activity which in turn give new insights into human diseases associated with lipid dysfunction. The study of fatty acid desaturases has important biotechnological applications involving the engineering of novel plant oils to meet the escalating demands of the chemical, pharmaceutical and nutraceutical industry. Identification of novel desaturases helps our understanding of evolutionary relationships between organisms and also provides valuable information on how different organisms have evolved distinct mechanisms of PUFA biosynthesis in adaptation to their environment (Pereira *et al*., 2003). As

structural characterizations of membrane bound desaturases are inadequate owing to the technical difficulties in obtaining large volumes of purified membrane bound proteins, molecular genetic approaches will be the alternative resource used to cram the structure function relationship of different membrane bound desaturases (Periera *et al*., 2003).

Over the last decade, major breakthrough in the cloning and identification of fatty acid desaturase genes from an array of different organisms has given new insights into the physiological functions, attributes as well as biosynthesis of PUFAs. A multitude of organisms including lower eukaryotes such as fungi, algae and protozoa are known to produce large amounts of PUFAs with chain lengths of C20 and greater (Singh and Ward, 1997a). The pathway for fatty acid desaturation and elongation from stearic acid (18:0) to LC-PUFAs in filamentous fungi has been elucidated both by biochemical means and by studying mutant strains isolated by classical mutagenesis (Ratledge, 1993; Certik *et al*., 1998). Thus far, cDNAs have been cloned for the Δ^9, Δ^6 and Δ^5 desaturases in different organisms. These include Δ^9 desaturases from rat (Thiede *et al.,* 1986), mouse (Ntambi *et al.,* 1988; Kaestner *et al.,* 1989) and humans (Zhang *et al.,* 1999). Δ^6 desaturase genes have been cloned from some GLA and STA producing species like borage (Sayanova *et al*., 1997), mosses *Physcomitrella patens* (Girke *et al*., 1998), *Ceratodon purpeureus* (Sperling *et al*., 2000), echium (Garcia Maroto *et al*., 2002), diatom (Domergue *et al*., 2002) and anemone (Whitney *et al*., 2003). These genes have also been isolated from *M. alpina* (Huang *et al*., 1999; Sakuradani *et al*., 1999b; Sakuradani and Shimizu, 2003), *Caenorhabditis elegans* (Napier *et al*., 1998), cyanobacteria (Reddy *et al*., 1993), rat (Aki *et al*., 1999), mouse (Cho *et al*., 1999b) and man (Cho *et al*., 1999a). The gene encoding Δ^5-desaturase (DES1) which converts dihomo-γ-linolenic acid (20:3) to arachidonic acid (20:4) has been isolated from several organisms (Knutzon *et al*., 1998; Domergue *et al*., 2002; Michaelson *et al*., 1998b; Saito and Ochiai, 1999).

All the genes encoding fatty acid desaturases and elongases enzymes in the PUFA biosynthetic pathway such as $\Delta^{5,}$ Δ^6, Δ^9, Δ^{12} and ω3 desaturases and elongases have been isolated and characterized in *Mortierella alpina* IS-4. Preliminary analysis of the *M. alpina* Δ^5 gene sequences available in data bank revealed considerable differences between the sequences although certain regions are highly conserved. This disparity may account for the differential ability to accumulate arachidonic acid

among the strains of *M. alpina*. We focussed on isolating the gene encoding this enzyme from *M. alpina* CBS 528.72 and to compare it phylogenetically with other *M .alpina* Δ^5 desaturase sequences available in Genbank. The phylogenetic relationship of Δ^5 sequences with other desaturases in *M. alpina* was also considered.

5.2 Materials and Methods

5.2.1Chemicals

Ethidium bromide, SDS, Tris-HCl, EDTA and PCR clean up kit were procured from Sigma chemicals, USA; Agarose, glucose and yeast extract from Hi media Pvt Ltd, Mumbai, India; Glacial acetic acid from s.d Fine chemicals Ltd., chloroform and iso amyl alcohol from Qualigens Fine Chemicals, India.

Preparation of buffers and ethidium bromide stock used in the study are given below. The protocol described in Sambrook and Russel (2001) were followed for the preparation of molecular biology working stocks.

5.2.1.1 Preparation of TE buffer

TE buffer consists of 10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0). The buffer was sterilized by autoclaving and stored at room temperature.

5.2.1.2 Preparation of TAE 50X buffer

24.2g Tris base, 5.71 mL of glacial acetic acid and 10mL of 0.5M EDTA (pH 8.0) were added to 80 mL of distilled water, pH adjusted to 7.2 and final volume made up to 100mL with distilled water. The buffer was sterilized by autoclaving and stored at room temperature.

5.2.1.3 Preparation of ethidium bromide stock solution

10mg of ethidium bromide (Sigma, USA) dissolved in 1 mL of distilled water. The solution was stored in microcentrifuge tube wrapped with aluminum foil at 4°C.

5.2.2 Organism and culture maintenance

M. alpina CBS 528.72 was grown in GY (Glucose-20g/L -Yeast extract-10g/L) medium at 28°C with shaking at 240 rpm as described in section 1.2.2.1. After 48h the actively growing mycelium was harvested and used for DNA extraction.

5.2.3 Genomic DNA isolation

DNA isolation was done according to the procedure described by Michaelson *et al*. (1998a). Approximately 5 g wet weight of mycelium was ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle. The ground tissue was added to 10mL of extraction buffer (10mM Tris –HCl, pH 8, 10mM EDTA, and

0.5% SDS), thawed and mixed gently by inversion. Phenol: chloroform: isoamyl alcohol (10mL) was added and mixed gently for 15-30 min. The organic and aqueous layers were separated by centrifugation and the aqueous layer was removed to a fresh centrifuge tube. The extraction was repeated until the interface between the two phases was clear. Further extraction was done with chloroform: isoamyl alcohol (24:1). DNA was concentrated by ethanol precipitation and stored in TE buffer.

5.2.4 Designing of oligonucleotides

Three oligonucleotide primers forward, reverse and semi-nested were synthesized based on sequences available in the GenBank (Accession nos AF054824, AF067654, AY464949 and AB188307). The nucleotide sequences of the primers are detailed in Table 5.1

Table 5.1 Primers used for the amplification of Δ^5 desaturase gene

Oligo name	5'-3'direction	Length	Position
NaF	CTCCTGCTCGGAGCTGGC	18mer	535-552
NaR	GGTCCAGAGGTGCGARTCG	19mer	1963-1982
NaNF	GCYTCCTACTACGCGCAGC	19mer	1152-1170

5.2.5 PCR amplification of Δ^5 desaturase gene (DES 5)

PCR was carried out by combining the following components in 50µl reaction (Table 5.2). 10X reaction buffer for XT-Taq sys (Bangalore Genei, India) contains 15mM $MgCl_2$ and 0.1% gelatin. The contents of the tube were mixed by a brief spin in a microcentrifuge. The reaction was carried out in a thermocycler Gene Amp PCR system 9700 (Perkin- Elmer, USA). After initial denaturation at 94°C for 5 min, amplification was performed in 35 cycles of 40s at 94°C, 40s at 61°C and 1.2 min at 72°C followed by a final extension at 72°C for another 15 min.

Table 5.2 Reaction components of PCR

Components	Vol(µl)	Final conc
Nuclease free water	37.5	
10X Reaction buffer	5.0	1X
dNTP mix (10mM)	1.0	0.2mM
XT-*Taq* sys (3U/µl)	0.5	0.03U/µl
Primer Na-F(Forward)	2.0	0.2µl
Primer Na-R(Reverse)	2.0	0.2µl
Template(≈100ng)	2.0	

5.2.6 Analysis of PCR product by agarose gel electrophoresis

The boat was sealed with an adhesive tape and the comb placed for the wells. 1.2% agarose was prepared in 1X TAE buffer. The mixture was heated to melt the agarose, cooled to 50°C, poured into the sealed boat and the gel allowed to polymerize. After removing the comb and the adhesive tape, the gel was placed in the gel tank of an electrophoresis unit (Bangalore Genei, India) with sufficient volume of 1X TAE to cover the surface of the gel. A 5µl aliquot of the PCR product and 2µl standard DNA marker were loaded in the wells and analyzed. The size of the partial Δ^5 desatuare gene amplicon was checked by comparing with the DNA marker.

