

Production, Extraction and Characterization of Astaxanthin from Microalgae

**BY
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COLLEGE OF BASIC SCIENCES & HUMANITIES
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2022

CERTIFICATE -I

This is to certify that this thesis entitled, “**Production, extraction and characterization of astaxanthin from microalgae**” submitted for the degree of **Doctor of Philosophy** in the subject of **Microbiology** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Mr. Satish Kumar**, Admn. No. **2017BS10D** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that this thesis entitled, **“Production, extraction and characterization of astaxanthin from microalgae”** submitted by **Mr. Satish Kumar**, Admn. No. **2017BS10D** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in the subject of **Microbiology**, has been approved by the Student’s Advisory Committee after an oral examination on the same.

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Microalgae are aquatic, microscopic photosynthetic organisms. Microalgae are diverse and exhibit different cellular forms like unicellular, multicellular, siphonaceous, or filamentous that can be categorized as prokaryotic and eukaryotic. Microalgae evolved and diversified from aquatic organisms around 3.5 billion years ago (Andersen, 2013). The group constitutes hundreds and thousands of species found in extreme environments, including acidic mine water, deserts, and snow (Seckbach, 1995, Leya *et al.*, 2009). Microalgae being at the bottom line of food chains, share an excellent part in total oxygen production. Microalgae are photosynthetic organisms that bring out carbon dioxide conversion into lipids, proteins, amino acids, carbohydrates, vitamins, and pigments (Novoveská *et al.*, 2019). Microalgae is well studied for its properties, including secretion of anti-cancerous, anti-inflammatory, and antifungal compounds, antioxidants, minerals, vitamins, and many more, making it a commercially important group (Borowitzka, 2013). Microalgae found applications in pharmaceuticals, nutraceuticals, cosmetics, biofertilizers, bioremediation, feed, biofuel, and proteomics. Only 40 microalgal strains are in commercial use at present. Around 44,000 species are taxonomically described, which constitute a small fraction, and a much smaller fraction is preserved in culture collection for research. A more significant fraction is still undiscovered, accounting for a considerable potential for economic exploitation (Day *et al.*, 2012; Guiry, 2012). Microalgae have driven the global market of pharmaceuticals and nutraceuticals and is expected to attain USD 3318.1 million by 2022 (Meticulous Research, 2018). Also, algal demand is elevated due to diverse applications in aquaculture and biofuel production.

Microalgae are high lipid-containing organisms and accumulate lipid and specific secondary metabolites under stress conditions like nutrient deficiency, acidic environment, temperature fluctuations, and light stress. Some antioxidants or polyunsaturated fatty acids like omega-3 fatty acids, phycobiliproteins, docosahexaenoic, eicosapentaenoic, and carotenoids can be obtained from microalgae and used for increasing immunity in humans and animals (Natrah *et al.*, 2007; Park *et al.*, 2018; Yarkent *et al.*, 2020). Among them, carotenoids are the most common naturally occurring terpenoid pigments. Carotenoids are tetraterpenes derived pigments constituted of 5 isoprene units, thus forming a 40-carbon polyene structure. More than 1100 carotenoids are produced by over 600 organisms, including plants, archaeobacteria, bacteria, fungi, and algae (Yabuzaki, 2017). Carotenoids can be categorized as xanthophyll and carotenes. Xanthophylls have oxygen in their structure, such as zeaxanthin and astaxanthin, while carotenes do not have oxygen in their structure, β -

carotene, and lycopene. Carotenoids are fat-soluble compounds known to absorb light within a range of 400-500 nm. Carotenoids provide yellow, orange, or red pigmentation in fruits and vegetables. The compound's color or pigmentation is determined by the number of conjugated double bonds present in the structure (Meléndez-Martínez *et al.*, 2007). Their two group endings determine the nomenclature of carotenoids. Carotenes have seven different group ends, which are represented by 7 Greek letters, for instance, β , γ , ϵ , ζ , κ , ϕ , and ψ . The name of carotenes has two Greek letters as prefixes for group ends such as β , ϵ carotene (α -carotene) contains one β ring and one ϵ ring. Another example is β , β carotene, commonly known as β -carotene, having two β rings. Similarly, 3,3'-dihydroxy- β , β' -carotene-4,4'-dione (astaxanthin) also have two β rings (Figure 1).

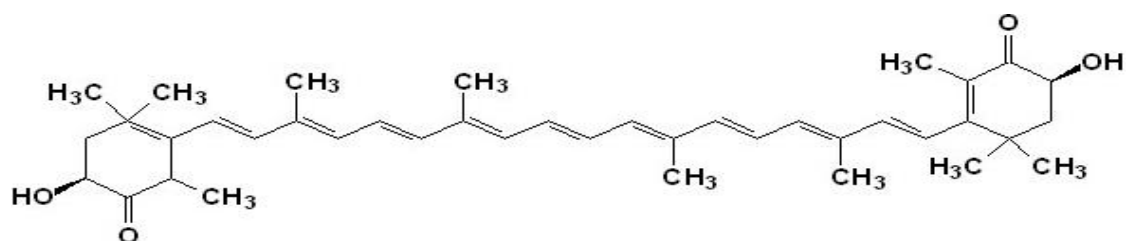


Figure 1. Molecular structure of optically pure (3S, 3'S) astaxanthin from *Haematococcus pluvialis*

Humans require carotenoids for body needs, which must be obtained from diet only as humans cannot synthesize carotenoids. In addition to pigmentation, carotenoids are associated with light-harvesting, ROS scavenging, excess energy dissipation, and quenching of the chlorophyll triplet system. Carotenoids prevent the oxidation of DNA, lipids, and proteins. Chromophores absorb sunlight and form singlet oxygen, which could be prevented by quenching. Carotenoids help in photoprotection and light-harvesting (Chen *et al.*, 2020; Yang *et al.*, 2020). Under low-intensity light conditions, carotenoid molecules perform light harvesting. The energy so produced is passed to chlorophyll, while under high light intensity, carotenoids take up the extra energy from chlorophyll and dissipate it to protect it from photodamage. Carotenoid quenches the triplet chlorophyll state and helps in membrane stabilization in the microalgal cell. Due to this enormous antioxidant activity, microalgae are exploited commercially for human use (Demmig-Adams and Adams, 2002). Carotenoids have unique biological properties and do not show cytotoxicity. Therefore, carotenoids have applications in pharmaceuticals, food, feed, and cosmetic industries.

Astaxanthin (3,3'-dihydroxy- β , β' -carotene-4,4'-dione) is one of the compounds produced by microalgae, yeast, bacteria, marine animals, and plants, which is attracting cosmetic, food, and pharmaceutical industries. Astaxanthin is a red-colored, super antioxidant, anti-inflammatory, and immunomodulatory compound (Shah *et al.*, 2016). It is a keto-carotenoid and acknowledged as a super antioxidant due to phenomenal antioxidant potential. Astaxanthin is 800 times stronger than coenzyme Q, 550 times than green tea

catechins, 6000 times than vitamin C, 11 times and 2.75 times as compared to beta carotene and lutein (Nguyen, 2013; Fakhri *et al.*, 2018). Carotenoid is synthesized from photosynthetic products glyceraldehyde-3-phosphate and pyruvate. Glyceraldehyde-3-phosphate and pyruvate both are passed to the non-mevalonate pathway to produce isopentenyl pyrophosphate (IPP). IPP is the critical intermediate compound for the synthesis of carotenoids (Rohmer, 1999). In aquaculture, astaxanthin is used as a pigment or color enhancer primarily for salmon, shrimp, trout meat of fish, Arctic char, red bream, krill, shrimp, lobster aquarium and ornamental fishes. It also enhances the color of egg, yolk, skin, and meat of broiler chicken carcasses (Lim *et al.*, 2018). Astaxanthin is approved as a food color for salmon and nutraceuticals for human consumption in Japan, the USA, and other European countries. It can prevent and cure cancer, diabetes, metabolic syndrome, hepatopathy, eye disease, gastrointestinal diseases, cardiovascular diseases, neurodegenerative diseases, and chronic inflammation diseases. Astaxanthin improves skin health, muscle health, brain function, and immune response (Yuan *et al.*, 2011; Visioli and Artaria, 2017; Yarkent *et al.*, 2020). Astaxanthin can treat spinal cord and central nervous system injuries, Alzheimer's disease, Parkinson's disease, and neural damage associated with age-related muscular degeneration (Chen and Kotani, 2017). Astaxanthin is also well recognized and exploited in the nutraceutical and pharmaceutical industry to fortify food and beverages.

Less than a percent of total astaxanthin is derived from microalgae *Haematococcus pluvialis*, which is costly. One kg of synthetic astaxanthin costs around \$1000, while natural astaxanthin is derived from a biological source. The sale value of this product tends to vary between USD 2000.00 and 15,000.00 Kg⁻¹, depending on the level of purity and the place of sale (Leu and Boussiba, 2014). Naturally derived astaxanthin is documented to have more excellent bioactivity and a better impact on human health than synthetic astaxanthin. Synthetic astaxanthin can be produced at a much lower rate than the biological one, and that's why the former shares 95% of the feed market. But people are stepping back from synthetic astaxanthin as it is derived from petrochemicals, and with increasing awareness, consumers are leaning towards natural astaxanthin, which results in increasing the demand. However, cosmetic, food and pharma industries only rely on natural astaxanthin. The global market of astaxanthin was worth USD 1 billion in 2019 as estimated by the Grand View Research and will be worth USD 3398.8 million by 2027 at an annual growth rate of 16.2% from 2019 to 2027 (Astaxanthin market report, 2020). By comparing both synthetic and natural astaxanthin, the latter is a 20 times stronger quencher of singlet oxygen and 20 times more potent in scavenging free radicals (Brotosudarmo *et al.*, 2020). There is an increasing demand for natural astaxanthin as a food supplement. Also, the antioxidant property of this compound has fascinated many scientists to carry out clinical research to treat diseases related to ROS,

including diabetes, cancer, neurodegenerative diseases, eye diseases, and atherosclerosis. Astaxanthin can tackle the gastric inflammations due to *Helicobacter pylori*. The US, Food and Drug Administration (FDA), and Brazil, which is already registered by ANVISA (Brazilian Agency of Health Surveillance), have given GRAS status to *Haematococcus pluvialis* derived astaxanthin (Capelli *et al.*, 2013; FDA, 2017; ANVISA, 2020). Astaxanthin can be produced by bacteria, algae, fungi, yeast, and the *Adonis* genus of higher plants.

Increasing astaxanthin demand and its high-cost shifts the interest to develop a better system for producing astaxanthin from microalgae. There are various methods reported for cultivating microalgae for producing astaxanthin. Different growth conditions like photoautotrophic, heterotrophic, and mixotrophic, both indoors; in open raceway ponds, or closed photobioreactors, can be applied using the batch or fed-batch approach (Kang *et al.*, 2005, 2010; Li *et al.*, 2011; Han *et al.*, 2013; Wang *et al.*, 2013a, b). However, a two-stage mixotrophic culture system is the most advanced strategy for astaxanthin production (Park *et al.*, 2014). *Haematococcus pluvialis* (belong to *Chlorophyceae* of algae) and *Phaffia rhodozyma* (a yeast) are the two microbes of commercial interest as they offer good astaxanthin accumulation. Astaxanthin production is affected by environmental conditions such as nutritional stresses (Chekanov *et al.*, 2014); pH (Hata *et al.*, 2001); light (Saha *et al.*, 2013; Park *et al.*, 2014), temperature (Yoo *et al.*, 2012); salt concentration (Kobayashi *et al.*, 1993) as well as various plant hormones and their derivatives (Yu *et al.*, 2015). After sufficient biomass accumulation, the process is directed towards efficient harvesting, cell disruption, dehydration, and recovery/extraction of astaxanthin. For producing maximum astaxanthin certain factors such as temperature, pH, light intensity, aeration, agitation, and nutrient composition in the medium should be considered to optimize the process. Medium composition influences the biomass yield, cell composition, and production of metabolites. All these stated parameters are well known to affect cell growth and yield significantly. The present study was undertaken to optimize growth conditions, including nutrient and cultural factors of microalgal strains for increasing astaxanthin production.

Keeping all these in view and the importance of natural astaxanthin following objectives of the research program were planned:

1. To isolate and screen microalgae for astaxanthin production
2. To optimize cultural conditions for maximum production of astaxanthin
3. To extract and characterize astaxanthin for antioxidant activity

2.1. General introduction to algae

Algae are lower plants with undifferentiated plant parts like roots, leaves, and stems. However, algae do not fit in the Plant Kingdom as per molecular phylogenetic systematic based on modern classification. Algae are grouped as eukaryotic, placed under Kingdom Protista, and prokaryotic algae placed under Kingdom Monera. Kingdom Protista includes microscopic and macroscopic algae, and the Kingdom Monera includes seaweeds. Algae can exhibit great diversity in size, from picoplanktonic form (0.2-2.0 μm) to giant kelps (60 m in length). Algae is a group of 10 million species majority of which are microalgae. Microalgae evolved more than 3.5 billion years ago in the aquatic environment (Zimmer, 2009) and constitute a single or small group of cells. More than 30,000 algal species are found in fresh water and marine water system. However, moist soils and rocks are also a habitat of microalgae (Seckbach, 1995; Leya *et al.*, 2009). The examples are *Spirulina*, *Chlamydomonas*, *Chloromonas*, *Nannochloropsis*, *Chlorella*, *Scenedesmus*, *Dunaliella*, *Chlorella*, *Botryococcus*, *Haematococcus*, and *Desmodesmus* *etc.* Microalgae has enormous potential for commercial exploitation, and many industries produce algae-related products dealing with cosmetics, feed, formulation of food, health products, fertilizers, wastewater treatment, and biofuel. They accumulate various biologically active compounds, including pigments, vitamins, minerals, proteins, polyunsaturated fatty acids, and extracellular compounds, such as oligosaccharides. The associated efficiency of (bio) synthesis in algae is higher than that of terrestrial crop plants. Polyunsaturated fatty acids (PUFA) like eicosapentaenoic (EPA), omega-3 fatty acids, docosahexaenoic (DHA), phycobiliproteins, and carotenoids are beneficial for the immunity of humans and animals (Park *et al.*, 2018). Microalgae produce certain compounds helpful in combating stress conditions. Its cultivation is carried out on a large scale to produce bioactive compounds. There is a better understanding or outlook of bioactive molecule production in microalgae with the advancement in molecular biology.

2.2. High-value pigments from microalgae

Carotenoids are the terpenoid compounds synthesized by all photosynthetic (plants, algae) and several non-photosynthetic organisms (bacteria and fungi). They are the most common naturally occurring compounds. Carotenoids are the conjugated compounds and derivatives of 5-carbon isoprene, enzymatically linked together to form a 40-carbon compound. Mostly carotenoids are hydrophobic compounds (Yabuzaki *et al.*, 2017). The compound's color depends upon the number of conjugated double bonds in its structure. The color of carotenoids ranges from yellow to orange or red depending on the absorbance of photons in blue or near UV regions by the conjugated double bond (Meléndez-Martínez *et al.*, 2007). Around 750 carotenoids exist and are

bifurcated into two classes: carotenes or primary carotenoids and xanthophylls. Carotenes are linear or cyclized compounds; for instance, lycopene is linear, while β -carotene and α -carotene are cyclic compounds. Xanthophylls are the oxygenated compound and are further classified under three groups containing: =O group (e.g., canthaxanthin), -OH group (e.g., zeaxanthin), or both of the two groups (e.g., astaxanthin and lutein). More than 600 compounds are known to derive merely by substitution of oxygen-containing groups (exclusive of *cis/trans* isomers) (Rodríguez-Concepcion *et al.*, 2018; Britton, 2020). Primary carotenoids are the essential structural and functional constituents of the photosystem apparatus, which are necessary for the proper assembling of photosystems (Pogson *et al.*, 2005; Li *et al.*, 2009). Primary carotenoids capture a broad range of light in the spectral region and transfer it to chlorophyll for photosynthesis (Polívka and Frank, 2010). Secondary carotenoids act as a response mechanism against stress conditions like high salinity, nutrient deficiency, and high light (Young and Britton, 1990; Lemoine and Schoefs, 2010). These are accumulated within the plastids or in the cytoplasm as oil droplets. Phenomenons like red snows and blood rains are due to the accumulation of these secondary carotenoids (Bidigare *et al.*, 1993). Carotenoids have tremendous antioxidant activity and prevent lipids, proteins, and DNA oxidation. They quench the singlet oxygen formed by the absorption of sunlight by chromophores (Collins *et al.*, 2011; Guedes *et al.*, 2011; Maoka, 2020). Also, clinical trials and epidemiological studies have proved a positive influence of carotenoids on mammals.

2.3. Astaxanthin

Astaxanthin (3,3'-dihydroxy- β , β' -carotene-4,4'-dione) is a red-colored fat-soluble compound. It is a keto-carotenoid related to zeaxanthin, β -carotene, and lutein. It has a chemical formula of $C_{40}H_{52}O_4$ and a molecular weight of 596.8 Da (Dalton) (Guerin *et al.*, 2003; Shah *et al.*, 2016; Oslan *et al.*, 2021). Astaxanthin can be found in some plants, marine animals, and microorganisms (Higuera-Chipera *et al.*, 2006; Ambati *et al.*, 2014; Li, 2019). It is categorized under the terpene group, and is soluble in the organic solvent while insoluble in water.

Astaxanthin has both carbonyl (C=O) and hydroxyl (OH) groups on each of its ionone rings, thus making it an excellent antioxidant compound for the free radical scavenging and reactive oxygen species (ROS) quenching. It aligns perfectly in the lipid bilayer due to the presence of both hydrophobic and hydrophilic components in its structure (Ambati *et al.*, 2014; Visioli and Artaria, 2017). By this arrangement, astaxanthin can effectively interact with cytoplasm and the external environment through polar parts, thus facilitating electron transfer *via* a double bond. This arrangement also allows the scavenging of radicals at the external and internal surface of the membrane (Goto *et al.*, 2001; Pashkow *et al.*, 2008; Ambati *et al.*, 2014). Astaxanthin interacts with the membrane *via* modulation of lipid peroxidation (McNulty *et al.*, 2007). Terminal rings of astaxanthin have hydrophilic groups attached, which offers an advantageous position in the cell membrane. It spans the cell membrane so that the hydrophilic terminal is exposed outside the membrane while lipophilic backbones are exposed to the inner

surface. Due to the properties discussed so far, astaxanthin is produced worldwide for human and animal applications (Higuera-Ciapara *et al.*, 2006; Yuan *et al.*, 2011). Around 17 different European countries, including France, Netherlands, Germany, and Denmark. The astaxanthin market produces 66 tons of algal biomass and is expected to reach 3.4 billion USD by 2030 (Borowiak *et al.*, 2021).

Astaxanthin can cure many diseases, including cancer, diabetes, hepatopathy, metabolic syndrome, gastrointestinal disorders, neurogenerative diseases, chronic inflammation, and cardiovascular diseases. It protects and promotes the immune system, brain, skin, and skeletal muscle health (Yuan *et al.*, 2011; Visioli and Artaria, 2017; Farruggia *et al.*, 2018; Sztretye *et al.*, 2019). Astaxanthin imparts color to crabs, shrimps, trout, lobsters, and the egg yolk of quail and chickens (Miki, 1991). Astaxanthin is widely distributed in some aquatic animals and helps in enhancing their growth and disease resistance, improves survival and immunity, and enhances reproductive capacity (Sheikhzadeh *et al.*, 2012; Zhang *et al.*, 2013; Wade *et al.*, 2015; Lim *et al.*, 2018; Ou *et al.*, 2019). Astaxanthin has been approved as a natural food additive for fish and animal feed and natural dye for animal and human diets by the US Food and Drug Administration (FDA) and European Commission, respectively (Brendler and Williamson, 2019; Singh *et al.*, 2019). The market value of astaxanthin is influenced by several factors like commercial model, production cost, and marketing strategies. The business-to-customer value of astaxanthin is 150 to 300-euro/kg, while the business-to-business value of astaxanthin is between 6.000 and 8.000-euro/kg (Araújo *et al.*, 2021). Astaxanthin has enormous applications in industries like aquaculture (Yu and Liu, 2020), pharmaceutical (Araújo *et al.*, 2021; Medhi and Kalita, 2021), health and personal care (Borowiak *et al.*, 2021), food and feed (Gateau *et al.*, 2017; Fernandez *et al.*, 2021; Kusmayadi *et al.*, 2021). Therefore, it is practical and profitable to harvest astaxanthin for commercial purposes.

2.4. Sources of astaxanthin and its structure

Astaxanthin is synthesized by bacteria, yeast, microalgae, marine aquatic animals, and plant-like *Adonis aestivalis*. Aquatic animals can also accumulate astaxanthin through the food chain (Fang *et al.*, 2019; Shao *et al.*, 2019). Natural astaxanthin can be consumed indirectly by adding fish and crustaceans to the diet. Astaxanthin is synthetically produced from chemicals and naturally extracted from microbes. Synthetic astaxanthin is produced *via* Wittig reaction of 3-methyl-5-(2,6,6-trimethyl-3-oxo-4-hydroxy-1-cyclohexenyl)-2,4-pentadienyltriaryl phosphonium and 2,7-dimethyl-2,4,6-octatrienedia (Ernst *et al.*, 1991; Krause *et al.*, 1997; Wan *et al.*, 2021). The global market of astaxanthin is dominated by synthetic astaxanthin. Still, with increasing awareness about its different stereochemical derivatives and potential carryover of its synthesis intermediates, synthetic astaxanthin is only limited to aquatic feed supplements. It is not used for human consumption (Shah *et al.*, 2016). For human consumption, natural astaxanthin producers are widely exploited. *Haematococcus pluvialis* (green microalgae), *Xanthophyllomyces*

dendrorhous (red yeast), and *Paracoccus carotinifaciens* (bacteria) are used in the commercial production of astaxanthin. However, *H. pluvialis* is believed to be an abundant source of astaxanthin which is also approved for human use (Ambati *et al.*, 2019; Wan *et al.*, 2021).

The structure of astaxanthin is composed of a polyene chain and an oxygenated ionone ring at both ends. Astaxanthin has a range of geometric isomers. Natural astaxanthin occurs in the most stable all-*trans* configuration (Figure 2). However, it is also found in the *cis* configuration, for instance, 9-*cis*, 13-*cis*, 15-*cis*, and 13, 15-di-*cis* isomers (Brotosudarmo *et al.*, 2020). There are two chiral centers (C-3 and C-3') in astaxanthin, giving three stereoisomers, 3R,3'S (meso form), and two enantiomers 3S,3'S, 3R,3'R. Fish has all three optical isomers of astaxanthin. 3S,3'S optical isomer is the dominant form of astaxanthin observed in salmon. *H. pluvialis* produces (3S,3'S)-isomer, *X. dendrorhous* produces (3R,3'R)- configuration of astaxanthin (Visser *et al.*, 2003). *Oncorhynchus mykiss* and *Euphausia superba* produce 3S,3'S and 3R,3'R isomers, respectively (Bjerkeng *et al.*, 1997; Cong *et al.*, 2019).

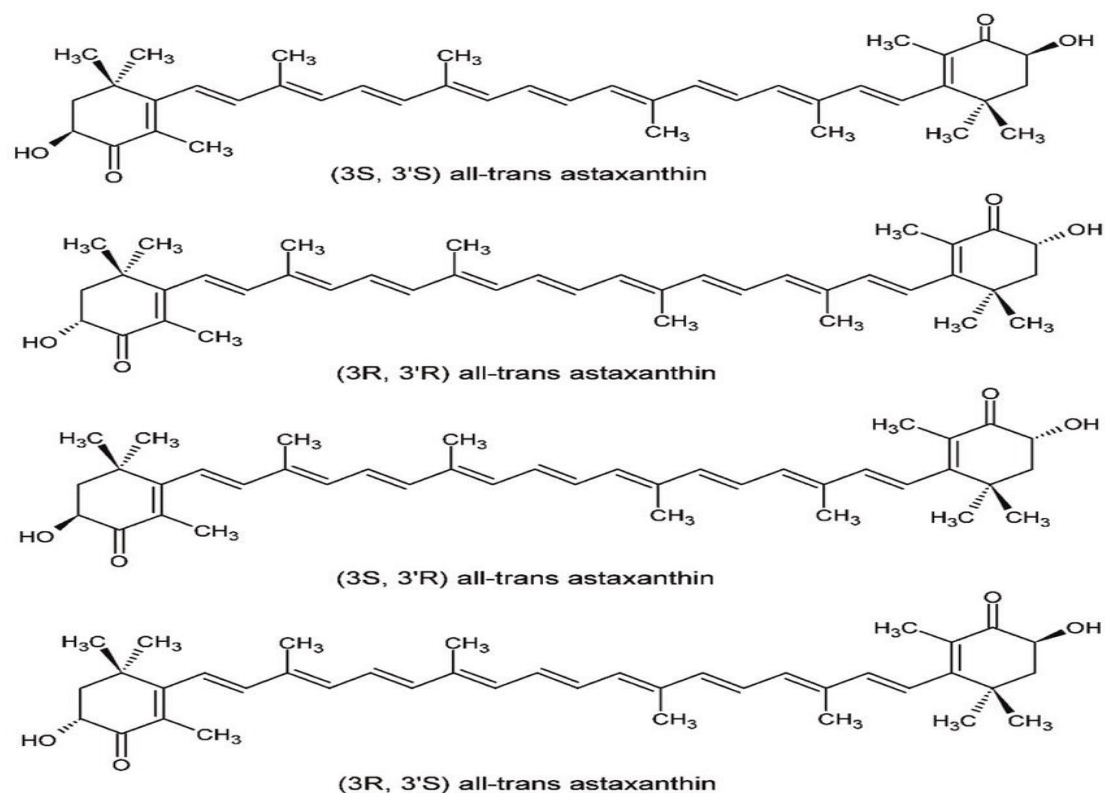


Figure 2: Configuration of different astaxanthin isomers in the trans form

Synthetic astaxanthin has all three isomers (3S,3'S), (3R,3'S), and (3R,3'R) in a 1:2:1 ratio, respectively. It exists in free form, while natural astaxanthin can be found in free form or bond with fatty acids like palmitic, oleic, linoleic, etc., to produce esters astaxanthin (Figure 3).

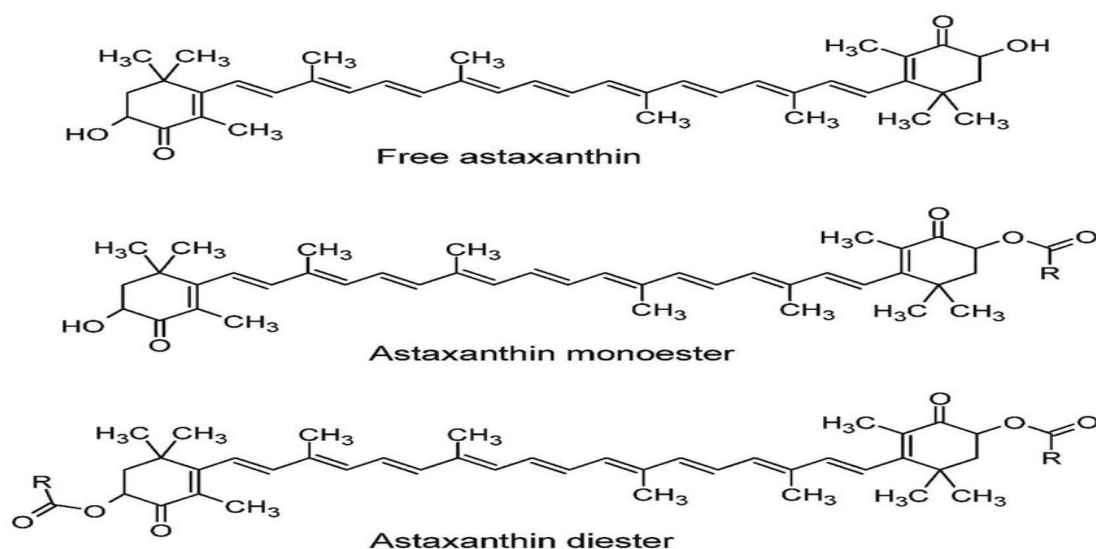


Figure 3: Free astaxanthin, mono, and diester forms of astaxanthin

Lower crustaceans, copepod *Calanus finmarchicus* (> 80%), and a tiny euphausiid species *Thysanoessa inermis* (> 50%) show the dominant presence of 3S,3'S, followed by 3R,3'R, and 3R,3'S isomers (Foss *et al.*, 1987a). *Exopalaemon carinicauda* and *Macrobrachium nipponense* are also confirmed for more than 90% of astaxanthin's 3S,3'S configuration (Matsuno *et al.*, 1984; Su *et al.*, 2018; Zhang *et al.*, 2018). Euphausiid species, Antarctic krill (*Euphausia superba*), majorly produces astaxanthin in 3R,3'R configuration (Fujita *et al.*, 1983; Arai *et al.*, 1987). Additionally, in the carapace of the crabs, *Sesarma haematocheir*, *Sesarma intermedia*, *Percon planissimum*, and *Portunus trituberculatus*, about 60–70% of the astaxanthin is 3R,3'R isomer, and a minor portion is 3S,3'S isomer (Matsuno *et al.*, 1984). Different configurations of astaxanthin are applied in aquatic feed; therefore, various stereoisomers are found in farmed rainbow trout, whereas such variation is not observed in fish like Atlantic salmon and tilapia (Yu and Liu, 2020).

2.5. Optimization of cultural conditions to induce biomass at the green stages

Temperature and light are the two significant factors influencing carotenoid production in both autotrophic and mixotrophic systems. Other factors like salinity, pH, and nutrition also affect xanthophyll production. Stimulants are added to the broth to induce hyper-production of carotenoids by involving biosynthetic enzymes. Therefore, proper adjustment or alteration of nitrogen, carbon, and salt sources, along with the addition of precursors and stimulators, can help to achieve better microbial production goals. Commercial processes are based on developing a cost-effective medium for xanthophyll production using microorganisms.

2.5.1. Effect of nitrogen sources

Nitrogen affects cell growth by increasing the cell size and enzyme activity in *H. pluvialis*, which is necessary for astaxanthin production (Scibilia *et al.*, 2015; Zhang *et al.*, 2018). Microalgae require nitrogen in the form of nitrate, ammonia, or urea (Wang *et al.*, 2019). Urea is a source of both carbon (20%) and nitrogen (46%), which serves the additional benefit of increasing microalgal biomass (Dhup, 2016; Minyuk *et al.*, 2020). Inorganic source like potassium or sodium salt of nitrate generally used in aquaculture provides only 16.5% of nitrogen (Minyuk *et al.*, 2019).

Ammonia is the easiest to uptake and metabolize as a nitrogen source for microalgae (Capelli *et al.*, 2013). At the same time, urea requires an energy-driven co-transport process, and after uptake, it is reduced into ammonium ions by urease and nitrate reductase (Buchheim *et al.*, 2013). Bold basal medium (BBM) and BG-11 medium are used for microalgae cultivation. The medium contains phosphorous, nitrogen, and essential trace minerals and is according to the requirement for the growth of microalgae. However, ammonium hydrogen carbonate causes acidification in the medium leading to cell death of *H. pluvialis* JNU35 (Wang *et al.*, 2019). Yoshimura *et al.* (2006) reported that urea is easily converted to carbon dioxide and ammonia by the action of urease without acid production, and thus pH remained constant. Wijanarko (2011) reported a 55-60% increase in lipid formation with the addition of 500 mg L⁻¹ of ammonia as a nitrogen source in *Chlorella vulgaris*. Ammonia was readily converted into nitrate and ammonia, which was taken up by the cells. Dhup *et al.* (2016) confirmed the increase in microalgal biomass accumulation under different trophic conditions with a urea-based medium containing nitrate and ammonium under light and dark conditions.

2.5.2. Effect of carbon sources

Acetic acid or acetate could help to obtain higher biomass of *H. pluvialis* through mixotrophic cultivation (Cui *et al.*, 2020). Research conducted by Pan-utai *et al.* (2017), 100 mM of potassium acetate as an organic carbon source induced astaxanthin accumulation in *H. pluvialis*. In another finding, 30 mM of sodium acetate increased the cell biomass by 0.243 g/L/day (Jeon *et al.*, 2006). Göksan *et al.* (2010) found that adding 1 g/L of sodium acetate at the initiation or at the end of the log phase of *H. pluvialis* cultivation resulted in increasing the cell number two-fold from 21.7 to 42.9 × 10⁴ cells mL⁻¹. However, when sodium acetate was added at the initial stage of cultivation, biomass accumulation was also better than the addition at the end as the increase in the latter case was only 1.2-fold. As an organic carbon source, Sodium acetate is an effective source for increasing the growth rate. Carbon-nitrogen balance can be manipulated using carbon dioxide, thus inducing cyst formation and astaxanthin accumulation (Jannel *et al.*, 2020). Also, carbon dioxide at a specific concentration of carbon removes the nitrogen deficiency. Nitrogen starvation is induced by increasing carbon through altering the C/N ratio in the system instead of replacing it with

nitrogen deficient media. Cheng *et al.* (2016) reported that addition of 6% of carbon dioxide resulted in maximum biomass accumulation and astaxanthin induction. Additional carbon shifts the C/N balance and causes nutrient deficiency leading to increased astaxanthin production. However, when CO₂ was increased by up to 20%, cell growth was reduced (Chekanov *et al.*, 2017), and induction of chloroplast inhibition eventually led to cell mortality (Chekanov *et al.*, 2017; Christian *et al.*, 2018). In a study conducted by Pang *et al.* (2019) cell number of *H. pluvialis* was increased up to 2.43×10^6 cells/ml at 2 g/L of sodium gluconate and $105 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$ of illumination intensity. Lu *et al.* (2019) experimented using a series gradient fed-batch strategy (SGF) and gradually controlled the C/N ratio from 10 to 50 led to 9.18 g/L of cells at the end of the green stage with sodium acetate and sodium nitrate as carbon and nitrogen sources. After that, high light intensity treatment was given in the second stage, which increased the astaxanthin productivity up to $15.45 \text{ mg L}^{-1} \text{ d}^{-1}$.

2.5.3. Effect of salinity

Salinity induces stress and affects microalgae's net lipid productivity (Minhas *et al.*, 2016). Sodium chloride (NaCl) destructs the reaction center of PSII (photosystem II), an oxygen-evolving complex, and restricts the electron transport at the donor and receptor end. It affects the absorption of light energy, transfer, and application, probably leading to inhibition of algal growth and cell death (Ji *et al.*, 2018). Extracellular ion, if in excess, disrupts the osmotic balance causing exosmosis (Annamalai *et al.*, 2015). Equilibrium of consumption and disruption of reactive oxygen species is destroyed by increased NaCl concentration (Guilian *et al.*, 2004). Gao *et al.* (2012) reported different adaptation mechanisms under salinity stress of 0.17 M ($\approx 1\%$) NaCl as supported by the growth of three Australian *H. pluvialis* isolates (Gao *et al.*, 2015). The application of cheap stock of NaCl enhances biomass, lipid, and carotenoid accumulation, thus reducing the cost of astaxanthin production (Ji *et al.*, 2018). Sodium chloride (0.25-0.5% w/v) is sufficient to induce astaxanthin accumulation. Astaxanthin production increases with the combined use of NaCl and 0.2 mM of sodium acetate (Sarada *et al.*, 2002). Salinity induces the buildup of reactive oxygen species; that is why salt is added to prevent growth and induce astaxanthin production (Boussiba and Vonshak, 1991). Tam *et al.* (2012) investigated the effect of different NaCl concentrations on *H. pluvialis* growth and astaxanthin production. Salinity stress resulted in decreased cell proliferation and increased carotenoid content per cell.

2.5.4. Effect of temperature

The temperature has a significant influence on the growth and development of living organisms. The optimum temperature range is between 20 to 25°C for microalgae (Li, 1980). However, for photosynthesis and cellular division, 15 to 35°C is the tolerable range. With increased temperature, cellular division and photosynthesis also increase until optimum temperature is attained above which growth starts decreasing. The decrease in growth with an

increased temperature above optimum temperature is due to the denaturation or inactivation of enzymes or proteins (Salvucci and Carfts-Brandner, 2004; Ras *et al.*, 2013). The maximum temperature for carotenogenesis in *H. pluvialis* is 30°C, while temperatures above 30°C, i.e., 35-40°C, cause cell lysis (Hong *et al.*, 2015; Shah *et al.*, 2016; Pereira and Otero, 2020). Giannelli *et al.* (2015) reported that astaxanthin accumulation was 37% more at 27°C as compared to 20°C in control under nitrogen starvation. Total cell count was more at 27°C than at 20°C, i.e., 9.8×10^6 cells mL⁻¹ and 9.1×10^6 cells mL⁻¹ for 27°C and 20°C, respectively. The combination of higher temperatures and nitrogen deficiency boosts astaxanthin synthesis. Furthermore, Hong *et al.* (2015) reported increased astaxanthin production with a slight increase in temperature from average temperature, i.e., from 23°C to 25-28°C. Under photoautotrophic conditions, biomass concentration was reduced by 20% at 30°C and by 48% at 36°C when compared to cells at 23°C. Photosynthesis in *H. pluvialis* was suppressed when the temperature rose from 30°C to 36°C. Photosynthesis is inversely proportional to carotenoid content.

2.5.5. Effect of pH

pH is another crucial factor for the growth of microalgae. During the daytime, cells perform photosynthesis and capture carbon dioxide, causing an increase in pH. In contrast, the opposite of this happens at night as only respiration takes place, causing a decrease in pH. Also, pH increases with an increase in cell density of microalgae (Bartley *et al.*, 2014; Pires *et al.*, 2017). Carbon dioxide is generally diffused in the bioreactor to maintain the pH for microalgae cultivation. Apart from carbon dioxide and sodium carbonate, HCl can also control pH (Giordano *et al.*, 2005; Chi *et al.*, 2011). The biochemical content of microalgae may be affected by pH. The optimum range of pH is 7.00-7.85 for the biomass accumulation and astaxanthin production in *H. pluvialis* (Hata *et al.*, 2001; Shah *et al.*, 2016).

2.6. Biosynthesis of astaxanthin

Microalgal-based production of astaxanthin requires microalgal cultivation followed by astaxanthin induction and ends with biomass harvesting (Figure 4). With the advancement in technologies, the process is highly automated. Microalgae culture inoculated in the medium supplemented with sufficient nutrients and incubated at optimum temperature for biomass accumulation. After biomass accumulation, astaxanthin-inducing conditions are provided, for instance, carbon addition and nitrogen starvation (Kang *et al.*, 2007; Kumar *et al.*, 2021). One stage process is based upon providing favorable conditions or essential nutrients for microalgae growth; with advancement in growth, these nutrients get depleted, which causes a pH change, thus creating adverse conditions (Han *et al.*, 2020). Upon adverse conditions, vegetative cells start changing into a resting cyst stage marked as the beginning of astaxanthin synthesis. Specific inducers are also added within the medium. However, inducers sometime negatively affect microalgal growth (Liu *et al.*, 2017). One stage process does not require a

workforce; therefore, it is a cost-effective option. Certain drawbacks are associated with the one-step process, and specific commercial units opt for a two-stage process for astaxanthin production. A two-stage approach is based on the green vegetative stage and red cyst stage of *Haematococcus pluvialis*. The green stage is characterized by biomass accumulation. In contrast, the red stage is characterized by loss of reproduction and motility marked by astaxanthin accumulation of up to 5% of dry weight (Han *et al.*, 2020; Scarsini *et al.*, 2020).

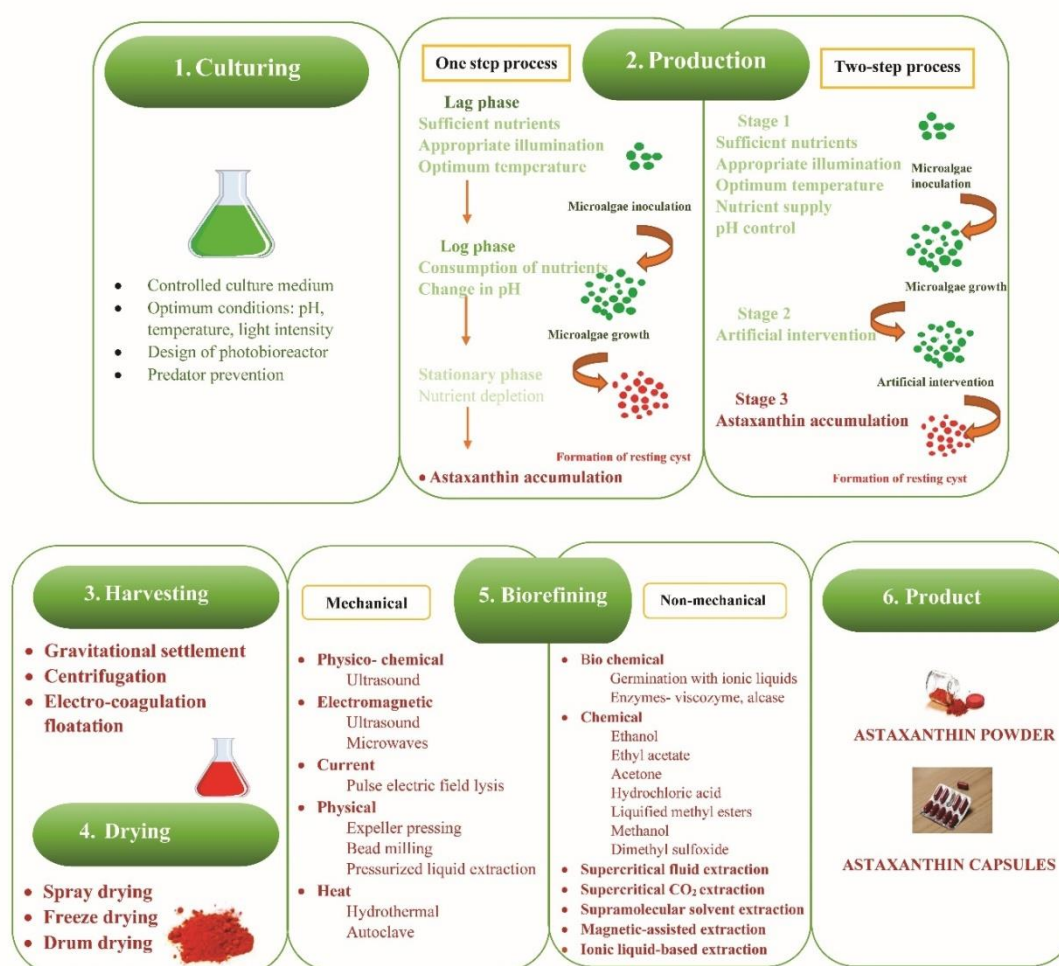


Figure 4. Overview of biosynthesis of astaxanthin by a two-stage process

Haematococcus is grown in enclosed photobioreactors, either mixotrophically or photo-trophically, until sufficient biomass is accumulated, later on cells are transferred to raceway ponds or large photobioreactors with nutrient limitations. Nutrient limitation conditions allow astaxanthin accumulation and phototropic encystment (Hu *et al.*, 2020; Du *et al.*, 2021). Stainless steel fermenters are used for the red stage process. Astaxanthin initiation requires three to five days and ends with transforming vegetative cells into aplanospores or cyst cells. Aplanospores are harvested through gravitation settlement followed by ultracentrifugation. These break down the cell wall, giving access to astaxanthin. Biomass is then dried by any method, including spray drying (cost-effective), drum drying, or freeze-

drying. The dried biomass is cracked either by high-pressure homogenizer or bead millers, followed by dehydration through spray dryers or belt dryers (Shah *et al.*, 2016).

Organic solvents like hexane, ethanol, propanol, and certain edible oils were initially used for astaxanthin extraction, but supercritical fluid extraction with carbon dioxide was applied (Liu *et al.*, 2017; Zgheib *et al.*, 2018). Aplanospores are highly resistant structures and indigestible which cannot be directly given to animals and humans. Therefore, astaxanthin is extracted and given as a food and feed supplement. Cost and biomass safety are the two crucial harvesting concerns process as 20-30% of the cost of astaxanthin comes from the harvesting process (Zhang and Hu, 2012). Harvesting costs must be lowered down for making the process cost-effective. The harvesting process must eliminate the toxic compounds from the biomass; for instance, astaxanthin accumulation occurs if harvesting is done using Al^{3+} (Oberholster *et al.*, 2012). Also, the process must reduce carbon footprint low energy and water consumption. To make the process sustainable and cost-effective, AlgaTechnologies apply solar energy (specified at 250 W, 15% conversion efficiency, 1.65 m^2) instead of electricity, thus reducing the cost (Panis and Rosales, 2016).

