Sustainable process development for high density protein rich microalgal cultivation and its application as aquafeed

A Thesis

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by

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STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the **Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati,** Guwahati, Assam, India under the supervision of **Prof. Debasish Das**. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled “**Sustainable process development for high density protein rich microalgal cultivation and its application as aquafeed**” by **Mr. Ratan Kumar** for the award of degree of **Doctor of Philosophy** is an authentic record of the results obtained from the research work carried out under my supervision in the **Department of Biosciences & Bioengineering**, **Indian Institute of Technology Guwahati**, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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ABSTRACT

Up to 7 % (v/v) of worldwide CO_2 emissions are caused by flue gases released by coal-fired thermoelectric facilities, with the flue gases emitted from the power plants consisting of 10 to 15 % (v/v) of CO_2. Increased CO_2 levels in the atmosphere are the cause of global warming and climate change. With a high dependence on fossil fuels, global CO_2 emissions continue to increase. An independent research perspective towards the development of a sustainable technology for CO_2 sequestration is being examined in light of the enormous need to capture and utilise CO_2 for lowering the environmental effect. Aligned with this initiative is the development of a microalgae-based carbon sequestration process, which can be attributed to the microalgae's inherent capability of growing at high CO_2 concentrations and accumulation of a high intracellular protein composition; providing an opportunity to be an attractive alternative aquafeed, thereby making the process sustainable and economically feasible. The development of an industrially practicable and economically sustainable process strategy for microalgal-based aquafeed production is currently hampered by a number of barriers. Such incipient bottlenecks can serve as motivation for the development of an aim for the selection of novel strains and the design of novel process strategies; which will boost research towards the industrialization of microalgal-based aquafeed.

The current research seeks to comprehend and address the aforementioned obstacles by screening the high CO_2-tolerant novel microalgal strain *Desmodesmus pannoniccus* CT01 and development of process engineering strategy for protein-rich high cell density cultivation. This research also focuses on real assessment of the biomass performance of novel microalgal species as an alternative raw material for aquafeed. These objectives were accomplished via the combined approach of (i) selection of potential high CO_2 tolerant microalgal strains and characterization under various nutritional and physicochemical parameters; (ii) real time evaluation of microalgae species performance as a feed supplement for aquaculture; (iii) process engineering for cultivation of high-density protein rich novel microalgal isolate and finally, (iv) demonstration of the integrated sustainable microalgal feed technology at large scale of 50 L photobioreactor.

The study begins with the screening of CO_2 tolerant strains present in the aqueous sample, collected from industrial hotspot. This was achieved by a novel CO_2 selection pressure-based screening strategy where the enriched mixed culture was exposed to sequentially elevated concentrations of CO_2 mixed with air stream starting from 5% to 25% v/v, with step-wise increase by 2.5% v/v. A unique indigenous freshwater microalgal strain *Desmodesmus pannonicus* CT01 (Accesion Number: OL470985) that could tolerate and sustain much higher CO_2 concentration of up to 25% v/v was isolated and identified. CT01 was further subjected to different suitable nutritional and growth conditions and characterization was carried out under different media compositions, various initial pH of the culture medium, nitrogen sources, and phosphate sources supporting maximum growth of CT01. Further to evaluate the application potential of the microalgal biomass as a carbon sequestration house, the influence of CO_2 concentration in the inlet gas steam on growth and CO_2 sequestration ability of the organism, experiments were performed under six different CO_2 concentration such as 0.03 (air), 5, 7.5, 10, 12.5, 15 and 20%, v/v. The strain unveiled optimal growth performance with CO2 concentration in the range of 10 -15%, v/v. The highest biomass titre and productivity of 1.42 g L-1 and 101.43 mg L-1 d-1, respectively was recorded with estimated CO_2 fixation rate of 159.91 mg L-1 d-1, when grown at 12.5% CO_2. The intracellular total protein content of 49.53 (w/w) was found to be highest in 12.5% CO_2 hinting the possible application of CT01 biomass as potential alternative aquafeed.Owing to its high protein content, CT01 biomass was evaluated as potential alternative to commercially available conventional fish feed. Three different feedstocks were considered to evaluate growth metrics and dietetics of *Hypophthalmichthys molitrix* (silver carp): microalgae feed (MiF), that is whole cell biomass of CT01; reference feed (ReF), composition of which is equivalent to the commercially used feed for silver carp, and mixed feed (MixF) comprising both microalgae and reference feed in 1:1 ratio. Mixture of microalgae with reference feed (1:1), resulted in significant improvement in growth matrices such as final body weight (mg), average weight gain (mg day^{-1}) and specific growth rate (% day^{-1}) of fish fry as compared to the reference feed. Similar to the growth performance, dietetics of the fish fry in terms of feed efficiency, protein efficiency ratio and protein productive value was found to be higher while fed on mixed feed as compared to the reference feed. The quality of fish in terms total protein content was estimated to be highest at 60% for microalga feed, followed by mixed feed at 59% and reference feed at 54%. This suggests better digestibility of microalga protein by the fish as compared to reference diet.

Further to enhance the cell density and protein content of CT01, a process engineering strategy was developed. At the onset, the media components, concentrations of initial phosphate, initial nitrate and trace and micro elements, were optimized for improved biomass titre and productivity using response surface methodology (RSM) based central composite design (CCD). Validation of the model was confirmed by comparing the predicted biomass concentration (0.9778 g L^{-1}) with the experimental value (0.9649 g L^{-1}) and predicted biomass productivity (32.11 mg L^{-1} d^{-1}) with the experimental value (31.39 mg L^{-1} d^{-1}) at optimized concentration of nitrate (0.79 g L^{-1}), phosphate (0.185 g L^{-1}) and TME (0.73-unit L^{-1}). CCD-RSM based optimization of the process variables resulted in 28 % increase in biomass titer and 27% increase in biomass productivity while compared to un-optimized condition. With the optimized medium, the CT01 was subjected to different light wavelength and intensity to improve the total protein content in the biomass composition. Experiments were performed under exposure to seven different wavelength of light combining mono and multi wavelengths *i.e* white, blue, green, red, red-blue, red-green and blue green and subsequently under exposure to four different light intensity ranging from 50, 100 ,150, 200 µE $m^{-2}s^{-1}$ to enhance the biomass productivity, total protein content and carbon sequestration ability of the strain. The biomass concentration and biomass productivity were found to be highest when exposed to combination of red-blue light wavelength and attain the maximum value of 1.38 g L^{-1} and 81.7 mg L^{-1} d^{-1}, respectively. As the CO_2 sequestration ability of the microalgae has a liner correlation with the growth kinetics, the highest CO_2 fixation rate of 144.15 mg L^{-1} d^{-1} was found when exposed to red-blue light. Total protein 54.6 % (w/w) was found to be highest with red-blue light wavelength enhancing the credibility for biomass as a suitable candidate for alternate aquafeed. Total protein of the biomass and carbon sequestration rate of CT01 were also found to be higher with the light intensity of 150 µE $m^{-2}s^{-1}$. The interdependent dynamics of light intensity, growth and culture pH indicated that the process engineering strategy, based on on-demand supply of CO_2 under optimized light wavelength and intensity, may results in improved biomass concentrations and productivity by maintaining optimal culture pH and eliminating CO_2 limitation. Two parallel batches of CT01 were performed where all the growth parameters were kept identical except in one batch, the pH of the culture was maintained at optimal value of 8 through cascade driven intermittent purging of CO_2 and in another batch, culture pH remained uncontrolled. The batch with controlled pH resulted in maximum biomass concentrations of 1.93 g L^{-1} , productivity of 145.4 g L^{-1} day^{-1}, CO_2 fixation rate of 259 mg L^{-1} day^{-1} shows improvement as in when compared with the batch with uncontrolled pH.

With the view of evaluation of developed process engineering strategy for CT01 at large scale, cultivation was carried out in a novel customised airlift photobioreactor (APBR) of 50 L volume. The scaling of APBR up by volume while trying to maintain sufficient CO_2, light availability to cells and minimal shear forces was the key areas to be considered for. The design of novel APBR was targeted to minimal the risk of scaling up without compromising the growth and intracellular properties of the strain. The biomass composition obtained from both batches suggest the reactor performs well and also successful in minimising the risk. Next to it, the high protein rich fresh microalgal biomass was subjected as aquafeed for the model fish *Cirrhinus mrigala* (mrigal carp). The fish growth performance was evaluated in terms of growth metrices and biomass composition. The live fresh biomass of CT01 showed no negative impact on the fish as compared to reference feed suggesting its potential to be an alternative aquafeed.

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CHAPTER 1

Introduction

1.1 Background and motivation

Carbon dioxide (CO_2) emissions are steadily increasing as a result of increased usage of fossil fuels and industrial activities, and are considered as major contributor to climate change. Human activities produce a minute percentage of CO_2, as relative to natural sources, but it upsets the natural equilibrium because latter can be balanced the amount of CO_2 produced and utilized (Walsh *et al.*, 2017). Global warming from increased greenhouse gases (GHGs) emissions, has resulted in the melting of polar ice, rising sea levels, ecosystem collapse, and extreme climatic catastrophes (hurricanes, droughts, and floods) (Harris, Roach and Codur, 2007; Tan *et al.*, 2012). Many initiatives were considered in different conferences like The Kyoto Protocol, the Paris Agreement and COP26 and established objectives and duties for participating countries in order to reduce CO_2 emissions and keep global warming to its minimum. A temperature increase of 1.5 °C has been targeted as compared to 2 °C, in order to keep the impact of temperature rise on climate change much lower (*COP26 Goals - UN Climate Change Conference (COP26) at the SEC – Glasgow 2021*; UNFCCC, 2015). As a result, it is critical to develop a variety of sustainable technologies capable of reducing CO_2 emissions and its capture from the atmosphere.

Biological and physicochemical are currently the two methods for capturing atmospheric CO_2 (Nanda *et al.*, 2016). Capturing and storing CO_2 in geological formations allows for the permanent removal of CO_2 from the atmosphere. Absorption, adsorption, and cryogenic distillation are the primary physicochemical approaches for CO_2 sequestration (Oschatz and

Antonietti, 2018; Song *et al.*, 2018; Zhai *et al.*, 2018). CO_2 capture involves several mechanisms, including CO_2 hydration to bicarbonate by carbonic anhydrase, CO_2 reduction to methane, and CO_2 conversion to formic acid and methanol (Zhang *et al.*, 2011; Schlager *et al.*, 2017). The key barriers towards implementing these technologies have been the high cost; stringent government regulations and subsidy to speed its deployment. In view of these bottlenecks; these technologies are still in the early stages and will require significant study to become a reality. Carbon capture and sequestration (CCS), based on biological systems is an environmentally benign method of reducing atmospheric CO_2 and its emission from stationary sources *e.g.*, coal-fired plants and the cement and steel industries. Biomass generated from sustainable growing and harvesting processes is considered as an alternative, since it takes CO_2 from the atmosphere (by photosynthesis) and can be utilised to transform into various useful products. This alternative is known as "bio-energy with carbon capture and storage" (BECCS), and it is anticipated that its careful implementation would result in low-carbon energy products and a negative CO_2 balance (Vergragt, Markusson and Karlsson, 2011). *Acetabacterium woodii*, *Clostridium aceticum*, *Clostridium kluyveri*, *Clostridium ljungdahlii*, *Chlorella vulgaris*, *Rhodococcus erythropolis*, *Ralstonia eutropha*, *Synechococcus elongatus*, and *Rhodobacter sphaeroides* are among the microorganisms that may capture CO_2 and has been utilised for production of various by-products like biofuels, fertiliser, etc. (Li *et al.*, 2011; Zheng *et al.*, 2012; Wong, 2014).

Among all the microbes, the usage of microalgae to bridge carbon reduction and bioenergy production is gaining popularity. Carbon collection and utilisation technique based on microalgae is eco-friendly and effective for CO_2 reduction. 55–65 % of anthropogenic CO_2 emissions are taken from the atmosphere by biological medium as natural sinks (Farrelly *et al.*, 2013). In addition, CO_2 is the most important resource for microalgae photosynthesis and carbon absorption, which accounts for 36–65% of the dry matter of microalgal biomass (Chae, Hwang and Shin, 2006). Biomass derived from microalgae is considered as a CO_2-neutral replacement for fossil fuel due to the net transfer of ambient CO_2 into biomass. Due to their unique properties, microalgae farming provides a viable alternative to CO_2 sequestration. Microalgae may live at a range of temperatures and thrive mostly in watery environments(Khan, Shin and Kim, 2018). Some species of microalgae thrive on dirt, deserts, hot springs, etc. Microalgae have a greater pneumatic productivity than other photosynthetic organisms, making them more attractive candidates for carbon sequestration. The primary advantage of cultivating algae over crops and agriculture is its resistance to salt water. In regions of the globe where freshwater is scarce, it is straightforward to cultivate algae to minimise freshwater usage (Rodolfi *et al.*, 2009). In addition, microalgae may be produced throughout the year because of their temperature tolerance. Another significant advantage of algae over crops is that no herbicides or pesticides are required to cultivate algae (Apandi *et al.*, 2019). With such modest development needs, microalgae are able to collect abundant amounts of protein, fat, and carbs, resulting in a biomass that is rich in growth-promoting nutrients. The protein content of most microalgal strains of *Spirulina*, a few strains of *Chlorella*, and *Nannochloropsis* ranges from 40 to 65 % (Vaz *et al.*, 2016). 10–25% of the dry weight of microalgae is composed of carbohydrates, depending on their development circumstances and the age of the cultures (Chacón-Lee and González-Mariño, 2010). They are present in microalgae as starch, cellulose, sugars, and other polysaccharides. Microalgae have the capability to acquire large amounts of lipid when growing under stress, and their fatty acid composition is nutritionally beneficial for human and animal consumption. The most important functional components of microalgal lipids are polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). *Schizochytrium limacinum* (DHA) and *Phaeodactylum tricornutum* and *Nannochloropsis sp*. (EPA) contain between 30 and 40 % omega-3 fatty acids out of their total fatty acids. Due to this balanced nutritional composition, microalgae are quickly gaining relevance as a feed supplement for animals, with the potential to replace the conventional protein and lipid sources and other traditional ingredients in aquaculture and animal feeds.

The aquaculture business is an efficient producer of edible proteins and is the fastest-growing food sector. Aquaculture is among the fastest-growing food industries worldwide. Aquaculture produces 50% of the fish for human consumption, and the trend has been rising steadily over the past decade (Prem and Tewari, 2020). In the past 50 years, global per capita fish consumption has climbed dramatically from 9 kg in 1961 to 20.9 kg in 2020 (Cordeiro, 2019). The existing aquaculture production capacity must be quadrupled by 2050 in order to meet global per capita fish consumption rates without placing further pressure on wild fisheries. The nutritionally balanced diet has emerged as a crucial limiting factor for increasing production quality and productivity. Consequently, the aquafeed industry has a global annual value of over $60 billion (Tibbetts *et al.*, 2015). Over 70 % of global aquaculture output is dependent on formulated feed, and 50–70 % of overall production costs are related with feed (Gong *et al.*, 2019; Ansari *et al.*, 2021). Proteins, lipids, carbs, vital amino acids, vitamins, minerals, and colours are some of the elements included in formulated diets. These elements come from numerous origins. Protein sources include fish meal (FM), chicken feather meal, and soy meal, while corn and wheat bran are utilised as carbohydrate sources. The amount of each ingredient (protein, oil, carbohydrate, mineral, vitamin, etc.) changes according to fish species and growth phases (i.e., larvae, fry, juvenile, adult, and spawning) (Kiron, 2012). Early development (e.g., larval or juvenile) has a greater protein demand because of a higher metabolic rate compared to the grower and finisher phases. Among all feed components, protein-containing substances constitute the largest contributor to total feed costs. Not only does the quality of fish food assure adequate development rates, but it also provides physiological benefits for fish. Consequently, it is essential to guarantee that these feed elements are pertinent (Haas *et al.*, 2016). The majority of traditional protein sources offer basic nutrition but are deficient in critical amino acids, long-chain polyunsaturated fatty acids (LC-PUFA), and colours. In addition, the addition of these costly key components substantially raises the price of fish feed (Cho and Kim, 2011). Due to its high digestible protein concentration (60–72 %), necessary fatty acids, and balanced essential amino acid profile, FM is one of the finest protein sources for fish feed (Palmegiano *et al.*, 2005). Fish oil (FO) includes ALA, EPA, and DHA in high concentrations. FM is also rich in natural vitamins (such as biotin, B12, and choline) and trace minerals (selenium and iodine). Therefore, FM and FO are considered critical components of the diets of certain fish species (carnivorous, omnivorous). For the expansion of the aquaculture business in the future, the supply of alternative high-quality feed ingredients will be severely limited (Macreadie *et al.*, 2019).

Important biochemical elements of microalgal biomass include lipid, protein, glucose, and different colours (Benemann, 1992). They are an important food source for fish in their natural environment, making them excellent components for aquaculture feed production. Algae supply virtually all needed elements, including polyunsaturated fatty acids, amino acids, vitamins, and minerals (Becker, 1994; Knuckey *et al.*, 2005; Khatoon *et al.*, 2010; Carneiro *et al.*, 2020). Due to their high nutritional content and adaptability, algae such as *Spirulina*, *Chlorella* sp., *Scenedesmus* sp., *Dunaliella* sp., and *Nannochloropsis* sp. are extensively used in aquaculture feed (Belay, Kato and Ota, 1996). Microalgae have been utilised extensively as feed additions for fish and shrimp larvae, crabs, and mollusks (Borowitzka, 1997). Numerous studies have evaluated alternative protein sources as partial or total replacements for fishmeal in the diets of various fish species, including salmon, rainbow trout, and chinnok salmon - *Onorhynchus* spp. (Higgs *et al.*, 1992; Bureau *et al.*, 2000), Java and Nile tilapia-*Oryochromis* spp.(El-Sayed, 1998), Indian major carp- *Labeo rohita* (Hasan *et al.*, 1994),*Cirrhinus mrigala* (Hasan *et al.*, 1988) and common carp-*Cyprinus carpio*.(Yilmaz *et al.*, 2003).

In order to develop any sustainable process for incorporating the efficient sequestration of atmospheric CO_2 via microalgae along with effective utilization of biomass, strain selection is a crucial aspect of creating a viable bioprocess (Mutanda *et al.*, 2011). Even though several microalgal strains have been identified for the synthesis of various metabolites, fresh strains must be routinely developed for resilience, high carbon sequestration rate, and high protein content. Indigenous microalgal strains will be excellent choice, since these organisms are able to adapt to the shifting environmental circumstances in that ecosystem. They may become the top producers in that environment due to their high biomass production, and as a result, they will be more useful for commercial activities (Mutanda *et al.*, 2011). Commonly, sequential step techniques were employed to boost biomass productivity by increasing the intracellular biomass composition. The biochemical engineering strategy include the identification of innovative engineering solutions to produce high cell density, protein -rich growth through process optimization and the creation of algae culturing techniques. To realise the full potential of this technology, more essential engineering innovation in process development is necessary, with a focus on basic biological concerns pertaining to intracellular component regulation and control. (Hu *et al.*, 2008).

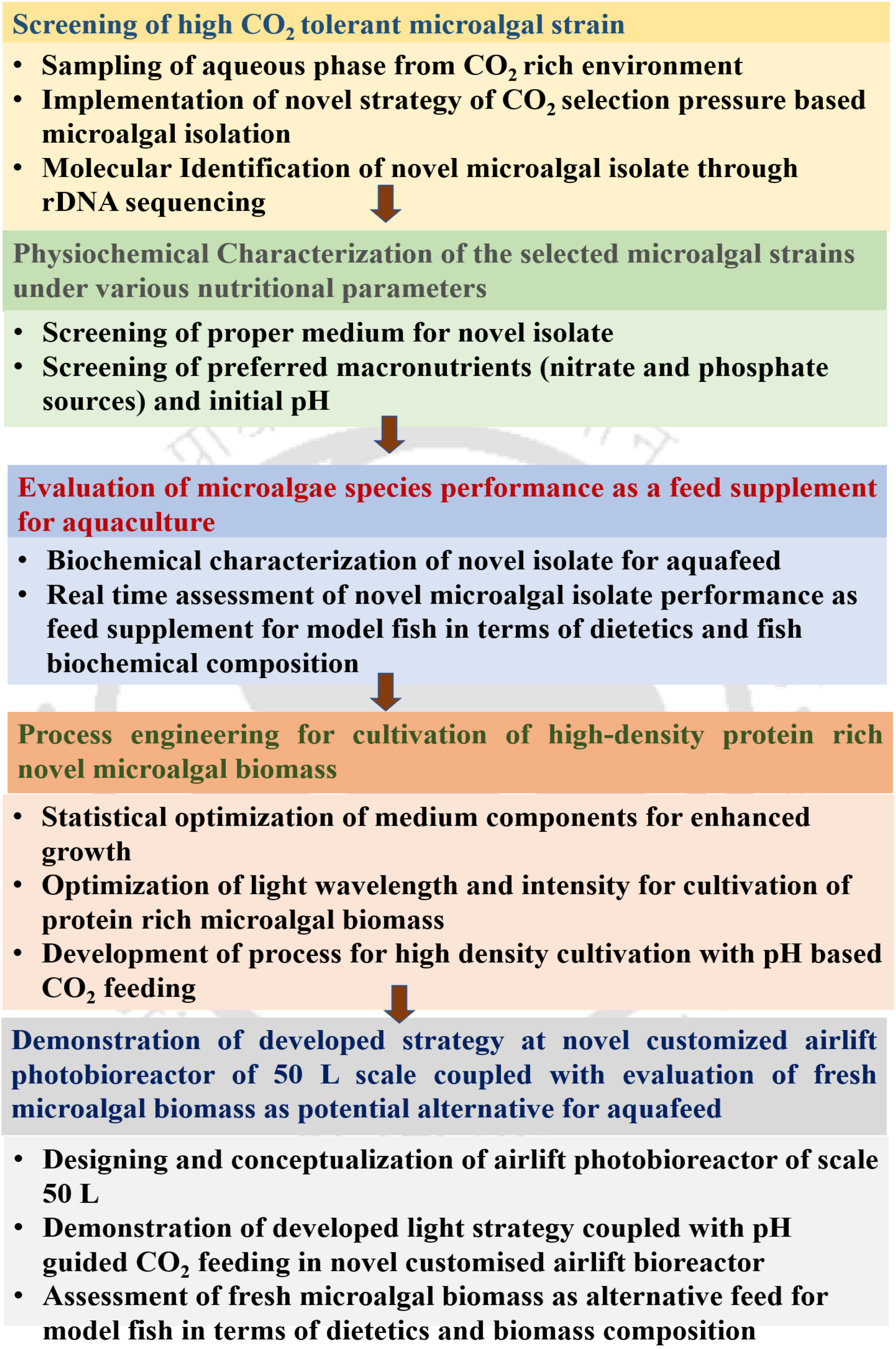
It is uncommon to cultivate microalgae on a large scale for aquafeed production, and estimating production costs is difficult since these processes are frequently strain-dependent and indigenous. Multiple barriers, ranging from strain selection to process development, have impeded the commercial adoption of microalgae as an alternative aquafeed. The development of eco-friendly aquaculture system composed of microalgae, zooplanktons, and fish/shrimp is becoming more and more popular in the circular economy. It is predicted that these new technologies and models will promote the extensive use of microalgae biomass and bio-products as aquafeed in the future (Chen *et al.*, 2021). When contemplating such an application of algal biomass, it would be preferable to prioritise the production of higher-value product, *i.e*. aquafeed, before biofuel generation. In other words, the idea of high-value product priority should apply (Li *et al.*, 2015). The availability of significant quantities of microalgal biomass may impede the growth of the aquafeed sector. Large-scale algal cultivation sustainability can be compromised by grazers, pests, and pathogens (Hannon *et al.*, 2014), but it is necessary to achieve successful large-scale cultivation of commercially important microalgae species in order to supply a large quantity of microalgal biomass for the aquafeed industry. Thus, the sustainability and economic viability of microalgae-based aquafeed necessitate a substantial improvement at both the strain and process levels.

1.2 Objectives of the study

The present study was carried out with the following objectives formulated based upon the current bottlenecks in microalgal research:

- *Sampling, isolation and selection of potential CO_2 tolerant microalgal strains.*
- *Characterization of the selected microalgal strains under various nutritional and physicochemical parameters.*
- *Detailed evaluation of microalgae species performance as a feed supplement for aquaculture.*
- *Process engineering for cultivation of high-density protein rich novel microalgal isolate.*
- *Demonstration of the integrated sustainable microalgal feed technology at large scale of 50 L Airlift Photobioreactor (APBR) coupled with application of fresh algal cell as live aquafeed.*

1.3 Approach



Screening of high CO_2 tolerant microalgal strain

- Sampling of aqueous phase from CO_2 rich environment
- Implementation of novel strategy of CO_2 selection pressure based microalgal isolation
- Molecular Identification of novel microalgal isolate through rDNA sequencing

Physiochemical Characterization of the selected microalgal strains under various nutritional parameters

- Screening of proper medium for novel isolate
- Screening of preferred macronutrients (nitrate and phosphate sources) and initial pH

Evaluation of microalgae species performance as a feed supplement for aquaculture

- Biochemical characterization of novel isolate for aquafeed
- Real time assessment of novel microalgal isolate performance as feed supplement for model fish in terms of dietetics and fish biochemical composition

Process engineering for cultivation of high-density protein rich novel microalgal biomass

- Statistical optimization of medium components for enhanced growth
- Optimization of light wavelength and intensity for cultivation of protein rich microalgal biomass
- Development of process for high density cultivation with pH based CO_2 feeding

Demonstration of developed strategy at novel customized airlift photobioreactor of 50 L scale coupled with evaluation of fresh microalgal biomass as potential alternative for aquafeed

- Designing and conceptualization of airlift photobioreactor of scale 50 L
- Demonstration of developed light strategy coupled with pH guided CO_2 feeding in novel customised airlift bioreactor
- Assessment of fresh microalgal biomass as alternative feed for model fish in terms of dietetics and biomass composition

Fig 1.1 Approach of thesis

The investigation starts with the screening and isolation of a high CO_2 tolerant strain, from an aqueous sample collected from industrial hotspot, based on a unique CO_2 selection pressure. Further, the unique novel microalgal isolate was evaluated for its growth performance and biochemical composition under various medium compositions, physiochemical parameters, and CO_2 concentration. Analysis of the biochemical content of new microalgae indicated its potential as an aquafeed. The novel microalgal isolate biomass was further considered for the real time evaluation as alternate feedstock for model fish *Hypophthalmichthys molitrix* (silver carp). Three different types of feedstocks were prepared and fed to the model fish and performance was assessed in terms of growth dietetics and carcass composition of fish. In the subsequent stage, a process engineering technique was established with the objective of producing a protein-rich, high-density microalgal biomass with enhanced CO_2 sequestration capability in a 50-L volume innovative airlift photobioreactor. The fundamental concept of the process engineering technique was to increase the protein content of biomass by modulating the wavelength and intensity of light. In the initial phase, a culture technique was established in the laboratory under varying light wavelengths and intensities. Optimized light wavelength and intensity were combined with pH-guided CO_2 feeding method to increase biomass density. Subsequently, the devised technique was assessed under a unique customized airlift photobioreactor by evaluating the growth performance of the organism at a scale of 50 L. In addition, the fresh microalgal biomass with high protein content was used as aquafeed for the model fish *Cirrhinus mrigala* (mrigal carp). The growth performance of fish was assessed using growth metrics and biomass composition.

1.4 Organization of the thesis

The thesis consists of seven chapters encompassing introduction to conclusions. **Chapter 1** primarily establishes the background and motivation for the current thesis with detailed emphasis on the existing gaps in present research, steps towards understanding hurdles to fill gaps and the futuristic objectives to resolve the problems in the current state of art technology. It highlights the strategies employed in the present study to counter the major bottlenecks assessed from existing microalgal cultivation and its applicability as aquafeed. **Chapter 2** discussed about studies about microalgal cultivation for decades as a potential cell factory for CO_2 bio fixation with multiproduct paradigm. Current commercial scale microalgae cultivation technologies are compared in order to determine the drawbacks of existing process engineering strategies. The role of environmental factors and nutrients as an influencer for enhancement of biomass composition and productivity has been thoroughly discussed**.** Current status of aquaculture feed and a light on the approach for finding an alternate feedstock for aquaculture was also discussed**.** **Chapter 3** describes the stepwise study comprising sampling, isolation and identification of high CO_2 tolerant strain from an aqueous sample collected from industrial effluent. A novel screening method via CO_2 selection pressure was developed for screening and isolation. Identification of novel isolate was performed via molecular analysis based on 18s rDNA sequence and, the isolated microalgal strain was identified as *Desmodesmus pannoniccus* CT01 (Accesion Number: **OL470985**). Furthermore, the isolate was characterized for different cultivation medium, initial pH, the phosphate sources and the nitrogen sources. The highest biomass titre was obtained using Basal Bold medium (BBM) with initial pH of 8. Potassium dihydrogen phosphate and sodium nitrate was found to be the most suitable phosphate and nitrogen source for CT01. CT01 was subjected to different % of CO_2 (v/v) concentrations in the air steam and its performance was evaluated. The microalgal growth performance was found to be highest in 12.5% CO_2 (v/v) concentration in the air stream. **Chapter 4** demonstrates the real time assessment of CT01 microalgal biomass potential as an alternative to commercially available conventional fish feed. Three different feedstocks were considered to evaluate growth metrics and dietetics of *Hypophthalmichthys molitrix* (silver carp): microalgae feed (MiF), whole cell dried biomass of CT01; reference feed (ReF), composition of which is equivalent to the commercially used feed for silver carp, and mixed feed (MixF) in 1:1 ratio of microalgal biomass and reference feed. Mixed feed (MixF) was found to be suitable feed in terms of fish growth dietetics. **Chapter 5** demonstrates the development of an integrated process engineering strategy which offered high density protein rich biomass with efficient CO_2 sequestration through optimisation of medium components, light wavelength and light intensities. The media components, potassium dihydrogen phosphate, sodium nitrate and trace metals and microelements (TME), were optimized via response surface methodology (RSM) based on central composite design (CCD) with dual objective of maximisation of biomass titre and productivity of CT01. Furthermore, the incident light wavelength was optimized for cultivation of CT01 for enhancing the biomass titre and total protein content of the biomass. Seven different light wavelengths including mono and multi wavelength was subjected to CT01 cultivation. The combination of red-blue wavelength was found to be suitable for CT01 for better production of protein rich high-density cells. Effect of incident light intensities (50 -200 µE m^{-2} s^{-1}) on the growth of CT01 was also optimized. The incident light intensity on the surface of the reactor of 150 µE m^{-2} s^{-1} in the ratio of 1:1 of red-blue light wavelength was found to be suitable for CT01 cultivation for enhance biomass titre. Additionally, to enhance the CO_2 sequestration ability of CT01strain, a novel strategy of pH-based CO_2 feeding was employed which helps eliminate both, pH stress to the organism and limitation of the carbon source. The strategy was able to improve the carbon sequestration ability and also enhance the biomass titre. **Chapter 6** demonstrates the developed process engineering strategy in a customised airlift photobioreactor (APBR) of 50 L capacity. The photobioreactor was customised to provide high CO_2 mass transfer rate and better light availability to the cells for enhanced microalgal growth. The performance of the reactor was found to be at par with lab scale mini reactor and hence depicting the capability of the reactor. The biomass titre and composition were found to be in similar with mini reactor. Furthermore, high density protein rich fresh microalgal biomass of CT01 was evaluated as alternative feed for the model fish *Cirrhinus mrigala* (mrigal carp). Three different feedstocks were considered to evaluate growth metrics and dietetics of a fish *Cirrhinus mrigala* (mrigal carp): Live microalgae feed (LiF), that is fresh cell biomass of CT01; reference feed (ReF), composition of which is equivalent to the commercially used feed for mrigal carp, and dry microalgal feed (DrF). Growth performance of fish fry of mrigal carp fed on diets with three different types of fish meal. **Chapter 7** comprises the overall conclusion and summarizes key research highlights obtained from the present study. The conclusion section is followed by the future prospects of the current thesis outcomes and scope for further investigation.

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CHAPTER 2

Review of Literature

2.1 Global CO_2 emission status

Carbon is an essential component of both the earth and the biosphere and also participates in a specific cycle of capture and accumulation, which is essential to the stability of the ecosystem (Schimel, 1995; Macreadie *et al.*, 2019). The presence of humans has had a profound effect on this equilibrium. As a result of the proliferation of industrialisation, adverse effects were experienced by the environment (Hunter, 2007; McMichael *et al.*, 2008). In addition, the uncontrolled exploitation of natural resources has had a devastating impact(Yang *et al.*, 2008; Sayre, 2010) . The generation of energy throughout the world still relies heavily on fossil fuels, which account for around 80% of total output. The burning of fossil fuels results in the emission of a significant quantity of CO_2 (~24 GT per year) (Sayre, 2010; Goli *et al.*, 2016). The rapid variations in the environment will have a significant effect on the whole globe, and the issue with global warming has already frightened the countries to the point that they are re-evaluating energy regulations such as the Paris Agreement and the Kyoto Protocol. With these all in practice, the Food and Agriculture Organization of United Nations have some key points in framing the sustainable development goals related to low cost protein feedstock for aquaculture include: No Poverty (SDG 1) - Low-cost protein feedstock can help make aquaculture more economically accessible to people in developing countries and reduce poverty. Zero Hunger (SDG 2) - By increasing the efficiency and productivity of aquaculture, low-cost protein feedstock can help meet the growing demand for food and contribute to achieving food security. Good Health and Well-being (SDG 3) - Aquaculture can provide a healthy source of protein that can contribute to better nutrition and improved health. Sustainable Cities and Communities (SDG 11) - By reducing the environmental impact of aquaculture, low-cost protein feedstock can help create sustainable and liveable communities. Life Below Water (SDG 14) - Aquaculture can help to protect and conserve marine and coastal ecosystems, by reducing the pressure on wild fish stocks, and low-cost protein feedstock can help make this more sustainable and economically viable. Partnerships for the Goals (SDG 17) - To achieve the goal of low-cost protein feedstock for aquaculture, it is important to foster partnerships between government, academia, industry, and civil society, to share knowledge and resources.(*Sustainable Development Goals | Food and Agriculture Organization of the United Nations*). Therefore, in order to reduce the effects of global warming and keep the levels of carbon dioxide in the atmosphere stable, some of the technologies associated with renewable and sustainable energy sources have been taken into consideration (Baena-Moreno *et al.*, 2018; Singh and Dhar, 2019). The increasing concentration of carbon dioxide in the atmosphere is responsible for a rise in the worldwide average surface temperature. This, in turn, has direct and indirect effects on the weather and climate phenomena that occur on a global scale, such as intense rainstorms and droughts(Z. Liu *et al.*, 2020). The Paris accord, which was approved by 196 nations to keep global warming to below 1.5 °C compared to the pre-industrial period, came into action in order to battle the growing temperature of the earth's surface. This was done in order to tackle climate change. This may be accomplished via implementation of the Nationally Determined Contributions (NDCs) to cut greenhouse gas emissions. When compared to 2018, the total amount of carbon dioxide emissions produced by the world has grown by 0.9% in 2019. According to the Emission Database for Global Atmospheric Research (EDGAR), the countries with the highest carbon emissions were as follows: China, the United States of America, India, the European Union 27 plus the United Kingdom, Russia, and Japan.(Crippa *et al.*, 2019; Crippa *et al.*, 2020). In terms of population, these nations account for 51 % of the world total, yet they are responsible for around 67 % of the world's CO_2 emissions. Surprisingly, as compared to that in 2018, the amount of CO_2 emission in 2019 climbed in China and India, while it declined in EU28, the USA, and Russia (Crippa *et.al.*, 2019; Crippa *et al.*, 2020). The demand for coal (8 %), oil (4.5 %), and natural gases (2.3 %) decreased during the first three months of 2020 in comparison to the first three months of 2019, which resulted in a 5 % decrease in global carbon emissions during this time period. In a separate analysis, the daily, weekly, and seasonal dynamics of CO_2 emissions were described. The authors of that analysis anticipated that there would be an 8.8 % decline in CO_2 emissions in the first half of the year 2020(Crippa *et al.*, 2019; Crippa *et al.*, 2020). The COVID-19 epidemic was the primary contributor to the reduction in worldwide CO_2 emissions that occurred in 2020. This reduction was the most substantial one seen since the conclusion of World War II. EDGAR anticipated that the year 2020 would show a decrease, with worldwide anthropogenic fossil CO_2 emissions being 5.1 % lower than in 2019. This would place them slightly below the level of 36.2 Gt CO_2 emissions that was documented in 2013. In 2019, global carbon emissions (fossil fuels) per unit of Gross Domestic Products (GDP) showed a declining trend reaching an average value of 0.298 tCO_2/k USD/yrs., while per capita carbon emissions remained stable at 4.93 tCO_2/capita/yrs., confirming a 15.9 % surge from 1990 (*World Energy Outlook 2020 – Analysis - IEA*, 2020; Crippa *et al.*, 2019; Crippa *et al.*, 2020).

2.2 Carbon sequestration technologies

There are many different physical, chemical, and biological approaches now in use with the purpose of lowering the release and concentration of CO_2 emissions into the atmosphere (Leung, Caramanna and Maroto-Valer, 2014; Osman *et al.*, 2020; Shreyash *et al.*, 2021). The processes of carbon sequestration or fixation are collectively referred to as carbon capture and storage/utilization (CCS/U) by the scientific community. The process of carbon capture and storage (CCS) involves a number of steps, including CO_2 collection and separation, transportation and usage, and storage of the captured gas). Major routes that is frequently used for carbon capture includes following: a) pre-combustion, in which CO_2 is removed before combustion and the fuel is broken down to yield synthesis gas, which is a mixture of CO, CO_2 and H_2; subsequently, CO_2 is separated into various processes, and H_2 is used as a clean fuel; (b) post-combustion, in which CO_2 is captured after the combustion of fuels through the use of chemical absorption; (c) oxy-fuel where the fuel is combusted in the presence of pure oxygen to produce high levels of CO_2; (d) chemical looping combustion, where oxygen carrier (solid metal oxides) particles are continuously circulated to supply oxygen to react with fuel, wherein the combustion of metal oxide and fuel produce metal, CO_2 and H_2O (Jerndal, Mattisson and Lyngfelt, 2006). The removal of carbon dioxide from flue gas is another critical step in the development of devices that collect and store carbon. There are several other methods of separation that are now in use, which include absorption, adsorption, membrane separation, and cryogenic distillation (Pires *et al.*, 2012).

2.3 Status of feed for Aquaculture

Aquaculture is one of the fastest growing sector in the food industry. It is expected that the value of the aquaculture business would reach 31.94 billion dollars in 2019 (*News Viewer - MarketWatch*, 2020). It is expected that over the period of 2020 and 2027, it will increase at the rate of approximately 7.1 % per year. Increasing demand from consumers and industry's general acceptance are the primary forces behind current boom in the aquaculture industry. During the course of the last several years, the sector has begun stocking a number of newly discovered species. The nutrition of fish has been optimised, which has reduced the amount of feed waste and improved industry's capacity to remain financially viable. A diet high in functional components such as omega-3 fatty acids, antioxidants, and prebiotic compounds has been shown to improve the productivity, survival rates, and overall quality of fish that are farmed. Aquaculture has recently gained a competitive advantage over the use of wild fish resources (Nagappan *et al.*, 2021). At the moment, commercial fish account for 70 % of the total catch from aquaculture operations (Tacon, 2019). Fish feed is essential to the survival of around 68% of all commercially important species (Tacon, 2019). Fish meal, which may be made from both very small fish and waste products from larger fish, has traditionally played a significant role as the principal component of fish feed. Fish meal is a component of fish feed that is in high demand due to the following qualities: (1) excellent digestibility and palatability for fish, which leads to increased growth; deformities are reported rarely or not at all; (2) the well-balanced composition and concentrations of protein, minerals, essential fatty acids, and essential amino acids; (3) a low feed conversion ratio, which means that a high percentage of feed is converted into fish biomass, which leads to less feed waste; and (4) Fish meal is an essential ingredient in animal feed, and demand for it has increased by a factor of three over the last decade (*Fishmeal - Monthly Price - Commodity Prices - Price Charts, Data, and News - IndexMundi*, 2022). In a similar vein, the prices of other important components like soy meal and fish oil are going up. It is projected that the growth of the fish farming industry will continue in the future. At the moment, fish meal and fish oil are both obtained from wild fish, harvesting of which is regulated and subject to uncertainty. In addition, pelagic fish are the source of fish feed; however, their populations are falling as a result of the influence of El Nino (Bakun and Broad, 2003) and the unsustainable practise of overfishing. Pelagic fish are becoming scarcer. As a consequence of this, a number of industries and academic institutions have already begun looking for replacements to fish meal, soybeans, and fish oil that are both sustainable and acceptable. It is very rare for aquatic organisms to get all of their nutrition from a single source. This is because a single source can fulfil all of an aquatic species' dietary requirements, including its carbohydrate, protein, fat, mineral, and vitamin requirements. This is the case because a single source can provide all of these components. Corn, wheat, rice, maize starch, and potato starch are all examples of common sources of carbohydrates (Hodar *et al.*, 2020). The meal includes protein that comes from a variety of sources, including plants, animals, insects, and microbes. Common sources of plant protein include soybean meal, guar meal (a by-product of guar gum), maize gluten, potato protein, wheat gluten, cassava, canola, and wheat gluten. Other sources include peas, co-products of sugar cane production, macroalgae, and canola (Montoya-Camacho *et al.*, 2019). The most major sources of animal-derived protein in animal feed are fish meal, feather meal, blood meal, animal waste, seafood waste, and fish silage. Other sources include animal waste, seafood waste, and animal waste (Mo, Man and Wong, 2018). Protein comes from a variety of microbiological sources, including bacteria, yeast, and microalgae, in fish food (Jones *et al.*, 2020). The following are some examples of sources of fats and lipids: fish oil, vegetable oil, soya oil, rapeseed oil, sunflower oil, and algal oil. Other components, including as fibre, vitamins, minerals, and amino acids, are vital to fish performance but are not required in order to get optimal results. Research is being conducted to determine whether or not genetically modified crops with enhanced properties, such as canola and camelina that have a high omega-3 fatty acid content, might serve as a viable alternative component in aquafeed (Jones *et al.*, 2020).The alternative feed components that have been outlined above each have the advantages of nutritional content, presence of bioactive compounds, and sustainable manufacture. Having these options available does, however, come with a few negatives that should be considered. One of the most significant downsides of these plant-based sources is the significant presence of anti-nutritive components and indigestible fibres (Kokou and Fountoulaki, 2018). Tannins, saponins, and soluble non-starch polysaccharides are examples of chemicals that inhibit nutrient absorption. This prevents the fish from developing properly and results in wasted nutrition. Bacterial meal and proteins derived from insects both come at a high cost. Omega 6 fatty acids may be found in high concentrations in oils extracted from plants. On the other hand, omega-3 fatty acids are hard to come by. Fish that has been infused with omega-3 fatty acids has a higher overall quality

(Shah *et al.*, 2017). There is a deficiency of vital amino acids and minerals in plant proteins and animal by-products. In spite of the fact that plant-based feed is beneficial to the growth of fish, this kind of feed is deficient in essential amino acids such as methionine, tryptophan, lysine, and threonine. It is possible that the quality of the fish may deteriorate since it is lacking essential amino acids. Additionally, it has been shown that the digestion of proteins derived from plants is a challenging process. The advantages and disadvantages of several alternate meals is shown in Table 2. 1. As a result of the growing need for alternate components in fish feed, a number of publications have been issued in response.

