The Screening, Fabrication and Production of Microalgae Biocomposites for Carbon Capture and Utilisation

A Thesis Submitted By

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Abstract

The use of microalgae for carbon dioxide sequestration and as a feedstock for biodiesel production has been a topic of active research since the late 1950s. It has not been adopted as a technology due to the difficulties in growing the microalgae, harvesting it and the excessive cost of the fuel produced via this route. This research work focuses on a novel idea of attached microalgae growth method to cultivate different species of freshwater and marine microalgae on a solid substrates to sequester carbon dioxide and use the biomass to produce biofuel. Initially, we undertake a study to prove the concept of nourishing microalgae cells attached to filter paper via capillary. The initial results indicate a good survivability of the immobilised cells with limited nutrients for 8 weeks. The average cumulative CO_2 fixation of *C. vulgaris* cells (3.33 mmol g^{-1} day^{-1}) attached to the paper was more than twice the suspended culture cultivation (0.924 mmol g^{-1} day^{-1}) using 5% CO_2/air mixture. The next stage in this research work investigated the use of binders for cell immobilisation on a biodegradable substrate. A binder screening protocol which took into account toxicity and adhesion strength was developed to produce a biocomposite using the best combinations of microalgae species and binders upon different substrates. We subsequently developed an experimental system to continuously sequester carbon dioxide for 6 weeks using biodegradable microalgae bio composites made from loofa sponge and latex binders. *D. salina* cumulative CO_2 fixation of 5.96 mmol/g day^{-1} when immobilised with latex binder Baymedix CH-120 polyurethane resin dispersion was 15 times higher than the 0.40 mmol/g day^{-1} recorded for suspended culture. This also translate to reduction in land and water usage when compared to open pond algae cultivation or photobioreactor. The immobilised cells lipid content production improved for two of the algae species (*C. vulgaris and D. salina*) that were tested. The lipid content was 69.38% and 66.22% biomass dry weight for *C. vulgaris* and *D. salina* respectively. This novel research work has the potential to substantially reduce the cost associated with biological carbon capture and biofuel production using microalgae when compared with the open ponds and photobioreactors.

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All thanks are to Allah who gave me the strength, health and opportunity to undertake this programme of study.

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Publications, Conferences and Visit

Publications:

- Abbas Umar, Gary S. Caldwell, and Jonathan G.M. Lee. "**Foam floatation can remove and eradicate ciliates contaminating algae culture system**" (Chapter 3 of this Thesis). Published by the Journal of Algal Research, Vol 29C, 2018, Pages 337-342.

- Abbas Umar, **Pichaya In-na, Adam D. Wallace, Micheal C. Flinkinger,** Gary S. Caldwell, and Jonathan G.M. Lee. "**Development of a loofah-based microalgae biocomposite for carbon capture applications"** (Chapter 5 of this Thesis). To be submitted to the Journal of Bioresource Technology.

Conferences:

- Abbas A. Umar, Muayad A.S. Al-karawi, Gary S. Caldwell, and Jonathan G.M. Lee. **Continuous harvesting of microalgae using dispersed foam flotation**. Oral Presentation at the 3rd European congress of applied biotechnology, Nice, France. September 27 – October 1st, 2015.

- Abbas Umar, Gary S. Caldwell, and Jonathan G.M. Lee. **Using sodium dodecylsulphate and foam column separation to remove ciliates from microalgae culture.** Poster presentation at the 1st international conference on bioresource technology for bioenergy, bioproducts & environmental sustainability, Sitges, Spain. 23-26 October 2016.

- Abbas Umar, **Pichaya In-na,** Gary S. Caldwell, and Jonathan G.M. Lee. "**Growth and maintenance of microalgae on a filter paper strip and its potential for biological CO_2 mitigation"** Poster presentation at the 10th World Congress of Chemical Engineering, Barcelona, Spain. 1st – 5th October 2017.

Visit:

- Two months academic visit to the laboratory of Prof Michael Flickinger, Department of Chemical and Biomolecular Engineering, North Carolina State University, United States. June – August 2017.

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Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CAPEX	Capital expenditure
CCAP	Culture collection of algae and protozoa
CCS	Carbon capture and storage
CCU	Carbon capture and utilisation
CMC	Carboxymethyl cellulose
EPS	Extracellular polymeric substance
FAME	Fatty acid methyl esters
FTIR	Fourier-transform infrared spectroscopy
GHG	Greenhouse gas
MEA	Monoethanolamine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
OPEX	Operational expenditure
PAR	Photosynthetically active radiation
PBR	Photobioreactor
ppm	Part per million
rANOVA	Repeated measure ANOVA
RE	Removal efficiency
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SPI	Soy protein isolate

SPSS	Statistical package for social science
TAG	Triglyceride/triglycerol
UVA	Ultraviolet radiation alpha
UVB	Ultraviolet radiation beta
WCP	Wet cell pellet

Chapter 1

Introduction

1.0 Background to the thesis

Over several decades microalgae has generated much interest from both academia and industry due to its wide range of potential applications; however, its cultivation and further processing are techno-economically demanding. Achieving products and processes from microalgae that are competitive against current market leaders continues to drive research and development in this field. Pond-based cultivation is widespread but has high land and water requirements, whereas photobioreactors, despite intensifying the culture process, suffer from high CAPEX and OPEX costs that limit their use for low-value high-volume products such as biofuel. Part of a solution to these economic conundrums likely lies in deriving multiple valorisable products and services from the same biomass (the so called biorefinery concept). One service that may be provided throughout the microalgae growth cycle is carbon capture from flue gas (with marketable products extracted from the biomass – carbon capture and utilisation as opposed to carbon capture and storage); however, there is much work to be done to commercialise this. Efforts to alleviate some of these challenges include growing algae on supportive substrates (scaffolds) that allow for enhanced carbon dioxide (CO_2) capture and utilisation. Unfortunately, there is a dearth of literature regarding pilot or commercial production of microalgae on scaffolds, despite its apparent benefit (reduced land and water use, flexibility of design, recyclable and biodegradable, greater CO_2 mass transfer efficiency, etc.). Most research has focused on culture contamination control but not for improved carbon capture and utilisation. The research presented in this thesis was tailored towards improving the potential for microalgae carbon capture combined with improvements in lipid yield for biofuel production, thereby addressing two vital dimensions of an eventual biorefinery concept.

1.1 Rationale and significance

Global energy demand is increasing due to rapid industrialisation combined with a growing consumerism culture. However, fossil hydrocarbon reserves are finite, there is a slowing of new oil discoveries, a geopolitical need for energy security to combat unstable supply chains, and all under the looming shadow of human induced climate change. The Intergovernmental Panel on Climate Change (2018) report stressed the need to limit the rise of global temperature

(caused by greenhouse gases) to below 1.5 ^{o}C before the year 2030, the consequences of which would be irreparable damage to the planet (IPCC, 2018). These factors have combined to fuel the need for reliable renewable fuel sources. Biofuels (fuels produced from biomass) have the capability to service most of our existing infrastructures (transport, industrial and domestic) without undergoing serious modification. This could also reduce the amount of CO_2 emitted into the environment from fossil fuel use. However, biofuel industries are facing a growing dilemma of meeting the demand for sustainable feedstock supply.

CO_2 sequestration using biological sources (e.g. microalgae) is of interest due to the perceived benefits associated with the procedure compared with current carbon capture methods. However, it is difficult to co-locate microalgae culture systems such as open ponds with existing CO_2 emission sources. Photobioreactors are costly and also require substantial water and energy use. Furthermore, photobioreactor processes need to be further intensified by addressing the challenges posed by CO_2 and light diffusion within the liquid medium to enhance algae metabolism. The growth and cultivation of microalgae for carbon capture and production of other valuable products on/within substrates (termed biocomposites) potentially gives algae biosequestration an advantage over other methods.

1.2 Hypotheses

Many microalgae species can grow naturally in open environments, sometimes growing on rock and building surfaces. We hypothesised that: 1) *aquatic microalgae with high CO_2 fixation rate and lipid content can be grown on a solid substrate*; 2) *growing microalgae outside the water medium will increase CO_2 consumption as a result of improved mass transfer efficiency*; and 3) *the biomass generated from this process can be converted to other useful products like biofuel*.

1.3 Aims and objectives

The primary aim of this work was to develop a technique that could be used to produce an environmentally friendly way of carbon capture using microalgae biocomposites with concomitant improved lipid production.
The specific objectives of this project were to:

a. demonstrate the feasibility of microalgae decontamination using foam column separation technique.

b. demonstrate that different microalgae species can be grown and maintained on a solid substrate being nutritionally nourished via capillary action similar to water transportation within the vascular system of plant woody biomass. This assumption is upon the fact that when a dry porous substrates is brought in contact with liquid medium it absorb the liquid at a certain rate over time depending on the temperature, humidity and permeability of the substrates.

c. establish a protocol to screen binders, substrates and coating techniques for microbial immobilisation on a supporting material (biocomposite formation) for enhance CO_2 fixation;

d. demonstrate the use of binders for microalgae immobilisation on a solid substrate (microalgae biocomposite formation);

e. demonstrate improved rate of CO_2 capture by microalgae biocomposites for 1000+ hours using a bioreactor with limited water and nutrient supply; and,

f. demonstrate that the modified culturing conditions within biocomposites could produce a high lipid yield.

1.4 Thesis plan and chapter details

This thesis was written as a series of standalone chapters formatted in the style of journal papers for publication. All papers were written by the primary author, Abbas Umar, and edited by Drs Jonathan Lee and Gary Caldwell. All experimental work and data collection was conducted by the primary author unless otherwise stated, such as collaborative work or work conducted by Masters students under Abbas Umar's supervision.

Chapter 2 presents a review of the literature most pertinent to the research topic, including various efforts undertaken by both states and non-state actors, scientists and academicians toward reducing carbon emissions from burning fossil fuels. After examining the pros and cons of current carbon capture technologies the review considers the option of CO_2 biosequestration using microalgae. Microalgae cultivation techniques and strain selection are discussed to highlight their shortcomings and possible remedies for effective utilisation and deployment or co-location with CO_2 emitting sources. Among the problems identified for algae CO_2 fixation was the mass transfer limitation in liquid culture media, and the need to grow algae on supporting materials. Microalgae immobilisation using different materials, techniques

and methods are also described, highlighting the shortcomings that the current project aims to address.

Chapter 3 describes the work initially outlined and agreed by my supervisors which was the use of foam flotation to decontaminate microalgae cultures infected by ciliates. Ciliates harvesting in liquid medium was initially conducted without microalgae to optimise and determine the main process variables necessary for effective ciliate extraction using negatively charged surfactants. The optimised variables were then applied to algae cultures. Various parameters such as surfactant concentration and air flow rates were investigated. Finally, a multistage ciliate death and surfactant recovery system was adopted for the algae culture decontamination process, highlighting its economic benefits and advantages over current chemical control methods targeted against ciliates.

Chapter 4 marks the beginning of a series of interrelated studies that addressed microalgae immobilisation on solid support materials. Chapter 4 specifically dwells on the growth and maintenance (8 weeks) of different microalgae species (freshwater and marine) on filter paper nourished with nutrients via capillary action. Various techniques such as chlorophyll *a* fluorescence and CO_2 fixation were used to monitor algae metabolism. At the end of the experiment, total lipid, protein and carbohydrates content of the immobilised cells were determined using FTIR spectroscopy. The results obtained highlighted some pertinent issues (cell-to-cell and cell-to-substrate binding affinity) that need to be addressed for effective microalgae immobilisation.

Chapter 5. Building upon the lessons learned from Chapter 4, we realised the importance of having long lasting biocomposites that can withstand various culturing, production and environmental stresses yet still continue to act as carbon sink and product source. The microbial encapsulation literature is rich, using different materials and techniques, but few combinations last the test of time due to their fragile nature. Thus, we set out to develop a broad range of protocols that could be used to screen adhesive binding materials and support structures to enable the growth of different freshwater and marine microalgae. Three stages were employed; 1) binder toxicity tests; 2) determination of suitable coating and drying techniques for different solid substrates (paper, cotton and loofah); and, 3) test the adhesion strength on the solid support for non-toxic binders. Some parts of this work were done in collaboration with the Flickinger Group at the North Carolina State University, United State of America.

Chapter 6 reports the results of batch and continuous CO_2 fixation of both freshwater and marine microalgae immobilised onto loofah sponge for 1000+ hours. The biocomposites produced using latex binders was tested (using a locally fabricated rig) for improved carbon capture compared to suspension culture controls. Total lipid yield of the immobilised cells was determined via a wet extraction method. Cell, substrate and binder arrangement was observed using high magnification SEM analysis.

Chapter 7 discusses a hypothetical techno-economic analysis of immobilised algae CO_2 sequestration with other carbon capture methods. Summary of major findings, including shortcomings were also highlighted, including proposed future directions for carbon capture using immobilised microalgae.

Chapter 2

Literature Review

2.0 Introduction

Global average temperatures have been increasing since the Industrial Revolution as a result of greenhouse gas (GHG) emissions (IPCC, 1995). The UN estimated that natural disasters stemming from manmade activities have quadrupled since the 1970s, some of which are directly or indirectly related to the anthropogenic use of fossil fuels and the concomitant release of GHGs. There is consensus that GHG emissions must be reduced, of which carbon dioxide (CO_2) contributes about 50-60% (Coyle, 2007; Lotfalipour *et al.*, 2010). Since the industrial age, the global atmospheric CO_2 concentration has risen from 280 ppm and has bypassed the symbolic 400 ppm level to about 407.7 ppm (Fig. 2.1A) (Betts *et al.*, 2016). Global population growth (with associated industrialisation) and CO_2 emissions are directly proportional (Zlotnik, 2007). It is estimated that population growth up to the year 2050 will increase atmospheric CO_2 concentration by up to 75 ppm. Any increase beyond 450 ppm could be catastrophic for the planet (Hansen, 2007; IPCC, 2018).

Despite massive investment in renewable energy and the political impetus of the Paris Agreement, if unchecked, the CO_2 concentration could reach 560 ppm in the next 50 years (Singh *et al.*, 2016). This can be translated to about 75% of the expected 1 ^{o}C rise in global warming (Fig. 2.1B); however, if nothing is done to curtail CO_2 emissions it could approach 1.9 ^{o}C and potentially increasing mean sea level by 3.8 m (Stewart and Hessami, 2005; Bacon and Bhattacharya, 2007). Similarly, acidification of the oceans due to high CO_2 concentration could have disastrous impacts on marine habitats and fundamentally alter biodiversity and food webs (Freund and Ormerod, 1997; Ormerod *et al.*, 2007; Mata *et al.*, 2010b). There is pressing need to curtail CO_2 emissions to not more than 50% above current levels, this involves reducing current emissions by at least 60% (IEA, 2012; IPCC, 2018).

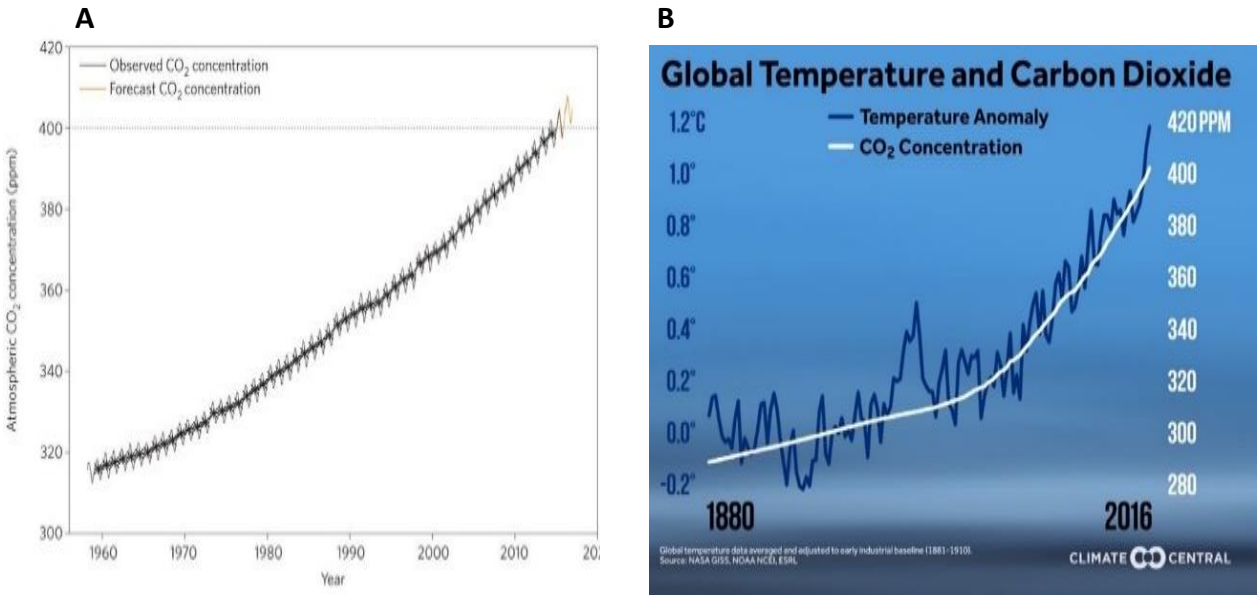


Figure 2.1: A. Monthly mean CO_2 concentrations, observed from 1957 to 2015 (black line) and hindcast/forecast for 2015 and 2016 (orange line), Adapted from (Betts et al., 2016). B. Correlation between CO_2 and rising global temperatures (Rising Global Temperatures and CO_2, 2017).

Coal accounts for 40% of point source CO_2 emissions (Fig. 2.2) with many new coal-fired power plants expected to come on line in the near future; although new plants are expected to be more efficient, making use of integrated gasification combined cycle technology. Most coal powered plants supply the base load for a country's electricity; this is difficult to completely replace by renewable sources. An alternative strategy would be to develop affordable, sustainable carbon-neutral fuels alongside carbon capture technologies; however, the techno-economic realities remain challenging.

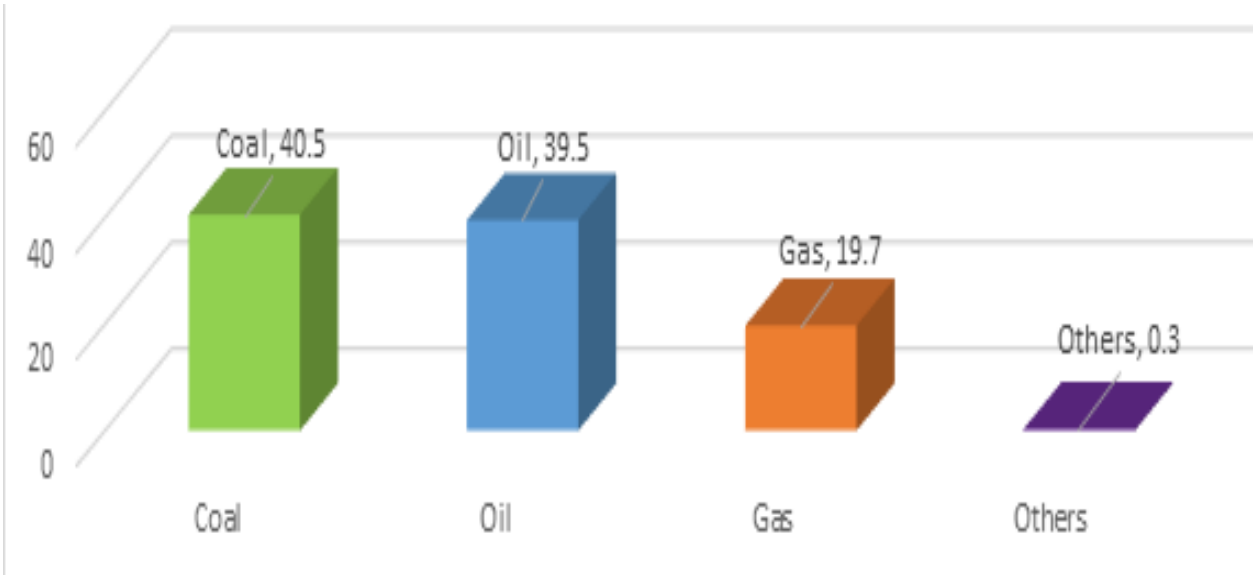


Figure 2.2: Percentage of global fossil fuel CO_2 emissions (mT/yr), International Energy Outlook (2016).

Carbon capture and storage (CCS) technologies can mitigate carbon emissions that would otherwise be released to the atmosphere, particularly from major point sources e.g. cement and steel manufacturing industries, and power plants. However, CCS implementation at large scale is far from realisation due to the associated costs (Herzog, 2009). There is also the difficulty in managing more diffuse CO_2 emitting sources, e.g. the transport sector which also has a substantial contribution to global carbon emissions. Nonetheless, finding comprehensive solutions to carbon capture remains a societal and political imperative.

2.1 Overview of current carbon capture technologies

There are three major types of carbon capture based on the stage at which the CO_2 is separated and capture (Fig 2.3), the pre-combustion, post-combustion and oxyfuel separation (Singh, 2013). The pre-combustion separation involves the gasification of the fuel source with steam to produce syngas (equation 2.1) containing CO and hydrogen molecules. The CO is later converted to CO_2 (and subsequently capture) by the addition of water in a reaction known as the 'water-gas shift' reaction (equation 2.2). Oxyfuel CO_2 separation, involves burning of fuel in the presence of air containing high percentage of O_2 which result in the formation of ultra-pure CO_2 ready for capturing. There is high energy cost in operating the air separation unit responsible for removing the air components such as nitrogen. Post-combustion carbon capture involves the active separation of CO_2 from flue gas after fuel combustion. This is a widely deployed technology for carbon capture and the different methods are further explained below.

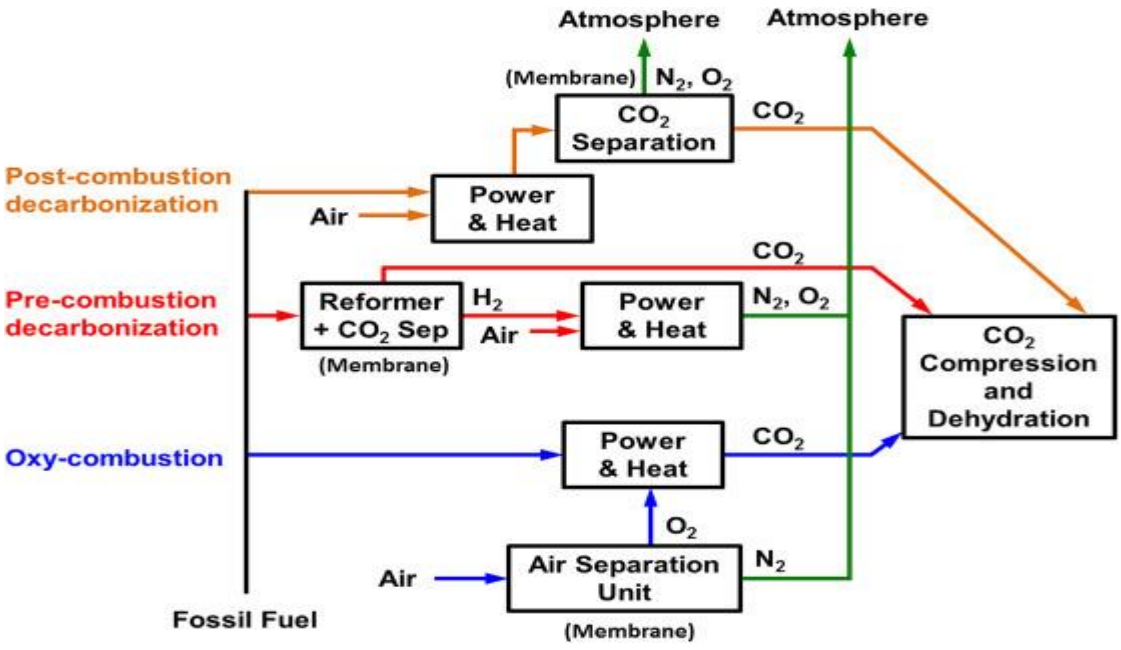


Figure 2.3: Overview of carbon dioxide separation routes from fossil fuel combustion. (Singh, 2013)

$$\underset{\text{Carbon (fuel)}}{C} + \underset{\text{Steam}}{H_2O} \rightarrow \underset{\text{Carbon monoxide}}{CO} + \underset{\text{Hydrogen}}{H_2} \text{ ----------(2.1)}$$

$$\underset{\text{Carbon monoxide}}{CO} + \underset{\text{Steam}}{H_2O} \rightarrow \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Hydrogen}}{H_2} \text{ ----------(2.2)}$$

The type of fossil fuel combustion process directly affects the choice of a suitable CO_2 removal procedure. Various CO_2 capture technologies are available (summarised in Fig. 2.4), unfortunately they tend to be costly, at a laboratory scale of development or are sometimes unsafe to operate.

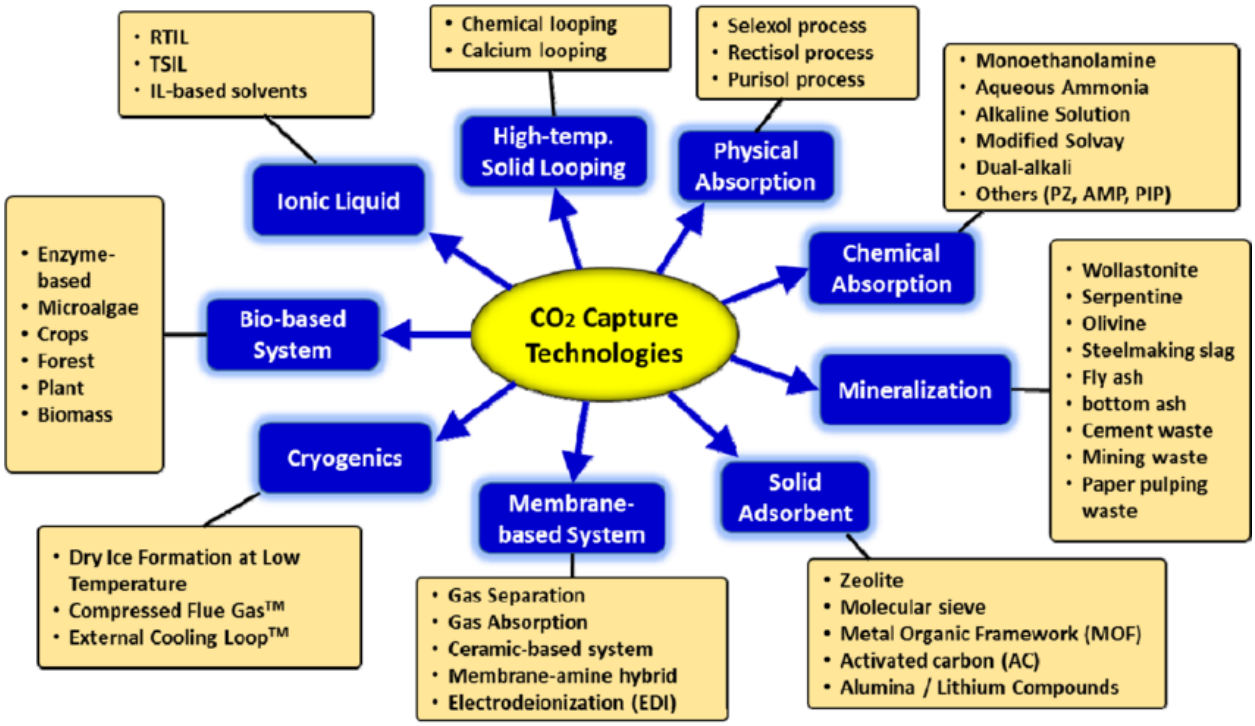


Figure 2.4: Different technologies for CO_2 capture. Adapted from (Li *et al.*, 2016).

