DISSERTATION REPORT - II

Value added products from *Spirulina*, extraction, estimation and biotechnological application

SCHOOL OF BIOENGINEERING AND BIOSCIENCE

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B1527-08

M.Sc Botany (Hons.)

Under the Guidance of-

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DECLARATION

I hereby declare that the project work entitled as 'Value added products from spirulina, extraction, estimation and biotechnological application' submitted to Lovely Professional University, is a record of an authentic work done by me, under the guidance of Dr.Devendra Kumar Pandey, Lovely Professional University, in order to fulfil the requirement of project. This work has not been copied from any source and whatever decoration and connection made in the circuit is a total dedicated work of mine.

Shashanka shekhar singh(11506936)

CERTIFICATION

This to certify that Shashanka shekhar singh is undergoing his project title 'Value added products from *spirulina,* estimation, identification and biotechnological application' under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of the project has ever been submitted for any other degree at any University.

The project is for the as part of the requirement necessary for the awards for the degree M.Sc Botany (H).

Date-

Signature of Advisor

Dr. Devendra Kumar Pandey

Assisitant Professor

Department of Bioengineering and Bioscience

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ACKNOWLEDGEMENT

It is my honour to present my project on "culture and quantification of valuable compounds of *Spirulina* species and its product".

I wish to express my sincere gratitude to my project supervisor Dr. Devendra kumar pandey, Assistant Professor, who gave me the motivation to take the project and inspired me each point of time. I deeply thank him for his dedication and patience provided for my project work.

Also, I sincerely thank all the faculty members who helped me at various stages of our project. My thanks also go out to Mr. Chirag Chopra, Head of Laboratory of Biotechnology for encouraging and supporting me during my project.

Last but not the least I would like to thanks my parents who always supported and encouraged me, that always helped me to move forward. I would like to thank all my friends for their kind support and stimulating thoughtful discussions that helped me so much in my project.

Shashanka shekhar singh (11506936)

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Abbreviations

1. gm (Gram)
2. mg (Milligram)
3. mcg (Micro gram)
4. µg (Micro gram)
5. ºC (Degree Celsius)
6. DPPH (2,2-diphenyl-1-picrylhydrazyl)
7. NaK (Sodium- Potassium tartarte)
8. $CuSO_4$ (Copper sulphate)
9. GAE (Gallic acid equivalent)
10. QE (Quercetin equivalent)
11. IC (Inhibitory concentration)
12. UV (Ultra violet rays)
13. Abs (Absorbance)
14. SD (Standard deviation)
15. OD (Optical density)
16. Spp. (Species)
17. pH (Potential of hydrogen or measure of acidity and alkalinity)
18. Na_2CO_3 (Sodium carbonate)
19. NaOH (Sodium hydroxide)
20. wt. (Weight)

Abstract

Studies on the identification of fresh water algae was made by making collection of algae from Lovely Professional University Punjab and nearby areas namely Phagwara and Jalandhar. In total four species belonging to the groups have been identified. Microscopic studies of *Spirulina platensis revealed* its structure and characteristics, to improve the production five culture practice, was done which were setup in agar plates, broth culture, photoautotrophic culture, mixotrophic culture and batch culture. In the study the quantification of the valuable nutrients like protein, carbohydrate and nutraceutical compounds such as phycocyanin, beta-carotene, flavonoids, phenols and amino acid were performed by using UV-vis spectrophotometry to show its value, which was found to be more better than any other source of vegetarian diet. Finally the thin layer chromatography analysis resulted in identification of amino acid like tryptophan, glycine, tyrosine and aspargine in the sample.

Introduction

Algae the collective term of those chlorophyll bearing organism which shows thalloid structure like pond scums, stone worts, sea weeds etc. they never form true roots, stems and leaves thus called as thallus, there are around 30000 species, predominantly aquatic and are found in fresh or salt water. In habit they may be motile(free swimming and free floating) or non-motile(attached to the bottom in the shallow water) some are also terrestrial and also grow in moist condition such as damp soil and damp shaded side of tree/ walls or even in rocks. Epiphytic as usual as epizoic condition are seen in algae. In brief they can be differentiated into aquatic algae, terrestrial algae, pterophytes, bryophytes, thermophytes. The major form of algae genera are aquatic which are completely submerged or free floating. *Chyladamonas, volvox, hydrodictyon a*re few example of fresh water algae and algal genera like *Phaephycaeae* and *Rhodocyceaea* are example of marine algae.

There are many types of algae all of them can be placed under microalgae or under macro-algae. Among the two, microalgae are the algae that can't be seen under naked eyes they need a aid like microscope to study the plays an important role in the sustaining the life on earth by providing the maximum amount of oxygen to the atmosphere by consuming carbon dioxide. They are placed at most bottom point of the food chain which sustain the ecosystem. Whereas if you considered the macro-algae they are large aquatic plants that can be seen without the help of the microscope they are also called as seaweeds the class of macro-algae can be again divided according to their photosynthetic pigments into four different phyla also with evolutionary history. The four phyla are the Cyanophyta, Rhodophyta, Chlorophyta and Ochrophyta which are commonly found deep in shallow water, marine water and few in fresh water with exception that grows on hard surface like rocks, dead coral and in mud and sand (Puliedo etal, 2008).

Microalgae along with the macro-algae contributes towards the aquatic biomass they ranged from 1-50 micro meter in diameter with 200000-800000 species among which around ten to thousand has been described occurring both in fresh and marine water. Since the classification, identification and genetic constitution of the microalgae by taxonomist and scientist is still under process but till now the microalgae can be distinguished into many groups like green algae, red algae, diatoms, brown algae, gold algae, yellow-green algae and blue algae or cyanobacteria (wolkers, etal).

Collection and screening of the algae helps in choosing the good quality and more production of the desired products like for the lipid and bio fuel production the algae are to be collected and isolated from elevated thermal gradients which are thermotolerant growing at temperature of 35 º C. The production quality differs in every case like local microalgae gain competitive advantage due to the local climate and ecological factors unlike collection the isolation has the key role for the potential production, the isolation can be traditional such as single cell isolation under microscope from the culture and modern techniques where properties like chlorophyll autofluorescence (CAF) and green autofluorescence (GAF) are used to distinguish and isolate many strains of algae species. (Duong et al., 2012).

Playing an important role in the ecosystem algae has other basic importance in day to day work, research cells, and medicinal field. Some of the application of these species are, they are used as food source, as fodder for cattle, as fertilizers(*Oscillatoria*, *Scytonema*, *Spirulina*, etc), in fish culture, used for reclamation of alkaline soils, in sewage disposal(*Pondorhina*, *Euridina* etc), in production of antibiotics like "chlorellin" which is effective against many pathogenic bacteria, in industry for production of iodine(*Ecklonia*, *Eisenia* and *Phyllophora*), in research as research material in the field of genetics and cytology(*Chlorella, Scenedesmus* and *Anacystis*) and moreover today they are used in space(*Chlorella* and *Synechococcus*) used for generating oxygen and also used as food and water recycling agent. Production of many important metabolites like proteins, carbohydrates, lipids, vitamins, phenols and flavonoids are obtained from these microalgae. Basic and fundamental attributes like, a wide area of genetic diversity with different physiological and biochemical characteristics, ability to produce different bioactive compounds, lipids, fats and sugars in different environmental factors and their effectiveness to incorporate their biomass with stable isotopes make them a potential source of many products (Priyadarshini and Rath, 2012).

We get many valuable products from the microalgae which help to meet our needs without affecting the environment so in these modern era the culturing of the microalgae is one of the modern technology that has been adapted. If you go on history the first micro/unialgal culture was achieved by Beijerinck(1890). Then afterwards many started to culture these algae for research and food purposes but the success of the culture depends upon many factors like processing, harvesting and proper maintenance more importantly it depends upon the land, labour, energy, nutrients, water and the constitution of algae. Mainly there are two system open and close system process for the production of the microalgae which includes pond, tank, tubes and fermenters (Borowitzka, 1998).

Spirulina species generally belong to the Cyanophyceae family which are rich source of blue pigments known as c-phycocyanin, giving them a blue appearance for which they are also referred as blue green algae. They are prokaryotic without nucleolus and nuclear membrane, mainly composed of mucopeptide in their walls. Most of them occur on fresh water like *Oscillatoria* and few are subjected to marine but are widely distributed around the earth, exhibiting oval or spherical shape, reproducing through akinetes, exospores, nannocyte and endospores. Due to their unorganised nucleus, absence of flagella and chromatophores, and incapability of sexual reproduction they are regarded as the primitive organism.

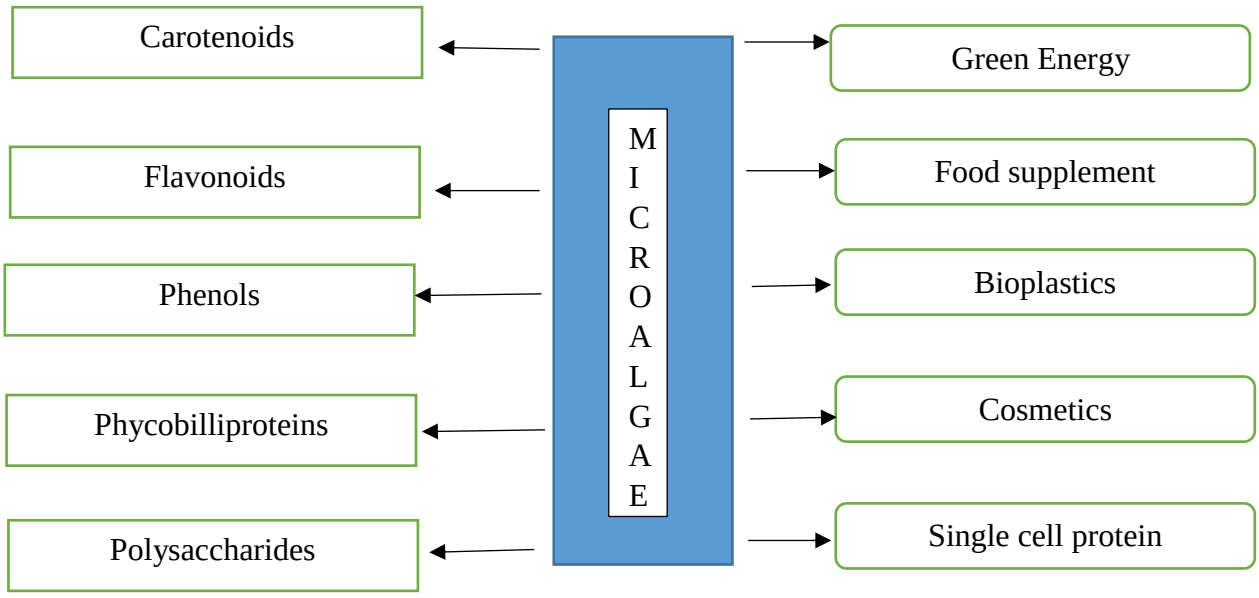


Fig 1: Schematic representation of importance and value added compounds obtained from microalgae.

Review of literature

A long been dietary supplement for people living near to the alkaline lakes region spirulina a multicellular, blue green filamentous algae has gained the popularity in dietary industry and act as a protein and vitamin supplement to humans and aquaculture life. It is very easy to grow and is very easily harvested and processed having high content of micro and macro nutrients.

Among all the algae *spirulina* has a great importance as it is considered to be the superfood due to its high protein content and other valuable vitamins present in it and also pigments like phycocyanin which has many important aspects . *Spirulina* were one of the ancient species existing from 3 billion years in these earth they are best situated in nature, mostly found in alkali, mineral rich lakes and water bodies that are not contaminated with pollution. Warm water ranging from 32-45 $^{\circ}$C favours the species to thrives and can withstand up to 60 degree Celsius it is being seen that some certain xerophytic adapted species survive when the habitat evaporate in the intense sun, creating a dormant state on rocks as hot as 70 degree Celsius where it turns into a frosted white and develop a sweet flavour as it protein structure is transformed into polysaccharides sugars by heat. In fact the more the mineral salts concentration increase due to evaporation of water the faster and prolifically Spirulina grows and the pH range between 8-11 also favours the condition.