Electrophoresis was carried out at 50V till the dye reached 3/4th of the gel. The gel was removed from the tank and stained by soaking in a solution of 0.5µg/mL ethidium bromide for 30 min at room temperature. The gel was destained with distilled water for 10 min at room temperature, examined on a UV trans-illuminator and documented using Gel documentation system (Herolab, Germany). Authenticity of the PCR product was confirmed through semi nested PCR using primers NaNF and NaR.

5.2.7 Purification of the PCR product

The amplicon of partial Δ^5 desaturase gene was purified using Gene Elute PCR cleanup kit (Sigma, USA) following the manufacturer's instructions. The purified PCR product was eluted in 50µl elution buffer and stored at $^{-}$20°C. The scheme for purification of the PCR product is given in Fig 5.1

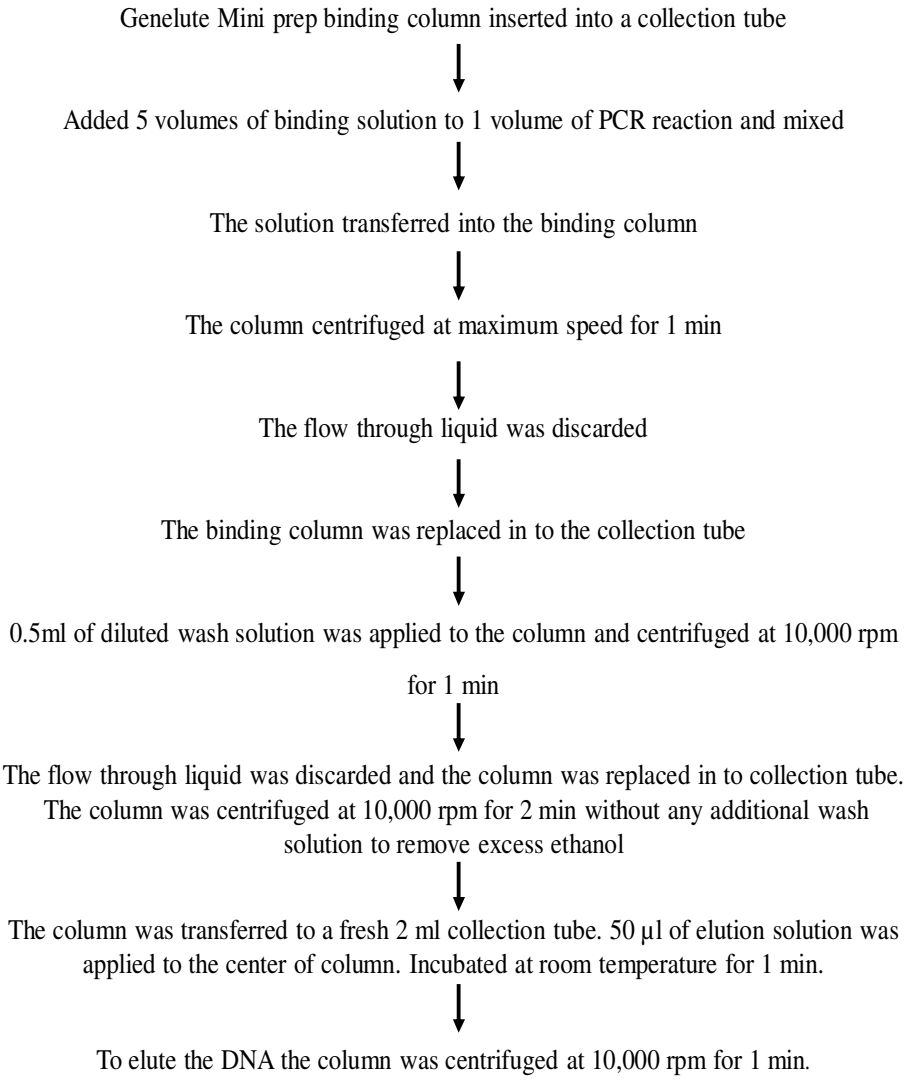


Fig 5.1 Purification of PCR product using GenElute PCR cleanup kit

5.2.8 Nucleotide sequencing

DNA Sequencing was done using an automated ABI 3100 Genetic Analyser (Applied Biosystems, USA) that uses Fluorescent label dye terminators or fluorescent label primers. ABI's AmpliTaq FS dye terminator cycle sequencing chemistry which is based on Sanger's Sequencing method was used (Sanger, 1977). The electropherogram was generated which represents a multicolour picture of the sequence showing coloured peaks that indicate the bases. The accessions in gene bank showing significant homology with the sequence were identified by Basic Local Alignment Search Tool (BLASTn).

Intron-exon border in the nucleotide sequence were identified by the gene finder program (Schiex *et al*., 2001) and by multiple sequence alignment with *M. alpina* cDNA sequences available in the data bank. Deduced amino acid sequence was obtained with the help of EXPASY DNA to protein sequence converter.

5.2.9 Homologs-PSI-BLAST search

Homologs for the deduced Δ^5 desaturase protein sequence were searched using EMBL-EBI PSI-BLAST. The sequences of the BLAST hits showing significant homology were aligned by Clustal W program (Thompson *et al.*, 1994) and a phylogenetic tree was constructed. Protein sequences were used for generating the phylogenetic tree using MEGA 4.0 software (Tamura *et al*., 2007). Neighbour joining method with 500 replicates of bootstrap values were used.

5.2.10 Cluster analysis

Cluster analysis of Δ^5 desaturase from different organisms was done by EXPASY Just another Classification of Protein (JACOP) programme (Sperison and Pagni, 2005). Clustering was based on amino acid sequence similarity. To identify the hydrophobic domains in the deduced amino acid sequence, hydropathy plot was generated (Kyte and Doolittle, 1982).

5.3 Results

5.3.1 PCR amplification of partial Δ^5 desaturase gene

PCR conditions were standardized for amplification of partial Δ^5 desatuarse gene from *Mortierella alpina*. Annealing temperature optimized was 61°C. Higher annealing temperature proved ineffective in amplifying the target sequence whereas at lower annealing temperatures, nonspecific amplification was prevalent. PCR fragments of the expected size were obtained from the genomic DNA of *M. alpina* using the primers detailed in Table 5.1. Primer combination of NaF and NaR resulted in amplification of a product 1.5Kb in size. The amplicon size was determined using a DNA ladder run alongside the sample. Confirmation of the PCR product was done by carrying out semi-nested PCR using the 1.5 Kb PCR product as template and NaNF and NaR as primers which resulted in an amplicon of length 830bp (Fig 5.2).

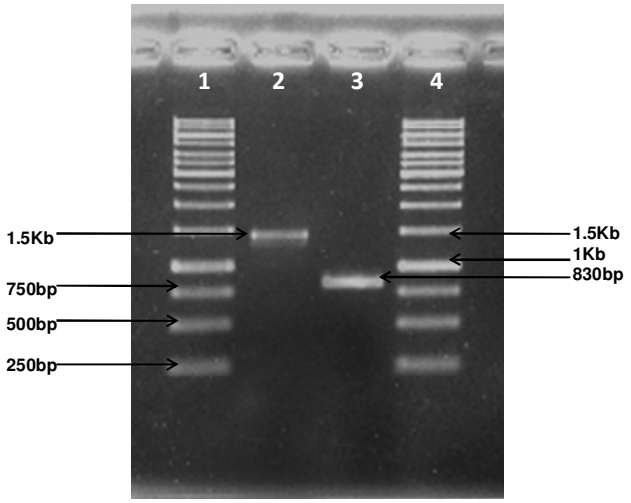


Fig 5.2 PCR amplification of partial Δ^5 desaturase gene from *Mortierella alpina*

lane 1 and 4-marker, lane 2-1.5Kb PCR product, lane 3-830 bp Semi-nested PCR product

5.3.2 Nucleotide sequencing and analysis

The purified PCR product of 1.5Kb (Fig 5.3) was sequenced using all the three primers. The partial genomic DNA sequence contained three stretch of introns. The sequence of introns had more A and T nucleotides than G and C. The introns showed no sequence similarity to each other.

Sequencing of the partial Δ^5 desaturase gene revealed homology to known *Mortierella alpina* sequences (Table 5). The sequence of Δ^5desaturase from the strain investigated was found to have 100% homology with that of *M. alpina* sequences AF067654.1 and AF054824.1 as determined using the BLASTn programme.

