STUDIES ON SELECTED PLANTS AND MICROBES WITH SPECIAL REFERENCE TO POLYUNSATURATED FATTY ACIDS

THESIS

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CERTIFICATE

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

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

Signature of Doctoral candidate

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Dedicated

To

My Parents

and

Teachers

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C O N T E N T S

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ABBREVIATIONS

ATP	citrate lyase
ACC	Acetyl CoA Carboxylase
ALA	Alpha linolenic acid
ALP	Alkaline phosphatase
AMP	Adenosine mono phosphate
AOAC	Association of Official Analytical Chemists
ARA/AA	Arachidonic acid
ATP	Adenosine triphosphate_
CBE	Cocoa butter equivalent
cm	Centimeter
C/N	Carbon/nitrogen
CO_2	Carbondioxide
CoA	Coenzyme A
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EL	Elongase
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
g	Gram
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GLA	Gamma linolenic acid
GRAS	Generally regarded as safe
h	Hour (s)
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
Kg	kilogram
LC-PUFA	Long chain polyunsaturated fatty aci

LA	Linoleic acid
LDH	Lactate dehydrogenase
LT	Leukotriene
M	Molar concentration
mg	Milligram
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimeter
mM	Millimolar
N	Normal
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGE1	Prostaglandin E1
PGE2	Prostaglandin E2
PUFA	Polyunsaturated fatty acid
rpm	Rotation per minutes
SFA	Saturated fatty acid
s	Second
SCO	Single cell oil
$SCCO_2$	Supercritical carbon dioxide
SD	Standard deviation
SCFE	Supercritical fluid extraction
sp./sps	Species
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TFA	Total fatty acid
TX	Thromboxane
UFA	Saturated fatty acid
UV-VIS	Ultraviolet-visible
U/L	Units per litre
v/v	Volume/volume
w/w	Weight/weight
ω	Omega

ABSTRACT

Fats and oil are the most essential nutrients for human health. Specialty lipids like GLA, DHA, EPA, and AA are important dietary supplements especially for infants and geriatrics and also recommended for many therapeutic purposes. Plant materials like seeds, dry nuts, pulp and leafy vegetables and certain oleaginous microbes were screened for oil yield and polyunsaturated fatty acids (PUFA) contents. Among the plant products avocado fruit pulp (*Persea americana* Mill.) and watermelon seeds (*Citrullus lanatus* (Thunb) Matsum and Nakai), showed higher oil yield and promising EFA contents. From the microbials source, *Rhizopus oligosporous* MTCC 556 was selected. The biomass, total lipid content and PUFAs values were analysed. Strain improvement by mutagenesis and optimization of culture conditions for higher oil and PUFAs yields were carried out. Fat Producing medium favoured the growth, biomass build up and lipid production than the normal cultivation medium (PDB). Similarly, nitrogen limiting medium favoured more PUFAs content in the total lipid than nitrogen non limiting medium. The culture was subjected to UV and EMS mutagenesis and 21 putative mutants were obtained. Three selective mutants possessed more lipid and GLA contents. The supercritical CO_2 extractions of avocado pulp oil and watermelon seed oil improved the oil yields, showed higher concentrations of PUFAs and are free from any solvent impurities. These quality oils were used for o/w emulsion preparations to serve as stabilized nutraceutical products. Also produced were ready to eat emulsion gels to be used in several food products in the form of thermo set gels, slurries, batters, disperses, solutions and emulsions which form a delivery system that can be eaten for health benefits. Thus the importance and utilization values of the ω-6 and ω-3 fatty acids from selected plant materials and a microbial source have been explored biotechnologically throughout the study.

CHAPTER-1

1.1 INTRODUCTION

Fats and oils are the most essential nutrients of both human and animal diets. They are collectively called lipids and do constitute a substantial portion of our diets. About 35-40% of dietary energy comes from fat. They are the sources of most concentrated form of energy i.e. 9 kcal/g of any food material and are calorically denser, compared to both protein and carbohydrate at about 4 kcal/g. About 93% of the fats are lipids composed of triglycerides. The remaining portion in our diet is made up of other lipids such as cholesterol and phospholipids. Lipids also supply essential fatty acids which are precursors for important hormones like prostanoids, serve as carriers for fat soluble vitamins, make foods more palatable and contribute towards the feeling of satiety after eating (Liu SC et al., 2009; Talbot, 2005).

Fatty acids are the building blocks of the majority of the lipids. Studies reveal that in plant oils, a predominance of three acids; oleic, linoleic, and palmitic together constituting for about 85% of all the fatty acids available from them. The balance is largely made up of linolenic, stearic, lauric and myristic acids. The total level of saturated acids is in excess of 28%. The major sources of these seven fatty acids is also worthy of attention. Palm oil is the major source of dietary oleic and palmitic acids while soybean oil provides almost half of the total supply of linoleic acid and is also the major single source of stearic acid. It is understood that soybean oil and canola oil are virtually the only source of dietary linolenic acid. The linoleic / linolenic ratio at this level of global calculations is ~9:1 (Gunstone, 2005). Nutritionally the essential lipids in our regular diets are the so called essential fatty acids, linoleic acid (ω-6) and linolenic acid and / or docosa hexaenoic acid (ω-3). Over the last 50 years and beyond, production and consumption of oils and fats have risen and also the production in 2004-05 was twice of that of 30 years earlier and it is expected that

both production and consumption (of food and non-food oils) will continue to increase. Most of this oil (80%) is used for food purpose and the remainder (6%) is applied as animal feed and is also used by the oleo chemical industry (14%). Hence the fats and oils is an important element of the food chain (Gunstone, 2005).

Saturated, mono-unsaturated and polyunsaturated fatty acids

Fatty acids consist of a hydrocarbon chain with a methyl group at one end of the chain and a carboxyl group at the other end. There are short (4-6 carbons), medium (8-14 carbons) and long chain (>16 carbons) fatty acids occurring naturally in foods. Fatty acids may be totally saturated (no double bonds), mono unsaturated (one double bond) or poly unsaturated (two or more double bonds). Examples of saturated (Stearic acid 18:0), mono unsaturated (Oleic acid 18 : 1 ω –9) and polyunsaturated (linoleic acid 18:2 ω-6 and linolenic acid 18:3ω-3) fatty acids are

18: 0 – Stearic acid $CH_3 (CH2)_{16} COOH$

18: 1ω- 9 – oleic acid $CH_3 (CH2)_7 CH = (CH2)_7 COOH$

18: 2ω- 6 – Linoleic acid $CH_3 (CH2)_4 (CH=CH CH2)_2 (CH2)_6 COOH$

18: 3 ω- 6 Linolenic acid $CH_3 CH_2 (CH = CH CH2)_3 (CH2)_6 COOH$

Saturated fatty acids tend to be solids at room temperature (with the exception of tropical oils, and coconut and palm oils), while monounsaturated and polyunsaturated fatty acids are liquid at room temperature. The naturally occurring unsaturated fatly acids in foods contain double bonds in the cis-configuration. Fatty acids containing double bonds in the trans configuration (mostly mono – unsaturated) are produced by the hydrogenation of vegetable oils to make margarines and by rumen bio – hydrogenation. The point of unsaturation of fatty acids is designated by its distance from the methyl end of the chain. Thus linoleic acid is designated as 18: 2 ω – 6 meaning that the first double bond occurs at the 6'th carbon from methyl end.

Fatty acids perform a number of functions. They are esterified via the carboxyl end of the chain to cholesterol to form cholesterol ester and are also esterified to a glycerophosphate back bone to form phospho-lipids. Cholesterol ester and phospho lipids are important structural components of cell membranes. The fatty acids themselves are an abundant energy source. Free or non esterified fatty acids in plasma can be utilized for energy by various tissues in the body especially heart, muscle and kidney. Fatty acids can be incorporated with triglycerides via etherification to glycerol by storage in adipose tissue. This is the pre-dominant storage form of energy in the body. Certain fatty acids are also precursors for eicosanoids which have a number of functions in the body themselves.

Polyunsaturated fatty acids / PUFAs

PUFAs are polyenoic acids having more than one double bond, generally in a cis- configuration and methylene interrupted. By convention, PUFAs are described as being ω-3 or ω-6 (n-3 or n-6) FA depending on the location of the first double bond in the third or sixth carbon atom away from the methyl carbon (ω-carbon), respectively. Additional double bonds are separated from each other by a methylene group.The ω-3 acids are derived from α-linolenic acid ALA/ LnA (C18:3 ω-3) by chain elongation and further desaturation and include eicosa pentaenoic acid EPA (C20:5 ω-3) and docosa hexaenoic acid DHA (C22:6 ω-3). On the other hand ω-6 acids are derived from linoleic acid LA (C18:2 ω-6) and include γ-linolenic acid GLA (C18:3 ω-6) and arachidonic acid AA/ ARA (C20:4 ω-6). The delta system defines the position of the double bonds from carbon atom i.e . the Δ-end of the molecule. Hence LA and ALA are represented as C18:2 $\Delta^{9,12}$ and C18:3 $\Delta^{9,12,15}$ respectively. Desaturases abstracting hydrogen atoms from adjacent CH_2 groups to produce double bonds, can be defined as Δ^{12} desaturase or a Δ^5 desaturase would introduce a double bond at the Δ^{12} or Δ^5 carbon atoms in the acyl chain respectively (Griffiths and Morse, 2006) .

The ω-6 and ω-3 essential fatty acids as established (Health and Welfare Canada 1990) are linoleic (C18:2 n-6) and α-linolenic (C18:3 n-3) acids respectively. These fatty acids are mainly available from vegetable oils. Linoleic acid is abundant in corn oil, safflower oil, peanut oil and sunflower oil. Vegetable oils high in linolenic acid content are canola oil, soybean oil and linseed (flax) oil. Linoleic and linolenic acids (to a much lesser extent) are components of membrane phospholipids in the tissues of the body (Liu SC et al., 2009). They are converted by desaturation and elongation to long chain polyunsaturated fatty acids namely arachidonic acid (AA/ARA 20: 4 ω- 6), eicosa- pentaenoic acid (EPA C20: 5 ω- 3) and docosa hexaenoic acid (DHA C22 : 6 ω –3). Although tissue levels of EPA are minor, AA is a major component of membrane phospholipids in most tissues, while DHA is abundant in retina and brain. In addition to the desaturation and elongation of essential fatty acids, AA may be obtained in the diet from animal meats and poultry, while EPA and DHA are abundant in fish and sea foods (Talbot, 2005). In human nutrition, plant lipids and seed oils are preferable to animal fats due to their low content of cholesterol and their generally high proportion of polyunsaturated fatty acids (PUFAs). Of which the predominant ones are linoleic acid (C18 : 2 ω-6) and linolenic acid (C 18 : 3 ω-6) both of these are of vital importance to human metabolism as we cannot form additional double bonds between carbon numbers 12-13 and 15-16 of an oleic acid molecule. All these essential fatty acids exist throughout in cis-form.

Deficiency symptoms

Linoleic and linolenic acids cannot be synthesized in the body. A lack of them in the diet leads to deficiency symptoms. Total essential fatty acid (EFA) deficiency (both ω-6 and ω-3) causes reduced growth, reproductive failure and dermatitis. These symptoms are prevented or reversed by feeding linoleic acid. Linolenic acid is also partially effective at preventing or reversing these symptoms but is not specifically required (Holman, 1968). The fact that ω-3 usage is essential to human health is well established (Shahidi, 2009).

In a non deficient state the long chain ω-3 fatty acid, DHA is abundant in the phospholipids of retina and brain (Neuringer and Conner, 1986). In addition, brain levels of DHA rise markedly, at the expense of ω-6 fatty acids, during the last trimester of pregnancy and the period after birth. As well premature infants at birth are deficient in DHA in the brain and liver (Simopoulos, 1989). Upon deficiency of ω-3 fatty acids in brain and retina, DHA is replaced in these tissues with long chain ω-6 fatty acids such as docosa pentaenoic acid (DPA C22:5 ω-6). Reduced visual activity of monkeys and lowering of learning ability in rats under ω-3 deficiency are also proven. In a human study a child on total parenteral nutrition (TPN) receiving a solution low in linolenic acid developed peripheral neuropathy and the subject had intermittent blurred vision. These symptoms disappeared when the subject received a high linolenic acid contained TPN solution (Holman, 1982)

Recommended intake levels of PUFAs

Currently the ratio of ω-6/ ω-3 in the diet has been recognized as important. A very high ratio as 50:1 in our diets may lead to tissue depletion of DHA. This is due to the ω-6 fatty acid inhibiting the conversion of LA to DHA via desaturases. The current ratio of ω-6/ ω-3 fatty acids in the western diets is estimated between 10:1 and 11:1 (Simopoulos, 1989). Based on human breast milk composition a ratio between 4:1 and 10:1 has been suggested as an optimum for our diet (Neuringer and Conner, 1986). Health and Welfare Canada (1990) has recommended intakes of ω-6 and ω-3 fatty acids at 3% and 0.5 % of calories respectively, this recommendation provides a ratio equal to 6:1. To prevent deficiencies in adults the optimal intake of linolenic acid has been estimated to be at 800-1100 mg/day. In addition an optimal intake of long chain ω-3 fatty acids (EPA and DHA) was recommended at 300-400 mg/day. But infant formulas should contain levels similar to human milk only (Simopoulos, 1989).

Therapeutic values of PUFAs

There is a great revolution in fatty acid therapies for a variety of human diseases. The importance and influence of various fatty acids over health aspects have become increasingly apparent. Fetal and infant nutrition studies have proved that FA status at birth can have life-long health implications affecting the eye and brain function, insulin resistance and blood pressure control. As well nutrition studies have identified dietary imbalances and deficiencies that have the potential to alter the health of future generations severely and to promote progression of age-related degenerative disorders. Mixtures of naturally occurring FAs can serve as therapeutic agents for a diverse range of health conditions like atopic eczema, rheumatoid arthritis, cardiovascular disease and neurological problems (Horrobin, 2000). Actually the addition of ALA as supplementation to human food chain converts it in to LCPUFAs (Brenna et al., 2009).

From 1990s through 2000s the advent of technologies has seen the formulations of newer pharmacologically active individual FA components as well as tailored combinations and has propelled the development of this new drug category (Gunstone, 1998). Under-pinning these pharmaceutical / nutraceutical applications of FA are the need of high quality fats and oils from natural sources. In the plant kingdom, well over 800 FA species have been identified; many of these are uncommon and are found in only a few species. Oil seed crops generally produce unsaturated FA having chain lengths up to C18, with linoleic (LA) and α-linolenic (ALA) acids being the most abundant. There is also a dietary requirement for more highly unsaturated and longer chain FA for optimal health benefits. Several PUFAs are recognized as 'essential fatty acids' in the normal diet for preventing nutrition-related illnesses. These are LA, GLA, ALA, and AA (WHO/FAO, 1977; Gurr and Harwood, 1991). Beside these, there are other PUFAs that are being investigated for their role in human health. In the latter category we have the fatty acids OTA, DHGLA, EPA, and DHA. GLA has been claimed to play a role in the development and prevention of some skin

diseases, diabetes, reproductive disorders, and others (Gunstone, 2005; 1998; Horrobin, 1992). The role of AA and DHA in the development of nervous system is well supported (Nettleton, 1993; Innis, 1991; Singh and Chandra, 1988), and DHA is additionally related to the development of retina (Brown, 1994). In fact, several health agencies have recommended that infant feed formulae be fortified with DHA and AA (Gill and Valivety, 1997). The role of EPA in the proper functioning of the circulatory system was first identified nearly 40 years ago (Nelson, 1972) and is now well supported (Iacono and Dougherty, 1993; Nettleton, 1993; Simopoulos, 1991; Singh and Chandra, 1988; Dyerberg, 1986). There is growing interest in the putative involvement of EPA in some cancers and other diseases (Simonsen et al., 1998; Wingmore et al., 1996a; b). It is hypothesized that there are few direct actions of these PUFAs, and their activities are mostly mediated by their transformation in to a number of metabolically active compounds collectively known as 'eicosanoids' (thromboxanes, leukotrienes, and prostaglandins). Eicosanoids are physiologically highly active and play diverse roles in human metabolism (Gill and Valivety, 1997; Peck, 1994; Parent et al., 1992; Innis, 1991;; Singh and Chandra, 1988; Dyerberg, 1986). Because of their significance in human health, many PUFAs and PUFA-based products are on the market. The market size is very vast and the production of some PUFAs is relatively well established, and intense research is underway to develop commercially viable production methods for others too. For instance, GLA production is relatively well known, and this fatty acid is commonly obtained from plant sources such as evening primrose (*Oenothera biennis*), borago (*Borago officinalis*), and black-currant (*Ribes nigrum*) (Gunstone, 1998). Also, attempts have been made to produce GLA from some fungi like Mortierella sp., *Mucor sp., Rhizopus sp.,* etc. (Ratledge, 1989, 1993). These unsaturated fatty acids reduce the risk of cardio vascular diseases and cancer in some animals too (Vanessa et al., 2009).

Nutritive and Market demands for PUFAs

Considering the availability of different fatty acids from the whole lot of global fats and oil production, there are about 13 or so vegetable oils in the market for proper utilization. Classification of such fatty acids in to the likes of saturated or unsaturated and even then monounsaturated, di-unsaturated and polyunsaturated varieties is a different but valuable kind of approach towards these plant produces. In general, the natural supply of saturated acids does not appear to be excessive. The availability of monounsaturated acids is lower than desirable and this is made worse by the fact that whatever the material available is not entirely a cis-isomer. Among the polyene acids, linoleic acid is too high and linolenic acid is too low and the ratio between these two is seriously different from the recommended value by nutrition experts (Gunstone, 2005). Polyunsaturated fatty acids (PUFAs) are thus valuable products because of their involvement in several aspects of human health. Market demand for the PUFAs is growing continually and current sources are considered insufficient / are yet to be exploited effectively. For satisfying this demand; alternative sources are also actively sought after. Oilseed plants can thus be a potential source of PUFAs if they are appropriately utilized for the supply of RDA (recommended daily allowance). Most of the basic tools for genetically engineering the oilseed plants for improving their ability to produce PUFAs are already being explored.

Current and potential sources

PUFAs are currently obtained from a number of sources including higher plants, animal viscera, and oily fish (Liu SC et al., 2009), but it has become evident that PUFA production from these sources is inadequate for supplying the ever expanding global PUFA market (Gill and Valivety, 1997; Napier et al., 1999). Additional and significant problems of the current PUFAs sources are seasonal and climatic variation in the oil composition, (Gill and Valivety, 1997; Yongmanitchai and Ward, 1989); low productivity of used and cultivated plants (Gill and Valivety, 1997; Sayanova et al., 1997); complex and expensive downstream processing (Belarbi et al., 2000; Gill and Valivety, 1997; Barclay et

al., 1994) resulting in inconsistency in the oil supply and quality. Some microorganisms, including bacteria, fungi, and microalgae, are considered alternative sources of PUFAs (Gunstone, 2005, 1998; López Alonso et al., 1999 1996; and 1992; Gill and Valivety, 1997; Barclay et al., 1994; Bajpai and Bajpai, 1993; Yongmanitchai and Ward, 1991; Shimizu et al., 1988;Yazawa et al., 1988). Already, several nutraceutical companies such as Martek Biosciences, USA, are marketing PUFA oils or PUFA concentrates obtained from microbial culture. Other potential sources are the genetically modified oilseed crops (Napier et al., 1999; Gill and Valivety, 1997). Because of these essentialities, it is highly imperative to identify newer sources of PUFAs (both plants and microbes), learn about their qualities and to explore the maximal possibilities of exploiting them through improvement of required qualities to enable human race to meet out the nutraceuticals demands to compete in cost and quality with the current sources. It is noteworthy to mention here that the Indian sub-continent has a huge reserve / treasury of vegetation which is yet to be utilized for such nutraceutical applications, and it's presumed that it will take decades even before we start using them to a fewer percent.

1.2. OBJECTIVES

Keeping the importance of plant and microbial PUFAs for human health purpose the following objectives were undertaken in this study.

1. Analyzing the types and quantities of PUFAs in selected plant materials and microbial species.
2. Selection of certain microbial strains for PUFA production and subject them to strain improvement through mutagenesis.
3. Optimization of culture conditions for PUFA production using the improved strain.
4. A detailed study on supercritical fluid extraction (SCFE) of selected plant oils and their characterization.
5. To make use of such supercritical oils for developing stabilized health / nutraceutical products.

1.3. SCOPE OF THE INVESTIGATION

- Administering the essential fatty acids which cannot be synthesized by the human systems, as recommended daily allowance (RDA) helps for the well being of the humans.
- The essentiality of PUFAs to human health is attributable to the nutritive as well as therapeutic values of unsaturated fatty acids.
- Screening and selecting the PUFAs producing plants and microbes help in identifying and exploiting them as easily available sources.
- The suitable microbial strain thus selected could be exploited by strain improvement through mutagenesis and optimization of culture conditions.
- Similarly novel solvent free extraction modes like supercritical CO_2 fluid ($SCCO_2$) extraction helps in obtaining nutraceutically valued plant oils in their near natural, pure and solvent free form
- Such valued oils could be used to develop stabilized and protected nutraceutical delivery systems.

CHAPTER-2

2.1. INTRODUCTION

Oleaginous plants are attractive because of their high oil productivity. Also, well-established methods exist for commercial and agronomic production of oilseeds for recovery of the oil. The nutritional value of nut and seed products is related to fatty acid content in their oils. Nuts in general offer abundant poly unsaturated fatty acids in the diet. High linoleic acid content reduces shelf life. Increasing oil to linoleic acid ratio produces more stable oil with a longer shelf life. Of more than 40 fatty acids prevalent, only a few are essential, like linoleic, linolenic and arachidonic acids (LA, LnA / ALA and AA / ARA). Generally PUFAs lower total blood cholesterol and low density Lipoprotein (LDL) levels leading to reduced risk of coronary heart diseases and atherogenesis (Horrobin,199 ; 2000). Mono unsaturated fatty acids are beneficial in lowering blood cholesterol (Sheppard and Rudolf, 1991). Replacement of double bonds in unsaturated fattty acids with an atom of oxygen leads to slight rancidity which doesn't affect the nutritional value of nuts but cosmetic characters like color, flavor, odor and total acceptability may be greatly degraded.

Microbial PUFAs

Similarly, microorganisms having oleogenicity have often been considered for the production of oils and fats as an alternative to agricultural and animal sources. However, owing to their resemblance to plant oils and animal fats, microbial oils will inevitably have to compete with those traditional lipid products if they are to be produced commercially. Therefore, for microorganisms to be accepted as sources of single cell oils then these oils must be highly specific and lesser expensive compared to those obtained from agricultural and animal sources. Although thereby the occurrence of an amazing diversity of fatty acid structures in the microbial kingdom (Ratledge,1989), many of the minor

fatty acids have potential uses but are not available in sufficiently large quantities. Hence the increasing demand for commercially valuable microbial lipids has resulted in the production of PUFAs for health and dietary applications. It is clear that with the lowering cost of plant oils and animal fats, there appears no prospect that microbial oils similar to these could ever be produced economically. However *Blakeslea trispora* is commercially produced for lycopene and β-carotene (Roukas and Mantzouridou 2001). Supercritical CO_2 extraction of lycopene from the same source is also effective for that purpose (Choudhuri and Singhal 2008).

The aims of the present study were to screen some of the microbes and plant products to learn about their saturated and unsaturated fatty acid compositions and select one / two microbes and plant products for further studies. Microbial species thus selected was subjected to strain improvement experiments for improved quality and yields of PUFAs. It was also planned to involve such an improved microbial strain for the optimization of culture conditions. Additionally the chosen plant materials were taken up for application purposes for valuable exploitations. Certain plant materials (such as leafy vegetables, fruits, seeds, nuts, pulses, cereals and spices) commonly used for human consumption were listed for basic PUFAs composition analysis. Similarly oleaginous microbial systems, which are edible, having GRAS (generally regarded as safe) status and commonly available for culture were also screened for PUFAs. Generally, steps for the optimization of extraction modes and analytical procedures (using chromatographic studies) for PUFAs were attempted. Effective extraction and production of PUFAs from the selected species (both Plants and microbes) for developing food, pharma, and therapeutical products having nutraceutical values and applications were attempted.

2.2. MATERIALS & METHODS

Plant materials

Selected plant materials like nuts (cashew nut, pistachio, almond, walnut- black and walnut-english), fruits (avocado), vegetable-seeds (cucumber, pumpkin, muskmelon, squash, vegetable-sponge and watermelon) and fruit-seeds (pomegranate) were selected for screening for unsaturated fatty acid contents. All the materials were sun-dried or oven dried as required and subjected to oil extraction using soxhlet apparatus and procedure.

Microbial Culture and Chemicals

Fungal strains *viz,Rhizopus oligisporus* MTCC 556, and *Rhizopus oligosporus* MTCC 286 were procured from Microbial Type Culture Collection Centre (MTCC), IMTECH, Chandigarh, India . These fungal strains were maintained as per the instructions given by MTCC catalogue. Other strains like *Rhizopus sp.1., Rhizopus sp.,2* and *Rhizopus sp.,3* along with *Rhodosporidium toruloides* and *Mucor hiemalis* were accessed from the culture collections maintained at the Food Microbiology Department of Central Food Technological Research Institute , Mysore, India.