The cyanobacterium, (*Oscillatoraceae* family) which has the ability of photosynthesis is the ancestor of higher plants. *Spirulina* has been derived from a Latin word means tiny spiral. It is a microscopic, spiral shaped vegetable bacteria. The genus spirulina comes under the phylum Cyanobacteria, though it is single celled attaining to size of 0.5mm makes some individuals visible to the naked eye. Spirulina algae, also known as one of the "superfoods", thought to have been the major food source of Aztecs in 16th century Mexicoas it is harvested from Lake Texcoco and called it as Tecuitlatl means the stone's excrement. The production of these in a large scale in early 1970 drew the attention and in today's world it is so well-written and understood by the scientist for its medicinal value and economic properties.

Distribution of *Spirulina* Species

These species are generally found in alkaline brackish and water with high salinity near the tropical and sub-tropical region, and are more common to Africa and Asian countries other than these they are also found in California and Mexico.

Structure of the *Spirulina*

They are the very organism which were curious to know due to their photosynthetic behaviour which are mostly absent in case of prokaryotes and also due to is basic composition and cell structure that are very much alike of Gram-negative bacteria which is an eukaryotes (Tornabene et al., 1985). Under the high resolution electron microscope it shows prokaryotic organization like capsule, ribosome, thylakoid, fibrils of DNA with numerous inclusions and pluri-stratified cell wall. It has irregular capsule around the filament which has a differentiating morphological characteristic. There is a change of shape from helical to spiral when it is transferred to a solid media from liquid media due to hydration or dehydration of oligopeptides in peptidoglycan layer. The cell wall of spirulina is formed of four layers which are made up of peptidoglycan, which are the main reasons for the easy digestion of spirulina by human. The thylakoid system contains chlorophyll, carotenes and phycocyanin which is our main concern and it also has the electronically transparent protein gas vesicles which help them to float. It also contains polyglucan granules, cyanophycin granules, lipid granules, polyhedral bodies and polyphosphate granules which are highly valuable for its chemical nature and pigments.

Chemical composition

The composition of the content in *Spirulina* species varies from each other due to factors like climate, nutritional factor and culture environment in the similar way the composition of the commercial product differs due to the processing and harvesting mechanism.

Table 1: Compound composition of *Spirulina* spp.in percentage (%)

Content of protein	55 - 69
Content of Lipids	5- 7
Content of Carbohydrate	7 - 15
Content of Minerals	6 – 9
Moisture content	2.5 - 6.0

Table 2: Phytopigments content in *Spirulina* spp. (mg / 100g)

Beta Carotene	150 – 250
Chlorophyll	1300 – 1700
Phycocyanin	15000 –19000
Zeaxanthin	125 – 200
Xanthophylls	250 – 470
Total Carotenoids	400 – 650

Table 3: Vitamin content of *Spirulina* spp. (per 100g)

Thiamine (Vit.B1)	0.5 mg
Riboflavin (Vit.B2)	4.53 mg
Niacin (Vit.B3)	14.9 mg
Pyridoxine (Vit.B6)	1 mg
Cobalamin (Vit.B12)	162 mcg
Folate (Vit.B9)	.05 – .3 mg
Inositol (Vit.B8)	70 – 90
Vitamin K	1090 mcg

Taxonomy and morphological characterization of *Spirulina*

Belongs to Phylum: Cyanophyta, Family: Oscillatoriaceae (Ciferri and Tiboni, 1985). A ubiquitous organism, found in brackish water, sand, marshes, soil, seawater and fresh water. Under the microscope appeared as cylindrical in shape with helicoidal trichomes which are unbranched also bearing motile filaments gliding along the axis and absence of heterocyst has been noticed (Ciferri, 1983). The genus has the helical shaped trichome as its special characteristic.

Value added substances in spirulina

The microalga spirulina has been cultivated for many purposes as it is an effective source of many valuable biochemical and a major source of protein. The biochemical substances which are found in spirulina are polysaccharides, linolenic acid, beta carotene, phycobiliproteins and chlorophylla. These biochemical are commonly used in many food and cosmetic industries, also used in pharmaceutical industries for medicinal purpose. Among the biochemical compounds GLA have the potential to fight with hypocholesterolmic, pre-menstrual problems and atopic eczema they are also found in many plants and fungi but the concentration level is low as compared to *Spirulina* species (Cohen et al., 1993).

Generally, carotene are the many related hydrocarbon having the general formula $C_{40}H_x$, which are most common in plants and photosynthetic bacteria. They carry out two essential task first contribute towards photosynthesis as an accessory pigment and in photo-protection. These two task are the result of the structure, which allow them to quench and absorb light also free radicals. Carotenoids are obtained to the human through various sources both in natural manner (fruits and vegetables) and artificial manner as food colourant. Although the function of these molecule is still unclear in human but it plays an important precursor to the vitamin A (retinol) which is highly recommended for the vision. There have been many observational studies on carotene which reveals its protective nature towards many human diseases like heart disease, cancer, immuno-modulation, aging, cataract, stroke and macular degeneration (Susan et al).

Bio fuel plays vital role in replacing the non-renewable fuel sources, *Spirulina* species due to their fast growing availability in few requirements produce large amount lipids and carbohydrates which were processed as biofuel and other valuable co-products (Brennan and Owende. et al., 2010). Moreover study conducted by Dartsch PC, 2008 revealed the antioxidant and anti-inflammatory property of these species which are far better than any plant.

The diversity in their chemical makeup made these organism more attractive for exploiting and bioprospecting as commercial and medicinal source. But the only barrier for it is the cost effective production of compounds mainly the biofuel products. They also produce sterols, polyhydroxyalkonates in general they are the source of cosmeceuticals, nutraceuticals and functional food (Borowitizka. 2013).

Why it is important?

This emerged as a wonder drug due to its diverse concentration of nutrients and its varied uses which increases the resistant factor to numerous infection in other words we can say that it boost the immunity. Moreover it is well known for its antioxidant and anticancer action. Also in diseases like diabetes, hypertension, anemia and others it plays an important role due to its multi beneficiary action it is an important natural product which also content the potential ability for production of biofuels because of high content of lipids and fatty acids.

Environmental factors influencing the growth of *Spirulina*

Temperature, light, pH and availability of carbon dioxide are the various environmental factors that controlled the microalgae growth and their chemical composition also nutrients play a major role. (Tzovenis et al., 1997; Zhu et al., 1997; Danesi et al., 2004; Belal et al., 2012). But in some cases salinity play the vital role (Chu et al., 1996).

Effect of various nutrients on *Spirulina*

Morphology of the *spirulina* can be altered due to the nutrient concentration and light intensity like in a nutrient deficit condition the degree of coiling increases as a result of which a tight trichome is formed, but in case of high nutrient concentration and high light intensity the opposite occurs that is the tight trichome losses themselves (Jeeji Bai and Seshadri 1980). For the cultivation of these algae carbon plays an essential nutrient which is being taken either in inorganic or organic form (Badger et al., 1994). For synthesis of proteins, amino acids and other cellular components nitrogen is required (Yang et al., 2010).Costa et al, (2003) reported the influence of nutrient for biomass production and growth rate of *Spirulina platensis*, in open raceway ponds.

Temperature effect on *Spirulina*

Biochemical composition of these algae can be altered by the temperature exerting different biochemical reaction (Hu, 2004). 35 to 38°C is the optimum temperature for their growth (Ciferri, 1983; Somasekaran, 1987). Oliveira et al, (1999) examined that temperature has a great impact on production of *S. maxima* and *S. platensis* used for food.

Effect of the pH

Vincent and Silvester (1979) reported that, the pH had a direct effect on the physiological properties of algae and the availability of nutrient. pH determined the solubility of carbon

source and minerals in the culture directly or indirectly. The optimal pH of the Spirulina nutrient medium was shifted from 8.4 to 9.5 during the mass cultivation, due to the consumption of bicarbonate and sodium ions. Spirulina grew well at pH values between 9 and 11, which are the limiting conditions for the cultivation of microalgae in open reactor (Subramaniyan and Jeeji Bai 1992, Volkmann et al., 2008). Pandey et al, (2010) examined the influence of pH in *Spirulina platensis* growth, protein and chlorophyll-a content. The dry weight of *Spirulina platensis* was 0.91g/500ml and protein and chlorophyll-a contents were 64.3% and 13.2mg g-1 respectively at pH 9.

Effect of light on *Spirulina*

Light intensity and the efficient utilization of it plays a vital role in increasing of biomass yield (Barbosa et al., 2001). Spirulina required light intensities during its growth phase. The optimal light intensity was between 20 and 30 K lux (Ogawa and Teuri 1970). Subramanian and Jeejibai, (1992) reported different effects on growth, pigment and protein synthesis caused of light quality in *Spirulina fusiformis*. The light was provided, with different coloured cellophane paper covering the fluorescent bulbs at 2 K lux intensity. Blue light yielded highest protein content followed by yellow, white, red and green light.

Effect of salinity on *Spirulina*

Imbalance of cellular ions result in osmotic stress and ion toxicity which can be caused due to salt stress which leads to growth retardation, directly or indirectly by oxidative stress, the salt stress can also alter the metabolic pathways leading to the death of the organism (Dahlich et al., 1983; Shalaby et al., 2010). The salt concentration (mostly carbonates and bicarbonates) plays a direct role in the growth of Spirulina.

Nutrient value of *Spirulina*

Spirulina is considered as the important natural sources of protein i.e meat protein content is five times less than spirulina and provides the essential and nonessential amino acids, having a well-balanced amino acid pattern and also the beta-carotene content is high which is a precursor of vitamin A. Moreover the content of vitamin B12 is 0.5 times the amount in liver and detected as only vegetable source of B12 and also act as the source of the precursor of hormones that involved in regulation of body functions that is essential fatty acids. It has been demonstrated that it has a source of vitamin A. It has a positive impact on weight and other parameters like arm circumference, height, albumin, prealbumin, protein and haemoglobin.

Improvement in nutritional status of malnourished HIV-infected patients has also been shown by supplementing the diet with spirulina. Moreover *Spirulina* has been recognized as the risk free food for children which is very much suitable.

Comparison of the nutrients in *Spirulina* with other;

Mainly cellulose is the major component of the cell walls of most vegetables are not digested by us. Mucopolysaccharides are the cell wall component of the *Spirulina* (complex sugars) that are easily digestible. It has been tested that cellulose of *spirulina* is 83 to 95% digestible. It has around 90% protein, which more than any standard standard protein content it has more than caseinthe). In addition to these most of the proteins of *Spirulina's* are already in the orthodox form .These building blocks of the body are called biliproteins and are considered to be the most profuse authority of biliproteins presently known. Products of *Glycine max,* soy flour, etc., are often instance as a high protein source. Out of 37%age of protein only 25% digestible (usable), an 8 ounce serving of *G. max* gives 74 ounces (21 grams) of passable protein. It has more usable protein than wheat flour that is 50 times more. Or, in other words, it takes only 1/6 ounce (4.5 grams) of *Spirulina* to get the same amount of protein as 8 ounces of whole wheat flour (provided it finds its way into people's stomachs). But in recent days the private and government agencies are annexing vitamins and minerals to flour as *Triticum* spp. are scandalously low in these part.

Essential fatty acids (EFA's) can't be produced by our body and are therefore "essential" in your diet. EFA's include linoleic, linolenic and arachidonic acids. It help to reduce total cholesterol and triglyceride levels (associated with arteriosclerosis and heart disease). Cell membranes of *spirulina* are largely composed of lipids. It has high lipid content that is 7% by volume.

It has a high content of calcium as much as milk, also the vitamin A content is 15 times more than carrot, 3 times the iron of sirloin steak, 2.5 times the vitamin B-12 of calves' liver, 3.7 times more potassium than rice. Being high in antioxidants such as Vitamin E, and ferrodoxin.

Spirulina has 16.5% carbohydrates, of which 9% is a rare sugar known as rhamnose. Rhamnose is more biologically active than other sugars because it combines more readily with other nutrients. Unlike sucrose, it does not upset the blood sugar balance or overwork the pancreas. The only vegetable that contains glycogen is spirulina. So, like its biliproteins, *Spirulina's* glycogen is ready made for direct assimilation and use. It also has glycosides and biflavonoids.