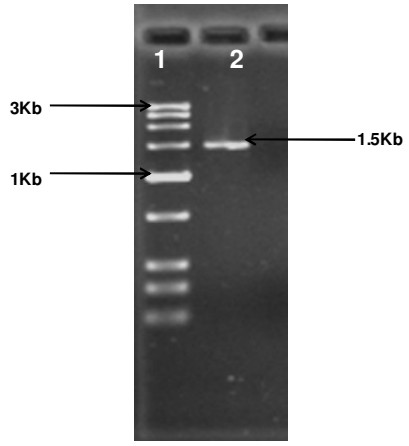


Fig 5.3 Partial Δ^5 desaturase gene of *M. alpina* purified by Gen Elute PCR cleanup kit
Lane 1-Marker, Lane 2-purified PCR product

5.3.3 Deduced amino acid sequence and homology

The deduced amino acid sequence (Fig 5.4) was identical with that of other *M. alpina* Δ^5 desatuarases over most of the protein. Comparison of the deduced amino-acid sequence of the Δ^5 fatty acid desaturase from *Mortierella* and those from fungi, algae, diatoms and other organisms revealed highly conserved regions with histidine residues at positions 189-193, 225-231 and 398-402. When we carried out a search for histidine box motifs essential to desaturase activity a diagnostic HPGG motif as well as three histidine boxes were identified in the *M. alpina* sequences. The ORF contained an N-terminal cytocrome b_5 domain which is identified by the diagnostic HPGG motif and the histidine boxes characteristic of all microsomal desaturases (Fig 5.5). Phylogenetic analysis revealed that the HPGG motif in the heme binding region was evolutionarily conserved in different organisms.

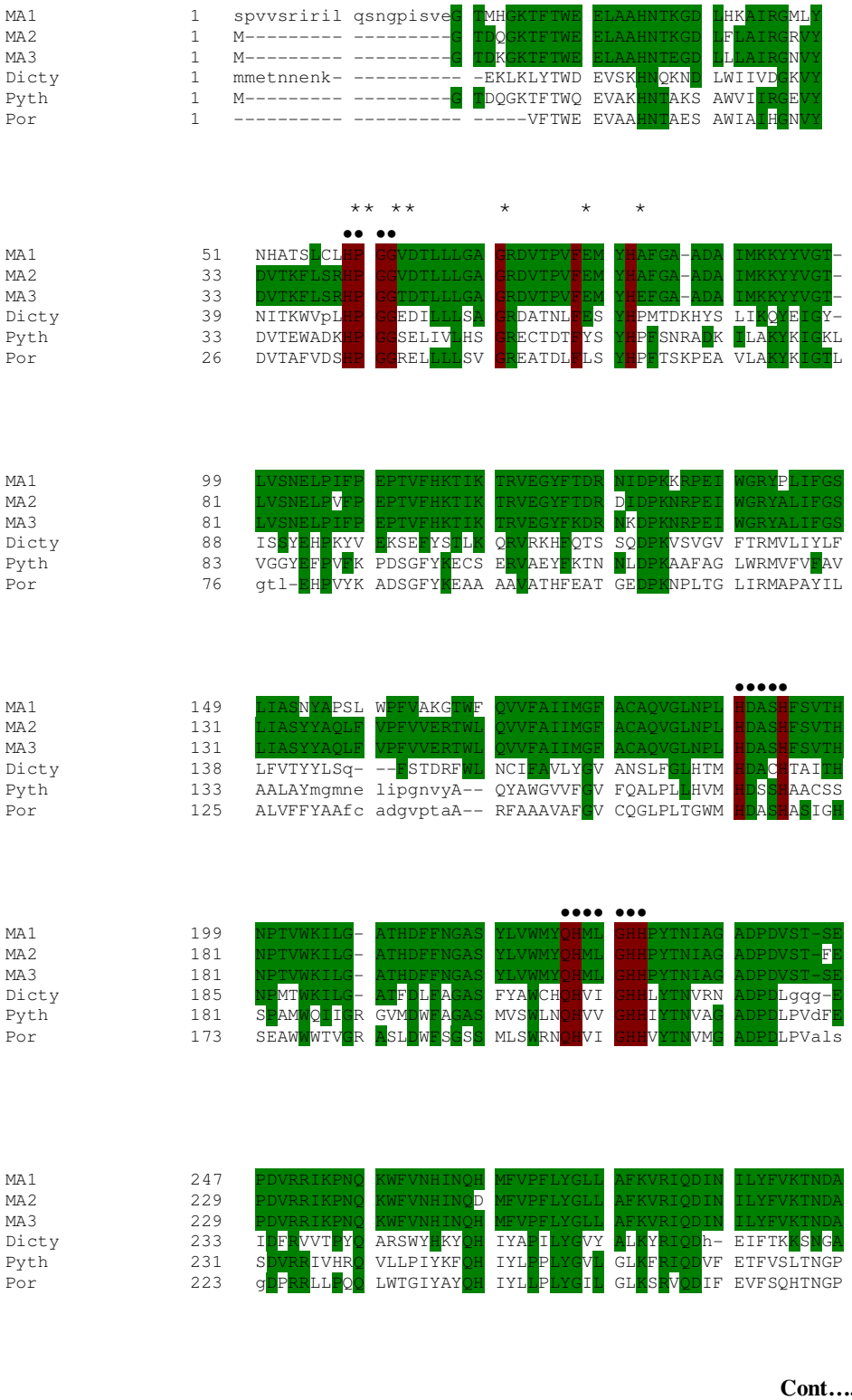
Table 5.3 Sequences in Genbank giving significant alignments with that of partial Δ^5 desaturase from *M. alpina* CBS 528.72

Accession No	Description	Max Score	Total Score	Query coverage	E value	Max Identity
AB188307.1	*Mortierella alpina* gene for delta 5 fatty acid desaturase ,complete cds	1443	1443	99%	0.0	84%
AB067654.1	*Mortierella alpina* delta 5 fatty acid mRNA, complete cds	991	1500	65%	0.0	100%
AF054824.1	*Mortierella alpina* delta 5 microsomal desaturase (DES1), mRNA complete cds	955	1449	66%	0.0	100%
AY464949.1	*Mortierella alpina* delta 5 fatty acid mRNA, complete cds	838	1247	65%	0.0	95%
FJ625826.1	*Mortierella alpina* strain M23 delta 5 fatty acid desaturase mRNA, complete cds	149	215	65%	0.0	93%
AM411536.1	*Mortierella alpina* delta 5 fatty acid mRNA, complete cds	46.4	46.4	12%	0.0	89%