Chemicals

All the chemicals and reagents used in this study were of analyltical grade and obtained from SD fine chemicals India, Merck India, Glaxco India Pvt, Ltd. Dehydrated media such as Yeast extract, Potato Dextrose Agar/broth CzepakDox Agar peptone etc were procured from Hi-Media company, India.

Culture Media
Potato dextrose broth: (PDB)

Potato dextrose broth was prepared by using fresh potato infusion by the following procedure: 200g of fresh potato after peeling of the skin, was cut into

big pieces and allowed to boil in 500 ml of distilled water. The infusion was filtered through a clean muslin cloth and 2% glucose was added and made up to one liter with clean tap water. In case of solid medium 1.5-2.0% agar was added prior to autoclave.

Estimation of dry weight of microbial cells

The dry weight of the cell biomass was carried out by the following method:

100 ml of culture broth was centrifuged at 6000 rpm for 15 min and the cells were washed twice with distilled water. The pellet was suspended in minimum volume of water and filtered through a pre-weighed Whatman No.1 filter paper. The filter paper was dried in an oven at 65^{o}C for nearly overnight or 12-16hrs and weighed till constant weights were obtained.

Estimation of cell dry weight

After fermentation, the mycelium was harvested from the culture broth by suction filtration using Whatman No. 1 filter paper. Cell dry weight was then estimated by washing the mycelia with distilled water twice and then dried at 55.2°C for 24 h and the fungal dry biomass was estimated.

Estimation of total lipids

A known amount of dried cells was wrapped well in Whatman No.1 filter paper, tied with a twine and hydrolyzed with 0.1N HCl over boiling water bath for 1 hour. It was then washed thoroughly in running water in order to remove excess acid and the filter paper packet was dried at 55^{o}C overnight. The lipid was extracted by using soxhlet apparatus by the following procedure:

These dried cells that had been hydrolyzed as described above was kept in the soxhlet apparatus and 150-200ml of hexane (60-80^{o}C) was added and extraction was done for more than 6 hours continuously. The solvent was recovered by distillation and the fat recovered in a minimum volume of hexane was transferred into a pre-weighed clean beaker. The solvent was evaporated to

dryness under vacuum or overnight in an incubator set at 40-45^{o}C and weighed till constant readings were obtained. Total lipid content of the micrcobials was estimated by gravimetric method of AOAC (1991). The lipid was extracted by solvent contact method in soxhlet apparatus. Analytical grade Chloroform and methanol (2:1 V/V) were used for extraction for 4-6 hours (Somashekar et al., 2003). About 2-3ml of solvent containing lipid fraction was transferred to pre-weighed beaker and refluxed with nitrogen gas and kept in desiccators under cold condition till the total lipid was estimated by gravimetric method.

Soxhlet Extraction

The powdered materials of plant products as well as dried biomass of fungal cultures were taken up in thimbles for soxhlet apparatus for the extraction of oils using hexane as the solvent. For each sample, about 100-120 ml of the solvent was used and the extractions were carried out for about 6' hours at 60-80^{0}C. Finally the oil was separated or decanted and subjected to solvent evaporation using a Rota vapor apparatus (Model Buchi VT111). The resultant oil was flushed with nitrogen gas for water removal. The final volume of the oil was collected in a vial. The oil was converted in to FAMEs (fatty acyl methyl esters) for fatty acid profile studies.

FAME Preparation

This was following Kates method (1964), which is otherwise known as Methanolic HCl method. A mixture containing 5ml of Acetyl chloride and 95 ml of Dry Methanol was made. Lipid samples of 0.1 – 0.2 µl were taken in 5ml of the reagent and was refluxed for 2 hours in a hot water bath. The lipid esters were extracted twice in 5ml of Hexane/ Chloroform and concentrated to 1 ml by flushing with nitrogen gas. The upper hexane layer containing the FAME was transferred to a small tube and stored at -20 ^{0}C for later analysis by capillary column gas chromatography and GC-MS analysis.

GC & GCMS analysis for lipid compositions

Gas Chromatography

The prepared fatty acyl methyl esters (FAME) were analyzed in a gas chromatograph (GC-Fisons-GC-8000 series, USA) provided with Flame Ionization Detector (FID) and an integrator. A capillary glass column of fused silica of model BP-1 (SGE, Singapore, length 30m; internal diameter 0.25mm with a film thickness of 0.2μm) was used. The flow rate of the carrier gas (nitrogen, hydrogen or helium) was allowed at a rate of 0.75 L/min. The split ratio of the injector was 100:1. The injector temperature was 250^0C and the detector temperature was adjusted to 260^0C.The oven program was to start with 140^0C (for 5 minutes) and was increased at a rate of 4 ^{0}C/ minute until 250^0C was reached, where it was retained for a 10 minutes period. The injection volume was 2μl and a blank was run every 10 analyses. Peaks were identified by comparison with known methyl ester standards (obtained from Sigma Co., St.Louis, MO,USA).

Gas Chromatography Mass Spectrometry (GCMS)

The identity of the fatty acid peaks obtained by GC was confirmed using a gas chromatograph coupled with a mass spectrometer (MS). The GCMS system was Auto System XL Gas Chromatograph comprised with Turbo mass Gold Mass Spectrometer (Perkin Elmer Instruments, Norwalk CT., USA). The samples were compared to the patterns obtained using pure fatty acid standards analyzed in the same apparatus at constant parameters. A capillary column of methyl silicone (Elite-1; length 30m; internal diameter 0.25 mm; thickness of the inner coated film 0.25μm) was used. The flow rate of the carrier gas Helium was 1mL/min. The split ratio was 1:40. The injector temperature was 240^0C and pressure at the head of the column was 48.9Kpa. The oven program was to start with 140^0C (for 5 minutes) and was increased at a rate of 4^0C/ minute until 250^0C was reached, where it was retained for a 10 minutes period. And the temperature of the source at the detector was 250^0C.

Oil contents in samples were determined using Methyl hepta-decanoate as internal standard. Kovats index calculations (KI) and mass spectral library were used to further confirming the peak identities. Identified peaks were taken in to account for further calculations. Duplicates were used for each sample and the mean values are reported in the tables. Variations among the duplicate samples were routinely less than 5%.

Table T 2.1 Fatty acid composition percentage of oils from nuts, fruits and vegetable and fruit seeds

Sl. No.	Common name	Botanical Name	Oil % / dw	16:0	16:1	18:0	18:1	18:2	18:3	Others
	Nuts									
1	Almond	*Prunus amygdalus*	52.7	6.7	0.52	1.2	66.3	22.3	T	T
2	Cashew nut	*Anacardium occidentale*	47.7	10.2	0.41	8.5	60.9	18.3	--	T
3	Pistachio	*Pistachia vera*	57.0	8.6	0.72	2.3	68.8	17.8	0.3	T
4	Walnut black	*Juglans nigra*	59.0	3.1	T	2.6	29.1	58.3	4.9	T
5	Walnut English	*Juglans regia*	67.4	7.3	0.17	2.3	19.1	57.4	13.1	T
	Seeds									
6	Pumpkin	*Cucurbita pepo*	42.0	12.0	--	4.7	24.8	58.3	0.1	--
7	Watermelon	*Citrullus vulgaris*	45.0	16.4	--	14.5	13.4	55.4	--	--
8	Muskmelon	*Cucumis maxima*	44.0	9.0	--	5.6	20.1	64.6	0.3	--
9	Squash	*Cucurbita maxima*	47.0	14.2	--	6.8	21.8	55.6	0.5	--
10	Vegetable sponge	*Luffa cylindrica*	42.0	13.6	--	6.5	10.3	68.4	--	--
11	Cucumber	*Cucumis sativus*	40.0	11.4	--	7.0	10.6	66.3	0.3	--
12	Pomegranate	*Punica granatum*	6.0	10.0	--	1.2	17.6	14.5	56.0	--
	Fruit									
13	Avocado	*Persea americana*	47.0	17.0	10.3	0.6	60.5	10.6	1.0	--

T-Trace quantities

Table T 2.2 Fatty acid composition percentage of oils from edible leafy vegetables

Sl No	Name of the leafy Vegetables	Oil mg/g (w/w)	C:12	C:14	C:16	C:18	C:18:1	C18:2	C18:3	Other LCFAs**
1.	*Coriandrum sativum*	12.08	29.3	15	13.5	16.5	4.2	13.2	1.9	Traces
2.	*Mentha virudis*	13.46	0.89	1.6	2.6	0.74	1.10	12.40	45.40	-
3.	*Murraya koenghii*	12.4	8.00	11.23	15.23	12.3	6.63	34.03	12.05	-
4.	*Coleus sp.*	11.31	39.2	10.4	4.3	10.12	4.09	20.2	5.5	-
5.	*Trigonella fenugraecum*	2.84	14.4	16.8	17.6	40.1	7.0	Tr	Tr	-
6.	*Centella sp.*	4.46	Tr	Tr	16.6	42.1	6.7	14.2	Tr	-
7.	*Solanum sp*	6.25	2.8	1.7	26.4	1.52	8.4	15.6	Tr	-
8.	*Solanum torvum*	4.2	8.9	1.76	2.6	0.74	1.4	2.26	22.4	-
9.	Other edible leafy vegetables *	2-4	-	-	-	-	-	-	-	-

Tr-Trace quantities.

*** Such as *Amaranthus virudis, Amaranthus sp.Alternanthera sp. and Talinum cuneifolium* contain more protein, amino acid and traces of other fatty acids.**

**** Other long chain fatty acids are in trace amounts only, hence not mentioned in the table.**

Table T 2.3 Biomass and lipid content of *Rhizopus oligosporus* MTCC 556 and other isolates

Sl No.	Cultures	Biomass (g/L) DWB	Total lipid (% w/w)
1	*R. oligosporus* MTCC556	11.28±0.96	42.2±1.37
2	*R. oligosporus* MTCC 286	12.56±0.87	40.3±1.71
3	*Rh. sp.1*	10.24±0.76	32.4±1.31
4	*Rh. sp.2*	10.04±0.86	31.86±1.25
5	*Rh. sp.3*	9.84±0.67	28.48±1.21
6	*Rhodosporidium toruloides CB*	8.84±0.61	38.46±1.59
7	*Mucor hiemalis*	12.46±0.93	26.86±1.41

Table T 2.4 Relative percentage of fatty acid profile of *Rhizopus oligosporus* MTCC 556 and other isolates

Sl No.	Cultures	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 n6	C18:3 n3	C20:0	C20:1	C20:2
1	*R. oligosporus* MTCC556	1.08±0.04	31.25±1.41	11.6±0.82	44.2±2.13	5.96±0.46	2.76±0.52	1.96±0.31	0.44±0.04	Tr	Tr
2	*R. oligosporus* MTCC 286	1.06±0.06	20.4±1.01	7.82±0.65	46.8±2.31	8.46±0.84	1.8±0.29	0.46±0.03	0.84±0.03	ND	ND
3	*Rh. sp.1*	0.28±0.01	17.41±1.02	1.97±0.06	51.62±2.54	7.45±0.73	7.46±0.76	Tr	ND	ND	ND
4	*Rh. sp.2*	0.80±0.05	28.21±1.67	2.41±0.10	56.34±2.31	4.85±0.42	2.42±0.23	Tr	ND	ND	ND
5	*Rh. sp.3*	1.10±0.05	22.42±1.31	4.14±0.41	54.84±2.26	2.85±0.39	3.84±0.42	0.3±0.04	ND	ND	ND
6	*Rhodosporidium toruloides*	0.84±0.04	16.24±1.00	2.84±0.23	51.44±2.09	17.46±1.01	ND	0.80±0.06	ND	ND	ND
7	*Mucor hiemalis*	1.24±0.07	10.46±0.60	6.84±0.74	44.86±2.03	12.08±0.96	ND	ND	ND	Tr	ND

Tr- trace quantities; ND- not detected

2.3. RESULTS

The results observed from the table (**Tables 2.1 and T 2.2**) show that the selected plant materials were with interesting compositions of unsaturated fatty acids. Most of these products are nuts, seeds of vegetables and fruits and fruit pulp in that order and mostly they are of about 40-69 % oil value. The unsaturated fatty acids contents show major concentrations in significant proportions denoting their nutraceutical values. Of which the nuts do have mostly monounsaturated fatty acid (MUFA) Oleic acid (C18:1 n-9) (60-70%) in great proportions followed by linoleic acid (C18:2 n-6) (20-60%), Palmitic acid (C16:0) (around 10%) and α-linolenic acid (C18:3 n-6) (about 13%) in that order. Trace amounts of Palmitoleic acid (C16:1) is also seen in the case of nuts. Whereas in the vegetable seed oils, the major proportion happens to be of linoleic acid (C:18:2n-6) (50-70%), which is a diunsaturated fatty acid (DUFA) followed by oleic acid and others. The occurrence of saturated fatty acids like stearic acid (C18:0) and palmitic acid (5-15%) are almost equal. As per the fruit seeds category the pomegranate seeds have registered very low oil content i.e. 6.0% only, but it has a major proportion (56%) of linolenic acid (C18:3 n-6) , which is otherwise referred to as Punicic acid or α-linolenic acid at various instances. The name of the fruit is also known as *Punica granatum* this is in connection to the compound present in the plant's fruit. As per the fruit pulp category avocado fruit which is otherwise known as butter fruit has recorded a major oil value of 47% which is great for any fruit pulp category. It is interesting to note that it contains MUFA (oleic acid) in major composition (60.50%) followed by DUFA (linoleic acid) and others. The EFAs (essential fatty acids) with nutraceutical, pharma, and therapeutical values (of MUFA, DUFA and PUFAs) have already been discussed elsewhere in the presentation.

Similarly from tables **T 2.3 & T 2.4** the microbial fatty acids have been analyzed using their respective oils and among those studied, *Rhizopus oligosporous* MTCC 556 was found to have interesting amounts biomass as well as

considerable values for gamma linolenic acid (GLA) content which is also a health benefiting fatty acid .

2.4. DISCUSSION

The oil content and the occurrence of the said proportions of saturated and unsaturated fatty acids of the experimented set of plant products like nuts, vegetable and fruit seeds, and fruit pulp all confirm the view that there are most of the natural resources which do have major oil values and interesting proportions of the nutraceutically valued EFAs for human betterment. Though most of the nuts are referred to as the costly food materials in the Indian scenario, a major population consumes it significantly. But it is true that these are not easily affordable to an average Indian to consume them often. As per fruit seeds though pomegranate records very low oil content it has major supply of tri-unsaturated fatty acid i.e. α-linolenic acid , which is not available in most of the plant products to that extent. But the oil yield from the seeds is not of considerable value. In the case of vegetable seeds, recorded are high oil values as well as major proportions of DUFA whose nutraceutical values are well documented and the availability and access of these are not difficult for any ordinary Indian. Because these vegetables are mostly the day today food supplement apart from the rice and wheat grains to any average consumer.

The feasibility of vegetable and fruit by products like seeds depends on various factors like the cost of processing, disposal cost of by products and leftovers, environmental costs, recovery costs, market factors, consumer acceptance of the products and the pricing patterns (Cahoon et al., 2007).. Still using these vegetables in the daily life of an average Indian is not hindered because of their low cost and food habits. If observed carefully it is easy to understand that most of these vegetables are consumed along with their seeds except that of watermelon. Hence for the present study **watermelon seeds** (*Citrullus lanatus* (Thunb) Matsum and Nakai (syn. *Citrullus vulgaris*. Schrad.) of cucurbitaceae),

which are disposed off during pulp eating has been taken up considering its commercial factors and easy availability and affordability. The price of these fruits is also very cheap during summer seasons. Moreover to exploit the wasted seeds to a greater extent, they were taken up for nutraceutical value based application studies.

Similarly the **avocado fruit** which has a vernacular name as butter fruit (*Persea americana* Mill. of family Lauraceae) also contains major oil yield from its pulp after drying about 80% of water content. This fruit also comes very cheap at seasons from July through September months of a year. Consuming this fruit as such is not attractive but addition of sugar and other spices along with salt and all provides an initial stimulus to make it an attractive item in the menu. To make it habitual to consume this fruit pulp needs some more promotions. This pulp contains the MUFA (oleic acid), which has recorded more therapeutical values. Based upon the cost and availability factors this avocado fruit is also taken up further application studies along with watermelon seeds.

Similarly from Table T 2.3 & T 2.4 the microbial fatty acids have been analyzed using their respective oils and among those studied, *Rhizopus oligosporous* MTCC 556 was selected for further improvement studies as it had interesting amounts of gamma Linolenic Acid (GLA) which is also a health benefiting fatty acid and hence was taken up for further strain improvement studies.

2.5. CONCLUSION

Plant materials like seeds dry nuts, pulp and leafy vegetables and certain oleaginous microbes screened for oil yield and unsaturated / essential fatty acid contents. Among the plant products it was the avocado fruit pulp(*Persea americana* Mill. of family Lauraceae) and watermelon seeds (*Citrullus lanatus* (Thunb) Matsum and Nakai of family cucurbitaceae), which showed higher oil yield and promising EFA contents (OA and LA) have been selected for further

application studies using solvent free Super Critical Fluid Extraction (SCFE) studies.

From the microbial strains *Rhizopus oligosporous* MTCC 556 was selected as it also gave higher biomass production as well as considerable amount of GLA which is an EFA with very good nutraceutical value.

Thus from the screening and selection of both plant products as well as microbials , the plant materials were planned for further application studies and the microbials were planned for strain improvement and optimization of culture condition studies for higher oil and PUFAs yields.

CHAPTER-3

3.1 INTRODUCTION

Microbes play a key role in the food industries, and also a variety of valuable enzymes, amino acids, antibiotics, polysaccharides etc. are obtained from microbial sources. In the recent past, many functional foods, nutarceutical and pharmaceutical products derived from microbial sources are used in daily life. In this study polyunsaturated fatty acids (PUFAs) and their medicinal and nutritional values are highlighted. Single cell oil (SCO) is a triacylglycerol type of oil from yeast and molds, generally analogous to plants and animal edible oils and fats. The technology of plant SCO consists of production of oil-rich biomass by fermentation of carbohydrate substrates by oleaginous yeasts or fungi followed by downstream processing that includes the cell and oil recoveries. Research on single cell oils (SCO), speciality lipids and/or structured lipids have been a subject of great improvement.

Specialty Lipids

The microbial specialty fats and oil denote lipids structured by environmental or genetic modification of an organism. Development of cocoa butter equivalents (CBEs) and also the trend to construct healthy fats to meet nutritional needs have created a great interest in oils rich in PUFAs because of their unique physiological activity in the human body. This resulted in the process for the production of specialty lipid products. The future for oleaginous microorganisms is linked to such lipid hormones as sterols, eicosanoids and valuable microbial metabolites not found in plants, eg. hydroxyl alkanoates or biosurfactants. The rich sources of ω-6 and ω-3 fatty acids have been discovered in recent years. In recent times scientists have induced infant formulae manufacturers to include docosa hexaenoic acid (DHA) as well as other fatty acids that are present in human milk but not yet a vital component of commercially available infant formulas available in the market. Though certain PUFAs are essential to human

nutrition, they are highly priced and unaffordable to common people. The ω-6 and ω-3 fatty acids series of PUFAs play important roles in the structural and functional aspects of biological membranes and also have other unique biomedicine activities such as lowering plasma cholesterol and triacylglycerol levels preventing atherosclerosis and cardiovascular diseases (Horrobin, 1993). These fatty acids are also precursors of various lipid hormones such as prostaglandins, leukotrienes and thromboxanes in human body. The ω-3 fatty acids from fish and fish oil tend to block the development of atherosclerosis and can prevent some cancers also (Shahidi, 2009). Thus the consumption of fish prolongs survival of those affected with coronary heart diseases and reduces the number of subsequent heart attacks (Vanessa et al., 2009).

ADVANTAGES OF MICROBIAL PRODUCTION OF PUFAs

- Microbial PUFAs are higher value oil sources rather than low priced commodities such as soy beans and sunflower oil.
- Their extremely high growth rates on wide varieties of substrates allow us to utilize low cost substrates.
- Oil production can be carried out throughout the year, there is no seasonal or climate dependence.
- Microbial sources can supply more concentrated pharma grade PUFAs than other sources with controlled quality.
- Enable the upgrading of PUFAs structure and allow biotransformation.
- Availability of numerous mutants defective in specific enzymes like desaturases improve production of tailor made oils.
- Oleaginous microorganisms can be considered as the appropriate hosts into which foreign genes could be cloned for the production of desired fatty acids.
- Microbes provide successful model for studying lipid biochemistry, metabolic control and function.
- Due to simplicity of microbial metabolic regulation they can be readily grown under controlled conditions

- Manipulation of lipid and profile can be easily done by genetic methods like induced mutation, protoplast fusion and DNA recombinations.
- Microorganisms are rich in protein, trace elements, vitamins, antioxidants, so they could be employed as micro- and macro-nutrients too.

Microbial Lipids

A variety of PUFAs have been detected in microorganisms including bacteria, fungi, algae, mosses and protozoa. Based on these observations several research groups have started for screening of microorganisms which accumulate lipids containing high PUFAs in order to develop large scale production of these specialty lipids.

Yeasts

The screening of yeasts for speciality lipids and biosynthesis pathway has been reviewed with particular emphasis on commercial aspects like production of triacylglycerols and other potentially useful materials (Davies et al 1990). Some of the yeasts screened for speciality lipids is given in **table T 3.1,** where palmitic acid (16:0) is usually between 15 and 20% of the total fatty acids and possibly can go up to over 30%, (*Lipomyces sp*.) and in one rare case it is over 60%. Stearic acid (18:0) is usually only a minor constituent and rarely exceeds 10% of the total fatty acids. Oleic acid (18:1) is usually the most abundant fatty acid as in some yeast, except for those where palmitoleic (16:1) is the major fatty acid, (e.g. *Schizosaccharomyces spp*, with over 80%. Species of *Saccharomyces, Schizosaccharomyces, Saccharomycodes and Hanseniaspora* are devoid of linoleic-acid (18:2) and all other polyunsaturated fatty acids. However, as 18:2 along with 18:3 residues are associated with phospholipid membranes, a high content of 18:2 indicates very low lipid content with the phospholipids being the only lipid type. Nevertheless, 18:2 may be up to 40% in a few species (Botha *et al*., 1995). The occurrence of di-homo-γ-linolenic acid (20:3) and arachidonic acid (20:4) in the yeast *Dipodascopsis uninucleata* is also true . The total lipid

content of the majority of yeasts is usually below 20% (w/w) and often less than 10 % (Rattray *et al*., 1988). Hence it is easier to classify yeasts as either oleaginous and non-oleoginous as in **table T 3.1.**

Table –T 3.1 Lipid content and fatty acid profiles of oleaginous yeasts*

Yeast species*	Maximum lipid content (% w/w)	**Relative % (w/w) of major fatty acyl groups**						
		16:0	**16:1**	**18:0**	**18:1**	**18:2**	**18:3**	**Others (%)**
Candida curvata D	58	32	--	15	44	8	--	
Candida curvata R	51	31	--	12	51	6	--	
Candida diddensii	37	19	3	5	45	17	5	18:4(1)
Candida sp 107	42	44	5	8	31	9	1	
Cryptococcus albidus var. aerius	65	12	1	3	73	12	--	
Cryptococcus albidus var. albidus	65	16	Trace	3	56	--	3	21:0(7) 22:0(12)
Cryptococcus var.laurentii	32	25	1	8	49	17	1	
Endomyces (Endomycopsis) magnusii	28	17	19	1	36	25	--	
Geotrichum candidum	50	No record*						
Hansenula saturnus	28	16	16	--	45	16	5	
Lipomyces lipofer	64	37	4	7	48	3	--	
Lipomyces starkeyi	63	34	6	5	51	3		
Lipomyces tetrasporus (= zygolipomyces lactosus)	67	31	4	15	43	6	1	
Rhodosporidium toruloides	66	18	3	3	66	--	--	23:0(3) 24:0(6)
Rhodotorula glutinis	72	37	1	3	47	8	--	
Rhodotorula graminis	36	30	2	12	36	15	4	
Rhodotorula mucilaginosa	28	No record*						
Trichosporon cutaneum	45	12	--	22	50	12	--	
Trichosporon fermentans	20	17	1	4	42	34	Trace	
Trichosporon pullulans	65	15	--	2	57	24	1	
Yarrowia lipolytica	36	11	6	1	28	51	1	

* **(Source: Ratledge, 1989)**

Molds

Molds show a greater diversity of lipid types and fatty acids than yeasts (Weete 1980). Nearly, 64 species from all orders and families of molds have been recorded as producing over 25% oil (Ratledge, 1989). It should be pointed out that C12 and C14 fatty acids are known to be produced in abundance by some species of *Entomophthora*, and the hydroxyl fatty acid, ricinoleic acid. 12-hydroxy-oleic acid is being produced by several species of *Claviceps.* Other molds produce branched-chain (Tyrrell, 1967) and a few produce short-chain (C4-C6) fatty acids .The main difference between the fatty acids of yeasts and molds is that the latter tend to produce a much higher proportion of polyunsaturated fatty acids and hence molds have become a target for intensive research. Molds produce both the ω-3 and the ω-6 series of fatty acids. The ω-6 fatty acids, confined to the lower fungi, also referred to as division "*Phycomycetes'* are comprised of two sub-divisions, *Mastigomycotina* and *Zygomycotina.* The ω-3 fatty acids, as typified by α-linolenic acid, are found in *Slime Moulds* (*Myxomycota*) and in the higher fungi which comprises the '*Ascomycetes*' (*Ascomycotina*), '*Basidiomycetes'* (*Basidiomycotina*) and the *'Fungi Imperfecti'* (*Deuteromycotina*). The difference between the distribution of the two isomeric 18:3 fatty acids (i.e. ALA and GLA) was first noticed and reported in molds (Shaw 1966). Although the occurrence of γ-linolenic acid is abundant in the seed oils of evening primrose, borage and blackcurrant, the prices of microbial oils are highly competitive and viable. Some important molds producing PUFAs are given in **table T 3.2**

Table –T 3.2 Lipid content fatty acids of some molds

Organism	Lipid Fraction (% cell dry wt)	Relative % (w/w) of major fatty acyl groups						
		14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**Others (%)**
Phycomycetes								
Entomophthorales								
Conidiobolus nanodes	26	1	23	15	25	1	4*	20:1(13) 22:1(8) 20:4(4)
Entomophthora coronata	43	31	9	2	14	2	1*	12:0(40)
Entomophthora obscura	34	8	37	7	4	Trace	Trace	12:0(41)
Mucorales								
Abisidia corymbifera	27	1	24	7	46	8	10*	--
Cunninghamella japonica	60	Trace	16	14	48	14	8*	--
Mortierella isabellina	86	1	29	3	55	3	3*	--
Rhizopus arrhizus	57	19	18	6	22	10	12*	--
Mucor alpina –peyron	38	10	15	7	30	9	1*	20:0(8) 20:3(6) 20:4(5)
Ascomycetes								
Asperigillus terreus	57	2	23	Trace	14	40	21	--
Fusarium oxysporum	34	Trace	17	8	20	46	5	--
Pellicularia practicola	39	Trace	8	2	11	72	2	--
Hyphomycetes								
Cladosporium herbarum	49	Trace	31	12	35	18	1	--
Ustilaginales								
Tolyposporium ehrenbergii	41	1	7	5	81	2	--	--
Clavicipitaceae								
Claviceps purpurea	60	Trace	23	2	19	8	--	12-HO-18:1(42)

* γ-Linolenic acid (18:3 ω-6) **(Source: Ratledge, 1989; Kendrick and Ratledge, 1992)**

Algae

Many of the lipids derived from algae are complex as they are frequently associated with the photosynthetic apparatus of algae. The distribution of lipids and fatty acids in algae has been well recorded. The oil content of algae, like that of other microorganisms, may exceed 70% of the cell biomass (Ratledge, 1989). However, due to the high cost of cultivating algae in sterile and illuminated

fermenters, it has been difficult to identify commercially useful strains as only a few algae can be grown outdoors in open ponds without becoming seriously contaminated with other algae, or even attacked by predatory protozoa. The occurrence of polyunsaturated fatty acids in algae has also been reviewed in conjunction with other potential sources (Yongmanitchai and Ward 1989).