Chlorophyll is an essential pigment. *Spirulina* is about 1% chlorophyll, which is 2 to 3 times more than most land plants. Bile pigment called phycocyanin makes up 7 to 18% of *spirulina's* weight which is not found in other food.

Importance of *Spirulina* towards health

Spirulina got its benefits that can't be achieved by any other organism in the present scenario it has many importance starting from the food to medicine. Due to his therapeutic values it is being used as dietary nutritional supplement and shows anti-inflammatory effect by inhibiting cytokine formation (CM et al., 2009). It also shows anti-arthritic effect by reducing beta-glucuronidase, histopathological and ultrastructural studies revealed these property, also shows the antioxidant side of the microalgae (Remirez D. et al., 2002). Due to its high content in proteins, amino acids, vitamins, carotenes and other compounds it is used as bioactive additive that help in increasing the immunity level by stimulating the production of antibodies and cytokines (BlinkovaLP.et al., 2001). Other than these studies *Spirulina* has many health benefits like,

• Helps in weight loss of the body.

• It has counter- mechanism for the toxins present in the body.

• It helps in purification of liver.

• Helps in lowering down the blood cholesterol level.

• Help to against the viral infections.

• Used in the treatment of radiation sickness

• Has the ability to generate new blood cells

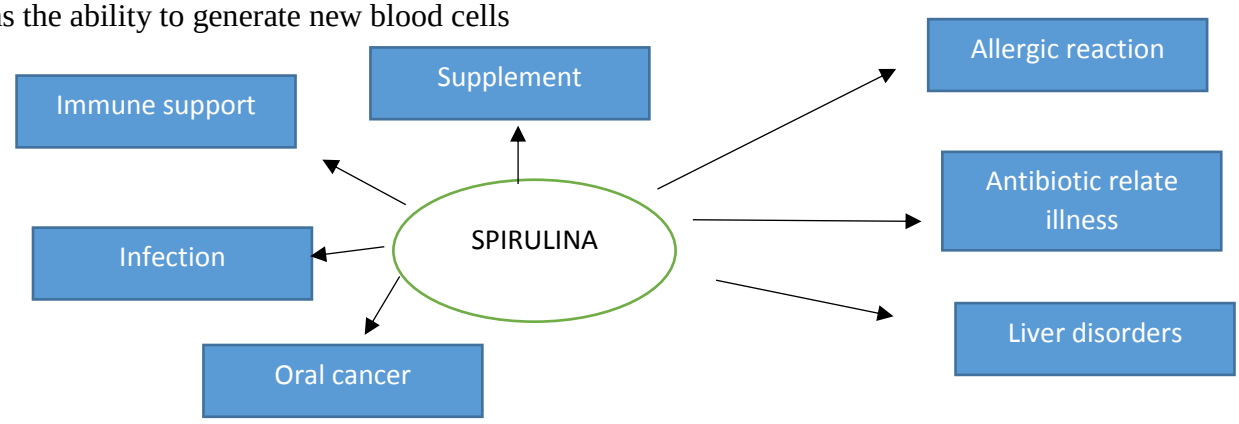


Fig 2: Importance of *Spirulina* in health

Culture of the *Spirulina spp.*

As the *Spirulina* strains are the potent source of many valuable nutrients and products that help to overcome the food problems and nutritional deficiency moreover due to presence many bioactive compounds like phenol, flavonoids, pigment-protein etc they are produced commercially for the human and animal feed and also in pharmaceuticals industry. So a cost effective comparison was done by growing it in zarrouk's medium which was considered as the standard control medium and in the cost reduced media with four different concentration of ammonium nitrate where it shows that the growth rate and the biomass are inversely correlated with the urea concentration (Madkour et al.,2012). Since years these species are grown in open ponds with low cell concentration but in recent studies it has been found that these organisms can use organic substance like glucose for their better growth and development with varying parameters like light intensity and glucose concentration (Chojnacka and Noworyta. 2003). Another method of culture is practiced known to be an alternative method for photoautotrophic culture using a fed-batch in a fermenter allowing to produce high cell density up to 10.24g dry biomass per litre, also with high concentration of phycocyanin (Chen and Zhang. 1997). A cost effective production by using NPK- 10:26:26 fertilizer and flue gas was obtained where the whole process is carried out in sintered disk chromatographic glass bubble column resulted in improved growth result up to 50% was successfully achieved as compared to the standard growth medium of the *Spirilina* species i.e Zarrouk's media (Kumari. et al, 2014). Microalgae is an effective way to reduce the green gas effects on the earth surface and the best alternative of fossils fuels by putting these in the view a high scale productivity is obtained by culturing the species in a bench-scale bioreactor with daily harvesting using electrostatic flocking cloth as substrate with a lower light intensity less than 200µmol m^{-2} s^{-1}, in CO_2 enriched air with an aeration rate of 0.0056ms^{-1} in sodium carbonate free zarrouk's medium (Lanlan et al., 2015)

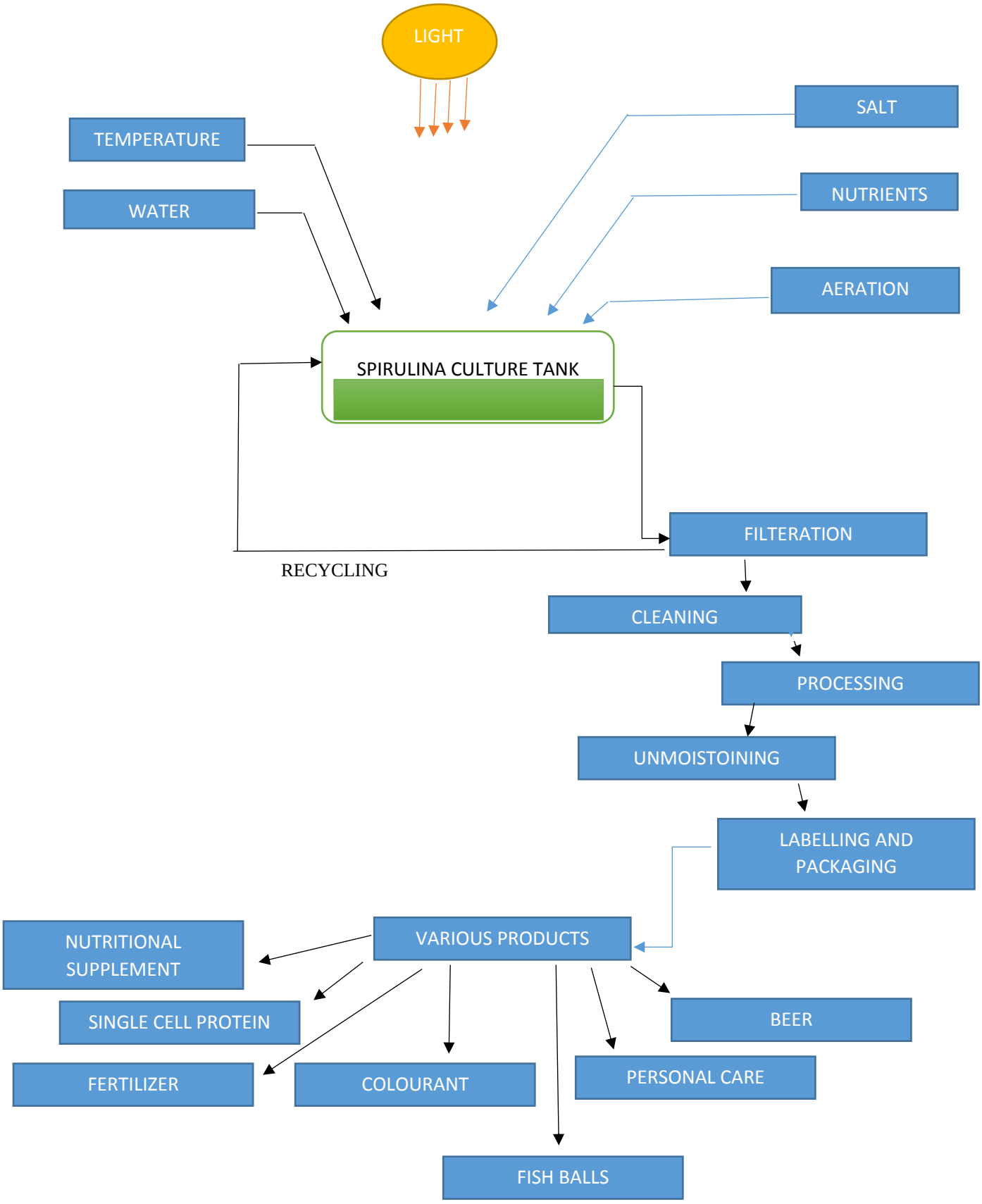


Fig 3: Schematic view of culture procedure and various product from *Spirulina* strains

Extraction

Generally the products that we get from the nature especially from the microorganism are limited in their availability, quality, yield, variability and the most important part is their extraction part which determine its value and effectiveness. So the extraction is the procedure that allow us to extract out the desired product from the organism or plant treating with different solvent and gaseous system followed by different physical and chemical processes. There are different extraction procedure with their advantage and disadvantage listed by different researchers but if you consider the procedure is valuable if it is environmental friendly, low time consumption and cost effective. If we go through the procedures of extraction it differ from each other based upon the compound and its need some of them are traditional solvent extraction, microwave extraction, pressurized liquid extraction, supercritical liquid extraction, solid/liquid extraction, detergent treatment extraction, lysozyme extraction and freeze-thaw (Kwei, 2012). For the bioactive compounds the extraction procedure may be done in two ways the first one is cold extraction (sonication, maceration) and the second one is the hot extraction which involves processes like soxhletation, hot water extraction etc (Agustini etal.,2015).

From years ago the species of spirulina has been used in various ways to meet the food requirement in many developed and under-developed countries. But in today's century the biomass of these species is being used in many cosmetic product. It contains a colorant compound which visualize a bright blue colour that exist in monomers, trimers or hexamers posing different absorbance at 620 and 650nm. Leaving his blue colour the phycocyanin exhibit many bioactivities including antioxidant, anti-tumour and anti-inflammatory effects (Chen et al., 2014).

For the production of high purity phycocyanin having the ratio more than 3.5 and higher yield of 30-40% the one-step chromatography process has been combined with flue gas from a power plant smokestack which then applied to the culture of *Spirulina paltnesis* as a result products were produced possessing physiological activities, beneficial to human health having the potential to induce secretion of inflammatory cytokines, which report the molecular mechanism of the phycocyanin in humans(Chen etal., 2014). Another study revealed where a simple protocol was developed for the ease extraction with higher percentage of purity from the species *S.platensis* by treating it with ammonium sulphate for precipitation, using DEAE cellulose membrane and acetate buffer for the single step chromatography as a result we get 80% of recovered phycocyanin with a purity of 1.5 (kumar et al., 2014). As a functional dye

known to be very unstable while performing normal extraction process, a conventional process using phosphate buffer has introduced where the value of extracted material increased to 3-5% more than the normal process with a purity rate of 0.909. In these the extraction of the material is done by the hexane combined with high pressure, the compound stability has been compared by DPPH scavenging activity that shows commercially standard phycocyain has 15% less activity than the extracted one (Seo et al., 2013). Since the phycocyanin belongs to the phycobiliprotein family which are pigment protein and amino acid storage complexes an effective process following four different extraction methods that are freeze-thaw method, homogenization by mortar and pestle, ultrasonic and polytron homogenizer. It has been noticed that the procedure with single freeze-thaw cycle followed by sonication yielded highest rate as compared to only freeze-thaw method (Horvath et al., 2013)