2.1.1 Chemical absorption-desorption process

Chemical absorption-desorption processes are frequently used to remove CO_2 from industrial flue gas. CO_2 is dissolved to saturation in a monoethanolamine (MEA) solution and later recovered via heating at around 150 ^oC (Pires *et al.*, 2011) to release the pure gas for further storage (Lam *et al.*, 2012). The main drawback is the energy required to regenerate the MEA (approx. 3.7 GJ/tonne (Knudsen *et al.*, 2009), accounting for 80% of the total energy consumption (Thomas *et al.*, 2016). For every tonne of CO_2 absorbed, another 352 kg of CO_2 will be emitted (assuming coal is used to generate the energy). Additionally, MEA can readily react with SO_2 (present in the flue gas) resulting in a permanent degeneration of the solution and production of salt (Cole *et al.*, 2011). Oxygen present in flue gas can react and corrode the

equipment, this is in addition to other factors which could exacerbate corrosion such as the increase in MEA concentration, solution velocity and process temperature (Kladkaew *et al.*, 2011; Kittel and Gonzalez, 2014).

2.1.2 Chemical looping process

This is a process where CO_2 is separated from other flue gas components using chemicals such as Fe-, Cu-, Ni-, Mn-, and Co-based metal oxides (Herzog and Golomb, 2004). The removal of other gases such as N_2 present in the air and allowing only O_2 to react with the fuel will result in nearly 100% CO_2 content within the flus gas Fig 2.5. Equation 2.3 shows how a metal oxide can be reduced by fuel such as natural gas containing methane to release water and CO_2 (Herzog and Golomb, 2004; Styring *et al.*, 2011).

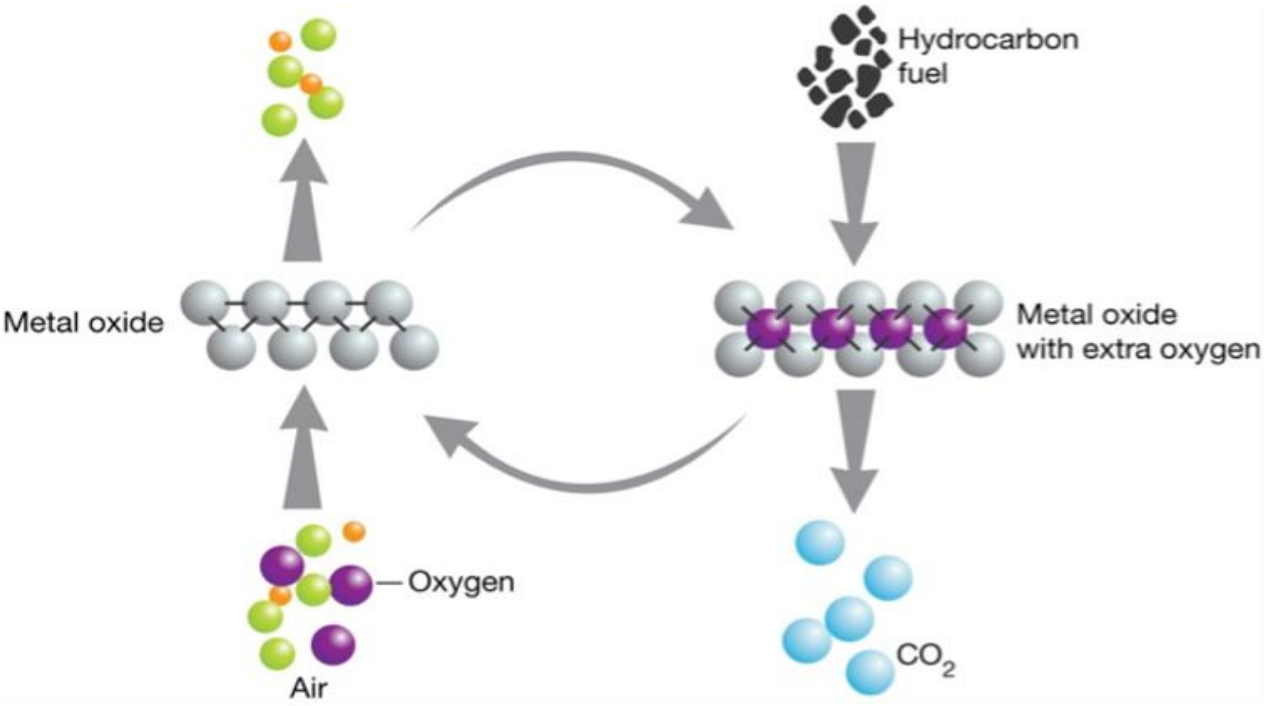


Figure 2.5: The chemical looping process utilising metal oxides to concentrate O_2 in the air for the production of high purity CO_2 in flue gas (Scholes, 2013).

$$\mathbf{CnH2m + (2n + m)\ Me_x O_y \rightarrow CO_2 + mH_2O + (2n + m)\ Me_x O_{y-1}} \quad \textbf{---------(2.3)}$$

However, large scale industrial application of this process requires that low cost O_2 carriers be developed with fast reaction times and reuse. Reactors and operating conditions need to be optimised. An additional issue that needs to be addressed is the abrasion of plant equipment by the metal particles, which constantly blast the internal structure of such plant (Scholes, 2013).

2.1.3 Solid adsorbents method

Solid adsorbents technology is a low energy and cost-effective process that is considered for large-scale CO_2 mitigation ((Hunt *et al.*, 2010; Pires *et al.*, 2011)). Zeolite 5A, activated carbon, Na_2CO_3, and CaO are some examples of CO_2 adsorbers (Hao *et al.*, 2011). This process boasts significant energy reduction compared to MEA regeneration, but the flue gas needs to be pre-treated before it is channelled to the adsorber, which nevertheless requires a substantial amount of power. Pre-treating the flue gas is designed to reduce the high moisture content of the gas which, in the presence of other contaminants, can affect the adsorbents. Li *et al.* (2008a), postulated that CO_2 recovery can drop from 78.5% to 60% when moisture is present as a result of thermal differences within the adsorption bed and also due to the sluggish desorption speed of the CO_2. Contaminating the adsorbent with SOx and/or NOx means frequent regeneration is needed. Other issues with this process include minimal adsorption ability of the adsorbent, and the need to create a high temperature or pressure environment which is largely determined by the adsorber setting and lifespan.

2.1.4 Ionic liquid separation

Ionic liquids such as 1-butyl-3-methylimidazolium (dicyanamide, acetate, trifluoromethanesulfonate and bis ((trifluoromethyl) sulfonyl) imide are salts with organic cations and an inorganic/organic anion that can be used for CO_2 separation from flue gas when immobilised on a suitable membrane (Dai *et al.*, 2016; Liu *et al.*, 2016). Ionic liquids have low melting point (<100^oC) and low volatility. Their selectivity and high solubility with CO_2 stand them out as a choice for CO_2 separation (Dai *et al.*, 2016). The advantage of using ionic liquid for carbon capture is its low vapour pressure, higher stability at room temperature and low energy requirement for solvent regeneration (Zhang *et al.*, 2013). However, high production cost, environmental concerns and high viscosity after reacting with CO_2 limit their application in CO_2 sequestration.

2.1.5 Mineralisation process

Direct reaction of some minerals such as calcium and magnesium with CO_2 to produce inert materials such as calcium and magnesium carbonates offer another pathway for CO_2 capture, storage and utilisation (Nanda *et al.*, 2016). This reaction is however very slow in nature and needs to be accelerated to be effective as a tool for carbon capture (Herzog and Golomb, 2004). The acceleration process requires high heat, pressure and sometimes mechanical treatment

(grinding) which are capital intensive (Styring *et al.*, 2011). Additionally, mining and transportation of these minerals will have other environmental impacts.

2.1.6 Membrane filter technology

Various gas mixtures can be effectively separated via membranes functioning as filters. This separation process is based on the selectivity and permeability of the membrane. The energy consumption of polymeric membrane CO_2 separation is lower compared to the MEA technique (Bounaceur et al., 2006). Nonetheless, the efficiency of this process is usually affected by temperature; thus, for effective performance the flue gas needs to be cooled before passing through the membrane (Pires et al., 2011). Similarly, high moisture content flue gas can affect the membrane porosity due to plasticisation of the polymer (Pires et al., 2011; Scholes et al., 2009). Other challenges associated with this process include fouling/corrosion of the membrane, high membrane production cost, quick failure, low permeability/selectivity and the large membrane surface area which is needed to accommodate the rate at which industrial flue gas is emitted.

2.1.7 Cryogenic separation process

Cryogenic separation was developed on the principle of cooling and condensation of gases at different condensation temperatures. This process has been used in the industrial sector for an extended period due to its effectiveness in air separation. However, similar to other separation techniques, high energy is required for refrigeration. Additionally, the system is susceptible to ice blockage if moisture within the flue gas is not removed; this adds to the operational cost (Kaewpintong et al., 2010). Another issue is the continuous build-up of CO_2 on the heat exchanger surface, which if not treated will reduce process efficiency.

2.1.8 Carbon dioxide storage in ocean and geological formations

CO_2 captured from power generating facilities can be compressed, stored and transported to the deep ocean or buried underground in geological formations. This technology has successfully been used (Sleipner in the North Sea, Salah in Algeria, Gorgon in Australia and Snohvit, of the shores of Norway) to capture and store carbon dioxide for decades (Benson and Cook, 2005; Furre *et al.*, 2017). However, any unforeseen CO_2 leakage due to transportation could acidify the ocean with detrimental consequences for the oceanic biodiversity. Additionally, land shortage near CO_2 emitting sources requires the transportation of the compressed gas over long

distances. Kadam (1997) estimated the cost of compression, drying and transportation over 100 km to be US$11.78 per m^3. Thus, in addition to safety, the high cost of CO_2 capture, compression, transport, storage, and other related difficulties, reduces the acceptability of this CCS method.

Safety concerns, health issues, transportation and energy/process cost implications of the current carbon capture methods described above make them unsustainable solutions for long term carbon mitigation. Biological entities such as higher plants, forestry, agricultural cultivation and algae have been at the forefront of carbon neutralisation for billions of years. There is growing research in purposeful biologically related carbon capture to complement existing natural system.

2.1.9 Natural biological carbon capture systems

Terrestrial plants fill an essential niche of capturing CO_2 and are widely recognised as an important eco-tech choice for CO_2 mitigation. Grasslands, agricultural lands, brush land, and forests (Fig. 2.6) are major contributors to carbon capture in nature (Tsai *et al.*, 2017). Forests absorb more CO_2 than other terrestrial plants such as agricultural crops. Oceanic phytoplankton also play a major role in natural carbon capture. It is estimate that about 40,000Gt of carbon is sequestered by the ocean compared to 2200 and 750 Gt by terrestrial sources and the atmosphere respectively (Pires *et al.*, 2011; Nanda *et al.*, 2016). By the year 2100, this oceanic carbon sequestration is expected at the rate of 5Gt annually (Cox *et al.*, 2000; Nanda *et al.*, 2016). However, most oceanic waters are nutrient depleted, specifically lacking iron. The deliberate addition of iron and nitrogen sources (e.g. urea) to oceanic systems (termed ocean fertilisation) has been shown to boost phytoplankton growth and increase the drawdown of CO_2 from the atmosphere (Yamasaki, 2003; Herzog and Golomb, 2004). However, the death and degradation of these phytoplankton could lead to the release of the stored CO_2 into the atmosphere (Nanda *et al.*, 2016). Additionally, these techniques are controversial and may not be accepted by environmentalists due to the possible growth of unintended species that may disrupt oceanic biodiversity. Microalgae is recognised as one biological carbon sequestration option that will not only sequester carbon from the atmosphere but could also provide biofuel to replace fossil fuels, especially in the transport sector.

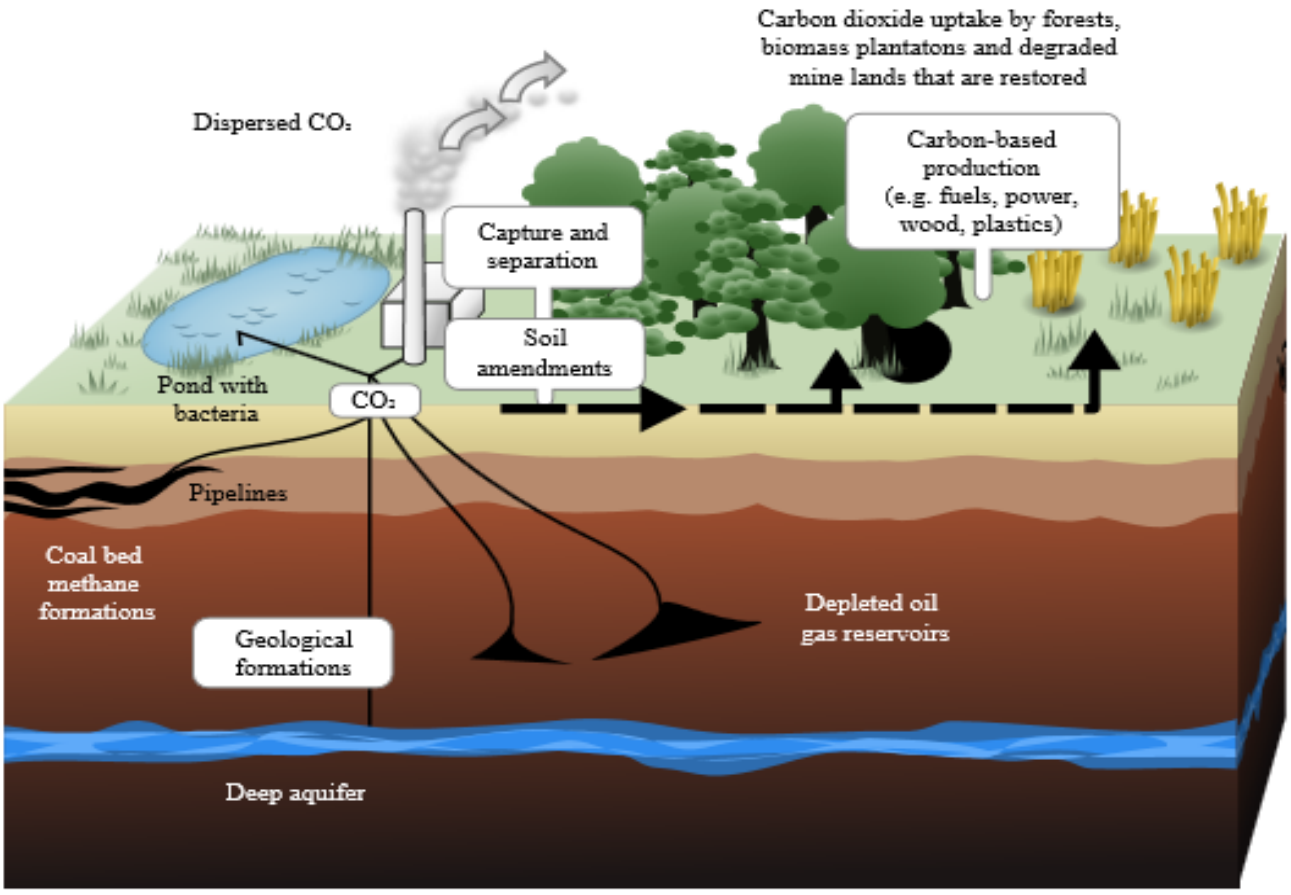


Figure 2.6: Schematic summary of CO_2 sequestration via different routes (Wikipedia, 2018).

2.2 Microalgae biology, photosynthesis and carbon fixation

Microalgae (including the cyanobacteria/blue-green algae) produce their food using light and CO_2 and in return produced oxygen in a process called photosynthesis. They were probably the first organisms to dominate both freshwater and marine environments (specifically the cyanobacteria). Microalgae exist as single cells (unicellular), or form filaments or colonies. Microalgae are more efficient than terrestrial plants in trapping and converting sunlight to produce their food. This particular advantage confers them with the ability to grow faster than higher plants, with some species capable of doubling their numbers within a few hours. Microalgae can propagate in three different ways, sexually, asexually or vegetative. In sexual reproduction, genetic materials are exchanged between cells to form a new cell combination. There are three stages to this method; oogamy, isogamy and anisogamy, which leads to the creation of a zygote. In some types e.g. the Cyanophyceae (blue green algae) where sexual reproduction does not take place, the exchange of genetic material is done via the formation of conjugate tubes. Asexual reproduction is done via the formation of different types of spore-like autospores and aplanospores, while vegetative reproduction occurs through binary fission.

Photosynthesis is a biological process whereby autotrophic organisms utilise CO_2 from the atmosphere in the presence of light and water to produce simple sugars as a source of energy; see equation 2.4 (Farrelly *et al.*, 2013). Photosynthetic organisms are equipped with specialised pigments (e.g. chlorophyll and carotenoids) that are responsible for the absorption of light at a particular wavelength. The pigments are arranged into two distinct photosystems located in the thylakoid membrane of the chloroplast; P700 (Photosystem I) and P680 (Photosystem II) (Zhao and Su, 2014).

$$\mathbf{CO_2 + H_2O + Light \Longrightarrow CH_2O + O_2} \quad \text{--------(2.4)}$$

Photosynthesis occurs in two stages; the light dependent reactions and light independent reactions (Fig. 2.7). The former uses light to convert ADP and NADP+ into ATP and NADPH through electron transfer, releasing oxygen in the process. The NADPH and ATP are used in the Calvin–Benson cycle to fix CO_2 as sugars using the enzyme ribulose 1, 5-bisphosphate carboxylase oxygenase (RuBisCO) (Rochaix, 2011; Zhao and Su, 2014).

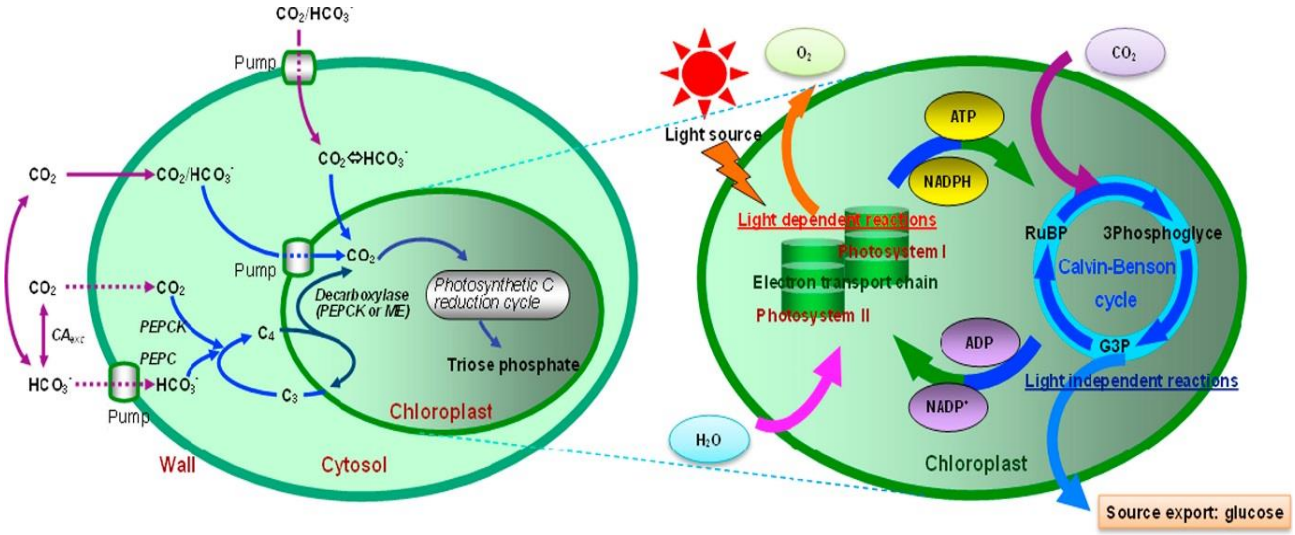


Figure 2.7: Calvin-Benson cycle: Photosystem II (PSII) releases protons into the chloroplast lumen and produces O_2, electrons and protons. ATP utilises the protons for ATP assembly. The electrons are moved via the electron transport chain protein complex, photosystem I (PSI), to ferredoxin (Fd). Lastly, ferredoxin-NADP reductase (FNR) mediates reduction of NADP to form NADPH. The Calvin-Benson cycle involves three phases: CO_2 fixation by RuBisCO, followed by reduction using NADPH and ATP and finally regeneration of precursors (Zhao and Su, 2014).

Algae are responsible for approximately 50% of the world's carbon fixation (Chung *et al.*, 2011). Besides their ability to sequester CO_2, the biomass produced by both macroalgae (seaweeds) and microalgae can be converted to many beneficial products and by-products; some of which are fuels and fuel additives. Fig. 2.8 shows a summary of inputs and outputs of photosynthesis in algae. These products include; hydrogen, oils, sugars, starch and proteins. Hydrogen is used as precursors in fuel cells, while oils, sugars and starch can be processed to

biodiesel, alcohol or methane respectively. High value products such as carotenoids, antibodies and organic acids can also be obtained from microalgae (Goel, 2014).

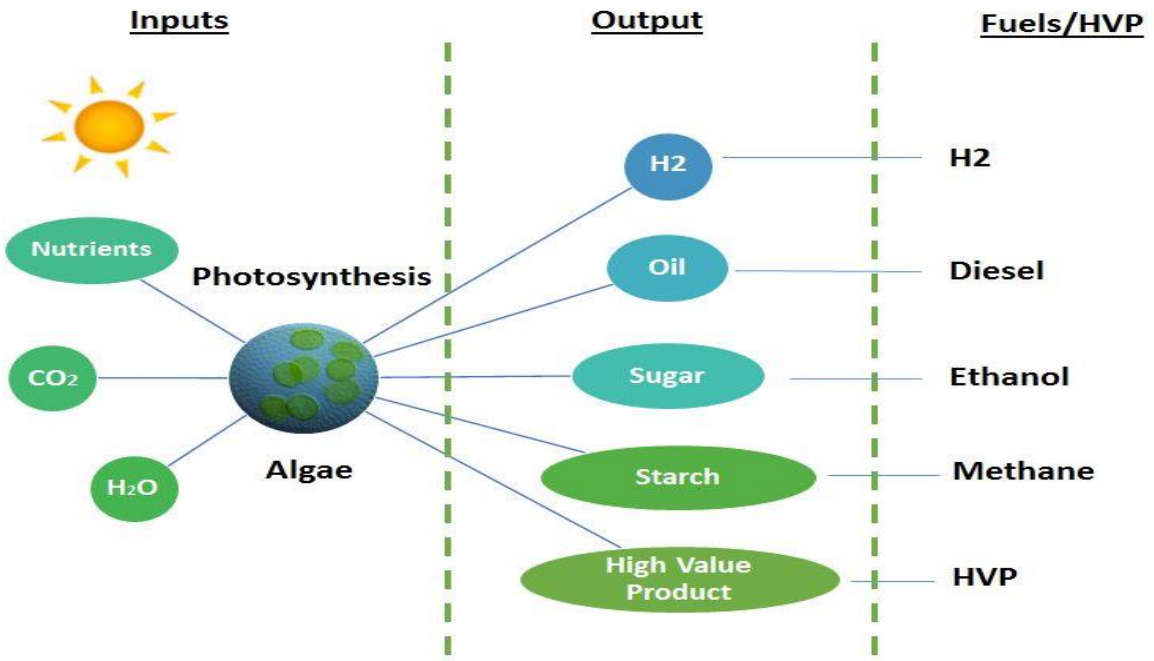


Figure 2.8: Overview of inputs and outputs of photosynthesis in algae. Light, CO_2 and water are utilised by the photosynthetic reactions to produce valuable products.

Microalgae consume substantial amounts of CO_2 during photosynthesis, e.g. *Chlorella vulgaris* and *Anabaena* sp. can fix up to 6.24 and 1.45 g L^{-1} d^{-1} respectively (Ghorbani *et al.*, 2014). It was estimated that 1 kg of microalgae could sequester 1.83 kg of CO_2 (Cheah *et al.*, 2015). Chen *et al.* (2005), demonstrated that *Chlorella* ZY-1 strain could grow up to seven times its initial biomass after six days of culturing using 70% inlet CO_2. The results of various studies conducted to assess the CO_2 mitigation potential of selected microalgae species is summarised in Table 2.1. It is obvious from this table that there are several microalgae species that can be used for carbon capture from elevated CO_2 sources such as flue gas from power plant. Microalgae such as *Chlorella*, *Dunaliella* and *Nannochloropsis* standout as best candidates among others due to their robustness, easy cultivation and high CO_2 fixation.

Table 2. 1: Microalgae biomass yield and their CO_2 fixation rate or removal percentage. Adapted from (Cheah et al., 2015). PBR = photobioreactor.