Bacteria

Bacteria are the simplest and smallest of microbial cells. They are divided into two groups: the *Archaea* / formerly *Archaebacteria*, and the true *Bacteria* / or *Eubacteria.* The *Archaea* are considered the progenitors of all living cells. They contain unusual lipids not found in other bacteria elsewhere in nature. These lipids are derived from isoprenoid chains and not fatty acyl groups; moreover, although the phytanyl units are linked to glycerol, this is via ether linkages and not by conventional ester linkages. Thus, these lipids are associated with halophilic, methane-utlilizing and thermo-tolerant microorganisms, some of which do not grow below 80^{o}C and may have temperature tolerances of up to 110^{0}C (Ratledge, 1989).

PROCESS OF MICROBIAL OIL ACCUMULATION

Physiological aspects

The term oleaginous refers to both plants and microbes producing oils. (Thorpe and Ratledge, 1972). In oleaginous microbes, the trigger for lipid accumulation is the exhaustion of a primary nutrient from the growth medium. Usually this is the supply of nitrogen, normally in the NH_4 form. The culture medium must be deliberately designed to be low in nitrogen and high in carbon availability. When the nitrogen is exhausted, the cells no longer proliferate as N is essential for both protein and nucleic acid biosynthesis. Hence, the excess carbon (usually glucose or other carbohydrates) continues to be assimilated by the cells and gets converted into fatty acids and then into triacylgycerols.

Lipid accumulation is a two stage process, and at the first stage, balanced growth occurs in which all nutrients are available to the cells. This phase ends when a chosen nutrient is exhausted and is then followed by the 'fattening' or lipogenic' phase. This phase continues until either the carbon supply itself becomes exhausted or some other nutrient needed for the process is taken up by the cells. If further nutrients, like carbon, are supplied to the cells, the lipogenic mode continues until very high contents of lipids are produced. In some cases, values of over 70% of the biomass have been recorded in both yeasts and molds (Ratledge, 1989). As all oleaginous microorganisms are aerobic, the supply of oxygen to the culture is the rate-limiting factor. However, as the process of lipid biosynthesis is largely independent of oxygen, lipid accumulating yeasts can grow to densities of 150g/l under carefully controlled conditions. Oxygen must therefore be regarded as a nutrient and its supply to the cells is paramount otherwise cell death occurs along with autolysis and loss of cell integrity.The low concentration of nitrogen in the medium is quickly exhausted by the avaricious cells in the vessel and the excess carbon is then assimilated as in a batch culture. Usually a C/N ration of about 30 to 50:1 is used. However, if the growth rate of the cells is too high, the cells have an insufficient residence time within the fermenter to utilize all the carbon and convert it in to lipids. Hence, higher the growth rate and lower the lipid content. The use of continuous culture techniques for the growth of oleaginous microorganisms has been found suitable (Ratledge *et al.* 1982). *Candida curvata*, also known as *Apiotrichum curvatum*, showed that lipid production was not just a matter of using a medium with any high C/N ratio together with a low dilution rate (Ykema *et al.* 1990). The accumulated lipid within oleaginous microorganisms is therefore a dynamic storage material; and it is produced in times of plenty, i.e. carbon sufficiency, and utilized in times of starvation, i.e. carbon deficiency. The organisms' growing conditions can modulate the type of lipid; and thus changes in organisms' growing growth temperature, pH and O_2 supply all have some effect on the proportions of the lipid types and on the nature of the fatty acyl groups that are being produced (Ratledge, 1986).

Biochemistry of lipid accumulation

Not all the microorganisms are oleaginous. The key oleaginous enzyme is ATP-citrate lyase (ACL), this enzyme provides acetyl-CoA to citrate in the cytosolic compartment of eukaryotic cells. The abundant supply of acetyl-CoA then ensures further fatty acid biosynthesis. The overall events are linked to the exhaustion of NH_4 from the culture medium and can be summarized as follows (Botham and Ratledge, 1979). When the microorganisms exhaust all fixed nitrogen from the culture medium, an intracellular scavenging for N takes place. This activates the enzyme AMP deaminase which catalyses reaction (Adenosine monophosphate -> inosine monophosphate + NH_3). As a result, the concentration of AMP within the cell rapidly drops (Boulton and Ratledge, 1983). The amount of NH_4^+ that is released is quickly consumed and the cells have only a brief respite from nitrogen deprivation.

Citrate is a direct product of glucose metabolism (isocitrate + NAD^+ -> α-ketoglutarate + CO_2 + NADH, citrate <-> isocitrate). The accumulation of citrate due to the equilibration of isocitrate and citrate via reaction is catalyzed by aconitase. Thus, as glucose continues to be assimilated by cells after nitrogen exhaustion, citrate exits from the mitrochondrion and is immediately cleaved by acetyl co-A lyase (ACL) into acetyl-CoA and oxaloacetate (Citrate + CoA + ATP -> acetyl-CoA + oxaloacetate + ADP + P_i), oxaloacetate reacts with NADH to form malate (OA+ NADH <-> malate + $NAD^{+)}$ and the malate then decarboxylated to pyruvate by malic enzyme (malate + $NADP^+$ -> pyruvate + NADPH + CO_2). Even in organisms that possess an active malic enzyme to produce NADPH and to recycle the oxaloacetate back into pyruvate, (Glucose -> triacylglycerol + $36CO_2$.) 16 moles of glucose may be needed to produce 1 mole of triacylgycerol; at best, the requirement cannot be less than 15.5 moles of glucose in order to provide both the necessary energy (ATP) and the reducing equivalents (NADPH). The regulatory controls of all the key enzymes have been elucidated (Evans and Ratledge, 1985; Ratledge and Evans, 1989).

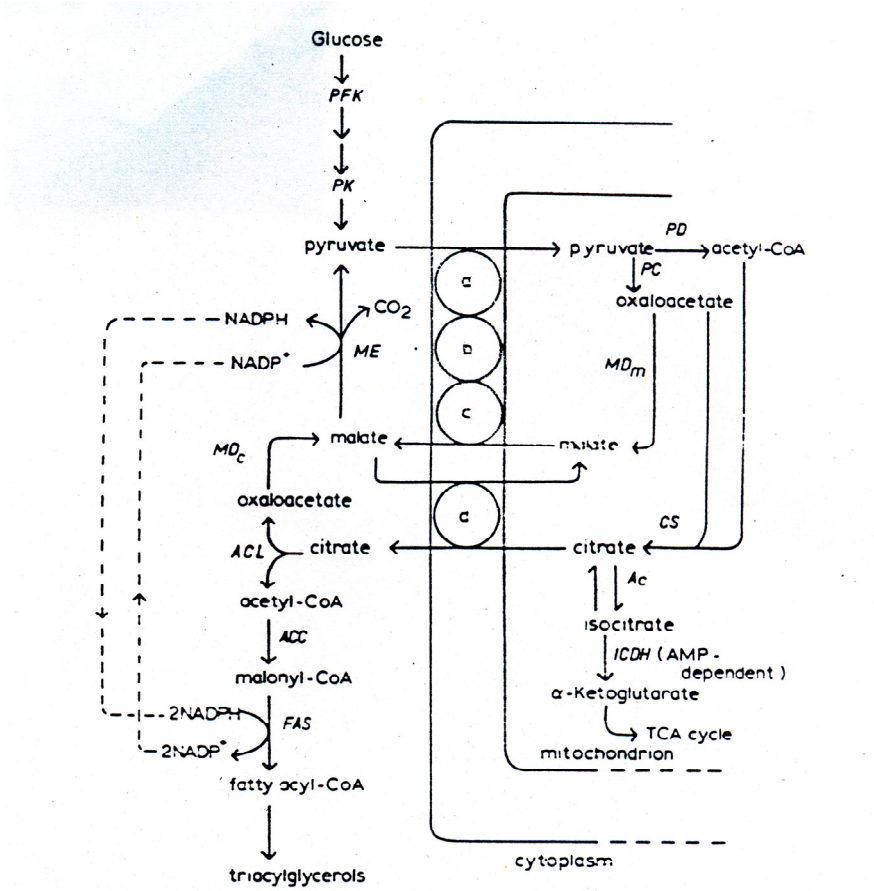

Fig 3.1. Fatty acid synthesis and intermediary metabolism as linked in oleaginous microorganisms.

Mitochondrial transport processes- a,b,c interlinked pyruvate-malate translocase systems; d.citrate –malate translocase. Enzyme AAC, acetyl – CoA carboxylase; AC, aconitase; CL ATP-citrate lyase; CS citrate synthase; FAS, fatty acid synthetase complex; ID, iso-citrate dehydrogenase; MD, malate –dehydrogenase(cytosolic); MDm malate dehydrogenase (mitochondrial); Malic enzyme ; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PFK, phosphor fructo kinase; PK, pyruvate kinase.

Source: Ratledge, 1989.

SOME IMPORTANT PUFAs (ω-3 & ω-6)

PUFAs like GLA, AA, DGLA, EPA and DHA are considered to be essential fatty acids as they are precursors for prostaglandins, thromboxanes, leukotrienes, prostacyclins and so on. All of which have hormone like activities. PUFAs exhibit several unique biological activities such as lowering of plasma cholesterol level, prevention of thrombosis etc. Some of the eicosanoids as supplements to infant formula have also been recommended for the growth of preterm infants.

γ-Linolenic acid (GLA 18:3 ω-6 / all cis 6,9,12 octadecatrienoic acid)

GLA is an intermediate in the transformation of linoelic acid into prostaglandins. Persons suffering from diabetes, cancer, virus infection and aging, the enzyme that converts LA to GLA (Δ^6 desaturase) is affected. Hence the need to supply GLA as health food is an important aspect (Horrobin 2000;1992). In Japan, certain strains of *Mortierella* were found to be suitable as a source of GLA. For the production of lipid containing oils of GLA glucose is ordinarily the source of carbon and ammonium nitrate and yeast extracts serve as N_2 sources. It yields a fungal mass of 40-156 g/L on dry basis containing 37-58% by weight of lipid corresponding to a yield of 13-83 g/L. The content of GLA in the fatty acids of the lipid was higher as 4-11% by weight. GLA was found equally distributed at the sn-2 and sn-3 positions of the mold oil, but at the sn-3 position was > sn-2 in evening primrose oil (EPO) and blackcurrant oil (BCO) and the sn-2 position > sn-3 in borage oil (BO). GLA prolongs fatty acid biosynthesis up to 20:5 and dihomo-gamma linolenic acid (DHGLA) is an intermediate between GLA and AA (Botha et al., 1995).

Eicosatrienoic acid (ETA 20:3 ω-9, 5, 8, 11-cis-Eicosatrienoic acid)

This unusual fatty acid was first described as Mead acid and was found to be probably involved in the formation of the ω-9 series of fatty acids whose nutritional status is still under study.

Arachidonic acid (ARA 20:4 ω-6)

Its current commercial source is from animal livers and recoveries are usually only 0.2% (w/w) or even less. Alternative sources would therefore appear attractive. Certain fungal cells show high productivity of AA. The fungus *Mortierella* spp. is a more promising source for commercial AA production. Among the strains of *Mortierella*, *M. alpina* was found to be accumulating 0.5 g/L (26.6 mg/g of dry matter of mycelium). The production of ARA/ AA by microorganisms (molds and algae) has been reviewed by Bajpai and Bajpai (1992).

Eicosapentaenoic acid (EPA 20:5 ω-3)

EPA is often found together with the other long chain polyunsaturated fatty acid, docosahexaenoic acid (DHA) in the oils of various marine fish which are the commercial sources of both acids. The nutritional and dietetic importance of these fatty acids is much revered. Both are members of the ω-3 series of polyunsaturated fatty acids. Other significant sources of these acids are marine bacteria, algae and fungi (Yongmanitchai and Ward 1989). Fungi belonging to the *Mortierella* subgenus *Mortierella* have recently been shown to produce EPA without the formation of DHA (; Bajpai *et al*., 1993; Shimizu *et al*., 1988). In *Pythium ultimum* EPA was maximal at 11% of the total fatty acids.

Docosahexaenoic acid (DHA 22:6 ω-3)s

The occurrence of the highly unsaturated fatty acids, EPA and DHA, in species of marine fungi having a natural low growth temperature and the halo- tolerance promote the formation of these long-chain PUFAs to ensure membrane fluidity. EPA and DHA thus as dietary supplements have been recommended for the prevention of heart diseases while GLA has been recommended for the relief of eczema.

Fungi produce a wide range of polyunsaturated fatty acids of current health interest, like members of both ω-3 and ω-6 series. As a number of fungi are easy to be modified genetically, it is possible to delete various desaturation reactions leading to increased accumulation of desired fatty acids, such as DHGLA which normally occurs only in small amounts. The key to industrial exploitation of molds usually lies not only in the specificity of the product but also in the rapidity of their cultivation. A high content of lipid is also usually required and species accumulating less than 20% are probably not commercially viable. The lipid, moreover, should be mainly a triacylglycerol but many of the PUFAs, like EPA and DHA, are predominantly associated with the phospholipid membranes of the cells. In such cases, release of the totally free fatty acids, as their ethyl esters, is the only satisfactory procedure. Successful exploitation of fungal oils or any microbial oil therefore depends upon very careful

identification of a market opportunity. By the genetic manipulation the quality of yeast fat is achieved (Ykema *et al*., 1990; Smit *et al*., 1992). Whatever the geneticists have achieved by mutation is also achievable by metabolic manipulations also in the microbial systems (Davies et al., 1990)

Future of Microbial Oils

The future prospects of microbial oils are probably very limited and are possibly confined to the specialty markets for the polyunsaturated fatty acids and perhaps one or two unusual lipids. Production of all the various PUFAs is feasible by microbial means; the basic problems are not scientific but of cost and public acceptability. Acceptability by regulatory authorities should not be difficult because of the high degree of safety associated with most microbial products. A significant cost difference would be a sufficient incentive but this would have to be coupled with a sensible and well-considered marketing plan.

OBJECTIVES

Hence in view of all the developments in the field of microbial oils and PUFA relevance it was decided to continue with the exploitation of the screened and selected microbial species *Rhizospus oligosporous* MTCC 556 by

- Strain improvement studies using mutagenesis for improved oil and GLA content
- And use such improved strains to optimize the culture conditions for higher and quality yield of oils with desired PUFAs rich compositions.

3.2 MATERIALS AND METHOD

Culture and Chemicals:

Already described in chapter 2 of section 2.2

CULTURE MEDIA

Czapek Dox Medium (CDM):	**g/L**
Sucrose	30.0
Sodium nitrate	3.0
Dipotassium hydrogen phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01
Agar-agar	20.0
pH	7.3± 0.2

Yeast extract peptone dextrose (YEPD) agar g/L	
Yeast extract	10
Peptone	20
D-Glucose	20
Agar	15
pH	5.5

Fat producing Medium (FPM)	g/L
Yeast extract	1.500
KH_2PO_4	0.750
$CaCl_2.2H_20$	0.400
$MgSO_47H_2O$	0.400
NH_4NO_3*	0.286
D-Glucose	40.000
pH	5.500

This medium is a modified medium of Enebo (1946) for production of fat by any oleaginous micro organisms.

*The above medium with 0.286g/L of NH_4NO_3 is nitrogen limiting. For rendering the medium nitrogen non-limiting, a higher concentration of 2.86g/l (10%) increase of Nitrate salt (NH_4NO_3) was used in this study.

Mutant Screening Medium (MYA)	**g/L**
Maltose	4.0
Glucose	10.0
Yeast extract	4.0
Peptone	6.0
Agar-agar	15.0
pH	5.8± 0.2

Cultivation of *Rhizopus oligosporus* MTCC556 in submerged fermentation

$1.5x10^6$ /ml spores suspension of selected culture was inoculated in 500-mL flask containing 100 mL of FPM and incubated in a rotary incubator shaker (Innova 4230, New Brunswick, USA) at 28±2ºC and 220 rpm for 7 days.

Fat Staining Technique

Fat staining methods is followed by the method of Burdon (1964) with a slight modification. The method is as follows:

a. Spread a small amount of wet mycelium on the clean microscopic slide
b. Air dry and heat fix the smear.
c. Wash the slide with distilled water thoroughly.
d. Apply a small amount of (drop) of Sudan Black IVB on the slide and leave it for 30min.
e. Remove the excess stain with 70% alcohol and dry the slide again.
f. Apply xylene a drop on the slide to wash off the remaining stain.
g. Blot-dry the slide and counter stain with safronin for 10-15 seconds.
h. Wash the slide again with running water blot-dry again and examine the slide under microscope under oil immersion lens. The lipid globules appear blue black or light colour while the non-lipid mycelia material is stained light pink.

Estimation of dry weight of cells,
Dry weight of the cell biomass,
Estimation of total lipids,
Oil extraction through soxhlet method,
FAME preparation and
Fatty acid analysis by GC and GCMS techniques

All the above procedures were performed as discussed in Materials and Method of Chapter2 section2.2

Mutagenesis of *Rhizopus oligosporus* MTCC556

a) UV Method

Spores of *Rhizopus.oligosporus* (10^6/ml) was grown in PDA medium for 24hrs and the whole plate was subjected UV irradiation with different time intervals (of 0, 10, 20, 30, 40, 50, 60 sec each). Exposure of the plates at 254 nm was performed using CAMAG UV lamp. During the course of irradiation, the plates were constantly agitated for better radiation. The irradiated plates were refrigerated at 4^oC overnight in the dark before keeping it for low temperature growth selection.

b) EMS Method

Spore suspensions of *Rhizopus oligosporus* was treated with different concentrations of EMS (from 0, 2, 4, 6, 8 and 10µl) and the treated cells were incubated at 4^oC for overnight. The suspensions were washed thoroughly and plated on the PDA medium and kept at low temperature selection growth for the period of 7 days.

3.3 RESULTS

The biomass content were observed as 11.2g/L (DWB) in N_2 limiting conditions. Whereas, in N_2 Non-limiting conditions it was 0nly 8.2g/L (DWB) .Similarly, under N_2 limiting environment, for *Rhizopus oligosporus*, the value of total lipid was 42.2% w/w basis. And under N_2 non-limiting condition the oil yield was 35.2% w/w **(Table T 3.3)**.

The figures **(Fig 3.1; 3.2)** show that UV and EMS mutagenesis results for *Rhizopus oligosporous* MTCC 556 strains. In both the cases mutagenesis effect on killing pattern appears to be steadily ascending, when the time (of UV treatment) and concentrations (EMS) of mutagens increases.

Further **(Table T 3.4)** the yield responses of the mutant strains on the biomass, oil, content were promising.

The maximal biomass producing strains were the parental *Rhizopus oligosporous* MTCC 556 and the mutants CFR M-1; M5; M13; M14 and M21 (11.2; 14.4; 14.46; 13.26; and 13.86 g/L on dry weight basis) in that order.
As per the lipid values the parental strain as well as mutants CFR M 5; M 13; M 14 appear to have higher yields (42.2;44.8;43.61; and 42.42 % of oil on w/w basis).

For oil productions and PUFAs contents the parental strain MTCC 556 and the mutants M13 and M14 proved valid. As for the GLA production is concerned, **(Table T 3.5)** the parental strain MTCC 556 and mutants M1, M9, M13 and M14 showed higher GLA product in that order (2.8%; 2.45% ,3.0% 1.5% and 1.4% respectively. But for the total biomass and oil yield wise both the mutant strains CFR M1 and M9 lose validation.

Hence the parental strain *Rhizopus oligosporous* MTCC 556 and the putative mutants CFR M13 and M14 appear to have been positive for GLA production.

