Enhanced production of *Scenedesmus acutus* extracellular polysaccharides exhibiting immunostimulation and antioxidant activity

A thesis submitted in partial fulfilment of the requirement for

the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

Tania

601604009

Under the guidance of

Dr. Manoj Baranwal

Associate Professor

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA (PUNJAB)-147004

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Certificate

This is to certify that the project entitled "**Enhanced production of *Scenedesmus acutus* extracellular polysaccharides exhibiting immunostimulation and antioxidant activity**" submitted by Tania in the partial fulfilment of the requirement for the award of degree of **Master of Technology** in Biotechnology to Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is a record of student's own work carried by her. The report has not been submitted for the award of any degree or certificate in this or any other University or Institute.

Dr. Manoj Baranwal
Associate Professor, Supervisor
Department of Biotechnology,
Thapar University, Patiala

Candidate Declaration

I, hereby declare that the work presented in the thesis entitled "**Enhanced production of *Scenedesmus acutus* extracellular polysaccharides exhibiting immunostimulation and antioxidant activity**" in the partial fulfilment of the requirement for award of the degree of **Master of Technology** in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is an authentic record of my work during the period of one year from June 2017 to June 2018, under the guidance of Dr. Manoj Baranwal, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other diploma or degree.

Tania

Tania **Place: Patiala**

601604009 **Date:** 04-09-2018

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Abstract

Microalgae biomass has been recognized and identified as a source of novel bioactive compounds for its great potential value with various industrial and health promoting applications in aquatic, human as well as animal lives. *Scenedesmus acutus* is a green freshwater microalgae which is known to sustain life even in low concentration of salts. Recently, the microalgae have been explored for the production of exopolysaccharides (EPS) with their promising applications in different industries. The scope of this study is assessment of antioxidant and immunostimulation activity on peripheral blood mononuclear cells of the EPS isolated from *S. acutus* and their enhanced production by cultivating the microalgae in nutritional stress conditions. Phenol-sulphuric acid assay confirm the presence of sugar content in alcoholic extraction rich EPS. The results of cell proliferation assay showed that EPS increased the cell proliferation of peripheral blood monocuclear cell (PBMC) in a dose dependent manner from 100 – 1000 µg/mL, after which the saturation for cell growth activity occurred. This result concluded the immunostimulatory activity of EPS extracted from *S. acutus*. Also DPPH assay have shown the anti-oxidant effect which increases with concentrations. Liquid chromatography-mass spectrometry results revealed the presence of different saccharide sugars composed of various hexoses (glucose, fructose, galactose, mannose) and pentoses (ribose, xylose, arabinose) with phenol groups (Glucogallin). Oligosaccharides (eight monosaccharide units) and tertasaccharides (four monosaccharide units) were also found to be present in EPS as per the mass spectrometry results. The presence of structure of alkyl halide and amine groups with anomeric carbon sugars were confirmed by ^{1}H NMR spectrometry. FTIR analysis showed the presence of amine, sulphate and halo groups due to the detection of N-H, S=O and C-Br bonds. The enhanced production of EPS (approximately 8 fold increase) was obtained when the culture was grown in sulphate stress (1.25g/L) and phosphate stress (0.6 g/L). However, highest sugar content (11% increased) was observed in sulphate (stress (1.25g/L) as compared with normal conditions ($MgSO_4$: 0.75 g/L and K_2HPO_4: 0.4 g/L) in BG-11 media. In few stress conditions, pigment (carotenoid) and lipids (waxes) were also observed to be increased. Hence, it is concluded that EPS isolated from *S. acutus* possess antioxidant and immunostimulatory activities that has the potential to hit the therapeutic industries.

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Chapter I

Introduction

1. Introduction

1.1 Preface of Microalgae

From the starting era of biotechnology, many researches have been carried out to utilize the living cells or their products for the benefit of mankind. With the wide range applications of microalgae, it has drawn the attention of many scientists to carry out biotechnological research which cover the sectors of food industry, nutrition, cosmetics, agriculture, biopharmaceuticals, nutraceuticals, aquaculture, medicines and energy production (Richmond 2008). For this purpose, a variety of microalage (*Spirulina, Nostoc, Chlorella, Ulva, Tetradesmus, Botryococcus, Scenedesmus and Dunaliella*) has been used and taken up from pilot to commercial scale for the achievement of desired outcome globally (Wolkers *et al.* 2011). From the pragmatic literature survey, it was studied that microalgae's are capable to yield and produce many biomolecules (proteins, phycobiliproteins, astaxanthin, carotenoids, fatty acids, polysaccharides, organic matter and lipids) that could be used for non-biological or biological activities (Yaakob *et al.* 2014). The biological active compounds from microalgae have the properties of antibacterial, antifungal, antioxidant, anti-inflammatory, immunomodulatory, antidiabetic and anti-cancerous (Miranda *et al.* 1998; Ghasemi *et al.* 2007; Wijesekara *et al.* 2011; Yuan *et al.* 2011)

1.2 Project Appreciation

Scenedesmus acutus is a green, unicellular, non mobile freshwater photosynthetic microalgae which is found to exist in colonies (Figure 1.1) of four to eight cells and sometimes they form the filaments (Trainor 1996). It belongs to the phylum Chlorophyta, order Sphaeropleales and family Scenedesmaceae (Kessler *et al.* 1997) and is distinguished by the presence of a rigid cell membrane (osmoregulation) and the presence of plastids and chlorophyll (photosynthesis) (Borowitzka & Siva 2007). A higher proportion of unicellularity, growth rate and non-colony formation of *S. acutus* is found at high light intensities and low temperatures. Thus, the successful growth, cell division and metabolite production of *S. acutus* depends mainly on the balance between maintaining ideal conditions (light intensity, temperature, pH) and nutritional conditions (Xin *et al.* 2010; Cordoba-Castro *et al.* 2012). The literature survey revealed that this algae has been studied to isolate various active compounds which have wide range of

applications in the fields of bioremediation, bio-fuel production (bio-hydrogen, bio-diesel, bio-ethanol), bio-pharmaceutical, waste water treatment and energy production (Martınez *et al.* 2000; McGinn *et al.* 2012; Kondaveeti *et al.* 2014).These active compounds (proteins, pigments, luetin, neoxanthin, lipids and fatty acids) are mostly produced intracellularly and have some commercial value. Thus recently, the focus has been shifted towards the isolation and purification of extracellular polymers. These extracellular polymers can be exopolysaccharide (EPS), proteins or lipids, which are secreted outside the cell wall of algae, when grown in specific growth media. The yield of EPS production mainly depends upon the growth rate and cell division of the cultivated algae.

Figure 1.1: Unicellular *S. acutus* growing in a colony of four cells

Immunomodulation is the process of any change with the immune response of the body which includes induction, inhibition or stimulation of immune cells. The compounds are called immunomodulators which have been categorised as either immunosuppressants or immunostimulants based on the effects given by them on the modulation of the immune system. Polysaccharides isolated from various microalgae are reported to exhibit immunomodulation activity (de Jesus Raposo *et al.* 2015). Polysaccharides isolated from *Chlorella* sp. have shown the activity of immunosuppression on macrophage cell lines whereas the exopolysaccharides isolated from *Phaeodactylum* sp. showed the immunostimulation activity (Guzman *et al.* 2003; Bae *et al.* 2006). Although, many microalgae have confirmed the biological activity of extracellular polymer secretions, still, none of them had been taken up for commercial exploitation.

As the diversified applications of microalgae isolates have been exploited, more research is going on for their enhanced production. *S. acutus* is mostly used in the fields which include the

production of intracellular products and most study on bioactivity of exopolysaccharides (EPS) is reported from other microalgae. Thus, the present study is done to elucidate the activity of EPS isolated from *Scenedesmus* as immunostimulants and antioxidants and to enhance the EPS content by growing the culture in different nutrient stress conditions. The EPS isolates were characterized by LCMS (Liquid chromatography mass spectrometry), Fourier transform infrared spectroscopy (FTIR) and ^{1}H NMR (Nuclear magnetic resonance spectroscopy) to identify the molecular masses, functional groups and structure of the compounds present in the EPS isolates. As exopolysaccharides have been proved to be commercialized for various applications, their isolation and enhanced production strategy from an organism whose market is not well established would be of highly economic value.

Thus, the objectives of this project are designed with the elucidation of bioactivity, characterisation and enhanced production of *S. acutus* exopolysaccharides.

Objectives of the study

1. Assessment of antioxidant and immunological effect of exopolysaccharides isolated from *Scenedesmus acutus.*
2. Enhanced production of the exopolysaccharides under different nutritional stress conditions.
3. Characterization of the exopolysaccharide by Fourier Transform Infrared Spectroscopy, Nuclear Magnetic Resonance spectroscopy and Liquid Chromatography-Mass Spectrometry.

Chapter II

Review of Literature

2. Review of Literature

2.1 Algae

2.1.1 Background history

Phycology is the study of algae, which are predominantly the diverse group of eukaryotic organism. They come under the category of oxygenic photosynthetic structural bodies which are located as the morphons of marine water, fresh water or terrestrial environment (Elster 1999; Elster 2002). They have been placed under the kingdom of Plantae and regarded similar to them due to their feature of photosynthesis (presence of chloroplast), but distinguished from them by the absence of well-developed and differentiated vascular systems, like, bast (phloem), roots and xylem (Gibbs 1970). They also differ from the chlorophyll containing plants by the feature and mode of reproduction (Bold & Wynne 1985). Algae ranged from unicellular to multicellular form. Most unicellular forms of algae are motile which can move freely in the growth liquid medium (*Chlorella, Dunaliella salina, Euglena, and Trachelomonas*) (Dodge 2012). Some of them are microalgae (microscopic), and some are macroalgae that may grow up to 30-60 m in length (Canter-Lund & Lund 1995). Among the colossal utilization of algae, one of the important area is "systems and healthcare" in biotechnological research (Bold & Wynne 1985). They had been proved to be the ideal experimental organism because of their smaller size, an easily cultivable organism in cheaper growth media and diversified applications.

Algae are one of the primary and largest producers of organic matter (*Euglena gracilisa and Microcystis aeruginosa*) in the ecosystem due to their photosynthetic ability. They aid in the oxygenation of water during daylight hours in the ecosystem, which helps in maintaining the oxygen balance in the aquatic environment (Haas *et al.* 2010). It also contributes as a member of the land food chain. In the early 1830's, algae were classified under the kingdom of protists due to the presence of different chloroplast pigments such as chlorophyll, carotenoids, and phycobilins. Later on, with the phylogenetic analysis of their photosynthesis capacity, they were shifted to the kingdom Plantae under eukaryotic morphons except for blue-green algae (cyanobacteria), which was classified as a prokaryote. Algae are divided as coenocytic, neustonic, cryophilic, corticolous, epilithic, parenchymatous, planktonic, thermophilic, epiphytic and chasmolithic on the basis of their complex morphology.

2.1.2 Phytoplankton/ Planktonic algae and divisions

Phytoplanktons are the microscopic and cyanobacterial algae and are photosynthetic in nature which tends to reside near fresh or marine water. They constitute the large mass of world's biomass and pigments such as phycobilins, chlorophyll or carotenoids in their cells. They have the ability to convert minuscule molecules into complex ones (inorganic to organic compounds). The cyanobacterial morphology ranges from unicellular (*Microcystis, Dunaliella*, *Chlorella, Spirulina*) to filamentous and multicellular (*Anabaena, Oscillatoria, Nodularia,*). On the basis of the pigment exhibition and colour differences, planktonic algae is grouped under various classes which are widely distributed in fresh or marine water environment, from arctic to tropical regions (Stewart & Mattox 1975; South & Whittick 2009). These groups are:

- Rhodophytae - Red algae
- Chlorophytae - Green algae
- Cyanophytae - Blue green algae (cyanobacteria)
- Xanthophytae - Yellow green algae
- Chysophytae - Golden brown algae
- Phaeophytae - Brown algae
- Euglenophytae – Euglenoids
- Diatoms – Bacillariophycea,Dinophyceae,Pyrrophyta
- Cryptophyta –Cryptomonas

Amongst all the divisions, Chlorophyta is the group which comprises approximately 15000 species of algae. It comprises most of the species for biotechnological research to study the system biology. The morphology of this division is identified by the presence of green coloured algae (motile or non-motile) and the occurrence of cellulose and pectin based double cell wall. The chlorophyll pigments reside in the organelle plastid (carotene, chlorophyll a and b, astaxanthin, carotenoids and xanthophyll). The division further comprises classes on the basis of cell shape and size, such as unicellular (*Chlamydomonas*), colonial (Hydrozoans), tubular (*Actebularia*) and filamentous (*Anabaena*) (Chaudhary *et al.* 2013).

2.2 Applications of microalgae - Global and Commercial

The three basic elemental aspects of microalgae have contributed in converting them to commercial and technical advantages (Radmer & Parker 1994).

- They genetically and morphologically comprise a diversified group of organism.
- They amount to the large group of organism which is an unexploited source of molecules (both organic and inorganic).
- They are able to assimilate the stable isotopes of ^{2}H, ^{13}C, ^{15}N, in the produced biomass.

The industrial and global market of dried microalgae biomass has reached the nearby amount of 5000 ton/yr, with the estimated annual turnover of 1.3 billion USD, approximately. Even though more than 2000 species of algae has been known, still relatively few species and their product have been exploited for biotechnological and commercial utilization (Pulz & Gross 2004). An overview on the examples of algae species that has been used to produce various metabolites for various biotechnological applications is provided in table (Table 2-1). Food additives for human and animals, pharmaceuticals, nutraceutical, industrial chemicals, waste water treatment with electricity production and biofuel production are among the few products that have been produced though the employment of biotechnology using microalgae (Raposo *et al.* 2013).

2.2.1 Medical applications

For past few years, microalgae have been in use as the natural source of traditional medicines, like ointments, food supplements (vitamins) and anesthetics to treat hypertension, cough, body pain, wounds and to prevent diet deficiencies. *Chlorella* and *Spirulina* have proven to be the source of food supplement and protein with commercial availability in the market. *Spirulina* also aids to balance of intestinal micro flora by supporting the growth of *Lactobacilli* present in the digestive tract of human beings (Moore 2001). Microalgae also possess antibacterial (*Laminaria japonica, Porphyridium cruentum*), anticancer(*Arthospira platensis*),antioxidant(*Haematococcus pluvialis, D. salina*), immunomodulation (*Chlorella, D. salina*) and antiviral (*Arthospira, Porphyridium, Spirulina platensis, Ulva fasciata*) properties.

2.2.2 Nutritional products and bioactive compounds

From 850 BC, microalgae are consumed as the part of an animal and human diet due to its containment of protein, flavor, nutritional compounds (vitamins, omega-3-fatty acids) and bioactive compounds (pigments, antioxidants, polysaccharides) (Becker 2004; Ishaq *et al.* 2016). Thus microalgae have been used for large-scale production of these nutritional compounds to be used as essential dietary supplements. Biomass of *Scenedesmus*, *Chlorella* and *Spirulina* is found to produce beneficial effect of human physiology and provides good immune response and

bioactivity against various micro-organisms. Today, microalgae biomass and the isolated compounds are being used for health food and available in the market in the form of capsules, tablets, powders, and liquid. Algae are also used to be added in semi cooked foods like pasta, macaroni, and drinks to be used as food colorants.

Table 2-1: Algae species exhibiting various biotechnological applications (Borowitzka 1992; Spolaore *et al.* 2006; Priyadarshani & Rath 2012).

Group	**Algae species**	**Product**	**Applications**
Chlorophyta	*Chlorella vulgaris*	Ascorbic acid	Cosmetics
		Dried biomass	Heath care
		Food additive	Food supplements
Chlorophyta	*Dunaliella salina*	B-carotenids	Food
		Exopolysaccharides	Healthcare
		(Immunemodulators)	Pharmaceutics
Cyanobacteria	*Spirulina platensis*	Biomass	Heath care
		Phyacocyanins	Food supplements
Chlorophyta	*Haematococcus pluvialis*	Asthaxanthin	Health care Feed additives Food supplements
Rhodophyta	*Porphyridium cruentum*	Polyssacharides	Cosmetics Heath care& Nutrition
Bacillariophyta	*Phaedactylum tricornutum*	Fatty acids Lipids	Fuel production
Chlorophyta/Haptota	*Isochysis galbana*	Biomass	Feed additive
		Fatty acids	Animal nutrition
Chlorophyta	*Muriellopsis* sp.	Lutein	Food supplements
		Carotenoids	Feed additives
Rhodophyta	*Porphyridium* sp.	Phycoerythin	Cosmetics
Bacillariophyta	*Odontella aurita*	Fatty acids	Cosmetics Pharmaceuticals

2.2.3 Aquaculture and food chain

Microalgae are the basic producers of a food chain in aquaculture (Muller-Feuga 2000). Microalgae added in the feed are used for fin and shell fish rearing at the juvenile (Brown 2002), and larval stages act as the source of lipids (fatty acids) and high-value proteins (Benemann 1992; Gladue & Maxey 1994). Different marine animals that have been farmed with feedstocks of microalgae at different stages for nutritious benefits have been provided in (Table 2-2). Before selecting the microalgae species to be used as feed for aquaculture, the important factors that should be checked are ease of cultivation, nutritional profile, growth rate and culture conditions.

Table 2-2: Marine animals which are farmed with microalgae feedstock in different stages (Olaizola & Huntley 2003)

	Growth Stages		
Marine animal	**Larvae**	**Juvenile**	**Adult**
Mussels	Yes	Yes	Yes
Oysters	Yes	Yes	Yes
Prawns	Yes	-	-
Lobsters	Yes	-	-
Scallops	Yes	Yes	Yes

2.2.4 Biofuel production and waste water treatment

With the considerable attention, unicellular microalgae have been used for the production of third generation biofuel (biodiesel and bioethanol) with the application of waste water treatment using microbial fuel cell technology (Pittman *et al.* 2011) (Figure 2.1). Many species (*Botryococcus*, *Tetradesmus*, *Chlorella*, *and Scenedesmus*) have been cultivating for the production of lipids, carbohydrates, and oils (long chain poly-unsaturated fatty acids (LCpUFA)) to produce renewable and eco-friendly fuels. Also, with declining water quality, they are used to make a microbial fuel cell, to produce bioelectricity along with the treatment of waste water (Christenson & Sims 2011).

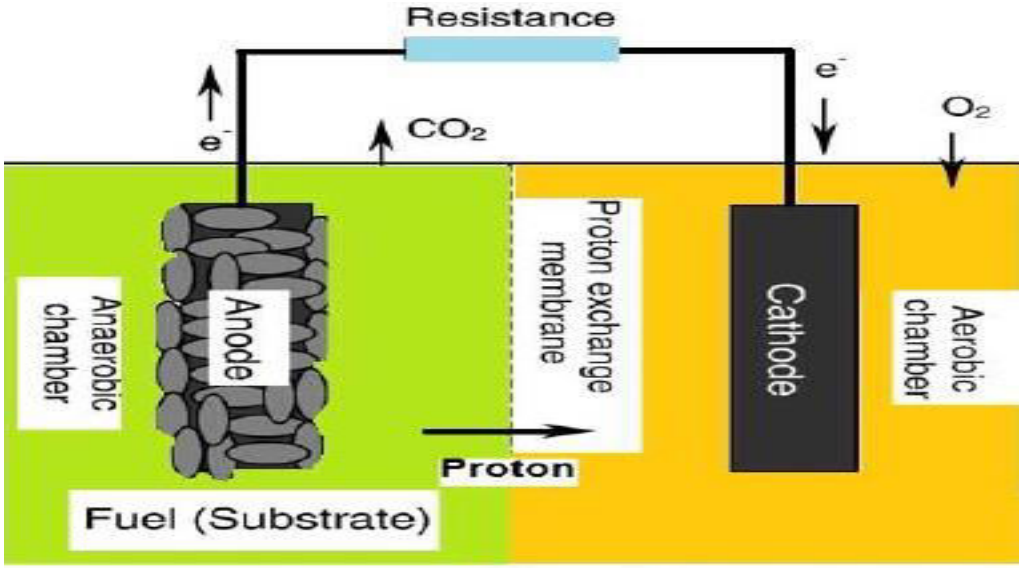

Figure 2.1: Microbial fuel cell for the treatment of waste water and bioelectricity production. Anode contains waste water for the utilization of bacteria, Cathode contains microalgae species to act as oxygen acceptor for electrons flow to complete the circuit.

2.3 *Scenedesmus acutus*

The current study is done on photosynthetic oxygenic microalgae, *Scenedesmus acutus*, which is a freshwater eukaryotic microalgae. *S. acutus* is commercialized for the isolation of valuable metabolites and bioactive compounds such as vitamins, pigments (astaxanthin, carotene), lutein, chlorophyll, polysaccharides, etc. The study focused on the isolation of exopolysaccharides for bioactivity.

2.3.1Classification and Taxonomic elucidation of *S. acutus*

S. acutus is a freshwater microalgae, placed under the phylum Chlorophyta and family Scenedesmusceae. Species of this genus is used worldwide for its great ability to adapt to harsh environmental conditions, grow rapidly and ease of cultivation and handling (Lürling 2003; Pulz & Gross 2004). *Scenedesmus* is a rich source of bioactive compounds, utilized for the benefit of human community. It also has been exploited for various industries including food, pharmaceutical, cosmetics, energy production and medicines.

Kingdom :Plantae

Phylum :Chlorophyta

Class :Chlorophyceae

Order :Sphaeropleales

Family :Scenedesmusceae

Genus :Scenedesmus

2.4 Mechanism of growth and reproduction in *S. acutus*

S. acutus belongs to the member of the genus which usually tends to grow in the colony of 4- to 8-celled form (Figure 2.2.b). They either grow as spiny or spine-less cells in the colony. Cells in the colony survive individually exhibiting spindle or elliptical shape (Trainor 1996). Each individual cell after being formed within the parent wall gets released into the environment. This mode of reproduction is also common to the *volvocalean* genera (*Volvox* and *Chlamydomonas*). *Scenedesmus* growing as an individual cell (non-flagellate) under *chlorococcalean* genera are called autospores (cells releases from the parent). The cells residing in the colony might have formed from the same cell at the same time, or might be the stockpile product of several cell divisions/generations or spore productions. The cells in the colony remain together by the formation of some matrix (gelatanious), tubes, specialized areas on the cell wall or exopolymeric substances secreted from cells outside in the medium (Trainor 1996).

Growth of the cells in the colonies occur via two type of mode, one is the non-coenobic form, where the number of cells increases in the colony with the increasing time, other one is the coenobic form (shown by *Scenedesmus* sp.) in which the number of cells remains fixed at the time of colony formation and once the number of cells gets fixed (2-, 4-, 8- celled) no additional cells are added for the lifetime of that colony. Coenobic type of cell growth is also termed as autocolony (cells resembles the parent cell) form of growth. In one study done on cytological data of coenobics, it was found that few individual coenobics could amount to the additional spines during their lifetime (Mishler *et al.* 1994).

2.4.1 Cell wall structure: Over the time, with the help of the light microscope, it is identified that the species of *Scenedesmus* contains three layered cell wall as sporopollium, cellulose and pectin/mucilage. Outer the cell wall, sometimes there are the presence of spines (made of hollow tubules) at the base of sine apex. They vary in thickness and length depending upon the growth conditions and helps in signaling from environment and movement (Figure 2.2.a). The elaborative description of the cell wall component is the prop, which contains ridges and warts (Komárek & Ludvík 1971; Pickett-Heaps 1975; Hegewald & Schnepf 1991). There might be one to three ridges present on the coenobium cells. Sometimes these short props, lead to the formation of bristles (100-200 µm in length) visible under the light microscope, also helps to the movement (floatation) and provide with the resistance to the colonial cells to settle down (Figure 2.2.a). They also keep cells stay a distance apart in the dense colonies of *Scenedesmus*.

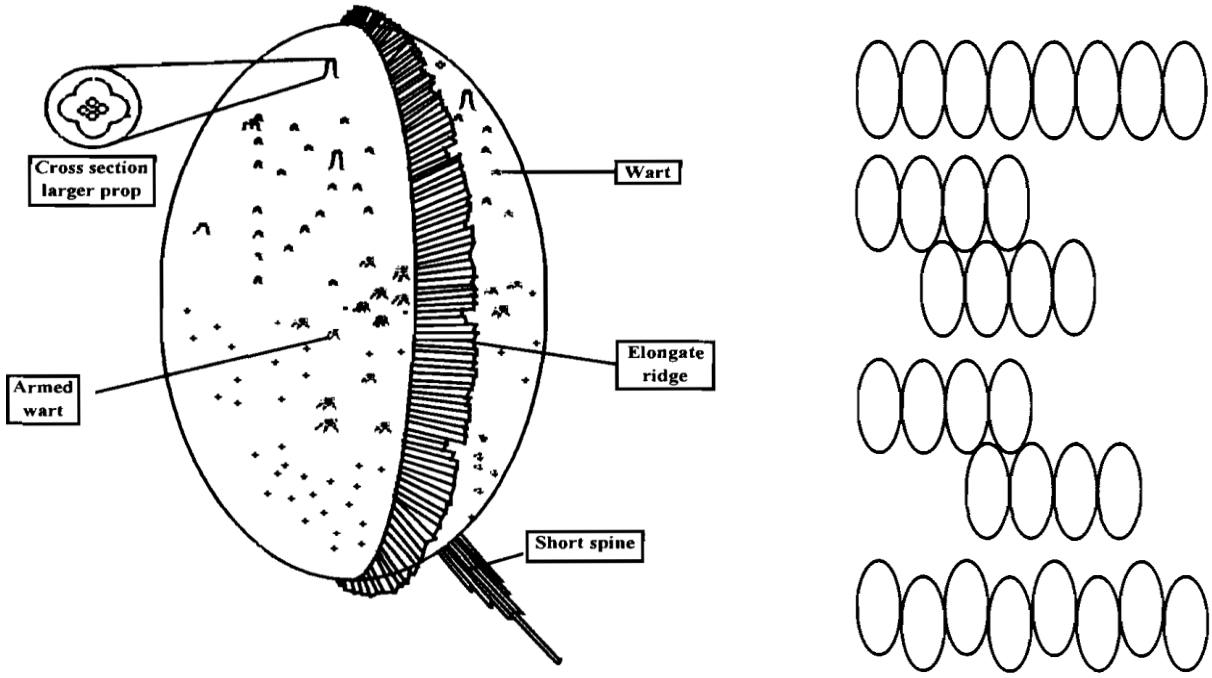

Figure: 2.2 (a) Schematic representation of basic cell wall structure of *Scenedesmus* (b) Cell arrangement in four to eight celled coenobic colonies (Trainor 1996).

2.4.2 Asexual reproduction: Asexual reproduction is one of the common modes of reproduction in *Scenedesmus* sp. (growing in nature or growth culture). This mode of reproduction involves the production of autocolonies/autospores though two non-vegetative successive cell divisions, where each cell almost produces unicellular or four-celled colony (Trainor 1993). There is the pattern of colony formation and reproduction which involves nuclear division followed by cytoplasm division (cytokinesis), organizing the cells into colonies (Figure 2.3). In one study on *S. communis* (*S. quadricauda*), it was revealed that colony formation takes place every two-three days, and the cultures were kept in light-dark conditions for photoperiodism (12/12 h).