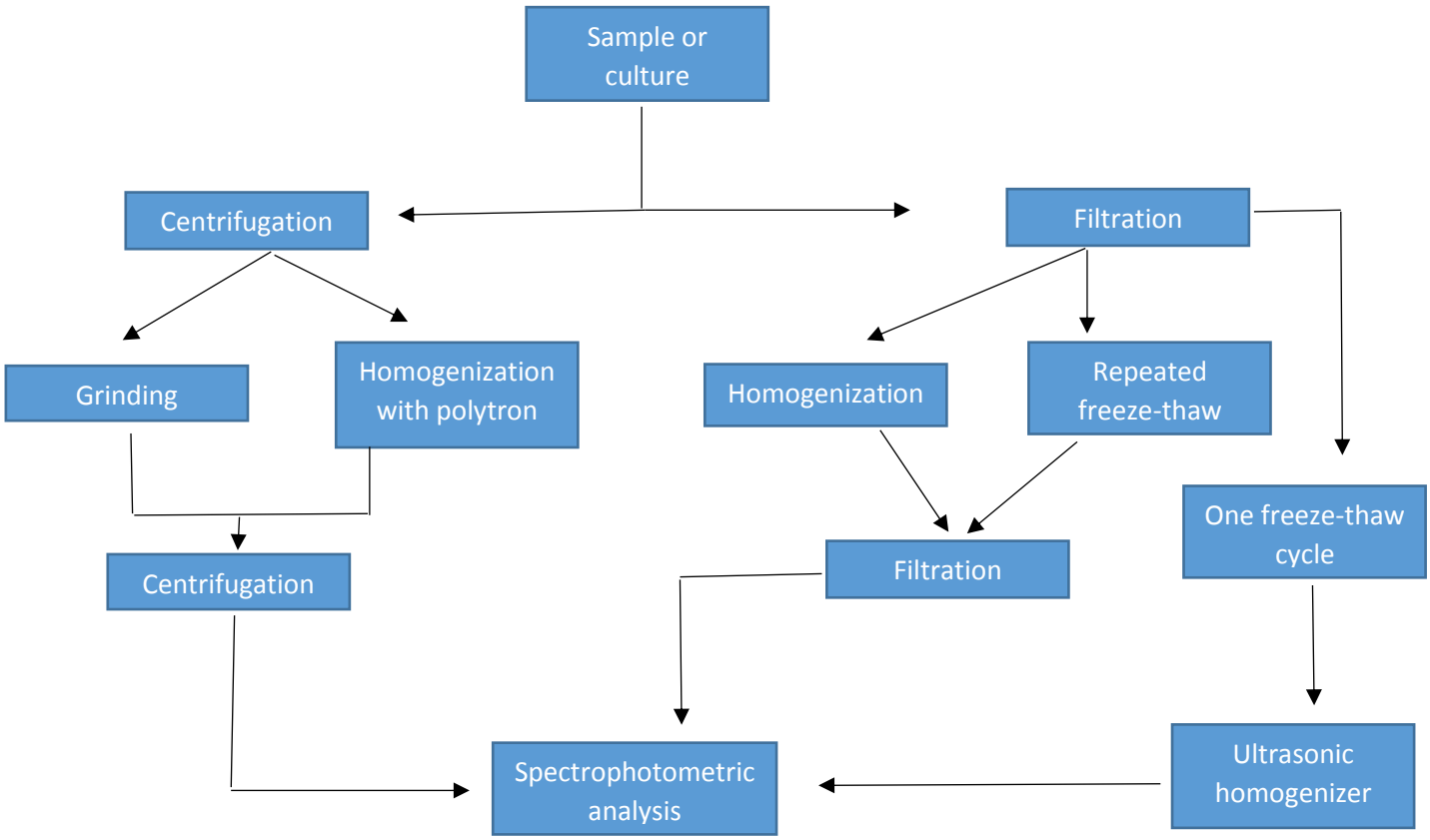


Fig 4: A simple schematic view of extraction and analysis of phycocyanin from *Spirulina* strain (Horvath et al., 2012).

Extraction of biofuel

The ability of the microalgae to develop rapidly and to accumulate lipids in a large amount in the form of Triglycerides make it a valuable source of biofuel (Duong 2016) and the unique characteristic of the *spirulina* to grow in high pH and salinity avoid the growth of the bacteria and moreover it is a microalgae containing typical carbohydrate and protein content, it shows the composition of glucose (54.4%), rhamnose (22.3%), mannose (9.3%), xylose (7%) and others which make them a potential source of chemicals and bio-fuels like ethanol (Sason et al.,2010).

Biofuel like biodiesel is an eco-friendly source of energy that can be used as an alternative, the major source of these friendly energy are the algae.

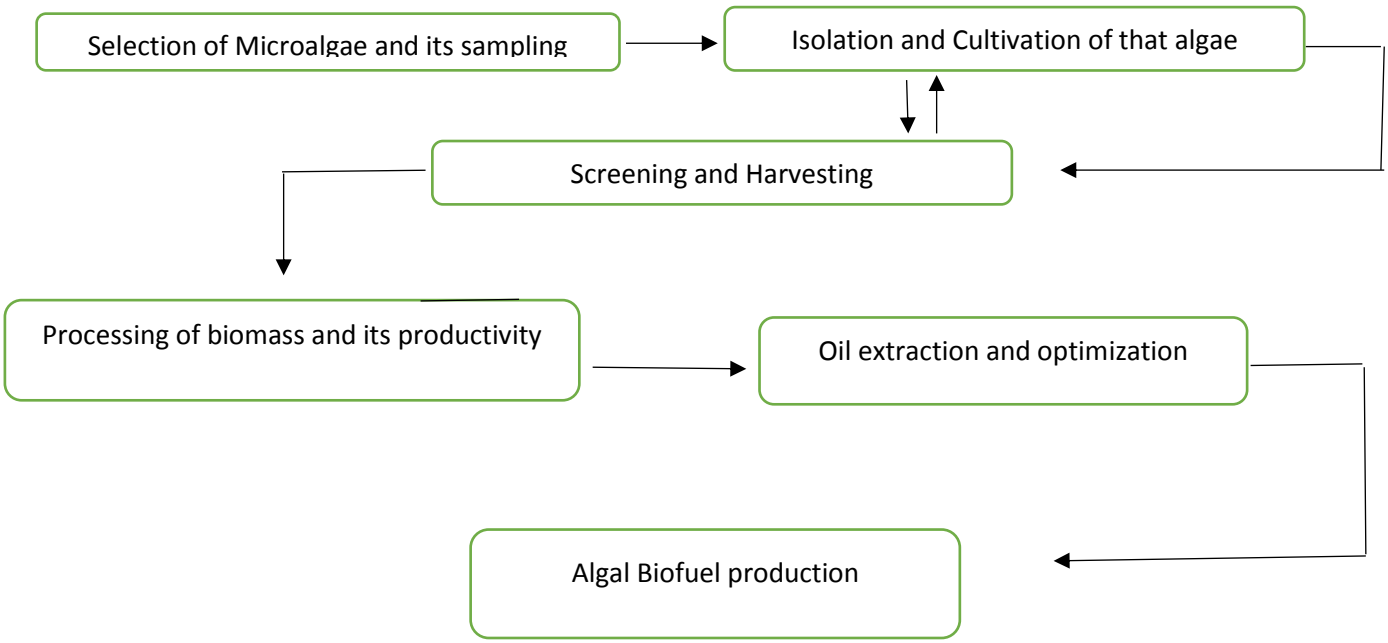


Fig 5: Production chain of Biofuel

Rationale and scope of the study

This study is related to the microalgae *Spirulina* and its product which is regarded as the superfood in today's time in many developed countries like Japan and other developed countries.

In this study different types of microalgae has being identified from different sources present in the locality in search of *Spirulina* species. The study guides us to extract different value added product from the species and their extraction and application for treatment of many diseases and controlling malnutrition. Protein and carbohydrate estimation is carried out to measure the amount in the species for references. The analysis of phycocyanin, phenols, flavonoids and amino acids is done for the quantification in the sample. The study also visualize the antioxidant property that is being carried out by DPPH scavenging activity.

Objectives of the study

- Culture of *Spirulina sp.*
- Quantification and identification of valuable products such as phycocyanin, proteins, carbohydrate, amino acid, phenols, flavonoids, Beta- Carotene, Fatty acids and other valuable products.
- To determine the antioxidant property of the species.

Materials and methodology

Table 4: Chemicals used

Serial no.	Chemicals	Company name
01	Acetic acid	Loba Chemie
02	Boric acid	Loba Chemie
03	Calcium chloride	Loba Chemie
04	Copper sulphate	Loba Chemie
05	Di methyl sulfoxide	Loba Chemie
06	Di potassium hydrogen phosphate	Loba Chemie
07	Ethylene diamine Tetra Acetate	Loba Chemie
08	Ferrous sulphate	Loba Chemie
09	Glycerol	Loba Chemie
10	Heptane	Loba Chemie
11	Magnesium sulphate	Loba Chemie
12	Methanol	Loba Chemie
13	Manganese chloride	Loba Chemie
14	Potassium Sulphate	Loba Chemie
15	Potassium hydroxide	Loba Chemie
16	Potassium nitrate	Loba Chemie
17	Sodium hydrogen carbonate	Loba Chemie
18	Sodium hydroxide	Loba Chemie
19	Sulphuric acid	Loba Chemie
20	Sodium molybedate	Loba Chemie

21	Sodium chloride	Loba Chemie
22	Sodium nitrate	Loba Chemie
23	Sodium Bicarbonate	Loba Chemie
24	Urea	Loba Chemie
25	Ethanol	Loba Chemie
27	Bovine serum albumin	Loba Chemi
28	Glycerie	Loba Chemi
29	Anthrone	Loba Chemi
30	Hydrochloric Acid	Loba Chemi
31	Folin-Coicaletu reagent	Loba Chemi
32	Potassium sodium tartarate	Loba Chemi

Table 5: Glassware used

Serial No.	**Name of Glassware**
01	Conical Flasks
02	Erlenmeyer Flasks
03	Graduated Pipettes
04	Volumetric flasks
05	Centrifuged tubes
06	Test tubes
07	Hach tubes

Table 6: Instruments Used

Serial No.	Name of the Instrument
01	Auto clave
02	Centrifuge
03	Laminar air floe
04	Metric balance
05	Water bath
06	Vortexer
07	UV spectra photometer
08	Pestle and mortar
09	Microscope

Table 7: Miscellaneous materials

Serial no.	Materials
01	Cotton
02	Aluminium foil
03	Butter paper
04	Forceps
05	Whatmann filter paper
06	Capillary tube

Research methodology

Research material – Spirulina product (commercial capsules) and different water from different water bodies for identification and isolation of microalgae. *Spirulina paltnesis* strains.

Microscopic studies

Collection of different water from different local water bodies were made. The available macro algae were separated and thrown out but the microalgae are separately collected in the specimen tube, kept in moist condition either in fresh water or collected water. After they are sorted out and identified in laboratory with high quality electron microscope of resolution at 10X, 40X and 100X by performing different staining procedure and applying glycerine for proper visualisation.

Culture:

The strains of the microalgae Spirulina platensis has been purchased from the IGCAR, Kalpakkam, Chennai, India. The strains are kept under optimal condition for cultivation in different culture medium and modified medium the basic media used for the cultivation is the Zarouk media. Four different setups has been done for the production of the species which are;

Culture 1- The strain is cultivated on the agar plates having the zarrouk medium as the media, a temperature of 25-30ºC with photoperiod of 12 hour dark and light is subjected to it and the observation has to be carried out after 30 days but it has to be observed on every week for checking the contamination in it.

Culture 2- These culture is exhibited in liquid/ broth medium, the media used is the zarrouk medium but without the agar in it, the whole process has carried out in 250ml flask and the condition is maintained in room temperature and manual agitation is done thrice in a day by shaking the flask, the culture has to be observed daily for the change in pH and viscosity, here the day-length varies.

Culture 3- The culture is a batch culture carried out in the conical flask, with constant light and a temperature of 35º C, the media is kept under constant agitation at 120rpm in the shaker and the growth is being observed after 30 days but weekly observation is done to see the viscosity.

Culture 4- These one is the photoautotrophic culture without any addition of sugar molecules, the initial pH has maintained at 9.5 and was subjected to light and temperature of the

surrounding, it is carried out in a 250ml flask the media used is the zarrouk's media of desired concentration and the culture is noted down for rest 30 days.

Culture 5- Glucose is added to the culture medium here the mixotrophic batch culture is maintained. All media and materials are autoclaved at 121ºC at 15psi for 15 minutes and a constant agitation and aeration rate is maintained in a room temperature. The culture is carried out in 250ml flask and the observation study is carried out for next 30 days for the production of biomass of the species.