Table 2. 1 Advantages and disadvantages of alternate fish feed

Alternate Feed	**Advantages**	**Disadvantages**	**References**
Guar meal	• Soy meal could be replaced with guar meal without affecting growth efficiency In some fishes,.	Anti-nutritional and anti-digestive compounds like Residual gum, saponin, phytate, and protease inhibitor tannin are present Slow rate of gastrointestinal evacuation- Poor in amino acid digestibility The supply of guar meal in the market is influenced by the oil industry's production and the amount of guar gum consumed.	(Nidhina and Muthukumar, 2015; Ullah *et al.*, 2016)
Macroalgae	• Apart from their nutritional value, macroalgae contain a variety of pigments, defensive compounds, and secondary metabolites that may benefit farmed fish.	Complex polysaccharides leads to poor digestibility Contains excess heavy metals Presence of anti-nutritional factors like phlorotannins, lectins, and phytic acids, trypsin inhibitors and amylase inhibitors	(Garcia-Vaquero and Hayes, 2015)
Soybean meal	• High protein content ranging from 44% to 48%	Anti-nutritional factors like lectin and non– starch polysaccharides are present; reduced feed intake Level of the amino acids like methionine, cystine lysine, and threonine and tyrosine are limited Low in phosphorous	(Goda *et al.*, 2007)

Canola meal and Corn gluten meal	• High protein content • Crude protein content ranging from 60% to 73% • Corn gluten meal is now commonly used in salmon and other aquatic fish such as gilthead seabream and European seabass and aquafeeds • highly digestible	Low in phosphorous Deficient in lysine	(Wickramasuriya *et al.*, 2015; W. Y. Liu *et al.*, 2020)
Cottonseed meal	• protein content of 40% can be used in aquaculture diets without causing growth inhibition	Presence of gossypol may be harmful	(Delgado *et al.*, 2021)
Peas/lupins	• High protein digestibility	Contain elevated amounts of non–starch polysaccharides lupins that are not metabolized; Anti–nutrient quinolizidine alkaloids are present Lysine and methionine are scarce	(Kokou and Fountoulaki, 2018)
Wheat	• Low in protein (<11)	Wheat is largely an energy source due to its high starch content (typically >70%); Lysine is a limiting amino acid.	(Sørensen *et al.*, 2011; Draganovic *et al.*, 2013)

Barley	• Well digested	Low crude protein content (9–15%); High fibre content; Low available phosphorous; Lysine and arginine can be limiting;	(Snow and Ghaly, 2007)
Hydrolysed feather meal	• protein content of hydrolyzed feather meal ranges from 74% to 91% crude protein, and it's high in cystine (4–5% crude protein)	Less digestible Low in lysine (2% of crude protein) and methionine (1% crude protein)	(Grazziotin *et al.*, 2008; Yu *et al.*, 2020)
Blood meal	• High protein content • Rich in lysine	Deficient in methionine; Heat sensitivity and drying conditions have a significant impact on protein digestibility.	(Hussain *et al.*, 2011; Aladetohun and Sogbesan, 2013)
Fish by-products from fish processing plants	• High digestibility • Good palatability	Potential viruses and contaminants that are toxic to both fish may be present.	(Hardy, 2000)

Insects	• Can be cultivated in food waste	Methionine and Cysteine were the most limiting amino acids for most insect meals Chitin is present which is an anti nutritional factor	(Bosch *et al.*, 2014)
Bacteria	• Rapid growth rate • Least explored • Can be grown in variety of substrates	The bacterial meal diet has a lower digestibility than the fish meal diet and can contain unidentified antinutrients.	(Skrede *et al.*, 1998)
Yeast	• Can grow in lignocellulosic wastes • Except low methionine content, yeast protein has a favorable amino acid composition for fish • Rapid growth rate	Production cost is high The sulfur-containing amino acids methionine and cysteine are usually low in yeast protein.	(Marques *et al.*, 2004; Blomqvist *et al.*, 2018)
Microalgae and Algal oil	• Rapid growth rate • Diverse species availability with wide range of characteristics • Rich in Omega-3 fatty acids • High in antioxidants, colouring compounds and probiotic effect	High production cost in case of formulated feed Selected microalgae have rigid cell wall leading to difficult in digestibility	(Madeira *et al.*, 2017; Arun *et al.*, 2020; Katiyar and Arora, 2020)

2.4 Microalgae: biology and classification

Microalgae, oxygen-producing, self-sufficient, photosynthetic bacteria are considered polyphyletic, which follow several separate evolutionary lineages(Barsanti and Gualtieri, 2005). The term "algae" refers to both the microscopic single-cell forms known as microalgae and the macroscopic multicellular loose or filmy conglomerations, matted or branched colonies, and more complex leafy or blade forms known as macroalgae. to the demand. The criteria for alternative components must be able to be met while maintaining environmental sustainability and maintaining economic viability. Microalgae are classified as being on the microscopic scale, while macroalgae are on the macroscopic scale. Microalgae have a diameter that ranges from 0.2 to 50 micrometres, whereas the diameter of macroalgae may grow to be as large as gigantic kelps that can be up to 60 metres in length. There are between one and ten million different species of algae on the Earth, of which only 40,000 have been described (Hu *et al.*, 2008), with the majority of these species still being unstudied. These creatures, in contrast to plants, do not exhibit significant diversity in the vegetative components that make up their bodies, such as their roots, stems, leaves, vascular systems, and sophisticated sex organs.

The issue of classifying algae is a challenging one, since more than 20 different types of algae have been reported ever since Linnaeus first introduced the concept of classification systems. In order to classify these complex systems at higher levels, many different approaches were utilised. These approaches can be broadly categorised as morphological concepts (organisation in vegetative states), ultra-structural concepts (basal body orientations in flagellated cells), and molecular concepts (smaller and larger subunits of Ribosomal DNA, 5.8S, including internal transcribed spacers ITS-1 and ITS-2, chloroplast and mitochondrial genes) (Brodie and Lewis, 2007). Cyanophyta and Glaucocystophyta are the two divisions that are used to classify the prokaryotic members of this assemblage (Croft, Warren and Smith, 2006), whereas the eukaryotic members of this assemblage are classified into nine different divisions: Glaucophyta, Rhodophyta, Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta. It is considered that the most fundamental forms of prokaryotic photosynthetic cyanobacteria are responsible for eliminating all of the harmful chemicals from the earth's atmosphere while also producing oxygen for the planet. These cyanobacteria are able to colonise a broad variety of settings, such as salty seas, hot springs, mountains, and glaciers, and they reproduce asexually by a process known as fission. There have been no reports of sexual reproduction (Barsanti and Gualtieri, 2005). In general, the vast majority of cyanobacteria are photoautotrophs, with just a few species capable of flourishing in heterotrophic and/or mixotrophic environments (Alagesan *et al.*, 2013). The formation of heterocysts, which are cells that do not participate in photosynthesis but are capable of fixing nitrogen, is a key trait that is innate to these organisms. Heterocysts are the source of nitrogen for all of the expanding photosynthetic cells in the system. Endosymbiosis, which occurs when prokaryotic cyanobacteria live inside of a non-photosynthetic eukaryotic host, is thought to have been the driving force behind the evolution of eukaryotic algal strains. This process is responsible for the formation of the three major groups of algae—glaucocystophyta, chlorophyta (green algae), and rhodophyta (red algae), as Chlorophyta is the biggest category of organisms that have been well studied and described. It is comprised of a great number of widespread species, including *Chlorella*, *Dunaliella*, *Hematococcus*, *Chlamydomonas*, *Tetraselmis*, and *Scenedesmus*. The other members of the group are produced as a result of the recurring endosymbiotic relationships that occurred between these variations (Croft, Warren and Smith, 2006). Because they include xanthophylls, haptophyta appear yellow, green, or brown in hue. Heterokontophyta contains phaeophyta, which are brown algae; xanthophyta, which are yellow algae; chrysophyta, which are golden algae; and diatoms (Bacillariophyta). Diatoms (Bacillariophyceae), which may store carbon in the form of natural oil or as a polymer of carbohydrate, are one of the algal communities that are actively engaged in the accumulation of lipids (Matsumoto *et al.*, 2010). According to the available research, these lipid-rich diatoms ultimately decomposed and gave rise to the traditional fossil fuel supplies (Ramachandra *et al.*, 2009). Green algae, also known as Trebuxiophyceae and Chlorophyceae, are most usually found in fresh water and brackish water, whereas only a very small number may be found in marine environments (Matsumoto *et al.*, 2010). They save their energy in the form of starch, and under specific situations, such as when they are under stress or when they are growing, they save neutral lipids (Illman, Scragg and Shales, 2000). Chlorophyta is the division that contains the oleaginous microalgal species that has been subjected to the greatest research (green algae). Golden algae, also known as Chrysophyceae, may have a golden, orange, or brown appearance and generate natural oils and carbohydrates that can be used as storage components. Microalgae have a unique and complicated biology and physiology due to the polyphyletic character of their phylogenetic tree. The schematic representation of the basic structure of a unicellular prokaryotic cyanobacteria cell and a eukaryotic green algal cell is shown in Fig 2.1. The absence of intracellular organelles and the presence of a prominent nuclear membrane are two distinguishing features of the prokaryotic blue-green algae. In addition, the thylakoid membranes are arranged as a network in the peripheral region of the cell, and they have phycobilisomes on their surfaces for the purpose of light harvesting (Fig 2.1 A). In cyanobacteria, this particular configuration of thylakoids is referred to as chromoplast. The granules of starch and the bodies of lipids may be observed dispersed throughout the cytoplasm. In contrast to many other higher algae, the cell walls of cyanobacteria are coated with an extracellular mucilage layer. This layer is not present in the cell walls of higher algae. On the other hand, in eukaryotic algae, the thylakoid membranes are stacked in a network that is similar to the thylakoids found in plants, and it also contains a brief section of chloroplast DNA (Fig 2.1 B). Eukaryotic cells need a number of intricate internal organelles in order to perform their functions properly. These organelles include the endoplasmic reticulum, golgi bodies, and mitochondria. Starch granules may be seen in the chloroplasts, while lipid bodies can be seen dispersed throughout the cytoplasm. The presence of mucilage layers in the cell wall of eukaryotic cells may or may not be present in their extracellular matrix, and this characteristic varies from species to species. It is possible for various species of microalgae to reproduce in a variety of ways, including vegetative via the division of a single cell or by the fragmentation of a colony, asexually through the formation of motile spores, or sexually through the union of gametes(Barsanti and Gualtieri, 2005). Auto sporulation is an asexual method of reproduction that is utilised by the

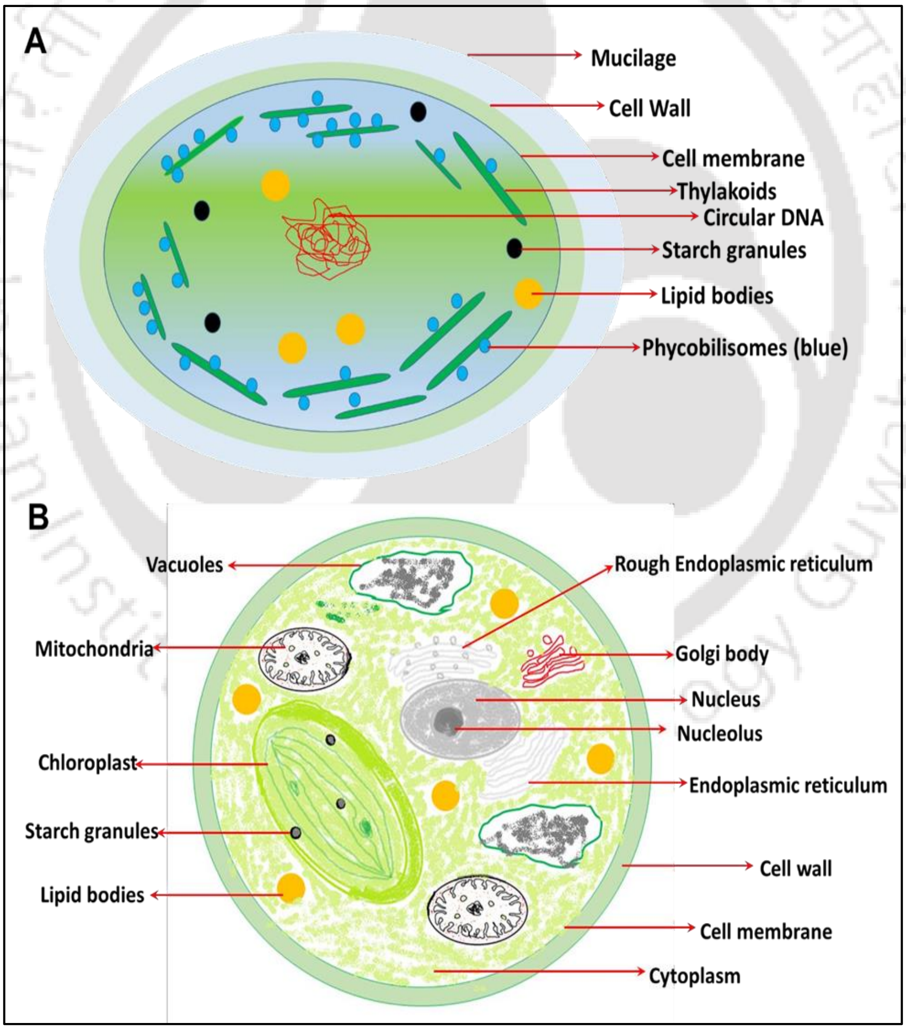

Fig 2.1 Generalized structural morphology of an unicellular (A) prokaryotic blue-green algae and (B) eukaryotic green algae (Adopted and modified from Barsanti and Gualtieri, 2006)

oleaginous green algae *Chlorella sp*. (Chlorophyta) and *Nannochloropsis* (Heterokontophyta). The mother cell divides into four daughter cells, each of which has its own cell wall, and then permits the daughter cells to develop inside. After maturation, the daughter cells are released from the mother's cell by the rupture of the cell wall, and the daughter cells then eat the remaining detritus that is left behind by the mother's cell (Safi *et al.*, 2014). The other types of asexual reproduction use a process called binary fission, in which the mother cell undergoes a division into two portions that are identical in size and contain the same amount of nucleic acid to produce two daughter cells. Within the vegetative mother cell of *Chlamydomonas* sp., the multiplication process involves the creation of flagellate motile spores known as zoospores. Zoospores are the reproductive units of the organism. Numerous algae species have been found to have distinct gametes that contain haploid genomes. This is the defining trait of sexual reproduction and is seen in all eukaryotic organisms.

2.5 Biochemistry of microalgae

Microalgae are known as the "cell factory" for the synthesis of a wide variety of biomolecules and compounds due to the complexity of their physiological diversity. More than 15,000 unique chemical compounds of varying degrees of relevance on the commercial market were extracted from the different strains of microalgae (Tabatabaei *et al.*, 2011). Under conditions of photoautotrophic nutrition, algae are able to synthesise such complex compounds by only utilising sunlight as the energy source, inorganic carbon dioxide as the carbon source, and some inorganic salts as the fundamental needs for development. This is the method of nourishment that is used most favourably for the development of algae. However, they are able to grow in heterotrophic environments by making use of organic carbon molecules as their primary supply of carbon as well as energy. Some photosynthetic microalgae have the ability to grow under mixotrophic condition, which allow them to carry out photosynthesis as well as make use of organic carbon molecules. Because of the organism's access to such a broad variety of dietary modes, it has successfully evolved to arrange metabolic pathways and transport machinery in order to operate well under a variety of culture settings. The photosynthetic activity of microalgae is responsible for more than half of all global photosynthesis. Photosynthesis is the process by which the energy of photosynthetically active radiation (PAR, in the wavelength band of 400-700 nm) is converted into biomass through oxidation and reduction reactions. Microalgal photosynthetic activity accounts for more than half of all global photosynthesis. The process of photosynthesis is comprised of two major reactions: I light dependent reactions, which involve the absorption and conversion of light energy into energy molecules NADH and ATP; and (ii) light independent pathways, which involve the fixation of CO_2 in the form of carbohydrates utilising the energy obtained from the light dependent pathways. Both of these reactions are necessary for the process of photosynthesis. A process that is light-dependent takes place in the photosynthetic complexes, which are composed of two protein complexes called PSI and PSII and light harvesting complexes (LHC) that surround them. This further excites the chlorophyll pigments to an excited state, which in turn initiates the transport of an electron across the thylakoid membrane along organic and inorganic redox couples that form the electron transfer chains. External photons that are available are absorbed by the photosystems PSI and PSII. The electron transfer chain results in the reduction of $NADP^+$, which results in the formation of NADPH. It also results in the development of a concentration gradient of H+ ions in the transmembrane area, which drives the ATP-synthesis process through the ATP-synthase enzyme. The photolysis of water ($2H_2O - 4e^- + 4H^+ + O_2$) is a thermodynamically demanding reaction that is catalysed by PSII. This reaction generates the electrons and protons that are further involved in driving photosynthesis by donating the electrons to plastoquinone. PSII plays the key role in this reaction and is responsible for catalysing it. PSI, on the other hand, is a redox enzyme that, while in its ground state, accomplishes the oxidation process by absorbing the electron from the cytochrome complex.

When it is in its excited state, however, it performs the reduction reaction by giving the electron to NADP. The Z scheme describes the movement of electrons from the PSII state to the PSI state in order to achieve the excited state. This collaboration between PSI and PSII results in the production of ATP and NADPH as the end products of light-dependent activities. These molecules are then made accessible for light-independent CO_2 fixation processes. Non-cyclic phosphorylation is the term used to describe the process by which ATP molecules are produced from the photolysis of water molecules. This is because the electrons that are liberated from water molecules and transferred to $NADP^+$ will not be returned to water molecules. When the terminal NADP is not accessible to take the electrons, the electron that is released from the PSI cycle is returned to the oxidised form of PSI through plastocyanin. This occurs when the terminal NADP is not available. It is referred to as cyclic phosphorylation, and the process by which ATP is created from this closed system is not one in which the reducing equivalent NADPH may be found as a result of photosynthesis.

2.6 Microalgal cultivation systems: growth parameters, nutrition and reactor types

Microalgae are capable of producing approximately 50 % of the oxygen that is produced on Earth through the process of photosynthesis (Khan, Shin and Kim, 2018), as well as a wide variety of bioproducts, such as polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds, and antioxidants. Because microalgae have such a wide range of uses, including renewable energy, medicines, and nutraceuticals, there has been a growing amount of interest in both the scientific and industrial domains on their potential. This is in contrast to the case of bioprocesses that use feedstocks, such as lignocellulosic biomasses, and require the transport to the biorefinery plants. In this scenario, fertile lands are required in order for the bioprocesses to function properly. This component provides a technique to simplify the pre-treatment operations, hence lowering both the total manufacturing costs and the worries about the environment. Cultivating microalgae calls for certain environmental requirements to be met, such as specific temperature ranges, light intensities, mixing conditions, nutritional content, and gas exchange. They may be cultivated

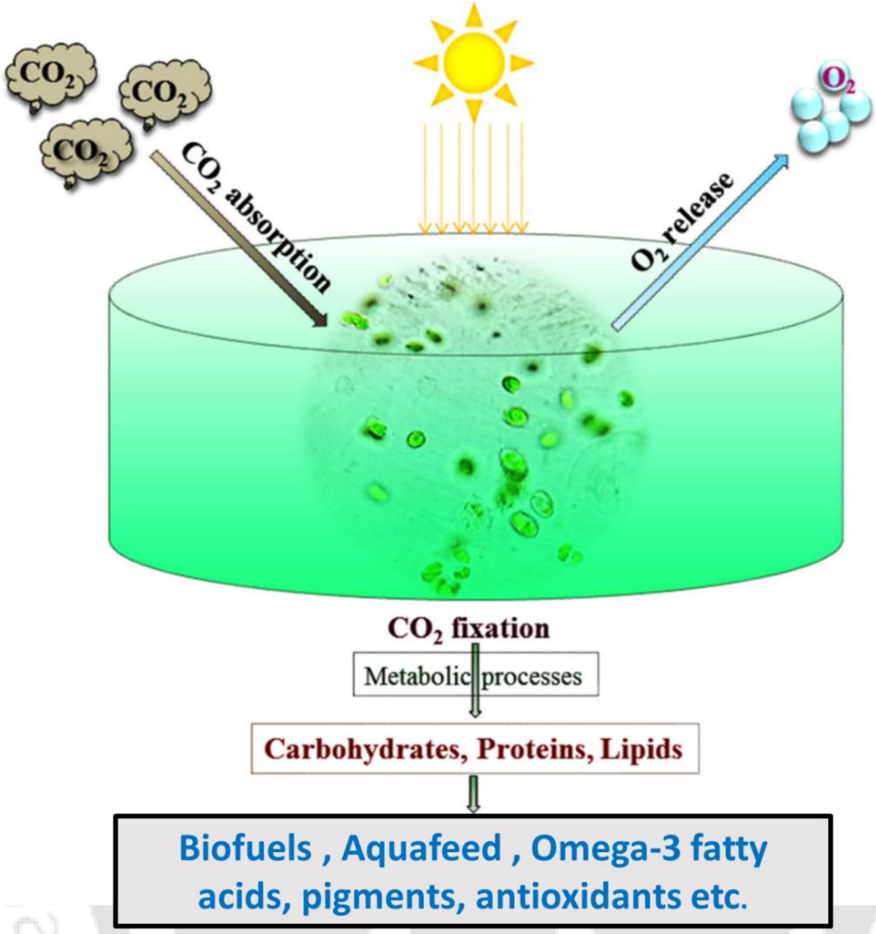

Fig 2.2 Schematic representation of microalgae-based carbon sequestration for production of various byproducts.

by a variety of metabolic pathways, including photoautotrophic, heterotrophic, and mixotrophic, as well as through a variety of cultivation techniques, which are often categorised as open and closed systems. The growth of microalgae is affected not only by a number of environmental factors such as temperature, light, nutrient availability, gas exchange, salinity, cell density, and pH, but also by a number of operational parameters such as fluid and hydrodynamic stress, mixing, culture depth, dilution rate, and harvest frequency (Bartosh and Banks, 2007).

2.6.1 Effect of temperature

Temperature has a direct and immediate influence on the physiological and morphological responses of microalgal growth (Venkata Mohan *et al.*, 2015). These reactions include photosynthesis and carbon fixation. It is possible to categorise the algae as psychrophiles (those that thrive in temperatures lower than 15 °C), mesophiles (those that thrive in temperatures between 15 and 50 °C), or thermophiles (those that thrive in temperatures higher than 50 °C). There have been no reports of any photosynthetic organisms being able to thrive at temperatures higher than 75°C owing to the instability of chlorophyll. The maximum temperature at which eukaryotic algae can survive is 62°C (Rothschild and Mancinelli, 2001). There is a connection between the carbon fixation process and the significance of the ideal temperature range. In point of fact, greater temperatures boost CO_2 uptake and fixation, but they also operate as an inhibiting factor for the metabolism of respiration and for the proteins involved in photosynthesis because they throw off the cells' delicate energy balance. Due to the affinity of ribulose for CO_2, cell size and the development of microbial biomass are both inhibited by high temperatures. This is especially true for culture systems that are carried out in the open air. The activation state of ribulose-1,5-bisphosphate (Rubisco), an enzyme that is capable of acting either as an oxygenase or as a carboxylase depending on the relative amount of oxygen and carbon dioxide present in the chloroplast, has been found to be affected by temperatures that are moderately higher than normal. The increasing temperature has a positive effect on the CO_2 fixation activity of the Rubisco enzyme up to a certain threshold, beyond which it begins to have a negative effect (Salvucci and Crafts-Brandner, 2004). The use of temperature as a kind of stress therapy may also be utilised to stimulate the creation of beneficial metabolites. It is difficult to assess the kinetic equation that is capable of describing this event due to the fact that temperature has an obvious influence on the process of photosynthesis.

2.6.2 Effect of light

There is a direct link between the three variables because changes in the amount of light and the length of cultivation can directly affect photosynthesis, the biochemical makeup of the plant, and biomass yield. Microalgae growth, light intensity, and cultivation duration all play a role in this relationship. For the most majority of microalgal species, the optimal level of light intensities ranges from around 200 to 400 mmol m^{-2} s^{-1} (Schuurmans *et al.*, 2015). However, for some microalgal species, this value might alternatively be equivalent to 100 mmol m^{-2} s^{-1}. The conversion of light energy into chemical energy in the form of molecules that may temporarily store energy is accomplished via the process known as light reactions. This is made possible by the fact that chlorophyll molecules are able to absorb photons, which in turn causes the excitation of a pair of electrons and ultimately results in the creation of ATP and NADPH. However, if the light intensity is allowed to continue to increase beyond a certain point, it may be the cause of photoinhibition and photooxidation, both of which have a detrimental effect on the development of cell density (Razzak *et al.*, 2013). In addition to this, the process of photoinhibition is connected in some way to the preexposure to either a high or low irradiance.

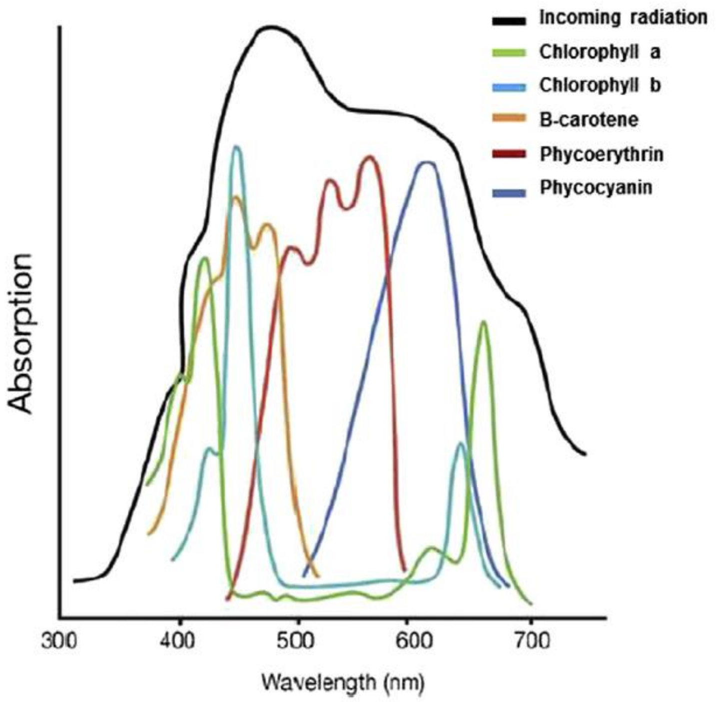

Fig 2.3 Absorption range of pigments and quantum photosynthesis rate of photosynthesis in microalgae. (Reproduced from P.J. le B. Williams and L.M.L. Laurens, Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics, Energy Environ. Sci. 3 (2010) 554e590.)

In point of fact, Beardall and Morris (Beardall and Morris, 1976) demonstrated a dynamic effect that is known as "hysteresis," in which a lower productivity of algae cells pre-exposed to high light intensity was observed when compared to the productivity of cells pre-exposed to a dark regime. This phenomenon can be caused to overestimate productivity, for example, when it is done during outdoor cultivation. On the other hand, the appropriate light intensity has to be empirically examined in each scenario in order to optimise CO_2 assimilation while minimising the rate of photorespiration and achieving the lowest feasible level of photoinhibition (Béchet, Shilton and Guieysse, 2013). The photoinhibition is linked to either self or mutual shading effects, in which cells that are near to the bottom are shaded by cells that are closer to the surface. The use of light emitting diodes (LEDs) rather of the more conventional fluorescent tubes is an example of one of the potential solutions that has been uncovered for this issue (Wu, 2016). The process of photosynthesis in microalgae has developed to function effectively in a spectrum of sun energy. However, an increase in solar UV radiation, which is caused by the depletion of the stratospheric ozone layer, may result in cell damage. This damage, however, can be avoided thanks to the presence of certain pigments that are able to increase the photosynthetic rate through resonance electron transfer with chlorophyll A. In any case, microalgae have physiological strategies that allow them to deal with an excessive amount of solar radiation. These physiological strategies include the production of antioxidants like b-carotene, astaxanthin, and a-tocopherol, as well as mechanisms for scavenging reactive oxygen species, which are produced when there are not enough electron sinks available.

2.6.3 Effect of nutrients

Microalgal cultures need necessary macronutrients, vitamins, and trace elements in appropriate numbers and in bioavailable chemical forms. However, the experience of cultivating microalgae in a variety of different types of media with different nutrient compositions, which diverged from the canonical Redfield ratio, suggested that the cultivation media could be flexible and adapted to microalgal metabolic needs, as well as particular environmental conditions (Arrigo, 2004).

2.6.3.1 *Carbon*

Carbon is the most abundant component of microalgal biomass, making about 65 % of the dry weight; but, depending on the growth circumstances and species, this number may be as low as 18 %. The majority of microalgal species contain approximately 50 % of carbon, but its increase is directly related to the limitation of other nutrients, such as nitrogen and phosphorus. This is the case because the majority of species also contain about 50 % carbon. Regardless of the pH of the solution, it is photosynthesis that is responsible for the majority of its uptake in its inorganic form. If the pH is lower than 6.5, H_2CO_3 is the predominant form; if the pH is between 6.5 and 10, HCO_3 is the predominant form. When the pH is more than 10, CO_3 is shown to be predominate. The Calvin cycle is responsible for the fixation of inorganic carbon inside the microalgal cells. Ribulose-1,5-bisphosphate carboxylase oxygenase is the enzyme responsible for the assimilation of carbon dioxide. When the intracellular concentration of CO_2 around Rubisco is high in comparison to its extracellular concentration, it is used as a substrate for its fixation (Price *et al.*, 2008). This prevents the presence of a respiration reaction, which is present when the intracellular concentration of CO_2 is low and the concentration of O_2 is high. In this particular instance, the Rubisco enzyme will react with oxygen instead of carbon dioxide. The uptake of inorganic carbon can either occur passively, through the process of membrane diffusion, with the carbon entering the cells as free CO_2, or actively, through the use of membrane pumps. The uptake of inorganic carbon is affected by the pH of the medium that is being used for cultivation. Carbon is metabolised primarily in alkaline environments through active transportation rather than diffusion. This results in the excretion of H+ ions, which can react with HCO_3 to produce CO_2. In response to the same environmental stimulus, microalgal cells are able to utilise homeostasis to control the pH level inside their cells. If the pH is low, the carbon dioxide can also be obtained through a process known as calcification. The precipitation of $CaCO_3$ from Ca^{2+} is the first step in the calcification process, which is utilised to both reduce the risk of intracellular toxicity and increase the efficiency with which nutrients are absorbed(Van Den Hende, Vervaeren and Boon, 2012). It is possible to get photosynthetic activity, and in particular the conversion of bicarbonate to carbon dioxide both extracellularly and intracellularly. Because there is no development of a hydroxylic group during the process of CO_2 fixation, the medium will not become alkaline as a result of the passive absorption of CO_2. When CO_2 is delivered in the form of gas, solubility and mass transfer both become important parameters to consider. The amount of CO_2 that can be dissolved in one litre of water is 1.5 g L^{-1} when the temperature and pressure are both set to 25 °C and 1 ATM. The rate at which mass is transferred from the gaseous phase to the liquid phase is another factor that affects the CO_2 fixation. The transfer is affected both by the particular contact area as well as the penetration gradient that exists between the two phases. The microalgal absorption of CO_2 cannot be compensated for because the mass transfer of CO_2 in solution is too modest. Because of this, the medium is purged with CO_2-rich gas, which has varying degrees of solubility depending on the pH of the surrounding environment. In point of fact, the rate of CO_2 mass transfer is higher at high pH than it is at low pH values. This is because the chemical interaction between CO_2 and OH^- occurs more quickly than the hydration of CO_2 to H_2CO_3, which slows the rate of CO_2 mass transfer. It is expected that the expenses associated with the creation of CO_2 for microalgae culture are about 50 % more than the costs associated with the production of biomass. Either the biogas that is produced as a by-product of anaerobic digestion and includes 20–40% carbon dioxide or the carbon dioxide that is produced as a by-product of alcoholic fermentation might be considered a low-cost alternative. One possibility is the flue gas that is produced by coal-fired plants, cement production plants, or natural gas combustion. This gas contains anywhere from 10 to 25% CO_2, as well as a concentration of inhibitors like

NO_x and SO_x, which can be removed via purifying processes like the reaction that forms sodium or ammonium bicarbonate and urea. When compared with CO_2, the most significant disadvantage is a rise in pH as well as an increase in ionic strength; nevertheless, there is also a benefit associated with an increase in solubility. The fact that the solubility of $NaHCO_3$ at 25 °C is more than 90 g L^{-1} makes these kinds of procedures appealing, with the exception of the economic effect, which is 3 times greater when compared with the utilisation of CO_2 (Suh and Lee, 2003; Chi *et al.*, 2014). Chisti et al. investigated the $NaHCO_3$ tolerance of a variety of strains, including *Chlorella sorokiniana*, *Synechocystis* sp. PCC6803, *Cyanothece* sp., *Dunaliella salina*, *Dunaliella viridis*, *Dunaliella primolecta*, and *Arthrospira platensis* (Chisti, 2013). The difficulty is in screening potential strains and identifying those that can thrive in environments with high levels of pH, alkalinity, and ion strength. In an ideal scenario, the rate of mass transfer of CO_2 and the rate at which it is assimilated should coincide with the activity level of the microalgae while concurrently regulating and adjusting the pH. On the other hand, the alkalinity and the partial pressure both have an impact on the theoretical equilibrium (Olaizola, 2003). In conclusion, there exist a great number of organisms that are capable, according to a heterotrophic or mixotrophic metabolism, of growing on organic substrates. Diffusion, active transportation, and phosphorylation are the three primary mechanisms that are responsible for the absorption of organic carbon into the cells (Perez-Garcia *et al.*, 2011). Monosaccharides, volatile fatty acids, glycerol, and urea are the primary chemical components. Glycerol is also present. Furthermore, the growth rate in the mixotrophic regime is higher than that in the autotrophic regime; however, an increase in the cost related to the organic carbon source supply must be taken into consideration. This is the case even though in some instances, for example, the support of various processes such as anaerobic digestion could represent a help because bacteria could convert organic matter into acetate or some other volatile fatty acids suitable for the growth of microalgae (Markou, Vandamme and Muylaert, 2014a) .

2.6.3.2 Nitrogen

Nitrogen is the second most abundant element in microalgal biomass, with a contribution of around 1 - 14 % of dry weight. Nitrogen is an essential biochemical compound that is present in DNA, RNA, proteins, and pigments, etc.The primary route via which nitrogen is metabolised is called the glutamine synthetase enzyme system. Through a reaction with ammonium, glutamine, an amino acid, is produced from glutamate (Markou, Vandamme and Muylaert, 2014b). Inorganic forms of nitrogen, such as NO_3, NO_2, and NH_4, as well as organic forms, such as urea and amino acids, are used to provide the element. Nitrate (NO_3) is typically distributed in the form of sodium nitrate ($NaNO_3$). Microalgal cells are able to tolerate concentration of nitrate up to 100 mM, but higher concentration have a negative impact on microbial growth. This is most likely caused by an increase in nitrate reductase activity as well as a simultaneous increase in the concentration of nitrite and ammonium in a range that could be toxic for the cells (Jeanfils, Canisius and Burlion, 1993). Nitrite (NO_2) is regarded to be an intermediate result of the nitrification processes that are caused by bacteria, which is the oxidation of ammonia to nitrate. However, nitrite may also be an intracellular intermediate product that is produced by the reaction of nitrate reductase. It is integrated by means of both active transportation and diffuse dissemination. In the presence of nitrite with a concentration of 4 mM, Yang et al. (Yang *et al.*, 2004) reported an increase in the length of the lag phase in cultures of *Botryococcus braunii*. They also observed a complete suppression of the development of microalgae when the nitrite concentration was increased to 8 mM. On the other hand, Taziki et al. (Taziki, Ahmadzadeh and A. Murry, 2016) found that *Chlorella vulgaris* is able to effectively remove and utilise both nitrate and nitrite from culture medium even at high concentration. This allows *Chlorella vulgaris* to simultaneously produce algal biomass for a variety of applications and be a promising candidate for the treatment of wastewater. High CO_2 concentration have a beneficial effect on nitrite absorption because they cause a rise in nitrite reductase activity, which in turn makes nitrite assimilation more efficient (Flores *et al.*, 1987). Nitrite oxide, also known as NO, is a nonpolar molecule that is very tiny and has the ability to diffuse straight inside the cells. It is a free radical that has the potential to cause damage to the cells, and its poor solubility might be another element that prevents its removal from the body. The addition of ferrous-complexed EDTA to the culture medium may increase the amount of NO that is soluble in the medium (Santiago, Jin and Lee, 2010). In the presence of dissolved oxygen, dissolved nitrogen monoxide may oxidise to either nitrite or nitrate, both of which are taken up by microalgae. However, since less energy is required for its digestion compared to the other nitrogen sources (Perez-Garcia *et al.*, 2011), ammonium and ammonia are the nitrogen sources that are most chosen by microalgae as a source of nitrogen. Ammonia has a high solubility for a gas, which implies that it is commonly found in liquid solution. Additionally, ammonia is a volatile gas. In each case, the presence of water triggers a chain reaction involving ammonia, which results in the formation of a system consisting of ammonia and ammonium and which is in equilibrium regardless of the pH. Because it is tied to the pH of the growth medium, the toxicity of ammonium ions is far lower than that of free ammonia.. Ammonium is assimilated by transportation mechanisms, which can positively control intracellular concentrations; on the other hand, since ammonia is metabolised through passive diffusion into cells, the control of intracellular concentration is more difficult. Ammonium is assimilated by transportation mechanisms, which can positively control intracellular concentrations. In addition to this, free ammonia has an effect on the photosynthetic system and, more specifically, it causes photodamage to photosystem II (Drath *et al.*, 2008). One method that may be used to prevent the adverse effects that are brought on by the presence of ammonia is the regulation of the pH. Another method is to gradually add ammonia to the medium in which the culture is being grown. It is possible for ammonia to be lost from the culture medium owing to volatilization if it is used as a source of nitrogen for the development of microalgae (Markou, Vandamme and Muylaert, 2014a), which is an additional limitation of employing ammonia as a source of nitrogen. It is possible for ammonia to combine with carbon dioxide to form bicarbonate or urea, either of which may be utilised as a source of nitrogen for the development of microalgae. Microalgae also have the ability to metabolise organic nitrogen, which may take the form of urea or amino acids that are actively carried into the cells. Urea, the most important source of organic nitrogen, is often hydrolyzed to produce ammonia and carbonic acid, both of which are consumed by microalgae. Urea is also the most abundant source of organic nitrogen.

2.6.3.3 Phosphorous

Phosphorus is another essential component for microalgal development since it is a limiting nutrient for microalgae and its concentration in biomass ranges from 0.05 -3.3 % .Phosphorus's content in biomass ranges from 0.05 to 3.3 %. Phosphorus is found in organic molecules such as RNA, DNA, membrane phospholipids, and ATP. Phosphorus comes mostly through the production of potassium, ammonium, and sodium phosphate from phosphate rocks. Microalgal cells may take in phosphorus in the form of orthophosphate either by the activity of enzymes found within the cell, enzymes found outside the cell, or directly connected to the cell wall. It is also possible for it to be metabolised as dissolved organic phosphorus via an extracellular mineralization process that is carried out by phosphate enzymes. Phosphorus absorption is controlled by available light, pH, temperature, ionic strength, and accessible ions (K^+, Na^+, and Mg^{2+}), all of which, in their own way, enhance the precipitation of phosphate (Cembella, Antia and Harrison, 2008). According to a pattern of behaviour known as "luxury," microalgae are able to store intracellular phosphorus reserves in the form of polyphosphate granules, which may then be exploited as a source of phosphorus (Powell *et al.*, 2009). It is used in the process of removing phosphorus from wastewater due to its capacity to hold an excessive amount of phosphorus.

2.6.3.4 Trace and micro nutrients

The culture medium needs the presence of a number of additional micronutrients, such as magnesium (Mg), which is mostly found in aqueous solution as Mg^{2+} and is delivered as $MgSO_4$ or $MgCl_2$, with a concentration that falls anywhere between 0.35-0.7 %. It is thought of as an activator for various enzymes, it is a component of the equipment involved in photosynthesis, which is chlorophylls, and it takes part in activities such as the generation of ATP (Markou, Vandamme and Muylaert, 2014b). When the pH is greater than 11, it precipitates as magnesium phosphate or magnesium hydroxide, both of which have the ability to induce the flocculation of microalgal biomass. Additionally important is the vitamin known as sulphur (S). Its concentration in microalgal biomass is between 0.15-1.6 % , and it is found in the form of amino acids, sulfur-based lipids, vitamins, and a variety of secondary metabolites that include sulphur (Melis and Chen, 2005). Sulfur is more often digested in the form of sulphate (SO_4^{-2}), but sulfite, which is its more poisonous form, is not(Oren, Padan and Malkin, 1979). The amount of calcium (Ca) that is present in the biomass of microalgae may vary anywhere from 0.1 to 1.4 %, but it can even exceed 8 %. It is capable of affecting both the process of cell division as well as the overall morphogenesis, and it is present in the form of Ca2+, however it is most often added as $CaCl_2$. When the intracellular Ca^{2+} value of the culture medium reaches high levels, it inhibits the development of microalgae and has a detrimental impact on the whole population. Due to the combination of high calcium concentrations in the cultivation medium and high pH values, the precipitation of $CaCO_3$ and various other calcium salts occurs. This results in a decrease in the alkalinity of the medium as well as the concentration of certain minerals, such as iron and phosphorus. Iron (Fe) is given as chelated complexes to boost its bioavailability and is engaged in essential enzymatic activities such as oxygen metabolism, electron transfer, nitrogen absorption, and chlorophyll synthesis (Markou, Vandamme and Muylaert, 2014b). Iron (Fe) is also involved in the production of chlorophyll.

2.6.4 Effect of pH

Because it controls the intake of ions, enzymatic activity, phosphorus availability, inorganic carbon availability, and ammonia toxicity (Havlik, Scheper and Reardon, 2016), pH is an extremely important factor in microalgal metabolism. There are two pH ranges that are often used for microalgal cultivation: 7.9-8.3 (for seawater) and 6.0-8.0. (for fresh water). The vast majority of microalgal species are pH-sensitive, and very few of them are capable of reaching the range that *C. vulgaris* can tolerate (Lam and Lee, 2012). Within an enclosed environment, the pH level might rise as high as 10. This rise in pH value may be stopped by flushing the system with CO_2 or by applying either inorganic or organic acids (Markou, Vandamme and Muylaert, 2014b). Combustion flue gas with high quantities of CO_2, which may lower the pH to 5, is one possibility; but, at increased acidity levels, the development of photosynthetic organisms is restricted. However, the simultaneous breakdown of metabolites, in addition to the release of multiple different organic acids, is a factor that has the ability to change pH levels. In contrast, the excess of OH that is present in alkaline solutions combines with CO_2 to generate $HCO^{3\ -}$, which leads to a larger bicarbonate-carbonate alkalinity and, as a consequence, a higher total carbon availability (Münkel *et al.*, 2013). It would seem that storing CO_2 is not only necessary for controlling the pH of the environment but also for preserving the overall carbon balance. In conclusion, a number of studies stated that high pH stress suppresses the cell cycle and induces the accumulation of lipids (Vuppaladadiyam *et al.*, 2018).

2.7 Microalgal cultivation system

In general, microalgae grow by converting inorganic carbons into CO_2 or sodium bicarbonate, and they get their energy from the absorption of light. This pathway is referred to be autotrophic. While doing so, they are able to make use of the organic substrate as a source of both energy and carbon under the heterotrophic regime. Lastly, they are able to cultivate using a mixotrophic method, which is when the microalgae execute photosynthesis as their primary source of energy, but both organic and inorganic (i.e., CO_2) are required for growth. The form of culture that one decide to use is a crucial choice.

2.7.1 Autotrophic mode

It is generally agreed that autotrophic culture is the most frequent mode of cultivation strategy for microalgae. The light can be supplied in the form of sunlight, which represents an additional advantage even if the availability of solar light is a limiting factor in zones where sunlight supply is not consistent and intense enough to support algae growth. Alternatively, the light can be supplied in the form of artificial light, such as light-emitting diodes (LEDs), which are a source of energy developed because of their low energy consumption and their range of lights (for example, red LED, 624-634 nm; green LED, Autotrophic cultivation is considered technically and commercially scalable, typically at outdoor environments, particularly to increase lipid productivity (by using 2% CO_2 in air) and also to recycle industrial CO_2 (Moreno-Garcia *et al.*, 2017; Saha and Murray, 2018). This is because autotrophic cultivation typically takes place in an outdoor environment. Nevertheless, in order to boost the development of autotrophic microalgae, it is necessary to solve the issue of self-shading brought on by photo acclimation and, at the same time, boost the net activity of Rubisco(Kenny and Flynn, 2017). According to Flynn and Raven's (Flynn and Raven, 2017) observations, a significant rise in the growth rate of microalgae seems improbable in the absence of a de facto artificial substitute for Rubisco, which is the single most important and abundant enzyme on Earth.

2.7.2 Heterotrophic mode

Due to the fact that certain microalgae are able to thrive in environments devoid of light, this procedure provides a solution to the issue of light availability. As an example of an organic carbon substrate, carbon and other energy sources are provided in this scenario. The most important benefits of heterotrophic culture are a high level of growth control and a high level of production. These benefits are achieved by using a two-stage cultivation process inside a fed-batch or a heterotrophic photoinduction regime (Li *et al.*, 2015). The use of low-cost carbon sources, such as glucose, acetate, and glycerol, which are often applied in fermentation processes and are obtained from low-cost feedstocks or wastewater. In addition, the high cell density that may be achieved makes it possible to lower the expenses that are involved with dewatering during the harvesting stage. Under these circumstances, it is only possible to culture a limited number of strains of microalgae, such as *C. vulgaris*, *Chlorella protothecoides*, *Crypthecodinium cohnii*, and *Schizochytrium limacinum* (Moreno-Garcia *et al.*, 2017; Vuppaladadiyam *et al.*, 2018). On the other hand, the presence of organic substrates and water are also possible sources of microbial contamination in culture media (Lowrey, Armenta and Brooks, 2016). Additionally, gloomy circumstances in heterotrophic environments might impede pigmentation and the formation of secondary metabolites.