Further, 1000-1500 tons of fresh water per ton of *H. lacustris* is required for biomass accumulation. This massive volume of water can be reduced either by simultaneously carrying out the red stage or using open ponds in which only 30% of water is used (Wan *et al.*, 2014). Biorefinery approach 'high-value product first' is opted for combined production of astaxanthin, polyunsaturated fatty acids, and phytosterols. The remaining biomass can be used as a biofertilizer or protein source (Bilbao *et al.*, 2016).

2.7. APPLICATIONS OF ASTAXANTHIN

Astaxanthin is a "super antioxidant" because it bridges biological membranes and protects them from peroxidation by scavenging free radicals. It protects lipids, notably phospholipids, against peroxidation. Astaxanthin is becoming more popular in the nutraceutical, cosmetic, culinary, and pharmaceutical industries due to its more potent antioxidant activity than any other carotenoids.

The significant applications of astaxanthin include

- Antidiabetic
- Cardiovascular diseases prevention
- Anti-cancerous
- Skin protective
- Application in cosmetics
- Food colorant
- Feed supplement

2.7.1. Antidiabetic activity

Diabetes mellitus creates hyperglycemia due to damage or ill-functioning of β -pancreatic cells, producing high oxidative stress in humans. The undesired generation of reactive oxygen species by neutrophils and macrophages causes oxidative stress. Astaxanthin helps the body by reducing oxidative stress and improves glucose and insulin levels. In a study, 54 patients with type 2 diabetes were given 12 or 6 mg of placebo and astaxanthin continuously for eight weeks, and patients were regularly monitored. It was observed that regular intake of astaxanthin decreased the plasma interleukin-6 level (IL-6), blood glucose levels, tumor necrosis factor- α (TNF- α), and HbA1c (%). If an increased dose is given, astaxanthin can reduce total cholesterol, LDL, plasminogen activator inhibitor, clotting factor (Factor VII), and plasma TG (Chan *et al.*, 2019). Similar results of decreased blood glucose levels were reported by Ursoniu *et al.* (2015) after studying and meta-analyzing data of 10 randomized controlled clinical trials (RCTs).

2.7.2. Cardiovascular disease prevention

Problems related to blood vessels or the heart are categorized as cardiovascular disease or disorders. Some cardiovascular disorders are peripheral heart disease, rheumatic heart disease, heart failure, congenital heart disease, and cerebrovascular disease (Jain *et al.*, 2015). The majority of people die from cardiovascular diseases, with 23.3 million people predicted to die by 2030, according to the WHO (Bansilal *et al.*, 2015; Brotosudarmo *et al.*, 2020). Cardiovascular diseases can be due to ROS, for example, myocardial infarction, heart failure, cardiomyopathy, ventricular remodeling, and ischemia/reperfusion injury. Specific compounds that can cure and prevent CVD include polyphenols, carotenoids, vitamins, ascorbic acid, and antioxidants (Bansilal *et al.*, 2015). Carotenoids help to reduce blood pressure and proinflammatory cytokines and enhance sensitivity towards insulin in adipose tissue, muscle, and liver. Astaxanthin can cure and prevent CVD and effectively improves blood profile (Gammone *et al.*, 2015). Astaxanthin affects blood fluidity and has antihypertensive effects by normalizing sensitivity to adrenoceptors (adrenoceptors) *via* the sympathetic route or restoring vascular tone *via* attenuation of angiotensin II and reactive oxygen species driven vasoconstriction (Hussein *et al.*, 2005). Kato *et al.* (2020) conducted a pilot clinical investigation on 16 heart failure patients. They were fed with a 12 mg ASX supplement for three months. They were tested for 6-minute walk distance, BMI, blood pressure, heart rate, left ventricular ejection fraction, and various oxidative stress indicators, such as biological antioxidant potential, urine 8-hydroxy-2-deoxyguanosine, and Dacron reactive oxygen metabolites (dROM). They discovered a higher concentration of ASX in plasma, which resulted in decreased oxidative stress and low dROM levels. Cardiac contractility and exercise tolerance were also enhanced in patients with left ventricular

systolic dysfunction. ASX lowers HDL cholesterol and serum TG levels while increasing adiponectin levels in persons with milk hyperlipidemia (Szentesi *et al.*, 2019).

2.7.3. Anticancer activity

Carotenoids are fascinating compounds with antioxidant activity, transformation into retinoids, and anticancer activity. Compared to β -carotene, some carotenoids are more effective anticarcinogens (Nishino *et al.*, 2002). Bertram *et al.* (2005) reported that β -carotene failed to cure people with lung cancer; instead, it initiated lung pathology. Therefore, to find a solution to this, scientists advised using astaxanthin, which does not have provitamin A activity; thus, retinoid toxicity can be tackled. Astaxanthin outperformed canthaxanthin, which has good impact on skin fibroblast cell development, growth regulation, intracellular communication gap junction, homeostasis, and connexin 43 phosphorylation. However, astaxanthin and some carotenoids promote intercellular communication of gap junction and induce connexin expression (Daubrawa *et al.*, 2005). Suppression of tumor by increasing levels of natural killer cells, and plasma interferon- γ were the common effects observed after giving astaxanthin before the beginning of tumor in BALB/C mice with the mammary tumor cell line. But the opposite was observed, including fastening the tumor's growth, increased plasma inflammatory cytokine IL-6, and tumor necrosis factor- α when astaxanthin was given after tumor initiation. Yang *et al.* (2018) studied the effect of 50 μ M of ASX as a 5-day treatment on mice. The therapy reduced the viability of epidermal JB6 P+ cells by 94% and prevented neoplastic transformation.

2.7.4. Skin protective effect

Reactive oxygen species create oxidative stress in the human body, resulting in skin damage and aging by producing metalloproteinase and inflammatory mediators, which degrade collagen. Exposure to UV-A is the primary reason for skin problems like skin sagging, dryness, laxity, wrinkling, and pigmentation. These can be cured by the oral uptake of astaxanthin (Singh *et al.*, 2020). Astaxanthin suppresses skin thickening and increases collagen fibers, ROS, capillary density, and epidermal thickening in the skin (Li *et al.*, 2020). Wrinkle formation and capillary density have an inverse relationship, whereas capillary regeneration and photoaging are directly related. For 16 weeks, Tominaga *et al.* (2017) studied the effects of 6 or 12 mg of ASX or a placebo on the skin of 65 females. There was decreased skin damage, and a positive result was observed in skin health.

Similarly, Ito *et al.* (2018) observed the effect of astaxanthin on 23 healthy humans. For nine weeks, all participants were given either a placebo or a 4 mg ASX pill. Skin texture improved with increased dose, and moisture loss was reduced from irradiated areas. As a result of the studies discussed above, ASX appears to be a protective agent against UV ray skin damage and aids in skin health.

2.7.5. Application of natural astaxanthin in the cosmetics industry

Cosmetic industries require synthetic colors to make their product pleasing to the eyes. However, the effect of artificial colors on the environment can't be avoided. Therefore, there is a great demand for natural pigments with increasing awareness. Microalgae serves the purpose, and that's why there is a great market value for microalgal pigments (Pangestuti and Kim, 2017). Microalgae have applications not only in food but in cosmetics also. Microalgal dyes are tested and proved to be human-friendly. They have many benefits, including antiaging, color, skin whitening, antioxidants, skin pigmentation, and protection. Microalgal pigments are used in sunscreen creams, antiaging products, anti-irritants, and emollients (Venkatesan *et al.*, 2015). Microalgal dyes are mainly sourced from *Haematococcus* and *Dunaliella* and include lutein, canthaxanthin, astaxanthin, and zeaxanthin (Ryu *et al.*, 2015). Tominga *et al.* (2012) reported the preventive effects on keratinocyte differentiation and cornification and the reversion of corneocyte condition to normal condition after treatment with astaxanthin. Astaxanthin from *Haematococcus pluvialis* has a protective effect against UV-A radiations on filaggrin metabolism, peeling in the epidermis and the extracellular matrix in the dermis (Komatsu *et al.*, 2017). Astaxanthin is a potent antioxidant compound with great potential in skincare (Guillerme *et al.*, 2017). Astaxanthin controls collagenases, ROS, MMPs, and inflammatory mediators; thus, effects like reducing wrinkles, preventing: aging, collagen breakdown, and age spots (Lephart, 2016; Davinelli *et al.*, 2018). Apart from antioxidant activity, astaxanthin can also be used as a colorant. It has additional health benefits like UV protection, antibacterial and antiaging (Chakdar and Pabbi, 2017).

2.7.6. Application of astaxanthin as a natural food colorant

Organoleptic characteristics of any food are essential for its acceptance by the consumers. So, food colors are generally used to give an appealing color to food and beverages (Martins *et al.*, 2016; Nwoba *et al.*, 2019). During food processing, different storage techniques and conditions like temperature, moisture, and light affect the color of the final food product. Food colorants possess a characteristic unpleasant taste. Food colorants are of two types synthetic or natural. Artificial food colors can have lead acetate (responsible for nerve damage) and other carcinogens, so synthetic food colors are banned in some countries. Therefore, with increasing knowledge, natural food colors have opted more as they are safe to consume (Carocho *et al.*, 2014; Carocho *et al.*, 2015; Rodriguez-Amaya, 2019). Microalgae-derived colorants offer the additional advantage of not depending upon season or climate conditions. However, further study on food stability, preservation, encapsulation, and storage is needed for a realistic and commercial approach to food. (Martínez-Delgado *et al.*, 2017).

2.7.7. Application of astaxanthin as a feed supplement in the aquaculture and poultry industry

Astaxanthin helps in enhancing immunity and thus can be used as a feed supplement for animal and aquaculture (Alishahi *et al.*, 2015; Rahman *et al.*, 2016; Biswas *et al.*, 2019). Astaxanthin can replace the use of antibiotics. It improves the pigmentation of ornamental fishes, rainbow trout, and other aquatic animals. Microalgae can be directly given as feed supplement in aquaculture without extracting astaxanthin which is a costly process. Astaxanthin is safe as a feed supplement and ensures no worries when delivered to the maximum dietary limit (Aquilina *et al.*, 2015). Synthetic and natural compounds are used in the aquaculture and poultry industry. However, natural astaxanthin offers colors and other health benefits to the aquaculture industry (Shah *et al.*, 2016). It decreases ROS activity by enhancing superoxide dismutase (SOD) and catalase (CAT) enzyme activity. Lim *et al.* (2019) reported an increase in lysozyme and phagocytic activity and an improvement in total immunoglobulin count. Li *et al.* (2014) compared the effect of natural astaxanthin and synthetic astaxanthin on the growth of sizeable yellow croaker fish. Natural astaxanthin increased the size of fish. It also improves yolk pigmentation, tissue in breast muscle, feed efficiency in broiler chicken, and decreases the mortality rate in chicken and hens (Inbarr and Lignell, 1997; Lignell and Inbarr, 1999; 2000; Yang *et al.*, 2006). Gao *et al.* (2020) randomly grouped the Hy-Line Brown layer (n = 288 50-week-old) into four different groups and observed the effect of astaxanthin. For six weeks, all hens gave astaxanthin @ 0, 25, 50, or 100 mg/kg of corn-soybean. Results showed improved antioxidant capacity and GSH-PX, SOD levels in plasma, yolk, and liver, and reduced MDA levels. Astaxanthin is a good source for increasing nutrient utilization in yellow catfish (Liu *et al.*, 2016). Cheng *et al.* (2018) studied the effect of astaxanthin @ 80, 160, and 320 mg/kg supplemented diet on pufferfish (*Takifugu obscurus*). There were increased growth rates, CAT, SOD, HSP70 mRNA, and plasma alkaline phosphatase activities. Astaxanthin reduced alkaline aminotransferase and plasma aspartate aminotransferase activity. The main reason for aquaculture development is natural ASX from microalgae; it improves the animal's color and immunity, thus, eliminating antibiotic overuse.

CHAPTER–III

MATERIALS AND METHODS

The present investigation was carried out in the Department of Microbiology, CCS Haryana Agricultural University, Hisar. The details of various materials and methods employed during the investigation are as under:

3.1. Materials

3.1.1. Chemicals:

Chemicals used for experiments were of analytical grade sourced from Hi-Media, E. Merck, Sigma-Aldrich, *etc.*

3.1.2. Instruments:

Different instruments used during the investigation were Laminar air flow, UV-Vis spectrophotometer, Autoclave, and B.O.D. incubator, Rotary shaker, Probe sonicator, *etc.*

3.1.3 Sample collection

Water samples were collected from the fresh water ponds of Himachal Pradesh and Haryana at different intervals.

3.1.4 Media Preparation

BG-11 medium was prepared in distilled water and autoclaved at 121°C (15 psi) for 20-30 minutes. The composition of the medium was as follows:

BG-11 Medium

Ingredients	g/L
Sodium nitrate	1.500
Dipotassium hydrogen phosphate	0.0314
Magnesium sulfate	0.036
Calcium chloride dihydrate	0.0367
Sodium carbonate	0.020
Disodium magnesium EDTA	0.001
Citric acid	0.0056
Ferric ammonium citrate	0.006
Trace elements	1 ml
pH	7.0
*Trace elements (for preparation of 1L stock solution)	
Boric acid (H ₃ BO ₃)	2.86
Manganese chloride (MnCl ₂ .4H ₂ O)	1.81
Zinc sulfate (ZnSO ₄ .7H ₂ O)	0.222
Copper sulfate (CuSO ₄ .5H ₂ O)	0.079

Cobalt nitrate	0.0494
Sodium molybdate (NaMoO ₄ ·2H ₂ O)	0.391
Agar	15

3.2. Methods

3.2.1. Identification of aquatic sampling sites

Different freshwater bodies of Palampur and Dharamshala of Himachal Pradesh and Tohana, Haryana, were selected to isolate astaxanthin-producing microalgae. Suseela and Toppo reported *Haematococcus*, a potent astaxanthin producer in Himachal Pradesh, in 2006. Palampur has located at 32°N latitude, 76°E longitude and 1300m above sea level. The city receives an annual rainfall of 250 cm. It is a cold climate city with a temperature of 19°C. Dharamshala is a town in Kangra district located at 32.22°N latitude 76.32°E longitude and 1457m above sea level. The chief water source is the glaciers of the Himalayan Dhauladhar Mountains. Tohana is a city in Haryana. It is in the northern region of India and different temperatures throughout the year.

3.2.2. Collection of water samples

In April 2019, water samples were collected in sterile bottles from various freshwater bodies of Himachal Pradesh and Haryana. The samples were stored and then later utilized to isolate microalgal strains.

3.2.3. Isolation, purification, and maintenance of microalgae

To isolate microalgal strains from collected water samples, techniques like the enrichment culture technique followed by agar plating, streaking and micropipette washing techniques were taken into practice (Anderson, 2005). As part of the micropipette washing technique, the glass Pasteur pipette was heated to soften on the flame, then stretched to form a thin capillary needle. With forceps, the tip of the pipette was broken and then plugged with cotton. The tip was then autoclaved. After sterilization, a soft rubber tubing was attached to it. The collected microalgal samples were added to BG-11 broth having a pH of 7.0. A single cell was picked up using a micropipette by try and error method and moved to watch glass containing BG-11 medium for the microscopic examination. Once the organism was confirmed, cells were again picked up using a micropipette and placed on a watch glass. The procedure was repeated to wash the cell and obtain a single pure cell. Isolated strains were grown and maintained on BG-11 medium, incubated at 25±1°C with a light intensity of 1.5 Klux. The lighting was provided by a cool white fluorescent unit (40 W Phillips India Ltd., India). A lux meter was used to measure light intensity (Sigma digital lux meter 1010D). Isolates were subjected to morphological identification and axenization.

3.2.4 Morphology

A microscopic study was carried out to observe the morphological characteristics of microalgae isolates. The cell cycle was also observed during the exponential growth phase of the microalgal life cycle. Micrographs were taken by Olympus E-450 digital camera.

3.2.5. Purification/Axenization to obtain bacteria-free cultures

Bacterial-free axenic culture of microalgal strains was obtained by supplementing the triple antibiotic solution with an isolation medium (Kaushik, 1987). The three antibiotics used were Penicillin G, Streptomycin sulfate, and Chloramphenicol.

3.2.6. Axenization process

In a 5 ml tube of BG-11 broth, a triple antibiotic solution with a concentration of Penicillin G (10 µg/µl), Streptomycin sulfate (5 µg/µl), and Chloramphenicol (10 µg/µl) was added and then inoculated with 0.5 ml of algal inoculum followed by incubation for 5 hours at 25±1°C. The cultures tubes were then taken out after 5 hours and decanted in sterile centrifuge tubes for the centrifugation at 3500 rpm for 5 min. The supernatant was discarded, and the pellet was re-centrifuged at 3500 rpm by adding 5 ml of distilled water. The process was repeated three times for the washing of the pellet. Finally, the pellet was inoculated in 5 ml of BG-11 medium and incubated for 14 days at 25±1°C under 1.5 Klux of light intensity. After 14 days of incubation, 5 ml of nutrient broth was inoculated with two loopful of culture, followed by overnight incubation at 25±1°C. The culture and control tubes were checked and compared for bacterial contamination. A solid agar-based medium was made for culture maintenance by dissolving 15 g of purified agar in 1 liter of BG-11 medium and autoclaved it before use.

3.2.7. Maintenance of axenized microalgal strains

Pure isolates were maintained on BG-11 medium at 25±1°C temperature under 1.5 Klux of light intensity. Sub-culturing was done at a one-month interval. Microalgal slants were kept at 4-10°C for further studies.

3.2.8. Measurements of growth and physiological parameters

Samples were withdrawn at different intervals to record the growth observation at the green vegetative stage, aplanospore, and red cyst stage.

3.2.8.1. Visualization under high resolution

The cells of microalgal strains were sampled from the exponential vegetative growth phase and red cyst phase. Morphological studies of microalgal strains were conducted using the High-Resolution Field Emission Scanning Electron Microscope (7610F Plus/JEOL).

3.2.8.2. Dry cell weight (Sorokin, 1973)

A pre-weighed Whatman no.42 filter paper was used to filter a particular amount of homogenized suspension on sintered glass equipment. The filter paper with filtrate was oven-dried at 60°C and cooled in a desiccator until it reached a consistent weight. The weight of

filter paper was subtracted from the recorded weight in the desiccator and expressed in terms of mg/ml.

3.2.8.3. Chlorophyll content (Lichtenthaler and Buschmann, 2001)

Ten milliliters of homogenized microalgal suspension were centrifuged at 3000 rpm for ten minutes. The pellet was utilized to extract chlorophyll using 95 percent methanol in a water bath at 60°C for 30 minutes. The sample was centrifuged, and the absorbance of the supernatant was measured against a blank of 95 percent methanol at 650 and 665 nm. The formula for calculating chlorophyll concentration was:

$$\text{Total chlorophyll (mg/mL)} = 2.55 \times 10^{-2} E_{650} + 0.4 \times 10^{-2} E_{665}$$

Where,

E₆₅₀= Absorbance at 650 nm

E₆₆₅= Absorbance at 665 nm

3.2.8.4. Carotenoids content (Jensen, 1970)

The microalgal suspension was centrifuged at 3000 rpm for 10 min. Traces of salts were removed from the pellet by repeated washing with distilled water. Two to three milliliters of 85% acetone were added to the pellet following repeated freezing and thawing until or unless acetone became colorless. The acetone fractions were added together, and the final volume was calculated. Absorbance was taken at 450 nm against 85% acetone as blank.

$$\text{Carotenoids (mg/mL)} = D \times V \times F \times 10 / 2500$$

Where,

D denotes absorbance at 450 nm

F = Dilution factor

V = Volume of the extract

The extinction coefficient of carotenoids is supposed to be around 2500.

3.2.9. Optimization of cultural conditions for enhanced astaxanthin production

3.2.9.1. Effect of different nitrogen concentrations on the growth of microalgae

Regarding standard nitrogen sources, *i.e.*, sodium nitrate in BG-11 medium, three different nitrogen sources (ammonium nitrate, sodium nitrate, and potassium nitrate) with five different concentrations (10, 15, 20, 25, and 30 mM) were taken and observed for growth. The microalgal cultures were inoculated, and flasks were incubated at 25±1°C for 15 days with a 16:8 h dark cycle and 1.5 Klux light intensity. The effects of nitrogen sources on growth were recorded by analysing dry cell weight, total chlorophyll, and total carotenoid content every two days. The experiment was performed in triplicates.

3.2.9.2 Effect of different carbon sources on growth of microalgae

Four different carbon sources, such as sodium carbonate, di-potassium carbonate, sodium bicarbonate, and glucose, with three concentrations of 3, 5, and 7 mM, were taken in reference to sodium carbonate (standard carbon source in BG-11 medium) and observed for growth. The flasks were inoculated and incubated at 25±1°C for 15 days with a 1.5 Klux light

intensity and a 16:8 h dark cycle. Effects of carbon sources on growth were recorded by analysing dry cell weight, total chlorophyll, and total carotenoid content at every two days intervals. The experiment was performed in triplicates.

3.2.9.3. Effect of different salt concentrations on the growth of microalgae

Five different salt sources, i.e., sodium chloride, calcium chloride, potassium chloride, magnesium chloride, and manganese chloride, with three concentrations of 16, 32, and 64 mM, were taken in reference to sodium ions (standard salt source in BG-11 medium) and observed for growth. The flasks were inoculated and incubated at $25\pm 1^\circ\text{C}$ for 15 days with a 1.5 Klux light intensity and a 16:8 h dark cycle. Effects of salt sources on growth were recorded by analysing dry cell weight, total chlorophyll, and total carotenoid content every two days. The experiment was performed in triplicates.

3.2.9.4. Effect of different pH and temperature

Four different pH, including pH 5, 6, 7, and 8 along with four different temperatures, 22°C , 25°C , 27°C , and 30°C , were observed for effect on growth in 100 ml BG-11 broth. After that, the flasks were inoculated and incubated at $25\pm 1^\circ\text{C}$ for 15 days with a 1.5 Klux light intensity and a 16:8 h dark cycle. The effect of pH and temperature on growth was recorded by analysing dry cell weight, total chlorophyll, and total carotenoid content every two days. The experiment was performed in triplicates.

3.2.9.5. Effect of nitrogen and phosphorous starvation and light intensity

Growing cultures studied the effect of light intensity on pigment accumulation under 3 Klux light for 15 days. Cultures were grown in a nitrogen and phosphorous limiting medium, and stress was induced by adding 0.2% NaCl and 4.4 mM sodium acetate. At the end of 15 days, cells reached the red cyst aplanospore stage and were observed for astaxanthin yield. Biomass was harvested by centrifuge dewatering process followed by lyophilization. The lyophilized biomass was stored at -20°C for further studies.

3.2.9.6. Effect of different LEDs wavelength shift strategies

Several LEDs' wavelength shift strategies for astaxanthin augmentation in microalgae were investigated using red LEDs for microalgae cell growth and then switching to blue LEDs after seven days to induce astaxanthin production. One set was exposed to 5000 lux of blue light. Sodium acetate (4.4 mM) was added to the experiment sets for the simultaneous application of blue LEDs and carbon sources and blue light intensities of 3000 and 5000 lux. The effect of different LEDs wavelengths on growth was recorded by analysing cell count, chlorophyll, and total carotenoid content every three days. The experiment was performed in triplicates.

3.2.10. Downstream processing of carotenoid from microalgae

The astaxanthin extraction from microalgal strains was carried out in different solvent systems reported by Sarada *et al.* (2006), i.e., acetone, methanol, DMSO, HCl, acetic acid, olive oil *etc.*

3.2.10.1. Treatment of cells and extraction of astaxanthin

- **Extraction in different solvents:** Astaxanthin was extracted from lyophilized dried biomass of *Desmodemus* sp. PLM2 used a mixture of polar and non-polar solvents. The experiment was carried out under light protection conditions. In centrifuge tubes, 5 ml of solvent was added to 10 mg of lyophilized biomass and kept at 70°C for 2 min. Tubes were then cooled down, followed by centrifugation at 5000 rpm for 5 minutes. Solvents-treated samples were rinsed twice with distilled water before resuspending in 1 ml acetone. The mixture was ultrasonically extracted for 30 minutes in an ice water bath and centrifuged for 5 minutes at 3500 rpm at 4°C. Steps were repeated until cell debris became colorless. The supernatant was used for the estimation of extractable astaxanthin. The solvents used were HCl, acetone, DMSO, methanol, and acetic acid.
- **Extraction in different edible oils:** Different edible oils like olive oil and soyabean oil in the ratio of 1:4 was used for astaxanthin extraction in 10 mg lyophilized dried samples. After that, samples were vortexed for 3 min and placed under low light for 2-3 h. For extraction, pulse sonication was carried out for 4-5 min and proceeded with centrifugation for 10 min at 5000 rpm. The supernatant was collected.

3.2.11. Analysis of carotenoids by UHPLC. Extracted carotenoid was characterized *via* UHPLC (Nexera 2 UHPLC/Shimadzu) analysis using a reversed-phase C18 column. Acetone and Methanol: H₂O (9:1) were taken as solvents. The flow rate was 1.25 ml/min. B was run at 80 to 20 % for 25 minutes, 20 % for 10 minutes, and 20 to 80 % for 5 minutes to generate a 40-minute gradient. A photodiode array detector was used to detect carotenoids and astaxanthin esters. To quantify astaxanthin, the peaks were aligned at 476 nm. The standard used was all *trans* astaxanthin (Sigma).

3.2.12. Identification of carotenoids by FT-IR. The FTIR micro-spectroscopy imaging measurements were carried out at the Central Laboratory (CCS HAU, Hisar) using an FTIR spectrometer (Perkin Elmer Spectrum, BX II).

3.2.13. Identification of carotenoids by Raman spectroscopy. The Raman spectroscopy imaging measurements were carried out at Central Instrumentation Laboratory (GJU, Hisar) using Raman Spectrometer (Alpha300/WITec).

3.2.14. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Free radical DPPH scavenging activity was estimated by the modified method provided by Rockenbach *et al.* (2011). Two milliliters of 0.2 mM ethanol stock solution of DPPH were added to 2 ml of extract and incubated for 30 min. Absorbance was noted at 517 nm. Blank was a solution of 2 ml ethanol and 2 ml solution of DPPH. DPPH free radical scavenging activity was estimated using the following formula:

$$\text{DPPH scavenging activity (\%)} = [(\text{Ac}-\text{As})/\text{Ac}] \times 100$$

The absorbance of the control is Ac, whereas the absorbance of the samples is As.

(IC50 was expressed as the concentration of 50% of DPPH radical-scavenging activity).

3.2.15. Hydroxyl (OH•) radical scavenging activity

The hydroxyl radical scavenging activity for extracted astaxanthin was calculated using a modified method by Valentão *et al.* (2002).

- **0.01M Phosphate buffer saline of pH 7-** Dissolved 1.44 g of disodium, 0.24 g of monopotassium phosphate, 0.2 g potassium chloride, and 7.9 g of sodium chloride in 1 L of distilled water.
- **0.75 M of Phenanthroline solution-** Dissolved 27.03 g of 1,10-phenanthroline monohydrate in 200 ml of ethanol.
- **0.75 mM of Ferrous sulfate -** Dissolved 0.114 g of ferrous sulfate in 1 L of distilled water.

Two milliliters of 0.75 M phenanthroline solution and 4 ml of 0.01 M phosphate buffer were mixed. Two ml of 0.75 M ferrous sulfate solution and 2 ml of sample were added to this mixture. After shaking the mixture, 2 ml of 0.01% H₂O₂ (w/v) was added and incubated at 37°C for 1 h, and Absorbance was noted at 536 nm. The same mixture with distilled water instead of sample and hydrogen peroxide was taken for blank. The Hydroxyl radical scavenging activity (HRSA) was calculated as follows:

$$\text{HRSA (\%)} = (A_0 - A/A_0) \times 100$$

A₀ = absorbance of blank;

A = absorbance of the test sample.

3.2.16. Storage of lyophilized biomass containing astaxanthin

The lyophilized samples were kept and stored in Ziploc bags in aliquots of 1 g each at -20°C, 4°C, 25 °C, and room temperature. The maximum temperature considered was room temperature (35-37°C) during summer. Astaxanthin was observed for degradation at various storage temperatures, and aluminium foil was wrapped all over the bags to avoid light. The study was carried out in triplicates, and after 4, 8, 12, 16, and 20 weeks, 10-20 mg of samples were withdrawn for observations.

3.2.17. Antibacterial activity

The spot test method on nutrient agar medium plates studied the antibiotic activity of astaxanthin with *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* (Sindhu *et al.*, 1999). About 0.1 ml of bacterial culture was spread over fresh nutrient agar medium plates. The solvent from extracted astaxanthin was evaporated by rotary evaporator. Ten µl of astaxanthin was spotted on each plate. The growth inhibition of bacterial cultures around the spotted astaxanthin was recorded after 48 h incubation at 28±2°C.

3.2.18. Authentication of identification by 18S rDNA gene sequencing

3.2.18.1. DNA extraction: Genomic DNA of the microalgae was extracted using the following procedure:

- In a mortar, 1 mL extraction buffer was used to homogenize the sample. Later the homogenate was transferred to a 2 ml microfuge tube.
- Phenol, chloroform, and isoamyl alcohol were added in a 25:24:1 ratio and gently mixed.
- The tubes were centrifuged at 14,000 rpm for 15 min at room temperature.
- The supernatant was transferred to a new tube containing an equal volume of Chloroform: Isoamyl alcohol (24:1)
- Tubes were then centrifuged for 10 minutes at 14,000 rpm.
- Again, the supernatant was transferred to another tube, and 0.7 ml of isopropanol and 0.1 ml of 3 M sodium acetate (pH 7) were added and incubated for 15 min for DNA precipitation.
- After that, the tubes were centrifuged for 15 minutes at 14,000 rpm at 4°C.
- DNA pellet was rinsed twice using 70% ethanol first and a second time with 100% ethanol.
- Tris-EDTA buffer was used to dissolve the DNA (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).
- 5 µl of DNase-free RNase A (10 mg/ml) was added to remove RNA contamination.

3.2.18.2. 18S rDNA amplification

For amplification of 18s rDNA, universal forward primer 5'-TCCTGAGGGAACTTCG-3' and reverse primer 5'-ACCCGCTGAACTTAAGC-3' were used. The 50 µl reaction mixture contained 1 µl DNA, 2 µl forward primer, 2 µl reverse primer, 2 µl dNTPs (2.5 mM), 4 µl Taq DNA polymerase assay buffer, 10 µl Taq DNA polymerase enzyme (3U/ml), and water. PCR conditions were initial denaturation: 94°C for 3 min, 30 cycles of denaturation (94°C for 1 min), annealing temperature (50°C for 1 min), extension (72°C for 2 min), followed by a final extension (72°C for 7 min). The PCR products were kept on hold at 4°C for 24 h. Gel electrophoresis of the PCR product was undertaken.

3.2.18.3. Sequencing

PCR products were purified and sequenced from a commercial facility (BioKart India Pvt. Ltd., Bengaluru, India) using Sanger sequencing.

3.2.18.4. Phylogenetic tree

The sequence obtained was deposited to NCBI, and the accession numbers were obtained. The phylogenetic tree was constructed by the maximum likelihood method using MEGA-X software.

3.2.19. Statistical analysis

The experimental data were analyzed using OPSTAT software available online on the university home page.

Data are represented as a mean and standard error where required.

4.1. Collection of samples

Three different sites with possibilities of microalgae producing red pigment were identified for sample collection located in Himachal Pradesh and Haryana (Plate 1). The location of freshwater bodies was presented in Table 1.



Plate 1: Freshwater pond with red cyst cells (A, B, C-Palampur, H.P. and D-Tohana, HR.)

Table 1: Isolation of microalgal strains from different regions of Himachal Pradesh and Haryana

Sr. No.	Isolate	Location	GPS coordinates
1	PLM1	Palampur, Himachal Pradesh	32°06'03.4"N 76°33'02.8"E
2	PLM2	Palampur, Himachal Pradesh	32°06'09.1"N 76°33'06.0"E
3	PLM3	Palampur, Himachal Pradesh	32°06'09.1"N 76°33'06.0"E
4	HAR1	Tohana, Haryana	29°35'16.1"N 75°56'07.9"E
5	HAR2	Tohana, Haryana	29°35'16.1"N 75°56'07.9"E

4.2. Isolation of microalgal strains: A total of five microalgal strains were isolated using BG-11 medium and coded as PLM1, PLM2, PLM3, HAR1, and HAR2 (Table 1). All five isolates were screened for astaxanthin production, and PLM1 and PLM2 isolates were found to be astaxanthin producers. However, only isolate PLM2 was investigated further due to its better astaxanthin production potential.

4.3. Morphological studies and life cycle of isolate PLM2: Isolate PLM2 is a unicellular green alga with a complex life history, commonly divided into two phases based on cell morphology and physiology: green vegetative phase and encysted red phase. Vegetative cells were spherical, ellipsoidal, or pear-shaped and between 8 and 20 μm long with a distinct gelatinous extracellular matrix of variable thickness (Plate 2 A-H). Under unfavorable environmental or cultural conditions, vegetative cell expands their cell size. They form an amorphous multilayered structure in the inner regions of the extracellular matrix or the primary cell wall as they developed into “palmella” and became resting vegetative cells (Plate 2 I-K). With the continued environmental stress (i.e., nutrient deprivation, high light irradiance, high salinity) and cessation of cell division, “palmella” transformed into the asexual “aplanospores.” At this stage, cells contain two distinct structures, a thick and rigid trilaminar sheath and a secondary cell wall of acetolysis-resistant material. Such cells became resistant to prevailing extreme environmental conditions (Plate 2 L). Aplanospore accumulated large amounts of secondary carotenoids, particularly astaxanthin, in lipid droplets deposited in the cytoplasm, which resulted in a characteristic bright orange-red color of these cells (Plate 2 M).

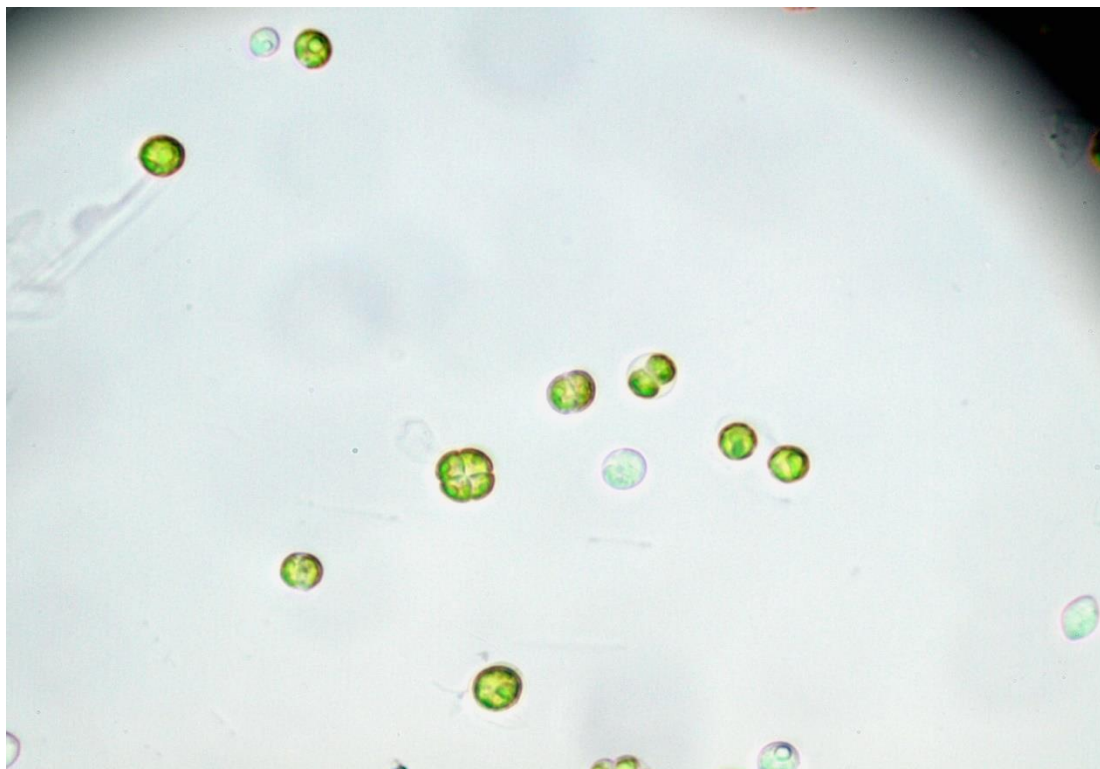


Plate 2(A): Vegetative reproduction by cell division exhibited by isolate PLM2

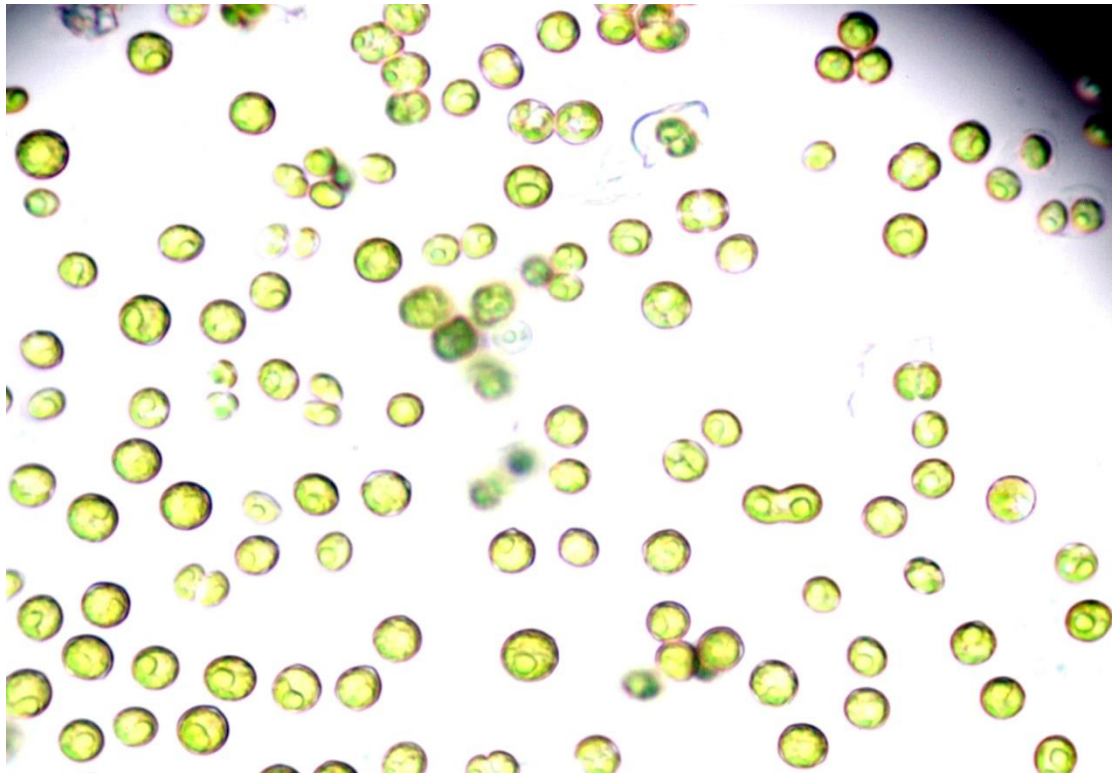


Plate 2(B). Sporangium with two cells of isolate PLM2

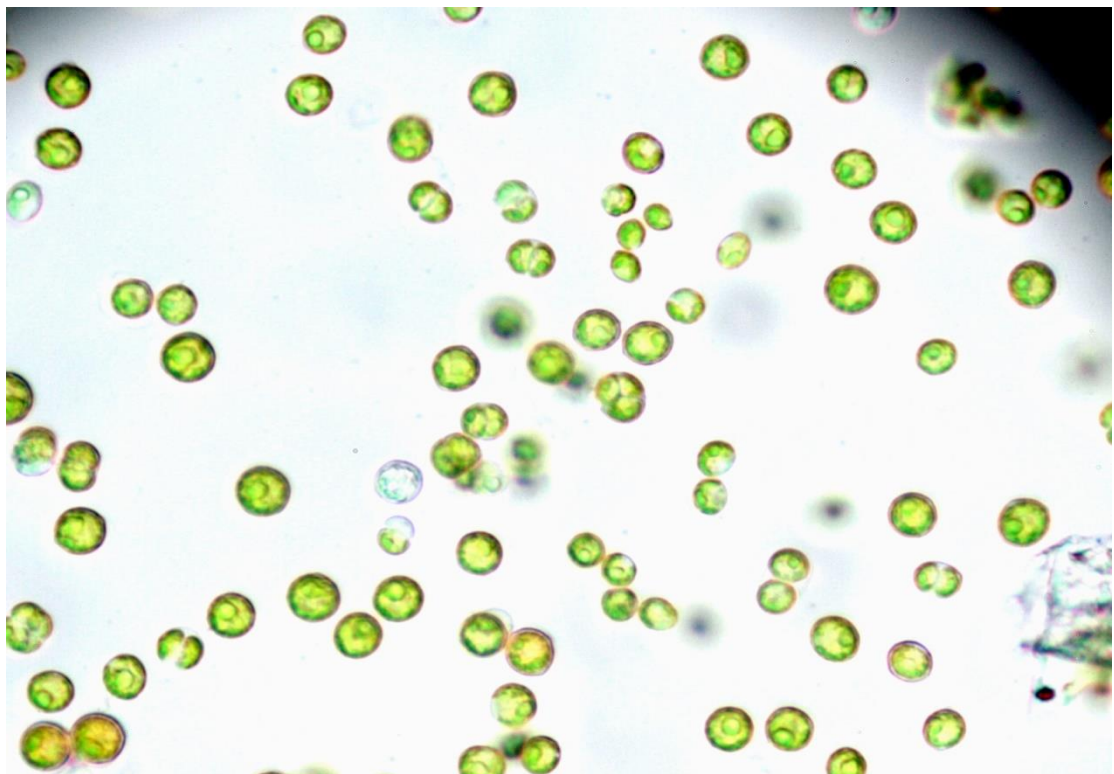


Plate 2(C). The first division of protoplast where mother cells get divided into two cells in isolate PLM2