In PSI-BLAST search the deduced amino-acid sequence of the partial Δ^5 desaturase revealed a maximum of 92% identity with that of *M. alpina* Δ^5 desaturase protein sequence available in the data bank (Acc No O74212). The deduced sequence also revealed 41% identity to *Dictyostelium discoideum* (Acc No O96099), 37% identity to *Pythium irregulare* (Acc No Q944W3), 35% identity to *Phytophthora megasperma* (Acc No Q8H0N8), 37% identity to *Porphyra yezoensis* (Acc No B1P8J9) and 37% identity with *Marchantia polymorpha* (Acc No Q696V6) sequences.

```
gatgggtacggaccaaggaaaaaccttcacctgggaagagctagcggcccataacaccaag
  M  G  T  D  Q  G  K  T  F  T  W  E  E  L  A  A  H  N  T  K
ggcgacctgcacaaggcgatccgcggcatgctatacaatcatgcaacttctttgtgcctc
 G  D  L  H  K  A  I  R  G  M  L  Y  N  H  A  T  S  L  C  L
catcctggtggagtggacactctcctgctcggagctggccgagatgttactccggtcttt
 H  P  G  G  V  D  T  L  L  L  G  A  G  R  D  V  T  P  V  F
gagatgtatcacgcgtttggggctgcagatgccatcatgaagaagtactatgtcggtaca
 E  M  Y  H  A  F  G  A  A  D  A  I  M  K  K  Y  Y  V  G  T
ctggtctcgaatgagctgcccatcttcccggagccaacggtgttccacaaaaccatcaag
 L  V  S  N  E  L  P  I  F  P  E  P  T  V  F  H  K  T  I  K
acgagagtcgagggctactttacggatcggaacattgatcccaagaaaagaccagagatc
 T  R  V  E  G  Y  F  T  D  R  N  I  D  P  K  K  R  P  E  I
tggggacgataccctcttatctttggatccttgatcgcttccaactacgcgccgagtctt
 W  G  R  Y  P  L  I  F  G  S  L  I  A  S  N  Y  A  P  S  L
tggcctttcgttgccaaaggcacatggttccaggtggtatttgcaatcatcatgggattt
 W  P  F  V  A  K  G  T  W  F  Q  V  V  F  A  I  I  M  G  F
gcgtgcgcacaagtcggactcaaccctcttcatgatgcgtctcacttttcagtgacccac
 A  C  A  Q  V  G  L  N  P  L  H  D  A  S  H  F  S  V  T  H
aaccccactgtctggaagattctgggagccacgcacgacttttcaacggagcatcgtac
 N  P  T  V  W  K  I  L  G  A  T  H  D  F  F  N  G  A  S  Y
ctggtgtggatgtaccaacatatgctcggccatcacccctacaccaacattgctggagca
 L  V  W  M  Y  Q  H  M  L  G  H  H  P  Y  T  N  I  A  G  A
gatcccgacgtgtcgacgtctgagcccgatgttcgtcgtatcaagcccaaccaaaagtgg
 D  P  D  V  S  T  S  E  P  D  V  R  R  I  K  P  N  Q  K  W
tttgtcaaccacatcaaccagcacatgtttgttcctttcctgtacggactgctggcgttc
 F  V  N  H  I  N  Q  H  M  F  V  P  F  L  Y  G  L  L  A  F
aaggtgcgcattcaggacatcaacattttgtactttgtcaagaccaatgacgctattcgt
 K  V  R  I  Q  D  I  N  I  L  Y  F  V  K  T  N  D  A  I  R
gtcaatcccatctcgacatggcacactgtgatgttctggggcggcaaggctttctttgtc
 V  N  P  I  S  T  W  H  T  V  M  F  W  G  G  K  A  F  F  V
tggtatcgcctgattgttcccctgcagtatctgcccctgggcaaggtgctgctcttgttc
 W  Y  R  L  I  V  P  L  Q  Y  L  P  L  G  K  V  L  L  L  F
acggtcgcggacatggtgtcgtcttactggctggcgctgaccttccaggcgaaccacgtt
 T  V  A  D  M  V  S  S  Y  W  L  A  L  T  F  Q  A  N  H  V
gttgaggaagttcagtggccgttgcctgacgagaacgggatcatccaaaaggactgggc
 V  E  E  V  Q  W  P  L  P  D  E  N  G  I  I  Q  K  D  W
```

Fig 5.4 Deduced amino acid sequence of cDNA from *M.alpina* CBS 528.72



Cont….

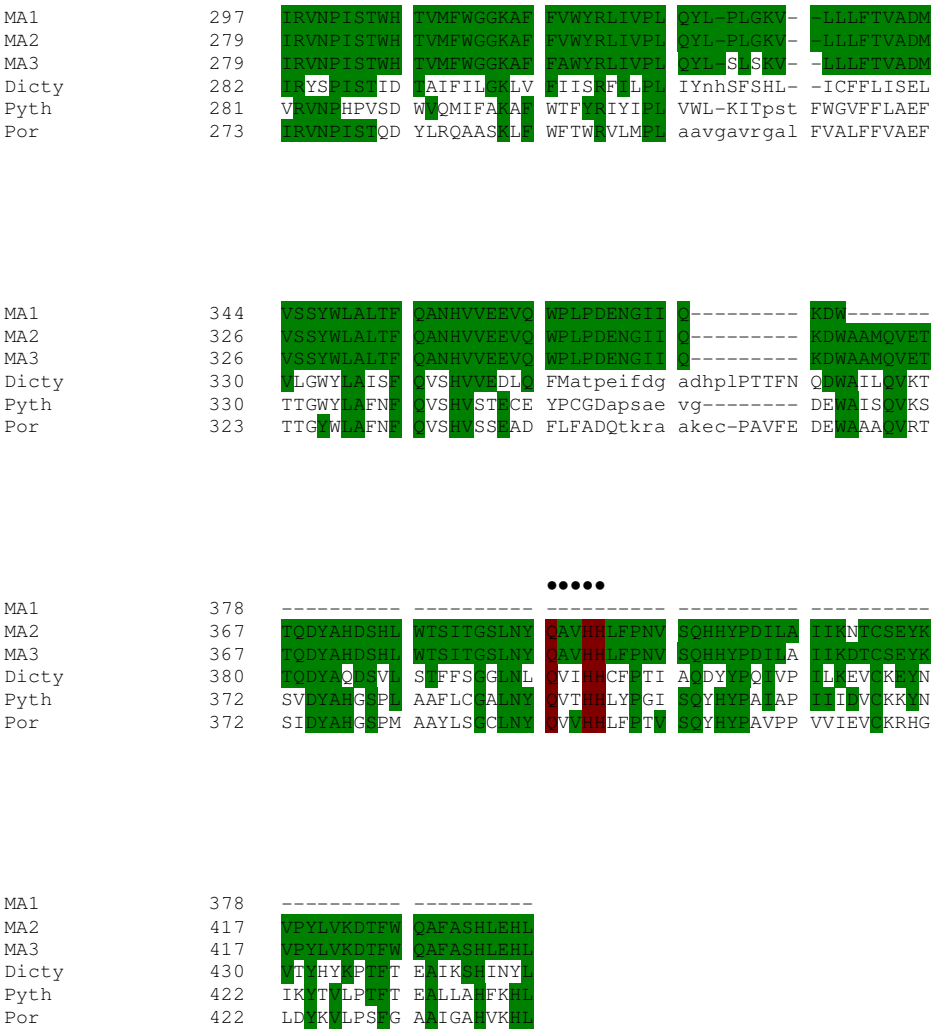


Fig 5.5 Comparison of deduced amino acid sequences of *M. alpina* Δ^5 desaturase with that of related organisms.

The translation of the coding sequence of *Mortierella alpina* CBS 528.72 (labelled as MA1), is aligned with MA2-*Mortierella alpina* O74212.1, MA3- *Mortierella alpina* Q6S5J3, Dicty-*Dictyostelium discoideum* O96099,Pyth-*Pythium irregulare* Q944W3 and Por-*Porphyra* B1P8J9. Identical or conserved residues are shaded. The HPGG motif and three histidine boxes are differentiated by ● symbol.* denote the conserved residues in the cytochrome b5 region

5.3.4 Cluster analysis and phylogeny

For classification of Δ^5 desaturases from organisms to different groups, hierarchical cluster analysis was done. Cluster analysis was done by EXPASY JACOP programme based on amino acid sequence similarity. Results indicated that the different Δ^5desaturases were clustered into 6 groups (Fig 5.6). *Mortierella* sequences in itself formed a strong cluster. The diatoms *Phaedactylum tricornitum, Thalassiosira pseudonana and Nitzschia closterium* were grouped together in a strong cluster. *Dictyostelium discoideum,* the slime mold formed a weak cluster with the red alga *Porphyra,* green alga *Osteococcus tauri,* liver wort *Marchantia* and fungi *Pythium* and *Phtophthora.* Sequences of olive baboon (*Papio Anubis*), atlantic salmon (*Salmo salar*) and rat (*Rattus norvegicus*) were clustered together which indicate the formation of a strong cluster by animal Δ^5 desaturases. The phylogenetic relationship of Δ^5 desaturase from different organisms is given in Fig 5.7

Cluster analysis of desaturases (Δ^5, Δ^6, Δ^9, Δ^{12}, ω-3, ω-9, Steroyl COA) from *M. alpina* was done to study the relationship between the different desaturases in involved in the fatty acid biosynthetic pathway. The Δ^5 and Δ^6 desaturases formed two distinct strong groups. ω-9, Δ^9 and steroyl COA desaturases were strongly clustered together. ω-3 and Δ^{12} desaturases were clustered together into an independent group. Alignment of the desaturase sequences from *M. alpina* revealed that the HPGG motif in the heme binding region of cytochrome b5 domain was specific to Δ^5 and Δ^6 desaturases (Fig 5.8). The phylogenetic relationship of desaturases from *M. alpina* is given in Fig.5.9.

When the deduced amino acid sequence was analyzed for the hydrophobic domains using Kyte -Doolittle hydropathy plot, two hydrophobic regions could be identified which formed two clear trans membrane domains (Fig 5.10).