Microalgae species	**Initial CO_2 (%) (v/v)**	**CO_2 fixation rate (g L^{-1} d^{-1})**	**% Removal achieved (v/v)**	**Biomass yield (g L^{-1})**	**Cultivation system**	**Reference**
Anabaena sp.	~0.03 (Air)	1.45	97	0.85	Bubble column	López et al. (2009)
Anabaena sp.	10	1.01	79	1.2	Bubble column	Chiang et al. (2011)

Botryococcus braunii	5	0.5	87.9	3.11	Fermenter	Sydney et al. (2010)
Chlorella vulgaris	0.03 (Air)	0.06	92.2	~0.315	Sequential bioreactor	Lam et al. (2012)
C. vulgaris	0.09 (Air)	3.55	96.9	0.9	Membrane-sparged helical tubular bioreactor	Fan et al. (2008)
C. vulgaris	2	0.43	57.03	2.03	Vertical tubular bioreactor	Yeh and Chang (2011)
C. vulgaris	5	0.25	86.7	1.94	Fermenter	Sydney et al. (2010), Chiu et al. (2008)
C. vulgaris	5	1.5	35	~0.73	Sequential bioreactor	Lam et al. (2012)
Chlorella sp.	0.038	0.06	60	0.4	Lab scale PBR	Ramkrishnan et al. (2014), Lam et al. (2012)
Chlorella sp.	0.106	3.55	80	0.7	Lab scale PBR	Ramkrishnan et al. (2014), Lam et al. (2012)
Chlorella sp.	1	6.24	59	0.19	Lab scale flask method	Ramanan et al. (2010), Lam et al. (2012)
Chlorella sp.	5	0.86	27	0.196	Bubble column	Chiu et al. (2008), Lam et al. (2012)
Chlorella sp.	5	0.7	1.5	2.02	Vertical tubular bioreactor	Ryu et al. (2009), Lam et al. (2012)
Chlorella sp.	5	0.2	51	0.28	Lab scale flask method	Ramanan et al. (2010), Lam et al. (2012)
Chlorella sp.	10	0.25	46	2.25	Lab scale flask method	Ramanan et al. (2010)
Chlorella sp.	10	0.13	63	5.15	Air lift PBR	Chiu et al. (2009a)
Chlorella sp.	15	0.75	85.6	0.95	Sequential bioreactor	Cheng et al. (2013)
Chlorella sp.	10	0.89	46	2.25	Open race-way pond	Ramanan et al. (2010)
Dunaliella tertiolecta	5	0.27	70.42	2.15	Fermenter	Sydney et al. (2010)
Nannochloropsis oculata	2–15	0.2 - 0.4	11–47	0.246–1.32	Cylindrical glass PBR	Chiu et al. (2009b)
Scenedesmus obliquus	10	1.15	64	0.653	Air lift PBR	Li et al. (2011)
S. obliquus	5.5	0.55	24	3.51	Glass-made vessel	Ho et al. (2010)
S. obliquus	20	0.39	17.5	2.63	Glass-made vessel	Ho et al. (2010)
Genetically modified *S. obliquus*	12	0.95	67	0.948	Air lift PBR	Li et al. (2011)
S. platensis	5	0.32	80.40	2.18	Fermenter	Sydney et al. (2010)
S. platensis	10	1.68	39	2.91	Lab scale flask method	Ramanan et al. (2010)
Spirulina sp.	6	1.39	53.29	3.40	Serial tubular PBR	De Morais and Costa (2007)
Spirulina sp.	12	2.38	45.61	3.50	Serial tubular PBR	De Morais and Costa (2007)

Spirulina obliquus	6	0.72	28.08	1.58	Serial tubular PBR	De Morais and Costa (2007)
Scenedesmus. obliquus	12	0.7	13.56	1.60	Serial tubular PBR	De Morais and Costa (2007)
Mixed *Chlorella, Scenedesmus* & *Ankistrodesmus*	5	0.98	59.80	4.90	Vertical PBR	Rinanti et al. (2014)
Mixed *Chlorella, Scenedesmus* & *Ankistrodesmus*	10	0.85	63.10	5.80	Vertical PBR	Rinanti et al. (2014)

Note:

a. These values (CO_2 fixation) differed from each other and may also not be directly comparable as the cultivation systems and the algae species differ from one another. Nonetheless, the Table can be used as a guide and illustration on the capability of microalgae to sequester CO_2 from different sources/systems at different concentrations.

Several microbes including microalgae where tested for CO_2 consumption at laboratory or pilot scale level, sometimes leading to novel invention that requires intellectual property protection. Focusing on the companies that dominate the development of biological separation of carbon dioxide, the firms that produced equipment that stand out are Alstom Technology Ltd. and CO_2 Solutions Inc., with 9% and 8% of the published patents, respectively. These companies are followed by Novozymes A/S, Akermin Inc., Morphic Technologies AG and Bio Fuel Systems SL, which together make up to 15% of the sector's activity. In this subgroup, the most frequently cited patent is US20080009055A1 (Lewnard, 2008) of Greenfuel Technologies Co., which proposes patented photobioreactor system designed as part of a flue gas scrubbing system which can partially remove certain unwanted pollutants from industrial/hydrocarbon gas effluents. Such pollutant could be CO_2 and/or NO_x and the resultant biomass produce thereof can be converted to other valuable by-products such as biofuels. Such uses of certain embodiments can provide an efficient means for recycling carbon, thereby reducing CO_2 emissions, fuel, and/or cutting stock. The term "phototrophic organism" or "biomass," used in the patent document, refers to any organism capable of photosynthetic growth in an aqueous phase (plant cells and uni- or multi-cellular micro-organisms, including algae, cyanobacteria, Lemna and Euglena) (Míguez *et al.*, 2018).

A patented work of Wei *et al.* (2013), provide a method of supporting growth of microalgae (*C. vulgaris*) using an absorption aqueous medium containing carbon dioxide. The process include contacting a gas with certain percentage of carbon dioxide with an absorbent liquid, allowing the absorbent liquid to absorb the carbon dioxide from the gas, and subsequently passing the carbon dioxide rich liquid into algae culturing system for biofixation and remediation. Chen *et al.* (2012), also reported the capture of CO_2 and converts same to

bicarbonate and used it as feed stock for *D. salina* cultivation. Then the carbonate is regenerated in the algae cultivation process as absorbent to capture more CO_2, which is converted to bicarbonate again for reuse as feedstock. This reduces energy requirement for the carbonate regeneration process. Additionally, transporting a solid or aqueous bicarbonate solution has a much lower cost than transporting compressed CO_2, and using bicarbonate provides a better alternative for CO_2 delivery to algae culture systems than supplying CO_2 gas (Avron and Ben-Amotz, 1978). A vertical tank photobioreactor utilising *N. oculata* as the microalgae feedstock was also developed by Collins (2015). The invention therein provided for the growth and maintenance of *N. oculata* for the production of feedstock for biofuel production, via conversion and recycling of waste gas such as carbon dioxide. This process promote the sustainable recycling of CO_2 using biological process.

2.3 Overview of microalgae cultivation systems

Carbon dioxide, water and nutrients in the presence of light are the basic requirements for microalgae cultivation. Cultivation is usually done in open aquatic environments usually referred to as "open pond systems" or closed systems called photobioreactors (PBRs). There are several issues affecting the deployment of algae culturing for large scale commercial and industrial use. These challenges include massive land and water requirement, high energy consumption, mixing time and rate (Acién Fernández *et al.*, 2012), CO_2 concentration, pH (Fan *et al.*, 2008), biomass density (Cheng *et al.*, 2013), bubble size, gas residence time (Van Den Hende *et al.*, 2012), mass transfer limitations (McGinn *et al.*, 2011), CO_2 solubility in water (Devgoswami *et al.*, 2011), air flow rates and CO_2–O_2 balance (Ho *et al.*, 2011). Considering co-locating algae cultivation near CO_2 emitting sources, other problems become manifest, such as high temperature of flue gas and the presence of unwanted compounds such as NOx and SOx that could hamper effective microalgae growth.

2.3.1 Open pond cultivation

Commercial algae production is mostly done using open ponds due to their simple construction and easy operation. Open ponds are mainly exposed to the open sky, with smaller scale operations under polytunnels. The cells obtain their CO_2 from the atmosphere once it is dissolved as bicarbonate (HCO_3^-) (Zhao and Su, 2014). There is little or no control of environmental factors in this type of cultivation, thus it is associated with low biomass yield and high contamination rates.

Natural water bodies such as ponds, lakes and lagoons or artificial water bodies like tanks can be used for open pond cultivation. They are constructed in different shapes and sizes as illustrated in Fig. 2.9. Due to their simplicity and economic consideration, non-stirred ponds (Fig. 2.9a&c) are favoured (Razzak *et al.*, 2013). Non-stirred ponds typically have a water depth of not more than 50 cm (Benemann, 2008). Problematic issues with open ponds include; cell sedimentation, limited light diffusion due to cell shading in high cell density culture, substantial land requirement, high water demand and evaporation, poor light and CO_2 diffusivity from the atmosphere. To address these, paddle wheels were introduced (Fig. 2.9b&d) and located at a suitable position to provide mixing and avoid sedimentation; this also improves CO_2 diffusivity. Additionally, invasion and competition by other unwanted algae species pose a serious challenge to the desired algae cultivated (Wang *et al.*, 2016; Umar *et al.*, 2018). Contamination by bacteria, fungi and ciliates also reduces the productivity of open ponds. To limit these challenges, farmers may resort to the use of plastic covers to improve growth, enhance CO_2 transfer and reduce evaporation (Razzak *et al.*, 2013). However, these add additional capital costs, increase pond temperature and make maintenance difficult. Plastic covers may also not address the issue of contamination (Jiménez *et al.*, 2003). Commercial open pond algae production is limited to specialised end uses like food supplements and cosmetics. Few algae species thrive in commercial scale ponds.

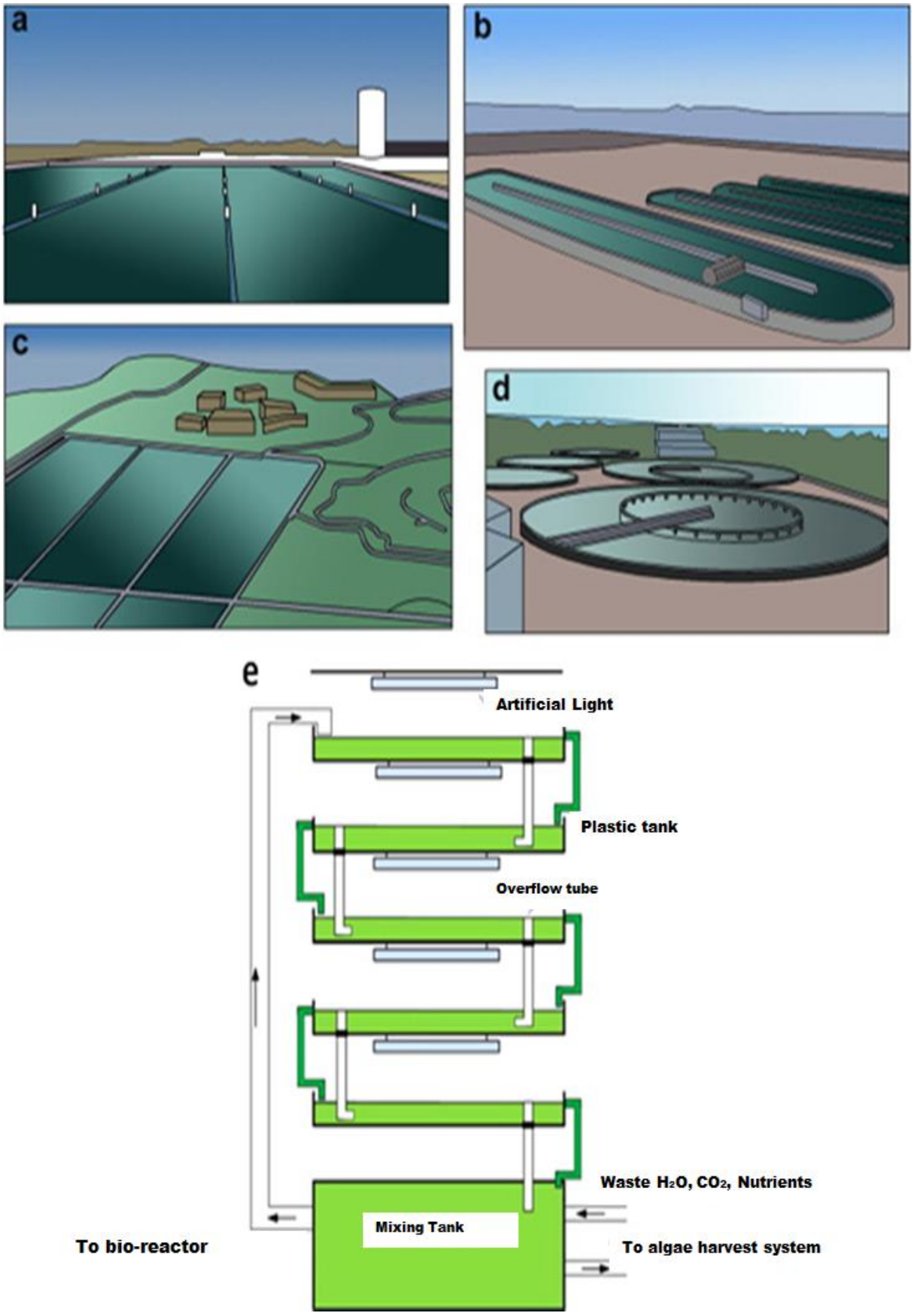


Figure. 2.9: Artistic impression of open cultivation systems: (a) open unstirred pond, (b) paddle wheel raceway pond, (c) partition open unstirred pond, (d) circular stirred ponds, (e) multi-layer open pond bioreactor. Adapted from Razzak et al. (2013) and Zhou et al. (2014).

An attempt has been made to address the land requirement of open pond cultivation by employing the concept of multi-layer open pond reactors (Zhou *et al.*, 2014). The pilot scale involved arranging several layers of tanks on top of each other, Fig. 2.9e. Up to 20,000 L capacity was constructed using artificial lighting to treat animal manure wastewater (Zhou *et al.*, 2014). Water flowed to the lower tank via gravity which also provided mixing and was pumped back to the top tank. However, this system was only able to address the land requirement and scale-up issues faced by open ponds by incurring addition energy costs for pumping.

2.3.2 Closed photobioreactors

Photobioreactors are becoming popular to address many of the challenges of open pond cultivation, especially in the production of high value algae products. PBRs have higher biomass yields due to improved control of the culture environment. A PBR in its most basic form is a reactor constructed using transparent materials to support algae cultivation using natural and/or artificial lighting. CO_2 enriched air is commonly injected directly into the system to improve yield and support mixing. Unlike open ponds, PBRs reduce the contamination rate and allow for the growth of a single species. The most commonly used PBRs include: horizontal tubular (Fig. 2.10a); bubble column airlift (Fig. 2.10b); helical–tubular (Fig. 2.10c); and hung plastic bags (Fig. 2.10d). Some of these are discussed below.

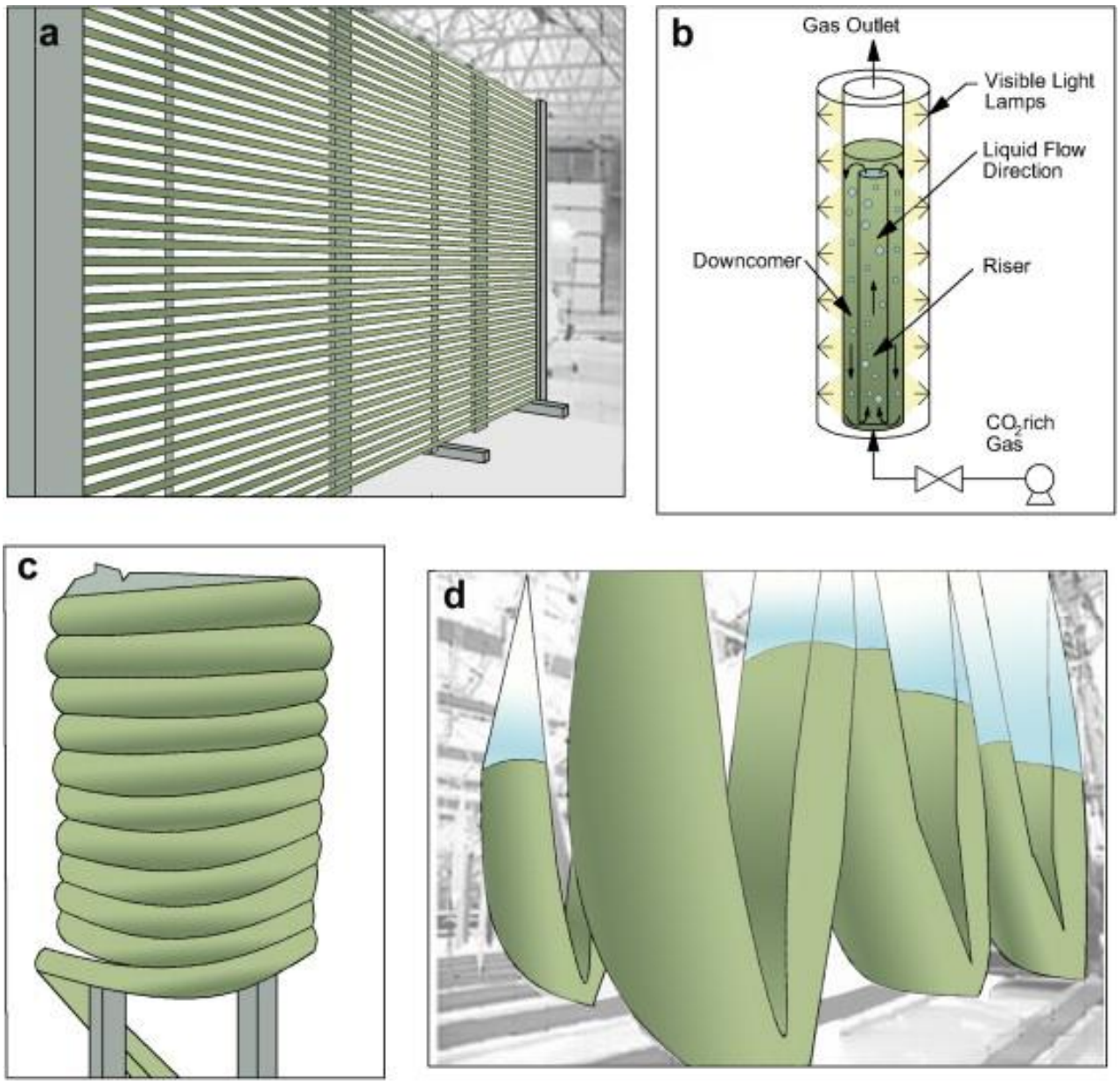


Figure. 2.10: Photobioreactor designs: (a) horizontal tubular PBR, (b) bubble column air-lift PBR, (c) helical–tubular PBR, (d) large-scale plastic bag PBR. Adapted from Razzak et al. (2013).

2.3.2.1 Tubular photobioreactors

Tubular reactors are constructed using glass or transparent plastics. Construction is done using tubes of 5–20 cm in diameter. They consist of a large reservoir and overhead tubes for effective circulation. Mechanical pumps are used to circulate culture from the reservoir to the tubes in a high turbulent flow. The flow (30 to 50 cm s^{-1}) provides mixing, avoids cell deposition and enhances trapping of solar radiation by individual cells (Razzak *et al.*, 2013). Part of the culture is periodically harvested after sufficient circulation through the tube. The usual installed capacity of the reactor is about 20L beyond which it is difficult to manage. Oxygen removal, reactor size, length and dimension are impediments to effective control of this type of reactor

(Ho *et al.*, 2011). The only practical solution for scale-up is multiple reactors connected together which will be expensive and can take up significant amounts of space.

2.3.2.2 Airlift bubble column photobioreactors

In these reactors the culture medium is divided into two interconnected areas via a baffle, with the culture flowing in a circular form due to air supplied from the reactor bottom (Razzak *et al.*, 2013). The circular motion of the liquid improves gas mass transfer, and exposure of cells to light which reduces photoinhibition and shading. Microbubbles can be generated using pressurised gas which will lead to fast bubble dissolution within the medium and prevent gas loss to the environment as a result of rising macrobubbles that burst at the liquid surface (Lam *et al.*, 2012). Despite these advantages, airlift bioreactors are difficult to scale-up because of high cell rupture, difficulty in regulating temperature and high operation costs. To address temperature control, Ferreira and co-workers (Ferreira *et al.*, 2015) designed a split column airlift PBR that supported a higher biomass yield compared to an unsplit airlift PBR, although the operational and scale-up issues remain a concern.

2.3.2.3 Flat plate photobioreactors

A flat plate PBR has a large surface area to volume ratio, providing enough space for light penetration which results in higher biomass yield. The reactor design ensures effective penetration of the short light path and can be fitted with baffles for air bubble aeration (Ho *et al.*, 2011). Aeration rates of 0.023–1.000 vvm were suggested for 5-10% CO_2/air mixture (Zhao and Su, 2014). This type of reactor is scalable up to 2000 L capacity but has difficulty in temperature control and slow growth around the container walls. Similarly, Razzak *et al.* (2013) reported problems with hydrodynamic stress affecting the cells.

2.3.2.4 Polyethylene bag photobioreactors

Transparent polyethylene bags of different capacity, being cheap and simple in construction, are most commonly used. The bags are compartmentalised into sections and hung outside. Air is introduced from the bottom of the bag and collected or exited via the other end to avoid cell settling and enhance mixing (Razzak *et al.*, 2017). It has low energy requirements compared to other PBRs, land requirement is also minimal compared to open ponds. However, bags can only be scaled to a certain level and have limited durability due to constant exposure to atmospheric elements.

2.3.2.5 Membrane photobioreactors

Membrane PBRs are modified versions of either the air lift or tubular PBR with the integration of a membrane to solve the problem of mass transfer within the system. The membrane generates fine bubbles that can quickly dissolve CO_2 in the medium. The bubble diameter generated by microporous membranes range from 5.5–10.1 mm (Lam et al., 2012); the smaller the pore diameter the better the mass transfer rate. A good membrane PBR should have good CO_2 distribution and limit oxygen accumulation. The membrane should be tough and resist corrosion and fouling. The drawback of this reactor is the presence of fine bubbles created by the membrane which result in cloudy conditions inside the reactor which may hinder effective light penetration. Lam *et al.* (2012), suggested separating the light and gas supply system into distinct components to eliminate cloudy culture; however, this will add to production/operational cost and restrict scalability.

2.3.2.6 Filtration photobioreactors

The concept of filtration PBRs was developed from the ability of some microalgae to attach to a supporting substrate using self-produced extracellular polymeric substance (EPS), forming a biofilm. This entails the use of membranes to partition two tanks (cultivation and recycle tanks) whereby the algae are trapped by the membrane as the culture medium is transferred from one tank to another. The algae inoculum is applied to the cultivation tank and allowed to reach their optimal growth. The culture is then passed via the membrane to the recycling tank where the cells are trapped on and attach to the membrane forming a thick biomass wall. The attached cell are easily harvested from the membrane via washing and the cycle continues with the return of the filtered medium back to the culturing tank. This type of reactor can reduced the energy requirement for biomass dewatering as the trapped cells can be concentrated during harvesting. Liu *et al.* (2013), grew *Chlorella* sp. using this method and achieved the highest productivity of 13.56 g m^{-2} d^{-1} in 7.5% (v/v) CO_2, at 35 °C. Care must be taken to regulate medium flow rate to avoid washing off the cells from the membrane. Membrane blockage and energy requirements for pumping medium are obvious drawbacks.

Overall, PBRs have, to a large extent, addressed some of the problems faced while cultivating algae using open ponds, but as indicated in Table 2.2, PBRs are also associated with many difficulties ranging from high energy consumption, limited light penetration in dense cultures, scale-up and cost. Additionally, CO_2 mass transfer in algae cultures remains one of the biggest challenges when growing algae for carbon capture and utilisation.

Table 2. 2: Microalgae cultivation systems and their respective setbacks. Modified from Cheah *et al.* (2015).

Cultivation system	Cost	Scale-up	Energy use	Space	Light efficiency	Growth rate
Raceway pond	Low	Easy	Low	High	Low	Low
Multi-layer bioreactor	Middle	Easy	Low	Low	Middle	High
Airlift PBR	High	Difficult	Middle	Low	High	High
Tubular PBR	High	Difficult	High	Low	High	High
Flat plate PBR	High	Middle	Middle	Low	High	High
Bag PBR	Low	Middle	Middle	Low	High	High
Membrane PBR	High	Difficult	Middle	Low	High	High
Filtration PBR	High	Middle	Middle	Low	High	High

2.4. Microalgae carbon capture: Promises and challenges

CO_2 capture methods are diverse, e.g. chemical absorption, membrane, cryogenic, storage in oceans/geological formations and biological. Technically, these methods are feasible for carbon capture despite variations in capture capability and efficiency. However, economically and from a safety viewpoint, most if not all have challenges. Thus, a viable cost-effective option is urgently needed to replace or compliment current methods.

Plants (including algae) have been the main CO_2 sink for millions of years. Macro- and microalgae dominate marine and freshwater ecosystems, producing their food via photosynthesis and consuming CO_2 in the process. Domestication of some algae species has seen the construction of high rate ponds and PBRs to satisfy demand for products such as food supplements, animal feed and cosmetics. There is now growing interest in combining algae cultivation with carbon capture and utilisation. According to Chisti (2007), microalgae biomass contains 50% carbon, with 1.83 kg of CO_2 captured by a kg of biomass produced. There are three main sources of CO_2 used for algae cultivation; atmospheric air containing 0.03-0.06% (v/v), flue gas from power plant (5-20%), and pure CO_2 of various concentrations mainly used for small scale laboratory experiments. Microalgae CO_2 capture processes using open ponds or PBRs have been widely studied to find solutions for global warming as their CO_2 fixation rate can be higher than terrestrial plants.

Compared with other carbon capture options, microalgae have many advantages such as; high photosynthetic rate which leads to fast growth, low cost of operation, is environmentally friendly and can provide many other useful products from the biomass e.g. biofuel. Microalgae CO_2 biofixation is regarded as a promising new method for post-combustion CO_2 capture and storage (Zhao and Su, 2014). In the last decade algae cultivation pilot plants have been operated to realise and optimise CCU. Algoland (a company in Degerhamn, Sweden), in conjunction

with Linnaeus University, Sweden, captures CO_2 from the cement industry using saline water from the Baltic Sea (Rathi, 2017). Large open ponds are also in operation in Israel for the production of microalgae (*Spirulina* sp.) for nutraceuticals with unintended CO_2 mitigation (Peretz, 2017). Several other plants are in operation in Australia (Sabinay, 2014), Japan (Herrador, 2016), Germany and Spain (Bancroft, 2016). Small scale PBRs are also being tested for carbon capture due to their high biomass yield, high CO_2 fixation, and flexibility in locating near CO_2 emitting points. A patented bioreactor is currently been tested for CO_2 capture at Michigan State University, U.S.A. (Sonal, 2016). de Morais and Costa (2007), used a three-stage bioreactor to sequester CO_2 using *Spirulina* and *Scenedesmus obliquus*. Similarly, Tang *et al.* (2011) used *Chlorella vulgaris* and *Scenedesmus* to remove up to 50% CO_2 from air at different concentrations.

However, despite its high efficiency (up to 80%) in carbon sequestration when compared to terrestrial plants, the employment of microalgae for industrial carbon capture from point sources with higher CO_2 concentration could not be realised due to its slow fixation rates (Sayre, 2010). The rate at which point sources emit flue gases is too fast for the microalgae to capture and utilise the CO_2 for its metabolic activity. This is partly due to the low carbon dioxide affinity of the enzyme RuBisCo responsible to fixing and converting CO_2 to other product within the algae cells. The major limiting factor for algae carbon sequestration also has to do with photorespiratation. Oxygen do compete with CO_2 for fixation by RuBisCo in a process called oxygenase reaction, which produces 3-phosphoglycerate and 2-phosphoglycolate. The phosphoglycolate is then metabolized to glycine, which, when mixed with another glycine molecule produce serine and subsequent loss of CO_2. This carbon loss (one carbon per two molecules of glycine) reduces the ability of the Calvin cycle to regenerate the five-carbon sugar substrate ribulose bisphosphate—required for CO_2 fixation by rubisco—further reducing the efficiency of photosynthesis and CO_2 fixation rate. The process of photorespiration reduces photosynthetic carbon fixation efficiency by 20% to 30% (Sayre, 2010). There were several attempts to improve the efficiency of RuBisCo in both higher and unicellular photosynthetic organism, but with no apparent success (Lin *et al.*, 2014b; Wei *et al.*, 2017).

