Basic lipid analysis of microalgae strain *Pectinodesmus* HM3, formation of phycosomes and *in vitro* drug delivery

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A thesis submitted in partial fulfillment of the requirements for the degree of

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IN THE NAME OF ALLAH, THE MOST BENEFICENT

AND THE MOST MERCIFUL

Read! And thy Lord is Most Honorable and Most Benevolent,

Who taught (to write) by pen, He taught man that which he knew not

(Surah Al-Alaq 30: 3-5)

Al-Quran

Declaration

I certify that this research work titled "**Basic Lipid Analysis of Microalgae strain *Pectinodesmus* HM3, Formation of Phycosomes and In-Vitro Drug Release**" is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Certified that the contents and form of thesis entitled "Basic Lipid Analysis of Microalgae strain *Pectinodesmus* HM3, Formation of Phycosomes and In-Vitro Drug Release" submitted by Dania Akram Kiyani, have been found satisfactory for the requirement of the degree.

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All efforts are in the name of **Allah (S.W.T)**, The Most Beneficent, and The Most Merciful, Who imparted in me the strength to overcome all hurdles in life and enlightened me with the greatest treasure of knowledge.

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ABSTRACT

Microalgal cells have been known to create a variety of substances like proteins, lipids, terepenoids, phenols, steroids, vitamins and minerals. Therefore the microalgae extracts are being purified or processed to produce multiple bioactive compounds or particles for use in the biotechnology industry. This study focuses on the lipid extract of microalgae strain *Pectinodesmus* HM3 and its general analysis. Lipid quantification was performed through gravimetric methods and qualification was done through FTIR and Gas Chromatography of the biodiesel created from the lipid extracted by ethanol and Folch solutions. The phospholipid or polar content of the lipid extract was used in the formation of algal liposomes or phycosomes. The phycosomes were characterized using various techniques. Curcumin loaded phycosomes were further used in drug delivery against brain cancer cell lines (u87). Results proved that the *Pectinodesmus* HM3 strain had a low lipid yield of 23% but the FAME content of the strain was 72%, this is a high biodiesel yield compared to majority microalgae strains present in the worlds algal database. The polar fatty acid content of the strain was found to be 20%. The unloaded phycosomes produced showed average size of 131 +/- 58 nm and average zeta potential of -34.1 +/- 4.74 mV. Negative charges on the phycosomes ensures that the particles will not form aggregates. The curcumin loaded phycosomes had an average size of 210 +/- 127 nm and zeta potential was an average of -48.6 +/- 5.28 mV. After drug loading, size of the phycosomes increased and zeta potential decreased as per previous studies on curcumin loading. Encapsulation efficiency of the phycosomes was 75%. Scanning electron microscopy images indicated dispersed and spherical phycosomes. Hemolytic activity of the phycosomes was 2% with a lipid

concentration of 0.025 mg/ml, this is within the measurement error range and hence the particles are safe for use.

The curcumin loaded phycosomes showed around forty percent higher anticancer activity as compared to unloaded phycosomes. These results indicate that phycosomes may be used as a potential drug carrier in the field of nanotechnology.

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Chapter 1

Introduction

1.0 Introduction

Microalgae have been harvested over time because of their ability to form diverse types of substances, all which can be used to produce various bioactive ingredients or products. Microalgae cells can be broken and processed for extracts like protein extracts, which have been shown to possess a high level of antioxidant activities, lipid extracts, which play a huge role in diet and formation of biodiesel fuels, phenolic extract, which have proven to have high antimicrobial, antioxidant and antiviral activities(Kim & Chojnacka, 2015), terpenoids and steroid extracts, which have shown to carry antioxidant, antiviral and anti-inflammatory activity (Kim & Chojnacka, 2015). Vitamins and mineral extracts play extensive parts in human diet and animal fodder (Kim & Chojnacka, 2015). This study will be focused on lipid extracts of *Pectinodesmus* HM3, specifically the application of polar fatty acids in liposomes and the acylglycerols for biodiesel production.

Liposomes are circular vesicles made up of lipid bilayers and are most commonly used as drug delivery systems. The vesicles membrane or boundary is made of phospholipids, which carry aqua philic heads along with an aqua phobic tail. The aqua philic head is attracted towards the liquid outside and the hydrophobic ends create a center away from the liquid surrounding them and hence spontaneously create vesicles (Prabhakar et al., 2019).. Liposomes play an important role in therapeutic drug transport methods because they help in controlled drug delivery, release and hydration. Changes in formulation of lipids, adjuvants and surfactants used allow lipids to carry multiple types of hydrophobic drugs, hydrophilic drugs, DNA, genes etc. Most of the phospholipids used for vesicle formation are present in cell membranes of cells of

various organisms (Prabhakar et al., 2019). Phosphatides with choline attached are usually called lecithins or phosphatidylcholines and phosphatides with ethanolamines attached are usually called cephalins. Phosphatidylethanolamines are principal components of bacterial cell membranes and phosphatidylcholines are principal components of animal cell membranes. Liposomes occupy a large sector of the global pharmaceutical market primarily for drug delivery reasons. According to a research conducted by Cientifica, drug delivery through nanotechnology has a current market of $136 billion, out of which nanocarriers occupy a $54.4 billion industry. Liposomes have a current market of $15billion in this empire (Prabhakar et al., 2019)..

Liposomes and liposomal based products dominate in USA with 41% demand in total world market, followed by Taiwan and Korea at 19% and 15% respectively. Next in line comes China with a 10% demand and Hong Kong and India bring up at the end (Prabhakar et al., 2019).

Liposome based drugs and products are extremely expensive because of their high cost of production, the major components of their production are either natural phospholipids (extracted from organisms and purified) or synthetic phospholipids (created industrially). Natural phospholipids are relatively cheaper and have a track record of being more biocompatible and less toxic (Prabhakar et al., 2019). Purified natural phospholipids are used in dermal, oral and parenteral liposomal products but the upscaling process cost a lot of money, for example 1 kg of purified natural phospholipids from soyabean costs 980 Euro (Aisha et al., 2014). Most liposomal applications are limited due to scaling up process and the cost of phospholipids. The cost of phospholipid is limiting even in the case of high value ingredients and bioactive

compounds. Still, majority studies use the same raw expensive materials as pharmaceutical industries like synthetic phospholipids or purified natural phospholipids (Yokota et al., 2012) and these phospholipids are produced commercially from only a few major industries in the world. Hence preparing liposomal systems is a continuous challenge in biological engineering (Gupta et al., 2008).

Although crude phospholipids are regularly extracted from multiple organisms like archaea (Benvegnu et al., 2009), bacteria (Gupta *et al.*, 2008), plant cells (Neill & Leopold, 1982), animal cells (Bittame, A., Lopez, J., Effantin, G., Blanchard, N., Cesbron-Delauw, M., Gagnon, J. and Mercier, 2016), egg (van Nieuwenhuyzen, 2015) and even milk fat globules (van Nieuwenhuyzen, 2015) and are being used in multiple liposomal studies, there is still no view of algal lecithin in the market or its reports in any literature for being used in liposomal studies (van Nieuwenhuyzen, 2015).

While working on microalgae the main aim was to analyze the lipid content and biodiesel potential of the strain. The major reason behind climate change, as identified by World Health Organization, is global warming caused by production of glasshouse gases from fossil fuel use. It is the need of the hour to develop techniques and processes which reduce the impact of fossil fuel emissions on global warming. On one hand using of fossil fuels is one problem, on the other hand the consistent depletion of fossil fuels is the problem. Unless alternative energy sources are created, we will be, very soon, looking at a future without energy. One solutions to this entire energy crisis is biodiesel .There are many advantages of extracting biodiesel from microalgae and using it as biofuel, it is nontoxic, renewable and biodegradable (Takisawa et al., 2014).

Most microalgae species have an unsaturated fatty acid content of 50-70% and few even 80% (B.braunii). This is higher than most plant lipid content or vegetable oil content. This makes Algae favorable as a source of biodiesel production. The most obvious benefit of expending microalgae for biofuel is that the amount of non-arable land required to grow algal biomass is only 6 million hectares i.e. 0.4% of the current total of fertile land on the earth (Khan et al., 2018).

Rationale of the Study:

High demand of lecithin in nanotechnology sector and lack of economical options for phospholipid has provided us with microalgae as a cheap and ecofriendly alternative source for lipid to be used in liposomes.

Aims and Objectives of the Study:

In this project we will strive to extract the lipid content of microalgal biomass from *Pectinodesmus* HM3, perform quantitative analysis through gravimetric method and qualitatively analyze the lipid extract through FTIR and GCMS analysis of the biodiesel. After minor treatment of the lipid extract, we will study its potential application in the development of phycosomes (algal liposomes).

The objective of this study was to explore the use of microalgae liposomes or phycosomes, prepared by lipid extraction of specie *Pectinodesmus* HM3, and relate them as possible vectors or carriers for cytoplasmic delivery of particles to cancer cells.

Chapter 2

Literature Review

2.1 Algae

Algae has no proper taxonomic standing but the term is used to identify polyphyletic, photosynthetic, non-cohesive organisms(Barsanti & Gualtieri, 2014)..

2.1.1 Organism structure

Algae are organisms which are quite similar to plants in their defense and storage strategies. The similarities end there. They don't have any defined features like roots, stems or leaves, or any other vascular tissues. Many seaweeds look like plants, but they still lack differentiation and specialization in their cells. Algae only carry fertile cells but no proper reproductive structures and no embryo formation occurs. They have both monogenetic and digenetic lifestyles (Barsanti & Gualtieri, 2014).

2.1.2 Taxonomic classification

There are estimated to be more than one million specie of algae. According to the AlgaeBase the recorded number of algal species within the database are 72,500. Algae may be classified into microscopic singular cells called microalgae, or macro algae which includes macroscopic cells in the forms of colonies of cells arranged as branches, circular bunched congregations or leaf like blades. Their sizes range from 0.2 um – 2.0 um as in the micro algal specie of picoplankton or to 60m frond length as in the macro algal specie of kelps. They may be prokaryotic or eukaryotic in nature, depending on their structure and morphology. The prokaryotic members of algae are grouped into the Kingdom Bacteria, within the Phylum Cyanobacteria, Class Cyanophyceae. The eukaryotic members of algae are grouped within the Kingdom

Plantae 9 (4 phyla), Kingdom Chromista (4 phyla) and Kingdom Protozoa (2 Phyla) (Barsanti & Gualtieri, 2014).

2.1.3 Distribution across environmental ecosystems

Algae, although commonly aquatic, can also be sub aerial. The aquatic ones thrive in various sorts of water bodies like ponds, lakes, rivers, streams. They are not very sensitive to pH, temperature, O2, CO2 or turbidity fluctuations and can survive in various ecosystems. Algae can be planktonic or benthonic, i.e. freely suspended in water bodies or attached to a specific substrate. Other than that, their region of growth within water bodies classifies them into superalitoral (at high tide), intratidal (where the tide touches the shore), sublittoral (below water level). Subaerial Algae species live on land, in places such as animal fur, tree trunks, hot springs, snow banks and even within desert rock. Where aquatic algae serve as a source of nutrition in aquatic ecosystems and more than 50% of the oceans oxygen content, subaerial algae serve various functions like reducing soil erosion, increasing water and nutrient retention, and also converting rock into soil etc. Most of their subaerial existence involves symbiotic relationships like lichens (Barsanti & Gualtieri, 2014).

2.2 The present status of microalgae and biotechnology

Microalgae is a highly neglected resource and area of science, of all the species discovered only 15 are in consistent use. The field of micro algal culture biotechnology is rapidly expanding due to the discovery of innovative processes and products. There

is a huge gap in food, pharmaceuticals and biofuels industry which micro algal biotechnology is trying to fill (Raja et al., 2008).

Now there are more than 110 micro algal commercial producers in the Asia-Pacific region, with 9/10 of algal production plants in Asia alone. Annual output from these cultivation plants is approximately 500 tons of dried algal biomass. The worldwide micro algal production is 5000 lots per year of dried mass (Raja et al., 2008).

Currently micro algal biomass is being used in production of various products and processes:

i) Direct usage of algal biomass:

Algal biomass is being used as or in human food, animal fodder or as food supplements for example in wine, noodles, beverages, cereals etc.

ii) Indirect usage of algal biomass:

Extracts of Algal Biomass are used as biofuels and bio products.

Biofuels include solid fuels in the form of biochar, liquid fuels in the form of bioethanol, biodiesel or vegetable oils. Gaseous forms of biofuels include biohydrogen and bio syngas.

Bio products include polyunsaturated fatty acids, antioxidants, coloring agents, vitamins, cosmetic agents, anticancer drugs, antimicrobial drugs and even drugs against diseases like cancer, AIDS and Cerebro vascular diseases. More than 75% of algal biomass is used in the health industry for manufacturing tablets, pastilles, powders and capsules (Khan et al., 2018).

2.3 Biodiesel

Climate change is the most critical global environmental problem. The potential threat of global climate change has increased, and much of the risk has been attributed to greenhouse gas (GHG) emissions by fossil fuel usage (Wuebbles et al., 2001). It has become necessary to develop techniques and to adopt policies to minimize impacts of global warming that result from the increase in anthropogenic GHG emissions. In 1997, the Kyoto Protocol called for a 5.2% reduction in GHG emissions from 1990 levels (Wang et al., 2008), and various technologies have been investigated to meet this goal. Another problem is a future energy crisis due to depletion of fossil fuels. The continuous use of fossil fuels as a primary source of energy is widely recognized to be unsustainable (Khan et al., 2009). Therefore, it is absolutely necessary to ensure new energy resources before the world is confronted with an energy crisis.

Biodiesel is a widely known alternative fuel. It can be produced from oils derived from plants, animals, or microbes (Graboski and McCormick, 1998), and currently represents 82% of total biofuel production (Bozbas, 2008). Biodiesel is usually defined as the monoalkyl esters of long-chain fatty acids derived from transesterification of renewable feedstocks(Takisawa et al., 2014).

There are three biofuel generations(Khan et al., 2018). The biofuel generation of first produced was from plants like rape seeds, palm oil, soybeans and sunflower seeds. The vegetable extract of these plants are Trans esterified to produce biodiesel (Khan et al., 2018). The problem with the first generation biofuels is that there is not enough arable land available to provide more than 10% of a country's biodiesel needs. Production of these biofuels also causes a rise in the cost of animal feed and consequently human

food. The second generation of biofuels is produced from cellulose extract of sawdust, agricultural debris (corn stalks, fast growing grasses, woody materials), construction debris etc. This type of biofuel is advantageous as it does not cause any interference in food production, these crops can be grown on land which is unfertile as well. The last generation of biofuels includes fuels formed from algal and cyanobacterial biomass (Khan et al., 2018).

The many advantages to integrate the use of microalgae as a basis for biofuel production include the fact that it is nontoxic, renewable and biodegradable (Takisawa et al., 2014). Most microalgae species have an unsaturated fatty acid content of 50-70% and few even 80% (B.braunii). This is higher than most plant lipid content or vegetable oil content. This makes Algae favorable for biodiesel production. It can be used in the motor car industry with little to no modifications, it can even be mixed with petroleum based fuel and used with no ramifications. Algal biofuels have low levels of Sulphur emission and oxygen levels of 10-45% during combustion while the exact opposite happens during fossil fuel combustion. They also have a high carbon dioxide fixation rate. It is reported that 1 kg of algal biomass can fix approximately 2 kg of carbon dioxide (Khan et al., 2018). The most obvious benefit of using microalgae is that the amount of non-arable land required to grow algal biomass is only $6x10^6$ hectares i.e. 0.4% of existing total of fertile land on the earth. As most biodiesel efficient algae species are marine, no freshwater would be required in culturing. Culturing can be carried out in saline / brackish water, shallow ponds or even hardpan soil (Arun & Singh, 2012). The only disadvantage of algal biodiesel is because of the high maintenance, operational, conversion and harvesting cost (Khan et al., 2018).

2.3.1 Biodiesel Production

Many species of plants and algae create a large amount of oils. Most of these oils are high in unsaturated fatty acids also known as TAGs (mainly triacylglycerols). Linoleic acid, arachidonic acid, docasahexaenoic acid, gammalinolenic acid and eicospentaenoic acid are some examples of commonly found unsaturated fatty acids in algae. The process of formation of biodiesel is called transesterification or alcoholysis. In this reaction methanol is combined with TAG's in the existence of a catalytic agent to produce biofuel or biodiesel and glycerine (Arun & Singh, 2012). The reaction is shown below.