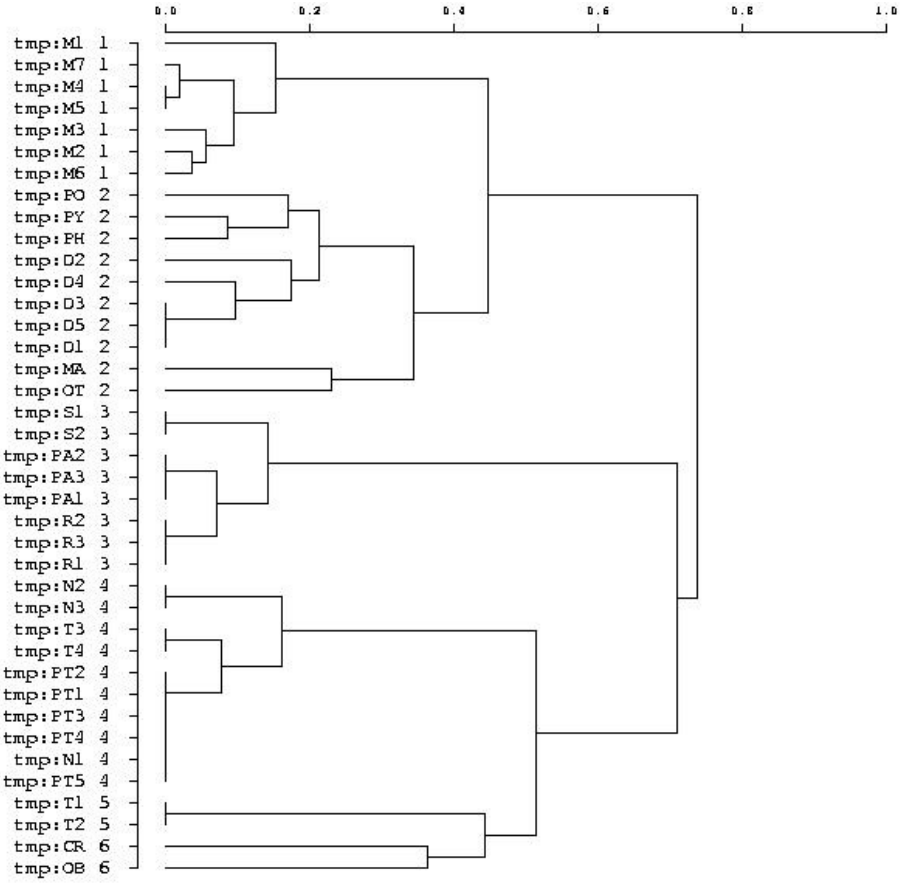


Fig 5.6 Cluster analysis of Δ^5desaturase sequences from different organisms

M1- *M. alpina* CBS 528.72, M2- *M. alpina* AAR28035.1, M3- *M. alpina* ACM89303.1, M4- *M. alpina* AAC39508.1, M5- *M. alpina* O74212.1, M6- *M. alpina BAD95486.1,* M7- *M. alpina* AAC72755.1, PO-*Porphyra yezeonsi*s ACB11556.1, PY-*Pythium irregulare* AAL13311.1, PH-*Phytophthora megasperma CAD53323.1*, D1- *Dictyostelium discoideum* BAA37090.1, D2- *D. discoideum* XP_001134469.1, D3- *D. discoideum* XP_640331.1, D4- *D. discoideum* XP_638329.1, D5- *D. discoideum* O96099.1, MA-*Marchantia polymorpha* AAT85663.1, OT *Ostreococcus tauri* CAL51370.1, S1-*Salmo salar* AAC82631.2, S2- *S. salar* NP00111704.1, PA1- *Papio Anubis* ABP06289.1,PA2 *P. Anubis* NP_001106097.1, PA3 *P. Anubis* A40V11,R1-*Rattus norvegicus* Q920R3.1, R2 *R. norvegicus* AAG35068.1, R3 *R. norvegicus* AAG 35068.1, T1- *Thalassiosira pseudonana* XP_002296094.1, T2- *T. pseudonana* AC164811.1, T3- *T. pseudonana* XP_002288842.1, T4- *T. pseudonana* EED94278.1, N1 *Nitzschia closterium* ABQ45365.1, N2 *N. closterium* AAX51386.1, N3 *N. closterium* AATO9160.1, CR-*Caligus*AC010922.1 , PT1- *Phaeodactylum tricornutum* ACE95865.1, PT2- *P. tricornutum* AAL92562.1, PT3- *P. tricornutum* ABP65280.1, PT4- *P. tricornutum* XP_002185732.1, PT5- *P. tricornutum* AC165202.1