In another study, it was observed that cells in each species are arranged in a specific manner and pattern (latitude). This pattern of cells arrangement in a colony (linear or alternate) is determined by the separating protoplast from neighbor protoplast in each individual cell in the colony.

After the colony formation two things can take place, before getting colony the actual morphological appearing characteristics:

a. The cells in the colony are joined together by extracellular matrices or spines and some gelatinous substances.

b. The cells remain individual unicellular cells while in the colony.

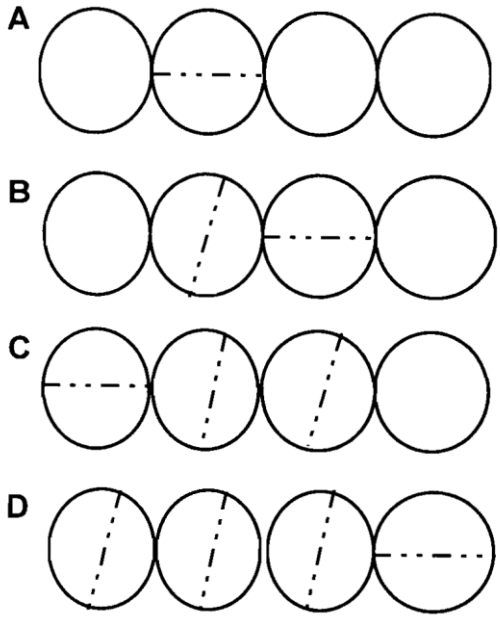

Figure 2.3: Division pattern of cells in *Scenedesmus* of a four-celled coenobium (a) Middle cell shows the first transverse cleavage (b) Two cells are dividing (c) and (d) In time, all coenobium cells have divided.

2.4.3 Sexual reproduction: Sexual reproduction though flagellates was first observed in the last of 20th century. It occurs in both natural/pond/open system and closed/bioreactor system. It is studied that in open system, *Scenedesmus* (spine less or spiny) reproduces sexually via gamete formation though flagellates.

2.4.3.1 Non-spine species sexual reproduction: Gametes formed are biflagellated, heterothallic, photosynthetic, without cell wall and are not produced in large numbers. The minus and plus matting gametes of *Scenedesmus* when become active, pair and fuses to form a zygote, which is quadriflagellated (Figure 2.4) (Trainor & Burg 1965a; Trainor & Burg 1965b). When this zygote enlarges by cell division, smooth cell wall starts forming up and it germinates (32 divisions) up till single cell results (Figure 2.4). From the pragmatic literature survey, it is also reported that the capacity of gamete formation (gametogenesis) could be lost over the time, which is also observed in the algae species (*Pandorina* and *Chlamydomonas*) but the maintenance of the gametogenesis can be assured by frequent cloning of the experimental material.

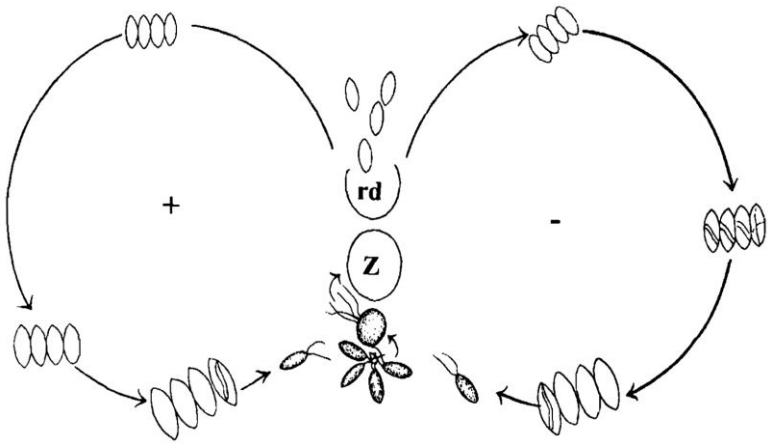

Figure 2.4: The sexual reproduction cycle of *Scenedesmus*, plus and minus mating gamete types fuses to form Zygote (Z) and after certain divisions of meiosis (rd), numerous progeny cells (unicellular) are released.

2.4.3.2 Spiny species sexual reproduction: Firstly the attempts made by researchers to produce motile cells or flagellates in *Scenedesmus* for gametogenesis reproduction was failed but over the time and conducted experimentations, quite success was observed provided with few conditions (nitrogen inhibitor free medium, preconditioning of the algae (25°C, 16:8 light-dark periodism), cool temperature, selection of specific ecomorph and changed light intensity) (Trainor & Burg 1965a).

2.5 Nutritional profiling of *S. acutus*

Although microalgae have been placed under kingdom Plantae, still their chemical and nutritional composition remains a bit different from other high order plants, due to different environmental and growth conditions like temperature, pH, growth chemicals requirement, CO_2 requirement, algae physiology and growth phases.

Temperature: Temperature also plays an important role in the growth of the algae which can survive in the range 0-45ºC (Ishaq *et al.* 2016). The temperature optimized for the growth of *S. acutus* is 28-30ºC (Hosseini Tafreshi & Shariati 2009). Temperature is controlled in bioreactors/closed systems by the use of thermostat and it is kind of impossible to control the temperature in open system, due to natural source of light (sunlight), whose intensity cannot be controlled and low temperatures occurring at night causes the algae culture to grow at slower rate. It has been reported in few cultures that at a temperature higher than 45ºC, glycerol from the

cells may leak out giving the opportunity for bacteria and fungus to grow in the medium. However, carotenoid production is enhanced at 40ºC.

Light: One of the primary requirements for the growth of oxygenic photosynthetic algae is light and energy source for the cellular metabolism. Depending upon the type of microalgae, the source of light varies, for example in ponds/open culture, sunlight is the main source and in the closed systems/photo-bioreactors, the light sources vary from fluorescent to high intensity white lamps (Österlind 1949). It is reported that the pigment production, growth of algae, photosynthesis and the production of metabolites is dependent on the duration, frequency and intensity of light, as with the increase of intensity, metabolite production might get increased (SENGER & Fleischhacker 1978).

pH value: *S. acutus* is adapted to survive in the pH range of 6-8 and it is optimized to keep the pH maintained in between 6.5-7.5 for its best growth. The pH can be maintained in ponds/open systems by HCl addition because of the pH increases by the fixation of CO_2 photosynthetically during respiration, due to which more of hydroxide ions are released in the growing medium (Grima *et al.* 2003).

Nutrient preconditions: Algae like other higher plants and organisms prerequisite nitrogen, carbon and energy sources to grow. As *S. acutus* is a photosynthetic alga, CO_2 and carbonate act as the carbon sources. Carbonate is provided as a $Na_2CO3/NaHCO_3$ source (0.02% concentration) and CO_2 bubbles are passed in the culture medium at the rate of 0.41/mL. Nitrate (1.5%) is the source of nitrogen for the growth. Reduction in the growth medium source (nitrate, carbon, phosphate, sulphate) might induce stress in the cell growth and cause them to produce more metabolites (carotenoids) but its limitation may lead to the slower growth rate and even death of the cells (Gour *et al.* 2018). Other macro-elements required are magnesium, chloride, potassium, molybdate and EDTA ions. Certain microelements are boric acid, manganese, copper and cobalt as nutritional element for the growth.

Microalgae are well known to biosynthesize, store and secrete a diverse group of metabolites (primary and secondary). The biomass of *S. acutus* usually is made up of proteins, lipids, oils and carbohydrates (Priyadarshani & Rath 2012) (Table 2-3). It has attracted the vision of researchers

for biotechnological advances due to its high nutritional profiling and bioactivities (Chacón-Lee & González-Mariño 2010; Guedes *et al.* 2011)

Some reasons why *S. acutus* came out to be such a commercially important microalgae due to its nutritional profiling includes : (1) contains favorable amino acid pattern to compete with other foods, (2) containment of high value protein content, serving as the source of protein, (3) provide with different forms of carbohydrates and sugars (glucose, starch, other polysaccharides), lipids (saturated or unsaturated) and oils for different industrial uses (4) its digestibility is highly easy and thus can be used in feed and food additives without much limitations (Cornet 1998; Becker 2004; Soletto *et al.* 2005)

Scenedesmus is one commonly used microalgae in the markets of food and health. In food sector it has drawn the attention to many researchers and manufacturers (Chacón-Lee & González-Mariño 2010). In a study done by Natrah *et al*. (2007), *Scenedesmus* sp. also exhibits few biochemical contents and antioxidant properties that could be useful in the food/nutraceutical industry. In another study, several toxicological assessments and high nutritional quality assays of *Scenedesmus* was done, which does not show any abnormality or toxic impact while experimenting with the test animals (Natrah *et al.* 2007). Gross *et al*. (1978) performed a nutritional study check on children (5 g/daily) and on adults (10 g/daily) by mixing *Scenedesmus* sp. (isolated biomass) into their diet and observation on slight increase in their weight was concluded. Similarly, an improvement in the weight of a child (four-year-old) was observed, when fed with microalgae diet as compared to normal diet (Gross *et al.* 1978).

Table- 2-3: Nutrient profiling of *Scenedesmus* spp. (% dry weight) (Ishaq *et al.* 2016)

Scenedesmus sp.	Carbohydrate	Protein	Lipid	Ash	Crude
S. acutus	**10-17**	**50-60**	**12-14**	**6-10**	**3-10**
S. obliquus	10-17	50-56	12-14	N/A	N/A
S. dimorphus	21-62	8-18	15-40	N/A	N/A
S. quadricauda	N/A	47	1.9	N/A	N/A

2.6 Global utilization of *S. acutus*

Globally *S. acutus* has been commercialized with its microalgae biomass in various industries (Table 2-4).They are produced in open culture system and close system, but in the open system, the chances of contamination are higher with protozoan's or other organisms, so better designing and precautionary measures are envisioned. The most part for its biomass commercialization has been covered by food, health and nutraceutical industries (Becker 2004; Toyub *et al.* 2008). Various useful and rich metabolic components have been isolated from *Scenedesmus* and these metabolites have been commercialized in bioremediation, aquaculture, pharmaceutical industries, and cosmetics. Its nutritional supplements like vitamins, protein, and amino acids have been added in human foods including candies, pasta, chewing gums, snacks and beverages. It has also become a potent source of biofuel (bioethanol and biodiesel), as this species contains high amount of oleic acid (52.8 %), makes it the most favorable producer of biofuel (Table 2-4).

It was reported that *Scenedesmus* can produce hydrogen and thus a new gateway was opened for its use to produce energy fuels from the conversion of solar energy and currently its feedstock is used for the production of biohydrogen as an energy fuel (biofuel) source to power marine engines, jets and transportation and duty vehicles (light or heavy). Various isolated compounds (MAA, β-carotene, carotenoids, astaxanthin, phycocyanine) from *Scenedesmus* is also being commercialized in cosmetics as skin care products, water binding agents and thickening agents. Not only in cosmetics, but β carotene is also used in many more applications due to which it has a trend of increasing demand in market with selling price of 300$/kg. Apparently algae have also been explored to secrete some extracellular polymeric substances and major one is the polysaccharides (Ishaq *et al.* 2016). These extracellular polysaccharides have various applications for different activities. The greatest advantage of isolating these extracellular productions is that being extracellular released in the culture medium; their cost of downstream processing (isolation, extraction and purification) is low as compared with the extraction cost of intracellular products. Also, the culture and extracellular productions are reproducible.

Table 2-4: Compounds isolated for industrial applications from *Scenedesmus*.

Nature	Compound	Application
Pigment	Astaxanthin	Medicine, human nutrition, cosmetics
Pigment	Chlorophyll a,b,c	Cosmetics, food colorants, pharmaceuticals
Organic compound	Vitamin B,C,E	Health, food additives, medicines, pharmaceuticals
Biomolecule	Polysaccharides	Medicines, Immunomodulation, $BioH_2$, Bioethanol
Biomolecule	Lipids and fatty acids (LCpUFA, oleic acid, lauric acid, stearic acid, linoleic acid)	Aquaculture, pharmaceuticals, biodiesel, animal nutrition
Glyco-protein	Haemaglutinin	Medicines
Pigment	Carotenoids	Medicines, pharmaceuticals, food colorants
Metabolites	Mycosporine- like amino acids (MAA), aminoacid(methionine,leucine,isoleucine,valie, alganine, aspartic acid), sporopollenin	Food industries and cosmetics (UV- screening)

2.7 Bioactive compounds isolated from *S. acutus*

Various bioactive compounds isolated from the species of *Scenedesmus* and used for various purposes include:

*Astaxanthin***:** It is a naturally occurring intracellular secretion keto-carotenoid pigment, which provides lightish pink or reddish color to the algae culture. In a literature survey, it is found that sometimes in stress conditions, this pigment is produced in the culture of *Scenedesmus* (Ambati *et al.* 2014). Astaxanthin is a lipid soluble pigment. It has hydroxyl (-OH) or ketone (C=O) with water loving polar ends and oil loving lipid backbone (Figure 2.5). It has reddish color due to the presence of conjugated double bond at its centre. This conjugated double bond chain is responsible for its antioxidant property.

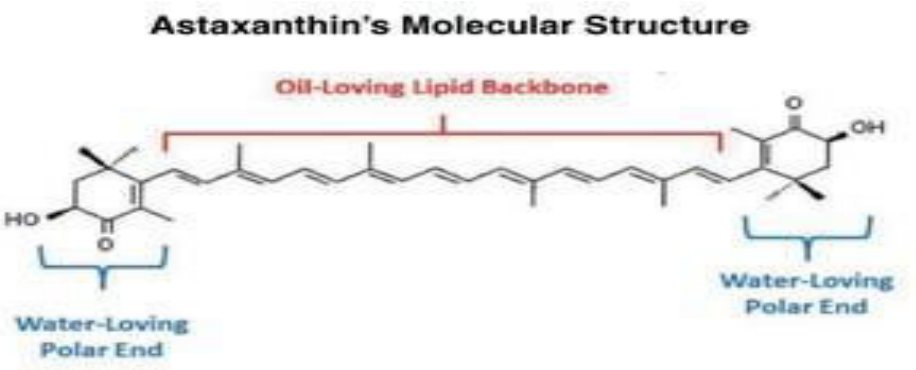

Figure: 2.5: Chemical structure of Astaxanthin showing its hydroxyl and ketonic groups

In a research study, it was observed that by the addition of NaCl, $NaNO_3$ or changed concentration of media chemicals, this pigment is produced in *Scenedesmus* sp. This pigment is also being commercialized from other microalgae species (*Haematococcus*) for various health and skin care benefits, as it acts as the best antioxidant, eye healthcare and skin care product.

Extracellular Proteins: These are the extracellular secretions of the species in the growth medium. It includes (1) exoenzymes (chitinases, alkaline phosphatases, proteases and -D-glucosidases) which helps in chemical signalling and can inhibit growth of microbes (Romani & Sabater 2000). (2) extracellular carbonic anhydrases (eCaAn), which helps in mechanism of CO_2 concentration. In some algae, it enhances the CO_2 uptake from external HCO_3 (Chen 2015) (3) extracellular proteases, which are found to be the target of drug development. These proteases are also produced by microalgae (*Dunaliella*, *Chlamydomonas coccoides* and *Chlorella sphaerkii*) (KELLAM & WALKER 1987)

β-carotene: It is also produced as a carotenoids pigment in the culture of *Scenedesmus* intracellularly. It helps in better functioning of vision (by getting oxidized into vitamin A). It contains aromatic and aliphatic ring of carbon with ketone (C=O) (Figure 2.6). It has also been commercialized by other species also (*D. salina, Chlorella).* It is industrialized in food, cosmetics and biopharmaceuticals industry as antioxidant and coloring agent. It has also been found to be an inhibitor of tumor growth. Its commercially available added liquid concentrations are 2% (beverages), 4% (food supplement) and 2-5 % (feedstocks of aquaculture life).

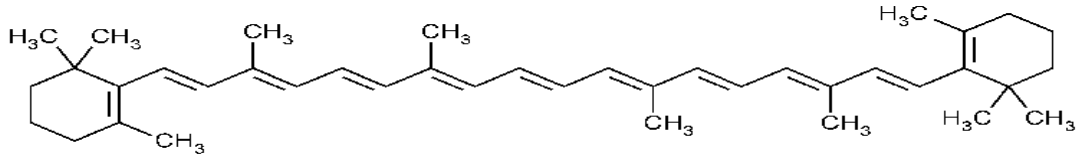

Figure 2.6: Chemical structure of carotenoids in the algae

Allelopathic Chemicals: Allelopathy is the term which describes the synergy between the organisms (microalgae to their target organism) regulated by the release of various biologically active chemicals by the microalgae in the surrounding environment. These compounds are phytotoxins/allelochemicals, which show stimulating or inhibitory effects. Several other microalgae species also releases these allelochemicals in the medium *(Chlorella vulgaris, Isochysis galbana, Anabaena flosaquae*). These chemicals include fatty acids, alkaloids and peptides (Inderjit & Nishimura 1999).

Organic compounds: These -CHO compounds could be produced intracellular or extracellular in the culture medium. In a report study, extracellular organic compounds production could be increased by adding NaCl in the medium, as it causes stress condition for the survival of freshwater algae. These compounds are vitamins, organic acids (lactic acid, oxalic acid, succinic acid), glycerophosphate, N-alkylglycine derivatives, organic phosphates, glycerol and its ester and myoinositol phosphate and carbohydrates (polysaccharides) (Inderjit & Nishimura 1999). Compound secreted outside the cell wall is shown in (Figure 2.7)

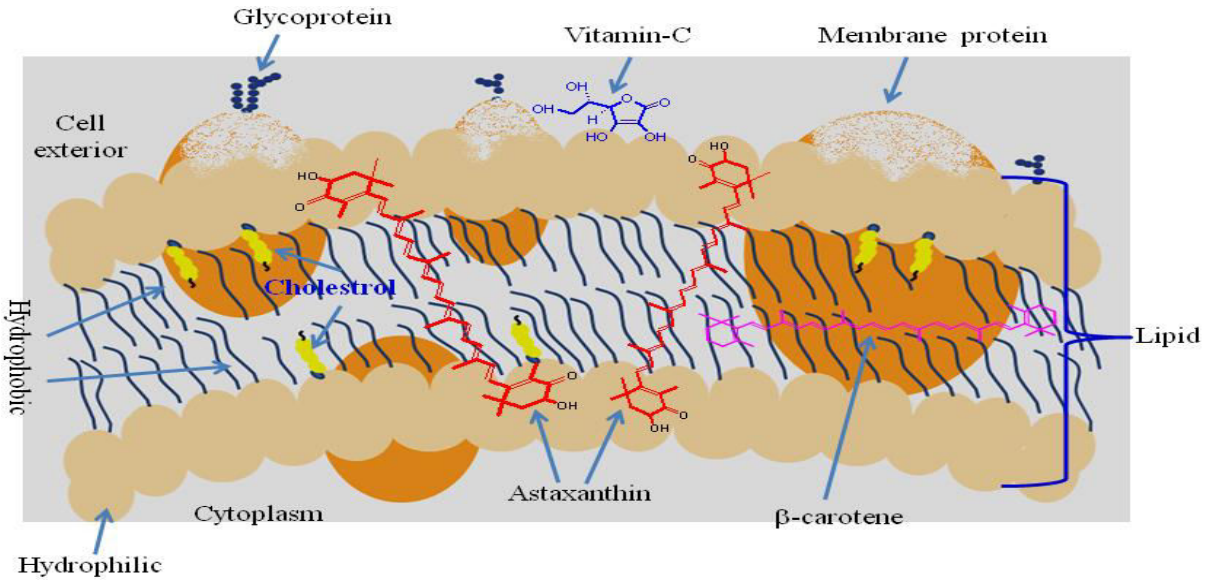

Figure 2.7: Superior position of bioactive compounds in the cell membrane

2.9 Polysaccharides

The bioactive compound that has been chosen for study and isolated from the green microalgae *S. acutus* is polysaccharide, which could be released intracellular (IPS) (inside the cell wall of

algae) or extracellular in the medium (EPS) (outside the cell wall). The downstream processing, cost of isolation, extraction and reproducibility is easy for EPS (Exopolysaccharide) as compared to the processes of IPS and have more therapeutic value and activity. Thus, the study is focused on exopolysaccharides.

2.9.1 Exopolysaccharides (EPS)

Exopolysaccharides are the extracellular polymeric substances that are secreted outside the cell wall of the algae. They are the high molecular weight naturally secreted carbohydrate polymers by the organisms (algae) into their surrounding environment (medium) during their propagation or growth phase. They comprise the polymerization of one or more mono-units of carbohydrate having glycosidic linkages. They can either be fully secreted in the medium (SMP) or can be attached loosely to cell wall (EPS) of the algae (Figure 2.8). Red algae, green algae or cyanobacteria are the primary producers of EPS that are structurally diverse in their nature. Polysaccharides can be homopolymers (consists single mono-units) like starch and glycogen which are polymers of glucose and heteropolymers (consists different mono- units) like hyaluronic acid which are the polymer of N-acetylglucosamine and D-glucuronic acid.

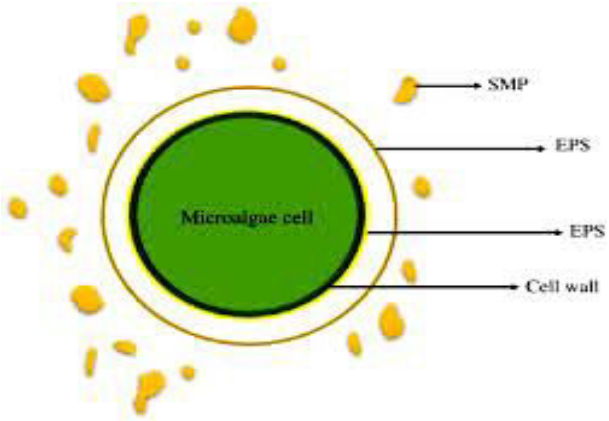

Figure 2.8: Microalgae showing EPS (Exopolysaccharide loosely bounded to cell wall) and SMP (Small polysachharides) secreted in the medium.

Exopolysaccharide is involved in various biological activities. They protect cells in stress conditions, helps in cell to cell interactions, biofilm formation and cell adhesion. Currently, EPS are greatly used in food industry as gelling agents, thickeners which can be used to improve the texture and quality of food products. Other than this, it contains various properties of antioxidant, antibacterial and anticancer which makes it a good pharmaceutical candidate. Different extraction methods have been employed till now for its downstream processing.

2.9.2 Physiological uses and Structure of EPS

The interaction and physiology of microalgae with its use and role of EPS is not completely known and understood. These EPS contains ketonic groups, CHO groups and hydroxyl groups in their basic structure (Figure 2.9). It is studied that in unfavourable conditions, algae can either produce high or lower amount of EPS for cell wall protection and are also involved to modulate the phytotoxin activities (Yang *et al.* 2015). Therefore it was reported that the response of *Chlorella vulgaris* and *Anabaena* was modulated by production of IPS and EPS on microcystin toxin from the extract of *Microcystis* under altered conditions of growth. The EPS secreted out in the medium can be simple or associated with sulphate and uronic acids. Therefore, it can be assumed that the EPS could be negatively charged contributing towards anionic property of polymer.

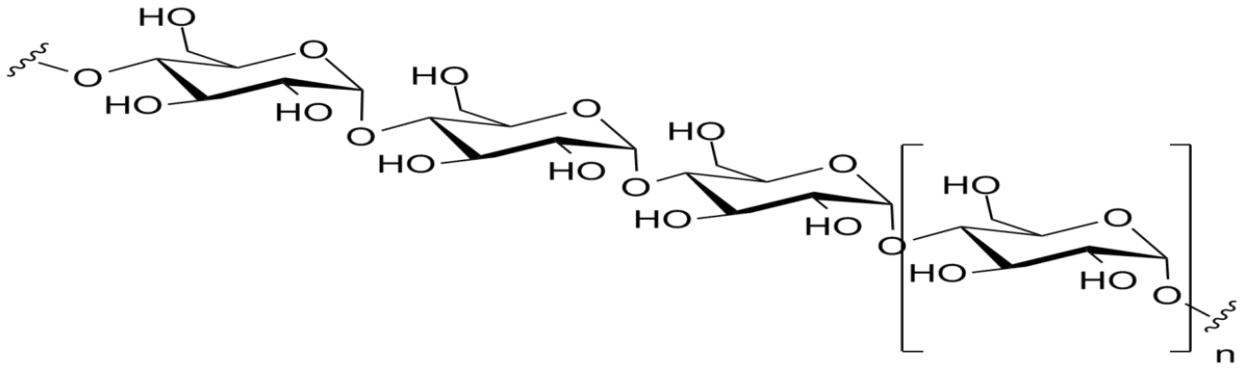

Figure 2.9: General structure of Exopolysaccharide, showing branched chain of sugars.

These EPS can be produced as homopolymers (produced by *Gyrodinium impudicum*, cyanobacteria) or heteropolymers (produced by *P. cruentum*), which might contains functional groups of acetate, sulfate, pyruvate, proteins and methyl. For example, *Arthospira platensis* EPS is heteropolymer associated with proteins (up to 55%). Several studies are still going on to understand completely the functions of EPS isolated from different microalgae and their productions.

2.10 Applications and bioactivity of EPS

The potential source of polysaccharides includes seaweeds, algae and bacteria, for example agars (Gelidium), carrageenans (Chondrus and Kappaphycus) and alginates (*Laminaria*) with costing global value of approximately 1 billion US$ (Mishra *et al.* 2011). These polysaccharides are

widely used in the food industries. Polysaccharides from microalgae are involved in following bioactive properties (Table 2-5):

- Anti-viral
- Antioxidant
- Immunomodulation and anti-inflammation
- Anti-lipidemic and Anti-glycemic properties
- Anti-tumour
- Nutraceuticals
- Nanofibre production
- Anti-thombotic and anti-coagulant properties
- Drag reducing capacity
- Anti- adhesives

Immunomodulatory activity*:* Polysaccharides from marine and freshwater microalgae shows different pharmacological properties (*D. salina, Phaeodactylum*, *Porphyridium and C. stigmatophora*). Few exopolysaccharides isolated shows immunomodulatory activity includes immunosuppressant activity, which suppresses/weakens the immune system of the host (*C. stigmatophora*) and immunostimulatory activity, which enhances/stimulates the immune system of host by increasing the activation of its immune system and cells (*P. tricornutum*) (Guzman *et al.* 2003). The agents showing Immunomodulatory activity are immunomodulators. According to one of the study, these immunomodulators are shown to possess immunomodulation by providing cytotoxicity against immune cells, helps in increasing reactive oxygen species (ROS), enhances the secretion of chemokines and cytokines, activates phagocytosis and increases nitric oxide (NO) production (*Tanacetum vulgare, D. salina, Chlorella stigmatophora).*

***Antioxidant (Free radical scavenging activity)*:** Photosynthetic algae may generate free radicals due to their constant exposure to light which can be overcome by scavenging complexes production. The antioxidant property of EPS prevents the oxidation of lipids into hydrogen peroxide and hydroxyl ions though reactive oxygen (RO) species in the pharma companies due to degradation of the quality and stability of nutritional products by oxidation of lipids. It is suggested that sulfur-polysaccharide and proteins may enhance the dose dependent activity of EPS. *Porphyridium creuntum and Rhodella reticulate* EPS can effectively scavenge DPPH (2,2-

diphenyl-1-picrylhydrazyl) (Guzman *et al.* 2003). Oxidative stress which is caused by the production of ROS to proteins, lipids and nucleic acids causes cell damage and various diseases, thus it also provides activity against this oxidative stress. The three classes of photosynthetic pigments in microalgae (chlorophyll, phycobilins and carotenoids) shows biological activity by providing prevention of chronic and acute coronary syndromes, atherosclerosis, cataract, muscular dystrophy, neurological disorders and rheumatoid arthitis (Mimouni *et al.* 2012).