Protein estimation:

Spirulina capsules has been collected from the market and were burst and the dry powder is weighed and kept in dry tube or bottle. 1gm of powder is taken and mixed in 10ml of methanol and another 1gm of powder is taken and mixed in 10ml of acetone in two different beaker for about 3 hours. The extract was then filtered out by the help of filter paper in test tubes.

Reagents preparation

A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% NaK Tartrate in water

C. 0.5% $CuSO_4$ in water

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

Preparation of standard BSA- 1 mg/ ml

Then different concentration of standard(200µg, 400µg, 600µg, 800µg and 1000µg) along with unknown concentrations are taken and mixed with distilled water to make it 1ml and then reagent C is added(4.5ml) in each tube and subjected to heat of 35ºC for 30 min in water bath and then reagent D is added of volume 0.5ml in each tube and subjected to 35ºC for 60min in water bath where the colour changes and after the absorbance is taken at 660 nm and graph is plotted from where the unknown concentration of the sample is calculated out by Trends formula.

Concentration of the sample- 16.6mg/ml

Carbohydrate Estimation (Anthrone Test):

Carbohydrates are hydrolysed by treating with acids to form furfurals which are condensed by anthrone reagent to form a blue green colour, where the intensity can be easily measured at 620-630 nm in spectrophotometer

200mg of anthrone is weighed and mixed with 95ml of concentrated sulphuric acid in 5ml of distilled water for the stock then 100mg of *spirulina* dry powder is weighed and placed in dry test tube, add 1ml of HCl in it and then add 4ml of anthrone solution.

Then different concentration (20µL, 40µL, 60µL, 80µL and 100µL) of the standard (glucose 1mg/ml) is taken with unknown concentration of the sample whose carbohydrate concentration is to be known is incubated in water bath at 60ºC for 60 minutes as a result of which deep blue green colour develops and its absorbance spectrum is taken at 630nm.

After the standard the sample absorbance is taken at three different concentration that is 20, 40 and 60 µl respectively and the OD is noted down.

Standard curve is drawn and the unknown concentration of the sample is calculated out by the trends formula.

Phycocyanin analysis:

Phycocyanin are the important accessory pigment that contributes towards the photosynthesis in a green plant, it belongs to the family phycobilliprotein but in case of algae like *Spirulina* it plays 50% role of the photosynthesis.

The phycocyanin concentration in the spirulina has been estimated by the principle of spectrophotometry at an absorbance 620nm. Before analysis the dried percentage of *Spirulina* dry mass has been noted by the following formula

Dry Weight Calculations

$$\text{Percent dry wt.} = \frac{\text{(pan (g) + dried powder (g)) - pan wt. (g)}}{\text{Powder wt. (not dried) (g)}}$$

The assay was performed by taking 40 mg of powder into a 15ml centrifuge tube with 10ml of 100mM phosphate buffer having the pH 7.0, vortex well to mix and kept overnight in the refrigerator. The next it was centrifuged at 3500rpm for 5 minutes and the absorbance has been taken at the 620nm. The readings are done in triplicates and the average absorbency has been noted down for the calculation of the estimated phycocyanin in the sample.

Derivation of pure C-Phycocyanin:

$$\%\ \text{pure CPC} = \frac{\text{A620 x (10) x (100)}}{\text{7.3 x (mg. sample). x (\% dry wt.)}}$$

Where,

7.3 is Extinction coefficient of C-PC at 620 nm

10 is total volume

100 represents 100%.

Derivation of crude C-Phycocyanin:

$$\%\ \text{crude C-PC} = \frac{\text{A620} \times (10) \times (100)}{[3.39 \times (\text{mg. sample}). \times (\%\ \text{dry wt.}]}$$

Where,

3.39 is Extinction coefficient of C-PC at 620 nm

10 is total volume;

100 represents 100%.

Beta carotene analysis:

Among all the pigments of carotenoids the highly pigmented molecule is the beta carotene, it is strongly red pigmented having the chemical formula C40H56 which structure was deduced by Paul Karrer in 1930 since it is a terpenoid, non-polar compound it dissolve in fats, lipids, and other non-polar solvents.

The assay was carried out on the basis of spectrophotometric principles, the experiment was performed in low light and temperature to avoid the denaturation of the compound as trhe compound is very sensitive to the above factors. It has two important part the extraction and the analysis. For the extraction 30mg *Spirulina* powder is to be dissolve in 2.5ml DMSO in tubes for proper mixture it is being subjected to vortex for 30 seconds, then it is placed into water-bath for 30minutes at 50 c. after removing from the bath 5ml methanol is added to the tube, vortexed for 30sec and subjected to centrifugation at 4200rpm for 3 minutes. Then the supernatant is to be removed to a volumetric flask and 4 ml methanol is added to the tube and subjected to the centrifuge at same rpm for same time interval after that the supernatant is again collected these procedure is followed until the methanol become clear. In the end the collected supernatant is brought up to 25ml by adding methanol.

After the extraction comes the analysis part where 8ml of the methanol extract is put into the 15ml centrifuge tubes and 5ml of heptane, 1.5ml of saturated KOH is mixed in it and allowed for saponification (30min) in dark, then the tube is subjected to the centrifuge at 4200rpm for 3 minutes and the heptane layer is removed, the interphase is washed with 1ml of heptane and add 3ml of heptane in it and invert it for 8 times and allow the mixture for 2 minutes for separation, then the heptane layer is collected in the tube and the volume is brought up to 10 ml by adding heptane into it. Analysis is done by taking the heptane extract and dissolving in DI water in the ratio 5:5. At 436nm the reading is noted down taking heptane as blank in spectrophotometer. The experiment is to be carried out in triplicates or duplicates.

Calculation

Percentage of beta-carotene =

$$\frac{Abs\ 436 \times 25\text{ml} \times 1.25 \times 100 \times 0.84}{196 \times (\text{wt} \times \text{dry wt.})}$$

Antioxidant assay:

Spirulina spp. exhibit many bioactive compounds that act as an antioxidant which are more efficient than the synthetic one and moreover they are non-toxic and safe to use.

Preparation of DPPH

1. 3.94mg of DPPH was mixed to 100ml absolute methanol to make a solution of 0.1mM DPPH solution.
2. After preparation of the solution it was kept at room temperature for half an hour in dark condition.
3. Then the absorbance was taken by spectrophotometer at 517nm.

DPPH Antioxidant Assay

1. Concentration ranging from 0.3-1.0 mg/ml of sample were taken and done in triplicates and 2 ml of the earlier prepared solution were added in each test tube.
2. Then it was kept under dark for 5-10 minutes at room temperature, it can be covered with a black paper for better results.
3. Optical density was recorded at 517nm.
4. All the tests were performed in triplicates.
5. DPPH radical scavenging activity was calculated using the following formula

Scavenging effect (%) = $\left(1 - \frac{As}{Ac}\right)$ x 100

Where, A_S = OD of sample with DPPH solution

A_C = OD of DPPH methanol solution. (Padmanadhan and Jangle, 2012).

Phenolic content analysis:

The total phenolic content of the spirulina powder that has been collected from the sample was analysed by Folin-Ciocalteu reagent method with some modification in it, Gallic acid has been used as the standard as a small amount of it indicates the presence of large amount of phenols in the ,material. A standard solution has been prepared in concentration of 5mg/ml from where (stock solution) 0, 2, 4, 6, 8, 10 ml has taken in 100ml volumetric flask where the volume was brought up by dissolving distilled water making the concentration 0, 100, 200, 300, 400, 1000 µg/mL Gallic acid and after that 40µL from each concentration is taken with 3.16ml DI and 200µL Follin reagent which is then mixed properly and kept for 5 minutes followed by addition of 600µL 20% sodium carbonate and kept for 30minutes in water-bath at 40ºC, a standard curve was obtained by measuring its absorbance at 765nm. For determining the phenolic content of the sample the same is followed by taking 40µL sample extract.

Concentration of the extracted sample- 16.6mg/ml

Flavonoid analysis:

The flavonoid content of the sample is recorded by the help of Quercetin as the standard of which the stock is prepared in a concentration of 1mg/ml in methanol, from this stock standard curve is made by taking 20, 40, 60, 80 and 100µg/ml. It is done by adding 900, 920, 940, 960 and 980µLmethanol in 20, 40, 60, 80 and 100µL of the stock. Then from each 0.5 ml is taken and mixed with 1,5ml methanol + 0.1ml 10% $AlCl_3$ + 0.1ml of 1M Potassium acetate + 2.8ml DI and kept for 30 minutes in room temperature. Absorbance taken at 415nm.

The same procedure is followed for plant extract by taking 0.5ml of the extract.

Concentration of the extracted sample- 16.6mg/ml.

Thin layer chromatography analysis for amino acids and vitamin:

The technique involve two phase which are mobile phase and the stationary phase it works on the principle of equilibrium distribution between the two components in the phase it separate according to that and the Rf value is noted down. The experiment is conducted to determine different amino acids and vitamins present in the sample extract for which the analysis is done on two plate one is the silica plate and the other is the commercially available TLC plate. The mobile phase prepared is the mixture of Chloroform, methanol and formic acid in the proportion of 35:15:1 and benzene, methanol, acetic acid and acetone in the proportion 70:20:5:5, the silica gel is prepared in the ratio of 2:1 in distilled water and plates were made which were kept under air for drying and after that in the oven for activation. For the commercial plate it is not necessary. The methanol extract of the sample is allowed to run in the plates with the standard prepared amino acid solution and after that the reagent i.e ninhydrin is sprayed and dried for getting different readings of amino acid in the sample (Bhawani. et al. 2012). But in case of vitamins the TLC plate is seen under the UV spectrum.

Results and Discussion

The studies area of the locality shows four specimen of microalgae which are *Gloeocapsa*, *Spirogyra*, *Dunaliella* and *Scenedesmus. Dunaliella* is common in the locality it is best adapted to the environmental factors of local surrounding than others.