2.7.3 Mixotrophic mode

Microalgae that are grown under mixotrophic circumstances are able to use both exogenous organic molecules (such as cheese whey permeate, sodium acetate, fruit peel, glucose, fructose, and glycerol) and carbon dioxide as a source of carbon. Light and inorganic carbon are used in the metabolic process of photosynthesis, while organic carbon is utilised during the process of respiration, where the CO_2 that is generated is, in part, utilised in the metabolic process of photosynthesis. Mixotrophy is a metabolic strategy that combines the positive aspects of autotrophy and heterotrophy, while also avoiding the drawbacks of autotrophy (Zhan, Rong and Wang, 2017). One further benefit has to do with the adaptability of the use of light, which is not a feature that is strictly restrictive in any way. If integrated with dark/light cycle, which allows the growth of autotrophy under their optimum conditions, the biomass and lipid content of the mixotrophic cultivation will not simply be a sum of autotrophy and heterotrophy (Zhan, Rong and Wang, 2017) the other hand, it could allow a reduction of photoinhibition phenomena, when compared with autotrophic and heterotrophic cultures. This is because mixotrophic cultivation is characterised by the presence of both autotrophic and heterotrophic components. *Spirulina platensis, Chlamydomonas reinhardtii, Chlorella sorokiniana, Scenedesmus obliquus,* and *C. vulgaris* all have metabolisms that are typical of mixotrophy (Moreno-Garcia *et al.,* 2017). In contrast to heterotrophy, mixotrophic cultivation mode allows for the valuable pigments and photosynthetic carotenoids to be maintained in illuminated conditions (Alkhamis and Qin, 2016). Despite this, the mixotrophic energy conversion efficiency is lower than that of the heterotrophic cultivation mode because of losses in photosynthetic activity.

2.8 Reactor systems used for algal culturing

Microalgae may be grown in a variety of environments, the most common of which being open and closed systems. An open system make use of sunlight and is broadly exposed to the surrounding environment, which results in a significant benefit as a consequence of the utilisation of unpaid natural resources. Closed systems, also known as photobioreactors (PBRs), may be broken down into four distinct categories: tubular, columnar, membrane, and flat plate. Every kind of agricultural production method has both benefits and cons.

2.8.1 Open ponds

The level of mixing and cultivation may be used to further categorise open ponds into three distinct types: unstirred, raceway, and paddlewheel open ponds. In general, they call for relatively low initial investments when compared to closed systems. They are typically built out of concrete or compacted earth, and they can take on a variety of shapes. They are typically able to meet fundamental criteria such as providing sufficient sunlight, an optimal hydrodynamic force, and a closed loop channel that is mixed to uniform the cells. They do not need area that can be farmed, but they do need a lot of water, which is a disadvantage. An outdoor culture of algae that is constantly mixed and maintained at a depth of 30 cm should be able to reach an average light-limited algal concentration of around 300 mg L^{-1} dry weight. Because the penetration of light is related to the log of its intensity an increase in the photon flux density only results in a marginal improvement in this regard. Turbulence is another factor that may boost the effectiveness of raceway open ponds. This factor does not harm the algal cells, but it is able to maintain the cells suspended, which prevents thermal stratification and makes it easier to remove oxygen that is produced by photosynthesis. The paddlewheel, which is situated dip inside the pond, is able to produce a flow and ensure that the mixing is achieved. The engineering design, which may include some of the elements listed above, is coupled to a number of environmental conditions, process parameters, and biological parameters that influence the amount of biomass produced. Accurate calculations of the amount of light that is being introduced and the temperature of the water are essential to the success of growth. The uncontrolled change in the ionic composition of the broth that occurs as a result of evaporation and the variations in seasonal circumstances is another significant factor to take into consideration since it may have an effect on the level of production. Because algae species thrive in the presence of microorganisms such as fungus, bacterium, virus, rotifers, *Cladocerans* (e.g., Daphnia), *Amebae*, *Cyclopoid copepods*, *Ciliates*, and *Chironomid midges*, the evolution of the microbial community has the potential to have an effect on the biomass production. Because of this, there has been a shift toward cultivating extremophile microalgae, which always have a lower risk of being eaten by predators when grown in large quantities outside. On the other hand, the interactions of the microbial community in open ponds are beneficial to both populations. For instance, the symbiosis that occurs between bacteria and microalgae results in the production of extracellular compounds that are advantageous. In addition, the use of cocultivation systems consisting of microalgae and bacteria may promote both the removal of nitrogen and phosphorus from wastewaters as well as simultaneous growth (Kim *et al.*, 2014; Kumar *et al.*, 2015) . More than 80 % of the world's algal biomass is produced in open ponds, which is mostly attributable to the lower level of investment necessary for these types of systems. On the other hand, it is anticipated that the usage of closed PBRs would increase by 2024 in terms of demand and sales as a result of the benefits that are associated with these production systems (*Algae Fuel Market - Global Industry Analysis 2023*).

2.8.2 *Photobioreactors*

The choice of PBRs is determined by a number of criteria, one of which is the productivity of the microalgal biomass, as well as the products that are ultimately produced. However, this style of culture involves higher expenses in illumination, carbon dioxide, and feeding of nutrients. On the other hand, it is easier to regulate when compared to open systems and is able to decrease issues linked to contamination. PBRs, on the other hand, are susceptible to a number of drawbacks, such as the formation of biofilms, which results in the accumulation of oxygen in the culture. This accumulation of oxygen has the potential to have a toxic effect on the growth of photosynthetic organisms, and light is still the primary factor that restricts growth. Although suitable technical solutions and subsequently alternative forms of PBRs, which are often more extensive and complicated, might help mitigate some of these issues, this should not be seen as a cause to lessen interest in the possible application of this technology on an industrial scale. The fundamental parameters for constructing a PBR are still connected to the roles that light and circulation play, the mass transfer that occurs, the materials that are utilised for the building, and the temperature. Because light may be absorbed or dispersed in the culture media, the amount of light that can pass through it is a limiting issue.

2.8.2.2 *Column photobioreactors*

Airlift and bubble column PBRs are simple cylinder devices with a radius that should not exceed 0.2 metres in order to avoid problems related to the light availability in the centre of the PBR, and a height limitation of approximately 4 metres for structural reasons, due to the strength of the transparent materials employed and to avoid shading effects. Both of these factors help to ensure that problems related to the light availability in the centre of the PBR are avoided. They have a low shear force, lack of wall development, high mass transfer and hence, great efficiency of CO_2 utilization. The effectiveness of the operation and the greatest amount of biomass that may be produced are directly proportional to the size of the column, as well as the specific growth rate of the algae strains, the amount of light that is present, and the surface area. A sufficient mixing, which can be achieved through aeration, is required in order to overcome these issues and the sedimentation of microalgal cells. This mixing must be capable of simultaneously ensuring an even exposure to light and nutrients, as well as facilitating the transfer of heat and the exchange of gases. Column PBRs may be broken down into bubble columns and airlift reactors, depending on the type of aeration being used. The size of the bubbles, on the other hand, is dependent upon a number of parameters, including the characteristics of the sparger, the physical properties of the liquid and gas phases, and the H/D ratio of the column. In addition to this, it is necessary to take into consideration the phenomena of bubble coalescence or breakage, as well as the possibility of clogging effects brought on by the presence of micron-sized algae, which are more likely to occur in environments with high biomass concentrations and high pressure drops. The bubble size distribution throughout the column is very significant because the size of the gas bubbles at the top of the column determines the down comer gas holdup, which in turn leads to a certain liquid circulation velocity and the light/dark cycle.

2.8.2.3 Tubular photobioreactors

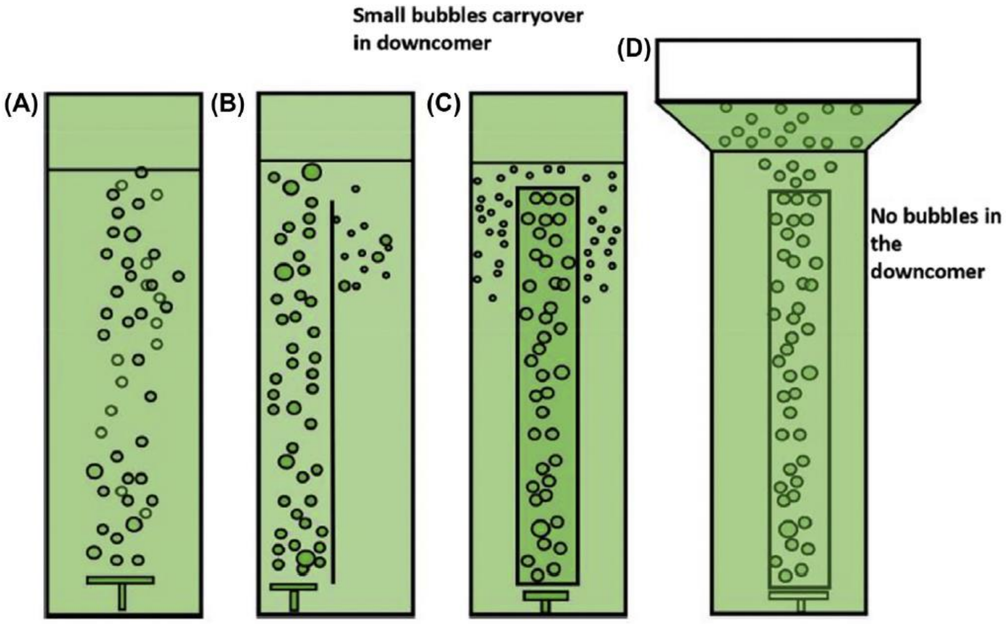

Fig 2.4 Schematic of different photobioreactors; (A) bubble column, (B) split column airlift, (C) internal loop airlift, and (D) internal loop airlift with gas separator. (Reproduced from S.B. Pawar, Process engineering aspects of vertical column photobioreactors for mass production of microalgae, ChemBioEng Rev 3 (2016) 101e115.)

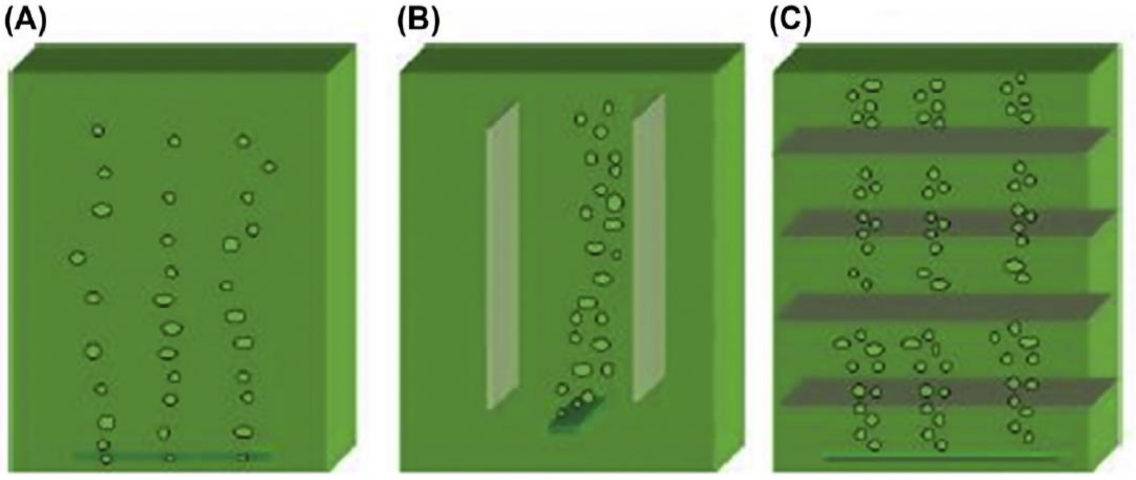

Fig 2.5 Flat plate photobioreactors; (A) simple flat panel, (B) flat panel with vertical baffles, and (C) flat panel with horizontal baffles. (Reproduced from S.B. Pawar, Process engineering aspects of vertical column photobioreactors for mass production of microalgae, ChemBioEng Rev 3 (2016) 101e115.)

Glass or plastic are the two most frequent materials utilised in the construction of tubular PBRs, which are employed mostly for bulk growing in outdoor settings. It is possible to arrange them in a variety of orientations, including as horizontal, inclined, vertical, or helical, in order to optimise the amount of sunlight that they absorb. The culture of microalgae is pumped into the reactors, which typically have a diameter ranging from 10 to 60 mm and a length that may even exceed 100 m. When the creation of large cell concentrations is the desired outcome, it is possible to build the system with a modest diameter of 10 mm (Posten, 2009). They have a number of drawbacks, such as a limitation in the photosynthetic efficiency, frequently in outdoor cultivations, for the buildup of oxygen, and also a very high energy consumption when compared with bubble column and flat plate PBRs. Both of these drawbacks are in contrast to the bubble column and flat plate PBRs. After just one minute of sitting in a tube with no gas exchange, there is a possibility that an inhibitory concentration may develop owing to the harmful effects of oxygen (Posten, 2009) In addition, the proliferation of the cells in the middle of the tube is constrained since it is subject to photo limitation as well as difficulties associated with mass transfer. Because of this, according to the idea, the width of the tubes has to be maintained as narrow as feasible based on the likely build-up of O_2 and the reduction of CO_2 levels. It is easy to see why this is the case given that CO_2, O_2 gradients, and pH should be carefully examined between the medium intake and the output (Huang *et al.*, 2017). Because it was demonstrated that the presence of external mass transfer resistance at biofilm surface is capable of creating profile switches of reagent concentration inside the biofilm, another disadvantage of these systems is the uncontrolled growth of pathogenic microorganisms in the inner walls and the formation of biofilms. Both of these factors influence the mass transfer of reagents. When compared to traditional PBRs, an unique tubular PBR that had the outer surface intermittently shaded was proven to improve the photosynthetic efficiency by improving the biomass output by 21.6 %. The development of an artificial light/dark cycle that is conducive to the expansion of microalgae is the fundamental cause of this rise (Liao *et al.*, 2014). In addition to this, the culture may reach a high temperature because to the restricted volume that is present as a consequence of the tiny diameters of the tubular PBRs. Last but not least, one of the most bothersome problems that arises with tubular PBRs is that of cleaning their walls, which is intimately related to the permeability of light. At the moment, the method that is used most often to do this washing is known as mechanical cleaning(Zhu, Rong and Zong, 2013).

2.9 Microalgae in aquaculture industry

There is a possibility that the use of microalgae might lessen aquaculture feed producers' reliance on traditional raw resources. Because of their high nutritional value and positive impact on the rate of growth of aquatic species due to increased triglyceride and protein deposition in muscle, improved resistance to disease, decreased nitrogen output into the environment, omega-3 fatty acid content, physiological activity, and carcass quality, the use of microalgae could have significant positive effects and could potentially replace or reduce common feed stuff (Becker 2004). Microalgae can grow in a wide variety of habitats, some species have several-fold higher biomass production than plants, they can divide quickly with simple nutritional requirements, they can accumulate useful metabolites, and their availability is not dependent on the harvesting of wild fish for fishmeal. These are just a few of the many advantages of microalgae (Hemaiswarya *et al.*, 2011). There are a few disadvantages and difficulties associated with using microalgae as a replacement for fishmeal and fish oil in the aquaculture industry. These include high production costs of microalgae (Becker, 2007; Sarker *et al.*, 2016) possibility that the cell walls of certain microalgae are not easily digestible (Skrede *et al.*, 2011), and availability of a large amount of biomass could be hampered by contamination. Microalgae have been the primary focus of technological advancements and commercial applications for the majority of the past decade. These developments and applications have primarily concentrated on the microalgae's beneficial properties rather than the microalgae's contribution of gross nutrients to the recipient animal. There has been a meteoric rise in the number of studies that investigate the possibility of using microalgae as a component in aquafeed for a variety of fish species. Several recent studies (Benemann, 1992; Hemaiswarya *et al.*, 2011; Yaakob *et al.*, 2014; Roy and Pal, 2015) have revealed that there is a significant potential for microalgae to be used as a bulk feedstuff for aquaculture diets. However, there is currently a limited amount of updated information and recent developments on the usage of microalgal biomass as a replacement for fishmeal and fish oil for the establishment of a sustainable aquaculture business.

2.9.1 Microalgal nutritional quality as fishmeal and fish oil supplement or feed additive

The use of microalgae as a source of protein, lipids, vitamins, carotenoids, and energy in feed has a lot of untapped potential. In general, microalgae in the late logarithmic development phase have a protein content of 30–40 %, a lipid content of 10–20 %, and a carbohydrate content of 5–15% (Brown *et al.*, 1997). The chemical and nutrient composition of microalgae, including proximate analysis, amino acid and fatty acid compositions, and vitamin and mineral contents, has been well documented in a variety of published reports (Brown *et al.*, 1997; Becker, 2007; Yaakob *et al.*, 2014; Kent *et al.*, 2015; Tibbetts, Milley and Lall, 2015). The protein level of microalgae is the primary factor that determines its nutritional value. The amount of polyunsaturated fatty acids (eicosapentaenoic acid, EPA); arachidonic acid, ARA; and docosahexaenoic acid, DHA) found in microalgae is the second most important factor (Reitan *et al.*, 1997).

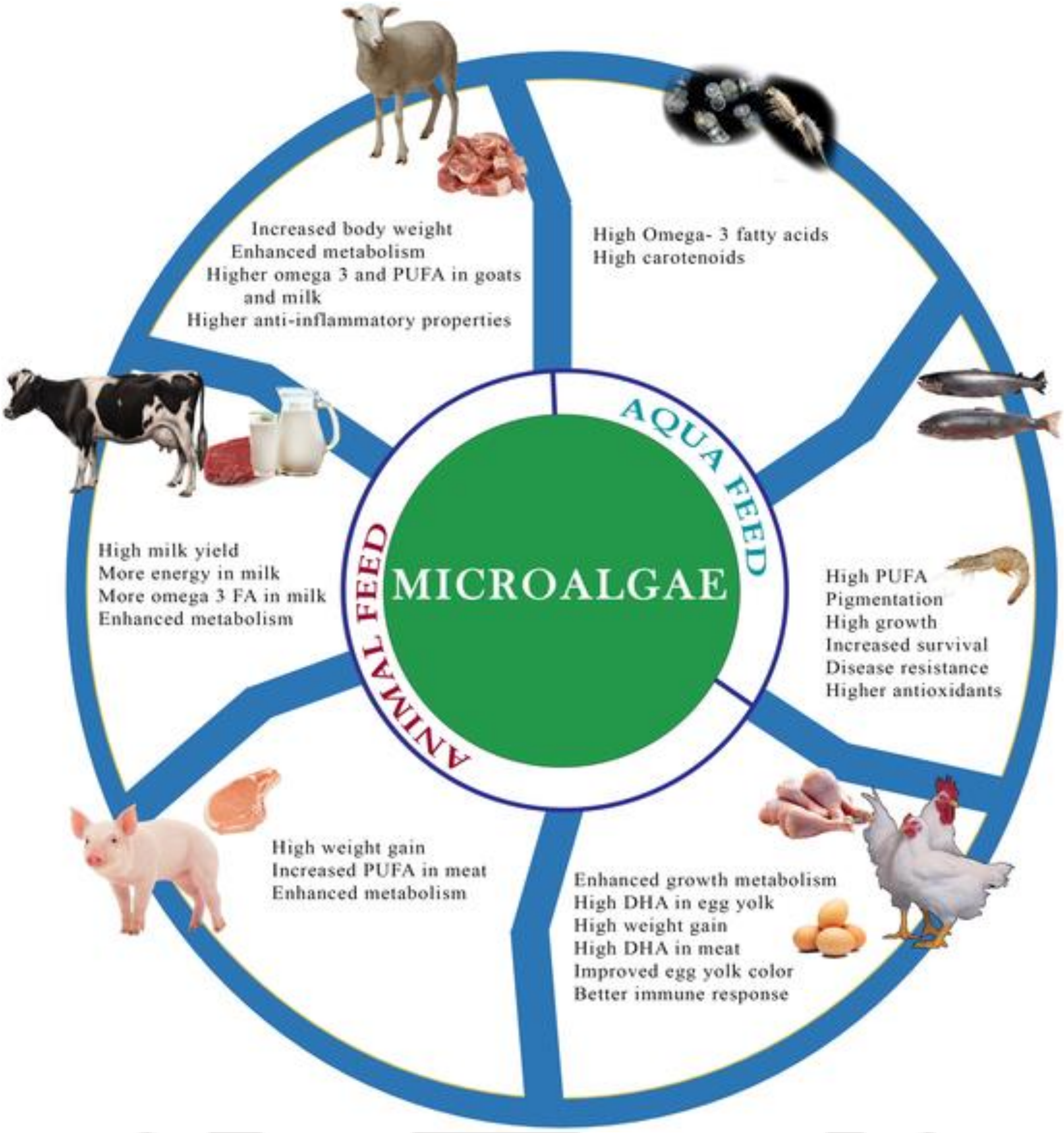

Fig 2.6 Graphical outline of the diverse application of microalgal feed and its advantages (reproduced from Dineshbabu, Gnanasekaran, Gargi Goswami, Ratan Kumar, Ankan Sinha, and Debasish Das. "Microalgae–nutritious, sustainable aqua-and animal feed source." Journal of Functional Foods 62 (2019): 103545.)

EPA and DHA are produced by a wide variety of autotrophic and heterotrophic microalgal species belonging to a variety of classes, but AA is often only found in trace levels (Bigogno *et al.*, 2002). *Bacillariophyceae* (diatoms) and *Chrysophyceae* species may be rich sources of EPA and DHA, as indicated by recent reviews of total lipid extracts; *Cryptophyceae, Prasinophyceae, Rhodophyceae, Xanthophyceae, Glaucophyceae,* and *Eustigmatophyceae* can represent interesting EPA sources; whereas DHA is found in significant amounts mostly in Dinophyceae (Hu *et al.*, 2008; Lang *et al.*, 2011). The nutritional characteristics of some microalgae, such *as Arthrospira (Spirulina), Dunaliella*, and *Chlorella*, have been well researched, and these microalgae have a long history of usage as sources of animal feed. The usage of microalgal biomass as a substitute for fishmeal or fish oil, or as a dietary supplement, has been the subject of a number of recent research, which are summarised in Table 2.2. In the following sections, we shall delve into the unique nutritional qualities of microalgae in more depth as well as their uses in aquafeed.

2.10 Current challenges

Microalgae have certain fascinating properties that make them potential candidates for use as alternative feedstocks in a wide variety of applications, including industrial and environmental uses. However, in order to be successful in overcoming various obstacles, such as controlling culture conditions by selecting favourable conditions that are able to increase productivity, and selecting systems that are designed specifically to meet needs such as CO_2 mitigation or wastewater treatment or photoinhibition problems or the cost of carbon substrates, efforts are required. In recent years, there has been a substantial increase in the production of microalgae; however, this improvement has not yet been accompanied by the anticipated increase in biomass productivity, which would result in enormous financial gains. In order for microalgal feed to successfully be used in conjunction with or in instead of normal feed, its cost must be comparable to that of regular feed. Because a higher biomass productivity results in lower production costs, the majority of research and development efforts are focused on developing algae strains that have a higher potential for productivity. Some of the few approaches that have been taken to produce improved productivity include the implementation of new cultivation systems, aeration and agitation tactics, lighting requirements, and medium engineering. It's possible that optimization will be required for each and every pressure, location, and amount of water used. According to Beal et al., however, even the promising solutions do not result in reduced selling prices that are greater than the prices that are already being paid on the market for petroleum and/or animal feeds (Beal *et al.*, 2015). There is a huge variety of microalgae that has not yet been investigated for their potential use as fish food. In spite of this, recent investigations on algae belonging to a variety of genera have uncovered a broad spectrum of feed conversion ratios, digestibility, nutritional, and functional properties. As a consequence of this, more screening tests of novel microalgae on fish meal selection might provide light on its genuine potential. In several instances, the microalgal feed resulted in a reduced fish consumption when compared to fish meal as a source of nutrition. However, if components such as taurine are added to the meal made from microalgae, fish will have a greater intake, which will result in higher growth performance (Takagi *et al.*, 2008). It will take some time before large-scale production systems, harvesting technologies, and processing methods can be optimised. In the future, creative manufacturing in feed paired with unique upstream and downstream processing technologies for the production of microalgae biomass may successfully replace fish meal and give a solution that is more environmentally friendly.

Table 2.2 Recent studies on applications of microalgae biomass as feed for aquaculture

Microalgae species	**Aquaculture species**	**% replacement of fish meal /fish oil/ dietary inclusion level**	**Effect of microalgae biomass**	**References**
Arthrospira platensis	Red tilapia (*Oreochromis sp*.)	30% inclusion in feed as carotenoid supplement	Improved fish color	(Ruangsomboon, Ganmanee and Choochote, 2013)
Dunaliella salina	Shrimp *Penaeus monodon*	5 to 10% inclusion in feed	Strongly enhanced the immunological and antioxidants factors (superoxide dismutase and catalase) and increased the surviving rate	(Madhumathi, 2011)
Nannochloropsis sp. and *Isochrysis sp*.	Juvenile Atlantic cod (*Gadus morhua*)	15% replacement of fish meal protein	Feed intake and growth improved in the fish. No differences in survival, feed conversion ratios, and omega-3 and omega-6 fatty acids in the muscle among the treatment groups	(Walker and Berlinsky, 2011)
Nannochloropsis gaditana, Phaeodactylum tricornutum, Tetraselmis chuii	Gilthead seabream (*Sparus aurata*)	0.5 and 1% inclusion in feed	Enhanced defense activity	(Cerezuela *et al.*, 2012)
Arthrospira sp.	Golden barb (*Puntius gelius*)	20% replacement of fishmeal in diet	Significantly increased growth rates of fish	(Hajiahmadian *et al.*, 2012)

Nanofrustulum sp.	Atlantic salmon (*Salmo salar*), common carp (Cyprinus carpio)	5 or 10% replacement of fishmeal	Growth performance and feed utilization did not exhibit any differences compared with fish meal-based feed, indicating algal meal as an effective replacement of fish meal.	(Kiron *et al.*, 2012)
Tetraselmis sp.	Pacific white shrimp (*Litopenaeus vannamei*)	5 or 10% replacement of fishmeal		
Haematococcus pluvialis	Pacific white shrimp (*L. vannamei*)	12.5% of the fishmeal protein replaced	No negative effects in shrimp performance and improved shrimp pigmentation	(Ju, Deng and Dominy, 2012)
Arthrospira maxima	Red tilapia fingerling (*Oreochromis sp.*).	Up to 30% replacement of fish meal	No negative impact on growth performance	(Rincón *et al.*, 2012)
Tetraselmis suecica	European sea bass (*Dicentrarchus labrax*)	Up to 20% replacement of fish meal protein	No negative impact of the growth performance and major quality traits of fish.	(Tulli *et al.*, 2012)
A. platensis	Ornamental carp (*C. carpio*)	7.5% inclusion in feed as carotenoid supplement	Improved pigmentation	(Sun *et al.*, 2012)
Arthrospira sp.	Tilapia (larvae/juveniles) (*Oreochromis sp.*).	Up to 43% replacement of fish meal	No negative impact on growth or feed intake and had a better FCR than a corn-gluten meal control	(Hussein *et al.*, 2013)
A. platensis	Nile tilapia (Oreochromisniloticus)	0.5 to 2% inclusion in feed	Positively improved the health conditions of fish through tissue protection and antioxidant effects	(Ibrahem, Mohamed and Ibrahim, 2013)

Arthrospira sp.	Parrot fish (Oplegnathusfasciatus)	5% replacement of fish meal protein	Significantly higher weight gain, protein efficiency ratios, feed intake, and lower feed conversion ratios than the fishmeal control	(Kim *et al.*, 2013)
Navicula sp. and *Nannochloropsis salina*	Juvenile Red drum (*Sciaenops ocellatus*)	Replacement of up to 10% of fishmeal	No any adverse effect found on the growth performance.	(Patterson and Gatlin, 2013)
Spirulina sp.	Rainbow trout(*Oncorhynchus mykiss*)	7.5% replacement of fishmeal	Highest weight gain observed	(Teimouri, Amirkolaie and Yeganeh, 2013)
Dunaliella sp.	Shrimp (*L. vannamei*)	1–2%inclusion of microalgal meal as carotenoid supplement	Survival rate of shrimp increased	(Medina-Félix *et al.*, 2014)
Nannochloropsis sp. And Schizochytrium sp.	Olive flounder (*Paralichthys olivaceus*)	100% replacement of fish oil	No negative effects on growth, feed efficiency or nutritive quality	(Qiao *et al.*, 2014)
Scenedesmus almeriensis	Gilthead sea bream (*S. aurata*)	38% replacement of fishmeal	The inclusion of algae meal did not affect the feed intake	(Vizcaíno *et al.*, 2014)
Schizochytrium sp.	Atlantic salmon (*S. salar*)	Up to 5% replacement of fish oil	No signs of toxicity, stress, inflammation, or any other negative effects of supplementation in diets; fillet quality good.	(Kousoulaki *et al.*, 2015)
Isochrysis sp.	European sea bass (*D. labrax L.*)	Up to 36% replacement of fish oil	No any adverse effect found on the growth performance.	(Tibaldi *et al.*, 2015)

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CHAPTER 3

Sampling, isolation and selection of potential CO_2 tolerant microalgal strains

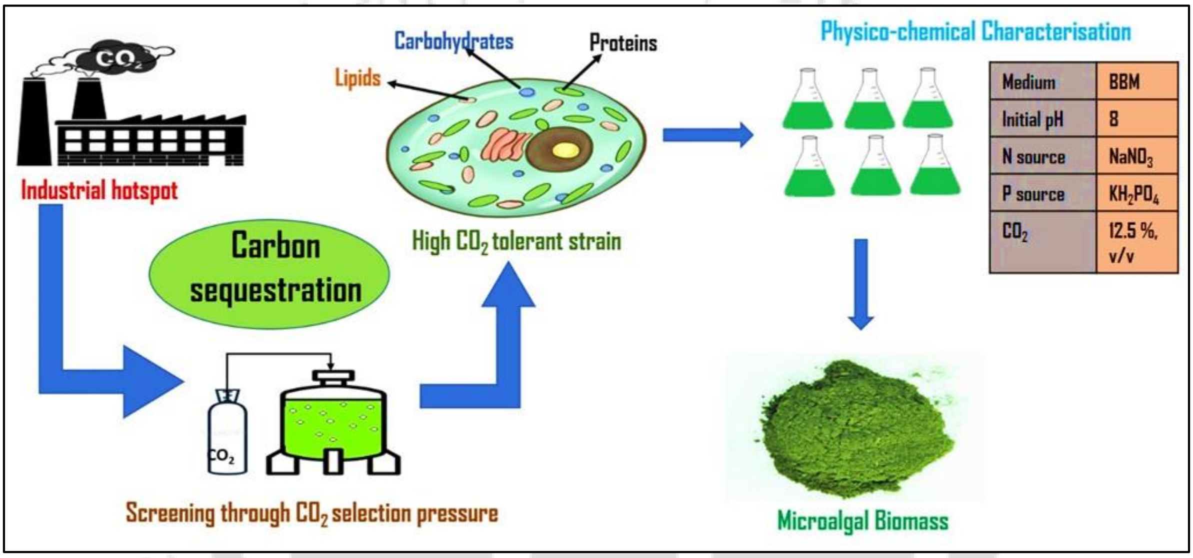

Fig 3.1 Sampling, isolation and screening of potential CO_2 microalgal strain followed by their characterization under different physiochemical conditions.

3.1 Background and motivation

Combustion of fossil fuels and fast industrialization have led to a substantial rise in CO_2 emissions into the atmosphere, propelling us towards serious climate change. Recent research indicates that roughly 33.4 giga tonnes of CO_2 are released every year as a result of human activities (Cheng *et al.*, 2019). Current efforts to reduce atmospheric CO_2 concentration involve technologies in three major categories, including pre-combustion, in which fuel is processed with steam and air to produce synthesis gas primarily consisting of CO and H_2; post-combustion, in which CO_2 is removed from flue gas using solvents, adsorbents, or membrane tubes; and oxyfuel combustion, in which the burning of fuel in the presence of pure oxygen reduces the CO_2 concentration in flue gas (Songolzadeh *et al.*, 2014; Wang and Song, 2020). However, the existing state-of-the-art methods for CO_2 sequestration suffer from a variety of drawbacks, such as the necessity of a greater amount of energy, sub-optimal performance of the system owing to the presence of pollutants (SO_x and NO_x) in the flue gas, limitation in the magnitude of scale-up, requirement of several processing stages, poorer CO_2 selectivity, high maintenance and operating cost, and hazardous waste stream (Osman *et al.*, 2020). Sequestration of CO_2 using microbes as biocatalysts is gaining prominence as an alternative to present technologies due to its eco-friendly character and economic viability (Das *et al.*, 2020). In the presence of sunlight and inorganic energy sources, photoautotrophs such as microalgae or cyanobacteria and chemoautotrophs such as acetogens, crenarchaeota and betaproteobacteria use CO_2 as their carbon source (Hu *et al.*, 2019; Zeng, Alain and Shao, 2021). However, among the various biological routes, microalgae-based CO_2 sequestration is gaining greater attention from the scientific community and industry due to its numerous advantages in terms of growth rate, photosynthetic efficiency (1,550 times that of terrestrial plants), biomass productivity, tolerance to high CO_2 concentration and extreme environmental stress, CO_2 sequestration capability, and multi-product paradigm (Yadav, Dubey and Sen, 2020; Zhao and Su, 2020). In addition, the GRAS (generally recognised as safe) status of microalgae, as granted by the United States Food and Drug Administration (FDA), has made it an industrially relevant production platform because it eliminates the need for costly downstream processing to refine high-value compounds (Marles *et al.*, 2011). The most important factor that must be satisfied for a microalgae-based CO_2 sequestration method to be effective is the presence of a robust strain. This strain must be able to resist any adverse effects that environmental changes may have on its growth rate. Not only should the strain have a high CO_2 tolerance, but it should also have the capacity to thrive when exposed to a high CO_2

concentration. Native strains are those that originate naturally in a particular environment and exhibit useful characteristics such as the capacity to tolerate and grow under higher CO_2 concentrations, fluctuating outdoor conditions such as seasonal and diurnal variations in temperature and light intensity, and the ability to offer products that are useful in the commercial sector.

This research seeks to build a cleaner production method for microalgae as an effective CO_2 biological sequestration technique. To this objective, a unique indigenous freshwater microalgal strain *Desmodesmus pannonicus* CT01 (henceforth CT01) that could tolerate and sustain much higher CO_2 concentrations of up to 25% v/v was isolated. The growth performance of the strain, CO_2 sequestration capacity, and amount of removal of macronutrients such as phosphate and nitrate of the strain were examined by growing it under a broad range of CO_2 concentrations ranging from 5% to 25%, v/v.

3.2 Materials and methods

3.2.1 Sampling, screening and isolation of potential CO_2 tolerant microalgal strain

Aqueous sample was collected from industrial effluent of Vananchal Steel Plant (22°54'35.7"N 86°02'46.2"E), located in Jharkhand, India and further used as inoculum for microalgal isolation in BG11 medium. The enrichment was performed in mini bubble column photobioreactor (Spectrochem Instruments Pvt. Ltd., India) of working capacity of 300 mL inoculated with 30 mL of collected aqueous sample. A novel strategy of CO_2 selection pressure-based screening was employed to screen high CO_2 tolerant microalgal strains where, the mixed culture was subjected to sequential increment of CO_2 concentrations in the inlet air stream from 5 to 25%, v/v, with step-wise increase by 2.5%. The reactor was provided with light intensity of 100 µE m^{-2} s^{-1} and light-dark cycle of 16:8 h at room temperature. The nitrate and phosphate concentration in the broth was maintained at 50% of their initial value via intermittent feeding to avoid possibility of nutritional stress (Fig 3.2 and 3.3). The profiles of nutrient utilization and pH were obtained via periodic sampling at the end of every light cycle. At the end of each sequential batch with specific CO_2 concentration, the screened culture was subjected to serial dilution and plating to isolate axenic colonies. Axenicity of the cultures was screened by growing in soya bean casein digest broth for 3 days at 37°C to check the bacterial contaminants and for 5 days at 28°C to check the presence of fungal contaminants. The isolated axenic algal strains were stored as glycerol stock at -80°C and as slants.

3.2.2 Identification of the isolated strain

The strain with the ability to tolerate maximum CO_2 concentration of 25 %, v/v was considered for identification and further characterization. Morphometric analysis of the strain was carried out under phase contrast microscope (Eclipse E200, Nikon, Japan) and Field-Effect scanning electron microscope (FESEM, Carl Zeiss SIGMA VP, Germany). Molecular level identification of the isolate was carried out via 18S rDNA sequencing. For molecular analysis, the cells were disrupted and the genomic DNA of the strain was extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The 18S rDNA sequence was further amplified by using special forward primer 5′GGTGATCCTGCCAGTAGTCATATGCTTG-3′ (ss5) and reverse primer 5′-GATCCTTCCGCAGGTTCACCTACGGAAACC-3′ (ss3) in a thermal cycler. Amplified PCR products were separated by gel electrophoresis and a gel elution kit (Sigma-Aldrich, St. Louis, MO, USA). Sequencing was performed using ABI PRISM 3700 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at Qube Biosciences Pvt Ltd, India and the similarity to sequences was determined using BLAST. A phylogenetic tree was constructed from the 18S rDNA sequences of the isolated strain and related species using the software ClustalX 2.1 and MEGA 5.0.

3.2.3 Characterization of the novel microalgal isolate under different physiochemical parameters

The effect of different medium composition, initial pH of the medium, nitrogen and phosphate sources was characterized for the novel microalgal strain. The strain was subjected to five different media (Table 3.1) and growth performance was evaluated in terms of maximum biomass titre. Further, growth performance of the strain was evaluated under different initial pH of the medium (4,6,8,10 and 12), different nitrogen sources (commercial grade urea (Urea (C), sodium nitrate (NaNO3), ammonium sulphate (NH4SO4), sodium nitrite (NaNO2), and analytical grade urea (Urea (L)) with equimolar nitrogen (0.11M) and different phosphate sources (monopotassium phosphate (KH2PO4), dipotassium phosphate (K2HPO4), and single super phosphate (SSP)) having equimolar phosphate concentration of 0.0015 M. The experiments were performed in 250 mL conical flasks with working volume of 100 mL. The flasks were kept in shaker incubator (Orbitek, Scigenics Biotech, India) at 28°C temperature with agitation of 150 rpm under light intensity of 100 μE $m^{-2}s^{-1}$ with a light: dark cycle of 16:8 h. 10 %, v/v of seed culture with absorbance of approximately 1 was used as inoculum for all the experiments. Samples were collected at the end of every light cycle for estimation of growth.

3.2.4 Growth of the organism and CO_2 sequestration under varied CO_2 concentration in the inlet gas stream

In order to understand the influence of CO_2 concentration in the inlet gas steam on growth and CO_2 sequestration ability of the organism, experiments were performed under six different CO_2 concentration such as 0.03 (air), 5, 7.5, 10, 12.5, 15 and 20%, v/v. The experiments were

Medium	**Suitable for Algal Family**	**Compositions (g L^{-1})***
Watanabe (AF6)	Euglenophyceae, Volvocalean algae, Xanthophytes, many Cryptophytes, Dinoflagellate and green ciliates; specific for algae requiring slightly acidic medium	$NaNO_3$ 0.14, NH_4NO_3 0.022, $MgSO_4$ 0.03, KH_2PO_4 0.01, K_2HPO_4 0.005, $CaCl_2$. 4H2O 0.01, ammonium ferric citrate 0.002, citric acid 0.002, biotin 0.002, thiamine 10 µg, vitamin B6 1 µg, vitamin B12 1 µg, Na_2-EDTA 0.005, $FeCl_3$ 0.098, $MnCl_2·4H_2O$ 0.18, $ZnCl_2·4H_2O$ 57 µg, $Na_2MoO_4·2H_2O$ 12.5 µg
Beijerincki (BJA)	Chlorophyceae	NH_4NO_3 0.15, K_2HPO_4 0.02, $MgSO_4·7H_2O$ 0.02, $CaCl_2·2H_2O$ 0.01, KH_2PO_4 0.363, K_2HPO_4 0.69, H_3BO_3 0.01, $MnCl_2·4H_2O$ 0.005, EDTA 0.05, $CuSO_4·5H_2O$ 0.0015, $ZnSO_4·H_2O$ 0.022, $CoCl_2·6H_2O$ 0.0015, $FeSO_4·7H_2O$ 0.005, $(NH_4)_6Mo_7O_{24}·4H_2O$ 0.001
BG 11	Cyanophyceae	$NaNO_3$ 1.5, $K_2HPO_4·3H_2O$ 0.04, $MgSO_4·7H_2O$ 0.075, $CaCl_2·2H_2O$ 0.036, Na_2CO_3 0.02, citric acid 0.006, ferric ammonium citrate 0.006, EDTA 0.001, and A5 + Co solution (1 mL L^{-1}) that consists of H_3BO_3 2.86, $MnCl_2·H_2O$ 1.81, $ZnSO_4·7H_2O$ 0.222, $CuSO_4·5H_2O$ 0.079, $Na_2MoO_4·2H_2O$ 0.39, and $Co(NO_3)_2·6H_2O$ 0.049
Bold Basal (BBM)	Broad spectrum medium for Chlorophyceae, Xantophyceae, Chrysophyceae and Cyanophyceae; unsuitable for algae with vitamin requirements	KH_2PO_4 0.175, $CaCl_2·2H_2O$ 0.025, $MgSO_4·7H_2O$ 0.075, $NaNO_3$ 0.25, K_2HPO_4 0.075, NaCl 0.025, H_3BO_3 0.011, $ZnSO_4·7H_2O$ 0.00882, $MnCl_2·4H_2O$ 0.00144, MoO_3 0.00071, $CuSO_4·5H_2O$ 0.00157, $Co(NO_3)_2·6H_2O$ 0.00049, Na_2EDTA 0.05, KOH 0.0031, $FeSO_4$ 0.005, H_2SO_4 1 µL
Algae Culture Broth (ACB)	Commercial medium obtained from Himedia Pvt. Ltd., India	$NaNO_3$ 1, $MgSO_4.7H_2O$ 0.513, K_2HPO_4 0.25, NH_4Cl 0.050, $CaCl_2.2H_2O$ 0.058, $FeCl_3$ 0.003

Table 3.1 Common growth media used for isolation of microalgal strains from freshwater habitats

performed in bubble column photobioreactor (Spectrochem Instruments Pvt. Ltd., India) of 500 mL with working volume of 400 mL. The organism was grown under constant light intensity of 100 µE m^{-2} s^{-1} and light:dark cycle of 16:8 h, with inoculum concentration of 0.1 g L^{-1}. Sampling was performed at a regular interval of 24 h to obtain dynamic profile of growth. The biomass productivity P (mg L^{-1} day^{-1}) and maximum specific growth rate μ_{max} (day^{-1}) was estimated as shown in Eq. (3.1) and Eq. (3.2), respectively. The carbon dioxide bio-fixation rate, R_{CO2} (mg L^{-1} day^{-1}) was estimated as described in Eq. (3.3) (de Morais and Costa, 2007).

$$P = \frac{(X_f - X_i)}{(t_f - t_i)} \qquad \text{(Eq 3.1)}$$

X_f and X_i represents the biomass concentration (g L^{-1}) at the final (t_f) and initial (t_i) days of the culture, respectively.

$$\mu_{max} = \frac{Log_e(X_1\,) - Log_e(X_2)}{T_2 - T_1} \qquad \text{(Eq 3.2)}$$

X_1 and X_2 are the concentration of cells at time point t_1 and t_2 , respectively.

$$R_{CO_2} = C_c \times \; P \times (\frac{M_{CO_2}}{M_c}) \qquad \text{(Eq 3.3)}$$

M_{CO2} and M_C are the molar mass of CO_2 and C (g mol^{-1}), respectively. C_c is the carbon content of microalgae cells at the end of batch (%, w/w) were estimated through a EuroEA elememtal analyzer.

3.2.5 Analysis of growth and substrate utilization of CT01

For monitoring the growth of the organism, absorbance of the culture was measured at 690 nm (A_{690}) using UV-Vis spectrophotometer (Cary 100, Agilent Technologies, USA). The absorbance values were converted into dry cell weight (DCW) using the correlation, one cell density = 0.202 g dry cells L^{-1} ($R^2 = 0.99$). Cell free supernatant obtained from centrifugation of the sample at 10000 rpm for 10 min was analysed for substrate utilization. Estimation of nitrate was carried out using salicylic acid method (Cataldo *et al.*, 2008) using sodium nitrate as the standard and phosphate was quantified using ascorbic acid method (Cataldo *et al.*, 2008) using dipotassium phosphate as the standard.

The phosphate utilization rate (PUR, mg L^{-1} d^{-1}) and nitrate utilization rate (NUR, mg L^{-1} d^{-1}) was calculated using the below equation:

$$\mathrm{PUR} = \frac{\mathrm{C_{pi}} - \mathrm{C_{pf}}}{\mathrm{t_b}} \qquad \text{(Eq 3.4)}$$

$$\mathrm{NUR} = \frac{\mathrm{C_{ni}} - \mathrm{C_{nf}}}{\mathrm{t_b}} \qquad \text{(Eq 3.5)}$$

Where $\mathrm{C_{pi}}$, $\mathrm{C_{ni}}$, $\mathrm{C_{pf}}$ & $\mathrm{C_{nf}}$ are intial and final concentrations (mg L^{-1}) of phosphate and nitrate in the medium respectively and t_b is duration of batch in days.

3.2.6 Statistical analysis

All experiments were determined in biological triplicate to ensure the reproducibility. Results were expressed as mean value ± standard deviation. All data are presented as means of standard error.