TRIGLYCERIDE (PARENT OIL) +ALCOHOL= GYCERIDE + BIODIESEL

2.4 Lipid Production in Microalgae

The reason algae can survive and adapt to multiple environment types is mostly due to their ability to modify their cellular lipids in response to environmental conditions. The lipids produced are usually neutral lipids, hydrocarbons, sterols, polar fats, prenyl derivatives and wax esters (Kim & Chojnacka, 2015) .

During prime growing situations the microalgae yields fatty acids principally for the membrane (Hu et al., 2008). The membrane lipids include glycosylglycerides and phosphoglycerides. Polyunsaturated fatty acids like palmitic acid and oleic acid undergo aerobic desaturation and chain elongation to form the glycerolipids in the membrane (Hu et al., 2008).

During unfavorable environmental conditions, specifically nitrogen limitation or an excess of carbon, there is a shift in algae metabolism, this causes an increase in neutral lipid production, mainly triacylglycerol's. These serve as a store of carbon and energy within the cell (Arun & Singh, 2012).

There are two steps before a lipid can be a part of the algal cell. The first is **fatty acid synthesis**, the second is **TAG synthesis**. A small view into how they are biosynthesized is as follows.

Fatty acid biosynthesis happens mostly in the microalgae cell chloroplast. This process includes a series of reactions. During reaction 1, Acetyl Coenzyme A is converted to the s –malonyl derivative also known as Malonyl CoA , the Malonyl ACP produced converts into 3 Ketoacyl ACP through a series of condensations i.e. (reaction 4) reduction, (reaction 5) dehydration and (reaction 6) reduction again. In the end of the process there is the creation of a C_{16} and C_{18} fatty acid, in some cases both may be created. Figure 2.1 shows a diagrammatic representation of the method (Hu et al., 2008).

Triacylglycerol biosynthesis occurs when fatty acids go through the glycerol pathway. Two fatty acids are relocated from Acetyl CoA to position 1 and 2 of G3P, glycerol tri phosphate. This forms phosphaditic acid (PA). Next, removal of phosphate from PA creates a molecule of DAG, diacylglycerol. Finally, fatty acid number three is relocated to the site 3 of the molecule DAG. Figure 2.2 represents a diagrammatic chart of the process.

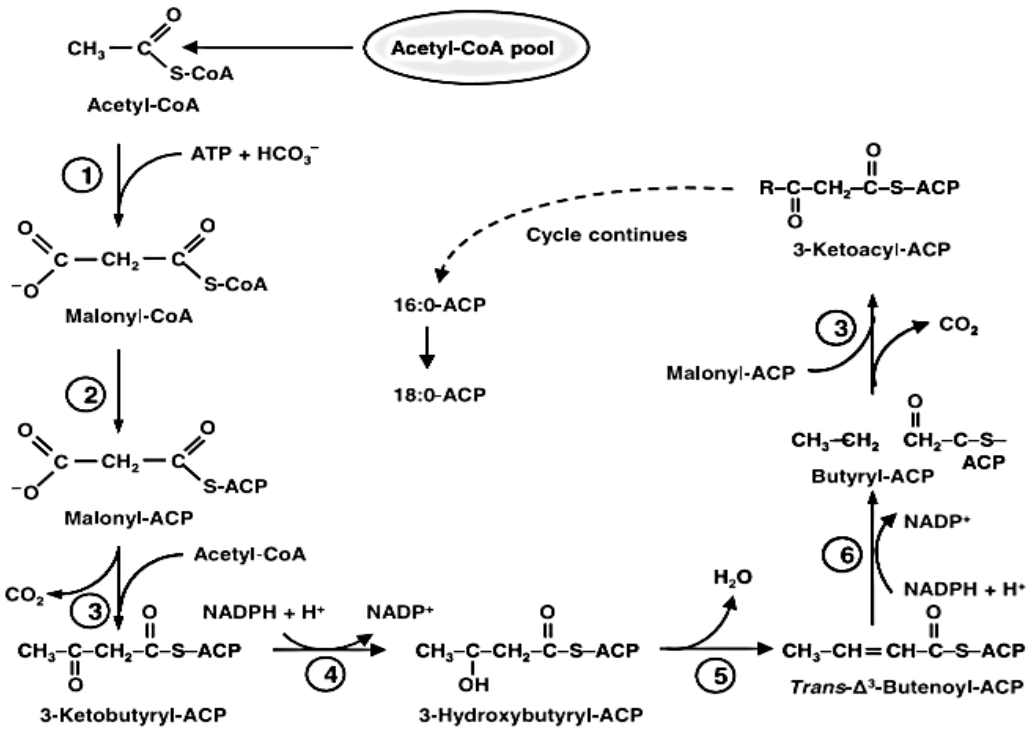


Figure 2.1 : Acetyl-CoA is converted into a C_{16} and C_{18} fatty acid through a series of condensation reactions (Hu et al., 2008).

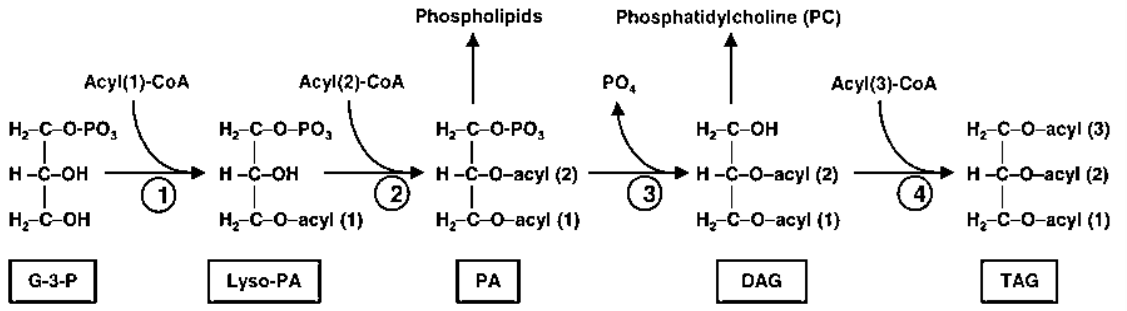


Figure 2.2: Triacylglycerol is formed by glycerol 3 phosphate condensation with 2 fatty acid molecules. This forms lysophosphatidic acid. A series of reactions, influenced by G3P acyltransferase to create TAG, diacylglycerol and phosphatidic acid. (Takisawa et al., 2014) (Hu et al., 2008).

2.5 Lipid Extraction in Microalgae

The critical point for any lipid related micro algal process, is the collection of a strain that gives a suitable fat yield, suitable meaning appropriate to the task at hand for example, if lipid is required for biodiesel formation, a microalgae strain with a higher amount of unsaturated fatty acids or TAGs (mainly triacylglycerol) is needed. On the other hand if algae lipid is required for use in the cosmetics industry there will be need of algae strains with other lipid specifications (Han et al., 2011)

A view on types of lipids existing within Microalgae are represented in figure 2.3.

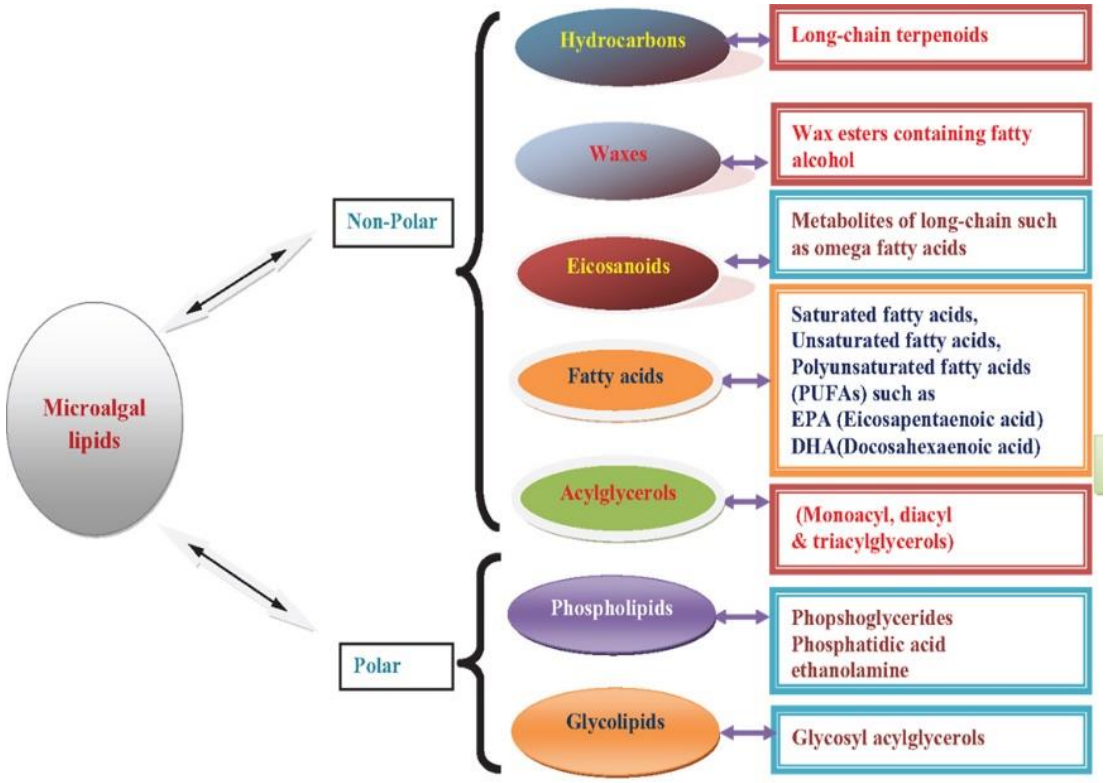


Figure 2.3: Classes of lipids within microalgae cells (Ranjith Kumar et al., 2015)

2.5.1 Algae Cell Breakage

The algae cells must be disrupted for the extraction of lipid. Major cell disruption methods being used include bead beating, expeller press, ultrasonic waves, microwaves, electroporation, osmotic pressure methods, chemical disruption and enzymatic disruption (Ranjith Kumar et al., 2015) .

2.5.2 Lipid Extraction Methods

Major lipid extraction methods being used include using organic solvents like hexane, chloroform, benzene, others methods employ solvents like supercritical CO^2 and liquid CO^2. Some methods employ solvents like ionic liquids which are basically organic salts with melting points below 100 degrees (Z. Chen et al., 2018).

2.6 Lipid Content analysis in Microalgae (Quantification / Qualification)

Lipid content analysis was started in the early 1930's. Now, the processes for content analysis have become quite accurate and evolved. The highest lipid percentages to be reported are from the microalgae *Schizochytrium* sp with a 50-77% lipid content and *Botryococcus braunii* sp with a 25-75% lipid content (Chisti, 2007).

Now, coming to various methodologies used for lipid content analysis. This process of lipid content analysis involves two parts (Han et al., 2011).

i) Quantification:

a) Gravimetric analysis

In this method lipids are extracted using any preferred method, the mixture of the solvent and biomass is centrifuged, algal biomass is discarded, the solvent is evaporated and the lipid is weighed on a weighing balance. The advantages of this method include its simplicity, the disadvantage includes the fact that this method requires the researcher to have a large amount of algal biomass and hence this process is a little time consuming, especially if the number of samples is more.

b) Staining Analysis

In this method, we use a lipophilic staining dye and a fluorospectrophotometer to detect the presence of lipid. The device is used to take readings before and after staining. The dyes typically used are Nile Red and BODIPY. Although this method is less time consuming, there is a slight problem with the accuracy level because the lipophilic dyes are more prone to detect neutral lipids then charged lipids (polar or non-polar)

c) Colorimetric SPV method

Colorimetric sulfo-phospho-vanillin (SPV) method is used for quick lipid quantification. It requires a small amount of sample, and so it is easier to process larger amounts of samples. The difference in coloration allows us to compare the lipid contents. Drawback this method is in sample preparation.

d) TD-NMR method

In this method, the different relaxation times of hydrogen nuclei are used to compare the substance being analyzed. For example, the relaxation time of proteins is short, and the relaxation time of lipids is about a hundred times less then proteins. This method is quite accurate and requires very little sample amount. But the cost of NMR is higher than other methods.

e) TLC/HPLC method

This method uses chromatography to compare retention times of different lipids to standards. This method has the added advantage of being qualitative as well as quantitative. This is because the standards used for comparison can indicate which lipid is present where in the chromatogram. This method is also slightly more expensive, due to the cost of chromatograph plates and standard.

ii) Qualification:

In this part of content analysis, the analysis of type of lipid is the focus. Major methods used for this are Thin-layer Chromatography (TLC), High-performance Liquid Chromatography (HPLC), Near Infrared Reflectance (NIR) and Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography Mass Spectrometry (GC-MS). The simplest method is Fourier Transform Infrared Spectroscopy (FTIR) due to its easy sample preparation and cost effectivity (Han et al., 2011).

2.7 Current trends in Nano Medicine

Nano medicine is a newly emerging and rapidly developing field of health sciences.

This field employs the use of nanotechnology in the field of medicine. The most ground breaking discoveries of this field include microarray tests, biosensors, tissue engineering, drug delivery systems(Soares et al., 2018). Treatments like biological agents, chemotherapeutic agents and immunotherapeutic agents for various chronic diseases have been discovered in the field of nanomedicine. Nanomedicine has helped revolutionize old and new medications. These small, circular particles have been proven to be very useful, because these can be maneuver between small articles, veins and membranes without disrupting normal bodily functions. Nanoparticles are used keeping in mind their magnetic, structural, mechanical, chemical, biological and electrical properties along with their possible cytotoxic levels(Soares et al., 2018)..

In Nano delivery systems, nanomaterials of the size range of 10-100nm are used to deliver drugs or beneficial agents to directed sites within the body in a controlled way. Materials being used in Nano delivery systems include chitosan ,alginate, xantham gum , cellulose, liposomes ,polymeric micelles, metals like gold , silver, iron , drugs in the form of nanocrystals, semiconductor nanocrystals known as quantum dots and even Nano peptides in the form of nanoparticles (Soares et al., 2018).

2.8 Liposomes as drug delivery vehicles

Discovered in 1960 by Alec Bangham, liposomes are bilayer lipid vesicles composed of steroids or phospholipids. Nano liposomes range from 45 nm- 450nm. These are considered to be one of the best drug delivery systems because their membrane structure is similar to membrane structure of cell membranes. They can be loaded with various types of drugs and are also biocompatible and biodegradable.

Liposomes, being minute spherical vesicles with both hydrophilic and hydrophobic properties, are the best structures for drug transfer. The characteristics of liposomes differ depending on the type of lipid used, the charge the vesicle carries on its surface, the technique of making or forming the vesicle, the size etc. For example, saturated phospholipids exhibit rigid bilayers, unsaturated phospholipids give stable and flexible bilayers. Phospholipids are known to spontaneously form closed structures in aqueous environments (Akbarzadeh et al., 2013).

Liposomes are being used as carriers in the field of cosmetics and pharmaceuticals. In the agriculture and food industry, they are being applied to trap unstable compounds, like antioxidants, antimicrobials, bioactive elements and flavors. Liposomes are also used to trap compounds, keep them from decomposing or effecting their surroundings before they reach their destination of release.

This liposomal encapsulation technology is the newest technique used in drug delivery systems. Besides having all these advantages liposomes have some disadvantages or drawbacks as well, like short half-life, high production costs and low solubility (Akbarzadeh et al., 2013). Liposome based drugs and products are extremely expensive because of their high cost of production, the major components of their production are either natural phospholipids (extracted from organisms and purified) or synthetic phospholipids (created industrially). Natural phospholipids are relatively cheaper and have a track record of being more biocompatible and less toxic (Prabhakar et al., 2019). Purified natural phospholipids are used in dermal, oral and parenteral liposomal products however the upscaling process costs a lot of money, for example 1 kg of purified natural phospholipids from soya bean costs 980 Euro (Aisha et al., 2014).

Most liposomal applications are limited due to the scaling up process and the cost of phospholipids. The cost of phospholipid is limiting even in case of high value ingredients and bioactive compounds. Still, majority studies use the same raw expensive materials as pharmaceutical industries like synthetic phospholipids or purified natural phospholipids (Yokota et al., 2012) and these phospholipids are produced commercially from only a few major industries in the world. Hence preparing liposomal systems is a continuous challenge in biological engineering (Gupta et al., 2008) .