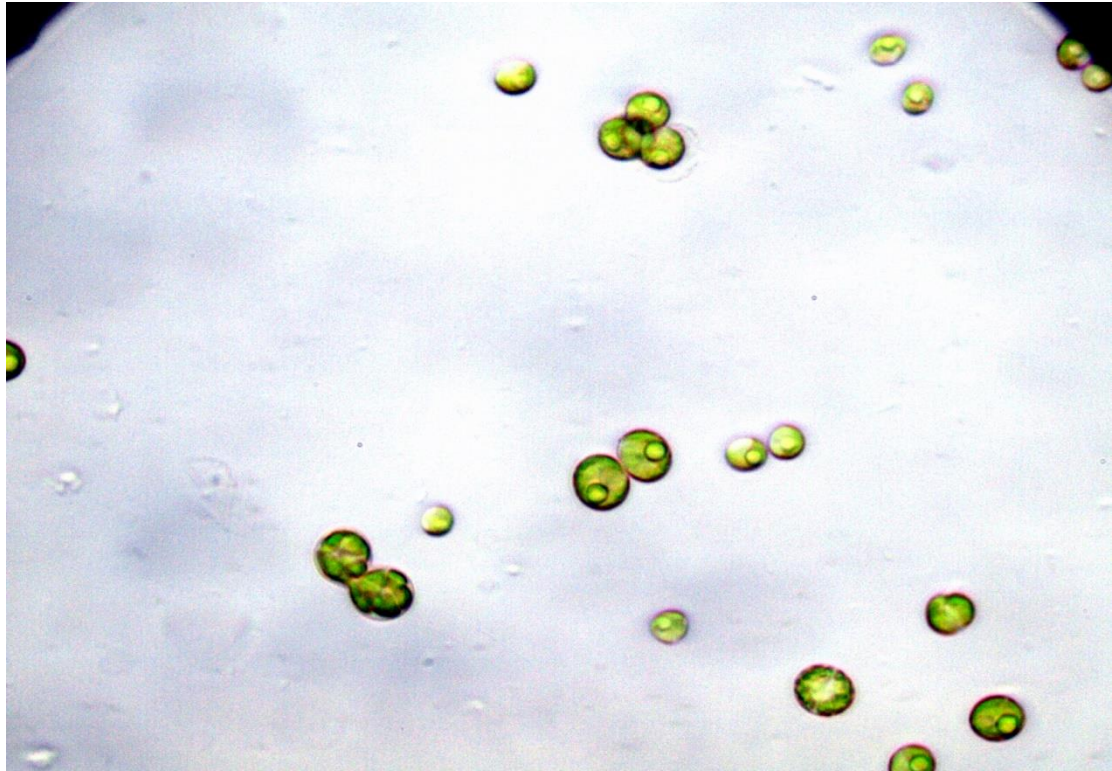


Plate 2(D). Putative isogamous sexual process in isolate PLM2

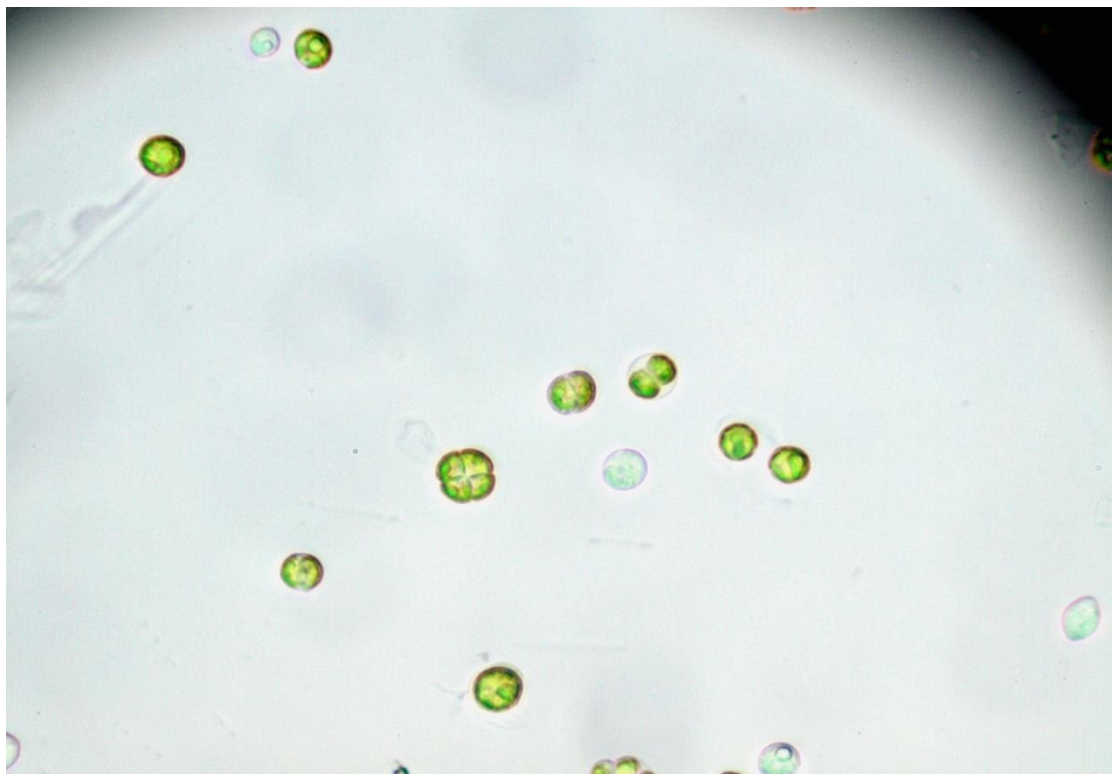


Plate 2(E). The second division of protoplast where two cells get divided into four cells in isolate PLM2

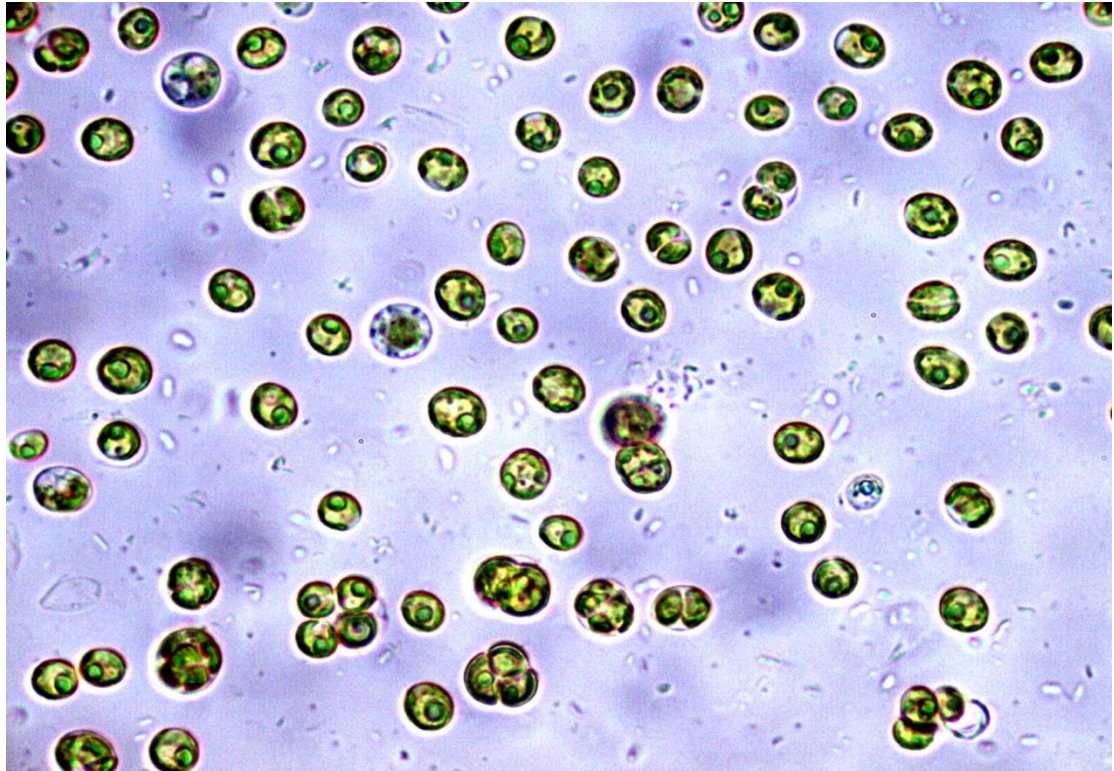


Plate 2(F). Sporangium with four cells in isolate PLM2

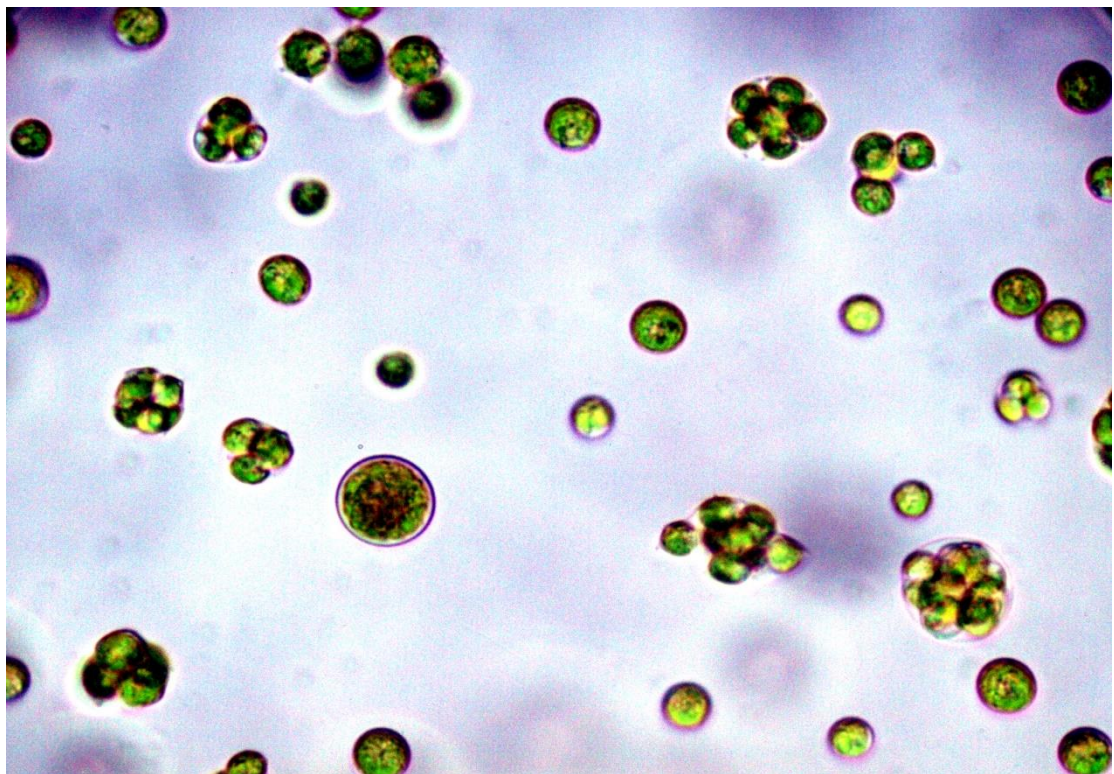


Plate 2(G). The third division of protoplast where four cells get divided into eight cells in isolate PLM2

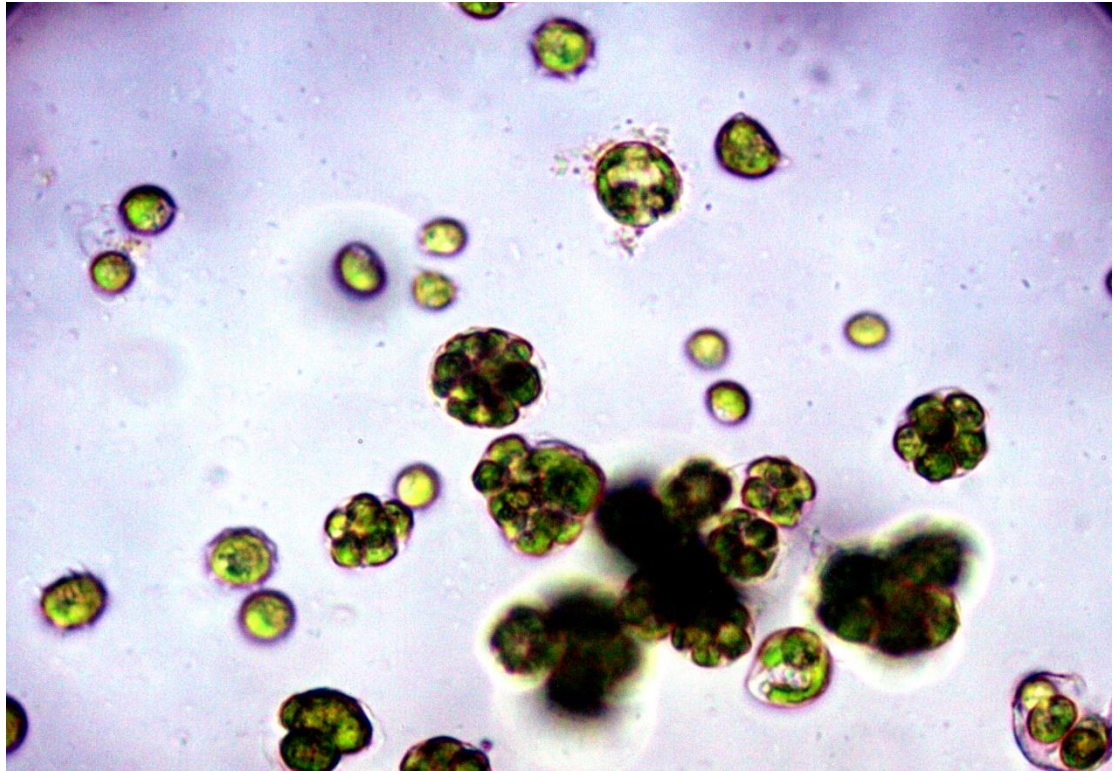


Plate 2(H). Forth division of protoplast where eight cells get divided into sixteen cells in isolate PLM2

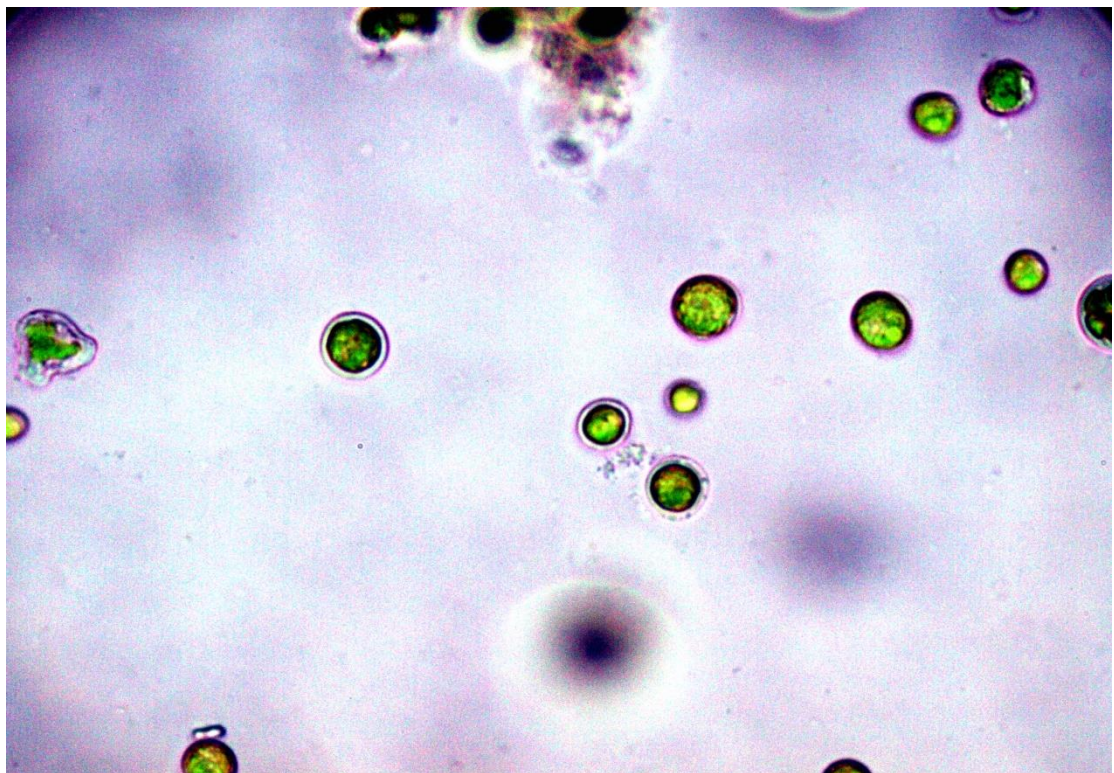


Plate 2(I). Isolate PLM2 in green young palmelloid stage

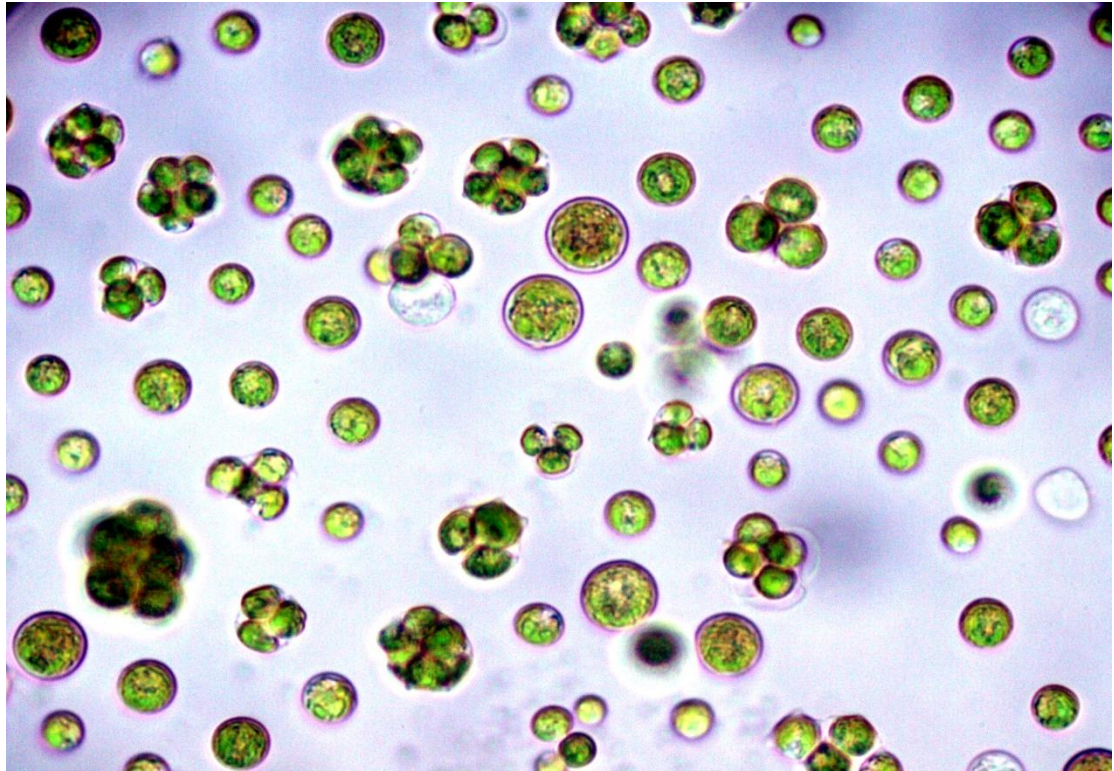


Plate 2(J). Isolate PLM2 in green adult palmelloid stage

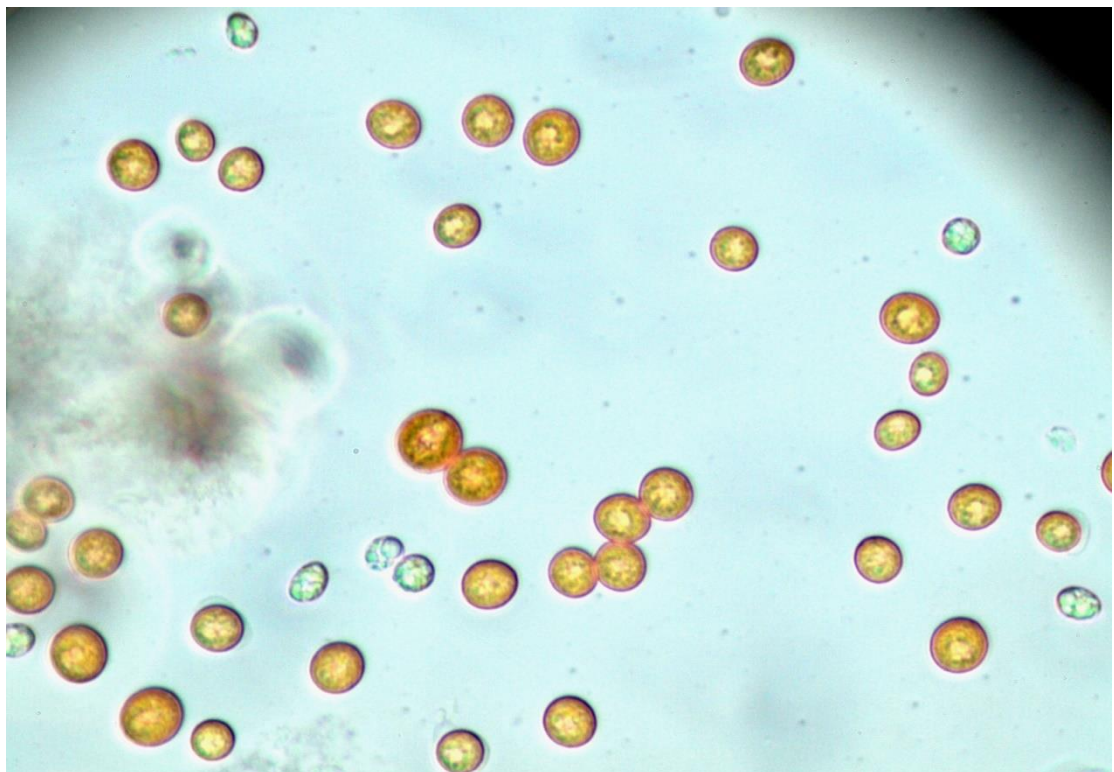


Plate 2(K). Isolate PLM2 in brownish adult palmelloid stage

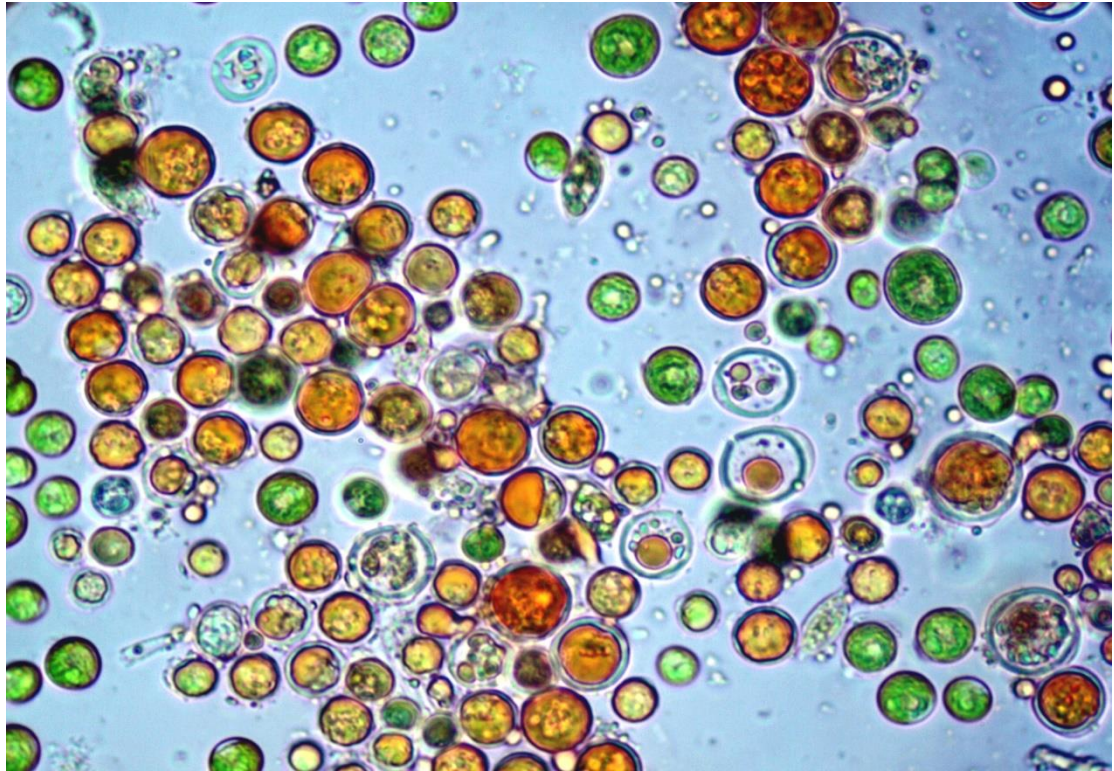


Plate 2(L). Isolate PLM2 green cells transformation into cysts form

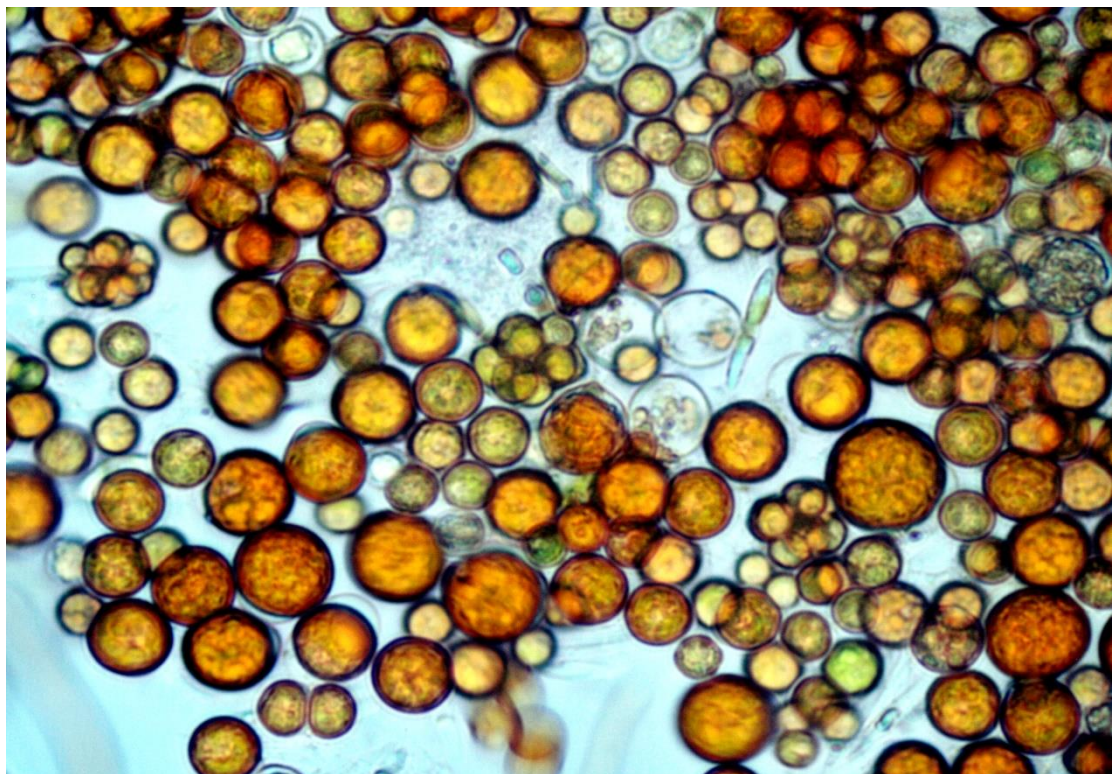


Plate 2(M). Isolate PLM2 in the red cyst stage

4.3.1. Ultrastructure

Field Emission-Scanning Electron Microscopy (FE-SEM) was conducted to observe the size and detailed cell structure. Isolate PLM2 had a spherical shape and 5-20 μm in diameter as predicted using FE-SEM (Plate 3 A, B, C). The red cysts were spherical and had a diameter of 10-50 μm (Plate 3 D). The red cysts cell showed defects on its surface (Plate 3 E), and the perforations were quite prominent in some cells (Plate 3 F).

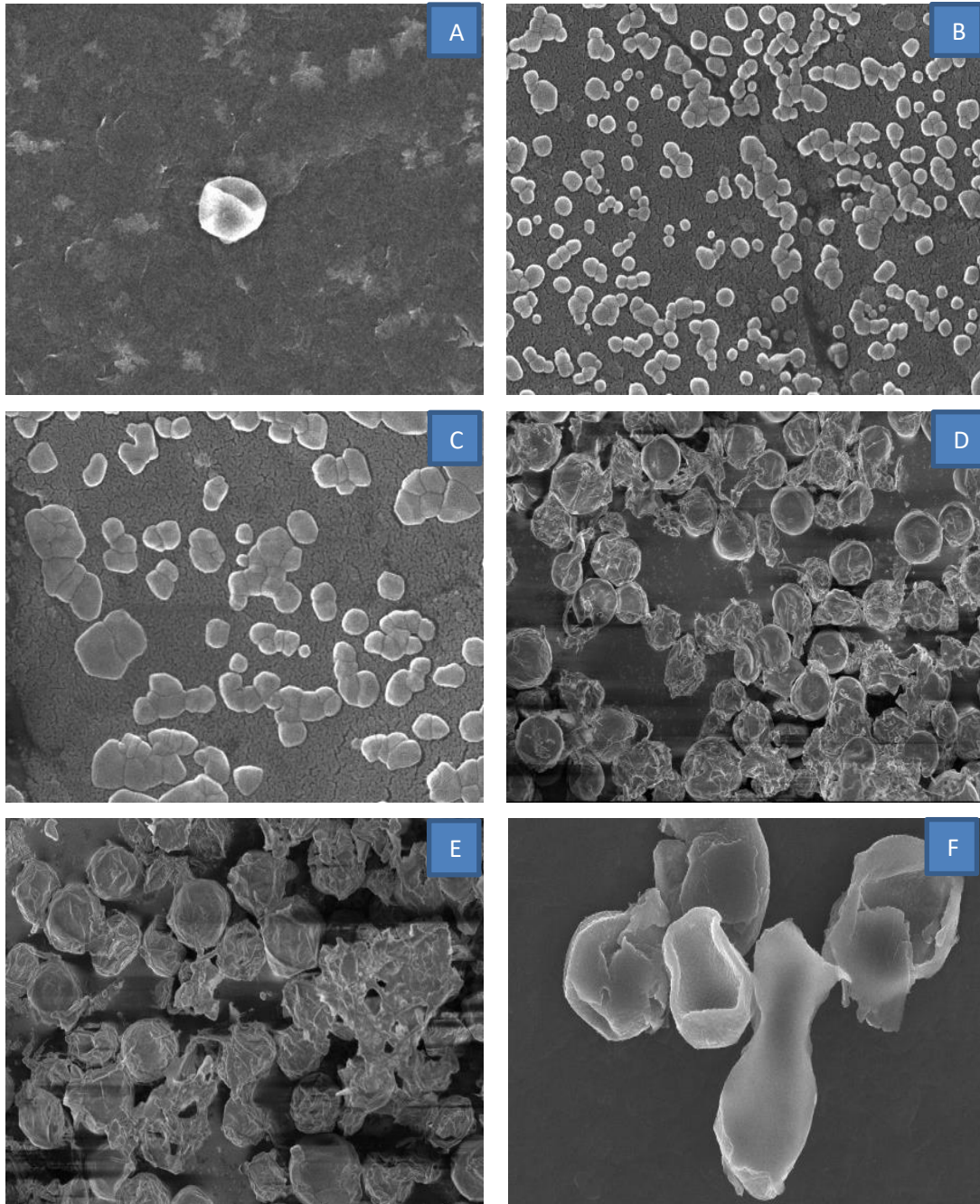


Plate 3. Field Emission Scanning Electron Microscopic (FE-SEM) images of green vegetative cells (A, B, and C) and red cysts of isolate PLM2 (D, E, and F)

4.4. Molecular identification of microalgal strains: PLM1 and PLM2 were identified using sequencing of 18S rRNA and ITS region. PLM1 was identified as *Desmodesmus* sp. nov. PLM1, while PLM2 was identified as *Desmodesmus* sp. nov. PLM2. Figure 5 and 6 represented the phylogenetic tree of *Desmodesmus* sp. nov. PLM1 and *Desmodesmus* sp. nov. PLM2 created by the neighbor-joining method using MEGA-X software. *Desmodesmus* sp. nov. PLM1 formed a separate clade and was supposed to be a novel species. *Desmodesmus* sp. nov. PLM2 was also considered to be a novel species. The 18S rDNA sequences were submitted to the NCBI database and received accession numbers accordingly (*Desmodesmus* sp. nov. PLM1-OL764500, *Desmodesmus* sp. nov. PLM2-OL764502 and for ITS of *Desmodesmus* sp. nov. PLM2-OM060397). Microalgal strains were deposited as novel species at the National Collection of Industrial Microorganisms (NCIM, Pune) with accession numbers NCIM-5807 and NCIM-5808, respectively.

Taxonomic classification of *Desmodesmus* sp. proposed as under

Kingdom : Plantae
 Subkingdom : Viridiplantae
 Phylum : Chlorophyta
 Class : Chlorophyceae
 Order : Sphaeropleales
 Family : Scenedesmaceae
 Genus : *Desmodesmus*
 Species : Unclassified

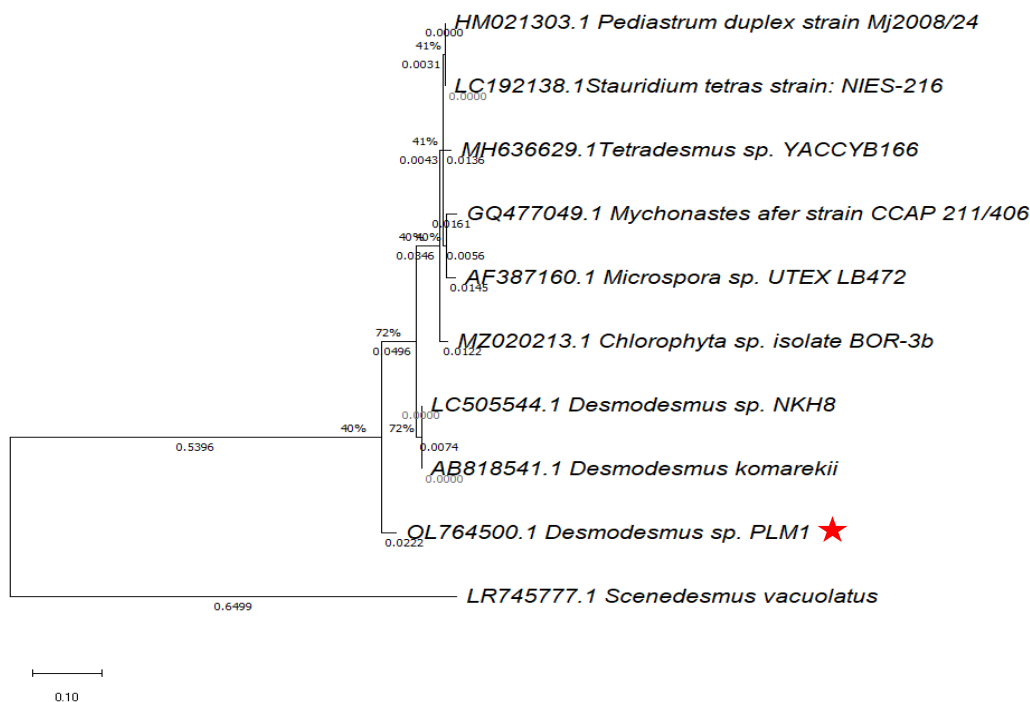
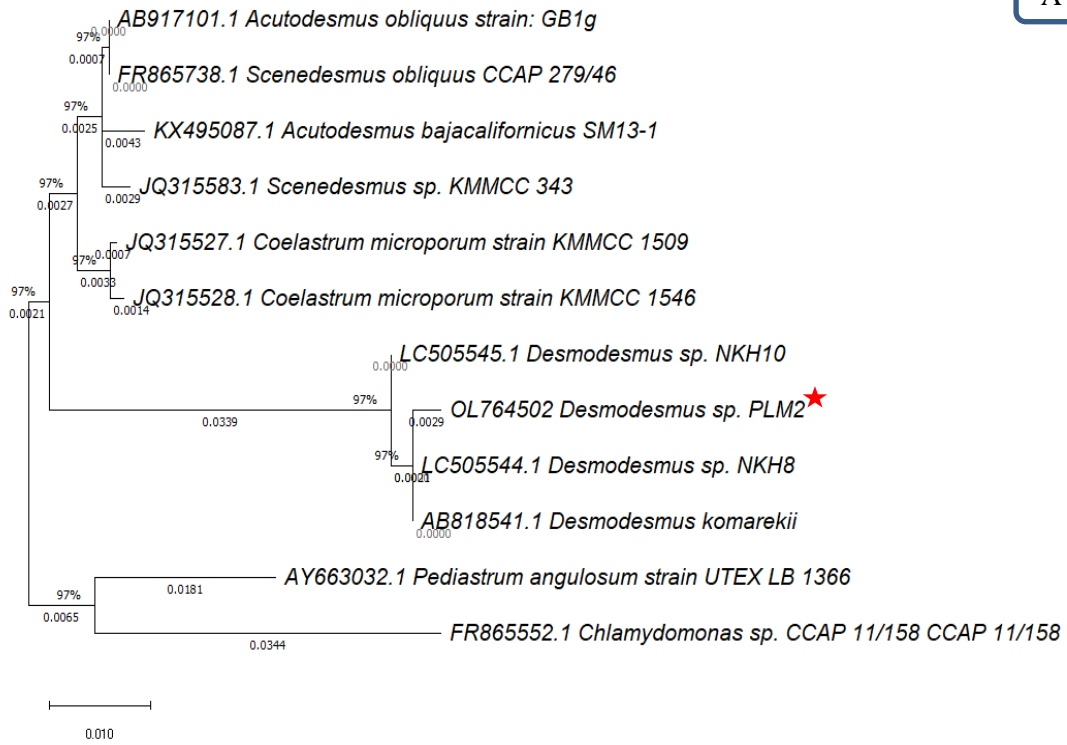


Figure 5. Phylogenetic tree of *Desmodesmus* sp. nov. PLM1 (OL764500)

A



B

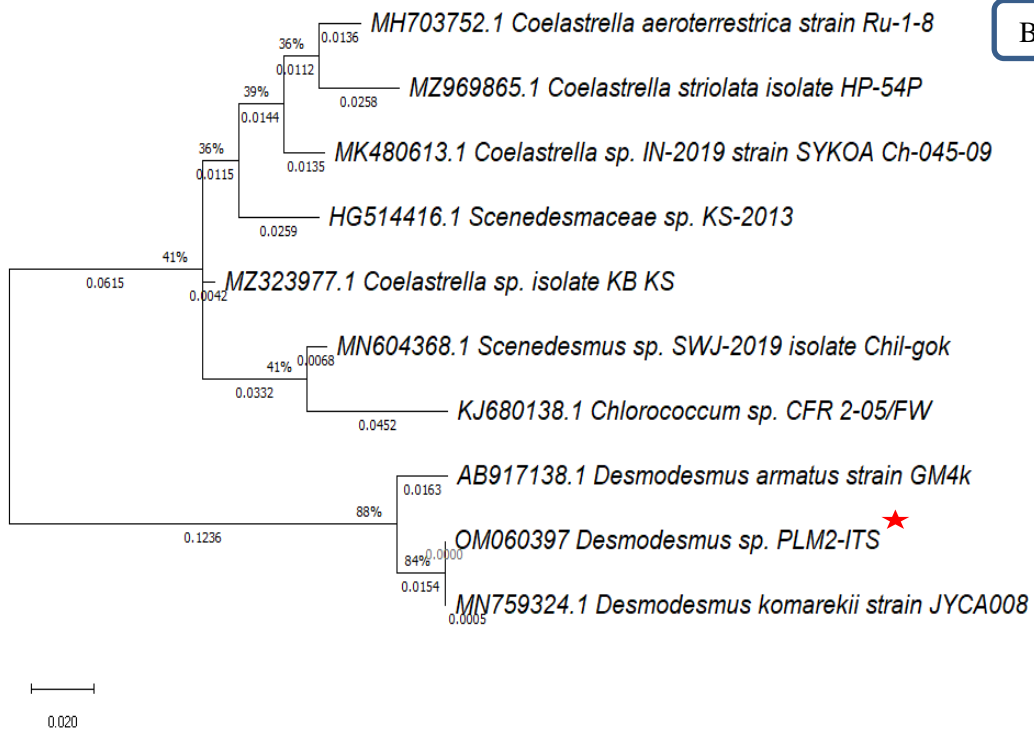


Figure 6. Phylogenetic tree of *Desmodesmus* sp. nov. PLM2 (A) 18S rDNA (OL764502) and (B) ITS (OM060397)

4.5. Monitoring of initial growth of *Desmodesmus* sp. PLM2:

Desmodesmus sp. PLM2 was grown in standard BG-11 media and incubated at 25°C with manual shaking twice a day in the presence of continuous white fluorescent light intensity of 1500 lux. The growth absorbance was recorded daily at 680 nm. The lag phase was from the 1-4th day, the exponential phase from the 4-12th day, the stationary phase from the 12-20th day, and the death phase from the 20th day onwards (Figure 7).

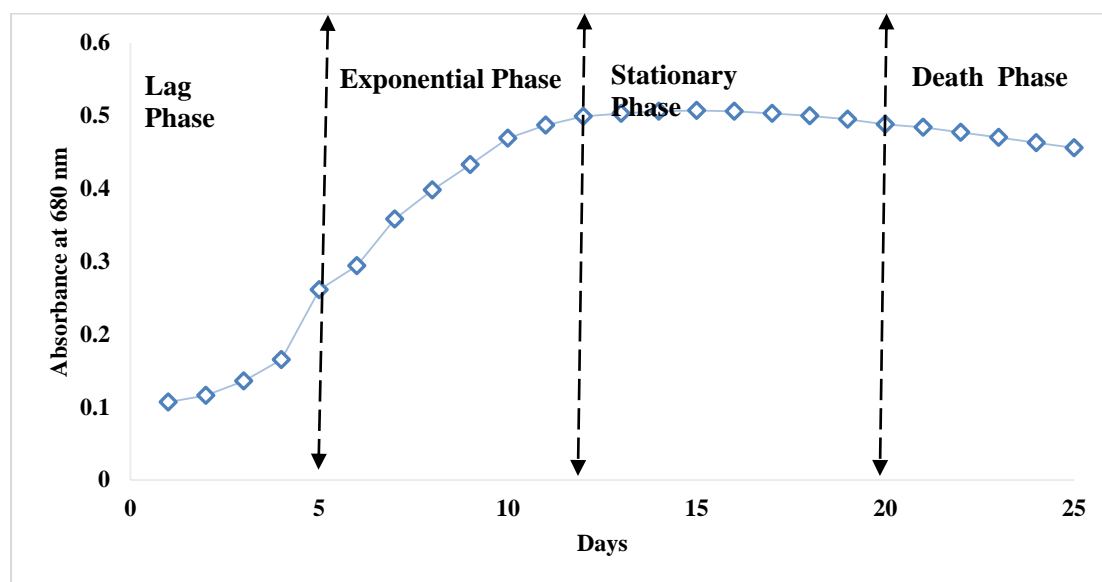


Figure 7. Initial growth curve of *Desmodesmus* sp. PLM2

4.6. Optimization of cultural conditions for biomass production

4.6.1 Effect of different nitrogen sources:

Three nitrogen sources, *i.e.*, potassium nitrate (KNO_3), sodium nitrate (NaNO_3), and ammonium nitrate (NH_4NO_3) were used for biomass production at variable concentrations, *i.e.*, 10 mM, 15 mM, 20 mM, 25 mM, and 30 mM. Effect of different nitrogen sources on growth, biomass yield, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid were observed.

4.6.1.1. Growth and biomass yield of *Desmodesmus* sp. PLM2: Sodium nitrate at 20mM concentration resulted in maximum growth and biomass yield, *i.e.*, 3.6 mg/ml, followed by sodium nitrate at 25 mM concentration, *i.e.*, 3.2 mg/ml. The lowest growth and biomass yield was exhibited using potassium nitrate (Figure 8, 9).