3.3 Results and Discussion

3.3.1 Screening, isolation and identification of CO_2 tolerant microalgal strain

The ability of microalgae to adapt and survive harsh environmental stress, makes them potential candidates to explore their role towards improvement in ecology of surroundings industrial areas. CO_2, a potent greenhouse gas has been used as the primary source of carbon for photoautotrophic growth of microalgae (Chen, Xu and Vaidyanathan, 2020). Henceforth, screening, isolation and identification of robust and CO_2 tolerant strain becomes the first essential step towards development of a large-scale sustainable process. In the current study, a rapid screening and isolation strategy was adopted which was based on CO_2 pressure-based selection of the CO_2 tolerant microalga strain. Sample from the reservoir of high carbon emitting industry was collected and exposed to sequentially elevated CO_2 concentration of 5 %- 25%, v/v, with a phase wise increment of 2.5 %. At the onset of process, in 5 %, v/v CO_2 in the air stream, the presence of four morphologically distinct microalgae strains were observed, in the sample containing various different group of organisms (Table 3.2). Growth of these four strains were monitored continuously by differential cell count, during the entire process of step-wise increment of CO_2 concentration. Amongst these four, only one strain not only could tolerate, but also exhibited substantial growth under highest inlet CO_2 concentration of 25%. The growth of the microalgae strains was observed to be correlated with the utilization of nitrate and phosphate during the entire period of screening. The culture pH was self-maintained within the range of 7-8, depicting the ability of the strain to utilize and grow even at higher concentration of CO_2 (Fig 3.2). The microalga strain, survived at highest concentration of CO_2 was considered for streak plate method to obtain axenic culture in BG11 agar plate. The identification of the strain was carried out at molecular level through 18s rDNA sequencing. Based on BLAST study, the novel strain was found to be the closest with *Desmodesmus pannonicus* GM4n with sequence similarity of 97%. A phylogenetic tree was prepared to understand the evolutionary position of the novel strain and similarities between the species (Fig 3.4). The partial 18s rDNA sequence coding for the ribosomal RNA of the strain was sequenced and further submitted to GenBank (Accesion Number: OL470985). The CO_2 tolerant microalga strain was further named as *Desmodesmus pannoniccus* CT01.

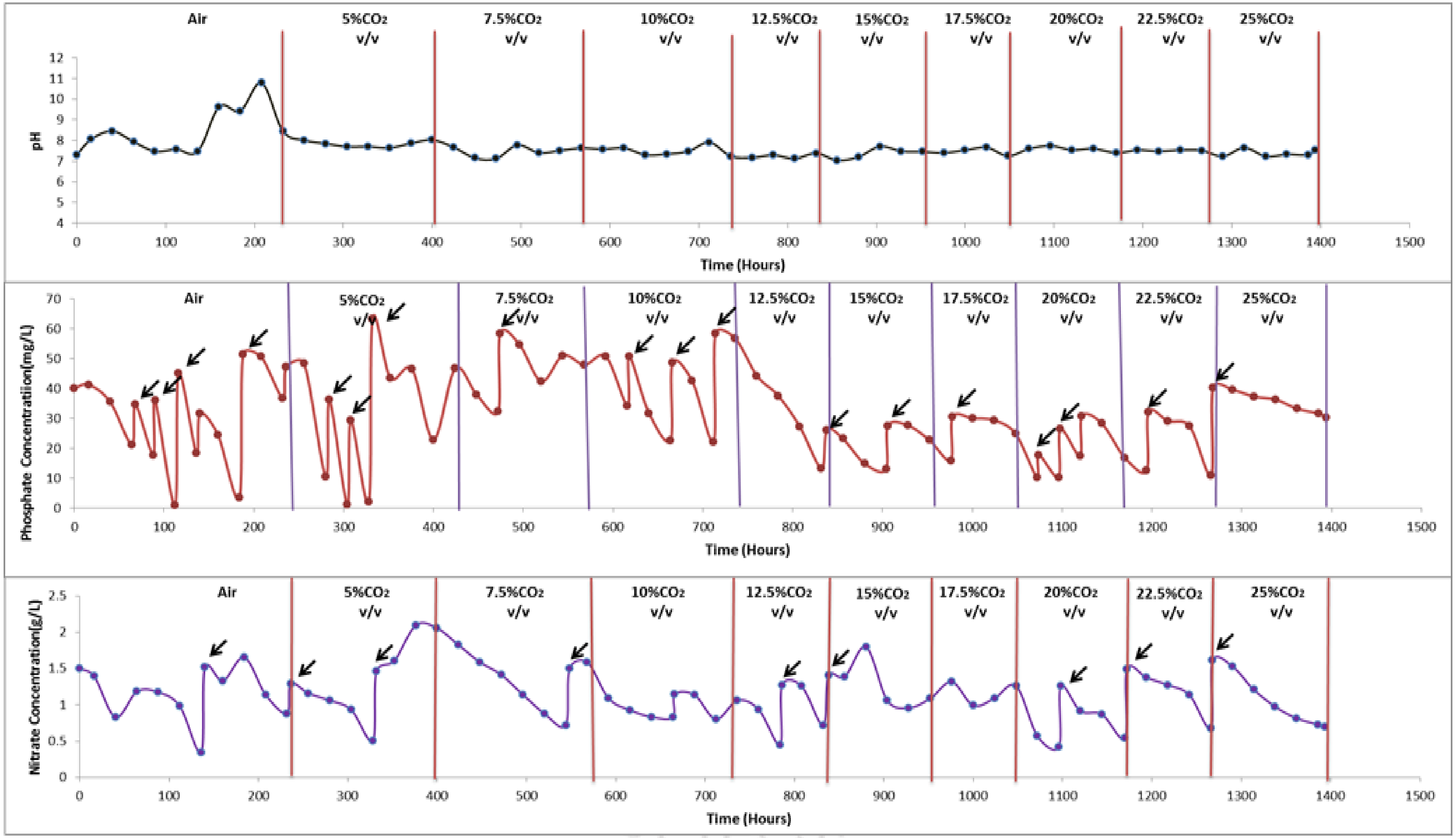

Fig 3.2 Dynamic profiles of (A) pH, (B) Phosphate concentration, (C) Nitrate Concentration for screening of aqueous sample under different CO$_2$ concentration in photoautotrophic growth condition. Arrows indicate intermittent feeding of nutrient in the reactor.

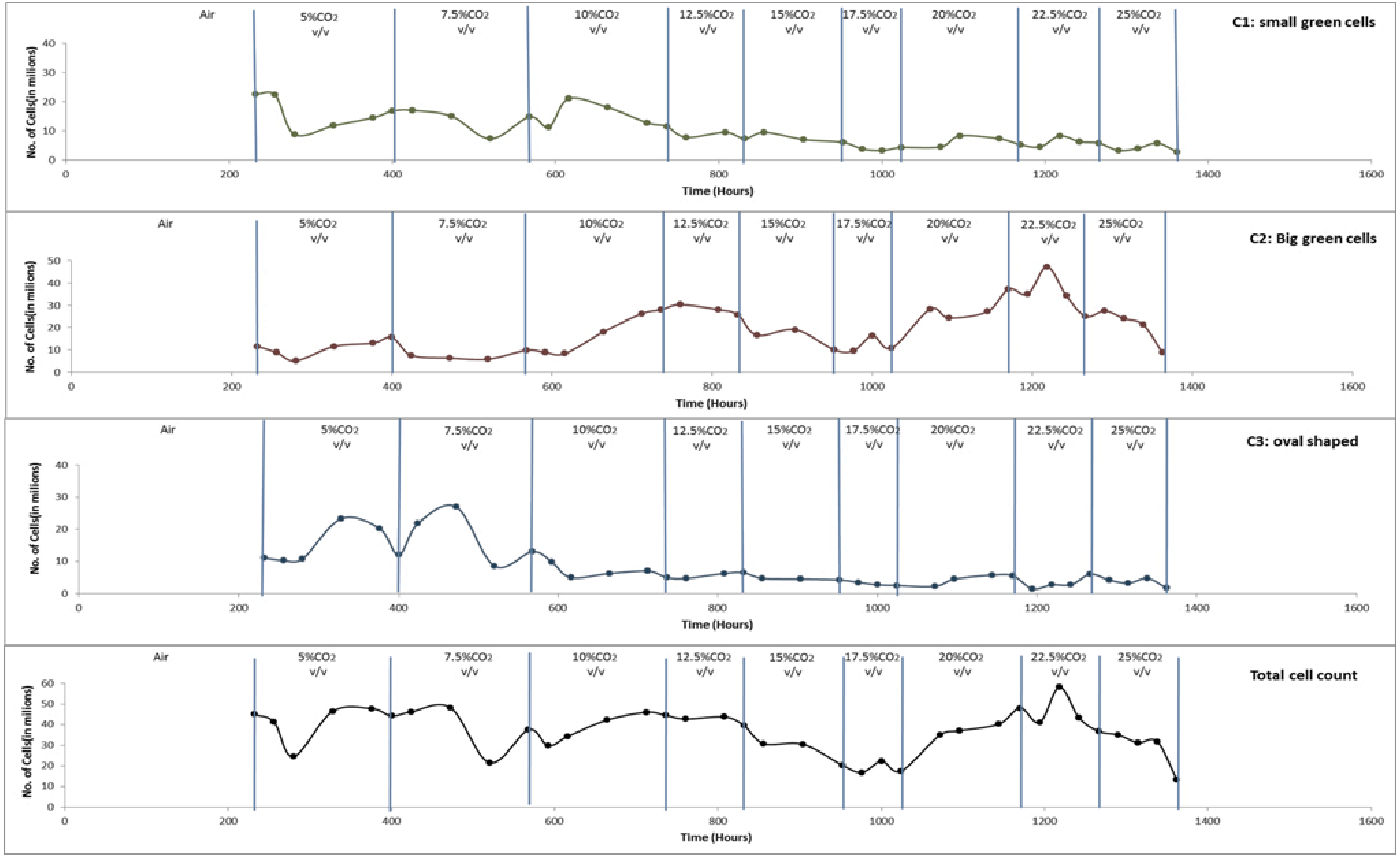

Fig 3.3 Differential and total algal cell count profile during screening of aqueous sample under different CO_2 concentration.

Table 3.2 Comparison of microscopic observation of different types of microalgae during selection of CO_2 tolerant strain form S10.

CO_2 selection pressure (% v/v)	0	5	7.5	10	12.5	15	17.5	20	22.5	25
Microalgae present in aqueous sample	1.big round 2.small round 3.oval clump 4.sharp oval 5.dot shaped 6. small oval	1.big round 2.small round 3.oval clump 4.sharp oval 5.dot shaped	1.big round 2.small round 3.oval clump 4.sharp oval	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump	1.big round 2.small round 3.oval clump

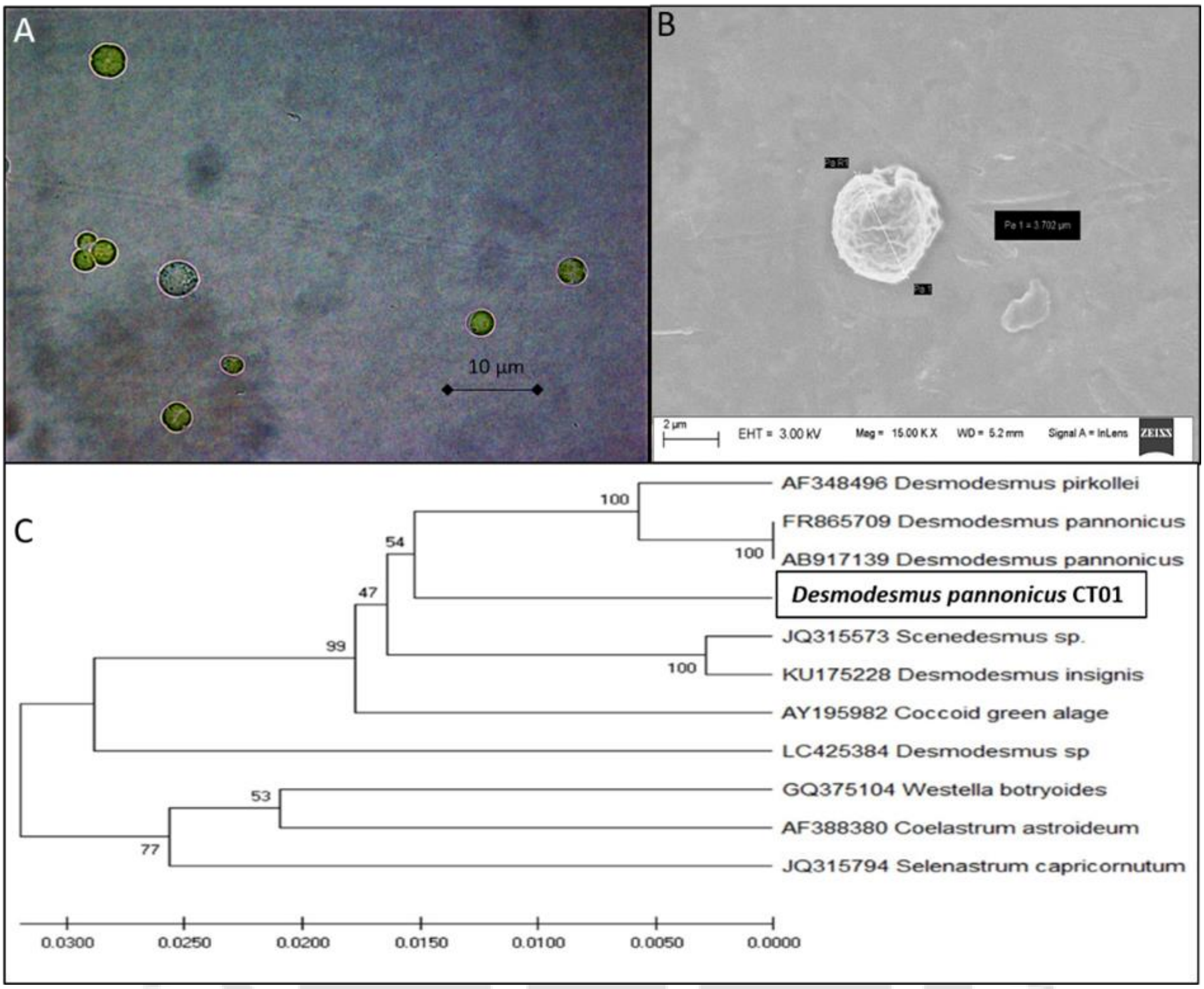

Fig 3.4 Morphological study of isolated microalgal strain under (A) bright field microscopy and (B) field emission scanning electron microscopy (FESEM). The molecular level identification of the strain was carried out based on phylogenetic tree generated using MEGA X (C). Neighbor-joining showing phylogenetic position of isolate and related taxa is based on partial 18s rRNA gene sequence comparisons. Bootstrap values are indicated at nodes. Representative sequences in the dendrogram were obtained from GenBank

3.3.2 Biochemical characterization of CT01

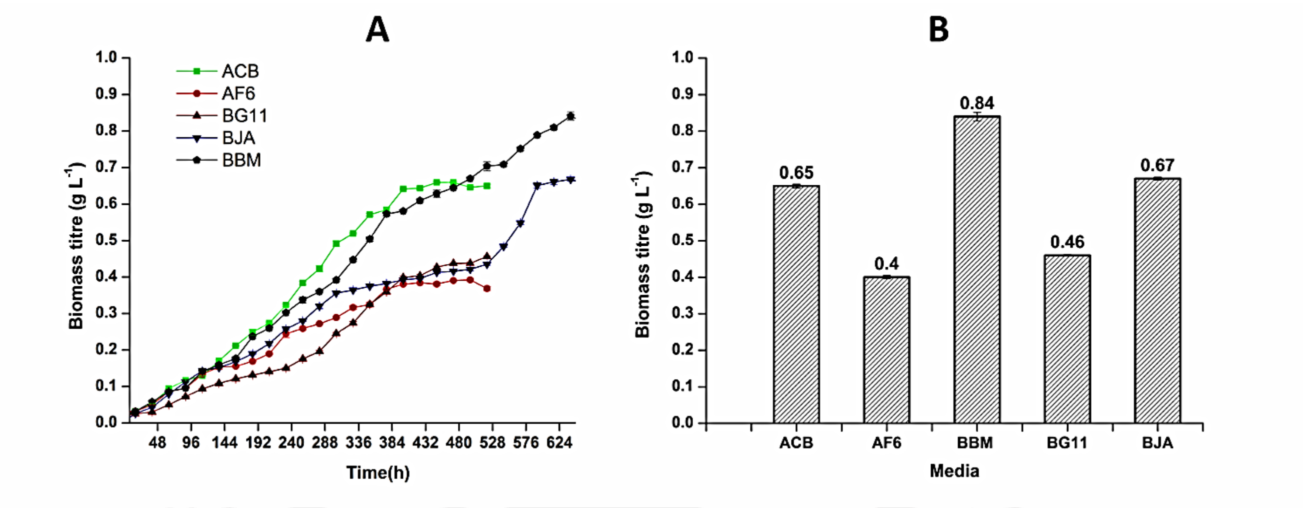

Fig 3.5 Dynamic profiles (A) biomass titre (B) of batches run on different medium for microlagae *Desmodesmus pannonicus* CT01

Being a new isolate, understanding the requirement of suitable nutritional and growth condition of the organism, biochemical characterization was performed under different pre-established medium composition, suitable pH range, nitrogen sources and phosphate sources of selected medium. While CT01 was able to grow in all medium compositions, the highest growth of 0.84 g L^{-1} was observed in BBM (Fig 3.5). This result can be correlated with previous reports where BBM was found suitable for growth of *Desmodesmus* or *Tetradesmus* species (Machegowda *et al.*, 2018; Rathnayake *et al.*, 2021).

Initial medium pH of 8 was observed to support optimal growth with highest biomass titre of 0.87 g L^{-1}. The growth of the organism was compromised when initial pH of the medium turned either acidic

(4-6) or alkaline (10-12) (Fig 3.6). Initial pH of the medium in the range of 7-8 was reported to support growth of the *Scenedesmus* species favorably, while alkaline or acidic pH induce formation of secondary metabolite (Xu, Shen and Chen, 2015). Carbon, nitrogen and phosphorus are the three essential nutrients for microorganism's biomass growth. Apart from carbon which can be obtained from atmospheric air or CO_2 sparging, microalgae assimilate sufficient nitrogen and phosphorus from medium for their metabolic activities. CT01 performs well in the presence of sodium nitrate with the maximum biomass concentration of 0.83 g L^{-1} as compared to other nitrogen sources (Fig 3.7). Earlier studies also identified sodium nitrate as favored nitrogen source for improved growth of microalgae (Arumugam *et al.*, 2013).

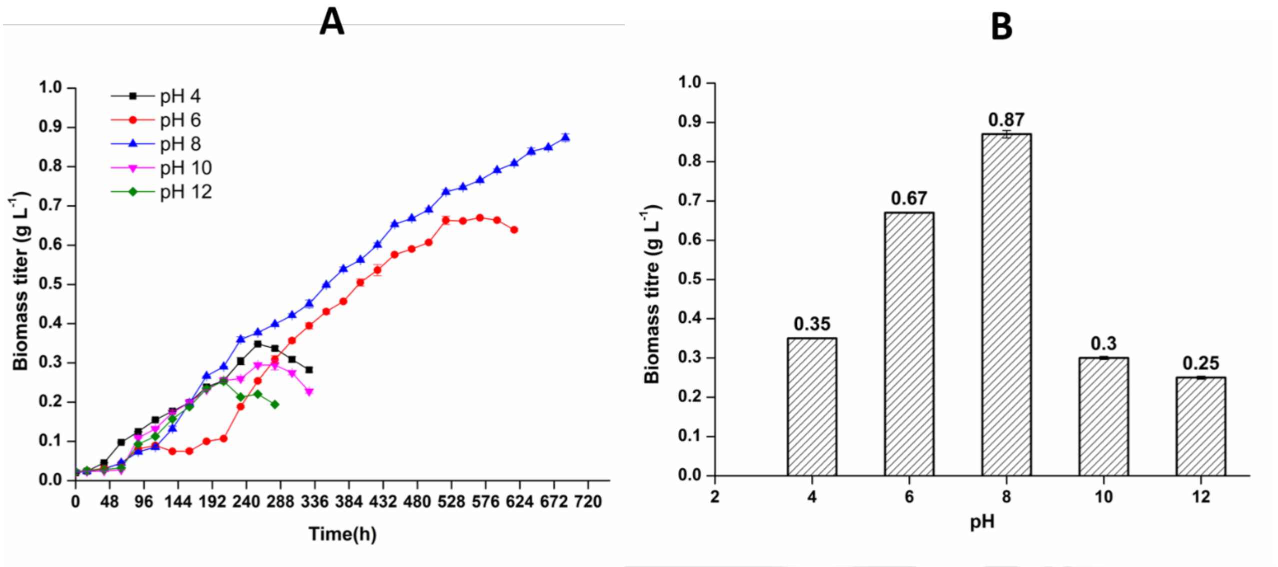

Fig 3.6 Dynamic profiles (A) biomass titre (B) of batches run on different initial pH for microlagae *Desmodesmus pannonicus* CT01

However, poor growth in presence of ammonium sulphate and urea may be attributed to the release of H^+ ions during ammonia uptake by the organism (Syrett and Biol 2016), which, in turn change the culture medium from neutral to acidic.

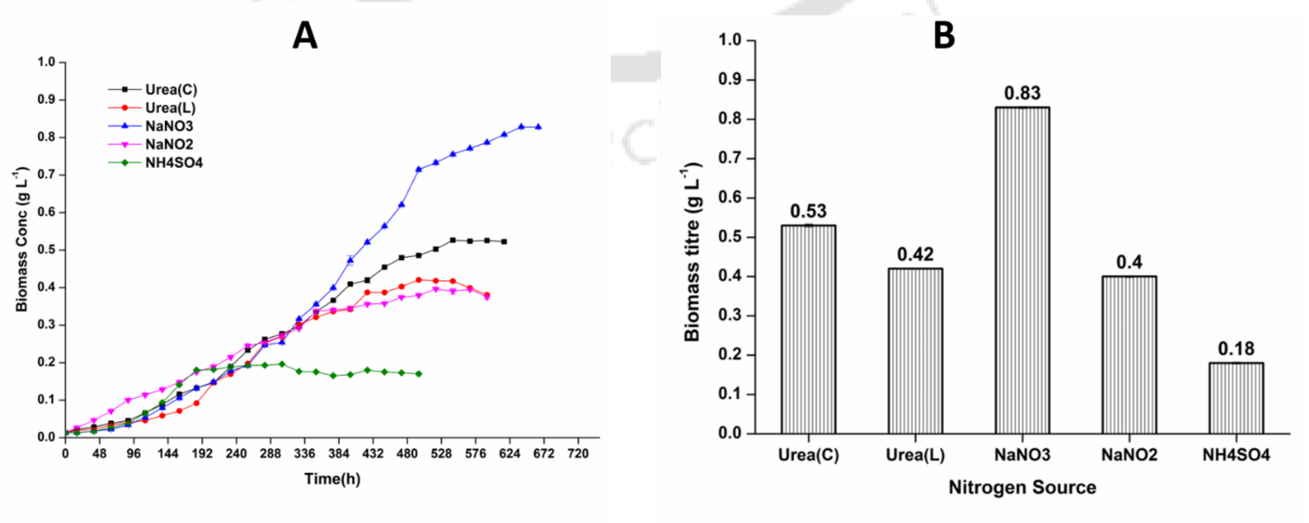

Fig 3.7 Dynamic profiles (A) biomass titre (B) of batches run on different nitrogen sources for microlagae *Desmodesmus pannonicus* CT01

Amongst three different sources, potassium dihydrogen phosphate satisfies the requirement of the phosphate more favorably towards photosynthesis, formation of DNA, ATP & cell membrane and other metabolic activities, supporting growth of the organism (Fig 3.8). Therefore, maximum biomass titre of 0.87 g L-1 was achieved at shake flask using Basal Bold medium containing sodium nitrate as nitrogen source, potassium dihydrogen phosphate as phosphate source and initial pH of 8.

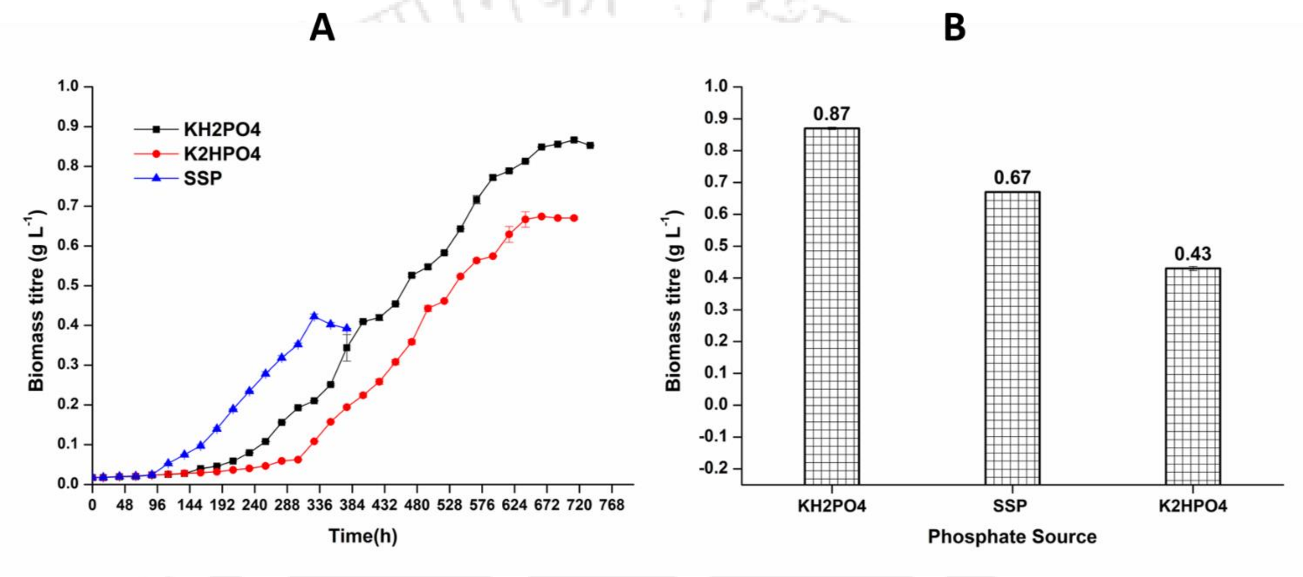

Fig 3.8 Dynamic profiles (A) biomass titre (B) of batches run on different phosphate sources for microlagae *Desmodesmus pannonicus* CT01

3.3.3 Growth linked carbon sequestration by CT01 under different concentration of CO_2

Owing to their ability to sequester CO_2, any bioprocess which involve the photoautotrophic growth of microalgae, is considered to be green process. CO_2, the major greenhouse gases, is predominantly present in the emissions of transportation sector and electricity generation with an average concentration of 16-36 % (2019) (Olivier, 2020). In order to develop a sustainable and cleaner process, CO_2 present in the emissions of various industries (flue gas or off gas) need to be coupled with the commercial scale cultivation of microalgae. Therefore, it is important to evaluate the growth performance of the selected organism under wide range of CO_2 concentration targeting the typical industrial emissions. The effect of different CO_2 concentrations on the growth kinetics of CT01 is shown in Fig 3.9.

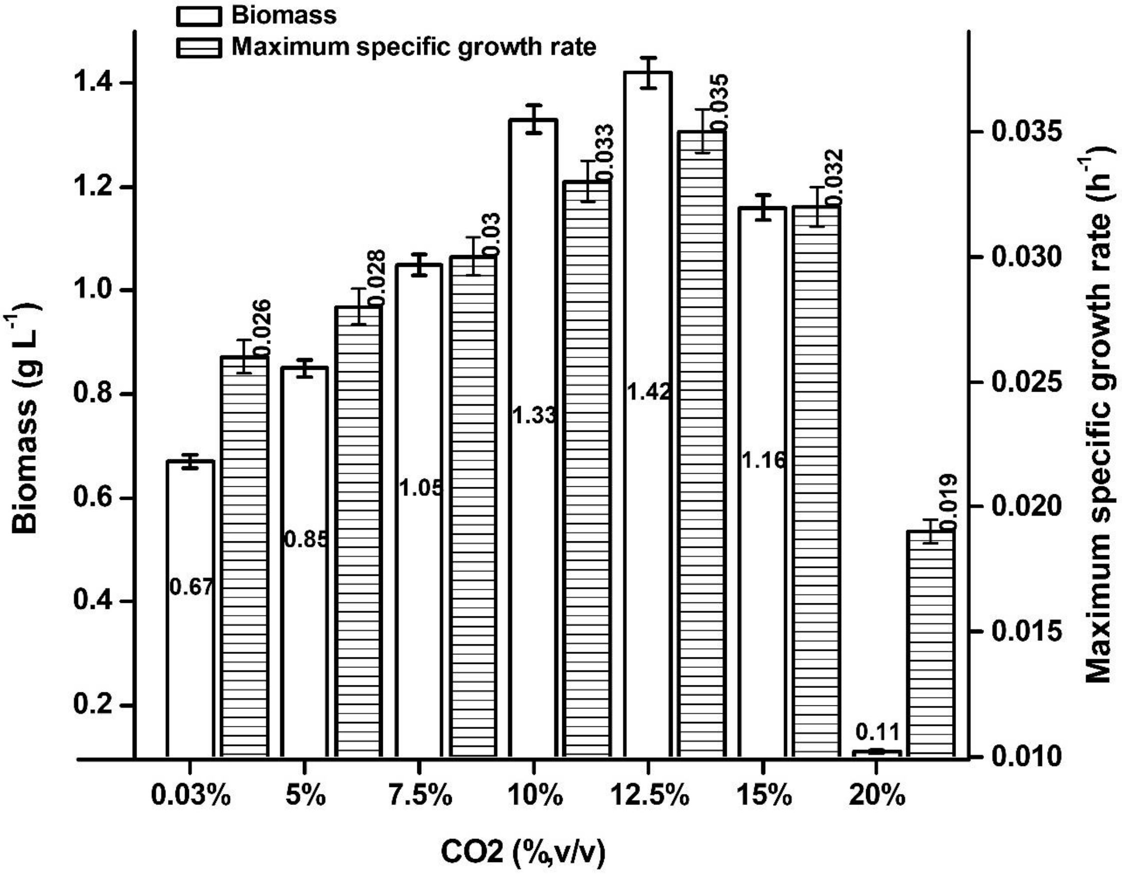

Fig 3. 9 Profiles of biomass titre and maximum specific growth rate of *Desmodesmus pannonicus* CT01 under different concentration of carbon dioxide, v/v.

The organism exhibited substantial growth even with the wide variation in inlet CO_2 concentration from 0.03% (air) to 20%, v/v. The biomass titre, maximum specific growth rate and biomass productivity was found to increase linearly with the increase in CO_2 concentration from 0.03% and attain the maximum value of 1.42 g L^{-1}, 0.035 h^{-1} and 101.43 mg L^{-1} d^{-1}, respectively at 12.5%, Further increase in CO_2 concentration beyond 12.5% resulted in decline in biomass titre, growth rate and productivity. It is important to note that the organism exhibited better growth performance when subjected to CO_2 concentration of 10 -15%, v/v, suggesting its possible application for large scale cultivation utilizing industrial flue gas. The growth performance of CT01, specifically at higher CO_2 concentration was found to be promising as oppose to many microalgae strains reported in the literature. For instance, *Chlorella* sp. and *N. oculata* showed optimal growth at lower CO_2 concentration of 2% and the growth was completely inhibited when the strains were subjected to CO_2 concentration more than 5% (Chiu *et al.*, 2008, 2009). The species related to CT01 such as *Scenedesmus* sp. exhibited similar growth pattern when subjected to varied CO_2 concentration in the range of 0.03 % to 50 %, with maximum biomass titre of 1.84 g L^{-1} at 10 % of CO_2 (Tang *et al.*, 2011). As the CO_2 sequestration ability of the microalgae has a liner

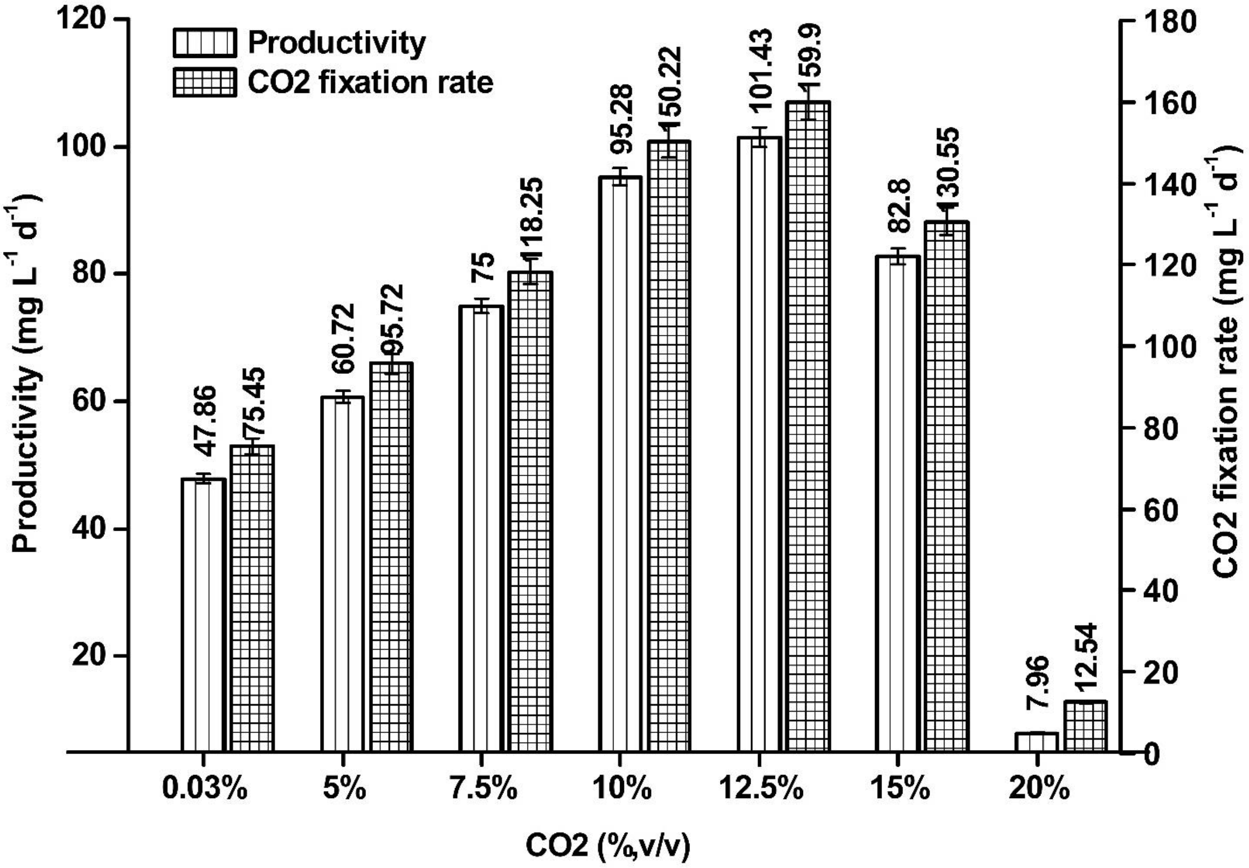

Fig 3.10 Profiles of productivity and carbon fixation rate of *Desmodesmus pannonicus* CT01 under different concentration of carbon dioxide, v/v.

correlation with the growth kinetics, the highest CO_2 fixation rate of 159.91 mg L^{-1} d^{-1} was found at CO_2 concentration of 12.5% (Fig 3.10). Similar to the growth pattern, reasonably higher CO_2 fixation rate in the range of 130.55 -159.91 mg L^{-1} d^{-1} was achieved at higher CO_2 concentration of 10-15% in the inlet gas stream. The reduced growth and lower CO_2 fixation rate at even higher CO_2 concentration of 20% may be attributed to induction of acidic pH of the culture medium and in turn, inhibition of carbonic anhydrase activity, the key enzyme of CO_2 sequestration. Many microalgae strains are reported to exhibit better CO_2 fixation efficiency in the range of 236 – 436 mg L^{-1} d^{-1} as compared to CT01 (Sydney *et al.*, 2010).

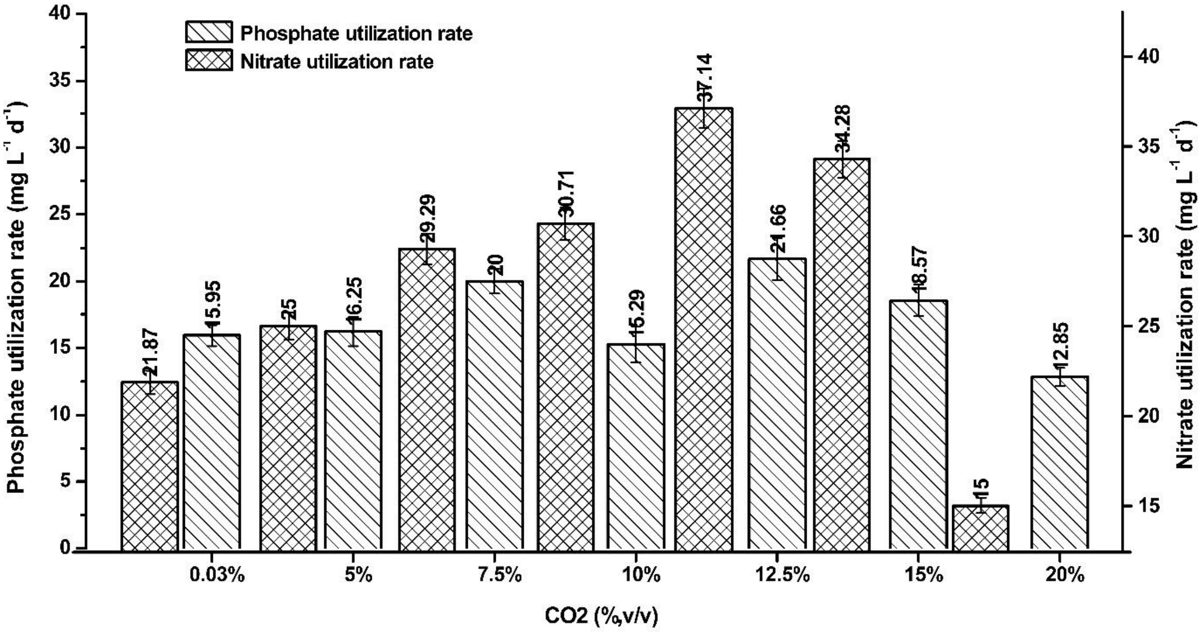

Fig 3.11 Profiles of nitrate and phosphate utilization rate of *Desmodesmus pannonicus* CT01 under different concentration of carbon dioxide, v/v.

anhydrase activity, the key enzyme of CO_2 sequestration. Many microalgae strains are reported to exhibit better CO_2 fixation efficiency in the range of 236 – 436 mg L^{-1} d^{-1} as compared to CT01 (Sydney *et al.*, 2010). It is important to note that, higher CO_2 fixation efficiency by these strains was achieved at 5% CO_2, lower than the average CO_2 concentration of industrial flue gas. Similar to other microbial biomass, the major elemental constituents of microalgae are carbon (C), nitrogen (N), oxygen (O), hydrogen (H) and phosphorous (P). Hence, similar to

CO_2 fixation, utilization of $NaNO_3$ and KH_2PO_4 or K_2HPO_4 as the sole source of nitrogen and phosphorous, respectively was linked to the growth of the organism. To that end, utilization rate of nitrate and phosphate was found to be correlated with the linear increase in the growth of CT01 with the increase in CO_2 concentration and attain a maximum value of 37.14 mg L^{-1} d^{-1} and 21.66 mg L^{-1} d^{-1}, respectively at 12.5% CO_2 (Fig 3.11).

3.4 Conclusions

A potential high CO_2 tolerant microlagal strain was isolated using the novel approach of CO_2 pressure-based screening process. The strain was identified as *Desmodesmus panoniccus* and therefore designated as *Desomdesmus pannonicus* CT01. Further characterization under different physiochemical conditions revealed the robustness of the strain in terms of growth under wide range of pH and nitrate and phosphate source. The generation of biomass as feedstock will offer cleaner production technology in terms of CO_2 sequestration even at higher CO_2 concentration of 10-15%, v/v, opening an avenue for its possible large-scale application utilizing industrial flue gas.

3.5 References

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CHAPTER 4

Evaluation of novel microalgae species performance as a feed supplement for aquaculture

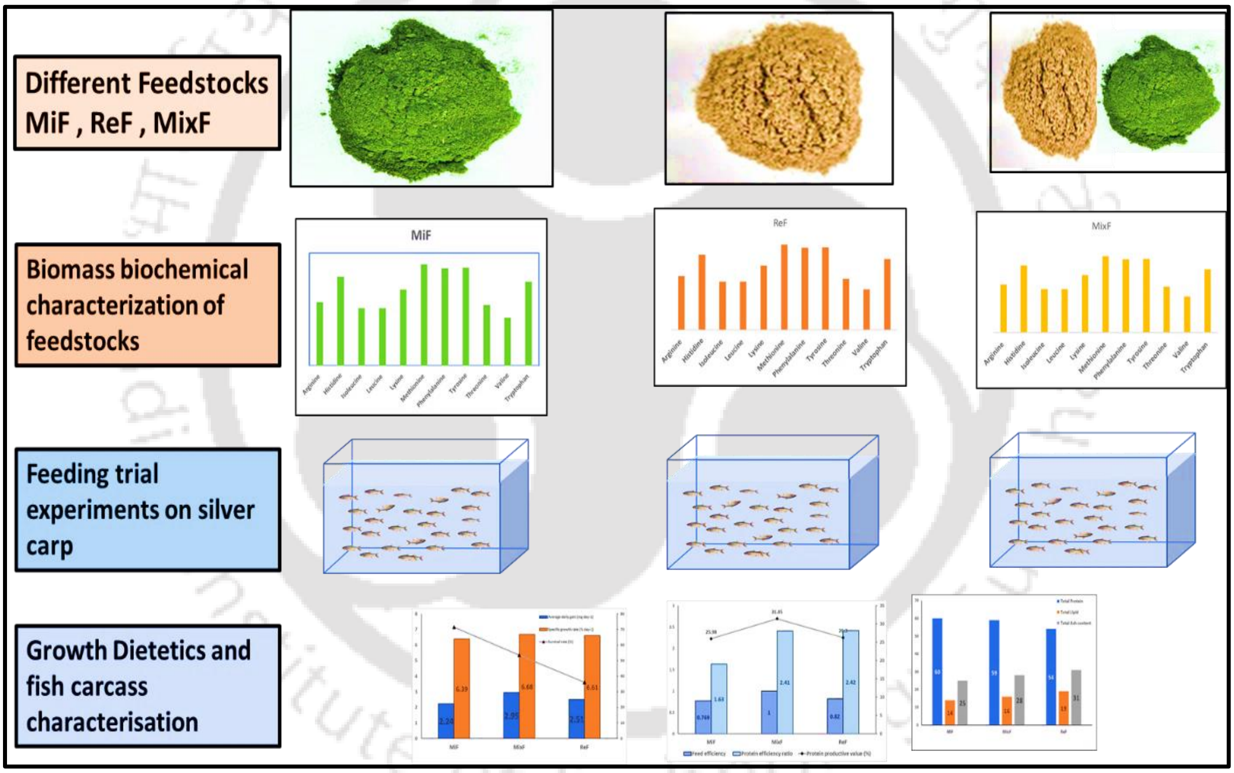

Fig 4.1 Flow diagram of evaluation of novel microalga CT01 as an alternative feedstock for fish *Hypophthalmichthys molitrix*

4.1 Background and Motivation

The world population is estimated to become 9.8 billion by the year 2050 and is expected to cause a surge in food demand over 60-100 % to that of reported in 2005 (Grafton, Williams and Jiang, 2015). It is also predicted that there can be an increase in demand of protein enriched food by 110% adding more demand on its conventional production (Tilman *et al.*, 2011). The current sources of protein enriched food are mostly plant sources and animal sources which constitute to (~57% of global protein and ~43% of global protein respectively. The plant sources include cereals, corn, rice millets etc. and animal sources include red meat, poultry meat, seafood, eggs and dairy products. With respect to seafood, wild caught and farmed fishes accounted for 10 % of the total animal products (1.7 billion tons per year) produced globally in 2016 (Henchion *et al.*, 2017). There has been an increased demand for fish food over the last 60 years from 20 million tonnes (1950) to 80 million tons (2016) with the rate of growth of 2.3 million tons per year (FAO 2018). Population explosion associated high demand for high-quality protein food and a simultaneous depletion in wild fish stocks caused an increase in demand of aquaculture source of fish to 37 % by the end of year 2030 (Draaisma *et al.*, 2013). This scenario shows the critical need to improve aquaculture-based food products in a sustainable way with a target to achieve food security. In this regard, the main component in cultivation of aquatic organism is the feed, which constitutes of fish meal and fish oil. In 2021, this sector almost consumed 70% of total global production of fishmeal and 73% of fish oil production(Hodar *et al.*, 2020). These fishmeal are rich in protein, easy to digest and provides essential nutritional components such as amino acids and fatty acids for the fish. Craig et. al (Craig, Kuhn and Schwarz, 2017). In the coming years, it is expected that, the amount of fish meal required for consumption, will not be met due to additional utilization of small fish for human consumption, negatively impacting supply/demand food chain. In this regard, expansion of aquafeed industry need a potential replacement of fishmeal without compromise in the quality of feed. Various alternative aquaculture feedstocks such as animal, plant, algal, and microbial sources, have basic difference in their biomass compositions (Khan, Shin and Kim, 2018; Jones *et al.*, 2020). Many terrestrial crops such as soybean, canola, corn, wheat, lupin and barley containing high percentage of protein are widely used as protein substitutes (Gatlin *et al.*, 2007; Burr *et al.*, 2011). But most of these lacks sufficient amount of fatty acids and essential amino acids preventing them from becoming a complete substitute. Few reports suggest presence of some toxic compound in plant feed which can affect the health of aquatic organism and in turn cause biomagnification upon consumption. The key challenge is to develop aquafeeds that can: (i) provide high nutritional quality; (ii) maintain sustainable food systems; and (iii) improved fish yields to meet increasing demand. The nutritional quality of feed ingredients is determined by their protein concentration, amino acid composition, and the extent to which they are used to fulfil the metabolic requirements of a specific species. However, the quality of an aquaculture feed is determined not only by its biochemical makeup, but also by its effects on the species. Aquaculture feeds are often composed of many nutritional components and frequently include a range of proteins with varying nutritive value, which eventually affects the bioavailability of amino acids (AA) (Figueiredo-Silva *et al.*, 2015). Various factors influence an animal's capacity to digest and absorb nutrients. Stone (2003) demonstrated that fish from lower trophic levels, such as tilapia, metabolize carbohydrates more efficiently than fish from higher trophic levels, such as salmon (Stone, 2003). Variations in protein digestibility may be attributed to changes in protein or cell wall matrices(Teuling *et al.*, 2017). In addition, various other parameters such as the type of fish (Teuling *et al.*, 2017), feeding habits, developmental stage of the fish, ambient temperature (Refstie *et al.*, 2006), nutritional needs of the animal (Peres, Santos and Oliva-Teles, 2013), and the health of the fish influence utilization of the nutrient. In order to compare the influence of different feedstocks on aquatic organism growth, it is also important to consider processing of feed ingredients, geographical location and time of harvest.