And although crude phospholipids are regularly extracted from multiple organisms like archaea (Benvegnu et al., 2009) (Gonzalez et al., 2009) (Ameri et al., 2016), bacteria (Gupta *et al.*, 2008) (Kargar, Moghimipour, Ramezani, & Handali, 2014), plant cells (Neill & Leopold, 1982) (Fork, van Ginkel, & Harvey, 1981), animal cells (Bittame *et al.*, 2016) (Downing, Abraham, Wegner, Willman, & Marshall, 1993), egg (van Nieuwenhuyzen, 2015) and even milk fat globules (van Nieuwenhuyzen, 2015). Although many of these are being used in multiple liposomal studies there is still no significance of use of algal lecithin in the forms of liposomes (Van Nieuwenhuyzen, 2015).

All of these papers did not use commercial or synthetic lipids however the lipids that have been extracted directly from the source organism i.e. crude lipids which are impure. The logics given behind this were mainly two. The first was to reduce production cost as industrially synthesized lipids are quite expensive, the second was that many extracted lipids have added functionality as compared to industrially synthesized lipids. For example, (Gonzalez et al., 2009) established that the total polar

lipids extracted from *Halorubrum tebenquichense* elicit antibody and cell mediated immunity and act as adjuvants.

Chapter 3

Materials & Methods

3.0 Materials and Methods

All chemicals used in the study were prepared according to standard WHO, FAO or Molecular cloning by Sambrook and Russel. Autoclaved distilled water was used to make dilutions of already sterilized stock chemicals. Unless otherwise stated. Table 1 enlists all the reagents and chemicals used in this study whereas Table 2 enlists the equipment.

Table 3.1: Chemicals used in the study

S.No	Chemicals	Company
Reagents used for Algal Culture Growth		
1	Bold Basal Media (BBM)	Made in Lab
2	Distilled Water	Local
3	NaOH pellets	Sigma Aldrich
Reagents used in Algae Processing		
1	Distilled Water	Local
Reagents used in Phytochemical Analysis		
1.	chloroform	Fischer Scientific
2.	10% ammonium solution	Sigma Aldrich
3.	10 % NaOH solution	Fischer Scientific
4.	distilled water	Local
5.	10 % ferric chloride solution	Fischer Scientific
6.	2N sodium hydroxide (NaOH)	Sigma Aldrich
7.	5% ferric chloride solution (FeCl3)	Sigma Aldrich
8.	Sulfuric Acid	Fischer Scientific

Reagents used in Lipid Extraction		
1.	Folch Solution	Sigma Aldrich
2.	Ethanol	Sigma Aldrich
3.	H_2SO_4 Solution	Sigma Aldrich
4.	NaOH Solution	Fischer Scientific
5.	Deionized Water	Local
6.	Hexane	Sigma Aldrich
7.	Distilled Water	Local
Reagents used in Biodiesel Formation		
1.	Methanol	Sigma Aldrich
2.	Sulfuric Acid	Fischer Scientific
Reagents used in Liposome Formation		
1.	Tween80	Fischer Scientific
2.	Cholesterol	Fischer Scientific
3.	Chloroform	Fischer Scientific
4.	PBS	Fischer Scientific
5.	Curcumin	Local
Reagents used in Hemolysis Assay		
1	Blood	Local
2.	Saline	Sigma Aldrich
3.	Triton	Fischer Scientific
Reagents used in MTT Assay		
1.	3-(4,5-dimethylthiazolyl-2)-2,5-	Sigma Aldrich

	diphenyltetrazoliumbromide (MTT)	

Table 3.2: List of Equipment used in the study

S.No	Instrument Name	Company	Model No
1	Centrifuge Machine	Sigma	1-16K
2	Incubator	K&K Scientific Supplier	K-DI8
3	Weighing balance	Sartorius	CP-153
4	Ph meter	HANNA Instruments	HI2211-01
5	Fridge	Dawlance	9175WBM
6	Vortex	SCILOGEX	MX-S
7	Autoclave machine	Hiclave	HV-25
8	Centrifuge Machine	Sigma	3-18K
9	Centrifuge Machine	DLAB	D1008
11.	Nano photometer	Implen	Pearl
12.	GC-MS	Shimadzu	QP 2010
13.	FTIR	Shimadzu	IRAffinity-1S
14.	SEM	Joel	56480LA SEM
15.	Zeta Sizer	Malvern Instruments	Zetasizer Nano ZS 90
16.	Magnetic Stirrer	VELP SCIENTIFICA	V230
17.	Optical Microscope	OLYMPUS	CX43
18.	Bath Sonicator	Elmasonic	E30H

20.	Water Bath	Memmert	SV 1422

3.1 Method of preparation of reagents

i) Bold Basal Media:

Bold Basal Medium (BBM) is an inorganic salts medium. A 1L water bottle was kept atop a magnetic stirrer, the solution stirred at 400rpm at 70 C°, while the ingredients listed in table 3.3 were added according to recipe mentioned in (Khalid et al., 2017).

Table 3.3: Recipe for Bold Basal Media

Ingredients	Concentrations (mg/L)
$NaNO_3$	95.2
$CaCl_2$	25
$MgSO_4.7H_2O$	75
K_2HPO_4	75
KH_2PO_4	175
NaCl	25
EDTA	50
$FeSO_4.7H_2O$	4.98
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.22
$MnCl_2.7H_2O$	14.4
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

KOH/NaOH	31
H_2SO_4	0.001 ml

The PH of the solution was adjusted between 6.4 and 6.8 with the help of NaOH pellets.

ii) Phosphate Buffered Saline:

PBS solution to be used in section 3.10, for phycosome formation was prepared using one tablet of PBS (Fischer Scientific), which was dissolved in 100ml of distilled water. The PH was adjusted to 7.4. The solution was transferred to a reagent bottle, autoclaved at 121ºC for 15 minutes and allowed to cool.

iii) Folch Solution:

Folch solution to be used in section 3.5.2.1, lipid extraction, was prepared using chemical grade chloroform and methanol which were mixed in the ratio of 2:1 (v/v) (Kumari et al., 2011), in a sterile bottle. The bottle was sealed and kept in the dark, at room temperature.

3.2 Sample Collection

Five Microalgae strains are currently present at the Nano Biotechnology Lab in Atta-ur-Rehman School of Applied Biosciences, NUST, and Islamabad. These strains have been recently identified as:

Strain 4: *Dictyosphaerium* sp. strain (DHM1) (Khalid et al., 2017)

Strain 5: *Dictyosphaerium* sp. strain (DHM2) (Khalid et al., 2017)

Strain 6: *Pectinodesmus* sp. strain (PHM3) (Khalid et al., 2017)

IESE: *Dictyosphaerium* sp. strain (DHS)

FFC: *Dictyosphaerium* sp. strain (DHSYM)

3.3 Inoculation in Bold Basal Media

Bold Basal Media is a growth media comprised of inorganic salts used for the growth of microalgae strains and other plankton like freshwater algae. Bold Basal Media is considered one of the best media for microalgae growth, especially in terms of biomass production (Klinger & Garoma, 2018).

Each microalgae strain was inoculated in a separate glass tank. Bold Basal Media was prepared according to recipe mentioned in section 3.1.1 Each 1L of water used to inoculate the algae contained 20 parts of inoculum, 50 parts of BBM and 920 parts of purified water according to ratio mentioned in (Khalid et al., 2017). Growth was aided by provision of air through pumps at the rate of 3.5L/min. Illumination was provided through 36W TLD fluorescent lamps 24/7 to keep the photosynthetic process going. The cultures were allowed to grow for 1 month, with pH maintained at 7.

3.4 Harvesting of Microalgae

The culture tanks were removed from their setups. The algae- water mixture was put into centrifuge tubes and spun at 6000rpm to settle the biomass. The supernatant was decanted and the pellet was again shaken and centrifuged with purified water to remove extra salts. After washing, the pellet was shifted into a glass dish, it left to dry

overnight in an incubator at 70C° to remove all moisture. The dried biomass was scraped, weighed, placed in a sealed container and frozen for further experimentation.

3.5 Lipid Extraction

3.5.1 Comparison of lipid yield of microalgae strains

Lipid was extracted from algal strains, mentioned in section 3.2, through Bligh and Dyers method using a small amount of biomass of each algal strain. The strain with the highest lipid quantity was used for further experimentation i.e. *Pectinodesmus* sp. strain (PHM3) (Khalid et al., 2017)

.

3.5.2 Extraction with different solvents

Before extraction the algal biomass was sonicated for 10 minutes at high intensity using a probe sonicater. Ultra-sonication has already proved to enhance lipid yield. This is because it increases the vibrations in the cell walls and helps destroy the cell structure. After the cells are lysed the lipids are more easily dispersed in the solvent (Naveena et al., 2015).

3.5.2.1 Folch Extraction

Modified versions of Bligh and Dyer method was employed for the withdrawal of fats from the microalgae cells. The method creates system carrying the solvents of water, methanol and chloroform in various ratios .The chloroform dissolves nonpolar lipids, methanol and water dissolve polar lipids or neutral fats (Bligh & Dyer, 1959).

Modified version of (Kumari et al., 2011) was used for lipid extraction, in which five parts of chloroform ,four parts of distilled water and , 10 parts of methanol were added to a cylinder holding 1 g of dried microalgae biomass. The samples were placed in a shaking incubator at 37ºC overnight. This allowed the solution to thoroughly dissolve the lipids. After this time period, equal amount of folch solution and aqua was further incorporated to the blend.

The cylinders were then centrifuged at 6000rpm for 10-15 minutes. Three layers were created, the upper being methanol and water with polar lipids, the middle layer with cell debris, and the final layer with chloroform carrying the major nonpolar lipid content. Supernatant must be discarded but in this case a little amount of sample was kept for FTIR analysis. The debris was also discarded and the lipid layer was dried, weighed and kept at 4ºC, dissolved in chloroform, for further analysis and experimentation. Experiment was run in triplicates for precise results.

3.5.2.2 Ethanoic Extraction

A modification of ethanoic extraction put forward by (Han et al., 2011) and (Fajardo et al., 2007) was used in which 5 g of algae mass was mixed with 70 ml of Ethanol (95% v/v) in a container, the container was kept at 60-70ºC , 400 rpm , over a magnetic stirrer for 8 hours approximately. It was then allowed to cool and the solution was transferred to centrifuge cylinders and rotated for 15 minutes at 6000rpm for to pellet debris. The extract was passed through a membrane filter to purify. The lipid layer was dried, weighed and kept at 4ºC, dissolved in chloroform, for further analysis and experimentation. Experiment was run in triplicates for precise results.

3.5.2.3 Acid Base Extraction

Modified versions of Acid Base extraction initially developed by (Duongbia et al., 2018) and (Sathish & Sims, 2012) were used for chemical extraction of lipid from cells. There were two major steps of this type of extraction. The first step was acid base hydrolysis in which 2ml of 1M Sulfuric Acid solution was added to 200mg of algae dry mass in a falcon tube. The tubes were put in a shaking water bath at a temperature of 90ºC for half an hour. The tubes were mixed by hand every quarter of an hour for correct distribution of acid solution. Subsequently, 2ml of 5M Sodium Hydroxide solution was added to the tubes, the tubes were shaken and heated to 90C° for 30 minutes. The tubes were shaken at every 15 minutes for proper dispersal of base solution. The samples were cooled and centrifuged to pellet the residual algal biomass (Duongbia et al., 2018).

The resulting supernatant phases were removed and collected from each sample in separate tubes and the residual hydrolyzed biomass pellet were vigorously mixed with 2 mL of deionized water. The resulting suspension was re-centrifuged and the liquid phase again removed and added to the corresponding tubes containing original supernatant. The second step was chlorophyll precipitation. To the supernatant phases collected in the previous step, Three ml of 0.5M H_2SO_4 is added to the supernatant we collected previously, this created a solid green precipitate. This is because when the pH goes under seven, the fatty acids revert into free form and create a white precipitate. Lipids form a complex with the precipitated solids. The mixture is centrifuged, supernatant is removed and the precipitates are collected. Five parts of chemical grade

hexane is added to the precipitate in the tubes. The tubes are then heated in a water bath at 90ºC, allowing the lipids to dissolve in the hexane (Sathish & Sims, 2012).

The hot samples from the last step are chilled or cooled in the fridge or in air and then centrifuged. The hexane portion of the sample is removed and shifted to clean flasks. The solid phase precipitate is removed from the system as a pellet of solid debris. The extract was passed through a membrane filter to purify. The hexane was dried, the lipid layer weighed and kept at 4ºC, dissolved in hexane again, for further analysis and experimentation. Experiment was run in triplicates for precise results.

3.5.3 Extraction using different mechanistic approaches

3.5.3.1 Soxhlet Apparatus

A variation of a protocol from (M. Chen et al., 2011) was used in which ten parts of algae was crushed and put in the thimble of the soxhlet apparatus. 100ml of ethanol was added in the apparatus. Reflux was run at 70ºC for 12 hours. Ethanol was added according to required volume. After the termination of the time period, the solvent was separated and dried. The lipid layer was weighed and stored.

The same experiment was run, only with folch solution as the solvent.

3.5.3.2 Continuous Agitation

A modification of protocol adopted from (M. Chen et al., 2011) and (Fajardo et al., 2007) was used in which 5 g of algae mass was mixed with 70 ml of Ethanol (95% v/v) in a container, the container was kept at 60-70ºC , 400 rpm , over a magnetic stirrer for 8 hours approximately. It was then allowed to cool and the solution was transferred to

cylinders and rotated at 6000rpm for quarter of an hour to pellet the debris. The extract was passed through a membrane filter to purify. The lipid layer was dried, weighed and kept at 4ºC, dissolved in chloroform, for further analysis and experimentation. Experiment was run in triplicates for precise results.

The exact experiment was repeated with folch solution as the solvent.

3.6 Analysis of Strain

3.6.1 Quantification of lipid Yield

We used the general formula in (Rai & Gupta, 2017) and various others for the quantific analysis of lipid yield. The mass of lipids obtained was checked using a weighing balance.

$$\text{Lipid yield (\%)} = \frac{\text{Mass of lipids extracted}}{\text{Mass of microalgae}} \times 100$$

3.6.2 FTIR of Lipid

Spectrum of multiple lipid extracts from various methodologies was created using Fourier-transform infrared spectroscopy. Small amounts of lipids were mixed with KBr, a disc of the mixture was created by adding pressure. Infrared rays were passed through the disc and an FTIR spectrum of their absorbance was generated. The machine was a model IRAffinity-1S present at School of Chemical and Mechanical Engineering, NUST, Islamabad. Wavelength was set at 500-4000$cm^{-1.}$

3.6.3 Phytochemical Analysis of Ethanolic Extract

Phytochemical testing of different extracts was checked by standardized methods. This testing allows us to analyze the presence or absence of terpenoids, alkanoids, coumarins, phenols, flavonoids, saponins, tannins, steroids, glycosides and carbohydrates. Phytochemical analysis was conducted as explained by (Deyab et al., 2016) and (Savithramma et al., 2011).

1. Steroid Test

The Liebermann-Burchard test is used to detect steroids. It uses 1 part of sulphuric acid and 2 parts of chloroform mixture which was then further mixed with 0.5ml of algae extract. Positive result is indicated by a red brown ring forming at the top of the algae extract and indicates the presence of steroids in sample (Savithramma et al., 2011).

2. Tannin Test

Ferric chloride or Tannin test is used to indicate the presence of phenolic compounds. This test uses 1ml of FeCL3 or ferric chloride which was then added to 1ml of algae extract. Positive result is indicated by creation of dark green, black or blue color and indicates the presence of Tannins (Savithramma et al., 2011).

3. Saponin Test

Saponin test is used to check for the presence of glycosides. In this test, 2ml of water and 3ml of algae extract were mixed together and shaken together for 15 minutes. This was done in a graduated cylinder, length wise .Positive result is indicated by the creation of a foamy layer of around 1cm above the mixture (Savithramma et al., 2011).

4. Flavonoid Test

Flavonoids are antioxidants found in plants. In this test, 2ml of algae extract and 1ml of sodium hydroxide were mixed. Positive result or presence of flavonoids is indicated by the appearance of yellow color (Savithramma et al., 2011).