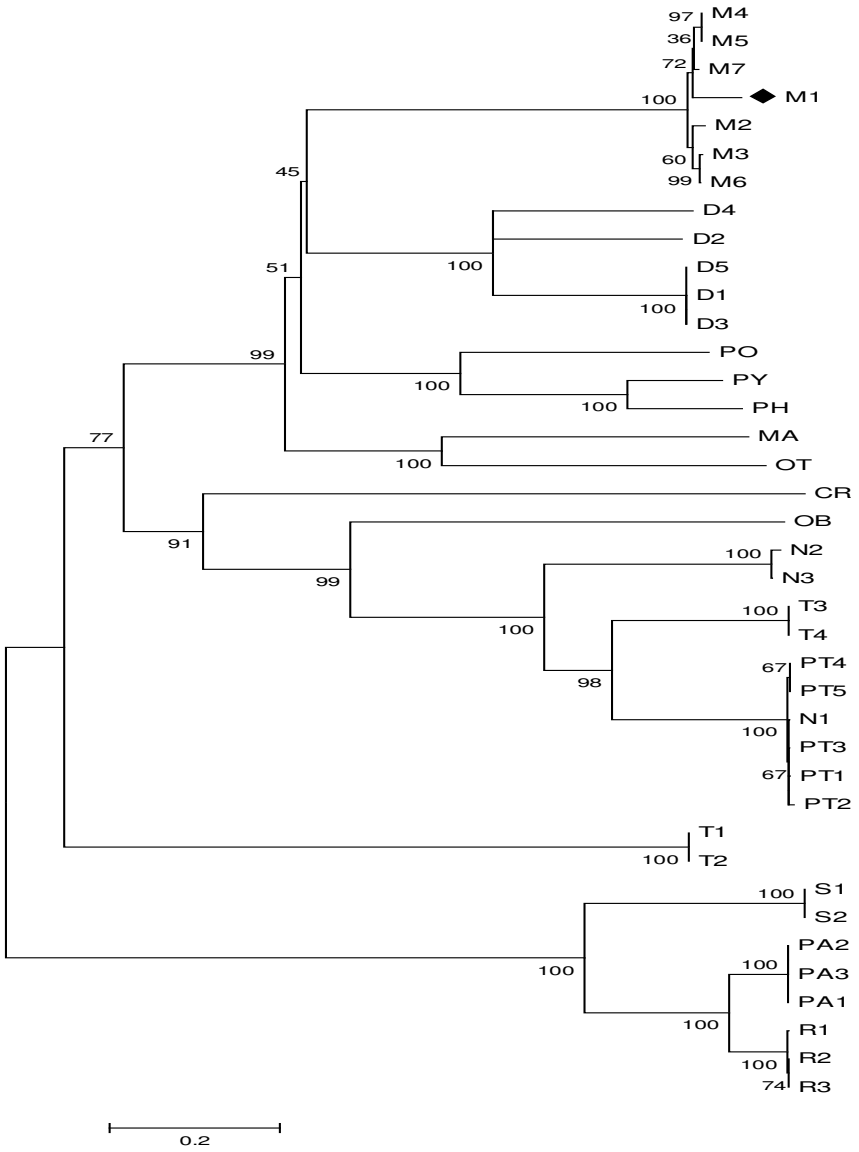


Fig 5.7 Phylogenetic relationship of Δ^5 desaturase from different organisms

M1- *M. alpina* CBS 528.72, M2- *M. alpina* AAR28035.1, M3- *M. alpina* ACM89303.1, M4- *M. alpina* AAC39508.1, M5- *M. alpina* O74212.1, M6- *M. alpina BAD95486.1,* M7- *M. alpina* AAC72755.1, PO-*Porphyra yezeonsis* ACB11556.1, PY-*Pythium irregulare* AAL13311.1, PH-*Phytophthora megasperma CAD53323.1*, D1- *Dictyostelium discoideum* BAA37090.1, D2- *D. discoideum* XP_001134469.1, D3- *D. discoideum* XP_640331.1, D4- *D. discoideum* XP_638329.1, D5- *D. discoideum* O96099.1, MA-*Marchantia polymorpha* AAT85663.1, OT *Ostreococcus tauri* CAL51370.1, S1-*Salmo salar* AAC82631.2, S2- *S. salar* NP00111704.1, PA1- *Papio Anubis* ABP06289.1,PA2 *P. Anubis* NP_001106097.1, PA3 *P. Anubis* A40V11,R1-*Rattus norvegicus* Q920R3.1, R2 *R. norvegicus* AAG35068.1, R3 *R. norvegicus* AAG 35068.1, T1- *Thalassiosira pseudonana* XP_002296094.1, T2- *T. pseudonana* AC164811.1, T3- *T. pseudonana* XP_002288842.1, T4- *T. pseudonana* EED94278.1, N1 *Nitzschia closterium* ABQ45365.1, N2 *N. closterium* AAX51386.1, N3 *N. closterium* AATO9160.1, CR-*Caligus*AC010922.1 , PT1- *Phaeodactylum tricornutum* ACE95865.1, PT2- *P. tricornutum* AAL92562.1, PT3- *P. tricornutum* ABP65280.1, PT4- *P. tricornutum* XP_002185732.1, PT5- *P. tricornutum* AC165202.1

Fig 5.8 Sequence alignment of desaturase sequences from *M. alpina*

The shaded letters denote the HPGG motif in the cytochrome b5 region which is specific to Δ^5 and Δ^6desaturases And highly conserved in different organisms

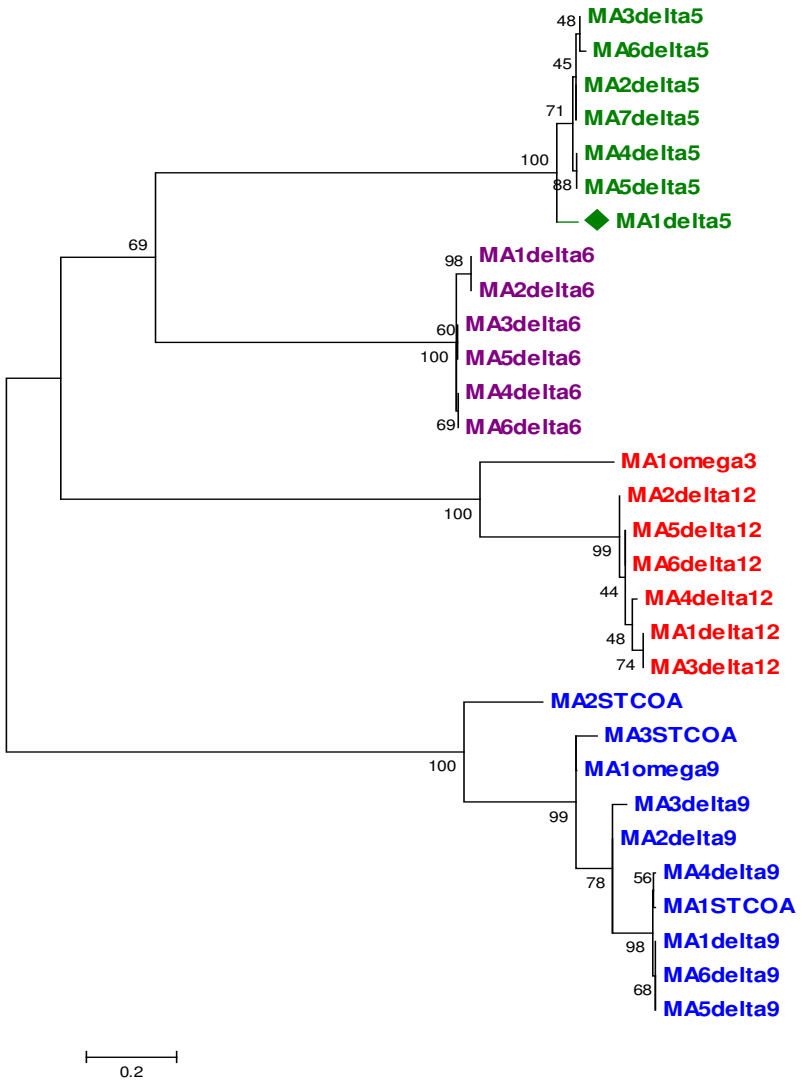


Fig 5.9 Phylogenetic relationship of desaturases from *M. alpina*.

MA1delta5-MA7delta5-delta 5 desaturase; MA1delta6-MA6delta6-delta 6 desaturases; Ma1 omega3-omega 3 desaturase; MA1delta12-MA6delta12-delta 12 desaturases; MA1STCOA-MA3STCOA-steroyl CoA desaturase; MA1omega9-omega9desaturase; MA1delta9-MA6delta9-delta9desaturases.Sequence alignment and phylogenetic tree construction was done by Clustal W and MEGA 4.0

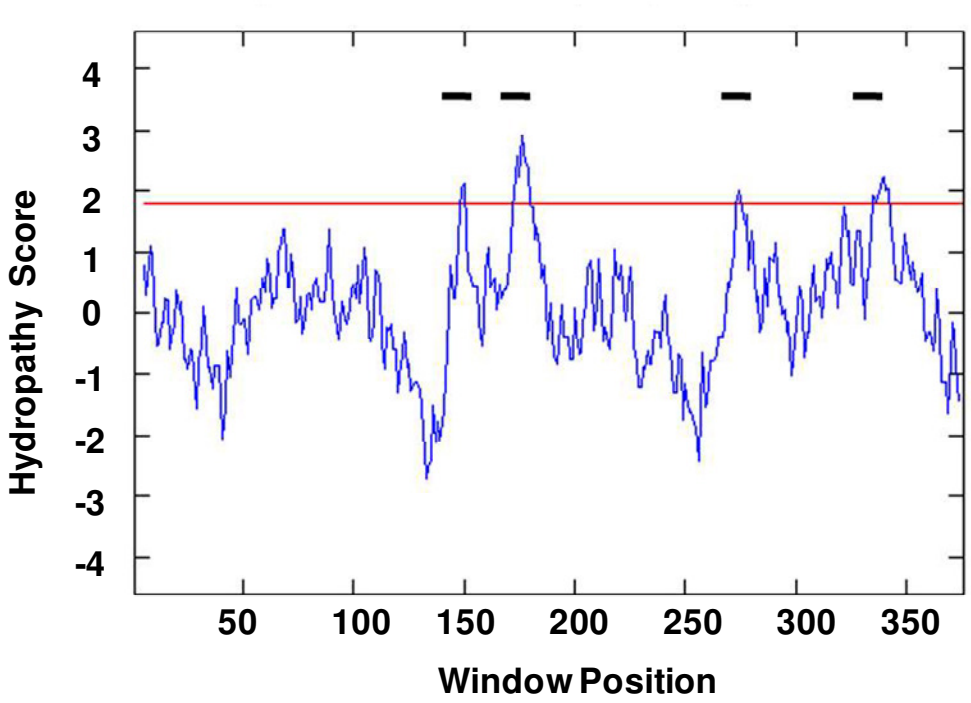


Fig 5.10 Hydropathy profile of *M. alpina* Δ^5 desaturase
The solid bars represent the trasmembrane regions

5.4 Discussion

Fatty acid desaturases which include soluble and membrane bound desaturases play essential roles in fatty acid metabolism and maintenance of proper structure and function of biological membranes in living organisms (Zhang *et al*., 2004). Membrane bound desaturases include the front end desaturases which introduce a double bond between the existing double bond and carboxy terminus of the fatty acyl chain.

The knowledge of enzymatic reactions and corresponding metabolic pathways in PUFA biosynthesis was well elucidated with the isolation of a number of desaturase genes from different organisms. During the last few years, many of the genes that are responsible for biosynthesis of PUFAs have been cloned from different organisms including fungi, algae, mosses, plants nematodes and animals (Sayanova and Napier, 2004).