Earlier reports have demonstrated microalgae as promising alternative to conventional aquafeed in terms of improved survival rates, better immune response and higher antioxidant content in various aquatic organisms (Chen *et al.*, 2021). Different feed experiments have been conducted using various species of algae as a protein replacement for soyabean meal, fishmeal or fish oil substitute and its performance was assessed based on growth dietetics. Kobbia et al. (2002), for instance, included different quantities of *Chlorella vulgaris* in the diet of silver carp (Kobbia *et al.*, 2002). Replacement of up to twenty percent fish diet with dried algal biomass resulted in improved growth and survival rate. Another research revealed that the addition of dried algae to fish meals considerably boosted growth parameters compared to the control (Fadda and Attalla, 2021b). Mukherjee et al. (2011) discovered that the incorporation of dried algae into fish diets boosted protein productivity (Mukherjee *et al.*, 2011). Therefore, more research should be directed towards evaluating microalgae biomass as a complete replacement of conventional aquafeed and its potential for development of sustainable aquaculture industry.

Present study evaluates the indigenous novel freshwater microalgal isolate *Desmodesmus pannonicus* CT01 as a potential alternative for aquaculture feedstocks. CT01 biomass was tested as an alternate aquafeed through growth metrices, feed efficiency (FE), protein efficiency ratio (PER) and protein productive value (PPV) of freshwater fish silver carp (*Hypophthalmichthys molitrix*) and the same was compared using conventional aquafeed as control. The fish carcass compositional analysis was performed to understand the feed effect on fish biochemical composition.

4.2 Materials and Methods

4.2.1 Feedstock preparation

In the present study, three different feedstocks were considered to evaluate growth metrics and dietetics of fish *Hypophthalmichthys molitrix* (silver carp): microalgae feed (MiF), that is whole cell biomass of CT01; reference feed (ReF), composition of which is equivalent to the commercially used feed for silver carp, and mixed feed (MixF) comprising both microalgae and reference feed in 1:1 ratio. MiF was prepared by growing the strain CT01 in BBM in 20 L transparent plastic jar with working volume of 16 L, illuminated from two sides using white tubelight of 20 W each (10 no.) resulting in average light intensity of 150 µE m^{-2} s^{-1} and a light:dark cycle of 16:8 h was maintained throught the cultivation period. CO_2 (12.5%, v/v) mixed with air was purged into the medium, with a flow rate of 0.5 vvm, to achieve homegeneous culture and continuous supply of carbon source. Late log phase culture was harvested using 1 µm Nylon filter (Nylon Fabrics, Delhi), followed by centrifugation at 3000 rpm for 10 min at room temperature. Finally, the biomass was freeze-dried and stored at -20 ^{o}C. ReF was prepared by mixing different ingredients in recommended amount as mentioned in Table 1. A dough of the composite mixture was prepared using distilled water, followed by autoclaving for 20 min to allow gelatization of complex carbohydrates and deactivaiton of microbes and antinutrients. The dough was processed through a hand pelletizing machine to prepare pellets of 1 mm diameter, which were then oven dried at 60°C for 3-4 h to achieve moisture content of less than 10%. Before feeding, these pellets were ground into fine powder. The powdered ReF was stored in airtight packets at 25 °C. ReF was used as control for the feeding experiments. Feed formulation and preparation is the process of combining different feed ingredients to form a complete mixture that meets the specific goals of production (Fig 4.3).

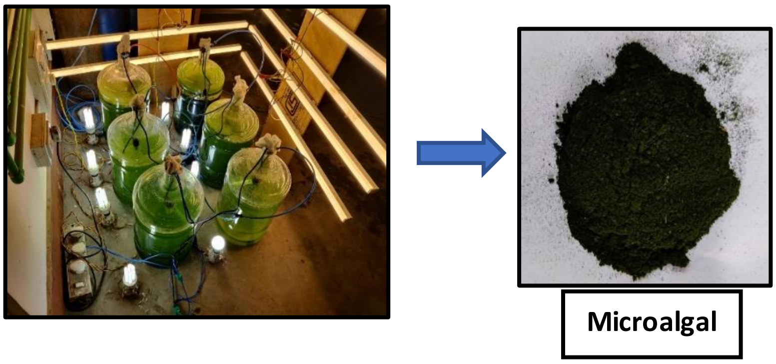

Fig 4.2 Representation of cultivation and dried biomass feed formation of CT01

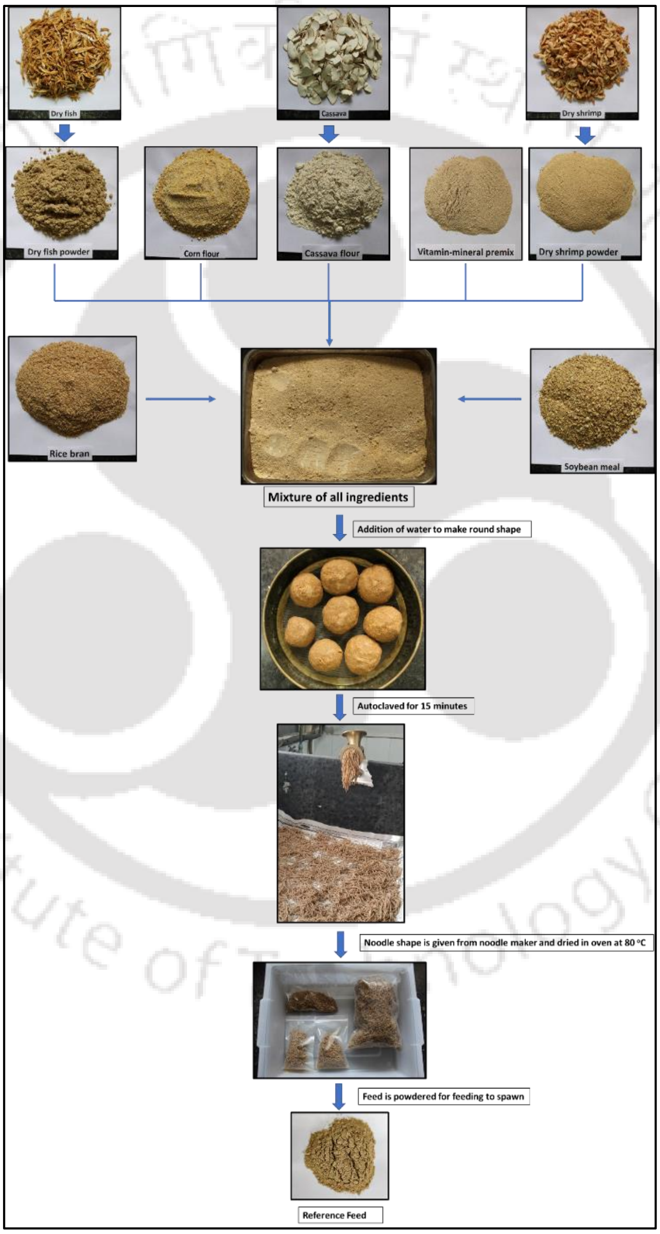

Fig 4.3 Stepwise representation of reference feed formulation for the feeding experiment of *Hypophthalmichthys molitrix*.

4.2.2 Assessment of feedstocks for fish growth metrices and dietetics

Effect of MiF, ReF and MixF on fish growth metrices and dietetics was assessed in rectangular glass aquarium, each with dimension of 1.5 (L) × 1.5 (B) × 1(H) ft and containing 55 L water. The aquarium was equipped with aeration device to provide oxygen in water. These aquaria were maintained at 12:12 h light-dark photoperiod. Three set of experiments was performed in parallel for three different types of feedstocks. 15-day old fry of *Hypophthalmichthys molitrix* (silver carp) procured from a nearby hatchery (JNB Aqua Agro Pvt

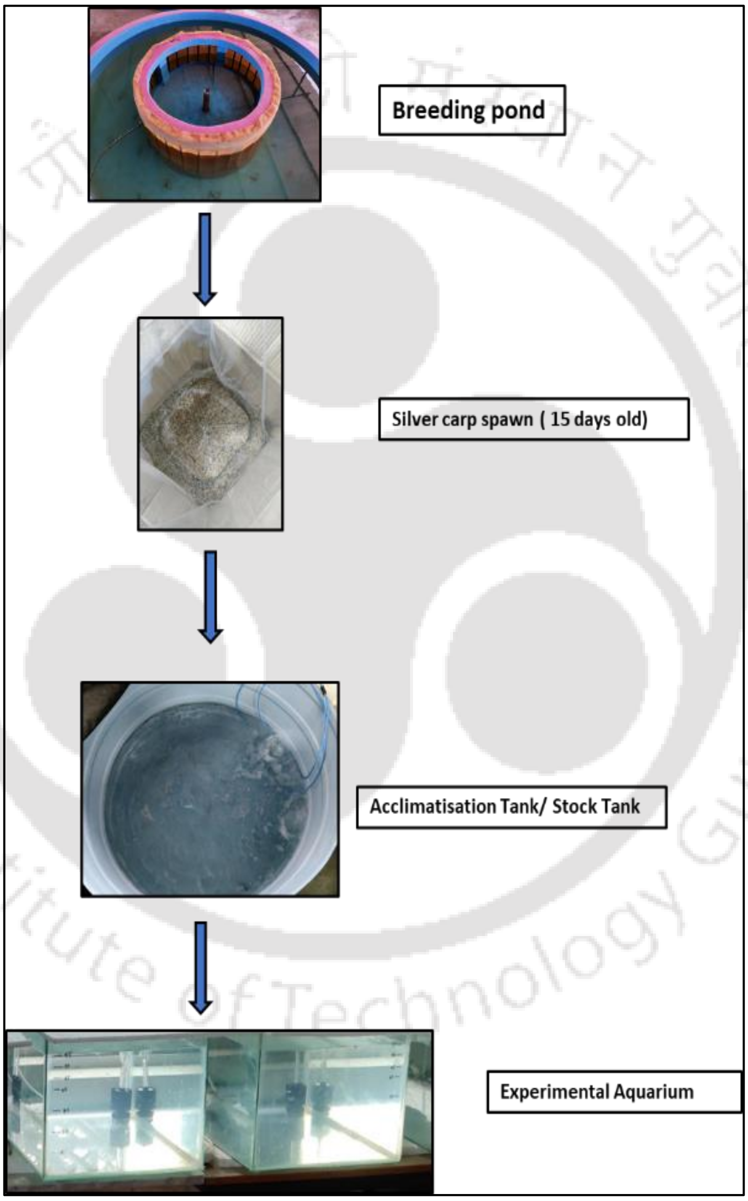

Fig 4.4 Schematic representation of step wise *Hypophthalmichthys molitrix* breeding and acquisition of fish from hatchery and cultivation aquarium

Ltd, Assam, India) was used a model fish. Silver carp was selected for the study considering its preference for phytoplankton as a natural feed. Before initiation of feeding trials, small fish fry were acclimatized for 72 h in a reservoir tank of 500 L capacity and were fed with the standard feed (ReF) followed by feed deprivation of of 24 h. Post-acclimatization, 500 number of fry were transferred to each aquarium in duplicates. The feeding trial was conducted for a duration of seven weeks (49 days). Fish were fed with respective feedstock thrice a day at 0600 h, 1200 h and 1700 h as per daily ration up to apparent satiation.

The water parameters such as pH, total dissolved solid (TDS) and salinity were kept within permissible limits by replacing about 10-15% of water at an interval of one week. The total ammonia, water temperature, dissolved oxygen (DO), TDS, salinity, conductivity and pH was measured throughout the course of feeding trials using water testing kit (Hach, USA)(Table 4.3). Estimation of nitrate was carried out using salicylic acid method (Cataldo *et al.*, 1975) using sodium nitrate as the standard and phosphate was quantified using ascorbic acid method (Parsons, Maita and Lalli, 1984) using dipotassium phosphate as the standard. In order to obtain initial body characteristics of the fish fry such as length, weight and total protein content, 150 individuals were randomly selected from the reservior and euthanized using clove oil (0.4 mL L^{-1}) on the first day of feeding trial. 10 numbers of fry from each treatment group were euthanized at an interval of seven days for profiling of growth metrices and dietetics such as weight (g), length (cm), average weight gain (AWG, mg day^{-1}), specific growth rate (SGR, % day^{-1}), feed eficiency (FE, %), protein efficiency ratio (PER), protein productive value (PPV, %) and survival rate (%).

$$\mathrm{AWG(mg\ day^{-1})} = \frac{\text{Total body weight gain}}{\text{Total culture days}} \tag{4.1}$$

$$\mathrm{SGR\ (\ \%\ day^{-1})} = \frac{Log_e\text{Final weight} - Log_e\text{Initial weight}}{\text{Number of days}} \times 100 \tag{4.2}$$

$$\mathrm{FE}\ (\%) = \frac{\text{Body weight gain (wet weight)}}{\text{Feed given (dry weight)}} \times 100 \tag{4.3}$$

$$\mathrm{PER} = \frac{\text{Net weight gain (wet weight)}}{\text{Protein feed}} \tag{4.4}$$

$$\mathrm{PPV}\ (\%) = \frac{\text{Amount of protein retained per fish}}{\text{Amount of protein fed per fish}} \times 100 \tag{4.5}$$

$$\text{Survival rate} = \frac{\text{Total number of fish harvested}}{\text{Total number of fish stocked}} \times 100 \tag{4.6}$$

4.2.3 **Biochemical composition of microalgal biomass, fish carcass and feedstocks**

For biochemical composition analysis of the microalgae biomass, stationary phase culture was collected and centrifuged at 7000 rpm at 4 °C for 10 min. Cell pellet was washed twice with distilled water and lyophilized overnight. To extract the intracellular protein, biomass resuspended in phosphate buffer (pH 6.8) was subjected to ultrasonication at 35% amplitude (a maximal power of 350 W) in a pulse mode (5 s on / 10 s off) under cold condition. Repeating 3 rounds of sonication for 5 min each, the supernatant was collected after every round. Total protein estimation of the whole supernatant was carried out by Bradford method (Bradford, 1976). The intracellular lipid was estimated by thermo-gravimetric analysis using Bligh and Dyer method (BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959)(BLIGH and DYER, 1959). Total carbohydrate content was estimated via phenol sulfuric acid method, using glucose as standard (Dubois *et al.*, 1956). Ash content of the biomass was calculated by subjecting 1 g of dry biomass to 575 °C in a muffle furnace for 4 h. The FAME content of the post extracted residual microalgal biomass was estimated using sequential two-step direct trans esterification method (Kumar *et al.*, 2014) and the FAME components were analysed using GC-FID (Agilent Technologies, USA). FAME dissolved in hexane was filtered through 0.22 micron nylon filter and injected to GC equipped with HP-5MS Ultra Inert column

(30 m × 250 µm × 0.25 µm). Helium was used as carrier gas and the split ratio was set as 10:1. Oven temperature was ramped from 70 °C to 180 °C at a rate of 15 °C min^{-1} and then to 260 °C at rate of 2.5 °C min^{-1} and the final temperature was held for 5 min. Supelco 37 component FAME mix (Sigma-Aldrich, USA) was used as the standard for quantification of compounds.

For different feedstocks, estimation of total protein, total lipid, total carbohydrate, essential amino acids and FAME content was carried out using the protocols mentioned for microalgae biomass. Total lipid, total ash and total moisture content of the fish body carcass was carried out using the protocol mentioned above. Total protein of fish body carcass was estimated using factor of 6.25 of nitrogen content. Total nitrogen of the fish carcass was estimated using Kjeldahl method (Barrins *et al.*, 2007). For amino acid estimation, samples were prepared and analyzed in triplicate. Replicates were prepared by weighing 15mg lyophilized algal biomass into vials. For hydrolysis, 1 mL 6 N HCl was added to each sample. Vials were capped with PTFE septa and vortexed for 20 s. Vials were hydrolyzed at 110 °C in a heating block for 24 h, then vortexed for 20 s, uncapped, and set to dry under nitrogen. One mL methanol was added to each vial, and samples were again vortexed for 20 s, then set to dry again under nitrogen. Samples were then placed under vacuum at 40 °C to finish drying overnight. Vials were returned to room temperature on benchtop (about 10 min). 1000 µL diluent was added to each sample, then immediately recapped. Samples were reconstituted by vortexing vials for 90 s each, then for an additional 20 s immediately prior to filtration through 0.2 µm PTFE syringe filters into clean vials. A total of 690 µL of biomass filtrate was diluted to final volume 5.0 mL in falcon tubes. Tubes were sealed and then vortexed to mix. This is the analytical sample stock. BSA samples were prepared using the same method, but with a dilution of 140 µL to 5 mL. These are the analytical stock solutions. Approximately 1.5 mL of analytical stock solution was transferred to clean, fresh vials to prepare individual replicates, capped and stored at −20 °C. OPA was prepared to a final concentration of 8 mM in water and sonicated to dissolve. To the same flask, 3MPA was added so that the final concentration would be 24 mM. Diluent (20% 0.2 M Sodium tetraborate, pH 9.3/80% Methanol) was prepared by dissolving 6 g boric acid in 480 mL water, and pH was then adjusted to 9.3 ±0.5 with 5 M NaOH. 400 mL of this solution was combined with 1600 mL methanol and stirred until the solution returned to room temperature. For the individual amino acid standards, the reaction mixture consisted of a total volume of 5 mL, including amino acids from the stock at concentrations between 20 and 100 µM diluted with diluent, and brought to final volume with 2.5 mL of the OPA/3MPA stock solution (final concentrations of 4 mM OPA and 12 mM 3MPA respectively). Samples were vortexed for 20 s after the addition of OPA/3MPA, and reaction times were measured relative to the completion of this step. For each of the hydrolyzed biomass samples, a total of 0.25 mL of analytical stock was combined with 2.25 mL diluent and 2.5 mL OPA/3MPA stock solution as described above (Cuchiaro and Laurens, 2019). Measurement spectra were normalized by using diluent, OPA, and sample blanks. Single-wavelength absorbance measurements were made at 334 nm.

4.2.4 Statistical analysis

All experiments were determined in biological triplicate to ensure the reproducibility. Results were expressed as mean value ± standard deviation. All data are presented as means of standard error. Tukey's multiple range tests were used to identify significant differences between any two means that differed at $p < 0.05$.

4.3 Results and Discussions

4.3.1 Feedstock Composition

The aim of the present study was to evaluate the whole cell biomass of CT01 as potential alternative to commercially available aqua feed as the source of key nutrients. To that end, microalgae feed (MiF) and mixed feed (MixF) was considered and compared with reference feed (ReF). In the first step, comparison was performed in terms of nutritional cpmposition followed by evaluation of these feedstocks for growth metrics and dietetics of the model fish silver carp. The chemical composition of all three types of feedstocks is shown in Table 1. Proximate analysis indicates, presence of higher fraction of crude protein both in MiF (49.63%, w/w) and MixF (41%, w/w) as compared to the ReF (35%, w/w). Carbohydrate and crude lipid content of ReF and MixF was found to be marginally higher as compared to MiF. Further, lower ash content for MiF and MixF (8-9.5%, w/w) should be advantageous as compared to the ReF (11%, w/w). The analysis revealed abundance of essential amino acids with lesser amount of FAME components for both MiF and MixF, as compared to ReF.

4.4.2 Assessment of feedstocks for fish growth metrices, dietetics and fish quality

Feed accounts for more than half of the variable operating costs in many aquaculture operations. Therefore, in the present study, the whole biomass of CT01 or its mixture with the reference feed was evaluated for their potential to be utilized as alternative to the commercially available aquafeed. Growth performance of fish fry of silver carp fed on diets with three different types of fish meal is shown in Table 4.3. While, growth of the fish fry on all three diets were found to be comparable, the parameters such as final body weight (mg), average weight gain (AWG, mg day^{-1}) and specific growth rate (SGR, % day^{-1}) was recorded to be highest at 150 ± 2.3, 2.95 ± 0.09 & 6.68 ± 0.44, respectively for MixF and lowest at 115 ± 1.3, 2.24 ± 0.08 & 6.39 ± 0.23, respectively for MiF(Fig 4.5 and 4.6). The results indicate that the biomass of CT01 alone, may not be suitable as complete replacement of reference fish feed. However, while biomass of CT01 was mixed with reference feed at a ratio of 1:1, the growth performance of fish fry of silver carp was found to improve significantly as compared to the reference feed. Similar to the growth matrix, dietetics of the fish fry was found to be superior while fed on MixF as compared to the ReF. For instance, FE, PER and PPV was estimated to be highest at 100%, 2.41 and 31.45%, respectively in case of mixed feed. When compared with the reference feed, the feed efficiency and protein productive value was found to be improved by 21.9% and 19.5%, respectively in case of mixed feed (Fig. 4.7). However, protein efficiency ratio remained same for both the feedstock. While, biomass of CT01 was used as complete replacement of reference feed, the dietetic values of the fish fry were found to be compromised.

Table 4. 1 Biochemical characterization of three different types of feed: MiF (Microalgae Feed), MixF (Microalgae mixed with reference feed), and ReF (Reference Feed).

Ingredients (%)	Experimental Diets		
	MiF	MixF	ReF
Microalgae	100	50	0
Dry fish powder	0	8.5	17
Dry shrimp powder	0	9.0	18
Soybean meal	0	13.0	26
Cassava flour	0	5.0	10
Corn flour	0	5.0	10
Rice bran or wheat bran	0	8.5	17
Vitamin-mineral premix	0	1.0	2
Proximate Analysis (%, dry matter basis)			
Dry matter	92±0.07^{a}	91.5±0.06^{ab}	91±0.05^{c}
Moisture	12±0.04^{a}	9.5±0.01^{b}	7±0.02^{c}
Crude protein	49.6±0.22^{a}	41±0.32^{b}	35±0.20^{c}
Crude lipid	4.6±0.01^{a}	6 ±0.02a^{b}	6.5±0.05^{c}
Ash	8±0.03^{a}	9.5±0.04^{b}	11±0.04^{c}
Total carbohydrates	3±0.07^{a}	6±0.12^{b}	9±0.02^{c}
Essential Amino Acid			
Arginine	5.09±0.03^{a}	3.73±0.04^{b}	2.37±0.02^{c}
Histidine	7.11±0.07^{a}	5.21±0.03^{b}	3.32±0.05^{c}
Isoleucine	4.58±0.22^{a}	3.36±0.36^{ab}	2.14±0.13^{c}
Leucine	4.58±0.16^{a}	3.36±0.19^{b}	2.14±0.41^{c}
Lysine	6.08±0.08^{a}	4.46±0.09^{b}	2.84±0.06^{c}
Methionine	8.09±0.07^{a}	5.93±0.18^{b}	3.78±0.03^{c}
Phenylalanine	7.78±0.11^{a}	5.71±0.21^{b}	3.63±0.09^{c}
Tyrosine	7.83±0.22^{a}	5.74±0.11^{b}	3.65±0.41^{c}
Threonine	4.85±0.19^{a}	3.56±0.09^{b}	2.26±0.36^{c}
Valine	3.83±0.23^{a}	2.81±0.14^{b}	1.79±0.2^{c}
Tryptophan	6.70±0.21^{a}	4.91±0.27^{b}	3.13±0.19^{c}
Major Fame components			
Linoleic acid (18:2n-6)	1.3±0.07^{a}	2.15±0.07^{b}	3±0.03^{c}
alpha- linolenic acid (18:3n-3)	0.06±0.11^{a}	1.03±0.19^{b}	2±0.07^{c}

Values (mean ± SD of three replicates) in the same row with different superscripts show significant difference ($P < 0.05$).

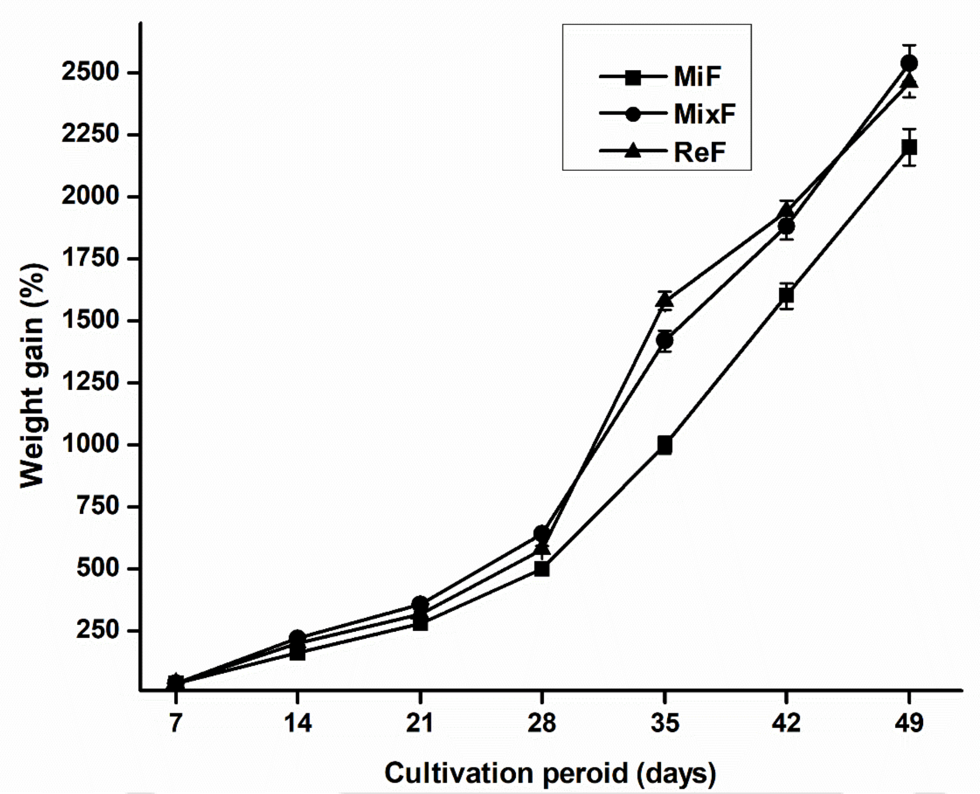

Fig 4.5 Profiles of weight gain of fish species *Hypophthalmichthys molitrix* under three different type of feeds: MiF (Microalgae Feed), MixF (Microalgae mixed with reference feed), and ReF (Reference Feed).

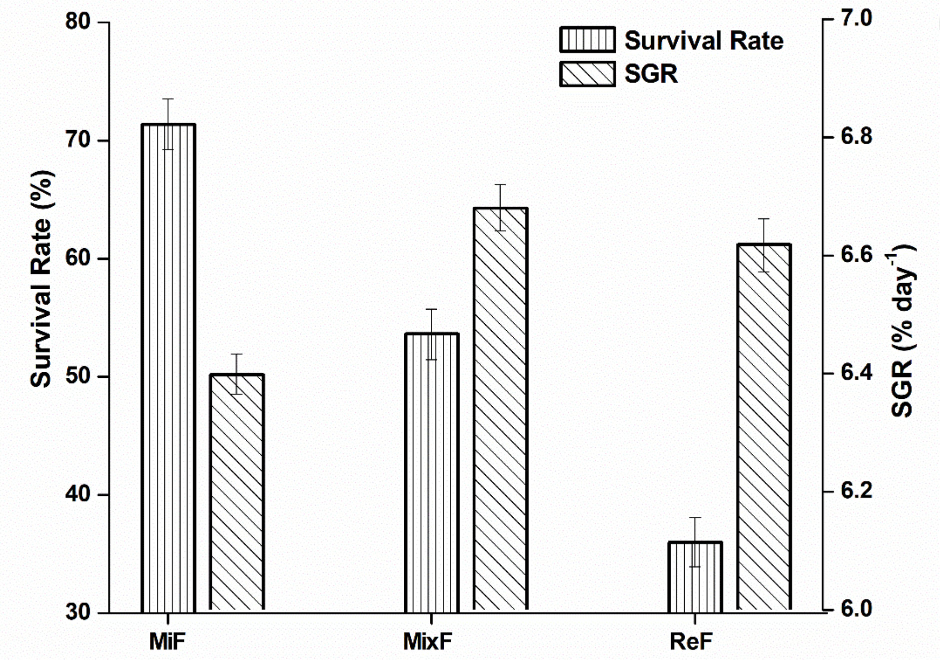

Fig 4.6 Profiles of growth characterization of fish species *Hypophthalmichthys molitrix* under three different type of feeds: MiF (Microalgae Feed), MixF (Microalgae mixed with reference feed), and ReF (Reference Feed).

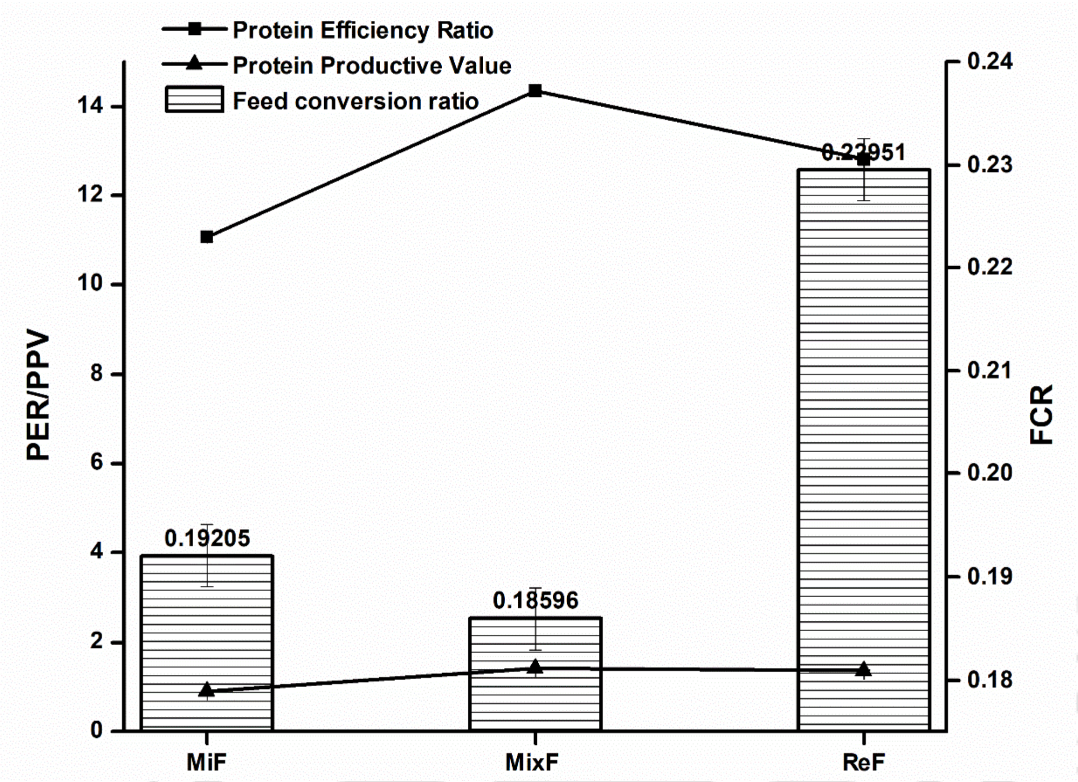

Fig 4.7 Profiles of PER, FCR and PPV of fish species *Hypophthalmichthys molitrix* under three different type of feeds: MiF (Microalgae Feed), MixF (Microalgae mixed with reference feed), and ReF (Reference Feed).

Table 4. 2 Characterization of growth metrices of fish species *Hypophthalmichthys molitrix* under three different type of feeds.

Item	Experimental Diet		
	MiF	MixF	ReF
Initial body weight (mg)	5.00 ± 0.02^{a}	5 ± 0.02^{b}	5 ± 0.02^{c}
Final body weight (mg)	115 ± 1.3^{a}	150 ± 2.3^{b}	128 ± 3.3^{c}
Average daily gain (mg day^{-1})	2.24 ± 0.08^{a}	2.95 ± 0.09^{b}	2.51 ± 0.10^{c}
Feed efficiency (%)	0.769 ± 0.04^{a}	1 ± 0.06^{b}	0.82 ± 0.07^{c}
Specific growth rate (% day^{-1})	6.39 ± 0.23^{a}	6.68 ± 0.44^{b}	6.61 ± 0.57^{c}
Protein efficiency ratio	1.63 ± 0.04^{a}	2.41 ±0.09^{b}	2.42 ± 0.11^{c}
Protein productive value (%)	25.98 ± 1.09^{a}	31.45 ± 2.11^{b}	26.30 ± 3.13^{c}
Survival rate (%)	71.4 ± 3.22^{a}	53.6 ± 3.16^{b}	36 ± 2.31^{c}
Average Initial fish Length (mm)	10 ± 0.12^{a}	10 ± 0.12^{b}	10 ± 0.12^{c}
Average Final fish Length (mm)	52 ± 1.89^{a}	59 ± 2.59^{b}	57 ± 2.66^{c}

Values (mean ± SD of three replicates) in the same row with different superscripts show significant difference ($P < 0.05$).

Table 4.3 Details of water quality parameters of three set of aquariums feed with the three different type of feedstocks

Water quality parameters (ppm)	**Experimental Diets**			**Days**
	MiF	**MixF**	**ReF**	
TDS	90	90	90	**0**
DO	8.4	8.4	8.4	
Total Nitrate	25	25	25	
Total Ammonium	31	31	31	
Total Phosphate	18	18	18	
TDS	97	112	131	**10**
DO	9.31	7.61	6.98	
Total Nitrate	32	44	53	
Total Ammonium	45	67	73	
Total Phosphate	22	38	46	
TDS	100	123	146	**20**
DO	9.55	7.51	6.87	
Total Nitrate	35	56	67	
Total Ammonium	47	71	77	
Total Phosphate	24	42	49	
TDS	102	131	151	**30**
DO	10.21	7.55	6.63	
Total Nitrate	44	67	73	
Total Ammonium	51	84	91	
Total Phosphate	26	51	55	
TDS	105	138	155	**40**
DO	10.55	7.67	6.58	
Total Nitrate	46	77	83	
Total Ammonium	50	91	98	
Total Phosphate	25	57	63	
TDS	111	152	171	**49**
DO	10.42	7.42	6.45	
Total Nitrate	47	82	96	
Total Ammonium	53	96	106	
Total Phosphate	27	66	72	

Chemical composition of the whole fish as affected by different types of feedstock at the end of experimental period is shown in Table 4.4. The protein content was found to vary in the range of 54-60% depending on the feedstock. It is important to note that, while growth matrix and dietetics was found to be inferior in case of MiF, the quality of fish in terms of total protein content was estimated to be highest at 60%, followed by MixF at 59% and ReF at 54%. This suggests better digestibility of microalgal protein by the fish as compared to reference diet. Total lipid content was found to be in reverse order with, lowest value of 14% in case of MiF followed by 16% in case of MixF and 19% for ReF. This reverse trend of lipid content may be attributed to lower crude lipid content present in microalgae feed. Similar to the lipid profile, the lowest ash content in the MiF fed fish corroborates well with the feed ash content. The total dry matter content of the fish was found to be similar irrespective of feedstock type. The study indicates that while pure biomass of CT01 may not be suitable for complete replacement of reference feed, equal mixture of CT01 and reference feed is a better alternative to reference feed offering improved growth, dietetics and fish quality for silver carp.

Feasibility of considering microalgae as potential supplement or replacement of conventional aquafeed would largely depend on both biomass composition of microalgae species and the species of fish under consideration. For instance, the results obtained in the present research corroborate well with the study reported by Fadda (2021), where addition of *Chlorella vulgaris* in fish diets improved growth performance of silver carp (*Hypophthalmichthys molitrix*) and a linear increase in body weight gain was observed with increase in the level of algae in the fish diet (Fadda and Attalla, 2021a). Further, supplementation of *Chlorella pyrenoidosa* as feed additive at a concentration of 5-7.5 g per kg of diet resulted in improved PER and food conversion ratio for common carp (*Cyprinus carpio* L.) (Mohi *et al.*, 2019). Another study reported better food conversion ratio and PER, when Nile Tilapia was maintained on artificial diets with 10-20% dried microalgae *Scenedesmus* sp. (Badwy, Ibrahim and Zeinhom, 2008).

With the increase in inclusion percentage of *Spirulina platensis* in the diet of Caspian brown trout, total protein and polyunsaturated fatty acid content was reported to increase linearly (Roohani *et al.*, 2019). Pool of literatures also reported no negative effect on growth and quality traits of different fish species such as Red tilapia (*Oreochromis* sp.) or European sea bass (*Dicentrarchus labrax*) when their conventional meal was supplemented with various microalgae species (Rincón *et al.*, 2012; Tulli *et al.*, 2012b)..

Table 4.4 Carcass proximate chemical analyses (%; on DM basis) of fish fed with three different types of feedstocks MiF, MixF, and ReF.

Item	Experimental Diet		
Proximate Analysis (%, w/w)	MiF	MixF	ReF
Total Protein$^{\#}$	60 ± 0.03^{a}	59 ± 0.07^{ab}	54 ± 0.08^{c}
Total Lipid$^{\#}$	14 ± 0.11^{a}	16 ± 0.09^{ab}	19 ± 0.06^{c}
Total Ash content	25 ± 0.11^{a}	28 ± 0.09^{b}	31 ± 0.05^{c}
Total Dry Matter	79 ± 0.81^{a}	78 ± 0.65^{b}	79 ± 0.71^{c}

Values (mean ± SD of three replicates) in the same row with different superscripts show significant difference ($P < 0.05$).

$^{\#}$ Total protein and total lipid composition were calculated for ash free dry weight of carcass *i.e.* % (Total dry weight – Total ash content), w/w

4.4 Conclusions

Owing to its high protein content, biomass of the alga was evaluated for its application as alternate aquafeed either as sole feed or as a mixture with an artificial (reference) feed. Fish growth matrices, dietetics and quality for microalga feed and mixed feed groups were compared with the reference feed. Mixture of microalgae with reference feed (at 1:1 ratio) resulted in significant improvement in growth matrices such as final body weight, daily weight gain and specific growth rate of young fish (i.e., fry) as compared to the reference feed. Similar to the growth performance, dietetics of the fish fry in terms of feed efficiency, protein efficiency ratio and protein productive value were found to be higher while fed on mixed feed as compared to the reference feed. The quality of fish in terms total protein content was estimated to be highest (60%) for microalga feed, followed by mixed feed (59%) and reference feed (54%). This suggests better digestibility of microalga protein by the fish as compared to reference diet.

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CHAPTER 5

Process engineering strategy for cultivation of high-density protein rich CT01 microalgal biomass

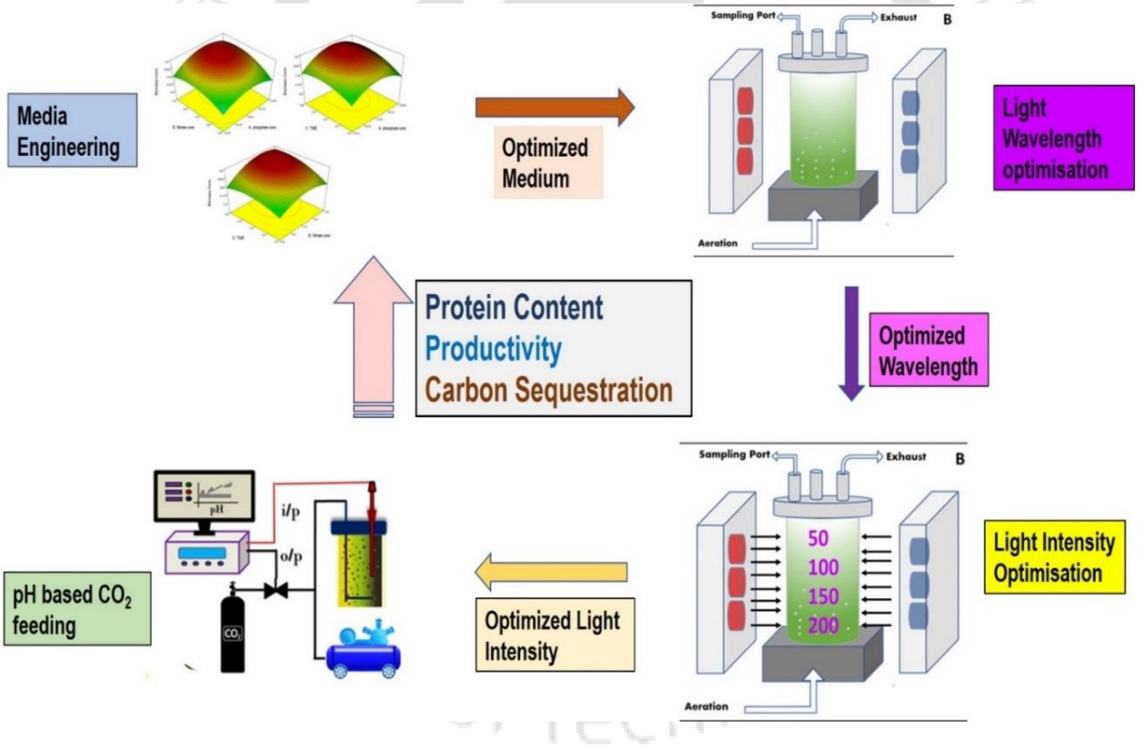

Fig 5.1 Schematic representation of developed process engineering strategy for CT01

5.1 Background and Motivation

Even though, many species of microalgae are shown to have potential as live feedstock in aquaculture, the high cost of biomass production and lower biomass concentration remain key concerns to compete with conventional fish meal (Han *et al.*, 2019). Among the various requirements, nutrients are the most cost-involving component, as the growth of microalgae involves huge number of natural resources and extensive energy utilization. Optimizing the nutrients required for algal growth can mitigate their production costs and significantly improve the process economics (Blair, Kokabian and Gude, 2014). Also, the media and conditions established for the general culturing of microalgae are not species specific, resulting in sub-optimal productivity. Therefore, species specific optimization of medium composition, it's concentration and culture conditions can improve growth performance and thereby making the process economically viable and feasible (Shin *et al.*, 2018). Light intensity and wavelength are another key cultivation parameters and primary limiting factor, which not only regulate the biological process, but exert significant influence on biomass composition and its production. For instance, with the increase in light intensity, growth rate of microalgae increases up to a certain level, while high irradiance may result in photo inhibition (Carvalho *et al.*, 2011; Metsoviti *et al.*, 2019). Light-emitting diodes (LEDs) of different wavelengths offer opportunities for delivering efficient, durable, reliable, economical and controllable light source in the cultivation of microalgae, and their use has resulted in increased productivity and enhanced biochemical compositions (Sui and Harvey, 2021). Variation in light intensity reported to modulate the total protein content in various microalgae strains such as *C. vulgaris* (Metsoviti *et al.*, 2019), *Scenedesmus* sp. (Gris *et al.*, 2014), etc. Photoautotrophic growth is vitally dependent on appropriate availability of light as an energy source and as well as availability of ample CO_2 as a carbon source. Therefore, it is critical to develop an efficient process engineering solution aimed at increasing biomass concentration or productivity, in conjunction with enhancing CO_2 sequestration. Efforts are being made to upregulate parameters such as the CO_2 fixation rate (Cabello, Morales and Revah, 2017), substrate utilisation (Goswami *et al.*, 2019), and light availability to cells (Muthuraj *et al.*, 2015) and mass transfer of carbon dioxide (Gonçalves *et al.*, 2016; Goswami *et al.*, 2019) toward enhanced biomass production.