Microalgae CO_2 fixation rates differs based on species, the state of the algal physiology, pond chemistry, cultivation type and temperature. For microalgae to be used for carbon capture the capture rate has to be significantly improved from the current range of 20 - 40% (v/v) to about 90% (v/v) based on our current experimental data. This may require significant improvement in algae genetic manipulation and biological carbon capture engineering designs.

2.4.1 Land requirement and associated cost

Open ponds are the main method of microalgae cultivation; however, ponds have low biomass productivity and require large areas for cultivation compared to PBRs. It was hypothesised that a 1MW plant emitting 8323 metric tons of CO_2 would need around 64 ha of algae cultivation area producing 20 g m^{-2} day^{-1} dry weight or a bioreactor occupying 16 ha producing 80 g m^{-2} day (Milne *et al.*, 2012). It is rarely possible to find such vacant land located near a power plant and transportation of the gas either via pipes or vehicle will add extra cost to the overall process. The cost of CO_2 transportation over 100km by canal and water tunnel is approximately US\$0.05–0.06/m^3 and US\$0.104– 0.125/m^3 respectively (Chi *et al.*, 2011). These costs increase significantly to US\$11.78 if the CO_2 had to be compressed, dried and transported over the same distance (Kadam, 1997). Thus, were land is unavailable near the CO_2 emitting source, algae growth has to be conducted in PBRs to save transportation costs. PBRs will reduce the cultivation footprint and increase the volumetric productivity, but scale-up, capital and operating costs must be considered.

2.4.2 Water use efficiency and availability

Open ponds are water intensive as water is frequently added to compensate for evaporative losses. Similarly, water is sometimes required to cool PBRs in hot climates. This water requirement may conflict with other needs, such as human consumption and crop irrigation. It was suggested that sea and brackish waters could be used to grow microalgae (if available near the cultivation site) but this may require further treatment which adds to energy consumption and cost (Darzins *et al.*, 2010). Additional cost will also be incurred for water transportation, plumbing and pumping if there is no water source near the cultivation site (Lundquist *et al.*, 2010). Energy and cost could be saved by reusing cultivation water, however, this comes with a greater risk of contamination. Other factors that need to be considered include the production and use of plastic liners and water treatment and disposal (Murphy and Allen, 2011).

2.4.3 High energy requirement

PBRs have a higher energy requirement than open ponds. About 84% of total energy consumption comes from cultivation and harvesting (Fig. 2.11) (Slade and Bauen, 2013). CO_2 procurement, compression and transportation also contribute around 40% of energy consumption (Clarens *et al.*, 2010); additionally, nutrients add 18.8-30.0%. Substantial amounts of energy (>60%) are required for biomass harvesting and drying (Fig.2.11).

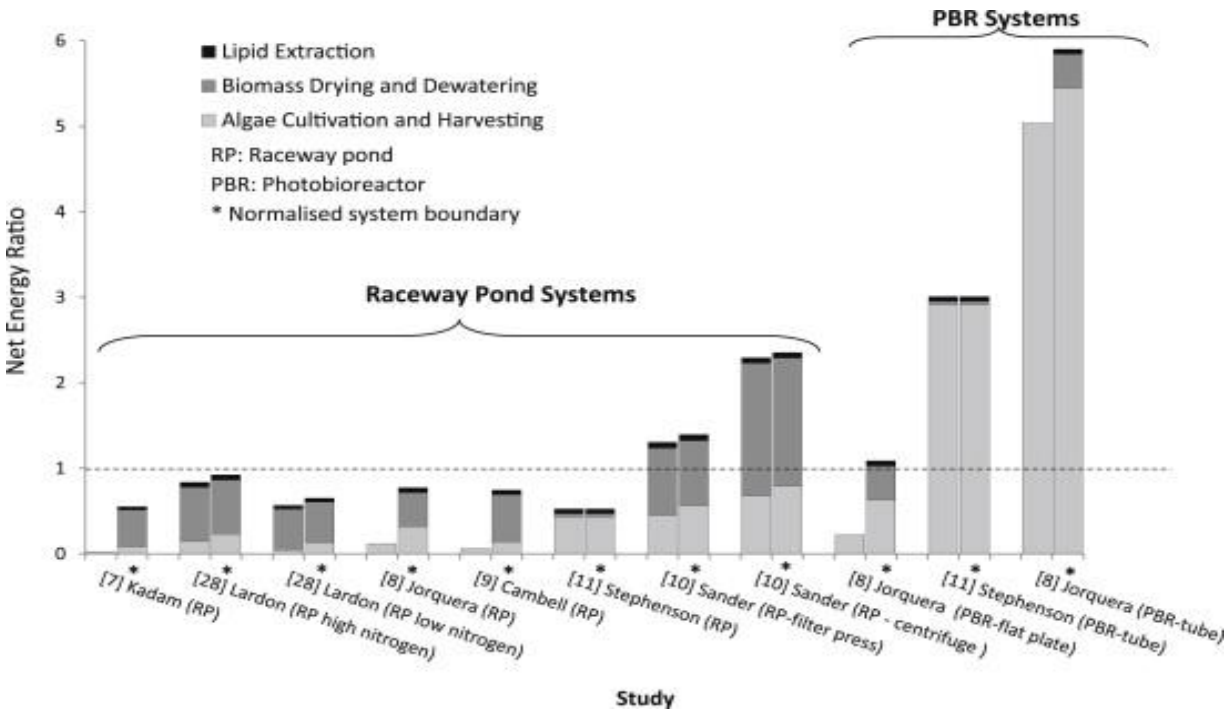


Figure. 2.11: Net energy ratio (sum of cultivation, harvesting and drying energies divided by the biomass energy) for microalgae biomass production: comparison of published values with normalised values. Adapted from Slade and Bauen (2013). NER of < 1 is required for the process to be economical.

2.4.4 Continuous mixing of algae cultures

The performance of open ponds is generally determined by efficient mixing, which also ensures effective light distribution/utilisation, CO_2 dissolution and avoids algae sedimentation. CO_2 solubility in water is low, which directly affects mass transfer from gas to liquid phases. Mass transfer limitation within the culture medium affects CO_2 diffusion and thus hinders cell growth. To improve mixing, different cultivation strategies are employed, such as mechanical stirring using paddles and baffles, membrane sparged devices and bubble gas injection using diffusers. These methods improve cell exposure to solar radiation, efficient nutrient distribution, cell suspension and pH control. Others are improved CO_2 dissolution and timely removal of accumulated oxygen to minimise photo-oxidation and photoinhibition (Chiaramonti *et al.*, 2013; Kumar *et al.*, 2014; Prussi *et al.*, 2014). Zhao and Su (2014), improved the performance of *Chlorella* sp. cultures by constant mixing and aeration. CO_2 biofixation efficiency was improved by 58, 27, 20 and 16% under the CO_2 concentrations of 2, 5, 10 and 15% (v/v), respectively. Mixing systems, although contributing in CO_2 utilisation for photosynthesis, suffer some drawbacks which include; loss of non-fixed CO_2 to the atmosphere; bio-fouling of membranes and diffusers; shear stress damage to cells and a high energy requirement (mixing accounts for nearly 69% of total utility costs in algae cultivation (Hreiz *et al.*, 2014)).

2.4.5 High CO_2 concentration in flue gas and acidification of the growth medium

Microalgae growth under ambient CO_2 conditions (350-400 ppm) can be affected by carbon limitation, whereby the carbon demand for photosynthesis exceeds the CO_2 gas-liquid mass transfer within the medium; this affects critical steps needed to complete the Calvin Cycle. Therefore for optimum growth there is a requirement to increase the level of CO_2 pumped into algae cultures beyond atmospheric levels (Chiu *et al.*, 2009; Ho *et al.*, 2011; Tang *et al.*, 2011). Microalgae growth is directly proportional to the CO_2 concentration of the inlet gas up to a certain percentage (assuming no other limiting factors), although this may differ between species (Table 2.3); for example, biomass productivity of *Nannochloropsis* sp. was improved by 58% when atmospheric air was substituted with 15% CO_2 (Pires *et al.*, 2012).

Tang *et al.* (2011), studied the carbon content and CO_2 capture rate for *Scenedesmus obliquus* and *Chlorella pyrenoidosa* in a modified BG11 medium (Fig. 2.12) using a range of CO_2 concentrations from 0.03 up to 50%. Biomass yield increased up to a 10% CO_2 concentration but decreased beyond this.

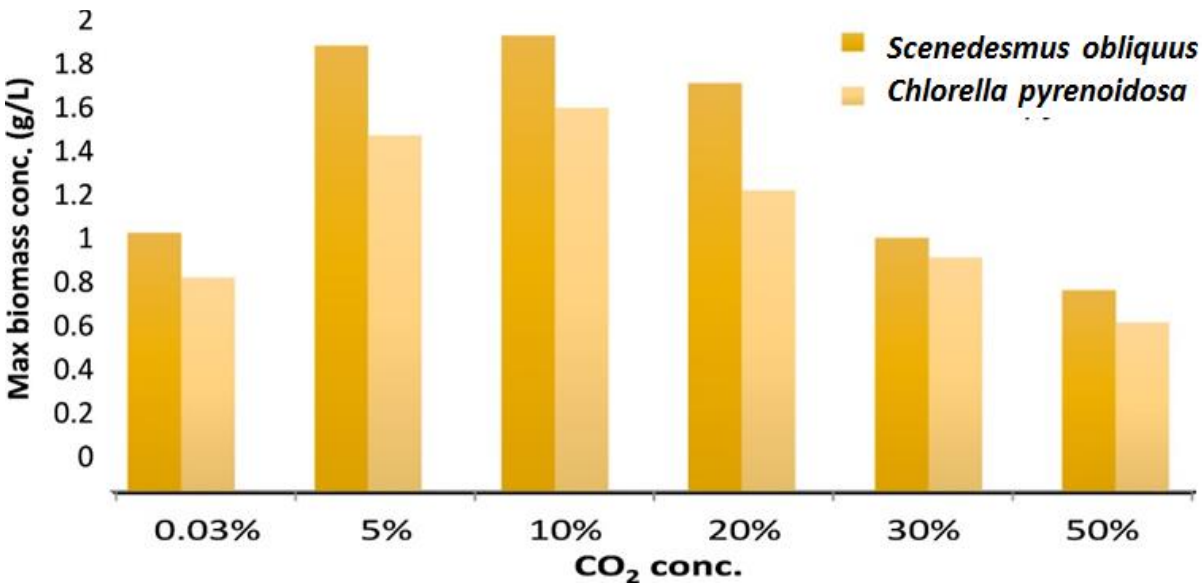


Figure. 2.12: Effects of CO_2 concentration on the biomass accumulation of two microalgae species (*S. obliquus* and *C. pyrenoidosa*). Both algae produced the highest biomass at CO_2 concentrations of 5-10%; Tang et al. (2011).

There are many unanswered questions relating to microalgae cultivation at high CO_2 concentration. The most important being the critical determination of the amount of CO_2 that microalgae can remove from a given concentration of input flue gas. Some researchers argue that no more than 5% could be removed from a flue gas stream of more than 1% CO_2 (Fig. 2.13) provided that the cells are at a modest concentration (Vance and Spalding, 2005). In the same vein, others suggest that cyanobacteria could take up to 70% CO_2 from a flue gas containing 2% CO_2 concentration (Sung *et al.*, 1999; Pires *et al.*, 2012).

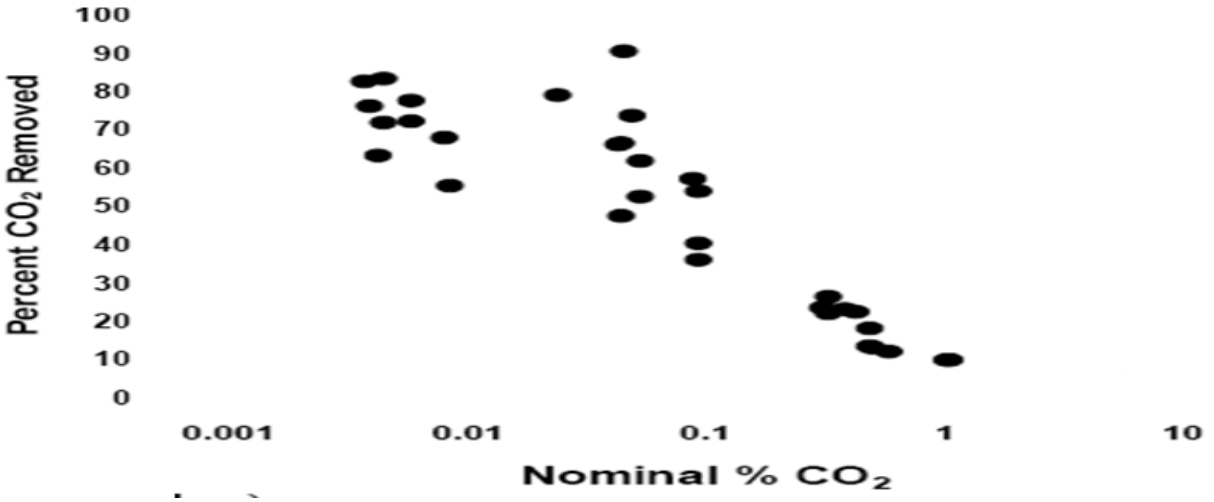


Figure 2.13: The amount of CO_2 sequestrated from the inlet bioreactor gas stream by *Chlamydomonas* over different CO_2 concentrations of ≤ 1% (Vance and Spalding, 2005).

Tolerance of microalgae to CO_2 concentration is species dependant (Table 2. 3), while some can withstand high CO_2 concentrations, others suffer growth retardation and subsequent death. High CO_2 concentration is related to media acidification. Low pH also affects the functioning of extracellular carbonic anhydrase within the cells which functions in the carbon concentrating mechanism (Tang *et al.*, 2011). For instance, slow growth and low productivity was recorded for *Chlorella* sp. when cultured above 2% CO_2 (Chiu *et al.*, 2009).

Table 2. 3: CO_2 uptake and tolerance of selected microalgae species (Pires *et al.*, 2012).

Microalgae	**Maximum CO_2 uptake concentration (%)**	**Maximum CO_2 % (v/v) tolerance**	**Reference**
Tetraselmis sp.	14	14	Matsumoto *et al.* (1995)
Chlamydomonas sp.	15	15	Miura *et al.* (1993)
Nannochloris sp.	15	15	Yoshihara *et al.* (1996)
Dunaliella tertiolecta	15	15	Tsuji *et al.* (2003)
Eudorina sp.	20	20	Sung *et al.* (1999)
Chlorella sp.	40	15	Sung *et al.* (1999)
Chlorella sp.	-	40	Sung *et al.* (1999)
Euglena gracilis	45	45	Kimura *et al.* (1996)
Synechococcus elongatus	60	60	Miyairi (1995)
Chlorococcum littorale	60	70	Sasaki *et al.* (1998)
Scenedesmus sp.	80	80	Sung *et al.* (1999)
Cyanidium caldarium	100	100	Seckbach and Kaplan (1973)

If algae is to be used for carbon capture from flue gas, it is imperative to identify and isolate those that show a positive tolerance toward high CO_2 concentration. Flue gas CO_2 concentration differs depending on the fuel source; however, 10–20% v/v is generally assumed. Using CO_2 from flue gas has many advantages over pure CO_2. Utilising pure CO_2 in microalgae cultivation

would increase the greenhouse effect and is not economically viable (Acién Fernández *et al.*, 2012). The price of pure CO_2 per ton is between £31–40 (Van Den Hende *et al.*, 2012). Therefore, CO_2 uptake by microalgae within a range of 20 and 70% translates to 0.05 and 0.48 £/kg of dry biomass (Van Den Hende *et al.*, 2012). The use of CO_2 from flue gas is however not as easy as it is reported.

2.4.6 Presence of nitrogen and sulphur oxides in flue gas

One pertinent issue that needs to be addressed is the presence of other gases (e.g. NOx, SOx) and heavy metals such as mercury (Hg) within a flue gas. Currently, there are energy penalties involved in controlling these toxic emissions beyond 0.1, 1 and 2% for Hg, NOx and SOx respectively (Milne *et al.*, 2012). Certain microalgae can tolerate these chemicals (within specific limits), for instance, concentrations up to 100 ppm for NO and 400 ppm for SO_2 can be accommodated if medium acidification is avoided (Fig. 2.14). These concentration limits are typical levels found in coal power plants (Matsumoto *et al.*, 1997; Lee *et al.*, 2002). The deployment of microalgae as a bio-CCU remediation technology with the ability to control NOx and SOx emissions could mean that no additional removal systems need to be employed. However, more research is needed to find suitable species with high tolerance to both NOx and SOx.

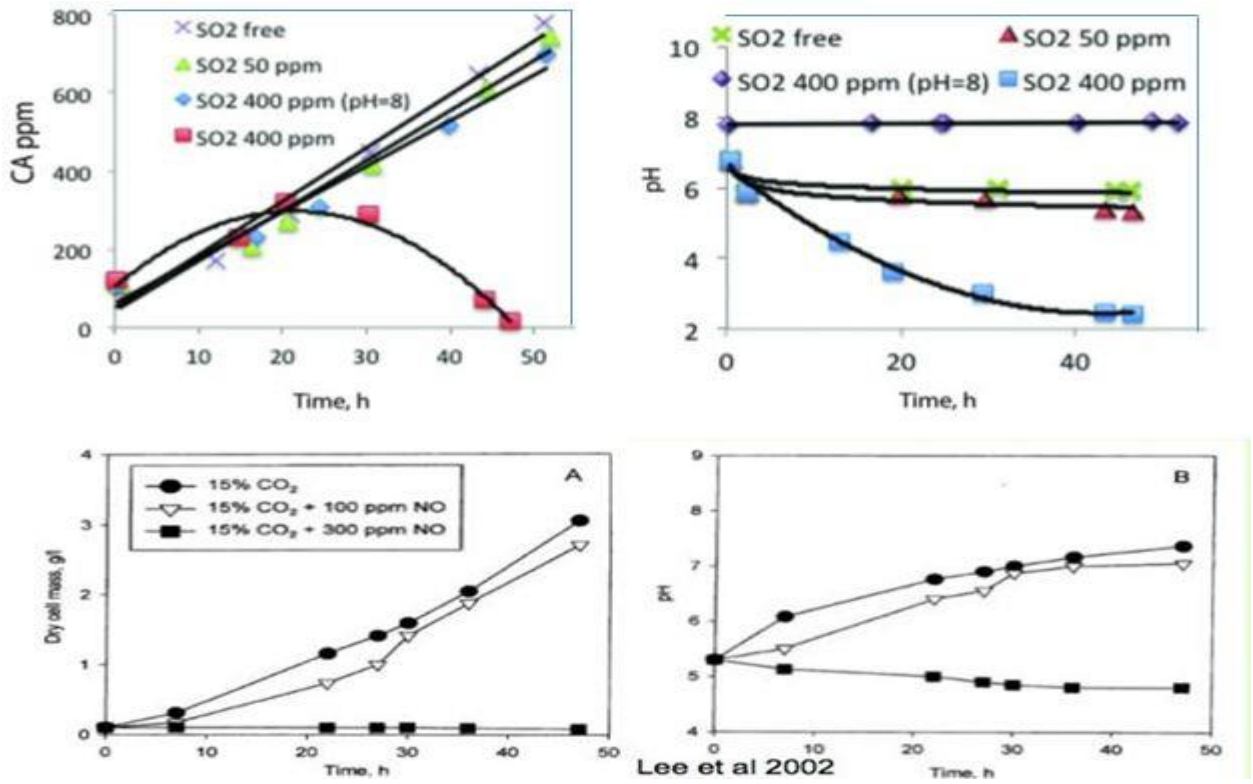


Figure 2.14: Microalgae growth in a simulated flue gas. **Top**: Tolerance of *Nannochloropsis salina* in the presence of SO_2; SO_2 concentrations above 400 ppm acidifies the medium and decreases productivity (Matsumoto *et al.*, 1997). **Bottom**: Tolerance of *Chlorella* sp. to NO (Lee *et al.*, 2002). Microalgae growth is not substantially affected by the presence NO and SO_2 at constant pH.

2.4.7 High flue gas temperature

The temperature of flue gas emitted from a power plant is usually up to 70 ^{o}C, this requires cooling before channelling to algae cultivation systems. To reduce cost of flue gas cooling, it is vital to identify and screen high-temperature tolerant microalgae that can grow optimally under high temperature. The usual upper temperature tolerance of most microalgae is between 20–35 ^{o}C (Pires *et al.*, 2012). However, it is not unusual to find thermotolerant algae thriving in some high temperature regions of the world (Table 2.4). For example, a *Chlorella* species that could withstand up to 42 ^{o}C and grow in air containing up to 40% CO_2 was isolated in Japan (Sakai *et al.*, 1995). Similarly, some species of *Scenedusmus* were found to grow at temperatures up to 75 ^{o}C. Using this kind of algae could ameliorate the need for flue gas cooling, and thus improving the economics. However, care must be taken as solubility of gases usually decreases with increasing temperature (Peary and Castenholz, 1964; Brock, 2012).

Table 2. 4: Summary of some microalgae strains that can be cultivated under high-temperature using air and simulated fluegas (Pires *et al.*, 2012)**.**

Microalgae	**CO_2 (%)**	**T (^{o}C)**	**Reference**
Chlorococcum littorale	40	30	Iwasaki *et al.* (1998)
Chlorella kessleri	18	30	de Morais and Costa (2007)
Chlorella sp. UK001	15	35	Murakami and Ikenouchi (1997)
Chlorella vulgaris	15		Yun *et al.* (1997)
Chlorella vulgaris	Air	25	Scragg *et al.* (2002)
Chlorella sp.	40	42	Sakai *et al.* (1995)
Dunaliella	3	27	Kishimoto *et al.* (1994)
Haematococcus pluvialis	16-34	20	Huntley and Redalje (2007)
Scenedesmus obliquus	Air	30	Gomez-Villa *et al.* (2005)
Botryococcus braunii	-	25-30	Murakami and Ikenouchi (1997)
Scenedesmus obliquus	18	30	de Morais and Costa (2007)
Spirulina sp.	12	30	de Morais and Costa (2007)
Scenedesmus (Bead Geyser)	Air	45-75	(Peary and Castenholz, 1964; Brock, 2012)

2.4.8 Mass transfer limitations and carbon dioxide solubility in water

In dense algal cultures, CO_2 mass transfer from the atmosphere or flue gas can pose a significant limitation to growth. Factors such as gas flow rate, CO_2 partial pressure and bubble diameter can affect mass transfer. CO_2 diffusion is 100 times faster in the gaseous phase than the liquid medium (McGinn *et al.*, 2011), leading to an inadequate supply of CO_2 for photosynthesis. This is further exasperated if atmospheric air is used as it contains only 0.04% CO_2 (Lam and Lee, 2012). The reduced solubility of CO_2 in water, which is approximately 1.45 g/L at 25 ^{o}C and 1 atm (Devgoswami *et al.*, 2011) can also be influenced by water chemistry and thus affect the

mass transfer capacity. After dissolving, CO_2 can react with water forming carbonic acid (H_2CO_3) and the equilibrium shifts towards the bicarbonate (HCO_3^-) as the pH shifts to neutral (Mario *et al.*, 2005). CO_2 penetrates algal cells via diffusion while HCO_3^- is actively transported into the cells (Fig. 2.15). The pH of growth medium thus plays a key role in mass transfer.

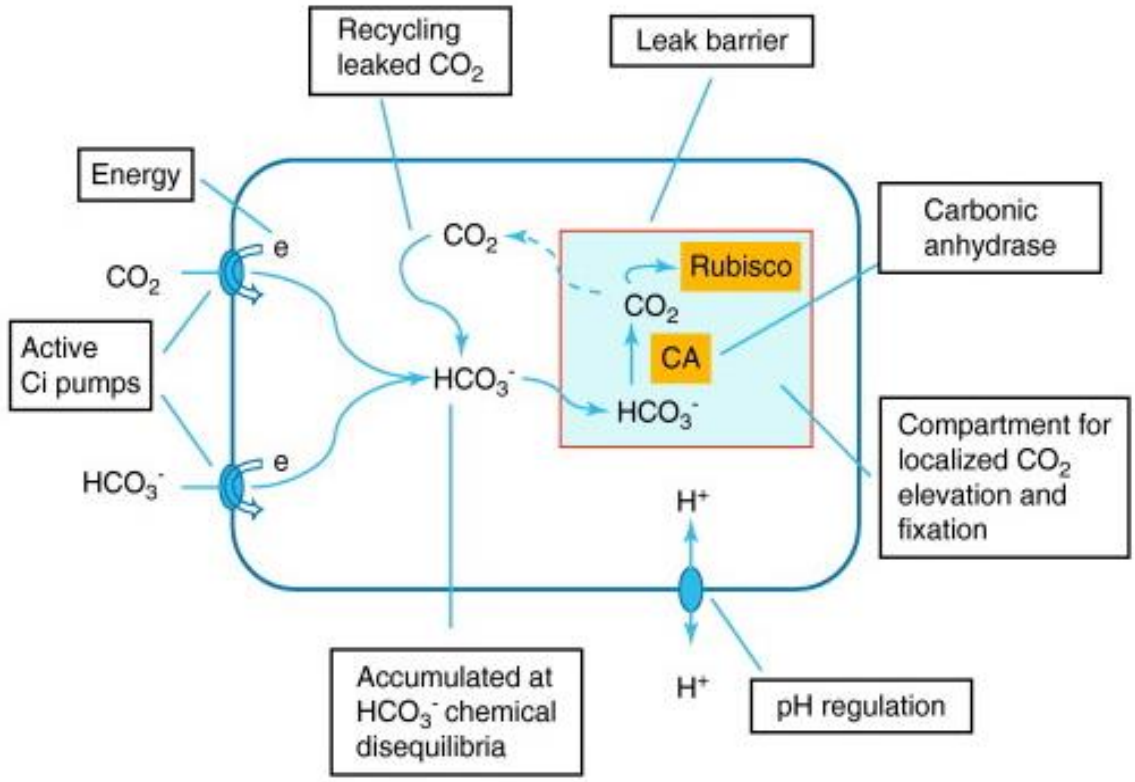


Figure 2.15: CO_2 concentrating mechanism in a green alga. CO_2 is conveyed into the cell cytoplasm and converted into HCO_3^- via redox reaction. The HCO_3^- is transported via carboxysome, a sub-compartment housing carbon fixing enzymes (RuBisCO). Dissolved HCO_3^- present in the medium is also actively transported to the carboxysome where the HCO_3^- is reduced to CO_2 and fixed to simple sugars by carbonic anhydrase (Price et al., 2008).