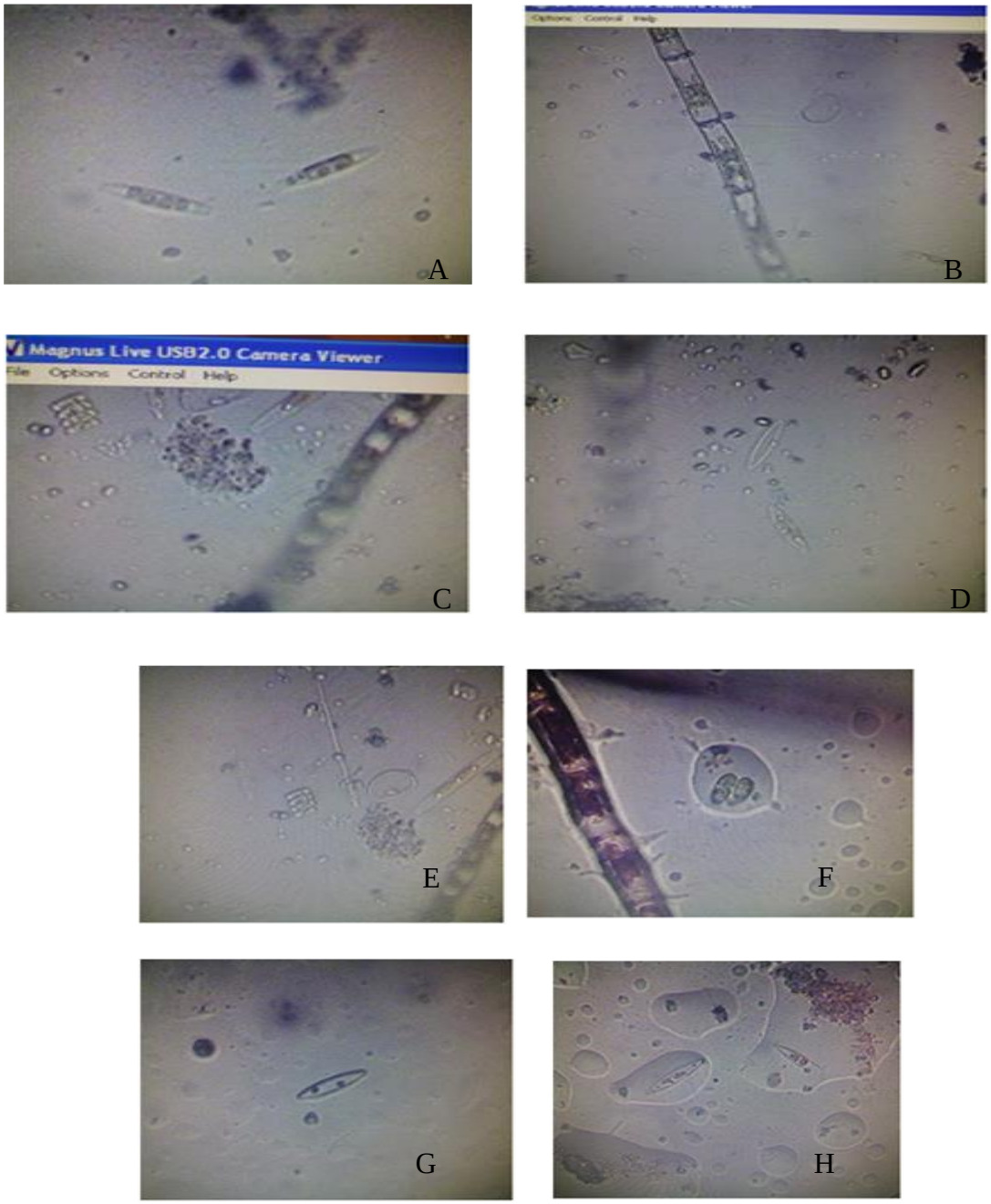


Fig 6: Represents four different strains of microalgae identified under the microscope which are more commonly found i.e *Spirogyra (B and C), Scenedesmus* (C and E), *Dunaliella*(G,H and D) and *Gloeocapsa* (F).

Microscopic study on *Spirulina paltensis.*

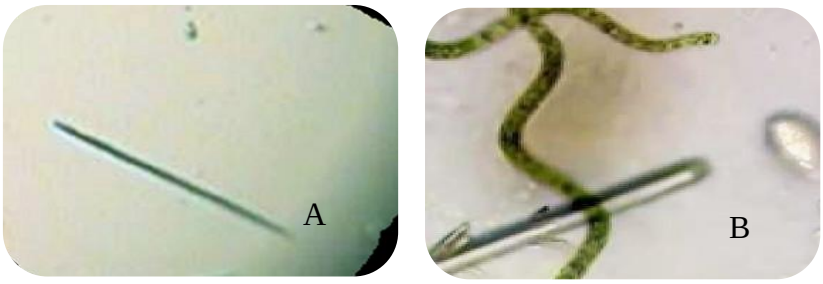
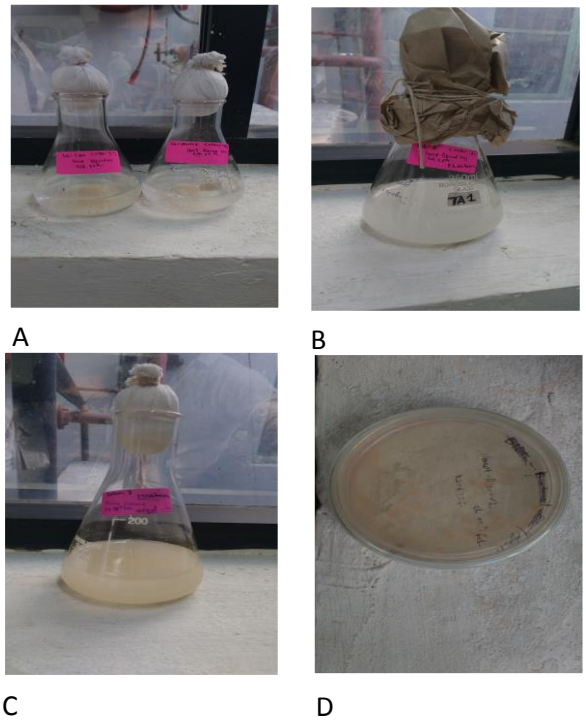


Fig 7: A and B shows pictorial view of *Spirulina platensis*

By comparing the results obtained with recently studies conducted so far it was confirmed that the sample studied was *Spirulina platensis*. Using glycine to visualise the morphology of the sample it was observed that the sample morphology and the morphological features suggested by the recent studies were the same. The comparative morphological studies gave results similar to the studies held recently on *Spirulina platensis*.

CULTURE:

The culture setup of the species didn't responded to the existing environment and climate, it fails to adapt it and moreover the system was affected by surrounding culture, at first the culture shows good response by increasing the pH of the medium, viscosity and the culture on agar plates started to form colonies but later on contaminated due to temperature fail and exposure to more darker periods. Also the failure of the production can be concluded due to disturbance in the light and dark period and agitation rate. So for the proper production all the factors should be processed carefully and efficiently slight disturbance in it fails the production procedure.



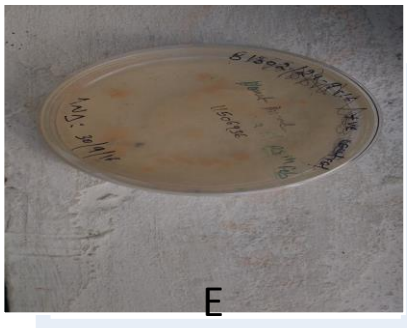


Fig 8: A-E, Shows culture of *Spirulina platensis* done on different media composition and factors.

Protein estimation:

Table 8: Absorbance data of protein content

Vol. of BSA (mL)	Conc. of BSA(µg)	Vol. of DW (mL)	Vol. of reagent C (mL)		Vol. of reagent D (mL)		O.D at 660nm
blank	0	1	4.5	**Incubation for 10 min.**	0.5	**Incubation for 30min.**	
0.2	200	0.8	4.5		0.5		0.361
0.4	400	0.6	4.5		0.5		0.460
0.6	600	0.4	4.5		0.5		0.545
0.8	800	0.2	4.5		0.5		0.661
1	1000	0	4.5		0.5		0.846
spirulina sample							
0.1	X	0.9	4.5		0.5		0.749
0.2	X	0.8	4.5		0.5		0.994
0.3	X	0.7	4.5		0.5		1.92
0.4	X	0.6	4.5		0.5		2.73

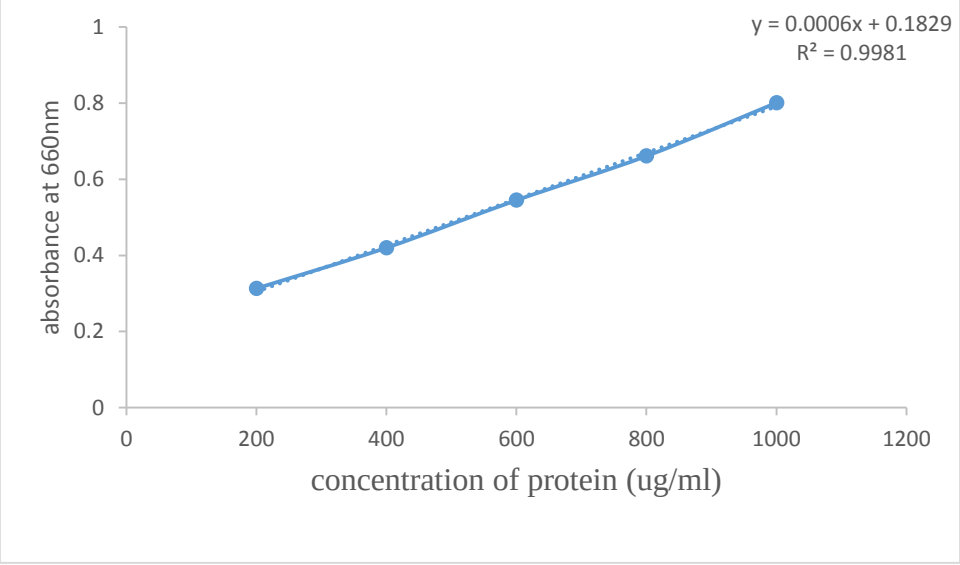


Fig 9: Standard curve of protein

- From the standard curve of protein and by the help of TREND formula it is estimated that the protein content in 100. 200, 300 and 400 µL of methanol extract sample are 929.68, 1331.53, 2850.36 and 4178.92 µg/ml indicating (54±9.83) % of protein in the commercial product of *Spirulina* species.

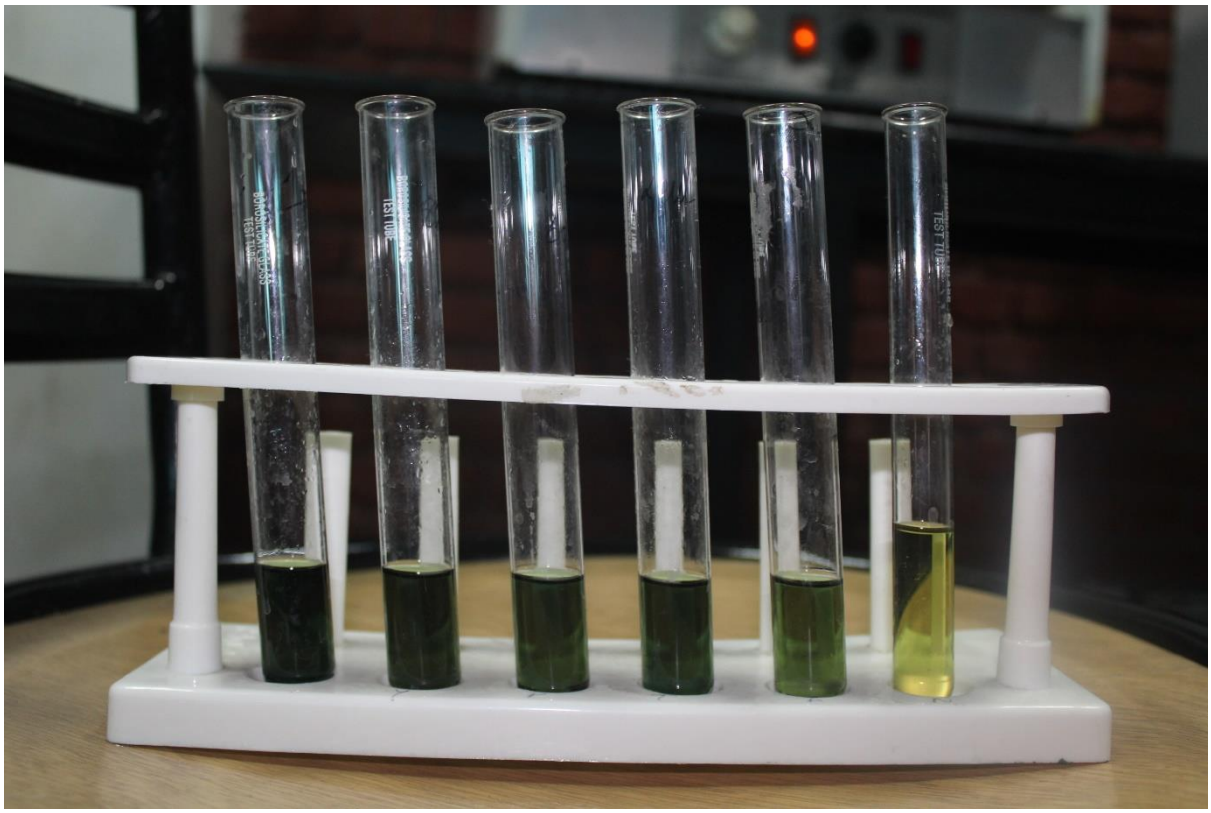


Fig 10: Image depicting protein content in tubes

The result obtained indicates high percentage of protein content in the sample. There are many methods that shows high content of protein in the species of *Spirulina* like there was an recovery of 83.5% by high pressure homogenization and 69.9% by ultra-sonication, it is also been noticed that if the factors are in optimized condition the average yield obtained was 60.7 % (Parimi et al., 2015).