5. Phenol Test

In this test, 1ml of algae extract, 2ml distilled water and ten percent ferric chloride was mixed together. The ferric chloride was added dropwise. Positive result is indicated by the creation of blue or green color in the algae extract (Savithramma et al., 2011).

6. Coumarin Test

Coumarins are a family of benzopyrones present in plants and algae, they are highly beneficial against viral disease, cancers, diabetes and cardiovascular diseases. In this test, 1ml of algae extract and 1ml of 10% NaOH were mixed together. Positive result, or presence of coumarins is indicated by creation of yellow color (Savithramma et al., 2011).

7. Test for glycosides

In this test, 3ml chloroform was mixed with ten percent ammonium solution and 2 ml algal extract. Positive result is indicated through the development of pink color in the algal extract (Savithramma et al., 2011).

3.6.4 Gas Chromatography Analysis of Ethanolic Extract

The dried biomass (100mg) was extracted using ethanol and water in equal parts volume. The impure organic part obtained from extraction was sieved using a 0.22um membrane filter and concentrated to dryness using a rotary evaporator machine at low

pressure. The fraction present after evaporation was examined by GC-MS (Rodeiro et al., 2015) .

The machine used was a Model QP 2010 series from Shimadzu, Tokyo, Japan. It has an auto sample model AOC-20iandanRTX-1 and a fused capillary column made of silica, with a length of 30min, diameter of 0.25mm and film thickness of 0.1um. The temperature was programmed to rise from 50C to 300C according to 2C per minute rise. Sample components were ionized in electron impact mode (EI, 70eV). The temperature of the detector was fixed at 310C and the temperature of the injector was fixed at 300C. The carrier gas was Helium with a purity of 99.995%. The flow rate was fixed at 1ml/min. Scanning rate was set at 3.0 scan/s at mass range of 40 to 1000m/z. One microliter of extract of PHM3 was injected into the GCMS system using a Hamilton syringe, using a split injection (1:40) for total ionic chromatographic analysis. The GC-MS system was run for 15 minutes. Analog data was converted to digital data using GC solution software.

3.7 Transesterification of Lipids

In order to avoid the subsequent saponification as a result of hydrolysis of esters due to water generation from the reaction between hydroxide and alcohol during basic catalyzed reactions, acid catalyst sulfuric acid was used for the one step transesterification of algal lipids.

Another benefit of the heterogeneous acidic catalyzed process is its ability to catalyze transesterification of lipids with high amount of FFA’s. Major problem in soap

formation and transesterification is the high amount of free fatty acids (Tran, D., Chen, C., Chang, J., 2013)(Tran, D., Chen, C., Chang, J., 2013).

The oldest gold standard used for the transesterification was by(Christie, 1998). With modifications the following method was adopted.

Lipids were heated in a small beaker placed on the hot plate, any solvent present in it was evaporated at 60 ºC. Methanol used in (40% v/v) and Sulfuric acid used in (5% v/v) were mixed separately and added to the lipid present in the beaker. There was 1.5 ml/1500 ul/1.6g of lipid, 40% /0.7 ml/ 700ul of methanol and 5%/ 0.075 ml/75ul of sulfuric acid.

The mixture was placed on the plate for two hours at 60 ºC and stirred at 400rpm with a magnetic stirrer. After the time period of two hours, the mixture was allowed to settle, this caused the phases to separate, in this system the lower darker brown phase was of biodiesel.

The lower brown phase was collected and measured through the equation used to check biodiesel yield as referenced in(Gandure et al., 2017; Tariq et al., 2011) and mentioned below.

$$\text{Biodiesel yield\%} = \frac{\text{Amount of biodiesel produced}}{\text{Amount of lipids oil used}} \times 100$$

3.8 Quantification and Qualification of Alkyl Esters through GC-MS

Biodiesel primarily consists monoalkyl esters having long chain fatty acids (Bajpai and Tyagi, 2006). To quantify the alkyl esters in the biodiesel GC-MS was used , as it is a highly recommended tool for monitoring organic compounds and is specifically used

for the analysis of fatty acids, esters, alcohols , terpenes and aldehydes (Johnson and Wen *et al*., 2009).

Analysis was carried out in biofuels lab, UPCAS-EN, NUST, Islamabad. The machine was a Shimazdu GC-MS QP2020 with SH-Rxi-5Sil MS silica based capillary column. GC-MS carried an automatic split injector at 250 C. Solvent used was ethyl acetate. The carrier gas was helium, with a flow rate of 1.78ml/min. Temperature increase was set at 7C/min. Initial temperature was 40C for 5 minutes, raised to 300C and then maintained for 5 minutes. All the compounds were identified according to inbuilt library and added to determine the total alkyl ester yield (Rahman et al., 2017) .

3.9 Lipid Processing

The lipid extract from Acid Base Hydrolysis was processed according to the modified phosphatide separation protocols present in papers like (Aylward & Showler, 1962) (Patil et al., 2010). This was to extract the phosphatides from the total lipid content. The protocol used by (Neill & Leopold, 1982), compared two procedures , one using solvent extraction and the other using adsorption chromatography. Since both the procedures gave very similar results and only differed in feasibility, therefore we used the method of solvent extraction. In this procedure the phosphatides were precipitated by dissolving the lipid extracted in a mixture of super cold acetone and 10% MgCl2. 6H2O in the ratio of 1:20:0.4 accordingly. The mixture was stirred for 15 minutes and kept for 2/3 hours in deep freeze. After the phosphatides had precipitated, the suspension was centrifuged and washed, the precipitates were dissolved in chloroform until later use. FTIR was used to analyze the content of the precipitates.

3.10 Phycosome Formation

Phycosomes were prepared through thin film hydration method. Algal Lipid, Tween 80 and Cholesterol were added in the ratio of 5:3:1 in a round bottom flask. 10ml chloroform was added to the flask. The chloroform in the solvent was vaporized using a rotary evaporator at 60ºC to create a thin film over the sides of the flask. Once the chloroform had vaporized the flask was left to cool and 8ml of phosphate buffer saline was further supplied. The concoction was left to incubate at 25ºC for half an hour. Flask shaking was performed for 20 minutes, the suspension was passed through a membrane filter of 0.2um. After that the suspension was sonicated using a probe sonicator for one minute at 70% amplitude. The prepared phycosomes were kept at 4ºC until further usage (Behera *et al.,* 2011).

Similarly, drug loaded phycosomes were prepared according to protocols in (Roy et al., 2016) and (Aditya et al., 2012). The drug used was curcumin, a well reputed anticancer drug. Curcumin was chosen because it is the most common and cheap drug being used for experimental testing of liposomes against anticancer cell lines. Curcumin was mixed in ethanol (curcumin is hydrophobic) and added to the concoction before rotary evaporation. Rest of the protocol was similar to the above methodology. Both empty and loaded phycosomes were further stored for characterization according to protocols developed by (De Leo et al., 2018)and (Feng et al., 2017) .

3.11 Phycosome Characterization

3.11.1 Optical Microscopy

A drop of the liposomal formulation was positioned on the glass slide and dispersed. The slide was inspected at 100X magnification with an optical microscope (OLYMPUS CX43). The formulation was examined before filtration or sonication as to observe the liposomes before they reached nano size as optical microscope cannot examine a nano formulation. Pictures were taken using a mobile camera.

3.11.2 Zeta Analysis

Particle diameter and distribution of the samples along with their zeta potential was checked using DLS.

The particle size, distribution and zeta potential of the samples were determined using dynamic light scattering utilizing the Zetasizer Nano ZS 90 (Malvern Instruments; Worcestershire, UK), equipped with software (version 6.34) and a He-Ne laser at a wavelength of 635 nm and static scattering angle of 90 degree. Briefly 10 µl of the sample was mixed with 1 ml of deionized water and vortexed for 2 minutes followed by analysis with zetasizer. Each result displayed was measured in triplicates(Din FU, Rashid R, Mustapha O, Kim DW, Park JH, Ku SK, Kim JO, Youn YS, Yong CS, 2015).

3.11.3 Scanning Electron Microscopy

The liposome suspension was diluted in a ratio of 1:1 with purified, autoclaved aqua. One droplet of the mixture was dispersed over the slide. The slide was allowed to air

dry overnight. Subsequently, the slide was inserted for gold sputtering, for a total of 5 minutes. Once the sputtering was complete, the slide was inserted onto an electron microscope disc and inserted into the scanning electron microscope machine. The slides were analyzed at 10kV. The electron microscope was Joel 56480LA SEM, present at NUST, Islamabad.

3.11.4 Ultraviolet–visible spectroscopy (UV-Vis)

Absorbance spectra of both liposome and curcumin loaded liposomes was performed to observe stability. The machines used was a Cary 300 SCAN UV-vis-NIR. Quartz cuvettes were loaded with the samples and their absorption was checked in a wavelength range from 200-400nm.

3.11.5 Fourier-transform infrared spectroscopy (FTIR)

Suspension of liposome and drug loaded liposomes were mixed with KBr, a disc of the mixture was created by adding pressure. Infrared rays were passed through the disc and an FTIR spectrum of their absorbance was generated. The machine was a model IRAffinity-1S present at School of Chemical and Mechanical Engineering, NUST, Islamabad. Wavelength was set at 500-4000$cm^{-1}$and a spectrum was generated using the OriginPro software.

3.11.6 Encapsulation Efficiency

Protocol mentioned in (Mahmud et al., 2016) was used to confirm encapsulation efficiency. The suspension of drug loaded phycosomes was centrifuged at 6000rpm for

45 minutes. After the material was centrifuged the supernatant was separated, dissolved in diluted ethanol or methanol (to remove and lipid vesicles and dissolve curcumin) and analyzed at 425 nm using a spectrophotometer (Kargar et al., 2014).

The entrapment, or also named encapsulation efficiency (EE %) was measured using the formula mentioned in (Cheng et al., 2017) written as follows:

$$EE\% = \frac{T-S}{S} \times 100$$

Where

T= total volume of curcumin present mixture

S = total volume of curcumin present supernatant

3.11.7 Hemolytic assay

Human blood was obtained from healthy colleagues. Volume of blood was extracted into 15ml tubes. The falcons were rotated for a total of 5 minutes at 1600rpm. An upper layer of plasma was thrown away, and a lower layer of red blood cells which constituted the pellet were rotated through centrifuging with saline solution. The pellet of red blood cells was diluted with a normal NaCl solution. Nano liposome suspensions in saline solution (1 ml) (concentrations between 1.5µg/ml and 3.125mg/ml) were added in 1 mL of RBC suspension. Positive control which caused total lysis was created by adding 1% Triton (lysate), and negative control which gave zero lysis was prepared, this carried no nanoparticle. The samples were kept at 25 ºC for one day, the 96 well plate was shaken once every 30 min. After 24 hours, the samples were rotated

using a centrifuge at 1,600 rpm for a time of 5 min. The process was repeated with each sample thrice. The absorbance of the 96 well plate was checked with a BIO-RAD PR 4100 microplate reader at 550 wavelength (Mourtas et al., 2009).

The hemolysis percentage was measure for each sample with the equation put forward by (Choi et al., 2011) :

$$\text{Hemolysis (\%)} = \frac{\text{Abs sample} - \text{Abs 0\% Hemolysis}}{\text{Abs 100\% Hemolysis} - \text{Abs 0\% Hemolysis}} * 100$$

Where:

$\text{Abs}_{\text{sample}}$ = Absorbance of sample in well

$\text{Abs}_{\text{0\% Hemolysis}}$ = Absorbance of negative control

$\text{Abs}_{\text{100\% Hemolysis}}$ = Absorbance of positive control

3.12 MTT Assay on cancer cell lines

The effect of empty and loaded phycosomes on viability of cancer cells was checked through the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) method using the protocol mentioned in (Sinjari et al., 2019). The MTT analysis is the most common type of Viability Assay performed involving cell lines. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a dye used for the measurement of in vitro cell proliferation. Tetrazolium salts have been widely used tools in cell biology for determining the metabolic activity of cells ranging from microbial origin to mammalian cells. For this purpose, u87 cells were cultured, using

DMEM Supplemented with 10% FBS and 1% Penicillin-Streptomycin solution, at 37°C for 24 hours. When the cultures achieved desired confluence, 10,000 cells were plated in each well of a 96-well culture plate. The plate was incubated in CO_2 incubator for 24 hours. The concentrations of the compounds were prepared by dissolving them in deionized water and making subsequent dilutions. The drug dilutions along with the solvent control were loaded in the 96-well culture plate with the final volume of 200ul per well and the plate was incubated for another 24 hrs. MTT was prepared in the medium to a final concentration of 5 mg/ml. The plate was then inoculated with 15ul of prepared MTT in each well and incubated for 3 hours at 37°C, until intracellular purple formazan crystals became visible under microscope. Then MTT was removed and solubilizing solution i.e., 150 ul DMSO was added in every well. The Incubation at room temperature was done for few minutes while pipetting up and down the materials of each well, so that the cells had been lysed and purple crystals had been dissolved. The plate was covered in foil to protect from light and immediate measurement of the absorbance in Spectrophotometer was carried out at 550nm wavelength. The experiment was performed in triplicates and the results are the average of the three experiments. (De Leo et al., 2018) .

Chapter 4

Results

4.0 Results

4.1 Comparison of Lipid yield of different strains

Using Bligh and Dyers method the lipid yield per gram in each strain was measured. The highest lipid yield was of *Pectinodesmus* (PHM3). It had an average lipid yield of 23% lipid yield per gram as visible in figure 4.1. Due to this reason, all further experiments were conducted with *Pectinodesmus* (PHM3). Considering microalgae strains this is an average lipid yield, the highest lipid yields are 75-80% (*Schizochytrium* sp and *Botryococcus braunii* sp) and average yields are 15-20% lipid per gram (Christie, 2007).

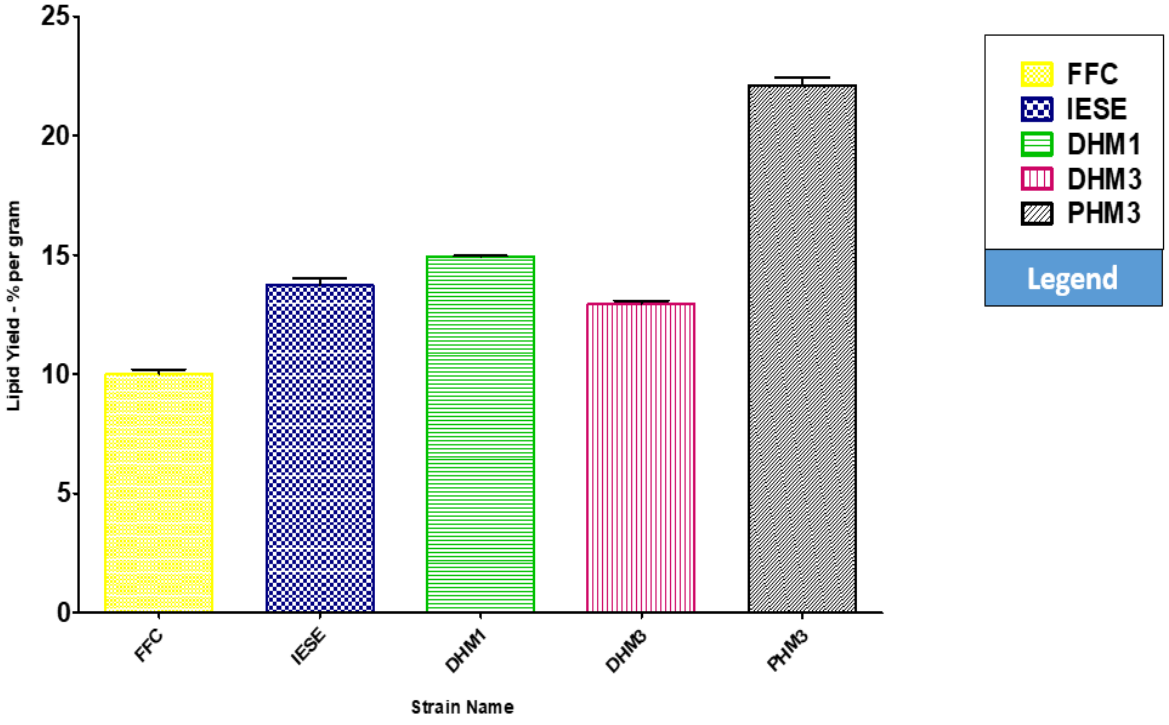


Figure 4.1: Lipid yield from FFC, IESE, DHM1, DHM3 and PHM3 strains. The highest lipid yield is of *Pectinodesmus* (PHM3)

4.2 Lipid Yield of *Pectindodesmus* HM3

The highest amount of lipid extracted was through continuous agitation using Folch solution as indicated in figure 4.2. This is to be expected because the highest surface area contact between microalgae biomass and solution occurs through continuous agitation and folch solution and this result is in accordance with (Kumari et al., 2011). The second highest lipid levels extracted were from continuous agitation in ethanol solvent and acid base hydrolysis. Ethanol is not a purely lipid extraction solvent like chloroform and extracts multiple substances like proteins, tannins, steroids and phenols and hence the extract would contain a lesser amount of lipids (van Nieuwenhuyzen, 2015).