This study demonstrates a sequential development of process engineering strategy to enhance the protein content and biomass productivity of the microalgal strain CT01 for the application as alternate aquafeed. First of all, to enhance the biomass concentration and productivity of CT01 strain, statistical optimization of Basal Bold Medium nutrients *i.e.* phosphate, nitrate and trace and micro elements (TME) was performed. The use of appropriate wavelengths of light is an effective way to enhance growth parameters and alter biochemical constituents of microalgal species. Effect of seven different combinations of light wavelengths on growth kinetics and biochemical composition of the strain was studied. Further the CT01 strain was subjected to different range of light intensities to further improve the biomass productivity, carbon sequestration rate and total protein content. Finally, a pH-based CO_2 feeding strategy was adopted in conjunction with optimized medium and illumination strategy to further improve the biomass productivity and carbon sequestration rate.

5.2 Material and methods

5.2.1 Microorganism and inoculum preparation

The indigenous CO_2 tolerant microalga strain *Desmodesmus pannonicus* CT01 was subjected to process engineering strategy for improved growth performance and intracellular protein content. Inoculum for all experiments were cultured in Basal Bold's medium (BBM) with an initial pH of 8. The cultures were grown in 250 mL Erlenmeyer flasks with working volume of 100 mL, incubated at room temperature and 150 rpm agitation in a shaker incubator (Multitron-Pro, Infors HT, Switzerland) in presence of warm white light of 50 µE m^{-2} s^{-1} intensity with a light and dark cycle of 16:8 h. As and when required inoculum of specific volume was cultured and for experimentation. The inoculum cell density of 0.404 g L^{-1} was used for inoculation of all experiment's batches.

5.2.2 Media optimization for maximization of biomass titer and productivity

With the dual objective of improving biomass titer and productivity, Response Surface Methodology (RSM) based on 3 factors and 5 levels Central Composite Design (CCD) was used to optimize key media components such as concentration of nitrate ($NaNO_3$), phosphate (KH_2PO_4) and TME. The design consisted of 20 experimental runs at three coded levels, where +α, +1 (high); 0 (medium); and −1, −α (low); correspond to high, medium, and low levels of the variables, with 5 replicates at the centre point to estimate the experimental error (Table 5.1 and 5.2).

Table 5.1 Actual and coded levels of the selected Basal Bold's media components for the maximization of biomass titer and productivity.

Factors			**Levels and corresponding actual values**			
Code	**Media Components**	**units**	**-1 Level**	**+1 Level**	**- alpha**	**+alpha**
A	**Initial KH_2PO_4 concentration**	**mg L^{-1}**	130.40	219.60	100	250
B	**Initial $NaNO_3$ concentration**	**g L^{-1}**	0.45	1.05	0.25	1.25
C	**Initial TME units**	**units**	0.40	0.85	0.25	1

The CCD predicted 20 experimental combinations comprising eight factorial points, six axial points and six replicates of centre points. A second-order polynomial equation was used to express the mutual interaction among the variables and their corresponding optimum levels. The general form of this equation is as follows:

$$Y = \beta_O + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \varepsilon \qquad \text{Eq. 5.1}$$

Where Y is the predicted response, β_O is the intercept term, β_i, β_{ii} and β_{ij} are the linear, quadratic, and interaction effects, respectively, X_i and X_j are the factor independent variables (regression coefficient of the model), k is the total number of parameters and ε is the error. Variable $i = 1,2,3$ represents phosphate, nitrate and TME, respectively. Multiple regression analysis was used for the analysis of experimental data obtained from the CCD to determine the significant differences ($p \leq 0.05$) in responses at various conditions. The 3D surface plots were generated for visualization of the relationships between the responses and the independent variables and to determine the optimum conditions. The best fit of the model was determined based on the coefficient of determination (R^2), and ANOVA as well as its statistical significance were checked by F test quadratic polynomial equations that were attained by holding one of the independent variables at a constant value and changing in the other variable level. Statistical significance was checked by F test quadratic polynomial equations that were attained by holding one of the independent variables at a constant value and changing in the other variable level. Design Expert Software Version 7.0.0 (Stat-Ease Inc., Minneapolis, USA) was used for all design and analysis. The predicted and actual values were compared to determine the accuracy and validity of the model. Experiments were performed in shake flask and was maintained at similar conditions as detailed in above section and samples were withdrawn at the end of every light cycle to monitor the growth. All subsequent experiments were performed using optimized BB medium.

Table 5.2 CCD matrix of the media components used in RSM with corresponding experimental and predicted measurements for biomass titer and productivity

Std. No.	**KH_2PO_4 (mg L^{-1})**	**$NaNO_3$ (g L^{-1})**	**TME (units)**	**Experimented**		**Predicted**	
				Biomass (g L^{-1})	**Productivity (mg L^{-1} day^{-1})**	**Biomass (g L^{-1})**	**Productivity (mg L^{-1} day^{-1})**
1	130.405	0.4527	0.40202	0.6625	20.3	0.66	20.21
2	219.595	0.4527	0.40202	0.661	22.45	0.66	22.41
3	130.405	1.0473	0.40202	0.6879	25.81	0.69	25.77
4	219.595	1.0473	0.40202	0.7812	23.78	0.78	23.84
5	130.405	0.4527	0.84798	0.6818	20.61	0.68	20.56
6	219.595	0.4527	0.84798	0.7539	28.39	0.75	28.45
7	130.405	1.0473	0.84798	0.7821	24.15	0.78	24.21
8	219.595	1.0473	0.84798	0.9512	27.87	0.95	27.97
9	100	0.75	0.625	0.6415	18.41	0.64	18.49
10	250	0.75	0.625	0.786	23.6	0.79	23.5
11	175	0.25	0.625	0.6516	22.16	0.65	22.24
12	175	1.25	0.625	0.8412	26.61	0.84	26.51
13	175	0.75	0.25	0.7299	26.47	0.73	26.54
14	175	0.75	1	0.8878	30.4	0.89	30.31
15	175	0.75	0.625	0.9555	31.66	0.96	31.59
16	175	0.75	0.625	0.9562	31.61	0.96	31.59
17	175	0.75	0.625	0.9553	31.58	0.96	31.59
18	175	0.75	0.625	0.9571	31.63	0.96	31.59
19	175	0.75	0.625	0.9567	31.52	0.96	31.59
20	175	0.75	0.625	0.9577	31.51	0.96	31.59

5.2.3 Effect of light wavelength and intensity on growth kinetics and biochemical composition of CT01

In the first stage, to evaluate the effect of light wavelength, seven sets of experiments were carried out where the CT01 strain was grown under different categories of illumination: three monowavelength (Red, Green and Blue), one multiwavelength (White) and three mixed combination of wavelength in 1:1 ratio (Red-Blue, Blue- Green and Red-Green). The incident light intensity on the surface of the mini reactor was manitained at 100 µE m^{-2} s^{-1} for all wavelength batches. A customized cylindrical membrane bubble column airlift photobioreactor (CM-APBR) made of flanged cylindrical acrylic tube (O.D: 75 mm, I.D.: 69 mm, H: 175 mm) with working volume of 500 mL and equiped with a membrane sparger, was used for the cultuvation. Porous neoprene rubber sheet of thickness 2 mm was used as membrane sparger. A LED panels of white light (3×50 W) , red light (3×50 W) , blue light (3×50 W) and green light (3×50 W) was installed parallel to the surface (one side) of the reactor for study of individual wavelength with uniform light intensity of 100 µE m^{-2} s^{-1} (Fig 5.2 A) and for study of mixed wavelength, two different LED panels were installed from the two sides of the reactor with photon flux density ratio 1:1 (Fig 5.2 B) maintaing the same intensity. A DC voltage regulator was connected to each LED panel to modulate the light intensity as per requirement. In the next step, with the best combination of light wavelength, the growth performance and biochemical composition of the CT01 strain was assessed under four different light intensities of 50, 100, 150 and 200 µE m^{-2} s^{-1}. All the experiments were performed using optimized BBM medium, with air flow rate of 0.5 vvm and light: dark cycle of 16:8 h. Sampling was done at regular time interval to monitor the growth and composition of biomass.

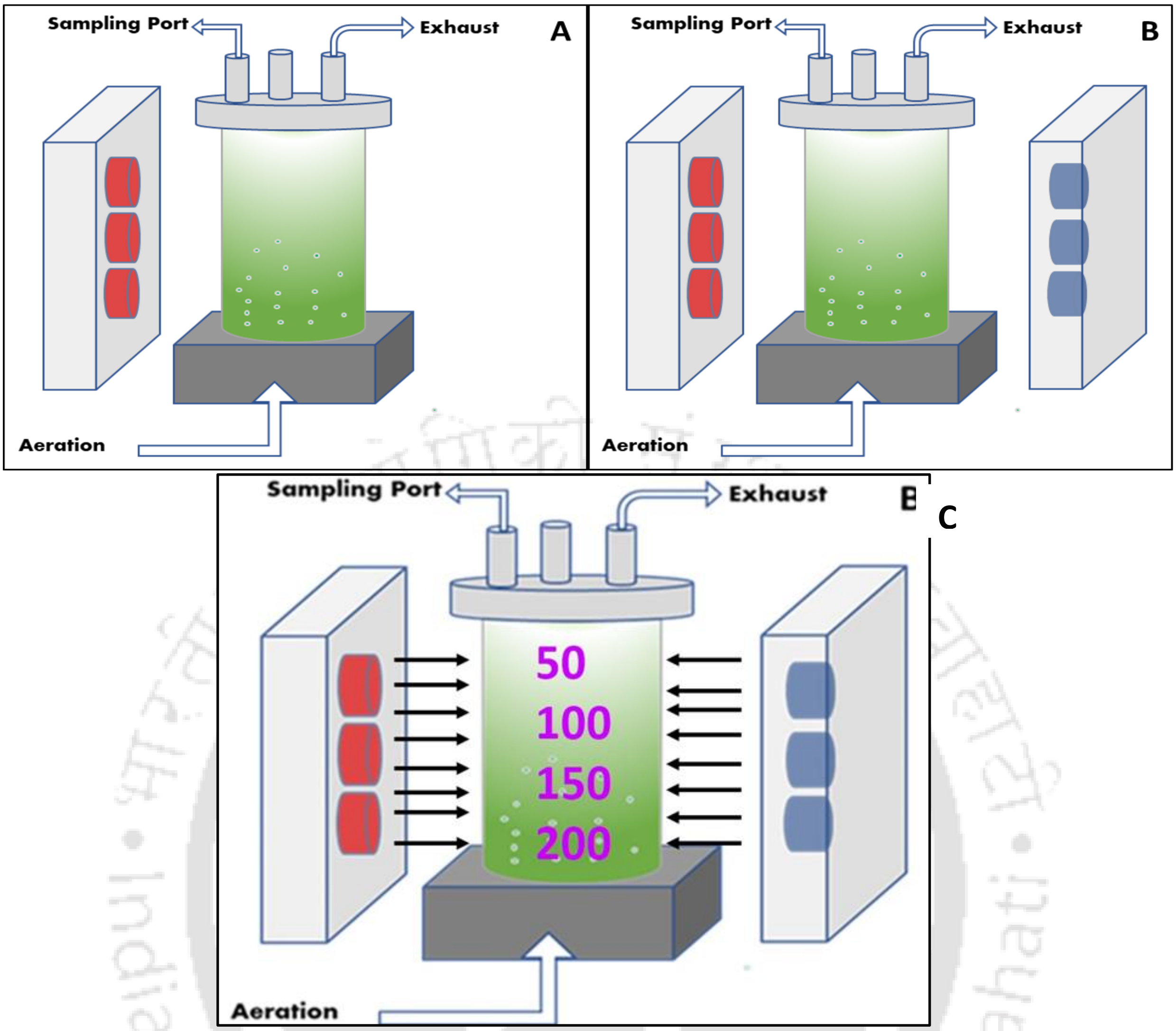

Fig 5.2 Schematic diagram of experimental setup of mini reactor: A) cultivation under mono light wavelength, B) cultivation under mixed light wavelength and (C) cultivation under different light intensity.

5.2.4 Growth of CT01 with pH based CO_2 feeding

Following optimization of media components and dvelopment of illumination strategy, with the aim of further improvement in biomass titer and productivity, a process engineering strategy was employed based on on-demand feeding of CO_2 to maintain the culture pH within the optimized range. The culture was grown in mini reactor equiped with a mixture of optimized light wavelength of 100 µE m^{-2} s^{-1} intensity, using optimized BBM medium and maintaining the optimum aeration rate of 0.5 vvm. The pH of the culture was maintained at its optmal range of 8-8.5 throughout the entire course of cultivation via cascade control with CO_2 feeding using solenoid valve, guided by the pH sensor (Sinha *et al.*, 2022). Two different batches were run, one without pH control cascade and the other with pH control.Sampling was performed at regular time interval to obtain dynamic profile of growth and the culture pH was recorded at every 10 min interval for the entire batch duration. The batch was terminated at the onset of stationery phase.

5.2.5 Analysis of growth, biomass composition and substrate utilization

The sample collected at regular time intervals was centrifuged at 8000 X g for 10 min at 4 °C to separate supernatant and cell pellet. While the pellet was used for estimation of growth and biomass composition, the supernatant was used for the analyses of substrate utilization. Cell growth was obtained by measuring optical density of the cells as mentioned in the section 3.2.4. Growth kinetics were calculated as per the formula mentioned in the section 3.2.3. The total lipid, total protein and total carbohydrate content of the biomass was estimated as explained in the section 4.2.3. The utilization profile of the nutrients such as nitrate and phosphate were carried out as mentioned in the section 3.2.5. To estimate chlorophyll content, the cell pellet was extracted with 90%, v/v methanol for 30 min at 45 °C, in amber tubes, followed by centrifugation at 10,000 rpm for 10 min at 4 °C. Chlorophyll a (C_a, µg mL^{-1}), chlorophyll b (C_b, µg mL^{-1}) and total carotenoid (C_{x+c}, µg mL^{-1}) concentrations was estimated using the following equations (Tandeau De Marsac, 1977).

$$C_a = 15.65\ A_{666} - 7.33\ A_{653} \tag{Eq.5.2}$$

$$C_b = 27.05\ A_{653} - 11.21A_{666} \tag{Eq.5.3}$$

$$C_{(x+c)} = \frac{(1000\ A_{470} - 2.86\ C_a - 129.2\ C_b)}{245} \tag{Eq. 5.4}$$

5.2.6 Statistical analysis

All the data obtained in this studies were performed in duplicate. All data are presented as means of standard error.

5.3 Results and Discussions

5.3.1 Statistical optimization of media for CT01 for maximisation of biomass titer and productivity

The CCD of RSM was employed to optimize the concentration of significant variables to maximize the biomass concentration and productivity of CT01 and to understand the significant interaction among different nutrients *i.e* potassium dihydrogen phosphate, sodium nitrate and trace and microelements (TME), which affect the growth parameters. The results were analysed using standard analysis of variance (ANOVA), all experimental data were fitted with second order polynomials (Eq 5.1).

$$Y_{\mathrm{titer}} = -1.10191 + 0.013478 * A + 0.94861 * B + 0.97357 * C + 0.0181 * AB + 0.0188 * AC + 0.28661 * BC - 0.000043 * A^2 - 0.83767 * B^2 - 1.0451 * C^2 \qquad \text{(Eq. 5.5)}$$

$$Y_{\mathrm{Productivity}} = -61.17632 + 0.66114 * A + 65.62309 * B + 13.46874 * C - 0.077687 * AB + 0.14306 * AC - 7.20306 * BC - 0.00188 * A^2 - 28.83612 * B^2 - 22.46421 * C^2 \qquad \text{(Eq. 5.6)}$$

Where, Y represent the predicted response and A, B, and C are the values of phosphate concentration, nitrate concentration, and TME, respectively.

Table 5.3 ANOVA for the quadratic regression model obtained from CCD-RSM employed in optimization of media components for biomass productivity

Source	*Sum of Squares*	d_f	*Mean Square*	*F-Value*	*P-Value prob > F*	
MODEL	0.3	9	0.033	19120.67	< 0.0001	significant
A-KH_2PO_4	0.024	1	0.024	14122.91	< 0.0001	
B-$NaNO_3$	0.043	1	0.043	24719.39	< 0.0001	
C-TME	0.03	1	0.03	17541.21	< 0.0001	
A2	4.60E-03	1	4.60E-03	2673.06	< 0.0001	
B2	2.79E-03	1	2.79E-03	1621.86	< 0.0001	
C2	2.89E-03	1	2.89E-03	1678.8	< 0.0001	
AB	0.11	1	0.11	61360.25	< 0.0001	
AC	0.079	1	0.079	45924.04	< 0.0001	
BC	0.039	1	0.039	22618.14	< 0.0001	
Residual Error	1.72E-05	10	1.72E-06			
Lack-of-Fit	1.29E-05	5	2.58E-06	2.97	0.1284	not significant
Pure Error	4.33E-06	5	8.66E-07			

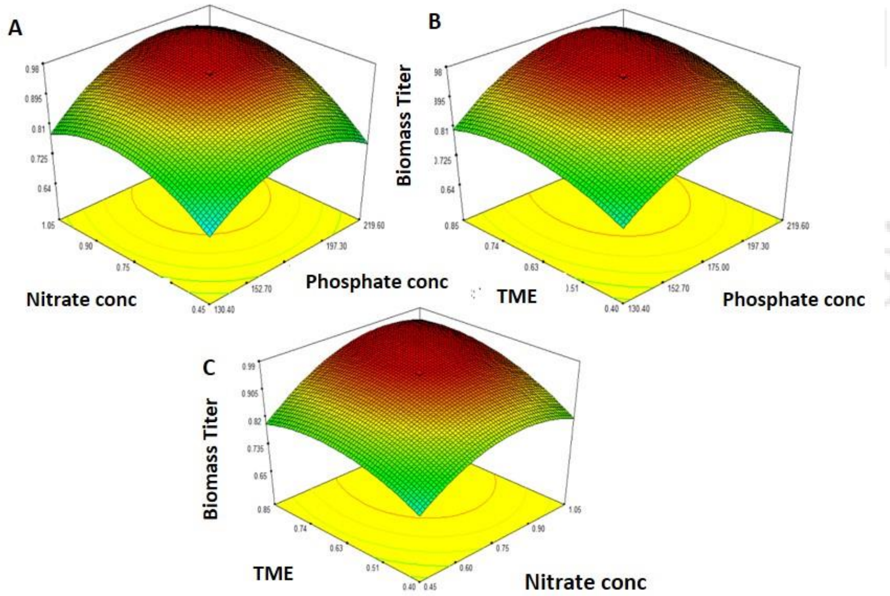

Fig 5.3 Response surface plots representing the interaction effect of (A) phosphate and nitrate, (B) TME and phosphate, and (C) TME and nitrate on biomass titer. For the interaction of two variables, third variable were kept at constant middle value. The middle values of phosphate, nitrate and TME were 0.175 g L^{-1}, 0.75 g L^{-1} and 0.63 units, respectively.

The ANOVA of quadratic regression models for growth and productivity (Tables 5.3 and 5.4) had prob > F < 0.0001, meaning that the models agreed the significance of experimental data. The lack of fit value was insignificant for all the responses and the fitness of the model checked by coefficient of determination (R^2). The R^2 value was 0.9999 for biomass growth, and 0.9997 for productivity respectively, which aligned with the goodness of fit of the models. The significance of each variable A, B and C representing nitrate concentration, phosphate concentration, and TME, respectively, was determined by the P values, and the variables with P ≤ 0.05 were considered to be statistically significant. All the three linear coefficients A, B and C, three squared coefficients (A^2, B^2 and C^2) and the interaction coefficient were determined to be significant for biomass concentration (Table 5.3). The linear coefficients A, B, and C, squared coefficients A^2, B^2, C^2 and the three-interaction coefficient were statistically significant for biomass productivity (Table 5.4). The response surface plots were generated to interpret and understand the interactions of those variables that were determined to significantly affect the biomass

Table 5.4 ANOVA for the quadratic regression model obtained from CCD-RSM employed in optimization of media components for biomass titer

Source	*Sum of Squares*	d_f	*Mean Square*	*F-Value*	*P-Value prob > F*	
MODEL	371.35	9	41.26	4210.24	< 0.0001	significant
A-KH_2PO_4	30.32	1	30.32	3093.74	< 0.0001	
B-$NaNO_3$	22.03	1	22.03	2247.58	< 0.0001	
C-TME	17.12	1	17.12	1746.63	< 0.0001	
A2	8.49	1	8.49	866.03	< 0.0001	
B2	16.19	1	16.19	1651.82	< 0.0001	
C2	1.82	1	1.82	186.13	< 0.0001	
AB	201.99	1	201.99	20610.78	< 0.0001	
AC	93.62	1	93.62	9552.91	< 0.0001	
BC	17.98	1	17.98	1834.38	< 0.0001	
Residual Error	0.098	10	9.80E-03			
Lack-of-Fit	0.08	5	0.016	4.4	0.0649	not significant
Pure Error	0.018	5	3.63E-03			

production. The interaction effects of all three factors on biomass production and productivity, while maintaining one factor at an optimum level, is shown in Fig 5.3 and 5.4. Based on the regression equations above, the optimum concentrations of major nutrients in the Basal Bold medium for maximum biomass production and productivity of CT01 were determined to be 770 mg L^{-1} of sodium nitrate, 174 mg L^{-1} of potassium dihydrogen phosphate, and 0.77 units of TME. The optimization of medium concentration for the maximum biomass production and better productivity using RSM design provided an efficient medium with altered concentration of nutrients. The model was experimentally validated using the determined optimum conditions and the validation experiments yielded a biomass concentration and productivity of 0.97 g L^{-1} and 31.39 mg L^{-1} d^{-1} respectively. The obtained results from validation experiments were close to and in agreement with the predicted values of a biomass concentration and productivity of 0.9674 g L^{-1} and 31.83 mg L^{-1} d^{-1} respectively Similar kind of studies has been done for *S. vacuolatus* suggesting importance of optimization of nitrate and phosphate concentration on the growth of microalgal species (Ghosh *et al.*, 2020). Substantial growth was observed with the increase in concentration of nitrate and phosphate. Various studies suggested that low concentration of nitrate or phosphate can affect the growth of microalgal species, making them key nutrients to manipulate for the growth and productivity (Prathima Devi, Venkata Subhash and Venkata Mohan, 2012; von Alvensleben, Magnusson and Heimann, 2016). Studies by Ruangsomboon et al. (2013) on *Scenedesmus dimorphus* KMITL proposed that high concentration of phosphate affect production of biomass positively (Ruangsomboon, Ganmanee and Choochote, 2013). The obtained biomass was 28.3 % higher than that of unoptimized medium (0.7541 g L^{-1}) and productivity was also found to increase by 26.67 % in comparison to unoptimized condition, and hence validating the successful implementation of applied statistical method.

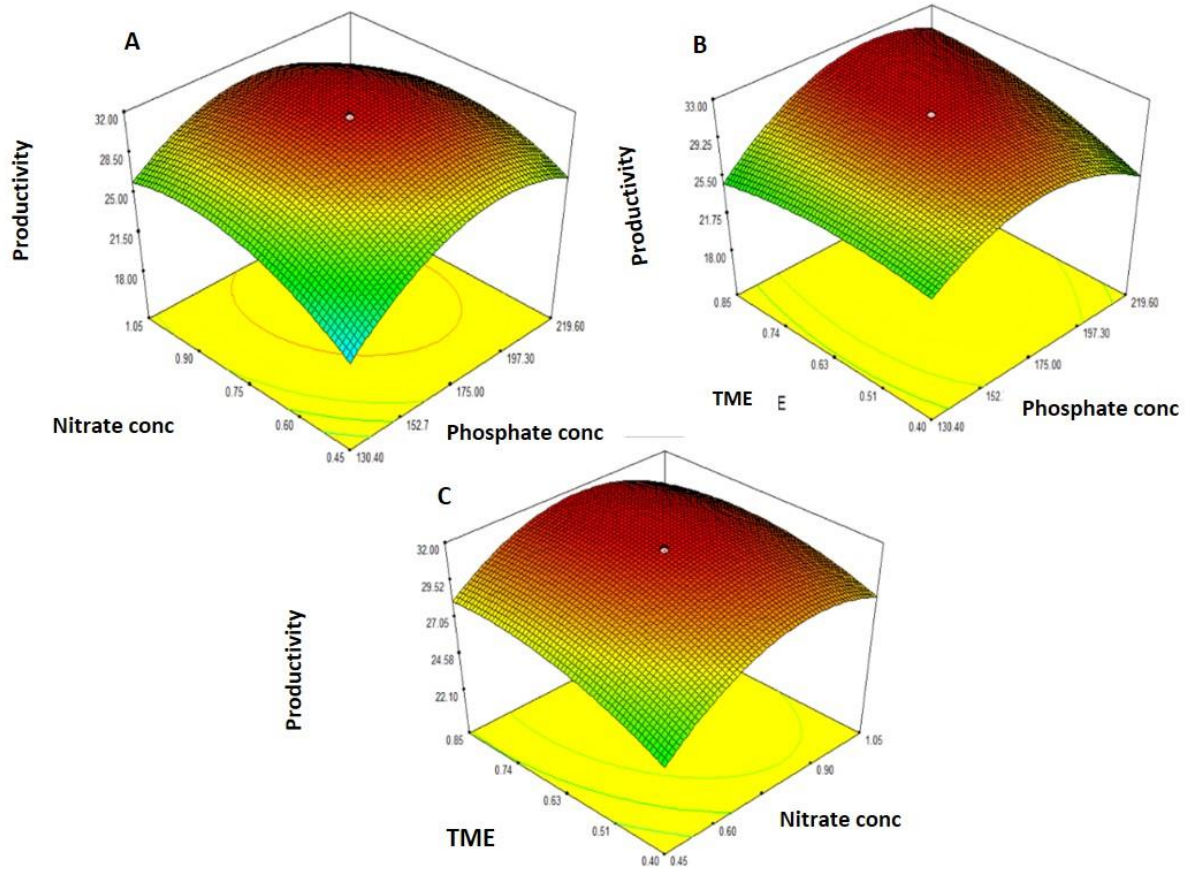

Fig 5.4 Response surface plots representing the interaction effect of (A) phosphate and nitrate, (B) TME and phosphate, and (C) TME and nitrate on biomass productivity. For the interaction of two variables third variable were kept at constant middle value. The middle values of phosphate, nitrate and TME were 0.175 g L^{-1}, 0.75 g L^{-1} and 0.63 units, respectively.

5.3.2 Combinatorial effect of light wavelength and intensity towards production of high-density protein rich biomass

With the aim of developing the strain CT01 as an alternative to conventional aquafeed, it is necessary to enhance not only the growth performance but also intracellular protein content of the biomass. In order to attain our objective, the CT01 strain was subjected to different light wavelength and intensity. Fig 5.5 shows the growth parameters of CT01 cultured under different light wavelength spectra, including biomass productivity, carbon sequestration rate and total protein content of the biomass. Fig 5.5 indicate that CT01 cultured under the combination of red-blue (1:1) wavelength had higher biomass productivity (81.7 mg L^{-1} d^{-1}), carbon sequestration rate (144.1 mg L^{-1} d^{-1}) and total protein content (54.6 %, w/w) as compared to the other light wavelength spectra. This result was corelated with findings from the earlier studies where it has been shown that combination of red and blue light wavelength performs better (Yan *et al.*, 2013). It has been observed that among monowavelength, the growth performance of red light

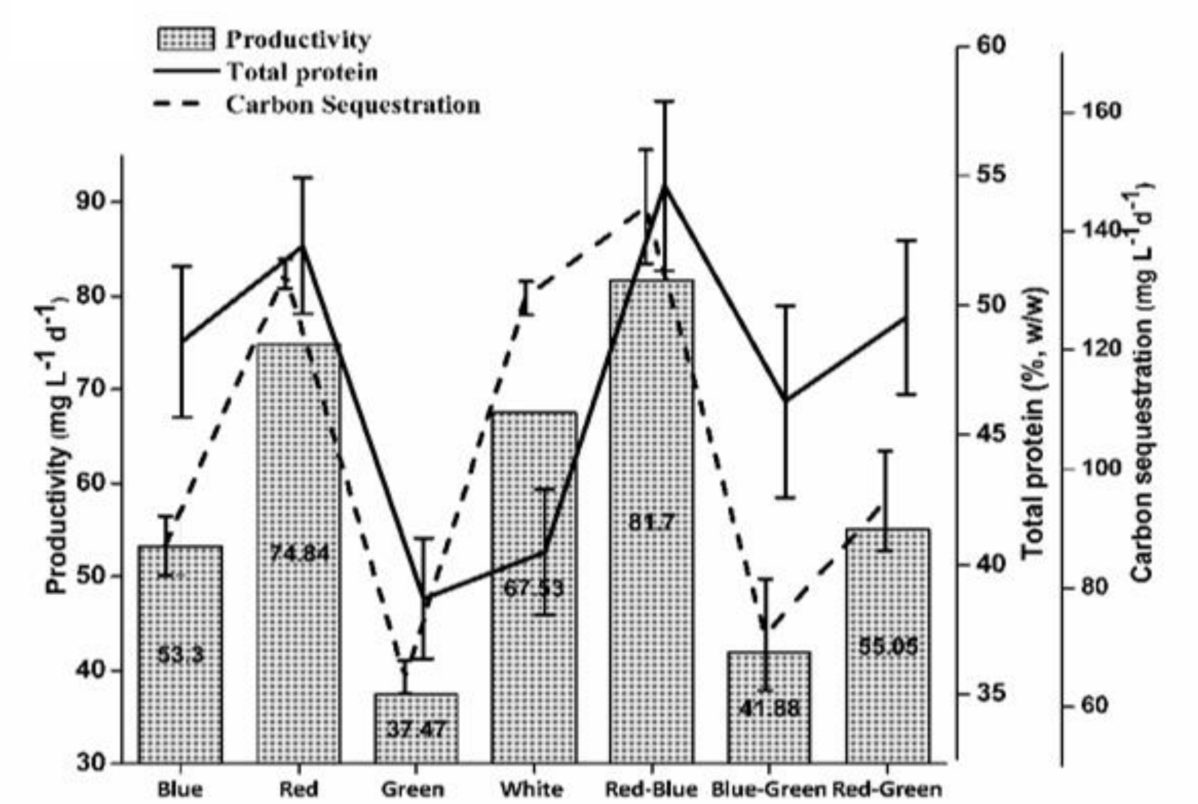

Fig 5.5 Biomass productivity, total protein content of biomass and carbon sequestration rate of *Desmodesmus pannonicus* CT01 under the exposure of various light wavelength.

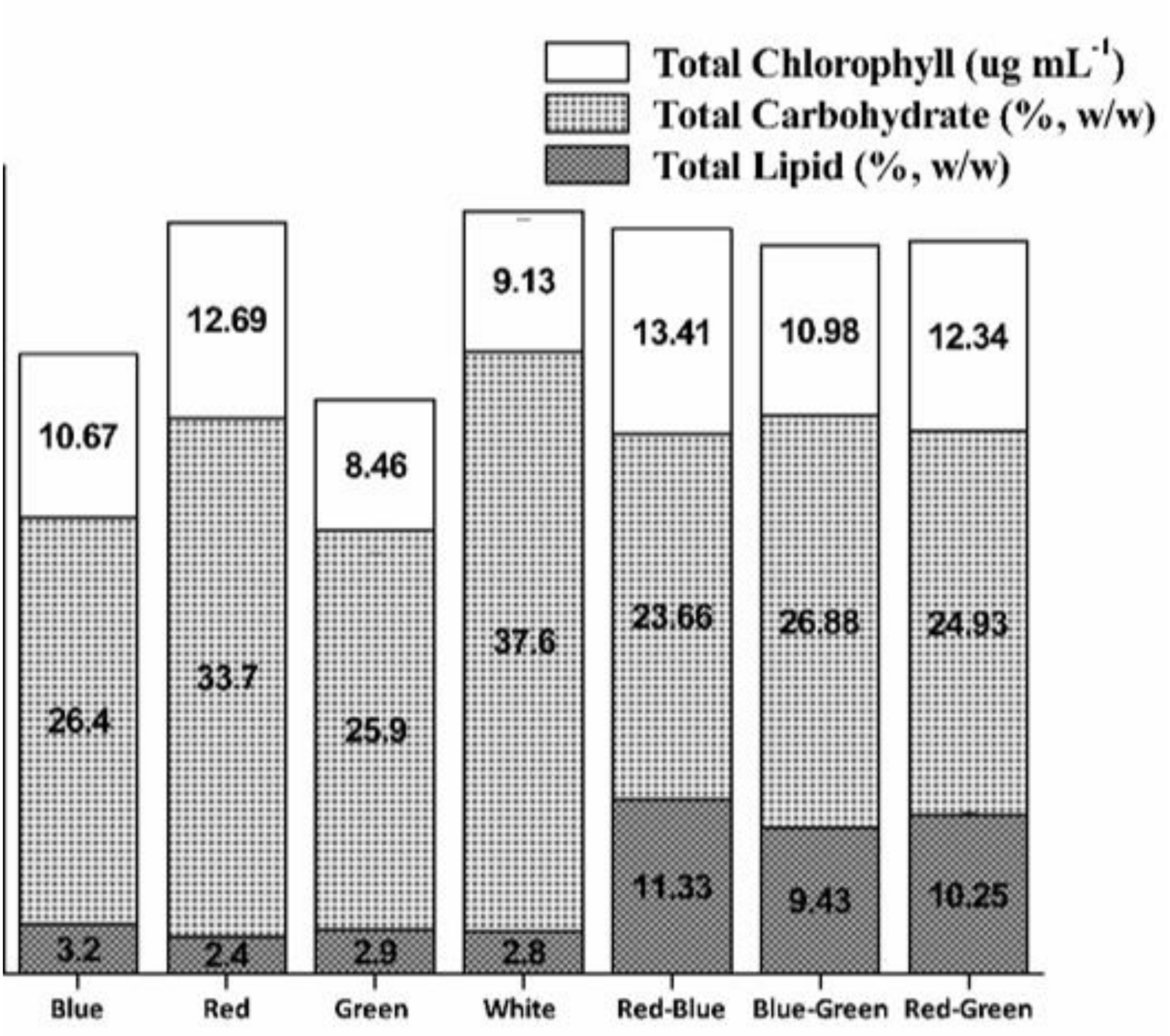

Fig 5.6 Total chlorophyll , total carbohydrate and total lipid content of *Desmodesmus pannonicus* CT01 biomass under different exposure of light wavelength.

was followed by white light then blue light. It is interesting to note that even when white light comprises of full visible wavelength spectra, the combination of red-blue light wavelength performed better in terms of growth kinetics and was found to be 10 % higher in terms of biomass productivity. The growth performance of CT01 in presence of green wavelength or combination of red- green or blue-green wavelength was found to be inferior. As carbon sequestration rate is related to productivity of CT01 cells, similar trends were found among all spectrum of light wavelength. These findings can be corelated with one of the studies where

Chlorella sp. cultured under the blue and red LEDs resulted in higher biomass concentration, productivity and specific growth rate as compared to the white and green LEDs (Choi *et al.*, 2013). However many studies also reported unsuitability of green light for growth of microalgae (Xu *et al.*, 2013; Abiusi *et al.*, 2014). As per previous reports it can be understood that there is a variability among different microalgal species to adopt different light spectra and hence the optimization of illumination is necessary (Ying and Dobbs, 2007; Vadiveloo *et al.*, 2015). It has been earlier reported that sufficient red and blue light should be provided for adequate photosynthesis of microalgae and plants (Kim *et al.*, 2013).

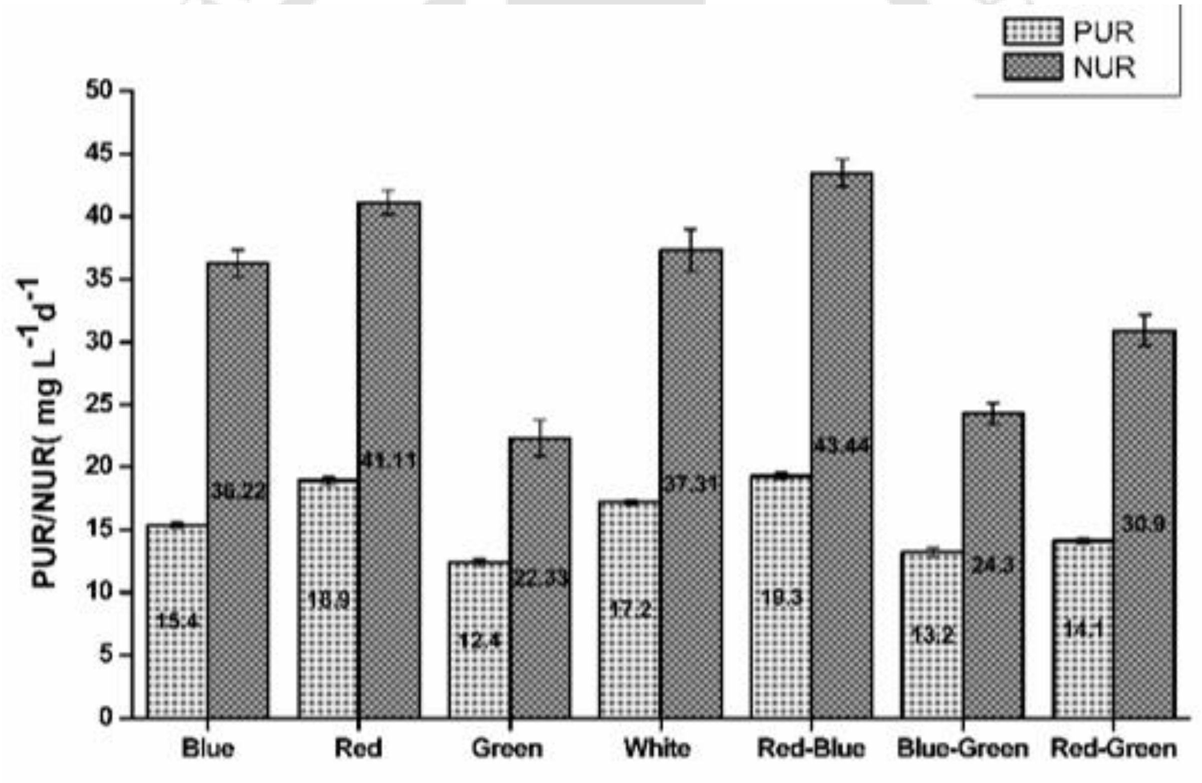

Fig 5. 7 Nutrient utilization rate profile of *Desmodesmus pannonicus* CT01 biomass under the exposure of different light wavelength.

For instance, in *Scenedesmus* sp., chlorophyll a and chlorophyll b absorb light at wavelengths of 450–475 nm and 630–675 nm, respectively, which is required for efficient photosynthesis and hence photosynthetic efficiency was improved when red and blue light wavelength were provided simultaneously. Consequently, microalgaegrowth rate in presence of red-blue light were found to be better than that recorded for red light, blue light, white light, or combination of other wavelength (Kim *et al.*, 2013). Induction of different light spectra to CT01 was performed to enhance the biomass quality in terms of total protein content. Fig 5.5 and 5.6 displayed the biomass composition profile of CT01 strain under different light wavelength. Similar to the growth performance, combination of red-blue light resulted in the highest percentage of total protein (54.6% ,w/w), total chlorophyll (13.41 µg mL^{-1}) and total lipid (11.33%, w/w). However, total carbohydrate content was found to be least in case red-blue light as compared to other spectra. One of the possible reasons can be that blue light induces the upregulation of phytoene synthase and phytoene desaturase, enzymes involved in the production of carotenoids and also photoreceptors of cryptochrome are sensitive to blue light. This might stimulates the production of chlorophyll, light-harvesting complexes, and enhance nitrogen metabolism (Ramanna, Rawat and Bux, 2017). These findings can be corroborated with nitrate utilization rate by CT01 which was also found to be higher for red-blue light as compared to other light spectra (Fig 5.7) . In a similar study the combination of red-blue (7:3) performed well for *Chlorella sp.* in terms of nitrogen removal in wastewater as compared to other light spectra (Zhang *et al.*, 2017). The development of microalgae may be influenced by a variety of external elements (Dean *et al.*, 2010), with the incident light intensity over the cells, being among the most significant. Microalgal autotrophic growth cannot occur without the presence of light, which is also the single most crucial component for photosynthetic activity (Sharma, 2012). It has a role in the proliferation of cells, as well as respiration and photosynthesis(Daliry *et al.*, 2017). Light is required for the production of ATP and NADPH as well as the synthesis of other important chemicals for microalgal development (Singh and Singh, 2015).

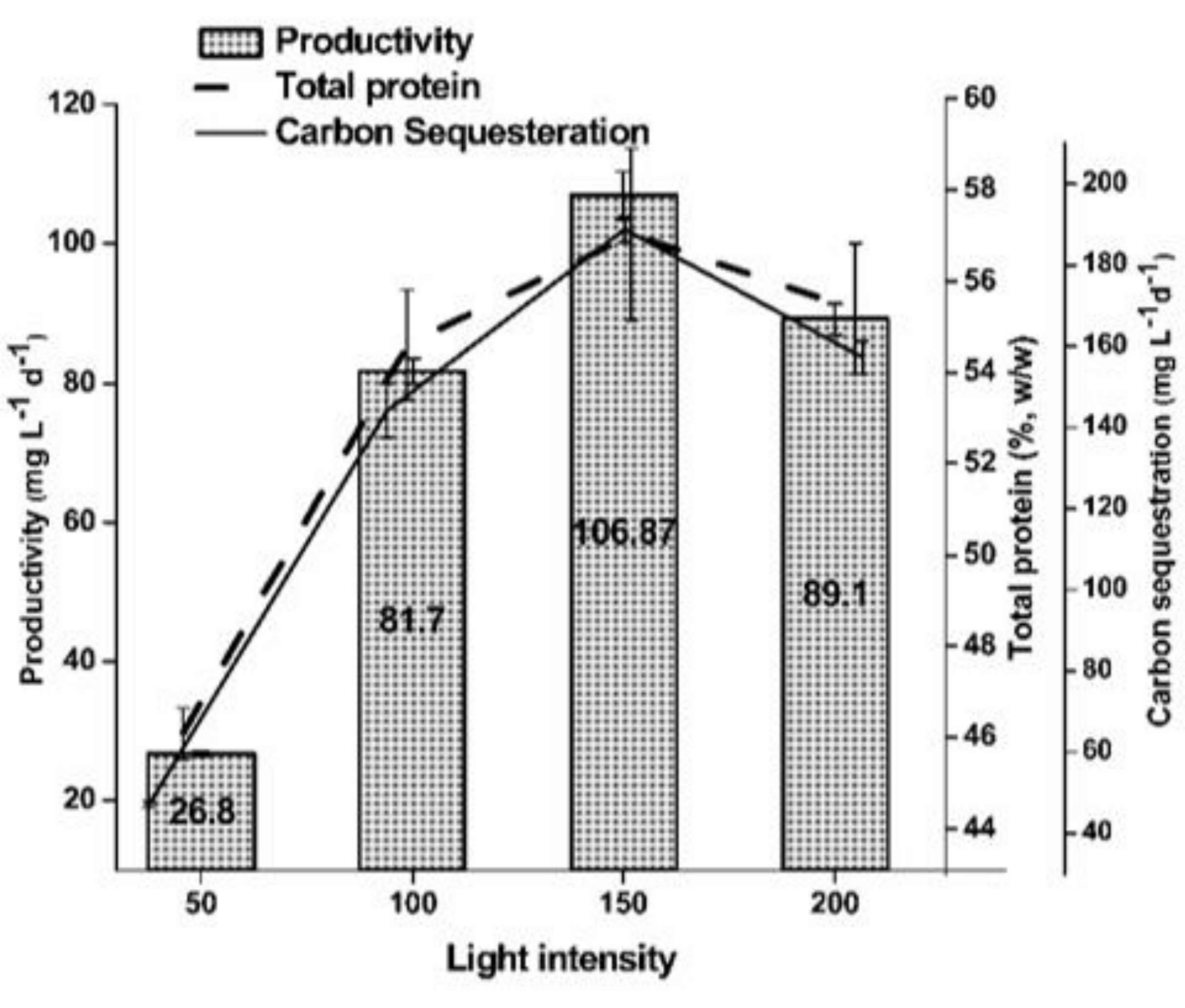

Fig 5.8 Biomass productivity, total protein of biomass and carbon sequestration rate profile of *Desmodesmus pannonicus* CT01 under the exposure of different light intensity.