Carbohydrate estimation:

Table 9: Absorbance data of Carbohydrate content

Vol. of Glucose (µL)	Con. of glucose (µg)	Vol. of distilled water (µL)	Vol. of anthrone (µL)		O.D at 630nm
20	20	980	4000	**Cover test tube and put in water bath for 10min.**	0.105
40	40	960	4000		0.226
60	60	940	4000		0.341
80	80	920	4000		0.450
100	100	900	4000		0.540
spirulina sample					
20	x	980	4000		0.300
40	x	960	4000		0.505
60	x	940	4000		0.692

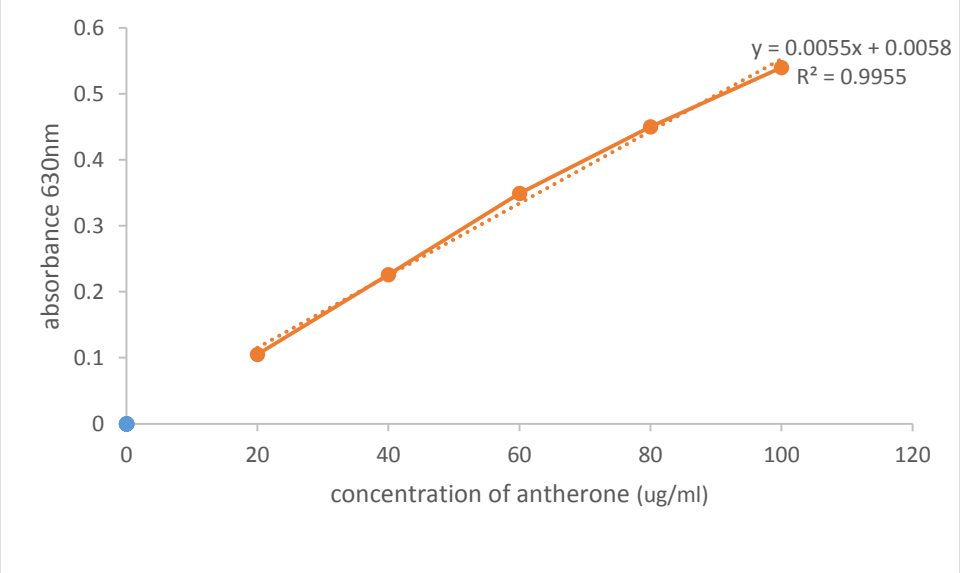


Fig 11: Standard curve of carbohydrate

- From the standard curve of carbohydrate and by TREND formula it has been estimated that the concentration of carbohydrate in methanol extract of *spirulina* powder are 53.82, 91.12, and 125.15 µg/ml in 20, 40 and 60 µL of the extracted sample solution respectively indicating (14.16±1.86)% of the carbohydrate in the product of the *Spirulina.*

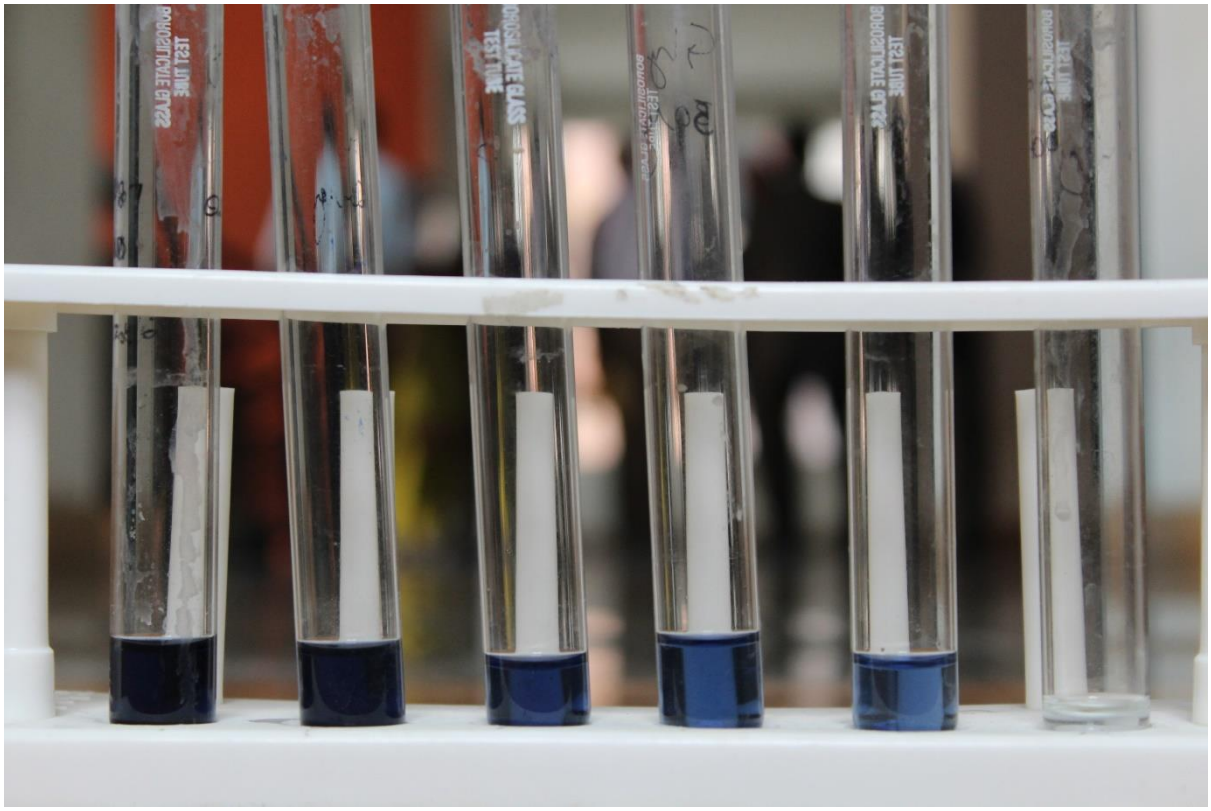


Fig 12: Image depicting carbohydrate content in tubes

As the species of algae contains high amount of polysaccharides, lipids and fatty acids which are used in the production of bio-fuels which is economically beneficial to environment and society. It has been noticed that the carbohydrate concentration range from 10.99 to 66.60% in different culture system with subject to variable factors (Markou et al., 2012).

Phycocyanin analysis:

Absorbance at 620nm = 0.177

%dry weight measured = 0.08

According to the formula the content of pure phycocyanin is 7.57% and the crude phycocyanin is 16.32% in the sample. The results obtained from the study confirm that the spirulina contains efficient content of phycocyanin. So the spirulina can be recommended for the culturing for industrial production of phycocyanins.

Beta carotene analysis:

Absorbance at 436nm = 0.273

According to the formula the content of Beta-carotene in the sample is 0.12%. *Spirulina* is source of many carotenes, the major components among then are the beta-carotene and zeaxanthin, the experiment didn't recover full beta- carotene estimation but according to the studies it ranges up to 3% which has been validated by HPLC. The percentage revealed in the study shows a very minimum value which may be due to the exposure of the sample extract to light and temperature more than requirement as they are sensitive to these factors.

Phenolic analysis:

Table 10: Absorbance data of phenolic content

Serial no.	Concentration of Gallic acid(µg/mL)	Absorbance at 765nm			Mean± SD
		1st	2nd	3rd	
01	100	0.172	0.168	0.171	0.170±0.002
02	200	0.295	0.298	0.296	0.296±0.001
03	300	0.404	0.454	0.428	0.428±0.025
04	400	0.615	0.522	0.598	0.578±0.049
05	500	0.684	0.691	0.687	0.687±0.003
Test sample		0.170	0.177	0.166	0.171±0.005

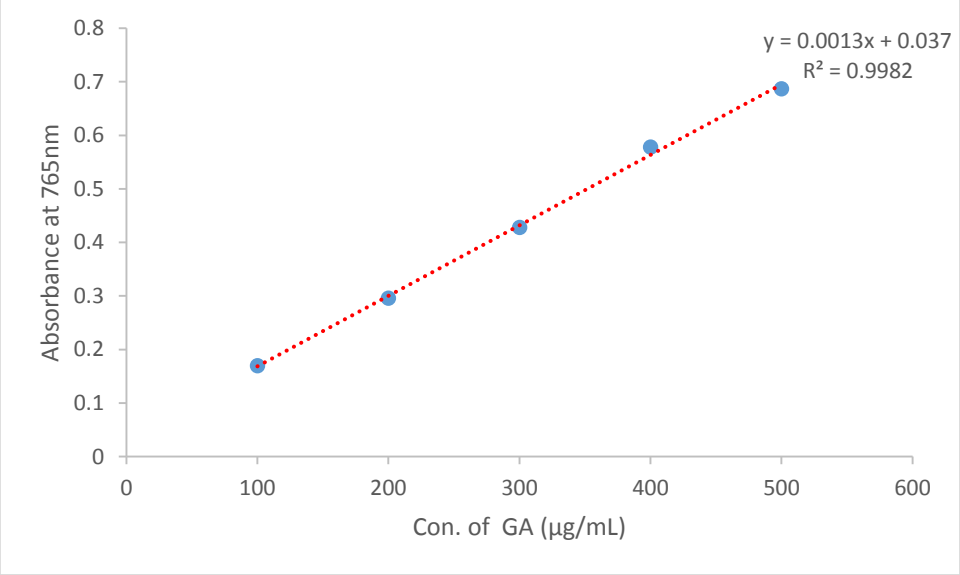


Fig 13: Standard graph of Phenol content

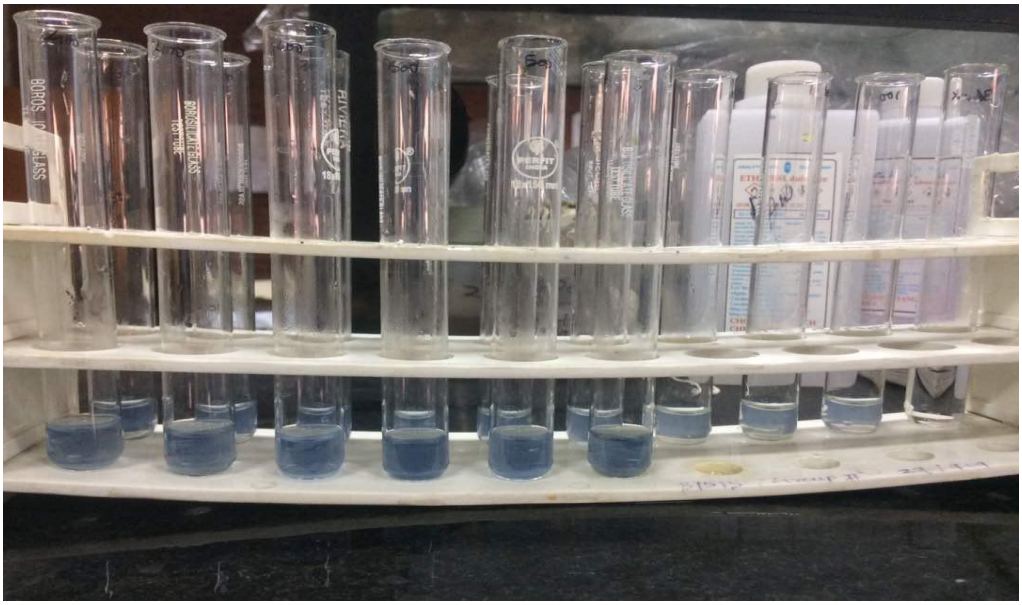


Fig 14: Image depicting phenol content in tubes

Unknown absorbance = 0.171

Concentration of the phenol in the sample = 103.03±0.005µg GAE/mg dry weight sample

It work on the principle of oxidation and reduction the reagent reacts with phenolic content of the sample and reduce to blue colour showing the presence of phenol in it the result came to be in GAE (Gallic Acid Equivalent) which is and the concentration percentage of phenol in the extract is 15%. These phenolic compounds plays the major role towards the anti-oxidant activity with other biological activities, the compound was analysed by spectrophotometric principle which shows that the phenolic content of the *Spirulina platensis ranges* range from 17.0-43.2 % with different solvent extraction procedure (Machu et al., 2015).