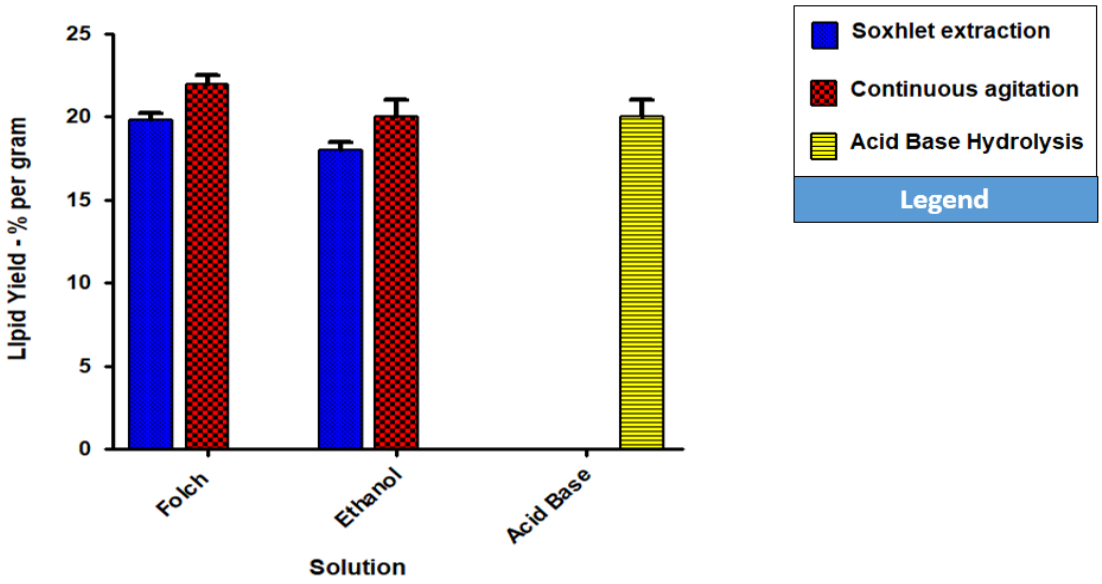


Figure 4.2: Lipid yield of *Pectinodesmus* HM3 using Folch solution, ethanol and acid base hydrolysis

4.3 FTIR study of different lipid extracts

Ethanolic lipid extract, Folch lipid extract and Acid Base lipid extract were examined through Fourier Transform Infrared Spectroscopy, and compared with the FTIR of the biomass according to the method given by (Pastierova *et al*., 2015) and (Dharani, 2013). These studies have proved that lipid peaks at a wavelength of 1700-2800 cm^{-1}. If we observe the FTIR of the biomass (figure 4.3) it shows two miniscule peaks at 1740 and 2870. These are implicative of lipid within the biomass (Mayers *et al*., 2013). Looking at the graph (figure 4.3) we observe that all three solvent extracts are showing a peak between 1700cm^{-1} and 2800cm^{-1} indicating that the extract is indeed lipid in nature.

A major peak is present at 1035cm^{-1} in the acid base extract and the ethanolic extract. This peak is indicative of the presence of phosphatides. This peak is present because ethanol extracts contain high levels of polar lipids (Dean et al., 2010) and acid base hydrolysis extracts contain the entire lipid content of a cell i.e. both polar and nonpolar. And as phosphatides i.e. polar lipids, are known to form a peak between 1018cm^{-1} and 1050cm^{-1} (Jebsen et al., 2012), we can say that the 1035cm^{-1} peak is indicative of polar lipids or phosphatides. This is also why the Bligh Dyer extract graph does not show the presence of any polar lipids, or peaks in the 1000cm^{-1} range because folch solution extracts nonpolar lipids through its chloroform solvent (Sudhakar & Premalatha, 2015).

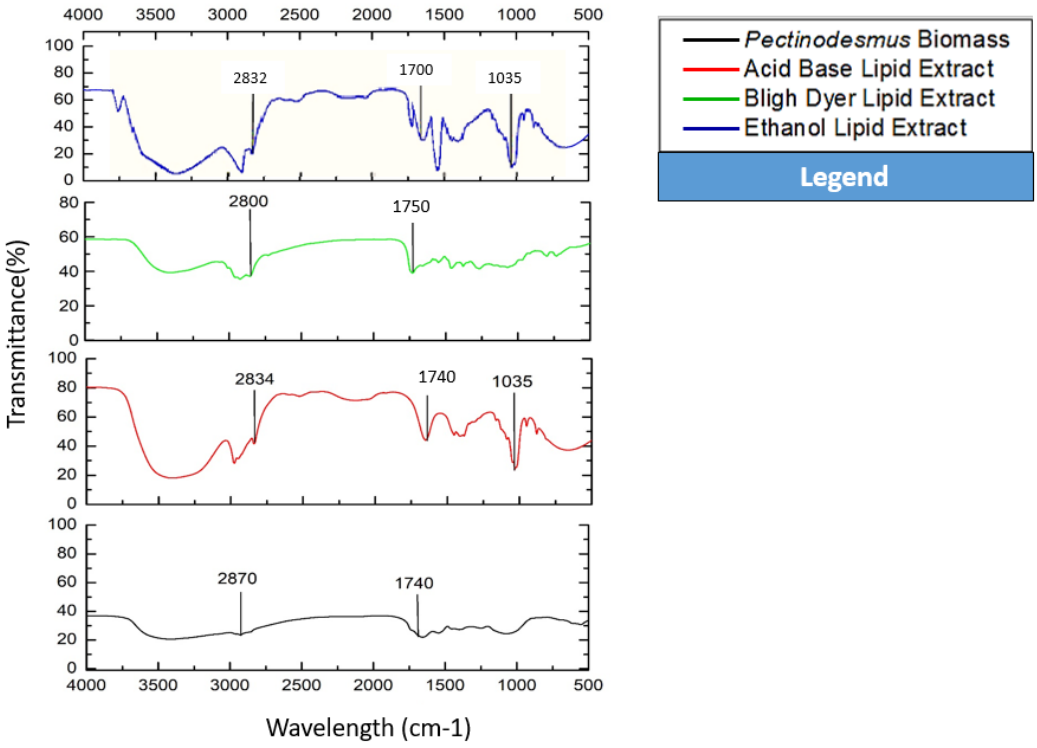


Figure 4.3: FTIR Spectrum of ethanol, Folch and acid base lipid extracts of *Pectinodesmus* HM3 and comparison with FTIR of its dry biomass. All peaks between 1700 and 2800 indicate presence of lipid.

4.4 Transesterification of Lipid

The biodiesel yield of *Pectinodesmus* HM3 was observed to be 7% per gram dry weight, the average biodiesel yield of microalgae species is 2-3% of dry weight according to (Christie, 2007). These results indicate that the biodiesel yield of PectindodesmusHM3 is slightly above an average biodiesel yield.

4.5 GC-MS analysis of Biodiesel

Biodiesel Analysis of *Pectinodesmus* HM3 indicates that it has a very high quantity of unsaturated fatty acids or TAG's which are converted into biodiesels or ethers i.e. 72% biofuels in the form of di propyl ethers, ethyl butyl ethers, methyl butyl ether ,propyl butyl ether and the 1% unsaturated fatty acids which weren't converted. This shows a high transesterification rate and good biodiesel content considering that the average FAME contents of microalgae are between 15-20% and the highest FAME contents are recorded to be around 85% (Christie, 2007) of *Botryococcu*s Braunii. The Chromatogram created by the machine is available in figure 4.4 and partial list of substances analyzed by Gas Chromatography are listed in the table 4.4.

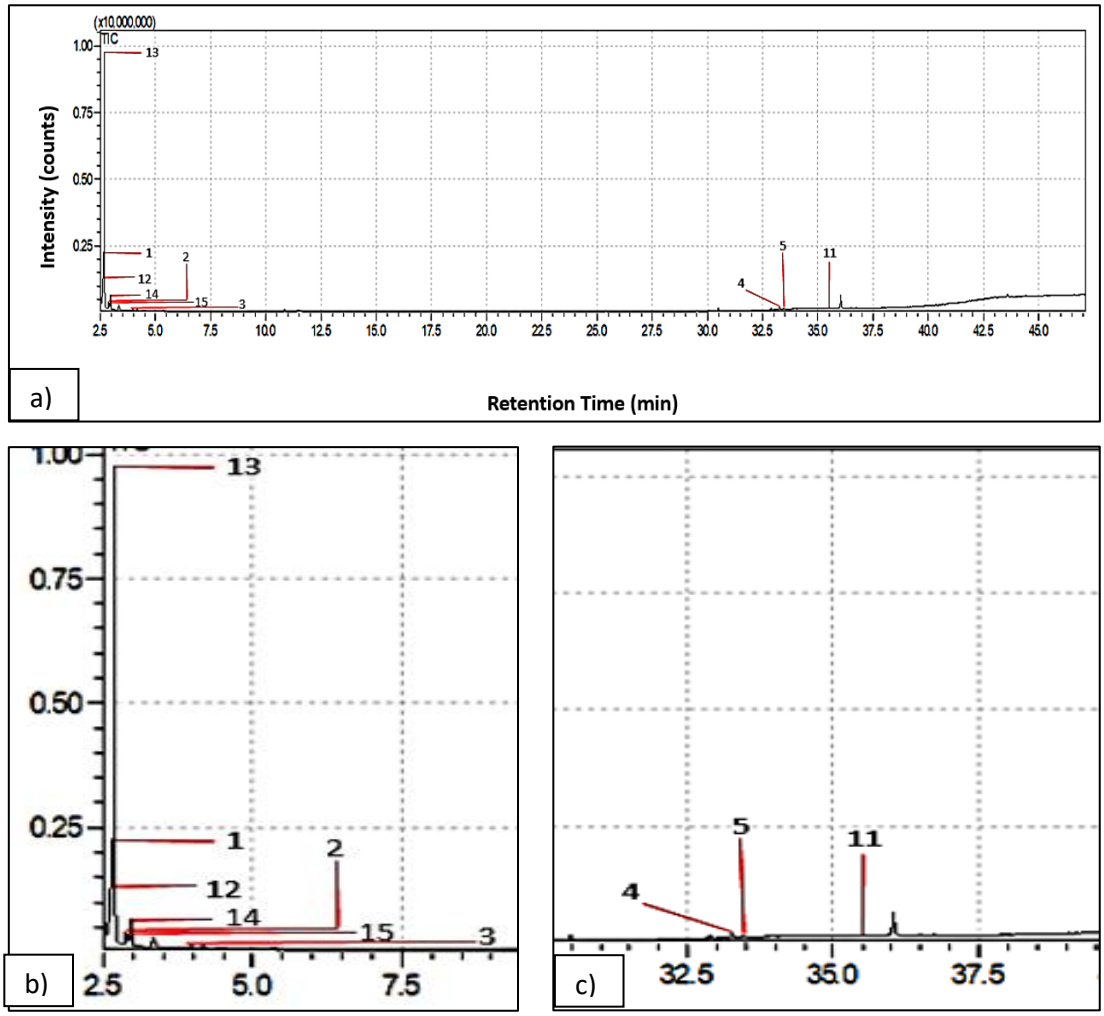


Figure 4.4: a) GC-MS Chromatogram of Biodiesel Trans-esterified from Acid Base lipid extract, b) Visible peaks listed in table 4.3, c) Visible peaks listed in table 4.3

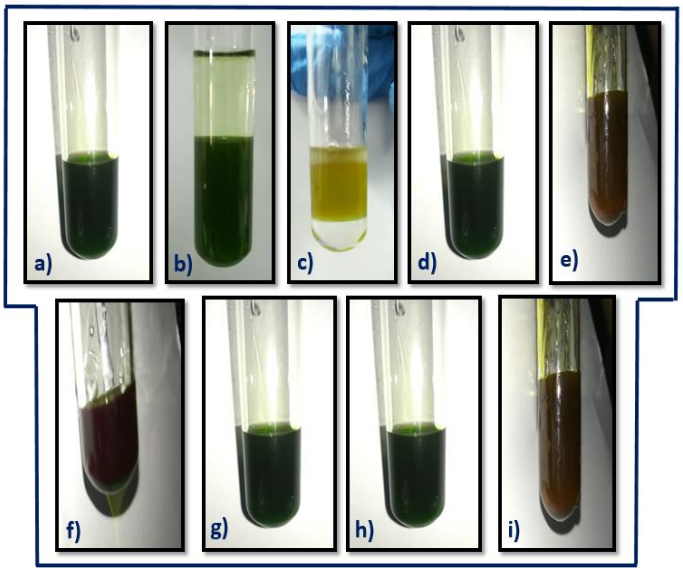
Table 4.3: Major fatty acids present in the biodiesel content. It can be seen that *Pectinodesmu*s HM3 has a high level of Ethers.

Sr no	Compound name	Percentage in sample
	Saturated fatty acids	
1	Propanoic acid	5.42
2	Acetic acid	0.20
3	Butyric acid	0.24
4	Palmitic acid	1.68
5	Stearic acid	1.3
6	Carbonic acid	0.15
7	Carboxylic acid	0.07
8	Glutaric acid	0.05
	Unsaturated fatty acids	
9	Fumaric acid	0.04
	Others	
10	Silicic acid	0.32
11	Sulfurous acid	1.56
	Ethers	
12	Di propyl ether	4.76
13	Ethyl butyl ether	60
14	Methyl butyl ether	3.30
15	Propyl butyl ether	1.56
Total Saturated Fatty Acids : 20% **Total Unsaturated Fatty acids : 1%** **Ethers : 72%** **Others : 7 %**		

Table 4.3 lists major peaks highlighted in figure 4.4. Contents of the biodiesel indicate that *Pectinodesmus* HM3 has a high ether content, this content can be used in the biofuel industry. Methyl butyl ether and ethyl butyl ether are already being used in the automobile industry (Christie, 2007). The total amount of saturated fatty acids is lower than ethers and total saturated fatty acids are negligible i.e. only 1%.

4.6 Analysis of PHM3

4.6.1 Phytochemical Analysis of Extract



Picture	Chemical	Presence
a)	Flavanoids	−
b)	Steroids	+
c)	Coumarins	+
d)	Glycosides	−
e)	Tannins	+
f)	Phenols	+
g)	Proteins	−
h)	Saponins	−
i)	Carbohydrates	+

Figure 4.5: Phytochemical analysis of *Pectindoesmus* HM3

The phytochemical analysis indicated the presence of steroids (a reddish brown ring over the interface), of coumarins (yellow coloring produced), presence of tannins (greenish black color), presence of phenols (blue green color) and presence of carbohydrates (brownish tint). Tabular presentation of results along with photographs of results are given in figure 4.5. The appearance of phenols indicates high antioxidant activity and the presence of carbohydrates indicates that *Pectindoesmus* HM3, while the appearance of brownish tint as indicated in figure 4.5 (d) shows the presence of lipids or fatty substance which means the strain is suitable for lipid extraction.