There are several trials to improve mass transfer within the liquid medium include using $NaHCO_3$ to generate a reaction that will improve CO_2 concentration or using fibre membranes to increase the gas-liquid contact area (Zhao and Su, 2014). Other theoretical means of enhancing CO_2 mass transfer were developed using models of CO_2 mass transfer within algae cultures. The models aimed to establish a relationship between the volumetric gas–liquid mass transfer coefficient, the gas holdup, the superficial aeration velocity and other principal operation variables. Similarly, other areas involved the use of unbalanced mathematical models of CO_2 dynamics bearing in mind the hydration of dissolved CO_2 to the bicarbonate ion (Razzak *et al.*, 2013).

Three alternatives are now being investigated by researchers to enhance CO_2 solubility in water: (i) microbubble technology, which reduces the air/CO_2 bubble sizes during injection into the medium; (ii) membrane PBRs which generate fine bubbles; and (iii) sparging of high CO_2 concentration. These technologies enhance the surface to volume ratio, slow the rising velocity in the liquid phase, and promote faster dissolution rates of gas (Lam *et al.*, 2012). For example, Fan *et al.* (2008) used an 800 ml membrane-sparged helical tubular PBR fitted with hollow fibres to improve CO_2 fixation and oxygen removal. However, scale-up of these systems will be a difficult task. Increased sparging frequency to generate fine bubbles can improve CO_2 dissolution. It is also suggested that high CO_2 concentration could increase mass transfer but will invariable decrease pH with a subsequent decline in growth rate (Cheah *et al.*, 2015). High CO_2 concentration does not guarantee the uptake and utilisation of all the injected CO_2 by the algae, with undissolved CO_2 finding its way back to the atmosphere. Channelling effluent CO_2 to the sequential algae bioreactors was proposed to address the issue of unutilised CO_2 (Lam and Lee, 2013) achieving higher CO_2 fixation rates and improved biomass yields compared to using only one reactor. In Fig 2.16, the productivity in reactors 2-5 were comparable to reactor one, with CO_2 fixation efficiency improved from 1.5 to 7.4%. It was hypothesised that adding more bioreactors will further increase the fixation rate up to 100%. However, increasing unit numbers means more space is needed and thus extra cost. Additionally, energy cost for sparging and aeration will be increased.

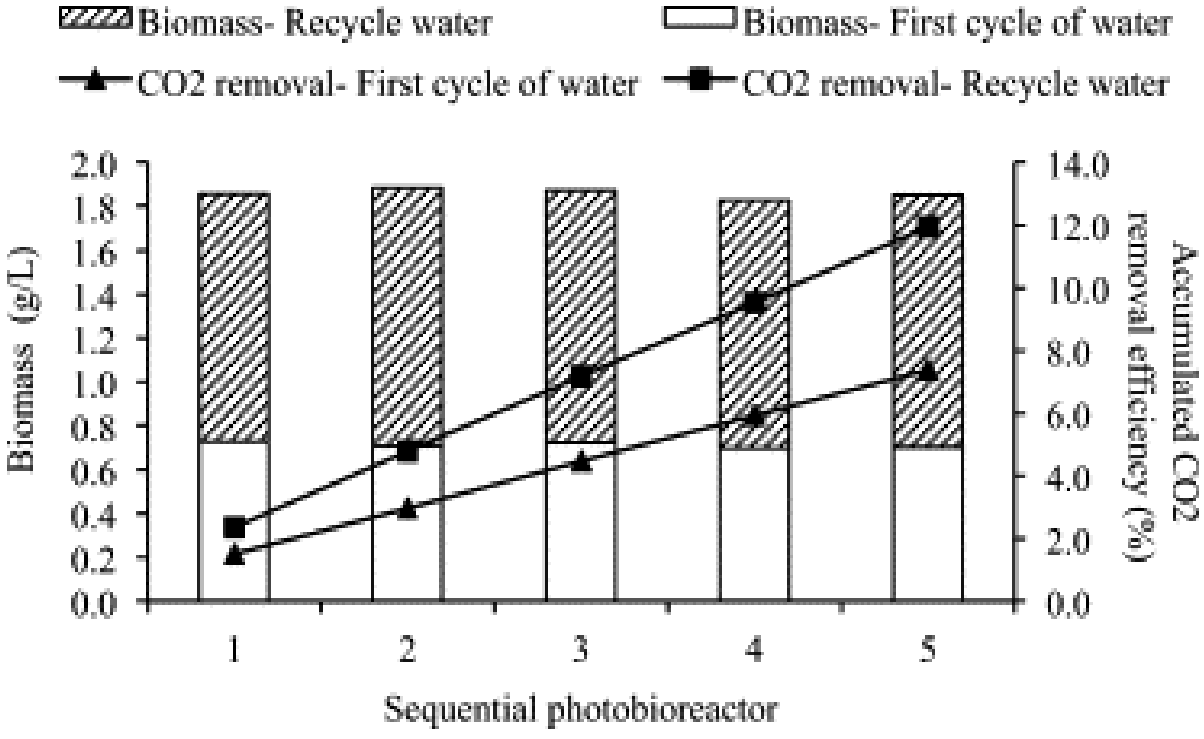


Figure. 2.16: Effect of sequential PBRs on CO_2 removal efficiency with 5% CO_2 supplement (Lam and Lee, 2013).

2.4.9 Air flow rate and bubble rising velocity

Reduced air flow rate means a longer gas residence time within the culture and hence, high probability of CO_2 fixation. Slow flow rate is also responsible for a slow bubble rising velocity. The longer a bubble remains under water the higher the chance of coalescence before reaching the surface (Fig. 2.17). Smaller sparger holes could also produce smaller bubbles that can easily coalesce before reaching the liquid surface (Fig. 2.17).

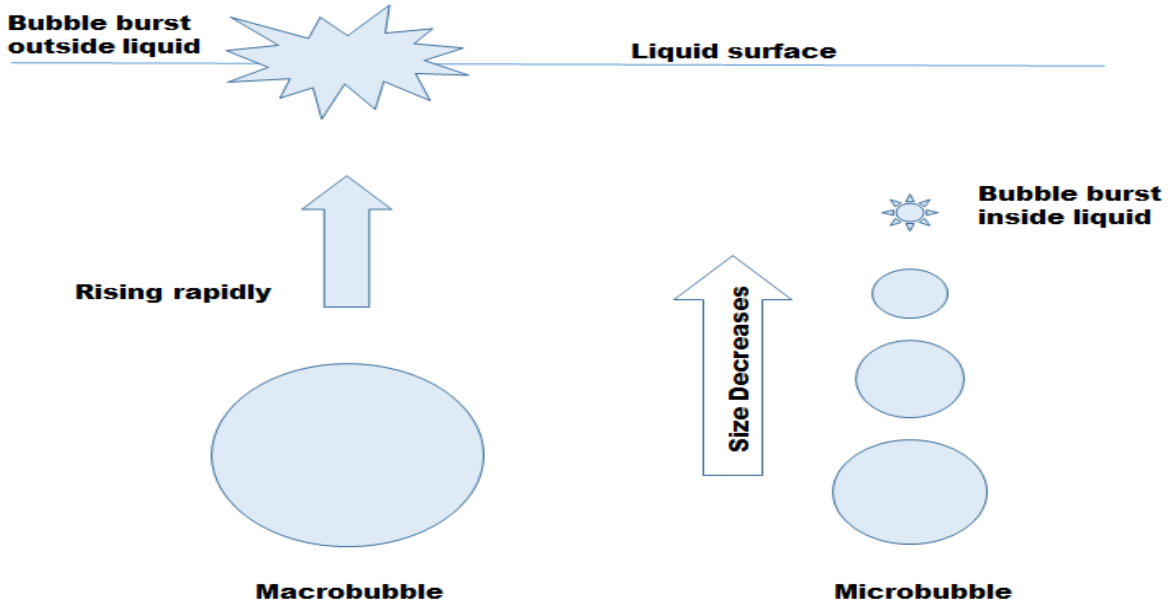


Figure 2.17: The quick rising of a macrobubble to the liquid surface and bursting to release CO_2 to the atmosphere, while a microbubble has a slow rise and bursts within the liquid to increase CO_2 dissolution (Lam *et al.*, 2012).

Conversely, significantly reducing flow rate could adversely affect microalgae cultivation as it affects mixing and causes cell sedimentation. It was reported that halving the flow rate of 5% CO_2 could not generate bubbles for mixing and caused cell sedimentation after two days.

At present, there is no single direct method of addressing the problems of carbon capture using microalgae, be it open pond, PBRs or hybrid systems. Land requirement, water usage, CO_2 diffusion and medium acidification all need to be addressed. There is a pressing need to develop alternative algae-based methods. One option could be microalgae immobilisation as has previously been done for other microbes in the fields of enzymology, hydrogen production and environmental bioremediation (Anh and Bui, 2013; Partovinia and Rasekh, 2018; Sekoai *et al.*, 2018).

2.5 Microbial immobilisation

Some microorganisms (including microalgae) have an affinity for growing in or on a supporting material, either alone or in the presence of other microbes. This can be in or outside aquatic

environments. These behaviours have been studied and mimicked by scientists to grow microorganisms on supporting materials.

2.5.1 Microalgae immobilisation in nature

Microalgae can be divided into two major groups based on habitat; aquatic microalgae that grow in water environments, and aeroterrestrial microalgae which growth outside water bodies (usually on natural or artificial substrates). These substrates could be rooftops, building facades, trees and rocks where humidity is relatively high (Gaylarde and Gaylarde, 2005; Görs *et al.*, 2007; Aburai *et al.*, 2013). They can live alone or form biofilm with other organisms such as fungi, bacteria and protozoa (Eggert *et al.*, 2006; Görs *et al.*, 2007; Patrick, 2016); examples include; *Apatococcus* sp., *Coccomyxa* sp. and *Stichococcus* sp. (Gaylarde and Gaylarde, 2005; Häubner *et al.*, 2006; Elster *et al.*, 2008; Gladis *et al.*, 2010). The majority of aeroterrestrial algae are green algae such as the Chlorophyceae and Trebouxiophyceae (Boedeker *et al.*, 2013). There is limited study on the beneficial aspects of aeroterrestrial microalgae (Hallmann *et al.*, 2013), with most research involving problematic aspects of their presence, especially on buildings, ships and industrial surfaces.

Aeroterestrial algae can withstand desiccation for long periods (Mary and Robert, 1985; Katarzyna *et al.*, 2015). They can also alter their reproductive processes to withstand the effects of drought. Compared to aquatic species, aeroterrestrial algae can shrink to about 60% of their original size during dry seasons (Holzinger *et al.*, 2011). Aquatic species such as *Nannochloropsis* struggle to survive prolong desiccation (Anandarajah *et al.*, 2011), whereas aeroterrestrial species e.g. *Chlorella luteoviridis* can withstand prolonged desiccation and be revived almost immediately upon hydration as they can withstand the effects of high temperature, salinity and UV light. They also have high survival rates under extremely low temperature, e.g. Lukesova *et al.* (2008) obtained 50% survival of 27 cryopreserved aeroterrestrial species. This character could be mimicked to pave the way for the immobilisation of both living and non-living cells on different substrates (Codd, 1987; Katarzyna *et al.*, 2015). Many factors influence the formation and survival of aeroterrestrial microalgae on surfaces, including: species, biofilm formation process, EPS secretion and cell deposition style. Other environmental factors such as substrate properties and surface pH, light intensity and fluidic movement determine the formation and survival of aeroterrestrial microalgae in nature. Knowledge of these underlying factors could help in the development of microalgae biocomposites.

2.5.1.1 Microalgae species

Microalgae species and strains can differ regarding how they are grown and maintained on supporting substrates with some strains preferring surfaces to liquids and vice versa, for instance, the diatom *Amphora* sp. grows well in non-suspended culture while *Nitzschia* sp. grows well in suspended culture medium (Silva-Aciares and Riquelme, 2008). Additionally, medium properties such as sterilisation could affect growth. *C. vulgaris* can form thick biofilm in unsterilised medium in contrast to the declining growth observed with *S. obliquus* (Silva-Aciares and Riquelme, 2008).

2.5.1.2 Biofilm formation

Symbiotic relations between microalgae and other microorganisms such as bacteria, fungi and protozoa often result in biofilm formation (Fig. 2.18) (Sekar *et al.*, 2002; Kanavillil and Kurissery, 2013; Patrick, 2016). Microbial biofilm can be found thriving in some of the world's most inhospitable environments e.g. thermal vents and even nuclear power plant. The formation of biofilm is a complex procedure while the adhesion process is not well understood (Latour, 2005; Genzer and Efimenko, 2006). It is generally hypothesised that the hydrophobic nature of algae cells are the driving force behind biofilm formation on supporting substrates (Cowling *et al.*, 2000).

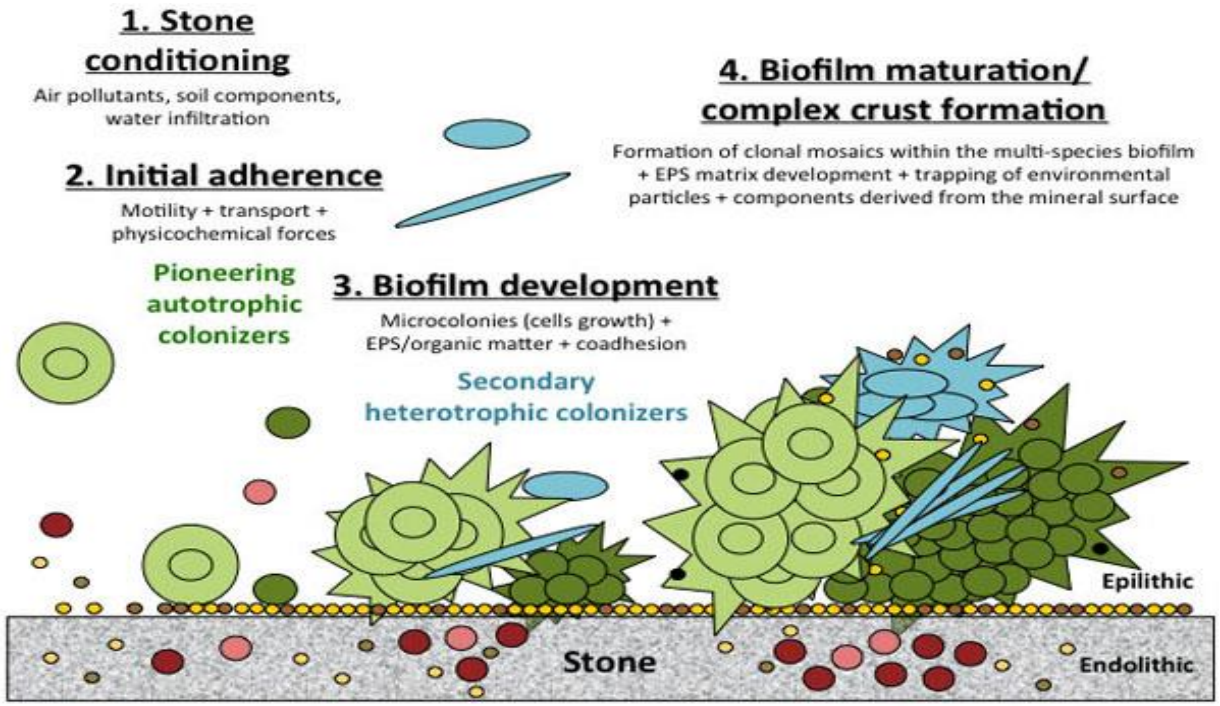


Figure 2.18: Steps in the development of biofilm on substrate. Organic, inorganic molecules and ions are depicted in yellow, brown and dark circles respectively. Pioneering autotrophic colonisers (cyanobacteria and microalgae) are respectively depicted with single and double green circles, while colonisers such as bacteria and fungi are denoted with blue coloured cells. (Patrick, 2016).

The likely first stage of biofilm development is the formation of a conditioning film on endolithic substrates (Fig. 2.18) which is the foundation upon which microorganisms grow (Lindley, 2001; Flemming, 2011). Although there is no hard evidence to suggest the formation of a conditioning film before a biofilm is produced, its presence helps promote cell deposition (MO Pereira, 2001; Donlan, 2002). The formation of a conditioning film starts immediately upon immersion of the substrate in the medium, where layers of molecules and ions are formed (Lindley, 2001). Microorganisms start their attachment immediately after the creation of a conditioning film. It takes only a few hours for microalgae to attach to a substrate in marine environments (Murray *et al.*, 1986). The attached biofilm continues to grow due to cell division of the attached cells rather than the continued deposition of free-floating cells/particles from the surrounding environment. Microalgae undergo a lag phase just before the exponential growth phase which is characterised by the formation of new cells (Doiron *et al.*, 2012). In one such example, initially the biofilm is dominated by the primary colonising algae specie (step 1 of Fig. 2.18), but with time other species take over (step 2 of Fig. 2.18), and finally the biofilm will be dominated by the climax community (in microalgae biofilms this is often diatoms, cyanobacteria or a combination of the two) (step 3 of Fig. 2.18), (Sekar *et al.*, 2002). The microalgae biofilm growth curve is similar to that of aquatic algae (Fig. 2.19). The lag phase is followed by exponential growth, the biofilm reaches its maximum growth and start to slough.

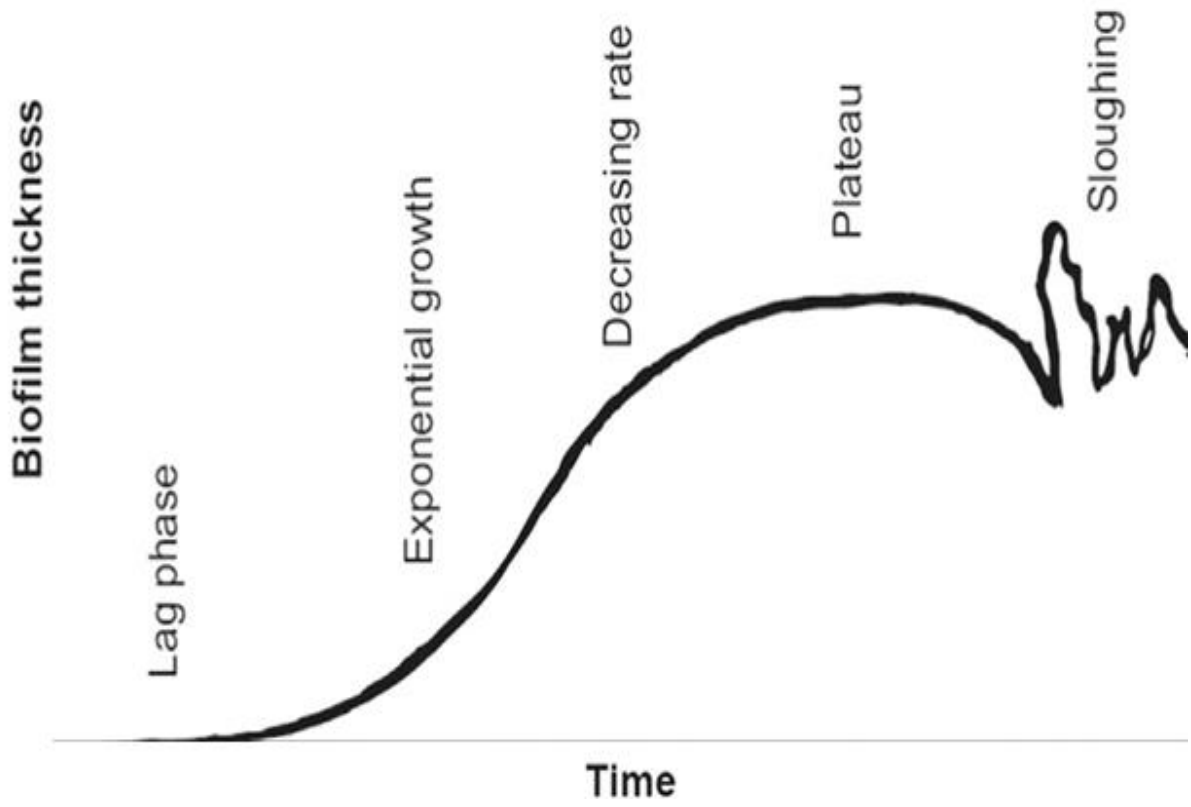


Figure 2.19: Growth of microalgae biofilm (from lag phase to sloughing and subsequent death) (Bott, 2011).

2.5.1.3 Cells deposition factors

The most important factors in biofilm production are light and nutrients (Barranguet *et al.*, 2005; Flemming, 2011), while water and substrate physicochemical properties also play important roles for the growth of aeroterrestrial microalgae (Lindley, 2001; Gaylarde and Gaylarde, 2005; Häubner *et al.*, 2006; Gladis and Schumann, 2011; Hallmann *et al.*, 2013). Porous or semi-porous materials aid in water retention. Aeroterrestrial microalgae thrive on rough porous surfaces under low temperature which help to minimise water evaporation (Ortega-Calvo *et al.*, 1995; Barberousse *et al.*, 2007). Other factors determining cell deposition include; external disturbances (Biggs Barry and Smith Robert, 2002), irradiance (Pillsbury and Lowe, 1999), pH (Sekar *et al.*, 2002), fluid velocity (Chen *et al.*, 2005) and the amount of free floating cells (Lamb, 1987).

2.5.1.4 Extracellular polymeric substances

Extracellular polymeric substances (EPS) are materials released by cells during biofilm production to bind the structure together (Flemming, 2011). EPS creates an enabling environment for further particle attachment; it also enhances cell growth and development (García-meza *et al.*, 2005). EPS comprises various groups (such as phospholipids, proteins and polysaccharides) which function as metal binding sites. EPS helps in cell cohesion (cell/cell binding) and adhesion (cell/substrate binding); attachment of *C. vulgaris* onto substrate is affected by its inability to produced EPS. EPS also plays an important role in nutrient exchange (Geesey, 1982; Katarzyna *et al.*, 2015) and protects the formed biofilm from the effects of the external environment such as grazing (Pajdak-Stós *et al.*, 2001; Matz *et al.*, 2002) and biocide exposure (Wingender, 1999). The production of EPS by immobilised microalgae also helps in protecting the cell from desiccation by helping conserve water within the biofilm matrix.

2.5.1.5 Substrate properties

Substrate characteristics such as texture, hydrophobicity and surface energy contribute to microalgae immobilisation (Fig. 2.20). Many studies have shown that microalgae prefer attachment on rough rather than smooth surfaces. Similarly, hydrophobic substrates tend to draw microalgae toward their surfaces better than hydrophilic objects (Cooksey and Wigglesworth-Cooksey, 1992; Katarzyna *et al.*, 2015). Microalgae also accumulate more biomass on surfaces with low surface free energy (Fig. 2.20). The contribution of surface free energy towards immobilisation surpasses that of surface roughness (Cui (2013); Fig. 2.20).

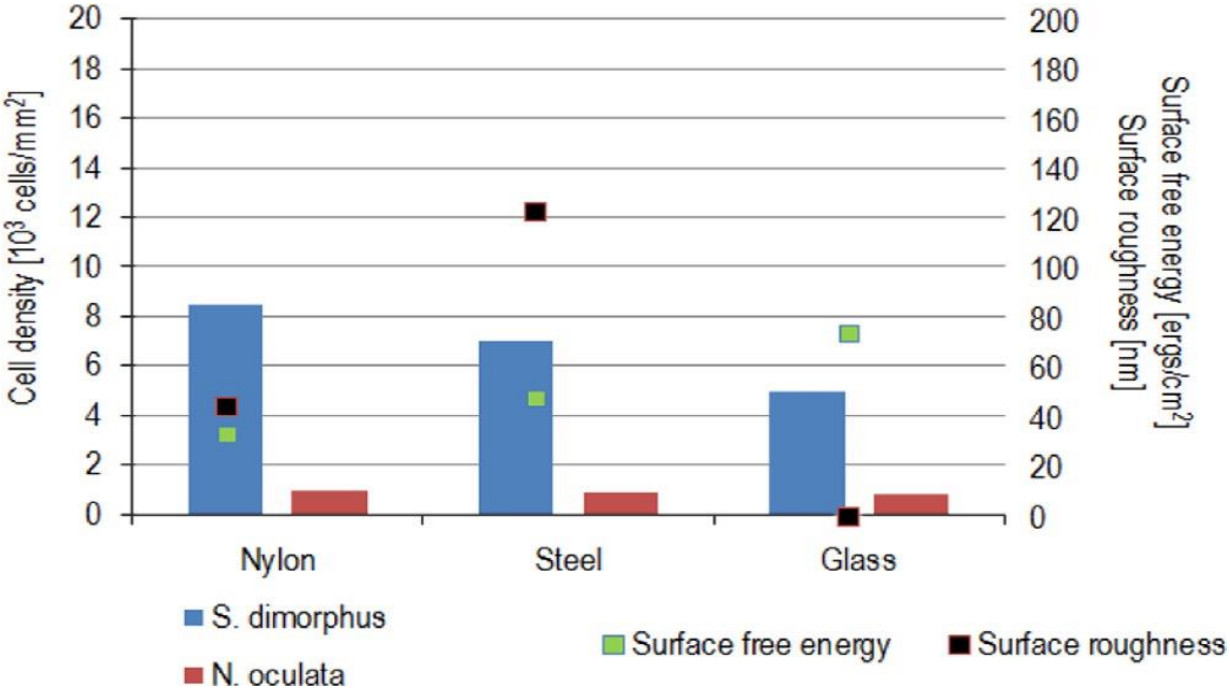


Figure 2.20: Growth of *Scenedesmus dimorphus* and *Nannochloropsis oculata* on nylon, steel and glass. Surface free energies and surface roughness are given. Data based on work conducted by Cui (2013).

Gross *et al.* (2013), demonstrated that the best material for algae deposition is cotton compared to nylon, fiberglass and vermiculite (hydrous phyllosilicate mineral). Similar conclusions were reached by Christenson Logan and Sims Ronald (2012) when they observed that cotton cord was more effective in cell deposition than nylon, jute, acrylic or polypropylene.

2.5.1.6 Nutrient concentration

Algae immobilisation and biofilm development is dependent upon the amount of nutrients received by the cells, which should be sustained at optimum level. Unnecessary increases in the amount of nutrients slows down biofilm formation and activity (MO Pereira, 2001). Some immobilised algae can withstand harsh environmental conditions, though nutrient rich substrates are more promising in maintaining cellular activity. Cells close to (impermeable) substrate surfaces in matured biofilms receive less nutrients which result in sloughing of the biofilm and its eventual death (Flemming, 2011). However, this assertion may not be true for porous permeable substrates that can absorb and retain some nutrients within its matrix which could be used by the immobilised cells. Nutrient requirement for both aquatic and aeroterrestrial algae are the same (but unique for each species).