Flavonoid Analysis:

Table 11: Absorbance data of flavonoid content

Serial no.	Con. of Quercetin (µg/ml)	Absorbance at 415nm			Mean ± SD
		1st	2nd	3rd	
01	20	0.035	0.030	0.025	0.03±0.005
02	40	0.066	0.067	0.065	0.066±0.001
03	60	0.089	0.083	0.087	0.084±0.003
04	80	0.119	0.112	0.117	0.116±0.003
05	100	0.151	0.150	0.145	0.148±0.003
Test sample		0.523	0.556	0.569	0.549±0.023

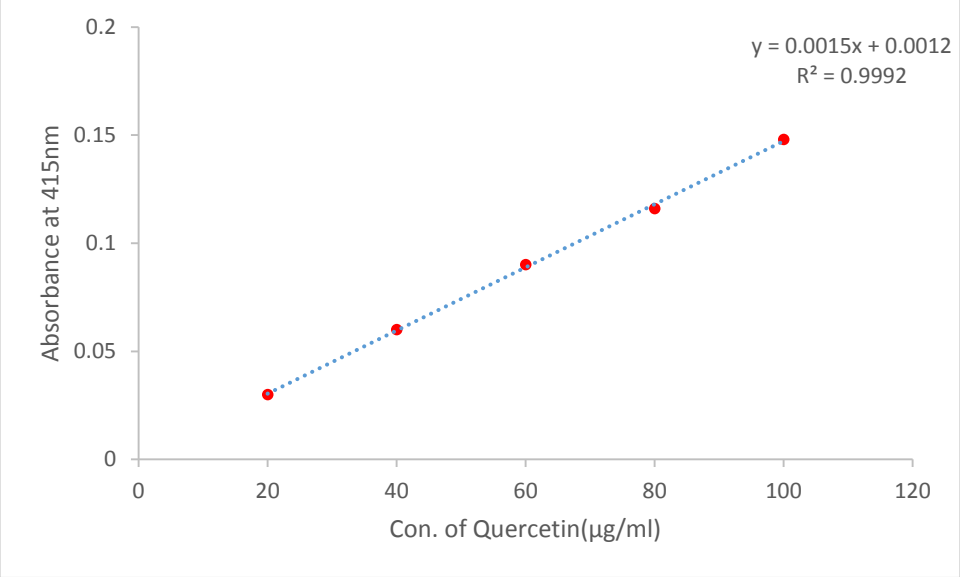


Fig 13: Standard graph of Quercetin

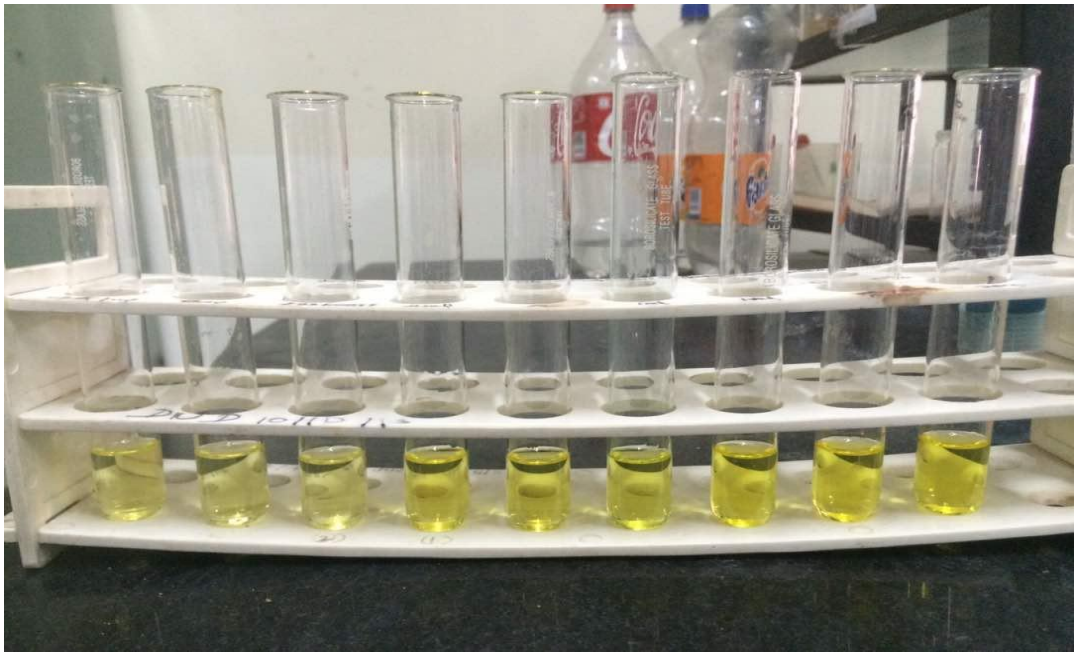


Fig 16: Image depicting flavonoid content in tubes

Unknown absorbance = 0.549

Concentration of the flavonoid in the sample = 374.97µg/ml

The flavonoid concentration in the 500µL of the methanol extracted sample was found out to be 374.97µg indicating 4.51% of the flavonoid in the product. Microalgae shows good amount of bioactive compounds in them among which flavonoid is one of the part, it is been useful to mankind during recent years due to its free radical and antioxidant effectiveness and also their high binding ability to bind with specific proteins. For these above features nowadays large scale production of flavonoids were tried by introducing genetically modified strains where the optimization is done by L- phenylalanine, moreover they can also be exploited as anti-cancer, anti-bacterial and antiviral (Baviskar and Khandelwal. 2015).

Antioxidant Assay:

Table 12: Absorbance data of antioxidant assay

Sample (µL)	Methanol (µL)	DPPH (ml)	Conc. of sample (mg/ml)	Absorbance at 517nm			Mean± SD	%Scavenging Activity
				1st	2nd	3rd		
40	60	2	0.332	0.661	0.667	0.670	0.665±0.004	20
60	40	2	0.498	0.506	0.519	0.560	0.528±0.028	33
80	20	2	0.664	0.401	0.404	0.402	0.402±0.001	49
100	0	2	0.830	0.386	0.385	0.383	0.384±0.001	51

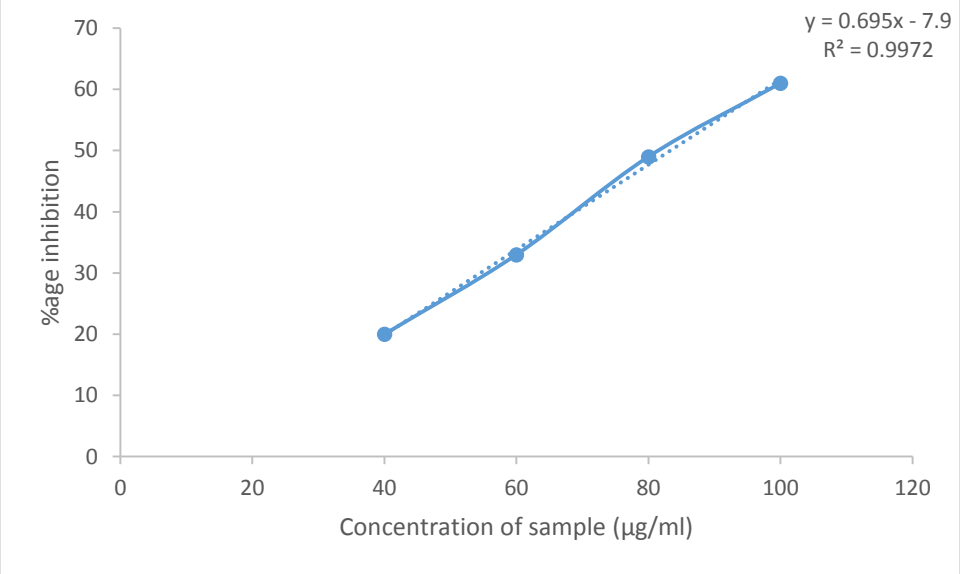


Fig 17: Graph showing DPPH scavenging activity

Concentration of stock solution = 16.6mg/ml

OD of DPPH at 517nm = 0.791

IC50 = 83.30mg/ml

From the above information we can say that the product has high anti-oxidant activity which is highly helpful to maintain a healthy body.

TLC analysis:

The analysis of the extracted sample on silica plate shows three amino acids which were allowed to run in the solvent chloroform, methanol and formic acid in the ratio 35:15:1. The result was analysed by measuring the Retention factor of the amino acid travelled in the plate and by comparing it with the standard that were simultaneously done in the plate.

Try Gly Trp test

Fig 18: TLC of amino acids on prepared silica plate

Fig 19: TLC of amino acids on commercial silica plate

Distance travelled by the solvent = 11.2

Distance travelled by the standards: Tyrosine = 5.1

Glycine = 2.8

Tryptophan = 6.9

It was observed by running TLC on a freshly prepared silica gel plate that rf values for Tyr, Gly and Try were 0.55, 0.25 and 0.61 respectively. These values are very much close to the real reference values 0.45, 0.26 and 0.66 respectively. The sample values were compared with the standard values obtained above. It was confirmed that the sample contains glycine and

tyrosine. The spirulina contains numerous amino acids but the other amino acids were not observed because of the presence of various chemicals present in the sample.

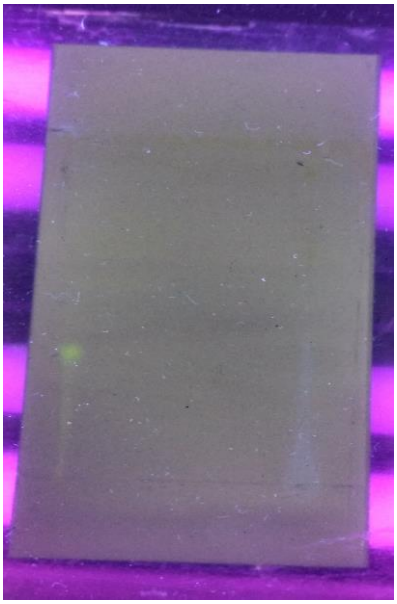


Fig 20: TLC of vitamin on commercial silica plate

The sample was allowed to run with three standard out of which the riboflavin shows positive result but the other two ascorbic acid and pyridoxine doesn't revealed any fluorescence under the UV spectrum due to the solvent system used and moreover the other vitamins may be masked out by the other constituents of the sample for which it fails to show other vitamins in it. Since the *Spirulina* Spp. are rich in riboflavin content, the product shows positive result to the analysis.

Table 13: Composition content analysed in the product of *Spirulina.*

Serial no.	Compound	Content percentage (%)
01	Protein	54±9.83
02	Carbohydrate	14±1.86
03	Phycocyanin	16.52
04	Beta- carotene	0.12
05	Phenol	15±0.005
06	Flavonoids	4.51±0.023

Conclusion and Future scope

Microalgae shows that they are the sources of many biological active compounds and nutrients which drives the attention due to its health benefits, the compounds residing in them ranges from phenol to flavonoids to essential amino acids which in near future will play key role in deleting lethal diseases from the society. The experimental study done on the *Spirulina platensis* revealed its importance to the human kind and the environment, also it is going to play a very crucial role in the near future for its nutritional value and for its pharmaceutical value. They are very common to the marine environment but few of them also resides in fresh water. Collection and sampling of nearby water bodies doesn't revealed the presence of the species as it might be that the optimum condition for the growth and development of these microalgae is not suited here but it shows the presence of other form of microalgae. The production of these microalgae depends upon the temperature and pH of the surrounding with the availability of the sunlight for its production of its biomass.

The experiment shows a good concentration of protein and carbohydrate, it also summarize the phenolic and flavonoid content of the species in a good manner. It has high scavenging activity revealing its antioxidant property. The species has a favourable concentration of phycocyanin and beta-carotene in it that can be very beneficial to the human kind to meet the food demand and to remove malnutrition diseases from the world.

Due to the increasing population and global warming the earth is losing its land surface, in these case *Spirulina* can be used as crop to meet the global demand to eradicate the nutritional deficiencies and can be cure to many diseases. It can be an ideal food source which can be cultivated on space by International space station which will be beneficial for future space exploration. Moreover it can be an alternative for fishermen to sustain their livelihood.

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