The Δ^5 desaturase enzyme mediates the Δ^5 desaturation reactions of DGLA to ARA and ETA to EPA in the n-3 and n-6 pathways respectively. The first Δ^5 desaturase was cloned from the oleaginous fungus *M. alpina* (Knutzon *et al*., 1998; Michaelson *et al*., 1998a). Δ^5 desaturase gene has been cloned from the diatom *Phaeodactylum tricornutum* (Domergue *et al*., 2002) as well as from *C. elegans* (Michaelson *et al*., 1998b; Watts and Browse, 1999), *Bacillus subtilis* (Aguilar *et al*., 1998), human (Cho *et al.,* 1999a), Dictyostelium *discoideum* (Saito and Ochiai, 1999; Saito *et al*., 2000), rat (Zolfaghari *et al*., 2001) and *Oblongichytrium* (Kumon *et al*., 2008). Gene encoding a Δ^5 desaturase of another type which catalyzes desaturation of monoenoic and dienoic fatty acids has been identified from *Limnanthes douglasii* (Cahoon *et al*., 2000)

Heterologous reconstitution of the PUFA biosynthetic pathway has been demonstrated in yeast (Sayanova *et al*., 2001), *Aspergillus oryzae* and plants including *Nicotiana tabacum* (Sayanova *et al*., 1997; Hamada *et al*., 1998) and *Arabidopsis* (Zou *et al.*, 1997). Arachidonic acid and EPA were produced in *Arabidopsis thaliana* leaves using an alternate synthetic pathway that expressed the Δ^9elongase, Δ^8desaturase and Δ^5desaturase (Qi *et al*., 2004). Kajikawa *et al.* (2008; 2004) successfully isolated and characterized Δ^6 desaturase, an ELO like enzyme and Δ^5 desaturase from the liverwort *Marchantia polymorpha* and produced ARA and EPA in plants and yeast respectively. Arachidonic acid has been produced in the seeds

of soybean (*Glycine max*) by seed specific expression of the genes encoding Δ^6 desaturase, fatty acid elongase and Δ^5 desaturase from *M. alpina* coupled to down regulation of Δ^5 desaturase gene of *Glycine max* (Chen *et al*., 2006). Abbadi *et al.* (2004) and Kinney *et al*. (2004) reported the production of very long chain PUFA in seeds of linseed and somatic embryos of soybean.

M. alpina has been used not only for oil production but also for the elucidation of PUFA biosynthesis in microorganism. Notable accumulation of several PUFAs in this organism divulges that *M. alpina* desaturases are expected to have high activity as to fatty acid desaturation. In the present study, partial Δ^5 desatuarse gene was cloned from *M. alpina* 528.72 by PCR using degenerate oligonucleotide primers. The PCR product of expected size (1.5Kb) was obtained by amplification. Authenticity of PCR product was confirmed by a semi-nested PCR, with the PCR product as template and an amplicon of size 830bp was obtained. The predicted amino acid sequence encoded from the cDNA revealed the highest homology to previously reported *M. alpina* Δ^5 desaturases although similarity with other fungal and algal membrane bound desaturases is observed as well. The Δ^5 desaturase sequences had two features which distinguished it from Δ^{12} and Δ^{15} desaturases. The presence of the N terminal cytochrome b5 domain is of functional significance due to the role of cytochrome b5 in electron transport of atleast some membrane bound desaturases and related hydroxylases (Smith *et al*., 1990; Smith *et al*., 1992). However it is not clear if *M. alpina* uses only this domain for desaturation or uses free cytochrome b_5 similar to some microsomal fatty acid desaturases from plants (Smith *et al*., 1990; Smith *et al*., 1992). The second distinguishing character ie., the presence of QXXHH sequence at the third histidine box is functionally significant due to the proposed role of these histidine regions in the ligation of iron atoms (Shanklin *et al*., 1994).

The Δ^5-desaturase from *M. alpina*, therefore, appears to be the first fungal fatty acid desaturase described with an N-terminal cytochrome *b*5 domain and in this respect is similar to the "front end" Δ^6-desaturase of *B. officinalis* (Sayanova *et al*., 1997; Napier *et al*., 1997) and the Δ^6-desaturase of *C. elegans* (Napier *et al*., 1998). Alignment of the sequences indicated that homology mainly occurred in the cytochrome b5 like domain and the three conserved histidine rich motif area. Analysis of the deduced protein sequence revealed that of the 8 conserved residues

among the cytochrome b_5 superfamily, 7 are conserved whereas one residue is substituted in *M. alpina*. Presence of a glutamine residue in the third histidine box, a characteristic of front end desaturase was also observed (Wongwathanarat *et al.,* 1999).

Phylogenetic analysis revealed that Δ^5 desaturases from *Mortierella* were more related to slime mould, fungal and algal desaturases and were significantly different from animal Δ^5 desaturases. It is interesting that Δ^5 desaturases of animal species are more closely related to each other than to those from other species. Non-animal desaturases were so divergent from each other and it can be assumed that the divergence of fungi and plant desaturase genes occurred independently long before that of animal front end desaturases (Kajikawa *et al*., 2004).

In phylogenetic analysis, the Δ^5 desaturase were found to be more structurally related to Δ^6 desaturase and the conserved HPGG motif was specific to these desaturases. The omega 3 desaturase and Δ^{12} desaturases from *Mortierella* exhibited similarity and it is reported that both these desaturases introduce double bonds at C termini of fatty acids (Sakuradani *et al*., 2005). Analysis further confirms that the sequences of these proteins include a highly conserved domain which determines the differences in specificity and regioselectivity found in these enzymes.

A hydropathy plot of the deduced amino acid sequence was used to find the clusters of hydrophobic amino acids that indicate the trans membrane regions. A trans membrane protein has hydrophilic parts which protrude out on either side of the cellular membrane and a hydrophobic centre which lies within the membrane. The predicted hydrophobicity plot for the Δ^5 desaturase from *M. alpina* revealed a profile characteristic of a fatty acid desaturase. The two conserved hydrophobic regions assumed to form two membrane spanning domains in the endoplasmic reticulum are present (Shanklin *et al*., 1994). This domain in the desaturase probably plays a role of a unique electron transport system binding a heme group and therefore belongs to cytochrome b5 superfamily.

5.5 Conclusion

Δ^5 desaturase (DES) gene was isolated from the arachidonic acid producing fungus *Mortierella alpina* by PCR based approach. Deduced amino acid sequence of Δ^5 desaturase gene from *M. alpina* was identical to that of other *M. alpina* Δ^5

desaturases over most of the protein and analysis of the sequences revealed high homology at the cytochrome b5 region with the HPGG motif and three histidine boxes conserved in different organisms. The Δ^5 desaturase from selected organisms was clustered into 6 distinct groups and Δ^5 from *Mortierella* were phylogenetically more related to algal, fungal and slime mould desaturases. Analysis of the delta, omega and stearoyl CoA desaturases in *Mortierella* confirmed that Δ^5 desaturases were phylogentically related to the Δ^6 desaturases with a common HPGG motif, absent in other desaturases. The hydropathy profile of the amino acid sequences indicated that the two conserved hydrophobic regions assumed to form two membrane spanning domains which probably play the role of a unique electron transport system. Thus we successfully isolated partial Δ^5 desaturase gene (DES 1) from *M. alpina* by a PCR based approach. The results clearly established that Δ^5 desaturase gene from fungi, algae, slime mould etc., are phylogenetically related with four highly conserved regions which probably play a functional role as catalytic domains of the enzyme.

Summary and Conclusions

There is an unprecedented surge of public interest in the applications of PUFAs of n-3 and n-6 series as nutraceuticals which promise to extend our therapeutic horizons with potentially fewer side effects in chronic diseases. This interest has led to the commercial exploitation of microbial sources of these fatty acids and such lipophilic compounds derived from microbial sources are referred to as single cell oils (SCOs). For an economically viable process for SCO, it is crucial that the respective oils are produced in high titres. The final dry biomass, oil content and fatty acid profile of oil determine volumetric productivity. Lipid accumulation in an oleaginous microorganism is a dynamic process which depends on a multitude of variables and hence proper selection of organism and process optimization are essential for efficient SCO production.