4.6.2 GC-MS of Ethanolic Extract

GC-MS analysis of the biomass ethanol extract indicated the presence of a mass of compounds, refer to figure 4.6 and table 4.4 for details. Some substances were present in high amount in the chromatogram. Phenol with total of 8 % in sample and Tris (2,4-di-tert-butylphenyl) phosphate with 1.4% in the sample as seen by the two high peaks in figure 4.6. The rest of the compounds were in minor amounts and the type and quantity of fatty acids in the extract are given in the table 4.4. We have short listed fatty acids for the lipid analysis of the *Pectinodesmus*HM3 strain. High amount of phenol in extracts indicates that the extract is high in oxidative properties and a good precursor for nanoparticle formation. High phenol content in extracts leads to better nanoparticle formation as stated by (Van Nieuwenhuyzen, 2015)

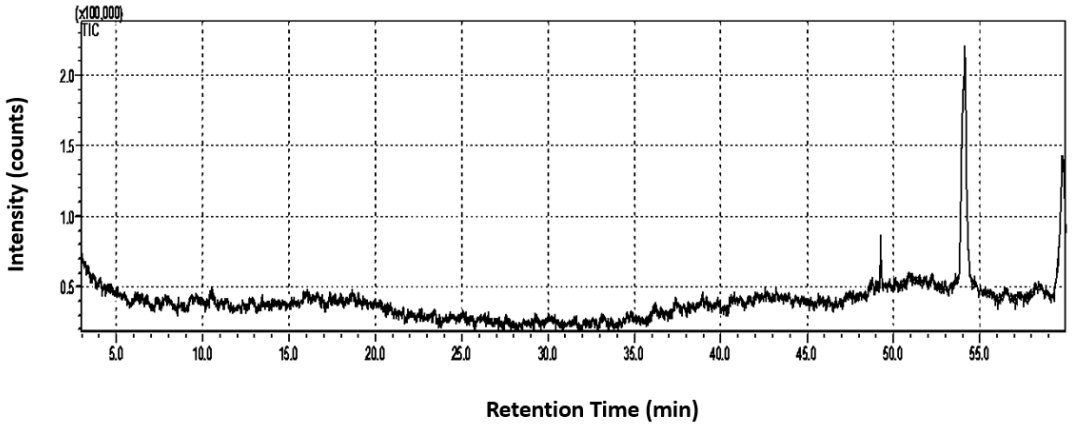


Figure 4.6: GC-MS Chromatogram of Ethanolic Extract. Peak at 54 minutes indicates presence of phenol, peak at 59 minutes indicates presence of Tris(2,4-di-tert-butylph enyl) phosphate.

Table 4.4: Major fatty acids and compounds with higher presence in Ethanolic extract

Peak number	Compound name	Percentage in sample
2	Propanoic acid	0.16

9	Phosphinous acid	0.15
35	Glycolic acid	0.7
45	Carboxylic acid	0.29
133	Butenoic acid	0.5
134	Hexenedioic acid	0.29
139	Nonynoic acid	0.15
222	Hexynoic acid	0.15
240	Benzoic acid	0.4
296	Tetradecanoic acid	0.09
324	Hydrastininic acid	0.09
380	Glutaric acid	0.3
501	Fumaric Acid	0.21
513	Diglycolic acid	0.12
514	Octadecanoic acid	0.27
543	Docosanoic acid	0.11
551	Silicic acid	0.2
562	Pentadoic acid	0.15
577	Phtalic acid	0.14
585	Fumaranic acid	0.23
615	Lysergic acid	0.08
622	Succinic acid	0.09
623	Docasanedioic acid	0.12
654	Propylgl	0.1
674	Citamelic acid	0.15
677	Pimelic acid	0.13
680	Heptanoic acid	0.12
692	Hexadecanoic acid	0.13

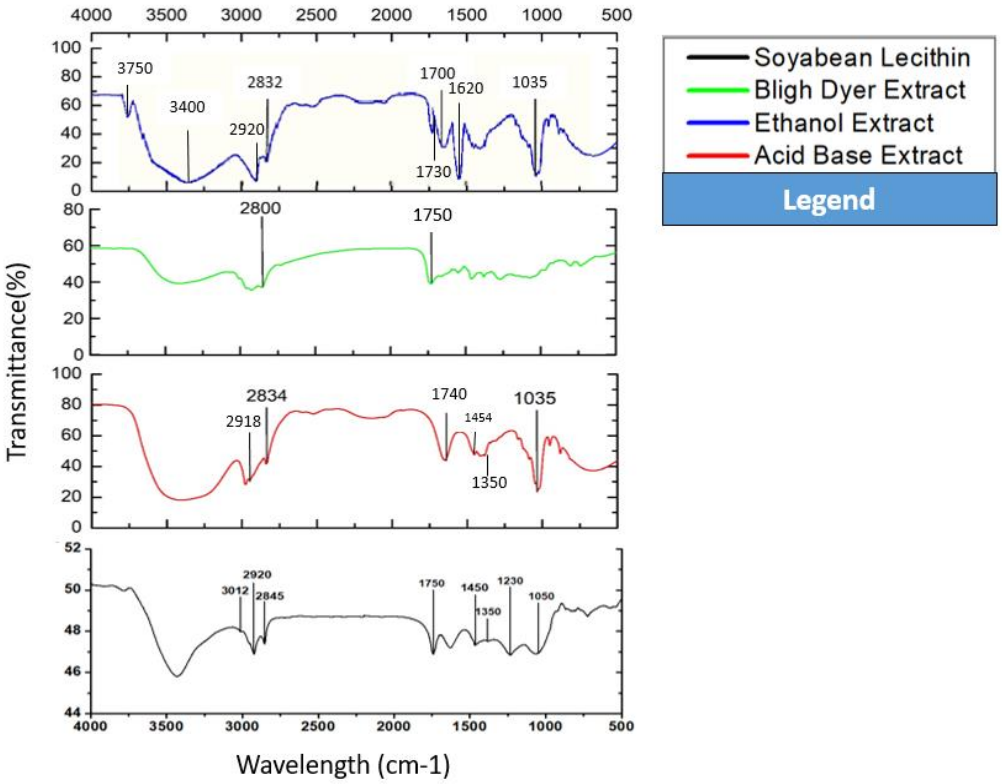
As we can see from table 4.4 majority of the fatty acids, like hexanedioic acids, butenoic acid, phosphinous acid etc present in the ethanol extract are carboxylic acids.

Carboxylic acids are commonly used in the production of antimicrobials in the pharmaceutical industry (Dean et al., 2010).

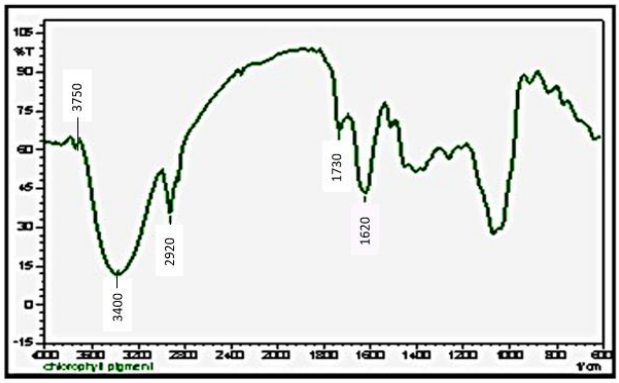
4.7 Lipid Processing

The FTIR's of lipid extracts from ethanol, folch solution and acid base hydrolysis in figure 4.6 (a) showed a high similtude with FTIR of pure soyabean lecithin figure 4.6 (c) . The FTIR's of all three extracts were compared in detail to see which one showed the highest similtude with lecithin. The first one, i.e. the ethanol extract showed a high amount of compounds in its extract , most of its peaks matched the peaks of chlorophyll (Behera *et al.,* 2011) and amino acids as shown in figure 4.6 (b) . The extract from folch solution showed a high amount of lipid but showed little to none phosphatide prescence which shows an un ideal condition for liposome formation as phosphatides form the most stable vesicles. The highest similtude was shown by acid base extract, there were no traces of chlorophyll in the FTIR and least amount of protein content. This is to be expected because the acid base lipid hydrolysis protocol removes chlorophyll and maximizes lipid presence. In case of ethanol lipid extracts, ethanol dissolves multiple types of compounds as per its alcoholic nature so the number of impurities are higher. In the case of folch solution, although the solution dissolves the most lipid content , the choloroform in in the solution does not dissolve the polar lipids i.e lipids like phosphatides. The extract after being treated with acetone was again tested with FTIR and there was a shift in the peaks. The phosphatide peak at 1050cm^{-1} was more defined and there was significant reduction in noise in the lipid range between 1700cm^{-1} and 2800cm^{-1}. Most of the peaks which showed minor curves before processing showed more defined peaks after processing like the 3014cm^{-1}, 2918 cm^{-1}, 1450 cm^{-1}, 1350 cm^{-1} and 1250 cm^{-1} peak.

a)



b)



c)

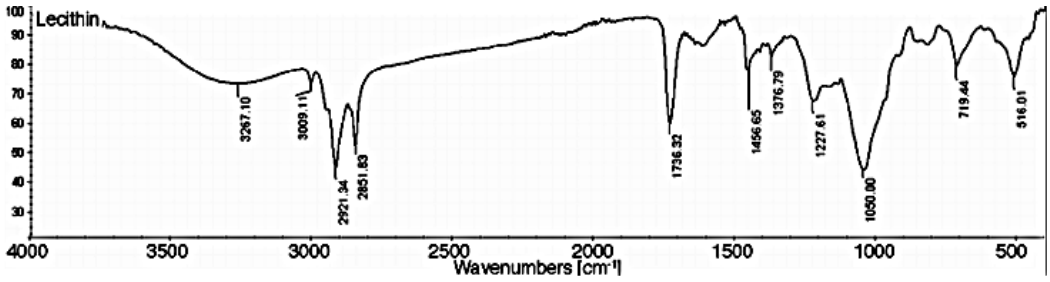
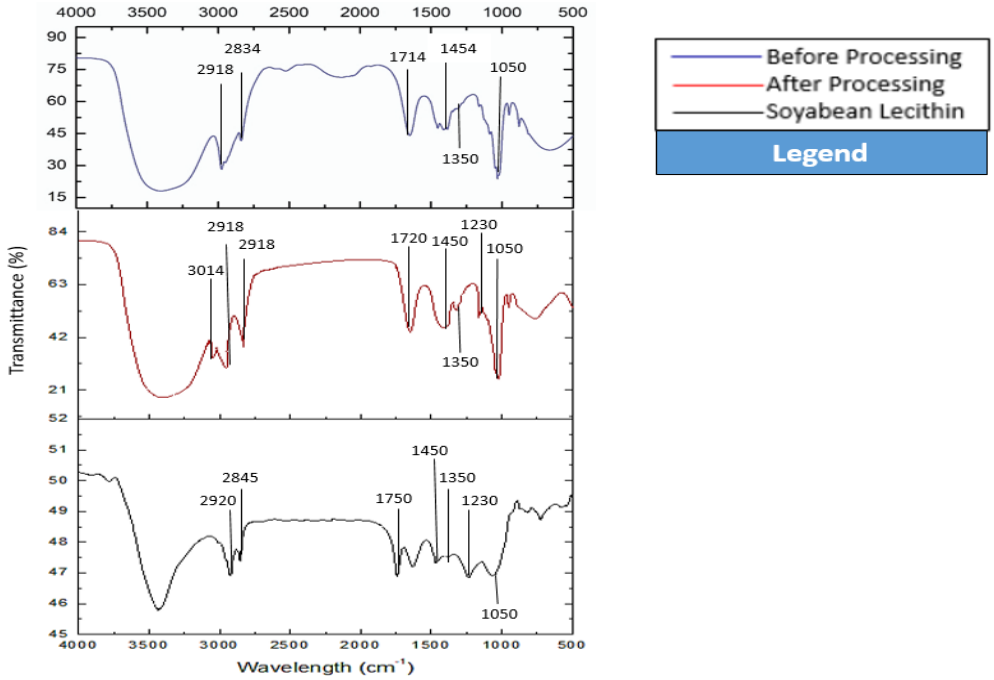


Figure 4.6: a) FTIR spectrums of Ethanol Extract, Bligh Dyer Extract and Acid Base Extract, b) Reference Image of Chlorophyll FTIR *(Behera* et al., *2011)* ,c) Reference image of Lecithin FTIR(Aisha et al., 2014)

a)



b)

c)

Figure 4.7: a) FTIR of lipid extract before and after acetone processing b) Lipid extract from Acid Base method c) Lipid extract after processing through acetone precipitation

The extract after being treated with acetone was again tested with FTIR and there was a shift in the peaks. The phosphatide peak at 1050cm^{-1} was more defined and there was significant reduction in noise in the lipid range between 1700cm^{-1} and 2800cm^{-1}. Most of the peaks which showed minor curves before processing showed more defined peaks after processing like the 3014cm^{-1}, 2918 cm^{-1}, 1450 cm^{-1}, 1350 cm^{-1} and 1250 cm^{-1} peak. As shown in figure 4.7 (a). This figure indicates there was a major difference in the lipid type and it was closer to phosphatides than before processing. Figure 4.7 (b) and (c) show a before processing lipid sample and an after processing lipid sample. We can see the oily nature of the lipid shifted to a more precipitate form, this is common when a mixture of lipids is converted to phosphatides.

4.8 Liposome Characterization

4.8.1 Ultraviolet – visible spectroscopy (UV-Vis)

UV-vis of unloaded phycosomes indicated a peak at 223cm^{-1} while UV-vis of curcumin loaded phycosomes indicated two peaks at 422 cm^{-1} and 187 cm^{-1}. Since the results of our extracted lipids FTIR is similar to FTIR of soya lecithin ,which is a plant derived phospholipid, it can be said that the phycosomes formed would form peaks in similar ranges of UV-Vis and FTIR as soyabean lecithin liposomes do. These results agree with (Aisha et al., 2014) and (Prabhakar et al.,2019) **,**where soyabean lecithin liposomes are known to show absorbance peaks between 200 cm^{-1} and 250 cm^{-1} .After loading the size of phycosomes increases, hence decreasing the absorbance to 187 cm^{-1} (in upper red graph) along with creating a second peak at 422cm^{-1}as seen in figure 4.8. The standard UV-Vis of curcumin , which has already been reported in papers like (Rahul et al., 2017), is 424cm^{-1}.The second peak in the upper red graph is hence curcumin.

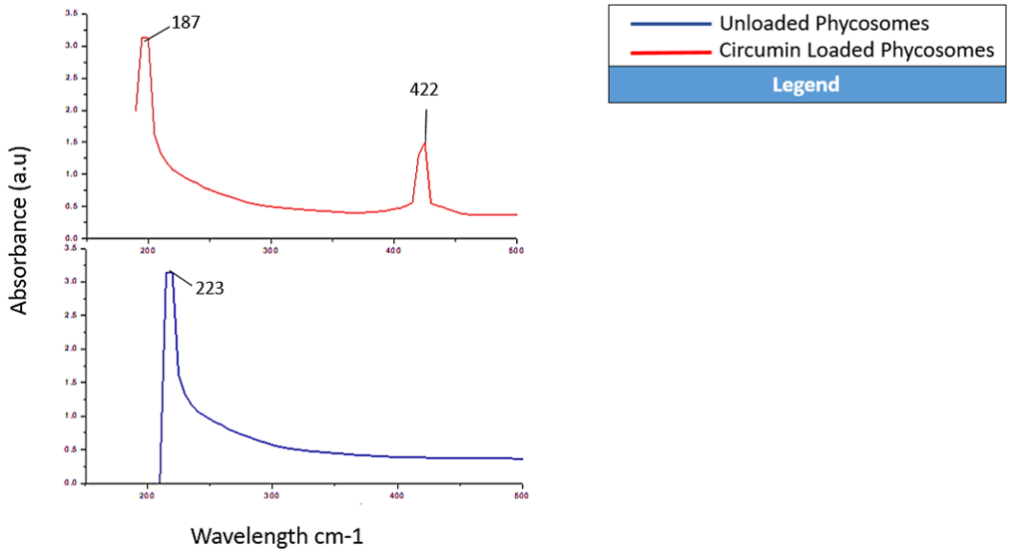


Figure 4.8: UV-Vis of unloaded phycosomes (lower graph) and curcumin un loaded phycosomes (upper graph)

4.8.3 Optical Microscopy

In (figure 4.10 a) phycosomes are visible before size modification through membrane filtration and sonication. This was performed because the optical microscope has a maximum magnification of 100X and can't observe particles in the Nano range. We can see circular vesicles made of lipid and are of various sizes, very much dispersed. In figure 4.10 (b) phycosomes after size modification through membrane filtration and sonication and are barely visible except as tiny dots in the liquid. The morphology of phycosomes is similar to morphology of liposomes produced using commercial lipid DOPS and characterized through optical microscopy as seen in the reference paper by (Jiménez et al., 2017).