There are many distinct species of microalgae, each of which has its own optimal light intensity for growth and biomass production. This optimal light intensity also relies on other conditions, such as the temperature and the availability of nutrients in the culture medium (Li *et al.*, 2012). The greater the light intensity, the more likely it is will produce more biomass. This is because the photosynthetic equipment will be able to absorb and use the light more efficiently. However, photo-inhibition was found at high light intensities, beyond the point when the cell has reached its point of saturation (Khoeyi, Seyfabadi and Ramezanpour, 2012). This is because photo-oxidation events are taking place within the cell. This saturation threshold is

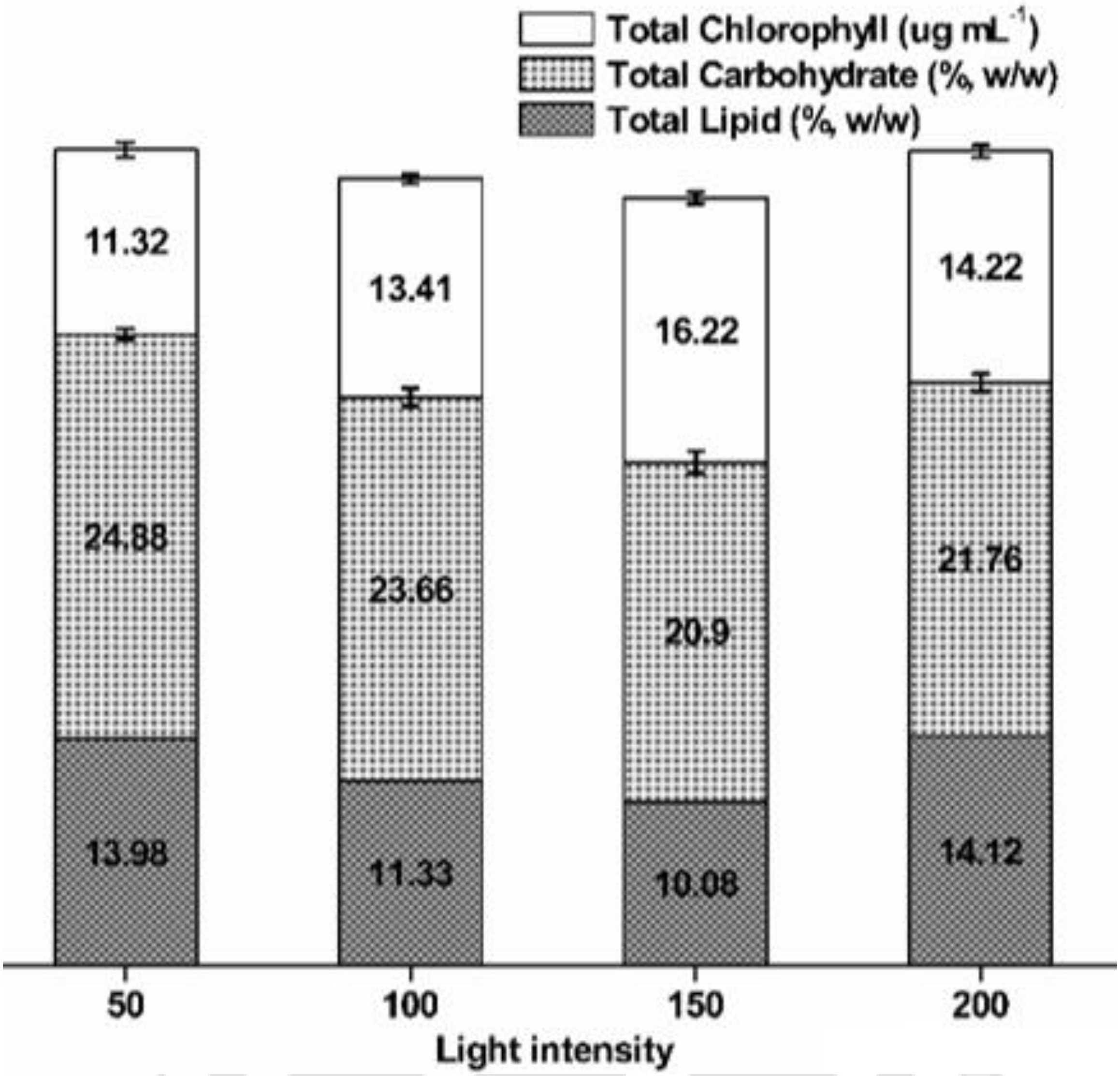

Fig. 5.9 Total chlorophyll, carbohydrate and lipid content of *Desmodesmus pannonicus* CT01 biomass under different exposure of light intensity.

dependent on the specific algae species in combination with growth conditions. In the present study, CT01 strain was subjected to four different light intensities of red-blue light wavelength in the ratio of 1:1. It has been observed that with light intensity of 150 µE m^{-2} s^{-1} the CT01 have highest productivity (106.87 mg L^{-1} d^{-1}), carbon sequestration rate (188.56 mg L^{-1} d^{-1}) and total protein (57 % , w/w) of the biomass as compared to other light intensities including 200 µE m^{-2} s^{-1} . However, total lipid was found to be highest in 50 µE m^{-2} s^{-1} suggesting the stress to the cells because of unavailability of adequate light for the growth. In a similar study where the growth performance of C. *vulgaris* increased when the light intensity with blue LED lamps increased from 100 to 200 μE m^{-2} s^{-1}. However, further increase of light intensity to 200 μE m^{-2} s^{-1} resulted in a decrease in the growth rate (Atta *et al.*, 2013). With the incident light intensity of 150 μE m^{-2} s^{-1} the protein content of CT01 was found to be highest at 57%, w/w. This result can be corelated with one of the studies where total protein content increase with increase in irradiance (Chrismadha and Borowitzka, 1994). Ogbonda et al. (2007) noted that increase in irradiance may result in increase in biomass production and protein biosynthesis in *Spirulina sp.*(Ogbonda, Aminigo and Abu, 2010). The utilization of nutrients was increased linearly with the increase in light intensity, declining at 200 μE m^{-2} s^{-1} (Fig 5.10). This can be explained as utilization of macro nutrients corelated with growth of microalgal cells. Various studies reports with increase in irradiance , the phosphate and nitrate consumption enhances and hence resulting in high density of cells (Dogaris *et al.*, 2015).

5.3.3 Growth performance of CT01 with pH based CO_2 feeding strategy

In case of phototrophic growth of microalgae, pH of the broth provides indirect measurement of CO_2 availability to the organism. The dissolved inorganic carbon is primarily present in the system in three different forms: aqueous carbon dioxide (CO_2 (aq)), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}). The majority of the microalgae are able to use CO_2 (aq) and HCO_3^- as a source of carbon for photosynthesis. However, some species of algae only need HCO_3^- to supplement their intake of CO_2, while others are able to effectively use HCO_3^- on its own (De Paula Silva *et al.*, 2013). The pH has an effect on the distribution of different inorganic carbon species. When the pH is between 6 and 8, either CO_2 (aq) or HCO_3^- may be found in equal amounts. However, when the pH is between 8 and 9, the HCO_3^- form predominates. As the microalgae multiply, the total amount of dissolved inorganic carbon in the water drops, and the pH gradually rises. Following the addition of more CO_2 to the system, the pH will instantly begin to fall until it reaches the target level, and the balance between the various carbon species will be restored correspondingly. Hence to enhance CO_2 utilisation and avoiding any carbon source limitation, a strategy of pH-

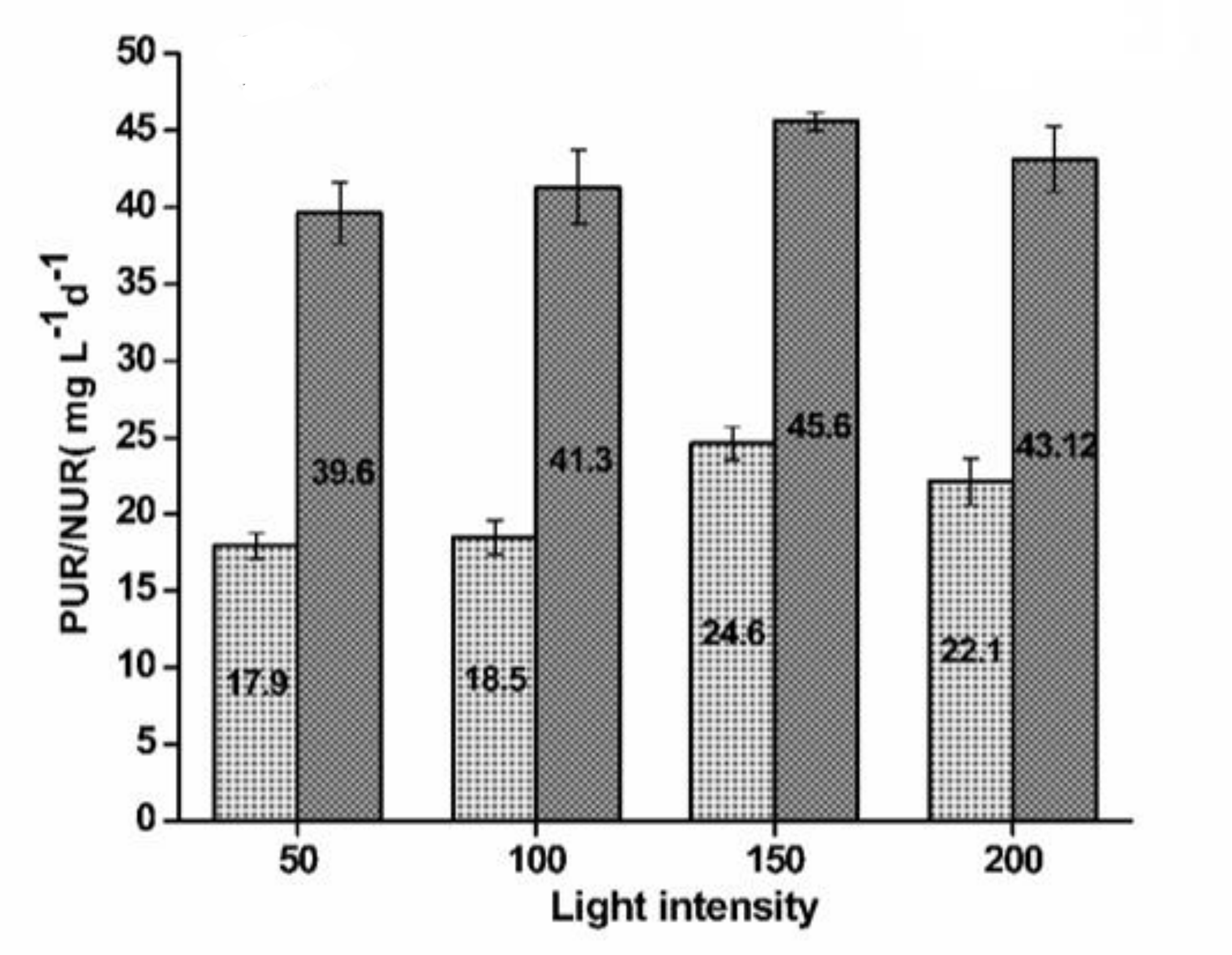

Fig 5. 10 Nutrient utilization rate profile of *Desmodesmus pannonicus* CT01 biomass under different exposure of light intensity.

based CO_2 was employed for CT01 strain. The strategy employed here was successfully demonstrated in our earlier studies (Sinha *et al.*, 2022). A combinatorial approach of combining developed light strategy with pH-based CO_2 feeding was successfully demonstrated. With all optimised culture conditions and developed illumination strategy, two different batches were run *i.e.* one without pH control and other with pH control. The biomass productivity and carbon sequestration rate of pH controlled batch *i.e.*145.37 mg L^{-1} d^{-1} and 259.17 mg L^{-1} d^{-1} respectively was found to be superior when compared to batch without pH controlled i.e 97.93

mg L^{-1} d^{-1} and 174.57 mg L^{-1} d^{-1} (Fig. 5. 13). The results can be corelated with previous studies reporting increment in productivity and carbon sequestration rate when CO_2 was introduced additionally (Choi *et al.*, 2019). The pH profiles of batch were shown in Fig. 5.12 and increment in growth performance could be contributed by maintenance of optimal pH of the culture medium throughout entire course of fermentation. Moreover, no significant difference was found in CT01 microalgal biomass composition in terms of total protein content. However, slight increment of approximately 15% was found in total carbohydrate content in pH-controlled batch. These findings can be corelated with the earlier studies where carbohydrate content in the biomass enhanced with increment in the CO_2 percentage in air inlet stream (Ji *et al.*, 2017). With pH-controlled batch it was observed that the nutrient utilization rate also enhanced by approximately 30% and 20% in terms of nitrate utilization and phosphate utilization, respectively, when compared to uncontrolled batch. In one of the studies, the increment in nitrogen and phosphorous removal efficiency was observed when CO_2 concentration was increased in inlet air stream(Ledda *et al.*, 2015). Nutrient utilization was also found to be enhanced after implementation of CO2 based pH control suggesting favourable condition for the CT01 to proliferate and hence increased growth and productivity along with carbon sequestration rate (Fig 5.14). However, CT01 biochemical composition was found to be almost similar in for both batches i.e pH controlled and without pH controlled.

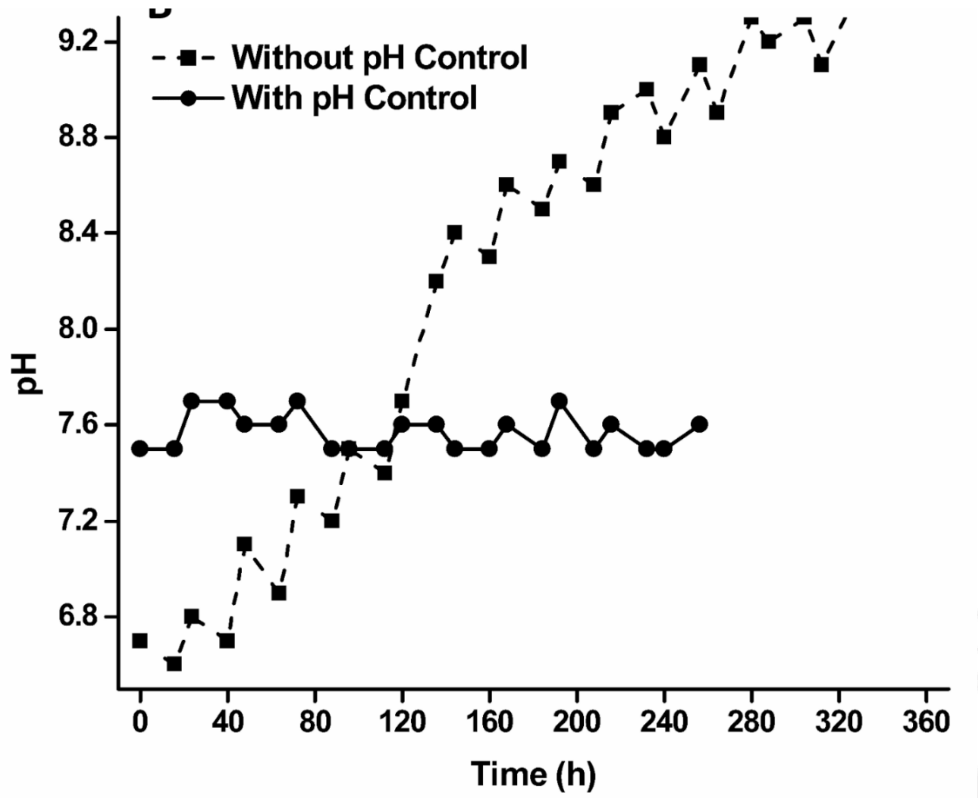

Fig 5. 11 Dynamic profiles of growth of CT01 cultivated without and with pH control employed in combination with the previously developed process engineering strategy for light wavelength and intensity

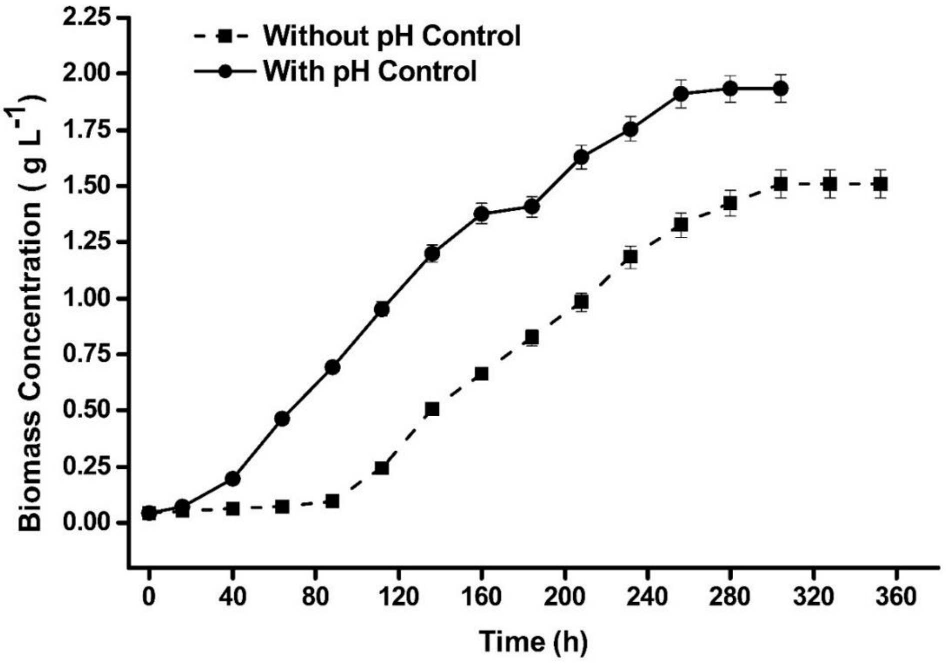

Fig 5. 12 Dynamic profiles of pH of the broth when CT01 cultivated without and with pH control, employed in combination with the previously developed process engineering strategy for light wavelength and intensity

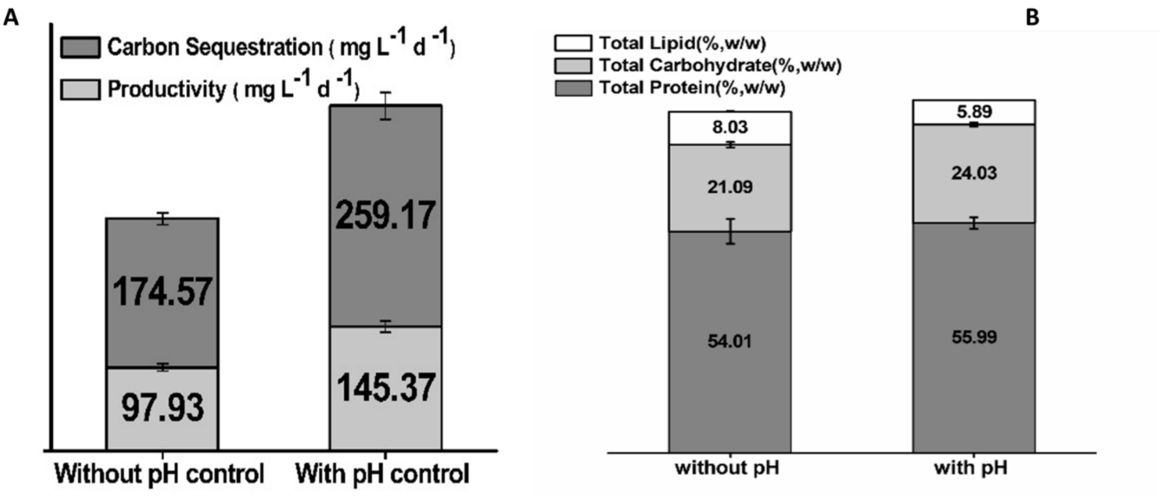

Fig 5. 13 (A) Carbon Sequesteration rate and Total productivity (B) Biomass composition of CT01 batches cultivated without and with pH control employing the previously developed process engineering startegy for light wavelength and intensity.

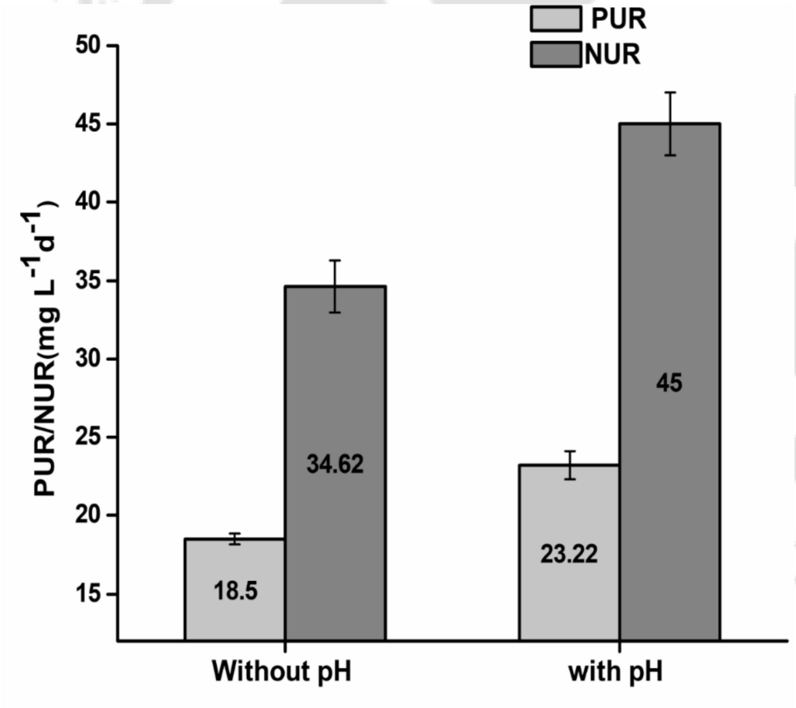

Fig 5. 14 Nutrient utilization rate profile of CT01 batches cultivated without and with pH control employing the previously developed process engineering startegy for light wavelength and intensity.

5.4 Conclusions

Initial concentrations of sodium nitrate, dihydrogen potassium phosphate, and units of TME of BB media were optimized via CCD for maximization of biomass titer and productivity.

CCD-RSM based optimization of the process variables resulted in 28 % increase in biomass concentrations and 27% increase in biomass productivity while compared to un-optimized condition. With optimized BB medium, the CT01 was further subjected to seven different combinations of mono and multi wavelength of light for enhance in the growth parameters and intracellular biomass constituents. The combinations of red-blue light wavelength were found to be optimized for the CT01 in terms of biomass productivity, carbon sequestration rate and total protein content. CT01 was then subjected to different light intensity in presence of optimized medium and combinations of red-blue light wavelength. The light intensity of 150 µE m^{-2} s^{-1} displayed a significant enhancement in biomass productivity and carbon sequestration rate capability of CT01 strain. However, a marginal increment was found in terms of total protein content of the microalgal biomass. The interdependent dynamics of light intensity, growth and culture pH indicated that the process engineering strategy, based on on-demand supply of CO_2 under varied light intensity, may results in improved biomass concentrations and productivity by maintaining optimal culture pH and eliminating CO_2 limitation. The batch with controlled pH resulted in maximum biomass concentrations of 1.93 g L^{-1}, an improvement by 28% when compared with the batch with uncontrolled pH. The phosphate utilization rate and nutrient utilization rate has been found at the highest for pH-controlled batch. It has been observed that nutrient utilization also enhances with the developed strategy suggesting its better application in the areas of wastewater treatment.

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CHAPTER 6

Demonstration of an integrated sustainable microalgal feed technology at large scale of 50 L Airlift Photobioreactor (APBR) towards application of fresh algal cell as live aquafeed

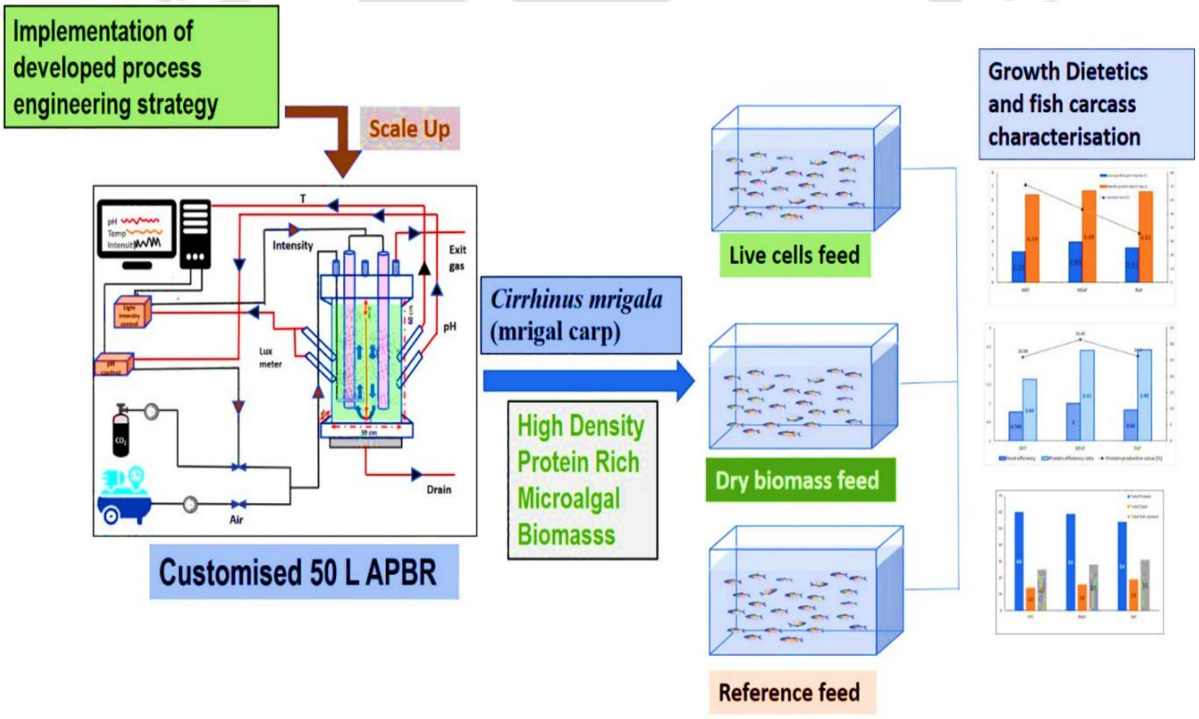

Fig 6.1 Schematic representation of sustainable microalgal feed technology at large scale airlift photobioreactor and its application as live aquafeed

6.1 Background and Motivation

The developed process engineering strategy for the production of high cell density protein rich CT01 biomass was successfully achieved in laboratory scale mini reactor setup of 500 mL working volume. However, to utilize high density protein rich microalgal biomass as aquafeed at commercial scale, there is a dire requirement to successfully implement the strategy at large scale cultivation system. This necessitates designing of a customized photobioreactor (PBR). Designing PBR for microalgal culture remains challenging. Most of the reactors are developed and scaled up using semi-empirical methods (Huang *et al.*, 2017), making them inefficient and expensive. PBR provides several benefits over open-air systems, including greater photosynthetic efficiency, better biomass concentration and areal production, reduced contamination, elimination of evaporation-caused water loss, and perfectly regulated growing environment (Voloshin *et al.*, 2016).. As optimum biomass productivity must be maintained to counteract high production costs; key process parameters such as mass transfer, sufficient mixing and adequate light availability to cells must be efficient(Wang, Lan and Horsman, 2012). In auto phototrophic cultivation, a typical PBR is a three-phase system consisting of culture media as the liquid phase, the cells as the solid phase, and CO_2-enriched air as the gas phase. As a distinguishing characteristic, the light in a PBR is a radiation field with a superimposed fourth phase (Posten, 2009). Due to the absorption and scattering of light by fluids, the radiation field in a PBR is very heterogeneous. It is well known that light availability is the limiting element in PBR cell development (Richmond, 2004; Huang *et al.*, 2011). Most outdoor photobioreactors, including flat-plate, horizontal, and inclined tubular photobioreactors, have lightning-exposed surfaces. Bubble-column, airlift, and stirred-tank photobioreactors provide excellent scalability, but their modest light surface areas restrict their use in outdoor cultures (Oncel and Sukan, 2008; Ranjbar *et al.*, 2008; Kumar *et al.*, 2011; Zhang, 2013).

Microalgae can be used in aquaculture feed in different forms, and the mode of application depends on the fish, type of aquaculture, and product. The dried microalgal biomass has been already established as a potential alternative to conventional feeds available for aquaculture in terms of improved palatability and feed nutritional quality (Gamboa-Delgado and Márquez-Reyes, 2018). The sustainability of this process is still in the questions because, cultivation of microalgae and then further its alteration as an aquafeed is still a luxurious outflow, creating burden to its commerciality (Ferreira *et al.*, 2019; Carballeira Braña *et al.*, 2021). Utilizing live microalgae as feed has several benefits. All nutrients and beneficial compounds are retained in active microalgae, which may be fed directly to fish. Protein, vital amino acids, oil containing PUFAs, carbohydrates, pigment, minerals, and vitamins may be obtained from live microalgae. Various attempts have been taken in the past to develop an economically feasible processes such as feeding live microalgal cells to zooplanktons (Kandathil Radhakrishnan *et al.*, 2020), a natural feed for fish, co cultivation of fish and microalgae (Benemann, 2013), etc. These attempts have been done in laboratory scale and hence never comes to scale of commercialization because of limitations in implementation and instability of expected outcomes.

In the present study, a novel large-scale airlift photobioreactor (APBR) was designed and customized considering critical factors such as mass transfer, mixing and efficient illumination strategies. Further, a process engineering strategy with a combinatorial approach of optimized culture medium, manipulation of light wavelength-intensity and pH guided CO_2 feeding strategy was employed for the cultivation of the strain in the customized photobioreactor. A detail characterization of growth kinetics and nutritional composition of the biomass was carried out and compared with the performance of that achieved at laboratory scale. Furthermore, the protein rich fresh CT01 biomass cultivated in APBR was evaluated for its application as alternative live feedstock for model fish *Cirrihinus mrigala* (mrigal carp).

Growth dietetics performance and total carcass composition of the mrigal carp was assessed and compared with dried microalgal biomass of CT01 and reference diet.

6.2 Materials and methods

6.2.1 Inoculum preparation

Inoculum of CT01 strain was prepared in optimized BBM as described in the section 3.2.1. Active log phase culture of microalgae was used as inoculum for all the experiments. For experiments in 50 L customised airlift photobioreactor, inoculum was prepared using optimized BB medium in 7.5 L automated photobioreactor (Bioflo115®, Eppendorf, Germany) with 5 L working volume, operated at 200 rpm agitation, 0.5 vvm (volume of air per volume of culture per min) aeration rate and total incident light intensity of 150 µE m^{-2} s^{-1} with the combination of red-blue wavelength in the ratio of 1:1 with the photoperiod of light:dark 16:8 h. Inoculum size of 10 % with cell density of 2 g L^{-1} was used in every batch. The experiments were carried out in customised photobioreactor mentioned in the section 6.2.2. All the experiments were performed in room temperature and under no pH control.

6.2.2 Cultivation of CT01 in customised 50 L arlift photobioreactor

The growth performance of CT01 was evaluated under optimized cultivation condition in customised stainless-steel airlift photobioreactor (APBR) of 50 L with working volume of 35 L. The APBR (Fig 6.2) was built using 4 mm thick stainless-steel panel with dimensions of 60 (H)× 40 (L)× 30 (W) cm and equipped with the automated CO_2-based pH control and pH data logging facility. The reactor was equipped with *in-situ* sterilization facility via steam generator and all the ports were air tight to eliminate the contamination from external environment. One of the key design considerations of APBR is the placement of hanging tubes to accommodate LED light panel for providing light into the culture medium. These hanging tubes are placed inside the reactor and are submerged into the culture medium. There was a total of eight LED panels equipped with 16 15W LED cells (8 each of red and blue colour LED). With all the hanging tubes equipped with LED panels, the average light intensity inside the reactor was measured in between 100- 400 µE m^{-2} s^{-1}. The temperature of LED hanging down tubes were controlled by circulating water through glass pipes to maintain the temperature around 25 °C on the surface to avoid the damage to LED panels and microalgal cells. The light intensity inside the APBR was controlled and maintained using SCADA based controlled system. The temperature data was measured and stored using the same SCADA system. The cells were kept in suspension by aeration using tube sparger made up of soft PVC pipe with pore size of 100 µm. The sparger was mounted on head plate with the help of stainless steel pipe and was placed 10 cm above the bottom surface of the reactor. A transparent removable square baffle made up of polycrylic sheet of dimesnsion (30 cm ×40 cm) was placed at the the centre of the reactor fixed with side walls and 5 cm above the bottom surface of the reactor. Aeration rate required for the scaled-up condition was calculated based on the criteria of equal volumetric power consumption rate (detailed calculation is given in the Appendix section A 2). The culture was grown in optimized BBM medium and on-demand CO_2 feeding was performed to maintain the culture pH at its optimal range of 8-8.5 throughout the entire course of fermentation. Sampling was performed at regular time interval to obtain growth kinetic of the organism. The reactor was equipped with the facility to control the temperature inside the reactor. However, in these experiments temperature was not controlled and was found to be in the range of 26-30 °C which is suitable for the CT01 growth. To harvest the cells or drain the culture, a controllable manual valve was provided at the bottom of reactor.

6.2.3 Assessment of different types of feedstocks for fish growth metrices and dietetics

Study on live microalgal feed (LiF), dry microalgal feed (DrF) and reference feed (ReF) for fish growth metrices and dietetics was carried out in duplicate in rectangular glass aquarium. Each aquarium (1.5 (L) × 1.5 (B) × 1(H) ft) containing 55 L water, was equipped with aeration device to provide oxygen in water and was kept under 12:12 h light-dark photoperiod. 25-days old *Cirrihinus mrigala* (mrigal carp) procured from a nearby hatchery JNB Aqua Agro Pvt Ltd, India and used as a model fish for this study. LiF was prepared by cultivation of CT01 in 50 L customised APBR followed by harvesting using

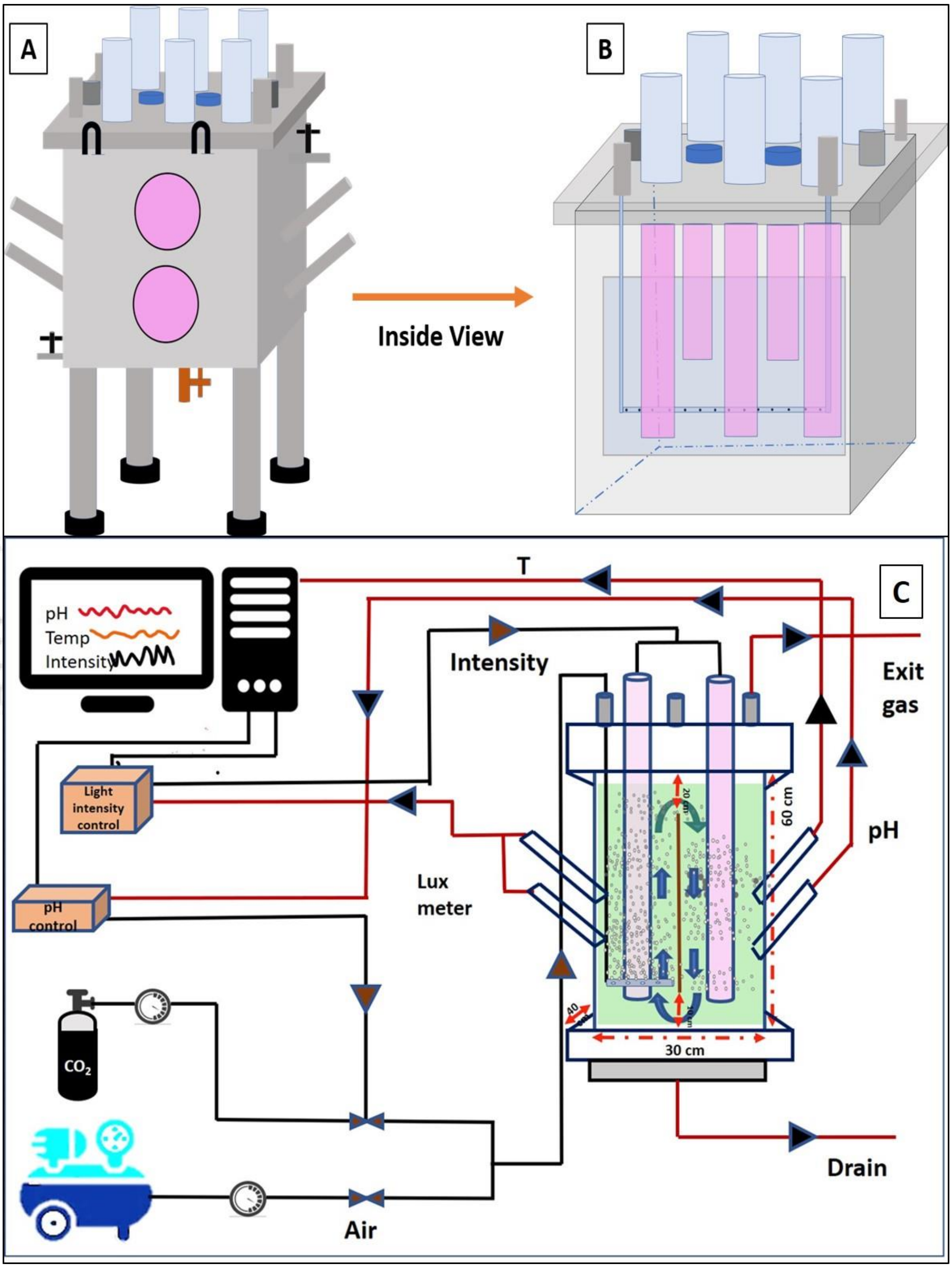

Fig 6.2 Schematic diagram of 50 L APBR showing working principle of the bioreactor including pH based CO_2 feeding system and mixing pattern.

nylon sterile filter at the end of active log phase and sebsequent concentration by centrifugation at 3000 g for 5 min. The LiF was stored at 4 °C for subsequent experiments. 20 mL of LiF (maximum of three days old) with desired cell density was fed every time to fish as per the requirement of daily ration. In case of DrF, post centrifugation, the microalgal cells were lyophilized and stored at 4 °C. ReF was prepared as reported in the section 4.2.4. Before initiation of feeding trials, small fish fry were acclimatized for 72 h in a reservoir tank of 500 L capacity and were fed with the standard feed (ReF) followed by feed deprivation for 24 h. Post-acclimatization, 150 number of fry were transferred to each aquarium from the reservior. The feeding trial was conducted for the duration of five weeks (35 days). Each of the three tanks (in duplicate) were fed with their respective feedstock thrice a day at 0600 h, 1200 h and 1700 h as per daily ration up to apparent satiation. The parameters such as dissolved oxygen (DO), total dissolved solid (TDS) and concentrations of total nitrate , ammonium and phosphate of the water were kept within permissible limits by replacing about 10-15% of water at an interval of one week. The temperature, DO and TDS of the water was measured throughout the course of feeding trials using water testing kit (Hach, USA). The concentration of total nitrate and phosphate of water were measured as per section 3.2.5. Concentertaion of ammonium was measured using the water testing kit (Hach, USA). The temperature of aquarium was found to be in the optimal range of fish growth. In order to obtain initial body characteristics of the fish fry such as length, weight and biochemical compositions, 15 individuals were randomly selected from the reservior tank and euthanized using clove oil (0.4 mL L^{-1}) on the first day of feeding trial. 10 numbers of fry from each treatment group was euthanized at an interval of seven days for profiling of growth metrices and dietetics such as weight (g), length (cm), average weight gain (mg day^{-1}) , specific growth rate (SGR, % day^{-1}), feed eficiency (FE, %), protein efficiency ratio (PER), protein productive value (PPV, %) and survival rate was estimated as mentioned in the section 4.2.2.

6.2.4 Analysis of growth of CT01 and its substrate utilization

The sample collected at regular time interval was centrifuged at 8000 × g for 10 min at 4 °C to separate supernatant and pellet. While the pellet was used for estimation of growth and biomass intracellular constituents' measurement, the supernatant was used for the analyses of substrate utilization. Cell growth was obtained by measuring optical density of the cells as mentioned in the section 3.2.4. Growth kinetics were calculated as per the empirical formula mentioned in the section 3.2.3. The utilization profile of nutrients such as nitrate and phosphate were carried out as mentioned in the section 3.2.5.

6.2.5 Biochemical composition of microalgal biomass and fish carcass

Total lipid, total protein and total carbohydrate of microalgal biomass was estimated as explained in the section 4.2.2. Total protein, total moisture content, ash content and total lipid of fish carcass was estimated as mentioned in section the 4.2.2.

6.2.6 Statistical analysis

All experiments were determined in biological triplicate to ensure the reproducibility. Results were expressed as mean value ± standard deviation. All data are presented as means of standard error. Tukey's multiple range tests were used to identify significant differences between any two means that differed at $p < 0.05$.

6.3 Results and discussions

6.3.1 Development of customised airlift photobioreactor clubbed with pH guided CO_2 feeding strategy for large scale cultivation of CT01

Light penetration and distribution inside the reactor are the determining variables in the construction of an effective PBR for microalgal cultivation. Furthermore, efficient mixing and mass transfer, as well as appropriate temperature and pH, may considerably enhance the development of microalgae. Previous reports have shown that the airlift bioreactor is one of efficient reactor for microalgal cultivation, owing to its relatively better mixing efficiency and gas-liquid mass transfer (AL-Mashhadani, Wilkinson and Zimmerman, 2015). CT01 growth profiles (Fig 6.3), pH profiles (Fig 6.4), biomass compositions (Fig 6.5) and nutrient utilisation rate (Fig 6.6) when cultivated in customised APBR are shown. When cultivated with uniform light intensity of 150 μE m^{-2} s^{-1}, gas flow rate of 6 L min^{-1} along with pH based CO_2 feeding, the maximum biomass concentration of CT01 of 1.75 g L^{-1} with biomass productivity of 142.21 mg L^{-1} d^{-1} and carbon sequestration rate of 253.52 mg L^{-1} d^{-1} was achieved, which, is approximately 113% , 136 % and 137 % higher than the batch run without pH control. The nutrient utilization rate was also found to be higher in the case of pH-based CO_2 feeding batch. pH profile of both batches advocates the importance of maintaining optimal pH throughout entire cultivation period. Even with large scale cultivation with working volume of 35 L, the growth performance of CT01 was found to be similar to the laboratory scale batch of 500 mL. A suitable explanation of such negligible difference in growth parameters, even with scale up, can be attributed to characteristics of large scale photobioreactor in terms of efficient availability of photons to the microalgal cells, proper mixing through the baffle placement and adequate aeration rate. Table 6.1 shows comparison between current study and other similar studies on different species and reactor types and its impact on microalgal growth and biomass composition. The performance of current customised reactor was found to be at par with other similar studies in terms of carbon sequestration rate and protein composition of CT01 strain. However, there are very few reports which showed better results compare to current study and it is important to note that the performance of the reactor and strains are very specific in nature as mostly designed or cultivate depending on the objective of that particular study. Our research demonstrated the viability of establishing a reasonably simple and inexpensive photobioreactor for the production of CT01 strain. Similar bioreactor may potentially be appropriate for other microalgal species. Compared to washing, sterilising, and irradiance source, this APBR lowers labour and downtime. Internal lighting and automatic CO_2 injection/pH management allow for increased energy efficiency and higher cell densities. The larger volume of the PBR makes monitoring and control devices economically viable, enabling higher cell densities and a smaller footprint. Further development of the PBR might enable continuous production in which the ideal density of the culture is maintained by constant harvesting and water replenishment. Even while environmental control in our system was not very precise, it was nonetheless reliable in generating quantities of harvestable algae that were significant enough for commercial purposes.