Table: 2-5 Microalgae species producing EPS, with their bio-activities and applications (Liu *et al.* 2016)

Group	**Species**	**Monosaccharide units**	**Bioactivity and Applications**
Chlorophytes	*Dunaliella salina*	Galactose, glucose, xylose, fructose	Immunomodulation antioxidant
Cyanophytes	*Anabaena spiroides*	Glucose, fucose, arabinose, mannose	Metal-binding antioxidant anticoagulant antibacterial
Dinoflagellates	*Gyrodinium impudicum*	Mannose, galactose, glucose	Antiviral activity
Chlorophytes	*Chlorella stigmatophora*	Glucose, xylose fucose	Metal-binding `
Rhodophytes	*Porphyridium cruentum*	Xylose, galactose, glucose	Immunomodulation Antiviral, antibacterial activity
Chlorophytes	*Chlorella. Vulgaris*	Rhamnose, arabinose, galactose, rhamnose	Metal-binding
Rhodophytes	*Rhodella reticulata*	Glucose, galactose	Drag-reducing effect Free radical scavenging
Rhodophytes	*Porphyridium* sp.	Galactose, glucose	Hypocholesterolemic effect Intestinal morphological modification
Chlorophytes	*Arthospira platensis*	Rhamnose, glucose, xylose ,fructose, galactose,mannose and arabinose,	Antioxidant Antiviral antibacterial

2.11 Strategies for Yield increase of EPS

From the pragmatic literature survey, it is found that EPS extracted from microalgae have various beneficial applications, but still suffering from their low yield production limitation. The amount, type and yield of extracted EPS depends upon few environmental and culturing conditions, as growth conditions (pH, temperature, time), culture conditions, culture system design and down streaming process handling. Few strategies that have been employed for its higher production includes, providing stress conditions, altered growth conditions, use of response surface methodology, co-culturing and optimization with media composition (Table 2-6). In one study on *Chlamydomonas reinhardtii,* the EPS yield reached upto 628mg/L was optimizing media composition. Similarly, on addition of Mg2+ and sulphate increases the EPS yield of *P. Cruentum*. Co-culturing of *Spirulina* and *Chlorella* with *Trametes versicolor* (Basidiomycetes) increases the yield of EPS by 33%, which was a great achievement by the researchers. The recent advancement in the strategies is the use of novel tool causes mutagenesis, atmospheric and room temperature plasma (ARTP), which uses helium radio frequency, when employed to cultures of *Crypthecodinium cohnii*, creates ten mutants and causes them to yield higher amount of EPS (34% increased fold with 1.02g/L volumetric yield). A study in 1939 also showed the effect of nutritional stress on EPS yield effect, where with increasing concentration of $MgSO_4$, the cell replication and cell size increases. The cell replication is found to be enhanced when $MgSO_4$ concentration is increased whereas, low concentration yields increased cell size). Yet another study from China showed that high phosphate concentration would enhance chlorophyll content in the cells and thus yield to higher cell numbers and EPS yield.

Table: 2-6 Strategies employed to increase EPS yield

Species	**Method adopted**	**EPS Yield (mg/L)**	**Reference**
Botryococcus braunii	Medium optimisation	44	(Bayona & Garcés 2014)
Chlamydomonas reinhardtii	Response surface methodology	628	(Mona & Kaushik 2015)
Chlorella vulgaris	Co-culture	7100	(Angelis *et al.* 2012)
Crypthecodinium cohnii	ARTP	1020	(Liu *et al.* 2015)
Phaeodactylum cruentum	Medium optimisation	NA	(Serive *et al.* 2012)

According to best of our knowledge, there is less information available regarding the immunodulatory activity of EPS isolated from *Scenedesmus acutus.* Thus, the focus of this study is towards the strategy of mass production, antioxidant and immunostimulatory activity of EPS.

Chapter III

Material and Methodology

3. Project methodology

I. Materials

Table 3-1: List of chemicals and reagents used

No.	Chemicals/ Reagents	Company
1	Amphotericin B	Sigma Aldrich, USA
2	Concanavalin A	Sigma Aldrich
3	DMSO (Dimethyl Sulphoxide)	Merck, Germany
4	Ethanol	
5	Foetal bovine Serum	Gibco®Life Technologies, USA
6	Histopaque® -1077	Sigma Aldrich
7	MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide)	Sigma Aldrich
8	Penicillin Sodium	Himedia
9	Potassium Chloride (KCl)	Himedia
10	Potassium phosphate monobasic (KH2PO4)	Himedia
11	Rosewell Park Memorial Institute (RPMI)-1640 medium	Sigma Aldrich
12	Sodium Bicarbonate (NaHCO3)	Himedia
13	Sodium Chloride (NaCl)	Himedia
14	Sodium phosphate dibasic (Na2HPO4)	Himedia
15	Streptomycin	Sigma Aldrich
16	Trypan blue	Himedia

II. Methodology:

3.1 Procurement of culture

3.1.1 Algal genotype used for the study:

The microalgae culture of *Scenedesmus acutus* (*S. acutus*) was procured from Counsil of Scientific and Industrial Research-National Chemical Laboratory, Pune, India.

3.2 Maintainance of the culture

3.2.1 Culture conditions of the microalgae

Preparation of media: The procured microalgae *S. acutus* was established in the laboratory using two different medium, with different salt compositions in 250 mL Erlenmeyer flask containing 100 mL medium. One of the medium used was Bristol liquid medium by adding Thiamine (2.2mg/mL) and Biotin (0.24mg/mL) (Table 3-2) and another medium used was BG-11 (Table 3-3), which is a widely used medium for most of the cyanobacteria and green algae species. For the preparation of these two different medium, their varied salt compositions with specific molarities were mixed according to their concentration and the final volume of the medium was made up with double distilled water. The pH was adjusted and both media were autoclaved for sterilization at 20 psi, 121°C temperature with the holding time of 15 min. The prepared medium was used for microalgae culturing.

Cell morphology: Cell morphology was visualized under microscope on glass slide from Bristol agar and BG-11 agar media prepared in conical flask by adding 1% agar to the liquid medium and autoclaved. After autoclaving medium was poured in the petri-plates and allowed to cool in laminar air flow (LAF) to maintain aseptic conditions.

Inoculation of the culture: The culture flask containing autoclaved liquid medium (Bristol and BG-11) was inoculated from the procured algal slant using autoclaved inoculating loop, capped and labelled. The inoculated flasks were incubated in the temperature of 28±2°C with12/12 h of light-dark condition for the proper growth of the algae species. *S. acutus* is a photosynthetic oxygenic species, and thus light and dark conditions are necessary to provide photoperiodism. The growth was observed for 20 days and culture was constantly shaken manually. The initial 100 mL of the culture served as the mother culture (Mishra *et al.* 2011).

Table 3-2: Chemical composition for Bristol liquid medium

Salt	Concentration (g/L)
NaNO3	0.25
K2HPO4	0.075
MgSO4.7H2O	0.075
CaCl2.2H20,	0.025
NaCl	0.025
KH2PO4	0.175
Trace metal solution (PIV Metal solution)	6 mL

PIV Metal solution	Concentration (g/L)
$Na_2EDTA•2H_2O$	0.75
$MnCl_2•4H_2O$	0.041
$FeCl_3•6H_2O$	0.097
ZnSO4	0.005
$Na_2MoO_4•2H_2O$	0.004
$CoCl_2•6H_2O$	0.002

Note: After adding all the salts mentioned in distilled water, make up the volume upto 1 liter and the pH was adjusted to 6.4.

Table 3-3: Chemical composition for BG-11 Medium

Salt		Concentration (g/L)
NaNO3		1.5
Na2CO3		0.02
Stock 1	EDTA (disodium)	0.01
	(NH4)5[Fe(C6H4O7)2	0.06
	C6H8O7	0.06
	Final added volume	0.36
	CaCl2.2H20,	10mL/L
Stock 2	MgSO4.7H2O	0.75
	Final added volume	10mL/L
Stock 3	K2HPO4	0.4
	Final Added volume	10mL/L
Stock 4	H_3BO_3	2.86
	$ZnSO_4 \cdot 7H_2O$	0.222
	$CuSO_4 \cdot 5H_2O$	0.079
	$MnCl_2 \cdot 4H_2O$	1.81
	$Co(NO_3)_2 \cdot 6H_2O$	0.0494
	$NaMoO_4 \cdot 2H_2O$	0.39
	Final Added volume	1mL/L

Note: After adding all the salts mentioned in distilled water, make up the volume upto 1 liter and the pH was adjusted to 7.2.

3.2.2 Mass production and maintenance of the culture

Sub-culturing: The mass production of algae was done in 500 mL Erlenmeyer flask containing 250 mL medium by sub-culturing after every 20th day. For sub-culturing,10 % of the previously cultured medium with the cell density of 2-4×10^{5}cells/mL was used .The final volume of culture for experiment work was kept 1000 mL in replicates.

Maintenance and preservation of the Germplasm: The axenic cultures of *S. acutus* were preserved on 1.5% agar slants of BG-11 medium. The cultures were kept at 28±2°C under 12/12h of photoperiodism (light and dark) and then shifted to 4°C for preservation.

3.3 Modification of Culture Conditions for the effect on Biomass production

Two parameters, nutrient stress and pH stress were considered to assess their effect on cell growth and biomass production of *Scenedesmus* cells.

3.3.1. Effect of nutrient stress

In order to determine the effect of nutrients on growth and biomass production of algal cells, the nutrient stress (Citric acid, Nitrogen and Ferric ammonium stress) were incorporated in the media.

3.3.2 Effect of pH stress

The effect of pH change (7.1-7.4) was incorporated in the media (BG-11), to study the effect of pH on biomass accumulation (cell number and growth).

3.4. Measurement of the growth of *S. acutus*

3.4.1. Cell morphology study

The growth morphology of *S. acutus* in petri-plates containing solidified medium was observed under inverted microscope (Nikon Eclipse E200 Biological microscope) after every 5^{th} day. The change of colony color was also observed during this time period from red to white and to green color finally.

3.4.2. Growth curve study

The growth curve of the culture was measured at different optical densities (520, 580, 590 and 620 nm). As the maximum OD was obtained at 620 nm, it was selected for growth curve. The OD was taken every three-four days for 30 days to obtain the growth curve (Figure 3.1) pattern.

Lag phase/induction phase: In this phase, culture is transferred to liquid broth from the agar plate and cells prepares for division.

Log phase: In this phase, the cell density and number increases exponentially as the function of time.

Stationary phase: It is observed that during this phase the cell division is slowed down due to the exhaustion of nutrient media, decreased CO_2, pH disbalance, toxins produced by the cells.

Death phase: It is the final stage of growth curve, where cells are incapable to sustain their growth.

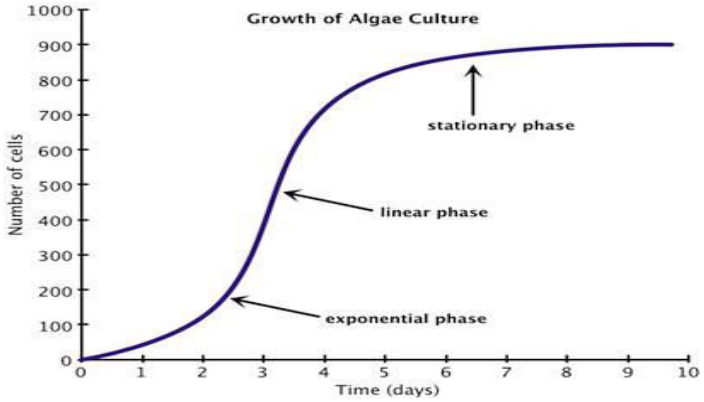

Figure 3.1 Typical growth curve pattern of cells with different phases.

3.4.3. Cell count study

The cell growth of the algae was measured by counting the cell number using nauberg haemocytometer, after every three-four days for 20 days of culturing. The cells of *S. acutus* were either fixed by lugol's iodine solution before counting or directly mixed with Trypan blue dye and counted on haemocytometer (Auinger *et al.* 2008).

3.4.4. Measurement of per day dry weight of algae cell (PDW)

For the determination of algae cell PDW (mg L^{-1}), 2mL aliquots of algae culture samples were collected in pre-weighed and pre-dried micro centrifuge tubes, put in the oven at 80°C for complete drying and the tubes were weighed again.

3.4.5. Specific growth rate

Specific growth rate (μ day^{-1}) was calculated though the data obtained from growth curve readings using the understated equation as (Singh *et al.* 2014).

$$\mu\ (\mu\ day^{-1}) = 3.3(\ln N_t - \ln N_o) / t_2 - t_1$$

Where,

μ = specific growth rate

ln N_t = Final reading at time t_2

ln N_o = Initial reading at time t_1.

3.5 Pigment Estimation and analysis

3.5.1 Estimation of chlorophyll content

S. acutus cells in their log phase were taken in micro-centrifuge tubes and subjected to centrifugation at 8000 rpm/15 min. Supernatant was discarded and pellet was mixed with 80% acetone diluted with autoclaved distilled water. Then, the mixture was vortexed for 30-40 seconds and the tubes were kept for 24 h (dark conditions). After 24 h, tubes were taken out and centrifuged again at 3000 rpm/10 min. The supernatant was taken and absorbance was measured by spectrophotometer at 645 nm and 661.5 nm. Chlorophyll a, b and total chlorophyll concentration was measured using the equations (Singh *et al.* 2014).

$$\text{Chl a (mg/mL)} = 11.24 * O.D661.5 - 2.04 * O.D645.0$$

$$\text{Chl b (mg/mL)} = 20.13 * O.D645.0 - 4.19 * O.D661.5$$

$$\text{Total chlorophyll content (mg/mL)} = \text{Chl a} + \text{Chl b}$$

$$= 7.05 * O.D661.5 + 18.09 * O.D645.0$$

3.5.2 Estimation of Carotenoid content

Carotenoid content in the algae cells were also estimated in 80% acetone using same protocol as mentioned for chlorophyll estimation and analyzed via spectrophotometer by measuring absorbance at 450nm (Singh *et al.* 2014) , further evaluated using Davis formula and extinction coefficient (2500).

$$\text{Carotenoid (mg/mL)} = (O.D450 * \text{Volume of sample taken}) / 2500$$

3.6. Downstream processing of *S. acutus cells* to extract extracellular polymeric substances

3.6.1. Extraction

After 20 days of the growth, the algal culture was taken and centrifuged at 10000 rpm/30 min to separate the debris and cells. The supernatant was transferred to fresh tube and algae pellet was processed separately for dry weight measurement. Supernatant was then filtered using Whattman filter paper and heated on the hot plate at 50-70°C temperature till the final volume reached to $1/6^{th}$ of the original volume. The concentrated volume was then proceeded for alcoholic precipitation, where the equal volume of chilled ethanol was added to the concentrated supernatant for the precipitation of extracellular polymeric substances and kept in chilled

condition (4°C) for16-18 h. Centrifugation at 10000 rpm/10 min was again done to remove the ethanol and the obtained pellet was dissolved in MQ (Milli-Q/ ultrapure water) after washing it thrice with the absolute ethanol (99.99%). The dissolved pellet was heated to ensure the dissolvation of any clump left and filtered (Mishra *et al.* 2011).

3.6.2 Total dry weight of algae cells

To determine the total algal cell dry weight (mg/L), the cells collected after centrifugation were taken in a fresh pre-weighed tube after washing thrice with the distilled water (to remove extra chemicals, debris and salts), and were put in the oven for drying at 80°C until the constant weight was obtained.

3.6.3 Dialysis

The dissolved pellet in MQ water was dialyzed to remove the salts and chemicals against distilled water (48 h). The water was changed after every 8 hours. The membrane tubes used for dialysis were activated beforehand (Marusyk & Sergeant 1980).

Tubing preparation and membrane activation: The dialysis membrane with pore size of 12 kDa was purchased and tubing of 7-8 cm was cut. These tubings were immersed in 2% sodium bicarbonate solution and boiled for 10 min for activation. The solution was discarded and membranes were again kept in boiling distilled water for 10 min. The activated membrane can be immersed in 50% ethanol and stored at 4°C.

3.6.4 TCA (Trichloro-acetic acid) precipitation for proteins

The dialyzed liquid was subjected to TCA precipitation to remove the proteins if present (Link & LaBaer 2011). For precipitation, 20% working concentration of TCA was prepared freshly. Then, TCA was added to the liquid and incubated for 2 h followed by centrifugation at 14000 rpm/10 min. The pellet of proteins was discarded.

3.6.5 Lyophilisation

The TCA precipitated samples were subjected to the process of lyophilisation at -58ºC in the vacuum created by the vacuum pump for evaporation of the liquid to obtain the powdered sample (Coppa *et al.* 2001).

3.7 Analysis and estimation of bioactive compounds (Biomolecules) in extracellular polymeric substances

3.7.1 Estimation of glucose in exopolysaccharide content

The exopolysaccharide content present in the extracted extracellular polymeric substances was estimated by phenol-sulphuric acid assay (Masuko *et al.* 2005). The principle of this assay is that in the acidic condition, the polysaccharides are hydrolyzed in the monosaccharide units and their reducing ends are exposed. Then the hydrolyzed monosaccharide units react with the added phenol in the mixture to develop the yellow golden color.

Standard preparation: Glucose, taken as the standard was prepared in the range of 5-200 µg/mL in autoclaved distilled water (DW).

Sample preparation: 250 µg/mL of the powdered sample was prepared in autoclaved distilled water.

Estimation of glucose content on microtitre plate: The prepared sample and glucose standards were added in each well of 96 well microtitre plate and the volume was made up to 50 µL using DW. Following the step, 150 µL sulphuric acid was added. Then, immediately 30 µL of 5% phenol (prepared in DW) was added. The microtitre plate was incubated at 70°C/15 min in water bath. The plate was taken out of the water bath and cooled to the room temperature. Then, the absorbance was taken at 492 nm. The amount of glucose equivalence was calculated by the obtained curve equation.

3.7.2 Nucleic acid assessment in extracted EPS

The presence of nucleic acids (DNA and RNA) in the extracted sample was assessed by the method using spectrophotometeric absorbance (Mackey & Chomczynski 1997). The absorbance was observed at 260 and 280 nm for the extracted sample and the ratio was calculated. The calculated ratio of 1.8 confirms the presence of pure nucleic acid and higher than 2 confirms the presence of proteins.

3.7.3 Estimation of total protein content

Bradford assay is a rapid method for the protein estimation (Bradford 1976). Dye (Coomassie brilliant blue G250) was used to detect the proteins present in the sample, as this dye binds to

amino acids and leads to the complex formation with the increased molar absorptivity from 465 to 595 nm. And thus the absorbance is taken at 595 nm.

Standard preparation: Stock of 1 mg BSA (bovine serum albumin) in 1mL DW was made for the standard. Further standard dilution was prepared in the range of10-100 µg/mL.

Sample preparation: Sample was prepared as 250 µg/mL in DW.

Microtitre estimation: The test was done in microtitre 96 well plate. The prepared sample and BSA standards were added in the well as per their range concentration. After this, DW was added in each well to make the volume up to 40µL. Then, 160 µL of prepared Bradford reagent was added in each well and incubated in dark. After incubation of 5 min at room temperature, the absorbance was observed at 595 nm. The standard curve of BSA was plotted at different concentrations and the protein concentration was calculated by the obtained equation.

3.8. Biological Activity of extracellular polymeric substances

3.8.1 Free radical scavenging (antioxidant) activity of extract of S. acutus

To know the antioxidant ability of extracted EPS from *S. acutus,* it was subjected to the antioxidant assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) protocol. The underlying principle is the reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) to 2,2-diphenyl-1-picrylhydrazine when a hydrogen donor is added in the mixture and colorimetric change is observed from violet to pale yellow color (Free radical to non-radical) (Molyneux 2004) (Figure 3.2).

Sample preparation: Sample was prepared in autoclaved DW with the stock of 5mg/mL in the range of 100-1500 µg/mL

Standard preparation: Ascorbic acid (100µM) was prepared in autoclaved DW, which makes the calculation of 1mg/10mL as the standard.

DPPH preparation: DPPH stock of 1mM in methanol was prepared (3.94 mg/10mL). From this stock, working concentration of 100µM was made in methanol (1mL from stock and 9 mL methanol).

DPPH assay on microtitre plate: Prepared sample stock was added in each well with the range concentration of 100-1500 µg/mL in the 96 well plate. Similarly, 30 µL of prepared Ascorbic acid (100µm) was added in the well to serve as standard. Then, 150 µL of prepared DPPH

(100µM) in methanol was added to each well and volume was made up to 200 µL using methanol. A blank of methanol, methanol and DPPH and DW was also run with the test. Then, the plate was kept for incubation in dark for 45 min and the absorbance was measured at 517nm. Radical scavenging activity was calculated using the following formula,

Scavenging activity (%) = (Control abs – Sample abs / Control abs) × 100

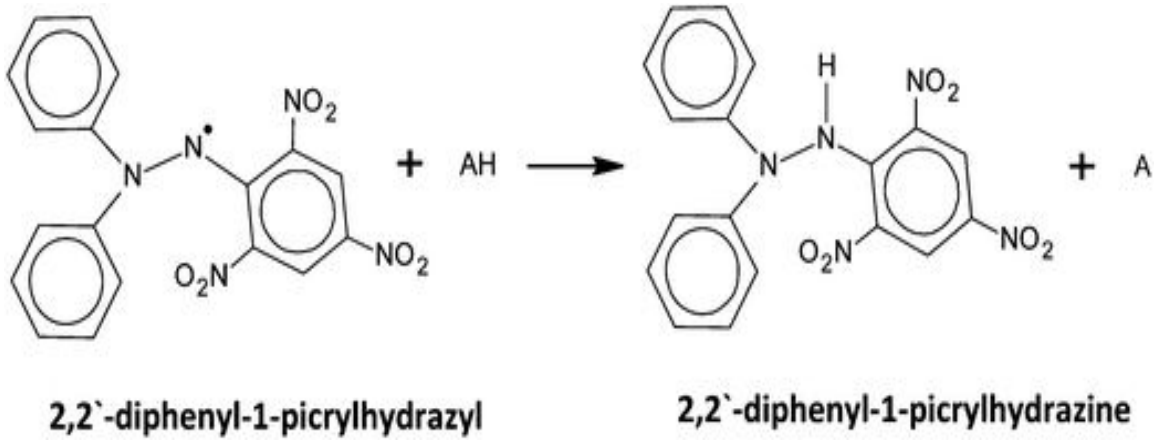

Figure 3.2: DPPH assay working and reduction of DPPH (2,2-diphenyl-1-picrylhydrazyl) to 2,2-diphenyl-1-picrylhydrazine.

3.8.2 Cell proliferation assay- The effects of extracted sample from *Scenedesmus* were evaluated on healthy immuno-competent peripheral blood mononuclear cells from human volunteers.

3.8.2.1 Preparation for experimentation

Media preparation: RPMI 1640 is a best suited media for the suspension cultures as it contains high amount of glutathione and vitamin (vitamin B12, biotin, choline and inositol). For the present work, RPMI 1640 media was used for the culturing of PBMC (peripheral blood mononuclear cells).

The media was prepared as per the instructions by manufacturer. Briefly, 9.6 g of powder media was dissolved in 900 mL of MQ water. The pH of the media was adjusted to 4 before adding 20 mL of 200 mM glutamine solution and 26.5 mL of 4% sodium bicarbonate solutionto the media. Then, final pH of the media was kept at 7.4 using 1M HCl and 1N NaOH. 10 mL of antibiotics solution containing 100 µg/mL streptomycin, 100 IU/mL penicillin and 2.5 µg/mL amphotericin was also added to the media. The final volume of media was adjusted to 1000 mL with MQ

water. Then, media was subjected to filtration under vacuum conditions using 0.22 µm filter membrane.

Preparation of phosphate buffer saline (PBS): One liter (1x) PBS was prepared by mixing 0.2 g KCl, 8 g NaCl, 0.24 g of KH2PO4 and 1.44 g Na2HPO4 in 900 mL of autoclaved DW and the pH of the solution was adjusted to 7.4 using 1N HCl and 1N NaOH. Final volume was raised to 1000 mL and was autoclaved.

3.8.2.2 Cell enumeration

Cell counting was done with the help of trypan blue exclusion assay using haemocytometer . The principle behind this assay is that the dead cell accumulates the dye on their cell wall and appears blue whereas the living cells appear shiny in visibility.

Slide preparation: 10µL of cell suspension was diluted with 10 µL of 0.4% trypan blue dye (prepared in PBS) and 80 µL of the media was added. After loading, 10 µL of this mixture on haemocytometer, the slide was kept under light microscope under 40 x magnifications (Nikon Eclipse E100 LED) and the viable cells were observed. The viable cells were counted in all of the four squares of haemocytometer and the calculations were made as per the equation given below (Figure 3.3).

$$\text{Cell count } (mL^{-1}) = (A \times B \times 10^4) /4$$

Where,

A = Number of cells counted in the four squares

B = Dilution factor

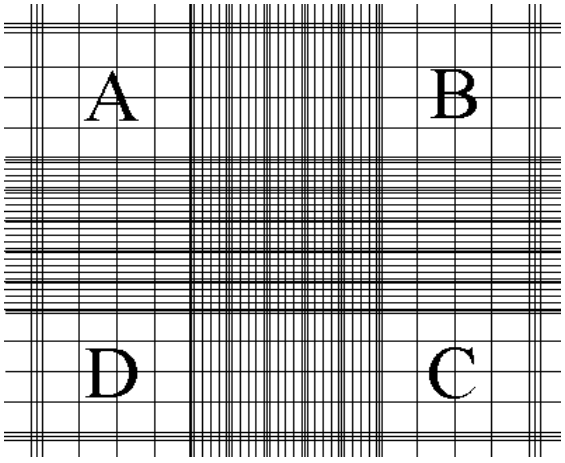

Figure 3.3: Schematic representation of haemocytometer, where A, B, C and D represents the four square chambers into which the counting of the viable cells has been done.

3.8.2.3 Isolation of Peripheral Blood Mononuclear cells:

5 mL of fresh blood was drawn from healthy donors using veni puncture, handled by the trained technicians from Lifeline blood bank, Patiala in EDTA coated tubes (BD vacutainer ® Tubes). The informed consent was taken from all three donors for the experiment work.

Isolation of PBMC: PBMC were isolated with the help of ficoll density gradient method. In the fresh 15 mL falcon, histopaque 1577 was added, onto which the collected blood was layered carefully in the ratio of 1:1. Then centrifugation at 450 g (30 min at room temperature) in swinging bucket rotor (Thermo scientific Biofuge Stratos) was done. After centrifugation, plasma layer was discarded and the buffy coat containing PBMC was collected very carefully (English & Andersen 1974). The cells were washed twice by adding 6 mL of prepared 1x PBS and again centrifuged at 330 g (/10 min). The pellet was then resuspended in prepared 1mL complete RPMI 1640 media containing FBS 10% (v/v).