2.5.1.7 Light intensity and pH

Light intensity generally influences cell growth in photosynthetically active organisms. Light can also determine the strength of cell adhesion in immobilised algae (Flemming, 2011).

Limited light intensity weakens cell adhesion while stronger light promotes stronger adhesion. However, higher light intensity also causes photoinhibition, some species can even stop cell division under the presence of ultraviolet radiation (A and B). Conversely, a study on immobilised microalgae such as *C. luteovirdis* indicates high tolerance to UVA and UVB (Table 2.5). High light tolerance of aeroterrestrial microalgae is a promising step towards algae immobilisation and biocomposite development.

Table 2. 5: The effect of photosynthetically active radiation (PAR), UVA and UVB radiation on selected algal species (Karsten *et al.*, 2007).

Algae	Type	Growth in PAR + UVA	Growth in PAR+UVB	Recovery in PAR+UVA	Recovery in PAR+UVB
Stichococcus sp.	Aeroterrestrial	No change	No change	Full	Full
Chlorella luteoviridis	Aeroterrestrial	No change	No change	Full	Full
Myrmecia incise	Aeroterrestrial	30% decline	43% decline	Full	Full
Desmodesmus subspicatus	Aquatic	30% decline	Inhibition	Full	80%

Microalgae growth and development is influenced by pH. The favourable pH range during algae cultivation is between 7-9 (Baert *et al.*, 1996), but sometimes there could be variation between the pH of the surrounding medium and that of the immobilised cells. In general, neutral pH supports cell immobilisation and enhances cell growth and development.

2.5.1.8 Fluidic motion

One of the most important factors affecting adhesion strength, size and thickness of biofilm is the flow of medium around the immobilised cells and biofilm (Chen *et al.*, 2005; Flemming, 2011). Thick biofilm layers are usually produced by laminar flow at low velocity which also accumulate cells and particles in a dispersed manner. Increases in fluid velocity also intensifies particle mass transfer but also increases the cell removal rates from the biofilm. The optimum fluid velocity for *Pseudomonas fluorescens* biofilm is between 0.7-1.0 m/s (Fig. 2.21), fluidic velocity beyond 1.2 m/s erodes the attached cells and reduces biofilm thickness (Nesaratnam, 1984). Aeroterrestrial microalgae are unaffected by fluidic motion as they usually grow outside a liquid medium, but they may be affected by rains, snow and strong air currents (Häubner *et al.*, 2006).

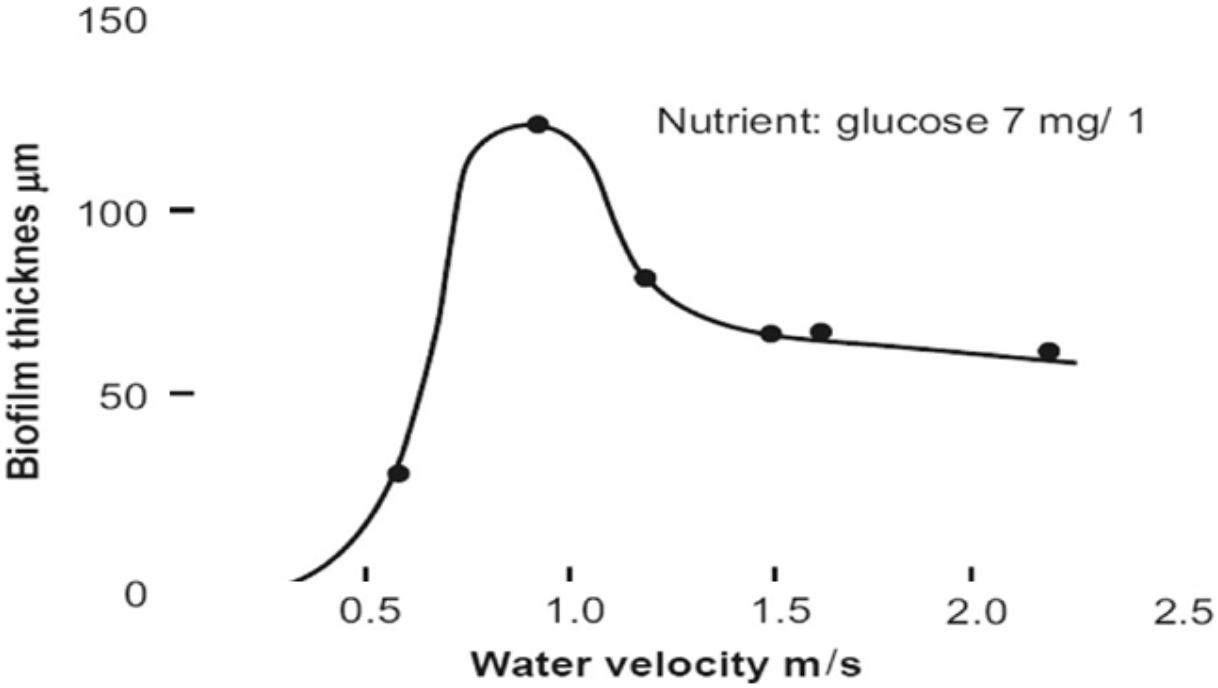


Figure 2.21: Dependence of biofilm thickness on water velocity (Nesaratnam, 1984).

Natural biofilms are cheap with long term reactivity and enhanced resistance to toxic elements which can tolerate structural stability and porosity for wastewater treatment applications (Lear, 2016). Their ability to produce EPS helps them to grow and adapt to their environment (Rosche *et al.*, 2009). Their shortcomings however include lack of controlled cell deposition, high film thickness, sloughing and cell release which could block reactors.

2.5.2 Artificial microbe immobilisation

In the early sixties, novel techniques for immobilising microbes as biocatalysts (from enzymes to whole cells) began to spread in the literature (Papageorgiou, 1987); since then, the use of immobilisation has diversified. At the same time the use of microalgae in biotechnology was gaining momentum, as these organisms are being used in the food, cosmetic, aquaculture and pharmaceutical industries (Borowitzca, 1988). Immobilisation techniques used for microalgae include the use of synthetic resins, polysaccharides (carrageenan, agar, chitosan and alginate) and latexes.

2.5.2.1 Synthetic resin immobilisation

The use of epoxy resin (two or more components reacting to produce a solid inert structure) for encasing living cells was reported by Blanco *et al.* (1999), who successfully encased the cyanobacterium *Phormidium laminosum* to assess metal biosorption capacity, finding that

metal accumulation was directly proportional to the number of encased cells. However, harmful effects of epoxy resins make them unsuitable for living cell encasement or if the end product is meant for human or animal consumption. The same species was also encased within polyvinyl (PV-50) and polyurethane (Hypol FHP) foams to remove nitrate from wastewater (Garbisu *et al.*, 1991), but cellular mortality was too high other than during slow passive colonisation of the treated foam. Polysaccharides were produced from *Porphyridium cruentum* after four days of being encased in polyurethane foam (Thepenier *et al.*, 1985). It was suggested that *P. cruentum* was forming a protective layer against the harsh environment, with some cells surviving the polymerisation procedure and serving as inoculum for the subsequent generation of colonies after most of the toxicity of the foam had been removed. There are several other chemical resin encasement procedures reported in the literature, such as immobilising *E. coli* on poly(*N*-benzyl-4-vinylpyridinium bromide) containing styrene (Kawabata *et al.*, 1990), the bacteria *Paracoccus denitrificans* immobilised with polycarbamoylsulphonate (Willke *et al.*, 1994), and the use of silica gel to immobilise *Spirulina platensis* (Rangasayatorn *et al.*, 2004). However, the cell survivability during these experiments were not mentioned.

Robertson and Phillips (1987), in their patented work were able to immobilise a hydrocarbon consuming microbes on a plastic carrier (polytetrafluoroethylene), to which hexadecane or dodecylcyclohexane were mixed with small amount of water. Presence of the water-immiscible hydrocarbon substrate results in increased loading of microorganisms on the carrier. The volume of water-immiscible hydrocarbon substrate to be added to the nutrient aqueous medium should be around of 0.1-3 ml per 100 cm^2 plastic carrier surface area at a temperature ranging between 25-37 ^oC. Microbes such as yeast, bacteria and microalgae could effectively be immobilised using this novel process, and can be used as a catalyst for heavy metal removal or conversion of hydrocarbon to acids. An immobilisation work utilising transparent polyurethane foam to immobilised microbes for solar light harvesting was also reported as a patent by Adet *et al.* (1987). The wall of the form has numerous pores which allows for liquid and gas permeation but not the immobilkised microbes. Thus, by circulating a liquid nutrient medium and a gas along the one face of the wall and exposing the other face thereof to light, it is possible to culture microorganisms and collect the metabolites formed by them in the liquid medium.

Two types of organic resins (biosorbent, milk casein floccules and glutaraldehyde) were investigated by Seki and Suzuki (2002) to remove Cd and Pb from aqueous media using inactive *Heterosigma akashiwo*. The procedure involved the use and immobilisation of up to 60% of the marine microalgae which proved effective as a biosorbent. Chemical interactions due to cross-

linking, such as covalent bonding involving resins may not be suitable for live cell immobilisation techniques and may only be suitable where non-living cells can be utilised.

2.5.2.2 Carrageenan immobilisation

Carrageenan is a complex polysaccharide derived from the biomass of red macroalgae of the class Rhodophyceae (Gigartinacieae and Solieriaceae) via alkaline extraction. It is made up of 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopiranose units. Carrageenan forms a stable gel upon precipitation in the presence of cations such as amines, amino acids and water soluble solvents (Tosa *et al.*, 1979). There are different types of carrageenan (e.g. ι, κ, and λ) depending on their origin (Burdin and Bird, 1994). $Al(NO_3)_3$ has been used to improve the strength of carrageenan structure. Increased cellular retention was observed by Chamy *et al.* (1990) when they used $Al(NO_3)_3$-treated λ-carrageenan to immobilise *Saccharomyces cerevisiae* with an improved gas absorption from a reactor. Carrageenan was also used to immobilise bacteria for periods of years (Cassidy *et al.*, 1997). Nutrient removal efficiencies of *Chlorella vulgaris, C. kessleri* and *Scenedesmus quadricauda* were assessed when immobilised on different materials including λ-carrageenan coagulated using KCl. The results however demonstrated the fragility of λ-carrageenan when the structure started breaking a week after the immobilisation, leaching the immobilised cells into the medium.

2.5.2.3 Agar immobilisation

Agar is a sulphated galactan, thermoreversible gel derived from red macroalgae of the genera *Gelidium*, *Pterocladia* or *Gracilaria* (Burdin and Bird, 1994). Agarose, which is the gel making part of agar, is made up of linear chains of (1–3)-linked-β-D-galactopyranosyl units and (1–4)-linkages to 3,6-anhydro-α-D-galactopyranosyl units. Agar is a good material for immobilising microalgae (Robinson *et al.*, 1986; Codd, 1987; Papageorgiou, 1987). Agarose was used to immobilise *C. vulgaris* (Aksu *et al.*, 1998); however, its thermoreversible property presents a major challenge to the immobilisation procedure. Agar is solid at room temperature but melts above 80 ^{o}C. Authors such as Bayramoğlu *et al.* (2006) Mallick and Rai (1994) postulates that microalgae immobilisation with agar increases the metal uptake capacity when compared to free cells, but no efforts were made to ascertain the viability of the immobilised cells (Eroglu *et al.*, 2015).

2.5.2.4 Chitosan immobilisation

Chitosan is a polysaccharide of β-D-glucosamine (2-amino-2-deoxy-β-D-glucan) units joined by (1→4) bonds (Oungbho and Müller, 1997). Chitosan is obtained from chitin present in crustacean exoskeletons. It is biodegradable and water soluble and can be employed in microalgae harvesting (Moreno-Garrido, 2008). The positively charged amino group in chitosan attracts negatively charged microalgae cells which is useful in many industrial applications (Lubián, 1989). Substantial amounts (70-75%) of *Euglena gracilis* was successfully removed from the media using 200 mg L^{-1} of chitosan at pH 7.5 (Gualtieri *et al.* (1988). Chitosan immobilisation suffers a setback due to its unstable nature. Kaya and Picard (1996), tried adjusting the rheological properties of chitosan by mixing with konjac (*Amorphophallus konjac*) flour to increase its viscosity and enhance the immobilisation of *Scenedesmus bicelularis*, however there was no noticeable improvement, although the chemical stability of the chitosan was said to be improved when the viscosity was 2% w/v ratio. This may be as a result of phosphate anions in the media which helped maintain its stability (Moreno-Garrido, 2008). Some studies suggest that chitosan decreases cell growth, e.g. the growth rate of chitosan immobilised *Phaeodactylum tricornutum* was slower than those immobilised using alginate cross linked with $CaCl_2$ or $SrCl_2$ (Moreno-Garrido, 2008).

2.5.2.5 Alginate immobilisation

Alginate is the most commonly used polysaccharide for cell immobilisation. They are derived from brown macroalgae; *Laminaria* (*L. hyperborea, L. digitata, L. japonica*), *Macrocystis pyrifera, Ascophyllum nodosum* and *Lesonia negrescens*. The alginate content differs according to species, some reaching up to 40% biomass dry weight (Ertesvåg and Valla, 1998). Some bacteria also produce alginate, such as *Azotobacter chroococcum* (Ertesvåg and Valla, 1998) and *A. vinelandii* (Smidsrød and Skjåk-Bræk, 1990). The addition of some metals can also trigger alginate production in some organisms, as Kidambi *et al.* (1995) reported the release of alginate by *Pseudomonas* in response to copper addition. The alginate backbone is made up of unbranched binary copolymers of 1–4-linked-β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequences (Smidsrød and Skjåk-Bræk, 1990).

Alginate cell immobilisation is preferred over other polysaccharide immobilisation techniques because the entrapped cells do not suffer serious stress during the immobilisation process. Additionally, alginate offers a favourable environment for the immobilised cells due to its air/water permeability, null toxicity and transparency (Smidsrød and Skjåk-Bræk, 1990; Araujo

and Andrade Santana, 1996; Moreno-Garrido, 2008). Alginate forms a thermostable structure when cross linked with monovalent salts. The commonest cation used for the alginate polymerisation is Ca^{2+}. The gelation process involves dissolving the alginate powder in water at room temperature for 3-4 hr and mixing with the required microorganism. The mixture is then dropped gently into a vessel containing Ca^{2+} (usually $CaCl_2$ solution). The stable gel can be dissolved to free the immobilised cells using sodium citrate or phosphate (Hertzberg and Jensen, 1989). It should be noted that sodium alginate does not dissolve in seawater due to the high salt content mixed with other impurities. Therefore, to immobilise marine microalgae alginate powder has to initially be mixed with artificial seawater (containing 35 g/L NaCl) using deionised water before the solution can be mixed with microalgae and subsequently hardened using $CaCl_2$ solution. This technique was used to assess the growth pattern of immobilised marine microalgae using Na-alginate (Hertzberg and Jensen, 1989; Moreno-Garrido *et al.*, 2005), concluding that the stability of the alginate beads depends on the type of microalgae species immobilised. Additionally, it was reported that the lifespan of beads formed using the marine immobilisation procedure fell short of what was observed for freshwater microalgae. Ca-alginate beads of approximately 5 mm in diameter can be quickly dissolved in seawater within 24 h. This is a result of the substitution of divalent cations in the beads by Na cations present in the seawater and leads to structural destruction (Moreno-Garrido *et al.*, 2005). To address this problem, Moreira *et al.* (2006) and Santos-Rosa *et al.* (1989) measured the stability of two types of alginate salt isolated from different algae species (*M. pyrifera* and *L. hyperborea*) and coagulated using Ca^{2+}, Ba^{2+} or Sr^{2+}, immobilising cells of *Phaeodactylum tricornutum*. These authors reported the preference of beads developed from *L. hyperborea* using 4.9% alginate and solidified with 4% Sr solution.

Other techniques used to improve the structural integrity of the alginate beads were reported by Joo *et al.* (2001), where they mixed 2% sodium carboxymethyl cellulose, 2% $CaCl_2$ and microalgae in Na-alginate solution to form beads. The beads were washed with deionised water and re-submerged in $CaCl_2$ for 20 min to increase their stability. The authors confirmed that this method increased bead stability compared with the traditional hardening procedure. Overall, alginate immobilisation presents a superior argument due to its low cost, availability, non-toxic nature, transparency and stability. However, similar to other polysaccharide immobilisations, alginate hydrogels have short operational life spans, need frequent hydration to sustain cells, release encapsulated cells within the medium, impose serious mass transfer limitations due to the additional layer, and are not desiccation tolerant.

In another patented alginate microbes immobilisation, (Chang *et al.*, 1998) were able to optimise the process by first mixing the microbes with CaCl solution and adding small quantity of xanthan before the alginates immobilisation. Additionally, some doses of surfactant were added to the alginate mixture before the cross-linking polymerisation. The addition of the surfactant provides higher porosity to the capsules which prevent it from swelling due to CO accumulation while retaining the immobilised microbes. Another microbe/alginate patented work by Yuan (Yuan, 2000) was also used in the removal of inorganic nitrogen and organic carbon from waste water by addition of polyethyleneglycol (PEG) and polyethylene imide (PEI) to the calcium alginate polymerisation process.

2.5.2.6 Latex polymer immobilisation

Biocomposites (also known as biocoatings, synthetic biofilms, cellular composite coatings, microbial paints and inks, biocatalytic coatings, nanobiocomposites, biomimetic leaves and biocatalytic latex nanocoatings (Cortez *et al.*, 2017)) are fabricated using latex adhesive materials, living microbes and support materials (Fig. 2.22). They can be made into single or multiple layers of metabolically active organisms (Lyngberg *et al.*, 2001). A wide array of readily available inexpensive materials can be used in forming biocomposites such as wood, chippings, paper, fibres and polyester sheets (Bernal *et al.*, 2014). Living biocomposites are designed to have nano- and microporosity for efficient gas and liquid diffusion. Porosity is determined by many factors such as latex particles/cells sizes and their ratio, and coating and drying technique. According to Mota *et al.* (2001), there is an inverse relationship between total porosity ($\varepsilon/\varepsilon_0$) and the volume fraction of the larger particles in the biocomposite (x_D) up to a certain extent (~70%) before reversing the trend to a direct relationship (Fig. 2.23) Additionally, particle and cell size also influences the total adhesion strength of the biocomposites (Cortez *et al.*, 2017).

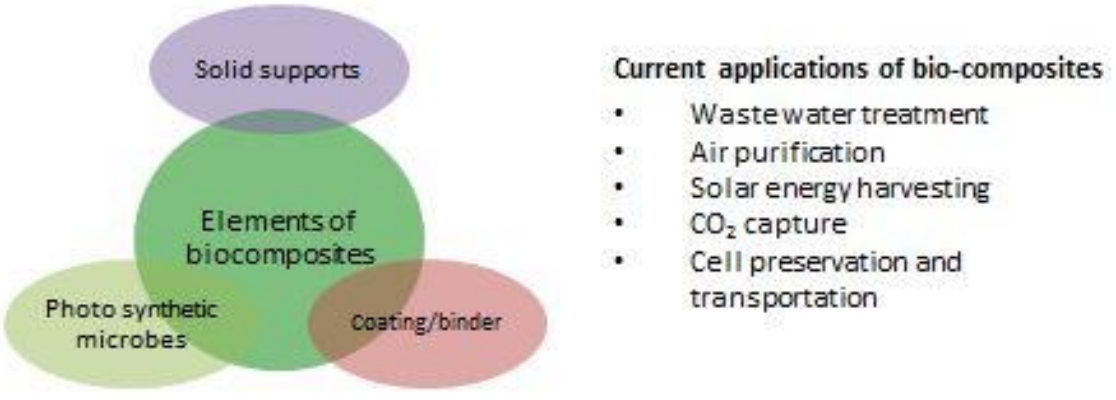


Figure 2.22: Basic elements (solid support, binder and photosynthetic microbes) that make up photosynthetic biocomposites and their potential application.

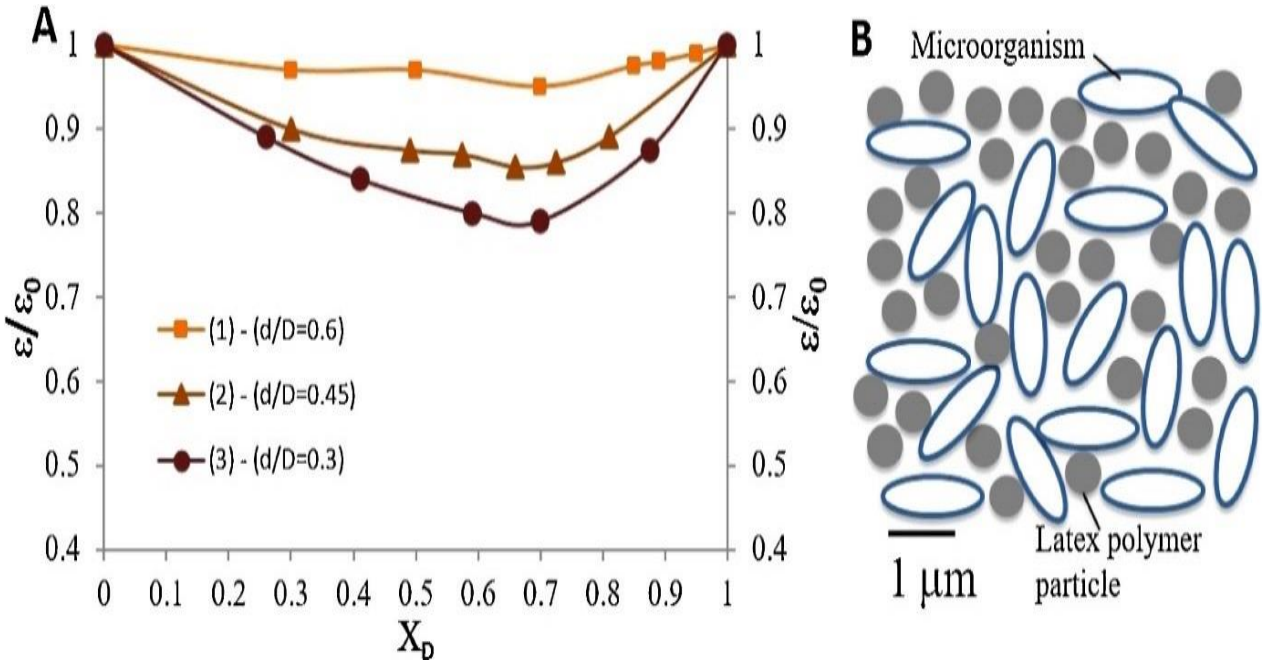


Figure. 2.23: Biocoating porosity is determined by particle size ratio and cell concentration. A. inverse relationship between porosity (ε/ε0) and particle size (xD) for different particle size ratios d/D, where d = latex particle size, D = microbe cell size. B, schematic of latex particles-microbe relationship. Adapted from Cortez et al. (2017).

The first microbial (fungi, yeast and bacteria) immobilisation using latex binders was done by Lawton and Bunning (Bunning *et al.*, 1991), using a polydispersed acrylate/vinyl acetate copolymer with an average particle size of 260 nm at 13 ^{o}C glass transition temperature (T_g) (Cortez *et al.*, 2017). Calcium carbonate was incorporated into the mixture to generate porosity for microbial immobilisation and growth. The mixture of two or more particle sizes (bimodal blend) with different physical properties was used by Cantwell. J.B. (1995) to immobilise living cells in the form of flocculates about 2 mm in diameter. However, there was no data on microbial condition and permeability of the aggregates due to its thick layer (2 mm).

Methylmethacrylate and butyl acrylate copolymer were used to immobilise *Synechococcus* on a carbon electrode, preserving virtually all of the cells with good photosynthetic efficiency after rehydration and illumination (Martens and Hall, 1994). These early biocomposite efficiencies and acceptability for microbial immobilisation suffered a series of setbacks such as poor thickness and porosity uniformity, weak mechanical strength, poor microbial viability and repeatability (Flickinger *et al.*, 2007). To address some of these challenges, Mayer rod drawdown coating methods were employed by Swope and Flickinger (1996) to immobilise *E. coli* using acrylate/vinyl acetate copolymer latex binders. Near thickness uniformity (~80 µm) was achieved. The cell/latex sheet was protected from the top with a nanoporous latex/glycerol (osmoprotectant) layer which enhanced cell viability upon rehydration. Total cell viability (~95%) was reported by these authors for several weeks after rehydration. However, the

procedure for determining cell viability (differential viability staining and laser scanning confocal microscopy) had limited penetration with the biocomposites due to photobleaching (Swope and Flickinger, 1996). Similar work using *E. coli* conducted by Lyngberg *et al.* (1999b) indicated the elimination of coat blistering and enhanced porosity of biocomposites by the addition of glycerol and sucrose followed by a controlled drying process, as well as using the appropriate cell/polymer particle ratio. Addition of sugars within the biocomposite also acted as porogens which helped protect the cells against desiccation (Cortez *et al.*, 2017). The pressure exerted during biocomposite drying causes the entrapped cells to leach from the edges, which was addressed by employing a pressure sensitive vinyl mask to arrest cell release (Lyngberg *et al.*, 1999a). Multi-layered coatings were successfully conducted by the same authors using the drawdown methods with coating thickness of 5-65 μm and patch diameter of 12.5-35 mm (Lyngberg *et al.*, 1999a). These studies demonstrated the feasibility of biocomposite fabrication with smaller coating thickness (∼2 to ∼75 μm) compared to the traditional hydrogels and natural biofilms. The methods also afford greater flexibility in latex coating porosity during desiccation and rehydration at ambient temperature. Good adhesion upon desiccation and rehydration with higher cell viability (> 60%) is an added advantage not found in other microbial immobilisation techniques (Cortez *et al.*, 2017).

Latex biocomposites differ significantly from natural biofilms or cells immobilised in hydrogels. Biocomposites made from adhesive porous polymers incorporating latex particles within a confined space with a thin (2–<50 μm) protective layer are used for gas and element diffusion or excretion, and can be used to stabilise non-growing microorganisms at high concentration (500–1000-fold) to a high fraction (>50% v/w) (Lyngberg *et al.*, 1999a; Lyngberg *et al.*, 2001). These biocomposites can intensify biological reactivity by several fold higher than freely suspended cells (End and Schöning, 2004; Flickinger *et al.*, 2007; Flickinger *et al.*, 2009; Gosse *et al.*, 2010). Osmoprotectants (carbohydrates and glycerol) may be added to stabilise and protect the immobilised cells during drying (Flickinger *et al.*, 2007; Jenkins *et al.*, 2012). Microbial activity can continue for 1000s of hours upon hydration of the biocomposites (Gosse *et al.*, 2010).