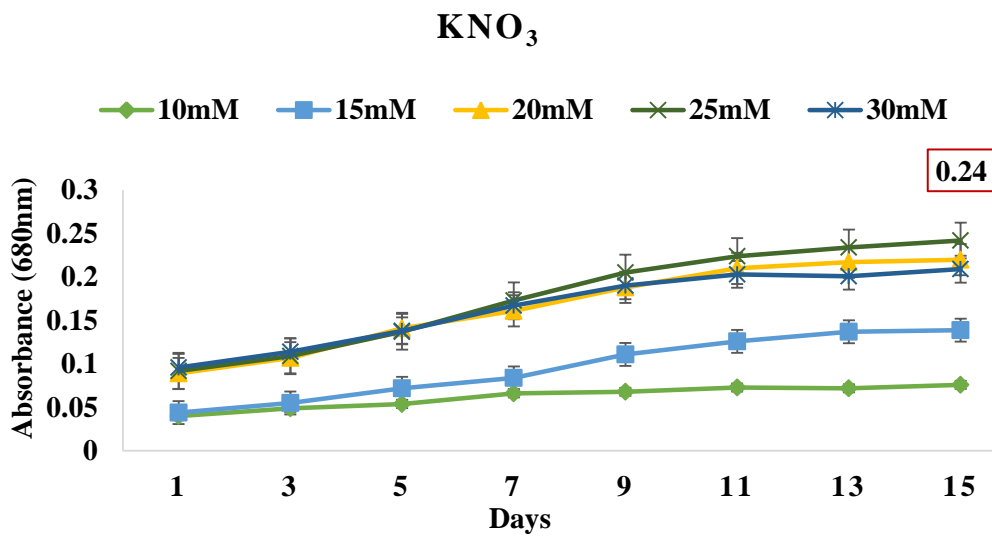
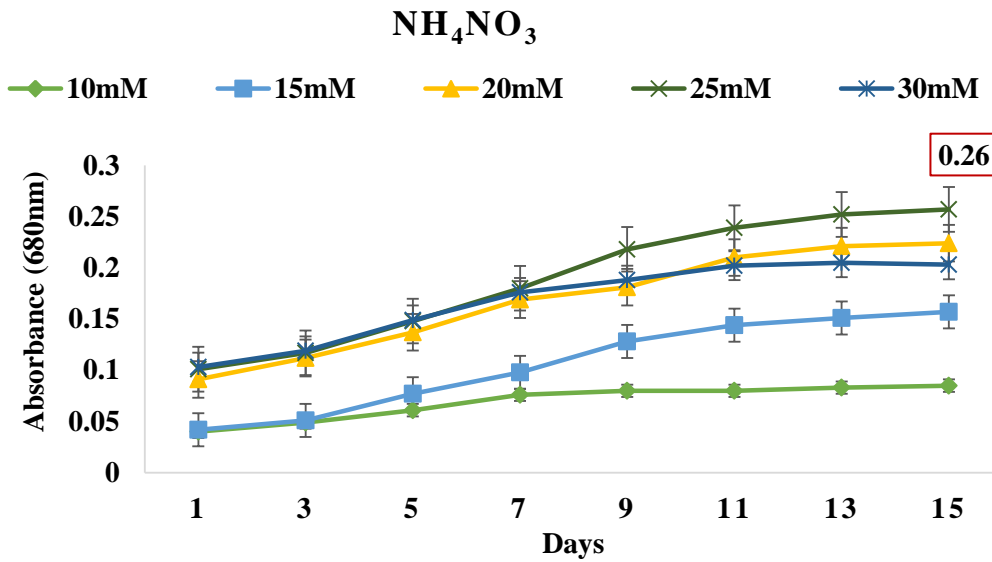
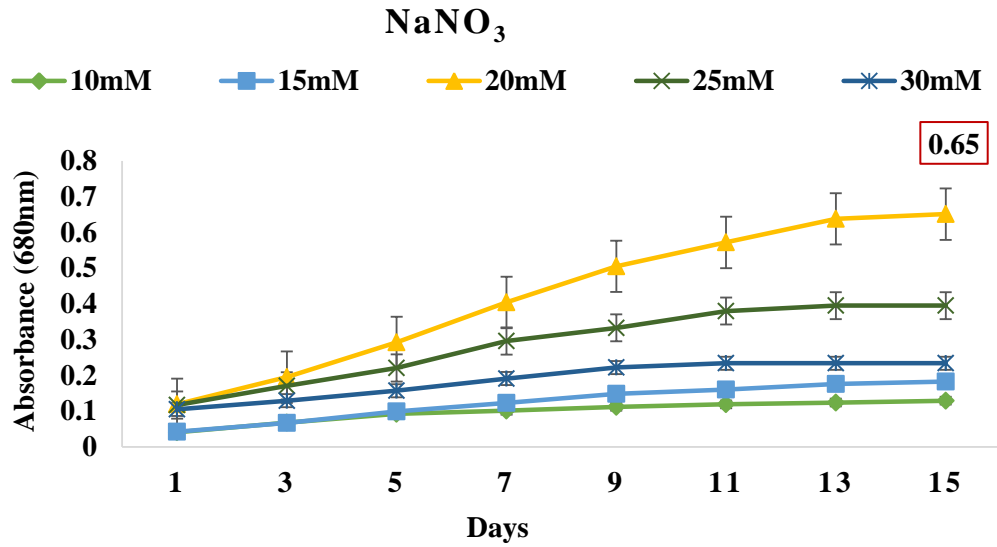


Figure 8. Effect of different concentrations of nitrogen sources on growth of *Desmodemus* sp. PLM2

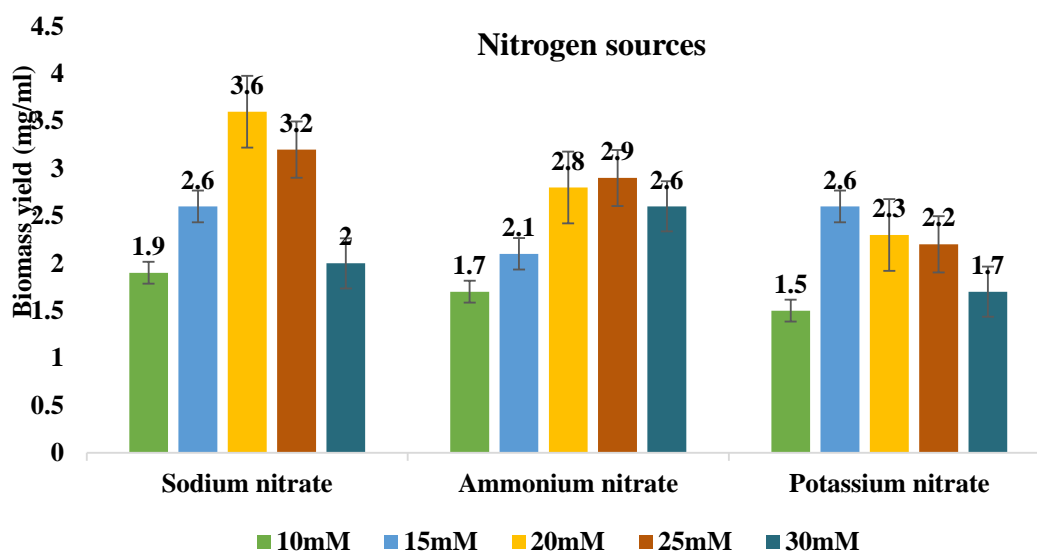


Figure 9. Effect of different nitrogen sources on biomass yield of *Desmodium* sp. PLM2

4.6.1.2. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content of *Desmodium* sp. PLM2: Sodium nitrate at 20 mM concentration resulted in maximum chlorophyll a (9.32 $\mu\text{g/ml}$), chlorophyll b (3.77 $\mu\text{g/ml}$), total chlorophyll (13.10 $\mu\text{g/ml}$) and total carotenoid (3.64 $\mu\text{g/ml}$) on 15th day followed by sodium nitrate at 15 mM concentration (chlorophyll a (5.83 $\mu\text{g/ml}$), chlorophyll b (3.06 $\mu\text{g/ml}$), total chlorophyll (7.69 $\mu\text{g/ml}$) and total carotenoid (2.10 $\mu\text{g/ml}$). The lowest chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content were exhibited by using potassium nitrate (Figure 10, 11, 12, 13).

4.6.2. Effect of different carbon sources:

Four different carbon sources, *i.e.*, sodium carbonate (Na_2CO_3), di-potassium carbonate (K_2CO_3), sodium bicarbonate (NaHCO_3), and glucose were used for biomass production at variable concentrations, *i.e.*, 3 mM, 5 mM, and 7 mM. Effects of these carbon sources on growth, biomass yield, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids were observed.

4.6.2.1. Growth and biomass yield of *Desmodium* sp. PLM2: Glucose at 3 mM concentration resulted in maximum growth (Figure 14), and biomass yield (4.1 mg/ml) which was observed on the 15th day, followed by glucose at 5 mM concentration (3.2 mg/ml). The lowest growth and biomass (1.4 mg/ml) were exhibited by using di-potassium carbonate at a 7 mM concentration of *Desmodium* sp. PLM2 (Figure 15).

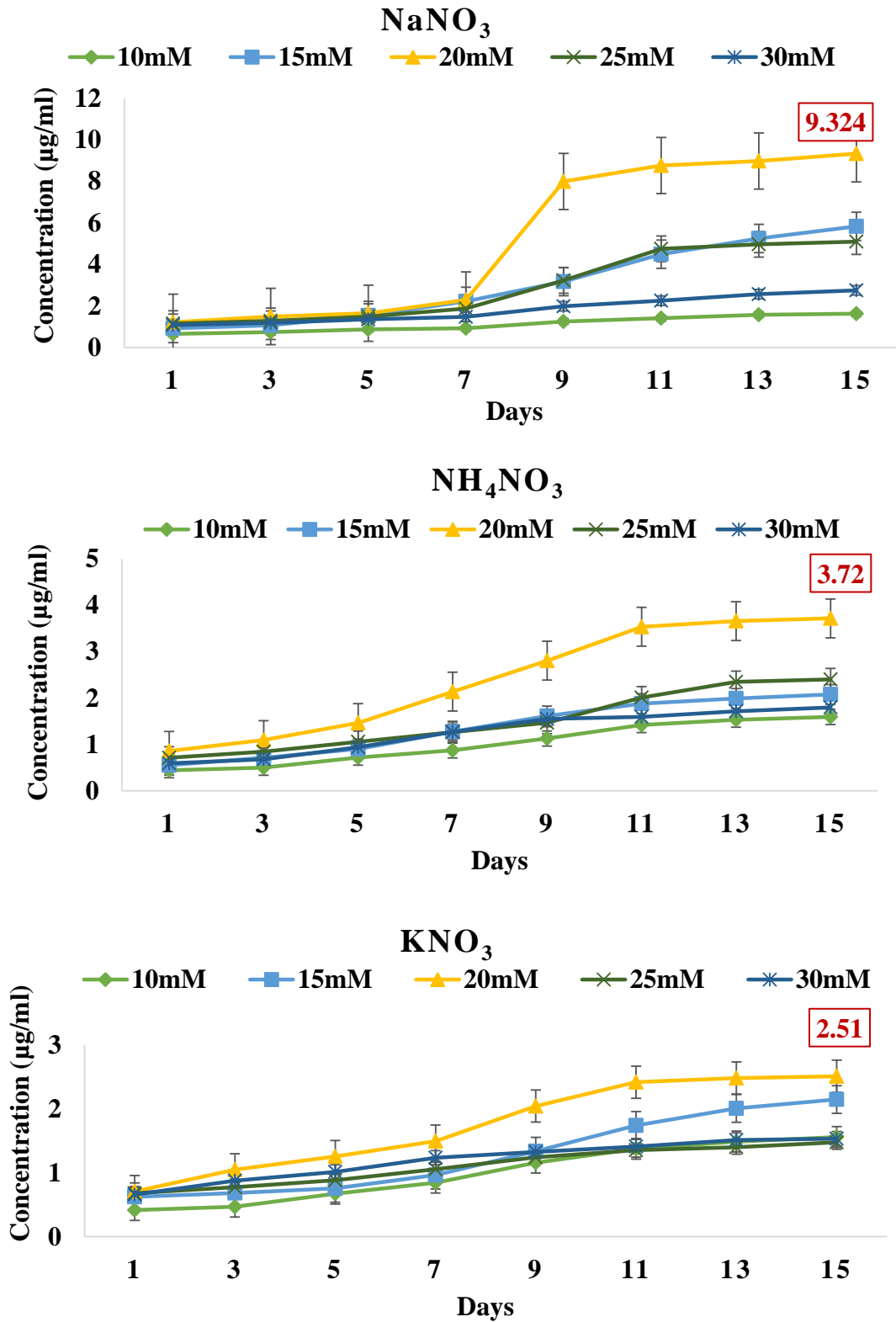


Figure 10. Effect of different nitrogen sources on chlorophyll a content in *Desmodemus* sp. PLM2

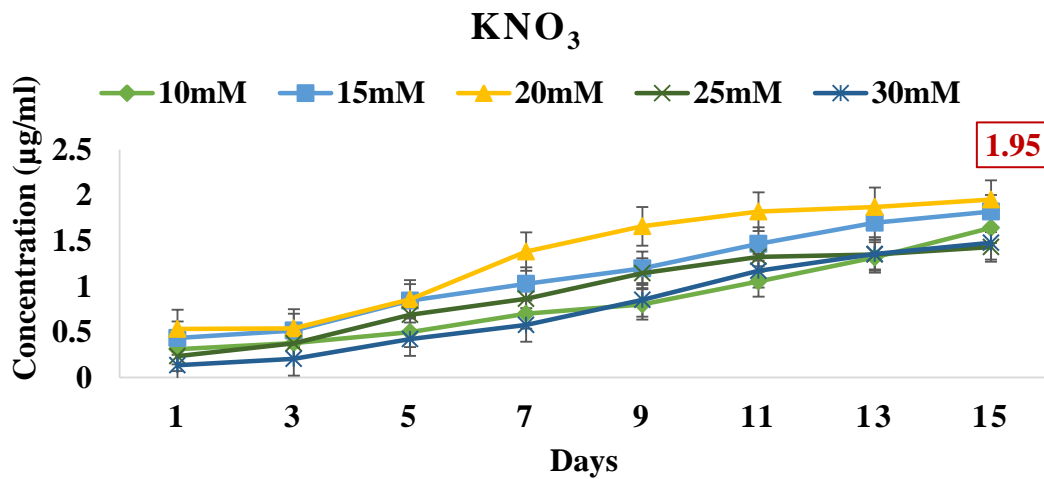
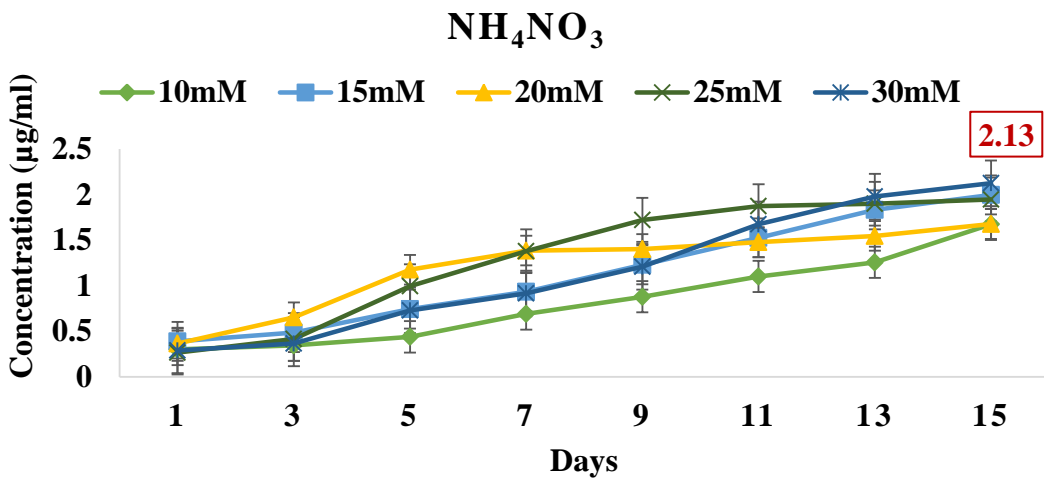
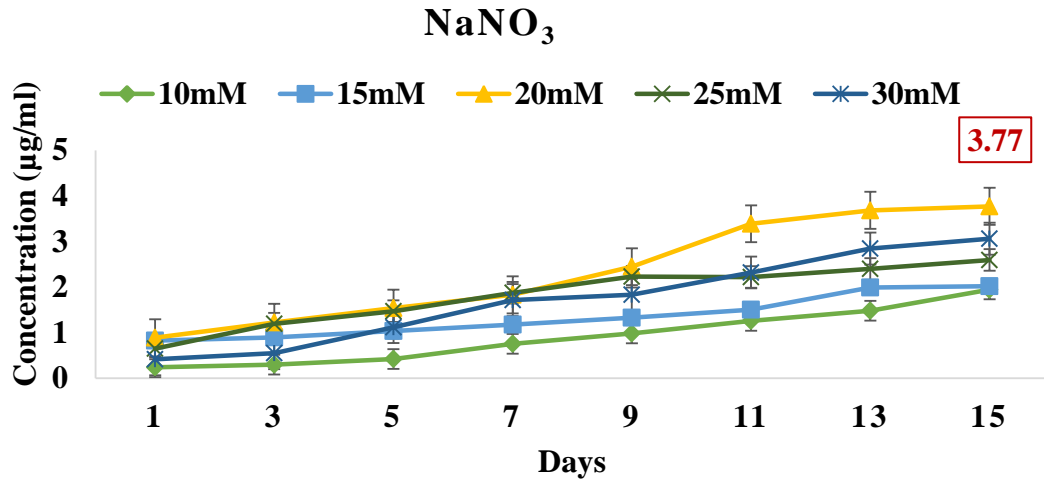


Figure 11. Effect of different nitrogen sources on chlorophyll b content in *Desmodemus* sp. PLM2

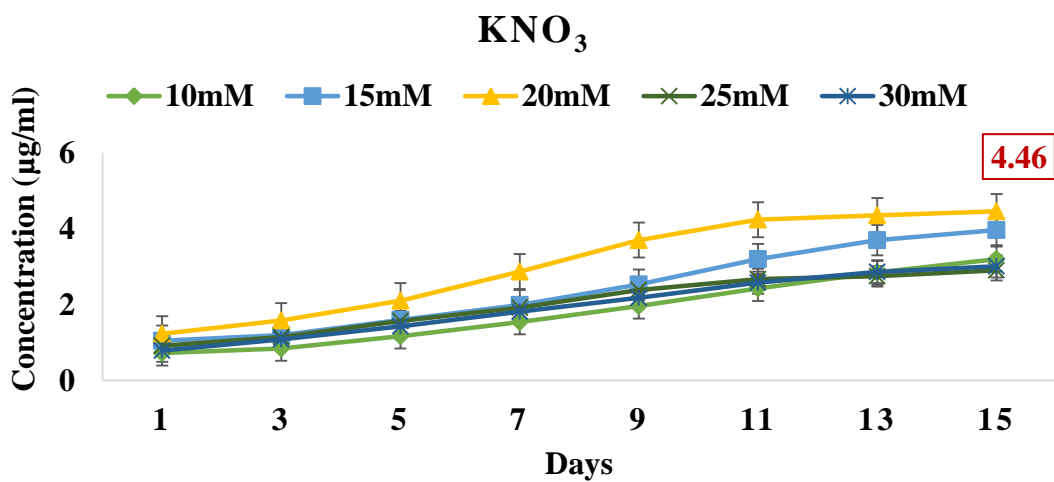
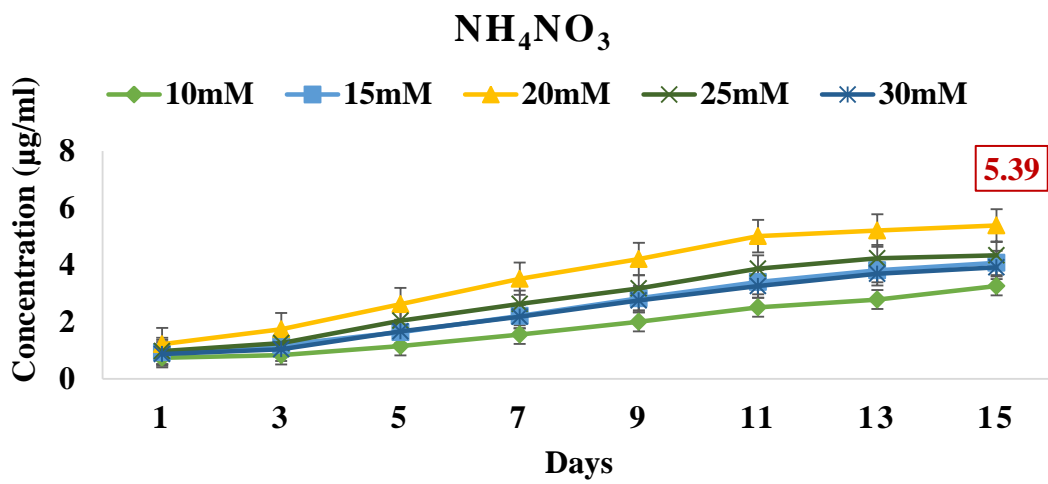
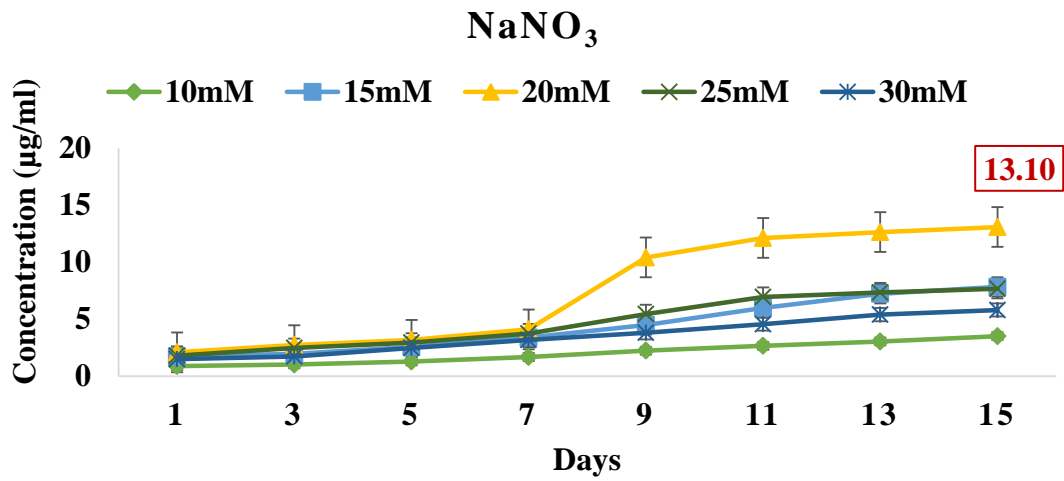


Figure 12. Effect of different nitrogen sources on total chlorophyll content in *Desmodesmus* sp. PLM2

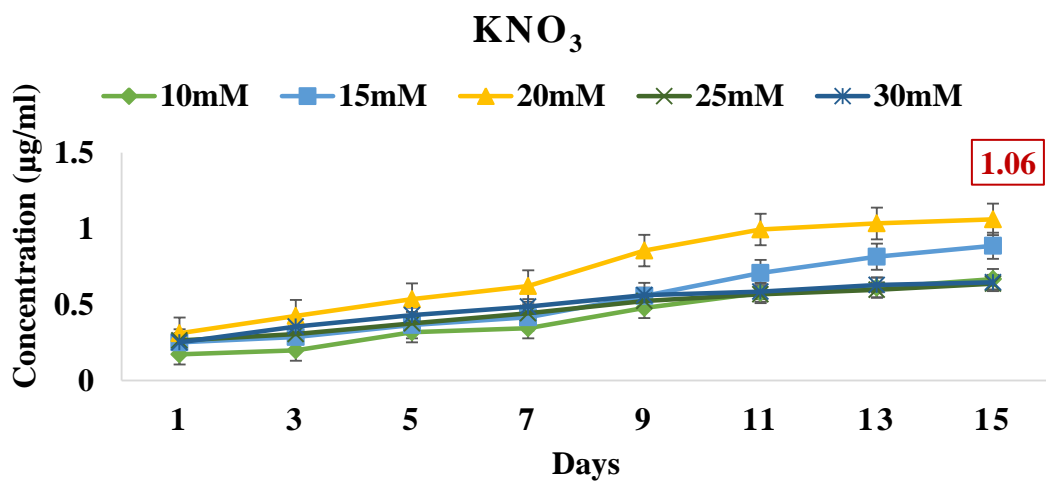
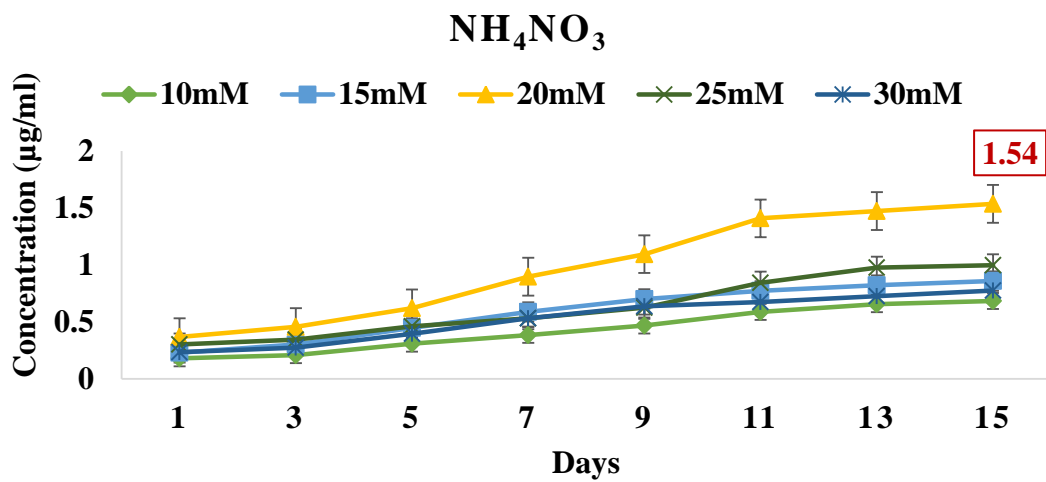
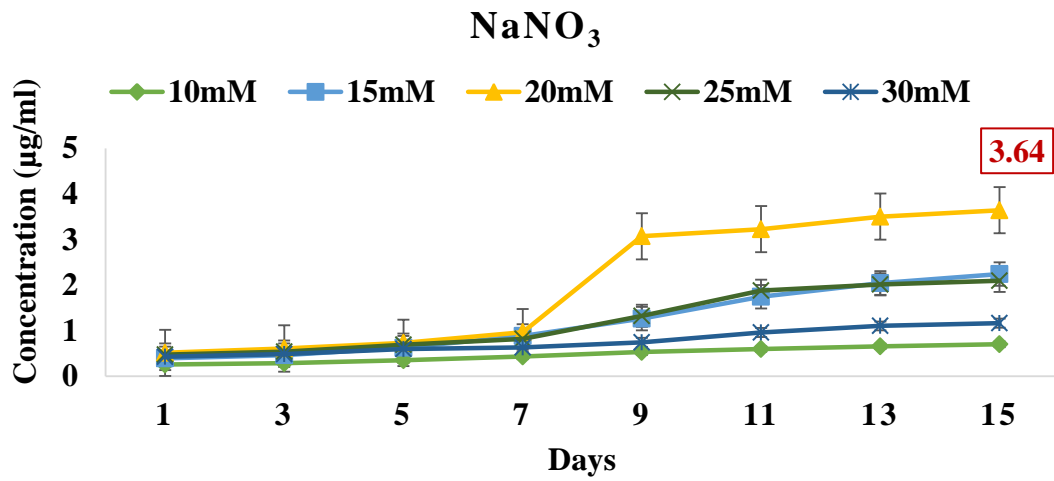


Figure 13 Effect of different nitrogen sources on total carotenoid content of *Desmodemus* sp. PLM2

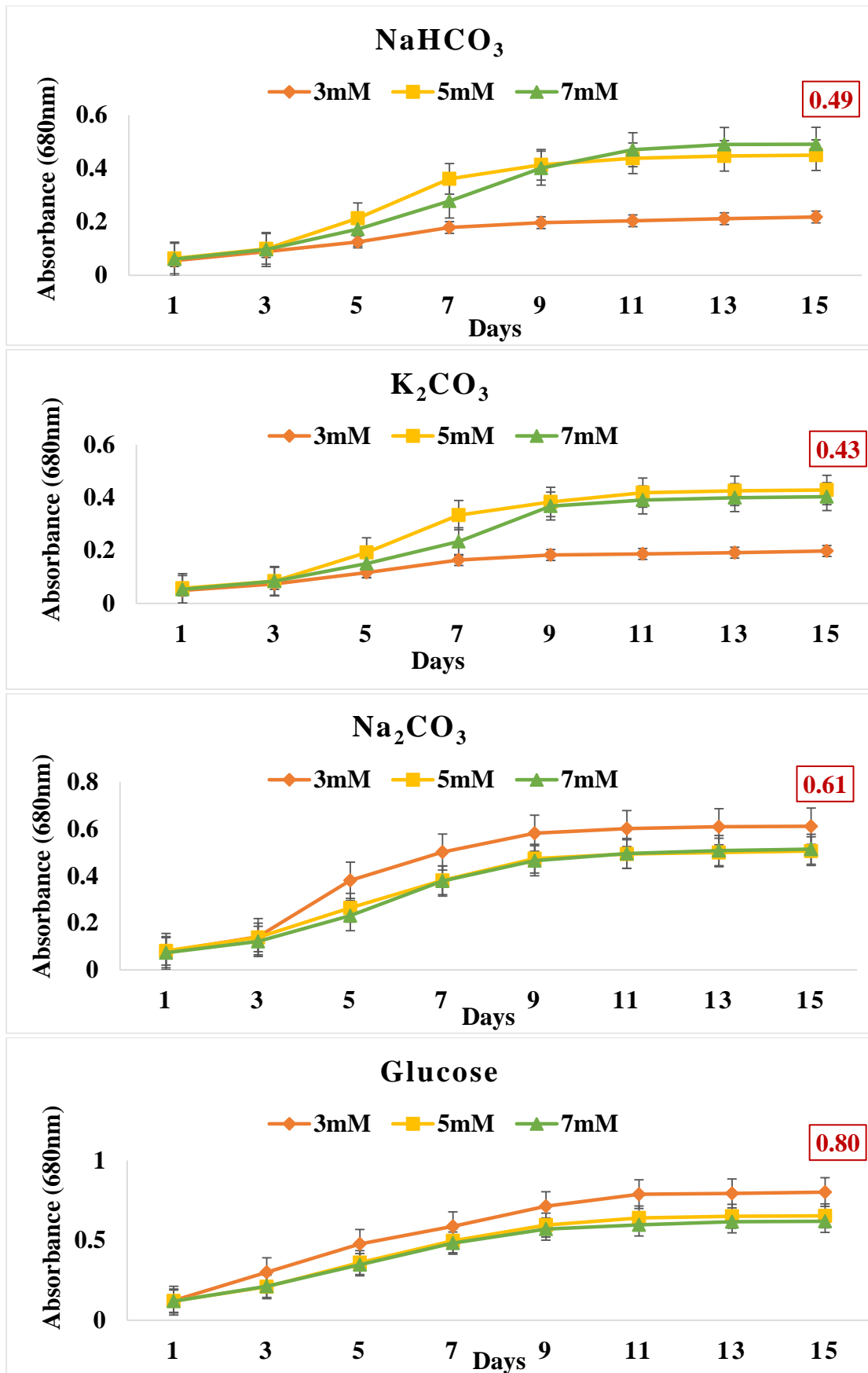


Figure 14. Effect of different concentrations of carbon sources on growth of *Desmodemus* sp. PLM2

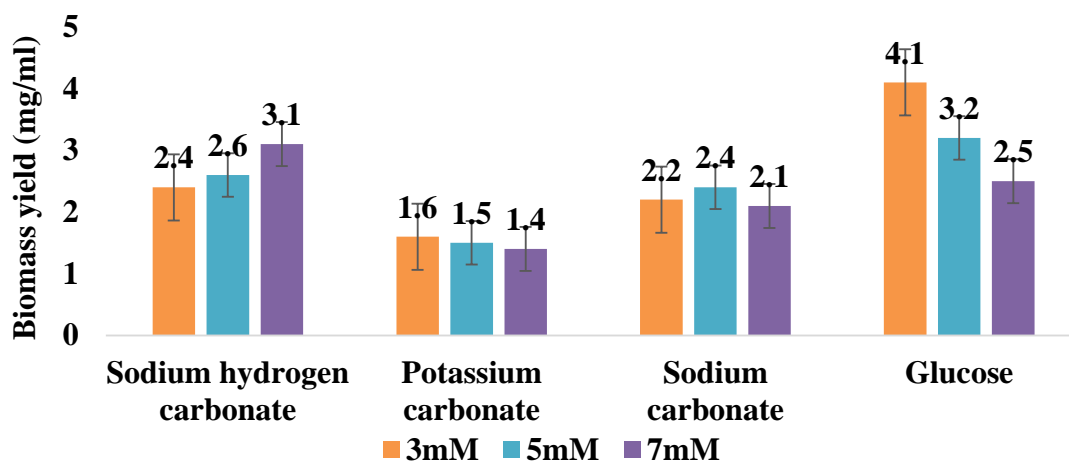


Figure 15. Effect of different carbon sources on biomass yield of *Desmodium sp. PLM2*

4.6.2.3. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content of *Desmodium sp. PLM2*: Glucose at 3 mM concentration resulted in maximum chlorophyll a (8.18 µg/ml), chlorophyll b (3.52 µg/ml) and total chlorophyll content (11.69 µg/ml) followed by sodium bicarbonate at 3 mM concentration (7.98 µg/ml, 3.38 µg/ml, 11.36 µg/ml, respectively). Minimum chlorophyll a, chlorophyll b and total chlorophyll were exhibited by using di-potassium carbonate at 7 mM concentration (3.69 µg/ml, 1.57 µg/ml, 5.31 µg/ml) (Figure16, 17, 18).

Carotenoid content also followed the same trend. Glucose at 3 mM concentration resulted in maximum total carotenoid content (3.51 µg/ml) followed by sodium bicarbonate at 3 mM concentration (3.11 µg/ml). Minimum total carotenoid content was exhibited by using di-potassium carbonate at 7 mM concentration (1.52 µg/ml) (Figure 19).

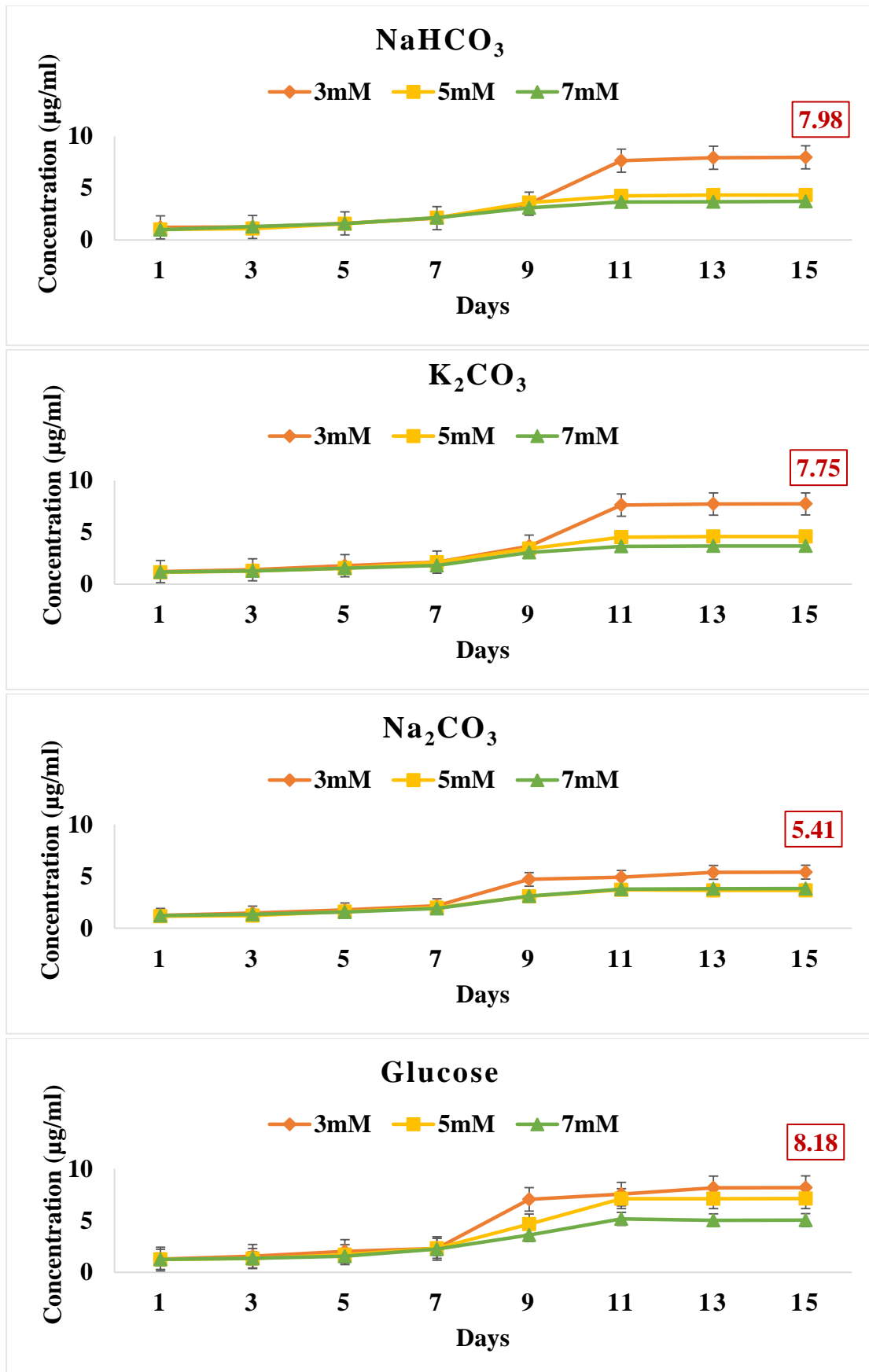


Figure 16. Effect of different carbon sources on chlorophyll a content in *Desmodemus* sp. PLM2

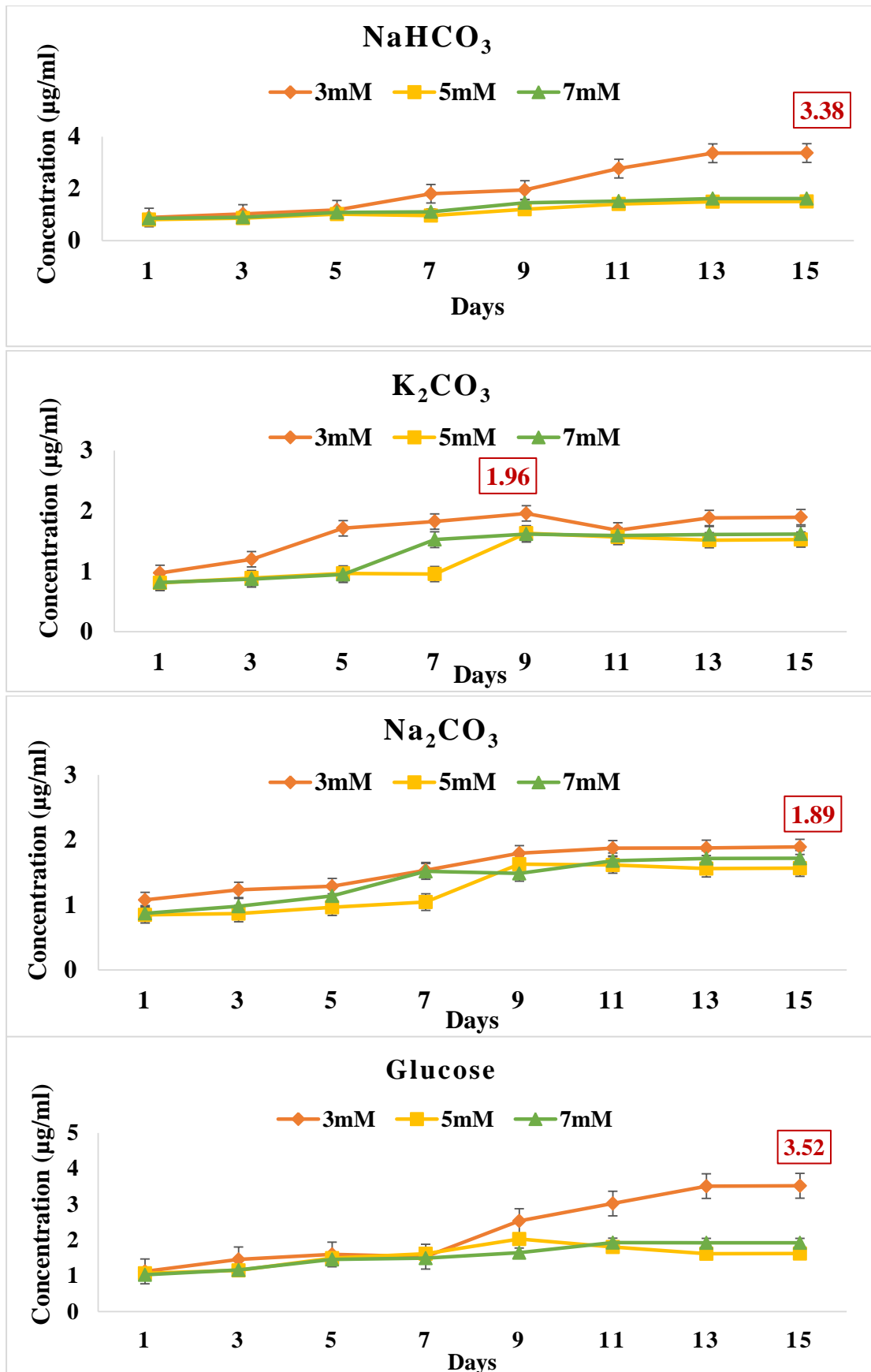


Figure 17. Effect of different carbon sources on chlorophyll b content in *Desmodemus* sp. PLM2

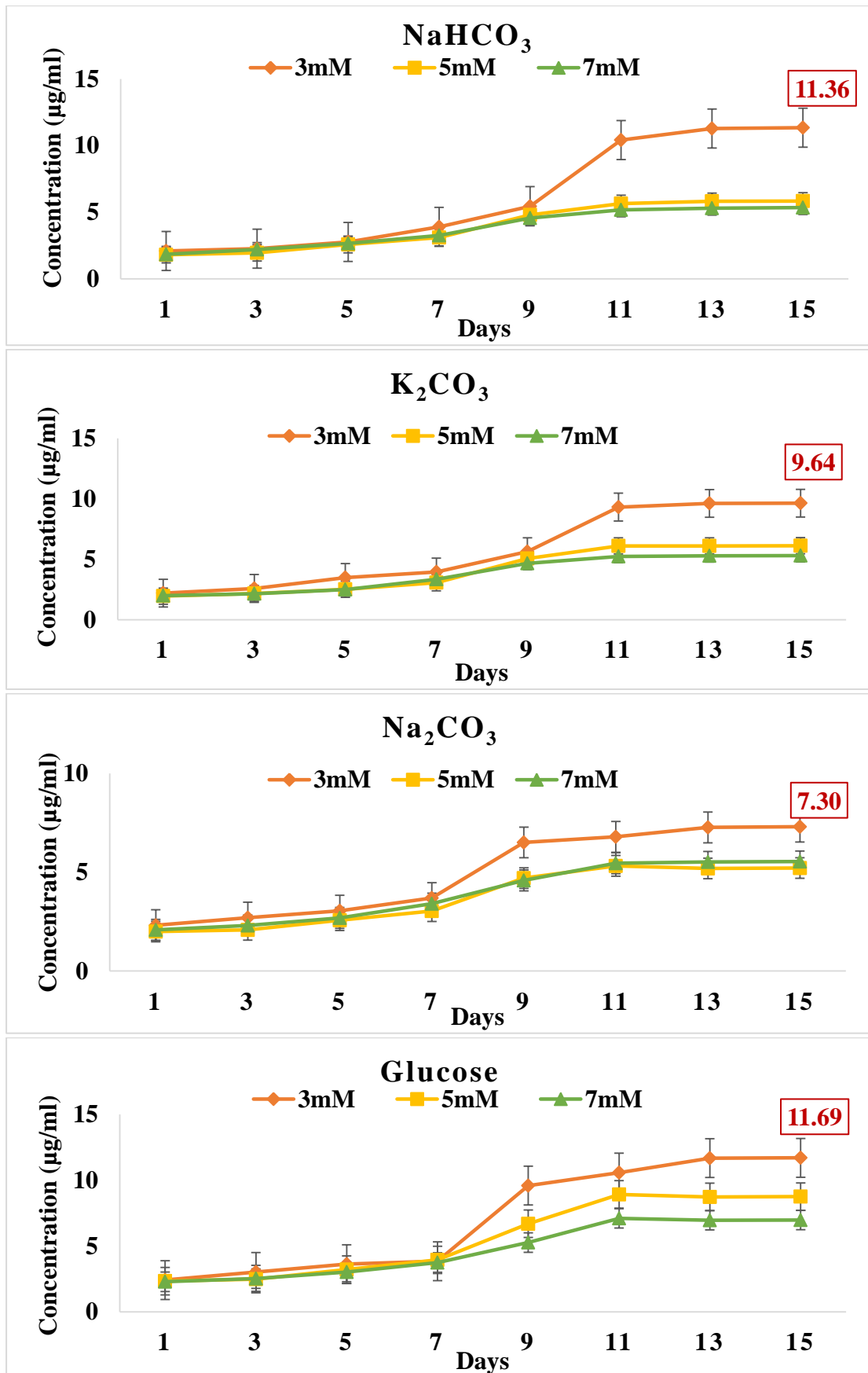


Figure 18. Effect of different carbon sources on total chlorophyll content in *Desmodemus* sp. PLM2

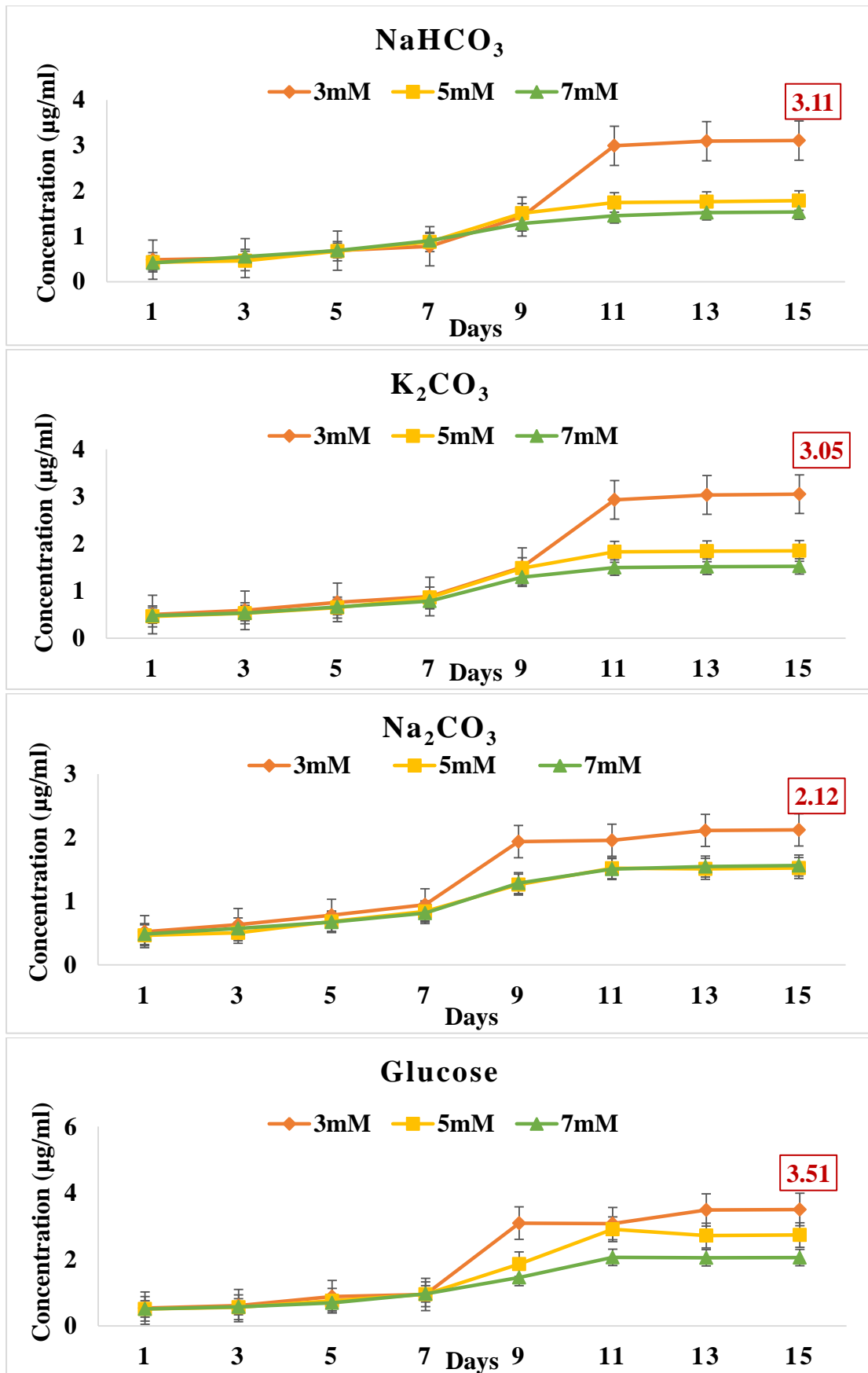


Figure 19. Effect of different carbon sources on total carotenoid content in *Desmodemus* sp. PLM2

4.6.3. Effect of different salt sources:

Five different salt sources, *i.e.*, sodium chloride (NaCl), calcium chloride (CaCl₂), potassium chloride (KCl), magnesium chloride (MgCl₂), and manganese chloride (MnCl₂) were used for biomass production at variable concentrations, *i.e.*, 16 mM, 32 mM, and 64 mM. Effect of different salt sources on growth, biomass yield, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids were observed.