3.8.2.4 Assessment of cell proliferation by MTT assay

The effect of extracted EPS on PBMC cell proliferation was estimated by calorimetric based MTT (3- 4, 5 dimethylthiazolyl-2-2-5, diphenyltetrazolium) assay (Borenfreund *et al.* 1988). The principle behind this assay is that on the reduction of MTT, a purple colored product, formazan is produced by the presence of an enzyme (succinate dehydrogenases), which is secreted by the metabolically viable active cells from mitochondria (Figure 3.4). The crystals produced are dissolved in dimethyl sulfoxide (DMSO). This assay accurately quantifies the changes in the rate of cell proliferation, as it gives the linear relationship between cell number and the signal produced.

Sample preparation: EPS was prepared as a stock of 5mg/mL, out of which it was diluted and prepared with the concentration range from 100-1000 µg/mL in autoclaved DW.

Positive control preparation: Concanavalin A (ConA) with the concentration of 10 µg/mL served as the positive control.

MTT assay in Microtitre plate: Isolated PBMC cells were seeded in 96 well microtitre plate at the fixed density of 2×10^5cells/well. Then, the prepared EPS extract and ConA was added in each well and volume was made upto 200 µL using complete RPMI 1640 (by adding 10% FBS). Only media served as the control for the experiment. The experiments were performed in triplicates. The plate was incubated for 48 h at 37ºC provided with 5% CO_2 and after the

incubation, 20 µL MTT (5 mg/mL in PBS) was added in each well. Then, the plate was again incubated for 4h for the reaction to occur. 170 µL media was discarded from each well and 100µL of DMSO was added. The final absorbance was taken at 570nm taking 620nm as the reference wavelength on ELISA plate reader (iTecan Infinite Pro ELISA reader).

The cell proliferative index was calculated as the function of absorbance taken by the equation below,

$$\text{Proliferative Index} = Abs_{sample}/Abs_{control}$$

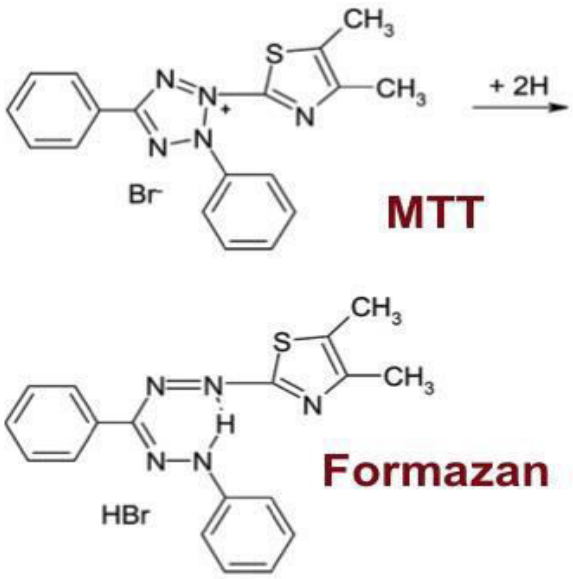

Figure 3.4: Principle of MTT assay

3.9 Stress conditions for enhanced production of exopolysaccharides (EPS) present in extracellular polymeric substances

3.9.1 Different conditions subjected to enhanced production of EPS

S. acutus is known to survive in less or more range of nutrient concentrations which can also put the algae to survive under stress conditions (Mishra *et al.* 2011). This leads to either increase or decrease in their cell growth, biomass production, metabolite production (EPS, carotenoids, chlorophyll content and other pigments) and specific growth rate. After a detailed study, to enhance the production of EPS, two nutrient stresses (Sulphate ($MgSO_4$) and Phosphate (K_2HPO_4) were provided in different concentration range.

Magnesium sulphate: To measure the growth rate and fluctuation in cell division rate and EPS yield, five different concentrations of $MgSO_4$ stress were introduced in the normal BG-11 media (Table 3-4)

Potassium- Hydrogen phosphate stress: Five different combination of phosphate stress have been introduced along with normal concentration of BG-11 media (Table 3.4)

Table: 3-4 Different concentrations of magnesium sulphate and potassium-hydrogen phosphate.

S. NO.	$MgSO_4$ (g/L)	K_2HPO_4 (g/L)
1	0	0
2	0.25	0.2
3	0.5	0.6
4	1.0	0.8
5	1.2	1.0

3.10. Characterization of the obtained EPS

3.10.1. Fourier transform infrared spectroscopy (FTIR)

FTIR is the method of passing infrared rays though the sample to get the imprinted pattern of absorption and transmission (Nicolet & All 2001). It identifies the organic and few inorganic chemicals in a whole range of applications. It works on the principle that most of the molecules have chemicals bonds that absorbs light of the IR region within the electromagnetic spectrum. The frequency range is given by the wave numbers (cm^{-1}) over the wide range between 4000 – 600. As, each molecule has its own chemical bond arrangement pattern with specific vibration energy. These bonds absorb light at particular wavelength and transmit it at another wavelength that leads to the generation of a specific spectrum where peaks at particular wavelength represent the particular chemical bond and its length represents the abundance. The resultant absorption indicates the presence of various functional groups and chemical bonds in the sample. As no two molecules could share the same IR absorption pattern, the obtained spectrum is unique to every compound. The basic instrumentation flow of FTIR includes interferometer, a source, sample, mirrors, beam splitter, detector and the computer to catch the transmittance pattern (Figure 3.5). The IR rays are generated from a source, pass though the interferometer (mirrors and beam splitter) and the sample, goes to the detector. The detector measures the transmission signal and shows on the computer in the form of readable spectrum.

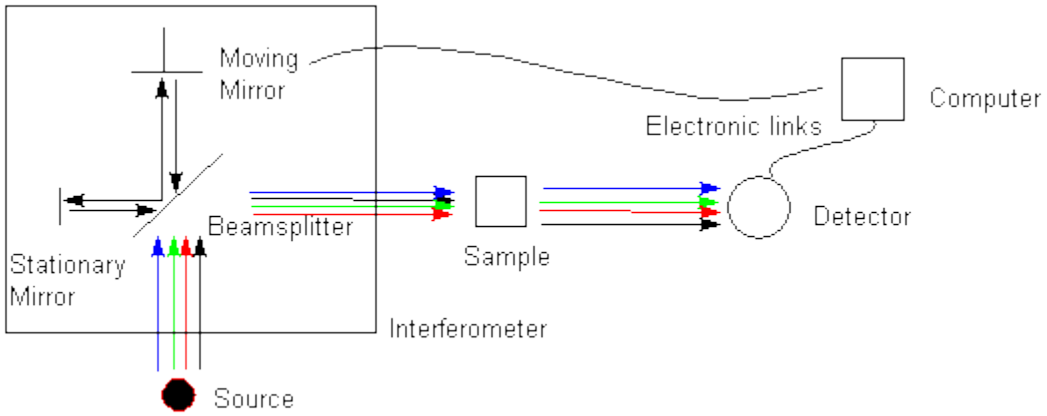

Figure: 3.5 Principle working of FTIR

3.10.2 *^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy*

NMR spectroscopy is a technique which is done to determine the structure of a compound. It characterizes the carbon and hydrogen bonds of an organic compound. This method along with other methods like, IR and mass spectrometry, the entire structure of a molecule can be determined.

The fact behind this method is that the spinning charged atomic nucleus generates a magnetic field. Without the influence of an applied external magnetic field (B_0), the nuclear spins are arranged in random direction. But, when B_0 is applied, the nuclear spins align themselves in the direction or against the direction of applied magnetic field. The working model of NMR contains radio frequency transmitter, sample tube holder, magnet, detector and the computer (Figure 3.6). Briefly, the sample in a tube is placed in the applied magnetic field and the NMR signal is generated by excitation of the nuclear spin from radio waves into nuclear magnetic resonance, which gets detected by the sensitive radio receivers. Thus, it leads to the change in the resonance frequency of the intramolecular magnetic field of atom and details about the functional group of a molecule and its electronic structure determined. The NMR spectrum tells about the no. of protons and positions of them (chemical shift) (Holger Försterling 2010).

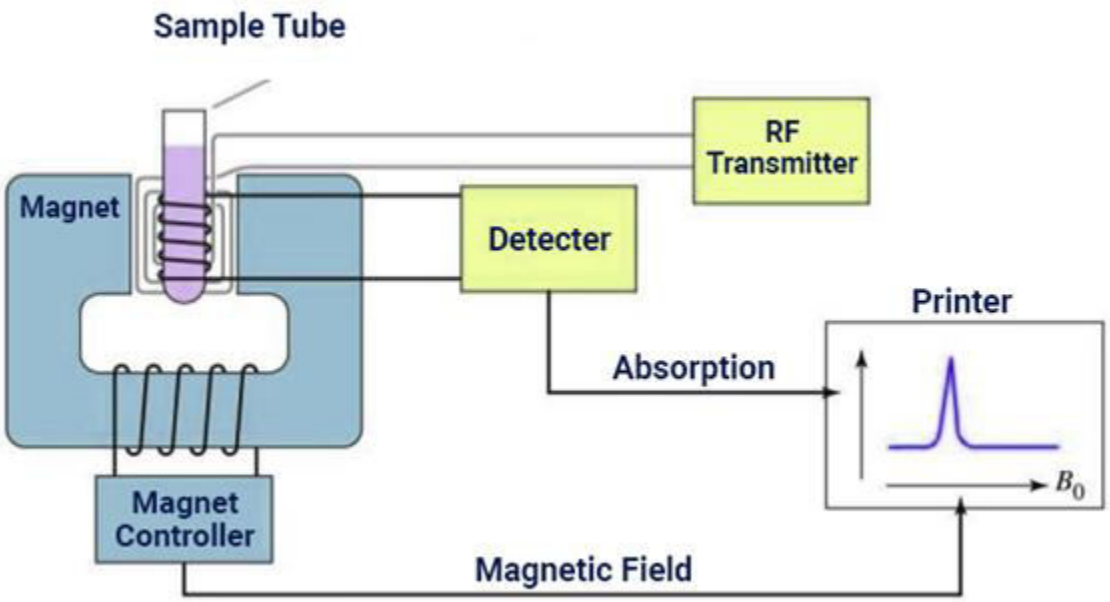

Figure: 3.6 Working principle of NMR (source https://byjus.com/chemistry/nmr-spectroscopy)

3.10.3 Liquid chromatography-Mass spectrometry (LCMS)

LCMS is the technique which combines the principle of separation of chemical compounds using liquid chromatography and with their mass analysis. In this technique the compound ionizes into ions which are sorted on the basis of m/z (mass to charge) ratio (Herbert & Johnstone 2002).

Liquid chromatography

In LC, the fixed amount of sample is directly injected into the mobile phase which is delivered though a high pressure pump. The mobile phase containing the analytical sample (analytes) moves to the stationary phase column. The components in the compound mixture are separated on the basis of their affinity with both the phases.

Mass spectrometry

After separation though LC, the analytes moves to the MS section which measures m/z ratio of the ions (charged particles).The basic components of MS include the ion source, mass analyzer, detector and the vacuum systems (Figure 3.7). The ion source provides the components of a sample in a MS system which is ionized by the beam of electrons, photons and laser. In the case of electrospray ionization (ESI), the ion source converts the liquid analytes into gaseous phase. And detector gives the spectrum of the compound. Thus, the spectrum obtained is helpful to measure the molecular mass and structure of the compound.

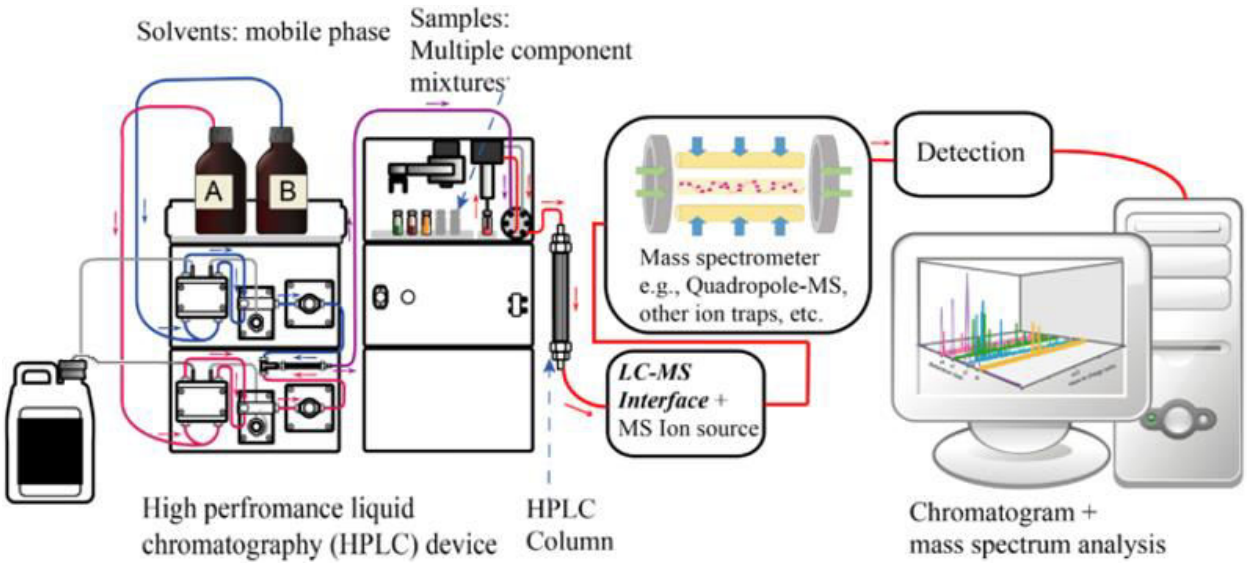

Figure 3.7: The components of a mass spectrometer

In the present study, LCMS of the sample was done in SAIF labs, Punjab University, Chandigarh. The details of the used technique are given in Table 3-5.

Table 3-5: Details of the instrument and parameters used of performing mass spectrometry

Instrument	**Waters, Micromass Q-TOF micro**
Separation Module	Waters Alliance 2795
Ionization	Electro spray Positive (ES+)
Acquisition	MRM, unit resolution
Injection Volume	20 micro litres
Flow rate	0.4 mL/min
Desolvation Gas	550 L/h
Cone Gas	30 L/h
Desolvation Temperature	300 ºC
Source Temperature	110 ºC
Capillary Voltage	3000V
Cone Voltage	30V

3.11. Statistical analysis

All the data were expressed with the independent experiments as the mean ± standard error of mean in PRISM software. Data were analysed using analysis of variance (ANOVA) and the means were compared using Tukey's test at $p < 0.05$.

Chapter IV

Results

4. Results

Objective1: Assessment of antioxidant and immunological effect of exopolysaccharide isolated from Scenedesmus acutus.

4.1 Optimization of growth, cultivation and nutritional Compositions of *S. acutus*

In this objective, the growth pattern (growth rate, biomass accumulation, cell count), pigment estimation, extraction of biomolecular content (extracellular polysaccharide substance) and their bio-activity was studied.

4.1.1. Maintenance and Production of Scenedesmus acutus

The suspension cultures of *S. acutus* were initially maintained in 250ml Erlenmeyer flask containing 100 ml of Bristol and BG-11 media respectively (Figure 4.1). These flasks served as the mother cultures and were incubated at 28±2°C under photoperiodism of light and dark conditions (12/12 h). Cell growth was monitored for 30 days and the mother cultures were sub cultured and further various nutritional and pH stress conditions were incorporated in the media with *S. acutus* (2-3 x 10^5 cells/ml) as inoculum.

S. acutus cells were also maintained on agar media test tube slants for preservation (Figure 4.1). The slants and plates were incubated at 28^{o}C for 30 days, under continuous light-dark photoperiodism and finally later stored at 4^{o}C.

Figure: 4.1 a). Mother culture of *S. acutus* b). Preservation of the culture in test tube slant.

4.2 Measurement of growth and productivity

4.2.1 Study of cell growth under microscope

Growth of *S. acutus* was observed on the streaked petri-plates of Bristol and BG-11 media, where for initial 10 days, the red coloured colony was observed which turned to white and finally green colour was observed after 22nd day. The green coloured colony of *S. acutus*, under Nikon Eclipse E200 microscope was observed to be non motile (non-flagellated), unicellular and exhibiting spherical shape (Figure 4.2). They were also observed to occur in the colony of four cells and the red coloured carotenoids pigments were clearly observed.

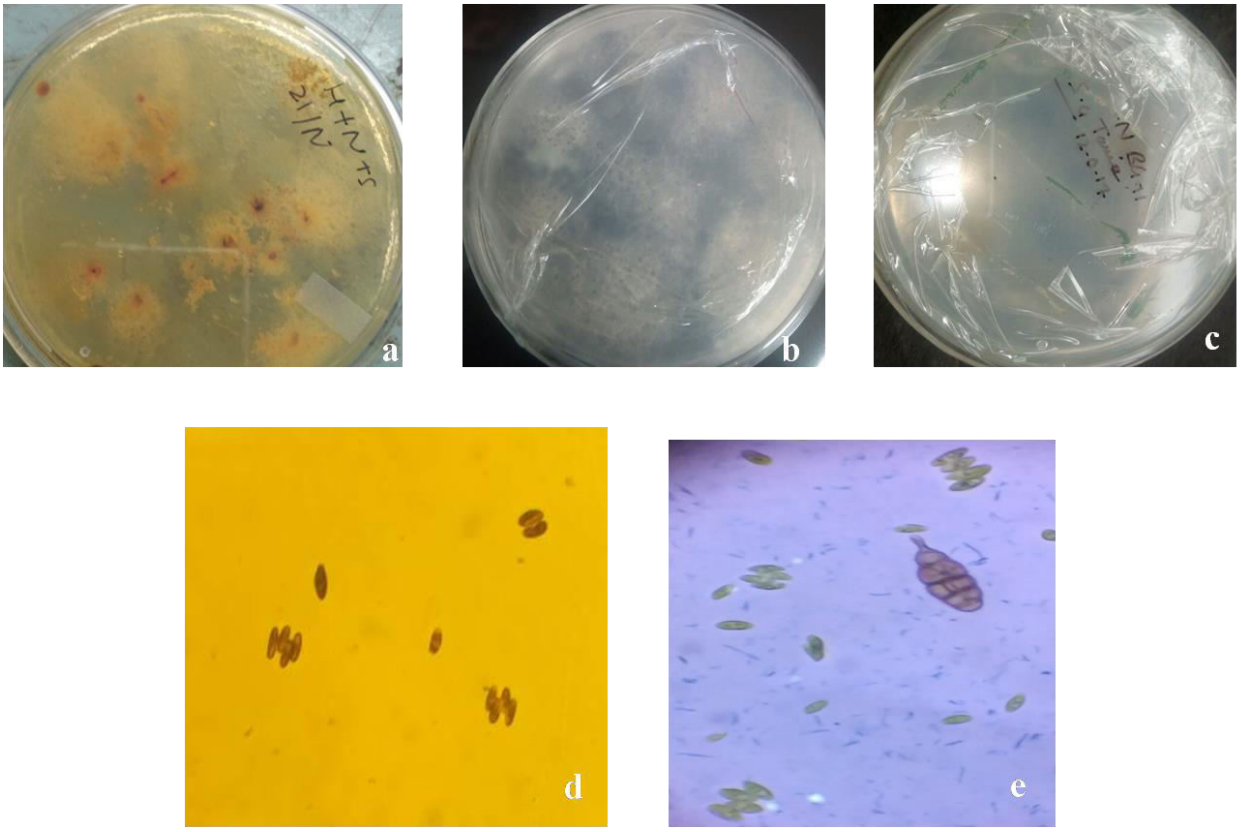

Figure: 4.2 Observation of growth pattern of *S. acutus* at different interval of time a).Red coloured streak for initial 10 days b). White coloured streak during 20 days c). Green coloured streak after 22nd day d). Unicellular *S. acutus* existing as four cell colony fixed in lugol's iodine solution e). Red coloured carotenoids pigment and green coloured unicellular cells.

4.2.2. Study of growth curve

The growth curve of *S. acutus* cells on Bristol and BG-11 media was estimated spectrophotometrically at optical densities 560,580,600 and 680 nm. The maximum OD was observed at 680 nm, thus it was fixed for further study. The growth curve of *S. acutus* was studied for 30 days till the stationary and death phase were achieved (Table 4.1). It was observed that cells attained stationary phase approximately after 20-22 days of the inoculation (Figure 4.3), hence further analysis was carried out till 20 days.

Table 4.1 Growth of *S. acutus* on BG-11 and Bristol media

	A_{680nm}(Mean± SD)	
Days	**BG-11**	**Bristol**
0	0.05± 0.014	0.051± 0.024
2	0.06± 0.12	0.059 ± 0.15
4	0.08± 0.08	0.078± 0.18
7	0.1± 0.13	0.099± 0.08
11	0.14± 0.11	0.13± 0.034
14	0.16± 0.04	0.15± 0.12
17	0.18± 0.09	0.17± 0.16
19	0.193±0.15	0.18± 0.07
20	0.196± 0.21	0.19± 0.056
22	0.197± 0.17	0.192± 0.0182
24	0.201 ± 0.089	0.191± 0.0134
28	0.2016± 0.076	0.189± 0.14
30	0.2± 0.045	0.187± 0.019

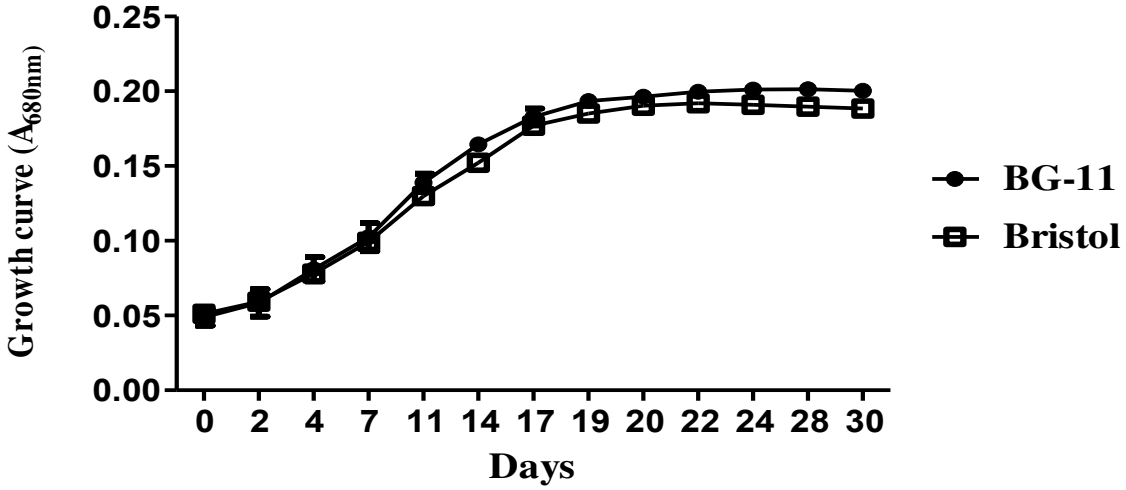

Figure 4.3: Growth curve study of *S. acutus* on BG-11 and Bristol media

4.2.3 Measurement of the Cell Count

Under optimum growth conditions, the cell count and density of *S. acutus* was observed to increase linearly and attained the log phase on the 4thday. The maximum cell count was seen to be 8.2 x 10^6 and 7.8 X 10^6 cells/ml on BG-11 and Bristol media respectively (Figure 4.4 and Table 4.2)

Table 4.2: Cell count of *S. acutus* on BG-11 and Bristol media, showing comparative study.

	10^6 cells /ml(Mean± SD)	
Days	**BG-11**	**Bristol**
2	2.6±0.12	2.5±0.25
4	3.6 ± 0.08	3.4± 0.08
7	4.8± 0.09	4.5± 0.14
11	6.2± 0.15	5.9± 0.03
14	7± 0.21	6.7± 0.16
17	7.5± 0.17	7.1± 0.27
20	8.2± 0.18	7.8± 0.07

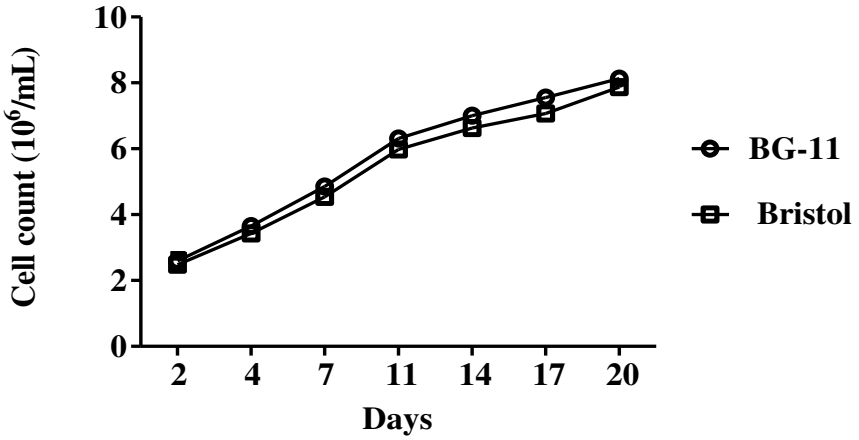

Figure 4.4: Cell count study of *S. acutus* on BG-11 and Bristol media

4.2.4 Measurement of dry weight of S. acutus cells

The dry weights of the algae cells were taken after every 2-3 days and the weight of biomass accumulation was recorded for 20 days (Figure 4.5). The pattern of biomass accumulation was found similar to cell count and growth in both media (Table 4.3).

Table 4.3 Dry weight biomass of *S. acutus* on BG-11 and Bristol media

Dry biomass(mg/ml)(Mean± SD)		
Days	**BG-11**	**Bristol**
2	0.20± 0.17	0.16± 0.12
4	0.37 ± 0.078	0.31± 0.13
7	0.51± 0.13	0.42± 0.20
11	0.74± 0.18	0.72± 0.098
14	0.89± 0.11	0.82± 0.14
17	0.94± 0.14	0.89± 0.036
20	0.95± 0.056	0.86± 0.09

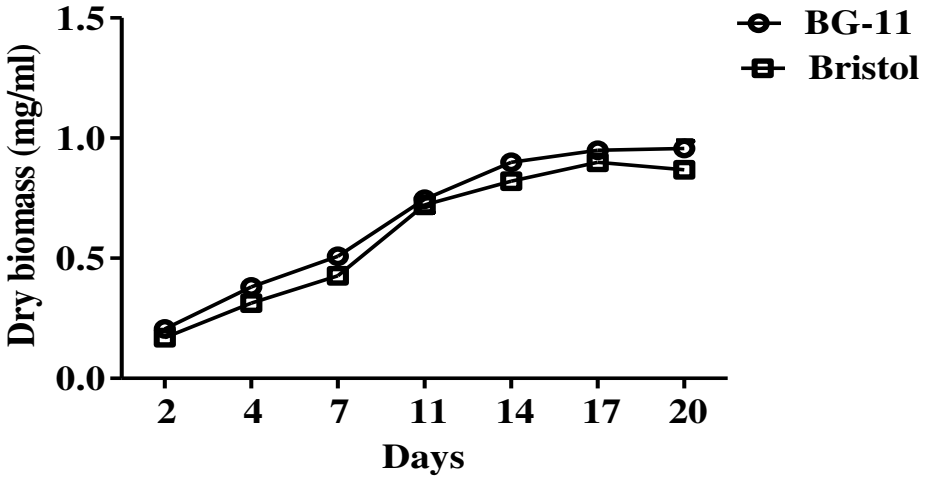

Figure 4.5: Dry weight biomass comparative studies of *S. acutus* on BG-11 and Bristol media

4.2.5 Measurement of growth rate

Under optimum growth conditions of 28^{o}C, the specific growth rate was calculated. The algae strain in both the media (BG-11 and Bristol) were found to grow with medium rate. Although the growth rate of cells in BG-11 medium was somewhat higher than that of the cells inoculated in Bristol medium. For first 2 days, the growth rate was observed to be decreased and thereafter it increased. The exponential/log phase was reached approximately after the 4-5th day (Figure 4.6). Maximum growth rate was observed to be 0.24± 0.18 (Table 4.4).