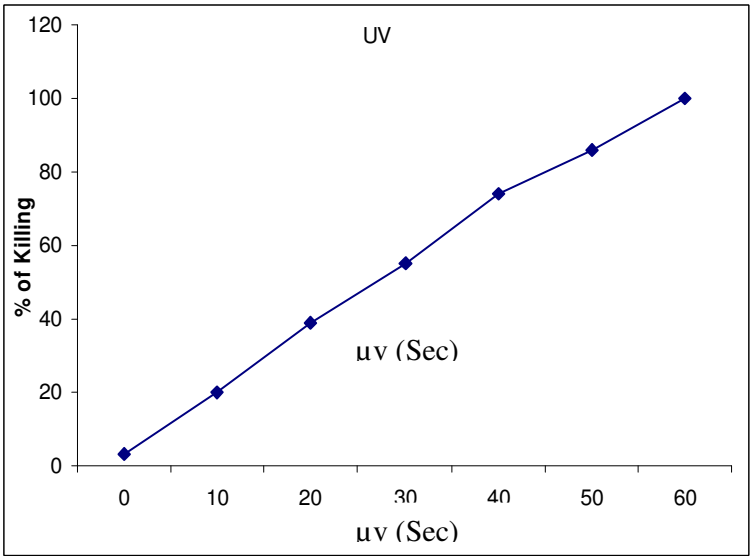

Fig 3.1 UV Mutagenesis of *R. oligosporus* MTCC 556

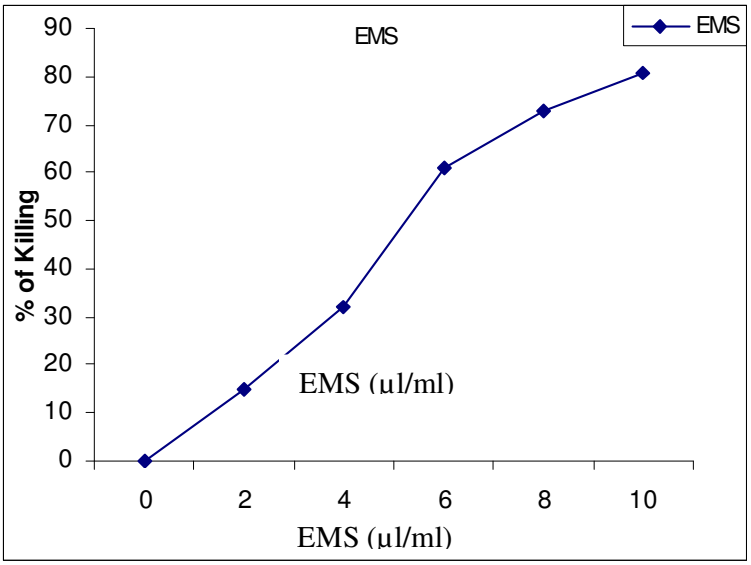

Fig 3. 2 EMS Mutagenesis of *R. oligosporus* MTCC 556

Table T 3.3 Fatty acid profile of lipid of *Rhizopus oligosporus* MTCC 556* grown under N_2 limiting and N_2 non-limiting conditions

Fatty acid profile	% (w/w) content	
	N_2 -limiting	N_2 non-limiting
C12:0	ND	0.61±0.16
C14:0	1.8±0.06	1.3±0.31
C16:0	8.4±0.57	19.2±0.88
C16:1	1.46±0.06	3.47±0.42
C18:0	15.48±0.89	42.86±1.31
C18:1	32.84±1.21	55.76±2.31
C18:2	27.71±1.07	8.61±0.58
C18:3n6	2.48±0.18	2.24±0.28
C18:3n3	8.14±0.98	3.86±0.31
C20:0	1.26±0.23	ND
C20:4	0.06±0.02	0.46±0.05

***Biomass: 11.2 g/L DWB in N_2 limiting condition; 8.2g/L in N_2 Non limiting condition; Total lipid in N2 limiting medium - 42.2% (w/w); in N_2 non limiting medium - 35.2%(w/w)**

Table T 3.4 Biomass and lipid contents of *Rhizopus oligosporus* MTCC 556 and its mutants CFR-M1 to CFR-M22 grown in FPM

Sl No.	Cultures	Biomass (g/L) DWB	Total lipid (% w/w)
1	**Parent (*R. oligosporus* MTCC556)**	11.2±0.98	42.2±1.32
2	**CFR-M1**	14.8±0.97	38.6±1.21
3	**CFR-M2**	12.8±1.01	34.2±1.24
4	**CFR-M3**	10.5±0.85	32.2±1.41
5	**CFR-M4**	11.5±0.92	36.8±1.48
6	**CFR-M5**	14.4±1.05	**44.8±1.63**
7	**CFR-M6**	11.01±1.03	32.6±1.13
8	**CFR-M7**	10.47±0.83	31.4±1.01
9	**CFR-M8**	12.46±1.01	33.4±1.35
10	**CFR-M9**	11.4±1.01	31.46±1.27
11	**CFR-M10**	8.46±0.86	28.41±1.29
12	**CFR-M11**	10.94±0.96	31.78±1.09
13	**CFR-M12**	11.0±1.01	32.46±1.11
14	**CFR-M13**	14.46±1.15	43.61±1.74
15	**CFR-M14**	13.26±1.11	**42.42±1.26**
16	**CFR-M15**	11.04±1.01	28.46±1.13
17	**CFR-M16**	9.46±0.79	29.42±1.15
18	**CFR-M17**	10.14±0.83	29.71±1.21
19	**CFR-M18**	9.46±0.67	30.16±1.42
20	**CFR-M19**	11.24±0.92	31.26±1.25
21	**CFR-M20**	13.21±1.01	32.41±1.42
22	**CFR-M21**	13.86±1.03	33.64±1.51
23	**CFR-M22**	10.6±0.95	31.05±1.42

Table T 3 .5 Fatty acid profile of *Rhizopus oligosporus* and its mutants

Fatty acid profile	*R. oligosporus* MTCC 556 parent	CFR-M1	CFR-M2	CFR-M3	CFR-M4	CFR-M5	CFR-M6	CFR-M7
	% (w/w) content							
C12:0	0.85±0.05	Tr	Tr	Tr	0.81±0.08	1.56±0.14	1.04±0.14	0.32±0.05
C14:0	1.08±0.09	1.8±0.04	0.38±0.02	1.37±0.34	2.96±0.29	0.9±0.06	2.24±0.36	1.97±0.42
C16:0	31.25±1.45	30±1.03	15.81±0.89	16.6±1.01	14.74±0.9	16.85±0.98	18.44±1.06	28.96±1.06
C16:1	0.53±0.03	4.6±0.53	4.0±0.23	4.8±0.35	5.61±0.78	3.45±0.56	4.64±0.69	1.53±0.46
C18:0	11.59±0.34	44.6±1.31	42.6±1.34	50.2±1.56	18.94±0.79	17.47±0.93	14.42±0.89	12.1±0.78
C18:1	44.19±1.02	17±0.4	28.1±1.01	16.1±0.98	42.79±1.556	42.62±1.34	50.42±1.81	42.21±1.67
C18:2	5.96±0.5	1±0.04	1.0±0.09	2.5±0.29	8.46±0.67	6.56±0.89	6.47±0.91	4.25±0.51
C18:3 n6	2.76±0.18	2.4±0.12	2.21±0.21	1.8±0.19	2.24±045	0.74±0.09	0.74±0.04	1.25±0.09
C18:3 n3	1.96±0.06	1.2±0.013	1.46±0.015	0.81±0.06	0.72±0.09	1.78±0.09	3.24±0.87	1.68±0.13
C20:0	0.44±0.05	Tr	1.78±0.11		2.11±0.23	0.26±0.09	Tr	Tr
C20:1	ND	ND	-	-	-	-	ND	ND
C20:2	ND	ND	-	-	-	-	ND	ND

Tr-Traces ND-Not detected

Continuation…….

Continuation of Table T 3. 5

Fatty acid profile	CFR-M8	CFR-M9	CFR-M10	CFR-M11	CFR-M12	CFR-M13	CFR-M14	CFR-M15
	% (w/w) content							
C12:0	0.70±0.08	0.39±0.05	0.97±0.07	0.41±0.03	1.06±0.05	2.21±0.42	1.29±0.17	0.85±0.12
C14:0	0.81±0.08	2.14±0.32	1.03±0.10	1.85±0.05	1.8±0.21	3.75±0.21	2.14±0.19	2.46±0.32
C16:0	22.54±1.32	24.3±1.02	24.86±1.23	26.8±1.04	20.4±1.03	30.0±1.34	25.34±1.24	28.25±1.21
C16:1	3.68±0.35	1.76±0.26	5.4±0.57	0.85±0.15	1.26±0.21	1.7±0.04	3.0±0.38	2.53±0.34
C18:0	7.49±0.67	3.36±0.19	0.68±0.04	4.74±0.59	6.42±0.65	3.46±0.33	6.48±0.51	10.49±0.89
C18:1	55.01±2.10	46.65±1.43	59.08±1.97	47.91±1.24	49.47±1.67	50.04±1.98	48.48±1.97	46.04±1.79
C18:2	1.75±0.23	2.59±0.34	1.24±0.07	10.95±0.76	7.81±0.65	1.26±0.21	5.8±0.54	6.96±0.67
C18:3 n6	1.21±0.12	3.05±0.52	1.39±0.05	1.04±0.03	1.21±0.15	1.48±0.31	1.42±0.13	1.16±0.08
C18:3 n3	0.68±0.07	1.78±0.09	Tr	0.96±0.06	0.47±0.06	1.76±0.06	1.92±0.19	0.46±0.07
C20:0	1.01±0.08	2.9±0.14	0.41±0.02	Tr	Tr	2.46±0.08	1.47±0.14	Tr
C20:1	0.17±0.02	2.57±0.21	1.19±0.14	Tr	ND	1.74±0.04	1.21±0.14	Tr
C20:2	1.2±0.06	0.71±0.07	1.29±0.21	ND	0.85±	Tr	0.86±0.09	1.24±0.06

Tr-Traces ND-Not detected

Continuation of Table 3.5

Fatty acid profile	CFR-M16	CFR-M17	CFR-M18	CFR-M19	CFR-M20	CFR-M21	CFR-M22
	% (w/w) content						
C12:0	0.72±0.05	1.06±0.08	2.44±0.34	0.64±0.04	1.13±0.06	0.42±0.03	1.86±0.08
C14:0	2.41±0.34	0.28±0.02	0.80±0.22	1.24±0.07	4.13±0.51	1.84±0.15	2.04±0.63
C16:0	22.25±1.23	17.41±1.02	18.46±1.01	18.74±0.97	15.46±1.08	18.84±1.13	30.46±1.45
C16:1	2.14±0.42	1.97±0.32	3.46±0.21	1.26±0.08	6.71±0.78	10.46±1.01	ND
C18:0	8.59±0.65	8.60±0.92	9.41±0.96	5.54±0.29	1.47±0.07	6.24±0.67	6.72±0.73
C18:1	47.19±1.67	51.45±2.31	57.64±2.23	50.42±2.12	48.48±1.74	39.48±1.43	31.46±1.45
C18:2	11.44±0.78	7.14±0.54	2.46±0.54	14.26±1.01	17.42±1.04	12.48±1.13	17.46±1.03
C18:3 n6	2.04±0.25	1.21±0.25	1.47±0.29	2.48±0.53	1.47±0.07	1.98±0.11	1.24±0.14
C18:3 n3	1.05±0.09	0.94±0.09	1.84±0.31	0.48±0.05	0.78±0.05	0.84±0.08	0.44±0.05
C20:0	0.53±0.04	0.56±0.06	1.47±0.12	0.71±0.03	1.1±0.13	0.64±0.05	1.28±0.06
C20:1	Tr	Tr	3.26±0.25	Tr	0.58±0.02	0.84±0.06	1.79±0.08
C20:2	ND	ND	ND	ND	Tr	Tr	0.84±0.05

Tr-Traces ND-Not detected

Growth conditions of *Rhizopus oligosporus* MTCC 556

In this study, the growth of this fungus was normally carried out with 100 ml medium in a 500ml capacity conical flask with sufficient aeration at 220rpm at 30^{o}C for 7-10 days and pH of the medium was adjusted to 5.8. The result indicated that under the control medium i.e. the potato dextrose broth (PDB) the biomass, lipid and the GLA contents were found to be 8.2-8.5g/L, 35.8-36.2% and 0.27 % respectively.

Normally, oleaginous microorganisms are grown in a defined medium for fat production. The defined medium is fat producing medium (FPM) which contains more sugar and limited nitrogen source. *Rhizopus oligosporus* MTCC 556 was subjected to the above growth condition and the biomass, lipid and GLA yields were found to be 11.2g/L, 42.2% and 2.76 % of GLA respectively. This clearly indicated that under the excess carbon and limited nitrogen conditions the fungus produce more biomass and lipid.

Further, to clearly differentiate the growth and lipid production in different nitrogen status in the culture medium, the fungus was grown in nitrogen limiting and nitrogen non-limiting growth conditions. The nitrogen status has caused a change in fatty acid profile of *Rhizopus oilgosporus* MTCC 556 It was found that this fungus when grown under nitrogen limiting conditions accumulated 42.2% (w/w) intracellular lipids, whereas in the nitrogen non-limiting conditions it accumulated only 35.2% (w/w) lipids. Similarly, the presence of GLA also varied from these two conditions. The results are indicated in table (**T 3.5**).

Mutation studies

Improvement of microbial strains for the over-production of nutraceutically important products has been the hall mark of industrially important microorganisms. With this background, *Rhizopus oilgosporus* MTCC 556 was

subjected to UV and EMS mutagenesis to get the improved yields of biomass, lipid and certain ω-6 products.

Ultraviolet light of wavelength on 254nm results in a rapid inactivation of cells of *Rhizopus oligosporus* and consequently yields a low percentage of mutants among survivors **(Fig.3.1).** Similarly, the cells treated with EMS mutagen yielded about 82% killing leaving 18% survival of cells **(Fig.3.2).** As a result, about 21 putative mutants were screened for biomass, lipid and GLA contents. The results are given in tables (**T 3.3 T 3.4** and **T 3.5).**

3.4 DISCUSSION

Microorganisms, especially fungi have been utilized extensively in the preparation of many traditional, particularly oriental food stuff, biotechnological products of acidulates, enzymes, antibiotics, vitamins etc. Polyunsaturated fatty acids (PUFAs) are one among the products which gain immense importance as nutraceutical and possess high medicinal value too. The present source of gamma linolineic acid (GLA) is the largely the evening primrose oil and it is a highly priced product. Preliminary studies carried out at the institute have indicated that fat producing microorganisms especially the *Mucor spp, Mortierella spp* and certain oleaginous yeasts can be employed for the production of lipids rich in unsaturated fatty acids. These organisms produce 10-12% of GLA in their total lipids which are considered to be the promising organisms for scale-up studies.By controlling the pH, temperature, carbon and nitrogen sources and oil supplementation, the potential for growth, lipid yield and absolute GLA content can be improved in the fungal strains (Dyal et al., 2005). The mutagenesis provided three improved strains having much oil production with desired PUFA i.e. the GLA content. When these cultures / strains received optimization treatments they showed effective results. The nitrogen limiting yet the carbon surplus medium or fat producing medium promoted both the biomass and oil yield along with a considerable GLA content.

Medium composition, concentration of carbon, nitrogen sources and growth temperature all influence cell growth, lipid accumulation and GLA production in *Mortierella ramanniana* (Hansson and Dostalek 1988*)*

It is also assumed that strains while producing lipid with low GLA content, should accumulate large quantities of lipid in order to synthesize quality GLA indispensible for their membrane functions (Kavadia et al., 2001). Generally researchers tend to enhance the lipid accumulation in the fungal mycelium , but this is a simple cultivation technique rather than attaining a desired PUFA content like GLA (Papanikolaou 2004).Earlier studies for GLA in microbial oils also have proved that the GLA thus made out, when stored in air, sun light direct and also at room temperature stood viable for up to 15 years of time. This character also is attributable to the antioxidants which might have been present along with fatty acid contents.

Fats are found in a wide range of both animals and vegetable foods and serve many functions in the body. Lipids are principal components of cell membrane and are crucial for the proper functioning of organs and tissues. In the recent past, certain fatty acids are recognized for specific nutritional and therapeutic needs. These specialty fatty acids belong to the ω-3 and ω-6 group of fatty acids. Normal individual can convert the dietary lipids into gamma linolenic acid (GLA) by the action of Δ^6-desaturase enzyme. However, it has been estimated that about 10% of human population comprising of diabetics, the aged, menstruating women and certain cancer patients have deficiency of the appropriate enzyme to make sufficient GLA. Dietary supplementation of specialty lipids like GLA, DHA and EPA are known to alleviate and reduce the symptoms of above disorders. Certain oleaginous micro organisms, especially moulds have the capacity to produce oils containing specialty lipids.

Considering the above facts, *Rhizopus oligosporus* MTCC 556 strain was subjected to different culture conditions to obtain good biomass, lipids and ω-3 and ω-6 fatty acids.

3. 5. CONCLUSION

Specialty lipids like GLA, DHA, EPA, and AA are important dietary supplements both for infants and geriatrics and are now a days recommended for many diseases.

Rhizopus oligosporus MTCC 556 culture was subjected to various cultural conditions to obtain maximum biomass, total lipids and some PUFAs.

Fat Producing medium favored the growth, biomass build up and lipid production than the normal cultivation medium Potato dextrose broth (PDB) medium.

Nitrogen limiting medium favored more PUFAs content in the total lipid than nitrogen non limiting medium.

Optimal killing rate was obtained when the culture was subjected to UV and EMS mutagenesis and upon the low temperature growth, 21 putative mutants were obtained.

Three selective mutants possessed more lipid and GLA contents.

Future commercial potentials for the production of GLA and other important PUFAs would be expected to increase mainly of the ever increasing nutritional and medical awareness, modern food habits and prolonged life span of human beings. The importance of quality lipids with no rancidity and higher degree of PUFAs in foods has already been accepted as an important nutraceutical criterion.

CHAPTER-4

4.1 INTRODUCTION

The rapid emergence of ***nutraceuticals*** as health products has created a new trend for food processing industries. The global market size of nutraceuticals is about $250 billion and the leading markets are US, Japan and European community. Growing demand for instant and processed foods, ever bulging cost of organic solvents, and thereby the worrying health concerns related to them and awareness for environmental safety have led to the development of newer, sophisticated and quality separation/ extraction techniques for industries of food processing and pharma product ranges. Conventional separation techniques like leaching, adsorption, extraction, distillation, etc., involve high temperature operations and usage of toxic solvents leading to the deterioration, and instability of various food constituents. To overcome this, efforts have been made to develop newer separation techniques like super critical fluid extraction (SCFE). Some of the advantages of supercritical fluid extraction over conventional methods are high mass transfer rate, ease of product separation from residual solvent, selective product separation and better product quality. Supercritical fluid is a fluid at a temperature and pressure above its critical point. The critical point of a substance is one at which vapor phase and liquid phase become identical (single phase). A critical temperature is the temperature above which it is almost impossible to obtain two phases (liquid and vapor), whatever the pressure is applied and the pressure at that temperature is the critical pressure.

Other extraction techniques

An extraction is usually the first step in analytical procedures applied to the determination of organic compounds in solid matrices. Several efficient extraction techniques have been developed and are commonly used for analyte isolation from solid matrices.

Conventional methods of separation

Most commonly used methods of separation are like Steam distillation for volatiles and Solvent extraction using Soxhlet apparatus for fixed oils and non volatile compounds.

Steam distillation

In this method heat energy is required to separate the low and medium molecular weight compounds. The raw material is subjected to a temperature of 100^0C by steam. This causes alteration of the essential oil constituents that are often thermo labile. In addition, the water can exert a hydrolytic influence that may bring about chemical changes in the oil. This method is more time and energy consuming for separating essential oils from plant products.

Solvent extraction

Solvent extraction is a principal mode for separating mixtures by using the differences in the solubility of the components. It is an efficient and complete mode of oil extraction, preferred by large cooking oil manufacturers. The oil-solvent mixture here is heated to about 300°F (150°C) to evaporate out the solvent (e.g. Nuts and oil seeds) to obtain oil devoid of solvent.

Soxhlet extraction

In this method, the sample is dried, ground, taken in a porous cellulose thimble, which is placed in an extraction chamber containing the solvent. After a few hours at the end of the extraction process, which lasts a few hours (9-18 hrs), the flask containing the solvent and lipid is removed. The solvent in the flask is then evaporated and lipid is quantified. The disadvantages here are poor extraction of polar lipids, longer duration and larger volumes of solvents involved causing health and environmental hazards. Despite the disadvantages, several automated extraction instruments of this procedure are available for faster and safer extraction of oils.

Supercritical fluid extraction

The Supercritical fluid extraction (SCFE) is a quality separation technique to extract the active ingredients or to remove the unwanted principles from materials using fluids in supercritical phase. This technique is for separating non-volatile materials of less molecular weight as well as volatile flavor components, essential oils etc. and hence is a valuable separation technique in the areas of foods, flavors, pharmaceuticals, nutraceuticals, petroleum industries and as well as in waste disposal (Zhang et al., 2010). The separation here can be achieved at low temperatures, without any degradation of sensitive and thermo-labile compounds (Udaya Sankar 1991). The physico-chemical properties of supercritical fluids lie intermediate between liquids and gases. Higher densities nearer to liquids give a good solvent matrix. A combination of these properties enhances the rate of mass transfer of solute in supercritical fluids. These supercritical fluids as solvents are effectively used for the extraction of various bioactive constituents, heat sensitive materials, flavor components etc., from the food materials (Rizvi et al., 1986).

Supercritical fluid

A supercritical fluid is a state of any substance or compound at a temperature and pressure above its thermodynamic critical point (T_C and P_C, **Fig 4.1**). It has the unique ability to diffuse through matrix solids like a gas, and dissolve materials like a liquid. Additionally, it can readily change its density upon minor changes in temperature or pressure. These properties make it suitable as a substitute for organic solvents and carbon dioxide and water are the most commonly used supercritical fluids.

Principle

The basic principle of SCF extraction is that the solubility of a given compound / solute in a SCF solvent varies with both temperature and pressure. At ambient conditions (25°C and 1 bar) the solubility of a solute in a gas is usually related directly to the vapor pressure of the solute and is generally negligible. In a SCF, however, solute solubility is up by 10-1000 orders of magnitude greater than

those predicted by ideal gas law behavior. The critical point is marked at the end of the gas-liquid equilibrium curve, and the shaded area indicates the supercritical fluid region (**Fig 4.1**). It can be shown that by using a combination of isobaric changes in temperature with isothermal changes in pressure, it is possible to convert a pure component from a liquid to a gas (and vice versa) via the supercritical region without incurring a phase transition.Among various supercritical fluids, carbon dioxide as a supercritical solvent plays a key role in food, pharmaceutical and nutraceutical applications because of its inherent advantages. Carbon dioxide below its critical point (31.05°C, 7.38 MPa) exists as a liquid vapor and above its critical point it exists as a supercritical fluid (Udaya Sankar, 1991). It is inert, non-toxic, nonflammable, most economical to use, readily available, easy to handle and readily separates from the extract without giving any damage to the product, and avoids environmental pollution problems. The boiling point and heat of evaporation are low and hence it is easy to separate from the solute with a lesser expense of heat energies. The selectivity of extraction is due to a wide variation of density with a slight variation in temperature. As carbon dioxide is inert, the extraction takes place in an inert atmosphere devoid of the problem of residual solvent in the final product.. These key advantages offer carbon dioxide a valid status as a supercritical fluid for extraction purposes in food industries.

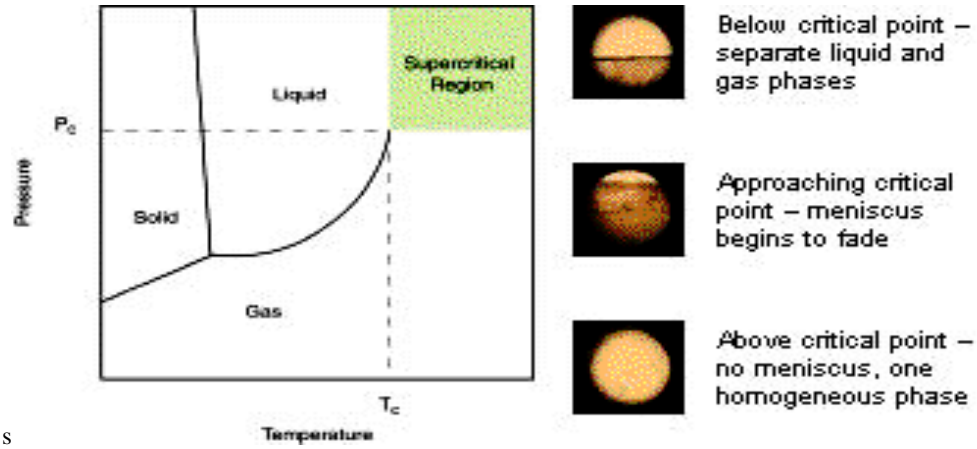

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Fig 4.1 Phase diagram and Disappearance of meniscus between liquid and gas at the critical point (Brunner 1994).

Physico-chemical properties of the supercritical fluids

A comparison of typical values for density, viscosity and diffusivity of gases, liquids, and SCFs is presented in **Table 4.1**(Brunner 1994; Rizvi et al., 1986)

Table T 4.1 Comparison of physical and transport properties of gases, liquids, and Supercritical Fluids (SCFs).

Property	**Gas**	**SCF**	**Liquid**
Density (kg/m3)	1	100-800	103
Viscosity (cP)	10-2	0.05-0.1	0.5-1.0
Diffusivity (m2 /s)	10-2 - 10-1	10-4 –10-3	10-5

Process description

The SCF extraction process essentially involves two steps, a **loading step**, in which the SCF solvent is brought into contact with the natural substance; and a **separation step** in which the material is separated by varying the physico-chemical properties of the solvent: this involves decreasing the temperature or pressure, or both; or by increasing the temperature alone; or even by entraining in a suitable adsorbent. The density can be varied by decreasing the temperature and pressure below the critical point; by increasing temperature, while keeping the pressure constant; increasing the temperature alone; and / or by increasing the pressure, so that there will be two separate liquid phases or gaseous phases. Thus, by varying the physico-chemical properties of the supercritical fluid, the material can be re-precipitated. Fractionation of the extract may also be possible. In **Fig. 4.1**, the variation of the solubility with pressure is used to separate the solute from the solvent. The solute-laden solvent is decompressed through an expansion valve, where the solubility decreases due to decrease in pressure, and the material separates out in the separator. The solute- free solvent is recompressed, and reused for extraction. In **Fig 4.2**, the temperature is varied to remove the desired extract from the SCF solvent. The extraction takes place at a temperature at which the solubility is at maximum, and the solute-laden solvent is then passed through a heat exchanger, where the temperature is so adjusted

that the solubility of the component is at a minimum. This allows the separation of the solute in the separator. The solute-free solvent is recompressed and reused.