4.6.3.1. Growth and biomass yield of *Desmodemus sp. PLM2*: Potassium chloride at 32 mM concentration resulted in maximum growth and biomass yield (4.4 mg/ml), followed by potassium chloride at 16 mM concentration (3.8 mg/ml). The lowest growth and biomass yield (2.2 mg/ml) was exhibited by using magnesium chloride as a source of salt (Figure 20, 21). Manganese chloride was toxic to the *Desmodemus sp. PLM2*, and no growth was observed.

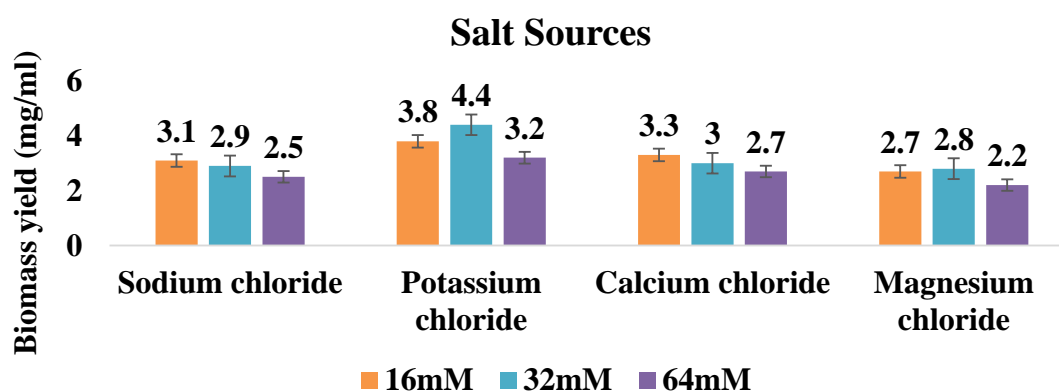


Figure 20. Effect of different concentrations of salt sources on biomass yield of *Desmodemus sp. PLM2*

4.6.3.2. Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content of *Desmodemus sp. PLM2*: Potassium chloride at 32 mM concentration resulted in maximum chlorophyll a (17.38 µg/ml), chlorophyll b (6.86 µg/ml) and total chlorophyll content (24.24 µg/ml) followed by potassium chloride at 64 mM concentration (12.22 µg/ml, 5.44 µg/ml, 16.42 µg/ml, respectively). The lowest chlorophyll a (4.20 µg/ml), chlorophyll b (1.18 µg/ml) and total chlorophyll (5.40 µg/ml) were exhibited by using magnesium chloride at 32 mM concentration (Figure 22, 23, 24).

Carotenoid content also followed the same trend. Potassium chloride at 32 mM concentration resulted in maximum total carotenoid content (7.18 µg/ml) followed by potassium chloride at 64 mM concentration (6.00 µg/ml). The lowest total carotenoid content was obtained using magnesium chloride at 32 mM concentration (1.09 µg/ml) (Figure 25).

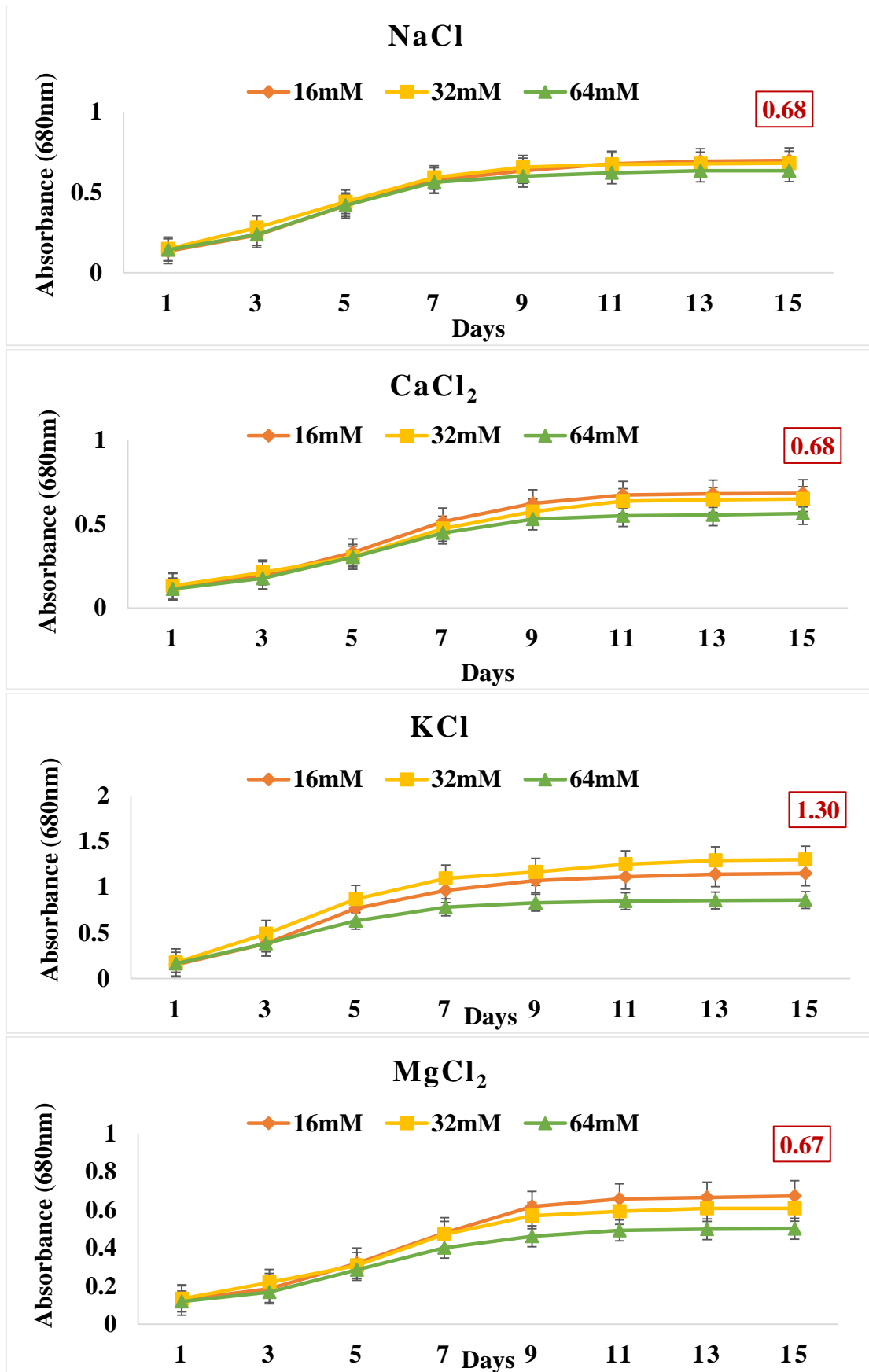


Figure 21. Effect of different concentrations of salt sources on growth of *Desmodemus* sp. PLM2

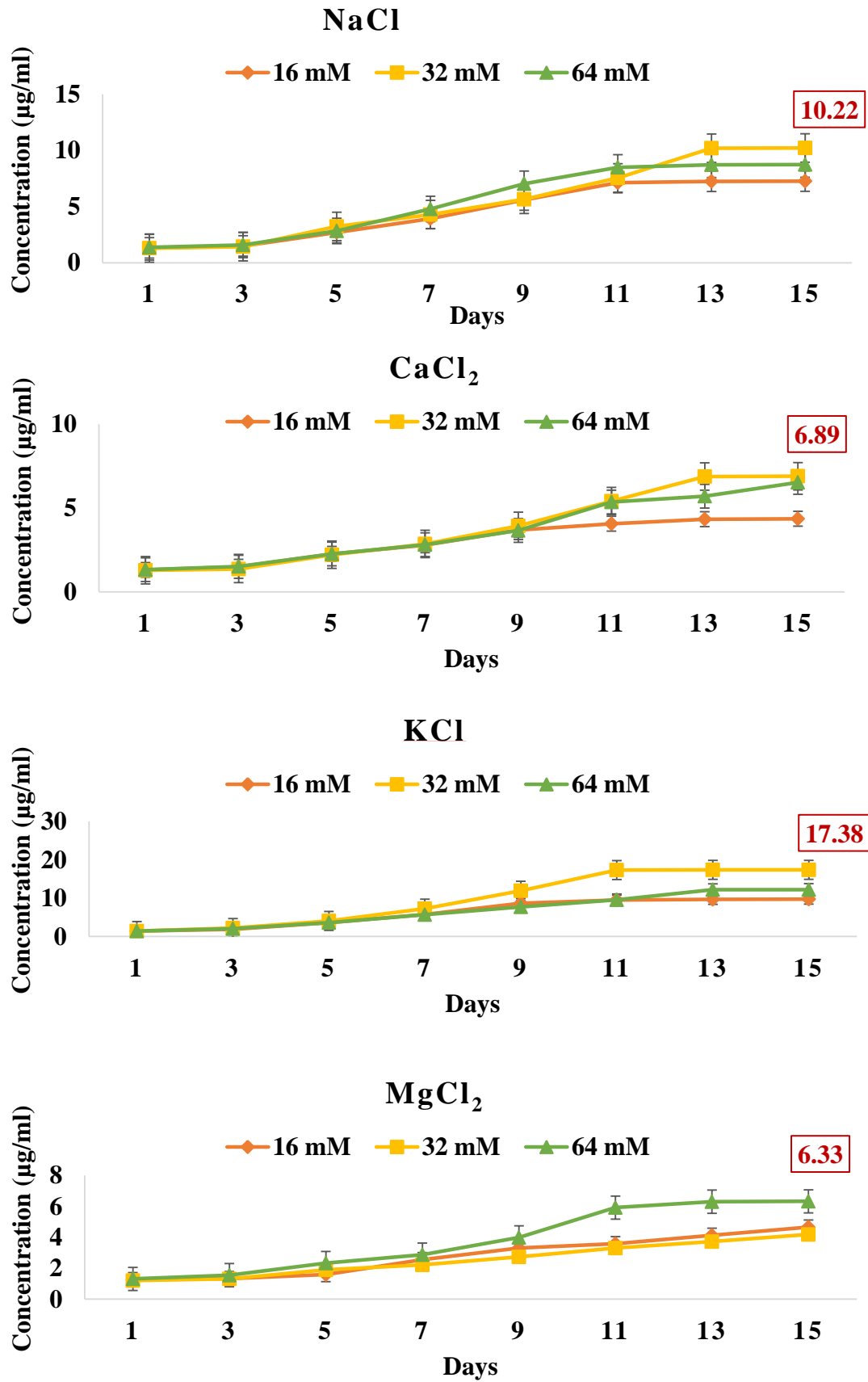


Figure 22. Effect of different salt sources on chlorophyll a content in *Desmodemus* sp. PLM2

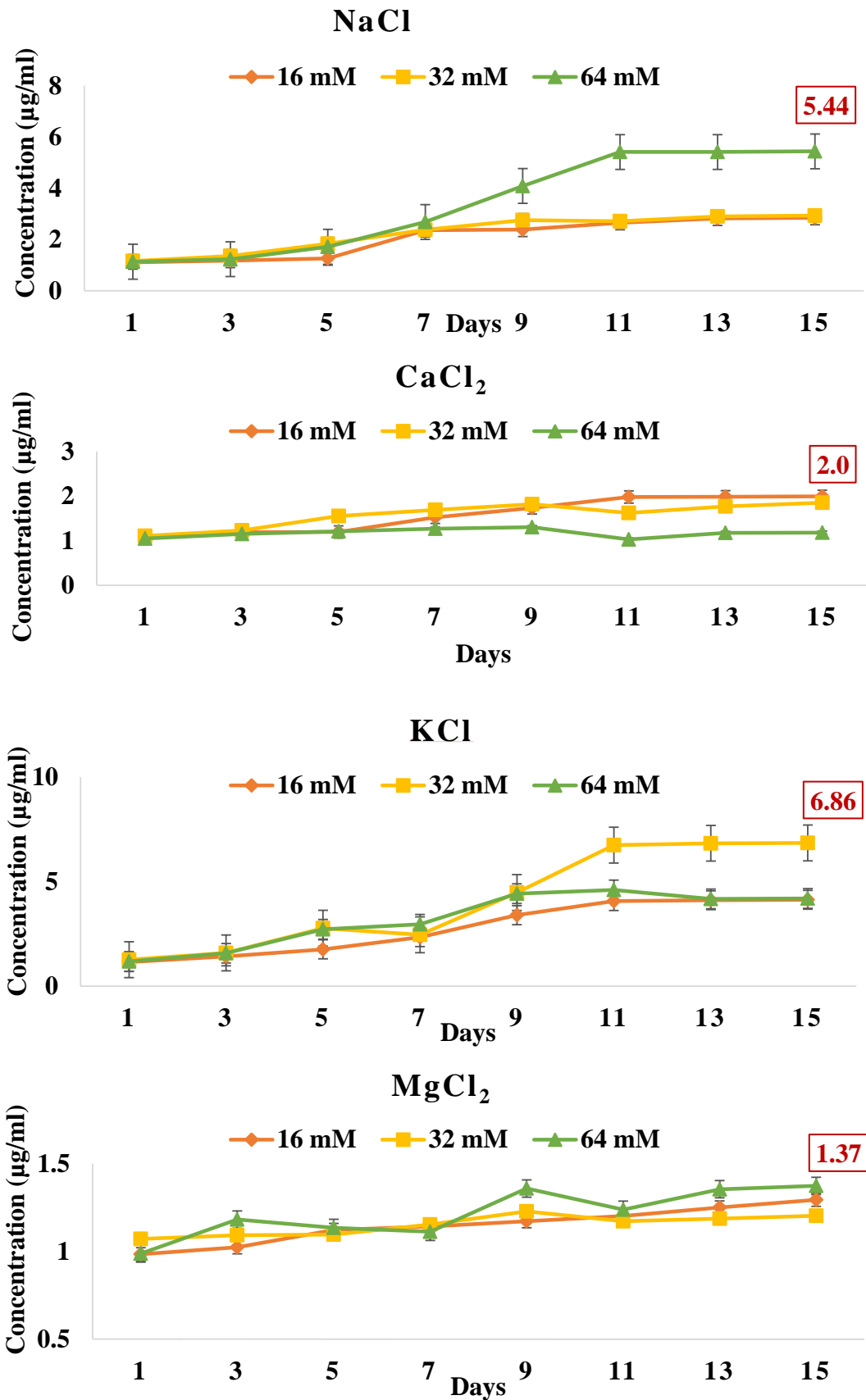


Figure 23. Effect of different salt sources on chlorophyll b content in *Desmodemus* sp. PLM2

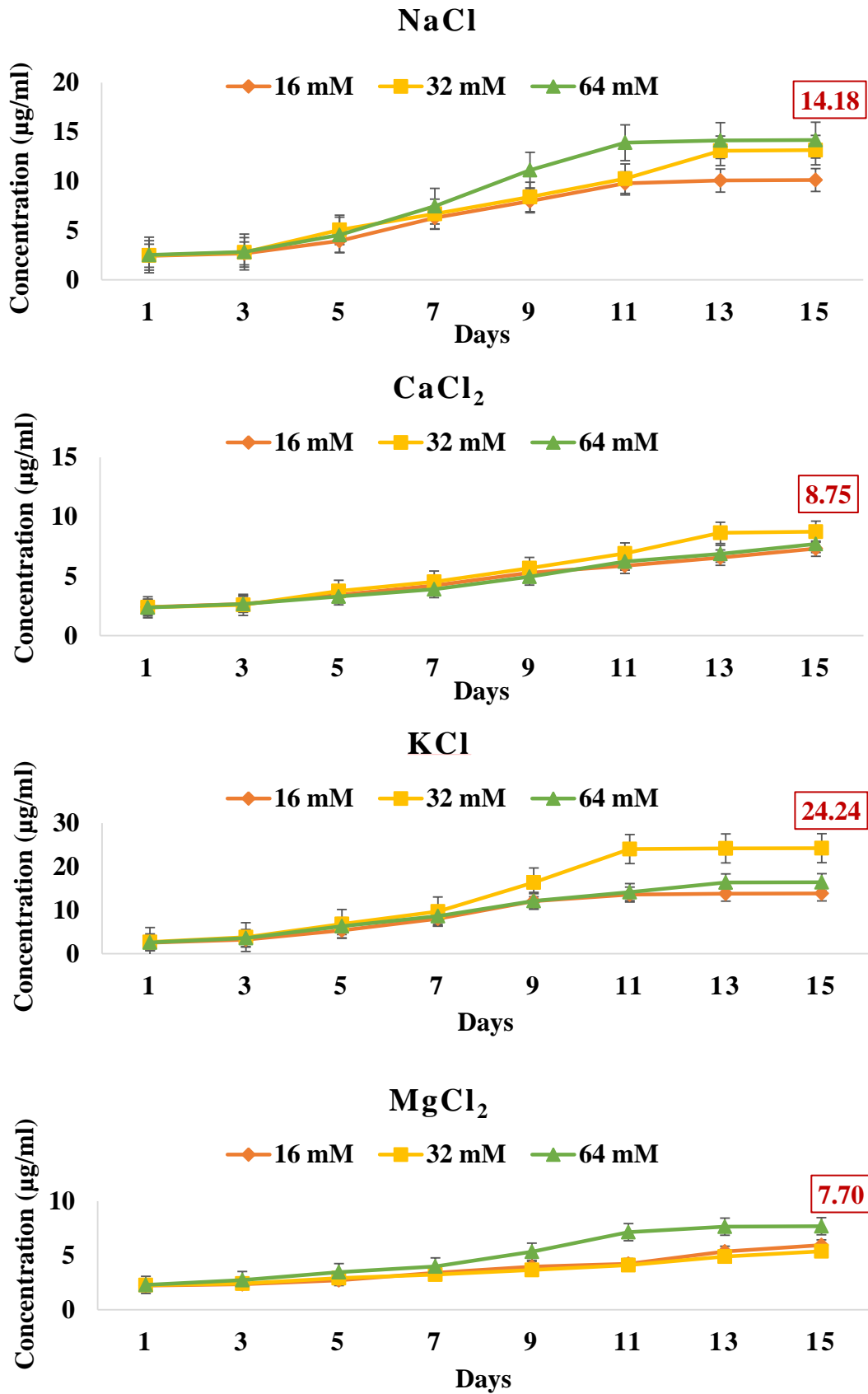


Figure 24. Effect of different salt sources on total chlorophyll content in *Desmodemus* sp. PLM2

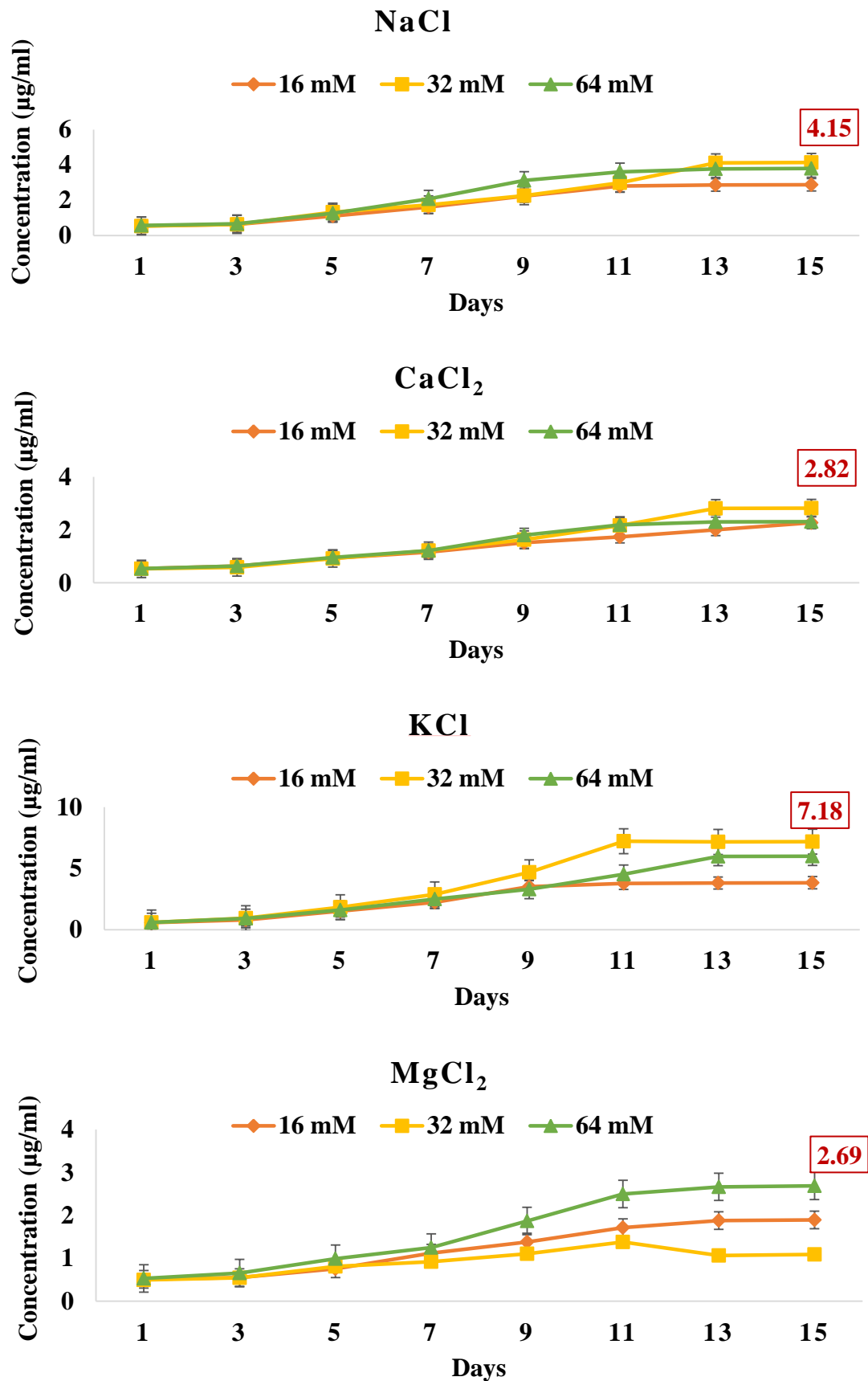
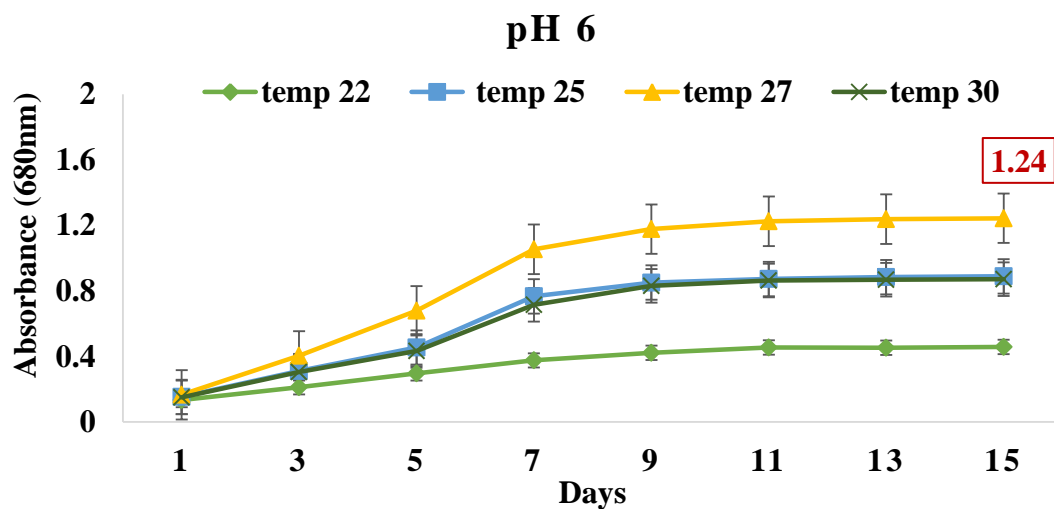
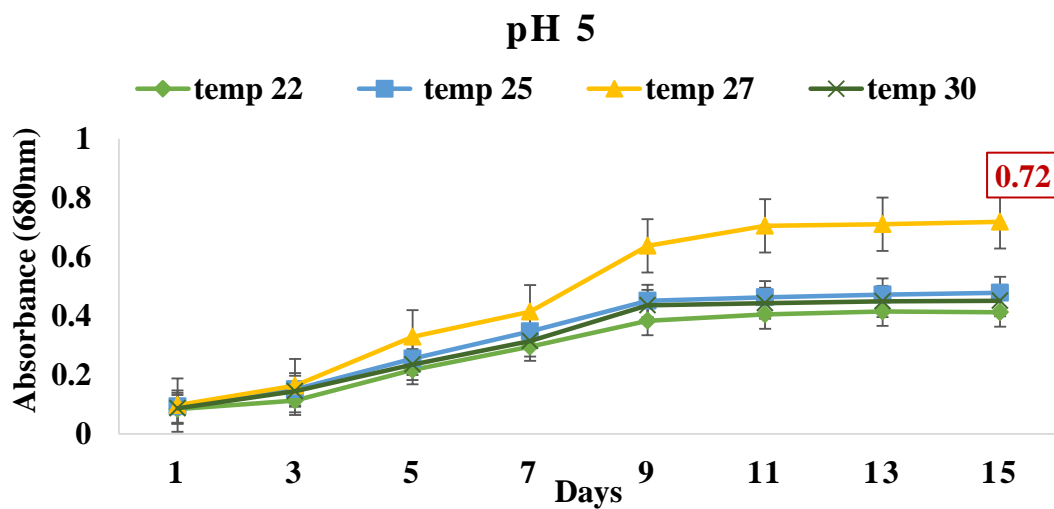


Figure 25. Effect of different salt sources on total carotenoid content in *Desmodemus* sp. PLM2

4.6.4. Effect of different pH and incubation temperature

Four different pH (5, 6, 7, and 8 pH) and four different temperatures (22, 25, 27, and 30°C) were taken into the study for maximum biomass production. Effect of varying pH and temperatures on growth, biomass yield, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids were observed:

4.6.4.1. Growth and biomass yield of *Desmodium* sp. PLM2: pH 7 and 27°C incubation temperature resulted in maximum growth and biomass yield (4.7 mg/ml), followed by pH 7 and 25°C (4.3 mg/ml). The lowest growth and biomass yield (1.6 mg/ml) were exhibited by using pH 5 and 22°C incubation temperature, respectively (Figure 26, 27).



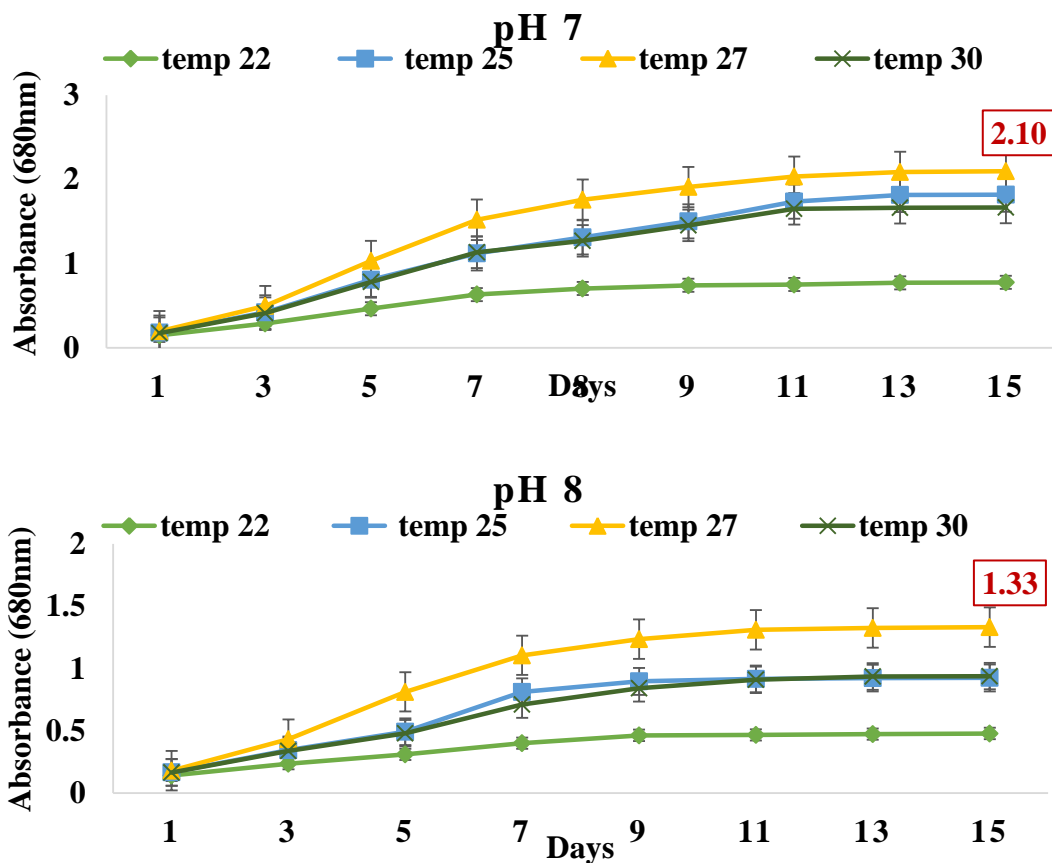


Figure 26. Effect of different pH and temperature on growth of *Desmodium* sp. PLM2

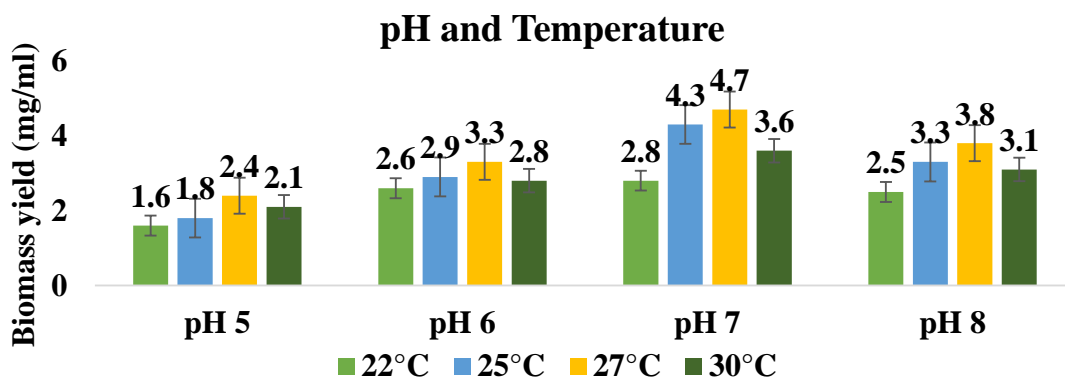
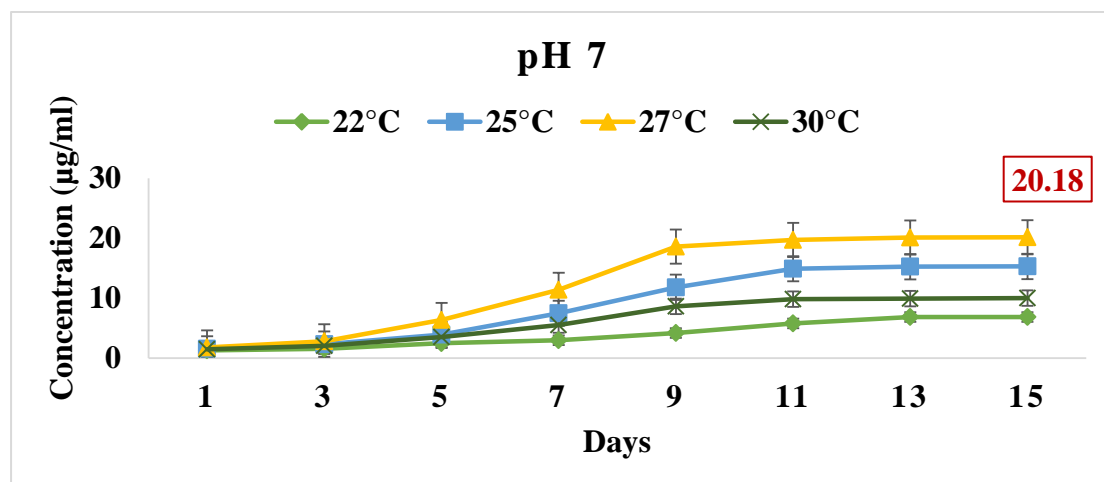
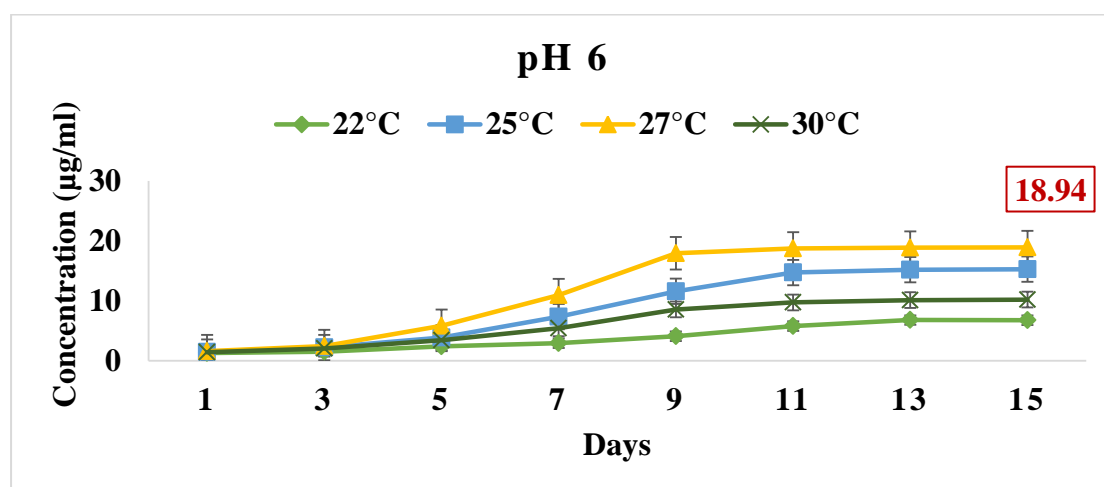
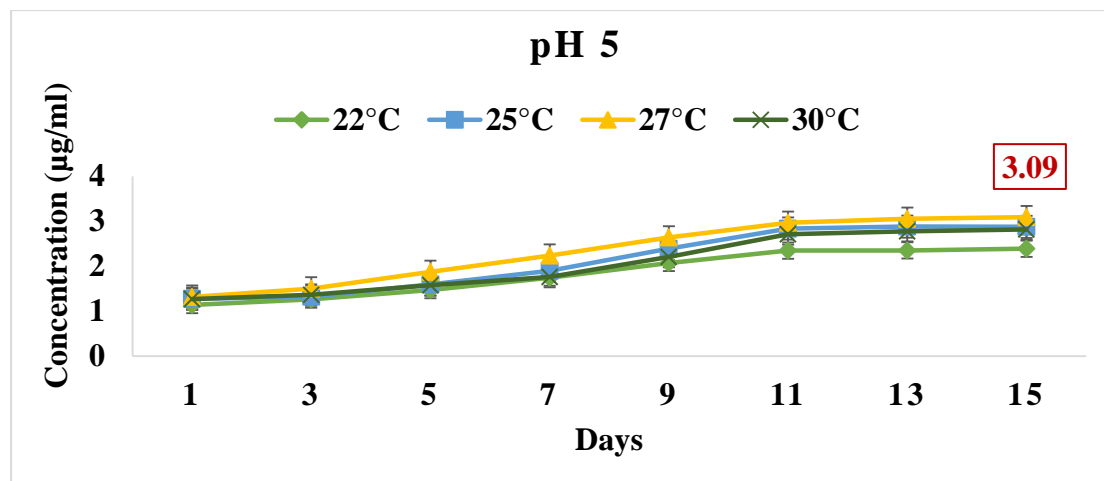


Figure 27. Effect of different pH and temperature on biomass yield of *Desmodium* sp. PLM2

4.6.4.2. Chlorophyll a, chlorophyll b, total chlorophyll and total carotenoid content of *Desmodium* sp. PLM2: pH 7 and resulted in maximum chlorophyll a (20.18 $\mu\text{g/ml}$), chlorophyll b (7.21 $\mu\text{g/ml}$) and total chlorophyll content (27.13 $\mu\text{g/ml}$) followed by pH 6 and incubation temperature 27°C (18.94 $\mu\text{g/ml}$, 6.95 $\mu\text{g/ml}$ and 26.15 $\mu\text{g/ml}$, respectively). The lowest chlorophyll a (2.40 $\mu\text{g/ml}$), chlorophyll b (1.03 $\mu\text{g/ml}$) and total chlorophyll (3.42

µg/ml) were exhibited using pH 5 and 22°C incubation temperature, respectively (Figure 28, 29, 30).

Carotenoid content also followed the same trend. pH 7 and incubation temperature of 27°C resulted in maximum total carotenoid content (7.88 µg/ml), followed by pH 6 and incubation temperature of 27°C (7.45 µg/ml). The lowest total carotenoid content was exhibited at pH 5 and incubation temperature of 22°C (1.16 µg/ml), respectively (Figure 31).