Biocomposites are used for many environmental applications (Fig. 2.24) such as biofuel production, gas and chemical synthesis, environmental remediation and as biosensors (Berger, 2009; Carballeira *et al.*, 2009; Demain, 2009; Rao *et al.*, 2009). Biocomposites could also be employed for even distribution of light over different layers of thinly fabricated coatings incorporating living microbes to produce or absorb gas (Flickinger *et al.*, 2009). Interest in

energy reduction during large scale capture and conversion of waste gases into useful products such as biofuels, will open a new dimension to the study of microbial immobilisation using latex as a biocatalyst (Bredwell *et al.*, 1999; Levin, 2004; Sipma *et al.*, 2006; Abubackar *et al.*, 2011; Gosse *et al.*, 2012). Thus, there is a growing need for a thorough understanding of biocomposite behaviour (gas–liquid–microbe mass transfer) within bioreactors as regards mass transfer, energy efficiency and utilisation (Flickinger *et al.*, 2009).

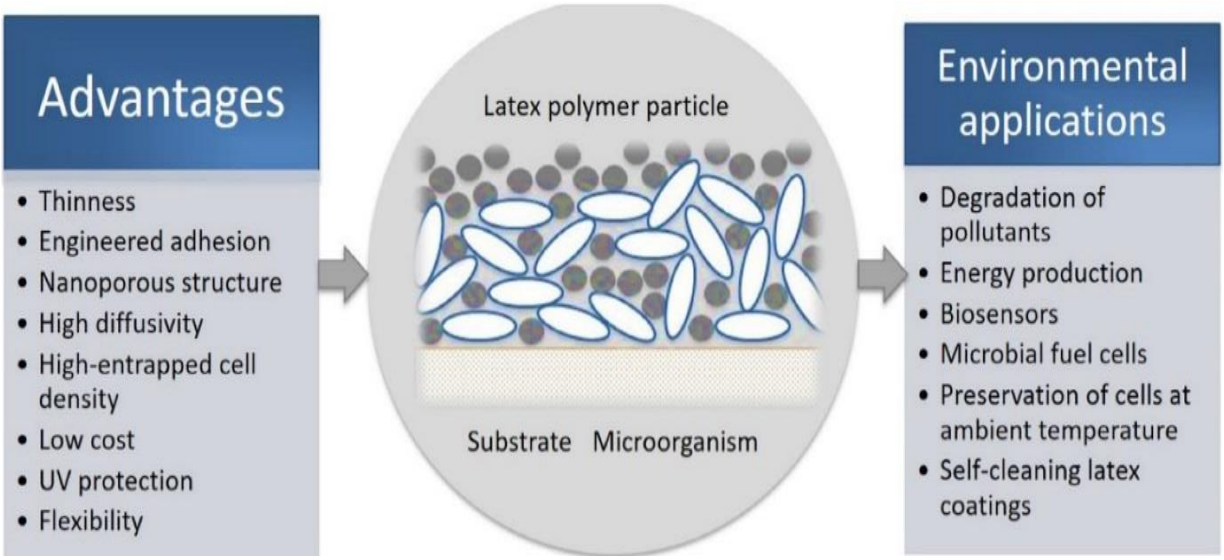


Figure. 2.24: Advantages and environmental applications of biocomposites. Adapted from (Cortez et al., 2017).

The employment of cheap (~\$1 m^{-2}) and readily available non-toxic waterborne latex binder emulsions in biocomposite fabrication is what drew the attention of the pioneers of biocomposites development (Guy, 2004; Flickinger *et al.*, 2009). Latexes can be applied in composites material using different applications such as printing or industrial coating techniques which ensure strong adhesiveness, greater flexibility and porosity (Thiagarajan *et al.*, 1999). The complex molecular interactions among the various components making up biocomposites (multiple polymer particles, osmoprotectants, water, microbes etc.) are not well understood, even though the field of polymer, film formation and adhesion using latex has been well studied in the painting and coating industries (Flickinger *et al.*, 2017).

2.5.3 Substrates used for microbial immobilisation

The physical and chemical properties of an immobilisation matrix are important aspects when fabricating biocomposites (Meunier *et al.*, 2010). Contrary to biofilms or hydrogels, an immobilisation matrix should possess good qualities of controlled porosity, strong adhesion when wet, and high microbial stability upon desiccation (Cortez *et al.*, 2017). Advanced natural or fabricated materials with the aforementioned characteristics should also possess a large area

to volume ratio or high interfacial reactivity to fulfil the requirement of a good material for microbial immobilisation (Kim *et al.*, 2008). The reason for having these properties is the leverage to control its physical (microstructure, porosity, mechanical strength and adhesion) and chemical environment when immobilised with living cells (Xu and Qu, 2014). Supporting materials for microbial immobilisation should be nontoxic, environmentally friendly, cheap, flexible, durable and active at room temperature, in addition to being mechanically and chemically stable (Cortez *et al.*, 2017). Other added advantages are the ability to accommodate a high cell volume of different shapes and composition which will also allow for gaseous/water diffusion when necessary (Cortez *et al.*, 2017). Other good qualities include; hydrophobicity, hydrophilicity, optical and or electronic properties which are all application dependent (Sinsawat *et al.*, 2003). Biocomposites material can be synthetic, porous glass, wood, ceramic, clay, paper, cotton and loofah (Mauter and Elimelech, 2008; Michelini and Roda, 2012).

Admiraal *et al.* (1999), measured the response of microbenthic algae attached to glass in response to metal pollution. The following artificial substrates in order of preference (glass > wood > plastic) were used to determine the settlement order of periphyton based on their attachment time (Danilov and Ekelund, 2001). Glass attached periphyton was also used to determine the effect of water currents on the structure of colonising periphyton (Ghosh and Gaur, 1998). Hydrogen production experiments were conducted by Robinson *et al.* (1986) using glass immobilised *Anabaena* sp. These support matrices are better used with algae with a natural affinity for adhesion such as diatoms and cyanophytes, if the substrate was not earlier treated or a flocculant was not used to aggregate the cells. Other support materials such as ceramics have also been used to immobilise bacteria without reference to any photosynthetic organism (Prieto *et al.*, 2002).

Diverse materials have been employed for microbial immobilisation for a range of applications (Table 2.6), such as polyvinyl alcohol with *Pseudomonas* sp. (Wang *et al.*, 1997), alginate/silica with *Spirulina platensis* (Rangsayatorn *et al.*, 2004), and polyurethane foam with *Mucor circinelloides* (Purwanto *et al.*, 2015).

Table 2. 6: Support materials employed in microbial immobilisation. Modified from (Cortez *et al.*, 2017).

Support material	Microbes	Remarks	Reference
Mixture of agarose (0.5%), polyvinylpyrrolidone (1%) and collagen (0.05%)	*Escherichia coli* and *Saccharomyces cerevisiae*	Biosensor for copper ion detection	(Michelini and Roda, 2012)
Polyvinyl alcohol	*Pseudomonas* sp.	Degradation of phthalic acid esters	(Wang *et al.*, 1997)

Loofa sponge	*Chlorella sorokiniana*	Nickel (II) accumulation	(Akhtar *et al.*, 2004)
Alginate Silica	*Spirulina platensis*	Cadmium accumulation	(Rangsayatorn *et al.*, 2004)
Silica sol-gel	Genetically modified *E. coli*	Degradation of atrazine	(Reátegui *et al.*, 2012)
Polyurethane foam	*Mucor circinelloides*	Recovery of omega-3 from fish oil waste	(Purwanto *et al.*, 2015)
Silica sol-gel	*Bacillus sphaericus*	Copper and uranium accumulation	(Raff *et al.*, 2003)
Alumina fibers reinforced sol-gel	*Rhodococcus rhodochrous*	Degradation of phenol	(Fiedler *et al.*, 2004)
Biocide-free polyvinylacetate-co-acrylate copolymer latex	*Rhodopseudomonas palustris*	Hydrogen production	(Gosse Jimmy *et al.*, 2007)
Solvent-free acrylate copolymer latex binder	*Synechococcus PCC7002*, *Synechocystis PCC6803*, *Synechocystis PCC6803*, and *Anabaena PCC7120*	Light harvesting for photosynthesis	(Bernal *et al.*, 2014)
Sugarcane bagasse	*Rhodotorula mucilaginosa*	Biodiesel production	(Surendhiran *et al.*, 2014)
Mussel adhesive protein (MAP)	Genetically modified *E. coli*	Biosensor for organophosphate compounds detection	(Kim *et al.*, 2013)
Polyethyleneglycol	Activated sludge	Nitrification	(Qiao *et al.*, 2010)
Rigid polyethylene biomass support	*Rhizopus oryzae*	Biodiesel production	(Athalye *et al.*, 2013)
Calcium alginate	Genetically modified *E. coli*	biodegradation of organophosphate compounds	(Ha *et al.*, 2009)
Pectin embedded on graphite electrodes	*Geobacter sulfurreducens*	Microbial fuel cell	(Srikanth *et al.*, 2008)
Sodium alginate, cellulose sulfate	Genetically modified *E. coli*	Cell preservation for long term storage	(Hucík *et al.*, 2010)
Polyurethane foam cuboids	*Aspergillus oryzae*	Biodiesel production	(Guldhe *et al.*, 2016)
Fe_3O_4 chitosan microspheres	*Pseudomonas mendocina*	Biodiesel production	(Chen *et al.*, 2016)
Alginate-polyvinyl alcohol (PVA)-clays	*Bacillus fusiformis*	Biodegradation of naphthalene	(Lin *et al.*, 2014a)
Ca-alginate (Ca)/poly-ϵ-caprolactone (PCL)/corn-cobs beads	*Ganoderma lucidum*	Remediation of anthracene	(Xie *et al.*, 2015)

Some authors have compared the performance of immobilised systems with standard PBRs (Lee Edmund and Bazin Michael, 1990; Sánchez Mirón *et al.*, 1999; Akkerman *et al.*, 2002; Kosourov Sergey and Seibert, 2009) (Table 2.7).

Table 2. 7: PBR geometry and associated illuminated surface area ratios. Adapted from Gosse *et al.* (2010).

PBR Geometry	Dimensions (cm)				Culture Vol. (cm^3)	Surface Area-to-		Reference
	L	W	Breadth	Diameter		Breadth (cm)	Vol. (cm^{-1})	
Flat-plate	200	100	10	NA	200,000	2,000	0.1	Carvalho Ana *et al.* (2006)
Stirred tank	9	NA	NA	10	707	NA	0.4	Carvalho Ana *et al.* (2006)
Helical	211	NA	NA	1	166	NA	1.27	Lee Edmund and Bazin Michael (1990)
Tubular	2,000	NA	NA	3	14,137	NA	1.33	Sánchez Mirón *et al.* (1999)
Coating (Alginate)	6	1	0.033	NA	0.198	182	30	Kosourov Sergey and Seibert (2009)
Coating (Latex)	6.25	1	0.00584	NA	0.036	1,070	171	Gosse Jimmy *et al.* (2007)
Coating m^2 (Latex)	100	100	0.00584	NA	58.4	1,712,329	171	Gosse *et al.* (2010)

There is some research involving the use of loofah sponge as a natural substrate for immobilising microorganisms. Loofah are the spongy part of the fruit of various plants of the genus *Luffa* (*L. aegyptiaca, L. cylinderica, L. operculata, L. acutangula*). The fruit is mechanically strong, non-reactive, and relatively safe for cell immobilisation (Liu *et al.*, 1998). *C. vulgaris* cell were safely immobilised on loofah sponge to remove nickel (II) from aqueous suspension with a higher rate of metal accumulation than the free suspended culture (Akhtar *et al.*, 2004). Other microorganisms have also been immobilised using similar techniques, e.g. a fungus (*Phanaerochaete chrysosporium*) was used to treat wastewater (Iqbal and Edyvean, 2004; Ahmadi *et al.*, 2006), while bacteria (*Zymomonas nobilis*) was used to produced sorbitol (Vignoli *et al.*, 2006) and other microbial bio-systems (Nagase *et al.*, 2006). Ogbonna (1996), reported the possibility of co-immobilising non-adhesive cells with chitosan to improve their flocculating efficiency over the loofah sponge. The adsorption efficiency between loofa and polyurethane was tested by Liu *et al.* (1998), and they concluded no meaningful difference existed between the two materials when *Coffea arabica* free cells were immobilised. Repeatability of experiments using loofah is seldom accurate due to the inconsistent sizes which differ between species and from plant to plant (Liu *et al.*, 1998).

Latexes are extensively used in paints and paper making industries to partially cover the surfaces of the building/paper but retain their internal porosity for breathing. Latexes are low toxicity, low cost and have fast drying properties (Sickler, 2012). Coating substrates with latex

binders does not affect their internal porosity and opens structures as demonstrated by Gosse *et al.* (2012). This knowledge can be extended to other substrates (textile and loofah) with similar properties to paper. Cellulose fibre matrices such as wood, chippings and saw dust are common substrates for microbial immobilisation in packed bed bioreactors and biofilters for air pollution control. Additionally, the natural growth of microalgae on tree trunks, rock and other supporting materials proves that most of these support matrixes are nontoxic. Thus, the combination of readily available substrate and inexpensive latex binders could reduce the cost of biocomposite fabrication.

2.5.4. Immobilised microbes as a catalysts

Catalyst could be any substance that increase the rate of reaction or conversion of one product to another without itself undergoing any permanent chemical change. Ca-alginate capsules were used in fermenters or reactors for a whole cell enzymatic reaction such as ethanol or lactic acid fermentation(Chang *et al.*, 1998). Microalgae by its nature can serves as catalyst for various purposes, such as degradation and sequestration of pollutant, secretion of various useful products and sunlight harvesting and conversion to energy among others, thus making microalgae a very important and useful catalyst, Algae cultivation as discussed previously is capital and energy intensive, thus stabilising, recycling and reuse of microalgae via immobilisation process could save both time and cost. It can also be isolated from a process in a simple manner.

2.6 Biofuel production

Biofuels may reduce CO_2 emissions if produced from renewable photosynthetic sources. Being biodegradable, any spills may be of less concern than fossil fuels (Zhang *et al.*, 2003). Also, the high flash point of biofuels compared to petroleum diesel makes them safer for transportation (Krawczyk, 1996). Similarly, the high viscosity can extend engine lifespan beyond what is obtainable in petroleum diesel engines (Zhang *et al.*, 2003). Biodiesel feedstock can be classified into plant oils, animal fats and waste cooking oils.

Plant oils obtained from agricultural crops such as canola, soybeans, corn, sunflower, palm and cottonseed are the most widely used biofuel feedstock (Briggs, 2004). Fig. 2.25 shows various feedstock and their corresponding potential oil yield. Nonedible plant oil sources such as neem, babassu and jatropha may yet replace edible sources.

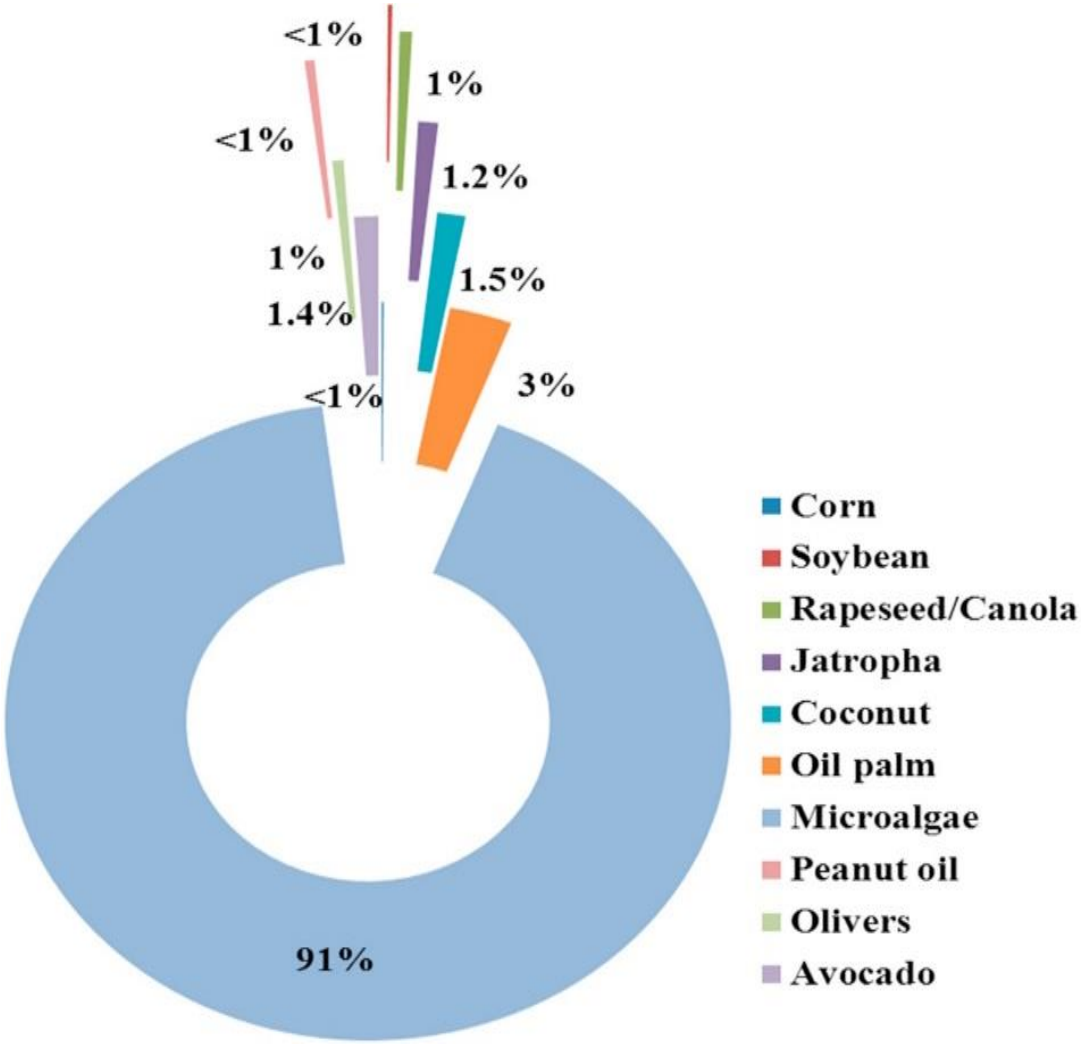


Figure. 2.25: The rate of production of biofuel (L/ha) from microalgae including other feedstock. Adapted from (Maity *et al.*, 2014b).

The second category of biodiesel feedstock is animal fats and tallow. However, animal fats contain significant amounts of saturated fats which cause the biodiesel to gel. This limits its wider application as a fuel source, especially in winter (Canakci and Van Gerpen, 2001).

The third category of biodiesel feedstock is used cooking oil; it includes both animal and plant fats. From an environmental viewpoint, converting used cooking oils is better than disposal in the sewage system which can pollute waterways and cause problems for wastewater treatment (Refaat *et al.*, 2008). These feedstocks have many impurities which require several purification steps. Alkali removal and glycerine purification also pose a challenge to the use of used cooking oil as feedstock (Zhang *et al.*, 2003). Other issues include alcohol recovery or an acid removal step, depending on the type of catalyst used for the transesterification process.

2.6.1 Biofuel from microalgae

Microalgae are regarded by some as the fuel for the future. The United State Aquatic Species Program conducted by the National Renewable Energy Laboratory (NREL), United State Department of Energy in the 1990s documented up to 300 species with biofuel potential. The close-out report of the NREL suggested that microalgae cultivation in open ponds could synthesise 30 times more oil per hectare compared to terrestrial plants. A more recent report suggests that microalgae cultivation can be more cost effective than conventional farming (Li *et al.*, 2008b). However, the harvesting and drying processes still pose a great challenge. It is envisaged that technological developments in the fields of genetic engineering and modified cultivation procedures could mitigate these challenges (Gouveia *et al.*, 2010).

2.6.2 Microalgae lipid synthesis

Triglycerides (TAG) form the major lipid found in microalgae. TAG is similar to those found in vegetable oil and act as energy storage compounds. In addition to TAG, microalgae also synthesise other lipids such as phospholipids found in cell membranes (phosphate group attached to two fatty acids on a glycerol), glycolipids which are molecules made of two fatty acids, glycerol and a sugar molecule, and sulphurlipids found in the chloroplast membrane which are sulphate esters of glycolipids.

The biosynthesis process consists of three steps: the production of fatty acids in the plastids, creation of glycerol-lipids in the endoplasmic reticulum, and the assimilation into oil bodies. Figure 2.26 represents fatty acid biosynthesis catalysed via the enzyme complex acetyl-CoA carboxylase (ACCase) that forms malonyl-coenzymeA from acetyl-coenzymeA and bicarbonate (Maity *et al.*, 2014a). ACCase is present in most prokaryotes and located in the chloroplasts of higher plants and algae. The enzyme catalyses the carboxylation reaction of acetyl-CoA to make malonyl-CoA via its bicatalatic centres, namely carboxyl transferase (CT) and biotin carboxylase (BC).

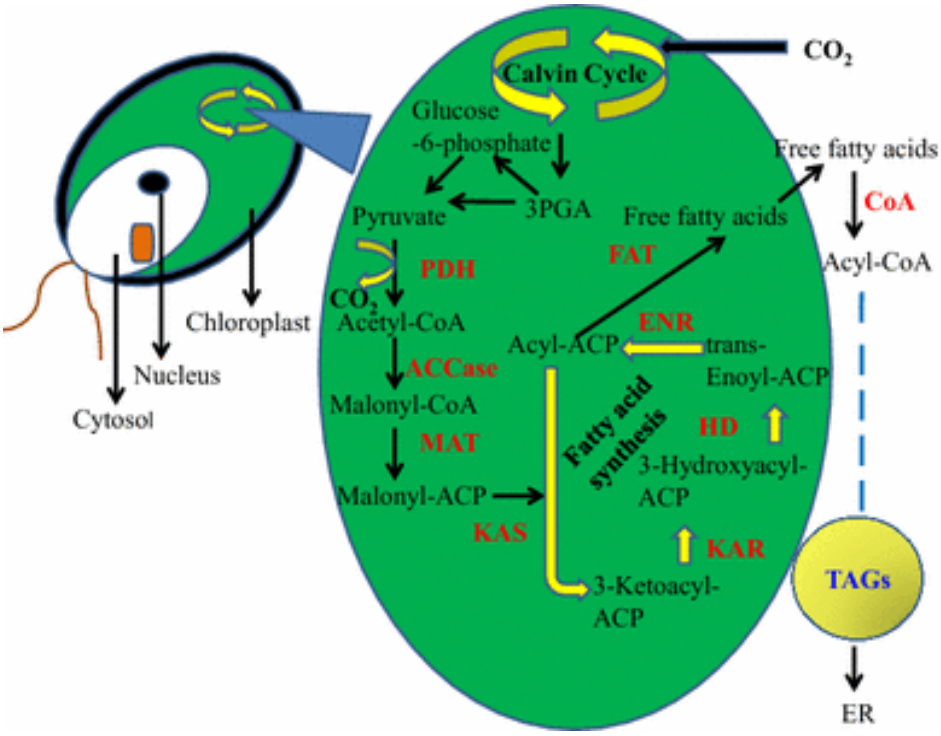


Figure 2.26: Diagrammatic annotation of microalgae lipid biosynthesis. Free fatty acids are synthesised in the chloroplast and the TAGs are assembled at the endoplasmic reticulum (ER). Enzymes are written in *red.* Adapted from (Maity *et al.*, 2014b).

Microalgae lipid content ranges from 1 to >50% depending upon species and growth conditions (Table 2.8). Under normal growth conditions the percentage oil of most microalgae ranges from 10-30% biomass dry weight, however this can reach up to 80% under stress cultivation conditions such as nutrient depletion and high light intensities (Schenk *et al.*, 2008; Ördög *et al.*, 2012). Eukaryotic microalgae have the highest amount of TAG, even though some bacteria can produce up to 80% TAG from their dry weight (Darzins *et al.*, 2010).

Table 2. 8: Microalgae lipid content in % biomass dry weight as reported by (Becker, 1994a; Moheimani, 2005).

Species	Lipid % dry weight
Botryococcus braunii	80
Chlorella protothecoides	57.9
Nannochloris sp.	30–50
Pleurochrysis carterae	30–50
Chlorella pyrenoidosa	46.7
Scenedesmus dimorphus	16–40
Prymnesium parvum	22–38
Dunaliella tertiolecta	35.6
Hormidium sp.	38
Chlorella vulgaris	14–22
Tetraselmis sueica	20
Euglena gracilis	14–20
Scenedesmus obliquus	12–14

2.6.3 Extraction of microalgae lipids

The first stage towards biofuel production in microalgae is extraction of the crude lipid and fatty acids from the biomass, usually via solvent extraction. The most frequently used solvents are ethanol and hexane or their mixture. Organic solvent extraction is less prone to reactivity and can be used directly on moist feedstock, however it is very slow and requires huge amounts of solvent. Ultrasonication of the cells can improve yield and reduce extraction time, e.g. from 4.8 to 25.9% from *Crypthecodinium cohnii* (Cravotto *et al.*, 2008; Mata *et al.*, 2010a).

Supercritical fluid extraction (SFE) is another technique, though on a small scale. This is a novel clean technology with the potential to compliment or replace traditional lipid extraction methods. Depending on the density, the extraction pressure and temperature can continuously be adjusted to suit the extracting ability of the supercritical fluid system. The physical characteristics of the supercritical fluids (liquid and a gaseous phase) allow for the efficient transfer of fluid via the cell matrices and result in higher lipid yield (Halim *et al.*, 2012; Santana *et al.*, 2012).

Table 2. 9: Summary of studies investigating the use of supercritical fluid extraction to recover lipid from microalgae biomass (Halim *et al.*, 2012; Santana *et al.*, 2012)**.**

Microalgae	Extraction pressure or *P* (bar)	Extraction temperature or *T* (°C)	Use of co-solvent	SC-CO_2 flow rate; extraction time	% of Lipid yield	Reference
Schizochytrium limacinum	350	40	Ethanol (95%, v/v)	Not specified; 30 min	33.9	Tang et al. (2011)
Spirulina maxima	100, 250, 350	50, 60	Ethanol; 10 mol % of CO_2	Not specified; Not specified	3.1	Mendes et al. (2006)
Botryococcus braunii	200–250	50–80	None	Not specified	17.6	Santana et al. (2012)
Hypnea charoides	241, 310, 379	40, 50	None	Not specified 1 l min^{-1}; 120	6.7	Cheung (1999)
Pavlova sp.	306	60	None	Not specified; 100	17.9	Cheng et al. (2011a, b)
Nannochloropsis sp.	400, 550, 700	40, 55	None	0.17 kg min^{-1}; 360 min	25	Andrich et al. (2006)

2.6.4 Conversion of microalgae oils into biofuels

The transesterification reaction is employed to convert algal lipid (mostly in the form of TAG) to fatty acid alkyl esters. Small traces of other compounds are also present in the lipid such as water, phospholipids and carotenoids (Bozbas, 2008). TAGs are preferred for biodiesel as they have higher fatty acids and no phosphates (Jin *et al.*, 2006; Mata *et al.*, 2010a). Table 2.10 shows the summary of different types of fatty acids found in different microalgae species.