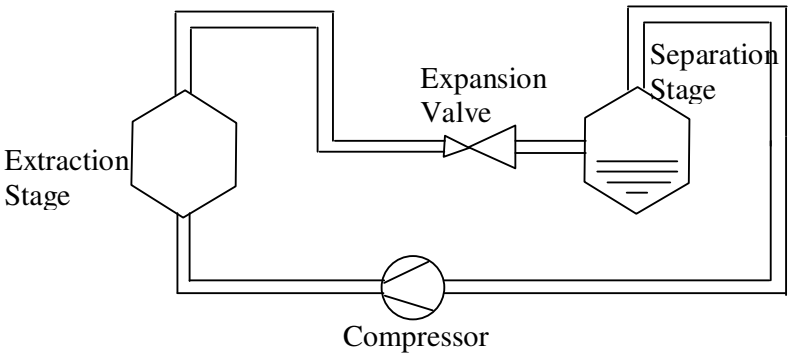

Fig 4.2 Schematic Process for supercritical fluid extraction utilizing pressure swing for separation of solvent and solute (Rizvi et al., 1986).

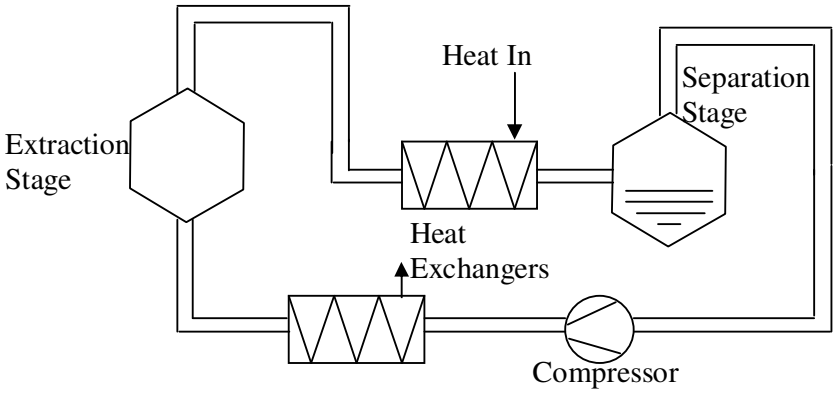

Fig.4.3 Schematic processes for supercritical fluid extraction utilizing temperature swing for separation of solvent and solute (Rizvi et al., 1986).

Advantages of supercritical fluid extraction (scfe)

By varying the pressures, temperatures and the composition of the solvent used in this method one can vary the selectivity and the capacity of the solvent over a wide range of natural substrates. The solvent can be recovered in a straightforward way and this can be effected by slight variation of pressures and temperatures that result in considerable solubility change, whereas for liquid

extraction, the separation of the micelle done by distillation, involving considerable thermal energies. In SCFE, solvent-solute separation may usually be accomplished by simple unit operations, like isothermal expansion and isobaric heating as stated earlier. Thus, the low viscosity and high diffusivity of supercritical gases allows excellent powers of penetration into solid matrix. SCFE is particularly suitable for processing easily oxidizable materials as the whole system is operated under pressure, hence no oxygen can enter the system.

Solvents of supercritical fluid extraction

The choice of the SCFE solvents is similar to the regular extraction and the principles of considerations are like good solving property, inert to the product, easy separation from the product and cheap costs. Carbon dioxide is the most commonly used SCF, primarily due to its low critical parameters (31.1°C, 73.8 bar), low cost and non-toxicity. Being non-toxic and physiologically harmless, the Supercritical carbon dioxide (SC-CO_2) has GRAS (Generally Regarded As Safe) status. Also, the energy costs associated with this novel extraction process are lower than the costs for traditional solvent extraction methods. However, several other supercritical fluids (SCFs) have also been used in both commercial and development processes. Beside CO_2, water is another increasingly applied solvent. One of the unique properties of water is that, above its critical point (374°C, 218 atm); it becomes an excellent solvent for organic compounds and a very poor solvent for inorganic salts. This property gives the chance for using the same solvent to extract the inorganic and the organic component respectively.

Phase diagram for supercritical CO_2

Two projections of the phase diagram of carbon dioxide are shown in **Fig.4.4** & **Fig 4.5.** In the pressure-temperature phase diagram (**Fig. 4.4**) the boiling line is observed, which separates the vapor and liquid region and ends in the critical point. At the critical point, the densities of the equilibrium liquid phase and the saturated vapor phases become equal, resulting in the formation of a single

supercritical phase. This can be observed in the density-pressure phase diagram for carbon dioxide, as shown in **Fig. 4.5**, where the critical point is located at 304.1^0K and 7.38 MPa (73.8 bar). With increasing temperatures, the liquid-vapor density gap decreases, up to the critical temperature, at which the discontinuity disappears. Thus, above the critical temperature a gas cannot be liquefied by pressure. However, at extremely high pressures the fluid can solidify. Many other physical properties also show larger gradients with pressure near the critical point, e.g. viscosity, the relative permittivity and the solvent strength, which are all closely related to the density. At higher temperatures, the fluid starts to behave like a gas, (**Fig.4.5)** and for carbon dioxide at 400 K, the density increases almost linearly with pressure.

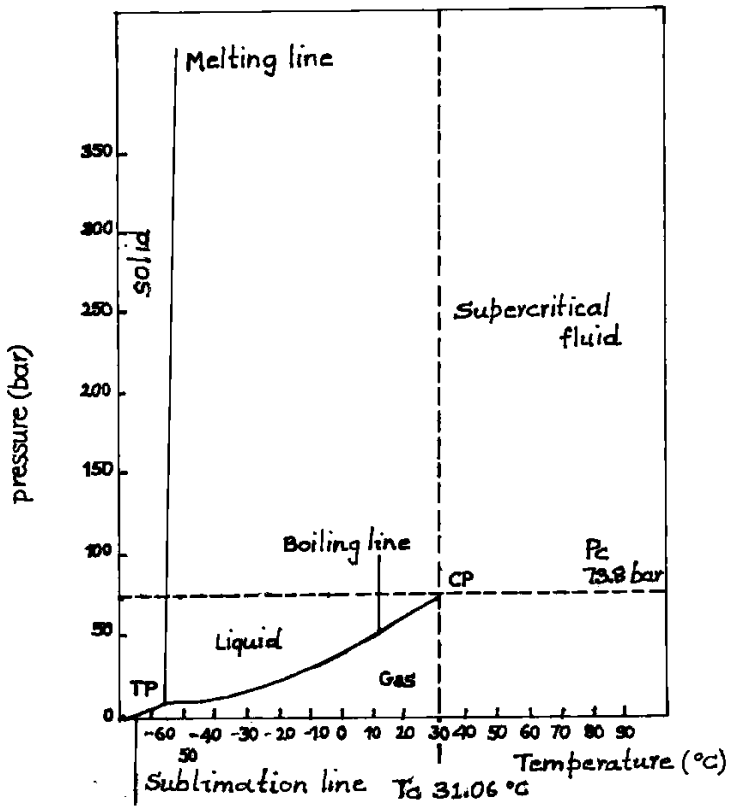

Fig.4.4. Pressure-temperature phase diagram for carbon dioxide(Bott 1982)

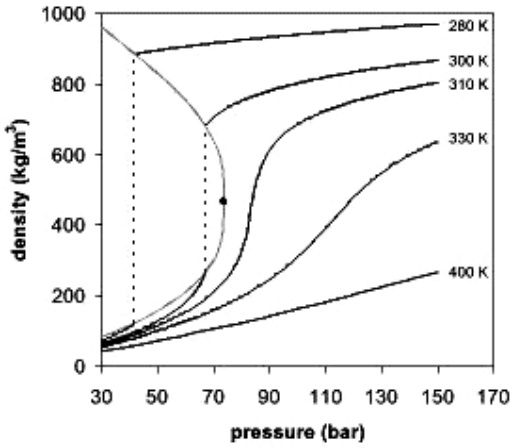

Fig.4.5. Carbon dioxide density-pressure phase diagram (Brunner 1994)

Since the solubilities of the components are usually reduced as the temperature decreases, most extractions with liquid CO_2 are carried out in the temperature range of -20°C to +20°C. However, when liquid CO2 is employed at a higher temperature range (25°C to 31°C), the rate of extraction generally slows down as the critical temperature is approached.

For a typical binary solid-fluid system, the solubility isotherms exhibit a remarkable pressure-dependent behavior. It can be seen that the solubility of a solid solute initially decreases, reaches a minimum, and then exponentially increases with pressure in the vicinity of the critical pressure in the SCF state.

Advantages of CO_2 as a supercritical fluid

Extraction with CO_2 usually takes place at low temperatures (35°C to 50°C), and in an inert atmosphere, whereby both thermal as well as oxidative degradations are prevented. The selectivity of SC-CO_2 can be very much varied by adjusting the pressure and temperature at supercritical conditions. Also, it is cheap and readily available. Moreover, it is completely harmless from the physiological point of view, and is even slightly germicidal. Further more, the energy consumption is low.

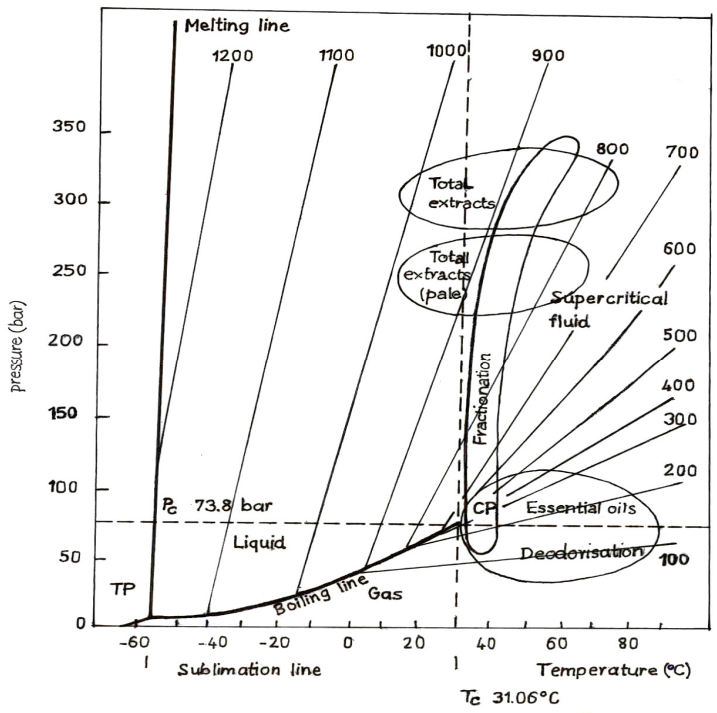

Fig.4.6. Phase diagram of carbon dioxide demarcated for processing (Bott 1982).

Commercial CO_2 required for SFE is already present in the environmental system; it may also be obtained as a byproduct of a fermentation process, or of the fertilizer industry. So, its use as an extractant does not cause any further increase in the amount of CO_2 present in the earth's atmosphere. Therefore, there is no additional 'green house effect' from using CO_2 as the SCF solvent. Supercritical carbon dioxide can be used in enhanced oil recovery in many oil refineries / industries.

Other SCFE fluids

Besides CO_2, the list of solvents which can be used as supercritical & sub critical fluids are given in **Table 4.2** (McHugh & Krukonis 1986). These fluids have been used for many applications such as in the extraction of paprika, carotenoids, tocopherols and capsaicinoids (Gnayfeed et al., 2001), and fluorinated hydrocarbons; their advantages over other solvents are, a low critical temperature and a high dissolving power, as also their chemical inertia. But, till now, these solvents have not been used in the field of food and fragrance extraction.

Table T 4.2 Critical constants of some compounds used as Supercritical Fluid (Reid 1989; Williams 1981;Mc Hugh & Krukonis 1986)

Compound	Critical temperature, T_c (°C)	Critical pressure, P_c (bar)
Benzene	289.00	48.30
$CClF_3$	29.00	38.00
Chlorotrifluoromethane	28.90	38.70
CO_2	31.10	73.00
Cyclohexane	280.30	40.20
Ethane	32.00	48.00
Ethylene	10.00	51.00
H_2O	374.00	218.00
Isopropanol	235.20	47.00
Methanol	240.00	78.00
N_2O	36.00	72.00
NH_3	132.00	112.00
p-Xylene	343.10	34.70
Propane	96.70	41.90
Propylene	91.90	45.60
Toluene	318.60	40.60
Trichloro fluoroethane	198.10	43.50

Applications of SCFE (supercritical fluid) CO_2

The combined effects of high hydrostatic pressure and low acidity of an SCF can be beneficially employed in water-containing systems to prevent food spoilage by destroying bacteria (Kamihira et al., 1987). Rapid decompression of dissolved gas is sometimes used to expand and disrupt the cell structure of natural materials; hence, this technique could also be used as a means of sterilization. Although SC-CO_2 can be an effective a polar medium for enzymatic reactions (Taniguchi et al., 1987; Van Eijs et al., 1988), it has also been used selectively to inactivate enzymes (Steytler et al., 1991; Taniguchi et al., 1987; Weber, 1980). In practice, this technique could be applied in situ, during an extraction process, or as a separate unit operation

Food and flavoring industry

The major application of SCFE is in food and flavoring industry like the decaffeination of tea and coffee, the extraction of essential oils and aroma materials from spices and the extraction of hop in the brewery industry (Vollbreacht, 1982). This method is used in extracting some edible oils from various seeds (Zhang et al., 2010) and also in the production of cholesterol-free egg powder.

Other applications of SCFE in food and flavoring industries are like extractions of hops, spices and tobacco (Hubert & Vitzthum, 1978),color from paprika (Coenen & Hagen, 1983), vanilla (Vidal et al., 1989), oil from oil seeds and oil bearing materials (Liu W et al., 2009; Chaoon et al, 2007; Egger et al., 1985; Bulley & Fattori, 1984; Friedrich & Pryde, 1984), flavors and fragrance (Caragary, 1981; Calame & Steiner, 1982), lipids and cholesterol from meat and fish (Chao et al. 1991; Froning et al., 1990), fractionation of fish oil esters (Nilson et al., 1989), fruit juices and concentrated essence, black pepper essential oil (Vidal et al., 1989), ,jasmine absolute (Udaya Sankar, 1991), mushroom oleoresins, antioxidants, de-oiling of crude lecithin (Stahl & Quirin, 1983), de-acidification of olive oils etc.

Pharmaceutical industry applications

These include production of active ingredients from herbal plants for avoiding thermo or chemical degradation and elimination of residual solvents from the products. Other applications are production of de-nicotined tobacco, environmental protection, elimination of residual solvents from wastes, purification of contaminated soil etc.

Work carried out

In the present study of plant oils extracted by soxhlet extraction (**Chapter 2 Tables T2.1 & T2.2**) the avocado pulp oil has oleic acid (60.50%) and linoleic acid (10.60%) and watermelon seed oil has linoleic acid (59.77%) and oleic acid

(12.35%) as major components respectively in that proportion. This is similar to avocado oil containing oleic acid (54-76%) and linoleic acid (11-15.60%) and watermelon seed oil containing linoleic acid (59.60%) and oleic acid (18.10%) as major components as per earlier findings (Vekiari et al 2004; El-Adawy & Taha 2001). Watermelon seed oil containing linoleic acid is being used as cooking oil as well as a food additive in Western Africa and middle - east countries. Avocado oil prevents human body from accumulating the undesirable low-density lipoprotein (LDL) cholesterol and promotes healthy high-density lipoprotein (HDL) accumulation, which is beneficial to the heart. Effective usage of such oils as nutraceuticals for the benefit of human kind is a major task to the present day scientific community (Chan 2005; Djordjevic et al., 2005)

The **avocado pulp** (*Persea americana* Mill.) and **watermelon seeds** (*Citrullus lanatus* Thunb. syn. *Colosynthis citrullus* Linn.) were extracted for their oils through soxhlet mode as mentioned in Chapter-II Based on the commercial and health based benefits and importance of food grade oils and antioxidants obtained from such edible plant products the present work is carried out with the following objectives to eliminate the solvent interference using SCFE modes..

- To extract both avocado pulp and watermelon seed oils using SC-CO_2 extraction and Soxhlet extraction systems to evolve an effective methodology.
- Characterization and comparison of these oils obtained by both SC-CO_2 extraction and Soxhlet systems by GC and GCMS techniques. This is to confirm the active compositions valuable for pharmaceutical,clinical, therapeutical and nutraceutical applications and practices.
- Arriving at and subjecting the proven compound containing SCFE -Oils for effective product developments.

4.2 MATERIALS & METHOD

Avocado fruits (*Persea americana* Mill.) and watermelon (*Citrullus lanatus* Thunb. Syn. *Colosynthis citrullus* Linn.) seeds were procured from the Horticulture Department, Mysore, Karnataka, India.

Freeze drying of pulp and seed material

Dry watermelon seeds were procured and stored at -20^{0}C in a deep freezer for one day prior to grinding employing a laboratory grinder (IKA mini mill, Germany). For avocado fruits containing about 80% water and 20% pulp (w/w), the pulp was converted into a puree by using a wet grinder followed by freeze drying by spreading in trays (45cm x 90cm) with a thickness of 2 to 3 mm. The puree was frozen at -30^{0}C and was freeze dried using a lyophilizer cum drier system (Model # Lyodrier LT5B, Lyophilization System, USA). The materials were freeze dried at a vacuum of 200-300μ with a drying temperature of -20 to 25^{0}C for a period of 12 hrs.

Moisture content estimation

The moisture content of the avocado pulp or watermelon seed powder was determined by *Toluene distillation method*. 5g of the powdered material was taken in a round-bottomed flask and about 200ml of toluene was added (along with boiling chips to avoid bumping), and placed in a heating mantle with a regulator. The flask was connected to the condenser through a calibrated water-trap. The freeze dried pulp or seed powder was refluxed for 2-3 hours. The amount of water collected was noted, and the percentage of the moisture in the pulp was calculated using the formula.

$$\% \text{ Moisture} = \frac{\text{Volume of water collected (ml)}}{\text{Weight of sample taken (g)}}$$

Supercritical carbon dioxide extraction of oil

Experimental procedure

The schematic diagram of supercritical carbon dioxide extractor is depicted in Fig.4.7. The freeze dried avocado pulp or watermelon seed powder of 100g was loaded into the extraction vessel, which is of 200ml capacity. The super critical carbon dioxide extraction of oils of both freeze-dried avocado puree and watermelon seeds was performed. The extraction was carried out by using a high pressure extractor (Model # EX - 4.2, Nova Swiss, Switzerland). The oils were extracted by using 100 g each of both avocado and watermelon materials separately below a temperature of 50^0C and a pressure of 240 bar were maintained. The extraction was completed over a period of 12 hours and about 55% of oils (dry basis) were recovered from both the samples. The samples were extracted on a pilot scale, with a high-pressure equipment (NOVA Swiss WERKE AG, EX 1000-1.4-1.2 type, Switzerland) designed for working pressures of up to 1000 bar and temperature up to 100°C. After attaining the desired temperature, the CO_2, which had been compressed to the set pressure, was allowed into the extractor. Fractions were collected from the separator at definite time intervals and the weights of the extracts were noted. The first sample collected was small due to the adhesion of the material to the walls of the separator vessel. The flow meters readings, i.e., the pressure, temperature, and frequency were noted at regular intervals and the samples were collected and weighed carefully on a balance to the accuracy of 0.01mg (METTLER AT201 Balance).

Soxhlet extraction, fame preparation and GC and GCMS analysis

All the above steps for studying the lipid profile were performed as described elsewhere in Chapter-II of the present work (See section 2. Materials and Method).

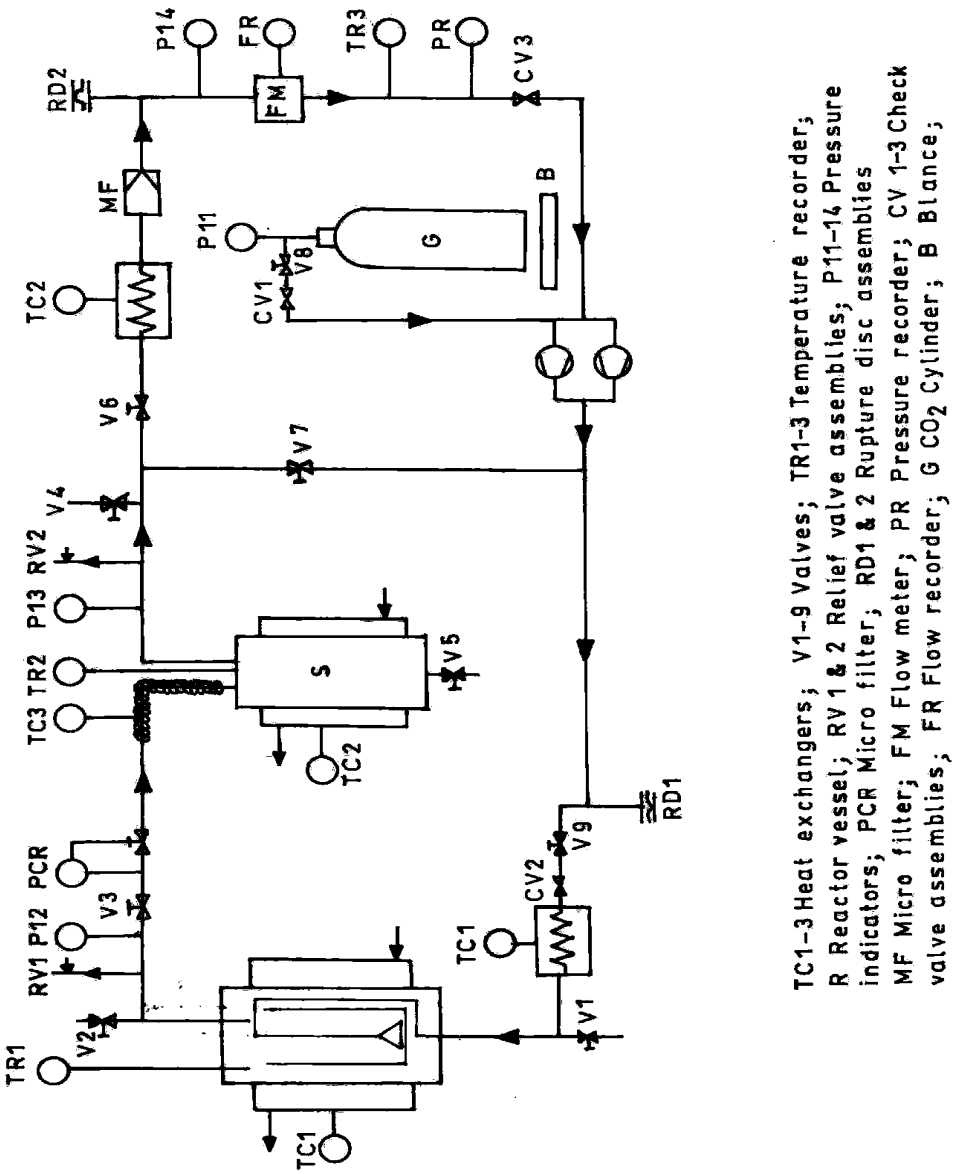

Fig.4.7 Schematic diagram of Supercritical carbon dioxide extractor

4.3 RESULTS

Table-T 4.3 Fatty acid composition of Avocado Pulp oil extracted under different extraction procedures

Fatty Acids	Soxhlet Extraction % w/w	SCF Extraction % w/w
Palmitoleic	10.30	9.00
Palmitic	16.97	14.00
Oleic	60.50	65.00
Linoleic	10.60	10.00
Linolenic	1.00	1.00
Stearic	0.63	1.00
	100.00	**100.00**
∑ Sat FA	17.60	15.00
∑ UFA	**82.40**	**85.00**
Sat FA / UFA	17.60 / 82.40	15.00 / 85.00
	0.214	**0.176**

Table T 4.4 Proximate composition of Avocado fruit pulp

Avocado pulp composition	Percentage (%)
Moisture	83.00
Fiber	2.00
Protein	1.99
Ash	2.00
Carbohydrate	2.00
Fat	8.00- 9.00 oil yield- 47% soxhlet; 50.20% by SCCFE

The table **(Table T 4.4)** for proximate analysis of the avocado pulp shows the moisture content to be of about 83% with a dry mass at 17%. Of the dry mass apart from other components like fiber, protein, carbohydrates, the fat / oil content is observed as 47% by soxhlet and about 50.20% by super critical carbon-di-oxide fluid extraction. The table **(Table T 4.3)** shows the fatty acid profile of the avocado oil obtained through soxhlet and super critical fluid CO_2 extractions by GC MS analysis of fatty acid esters. The FAME analyses were conducted using Gas chromatographiy apparatus and the components were confirmed precisely using Gas chromatography Mass Spectrometry (GCMS) by RRT and mass values. The results prove that saturated fatty acids and unsaturated fatty acids occur in a proportion typical for the avocado pulp oil i.e SFA at 15-17.6% and UFA at 82-85%. This is in conformity with the earlier reports (El Adawy and Taha 2001).