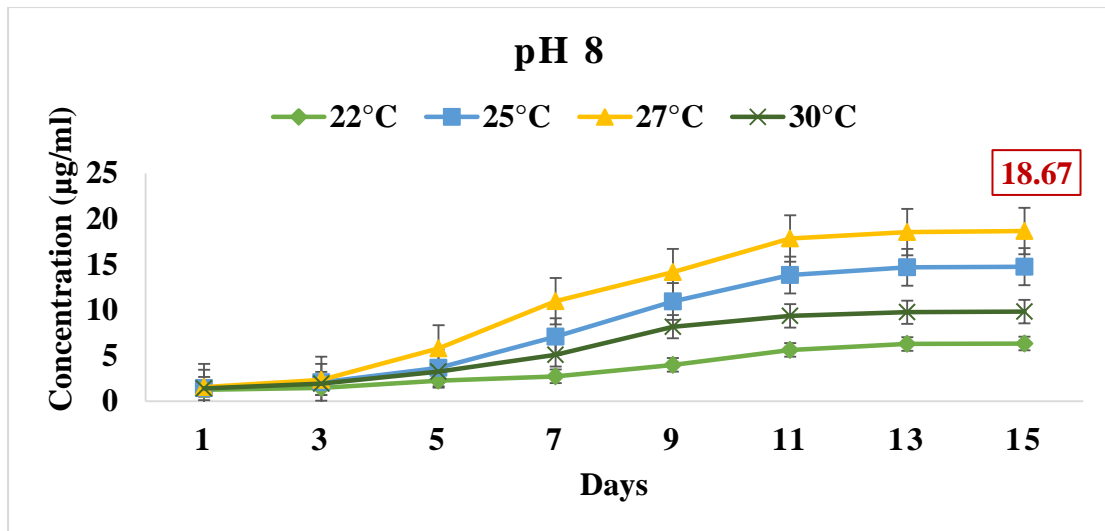
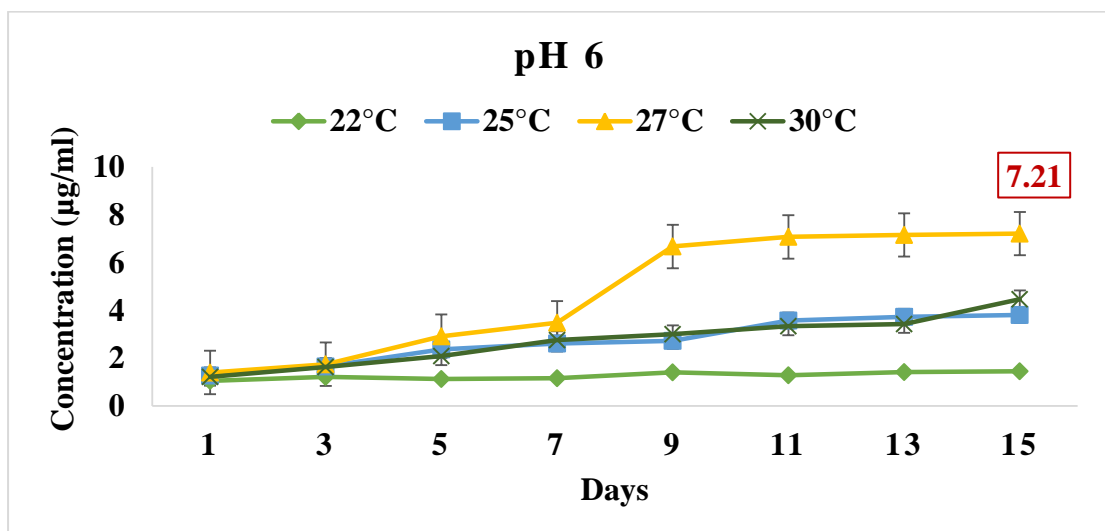
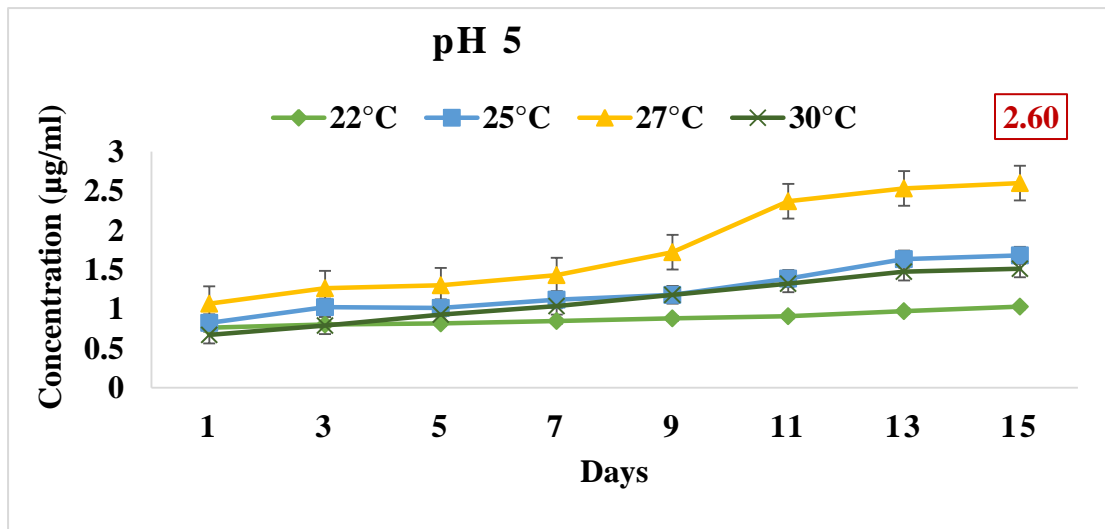


Figure 28. Effect of different pH and temperature on chlorophyll a content of *Desmodemus* sp. PLM2



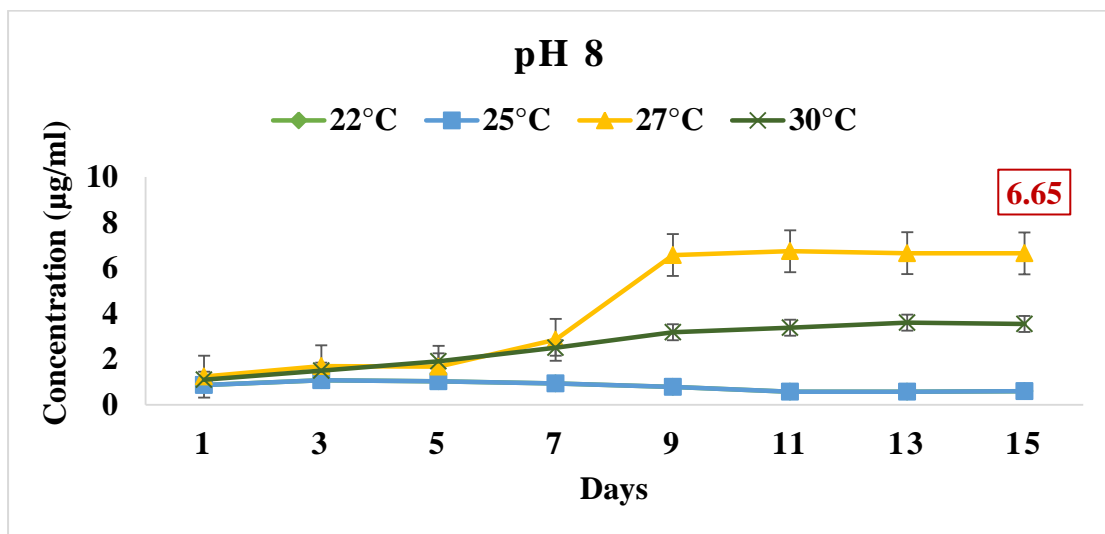
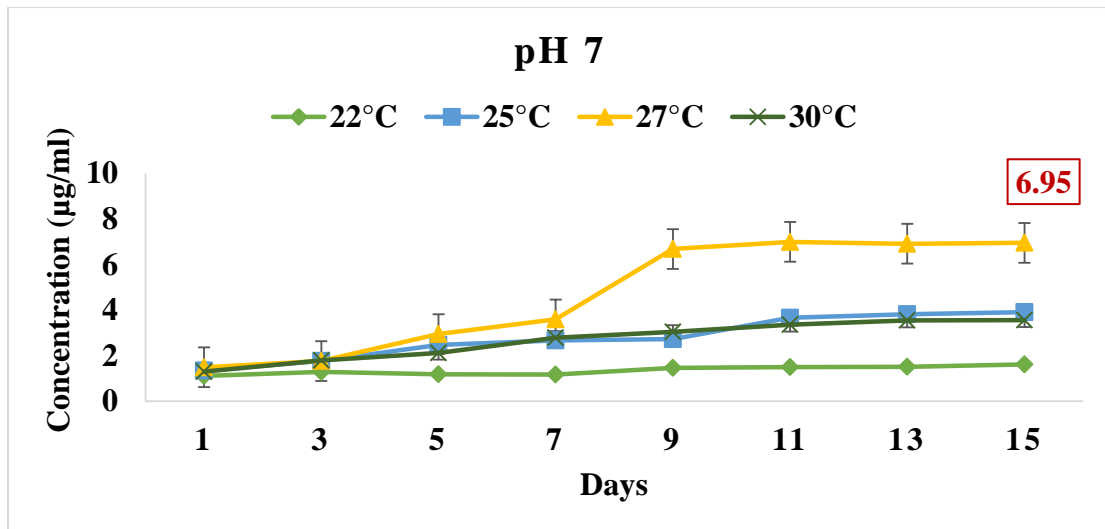
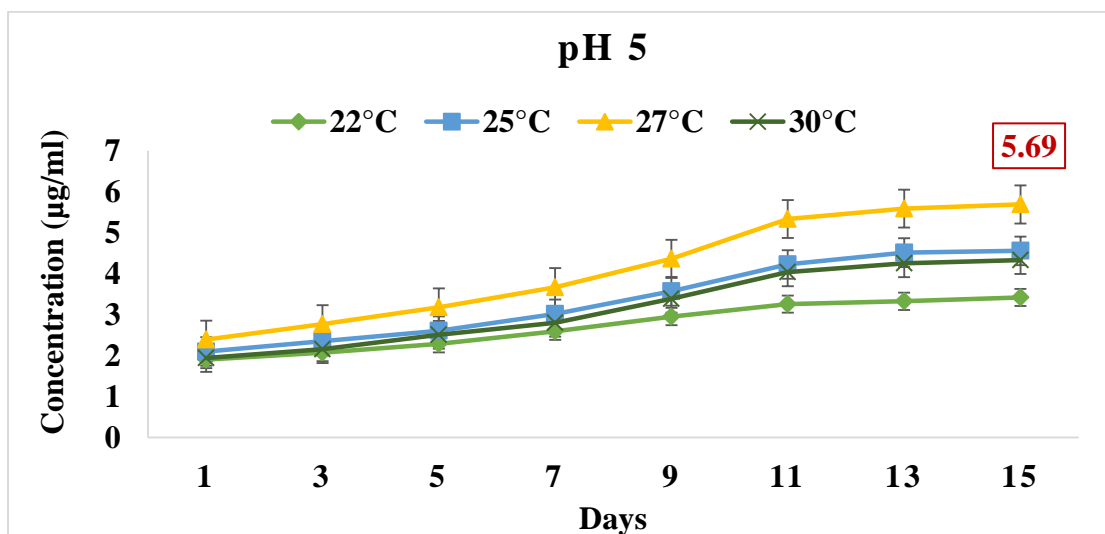


Figure 29. Effect of different pH and temperature on chlorophyll b content of *Desmodemus* sp. PLM2



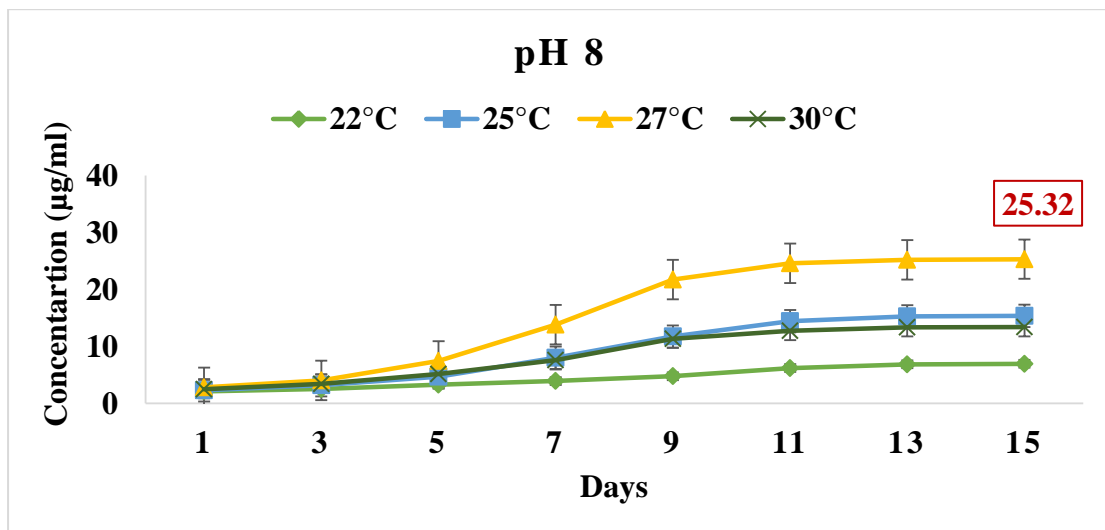
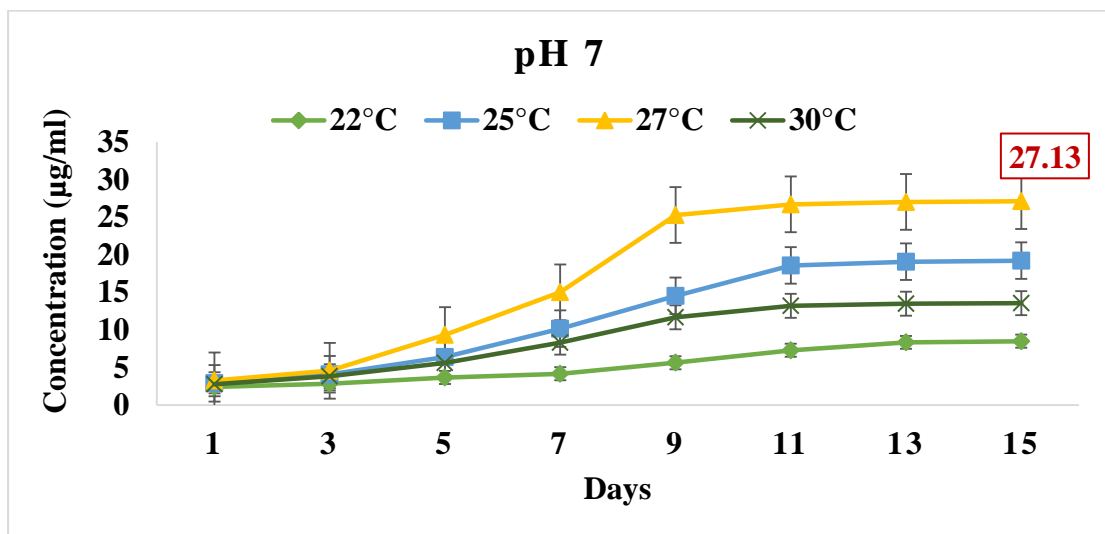
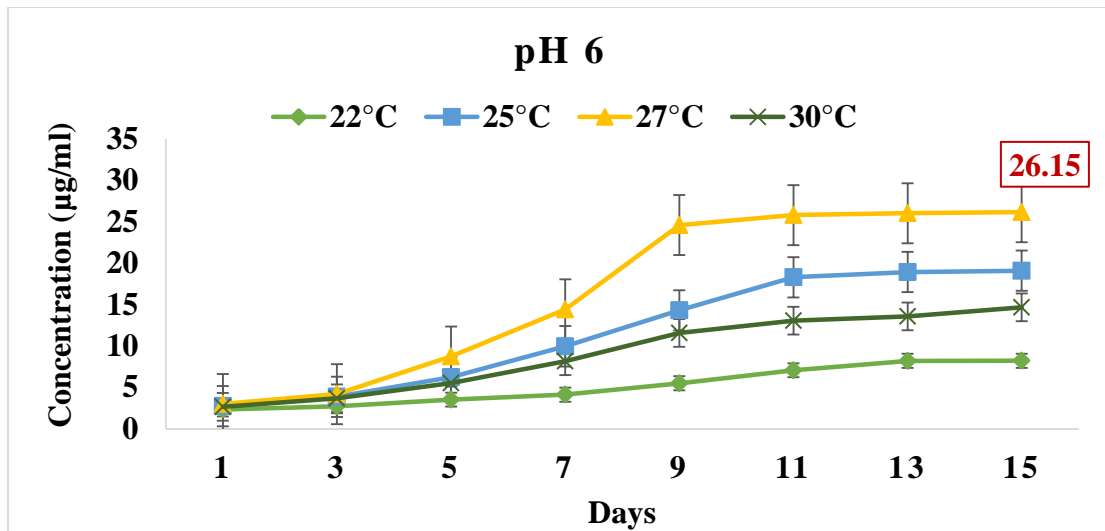


Figure 30. Effect of different pH and temperature on total chlorophyll content of *Desmodemus* sp. PLM2

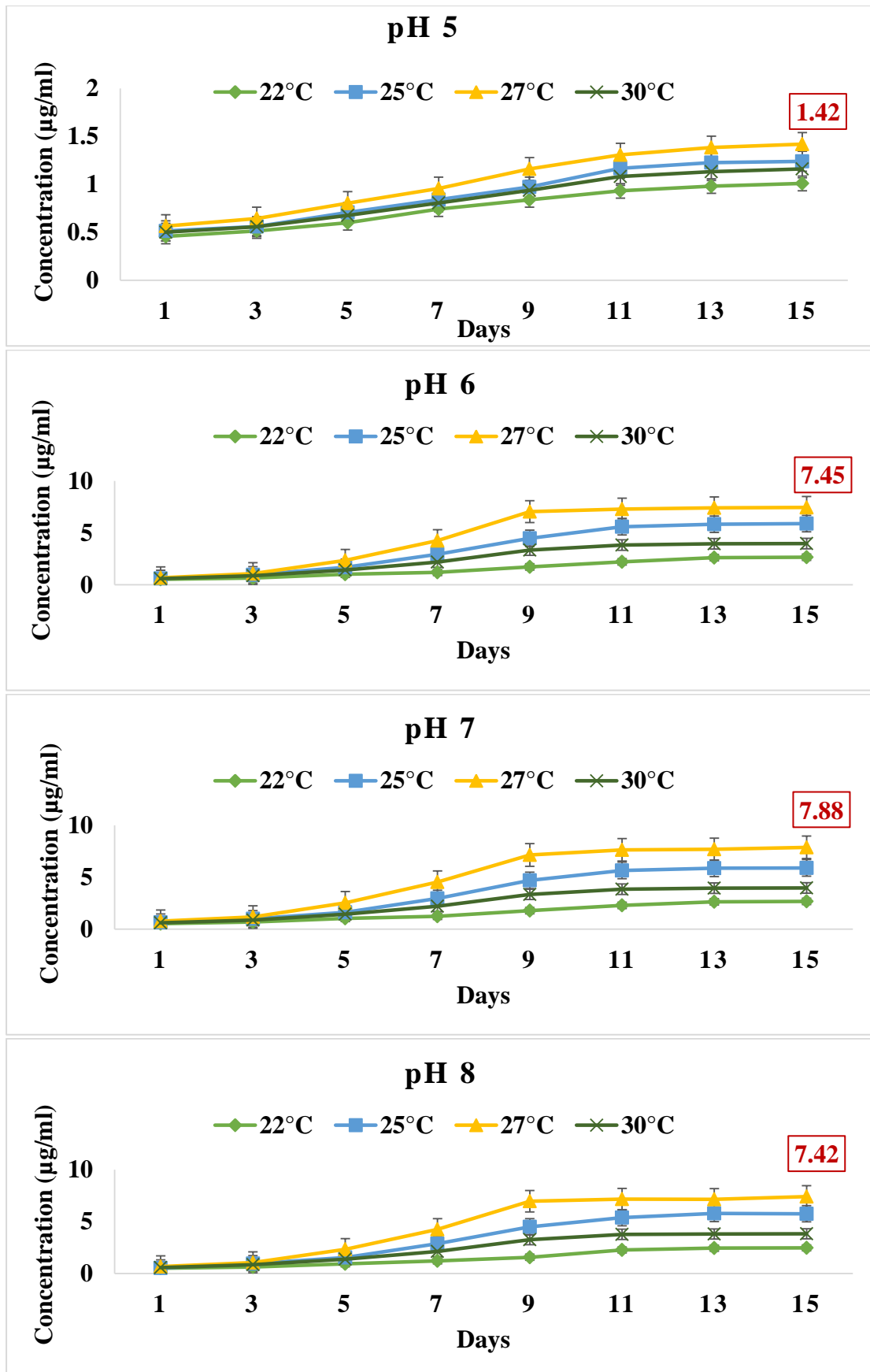


Figure 31. Effect of different pH and temperature on total carotenoid content of *Desmodemus* sp. PLM2

4.7. Optimized cultural conditions. The cultural conditions for the maximum growth, biomass, and chlorophyll content were optimized, and the medium was re-named as modified BG-11 medium or M-BG-11 medium (Table 2).

Table 2. Optimized medium composition and cultural conditions for the green stage

Component	g/L
Sodium nitrate	1.699
Glucose	0.5404
Potassium chloride	2.38
Di-potassium hydrogen phosphate	0.0314
Magnesium sulphate	0.036
Di-sodium magnesium EDTA	0.001
Citric acid	0.005
Ferric ammonium citrate	0.006
Trace element solution	1 ml
pH	7
Incubation temperature	27°C
Incubation time	15 days

4.8. Stress induction for astaxanthin accumulation: Generally, astaxanthin production occurred in two stages. The first stage was for the production or accumulation of green vegetative cells and the second stage involved the formation of red aplanospores and the beginning of astaxanthin accumulation (Plate 4). After the maturation of red aplanospores, biomass was harvested and extracted using different methods. The two-stage process provided a chance to optimize cell growth in the first stage (green stage) and thus, increase astaxanthin accumulation in the second stage (red stage). In this part of the study, astaxanthin accumulation was induced by inducing stress by adding 0.2% NaCl and 4.4 mM sodium acetate along with nitrogen and phosphorous starvation and increased light intensity at 3000 lux using optimized conditions for biomass production as described earlier.

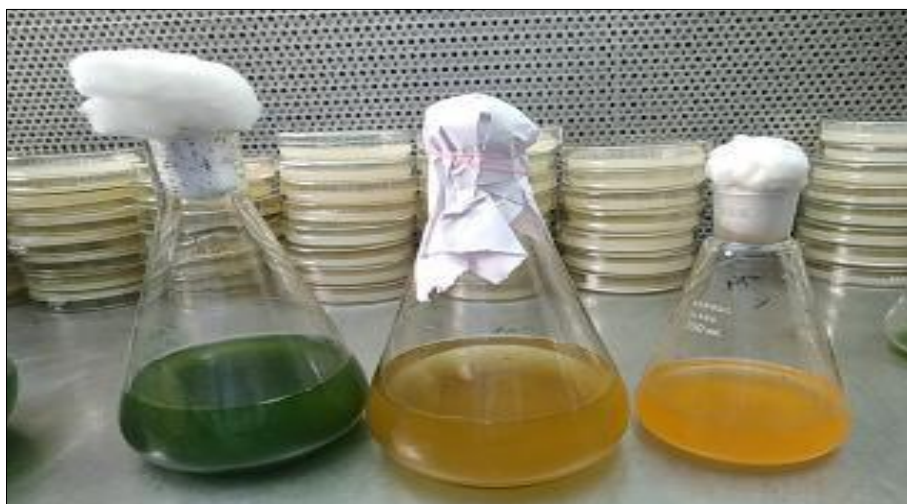


Plate 4. Effect of stress induction for astaxanthin accumulation

4.9. Extraction of astaxanthin: For extraction of astaxanthin from microalgae, cells were pretreated with different solvents and acids, followed by acetone (Plate 5). Microalgal cells were grown in two different media: BG-11 medium (standard medium) and M-BG-11 medium and lyophilized for astaxanthin extraction using different solvents. Maximum astaxanthin extraction resulted from cells grown in M-BG-11 medium was 21.13 ppm extraction using 4 M HCl followed by DMSO + Acetone (19.34 ppm). For the cells grown in BG-11 medium, astaxanthin extraction was maximum by using 4 M HCl (16.12 ppm) followed by DMSO + Acetone (11.07 ppm). Among oils, extraction was maximum using olive oil (Figure 32). When observed under a microscope, the cells after extraction appeared colorless with intact cell walls (Plate 6).

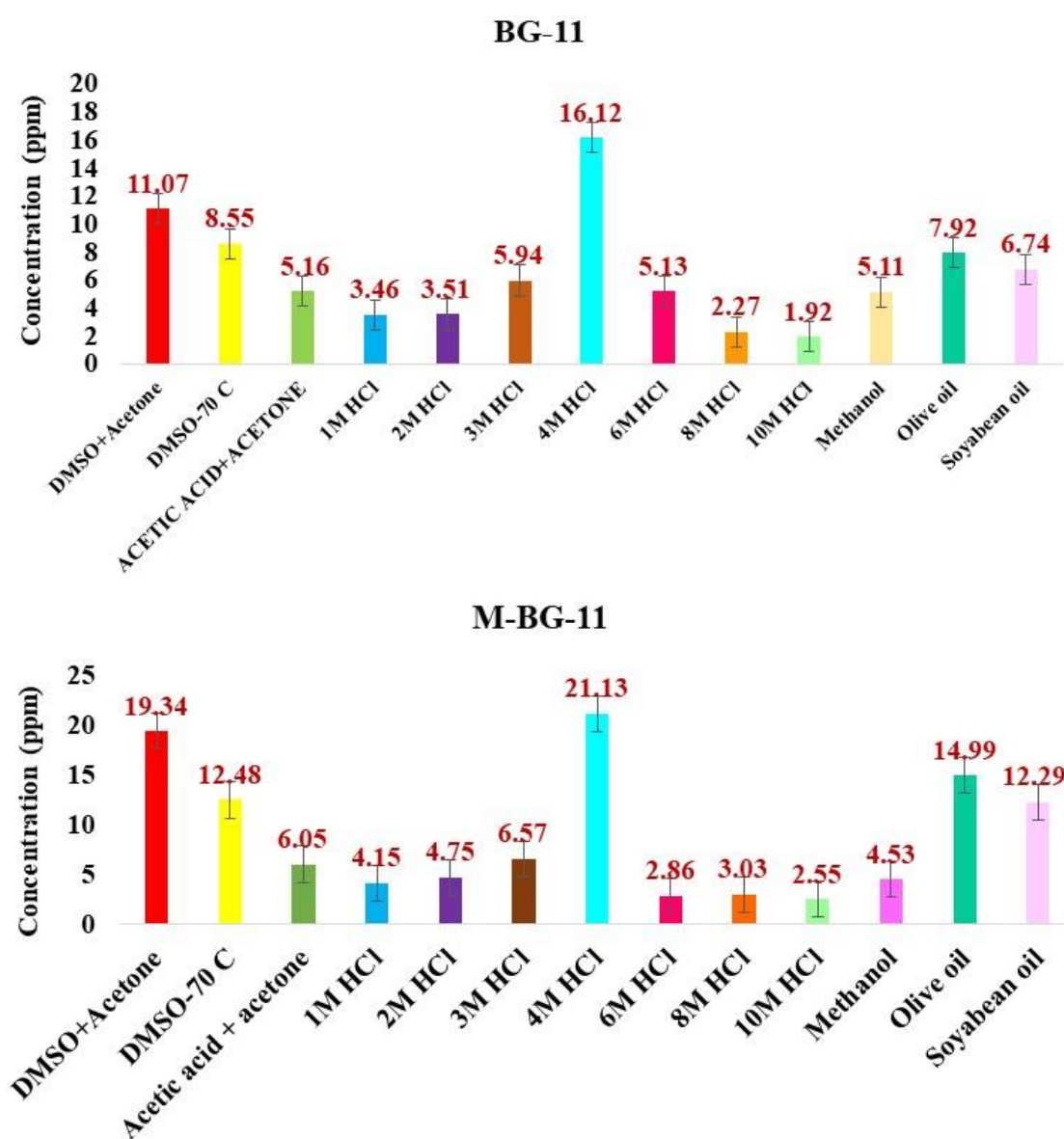


Figure 32. Extraction of astaxanthin using different solvents

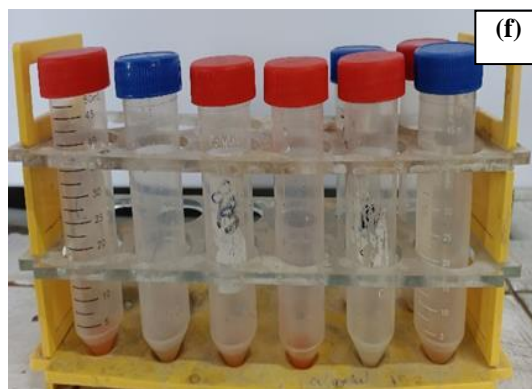
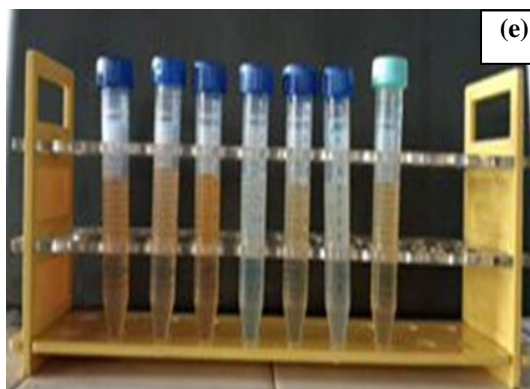
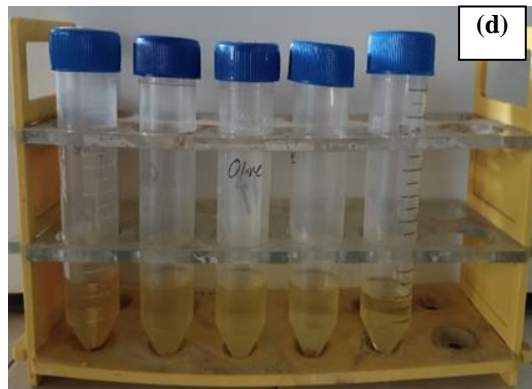
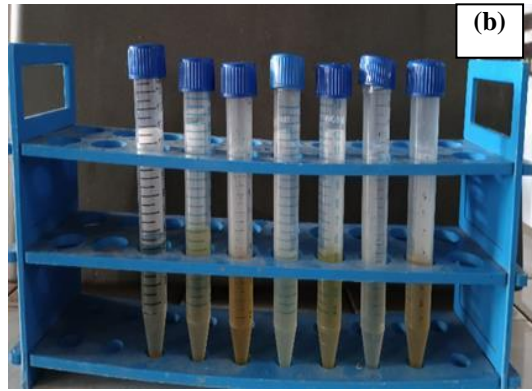
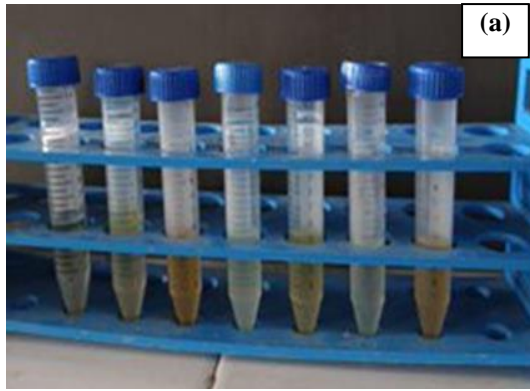
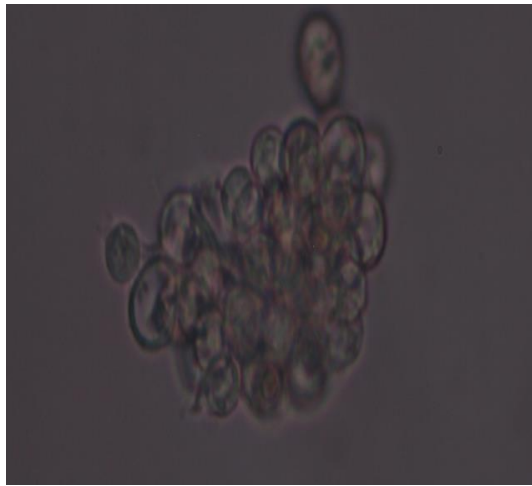
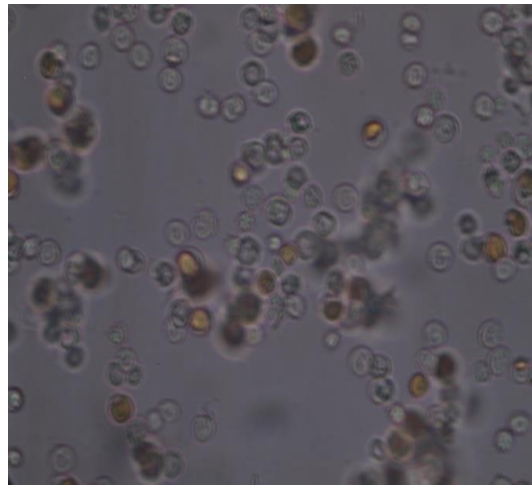


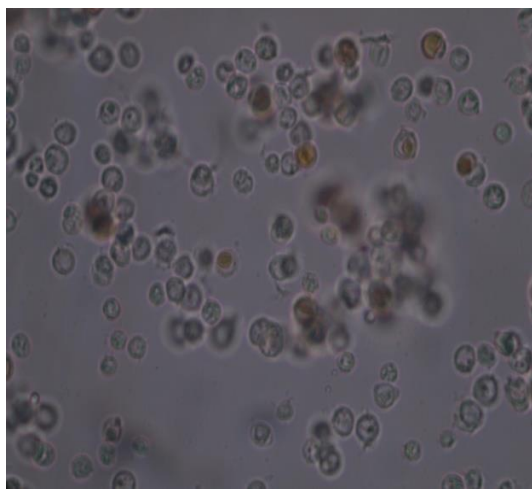
Plate 5. Extraction of astaxanthin using different solvents (a) Methanol, (b) Acetic acid, (c) DMSO, (d) Olive oil, (e) DMSO + Acetone and (f) 4 M HCl



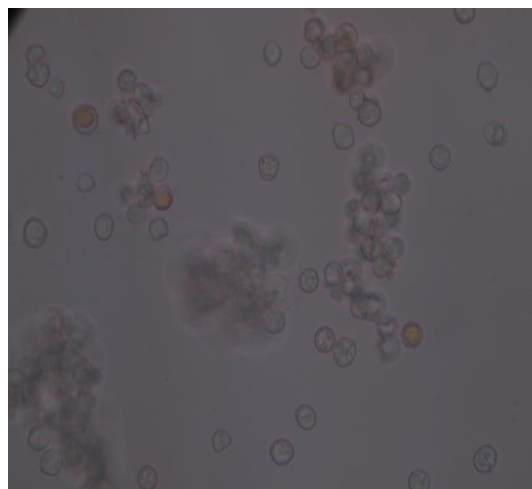
(a)



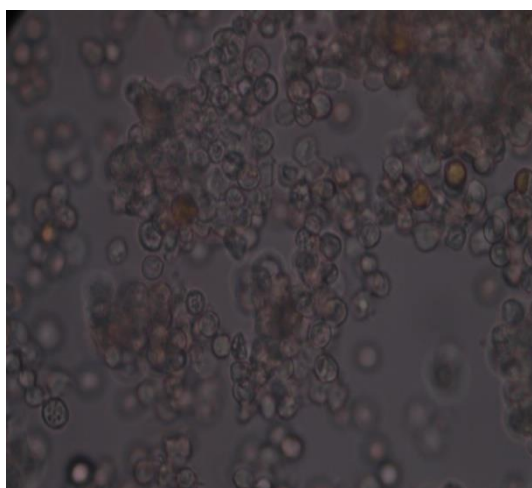
(b)



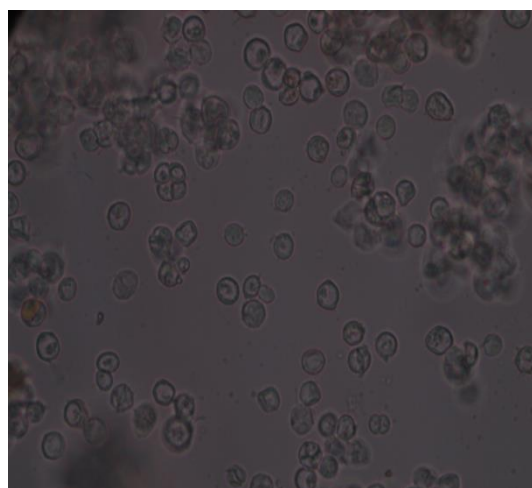
(c)



(d)



(e)

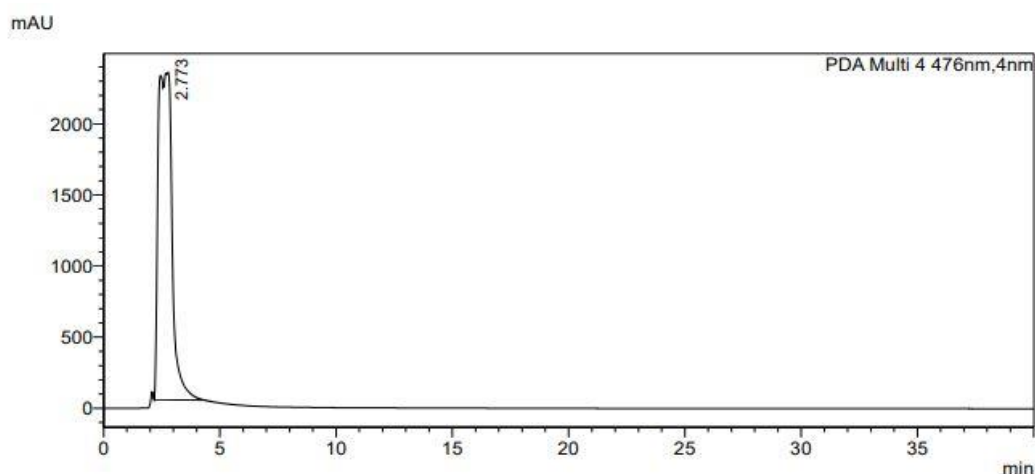


(f)

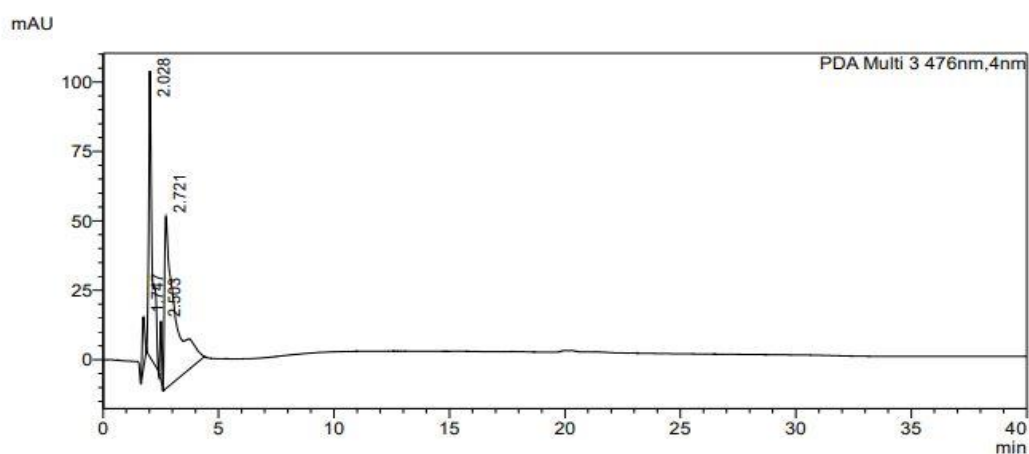
Plate 6. Microscopic image of empty cells after extraction with different solvents (a) 4 M HCl + Mechanical pre-treatment, (b) Acetic acid, (c) DMSO + Acetone, (d) Olive oil + Mechanical pre-treatment, (e) Methanol and (f) Soyabean oil + Mechanical pre-treatment

4.10. Identification and Characterization of astaxanthin: Different techniques like UHPLC, FTIR, and Raman spectroscopy were used to characterize astaxanthin.

4.10.1. Characterize and identification of carotenoid by UHPLC: Carotenoid extracts were subjected to UHPLC (Nexera 2 UHPLC/Shimadzu) analysis using a reversed-phase C18 column. Figure 33 depicted the absorption spectra of extracted astaxanthin from *Desmodesmus* sp. PLM2 and that of standard astaxanthin were similar, with a characteristic peak in the region of 476 nm having a retention time of 2.7 min.



(a) All-trans astaxanthin standard



(b) Sample

Figure 33. Ultra-High Performance Liquid Chromatography (UHPLC) analysis of carotenoid extracted from microalgae *Desmodesmus* sp. PLM2 (a) standard and (b) Sample

4.10.2. Carotenoid by FTIR: Different peaks were obtained using FTIR, trans astaxanthin peak at 3451 cm^{-1} , asymmetric and symmetric stretching vibration in CH_2 peaks at 2999 and 2915 cm^{-1} , trans conjugated alkenes 951 cm^{-1} (Figure 34, Table 3). Because of its conjugated system and, in particular, the existence of the H_2 bond in dimer form, pure astaxanthin showed a peak at 1708 cm^{-1} , which could be linked to a shift of the $\text{C}=\text{O}$ peak from 1740 cm^{-1} to low frequency.

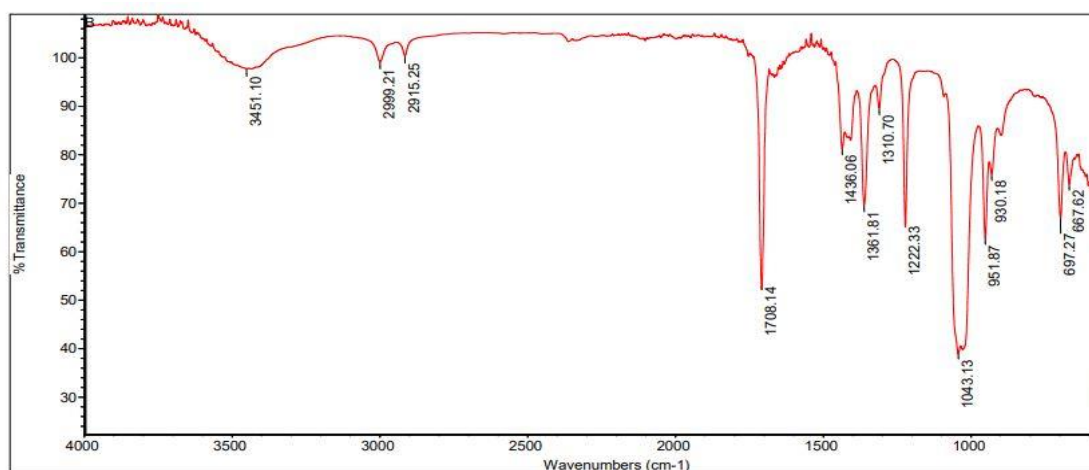


Figure 34. FT-IR spectra of carotenoid extracted from microalgae *Desmodesmus* sp. PLM2

Table 3. Peaks of *Desmodesmus* sp. PLM2 astaxanthin with corresponding functional groups

Wavenumber (cm ⁻¹)	Functional group
667.62	Strong, C=C bending
697.27	Strong, C=C bending
951.87	Strong, C=C bending
1043.13	Strong broad, CO-O-CO stretching
1092.33	Strong, C-O stretching
1222.33	Strong, C-O stretching
1361.81	Medium, O-H bending
1436.06	Medium, O-H bending
1708.14	Strong, C=O stretching
2915.25	Weak, C=C stretching
2999.21	Weak, broad O-H stretching
3451.10	Strong broad. O-H stretching

4.10.3. Carotenoid by Raman Spectrometer: Table 4 enlisted different bands and related compounds in Raman spectra. The Raman spectrum of astaxanthin from *Desmodesmus* was very similar to the Raman spectrum of astaxanthin from *Haematococcus pluvialis* as documented in the literature (Figure 35, Plate 7). The three peaks at 1010 cm⁻¹, 1161 cm⁻¹, and 1523 cm⁻¹ were the most noticeable. The results from UHPLC, FTIR, and Raman spectroscopy indicated the presence of astaxanthin.

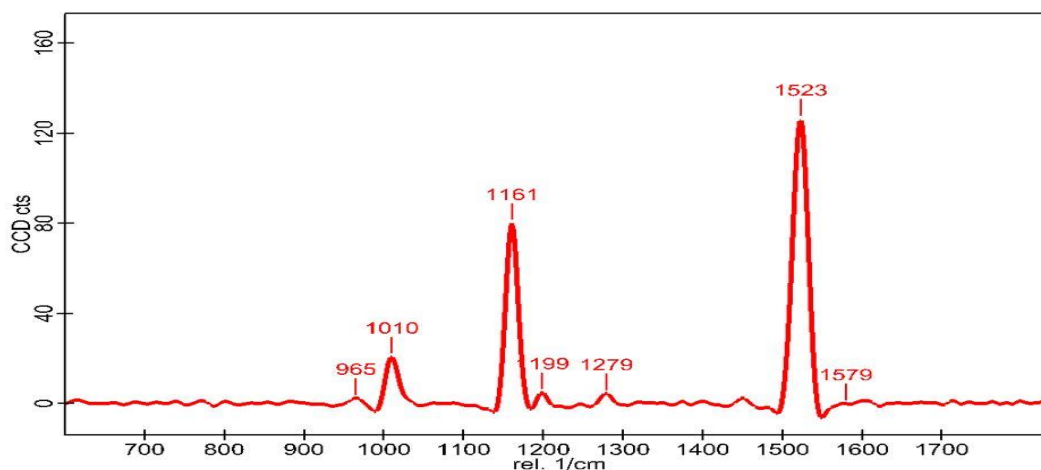


Figure 35. Raman spectra of astaxanthin extracted from microalgae *Desmodesmus* sp. PLM2

Table 4. Peaks of *Desmodesmus* sp. PLM2 astaxanthin with corresponding bonds and substances

Raman shift (cm ⁻¹)	Bonds	Substances
965	C-H ₃	-
1010	C-H bend, C-H ₃ , C-C	β-carotene, astaxanthin, lipids, etc.
1161	C-C stretching vibration	β-carotene and proteins
1199	C-H deformation vibration	β-carotene,
1279	C-C	Amide III
1523	C=C stretching vibration	Astaxanthin

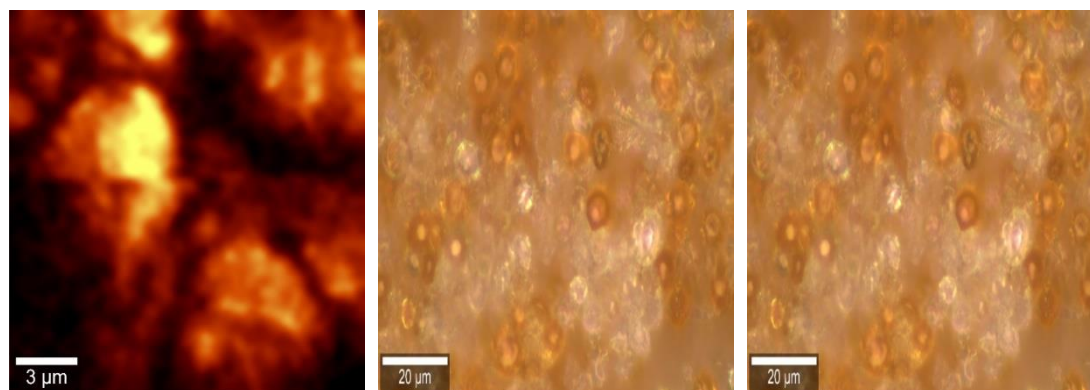


Plate 7. Concentration maps of spectral components representing carotenoids and astaxanthin in *Desmodesmus* sp. PLM2

4.11. DPPH Scavenging activity of astaxanthin: The extracted astaxanthin from *Desmodesmus* sp. PLM2 grown in two different media, BG-11 and M-BG-11, was evaluated and compared for DPPH (2, 2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity. Maximum scavenging activity was observed for astaxanthin from *Desmodesmus* sp. PLM2 grown in M-BG-11 medium, extracted using 4M HCl (78.69%) followed by extraction with DMSO + Acetone, *i.e.*, 77.56%, and similar results were obtained for astaxanthin extracted

from *Desmodium* sp. PLM2 grown in BG-11 medium. Maximum scavenging activity in the latter case was observed for astaxanthin extracted by using 4 M HCl (73.46%) followed by extraction using DMSO + Acetone (72.37%). Among oils, astaxanthin extracted using olive oil showed the highest scavenging activity (Figure 36).