Table 4.4: Specific growth rate of *S. acutus* on BG-11 and Bristol media, showing the comparative study.

Growth rate (μ,t^{-1}) (Mean± SD)		
Days	**BG-11**	**Bristol**
2	0.071± 0.01	0.073± 0.003
4	0.17±0.16	0.13± 0.078
7	0.23± 0.18	0.19±0.054
11	0.17±0.032	0.15±0.0087
14	0.06± 0.08	0.054±0.059
17	0.043± 0.038	0.04± 0.07
20	0.044± 0.013	0.03± 0.021

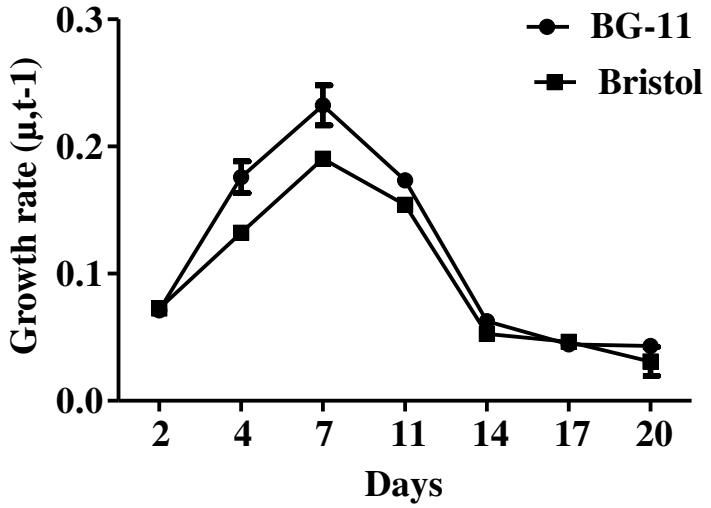

Figure 4.6: Specific growth rate of *S. acutus* on two different media (BG-11 and Bristol)

4.3 Pigment Estimation

Chlorophyll and carotenoid content of *S. acutus* were estimated for a period of 20 days. Total Chlorophyll (a and b) was found to be maximum at the 20th day (Figure 4.7), and the carotenoid content was found to be less than the chlorophyll content on 20th day (Figure 4.8). The accumulated data of chlorophyll and carotenoid is shown in Table 4.5 and 4.6.

Table 4.5: Comparative study of total chlorophyll content accumulation in *S. acutus* growing in BG-11 and Bristol media.

Total chlorophyll (mg/ml) (Mean± SD)		
Days	**BG-11**	**Bristol**
2	0.019 ± 0.0015	0.016 ± 0.0021
4	0.024 ± 0.002	0.018± 0.0005
7	0.025 ± 0.0040	0.019 ± 0.0002
14	0.034 ±0.002	0.028 ± 0.0014
20	0.04 ± 0.003	0.035 ± 0.0004

Table 4.6: Comparative study of total carotenoid content accumulation in *S. acutus* growing in BG-11 and Bristol media

Total carotenoid (mg/ml)(Mean± SD)		
Days	**BG-11**	**Bristol**
2	0.0018 ± 0.0003	0.012 ± 0.00015
4	0.002 ± 0.00014	0.0015 ± 0.00013
7	0.0021 ± 0.0001	0.0017 ± 0.00035
14	0.0027 ± 0.00012	0.0025 ± 0.0003
20	0.003 ± 0.000116	0.0026 ± 0.00016

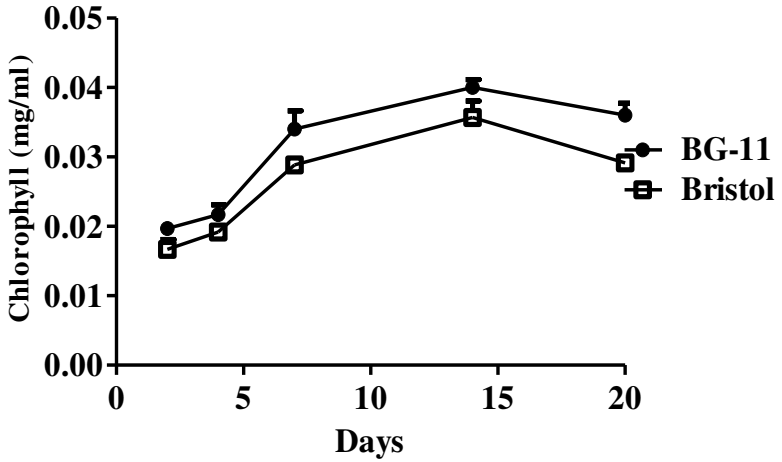

Figure 4.7: Total chlorophyll content of *S. acutus* growing in two different media (BG-11 and Bristol)

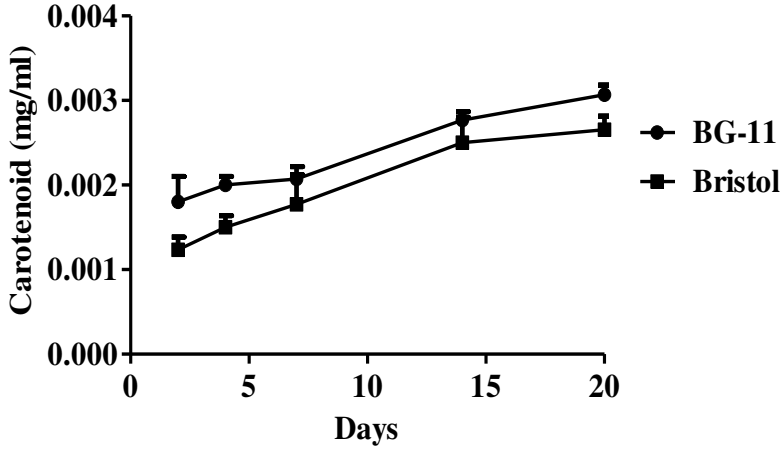

Figure 4.8: Total carotenoid content of *S. Acutus* growing in two different media (BG-11 and Bristol)

4.4 Extraction of exopolysaccharides from *S. acutus*.

After the optimization of growth and cultivation conditions, the culture of *S. acutus* was found to be suitable for extraction on 20th day and the exopolysaccharides **(EPS)** were extracted by alcoholic precipitation and protein was removed by TCA. The obtained liquid was processed and lyophilised to get the powdered form of extracellular polysaccharides.

4.4.1 Biomolecules content in extracellular polysaccharides

The extracted sample was analyzed for the presence of sugar using phenol-sulphuric acid method in which glucose was taken as the standard (Figure 4.9).The sugar was estimated quantitatively by the equation obtained from the standard graph (Figure 4.9).The obtained results showed the presence of sugar in the EPS (Table 4.7).

The EPS was also analyzed for the presence of other biomolecules i.e., proteins and nucleic acids by bradford and spectrophotometric analysis (A260/A280). No proteins and DNA was obtained, which confirmed that isolated EPS was free from DNA and protein contamination.

The EPS was also analyzed for the preliminary confirmation of polysaccharides by spectrophotometric analysis (UV-Vis) between 200-700 nm range. No absorption at UV range was found, which showed the confirmation for the presence of polysaccharides in the extracted EPS, as sugars are not UV active compounds.

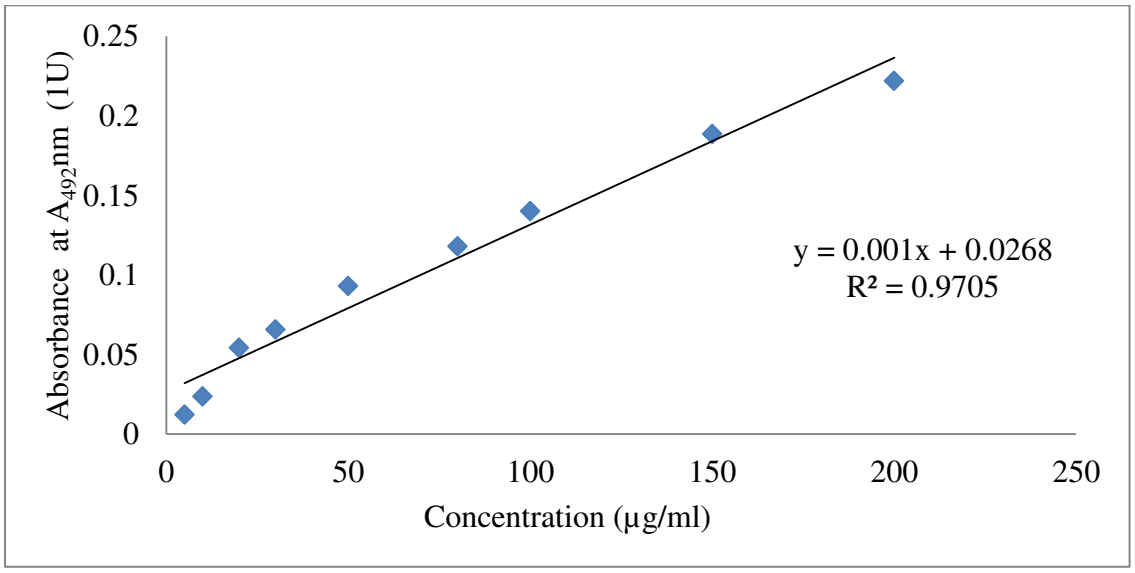

Figure 4.9: Glucose standard curve for the preliminary analysis of polysaccharides in extracted EPS.

Table: 4.7 Glucose content present in the exopolysaccharides (EPS).

Sample	**Glucose equivalence (µg) / EPS (250 µg)** (Mean± SD)	
	BG-11	**Bristol**
EPS	12.68 ± 1.57	9.33 ± 2.05

Due to the better growth rate and extractable quantity of EPS from *S. acutus,* the media BG-11 was chosen for further bioactivity experiments and enhanced production of EPS.

4.4.2 Anti-oxidant activity assessment of isolated EPS with BG-11

Free radical constantly generates in the body, which leads to various disorders (mutagenesis, carcinogenesis, aging and cell damage) and need to be removed. Antioxidants are the compound which exhibits the property of removing these generated free radicals from the body by interfering with the process of oxidative stress mediated by the free radicals.

The anti-oxidant activity of the obtained exopolysaccharides was analyzed with the assay of DPPH in which ascorbic acid served as the positive control. Free radical scavenging activity increased with increased concentrations and the maximum activity was found at the range of 750 µg/ml, which is 33.31± 2.02% (Table: 4.8). It appeared that these EPS have not much of the antioxidant activity (Figure: 4.10).

Table: 4.8 Table showing free radical scavenging activity of exopolysacchrides (EPS)

Sample (µg/ml)	**Antioxidant activity (%)**(Mean± SD)
AA*	82.74 ± 2.50
100	9.03± 1.1
250	19.66 ± 1.04
500	22.92 ± 1.7
750	33.31 ± 2.02
1000	31.68 ± 2.2
1200	25.87 ± 1.7
1500	26.03 ± 1.1

AA* = Ascorbic Acid (100µM)

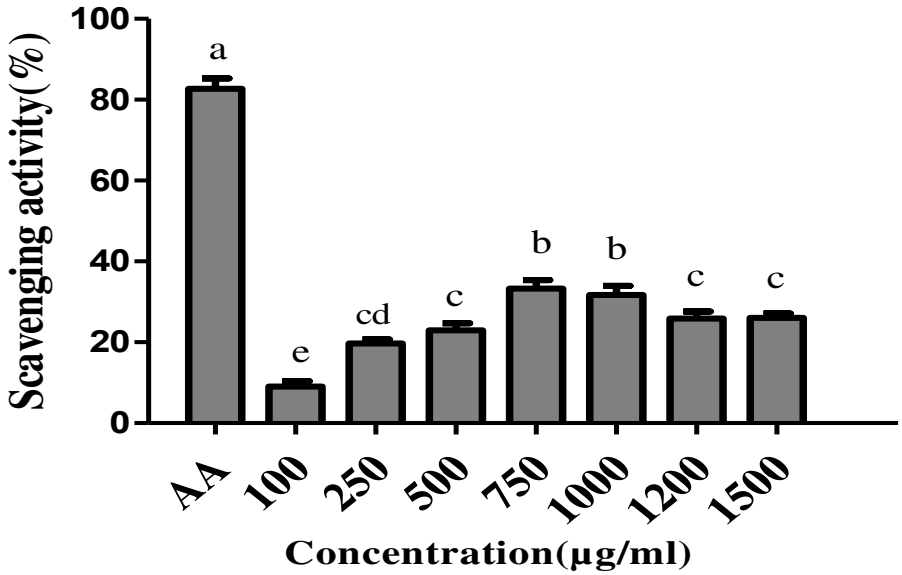

Figure 4.10: Free radical scavenging activity of EPS. Bars with the same lowercase letters are not significantly different at p>0.05.

4.4.3 Effect of EPS on peripheral blood mononuclear cells

The extracted EPS was assessed for their effect on peripheral blood mononuclear cells using MTT assay in the concentration range of 100-1500 µg/mL. It was observed that the proliferation index of PBMC increased with the increase in concentration till 750µg/mL and then it becomes relatively constant.(Figure 4.11).Proliferative index is more than one at all concentration which shows the enhanced cell proliferation of PBMC leading to the immuno-stimulatory activity of the EPS (Table 4.9).

Table: 4.9 Proliferative index showing immunostimulation activity of EPS from *S. acutus.*

Sample (µg/ml)	**Proliferative Index** (Mean± SD)
Con A*	1.38± 0.01
100	1.51± 0.02
250	1.52± 0.01
500	1.58± 0.008
750	1.97± 0.013
1000	1.96± 0.01
1500	1.94 ± 0.007

Con A*: Concanavalin A (10 µg/ml)

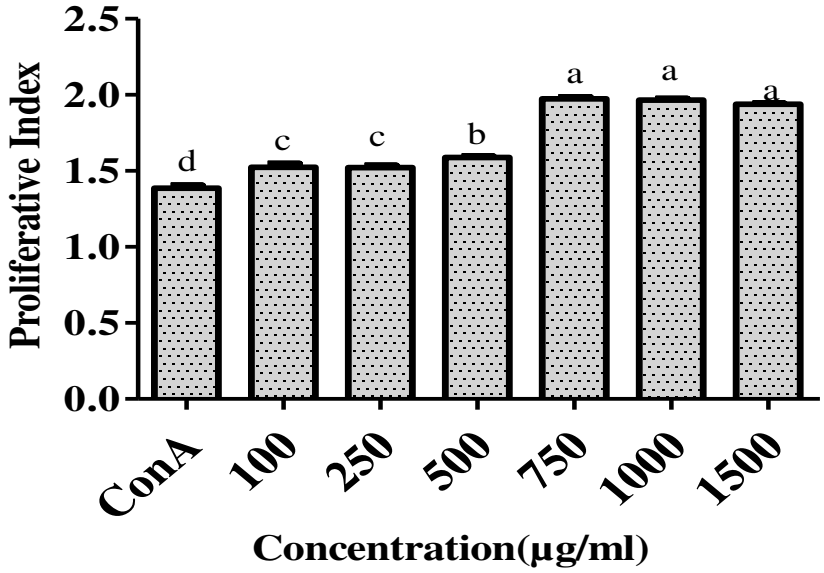

Figure 4.11: Proliferative index showing immunostimulation activity of EPS from *S. acutus.* Bars with the same lowercase letters are not significantly different at p>0.05.

4.5 Modification of Culture Conditions of BG-11 media for the effect on growth of *S. acutus*

To enhance the yield of the EPS, different nutritional and pH stress were introduced to study the effect of stress on cell growth and EPS yield using BG-11 media.

The effect of nutritional and pH stress on the growth curve and biomass production of *Scenedesmus* cells were studied, where citric acid deficient and ferric ammonium citrate (0.12g/L) (CC) stress condition led the cell culture to attain maximum growth for 12 days and then the growth curve started to fall leading to the total death after 20 days. Nitrogen (N-) deficient stress led to a negative shift in the growth curve compared to the nitrogen containing (N+) media (which is the normal BG-11 media). The pH stress (7.4 and 7.1) had the neutral effect on the growth curve (Figure 4.12).

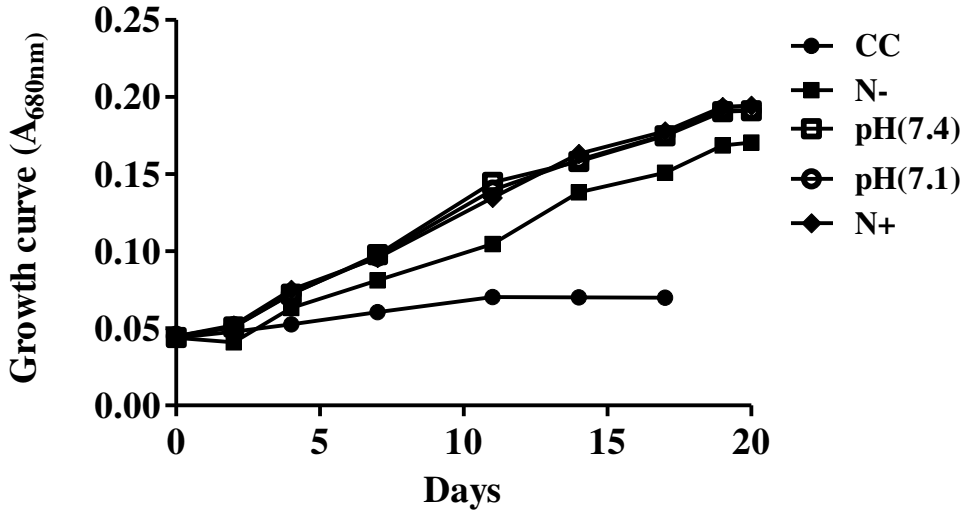

Figure 4.12: Growth curve in different stress conditions in BG-11 media.

Thus, the nutritional stress was observed to be playing more important role as compared to pH stress, so for further work, different nutritional stresses were taken.

Objective II: Enhanced production of the exopolysaccharides under different nutritional stress conditions.

4.6 Growth of *S. acutus* under different stress conditions

After the observation of bioactive results from EPS isolated from the algae grown in normal media, the next objective determined was to enhance their production. To achieve the objective, algae was grown in different nutritional stress conditions, sulphate ($MgSO_4$, 0-1.25 g/L) and phosphate (K_2HPO_4, 0-1.0g/L) with different concentration range and the effect of stress conditions in each parameter along with EPS production was studied.

4.6.1. Growth curve study

The study of growth curve of *S. acutus* on modified BG-11media consisting different $MgSO_4$ and K_2HPO_4 stress concentrations was done spectrophotometrically at absorbance 680 (Table 4.10 a. and b.). Results for the higher growth rate and biomass accumulation were also studied, which could affect the yield of EPS production. As compared to the normal BG-11 media, the enhanced growth was observed for concentrations, sulphate (1.25g/L) > phosphate (0.6g/L) > sulphate (1.0g/L) > sulphate (0.5g/L) > phosphate (0.8g/L) and the decreased growth was observed for concentrations, sulphate (0g/L) > phosphate (0g/L) > phosphate (0.2g/L) (Figure 4.13 a and b).

4.6.2. Cell count study

The cell count study was measured for 20 days and the comparative results were compiled (Figure 4.14). It was observed that the highest cell count for sulphate stress was obtained for concentration 1.25g/L with 14.575 x 10^6 cells/mL (Table 4.11 a) which is approximately twice the growth as compared to the normal condition cell count and the highest cell count for phosphate stress was obtained at concentration 0.6g/L with 13.525 x 10^6 cells/mL (Table 4.11 b) which is approximately 1.6 times higher of the normal condition cell count (Figure 4.14).

4.6.3. Specific Growth rate study

The growth rate was studied for all stress conditions and compared to the normal condition and it was observed to be reducing with time in all the stress conditions (Table 4.12 a, b and Figure 4.15 b a and b).

4.10 a). Readings of growth curve on modified BG-11 media with phosphate stress.

	A_{680nm}(Mean± SD)					
	K_2HPO_4 (g/L)					
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**	**1**
0	0.050±0.004	0.043±0.005	0.042 ± 0.003	0.046 ± 0.004	0.044 ± 0.001	0.043± 0.003
2	0.061±0.007	0.045±0.008	0.047 ± 0.006	0.051 ± 0.002	0.054 ± 0.006	0.048 ± 0.002
4	0.083±0.006	0.052±0.007	0.061 ±0.008	0.079 ± 0.001	0.079 ± 0.003	0.065±0.007
7	0.12±0.007	0.062±0.006	0.092 ±0.009	0.11 ± 0.005	0.10± 0.001	0.089 ± 0.009
11	0.14±0.004	0.082±0.004	0.13 ± 0.005	0.15 ± 0.003	0.14 ± 0.004	0.12 ±0.004
14	0.16±0.001	0.11±0.003	0.15 ± 0.007	0.19 ± 0.002	0.17 ± 0.007	0.14± 0.003
17	0.18±0.005	0.12±0.005	0.16 ± 0.008	0.23 ± 0.008	0.18 ± 0.002	0.16 ±0.006
19	0.193±0.002	0.136±0.008	0.19 ± 0.002	0.27 ± 0.004	0.19± 0.0024	0.18 ±0.009
20	0.197±0.002	0.14±0.006	0.193 ± 0.008	0.29 ± 0.007	0.20± 0.003	0.182 ± 0.008
22	0.20±0.0001	0.141±0.005	0.189 ± 0.0001	0.305 ± 0.009	0.203 ± 0.008	0.178 ± 0.002
24	0.201±0.001	0.141±0.00	0.1892 ± 0.004	0.309 ± 0.003	0.199 ± 0.005	0.18 ± 0.001
28	0.20±0.0003	0.14±0.0056	0.189 ± 0.005	0.302 ± 0.004	0.1992 ± 0.006	0.153 ± 0.004
30	0.19± 0.0008	0.139 ± 0.009	0.179 ± 0.003	0.308 ± 0.007	0.198 ± 0.005	0.15 ± 0.007

0.4*= Normal

4.10 b). Readings of growth curve on modified BG-11 media with sulphate stress.

	A_{680nm}(Mean± SD)					
	MgSO4 (g/L)					
Days	**0.75***	**0**	**0.25**	**0.5**	**1**	**1.25**
0	0.050±0.004	0.04± 0.003	0.046± 0.005	0.04±0.002	0.045±0.004	0.047±0.003
2	0.060±0.007	0.048±0.005	0.049±0.004	0.05± 0.007	0.051 ±0.006	0.063±0.007
4	0.08±0.006	0.051±0.001	0.06±0.003	0.06± 0.001	0.06±0.002	0.08±0.009
7	0.10±0.007	0.059±0.006	0.09±0.007	0.09±0.005	0.09± 0.09	0.15±0.004
11	0.14±0.004	0.079± 0.004	0.13± 0.009	0.15±0.003	0.14±0.001	0.19±0.001
14	0.16±0.001	0.11± 0.003	0.15±0.003	0.17±0.006	0.17± 0.004	0.24±0.005
17	0.18±0.005	0.12± 0.001	0.16± 0.001	0.18± 0.006	0.22± 0.002	0.29± 0.002
19	0.19±0.002	0.129 ± 0.002	0.18±0.005	0.19± 0.002	0.24± 0.004	0.31±0.004
20	0.197±0.002	0.131 ± 0.006	0.187± 0.006	0.199± 0.009	0.25± 0.001	0.33±0.007
22	0.1996±0.0001	0.123 ±0.007	0.197±0.002	0.205 ± 0.003	0.257±0.007	0.338± 0.005
24	0.200±0.001	0.124 ± 0.008	0.1971 ±0.004	0.201 ± 0.004	0.253± 0.006	0.332± 0.003
28	0.201±0.0003	0.122 ± 0.004	0.1961 ±0.001	0.202 ± 0.001	0.251± 0..02	0.322±0.009
30	0.199± 0.0008	0.121 ± 0.007	0.1954 ± 0.002	0.2 ± 0.007	0.248± 0.005	0.311 ± 0.004

0.75*= Normal

Table 4.11 a) Readings of cell count on modified BG-11 media with phosphate stress b). Sulphate stress

a)	**Cell count (10^6 cells /mL)** (Mean± SD)					
			K_2HPO_4 (g/L)			
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**	**1g/L**
2	1.8 ± 0.014	1.9 ± 0.001	2.2 ± 0.035	3.4 ±0.082	2.7±0.003	2.5±0.073
4	3.1 ± 0.017	2.3 ± 0.085	3.4±0.032	5.6 ±0.007	3.6 ± 0.001	3.4±0.008
7	4.02± 0.083	3.3 ± 0.003	5.2 ±0.075	7.7 ±0.001	4.9± 0.054	4.6±0.001
11	5.07± 0.006	4.2±0.011	6.3± 0.009	9.9±0.046	6.7± 0.003	6.2±0.045
14	6.7 ±0.064	4.8 ±0.078	6.6± 0.001	10.6± 0.002	7.2± 0.079	6.7±0.009
17	7.3 ±0.017	5.4±0.036	7.2 ±0.088	12.6±0.009	7.4 ±0.032	7 ± 0.008
20	8.4 ± 0.003	5.6±0.009	7.4 ±0 03	13.7±0.065	7.5 ± 0.001	7.1±0.083
b)						
			MgSO4 (g/L)			
Days	**0.75***	**0**	**0.25**	**0.5**	**1**	**1.25**
2	1.8 ± 0.028	1.8±0.003	1.4± 0.006	2.9± 0.72	2.8± 0.032	3.7± 0.0023
4	3.1 ±0.067	2.1 ± 0.005	2.7± 0.003	4.07± 0.054	3.8±0.021	6.2± 0.0054
7	4.02± 0.023	2.9±0.086	3.3± 0.028	5.2±0.001	5.2± 0.077	8.6± 0.032
11	5.07±0.016	4.02±0.005	4.6±0.009	7.05±0.054	7.0±0.093	10.8 ± 0.075
14	6.7 ±0.041	4.4 ±0.036	6± 0.038	7.5±0.092	7.4 ± 0.006	12.1± 0.002
17	7.3 ± 0.022	4.9 ±0.009	6.9± 0.023	7.8± 0.055	7.9±0.0034	14.3± 0.028
20	8.4 ± 0.005	5.1± 0.017	7.8± 0.005	8.5± 0.034	8.5± 0.001	14.5±0.019