In the soxhlet extracted avocado pulp oil the saturated fatty acids occur as low as 17.60 % and the unsaturated ones as high as 82.40%. On the saturated side of the oil the composition is of palmitic (16.97%) and stearic (0.63%) acids and on the unsaturated side the composition is of palmitoleic (10.30%), oleic (60.50%) linoleic (10.60%) and linolenic (1.00%) acids. Of the unsaturated ones monounsaturated fatty acids (MUFA), palmitoleic and oleic acids thus amount for 70.80%, diunsaturated fatty acid (DUFA) linoleic acid accounts for 10.60% and the triunsaturated fatty acid (PUFA) linolenic acid amounts for 1% thus totally comprising 82.40% of the oil as polyunsaturated fatty acids composition.

In the super critical fluid CO_2 (SCFE) extracted avocado pulp oil the saturated fatty acids occur as 15.00 % (palmitic (14.00%) and stearic (1.00%) acids) and the unsaturated occur as 85.00% (palmitoleic (9.00%);oleic (65.00%); linoleic (10.00%) and linolenic (1.00%) acids). Of the unsaturated fatty acids monounsaturated fatty acids (MUFA), palmitoleic and oleic acids amount for 74.00%, diunsaturated fatty acid (DUFA) linoleic acid accounts for 10.0% and

the polyunsaturated fatty acid (PUFA) linolenic acid amounts for 1% thus totally comprising 85.0% as unsaturated fatty acids' composition.

The monounsaturated fatty acids oleic and palmitoleic acids occur as 74% and 70.80% for supercritical and soxhlet oils respectively showing the superiority of the SCFE oil of the avocado pulp. Overall for the avocado pulp extracted oil the ratio of saturated fatty acids component to unsaturated fatty acids component is 0.176 for super critical fluid CO2 (SCF) extraction and 0.214 for soxhlet extraction**.**

The table for proximate analysis of the watermelon seeds **(Table T 4.6)** shows the moisture content to be of about 5.10%. Of the dry mass apart from components like fiber, protein, carbohydrates the fat / oil content values amount for about 45.31% for soxhlet extraction and about 55.84% for super critical carbon-di-oxide fluid extraction. The table **(Table T 4.5)** shows the GCMS fatty acid profile of the watermelon seed oil obtained through soxhlet and super critical fluid CO_2 extractions. The FAME analyses were conducted using Gas chromatographic apparatus and the components were identified by Gas chromatography Mass Spectrometry as done for the avocado oil. Here too the results prove that saturated fatty acids and unsaturated fatty acids occur in a proportion typical for the watermelon seed extracted oil. Here the SFA occur at 27-31% and the UFA occur at 69-73 % in conformity with earlier findings (Vekiari et al., 2004).

In the **soxhlet** extracted **watermelon seed oil** the saturated fatty acids occur as 31.18 % (palmitic (16.43%) and stearic (14.75%) acids) and the unsaturated occur as 68.82% (oleic (13.41%) and linoleic (55.41%) acids). Of the unsaturated ones monounsaturated fatty acid (MUFA) oleic acid amount for 13.41%, diunsaturated fatty acid (DUFA) linoleic acid account for 55.41% thus totally comprising 68.82% as unsaturated fatty acids' composition.

Table. T 4.5 GCMS Values Fatty Acid Composition of watermelon Seed oil Extracted under different Extraction Procedures

Fatty Acids	Soxhlet Extraction % W/W	SCF Extraction % W/W
Palmitic	16.43	12.17
Oleic	13.41	15.05
Linoleic	55.41	57.74
Stearic	14.75	15.04
	100.00	**100.00**
∑ Sat FA	**31.18**	**27.21**
∑PUFA	**68.82**	**72.79**
Sat FA / UFA	31.18 / 68.82	27.21 / 72.79
	0.453	**0.374**

Table T 4.6 Proximate composition of Watermelon seed

Watermelon seed composition	Percentage (%)
Moisture	5.10
Fiber	14.00
Protein	34.00
Ash	0.74
Carbohydrate	3.50
Fat	43.00 -53.00 oil yield- 45.31% soxhlet; 55.84% by SCCFE

In the super critical fluid CO_2 (SCFE) extracted watermelon seed oil the saturated fatty acids occur as 27.21% (palmitic (12.17%) and stearic (15.04%) acids) and the unsaturated occur as 72.79% (oleic (15.05%) and linoleic (57.74%) acids) and there is no linolenic acid as in avocado pulp oil.Of the un-saturated ones monounsaturated fatty acid oleic acid and diunsaturated fatty acid linoleic acid only account for 72.79% as unsaturated fatty acids composition.

The DUFA (diunsaturated fatty acid) linoleic acid occurs57.74% and 55.41% for supercritical oil and soxhlet oil respectively showing the superiority of the SCFE oil of the watermelon seed.Overall for the watermelon seed extracted oil the ratio of saturated fatty acids component to unsaturated fatty acids component is 0.374 for super critical fluid CO_2 (SCF) extraction and 0.453 for soxhlet extraction .The absence of MUFA palmitoleic and PUFA linolenic acids is a characteristic feature of the watermelon seed oil when compared to avocado pulp oil.

4.4 DISCUSSION

Oil contents and fatty acid compositions of plant materials (avocado pulp and watermelon seeds) considered in the work are listed in tables **(Tables T 4.3; T 4.4; T 4.5; and T 4.6).** The oil content ranged from values as low as 45.31 and 55.84 % in soxhlet and supercritical oils for watermelon. And for avocado oil it ranged as low as 47% in soxhlet and as high as 55% in supercritical extractions.

Both the oil species do contain fatty acids common to plant oils but in varying and characteristic proportions, such as saturated fatty acids like palmitic and stearic acids and unsaturated fatty acids like oleic, linoleic and linolenic acids along with palmitoleic acids. However, the avocado fruit oil has the highest MUFA contents (oleic and palmitoleic acids) both in soxhlet and supercritical oils (70.80% and 74%). Whereas the watermelon seed oil has highest linoleic acid (DUFA) content followed by oleic acid both under soxhlet and supercritical oils (50.41% and 57.74%).

Thus this study concludes that the extracted oil content values were higher in both the avocado fruit pulp and watermelon seeds under supercritical CO2 extraction compared to soxhlet extraction. The fatty acid profiles are in agreement with previous reports (Vekiari et al., 2004; El-Adawy and Taha 2001) that showed the presence of high amount of LA (linoleic acid (18:2 ω-6) as 57.74%) in watermelon seed oil and OA (oleic acid (18:1 ω-9) as 65%) in avocado pulp oil.

As per the fatty acid levels of avocado oil, the oleic acid reigned supreme followed by linoleic and palmitoleic acids and little of linolenic acid. Though this has been true for both soxhlet and supercritical extracted oils the SFA/UFA (saturated / unsaturated fatty acids) ratios (0.214 and 0.176) prove that the SCFE oils are effective for their health benefits due to their valued and higher PUFA contents.

In the case of watermelon seed oil linoleic acid is the dominant UFA followed by oleic acid and there is absence of palmitoleic and linolenic acids unlike the case of avocado oil. Here too the supercritical oils reign supreme than soxhlet oil in containing higher level of unsaturation among fatty acid components. The SFA / UFA ratios are found to be at 0.453 and 0.374.

Although seeds only represent major oil contents as reserve foods in the plant kingdom, exceptions like avocado fruits also do exist.

The higher oil contents and desired unsaturated fatty acid levels from both the avocado pulp and watermelon seeds have been achieved through supercritical extraction (SCFE) only. SCFE is considered to be a useful tool for the isolation and extraction of various plants, fungal and animal tissue materials, as under diffusion-controlled conditions, maximal solubility allows extracting oil more quickly with high yield. The present study is thus the first to record the complete nutraceutical values of total and individual fatty acids of supercritically extracted

oils of avocado pulp and watermelon seeds. These materials so far have not yet been put to any direct use for pharma related preparations because organic solvent extractions contain persistent residues undermining the oils' therapeutic values and properties.

Supercritical carbon-di-oxide ($SCCO_2$) is the most commonly used fluid for SCFE of a variety of separations in food, bio-processing and analytical applications, because of its non-toxicity, non-explosivity, non-flammability, safety and low cost. However, SCFE processes have been developed and standardized for varying plant materials (Zhang et al., 2010; Liu W et al., 2009; Manninen et al 1997; Cuperus et al 1996; Favati et al 1991;) animal tissues (Devineni et al 1997) and fungal cells (Sakaki et al 1990)

There is a confirmed negative correlation between oleic acid on one hand and linoleic and linolenic acids on the other as seen in avocado and watermelon seed oils where either oleic or linoleic acids are predominant in the total oil's percentage suppressing the other's genesis (Matthaus et al., 2003 ; Goffman, et al.,1999).The presence of C18 MUFA (oleic acid) and LC PUFAs like LA (Linoleic acid) or LNA (linolenic acid) as in other plants, makes them engage the desaturase and elongase enzymes to convert them in to long chain PUFAs of n-6 and n-9 origin. Hence n-3 pathways for the desaturation and elongation to produce EPA (eicosa pentaenoic acid) and DHA (docosa hexaenoic acid) are starved without the enough enzyme support (Shahidi, 2009).

Under SCFE, variations in the oil yield and its compositions are a function of pressure, temperature, time and the nature of plant materials leading to a higher extractability of lipid materials compared to solvent extraction. Employing SCFE technology in place of solvent extractions appears to be too expensive but the health benefits derived out of using the solvent free SCFE oils in pharmaceutical, medical and nutraceutical applications are highly valuable (Vanessa et al., 2009;Certik and Horenitzky.1999). The efficient extraction of fats from plant samples for analysis and application level studies must be rapid

to protect the loss of unsaturated fatty acids from the effects of the presence of oxygen, alkali and metal ions (Liu W et al., 2009 b; Lang et al., 1992) and the supercritical extraction achieves the same more effectively than the conventional solvent extractions

4.5 CONCLUSION

- The avocado pulp oil and watermelon seed oil have been confirmed to be containing their significant unsaturated fatty acid contents such as oleic acid and linoleic acid in major proportions..
- The supercritical CO_2 extractions of them have shown improved oil yields, higher concentrations of PUFAs and are free from any solvent impurities.
- It is appropriate at this juncture to study the possibilities of improving these oils' oxidative stabilities and subject them to develop effective application products for nutraceutical and therapeutical purposes.

CHAPTER-5

5.1 INTRODUCTION

The importance of unsaturated fatty acids in foods is well recognized. The classes of unsaturated fatty acids include MUFAs (monounsaturated fatty acids) like oleic acid (18:1) and PUFAs (polyunsaturated fatty acids) of ω-6 and ω-3 families. The major dietary ω-6 PUFAs include linoleic (18:2), gamma linolenic (18:3), and arachidonic (20:4) acids, whereas major ω-3 PUFAs include alpha linolenic (18:3), eicosa pentaenoic (20:5), docosa pentaenoic (22:5) and docosa hexaenoic (22:6) acids (Shahidi, 2009; Garg et al., 2006).

Oleic acid (OA) is a monounsaturated fatty acid (MUFA), present as a major constituent in ***avocado*** **oil.** It reduces the levels of an oncogene called Her-2/neu (also known as erb-B-2) and is effective in controlling breast cancer cells. High levels of Her-2/neu occur in more than a fifth of breast cancer patients and are associated with highly aggressive tumors that have a poor prognosis. Further, the presence of oleic acid boosts the effectiveness of the drug ***trastuzumab*** (Herceptin) and can help to prolong the lives of many such patients (Menendez et al., 2005). ***Watermelon seed oil*** containing linoleic acid is being used as cooking oil as well as a food additive in Western Africa and middle - east countries (Girgis and Said 1968).

Avocado oil prevents human body from accumulating the undesirable low-density lipoprotein (LDL) cholesterol and promotes healthy high-density lipoprotein (HDL) accumulation, which is beneficial to the heart. Studies also prove β-sitosterol in avocado oil helps in relieving the symptoms of prostate enlargement amongst men besides lowering the cholesterol build-up. It also contains other compounds like lutein and other color pigments, which are implicated in the eye disorders in older people (Chan, 2005).The consumption of long chain ω-3 fatty acids is effective in treating coronary heart diseases, type-2

diabetes, hypertension, immune response disorders and mental illness (Zhang et al 2010; Shahidi, 2009; Djordjevic et al., 2005). The required intake of PUFA can be achieved by consumption of PUFAs rich supplements. Problems are faced for the production, transportation and storage of these fortified foods as PUFAs are extremely susceptible to oxidative deterioration.

Temperature control, oxygen free atmosphere and addition of a lipid soluble antioxidant are the most common methods used in controlling the oxidation of PUFAs. Products like oil in water (o/w) emulsions are easy to produce and disperse into water based foods like beverages, dairy products, salad dressings and muscle foods than bulk oils that could physically separate from aqueous phase upon preparation and storage. Emulsions also maintain structural integrity when incorporated into water based foods.

The whey protein isolate /concentrate (WPI/WPC) is a common food additive often used to produce low viscosity ***oil-in-water emulsions*** at oil concentrations ranging from 5-30% having excellent physical stability against thermal processing (Djordjevic et., 2004). These emulsion based delivery systems need to be kinetically stable over a broad range of conditions they might experience during food preparations like sterilization, pasteurization, cooking heat, pressure and storage periods (Euston et al., 2000).

In the present study (as in Chapter 4) it is the super critical fluid CO_2 extraction (SCFE) of these oils from avocado pulp and watermelon seeds provide the maximum composites of these unsaturated fatty acids without any deterioration and contamination. These oils are used for the purpose of developing emulsions as effective nutraceutical delivery systems. These models would be of much value for clinical, therapeutical and nutraceutical applications and practices.

Here WPC stabilized oil-in-water emulsions as effective ingredient delivery systems to incorprate both MUFAs and PUFAs as nutraceuticals into functional

foods have been attempted. Hence, it would be advantageous to produce emulsions with high oil content to reduce the cost of transportation, maximize concentration of bioactive lipids and a low viscous behavior to facilitate ease of application. The developed product must also be stable towards oxidation and possess desirable sensory quality attributes with a good shelf life. This will help in developing physically and oxidatively stable ω-6 and ω-3 fatty acids delivery systems (like emulsions) which only need to be added to foods in small quantities as fortifiers or as direct dosages having nutraceutical, clinical and therapeutic applications.

The major use of an emulsion depends upon its rheological characteristics. An understanding of the rheological behavior of food materials like emulsions is required for the purpose of product development, process selection and standardization, and to know the stability of the samples during storage. An examination of the suitability of such rheological models is also required for characterizing the materials and for scale-up purposes. Though the importance of such studies lies in formulating nutraceutically valued emulsions (using avocado and watermelon oils), the rheological behavior of these preparations have not yet been reported. Thus it is important to understand the relationship between the rheology and sensory perceptions during the process of mastication. These sensory quality attributes may be described in terms of creaminess, stickiness, etc. which could be linked to pourability and spreadability of emulsions. These in turn are linked to rheology.

The concentration of protein in the o/w emulsion is critical for stabilizing the emulsion, and hence, affects the rheological status. Emulsified fat contributes to the creaminess of the system in addition to viscosity (Richardson and Booth, 1993). The size and number of oil particles, and their distribution pattern also impart a significant effect on the rheological characteristics; further, the state of aggregation of the droplets also possibly has an influence (Dickinson 2001).

In the present study, the system conditions have been optimized to avoid droplet aggregation or coalescence to explore the general rheological behavior aiming for a stability and quality of the product.

Hence the following studies were conducted.

a) To develop emulsions as delivery systems for nutraceutically valued PUFAs, made out of avocado pulp oil and watermelon seed oil using whey protein concentrate as the emulsifier.
b) to analyze the emulsion products for physico-chemical characterizations like visualizing the emulsion particles through microscopic techniques for particle sizing.
c) to assess and record the density, creaming index, stability and examine the surface tension properties over variable periods of storage of the emulsions for quality.
d) to study the suitability of these models through predictions of their rheological behaviors.
e) to prolong the oxidative stability of emulsions by assessing the peroxide values over different periods of storage time and
f) to examine the stability of these emulsions during storage and sensory attributions through a panel of experts over the relative periods of storage to recommend the preparations for nutraceutical applications.

5.2 MATERIALS AND METHOD

Materials

Whey protein concentrate (WPC), obtained from Mahaan Foods, New Delhi, India having about 93% protein, 5% moisture and 2% sulfated ash (dry weight basis), was used for the present study. Avocado fruits (*Persea americana Mill.*) and watermelon (*Citrullus lanatus Thunb.* syn. *Colosynthis citrullus Linn.*) seeds were procured from the Horticulture Department of Mysore, Karnataka, India.

Freeze drying of pulp and seed material and oil extraction

The above steps were conducted as mentioned earlier (Chapter-4, 4.2 Materials and Method).

Emulsion Preparation

Oil-in-water emulsions were made by following the method of Djordjovic et al. (2004). The SCF CO_2 extracted avocado pulp oil or watermelon seed oil was mixed with an aqueous phase having 10 mM sodium citrate buffer in the ratio 1:3 followed by adjustment of pH to 3.0, and WPC was added to obtain a final protein-to-oil ratio of 1:10 (i.e. 2.5 weight % protein for 25 weight % of oil). WPC was added with the buffer by stirring at 4^0C for 12 hours to obtain a uniform dispersion. Prior to homogenization, the aqueous phase was stirred for 1 hour at room temperature (25^0C) and was readjusted to a pH of 3.0 using 1.0 M HCl solution.

A high speed homogenizer model with cup and cone system (Model # RQ –127 A/D, Remi, India), having a speed regulator was used. The homogenizer had a toothed stator and a rotor that were separated by a small clearance to offer a wide range of fine globule/particle sizes. A coarse emulsion premix was obtained by employing this apparatus by homogenizing oil and aqueous phase at a moderate speed for 2 min at room temperature (25-27^0C). The coarse emulsion was passed through a homogenizer at high speed range of about 5000 rpm for a period of 20 min. immediately after homogenization; the formed emulsions were cooled by placing the samples in an ice bath.

Processing and storage of the emulsions

Once made, the emulsions were pasteurized by heating in a water bath up to 75^0C followed by a holding time of 30 min to avoid microbial contamination. Antioxidant in the form of mixed tocopherol isomers at a level of 200 ppm was added prior to homogenization. Then 10 ml of each sample was transferred to glass tubes with lids. The internal diameter of the tubes was 15 mm and a height of 125 mm, and the samples were stored at room temperature prior to further analyses.

Characterization of emulsions

Emulsion Density (ED)

It is one of the simplest methods of determining emulsion properties and it can be done by using simple equipments (Mc Clements et al 1994; Ozdemir et al 1994). The density studies were conducted on days 1,4,8,15,30 and 60 of storage period. Emulsion of 20 ml was pipetted out with the enlarged mouth side of the pipette and got weighed and the density was calculated as

ED = g of emulsion / ml of emulsion

Physical Stability of Emulsions / Creaming Index (CI)

The creaming stability / creaming index of emulsion samples (10 / 67 mm vials) were monitored at room temperature by visual observation. Here the susceptibility of liquid emulsions to creaming was ascertained for 1,4,8,15,30 and 60 days of storage, by measuring the height of the interface between the opaque layer at the top and the clear aqueous phase at the bottom and was reported as the creaming index .

Surface Tension Studies (ST)

The surface tension studies were conducted with the help of a tensiometer, provided with ring plate mechanism (Model Kruss, Germany). The platinum metal ring plate was used to touch the surface of the emulsion preparations to sense the tension range prevalent at the emulsion's surface that was exposed to air / atmosphere. The values were analyzed in accordance and standardization with surface tension values between pure water and air surface which was kept as a reference. The studies were conducted on days 1,4,8,15,30 and 60 of storage.

Microstructure of the Emulsions (Phase Contrast Microscopy)

The emulsion samples were kept as a small drop over a glass slide with a cover slip, and were viewed at a low magnification of 40X employing a phase contrast microscope (model # BX40/F4, Olympus Optical Company, Japan). The images of the emulsions were recorded using a manual camera and pictures were stored

on a computer. The respective particle sizes and their distribution patterns were calculated for a population of 20 particles at a constant range of magnification. This particle characterization is within the limits of phase contrast microscopy and the size recognition starts at about 2 μm only. The particle sizing was done on the 1^{st}, 30^{th} and 60^{th} days of storage.

Rheometer and Rheological Measurements

A controlled stress (CS) rheometer (Model # RT10, Haake GmbH, Karlsruhe, Germany) with a coaxial cylinder attachment having a ratio of 0.922 between the external diameter of the rotating bob and the internal diameter of stationary cylinder was employed to determine the rheological behaviour of the emulsions. The gap between the cup and rotor was 850 micron. A constant temperature of 20±0.1^{o}C was maintained during the measurement using a circulatory water bath. The controlled rate (CR) measurement technique was employed by progressively increasing the shear-rates to 500 $^{s-1}$ to obtain 50 shear-rate/shear-stress data points. All rheological measurements were conducted on triplicate samples.

Analysis of data and statistics

The shear-stress and shear-rate data were fitted to the power-law (Ostwald-de-waale model), as per the e.g. (1) as shear-thinning was observed but not the existence of yield stress. The flow behaviour index and consistency index were estimated by employing the non-linear analysis of shear-stress/shear-rate data by using the software supplied by the equipment manufacturer.

$$\sigma = \mathbf{k}\ (\dot{\gamma})^{\mathbf{n}} \qquad \textbf{(1)}$$

Here, σ is shear stress (Pa), $\dot{\gamma}$ is shear-rate (s^{-1}), **k** consistency index (Pas^{n}) and **n** is flow behavior index (dimensionless). The apparent viscosity of the samples was obtained as the ratio of the shear-stress and shear-rate when the latter was taken to be 50 s^{-1}. The suitability of the rheological model relating shear-rate and shear-stress values were judged by determining the correlation coefficient **(r)** and chi-square values.

Per-Oxide Value (PV) Studies

The per-oxide value studies over the emulsions to determine their oxidative stability were conducted following the AOCS official method Cd 8-53 (1997). In general about 10ml of the emulsion sample was added with 30ml of chloroform and shaken well to be kept for 5- 10minutes. To this was added 5g of anhydrous sodium sulphate and the solution was filtered to collect the filtrate. The filtrate was then divided into two equal halves and taken in two conical flasks. To one conical flask 1.5 times (the volume of extracted fat in chloroform) Glacial Acetic acid was added. To this mixture 1 ml of Saturated Potassium iodide solution was added, the conical flask was closed and kept in dark. After a delay of 60 sec. the lid was taken out and 30 -40 ml of distilled water was poured in and few drops of 1-2 % starch solution was added. The sample solution was titrated against an accurate normality (0.001N) Sodium thio-sulphate ($Na_2S_2O_3.5H_2O$) solution (standardized Vs. potassium dichromate), for a change from blue color to colorless condition. The volume of the sodium thio-sulphate (*V*) and the normality (*N*) were noted. The sample volume in the second conical flask was dried at 100$\pm$2 ^{0}C to evaporate all the chloroform and weighed to know the exact weight of the extracted fat (*W*). And the peroxide value (PV) was calculated following the formula

$$PV = \frac{1000 \times N \times V}{W}$$

Sensory Assessment (SA)

Super critical Carbon dioxide extracted avocado pulp oil / watermelon seed oil containing oil - in- water emulsions of known particle size distribution and viscosity were assessed by a trained sensory panel comprised of 14 assessors as conducted earlier (Akhtar et al 2005). Samples of the emulsions were prepared and stored at room temperature (20 $\pm$ 5 ^{o}C) prior to assessment by the panelists at the same temperature. The samples were presented in a regulated presentation sequence and coded with random numbers. The orders of the samples presented to the panelists were also randomized across sessions. Canned / mixed cocktails

were given to the panelists to eat between each tasting. Samples were analyzed in duplicates. Panel members were asked to rate the perception of aroma / flavor, creaminess, taste and thickness on a scale of 1-10, where 10 corresponds to the highest ratings. All the experiments were carried out in duplicates using freshly prepared and stored samples on days 1,4,8,15,30 and 60 of storage, and the results are reported as the mean and standard deviation of the measurements. The aroma / flavor, taste, creaminess and thickness were the sensory attributes studied upon with reference to pure milk sample as a standard reference.

5.3 RESULTS

Emulsion Density (ED)

The emulsion density studies have shown that the varying densities of avocado oil and watermelon oil made emulsions over the storage period were almost stable (**Fig.5.1**). The avocado emulsion had an average density of 0.99 values to pure water density value throughout the period of 60 days of storage and these values were almost uniform and steady. Whereas the watermelon oil based emulsion had around 0.96 as density profile throughout the entire study period. The keeping in of the density had neither a down nor an up but was almost steady throughout the entire storage period as indicated by the results from the graph (**Fig.5.1**). for both the emulsions.

Creaming index/ creaming stability of emulsions (CI)

During the storage period of 60' days, the susceptibility of liquid emulsions to creaming was ascertained by measuring the height of the interface between the opaque layer at the top and the clear aqueous phase at the bottom and reported as the creaming index. The Graph (**Fig 5.2)** shows that both the avocado and watermelon oil emulsions had creaming indices which were uniform, unaltered, and steady showing not much changes either on the higher or lower side proving the effective stability of the emulsions under the storage period. It is noteworthy to mention that both the emulsion products appear to have a uniform and steady

density values (0.96-0.99 gm/cc) for the whole of the storage period along with a similar quality reflected for the creaming status (58-65) too. This is in concomitance with the fact that as the emulsion density is stabilized, the creaming value of the emulsion also becomes stabilized to promote the viability of the products. Thus creaming is as such an important parameter endorsing the stability and utility value of o/w emulsions.