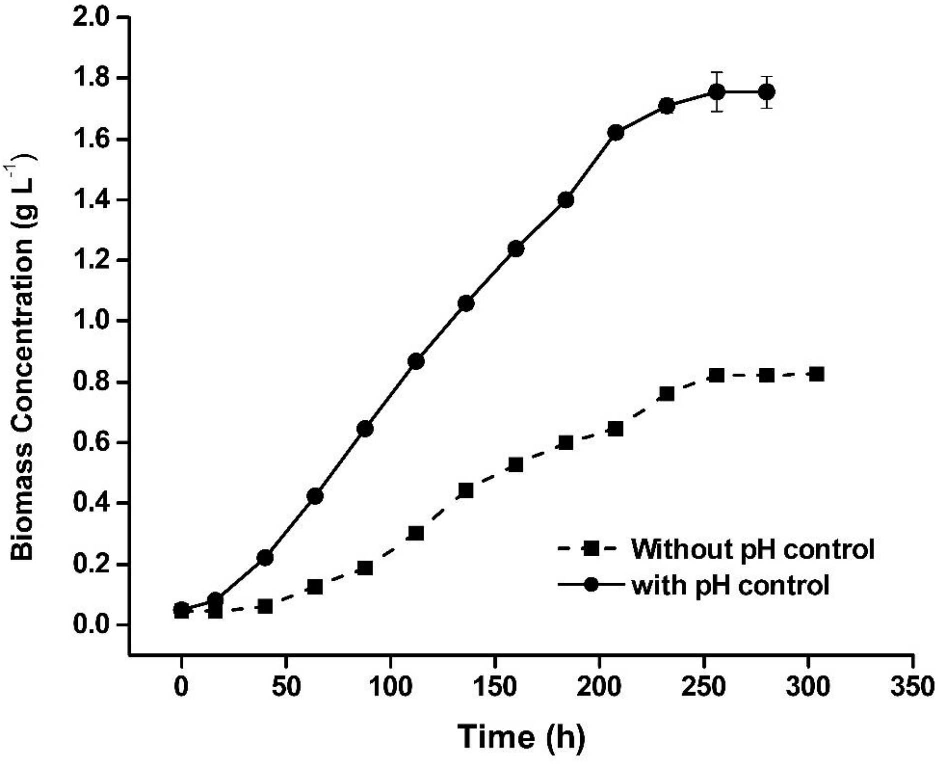

Fig 6.3 Dynamic profiles for growth of CT01 cultivated in APBR without and with pH control employing the previously developed process engineering strategy for light wavelength and intensity

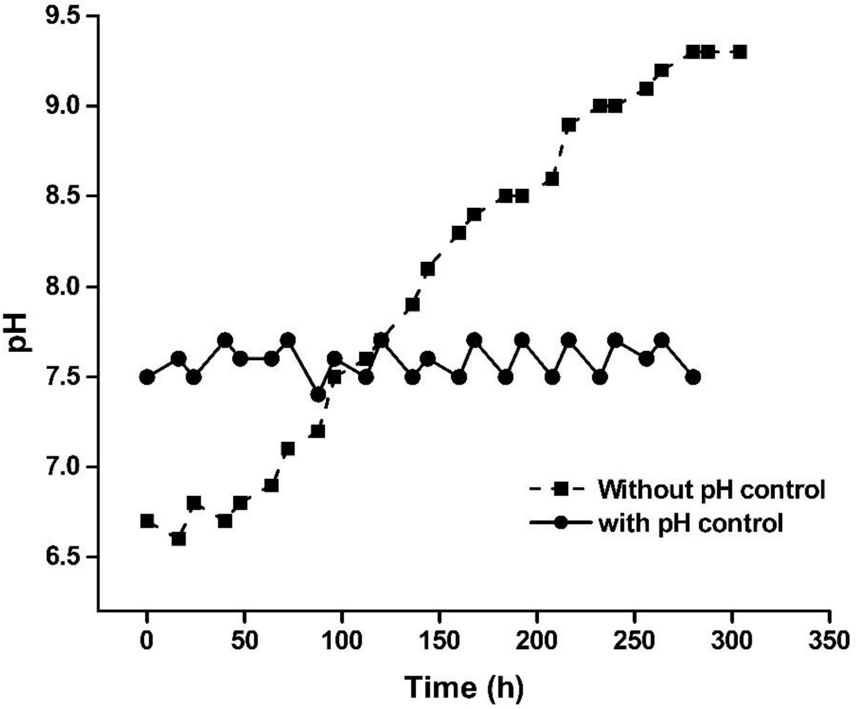

Fig. 6. 4 Dynamic profiles of culture pH when CT01was cultivated in APBR without and with pH control employing the previously developed process engineering strategy for light wavelength and intensity.

Table 6.1 Performance comparison of current customised airlift photobioreactor in terms of various parameters with reported studies.

Species	**Cultivation Time (Day)**	**Mode**	**Working Volume, L**	**CO_2 (% v/v)**	**Max. Biomass Produced**	**Biomass Productivity**	**CO_2 Fixation Rate (R_{CO2})**	**TC**	**TP**	**TL**	**Ref**
Chlorella sp.	8	Column Photobioreactors	0.8	2	1.21	0.15	0.28				(Chiu *et al.*, 2008)
Chlorella vulgaris	15	Bio Flow fermenter	8	10	1.94	0.13	0.25	16.74	40.95	9.95	(Sydney *et al.*, 2010)
Scenedesmus obliquus	6	Erlenmeyer flask	0.65	10	1.84	0.15	0.29			22	(Tang *et al.*, 2011)
Chlorella sorokiniana	8	Airlift photobioreactor	1.4	4	1.1	0.15				20.93	(Kumar *et al.*, 2014)

Scenedesmus sp.	7	Airlift photobioreactor	0.5	2.5	1.3	0.19	0.35	10.4	-	35.6	(Nayak, Karemore and Sen, 2016)
Scenedesmus sp.	7	Bubble-column photobioreactor	0.5	2.5	1.37	0.196	0.37			33.3	(Nayak, Karemore and Sen, 2016)
Acutodesmus sp.	5	Erlenmeyer flasks,	0.2	20	1.65	-		34.52	38.78	11.67	(Yadav *et al.*, 2015)
A. quadricellulare	6	Laboratoy scale photobioreactor	0.68	5	1.29	-	-	33.4	30.3	44	(Varshney *et al.*, 2016)
Desmodesmus sp. MCC34	18	Raceway pond	1000	-	1.9	-	-	-	-	0.103	(Nagappan and

											Verma, 2016)
Porphyridium cruentum		Airlift tubular,	200	-	3	1.5	-	-	-	-	(Yen *et al.*, 2015)
Chlorella sorokiniana		Inclined tubular	6	5	1.5	1.47	-	-	-	-	(Ugwu, Ogbonna and Tanaka, 2002)
Arthrospira platensis		Undular row tubular	11	-	-	2.7	-	-	-	-	(Carlozzi , 2003)
Phaeodactylum tricornutum	9	Outdoor helical tubular,	75	-	2.95	1.4	-	-	-	-	(Hall *et al.*, 2003)
Haematococcus pluvialis	16	Bubble-column,	55	-	1.4	0.06	-	-	-	-	(López *et al.*, 2006)

Chlorella pyrenoidosa	1.25	Tubular batch reactors,	0.66	10	-	0.11	0.096	-	-	-	(Kargupta, Ganesh and Mukherji, 2015)
Chlorella pyrenoidoisa ZU1	4.5	Cylindrical PBR	6	15		0.47	0.87	-	-	-	(Ye *et al.*, 2018)
Desmodesmus sp.	12	Loop photobioreactor,	26	0.03	0.96	0.018	0.013	14.6	14.4	15.5	(Anand *et al.*, 2021)
Desmodesmus sp.	12	Loop photobioreactor,	26	5	1.219	0.084	0.155	17.2	25	40	(Anand *et al.*, 2021)
Desmodesmus sp.	12	Loop photobioreactor,	26	10	1.903	0.185	0.333	20.7	32.3	42	(Anand *et al.*, 2021)

Desmodesmus pannoniccus CT01	14	Customisd Airlift photobioreactor	35	0.03	0.786	0.06	0.106	22.01	53.99	6.97	Present study
Desmodesmus pannoniccus CT01	12	Customisd Airlift photobioreactor	35	pH based CO_2 feeding	1.871	0.142	0.253	23.09	56.01	4.72	Present study

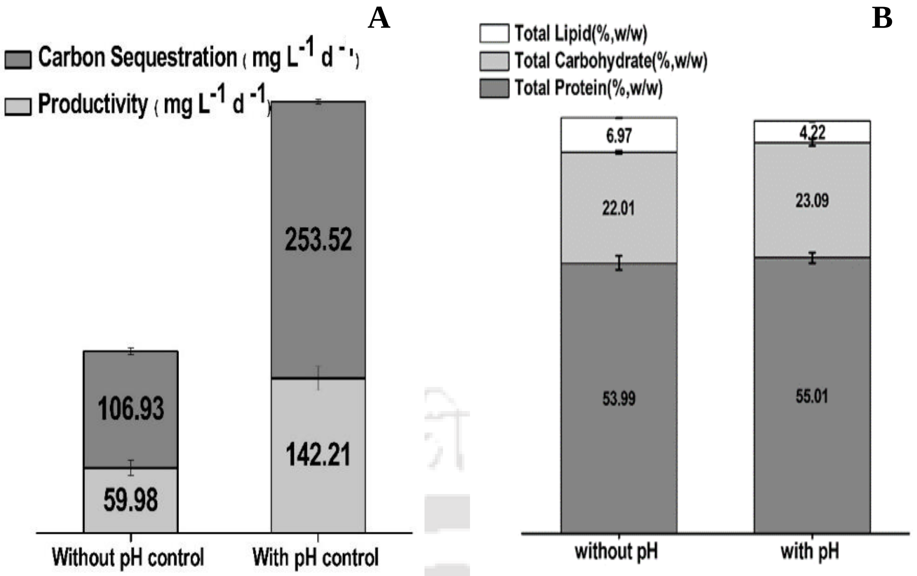

Fig. 6.5 Profiles of (A) carbon Sequesteration rate and biomassproductivity; (B) Biomass composition of CT01 batches cultivated without and with pH control employing the previously developed process engineering startegy for light wavelength and intensity

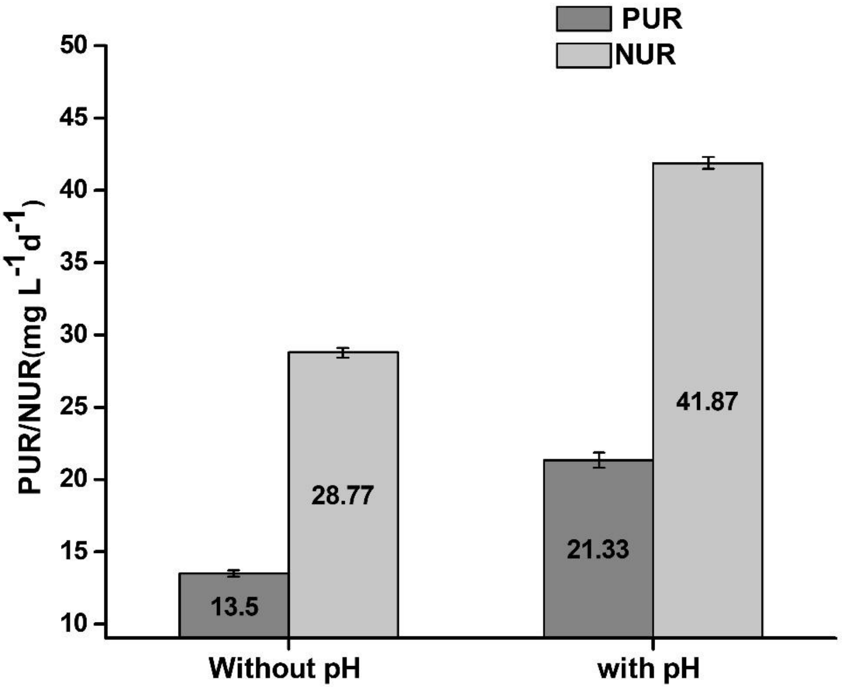

Fig 6.6 Nutrient utilization rate (Phosphate utilization rate (PUR) and Nitrate utilization rate (NUR)) profile of CT01 cultivated in APBR without and with pH control employing the previously developed process engineering startegy for light wavelength and intensity.

6.3.2 Evaluation of fresh microalgal cell as alternative aquafeed

The aquaculture industry is an efficient edible protein producer and grows faster than any other food sector. Therefore, it requires enormous amount of fish feed. Fish feed directly affects the quality of produced fish, potential health benefits, and cost. Determining relationships between dietary nutrient composition, juvenile growth and survival is essential to optimise feeding protocols that, in turn, support development of more appropriate procedures. An important part of this is to generate an understanding of the relative rates of consumption of particular diet that provide information on nutrient availability and the subsequent nutritional value of the diet. In the current study, the live fresh cells of CT01 was evaluated for their potential to be utilized as alternative to the commercially available fish feed. Growth dietetics of fish fry of mrigal carp fed on diets with three different types of fish meal is shown in the Table 6.2. While, growth of the fish fry on all three diets were found to be comparable, the parameters such as final body weight (mg), average weight gain (AWG, mg day^{-1}) and specific growth rate (SGR, % day^{-1}) was recorded to be in range of 312-347, 8.48 – 9.48 & 6.39-7.63, respectively (Fig 6.7). However, with performance in slightly lower side, the results indicate that the fresh cells of CT01 can be suitable as complete replacement of reference fish feed. The feed efficiency and protein productive value was found to be in the similar range for LiF when compared to DrF and ReF, however a significant difference was observed in terms of protein efficiency ratio for LiF and DrF with respect to ReF (Fig 6.8). One of the possible explanations could be the high content of protein present in the biomass of CT01 and it might not get utilized by the fish. The survival rate of fish was found to be similar in the LiF and DrF fed aquariums as compared to ReF fed. Enhanced immunity, post-trial test, was also evident from the fish fry fed with microalgal diets, as reflected by their high survival rates. Formation of natural food and interaction of the fish with other organisms may also be a factor that can enhance their growth, increase their capability to assimilate feeds efficiently, and promote high survival rates. There are limited studies regarding the dietary inclusion of live microalgal cells on juvenile fish as a completed replacement of fish feed. However, few studies demonstrated the ingestion of live microalgae and microalgae concentrates in the larvae of *H. scabra* and it has been observed that microalgae were digested rapidly in the older larvae hinting the microalgae as feed for juvenile fish(Duy, Pirozzi and Southgate, 2015).

Biochemical composition of the whole fish carcass fed by the different types of feedstocks at the end of experimental period is shown in Table 6.3. The protein content was found to be in the range of 42-46 %, w/w where LiF and ReF represent the highest and the lowest content, respectively. It is important to note that, while growth matrix and dietetics was found to be inferior in case of LiF, the protein content and total ash content founds to be better than any other feed. This suggests better digestibility of live microalgal protein by the fish as compared to dried microalgal and reference diet. Total lipid content was found to be in reverse order with, lowest value of 8 % in case of LiF followed by 12% in case of DrF and 21% for ReF. This reverse trend of lipid content may be attributed to lower crude lipid content present in microalgae feed. Similar to the lipid profile, the lowest ash content in the LiF fed fish corroborates well with the feed ash content. The total dry matter content of the fish was found to be similar irrespective of feedstock type.

Feasibility of considering live microalgae cells as potential supplement or replacement of conventional aquafeed would largely depend on both biomass composition of microalgae species and the species of fish under consideration. Our results showed a strong correlation between dietary protein level and performance of early mrigal carp juveniles. The studies for mrigal carp fed with live microalgae is scarce, however different species of live microalgal cells were studied with juvenile sandfish where concentrates support the growth rate and shows no negative effect on sandfish. (Duy, Francis and Southgate, 2017) Micro-algae such as *Chaetoceros muelleri* (Knuckey *et al.*, 2006)and *Tisochrysis lutea* (Cardinaletti *et al.*, 2018)have been used as a food for early juveniles of carp fish and displays no negative effect on the growth.

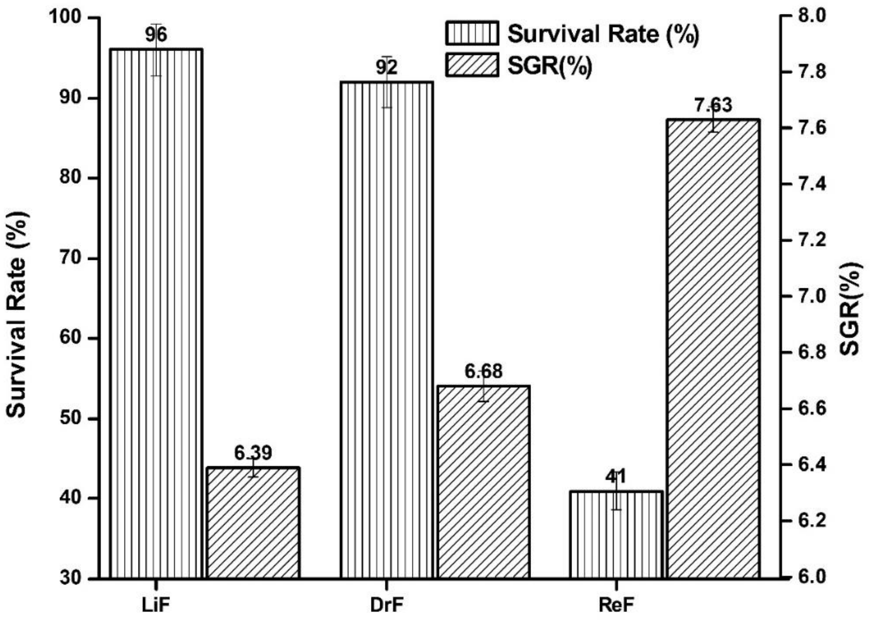

Fig 6.7 Profiles of growth characterization of fish species *C. mrigala* under three different types of feeds: LiF (Live Microalgae Feed), DrF (Dried Microalgae Feed), and ReF (Reference Feed).

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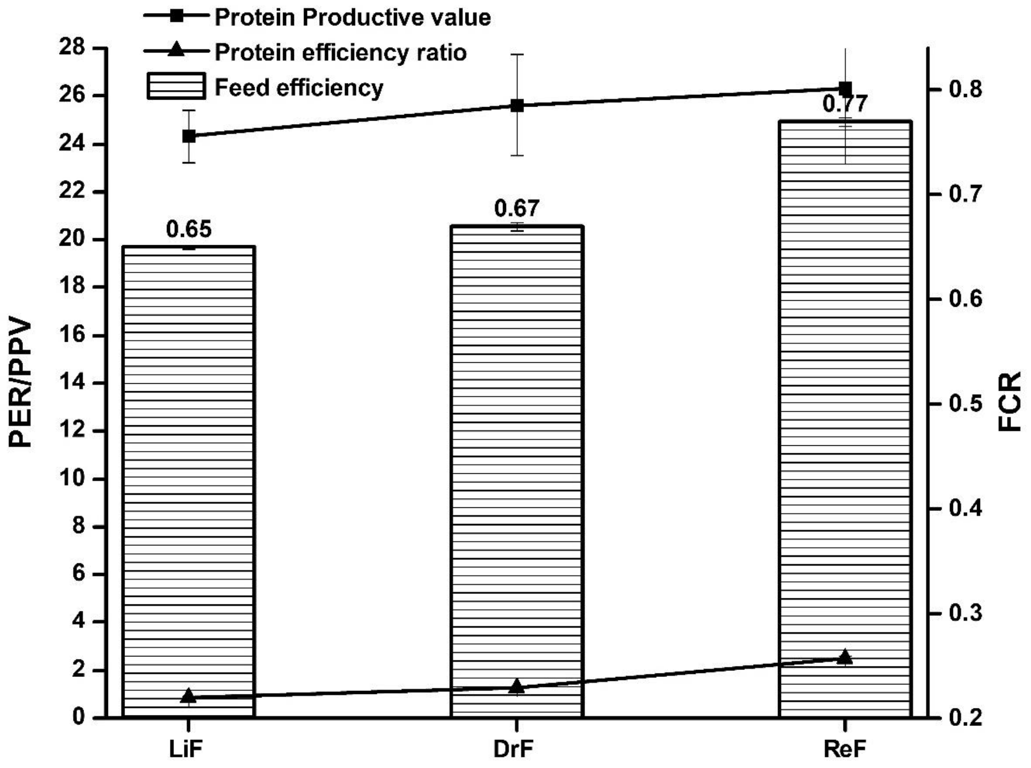

Fig 6.8 Profiles of PER, FCR and PPV of fish species *C. mrigala* under three different types of feeds: LiF (Live Microalgae Feed), DrF (Dried Microalgae Feed), and ReF (Reference Feed).

Table 6.2 Characterization of growth metrices of fish species mrigal carp under three different type of feeds.

	Experimental Diet		
	LiF	**DrF**	**ReF**
Initial body weight (mg)	15.00 ± 0.1^{a}	15 ± 0.11^{b}	15 ± 0.1^{c}
Final body weight (mg)	312 ± 6.3^{a}	322 ± 7.5^{b}	347 ± 4.2^{c}
Average daily gain (mg day^{-1})	8.48 ± 0.3^{a}	8.77 ± 0.2^{b}	9.48 ± 0.11^{c}
Feed efficiency	0.65 ± 0.002^{a}	0.67 ± 0.004^{b}	0.77 ± 0.004^{c}
Specific growth rate (% day^{-1})	6.39 ± 0.23^{a}	6.68 ± 0.44^{b}	7.63 ± 0.57^{c}
Protein efficiency ratio	0.87 ± 0.04^{a}	1.25 ± 0.09^{b}	2.51 ± 0.11^{c}
Protein productive value (%)	24.32 ± 1.09^{a}	25.62 ± 2.11^{b}	26.30 ± 3.13^{c}
Survival rate (%)	96 ± 3.22^{a}	92 ± 3.16^{b}	41 ± 2.31^{c}
Average Initial fish Length (mm)	35 ± 0.12^{a}	35 ± 0.12^{b}	35 ± 0.12^{c}
Average Final fish Length (mm)	63 ± 1.89^{a}	64 ± 2.59^{b}	71 ± 2.66^{c}

Table 6.3 Carcass proximate chemical analyses (%; on DM basis) of fish fed with three different types of feedstocks LiF, DrF, and ReF.

	Experimental Diet		
Proximate Analysis (%, w/w)	LiF	DrF	ReF
Total Protein$^{\#}$	46 ± 0.03^{a}	44 ± 0.07^{ab}	42 ± 0.08^{c}
Total Lipid$^{\#}$	8 ± 0.11^{a}	12 ± 0.09^{ab}	21 ± 0.06^{c}
Total Ash content	27 ± 0.11^{a}	28 ± 0.09^{b}	33 ± 0.05^{c}
Total Dry Matter	75 ± 0.81^{a}	78 ± 0.65^{b}	79 ± 0.71^{c}

Values (mean ± SD of three replicates) in the same row with different superscripts show significant difference ($P < 0.05$).

$^{\#}$ Total protein and total lipid composition were calculated for ash free dry weight of carcass *i.e.* % (Total dry weight – Total ash content), w/w

Table 6.4 Details of water quality parameters of three set of aquariums feed with the three different type of feedstocks

Water quality parameters (ppm)	**Experimental Diets**			**Days**
	LiF	**DrF**	**ReF**	
TDS	79			**0**
DO	9.1			
Total Nitrate	23			
Total Ammonium	24			
Total Phosphate	22			
TDS	83	87	110	**7**
DO	9.31	8.55	7.42	
Total Nitrate	32	36	51	
Total Ammonium	45	44	58	
Total Phosphate	17	25	37	
TDS	88	90	141	**14**
DO	9.55	9.54	6.95	
Total Nitrate	35	41	67	
Total Ammonium	47	43	77	
Total Phosphate	24	22	49	
TDS	93	95	153	**21**
DO	10.11	9.63	6.88	
Total Nitrate	44	51	76	
Total Ammonium	49	53	79	
Total Phosphate	24	33	49	
TDS	94	98	169	**28**
DO	10.55	9.65	6.76	
Total Nitrate	46	58	88	
Total Ammonium	50	61	109	
Total Phosphate	24	29	57	
TDS	96	105	184	**35**
DO	10.55	9.55	6.64	
Total Nitrate	48	63	96	
Total Ammonium	53	65	106	
Total Phosphate	27	38	72	

Values (mean ± SD of three replicates) in the same row with different superscripts show significant difference ($P < 0.05$).

[#] Total protein and total lipid composition were calculated for ash free dry weight of carcass *i.e.* % (Total dry weight – Total ash content), w/w

6.4 Conclusions

In order to understand the feasibility of developed technology in larger scale of cultivation, the strategy was deployed in a novel customised airlift photobioreactor of 50 L volume. The reactor was designed to provide better performance with respect to better mixing of CO_2, availability of light to the cells and better mass transfer of CO_2. With implementation of optimised cultivation medium and light wavelength and intensity, two batches were run: one without pH control and another with pH control through on demand CO_2 feeding. The performance of the large-scale reactor was found to be at par with lab scale setup in terms of biomass productivity and carbon sequestration rate, suggesting suitability of APBR at large scale without compromising the growth performance. Biomass composition of CT01 strain was found to be similar to lab setup suggesting absence of any stress to the cells caused mostly by scaling up. Furthermore, to minimize the cost and troublesome to develop a dry microalgal feed, an approach was targeted to utilize the protein rich high density fresh microalgal biomass as an alternative to current conventional feedstock for aquaculture. A model fish *C. mrigala* (mrigal carp) was fed with three different types of feedstocks: Live fresh microalgal biomass (LiF), dry microalgal biomass (DrF) and reference feed (ReF). The LiF and DrF had no distinctive negative impact on the growth dietetics of the fish, however ReF feed fish performed slightly better. The water quality of aquarium of LiF and DrF feeding experiments was found to be more suitable for the cultivation of mrigal carp and hence explain the more survival rate in presence of LiF feed. The biomass composition of mrigal carp suggested that digestibility of microalgal protein was better than conventional feedstock.

6.5 References

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Chapter 7

Conclusions

A high CO_2 tolerant microalgal strain was isolated using novel approach of CO_2 pressure-based screening process. The strain which could withstand upto 25% CO_2 (v/v) in air stream was isolated and molecularly identified as *Desomdesmus pannonicus* CT01. Further characterization of the strain under different physiochemical conditions revealed the robustness of the strain in terms of growth under wide range of initial pH, nitrate and phosphate sources. The generation of microalgal biomass as feedstock will offer cleaner production technology in terms of CO_2 sequestration even at higher CO_2 concentration of 10-15%, v/v, opening an avenue for its possible large-scale application utilizing industrial flue gas. Biochemical composition of strain revealed high protein content, making its suitability as an alternated feed to aquaculture.

In view of above observation, biomass of CT01 was evaluated for its application as alternate feed for fish species *H. molitrix* either as sole feed or as a mixture with an artificial (reference) feed. Fish growth matrices, dietetics and quality for microalga feed and mixed feed groups were compared with the reference feed. Mixture of microalgae with reference feed (at 1:1 ratio) resulted in significant improvement in growth matrices such as final body weight, daily weight gain and specific growth rate of young fish (i.e., fry) as compared to the reference feed. Similar to the growth performance, dietetics of the fish fry in terms of feed efficiency, protein efficiency ratio and protein productive value were found to be higher while fed on mixed feed as compared to the reference feed. The quality of fish in terms total protein content was estimated to be highest (60%) for microalga feed, followed by mixed feed (59%) and reference feed (54%). This suggests better digestibility of microalga protein by the fish as compared to reference diet.

To make the process more sustainable and feasible for commercial scale operation, a combinatorial process engineering strategy was employed with the aim of enhancing the biomass productivity coupled with carbon sequestration ability and protein content in microalgal biomass. In the first step, selected BB media components such as sodium nitrate, dihydrogen potassium phosphate, and TME were optimized via CCD for maximization of both biomass titer and productivity. CCD-RSM based optimization of the process variables resulted in 28 % increase in biomass titer and 27% increase in biomass productivity while compared to un-optimized condition. With optimized BB medium, the CT01 was further subjected to seven different combinations of mono and multi wavelength of light for improvement in the growth kinetics and intracellular protein content. The combination of red-blue light wavelength was found to be optimal for CT01 in terms of biomass productivity, carbon sequestration rate and total protein content. CT01 was then subjected to different light intensity in presence of optimized medium and combination of red-blue light wavelength. The light intensity of 150 μE^{-1} m^{-2} s^{-1} displayed a significant enhancement in biomass productivity and carbon sequestration rate capability of CT01. The interdependent dynamics of light intensity, growth and culture pH indicated that the process engineering strategy, based on on-demand supply of CO_2 under varied light intensity, may result in improved biomass concentration and productivity by maintaining optimal culture pH and eliminating CO_2 limitation. The batch with controlled pH resulted in maximum biomass concentration of 1.93 g L^{-1}, an improvement by 28% when compared with the batch with uncontrolled pH. The phosphate utilization rate and nutrient utilization rate has been found at the highest for pH-controlled batch. It has been observed that nutrient utilization also enhances with the developed strategy suggesting its better application in the area of wastewater treatment.

In order to understand the feasibility of developed technology at larger scale of cultivation, the strategy was deployed in a novel customised airlift photobioreactor of 50 L volume. The reactor was designed to provide better performance with respect to better mixing of CO_2, availability of light to the cells and reduced contamination issues. With implementation of optimised cultivation medium, light wavelength and intensity, two batches were run one without pH-based CO_2 feeding and the other with pH-based CO_2 feeding. The performance of the large-scale reactor was found to be at par with lab scale setup in terms of biomass productivity and carbon sequestration rate, suggesting efficient performance of the customized reactor at large scale without compromising the growth performance. Biomass composition of CT01 strain was also found to be similar to lab setup suggesting absence of any stress to the cells caused mostly by scaling up. Furthermore, to minimize the cost and additional processing step to develop a dry microalgal feed, an approach was targeted to utilize the protein rich high density fresh microalgal biomass as an alternative to current conventional feedstock for aquaculture. A model fish *C. mrigala* (mrigal carp) was fed with three different type of feedstocks: Live fresh microalgal biomass (LiF), dry microalgal biomass (DrF) and reference feed (ReF). The LiF and DrF has no distinctive negative impact on the growth dietetics of the fish, however ReF feed fish performed slightly better. The water quality of aquarium of LiF and DrF fed was found to be more suitable for the cultivation of mrigal carp and hence explain the better survival rate in presence of LiF feed. The biomass composition of mrigal carp suggested that digestibility of microalgal protein was better than conventional feedstock.

Future prospect

- Evaluation of CT01 strain in larger scale like open raceway ponds and validation of developed strategy to feed live microalgal cells to fish or aqua organisms in real time.
- Assessment of the strain's potential towards biorefinery approach to develop a sustainable technology
- Energy efficient harvesting strategy and downstream process technology for animal feed preparation need to be developed to attain net sustainability.
- Analytical methods which are applied to microalgae might be discussed, improved, developed and validated by technical working group in organizations such as Codex Alimentarius, International Standardization Organization (ISO).
- Extensive research and development are still required to make the use of algae as a fishmeal replacement in aquaculture feeds a feasible alternative

Appendix

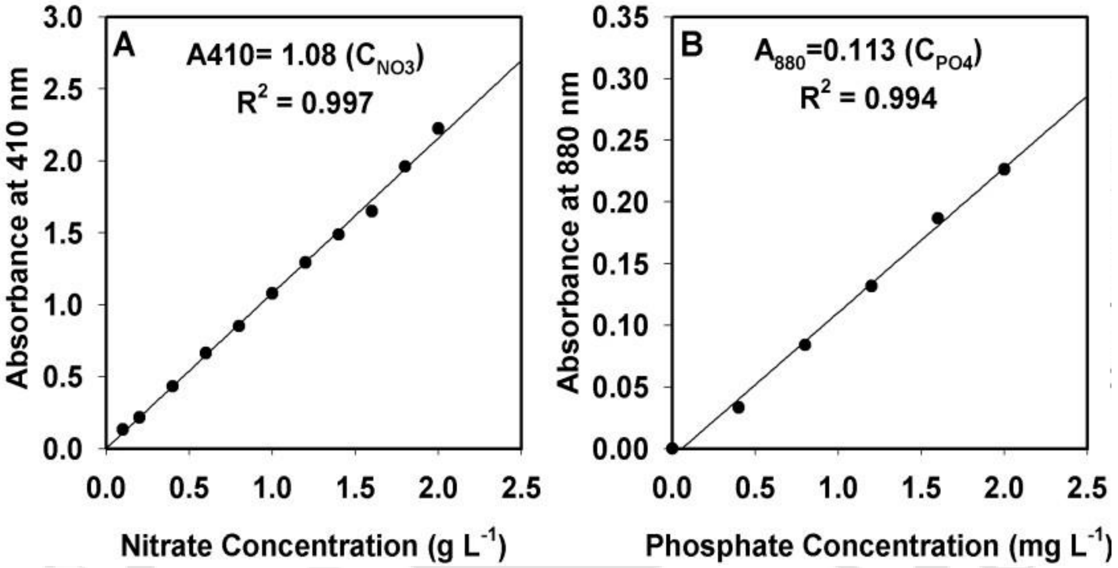

Fig A1 Correlation graph between concentration of the substrates and their respective absorbance in UV-Visible spectrophotometer for estimation of (A) Nitrate; (B) Phosphate

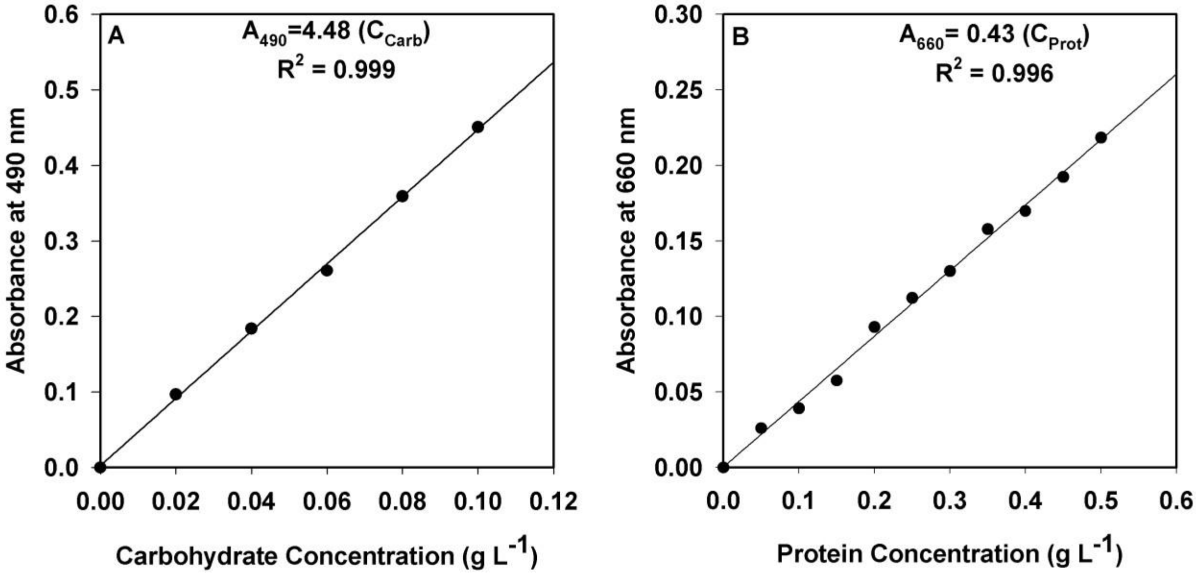

Fig A2 Correlation graph between concentration of the substrates and their respective absorbance in UV-Visible spectrophotometer for estimation of (A) Carbohydrate; and (B) Protein

A.1 Calculation of aeration rate for cultivation of CT01 in 50L APBR:

Key equations:

$$\frac{P_G}{V_L} = \rho_L \times g \times U_G$$

where P_G is the power supply by aeration (J s^{-1}), V_L is the volume of culture medium (L), ρ_L is the density of the liquid (kg m^{-3}), g is the gravitational acceleration (m s^{-2}), and U_G is the superficial gas velocity in the aerated zone (m s^{-1}).

$$U_G = \frac{Q}{A_C}$$

Where Q is the volumetric gas flow rate (m^3) and A_C is the cross-sectional area (m^2).

As the volumetric power requirement ($\frac{P_G}{V_L}$) is constant for both the bubble column photobioreactor and FP-ABR.

So, $\rho_{L_1} \times g \times U_{G_1} = \rho_{L_2} \times g \times U_{G_2}$

Where ρ_{L_1} = density of the liquid in bubble column photobioreactor

U_{G_1} = superficial gas velocity in bubble column photobioreactor

ρ_{L_2} = density of the liquid in FP-ABR

U_{G_2} = superficial gas velocity in FP-ABR

g =gravitational acceleration (m s^{-2})

For bubble column photobioreactor,

Radius (r) = 0.036 m

$$A_{C_1} = (\pi \times 0.036^2)\ m^2$$
$$= 0.0041\ m^2$$

$$Q_1 = 0.2\ L\ min^{-1}$$ (0.4 vvm for 0.5 L culture volume)

$$= 0.000003\ m^3\ s^{-1}$$

$$U_{G_1} = \frac{Q_1}{A_{C_1}}$$

$$= \mathbf{0.00082\ m\ s^{-1}}$$

Assuming negligible change in culture density compared to density of water for both the cultivation system,

$$U_{G_1} = U_{G_2}$$

For airlift photobioreactor (FP-ABR),

Length (L) = 0.4 m

Width (W) = 0.3 m

$$A_{C_2} = 0.4 \times 0.3\ m^2$$

$$= 0.120\ m^2$$

So, $Q_2 = U_{G_2} \times A_{C_2}$

$$= 5.904\ Lmin^{-1}$$

$$\approx 6\ Lmin^{-1}$$

Table A1 Average Molar Extinction Coefficients, Absorptivity (cm^{-1} M^{-1}) calculated for each amino acid

Amino Acid	**Molar extinction Coefficients**
aspartic acid	6522
threonine	5480
serine	7149
glutamic acid	6915
glycine	7367
alanine	7170
valine	7115
methionine	7108
isoleucine	6183
leucine	6818
tyrosine	5162
phenylalanine	6811
tryptophan	6404
lysine	11462
histidine	6250
arginine	7235

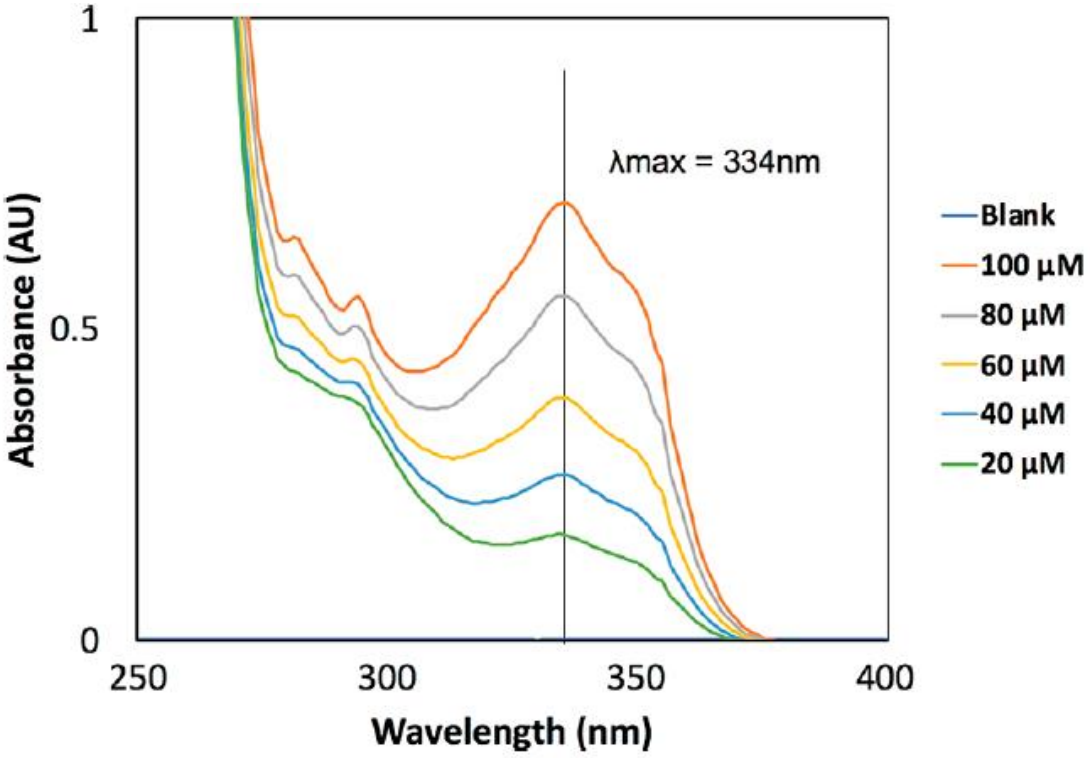

Fig A3 Characteristic absorption spectrum of AA/OPA/3MPA derivative. An absorbance peak was observed at 334 nm and selected as the analytical wavelength for subsequent analyses in this study.

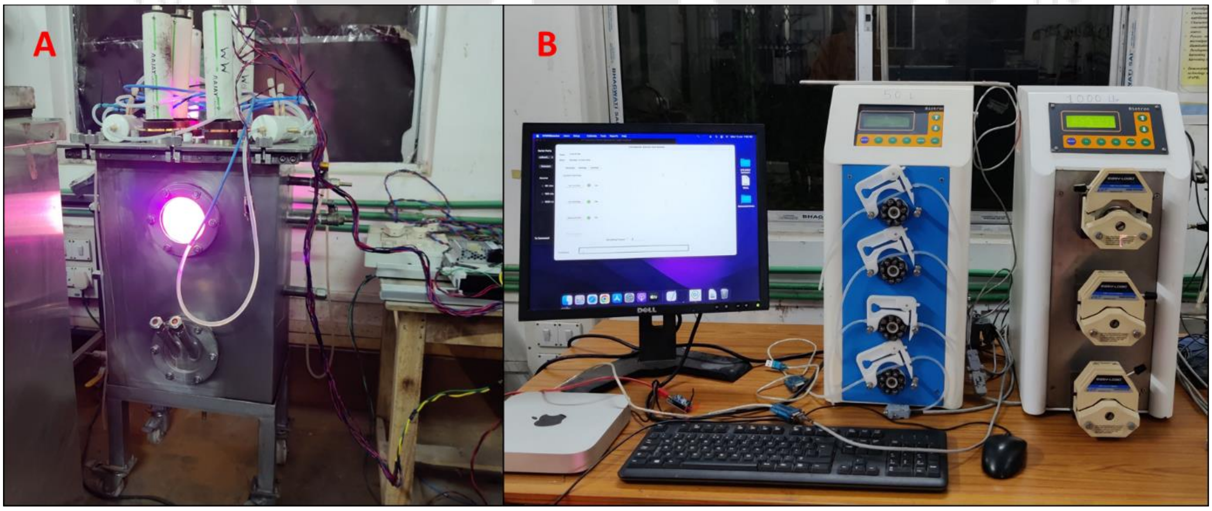

Fig. A4 A) 50 L customised airlift photobioreactor setup with illumination LED installed B) SCADA based controller with monitoring system

List of publications

Manuscripts under thesis work

1. **Ratan Kumar**, Gargi Goswami, Dipesh Debnath, Ankan Sinha, and Debasish Das. "Screening and evaluation of novel microalga Desmodesmus pannonicus CT01 for CO_2 sequestration potential and aqua feed application." Biomass Conversion and Biorefinery (2022): 1-12.
2. **Ratan Kumar**, Ankan Sinha, and Debasish Das. " Process engineering strategy for cultivation of high-density protein rich microalgal biomass and applicability of fresh microalgal cell as aquafeed." (Under review)

Articles from collaborative work

1. Sinha, Ankan, **Ratan Kumar**, Gargi Goswami, and Debasish Das. "Process engineering strategy for large scale outdoor cultivation of Tetradesmus obliquus CT02 coupled with pH guided CO_2 feeding." Journal of Environmental Management 318 (2022): 115539.
2. Goswami, Gargi, **Ratan Kumar**, Ankan Sinha, Boudhnath Birazee, Babul Chandra Dutta, Sanjay Bhutani, and Debasish Das. "ALGLIQOL: A two stage integrated process towards synthesis of renewable transportation fuel via catalytic hydrothermal liquefaction of lipid enriched microalgae biomass and distillation." Energy Conversion and Management 263 (2022): 115696.
3. Sinha, Ankan, Gargi Goswami, **Ratan Kumar**, and Debasish Das. "A microalgal biorefinery approach for bioactive molecules, biofuel, and biofertilizer using a novel carbon dioxide-tolerant strain *Tetradesmus obliquus* CT02." Biomass Conversion and Biorefinery (2021): 1-

14.

4. Goswami, Gargi, **Ratan Kumar**, Ankan Sinha, Soumen Kumar Maiti, Babul Chandra Dutta, Harendra Singh, and Debasish Das. "A low-cost and scalable process for harvesting microalgae using commercial-grade flocculant." RSC Advances 9, no. 67 (2019): 39011-39024.
5. Goswami, Gargi, Ankan Sinha, **Ratan Kumar**, Babul Chandra Dutta, Harendra Singh, and Debasish Das. "Process engineering strategy for cultivation of high density microalgal biomass with improved productivity as a feedstock for production of bio-crude oil via hydrothermal liquefaction." Energy 189 (2019): 116136.
6. Dineshbabu, Gnanasekaran, Gargi Goswami, **Ratan Kumar**, Ankan Sinha, and Debasish Das. "Microalgae–nutritious, sustainable aqua-and animal feed source." Journal of Functional Foods 62 (2019): 103545.

Patent applications from collaborative work

1. Goswami G, Kumar R, Sinha A, Dutta B C, Bhutani S, Das D, “Process for production of liquid transportation fuel via catalytic hydrothermal liquefaction of microalgae biomass.” Indian Patent Application No. 20211105535, filed on November 30, 2021.
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2. Webinar on “Sustainable Energy Technologies” organized by School of Energy and Sciences Technology, IIT Guwahati, 2021
3. Kumar Ratan, Das Debasish, “Microalgae for sustainable fuel and feed technology”, BESCON 2019, IIT Madras (Poster presentation)
4. GIAN course on “Biofuel Cell Technology: Fundamentals and Applications”; April 23-27,2018; IIT Guwahati
5. Kumar Ratan, Das Debasish, “Optimization of chemical flocculating agents for harvesting of *Chlorella* sp. FC2 IITG”, Bioprocessing India 2017, IIT Guwahati (Poster presentation)

Vitae

The author was born on February 6th 1991, in Godda,, Jharkhand,, India. He passed the All India Secondary School Examination conducted by the Central Board of Secondary Education, Deoghar, in 2006. He qualified the All India Senior School Certificate Examination conducted by Central Board of Secondary Education, Ranchi, in 2008. He completed his B. Tech in Biotechnology from Vellore Institute of Technology, Vellore, Tamil Nadu in 2014. He completed his M. Tech in Biotechnology from National Institute of Technology, Durgapur, Westbengal in 2016.

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