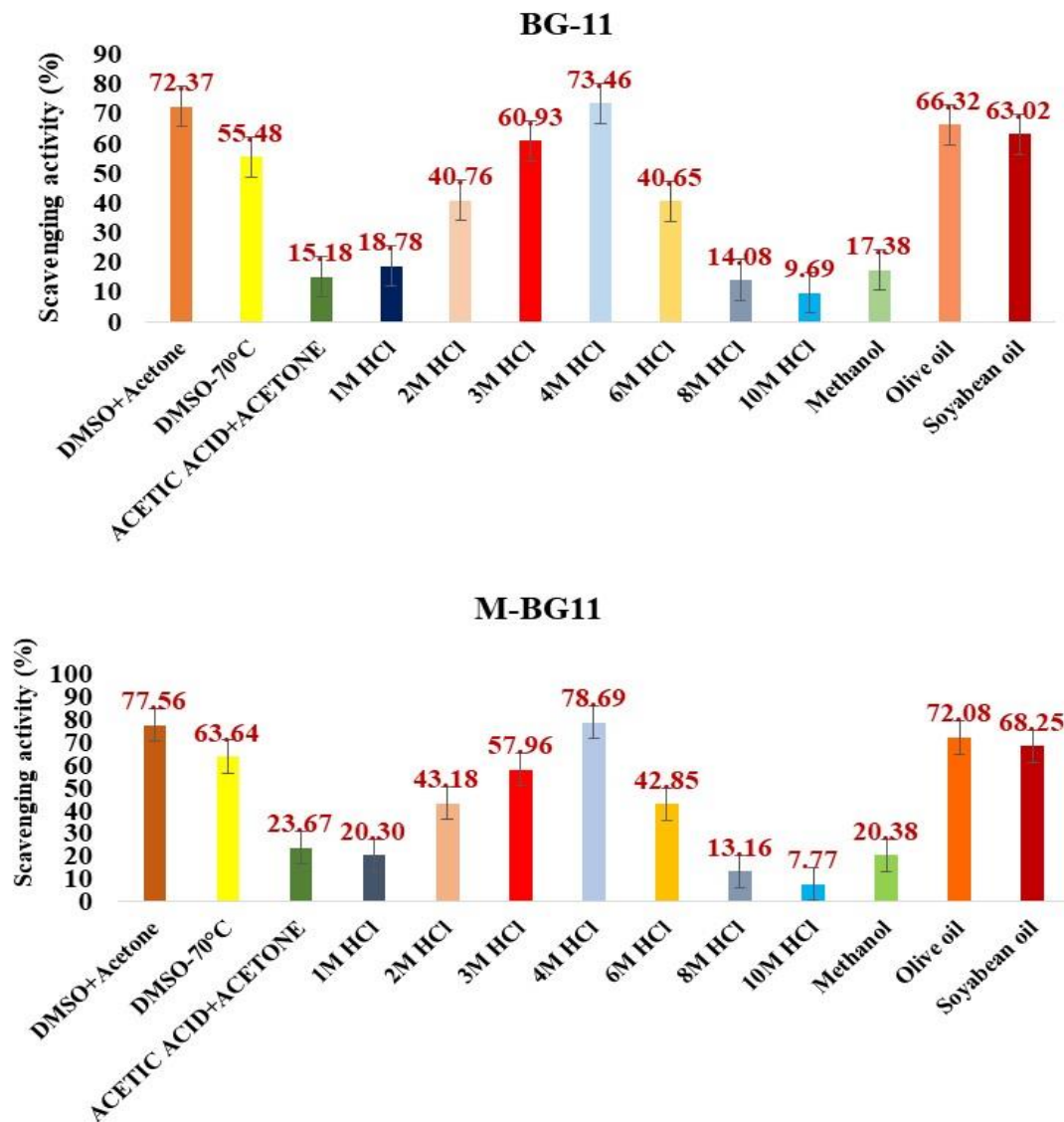


Figure 36. Effect of different extraction methods on DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of astaxanthin

4.12. Free Hydroxyl radical scavenging activity: A similar trend as DPPH scavenging activity was observed for hydroxyl radical scavenging activity. Maximum scavenging activity was observed for astaxanthin extracted from *Desmodium* sp. PLM2 grown in M-BG-11 medium extracted using 4 M HCl (37.81%) followed by extraction using DMSO + Acetone (29.39%). Maximum scavenging activity of astaxanthin was observed for astaxanthin extracted from *Desmodium* sp. PLM2 grown in BG-11 medium using 4 M HCl (27.04%)

followed by extraction using DMSO + Acetone (23.89%). Among oils, maximum scavenging activity of astaxanthin was observed with olive oil followed by soyabean oil (Figure 37).

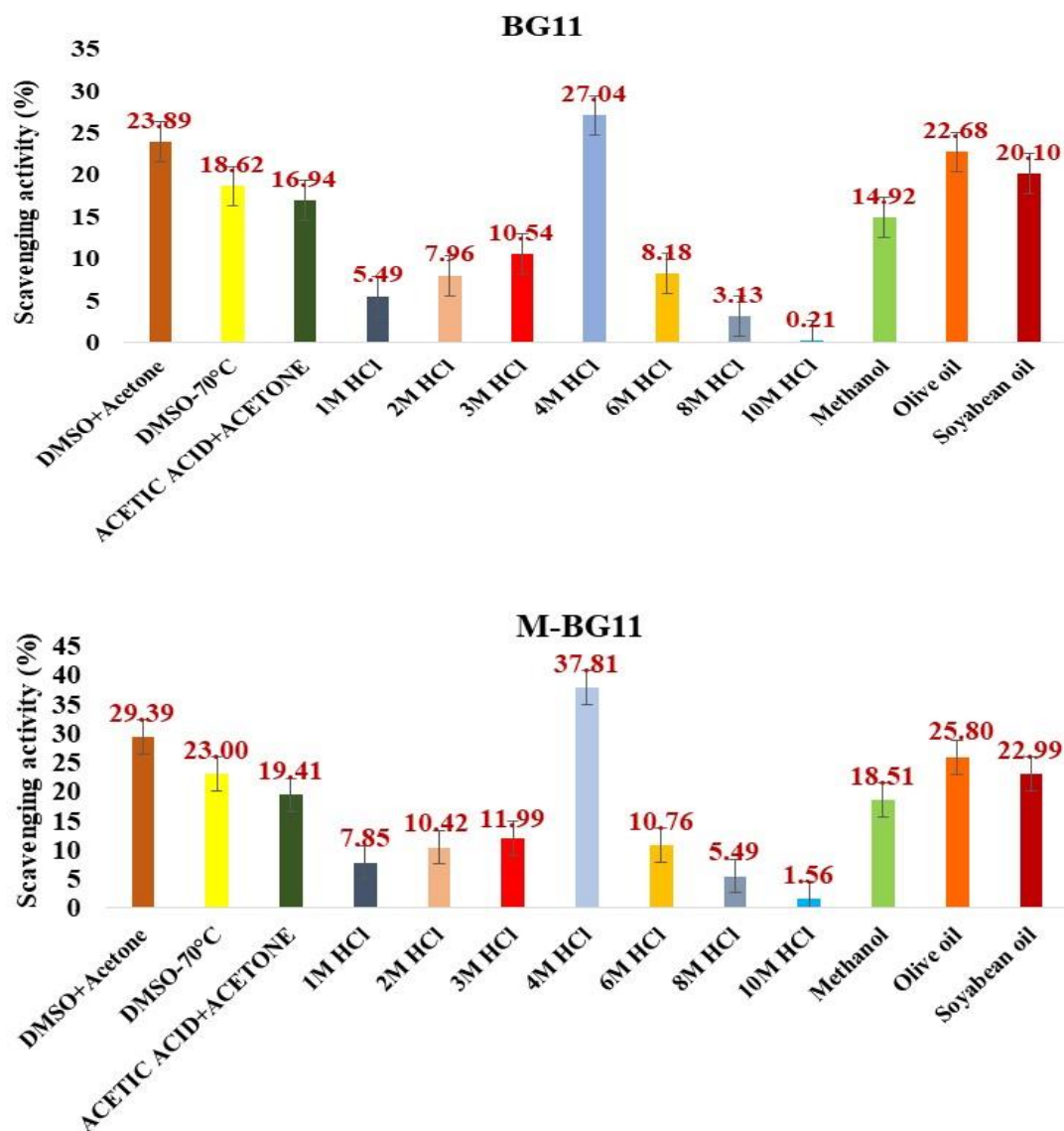


Figure 37. Scavenging activity of extracted astaxanthin on hydroxyl radical

4.13. Storage studies of extracted astaxanthin: The lyophilized dried red cyst cells collected from the two media were preserved in Ziploc bags kept at -20°C , 4°C , 25°C , and room temperatures. To avoid the effect of light, all of the bags were wrapped with aluminum foil. Sampling was done on the 4th, 8th, 12th, 16th, and 20th weeks after storing the dried powders. In the case of lyophilized dried cells from BG-11 medium stored at room temperature, degradation increased with time from 26.1% to 88.3% from the 4th week to the 20th week of storage. Samples were kept at 25°C , and degradation was observed from 16.5% to 72% on the 20th week of storage. The least degradation was observed when kept at -20°C (19.5%). In the case of lyophilized dried cells from M-BG-11 medium at room temperature, degradation increased with time from 22% to 86% from the 4th week of storage to the 20th

week of storage. Samples were kept at 25°C, and degradation increased from 15% to 70.5% during the 20th week of storage. The least degradation was observed for the samples from M-BG-11 kept at -20°C during the 20th week of storage (17%) (Figure 38).

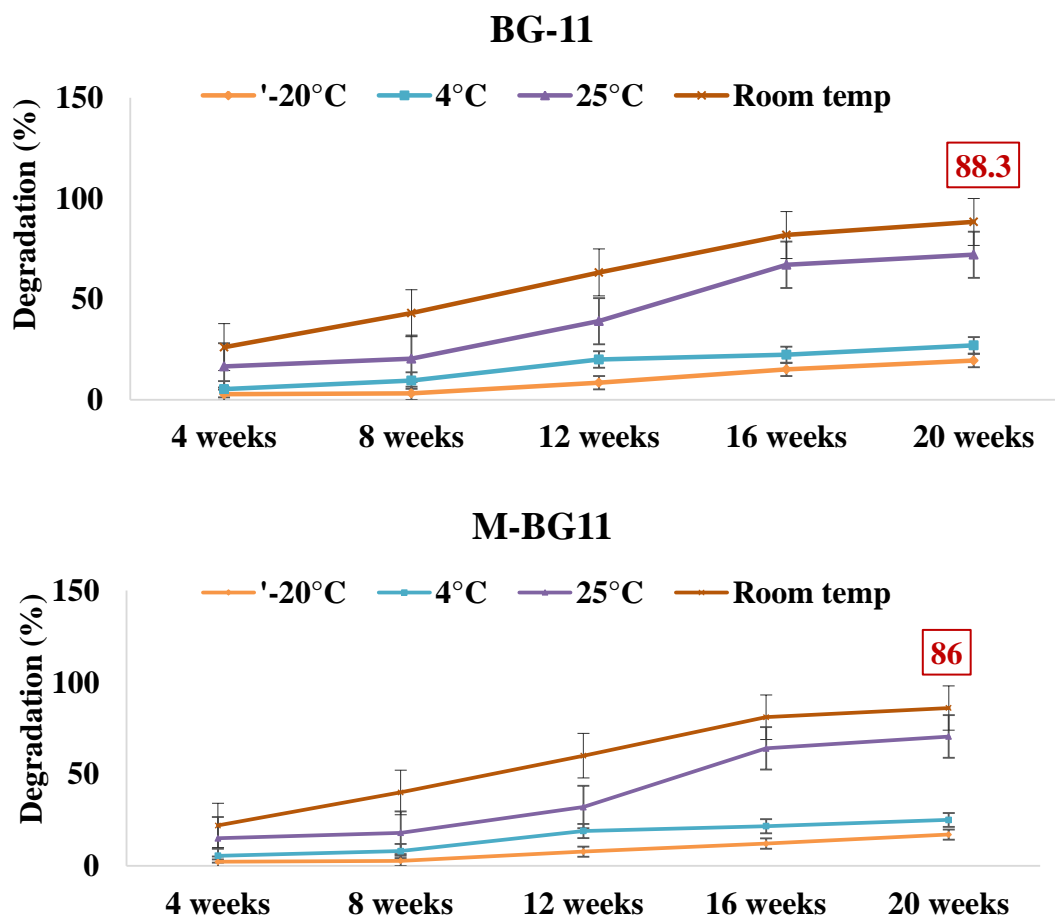


Figure 38. Degradation of total astaxanthin content (%) in relation to initial levels of lyophilized dried normal packed red cell powder stored at different temperatures.

4.14. Effect of red, blue, and white LEDs on growth and astaxanthin content of *Desmodesmus* sp. PLM2: In this experiment, the effort was made to establish a protocol based on the specific wavelength LEDs, *i.e.*, red (630-665 nm) for the cultivation and blue (430-465nm) for the production of astaxanthin using *Desmodesmus* sp. nov. PLM2. Red LEDs were used for microalgal growth and then shifted to blue LEDs to initiate astaxanthin production after seven days. After that, one set of samples was exposed to a higher intensity of blue light (5000 lux). For the experimental setups of the simultaneous application of blue LEDs and carbon source, a certain amount of sodium acetate 4.4 mM was added, along with the blue light intensity of 3000 lux and 5000 lux (Plate 8-10).



Plate 8. Setup for observing the effect of different light-shifting strategies on the accumulation of astaxanthin



Plate 9. Effect of different light intensity on the accumulation of astaxanthin (a) Green vegetative phase, (b) Red cyst phase



Plate 10. Photobioreactor under red and blue illumination

4.14.1. Effect of different light conditions on growth and cell count of *Desmodesmus* sp.

PLM2: Red light illumination resulted in maximum growth and cell count (76.6×10^5 CFU/ml) followed by white light (59×10^5 CFU/ml) and blue light (44.7×10^5 CFU/ml), respectively (Figure 39, 40).

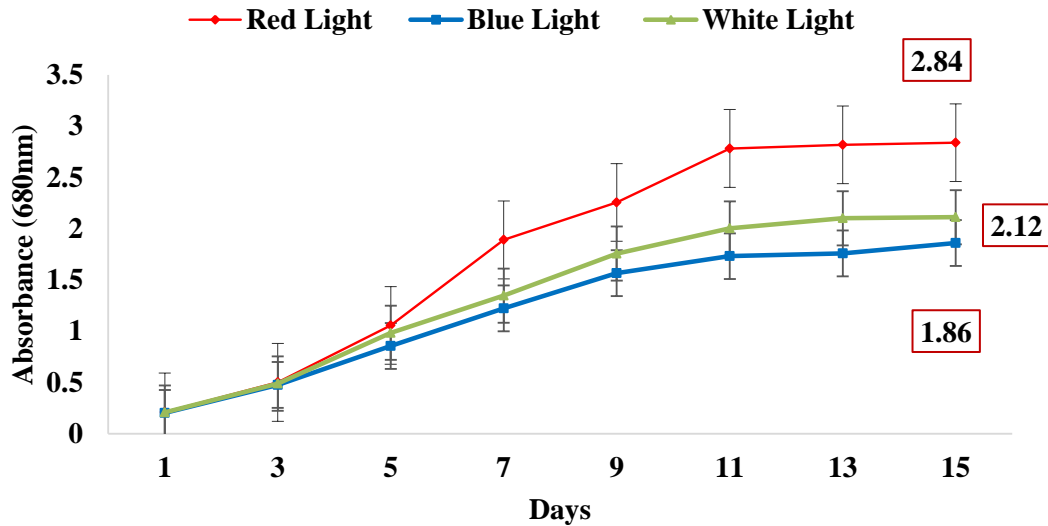


Figure 39. Effect of red, blue, and white LEDs on the growth of microalgae *Desmodesmus* sp. PLM2

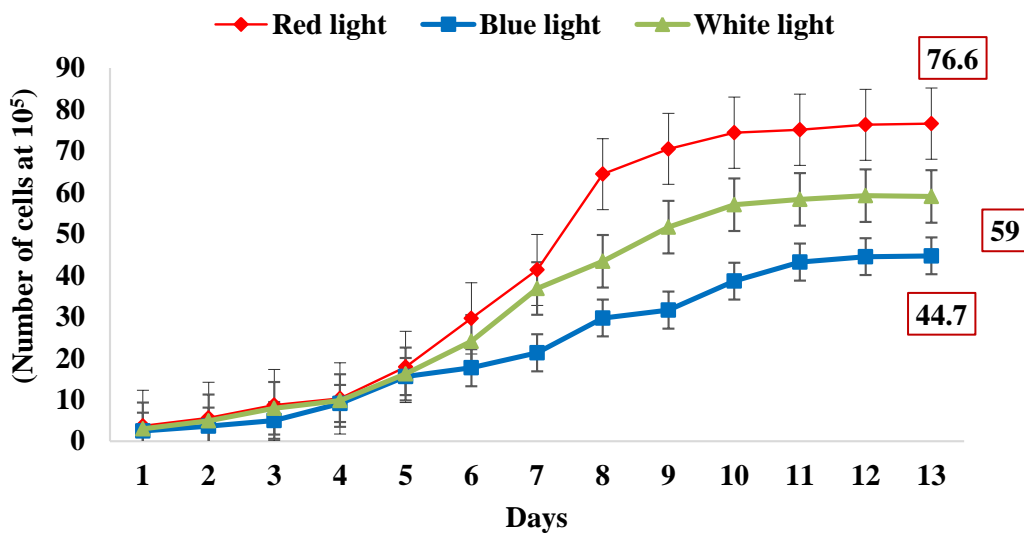


Figure 40. Effect of red, blue, and white LEDs on cell count of microalgae *Desmodesmus* sp. PLM2

4.14.2. Chlorophyll and carotenoid content of *Desmodesmus* sp. PLM2:

Red light illumination resulted in maximum chlorophyll a ($23.83 \mu\text{g/ml}$), chlorophyll b ($5.59 \mu\text{g/ml}$) and total chlorophyll ($29.41 \mu\text{g/ml}$) (Figure 41). The lowest chlorophyll a ($19.80 \mu\text{g/ml}$), chlorophyll b ($5.10 \mu\text{g/ml}$) and total chlorophyll ($24.90 \mu\text{g/ml}$) was exhibited by using blue light illumination (Figure 42). Similarly, blue light illumination resulted in total carotenoid

content (8.73 $\mu\text{g/ml}$) (Figure 42), and red-light illumination exhibited the lowest total carotenoid content (6.98 $\mu\text{g/ml}$) (Figure 41). The red-light illumination enhances the vegetative growth of *Desmodemus* sp. PLM2, whereas blue-light illumination resulted in stress in *Desmodemus* sp. PLM2, thereby triggering astaxanthin biosynthesis.

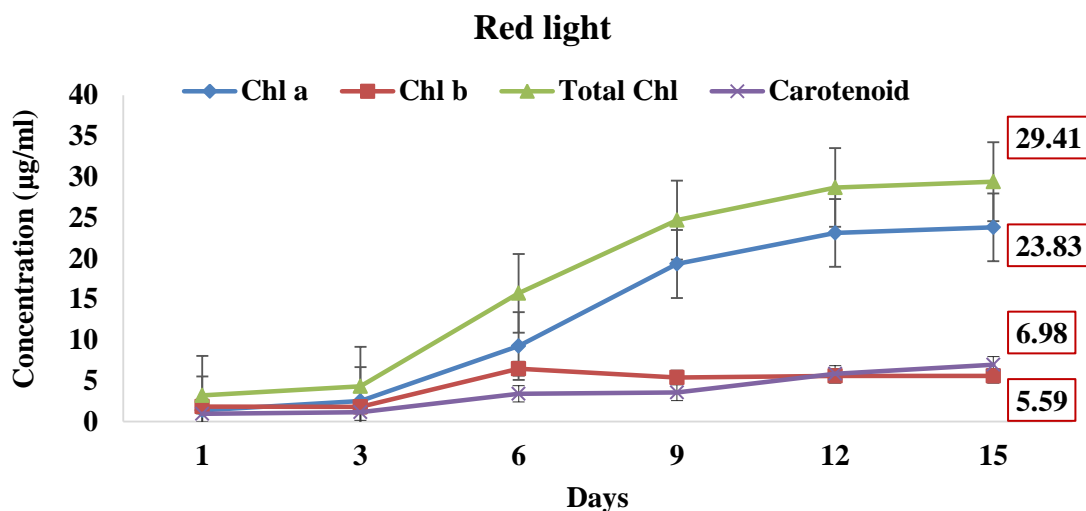


Figure 41. Effect of red LEDs on chlorophyll and total carotenoid content

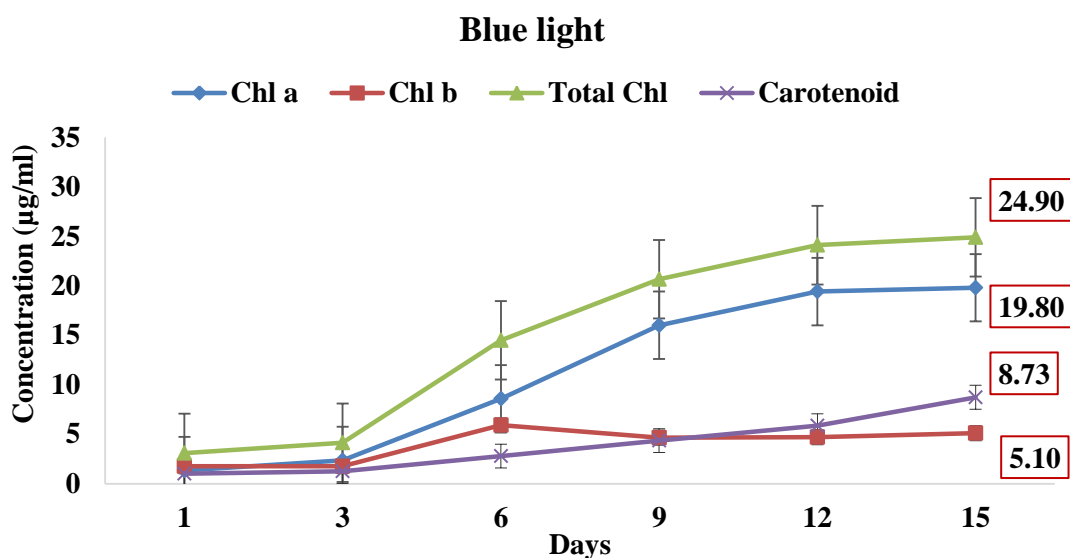


Figure 42. Effect of blue LEDs on chlorophyll and total carotenoid content

4.15. Astaxanthin extraction under different light conditions:

Microalgal cells are grown under different light illumination and lyophilized for astaxanthin extraction using different solvents and acids. Maximum astaxanthin extraction from cells grown under (5000 lux blue light + acetate) using 4 M HCl (28.68 ppm) (Figure 43) followed by cells grown under (blue light + acetate) with 4 M HCl (27.73 ppm) (Figure 44) and (white light + acetate) (24.44 ppm) (Figure 45). Minimum astaxanthin was extracted from cells grown under (5000 lux blue light) (Figure 46). Among oils, extraction was

maximum using olive oil. When observed under a microscope, the cells after extraction appeared colorless with intact cell walls (Plate 6).

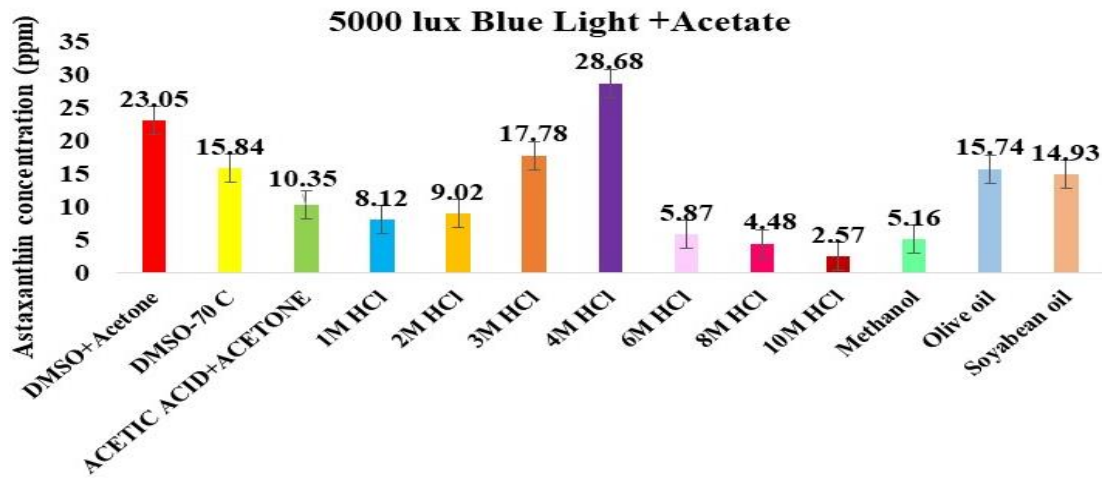


Figure 43. Extraction of astaxanthin with different solvents under 5000 lux blue LED + acetate conditions

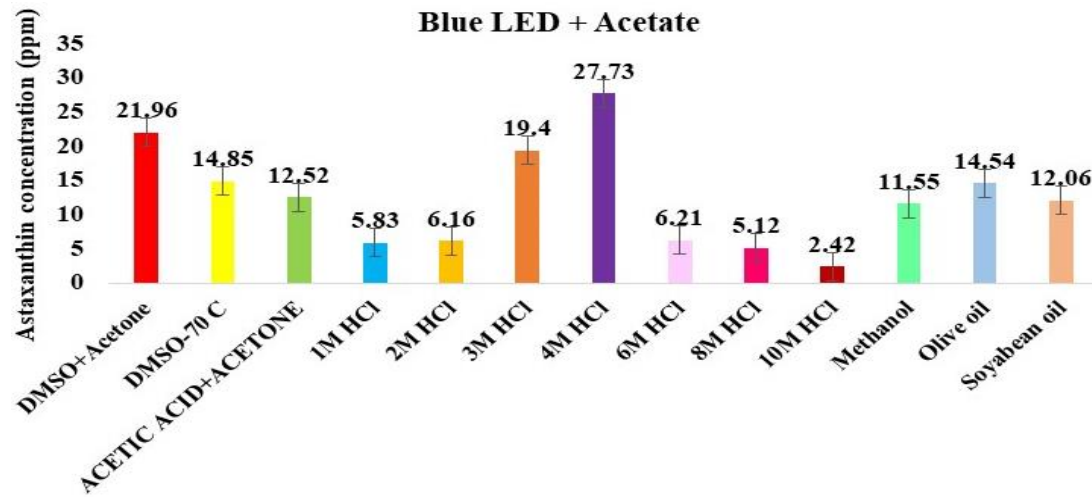


Figure 44. Extraction of astaxanthin with different solvents under blue LED + acetate conditions

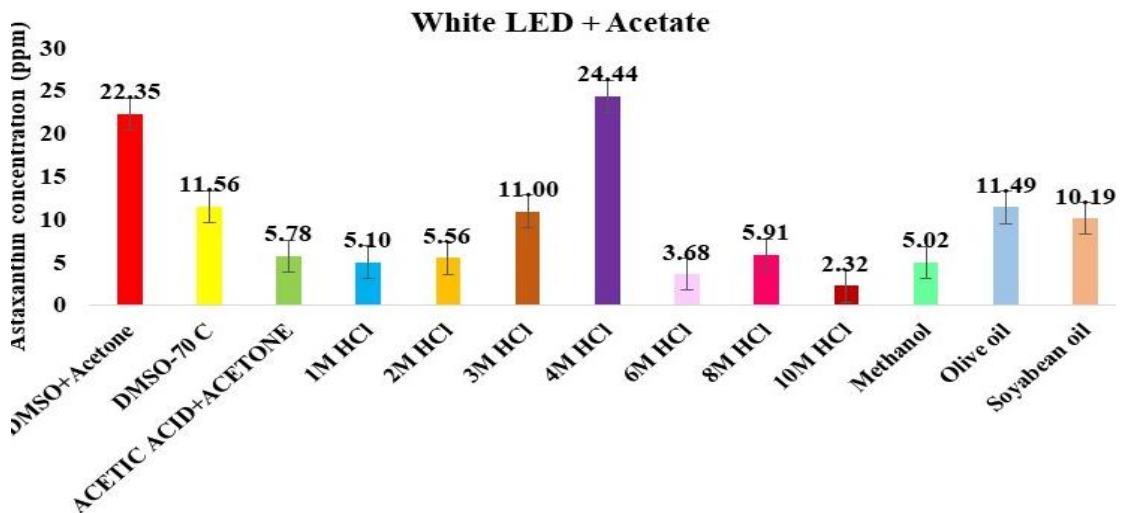


Figure 45. Extraction of astaxanthin with different solvents under white LED + Acetate conditions

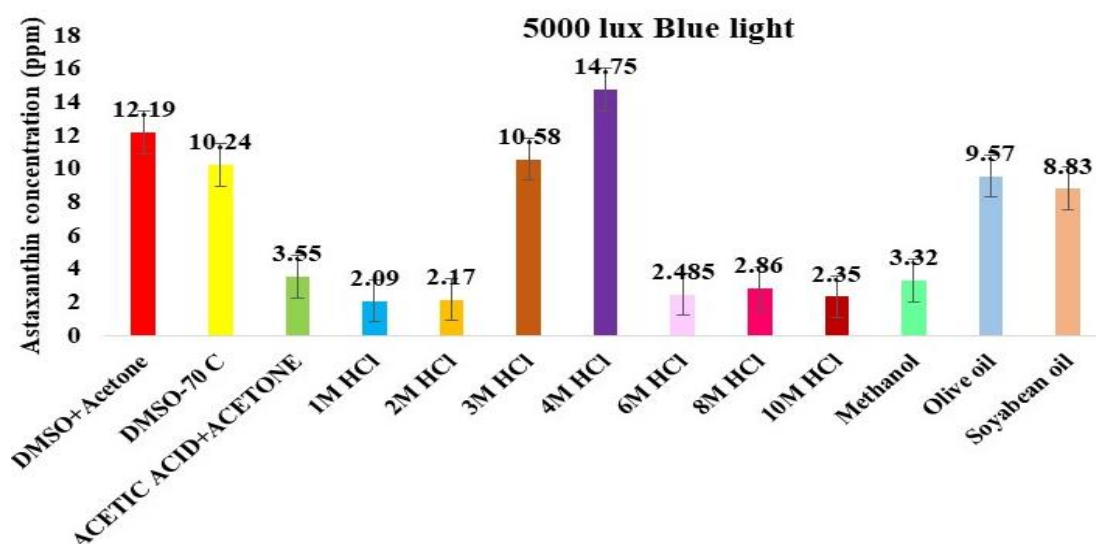


Figure 46. Extraction of astaxanthin with different solvents under 5000 lux blue light conditions

4.16. DPPH scavenging activity of extracted astaxanthin: Maximum scavenging activity was observed for astaxanthin from *Desmodemus* sp. PLM2 grown under (5000 lux blue light + sodium acetate), extracted using 4 M HCl (83.30%) (Figure 47), followed by astaxanthin from *Desmodemus* sp. PLM2 grown under (blue light + sodium acetate), extracted using 4 M HCl (82.87%) (Figure 48). The lowest scavenging activity was exhibited by astaxanthin from *Desmodemus* sp. PLM2 grown under (white light + sodium acetate), extracted using 4 M HCl (81.67%) (Figure 49) and 80.72% under (5000 lux blue light) (Figure 50).

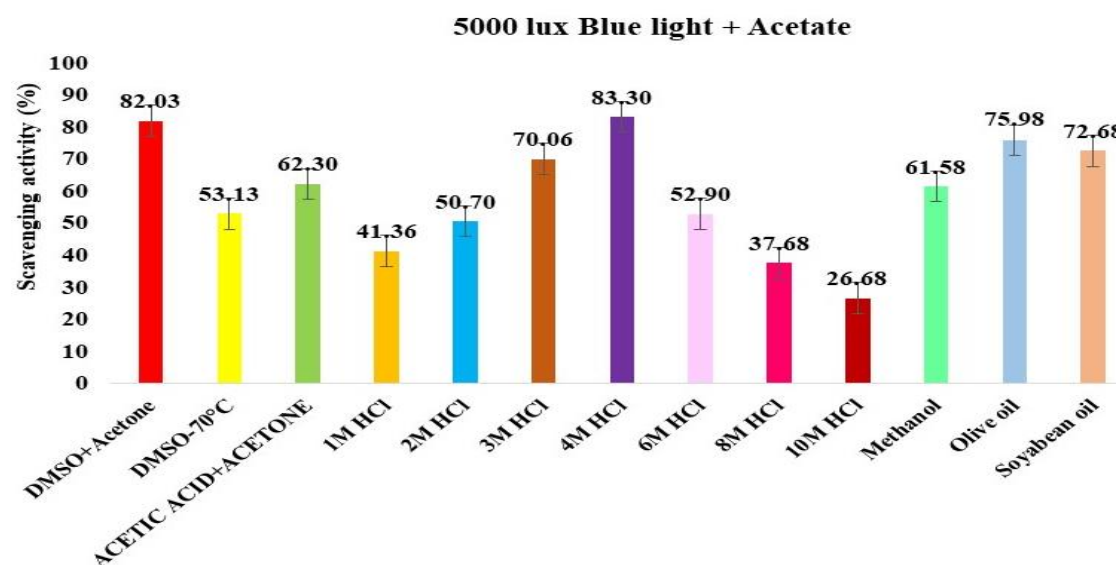


Figure 47. DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of astaxanthin under 5000 lux blue light + acetate conditions

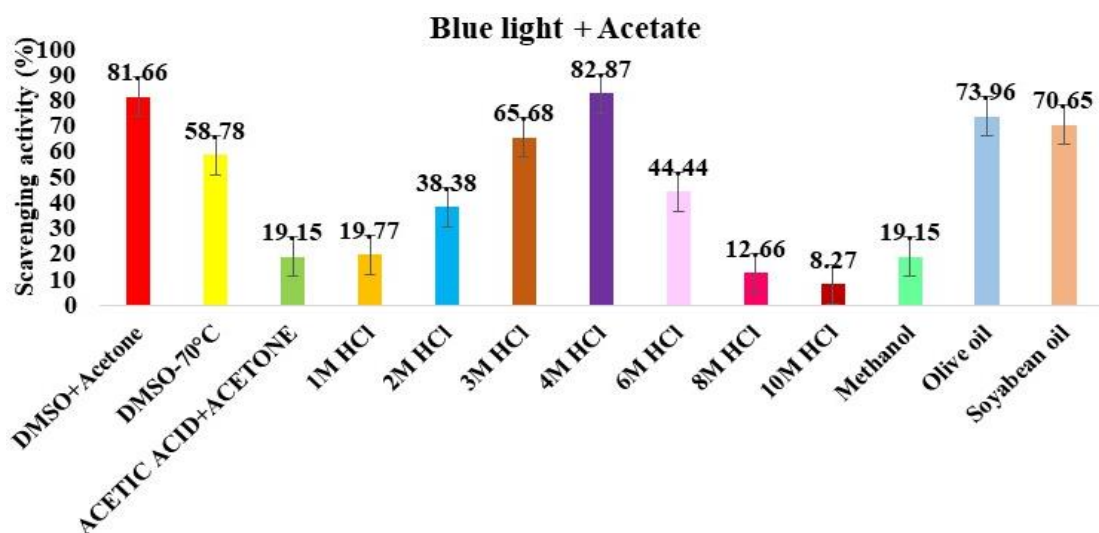


Figure 48. DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of astaxanthin under blue light + acetate conditions

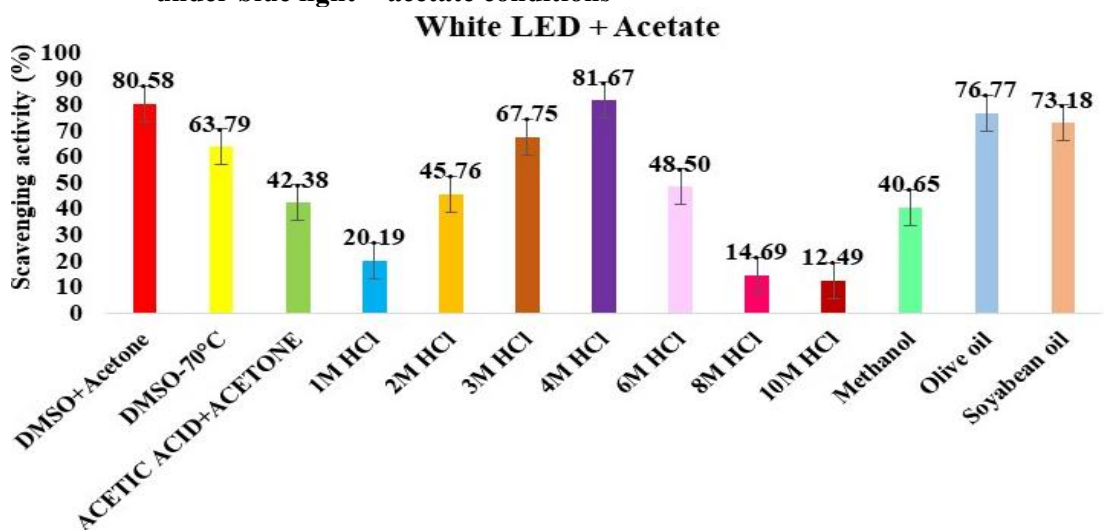


Figure 49. DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of astaxanthin under white LED + acetate conditions

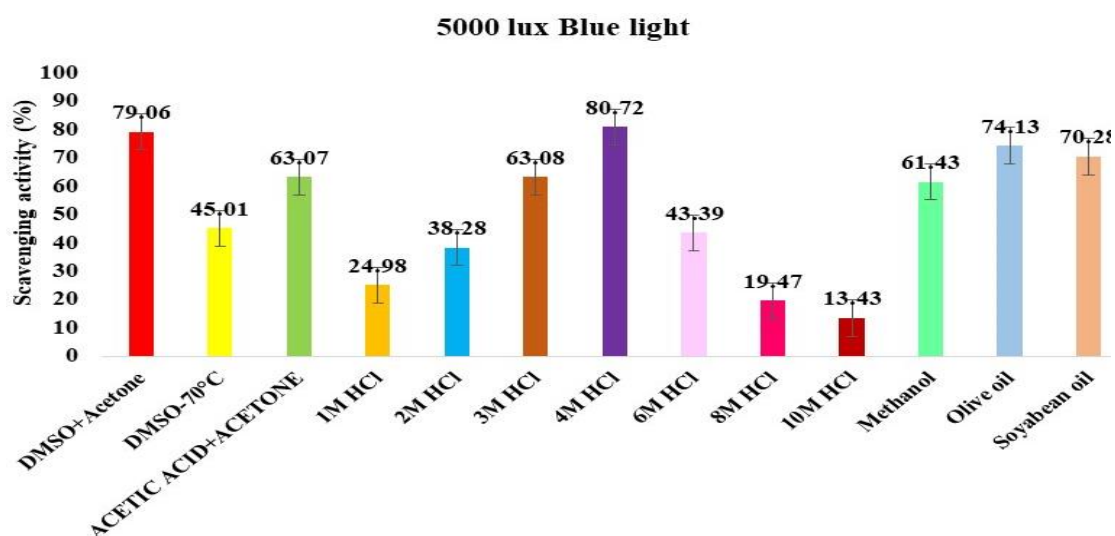


Figure 50. DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant activity of astaxanthin under 5000 lux blue light conditions

4.17. Hydroxyl radical scavenging activity of extracted astaxanthin: Maximum scavenging activity was observed for astaxanthin from *Desmodemus* sp. PLM2 grown under (5000 lux blue light + sodium acetate), extracted using 4 M HCl (43.98%) (Figure 51), followed by astaxanthin from *Desmodemus* sp. PLM2 grown under (blue light + sodium acetate), extracted with 4 M HCl (38.49%). (Figure 52). The lowest scavenging activity was exhibited by astaxanthin from *Desmodemus* sp. PLM2 grown under (white light + sodium acetate), extracted with 4 M HCl (37.92%) (Figure 53) and 28.61% under (5000 lux blue light) (Figure 54).