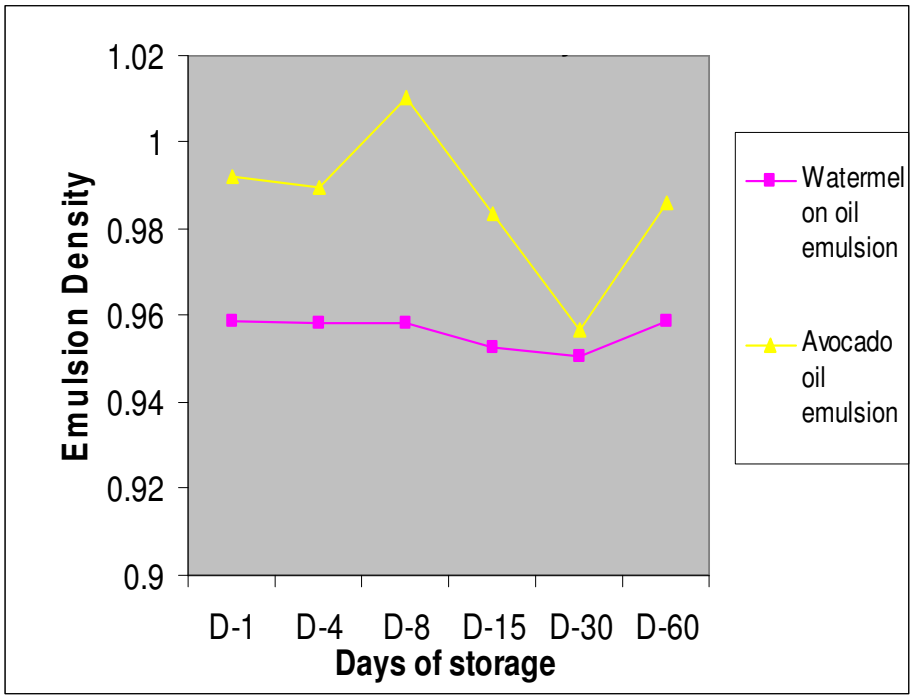

Fig 5.1 Emulsion density profile of avocado and watermelon emulsions over the 60 days storage period.

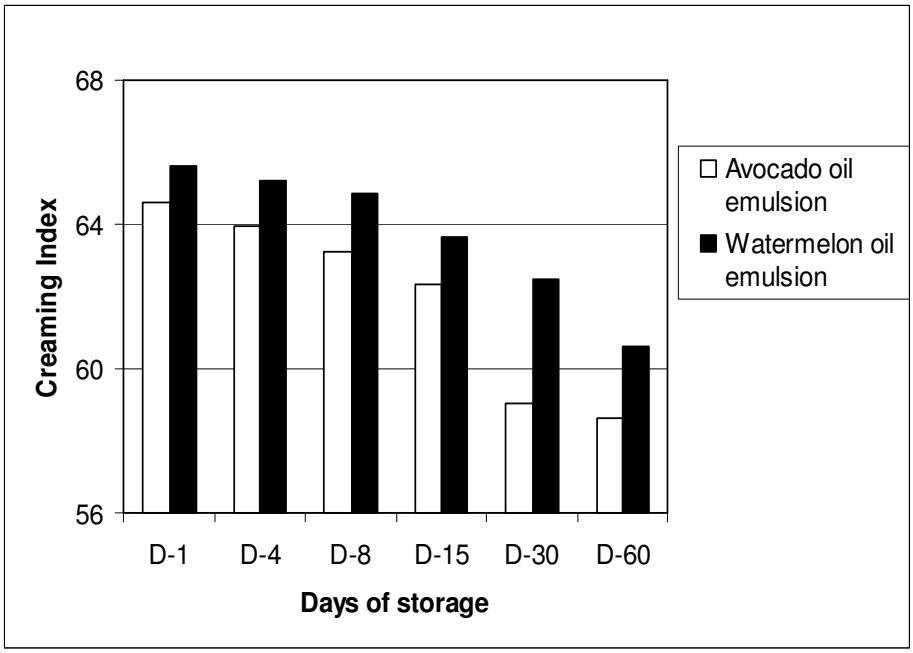

Fig 5.2 Creaming index studies profile for avocado and watermelon emulsions over the 60 days storage period.

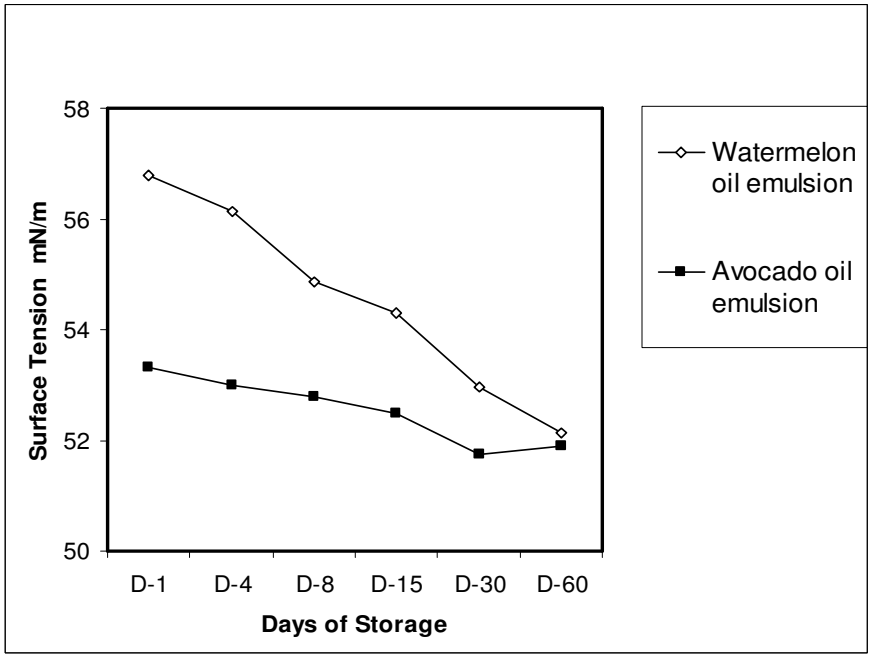

Fig 5.3 Surface tension studies and profile for emulsions of avocado and watermelon over the 60 days storage period.

Surface tension studies (ST)

The surface tension values range from 53.50 – 52.50 mN/m for the avocado emulsion and from 57.00 – 53.00 mN/m for watermelon emulsion for the whole of the 60 days storage period (**Fig 5.3**). This situation could be assessed as almost a steady state of quality for both the preparations. Though there is a slight trend of loss of surface tension, it is significantly negligible over the said period of storage (i.e. 60 days) proving that the products are viable notably over the entire period of storage.

Table T 5.1 Microstructure of the emulsions - particle sizing through phase contrast microscopy

Particle size* of emulsion droplets during storage

Range of particle size (μm)	Avocado Emulsion			Watermelon emulsion		
	Storage period (days)			Storage period (days)		
	1	30	60	1	30	60
< 5	100	100	70	90	85	70
5 to 10			30	10	15	30

*** Values are expressed as percentage distribution of size**

Microstructure of emulsions

Phase contrast microscopy & Particle Sizing

The Table (**T5.1**) shows the particle size data for the emulsions. The particle sizes were analyzed using the phase contrast microscopy (PCM) on the 1st, 30th and 60th days of storage. Figures (**Fig. 5.4 & 5.5**) show the phase contrast photomicrographs of avocado and watermelon emulsions, respectively kept for different storage periods. It is clearly evident that for the avocado pulp oil based emulsion (**Fig.5.4)** up to 30 days of storage the particle diameter size is unimodal and is on the range of less than 5 μm size and for the 60 days old sample only it showed a bimodal particle distribution with an additional occurrence of about 30

percent particles on the range of 5 – 10 μm diameter size. In the case of watermelon seed oil based emulsions (**Fig.5.5**), about 90% of the particles are below 5 μm in diameter prior to storage, which almost is maintained up to 30 days of storage. And in the 60 days old sample only it showed an increase in bimodal particle distribution with an increase in the size of the particles table (**T 5.1**) where about 30 percent of the particles are on the 5 – 10 μm diameter range and it hardly exceeds 10 μm. All these sizes are reproducible for the WPC (whey protein concentrates) based emulsions

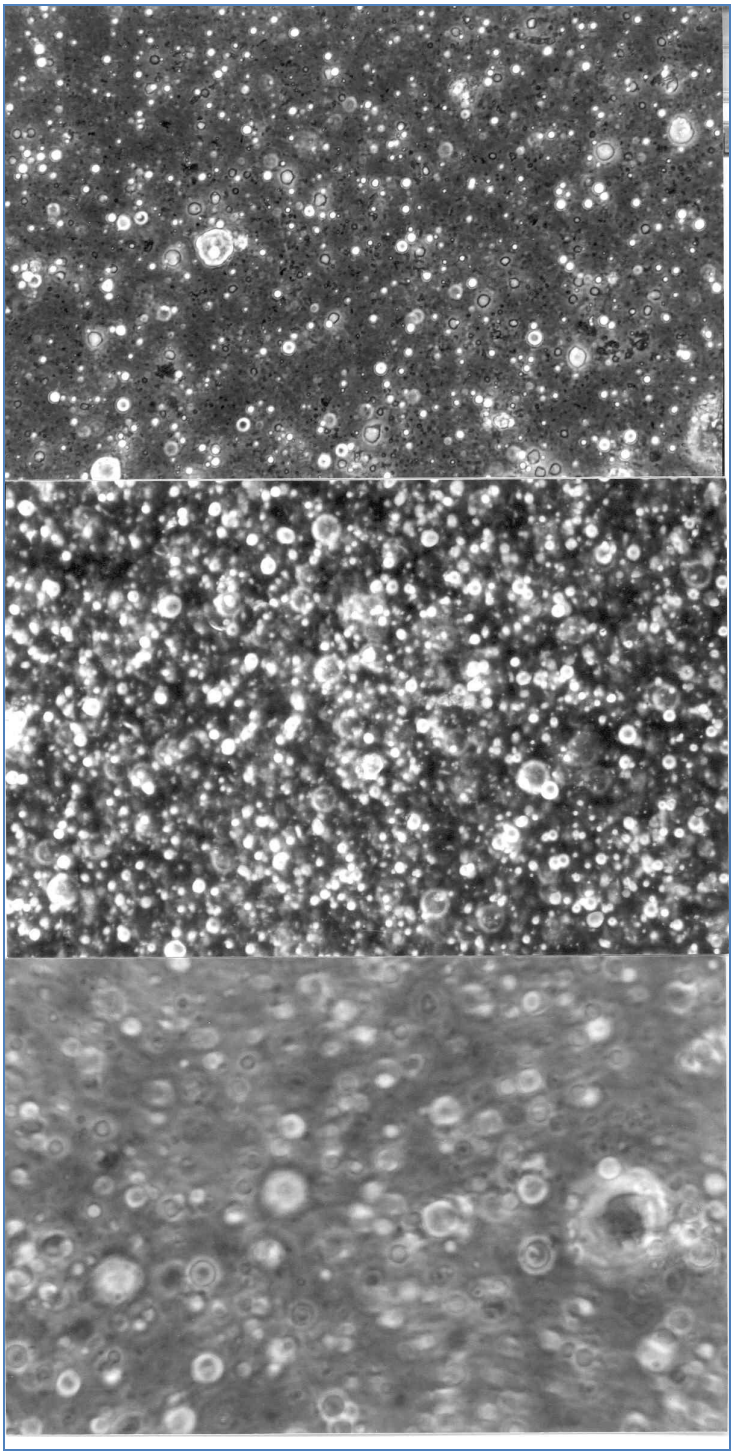

Fig.5.4 Photomicrographs of avocado oil emulsions at different days of storage (A) 1st day (B) 30th day and (C) 60th day

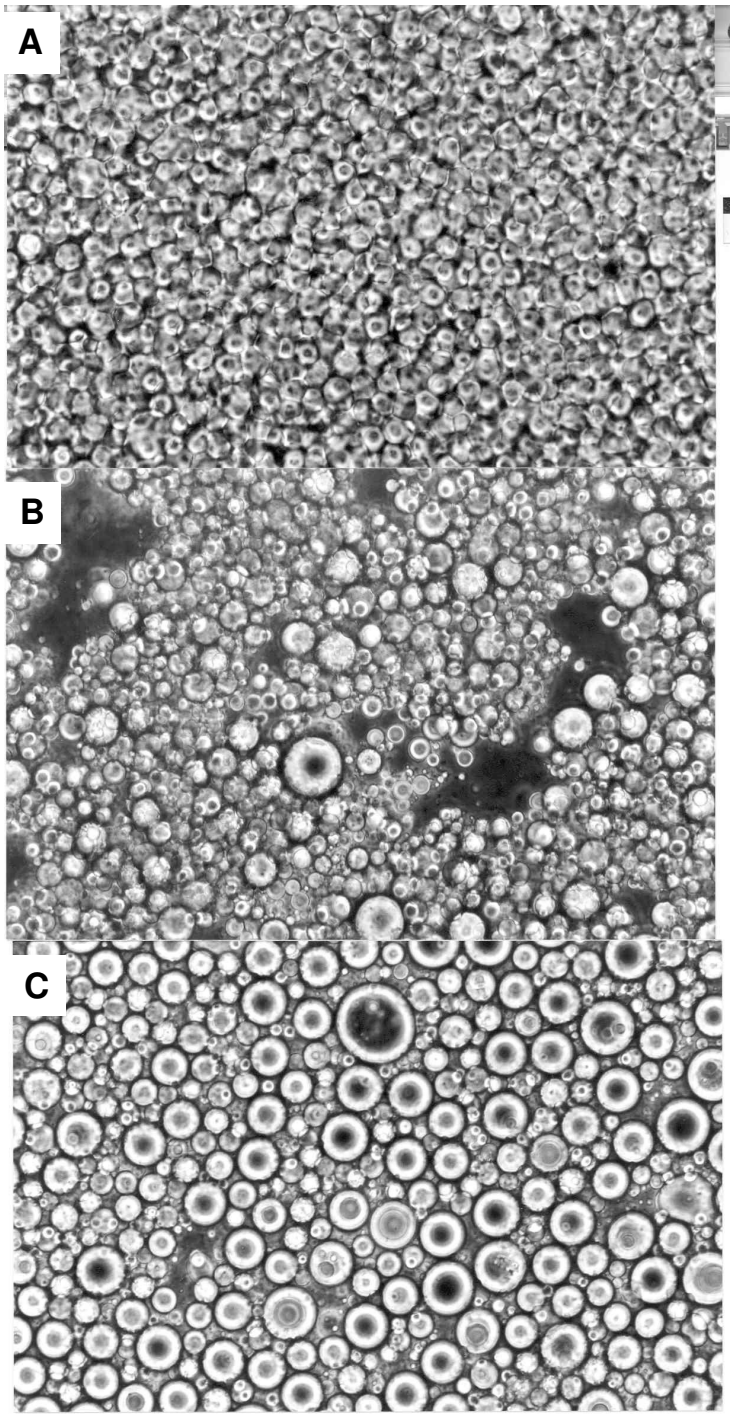

Fig 5.5 Photomicrographs of watermelon oil emulsions at different days of storage (A) 1st day (B) 30th day and (C) 60th day

Analysing the rheological data

The apparent viscosity of the samples was obtained as the ratio of the shear-stress and shear-rate when the latter was taken to be 50^{s-1}. The suitability of the rheological model relating shear-rate and shear-stress values were judged by determining the correlation coefficient (r) and chi-square values. As per the rheological status, the oils as well as the emulsions behave like non-Newtonian liquids having shear- thinning characteristics as shown by the sample rheograms (**Fig 5.6 & 5.7**). Both the avocado and watermelon oils are of moderate shear-thinning characteristics as the flow behavior indices (n) indicate (i.e. 0.86 and 0.88-n). The shear-rate/shear-stress data can be adequately fitted (r=0.997-0.999) to the common rheological equation like ***Ostwald-de-Waale or power law model*** (Avocado pulp oil is markedly more viscous than watermelon oil as evident from higher apparent viscosity (η), (0.165 and 0.112 Pas) and consistency index (k) values (0.260 and 0,191 Pas^{-n}). In addition, the apparent viscosity values show good repeatability with standard deviation values hardly crossing 3 mPas.

Interestingly, both the emulsions possess similar flow behavior index values. The emulsions made with whey protein concentrate (WPC) containing avocado pulp oil or watermelon seed oil (**Table- T 5.2 & 5.3)** also show shear-thinning behavior as indicated by the flow behavior indices (n), (0.570-0.619 and 0.534-0.633-n, respectively) that are lower than the indices of their corresponding oils (0.881 and 0861-n). This is because that the addition of buffer salts and WPC in emulsion makes the samples acquire non-Newtonian characteristics. The rheological behavior of both the emulsions, observed as apparent viscosity (η), is between 0.050 and 0.056 Pas over the entire period of storage time. This meager increase in viscosity values may be an effect of the size increase of particles. In avocado oil and watermelon oil based emulsions (**Fig 5.7)** about 100 % and 90% of the particles respectively were below 5 μm in diameter prior to storage. An increase in storage period increases the size of the particles (**Table T 5.1)** though it hardly exceeds 10 μm size.

Table- T 5.2 Rheology of oils used for the study

Type of oil	Apparent viscosity, η (Pas)	Flow behavior index, n (-)	Consistency index, k (Pas^n)
Watermelon	0.1125±0.001	0.8599±0.012	0.1906±0.011
Avocado	0.1647±0.027	0.8809±0.006	0.2595±0.053

Table- T5.3 Rheological status during storage of emulsions made using avocado pulp oil and watermelon seed oil

Storage period (Days)	Apparent viscosity, η (Pas)	Flow behavior index, n (-)	Consistency index, k (Pas^n)
Avocado pulp oil			
0	0.0504 ± 0.003	0.5934 ± 0.013	0.2383 ± 0.023
10	0.0561 ± 0.003	0.5718 ± 0.022	0.2859 ± 0.036
20	0.0540 ± 0.003	0.6192 ± 0.025	0.2159 ± 0.008
40	0.0535 ± 0.001	0.5794 ± 0.004	0.2641 ± 0.008
60	0.0566 ± 0.003	0.5701 ± 0.021	0.2976 ± 0.044
Watermelon seed oil			
0	0.0520 ± 0.001	0.6041 ± 0.018	0.2325 ± 0.024
4	0.0490 ± 0.001	0.6166 ± 0.021	0.2043 ± 0.026
8	0.0525 ± 0.001	0.5973 ± 0.027	0.2280 ± 0.046
15	0.0495 ± 0.002	0.6330 ± 0.010	0.1851 ± 0.008
30	0.0484 ± 0.002	0.5794 ± 0.018	0.2450 ± 0.015
60	0.0510 ± 0.005	0.5339 ± 0.048	0.2822 ± 0.087

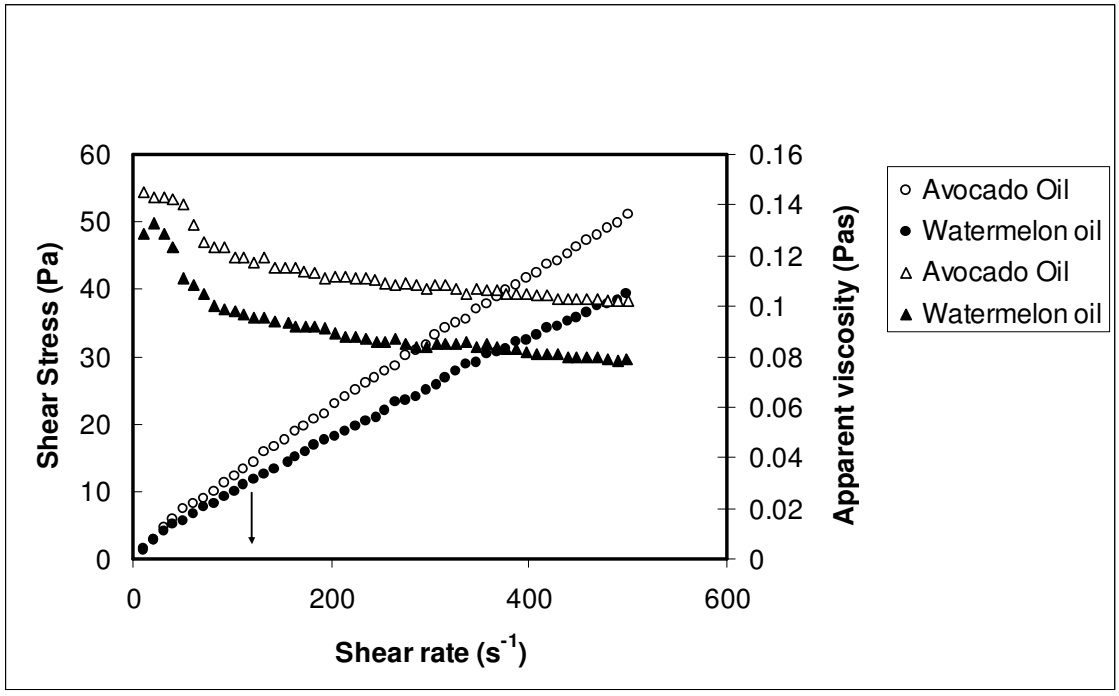

Fig 5.6 Rheograms for watermelon seed oil and avocado pulp oil

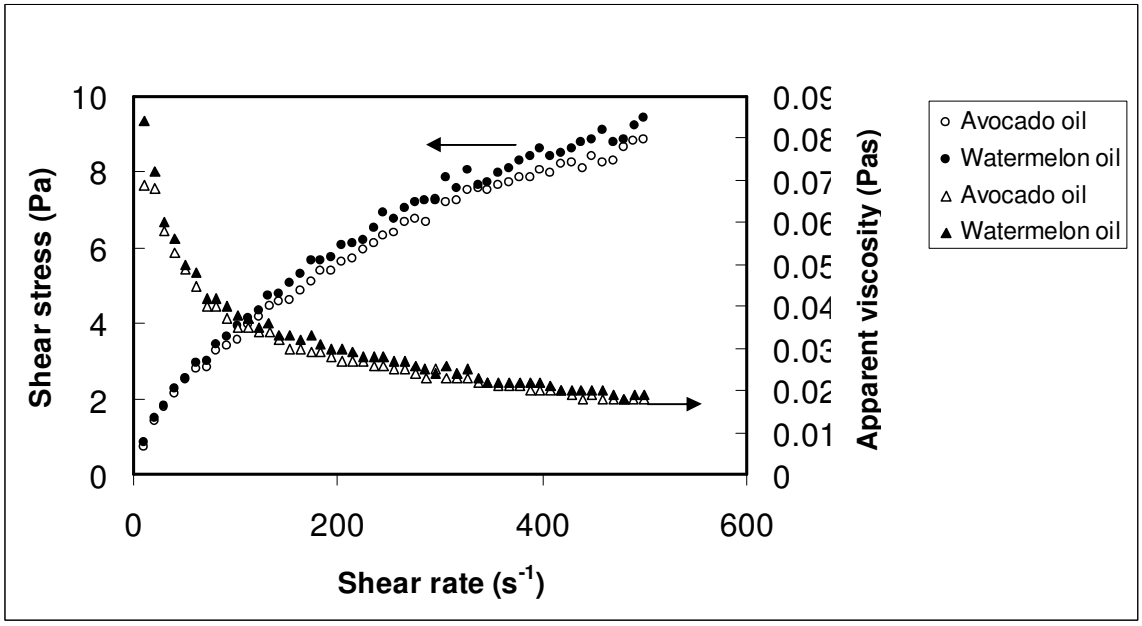

Fig 5.7 Sample rheogram for avocado and watermelon seed oils with whey protein concentrate at the beginning of storage period

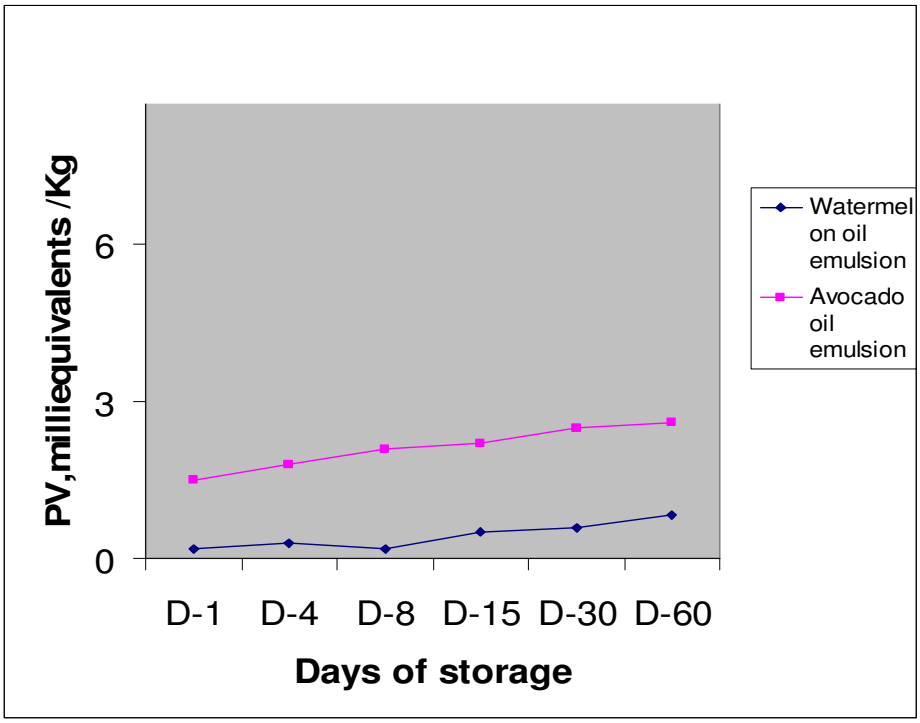

Fig 5.8 Peroxide value studies profile for avocado and watermelon emulsions over the 60 days storage period.