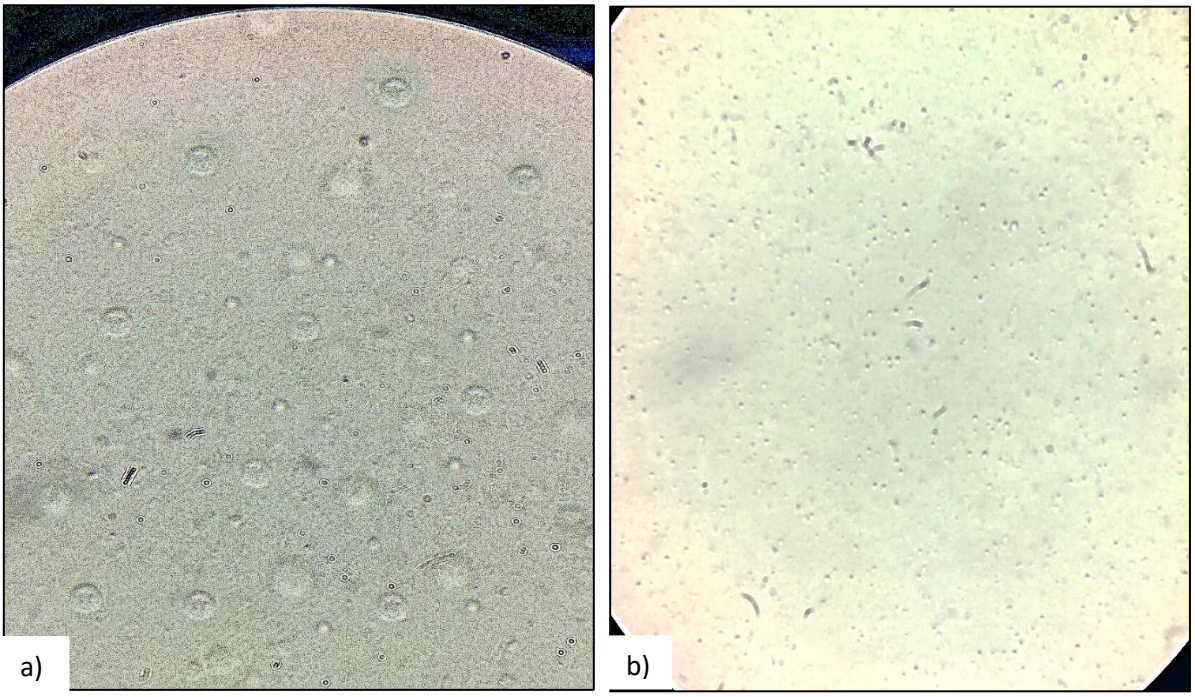
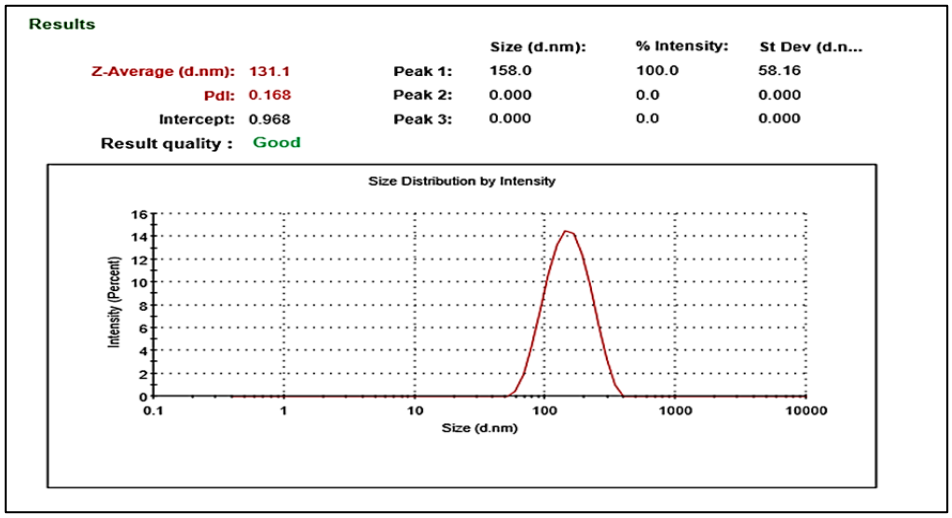


Figure 4.10: a) Phycosomes before membrane filtration and sonication b) Phycosomes after membrane filtration and sonication

4.8.4 Particle Size and Zeta Potential Analysis

The Unloaded phycosomes were shown to have an average size of 131 +/- 58 nm (see figure 4.11 (a)). The zeta potential was an average of -34.1 +/- 4.74 mV (see figure 4.11 (b)). The result indicated that the formulation has a negative charge, this is a good result because it indicates that the vesicles will have high repulsive forces between them and this will prevent them from fusion, sedimentation and aggregation (Liu et al., 2015). So the phycosomes are quite stable.

a)



b)

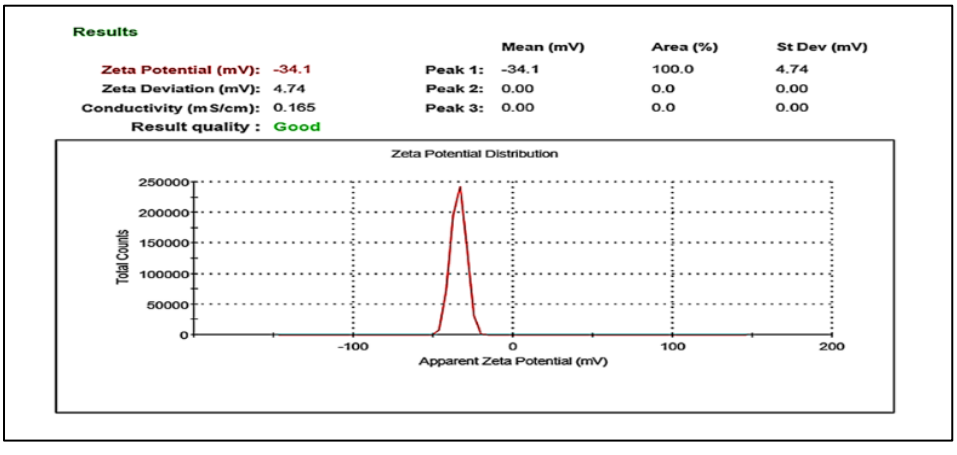
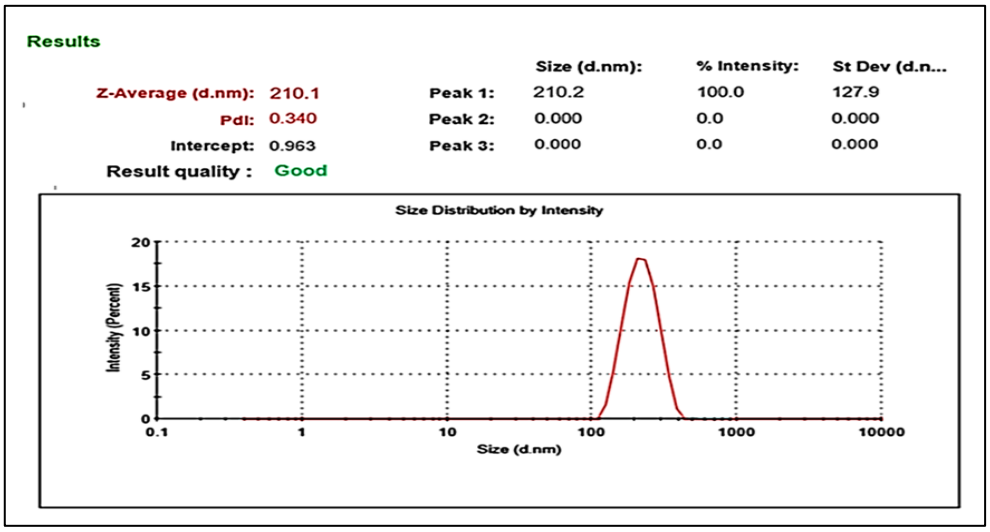


Figure 4.11: a) Zeta Size of unloaded phycoosmes and b) Zeta Potential of Unloaded Phycosomes

The curcumin loaded phycosomes were shown to have an average size of 210 +/- 127 nm (see figure 4.12 (a)). The zeta potential was an average of -48.6 +/- 5.28 mV (see figure 4.12 (b)). Results indicate that the size of the phycosomes increased due to the loading of the curcumin. The mean negative charge of the vesicles increased as well. These results agree with (Mahmud et al., 2016) and (Sinjari et al., 2019) the increase in negativity occurs because curcumin is a hydrophobic drug which integrates itself not in the center of a lipid vesicle as a hydrophilic drug would ,but within its lipid bilayer. This causes heads of the phospholipids present in the membrane to shift and deprotonation of phenolic moieties hence increasing the negative charge, curcumin also increases the size of the vesicle as compared to the charge of unloaded phycosomes(Cheng et al., 2017). The charge is still above -30mV which indicates a highly stable system. Particle distribution (PI) of both particles is below 0.3, which indicates that both loaded and unloaded phycosomes exist within a narrow size distribution (Jin et al., 2016).

a)



b)

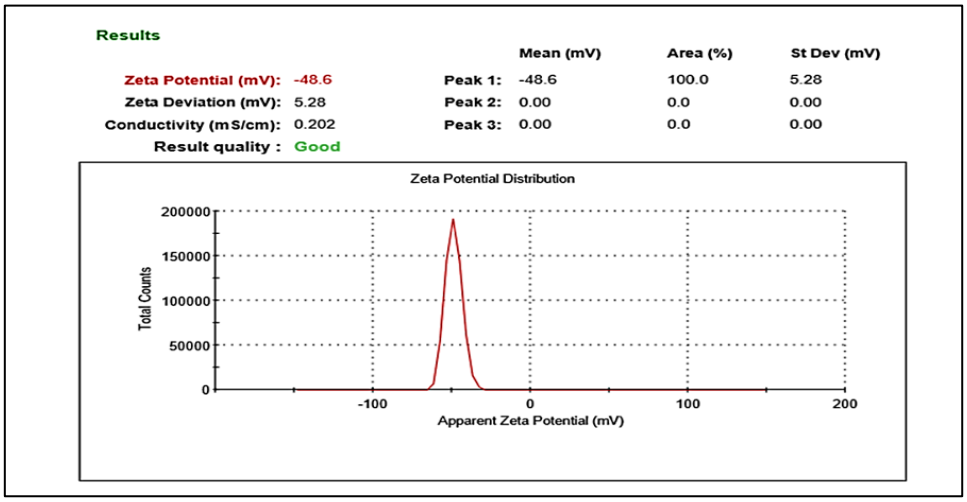
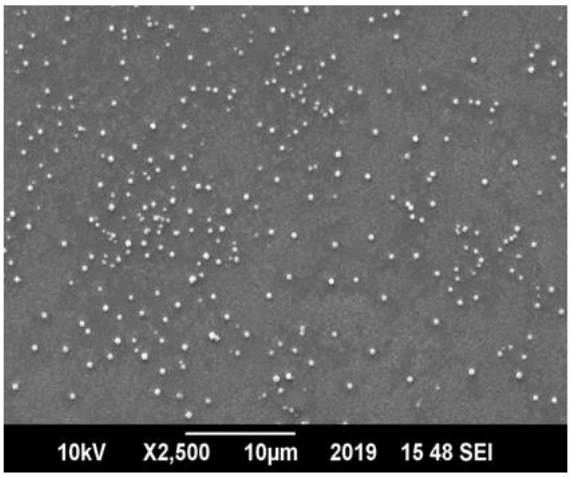


Figure 4.12: a) Zeta Size of curcumin loaded phycosomes and b) Zeta Potential of Curcumin loaded Phycosomes

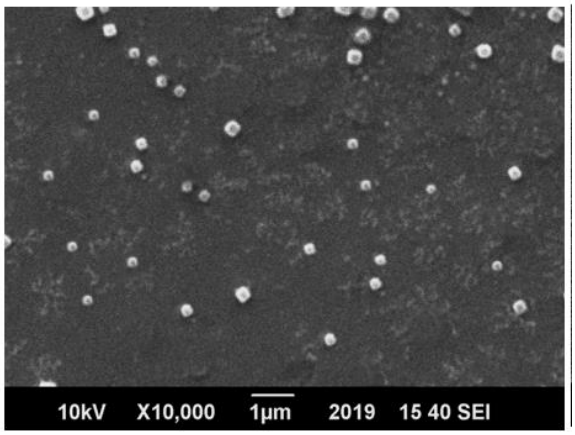
4.8.5 Scanning Electron Microscopy

Scanning Electron Microscopy Imaging depicted mostly circular vesicles, the vesicles were well dispersed, non-agglomerated hence indicating their stability (Surianarayanan et al., 2016) . The size range of the liposomes was between 150 +/- 100 nm. The images were taken at 10um, 1um and 0.2um as indicated in figure 4.13 (next page) and the size and shape of the phycosomes referenced in literature (Chandran & prasanna, 2015; Corrêa et al., 2019). Any voltage higher then 10kV caused the film to burn as most organic or biofilms do. The SEM images showed particle sizes between 150nm-220nm (next page).

a)



b)



c)

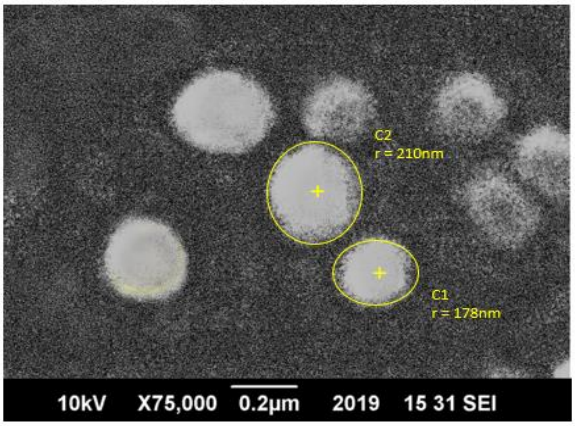
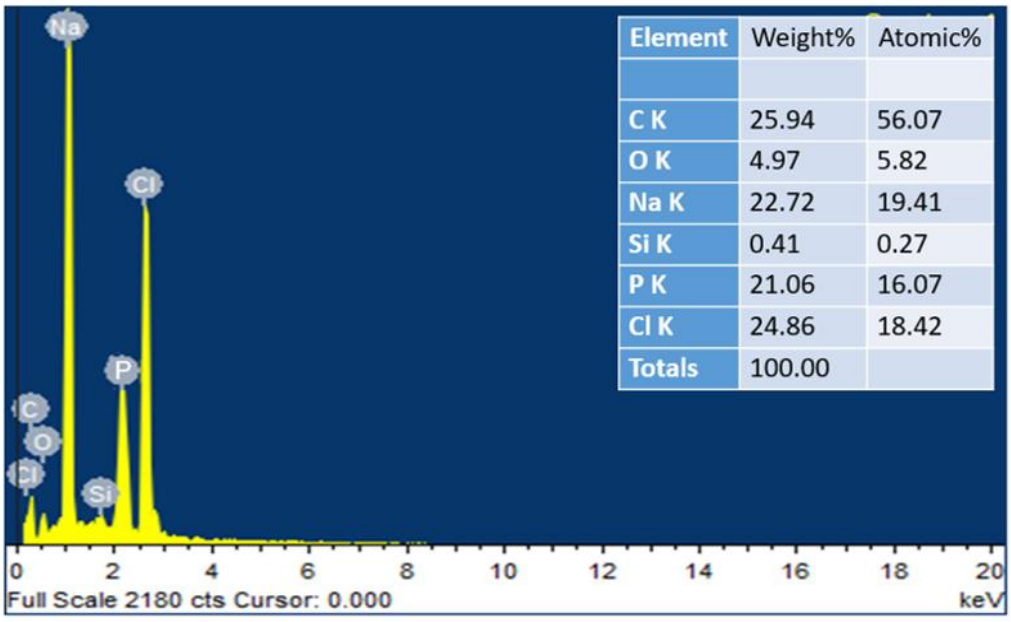


Figure 4.13: Scanning Electron Microscopy Images of Phycosomes at (a) 10um (X2500), (b) 1um (X10, 000) and (c) 0.2um (X75, 000) magnitude respectively

4.8.6 Energy Dispersive X-Ray Spectroscopy of Phycosomes

Energy Dispersive X-Ray Spectroscopy indicated the presence of carbon, oxygen, sodium, silicon and chlorine as shown in figure 4.14. Carbon and oxygen are present due to the organic lipophilic composition of the vesicles, Sodium and chlorine maybe due to the salt present in the PBS buffer when the liposomal suspension was prepared. Silicon is coming in the analysis due to the silicon composition of the glass slide. The basic elemental composition of the phycosomes is similar to elemental composition of liposomes referenced in literature (Wu et al., 2011) which show carbon , oxygen and phosphorus to be the main elements in the spectrum.