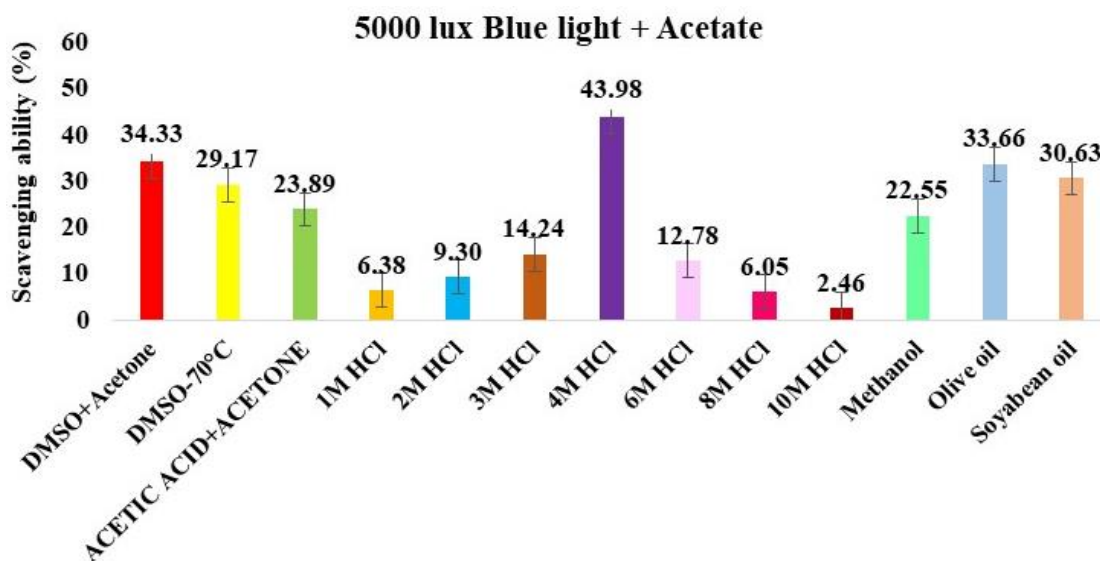


Figure 51. Scavenging effect of extracted astaxanthin on hydroxyl radical under 5000 lux blue light + acetate conditions

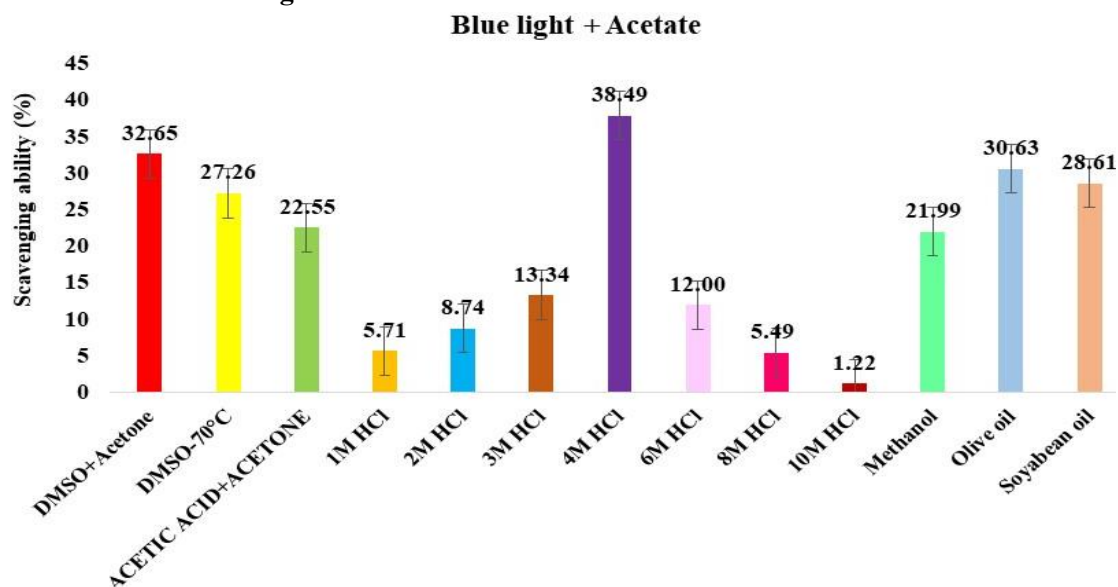


Figure 52. Scavenging effect of extracted astaxanthin on hydroxyl radical under blue light + acetate conditions

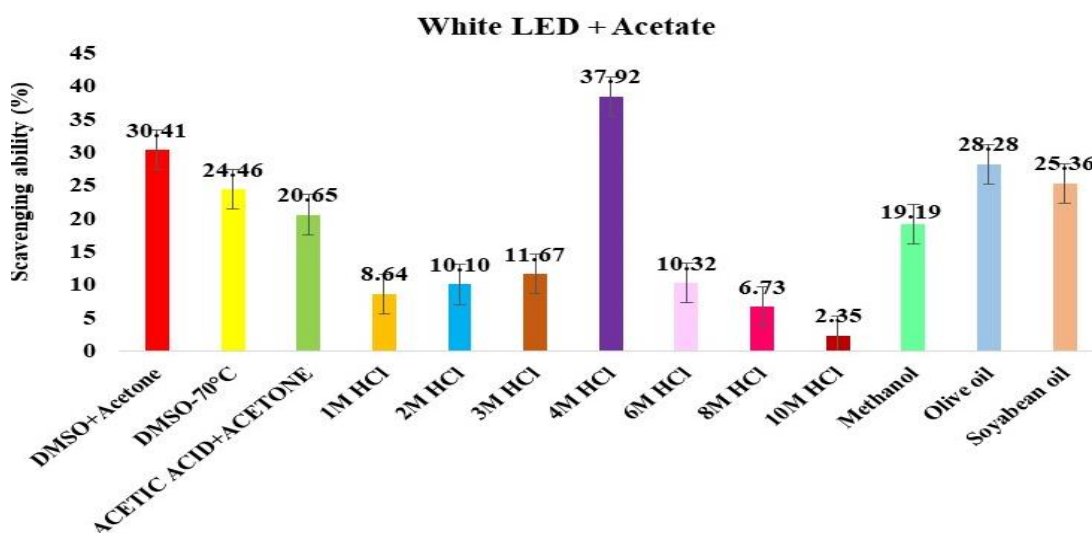


Figure 53. Scavenging effect of extracted astaxanthin on hydroxyl radical under white LED + acetate conditions

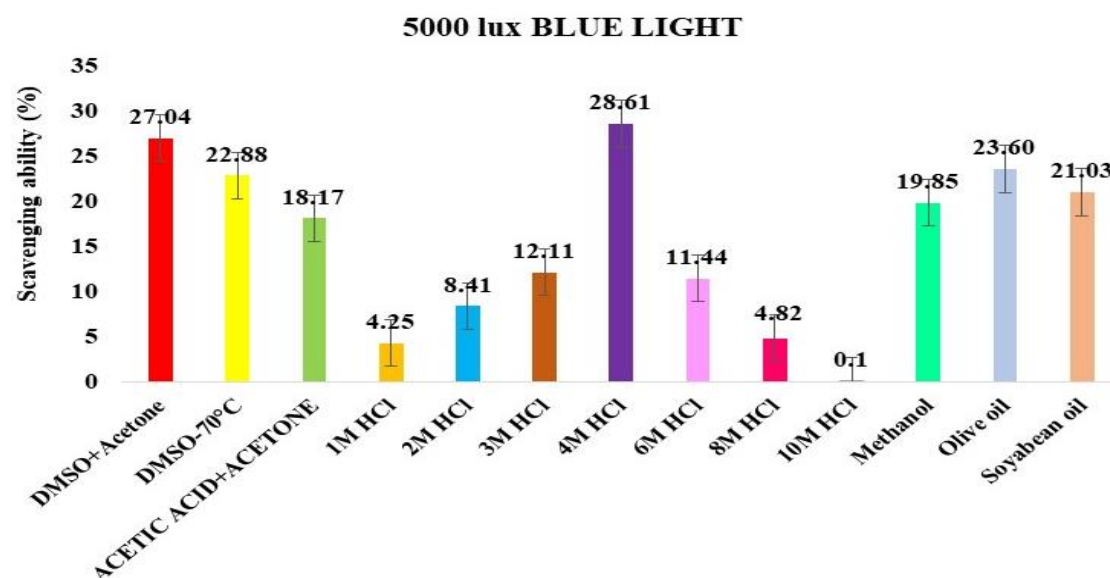


Figure 54. Scavenging effect of extracted astaxanthin on hydroxyl radical under 5000 lux blue LED conditions

4.18. Storage studies of extracted astaxanthin:

The lyophilized dried red cyst cells collected from the different light conditions were preserved in Ziploc bags kept at -20°C , 4°C , 25°C , and room temperatures. To avoid the effect of light, all of the bags were wrapped with aluminum foil. Sampling was done on the 4th, 8th, 12th, 16th, and 20th weeks after storing the dried powders. Maximum degradation of astaxanthin was in the case of lyophilized dried cells from 5000 lux blue light stored at room temperature, and degradation increased with time that is from 23% to 90.5% from 4th week to 20th week of storage, followed by blue light + acetate (84.2% at room temperature after 20th weeks of storage), white light + acetate (83% at room temperature after 20 weeks of storage) and 5000 lux blue light + sodium acetate (81% at room temperature after 20 weeks of storage) (Figure 55).

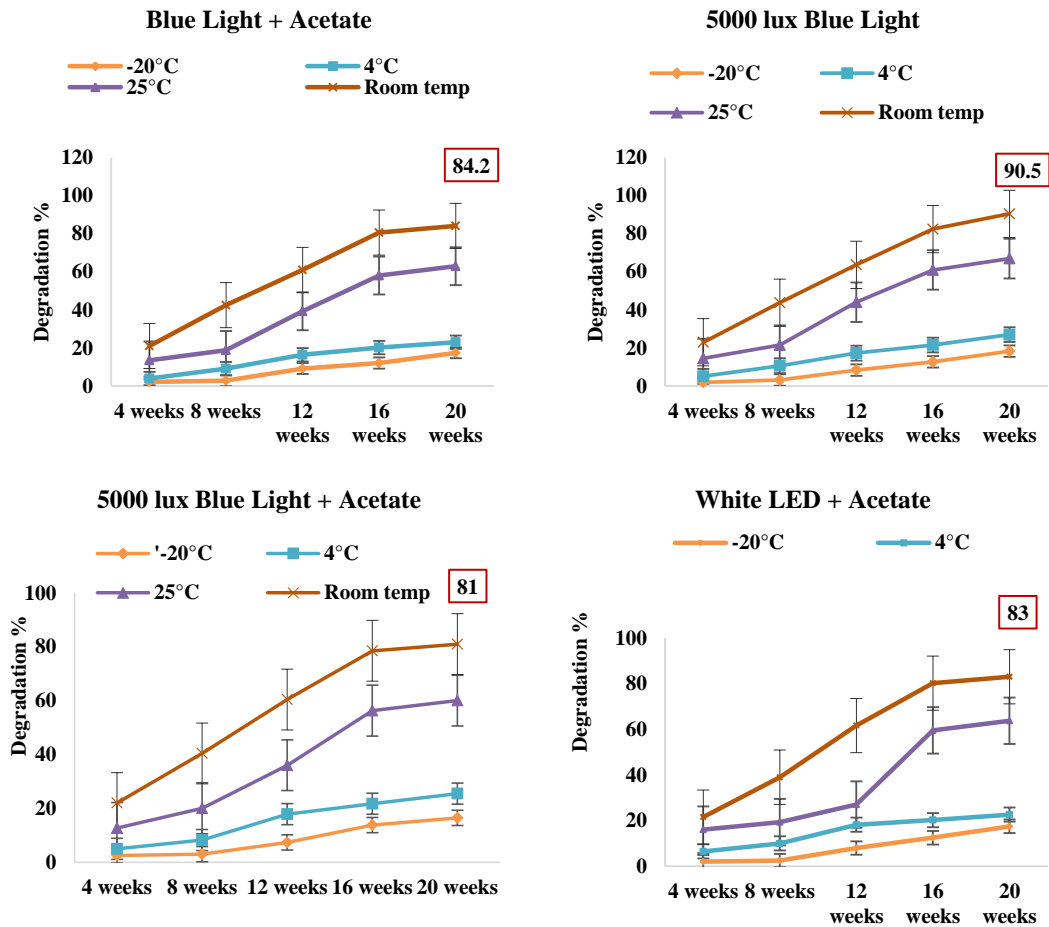


Figure 55. Degradation of total astaxanthin content (%) in relation to initial levels of lyophilized dried normal packed red cell powder stored at -20°C, 4°C, 25°C, and room temperature for 20 weeks

4.19. Antibacterial activity of extracted astaxanthin: Astaxanthin extracted from *Desmodium* sp. PLM2 was tested for the antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* (Plate 11). The results showed that *Desmodium* sp. PLM2 crude extract (10µl) possessed antibacterial activity by showing the inhibition zone. The highest antibacterial activity was a 28 mm zone of inhibition on *B. subtilis*, 25 mm zone of inhibition on *S. aureus*, and 24 mm zone of inhibition on *E. coli*.

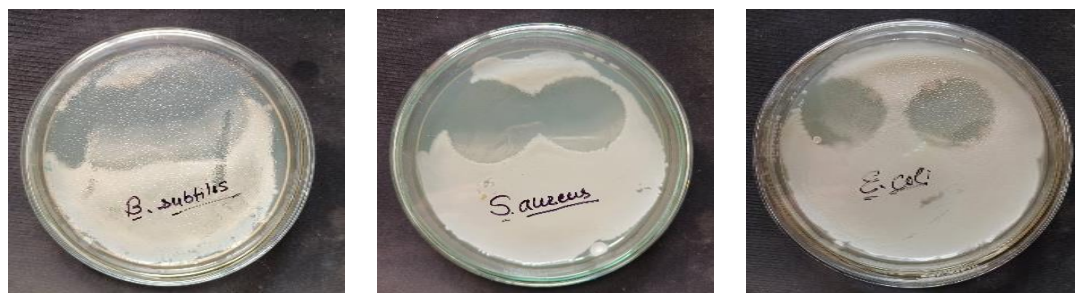


Plate 11. Antimicrobial activity of astaxanthin against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*

With the increasing population, food demand is also increasing. Therefore, alternatives are thought to alleviate the stress from crop land. One such alternative is the addition of microalgae to the diet, a rich source of protein, fatty acids, carbohydrates, minerals, and vitamins (Becker, 2007; Chronakis and Madsen, 2011; Wells *et al.*, 2017). Therefore, microalgae can be added to food to enhance the nutritional value of food. Polysaccharides and oligosaccharides compounds in microalgae are gaining attention due to their prebiotic applications (Jutur *et al.*, 2016; Raposo *et al.*, 2016; Moreno *et al.*, 2017). However, the price range of compounds like phycocyanin, astaxanthin, and β -carotene is high, ranging from hundreds to thousands of euros per kg depending upon the purity of the compound (Brennan and Owende, 2008; Mathys *et al.*, 2013). These compounds generate a high premium; thus, more and more people are coming into business related to such products. However, the microalgal cells-based food supplements market is less than 40 euros per kg (Brennan and Owende, 2008; Enzing *et al.*, 2014). Microalgae-derived food supplements are generally more expensive than other joint food supplements. The high cost is due to the increased cultivation and processing costs. The global market of microalgal biomass and related products is an expanding sector, thus attracting large enterprises and start-ups worldwide. Under stress conditions, microalgae accumulate several secondary metabolites, lipids, and carbohydrates (Markou and Nerantzis, 2013).

Among all the carotenoids produced by microalgae, astaxanthin is the most valuable compound with significant application in feed, food, pharmaceutical, and cosmetics. Astaxanthin is grouped under the xanthophyll family of carotenoids and provides characteristic pinkish-red color to salmons, shrimps, trout, lobster, and crayfish. It improves pigmentation and enhances immunity and fertility (Koller *et al.*, 2014). It has a vigorous antioxidant activity and scavenges free radicals within the human body (Kumar *et al.*, 2021). It prevents skin damage from UV-induced photooxidation. It also acts as an anti-cancer compound and is used to cure and prevent neural damage due to age-related muscular degeneration, Parkinson's disease, and Alzheimer's disease (Zhang *et al.*, 2017). Astaxanthin is used as a nutritional supplement to increase stamina, reduce muscle recovery time, and improve athletic performance (Capelli *et al.*, 2013). It can be obtained from bacteria, yeast, microalgae, aquatic fauna, and plants. Keeping this in mind, the present investigation entitled "Production, extraction, and characterization of astaxanthin from microalgae" was carried out to isolate and characterize astaxanthin-producing microalgal strains. Further, specific protocols were set up to enhance the pigment production potential of microalgal strain. The extracted carotenoid was further evaluated for antioxidant activity and antibacterial activity.

Five microalgal strains were isolated from water bodies of Haryana and Himachal Pradesh. All five isolates were screened for astaxanthin production. Only two isolates, PLM1 and PLM2, were astaxanthin-producing strains; however, only PLM2 was investigated for further studies due to better astaxanthin production potential. Phylogenetic analysis based on 18S rDNA; internal transcribed spacer (ITS) rDNA showed that PLM1 and PLM2 were distinct species with no known close relatives. PLM1 was identified as *Desmodesmus* sp. nov. PLM1 and PLM2 was identified as *Desmodesmus* sp. nov. PLM2. Both strains belong to the class *Chlorophyceae* and were novel species and astaxanthin producers. Many researchers have previously reported the isolation of novel astaxanthin-producing microalgal strains; for instance, Kawasaki *et al.* (2013; 2020) isolated *Coelastrrella astaxanthiana* sp. nov. strain Ki-4 from a dry surface of heated asphalt in Japan. Tharek *et al.* (2020) isolated two tropical microalgal strains, *Acutodesmus obliquus* and *Coelastrum* sp., from a sampling site at Hulu Langat River, Kuala Selangor, Malaysia, and screened for astaxanthin production under different stress conditions. Astaxanthin accumulation efficiency of isolate PLM2 was studied under lab conditions in BG-11 medium. The size/weight of microalgal strain directly relates to astaxanthin accumulation. With the increase in incubation time, dry cell weight also increased. A two-stage cultivation method was adopted to enhance astaxanthin accumulation. The first stage was directed for biomass accumulation, while the second or red stage is the beginning of astaxanthin accumulation induced by stress conditions.

Carotenogenesis is influenced by nutritional (nitrogen, carbon, and salt) and physical factors (temperature, pH, and light). The present study shows the effect of different concentrations of different nitrogen sources on growth, biomass yield, total chlorophyll, and total carotenoid content in *Desmodesmus* sp. nov. PLM2. Nitrate directly affects the cell division rate and carotenoid accumulation within the cell. Nitrogen is required for protein synthesis during astaxanthin biosynthesis. Therefore, nitrogen can enhance the total carotenoid content in microalgae. The present study depicted an increase in growth and biomass yield (3.6 mg/ml), chlorophyll a (9.32 µg/ml), chlorophyll b (3.77 µg/ml), total chlorophyll (13.10 µg/ml) and total carotenoid (3.64 µg/ml) at 20 mM concentration of sodium nitrate. The effect of nitrogen was concentration-dependent; higher concentrations inhibited the growth and markedly increased the carotenoid content of *Desmodesmus* sp. PLM2. Results were in accordance with the study of Hoys *et al.* (2021); they observed the effect of two different concentrations of sodium nitrate and observed green colored palmelloid cells when plenty of nitrogen was available in the medium (15mM sodium nitrate) while cells were palmelloid but red under nitrogen limiting nitrogen conditions (2mM of sodium nitrate).

Further, biomass concentration, astaxanthin content, and nitrogen content with 2mM concentration of sodium nitrate were observed to be 0.8g/L, 0.21%, and 5%, respectively.

Another similar series of experiments was conducted by Chen *et al.* (2017). They conducted a mixotrophic growth experiment in a modified basal medium with a high C/N ratio of 200 and containing 30g/L glucose and 0.6 g/L of NaNO₃. They observed rapid growth in the initial 192h, after which specific growth started decreasing due to advancement in nitrogen limiting conditions. The accumulated biomass was observed to be 3.27 g/L only. Further, they reported high astaxanthin content and decreased chlorophyll content with increased stress conditions, such as nitrogen limitation and high irradiation. They further reported a decrease in total chlorophyll content (a + b) from 31.9% at 146 h to 17.5% at 288 h, which eventually changed color from green to red. According to Droop (1954) and Borowitzka *et al.* (1991), the ratio of chlorophyll and carotenoid content signifies the physiological state of microalgal strain.

For 15 days, the influence of several carbon sources such as sodium bicarbonate, dipotassium carbonate, sodium carbonate, and glucose on growth, biomass output, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid content of *Desmodesmus* sp. PLM2 was investigated. Forty to fifty percent of algal biomass is carbon, which meets the requirement of inorganic carbon (Weissman, 1988). Among the carbon sources, glucose at 3 mM concentration increased the growth, biomass yield (4.1 mg/ml), chlorophyll a (8.18 µg/ml), chlorophyll b (3.52 µg/ml), total chlorophyll (11.69 g /ml) and total carotenoid content (3.51 µg/ml). Similarly, Yang *et al.* (2021) reported 3 g/L of glucose as an optimum carbon source to attain a biomass yield of 0.91±0.02 g/L. Results were supported by Sun *et al.* (2008) after evaluating the effect of six different sugars on astaxanthin biosynthesis in *C. zofingiensis*. They reported an increase in biomass yield and astaxanthin content from 10.3 g/L to 51.8 g/L and 10.5 mg/L to 32.4 mg/L, with glucose and mannose as carbon sources. Therefore, it can be concluded that glucose increased astaxanthin accumulation within the cell.

Among the salt sources, potassium chloride at 32 mM concentration increased the growth and biomass yield (4.4 mg/ml), chlorophyll a (17.38 µg/ml), chlorophyll b (6.86 µg/ml), total chlorophyll (24.24 µg/ml) and total carotenoid content (7.18 µg/ml). The proper salt concentration of an appropriate salt enhanced the algal growth; however, high salt concentration could induce stress conditions transforming vegetative cells into resting cysts and leading to the accumulation of certain metabolites (Cao *et al.*, 2018; Janchot *et al.*, 2019; Elloumi *et al.*, 2020; Li *et al.*, 2020; Lu *et al.*, 2021). Tharek *et al.* (2020) reported that 3 g/L of salt, 0.5 g/L of carbon, and 0.1 g/L of nitrogen led to a 2-fold increase in astaxanthin content in *Coelastrum* sp. Similarly, Li *et al.* (2022) reported an increase in biomass yield, chlorophyll content, and carbohydrate content in *H. pluvialis* under 12.5 mg/L of NaCl. Similar results were reported in *Scenedesmus* sp. *Tetraselmis* sp. and *Botryococcus braunii* (Rao *et al.*, 2007; Sing *et al.*, 2014; Elloumi *et al.*, 2020). A high concentration of NaCl

reduced the ROS level and increased the Ca^{2+} and GABA content. Also, with higher NaCl dose carotenogenesis genes, *CaM* and antioxidase expressions were upregulated, promoting astaxanthin accumulation in *H. pluvialis*.

Temperature and pH are the two crucial environmental factors determining microalga's morphological changes and carotenoid formation. Both the factors regulate the carbon dioxide and mineral solubility in the culture, affecting the metabolism directly and indirectly. Among the different pH and temperatures, pH 7 and 27°C incubation temperature enhanced growth, biomass yield, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid content. However, acidic pH reduced the growth and biomass yield of *Desmodesmus* sp. PLM2. At low pH, the lowest chlorophyll content may be due to the inhibitory effect of H^+ ions on chlorophyll formation. After the 15th day, a sharp decrease was observed in chlorophyll content at all pH conditions. This could be due to nutrient depletion in the media but not related with pH and temperature. Similarly, Giannelli *et al.* (2015) reported maximum enhancement in cell division rates and cell sizes and biomass yield of *Haematococcus* at 20°C and 30.5°C. Other researchers have also reported pH 7 and temperature 23°C optimum for increasing growth, biomass, chlorophylls 'a', 'b,' total carotenoids, and β -carotene to 1.72, 5.24, 1.65, 2.04, 2.18, 3.28 folds as compared to normal conditions (Reshma *et al.* 2021).

In this study, an initial optimization of growth conditions was carried out for both the green and red phases of *Desmodesmus* sp. PLM2. In the second stage, many environmental and nutritional factors, including nitrogen, carbon, salinity, and light radiance, affect microalgal growth and astaxanthin production (Oslan *et al.*, 2021). Studies on *Desmodesmus* sp. PLM2 showed that nitrogen and phosphorus limitation in culture media and stress induction by 0.2% NaCl and 4.4 mM sodium acetate under high light intensity improved astaxanthin production. Nitrogen deficiency was investigated using a half concentration of sodium nitrate in BG-11 medium because low nitrate inhibits chlorophyll production, which slow-downs the cell development (Garcia-Malea *et al.*, 2005). Energy derived from photosynthesis is used for cell growth and biomass accumulation. However, photosynthesis does not provide adequate energy for cell growth during nitrogen starvation, resulting in slow or less growth with an increase in photosynthates. Nitrate is required for chlorophyll and carotenoid production. In *C. zofingensis*, low nitrate condition resulted in reduced cell growth and a low ratio of lutein to astaxanthin. High nitrate conditions resulted in higher cell growth and lutein level and high lutein to astaxanthin ratio (Del Campo *et al.*, 2004). Naguib (2000) studied the effect of nitrogen starvation and high light irradiance on *C. zofingensis* strain UTEX 32. They reported a decrease in chlorophyll and carotenoid content (β -carotene, lutein).

However, the additional carbon source during the inductive stress condition resulted in a higher astaxanthin production. For improving biomass accumulation, the readily absorbable organic matter by *H. pluvialis* is NaAC under optimum light conditions (Naguib, 2000). Choi *et al.* (2002) also observed the enhanced astaxanthin accumulation in *H. pluvialis* by adding acetate supplemented with nitrogen starvation. Results signified that the carbon and nitrogen ratio in a medium could significantly affect carotenogenesis. Similarly, Cong *et al.* (2020) reported that 6g/L of sodium acetate and 12 days of stress for induction of biosynthesis of astaxanthin in *H. pluvialis*. Further, Wang *et al.* (2021) reported improved astaxanthin production in *H. pluvialis* with the addition of 0.17 mol/L of sodium acetate under 200 $\mu\text{mol}/\text{m}^2/\text{s}$ with 24 h continuous illumination. Also, an adequate amount of sodium acetate promotes *H. pluvialis* growth, while excessive sodium acetate acts as a stress inducer, prevents cell growth, and promotes astaxanthin production (Jeon *et al.*, 2006).

Exposure to higher intensity of light causes photobleaching and lowers the chlorophyll content. Under high light intensity conditions, initially, cells divide rapidly and form chlorophyll, but later, reactive oxygen species increase, leading to early carotenogenesis. Under the low light condition the organism grows more prolonged than usual and accumulates higher biomass. On comparing the astaxanthin accumulation under optimum and low light conditions, results were observed to be at par. Therefore, low light conditions can be applied for higher product recovery. High light intensity reduces the growth but enhances astaxanthin accumulation, while under low light conditions, growth is more. However, under extreme irradiation and nutrient limitation, cells can suffer from photooxidative damage due to the formation of excess photosynthates, which decreases electron carriers in the chloroplastic electron transport chain. To battle such circumstances, some of the unicellular phototrophs can transfer unneeded photosynthates to the synthesis of energy-rich storage lipids and are deposited in cytoplasmic lipid globules known as oil bodies. Another photoprotective mechanism is a coordinated synthesis of storage lipids and extra plastidic secondary carotenoids as observed in some algae belonging to genus *Haematococcus*, *Parietochloris*, *Chlorella*, *Scenedesmus*, and *Dunaliella*. This mechanism is further supported by changes in the optical properties of algal cells. Oil bodies capture excessive radiation in the blue-green region of the spectrum and serve as a depot of secondary carotenoids. A study was carried out by Liyanaarchchi *et al.* (2020) to observe the effect of phosphate limitation and light (2000 lux in the green stage and 5000 lux for induction of astaxanthin) in *H. pluvialis*. They reported high astaxanthin content and decreased chlorophyll a and chlorophyll b content.

The process of extraction determines the quantification of astaxanthin content. Algaenan, a sporopollenin-like substance found in microalgal cell walls, prevents astaxanthin extraction using solvents such as acetone, dichloromethane, and methanol. The final cost of

astaxanthin is determined by downstream processing. The technique for the downstream process is selected based on the methodology used. The carotenoid concentration of biomass decreases when it is dried. The direct oil extraction method opted for therapeutic use as oil can take up to 1200 ppm of carotenoids. For carotenoid extraction, several ways are used, including extraction using oils, organic solvents, and continuous extraction utilizing various solvents with biphasic systems. From this perspective, extracting astaxanthin from *Desmodesmus* sp. PLM2 cells using multiple techniques was a difficulty. In this study, microalgal cells were grown in two different media: BG-11 medium (standard medium) and M-BG-11 medium and lyophilized for astaxanthin extraction using different solvents. Maximum astaxanthin extraction from cells grown in M-BG-11 media was 21.13 ppm using 4 M HCl, followed by DMSO + Acetone (19.34 ppm). For the cells grown in BG-11 media, astaxanthin extraction was maximum using 4 M HCl (16.12 ppm) followed by DMSO + Acetone (11.07 ppm) under cool white fluorescent light. Olive oil had the highest astaxanthin extractability among the oils, which could be attributed to the chemical properties of these oils, implying that the chemical nature and natural antioxidant levels in this oil are suitable for stability. Results agree with the study of Sarada *et al.* (2006). They reported that 84-94% of astaxanthin was extracted using HCl. However, literature also presents acetone as a suitable solvent for astaxanthin extraction as Kobayashi *et al.* (1997) reported 70% extraction of astaxanthin from *H. pluvialis* cells with 40% (v/v) acetone treatment for 2 min at 80°C, followed by lyophilization or treatment of cells with specific lytic enzymes. Vegetable oils can also be used for more or less 88% extraction of astaxanthin in *H. pluvialis* as reported by Kang and Sim (2008). Another similar study was carried out by Wang *et al.* (2018). They treated the red cyst cells with 1 ml of DMSO at 60°C and acetone along with mechanical grinding followed by extraction with different solvents. DMSO can easily penetrate a cell. Generally, DMSO is used as a permeation enhancer with drugs or pesticides and a protection agent for cell cryopreservation. DMSO can ease the process of chlorophyll and carotenoid extraction from microalgae (Seely *et al.*, 1972; Boussiba and Vonshak, 1991). Tharek *et al.* (2020) treated the dried biomass of *Coelastrum* sp. with 5% KOH in 30% methanol at 70°C for 5 min, followed by extraction using 4N hydrochloric acid (HCl) to the cell and heated at 70 °C for 2 min. Astaxanthin extraction was continued by solvent extraction with acetone and set for one hour and recovered 14.44 mg L⁻¹ of astaxanthin. However, literature has several studies on astaxanthin extraction using different methods, yet there is no efficient method as the thick cell wall of an organism is still a hindrance.

Light is the most significant factor in inducing astaxanthin biosynthesis in microalgae. Previous studies showed that the conventional lights (e.g., fluorescent lamps, incandescent lamps, and cold cathode sources) are less economical and efficient for algal metabolism since the wavelength of traditional lights may not include the absorption bands of

the algal chlorophyll pigments or may contain only a combination of efficient and inefficient light spectra (Zhao *et al.*, 2011; Cheirsilp and Torpee, 2012) But there is still lack of knowledge to develop a system based on efficient light illumination for astaxanthin production. Light-emitting diodes (LED) are the next generation light sources and replace traditional light sources for astaxanthin production. Now it is feasible to use the light of a specific wavelength with low power consumption. LEDs are small chips and work for a longer duration, and that's why they are used in photo-bioreactors for biomass cultivation (Zhao *et al.*, 2011) and biochemical production such as astaxanthin production using *Desmodesmus* sp. PLM2. Therefore, it was a must to select a particular wavelength to influence the metabolism of *Desmodesmus* sp. PLM2 for improving biomass accumulation and biosynthesis of astaxanthin. The present study investigated the effects of specific wavelength LEDs (red LEDs, blue LEDs, white LEDs) on *Desmodesmus* sp. PLM2 biology includes growth, total chlorophyll content, total carotenoid content, and astaxanthin biosynthesis. Photosynthesis is majorly dependent on light corresponding to the red and blue regions of the light spectrum. Therefore, red and blue wavelengths were used in the present study.

Blue LEDs might have induced stress conditions for *Desmodesmus* sp. PLM2 delayed cell division. Red LEDs supported the vegetative stage of *Desmodesmus* sp. PLM2, by increasing cell number. However, blue LEDs lead to a lesser cell number. The observation is similar to Xi *et al.* (2016), which stated that the cell number of *Haematococcus pluvialis* grown under red LEDs was nearly 150% higher than the *H. pluvialis* grown under blue LEDs. Similarly, in *Chlorella vulgaris*, blue wavelength generated large-sized cells while red wavelength generated small cells with active division (Kim *et al.* 2014). Using blue wavelengths in conjunction with an additional carbon source could increase astaxanthin synthesis. Maximum astaxanthin extraction from cells grown under 5000 lux blue light + acetate using 4 M HCl (28.68 ppm). Blue wavelength light increased cellular stress in *Desmodesmus* sp. PLM2 and sodium acetate (4.4 mM) also likely aided cyst development by increasing cell size and improving astaxanthin accumulation capability. As a result, one of the most robust inductive variables for astaxanthin formation in *Desmodesmus* sp. PLM2 was successfully proven using a combination of blue wavelength and an additional carbon source such as acetate. Similarly, Xu *et al.* (2019) reported that blue light wavelength was optimal for phosphorus removal efficiency and accumulation of biomass and astaxanthin in *Scenedesmus obliquus*.

Further, the extracted astaxanthin was checked for its biological activity, such as antioxidant activity against DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity and hydroxyl radical scavenging activity. Scavenging activity refers to an antioxidant's ability to prevent oxidation. Thus, the higher the scavenging activity, the higher

the power of the antioxidant to scavenge the free radicals. The DPPH free radical-scavenging assay and hydroxyl radical scavenging activity assays were used to evaluate the effect of different extraction procedures on the quality of astaxanthin extracts from *Desmodesmus* sp. PLM2. It was suggested that astaxanthin was the prominent factor for the antioxidant character of extracts obtained by different extraction methods. The HCl-ACE extraction method is an efficient method for obtaining high DPPH free radical scavenging activity and reducing power scavenging activity in *Coelastrum* sp. (Kaha *et al.* 2021) and other species as well (Dong *et al.* 2014).

Astaxanthin is sensitive to light, temperature, and oxygen, and such extreme condition leads to its degradation. Therefore, much development is required to develop a method for its stability during long-term storage. To improve astaxanthin's solubility in water, it is reacted with different esters like di-sodium di-succinate, tetrasodium diphosphate, di-vitamin C di-succinate, and fatty acid (Tachaprutinun *et al.*, 2009). Miao *et al.* (2013) reported enhanced stability and protection against temperature when astaxanthin forms compounds with β -cyclodextrin, hydroxypropyl- β -cyclodextrin, and chitosan. Another efficient method for improving the solubility and stability of astaxanthin is encapsulation in compounds like poly (ethylene oxide)- 4-methoxycinnamoylphthaloylchitosan (PCPLC) or chitosan (Tachaprutinun *et al.*, 2009). Also, 90% of astaxanthin was retained when dried algal meal was stored under a vacuum (Gouveia and Empis, 2003). Similarly, Miao *et al.* (2013) kept spray-dried *H. pluvialis* powder for 96 weeks and suggested vacuum storage at 4°C in the dark as the most practical approach. Therefore, vacuum drying is better than hot air drying in retaining astaxanthin in shrimp (Niamnuy *et al.*, 2008). In this study, the biomass *Desmodesmus* sp. PLM2 was lyophilized and stored under normal packing at -20°C to room temperature (35-37°C) for 20 weeks. The highest astaxanthin stability was recorded at low storage temperature (-20°C, 4°C), with degradation as little as 17% in lyophilized dried biomass collected from modified BG-11 media, while in lyophilized dried biomass from BG-11 medium, the degradation was 19.5% at -20°C during 20 weeks of storage. Similarly, Ahmed *et al.* (2015) reported 41% higher astaxanthin recovery than spray drying in *H. pluvialis*. Low storage temperatures (-20°C, 4°C) and vacuum packaging improved astaxanthin stability, with as little as 12.3±3.1% degradation after 20 weeks.

In the present investigation, extracted astaxanthin was tested against three different strains for antibacterial activity. The crude extract (10µl) showed antibacterial activity by forming the zone of inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. The highest antibacterial activity was a 28 mm zone of inhibition on *Bacillus subtilis*, 25 mm zone of inhibition on *Staphylococcus aureus*, and 24 mm zone of inhibition on *Escherichia coli*. Similarly, Rather *et al.* (2021) tested the antibacterial activity of crude astaxanthin against four strains (*Escherichia coli*, *Salmonella typhi*, *Vibrio cholera*,

and *Staphylococcus aureus*). The highest antibacterial activity was recorded as 10.2 ± 0.20 mm for astaxanthin extracted with acetone on *E. coli*.

In contrast, the least antibacterial activity was 6.1 ± 0.0 mm for astaxanthin extracted with hexane on *Vibrio cholera*. The underlying mechanism of antimicrobial action of xanthophyll is yet to be explored. However, the mechanism might be similar to the mechanism of carotenoids and terpenoids of the xanthophyll group. Small terpenoids such as carvacrol directly damage the cell wall and cell membrane, causing leakage of cellular components (Gutiérrez-Del-Río *et al.*, 2018). Terpenoids can also permeabilize and depolarize the cytoplasmic membrane (Hayashi *et al.*, 2018).

The present investigation showed that *Desmodesmus* sp. nov. PLM2 is a potent candidate for astaxanthin production, being plausible for selectively favoring astaxanthin production through the adequate management of growth conditions. *Desmodesmus* sp. PLM2 has a complex life cycle; it was essential to optimize the cultural condition for maximum biomass yield in the green vegetative stage, improving astaxanthin production in the red phase. The improvement in the harvesting and extraction technology will accelerate the speed of the *Desmodesmus* sp. PLM2-derived astaxanthin from laboratory scale to commercial scale.

SUMMARY AND CONCLUSION

- In the present investigation, five microalgal isolates were retrieved from the freshwater ponds of Palampur, Himachal Pradesh, and Tohana, Haryana.
- Out of five microalgal isolates, two isolates, PLM1 and PLM2, were producing astaxanthin. However, the astaxanthin production potential of PLM1 was low; therefore, only PLM2 was selected for further studies.
- Based on molecular analysis, PLM1 and PLM2 were identified as novel species named *Desmodesmus* sp. nov. PLM1 and *Desmodesmus* sp. nov. PLM2.
- Optimized culture conditions for the growth of microalgal strain include sodium nitrate (20 mM), glucose (3mM), potassium chloride (32mM), pH (7), and incubation temperature (27°C) and 15 days of incubation time for the green and red stage. The medium with optimized composition was re-named as Modified BG-11 or M-BG11 medium.
- The vegetative cells were harvested and inoculated into nitrogen and phosphorous limiting M-BG11 medium supplemented with 0.2% NaCl and 4.4 mM sodium acetate and incubated at 27 ±2°C for 15 days under the illumination of 3000 lux of cool-white fluorescent light.
- Maximum astaxanthin extraction (21.13 ppm) resulted from cells grown in an M-BG-11 medium using 4 M HCl followed by DMSO + Acetone (19.34 ppm). For the cells grown in BG-11 medium, astaxanthin extraction was maximum by extraction using 4 M HCl (16.12 ppm) followed by DMSO + Acetone (11.07 ppm).
- Extracted astaxanthin was characterized using UHPLC, FTIR, and Raman spectroscopy, through which the presence of astaxanthin was confirmed.
- Maximum DPPH scavenging activity was observed for astaxanthin from *Desmodesmus* sp. PLM2 grown in M-BG-11 medium, extracted using 4M HCl (78.69%) followed by extraction with DMSO + Acetone, *i.e.*, 77.56%, and similar results were obtained for astaxanthin extracted from *Desmodesmus* sp. PLM2 grown in BG-11 medium. Maximum scavenging activity in the latter case was observed for astaxanthin extracted using 4 M HCl (73.46%) followed by extraction using DMSO + Acetone (72.37%).
- A similar trend as DPPH scavenging activity was observed by extracted astaxanthin for hydroxyl radical scavenging activity. Maximum scavenging activity was observed for astaxanthin extracted from *Desmodesmus* sp. PLM2 grown in M-BG-11 medium extracted using 4 M HCl (37.81%) followed by extraction using DMSO + Acetone (29.39%). Maximum scavenging activity of astaxanthin was observed for astaxanthin

extracted from *Desmodesmus* sp. PLM2 grown in BG-11 medium using 4 M HCl (27.04%) followed by extraction using DMSO + Acetone (23.89%).

- In the case of lyophilized dried cells from BG-11 medium stored at room temperature, degradation increased from 26.1% to 88.3% from the 4th week to the 20th week of storage. In samples kept at 25°C, degradation observed was 16.5% to 72% on the 20th week of storage. The least degradation was observed when kept at -20°C (19.5%). In the case of lyophilized dried cells from M-BG-11 medium at room temperature, degradation increased with time from 22% to 86% from the 4th week of storage to the 20th week of storage. In samples kept at 25°C, degradation increased from 15% to 70.5% during the 20th week of storage. The least degradation was observed for the samples from M-BG-11 kept at -20°C during the 20th week of storage (17%).
- An attempt was made to establish a protocol based on LEDs' specific wavelength, *i.e.*, Redlight (630-665nm) for the cultivation and blue light (430-465nm) for the production of astaxanthin using *Desmodesmus* sp. nov. PLM2.
- Red light illumination resulted in maximum growth and cell count (76.6×10^5 cfu/ml) followed by white light (59×10^5 cfu/ml) and blue light (44.7×10^5 cfu/ml), respectively.
- Red light illumination resulted in maximum chlorophyll a (23.83 µg/ml), chlorophyll b (5.59 µg/ml) and total chlorophyll (29.41 µg/ml). The lowest chlorophyll a (19.80 µg/ml), chlorophyll b (5.10 µg/ml) and total chlorophyll (24.90 µg/ml) was exhibited by using blue light illumination. Similarly, blue light illumination resulted in total carotenoid content (8.73 µg/ml), and red-light illumination exhibited the lowest total carotenoid content (6.98 µg/ml). The red-light illumination enhanced the vegetative growth of *Desmodesmus* sp. PLM2, whereas blue-light illumination caused stress in *Desmodesmus* sp. PLM2, thereby triggering astaxanthin biosynthesis.
- Maximum astaxanthin extraction was from cells grown under (5000 lux blue light + acetate) using 4 M HCl (28.68 ppm) followed by cells grown under (blue light + acetate) using 4 M HCl (27.73 ppm) and (white light + acetate) (24.44 ppm). Minimum astaxanthin was extracted from cells grown under (5000 lux blue light).
- The extracted astaxanthin was investigated for DPPH and OH free radical scavenging activity. Maximum DPPH and OH free radical scavenging activity were observed under (5000 lux blue light +acetate), *i.e.*, 83.30% and 43.98%, respectively.
- Maximum degradation of astaxanthin was observed in lyophilized dried cells from 5000 lux blue light stored at room temperature; degradation increased with the time that was from 23% to 90.5% from 4th week to 20th week of storage, followed by blue light + acetate (84.2% at room temperature after 20th weeks of storage), white light + acetate

(83% at room temperature after 20 weeks of storage) and 5000 lux blue light + sodium acetate (81% at room temperature after 20 weeks of storage).

- Astaxanthin possessed antibacterial activity by showing the inhibition zone around the colonies of *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. The highest antibacterial activity was found in a 28 mm zone of inhibition *Bacillus subtilis*, 25 mm zone of inhibition *Staphylococcus aureus*, and 24 mm zone of inhibition *Escherichia coli*.

Conclusion:

The vast biodiversity of the Himalayan region was utilized to isolate novel microalgae strains for astaxanthin production. *Desmodesmus* sp. nov. PLM2 was a potent candidate for astaxanthin production, and the combination of different stress factors was found to significantly increase the astaxanthin yield.

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ABSTRACT

Title of Thesis : **Production, Extraction and Characterization of astaxanthin from microalgae**

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In the present investigation, five microalgal isolates were retrieved from different regions of Himachal Pradesh and Haryana. Out of five, two isolates, PLM1 and PLM2, produced astaxanthin. Astaxanthin production potential of PLM1 was low; therefore, only PLM2 was selected for further studies. Based on molecular analysis, PLM1 and PLM2 were identified as novel species named *Desmodesmus* sp. nov. PLM1 and *Desmodesmus* sp. nov. PLM2. Optimized culture conditions include sodium nitrate (20 mM), glucose (3mM), potassium chloride (32mM), pH (7), incubation temperature (27°C), and 15 days of incubation time for the green and red stages. The media with optimized composition were re-named as Modified BG-11 or M-BG11 medium. After achieving maximum biomass yield, cells were harvested and inoculated in nitrogen, phosphorous limiting media supplemented with 0.2% NaCl and 4.4mM sodium acetate and incubated at 27°C for 15 days under the illumination of the white light of intensity 3000 lux. After 15 days, microalgal red cyst cells from BG-11 and M-BG11 medium were harvested, lyophilized, stored, and directed for astaxanthin extraction using different solvents. Maximum astaxanthin extraction resulted from cells grown in M-BG-11 medium was 21.13 ppm using 4 M HCl. The extracted astaxanthin was confirmed for its presence using UHPLC, FTIR, and Raman spectroscopy. Astaxanthin extracted from *Desmodesmus* cells grown in two different media was evaluated for DPPH free radical scavenging activity. Maximum DPPH free radical scavenging activity (78.69%) and free hydroxyl radical activity (37.81%) were observed for astaxanthin extracted using 4M HCl. The lyophilized dried biomass was stored and evaluated for degradation at different temperatures, including -20°C, 4°C, 25°C, and room temperature. Maximum degradation (88%) was observed at room temperature after 20 weeks of storage for the BG11 medium. The red-light illumination enhanced the vegetative growth of *Desmodesmus* sp. PLM2, whereas blue-light illumination caused stress in *Desmodesmus* sp. PLM2, thereby triggering astaxanthin biosynthesis. The extracted astaxanthin was inhibiting the growth of *Bacillus subtilis*, *Staphylococcus aureus*, and *E. coli*.

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- **Satish Kumar**, Rakesh Kumar and Jagdish Prasad (2019) Microbial flora and fauna of ponds in Hisar district of Haryana and their impact on water quality. Presented oral presentation during international conference on Climate Change towards Health and Agricultural Sustainability (CCHAS-2019) organized by Department of Environmental Science & Engineering, Guru Jambheshwar University of Science & Technology, Hisar, Haryana on February 18-20,2019.
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I, hereby, declare that all the information given in the resume is true to the best of my knowledge.

(SATISH KUMAR)

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I, **Satish Kumar**, Admission No. **2017BS10D**, undertake that I give copyright to the CCS HAU, Hisar of my thesis entitled **“Production, extraction and characterization of astaxanthin from microalgae”**.

I also undertake that patent, if any, arising out of the research work conducted during the program shall be filled by me only with due permission of the competent authority of CCS HAU, Hisar.

Signature of the student