* = Normal condition

Table 4.12 a) Readings of specific growth rate on modified BG-11 media with phosphate stress

a)	**Growth rate (μ, t^{-1})** (Mean± SD)					
	K_2HPO_4 (g/L)					
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**	**1**
2	0.072 ± 0.012	0.009 ± 0.0081	0.04 ± 0.0034	0.06±0.0047	0.10±0.089	0.06±0.0034
4	0.18 ± 0.0165	0.06± 0.078	0.13±0.0045	0.23±0.0012	0.19±0.024	0.14±0.026
7	0.24 ± 0.187	0.09± 0.023	0.20±0.0012	0.15±0.0084	0.18±0.015	0.16±0.087
11	0.17 ±0.032	0.14± 0.032	0.11±0.0098	0.11±0.0045	0.06±0.035	0.08±0.026
14	0.06 ± 0.08	0.17 ± 0.011	0.06±0.0087	0.16±0.0027	0.08±0.009	0.09±0.057
17	0.04±0.038	0.04± 0.032	0.03±0.0051	0.04±0.0031	0.051±0.023	0.04±0.044
20	0.042± 0.013	0.018± 0.0043	0.04±0.0065	0.05±0.0043	0.001±0.045	0.03±0.038
b)						
	MgSO4 (g/L)					
Days	**0.75***	**0**	**0.25**	**0.5**	**1**	**1.25**
2	0.072 ± 0.012	0.042±0.054	0.03± 0.043	0.08±0.0003	0.05±0.0021	0.13±0.00032
4	0.18 ± 0.016	0.043±0.038	0.16±0.026	0.14±0.051	0.13±0.036	0.18±0.037
7	0.24 ± 0.18	0.074±0.021	0.14±0.008	0.18±0.058	0.19±0.0053	0.2±0.062
11	0.17 ±0.03	0.093±0.034	0.11±0.001	0.15±0.081	0.12±0.0037	0.11±0.027
14	0.06± 0.08	0.07±0.076	0.06±0.014	0.06±0.056	0.1±0.099	0.09±0.053
17	0.045±0.03	0.03±0.091	0.03±0.072	0.023±0.034	0.04±0.038	0.056±0.043
20	0.046± 0.01	0.005±0.039	0.06±0.091	0.02±0.082	0.05±0.045	0.057±0.046

* = Normal condition

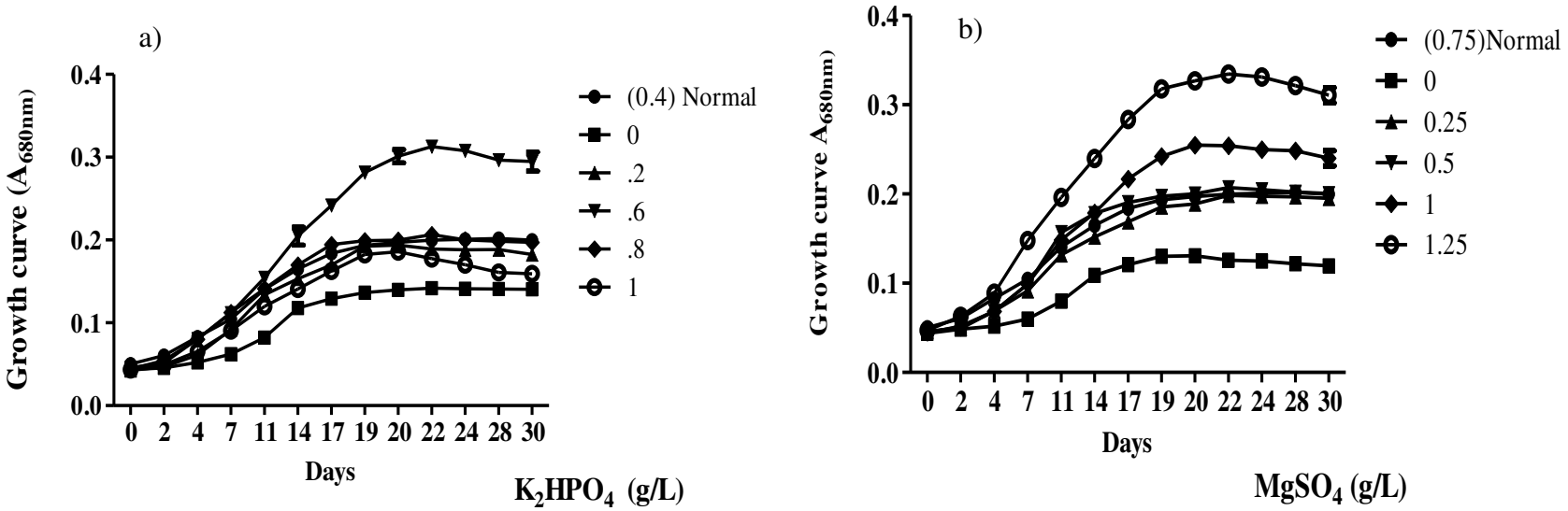

Figure: 4.13 Growth curves of *S. acutus* with a).Phosphate stress b).Sulphate stress

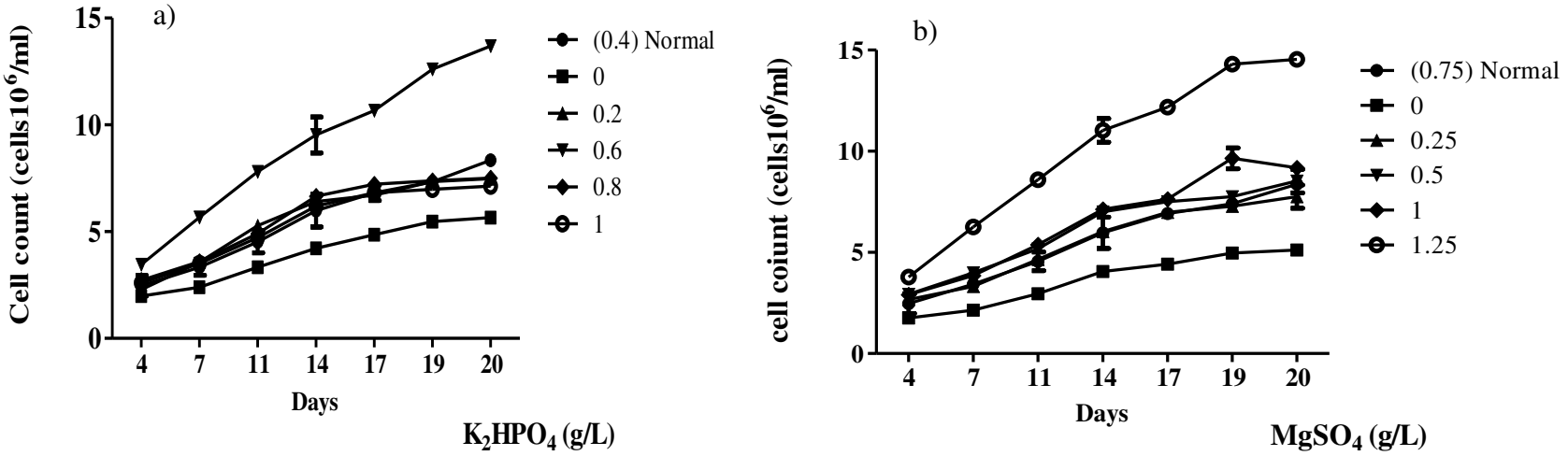

Figure: 4.14 A comparative study on the cell count of *S. acutus* a).Phosphate stress b).Sulphate stress

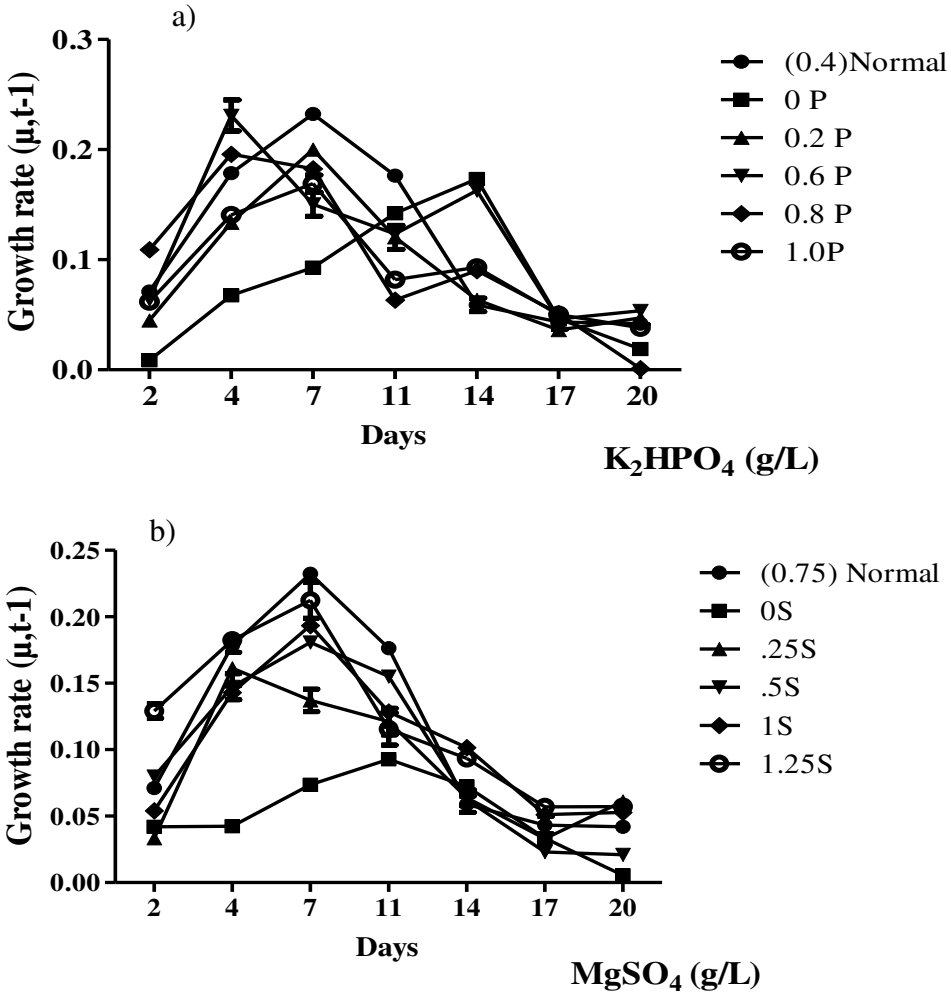

Figure: 4.15 Growth rates of *S. acutus* a). Phosphate stress b). Sulphate stress

4.7 Pigment Estimation

Chlorophyll and carotenoid content of *S. acutus* was estimated for all the stress. Total Chlorophyll (a and b) and carotenoid content was found to be increasing and decreasing in random manner as per the change in concentration of salts.

The highest chlorophyll content (Figure 4.16 a and b) was found in in sulphate and phosphate stress at concentration 1.25 g/L and 0.6 g/L The highest carotenoid (Figure 4.17 a and b) was found in sulphate at concentration 0g/L and phosphate at concentration 0g/L. An interesting observation about sulphate stress at concentration 0g/L was found that the chlorophyll content is too low at this concentration, that the culture's appearance was reddish-brown in colour after approximately 20^{th} day of culturing, indicating the accumulation of more amount of carotenoid pigments as compared to the chlorophyll. The comparative data accumulation of chlorophyll (Table 4.13 a and b) and carotenoid content is represented in (Table 4.14 a and b).

Table: 4.13 a) Total chlorophyll content on modified BG-11 media with phosphate stress b) Sulphate stress

a)	**Total chlorophyll (mg/mL)**(Mean± SD)					
	K_2HPO_4 (g/L)					
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**	**1**
2	0.019±0.0015	0.012±0.002	0.016±0.002	0.022±0.0015	0.019±0.001	0.017±0.0015
4	0.021±0.0025	0.016±0.001	0.020±0.0015	0.031±0.0030	0.023±0.0026	0.025±0.002
7	0.025±0.0045	0.019±0.001	0.031±0.0026	0.049±0.0020	0.041±0.0025	0.034±0.0020
14	0.034±0.002	0.023±0.00208	0.036±0.0020	0.06±0.002	0.042±0.0040	0.041±0.0025
20	0.04±0.003	0.024±0.0015	0.039±0.002	0.061±0.002	0.049±0.003	0.036±0.0015

b)						
	MgSO4 (g/L)					
Days	**0.75***	**0**	**0.25**	**0.5**	**1**	**1.25**
2	0.019±0.0015	0.008±0.0025	0.018±0.001	0.0193±0.0015	0.0216±0.0020	0.024±0.003
4	0.021±0.0025	0.014±0.001	0.021±0.001	0.021±0.001	0.025±0.003	0.035±0.001
7	0.025±0.0045	0.015±0.0017	0.026±0.0036	0.038±0.0015	0.0436±0.0015	0.05±0.0040
14	0.034±0.002	0.012±0.0020	0.031±0.002	0.041±0.001	0.0473±0.0015	0.0626±0.0035
20	0.04±0.003	0.0113±0.00208	0.037±0.0030	0.047±0.002	0.0526±0.0020	0.068±0.0025

* = Normal condition

Table: 4.14 a) Total carotenoid content on modified BG-11 media with phosphate stress b) Sulphate stress

Total carotenoid (mg/mL)(Mean± SD)						
	K_2HPO_4 (g/L)					
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**	**1**
2	0.0018±0.0003	0.0023±0.00021	0.0019±0.00015	0.0012±0.00015	0.0011±0.00015	0.0015±0.0002
4	0.002±0.0002	0.0024±0.00015	0.0021±0.0001	0.0018±0.00025	0.0017±0.00023	0.0019±0.0003
7	0.0021±0.0001	0.003±0.00014	0.0027±0.00014	0.0019±0.00018	0.0019±0.00014	0.002±0.0004
14	0.0028±0.0002	0.0032±0.0005	0.0030±0.00018	0.0026±0.00017	0.0020±0.00015	0.0021±0.0004
20	0.0031±0.0001	0.0034±0.0006	0.0032±0.00017	0.0027±0.00017	0.0021±0.00018	0.0022±0.0005

b)

	MgSO4 (g/L)					
Days	**0.75***	**0**	**0.25**	**0.5**	**1**	**1.25**
2	0.0018±0.0003	0.0027±0.0001	0.0016±0.0003	0.0019±0.00015	0.0015±0.0002	0.0015±0.0036
4	0.002±0.0002	0.0029±0.0006	0.0018±0.00015	0.0021±0.00016	0.0017±0.00025	0.0017±0.00025
7	0.0021±0.0001	0.0031±0.0007	0.0019±0.00011	0.0025±0.0002	0.0018±0.00022	0.0019±0.00018
14	0.0028±0.0002	0.0033±0.0004	0.0025±0.0009	0.0029±0.0001	0.0025±0.00008	0.0024±0.00013
20	0.0031±0.0001	0.0035±0.0003	0.0030±0.0007	0.0032±0.0001	0.0029±0.00002	0.0026±0.00012

* = Normal condition

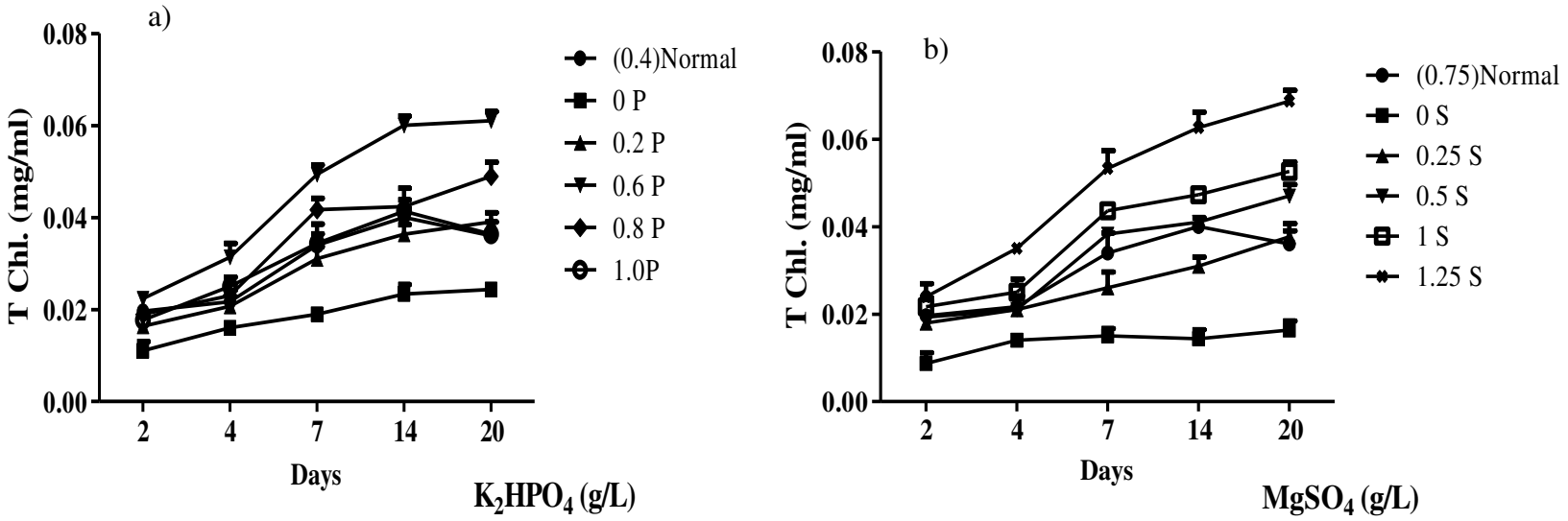

Figure: 4.16 Total chlorophyll content of *S. acutus* a) phosphate stress b) sulphate stress

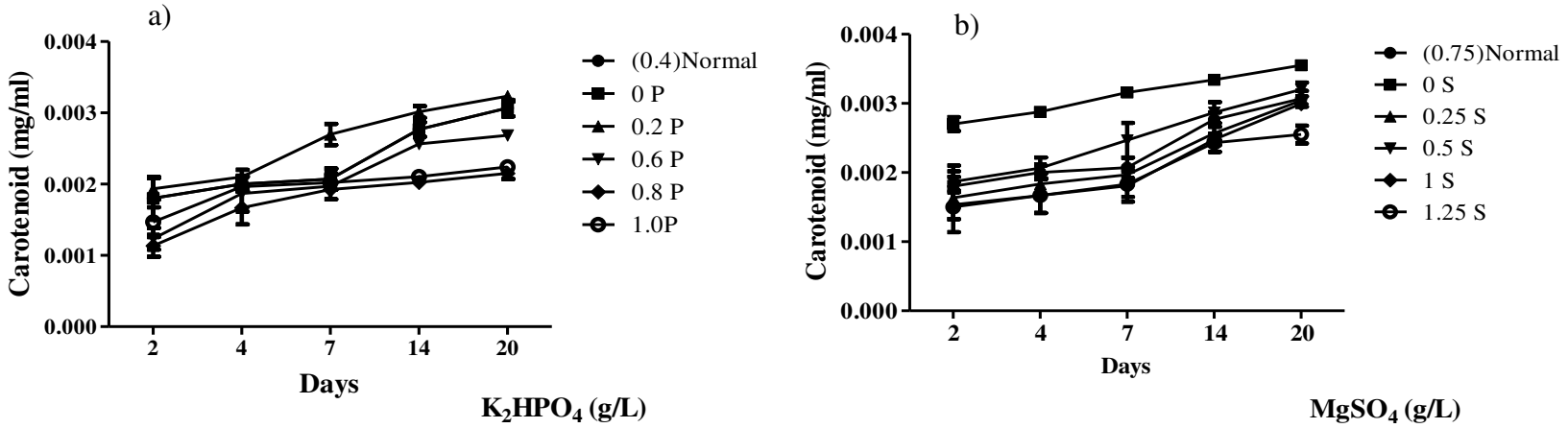

Figure: 4.17 Total carotenoid content of *S. acutus* a) phosphate stress b) sulphate stress

4.8 Extraction of extracellular polysaccharides from *S. acutus*.

4.8.1. Extracted sample (exopolysaccharides) from S. acutus.

The 20 days old culture of all stress conditions of *S. acutus* were taken for the extraction of exopolysaccharides by alcoholic precipitation (ethanol). The obtained liquid was lyophilised to get the powdered form of the sample and it was further assessed to detect the presence of sugars.

4.8.2. Measurement of Biomass accumulation of S. acutus cells

The dry weight biomass was recorded (after 20th day, when extraction was done) on modified BG-11 media with all phosphate (Table 4.15 a and Figure 4.18 a) and sulphate stress (Table 4.15 b Figure 4.18 b) to observe the increased or decreased biomass accumulation and its effect of the yield of EPS production by *S. acutus.* The maximum increased dry biomass was obtained for sulphate stress at concentration 1.25g/L with 2.42 g/L and the lowest biomass obtained was of sulphate at concentration 0 g/Lwith 0.66 g/L dry weight.

Table: 4.15 a) Dry weight biomass of *S. acutus* on modified BG-11 media with phosphate stress.

Dry weight(g/L)(Mean± SD)					
			K_2HPO_4 (g/L)		
Days	**0.4***	**0**	**0.2**	**0.6**	**0.8**
0.92±0.08	0.81±0.01	0.94±0.03	1.90±0.05	0.9±0.09	0.90±0.07

Table: 4.15 b) Dry weight biomass on modified BG-11 media with sulphate stress

			MgSO4 (g/L)		
Days	**0.75***	**0**	**0.25**	**0.5**	**1**
0.92±0.08	0.66±0.06	0.93±0.04	0.93±0.01	1.24.07	2.42±0.04

* = Normal condition

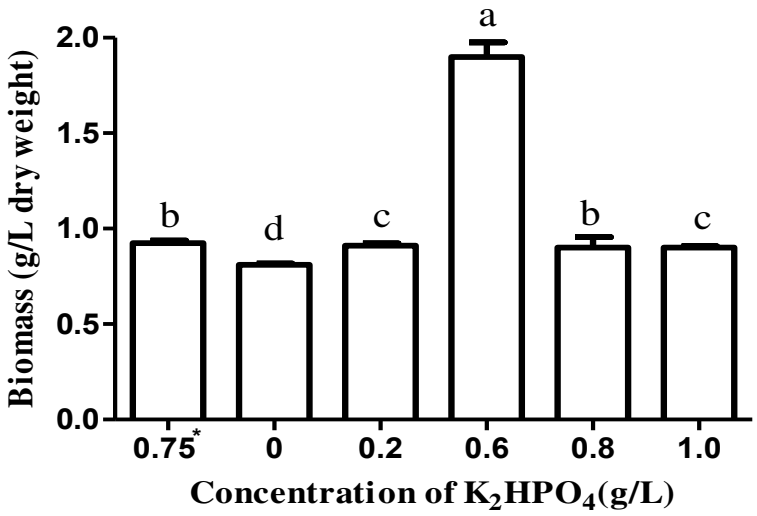

Figure: 4.18 a) Total dry weight biomass accumulation of *S. acutus* on modified BG-11 media with phosphate stress.

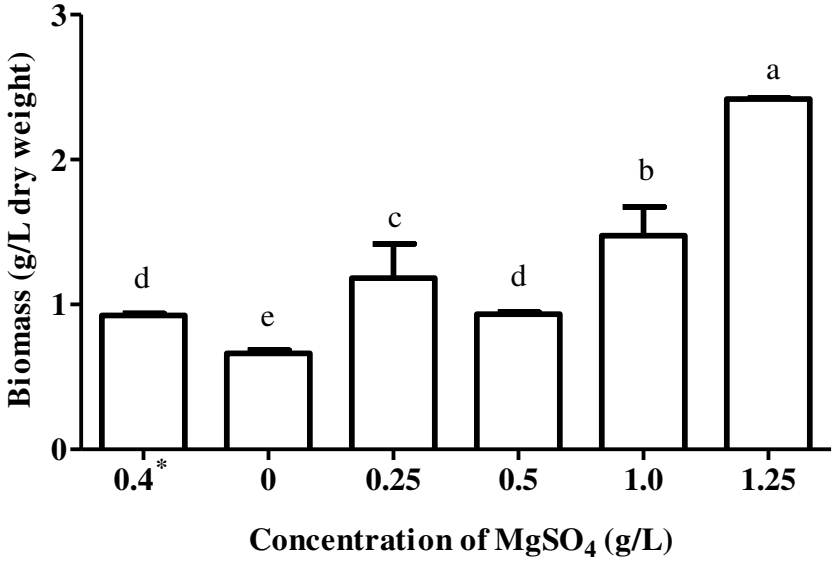

Figure: 4.18 b) Total dry weight biomass accumulation of *S. acutus* on modified BG-11 media with sulphate stress.