Element	Weight%	Atomic%
C K	25.94	56.07
O K	4.97	5.82
Na K	22.72	19.41
Si K	0.41	0.27
P K	21.06	16.07
Cl K	24.86	18.42
Totals	100.00	

Figure 4.14: Energy Dispersive Spectrum of phycosomes which shows the presence of carbon, oxygen and phosphorus.

4.8.7 Encapsulation Efficiency

Different formulations of phycosomes were created. Their curcumin encapsulation efficiency (EE %) was checked as described in materials and methodology, the EE was extremely low without any tween 80 or cholesterol. When tween and cholesterol were added to the formulation, there was an increase in encapsulation efficiency. In the next formulations, as lipid and tween were amplified, the encapsulation efficiency was amplified. The highest encapsulation efficiency was of F5, with a ratio of 5:3:1 of lipid, tween 80 and cholesterol. There came a limit where increasing lipid and tween became a limiting factor in encapsulation efficiency of formulation and instead caused it to decrease.

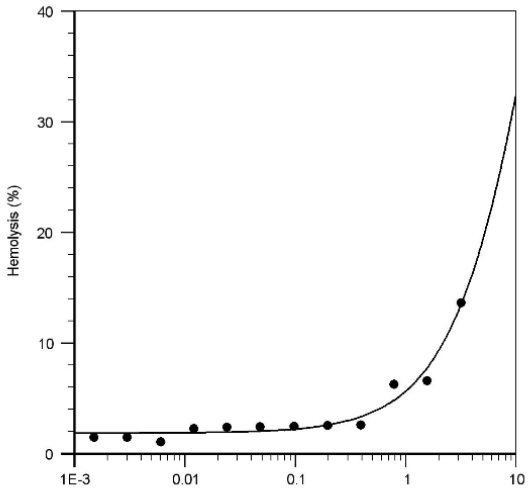
Formulation	Algae lipid: Tween 80: Cholesterol	EE%
F1	3:0:0	22%
F2	3:2:1	32%
F3	4:2:1	41%
F4	5:2:1	63%
F5	5:3:1	75%
F6	5:4:1	70%
F7	6:3:1	53%
F8	7:3:1	28%

Table 4.5: Different nano-phycosomal formulations and their curcumin encapsulation efficiency's

4.8.8 Hemolytic assay

Normally, the quantity of lipid present within a human body, after we administer ta drug like a liposome drug , is 0.025 mg lipid/ml blood (Mourtas et al., 2009). This is because a normal formulation carries a 100mg amount of lipid which disperses in 4 L of blood (Mourtas et al., 2009). As tests were conducted from 1.5µg/ml to 3.125mg/ ml amount of lipid, we see the hemolytic activity of the phycosomes was around two percent in a lipid concentration of 0.025 mg/ml, which is in the measurement error range and safe for use (Mourtas et al., 2009). The relative IC50 calculated by (AAT Bioquest, Inc.,2020) was 25.423, using the following formula.

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{X}{IC_{50}}\right)^{\text{Hill coefficient}}}$$



Lipid Concentration (ug/ml)	**Hemolysis** (%)
3.15	13.65
1.56	6.6
0.78	6.3
0.39	2.6
0.195	2.6
0.097	2.5
0.048	2.45
0.024	2.35
0.012	2.3
0.006	2.2
0.003	1.1
0.0015	1.48

Figure 4.15: Graph shows relation of amount of lipid plotted against Hemolytic activity caused by incubation of nano phycosomes,. Each dot on the graph is the mean of three independent tests. The table indicates the average of triplicate values between 1.5µg/ml to 3.125mg/ ml.

4.9 MTT Assay

Both unloaded phycosomes and curcumin loaded phycosomes were tested on brain cancer cell lines- u87. PBS and DI water were used in wells as controls.

The afore-mentioned results were obtained from spectrophotometric analysis after normalized interpretations. The normalization was done by subtracting the absorbance reading of the blank from all the samples. Absorbance readings from test samples were then divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. Absorbance values greater than the control indicates cell proliferation, while lower values suggest cell death or inhibition of proliferation. The results indicate that the curcumin loaded phycosomes can be used as a drug carrier because their effect on the cell viability is 40% higher than the effect of the unloaded particles. Type of drug and dosage can be adjusted according to use.

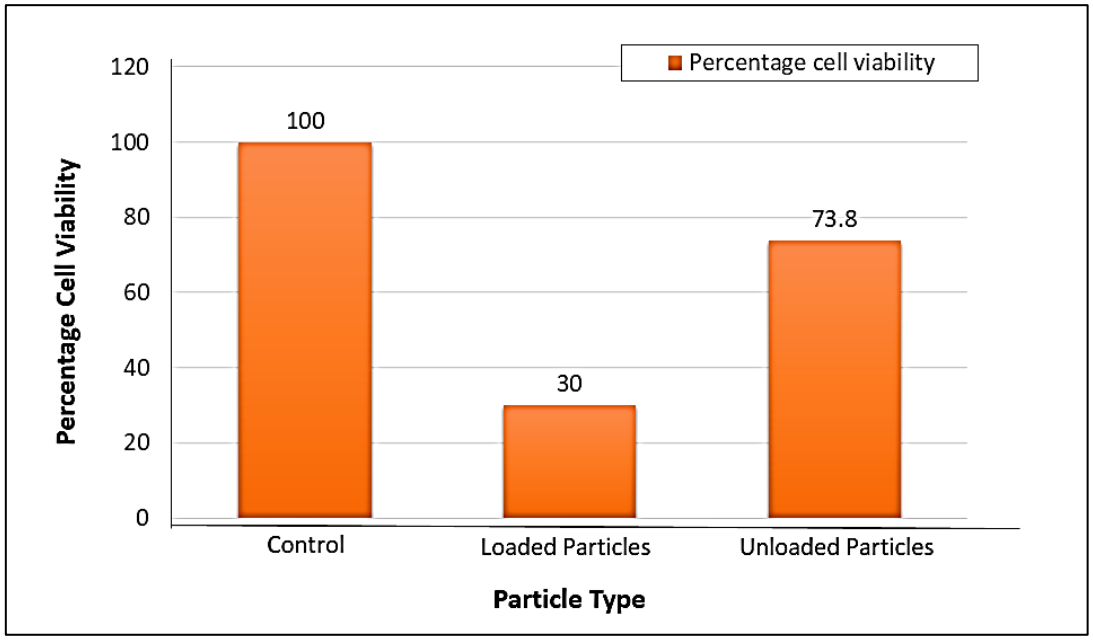


Figure 4.16: Cell Viability of control, curcumin loaded phycosomes, and unloaded phycosomes. Unloaded phycosomes have a higher viability then curcumin loaded phycosomes

Chapter 5

Discussion

In this study we extracted and analyzed the lipids present within *Pectinodesmus* HM3, the phosphatides present within the lipid extract were used in the formation of lipid vesicles, called phycosomes.

Liposome based drugs and products are extremely expensive because of their high cost of production, the major components of their production are either natural phospholipids (extracted from organisms and purified) or synthetic phospholipids (created industrially). Natural phospholipids are relatively cheaper and have a track record of being more biocompatible and less toxic (Prabhakar et al., 2019).Although crude phospholipids are regularly extracted from multiple organisms like archaea (Benvegnu et al., 2009), bacteria (Gupta *et al.*, 2008), plant cells (Neill & Leopold, 1982), animal cells (Bittame *et al.*, 2016), egg (van Nieuwenhuyzen, 2015) and even milk fat globules (van Nieuwenhuyzen, 2015) , there is still no reports of extraction of algal lecithin (Van Nieuwenhuyzen, 2015). This study reports one of the first uses of algal phosphatides for liposome/phycosome formation.

During lipid extraction, the highest lipid yield extracted from available microalgae strains was that of *Pectinodesmus* (PHM3). It had a lipid yield of 23% per gram. This was the reason all experiments were conducted with *Pectinodesmus* (PHM3). Considering microalgae strains referenced in literature, this is an average lipid yield, the highest lipid yields are 75-80% (*Schizochytrium* sp and *Botryococcus braunii* sp) and average lipid yields are 15-20% lipid per gram (Christie, 2007).

The highest amount of lipid extracted was through continuous agitation using Folch solution (23%). This result is in accordance with (Kumari et al., 2011) ,who extracted lipid through various methodologies. Kumari gives the reason for high lipid yield to be

the large surface area contact between microalgae biomass and solution which occurs during continuous agitation. The second highest lipid levels extracted were from continuous agitation in ethanol solvent and acid base hydrolysis. Ethanol is not a purely lipid extraction solvent like chloroform and extracts multiple substances like proteins, tannins, steroids and phenols and hence the extract contains a lesser yield of lipids as stated by (van Nieuwenhuyzen, 2015).

Examination of ethanolic, folch and acid base lipid extract using FTIR revealed their chemical nature. All three extracts exhibited singular large peaks between 1700cm^{-1} and 2800cm^{-1}. (Pastierova *et al*., 2015) and (Dharani, 2013) state that lipid substance forms peaks between 1700cm^{-1} and 2800cm^{-1} , this means the extracts are strongly lipid in nature. Whereas only ethanolic lipid extract contains peaks indicative of polar lipids or phosphatides, which are known to form a peak between 1018cm^{-1} and 1050cm^{-1} as mentioned by (Jebsen et al., 2012). This is why the ethanolic lipid extract was further transesterified into biodiesel for GC-MS analysis.

Biodiesel Analysis of *Pectinodesmus* HM3 indicated that it had a very high quantity of unsaturated fatty acids or TAG's which are converted into biodiesels or ethers i.e. 72% biofuels in the form of di propyl ethers, ethyl butyl ethers, methyl butyl ether ,propyl butyl ether and the 1% unsaturated fatty acids which weren't converted. This shows a high transesterification rate and good biodiesel content considering that the average FAME contents of microalgae are between 15-20% and the highest FAME contents are recorded to be around 85% (Christie, 2007) of *Botryococcu*s Braunii. The lipid extract obtained through Acid Base Hydrolysis was processed according to tried and tested phosphatide separation protocols (Aylward & Showler, 1962; Patil et al., 2010). This

was done to extract the phosphatide content from the total lipid content. FTIR was used to analyze the content of the precipitates. A significant shift in phosphatide peaks was observed after precipitation. The phosphatide peak at 1050cm^{-1} was more defined and there was a significant reduction of noise in the lipid range between 1700cm^{-1} and 2800cm^{-1}. The oily nature of the lipid shifted to a more precipitate form, this is common when a mixture of lipids is converted to phosphatides (Aylward and Showler, 1962).

The phosphatide extract used in the formation of liposomes / phycosomes was similar in chemical and physical nature to soyabean lecithins, as seen in section 4.7 of results.

UV-vis of unloaded phycosomes indicated a peak at 223cm^{-1} while UV-vis of curcumin loaded phycosomes indicated two peaks at 422 cm^{-1} and 187 cm^{-1}. Soyabean lecithin liposomes have been reported to show absorbance peaks between 200 cm^{-1} and 250 cm^{-1} (Aisha et al., 2014; Prabhakar et al., 2019)**,** hence validating the initial phycosome peak at 223cm^{-1}.After loading of the anticancer drug, curcumin, the size of phycosomes increases, hence the absorbance decreased to 187 cm^{-1}.Appearance of a second peak at 422cm^{-1} is indicative of the presence of curcumin , this is within the range of standard absorbance of curcumin mentioned in literature (Rahul et al., 2017) i.e. 424cm^{-1}. The Unloaded phycosomes were seen to have an average size of 131 +/- 58 nm and a zeta potential of -34.1 +/- 4.74 mV. The negative charge of the liposomes is indicative of their stability (Liu et al., 2015) because it indicates that the vesicles will have high repulsive forces between them which will prevent them from fusion, sedimentation and aggregation.

The curcumin loaded phycosomes had an average size of 210 +/- 127 nm and a zeta potential of -48.6 +/- 5.28 mV. The loading of the curcumin caused an increase in size and the mean negative charge of the vesicles. (Mahmud et al., 2016; Sinjari et al., 2019) state that the increase in negativity occurs because curcumin is a hydrophobic drug which integrates itself not in the center of a lipid vesicle as a hydrophilic drug would ,but within its lipid bilayer. This causes heads of the phospholipids present in the membrane to shift and deprotonates phenolic moieties (Cheng et al., 2017). Particle distribution (PI) of both particles is below 0.3, hence the phycosomes created fulfill the prerequisites of a narrow size distribution and stable system mentioned by (Jin et al., 2016). Scanning Electron Microscopy Imaging depicted mostly circular vesicles between 150nm-220nm, the vesicles were well dispersed and non-agglomerated hence falling within the parameters of stable liposomes (Chandran & prasanna, 2015; Corrêa et al., 2019; Surianarayanan et al., 2016).

Hemolytic activity of the phycosomes was almost two percent (Mourtas et al., 2009) states this percentage to be safe for use in in-vivo testing. MTT analysis of the phycosomes compared effect of unloaded phycosomes and curcumin loaded phycosomes in brain cancer cell lines. Results indicate significant activity of curcumin loaded phycosomes, hence reporting one of the first cases of production and use of algal liposomes being used to transfer anticancer drugs in-vivo.

Conclusion

Results proved that the *Pectinodesmus* HM3 strain had a low lipid yield of 23%. The FAME content of the strain was 72%, this is a high biodiesel yield compared to majority microalgae strains. The polar fatty acid content of the strain was found to be 20%. This indicates the strain is practical for biodiesel formation but not very feasible for phycosome formation because we require a lot of microalgae oil for phycosome formation. The unloaded phycosomes produced showed average size of 131 +/- 58 nm and average zeta potential of -34.1 +/- 4.74 mV. Negative charges on the phycosomes ensures that the particles will not form aggregates. The curcumin loaded phycosomes had an average size of 210 +/- 127 nm and the zeta potential was an average of -48.6 +/- 5.28 mV. After drug loading, size of the phycosomes increased and zeta potential decreased as per previous studies on curcumin loading. Encapsulation efficiency of the phycosomes was 75%. Scanning electron microscopy images indicated dispersed and spherical phycosomes. Hemolytic activity of the phycosomes was 2% with a lipid concentration of 0.025 mg/ml, this is within the measurement error range and hence the particles are safe for use. The curcumin loaded phycosomes showed around forty percent higher anticancer activity as compared to unloaded phycosomes. This means the phycosomes may be used as a potential drug carrier in the nanocarrier industry.

Chapter 6

References

6.0 References

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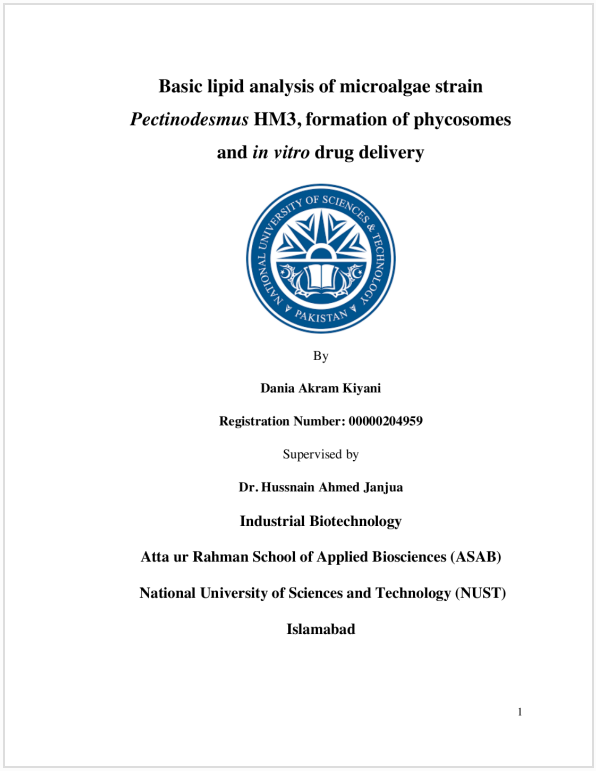

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