Per-oxide value (PV) studies

The whey protein concentrate stabilized (avocado pulp oil and watermelon seed oil containing) oil-in-water (O/W) emulsions at pH 3.0 show good physical characteristics and stability after pasteurization as evident from the peroxide value studies graph **(Fig 5.8).** Also evident is that the watermelon oil based emulsion is showing a lesser peroxide value over the said 60 days of storage after pasteurization than the same way prepared, treated and stored avocado oil based emulsion. Though the peroxide values of both the emulsions show an advancing trend towards lipid oxidation reactions, the values are kept well within an acceptable limit throughout the storage period.

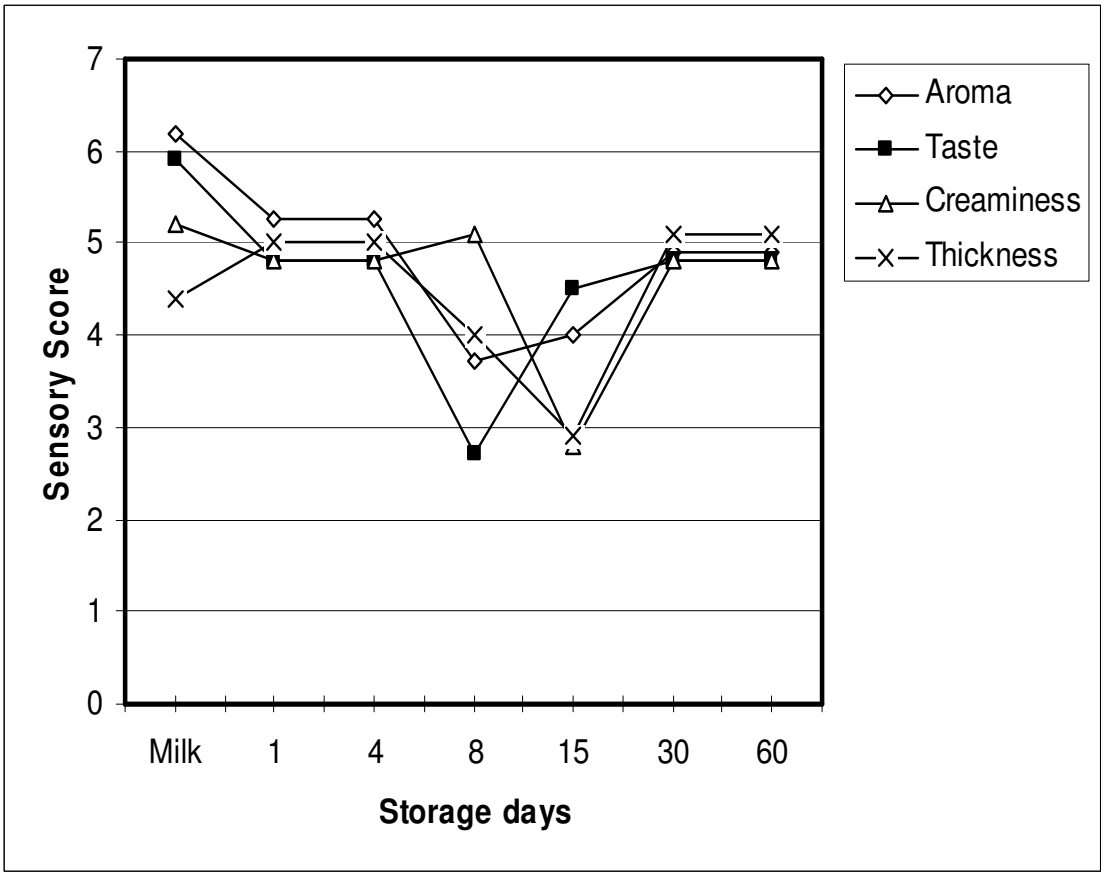

Fig 5.9 Sensory value studies profile for avocado emulsion over the 60 days storage period.

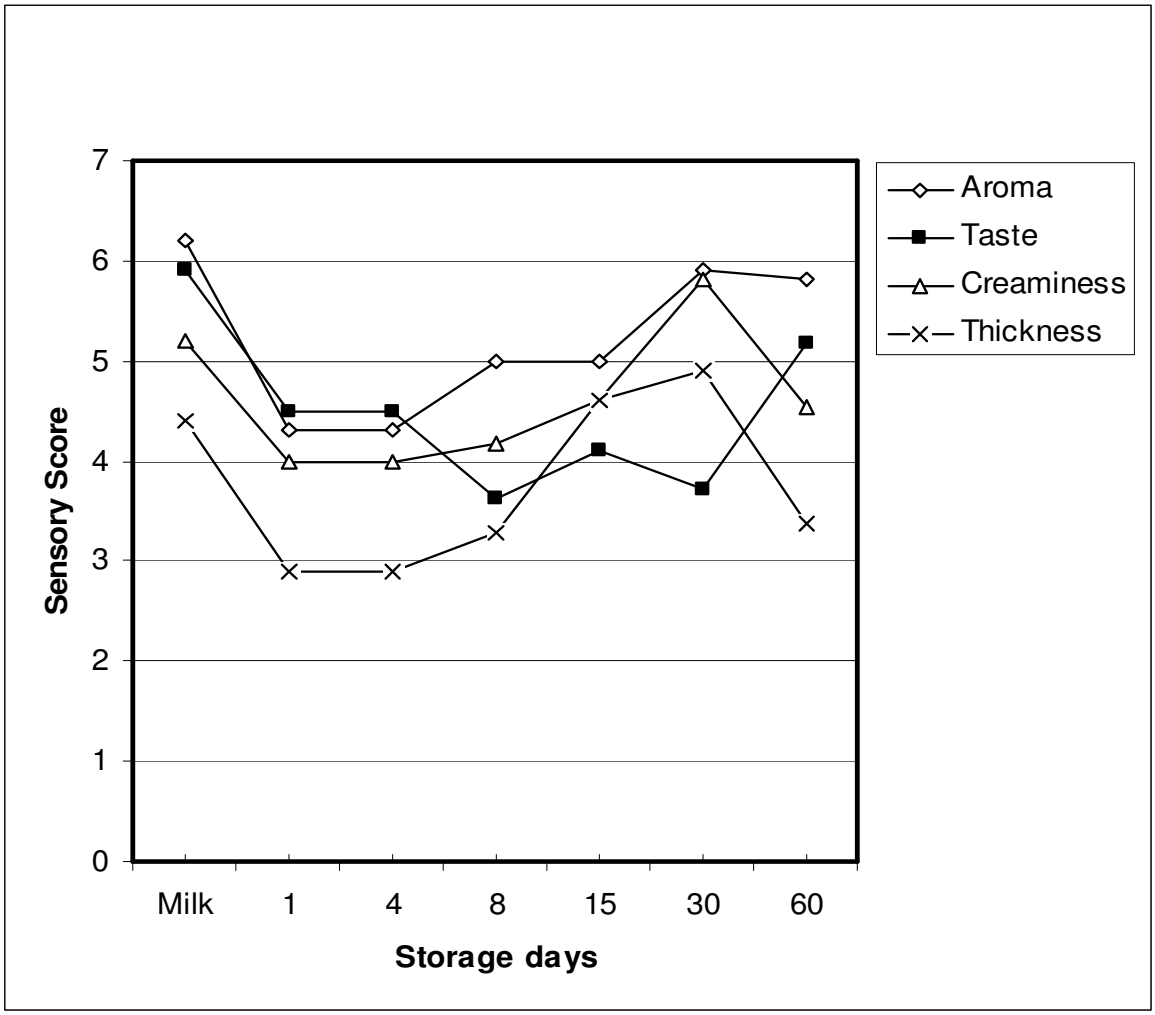

Fig 5.10 Sensory value studies profile for watermelon emulsion over the 60 days storage period.

Sensory Studies

Both the avocado and watermelon oil emulsions were compared with milk for sensory attributions on the first day of preparations and the analyses continued further for the emulsion samples for 4,8,15,30 and 60 days of storage. Such a comparison with milk, a natural emulsion and keeping it's acceptability as 100% with a sensory score of slightly above six (6.0), helps in understanding the sensory acceptability and feasibility of the products for various clinical, therapeutical, nutraceutical and commercial purposes and applications as PUFAs loaded delivery systems. It could well be visualized from the graphs(**Fig 5.9 & 5.10**) that the overall values of sensory attributes like flavor, taste, creaminess and thickness for both (avocado and watermelon oil based) emulsions, were almost 70 - 85%of that of milk over the entire storage period. The sensory values were almost uniform and the final scores were almost similar in both the emulsions. Here the thickness attribute of watermelon oil emulsion was only a half of that of milk, due to the composition of the emulsion product and it has nothing to do with the storability. This is evidenced by emulsion density, creaming index, surface tension values along with sensory evaluation results.

5.4 DISCUSSION

Emulsion stability

Generally, emulsions are thermodynamically unstable systems and tend to separate into a system that consists of a layer of oil (lower density) on top of a layer of water (higher density) so as to minimize the contact area between the two phases. Hence, they require high positive free energy to increase the surface area between the two phases to keep the emulsion droplets intact (Dickinson 1991; Mc Clements 1999). Surface active molecules like emulsifiers / surfactants used for homogenization adsorb to the surface of droplets to facilitate multiple breaking down and form a protective membrane to prevent aggregation (Dickinson 2001; Mc Clements 1999). In the present preparations the particle sizes are mostly in the <5μm range due to the high energy input used for

emulsification process (over a longer duration) resulted in a favorable emulsion stability. Hence creaming occurred with a low profile only in the products even after prolonged storage (i.e. 60 days).

Proteins and emulsion stability

The presence of a surfactant which can effectively reduce the interfacial tension (between the oil and aqueous phases) helps for the formation and stabilization of an oil-in-water emulsion. The surfactants may be small ones like lecithins, monoacylglycerol and so on, or macromolecules like proteins. Hence proteins with higher visco-elastic properties as in the presently used whey protein concentrate (WPC) can form a thicker and healthy interface and thereby reduce the possibility of flocculation and coalescence than the lower molecular weight surfactants. The size of emulsion droplets and the total interfacial area created under identical emulsification conditions are typical for different kinds of proteins. The droplet size increases in the order as soy proteins >whey proteins >sodium caseinate >soluble wheat proteins >blood plasma proteins (Walstra and de Roos1993)**.** Whey protein is a mixture of α-lactalbumin and β-lactoglobulin and several other minor proteins. The usage of whey protein concentrate (having sulfhydryl and disulfide groups in their structure) appears to be suitable in prolonging the life of the present avocado and watermelon oil based emulsions over a longer period of storage time as proved by the study.

Rheology

In o/w emulsions, the amounts of fat and protein and the properties of the later ingredient, and the particle sizes are expected to influence the rheological behavior. The emulsions made with whey protein concentrate (WPC) containing avocado pulp oil or watermelon seed oil also show shear-thinning behavior as indicated by the flow behavior indices that are lower than the indices of their corresponding oils. This is because that the addition of buffer salts and WPC in emulsion makes the samples acquire non-Newtonian characteristics.

The aggregation/coalescence of the droplets plays a substantial role for their stability during storage. The values of different droplet sizes and their distribution patterns, as per the phase contrast microscopy, are considered almost unimodal (i.e., between 2 and 10 μm). Such a narrow range of variations in the particle diameters as seen here may not markedly influence the particle behaviors and the incidence of aggregation, flocculation or coalescence is only marginal. Hence, the variations of droplet sizes in this range possibly do not have any significant effect on the rheological status of these emulsions for a period of 2 months.

Sensory perception

Aroma and taste

Both the emulsions had similar distribution of particle range initially as well as after prolonged storage with some minor slightings showing a consistent trend and it is the same for rheology also. The effect of rheological properties of emulsions and hydrocolloids over the aroma and taste release during mastication, swallowing and consumption in the mouth is the result of dynamic breakdown of food. Owing to the interrelationship between microstructure, in-mouth processing and oral physiology, the microstructures specifically control the delivery and release of aroma and taste during consumption (Malone et al., 2003). It is proved that binding of taste and aroma to biopolymers and the mouth coating behavior of such thickeners influence the flavor perception. And in some circumstances may also have a greater effect than viscosity. Here in the present work, whey protein concentrates are used in the place of biopolymers involving specific aroma–protein interactions

Creaminess and thickness

Creaminess, smoothness and sliminess are related to a pleasant sensation on eating, and are associated with rheological properties. Creaminess is an indicator of richness and quality of the food or nutraceutical products. Only preparations having heavy fat contents are perceived to be highly creamy. It is well proved

that emulsified fat contributes something to creaminess in addition to its effect on viscosity (Richardson and Booth 1993). The perceived creaminess is influenced by the viscosity of the continuous phase (Mela et al 1994). It is yet to be reported more on the effect of fat on taste perception. Another important factor which potentially influences the rheological and sensory behavior of emulsions is the state of aggregation of the droplets in the mouth. Oral viscosity plays an important role in texture perception of fluids and semisolid foods. Apparent viscosity is the most significant factor in determining creaminess perception. Fat content and oil droplet size are much less significant factors. It is proved that fat /oil content has a slightly positive effect over the perception of thickness and creaminess for high viscosity samples (Tornberg et al 2005).

Lipid oxidation in oil-in- water emulsions

Oxidation of lipids in foods produces undesirable effects like loss of fat- soluble vitamins, generation of off-flavors, palatability problems and even the production of toxins that cause food poisoning (Nawar, 1985: Shantha and Dekker, 1994).The peroxide value generally serves as an indicator for the oxidation of lipids, fats and oils. In the present models, the change in the peroxide values is minimal indicating the formation of stabilized and quality emulsions.

Lipid oxidation is a major cause of quality deterioration in many natural and processed foods (Mc Clements and Decker 2000). It can alter flavor and nutritional quality of foods and produce toxic compounds (Min and Boff 2002). Lipid oxidation involves reaction between lipids and oxygen. Oxygen is about three times more soluble in food oils than in water and so there is always enough oxygen present in the oil phase to propel lipid oxidation in emulsions. Hence an effective means of retarding lipid oxidation is to reduce the concentration of oxygen, for example by packing the preparation under vacuum or nitrogen. But placing the food preparations in exclusion of oxygen is not practical and once the preparation is opened in atmosphere the lipid oxidation gets initiated.

Further, creaming and serum separation of emulsions were not noticed during the storage period meaning the absence of major structural deterioration of emulsion particles and hence the stability of the emulsions are not affected during storage up to 2 months at room temperature. Here the minor variations in the particle size range, upon storage possibly do not have any significant effect on the rheological status of such emulsions (Logaraj et al., 2008) as well on the peroxide value criteria and sensory attributes. All these make the emulsions qualifiedly good enough to be popularized as clinically and therapeutically, valid products to serve as effective food grade nutraceutical delivery systems.

5.5 CONCLUSION

Creaming, flocculation and coalescence are the major deteriorative influences the emulsion products face during storage, which could be curtailed upon by the usage of emulsifiers and surfactants.

Proteins soluble in aqueous phase effectively reduce the interfacial tension between oil and aqueous phases to form and stabilize oil–in–water emulsions.

The physical and also the chemical / oxidative stability of oil-in-water emulsions are greatly influenced by several factors like minimization of droplet size (by involving more energy during emulsification), non covalent interactions, interfacial tensions, the magnitudes of attractive and repulsive forces between particles and interfaces, inter-facial pressure differences and so on.

The retention of surface tension values at a minimal range over a longer storage period and the usage of whey protein concentrates (having sulfhydryl and disulfide groups in their structure) appear to be suitable in prolonging the life of the manufactured avocado and watermelon oil based emulsions over a longer period of storage time as proved by the present study.

Lipid oxidation is controlled in this model o/w emulsion systems studied, making them as valued food and nutraceutical products with desirable sensory attributes for a considerable period of storage.

Rheological studies show that Avocado pulp oil and watermelon seed oil with a very narrow range of flow behavior index values, exhibit Marginal shear-thinning behavior and the emulsions formed with these oils incorporating WPC are pronouncedly non-Newtonian and shear-thinning.

Hence control of viscosity factors, fat fraction volumes, protein types like whey protein and their concentrations as emulsifiers, particle size range and interfacial layers and their compositions could all be kept at probable optimal levels to design and manufacture emulsion based delivery systems for nutraceutically valid MUFA and PUFA containing oils of avocado pulp and watermelon seeds.

The kinetic stability of the oil-in-water emulsions is affected by physical forces like the density differences between the two phases, inter-particle interactions, and the structure and visco-elastic properties of the surfactant film etc.,

A reduction in the size of the oil droplets and the viscosity of the medium reduces the rate of creaming leading to physical stability and upon storage at room temperature up to a period of 2 months showed consistency in their rheological status.

The minimal particle size range having an influence over the rheological properties controls the shear stress responses of the emulsion products, both in vitro and in mouth condition which is as well in vivo, accounting for the bio-sensory realizations.

Other influential factors like non covalent interactions, interfacial tensions, the magnitudes of attractive and repulsive forces between particles and interfaces, inter-facial pressure differences, surfactant film's visco-elastic properties etc. upon optimized conditions favor the essential oxidative or chemical stability of these emulsion products by inactivating the peroxyl radicals.

SUMMARY

Speciality lipids like GLA, DHA, EPA, and AA are important dietary supplements both for infants and geriatrics and now are learnt to be of therapeutic values.

Plant materials like seeds, dry nuts, pulp and leafy vegetables and certain oleaginous microbes were screened for oil yield and unsaturated / essential fatty acid contents. Of the plant products avocado fruit pulp (*Persea americana* Mill. of family Lauraceae) and watermelon seeds (*Citrullus lanatus* (Thunb) Matsum and Nakai of family Cucurbitaceae), showed higher oil yield and promising EFA contents (OA and LA). These two plant materials were selected for further application studies especially for the presence of PUFAs, using solvent free Supercritical Fluid Extraction (SCFE)..

Among the microbials, the strain *Rhizopus oligosporous* MTCC 556 was selected as it gave higher biomass production as well as considerable amount of GLA which is a PUFA with very good nutraceutical values. The biomass, total lipid content and PUFAs values have been analysed using various standard microbial methods. Strain improvement by mutagenesis and optimization of culture conditions for higher oil and PUFAs yields in *Rhizopus oligosporous* MTCC 556 were also carried out. Fat Producing medium favoured the growth, biomass build up and lipid production than the normal cultivation medium PDB (Potato dextrose broth). Nitrogen limiting medium favoured more PUFAs content in the total lipids than nitrogen non limiting medium. Optimal killing rate was obtained when the culture was subjected to UV and EMS mutagenesis and upon low temperature growth, 21 putative mutants were obtained. Out of these three selective mutants possessed more lipid and GLA contents.

The analysis of avocado pulp oil and watermelon seed oil have confirmed the presence of significant unsaturated fatty acid contents such as oleic and linoleic acids in major proportions. The supercritical CO_2 extractions of freeze dried

avocado pulp and watermelon seeds have shown improved oil yields, higher concentrations of PUFAs and are free from any solvent impurities.

Using such supercritical oils of free dried avocado pulp and watermelon seeds, ***o/w emulsions*** were made to serve as stabilised nutraceutical delivery systems. Proteins soluble in aqueous phase effectively reduce the interfacial tension between oil and aqueous phases to form and stabilize oil-in-water emulsions. The physical and the chemical or oxidative stability of oil-in-water emulsions are greatly influenced by several factors like minimization of droplet size (by involving more energy during emulsification), non covalent interactions, interfacial tensions, the magnitudes of attractive and repulsive forces between particles and interfaces, inter-facial pressure differences and so on. Creaming, flocculation and coalescence are the major deteriorative influences the emulsion products face during storage, which could be curtailed upon by the usage of suitable emulsifiers and surfactants.

Rheological studies showed that Avocado pulp oil and watermelon seed oil with a very narrow range of flow behaviour index, with marginal shear-thinning behaviour. The emulsions made of these oils incorporating WPC (whey protein concentrate) as emulsifiers are pronouncedly non-Newtonian and shear-thinning.

Lipid oxidation is controlled in the model o/w emulsion systems studied, making them as valued food and nutraceutical products with desirable sensory attributes for a considerable period of storage. A reduction in the size of the oil droplets and the viscosity of the medium reduced the rate of creaming leading to physical stability. Upon storage at room temperature up to a period of 2 months they showed consistency in their rheological status. The minimal particle size range having an influence over the rheological properties controlled the shear stress responses of the emulsion products, both in vitro and in mouth condition which is as well in vivo, accounting for the bio-sensory realizations.

Moreover, a process for the preparation of ***edible-emulsion-gels*** loaded with health benefitting PUFAs for ready to eat purposes for health and nutraceutical

values has also been described. Oil-in-water (o/w) emulsions, made by using the $SCCO_2$ extracted avocado pulp oil and watermelon seed oil along with soy protein in aqueous medium forms an emulsion gel under microwave treatment. These emulsion-gels can be used in several food products in the form of thermo set gels, slurries, batters, disperses, solutions and emulsions which form a delivery system that can be eaten for health benefits to deliver the RDA (recommended daily allowance) of nutraceuticals.

Thus the importance of ω-3 and ω-6 fatty acids from selected plant materials and also from a microbial source has been explored through this study.

Bitechnolgically, the microbe was subjected to strain improvement through mutagenesis for improved oil and PUFAs yield and the plant materials were made to yield supercritical oils. The plant oils also rich with PUFAs were in solvent free and pure form and hence with whey protein concentrates gave ***o/w emulsions*** to serve as stabilized nutraceuticals. Also by food engineering, using soy protein concentrate as an emulsifier the supercritical oils were made into ***emulsion-gels*** for ready to eat purposes. Both the products, emulsions and emulsion-gels can thus serve as ***nutraceutical delivery systems*** for the RDA of PUFAs for human health benefits.

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Appendix-I

A brief write up on the patent submitted

An edible emulsion-gel composition and a process for the preparation thereof

By

Logaraj.T.V., Udayasankar K., Venkateswaran G. and Bhattacharya

Indian patent filing No. 2009/DEL/ 2009

FT/PATFL/PMC/2009

PMC
12-10-2009

Sub: Request of Patent Filing number

With reference to your request note no. NIL dated 12-10-2009 the details of patent filing as received from IPMD, CSIR is given below for the purpose of submitting it to Doctoral committee for thesis submission of Mr. Logaraj (UGC-FIP Teacher Research Fellow).

Patent Title	An edible emulsion-gel composition and a process for the preparation thereof
Inventors	Logaraj TV, UGC-FIP Teacher Research Fellow, Food Microbiology, Udaya Sankar, Scientist, Food Engineering, Venkateswaran G, Scientist, Food Microbiology, Suvendu Bhattacharya, Scientist, Food Engineering.
Patent Filing Number	**2009/DEL/2009**
Patent Filing Date	24-09-2009

Head

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To:

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Through Head, FM Dept.

Abstract

A process for the preparation of edible emulsion-gel useful as ready-to-eat purposes containing poly unsaturated fatty acids for health benefits has been described by using oil-in-water (o/w) emulsions, made by using the SCF CO_2 extracted avocado pulp oil and watermelon seed oil along with soy protein in aqueous medium which forms an emulsion-gel when subjected to microwave treatment. The developed emulsion-gel, the minimum oil content that has been extracted by SCF method is 55% and these extracted oils contain a minimum of 65% oleic acid, 9% palmitoleic acid 10% linoleic acid, respectively in case of avocado pulp oil. For watermelon seed oil, these values are a minimum of 15% oleic acid, and 60 % linoleic acid as the constituents. The emulsion-gel can be used in several food products in the form of thermo-set gels, slurries, batters, disperses , solutions and emulsions such that they form a delivery system that can be used as a substance for health benefit.

Appendix II

A research paper published in Food Chemistry journal vide Reference Number is attached.

Title : **Rheological behavior of emulsions of avocado and watermelon oils during storage**.

Authors: T.V Logaraj., Suvendhu Bhattacharya., K Udaya Sankar., and G.Venkateswaran

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