4.8.3Sugar content in extracted exopolysaccharides

The extracted sample was analyzed for the presence of sugar, by the phenol-sulphuric acid method where glucose was taken as standard and the amount of glucose equivalence in produced EPS were calculated using the obtained standard equation (Figure 4.19). It was observed that as

compared to normal condition, variation (either decrease or increase) is observed in different stress conditions (Table 4.16). However, highest sugar content was observed in sulphate stress (1.25g/L) and phosphate (0.6 g/L) (Figure 4.20 a and b)

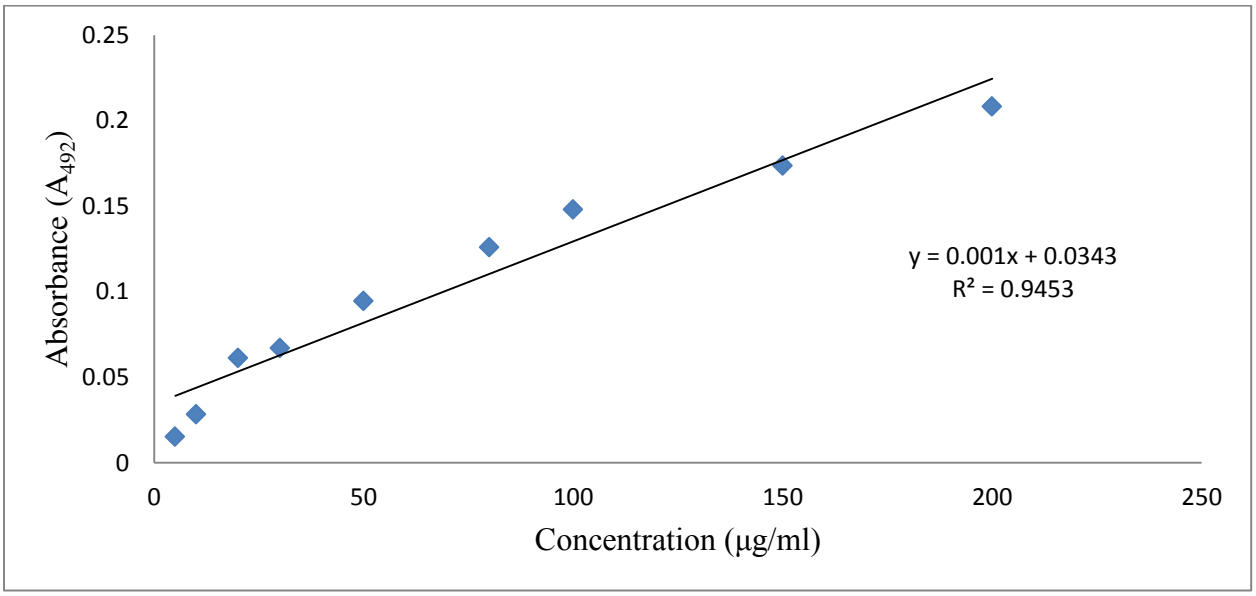

Figure: 4.19 Standard curve of glucose

Table: 4.16 Sugar content in the exopolysaccharides (EPS)

Stress conditions	**Glucose equivalence (µg) / EPS (250 µg)**	**Fold increase***
Normal*	12.68±1.57	
$MgSO_4$ (0g/L)	2.3±0.57	
$MgSO_4$ (0.25g/L)	3.1±1.2	
$MgSO_4$(0.5g/L)	27.15±1.23	2 %
$MgSO_4$ (1g/L)	44.3±2.42	3.5 %
$MgSO_4$ (1.25g/L)	136.2±2.39	11 %
K_2HPO_4 (0g/L)	8.36±0.55	
K_2HPO_4 (0.2g/L)	11.03±0.95	
K_2HPO_4 (0.6g/L)	129.76±1.86	10.5 %
K_2HPO_4 (0.8g/L)	28.24±3.19	2.3 %
K_2HPO_4 (1g/L)	12.36±1.58	

*Fold increase: Stress/Normal, (Results are expressed as mean value, ± the standard deviation)
Normal* = K_2HPO_4 (0.4g/L) and $MgSO_4$ (0.75g/L)

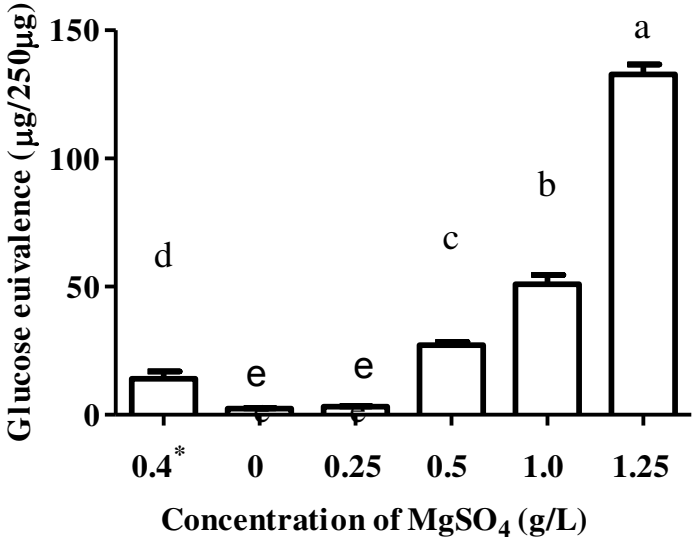

Figure: 4.20 a) Sugar content in EPS with sulphate stress

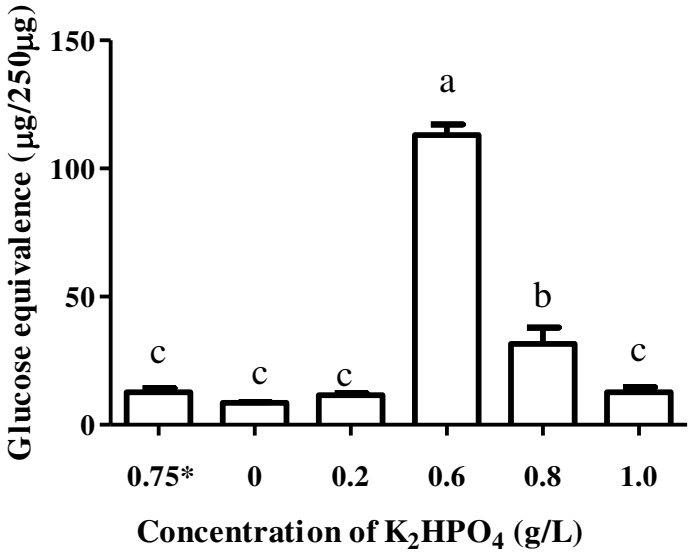

Figure: 4.20 b) Sugar content in EPS with phosphate stress

4.8.4 Anti-oxidant activity assessment of isolated EPS from stress conditions

Antioxidant activity of the obtained EPS from all the stress conditions of phosphate and sulphate was found to be more than the obtained EPS of normal conditions at concentration 1000 µg/mL (Table 4.17), which implied that the anti-oxidant activity increased as the concentration increased within the particular stress range.

However, the maximum enhanced activity of EPS was shown by the culture growing in sulphate stress (1.25, 1 g/L) and phosphate stress (0.6, 0.8 g/L) as the concentration increased (Figures 4.21 a and b)

Table 4.17: Free radical scavenging activity of the EPS isolated from different stress and normal conditions

Antioxidant activity (%)(Mean± SD)	
AA*	84.4 ± 2.5
Stress condition	**1000 µg/mL**
Normal*	31.68±2.2
K_2HPO_4 (0g/L)	21.17±1.71
K_2HPO_4 (0.2g/L)	28.18±1.60
K_2HPO_4 (0.6g/L)	43.14±1.91
K_2HPO_4 (0.8g/L)	34.88±1.38
K_2HPO_4 (1g/L)	30.89±1.86
$MgSO_4$ (0g/L)	21.17±1.71
$MgSO_4$ (0.25g/L)	16.88±2.60
$MgSO_4$(0.5g/L)	43.14±1.91
$MgSO_4$ (1g/L)	34.88±1.38
$MgSO_4$ (1.25g/L)	30.89±1.86

AA* = Ascorbic Acid
Normal* = K_2HPO_4 (0.4g/L) and $MgSO_4$ (0.75g/L)

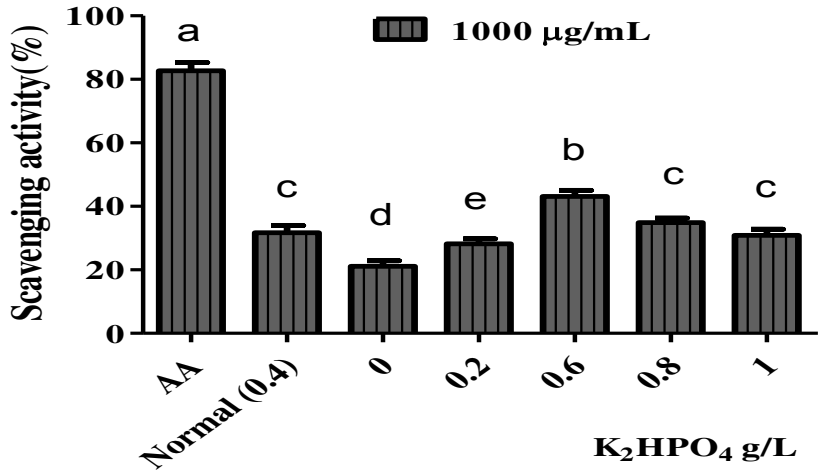

Figure: 4.21 a) Free radical scavenging activity in phosphate stress. Bars with the same lowercase letters are not significantly different at p>0.05. .*AA = Ascorbic acid

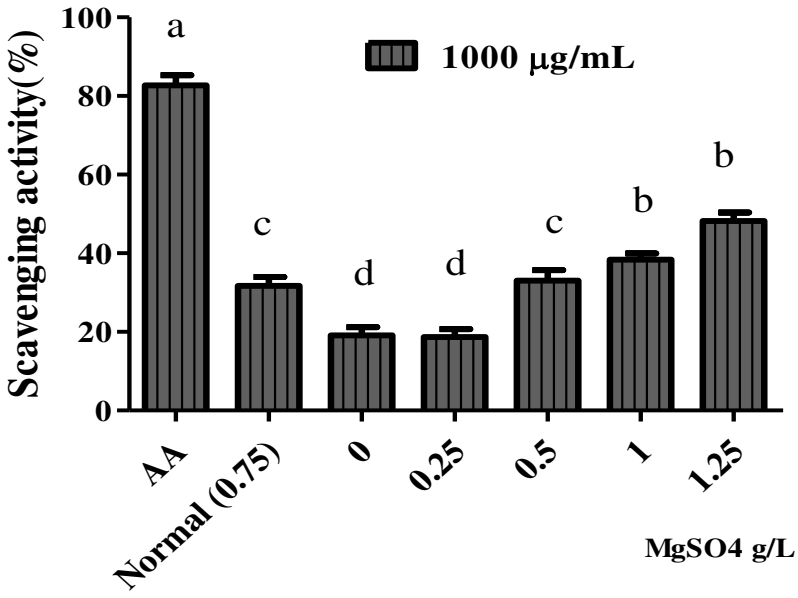

Figure: 4.21 b) Free radical scavenging activity (Antioxidant activity) of sulphate stress EPS. Bars with the same letters are not significantly different at $p>0.05$.*AA = Ascorbic acid

4.8.5 Effect of isolated EPS from stress condition on peripheral blood mononuclear cells

After the results of anti-oxidant activity, the obtained EPS from different stress conditions were assessed for their activity on PBMC (Table 4.18). It was found that the activity was increasing by the increased concentration, and the maximum activity was observed at concentration 750 and 1000 µg/mL in normal condition EPS, all the stress conditioned EPS was assessed for activity at 100, 250, 750 and 1000 µg/mL. Out of these, the maximum index was found for sulphate stress (1.25g/L) and phosphate stress (0.6g/L) (Figures 4.22 a and b).

Table: 4.18 PBMC activity, comparative study with all stress conditions.

PBMC activity(Mean± SD)				
Con A*		1.38±0.02		
Stress condition	**100 µg/mL**	**250 µg/mL**	**750 µg/mL**	**1000 µg/mL**
Normal*	1.53±0.03	1.55±0.03	1.9±0.05	1.91±0.007
K_2HPO_4 (0g/L)	1.33±0.03	1.37±0.03	1.47±0.04	1.56±0.009
K_2HPO_4 (0.2g/L)	1.42±0.02	1.45±0.01	1.56±0.03	1.58±0.06
K_2HPO_4 (0.6g/L)	1.67±0.03	1.83±0.03	2.37±0.06	2.37±0.04
K_2HPO_4 (0.8g/L)	1.55±0.05	1.94±0.04	2.1±0.08	2.07±0.11
K_2HPO_4 (1g/L)	1.48±0.02	1.75±0.05	1.79±0.09	1.75±0.05
MgSO4 (0g/L)	1.27±0.04	1.32±0.02	1.47±0.03	1.47±0.03
MgSO4 (0.25g/L)	1.31±0.02	1.35±0.01	1.53±0.1	1.49±0.08
MgSO4 (0.5g/L)	1.57±0.03	1.61±0.02	1.96±0.02	1.88±0.006
MgSO4 (1g/L)	1.72±0.05	1.87±0.01	2.31±0.17	2.2±0.02
MgSO4 (1.25g/L)	1.83±0.03	1.95±0.03	2.52±0.03	2.5±0.09

Con A : Concanavalin A (10 µg/mL)

Normal* = K_2HPO_4 (0.4g/L) and $MgSO_4$ (0.75g/L)

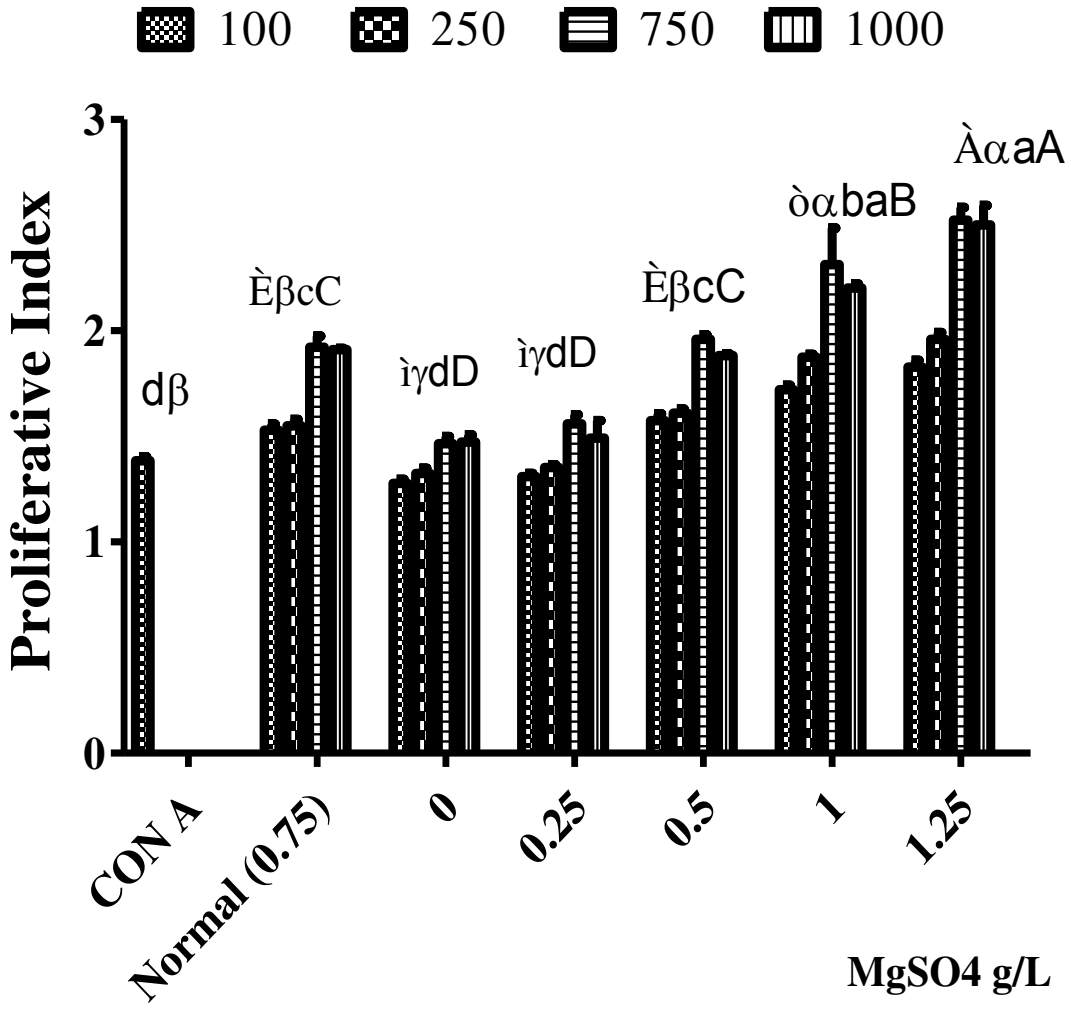

Figure: 4.22 a) Cell growth effect on peripheral blood mononuclear cells by EPS obtained from sulphate stress. Bars with the same lowercase and uppercase letters are not significantly different at p>0.05.ConA: Concanavalin A (10 µg/mL) was used as a positive control. Proliferation index is the ratio of absorbance of the Con A/EPS treated cell and the untreated cells (Cells only).

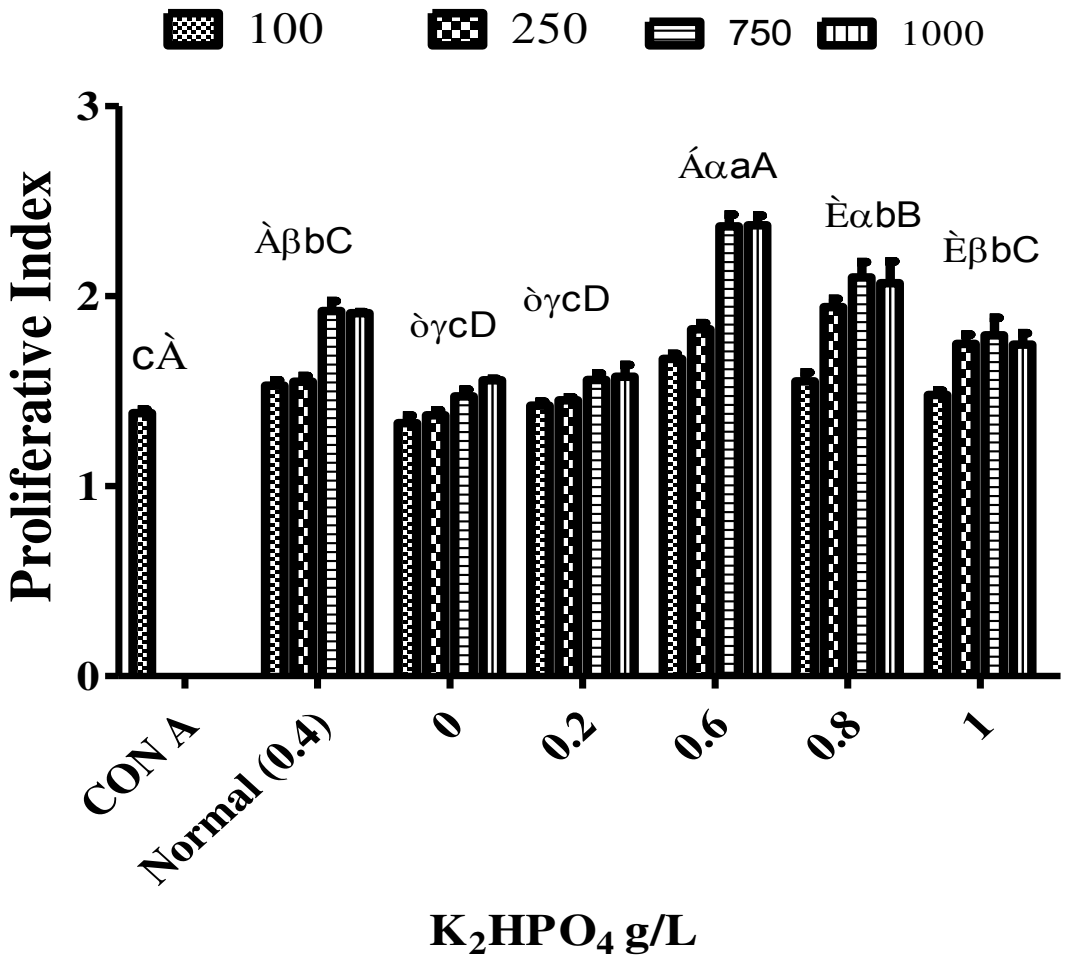

Figure: 4.22 b) Cell growth effect on peripheral blood mononuclear cells by EPS obtained from phosphate stress. Bars with the same lowercase and uppercase letters are not significantly different at $p>0.05$. Proliferation index is the ratio of absorbance of the Con A/EPS treated cell and the untreated cells (Cells only).

Objective 3

Objective 3: Characterization of the exopolysaccharide by Fourier Transform Infrared Spectroscopy, Nuclear Magnetic Resonance spectroscopy and Liquid Chromatography-Mass spectrometry.

4.9 Characterization of isolated EPS

The final objective of the study was to characterize the exopolysaccharides isolated from *S. acutus*; hence in this section the results of various methods (FTIR, NMR and LC-MS) have been explained.

4.9.1. FTIR spectroscopy

The FTIR Spectra of the isolated EPS was performed to detect the presence of functional groups in the sample, which displayed the intense characteristic absorption of polysaccharides between 1650 and 1050 cm^{-1} and O-H stretching band at 3339 cm-1. FTIR spectra also showed the presence of carbonyl and hydroxyl (C=O, C-OH) groups at various wavelength confirming the presence of polysaccharides (Figure 4.23).

The FTIR spectrum of EPS revealed some more characteristic functional groups, such as stretching C-H at 3339, 2900 and 2937 cm^{-1} and a carboxylic acid bending peak at 1422 cm^{-1}. Further, C-O stretching peak was noticed at 1261 cm^{-1} which corresponds to alkyl aryl ether group. Halo stretching and sulfone groups was also noticed at 630 and 1341 cm^{-1}, showed the presence of boron and sulphate group along with the polysaccharides (Table 4.19).

The final analysis of the spectra showed the wavelengths corresponding to the halo groups, carboxylic acid, alkenes, sulfone group and ether, which revealed that the polysaccharides were present with low amounts of sulfate, ether and lipid groups.

Table 4.19: FTIR analysis of EPS

Wavelength (cm^{-1})	**Range**	**Functional Groups**	**Absorption intensity**
630	690-515	C-Br (Halo compound stretching)	Strong
706	730-665	C-H/ C=C (alkene, bending)	Strong
778	770±20	C-H (bending)	Strong
818	820±20	C-H (bending)	Strong
875	880±20	C-H (bending)	Strong
901	920-900	C=C (alkene, bending)	Strong
917	920-900	C=C (alkene, bending)	Strong
1056	1070-1030	C-O (Primary alcohol)	Strong
1144	1150-1085	C-O (aliphatic ether, streching)	Strong
1203	1210-1163	C-O (ether, streching)	Strong
1261	1275-1200	C-O (alkyl aryl ether, streching)	Strong
1341	1350-1300	S=O (sulfone, streching) O-H (alcohol)	Strong, medium
1422	1440-1395	O-H (carboxylic acid, bending)	Medium
1647	1648-1638	C=O (α,β-unsaturated ketone)	Medium
2149	2149-2150	C=C=O (ketene)	Medium
2900	3000-2840	C-H (alkane, streching)	Medium
2937	3000-2840	C-H (alkane, streching)	Medium
3339	3550-3200	O-H (alcohol,streching)	Strong

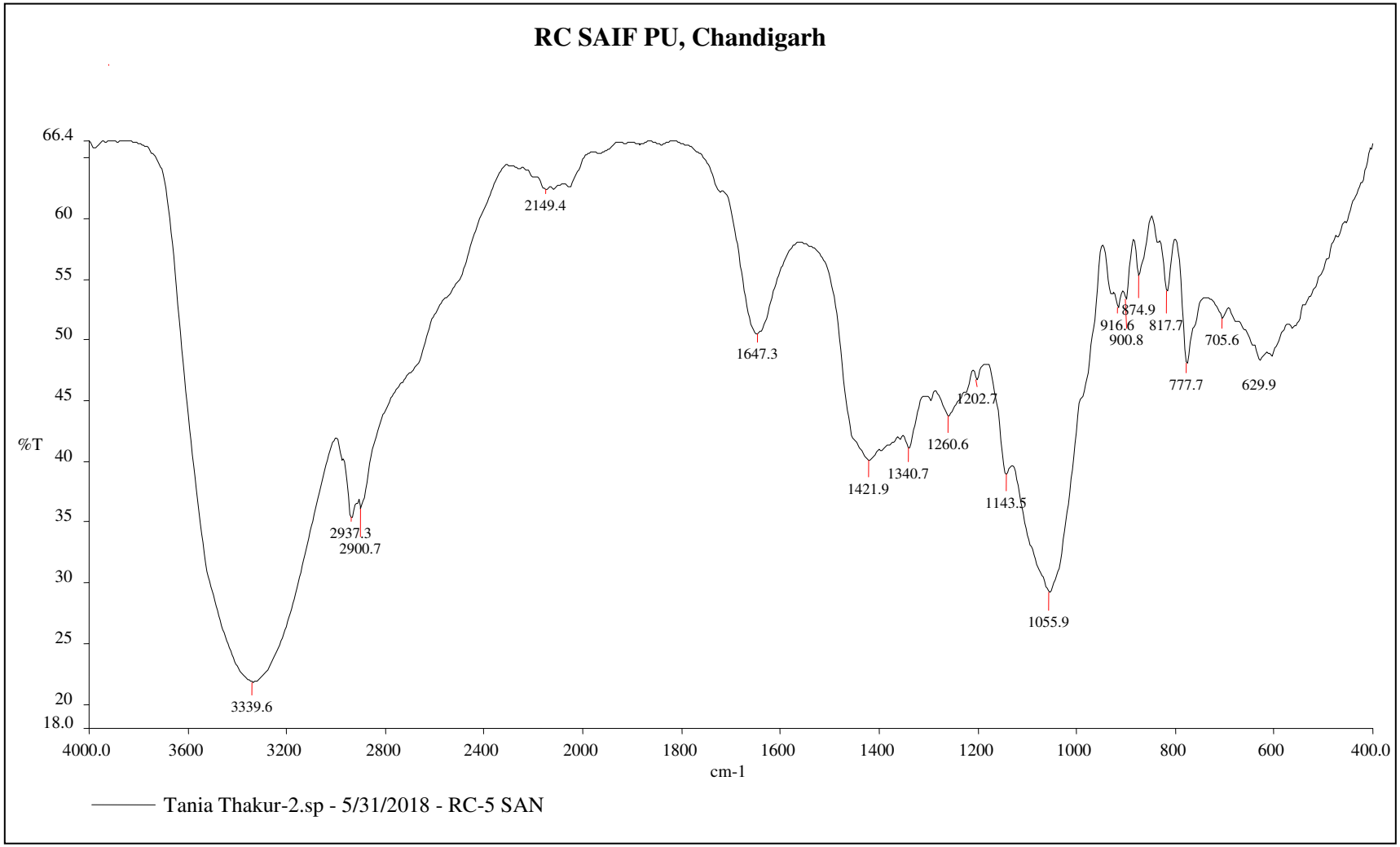

Figure4.23: FTIR Spectrum of EPS

4.9.2. ^{1}H NMR spectroscopy

The NMR spectroscopy was performed to determine the structure (protons) of the compound. The ^{1}H NMR spectra of obtained EPS revealed the chemical shifts (ppm) to their corresponding functional groups. In this analysis, 20 types of protons corresponding to different ppm peaks were identified (Table 4.20). The chemical shift obtained between 4.0 – 5.6 ppm atrributed towards the presence of β-anomeric (4.0-4.9) and α-anomeric carbon of pentose and hexose sugars respectively. This spectrum lead to the confirmation of presence of pentose (xylose/ribose/arabinose) and hexose sugars (glucose/galactose/mannose) in the sample. The ppm spectrum from 2.7-3.2 showed the presence of alkyl halide and acetyl amine groups, CH(NH). Ppm peaks from 3.2-3.4 showed the presence of CH_3O group, which revealed the presence of hydroxyl group, again leading for the confirmation of sugars (sachharide) along with few aliphatic and acetyl groups (Figure 4.24).