In this study the oleaginous fungus *Mortierella alpina* CBS 528.72 was exploited for the production of arachidonic acid- a nutraceutically and pharmaceutically important PUFA. Effect of culture variables on biomass, lipid and arachidonic acid production was investigated in this fungus under shake flask conditions and scale up study was carried out at 15L fermentor level. Optimization was done by one-factor-at-a-time and response surface methodology with the aim of maximizing arachidonic acid yield. Various approaches were used for SCO extraction from *M. alpina* biomass employing diverse solvents/solvent systems and extraction methods. Subsequent to optimization, strain improvement was carried out and efficient screening strategies were employed for selection of arachidonic acid hyper-producing mutants.

For assessing the safety of *M. alpina* as a source of arachidonic acid for use in infant and geriatric food formulations, the ARA rich biomass was subjected to acute and subchronic toxicity studies in Wistar rats. The experimental animals were given *M. alpina* biomass at different levels and were monitored for any overt signs of toxicity. Haematological, serum biochemical, histopathological and organ weight end points were evaluated after the experimental period.

PCR cloning and sequencing of partial Δ^5 desaturase gene was done to investigate the phylogenetic relationship of *M. alpina* Δ^5 desaturase with those of other organisms. The relationship between delta, omega and steroyl desaturases of *Mortierella* was also studied.

The following conclusions were drawn from this study.

- Biomass, total lipid and arachidonic acid yields in *Mortierella alpina* CBS 528.72 were influenced by several culture parameters viz., media, pH, temperature, carbon source, nitrogen source, oil as carbon source etc. These variables had a profound affect on biomass buildup, lipogenesis and ARA production in this strain and it was evident that fatty acid desaturation and elongation are finely tuned in this organism with a complex interplay between the culture variables.
- The results indicated that the most effective medium for growth and arachidonic acid production was Glucose Yeast extract (GY) medium. The optimum pH and temperature were found to be 6.5 and 28°C respectively which resulted in 0.92-1.0 g/L ARA.
- Glucose and yeast extract were found to be the most efficient carbon and nitrogen sources for maximum ARA yield. The efficacy of organic nitrogen sources over inorganic sources was also established.
- The biomass and ARA production declined drastically in a medium with vegetable oils as the sole carbon source but triggered the lipogenic pathway leading to higher accumulation of total lipids.
- Augmentation of glutamate at 0.8g/L concentration had a positive effect on ARA production. Replenishing the spent carbon by glucose fed batch significantly improved the biomass, lipid and ARA yield.
- Scale up of the process to a 15L fermentor level resulted in a marginal decline in biomass yield; the lipid and ARA yield remained unaltered.
- When yeast extract was replaced by corn flour in a 4 variable CCRD, cost of the production process was dramatically reduced. On optimizing the process by RSM, a significant increment in ARA yield was achieved when compared to that of yeast extract medium.

- RSM proved to be efficient in optimizing the parameters for maximizing ARA yield by relatively smaller number of experiments. The optimum conditions when employed resulted in maximum production of arachidonic acid (1.39 g/L) and the corresponding biomass and total lipid were 12.49 and 5.87 g/L respectively.
- Method of lipid extraction, solvents employed and pretreatment of biomass had a significant effect on lipid recovery from *M. alpina* biomass. All organic solvent methods except Bligh and Dyer method proved superior to supercritical extraction in terms of lipid recovery.
- The present study highlighted the efficacy of chloroform:methanol over hexane: isopropanol system for lipid extraction from *M. alpina* biomass. Lipid recovery from the HCl and enzyme treated biomass increased in proportion with the percentage of ruptured cells. Pretreatment of biomass by HCl or enzymatic means significantly improved the mass transfer of solutes thereby enhancing lipid recovery. A significant difference in color attributes was observed in the supercritical and solvent extracted oil.
- In spite of the limitations, conventional random mutagenesis still proved effective in developing hyper arachidonic acid mutants of *M. alpina.* 2, 3, 5-Triphenyl tetrazolium chloride (TTC) staining proved to be an efficient method for screening ARA hyper-producers from a large number of putative mutants. Maximum ARA yield was obtained in the NTG mutant M386 which showed an ARA productivity of 1.95g/L in GY medium.
- Proximate analysis revealed that in addition to lipids, *M. alpina* biomass is a good source of protein, fibre and micronutrients.
- Safety evaluation of arachidonic acid rich *M. alpina* biomass was carried out in Wistar rats by acute and subchronic oral toxicity studies. Results of the acute toxicity study revealed that the biomass was safe at acute doses and that the LD_{50} exceeded 5000mg/kg BW, the highest dose used in the study.
- Subchronic study indicated that biomass fortification had a positive influence on growth with no overt toxic effects on survival, food consumption and body weight gain throughout the treatment interlude.

- Few statistically significant changes in relative organ weights, serum biochemical and haematological indices in *M. alpina* fed groups were not concomitant with pertinent histopathological changes and hence toxicologically inconsequential. No microscopic or macroscopic lesions attributable to the treatment were manifested in the experimental groups. The results advocated the safety of *M. alpina* in rats at levels used in the present study.
- Δ^5 desaturase (DES) gene was isolated from *Mortierella alpina* by PCR based approach. Deduced amino acid sequence of Δ^5 desaturase gene from *M. alpina* was identical to that of other *M. alpina* Δ^5 desaturase in Gene bank over most of the protein. Analysis of the sequences revealed high homology at the cytochrome b_5 region with the HPGG motif and three histidine boxes conserved in different organisms.
- *M. alpina* Δ^5 desaturase were phylogenetically more related to algal, slime mould and fungal Δ^5 desaturases and were distinct from animal desaturases. This disparity probably arose due to earlier divergence of the fungal and plant desaturases than that of animal desaturases.
- Analysis of the delta, omega and stearoyl CoA desaturases in *Mortierella* confirmed that Δ^5 desaturases were phylogentically related to the Δ^6 desaturases with a common HPGG motif, absent in other desaturases.
- The hydropathy profile of the amino acid sequences indicated that the two conserved hydrophobic regions present assumed to form two membrane spanning domains which probably play the role of a unique electron transport system.

Future perspectives

The role of PUFAs in the pathophysiology of several chronic diseases has attracted considerable interest in recent years and comprehensive research into fatty acid biosynthesis and metabolism is vital. There is an escalating demand for tailor made PUFAs in both food and non-food applications and the oleaginous fungus *M. alpina* will become a more powerful producer of specific lipids, provided that suitable transformation and expression systems become available for this species. The industrial production of PUFAs other than ARA, appears to be the next step in utilizing this fungus as source of other PUFAs. To attain this, several variables must be standardized and the strains specific for the production of each fatty acid has to be decided. Nutritional and bioavailability studies has to be carried out with regard to the fatty acid they are used since a potent producer is effective only if the fatty acid it produces are utilized by the human body. Future prospects in biotechnological applications of oleaginous microbes like *Mortierella* are also related to commercially interesting products including hydroxyl-PUFAs, prostaglandins, leukotrienes, thromboxanes and other useful lipid classes which are extraordinarily expensive to produce by chemical synthesis. Manipulation and regulation of microbial lipid synthesis opens a large avenue for academic research and demonstrates the enormous potential in its application. Techniques dealing with overall analysis of gene expression ie., genomics, proteomics and metabolomics coupled with genome sequencing will pave the way to improvement in activity, specificity and stability of enzymes by directed evolution. Although recent progress in cloning and identification of genes encoding PUFA biosynthetic enzymes provided a potential mean to produce LC-PUFA rich transgenic oils, issues regarding the compatibility and safety of genes and accumulation of undesirable PUFAs have to be solved. The transgenic oils thus produced provide an economic alternative to naturally occurring oil and will satisfy the demand of chemical, pharmaceutical and nutraceutical industry for therapeutic and prophylactic use.

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I. PUBLICATIONS

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- **Nisha A,** Muthukumar SP, Venkateswaran G, 2009. Safety evaluation of arachidonic acid rich *Mortierella alpina* biomass in albino rats - a subchronic study. Regulatory Toxicology and Pharmacology 53,186-194.

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- **Nisha A,** Rastogi NK, Venkateswaran G, 2009. Optimization of media components for enhanced arachidonic acid production by *Mortierella alpina* under submerged cultivation. Communicated to Process Biochemistry.

- **Nisha A,** Udaya Sankar K, Venkateswaran G, 2009. Super critical CO_2 extraction of *Mortierella alpina* single cell oil: comparison with organic solvent extraction. Communicated to Bioresource Technology.

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- **Nisha A,** Venkateswaran, G, 2009. Effect of extraction methods on lipid yield and fatty acid composition of *Mortierella alpina*. (Under preparation).

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