Table 4.20 : NMR analysis

Chemical shifts (ppm)	**Types of protons**	**Probable group**
2.7-3.2	2	alkyl halide and acetyl amine
3.2-3.4	3	CH_3O
3.5-3.65	3	CH_3O, disaccharides
3.65-3.9	2	H-CN (amine groups)
4.0-4.45	3	β-anomeric carbon pentose/hexose sugars
4.45-4.65	2	β-anomeric carbon pentose/hexose sugars
4.65-5.0	3	β-anomeric carbon pentose/hexose sugars
5.0-5.6	1	α-anomeric carbon pentose/hexose sugars
5.8-7.0	1	$H\text{-}CO_2$

Disachharides (lactose/maltose/sucrose)

Hexose sugars (glucose/fructose/galactose/mannose)

Pentose sugars (xylose/ribose/arabinose)

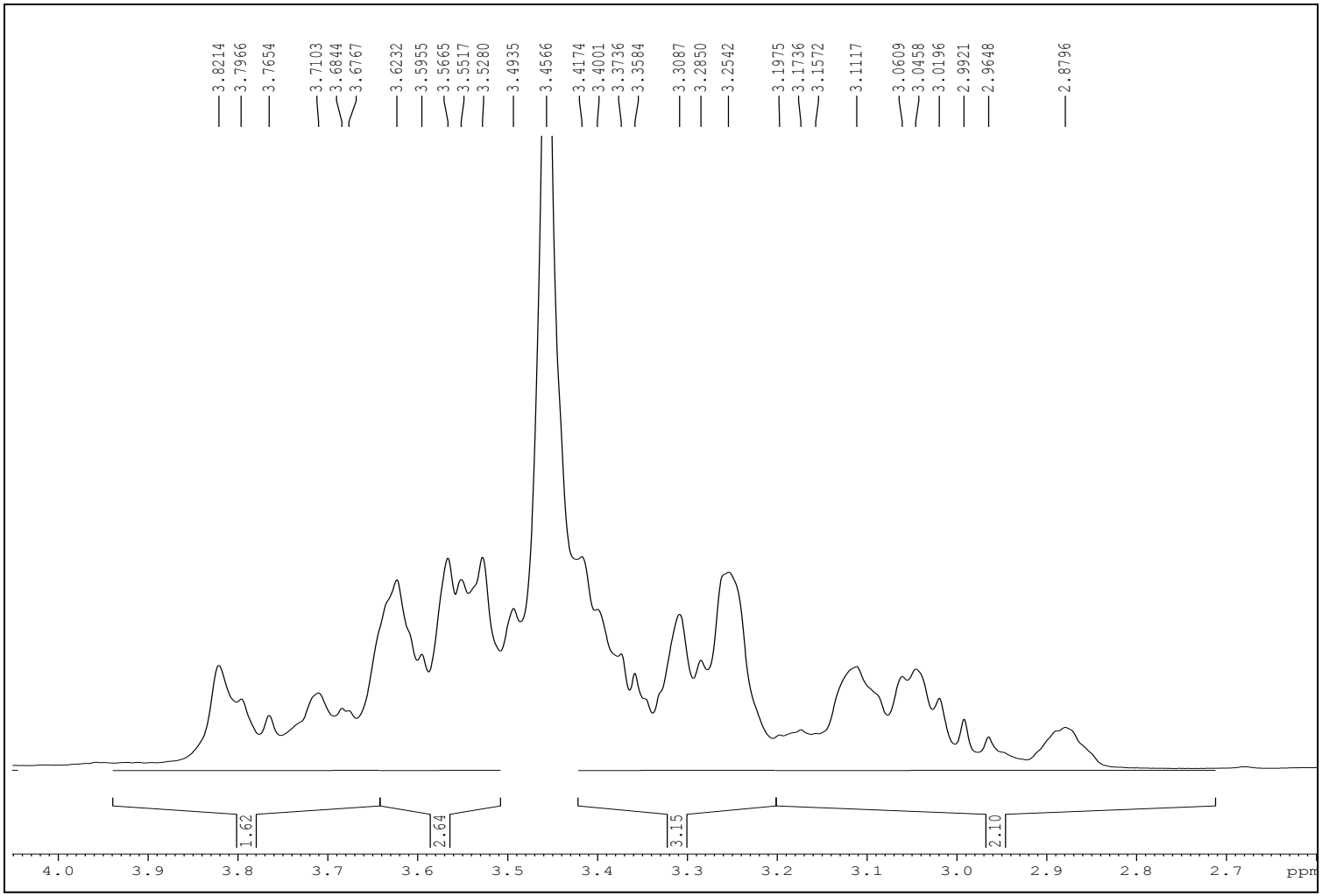

Figure 4.24 : NMR analysis of EPS

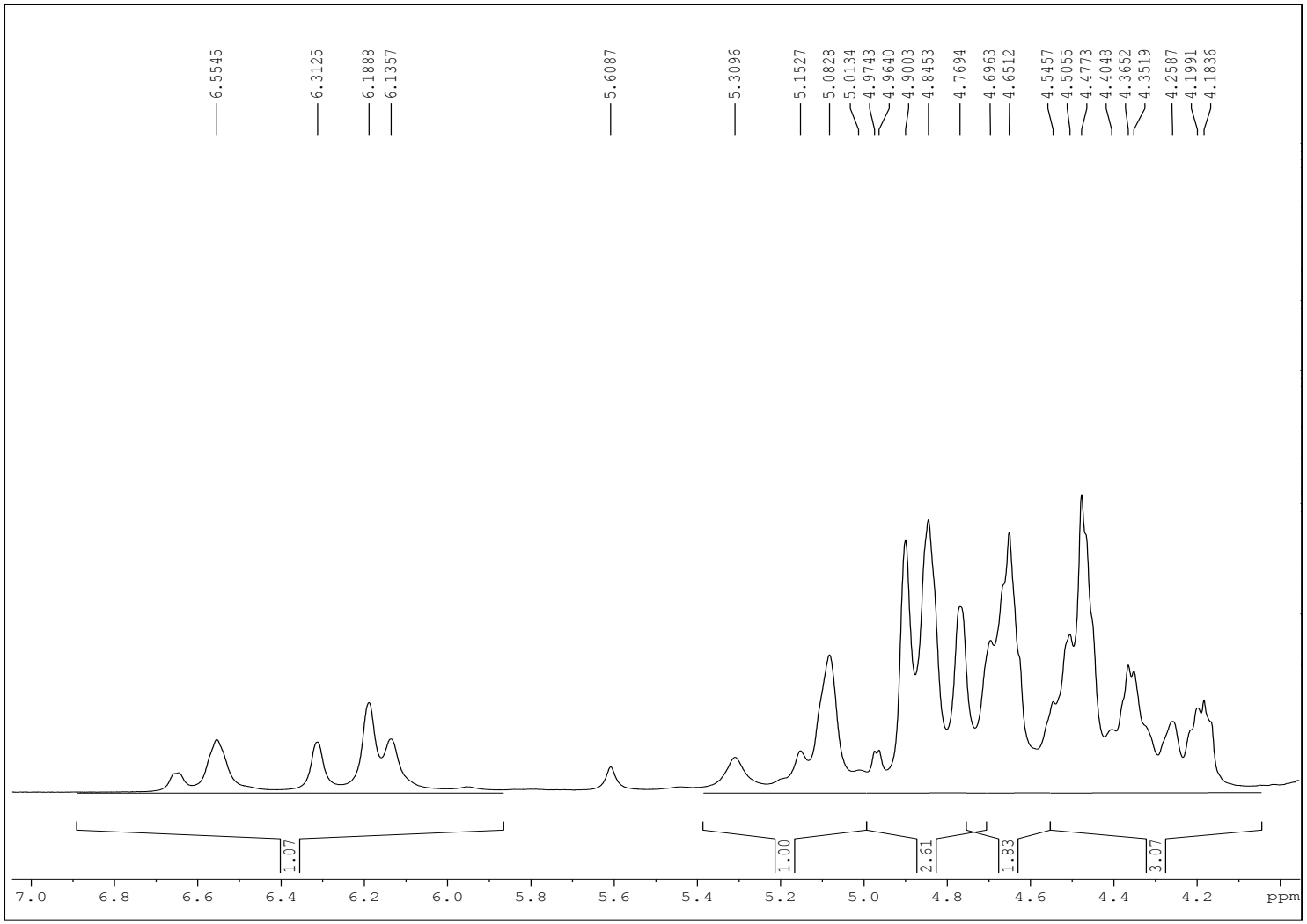

Figure 4.24: NMR analysis of EPS

4.9.3 LC-MS spectrometry

As, obtained EPS has shown remarkable immuno-stimulatory activity on PBMC cells. Thus, it was subjected to liquid chromatography-mass spectrometry (LC-MS) for the analysis of the compounds present in the EPS. The masses of different polysaccharides were analysed from the spectrum obtained of the EPS. The higher are the molecular weight of the polysachharides, the higher m/z ratio was obtained.

After the confirmation and probability of different sugars present in the sampleby FTIR and NMR, the analysis by LCMS revealed that the compounds that could be present in the sample included hexose (glucose/fructose/galactose/mannose) sugars at m/z ratio of 181, 163 and 183 (Table 4.21). The probability of pyranoside and hexose sugar containing phenolic group was found at m/z ratio of 301.16, at which glucogallin (gallic acid and β-D-glucose) andrahmnoseare the most probable compounds to be present. Other than the probability of various disaccahrides (Maltose/sucrose/ lactose), tetrasachharides and octasaccharides are there at m/z ratio of 347 and 358, 684 and 1356.28 (Figure 4.25).

Thus, the study revealed that exopolysaccharides contained the mixture of many sachharide units that together contribute to their bioactive property.

Table 4.21: Mass spectra analysis of EPS

m/z ratio	**Intensity**	**Probable compound**
181.07	327	Hexose sugar (Glu/Fruc/Galac/Mann)
163.05	1147	Anhydrous glucose
183	231.08	Hexose sugar associated with beryllium
347	496.47	Disaccharide (Maltose/sucrose/ lactose) associated with boron
385	469.45	Disaccharide (Maltose/sucrose/ lactose)
301.16	2765	Phenolic hexose/pentose sugars (Glucogallin), O-α-L-rhamnopyranoside
467	2456	O-galloyl-hexoside
684	9243	Tetrasaccharides
1356.28	864	Octasachharides associated with magnesium

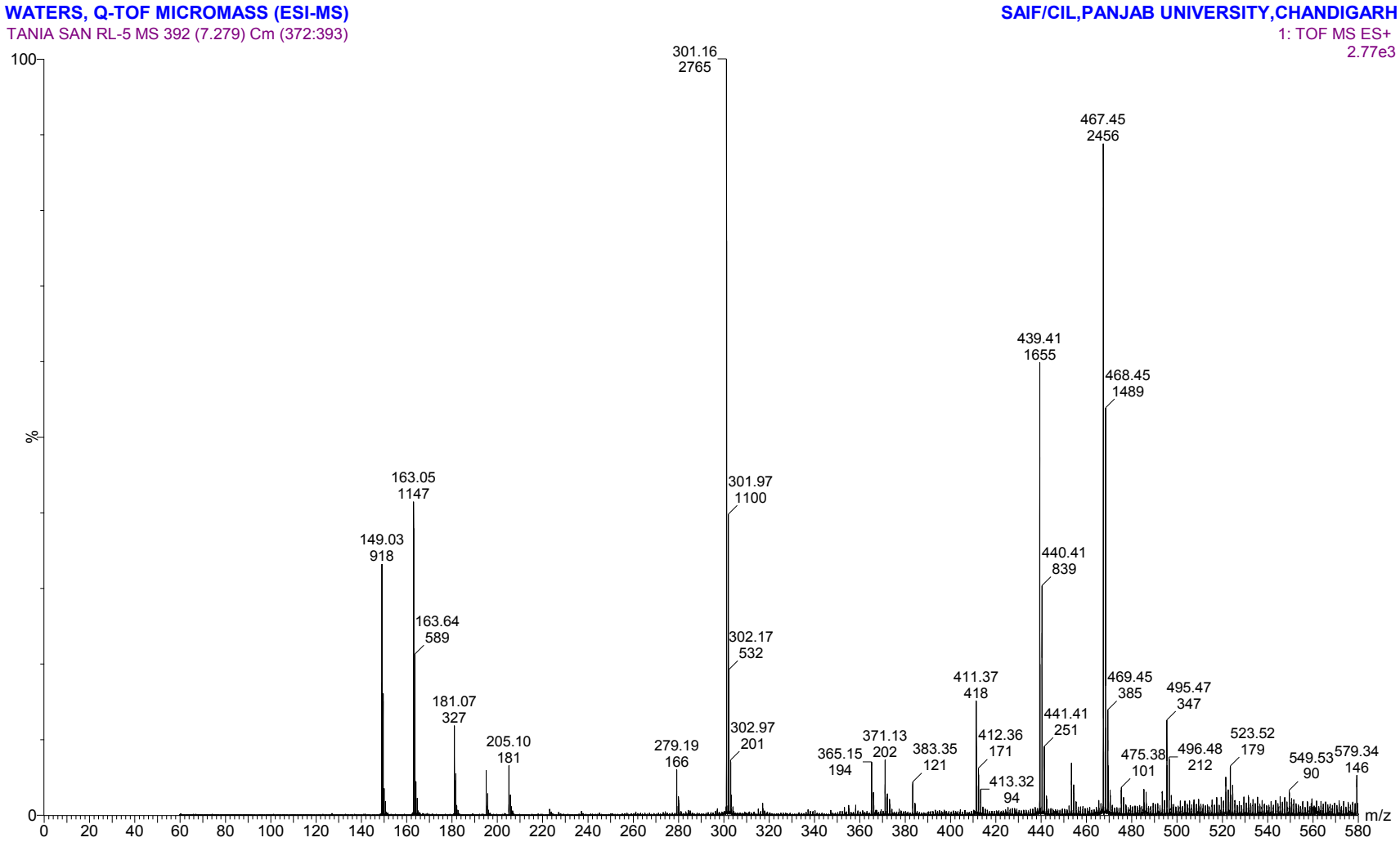

Figure 4.25: LC-MS spectrometry results

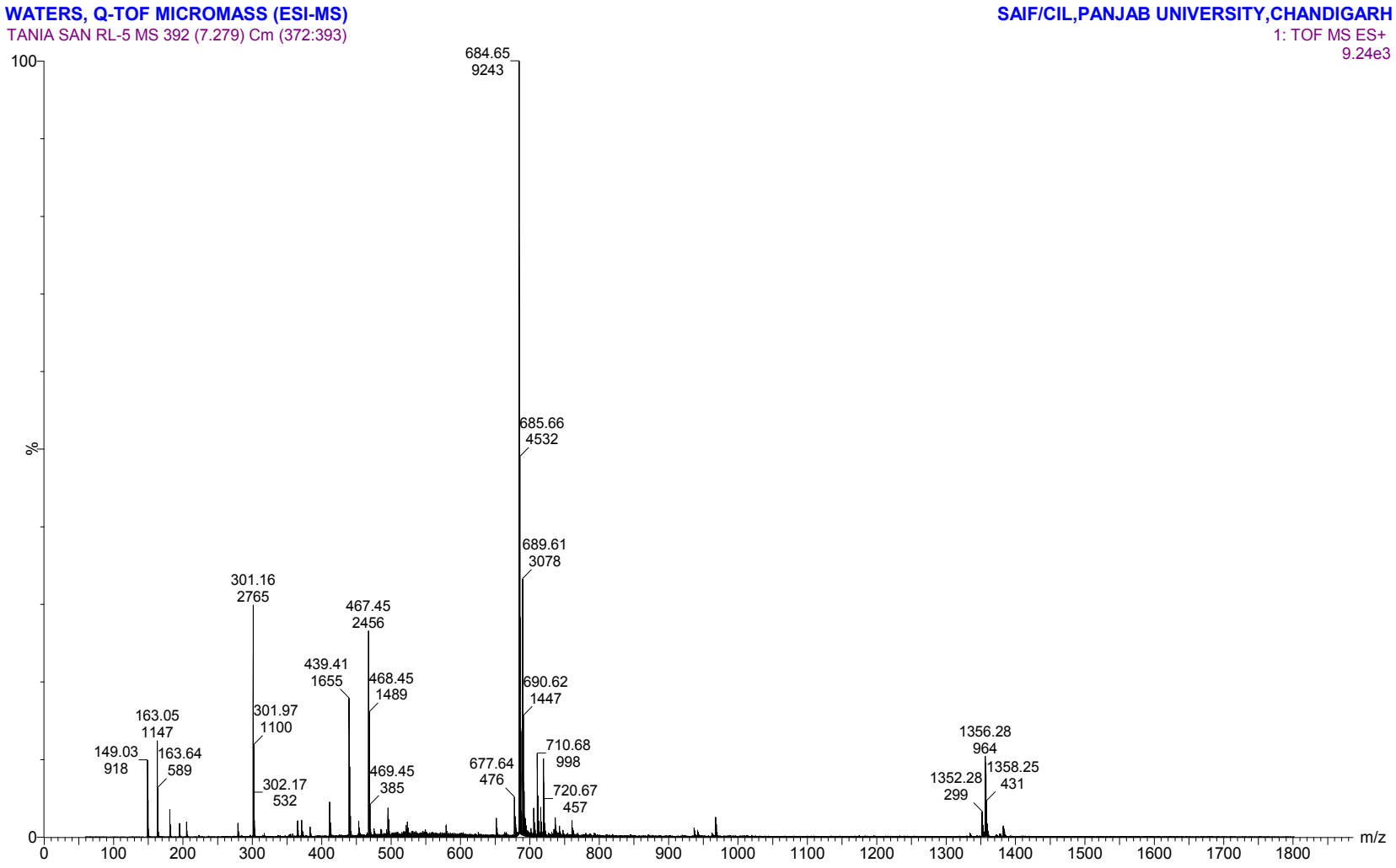

Figure 4.25: LC-MS spectrometry results

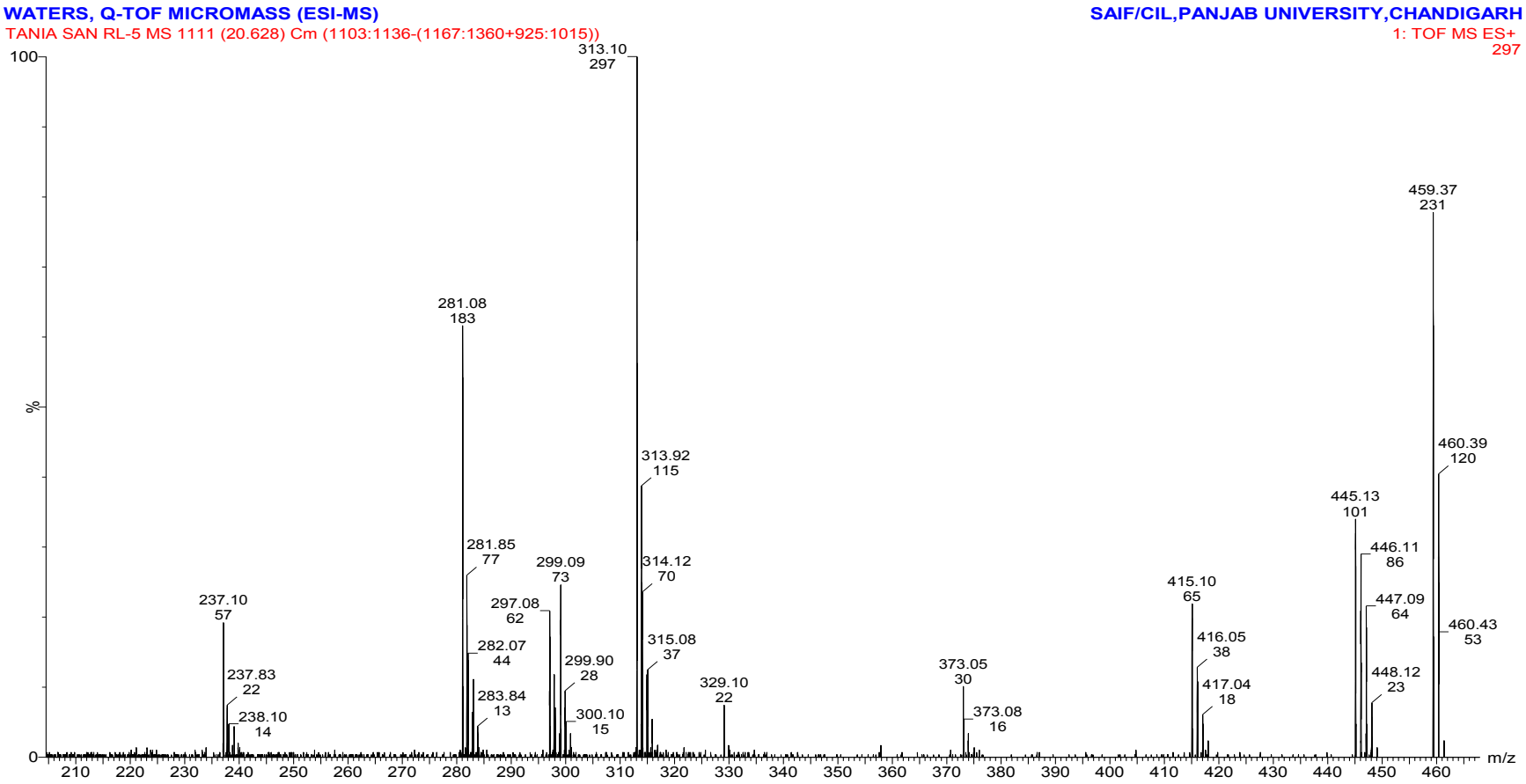

Figure 4.25: LC-MS spectrometry results

Thus, the characterisation from LC-MS revealed the major mixture of various pentose sugars in the EPS, along with the little mixture of hexose sugars.

Chapter V

Discussions

5. Discussion

Freshwater microalgae inhabitats the ecosystem ranging from lakes to wetlands and rivers to water streams (Guschina & Harwood 2006). They can live in a wide range of environment from saline to freshwater. From the past few years, they have been in study to secrete the extra polymeric substances which includes exopolysaccharides, proteins and lipids. Out of these extra polymeric substances, exopolysaccharides have gained recent attention by many scientists and industries for their uses in different sectors of biotechnology. Several microalgae (*Porphyra, Ascophyllum, Palmeria, Dunaliella, Chlorella, Scenedesmus*) have been explored to produce these exopolysaccharides that have anti-oxidant, anti-cancerous, anti-glycemic, anti-viral, anti-bacterial, anti-lipidimic and immunomodulatory activities. *S. acutus* is recently reported to produce these exopolysaccharides but not much reported to show the bioactive properties (immuno-modulation and antioxidant assessment) (Ishaq *et al.* 2016).

In the current study, the isolated EPS was explored which was found to exhibit immunostimulatory activity against PBMC and also possess anti-oxidant effect. Present studies also revealed that providing phosphate and sulphate nutritional stress conditions enhances or decreases the growth of *S. acutus* and effects the yield production of EPS. It was found that the production of EPS was enhanced in some phosphate and sulphate stress condition as compared to normal growth conditions. Liquid chromatography mass spectra analysis confirmed the presence and characterization of exopolysaccharides composed of various pentose sugars with little mixture of hexose sugars. FTIR results supported the results for the presence of various functional groups associated with these exopolysaccharides. NMR spectra gave an idea about the type of protons present in the exopolysaccharides along with the ppm peaks that revealed the chemical shifts associated with the probable sugars (hexose, pentose) and few groups (amine, alkyl halide) associated to them.

Immunomodulators are the bio-compounds which are known for their activity as stimulator or suppressor of immune system that have been used for several therapeutic purposes. The EPS isolated from *Chlorella stigmatophora* and *Porphyridiumt ricornutum* have shown good immunosuppression and immunostimulationactivity against PBMC respectively (Guzman *et al.* 2003). Sulfated polysaccharides from *Porphyridium* also had been explored to exhibit antioxidant (free radical scavenging) activity against auto-oxidation of linoleic acid (Tannin-

Spitz *et al.* 2005). The sulfated exopolysaccharide from *Rhodella reticulate* also has antioxidant activity (Sun *et al.* 2014) . In agreement with these reports, the isolated exopolysaccharides from *S. acutus* have shown the anti oxidant activity but has shown remarkable increase in cell proliferation activity for peripheral blood mononuclear cells (PBMC). The proliferative index was interestingly more than one at all the concentration of EPS which strongly indicated the immunostimulation potential. A study on other microalgae exopolysacchrides have revealed that the cell proliferation occurred by the increased level and secretion of cytokines (TGF-β, IFN-γ, and TNF-α) that regulate the immune system (Shin *et al.* 2007).This concentration dependent increased production of cytokines exhibits the increased cell proliferation in PBMC which lead to the activity of immunostimulation.

Several strategies have been employed till now on *Scenedesmus* sp. for the enhanced production of exopolysaccharides which includes providing nutritional stress, pH stress, light and temperature stress and a method using response surface methodology, where combination of different stresses are put at a time as input and their statistically significant production value for the experiment is checked. The type of stress condition to be provided depends upon the natural habitat and type of microalgae species. In this study, only nutritional stresses (phosphate and sulphate) have taken into account for the work.

Along with the enhanced production of EPS from *S. acutus* at some stress conditions, one very interesting observation was found that with each provided nutritional stress the amount of cell pigments (chlorophyll and carotenoids) varied in a wide range, as for sulphate stress (0g/L) and phosphate stress (0g/L) the chlorophyll content was too low, so the sulphate stress cultures appeared reddish-brown and phosphate stress cultures appeared green with small spots of visible red pigments in color (which are the color of carotenoid pigment) after 20 days of inoculation, which implied that these stress concentration could cause as much stress in the species that it yielded higher amount of carotenoids as compared to normal conditions which could be alpha-carotene, beta-carotene and astaxanthin (keto-carotene). Carotenoids have their own large global market for their remarkable property of anti-oxidant, anti-cancerous, medicinal (heart, eye, skin, male fertility) and as a food dietary supplement (Pangestuti & Kim 2011). Till now, microalgae species, *Haematococcus pluvialis, Dunaliella salina, Scenedesmus obliquus, Chlorella vulgaris, Coelastrella striolata, C. multistriata, Scenedesmus almeriensis* has been identified as the best source of Carotenoids.

Few carbohydrates and lipids have also been found in the extracellular polymeric secretions of the microalgae (*Scenedesmus, Tetradesmus*) which have been mainly used in the biofuel production industries. In our study, in one of the stress condition sulphate (0.25g/L) a good amount of lipids was also observed, which on getting wet in water gets solidified as a solid waxy type of material. The weight of yield was approximately

FTIR spectra reveals about the functional groups present in the compound. These spectra are interpreted by studying the correlation of the absorption spectra of unknown compound to the absorption spectra of known compounds. Previous studies on the characterization of EPS from *Scenedesmus* using FTIR and MS has revealed the presence of sugars at m/z ratio, D-ribose (87.0772), xylose (73.0842), D-galactose (101.0081), fructose (147.1506) and D-galacturonic acid (103.1000) with wavenumber stretches at 3445 (OH), 1645 (C=O), 2931 (OH), 1370 (S=O) groups (Angelaalincy *et al.* 2017). These results correspond with these study results confirming the presence of sugars in the exopolysaccharide yield.

S. acutus, being freshwater microalgae showed a wide range of different productions in different stress conditions. It has also been studied for the production of metabolite (β –carotene, astaxanthin, leutin) whose yield can be increased when the algae is put under stress condition. Likewise the EPS production is also studied to be influenced by the growth of algal cells in stress conditions. Thus, this study regarding EPS production and its bioactive property not only revealed about the conditions for enhance production of EPS but also to the change of growth pattern, pigment accumulation and lipids and carbohydrates yield also. Thus, *S. acutus* could be recognized as a potential source of various products in different sector of biotechnology.

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Chapter VI

Conclusions

6. Conclusion

Metabolites from *Scenedesmus* sp. have shown promising bioactive potential which provide an opportunity for their use in various applications. The wide range of application includes their use in cosmetics, pharmaceuticals, human nutrition, biofuel production and aquaculture. In the present study, the isolated EPS have shown immunostimulatory effect by enhancing the cell growth of PBMC. Anti-oxidant activity of these EPS has also been shown good results. Further, the characterization of the EPS by liquid chromatography-mass spectrometry revealed the presence of hexoses and pentoses in variable amounts. The FTIR data confirmed the presence of halo and sulphate groups associated with these EPS.^{1}H NMR spectroscopy revealed the type of protons and presence of hexose and pentose sugars associated with halide and amine groups with their particular chemical shifts. The study also reported the enhanced production of EPS by growing the culture in different nutritional stress conditions. Along with the EPS enhanced production, one interesting observation concluded from different stress condition of *S. acutus* culture was the production of various other pigments (carotenoids), lipids and carbohydrates which could have more other promising uses in different industries. Hence, *S. acutus* may be exploited commercially for different applications.

Chapter VII

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7. References

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