Table 2. 10: Summary of different microalgae fatty acid profiles adapted from (Sung *et al.*, 1999).

Microalgae	Saturated (%)	Monounsaturated (%)	Polyunsaturated (%)	Other (%)
Chlorella vulgaris	21	14	51	14
Neochloris oleoabundans	18	18	44	20
Phaeodactylum tricornutum	24	25	31	20
Nannochloropsis granulata	21	29	32	18
Isochrysis galbana	32	29	36	3
Botryococcus braunii	0	74	8	18

Transesterification, otherwise known as alcoholysis, is a multistep chemical reaction where TAGs are transesterified in the presence of catalyst to form esters. In the reaction, glycerine is removed from the free fatty acids in TAGs to form alcoholic esters. The initial transesterification is the conversion of TAGs to diglycerides, followed by conversion of the diglycerides to monoglycerides. Finally, monoglycerides are transformed to esters (biofuels) and glycerol as a by-product. The radicals *R*1, *R*2, *R*3 in Fig. 2.27 denote long-chain hydrocarbons, known as fatty acids during the transesterification reaction.

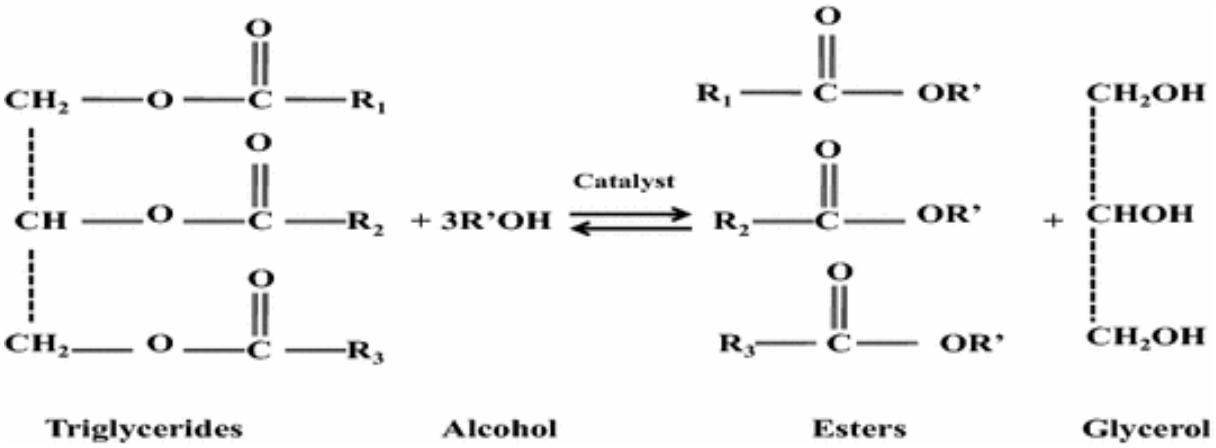


Figure 2.27: Transesterification reaction to produce biodiesel and glycerol as a bi-product.

An acid or base can be used for the transesterification reaction. Strong bases such as sodium or potassium hydroxide are usually employed as catalysts in large scale biodiesel production. Acid catalysed reactions are often used in the laboratory because it does not produce the soaps that are produced when using a base catalyst. However, recent studies have indicated the possibility of having enzyme catalysed transesterification reactions with high biodiesel yields. Teo *et al.* (2014), suggested the use of lipase derived from *Pseudomonas fluorescence*, *Rhizopus oryzae, Candida rugosa* and *C. antarctica.*

2.7 Summary

The need to find a lasting solution to the greenhouse gases (such as CO_2) affecting the planet has never been this urgent. The current chemical absorption and infusion methods for carbon capture and storage are either unsafe or prohibitively expensive. Biological carbon sequestration by microalgae faces numerous unresolved challenges such as excessive land and water requirements, as well as mass transfer limitations. Microalgae immobilisation could offer a solution by reducing land and water demand combined with enhanced mass transfer. Utilisation of the algae biomass into other useful products such as fuels could make this approach broadly appealing to the relevant stakeholders.

Chapter 3

Foam flotation can remove and eradicate ciliates contaminating algae culture systems

Abstract

We demonstrate, for the first time, the efficacy of a surfactant-aided foam flotation system to remove and eradicate ciliates contaminating microalgae cultures. Using sodium dodecyl sulphate (SDS) as the surfactant, ciliates removal efficiencies of up to 86.6% were achieved from pure ciliates cultures at an SDS concentration of 40 mg L^{-1}. At this concentration the majority of ciliates were lysed due to increase in SDS concentration in the collected foamate. The removal efficiency decreased to 55.0 % in mixed algae-ciliates cultures however, this was compensated by employing a multistage flotation and SDS (50 mg L^{-1}) reuse strategy that achieved removal efficiencies of 96.3%, lysing all collected ciliates, but not affecting microalgae growth. The chemicals and energy cost for the process was US$ 0.0025 per m^3, substantially less than comparator treatments. Building upon its applications in biomass dewatering, pre-processing and sterilising, we add metazoan contamination control to the utilitarian properties of foam flotation for the microalgae biotechnology sector.

3.1 Introduction

The industrial scale production of microalgae typically occurs in open culture systems such as ponds and raceways and in closed photobioreactor systems. Photobioreactors afford the grower much greater control over the culture environment, whereas open culture systems are, to all intents and purposes, a microcosm of natural waterways; incorporating the risks posed to microalgae from pathogens and grazers. In natural systems microalgae blooms are kept in check by an assortment of biotic actors, including; viruses (Brussaard, 2004), bacteria (Mayali and Azam, 2004), parasites (Park *et al.*, 2004), protists (including ciliates) (Sherr and Sherr, 2002; Tillmann, 2004), zooplankton (Landry and Hassett, 1982), and fungi (Hoffman *et al.*, 2008). However, such biological control can be catastrophic for algae culture systems, particularly when attempting to culture at industrial scale.

Reflecting the equivalence to natural systems, the type of contaminants found in ponds and photobioreactors (the ubiquitous bacteria and viruses aside) include; parasites (Carney and Lane, 2014), protists (Gong *et al.*, 2015), fungi (Letcher *et al.*, 2013), amoebae (Ma *et al.*, 2016), and

rotifers (Méndez and Uribe, 2012). There is therefore an imperative to develop cost-effective, robust and scalable culture management strategies that either prevent contamination, severely limit the population growth of the contaminant, and/or can safely remove the contaminating agent without harming the microalgae or necessitating major disruption to culture operations. This desire for contaminant control has vexed mass algae growers for over half a century (Loosanoff *et al.*, 1957; Fott, 1967; Lincoln *et al.*, 1983) and the microalgae industry continues to wrestle with contaminant management (McBride *et al.*, 2014; Montemezzani *et al.*, 2015; Montemezzani *et al.*, 2017), spawning growing research fields in monitoring and modelling of contamination scenarios (Day *et al.*, 2012; Forehead and O'Kelly, 2013; Fulbright *et al.*, 2014; Carney *et al.*, 2016; Flynn *et al.*, 2017; Wang *et al.*, 2017).

Current approaches to culture management include growing species or strains that tolerate abiotic factors (e.g. temperature and salinity) out with the tolerance range of their predators or competitors; *Dunaliella salina* culture being a prime example (Hosseini Tafreshi and Shariati, 2009). Rather more interventionist methods include dosing with chemicals such as pesticides (Huang *et al.*, 2014; Van Ginkel *et al.*, 2015; Xu *et al.*, 2015) and disinfectants (Moreno-Garrido and Cañavate, 2000; Park *et al.*, 2016); however, there remains the risk of causing inadvertent harm to the algae (Mostafa and Helling, 2002), or worse, to the culture personnel. Alternative ecological-based control strategies have been investigated (Bacellar Mendes and Vermelho, 2013; Smith and Crews, 2014; Mooij *et al.*, 2015; Wang *et al.*, 2016).

Foam flotation, a technique to separate solution components (including microalgae (Alhattab and Brooks, 2017) by exploiting the variation in their surface charges, is a hitherto unexplored technology for contaminant removal. The closest approximation is that of Kamaroddin *et al.* (2016) who used a microbubble-driven airlift loop bioreactor, switching between CO_2 and ozone as carrier gases, to demonstrate that it was possible to both disinfect and harvest *D. salina* cultures. Building upon work in surfactant-aided foam flotation which demonstrated the efficacy and economic merits of the approach for dewatering and pre-processing microalgae biomass (Coward *et al.*, 2013; Coward *et al.*, 2014; Coward *et al.*, 2015), the use of foam flotation for the removal of contaminating ciliates from cultures of *Chlorella vulgaris* was investigated.

3.2. Materials and methods

3.2.1 Microalgae and ciliates culture

Chlorella vulgaris (CCAP 211/63) was cultured in BG-11 medium in 10 L polycarbonate carboys (Nalgene) at 18 ± 2 °C and a 16L: 8D photoperiod with a mean luminance of 2,500 Lux. The cultures were continuously aerated using an aquarium pump (Blagdon KOI AIR 50). The microalgae were grown for three weeks, reaching a cell density of $3.28 \times 10^7 \pm 10^6$ cells mL^{-1}.

The ciliate *Tetrahymena pyriformis* (CCAP 1630/1W) was purchased from the Culture Collection of Algae and Protozoa, UK. *T. pyriformis*, despite not being a microalgae grazer, was chosen for this lab test as it is a well-researched organism that may be considered as a model to begin evaluating and optimising our foam column separation technique, which can subsequently be extended to other protozoa species that pose a threat to microalgae cultivation. One of the benefits of using *T. pyriformis* is its rapid growth rate, with a population doubling time of 5-7 hours under optimal conditions (Lukačínová *et al.*, 2007). The culture was grown axenically at 18 ± 2 °C, pH 6.5 – 7.5 in autoclaved proteose peptone yeast extract medium (PPY) containing 2% proteose peptone and 0.25% yeast extract (Sigma Aldrich, UK). The medium was stored at 4 ºC and subsequently adjusted to 15 ºC prior to use. A dense *T. pyriformis* culture was gently agitated to evenly mix the ciliates and 0.5 mL was transferred to 10 mL test tubes containing 5 mL of PPY medium using a sterile Pasture pipette. After seven days the density of each new culture was checked using a LEICA DM500 inverted microscope at ×200 magnification. The test tube junction cap was subsequently wrapped with white plastic nylon to reduce evaporation.

3.2.2 Foam flotation column

The foam flotation column was a modified version of that described by Coward et al. (Coward *et al.*, 2013) (Fig. 3.1); briefly, a sparger made from 6.0 mm thickness fine grade polyethylene sheet was used to provide bubbles with an average diameter of 1.13 ± 0.14 mm. The column section was made from a series of modular sections of 250 mm in length, 47.5 mm internal and 51.5 mm external diameter. The column height was 510 mm. A collection cup was attached to the top of the column to receive the foam. The total column volume was approximately 1.0 L. The foam harvester was designed as a foam separation column for the physical separation of the ciliates.

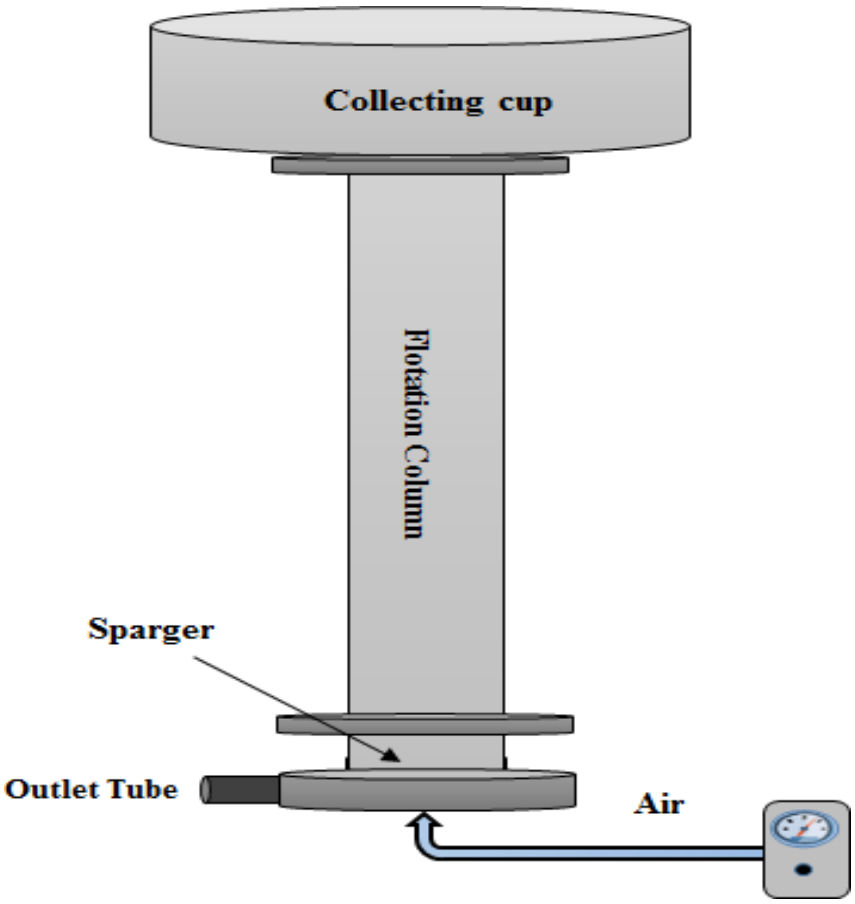


Figure. 3.1: The foam flotation column dimensions: foam collection cup, 300 mm diameter, 100 mm tall; flotation column, 510 mm tall, 51.5 mm outer diameter, 47.5 mm inner diameter; polyethylene sparger, 6.0 mm thickness.

3.2.3 Ciliates multistage harvesting using the foam column

Three sodium dodecyl sulphate (SDS) concentrations (20, 30 and 40 mg L^{-1}; Sigma Aldrich, UK) were made in reverse osmosis water and tested in a foam column operating in batch mode. Two experiments were conducted at airflow rates of 1.0 and 2.0 L min^{-1}. Air was fed to the sparger to produce bubbles which led to the formation of a surfactant stabilised foam. The foam flowed up the column and was collected in a collection cup. Before each trial, 50 mL of *T. pyriformis* culture was added to 950 mL of SDS solution. To ensure sufficient space within the column for foam to form, each run consumed 250 mL of the SDS-ciliates solution; allowing for four replicate runs per condition. The foam collected from each experiment was allowed to collapse, was collated and the volume determined. The *T. pyriformis* cell density in the foam and the remaining liquid phase was measured using an improved Neubauer haemocytometer and an inverted light microscope. A drop of Lugol solution was used to immobilise the ciliates for counting. Each column run lasted for a maximum duration of 30 min or until it was no longer possible to collect any more foam, whichever came first. The collated foam (ca. 10 mL) with an assumed high concentration of recovered SDS (including ciliates) was returned to the culture liquors and a further run was conducted as described above. In total, three runs were

conducted per SDS concentration as part of a multistage ciliates harvest (the fourth run did not produce any further foam). The aforementioned experiments were repeated but with the addition of *C. vulgaris* ($3.28 \times 10^7 \pm 10^6$ cells mL^{-1}). To control for any effect of air pressure on the ciliates, 100 mL of *T. pyriformis* culture was transferred into the foam column and subjected to 2 L min^{-1} air flow without any SDS for 30 min. The numbers of ciliates in the foamate and the Removal Efficiency (RE) were determined using equations 3.1 and 3.2 respectively.

$$\text{Ciliates in foamate} = A - B \text{ cells } mL^{-1} \quad \textbf{-----3. 1}$$

Where: *A* is the initial ciliates count prior to each run, and *B* is the final ciliates count after each run.

$$RE = \frac{Ciliates\ in\ foamate}{Initial\ ciliates\ count} \times 100 \quad \textbf{-----3. 2}$$

3.2.4 Determining the effect of SDS on the ciliates

Initial observations during harvesting suggested that the highest SDS concentration caused the ciliates to lyse; therefore an experiment was conducted to determine the lowest SDS concentration beyond which the ciliates could not survive. Four SDS concentrations (40, 44, 48 and 52 mg L^{-1}) were prepared and added to 1 L of algae/ciliates culture. The ciliates were monitored on an hourly basis using an inverted microscope and a haemocytometer.

3.2.5 Multistage foam column ciliates eradication using high SDS concentration.

Twenty millilitres of *T. pyriformis* (ca. $10{,}000 \pm 10^3$ cells mL^{-1}) was added to 980 mL of *C. vulgaris* and mixed with 50 mg SDS. The mixture was allowed to stand for two hours. A control sample was also prepared without SDS. The concentrations of both *C. vulgaris* and *T. pyriformis* prior to, and after the experiment were determined. The foam column described previously was used to recover the SDS (ca. 10 mL) from the culture. The recovered SDS was then added to fresh algae/ciliates culture without SDS making the whole volume to one litre. The mixture was allowed to stand for 12 hours before conducting another foam column experiment to recover the used SDS. The further two runs were conducted (each allowed to stand for 24 hours), thereby treating four litres of algae/ciliates culture with an initial SDS concentration of 50 mg with the total time taken to complete the experiment being 62 hours (Fig 3.2). The variation in timings from 2 to 24 hours before recovering the SDS for the 4 stages was to compensate for the assumed slight decrease in the SDS concentration within the collated foamate.

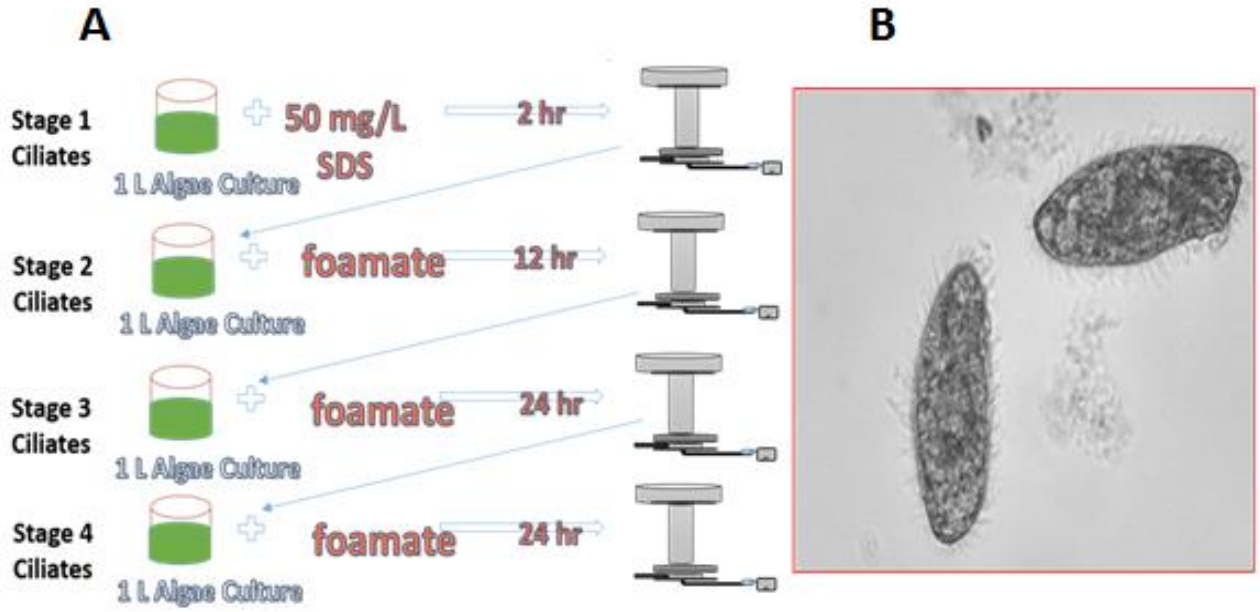


Figure 3.2: A, Multistage decontamination of microalgae culture using foam column and SDS. **B,** Image of microalgae grazers "T. *pyriformis*" taken using microscope x200 magnification, at ambiant temperature and pressure.

3.2.6 Data analysis

Data presented are the mean values of three separate experiments, with each experiment comprising four (250 mL) replicates. Statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) software analysis and Minitab v17. Data were tested for normality using a Shapiro-Wilk's Test and for equality of variance using a Levene's Test. Normally distributed data were compared using an Analysis of Variance (ANOVA) test. Data that were not normally distributed were analysed using the Levene's test. A repeated measures analysis of variance (rANOVA) was used to analyse the interaction of the three cycles of SDS reuse and between the SDS concentration and air flow rate on the ciliates removal efficiency. A General Linear Model was used to analyse ciliates regrowth with time and SDS concentration.

3.3 Results and Discussion

At industrial scales of microalgae production, contaminants such as ciliates are introduced via the air, the culture media and vessel, and the starter culture (Lavens and Sorgeloos, 1996), with heavy ciliates infection in outdoor microalgae cultures reported by Moreno-Garrido and Cañavate (Moreno-Garrido and Cañavate, 2000). Neutralising or removing these predator ciliates may enhance the growth rate, health and robustness of algae cultures without substantial disruption to farm operations.

3.3.1 Harvesting ciliates without microalgae

The addition of 20, 30 and 40 mg L^{-1} SDS into the *T. pyriformis* culture without *C. vulgaris* showed no immediate physical effect on the ciliates; however, after 60 min cellular debris of *T. pyriformis* cells began to appear in the 40 mg L^{-1} treatment. The initial harvesting trials yielded mean removal efficiencies of 56.95, 79 and 86.59% for 20, 30 and 40 mg L^{-1} SDS respectively at 2 L^{-1} air flow (Fig. 3.3A). Post harvesting, it was observed that many of the ciliates had lysed in the SDS treatments. This is likely due to an exponential increase in SDS concentration in the foamate, potentially in the order of a 400% increase (Coward *et al.*, 2013). The treatment without SDS (i.e. the air pressure control) resulted in no damaged ciliates.

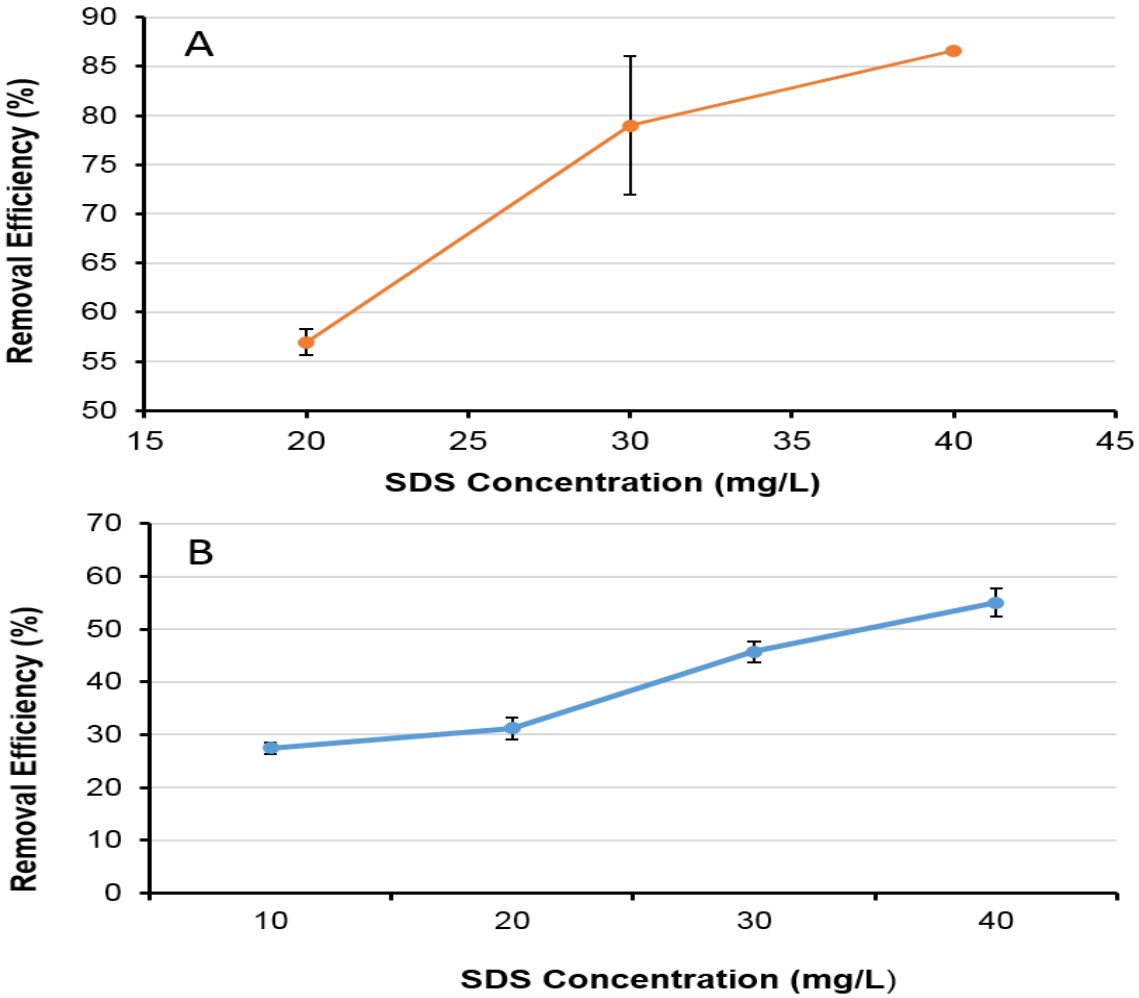


Figure. 3.3: Removal efficiency of *T. pyriformis* at an air flow rate of 2 L min^{-1} with different SDS concentrations: A) without the presence of microalgae; and, B) with microalgae. Mean ± St. Dev.

3.3.2 Harvesting ciliates with microalgae

Removal efficiency declined relative to the algae-free trials (Fig. 3.3B and first stage removal in Fig. 3.4) at all SDS concentrations. The added algal component interjects greater complexity to the suspension, which now involves ciliate−SDS−algae interactions rather than just

ciliate−SDS. The cations around the algae could interfere with the SDS−ciliate interactions, reducing the ciliates' contact area and thus decreasing removal efficiency. This was not possible when only SDS solution was used which resulted in a higher removal efficiency. Removal efficiency increased markedly (rANOVA F = 117.06, $p < 0.001$) following a multistage removal strategy at all SDS concentrations wherein the collected SDS was returned to the culture followed by two further column runs (Fig. 3.4). The highest removal efficiency (96.25%) was obtained on the third stage using 40 mg L^{-1} SDS at a 2 L min^{-1} airflow rate. To fully eradicate the ciliates from the algae culture and to eliminate recolonisation from within the same population, the ciliates RE must be 100%; regrettably this was not achieved using this approach.

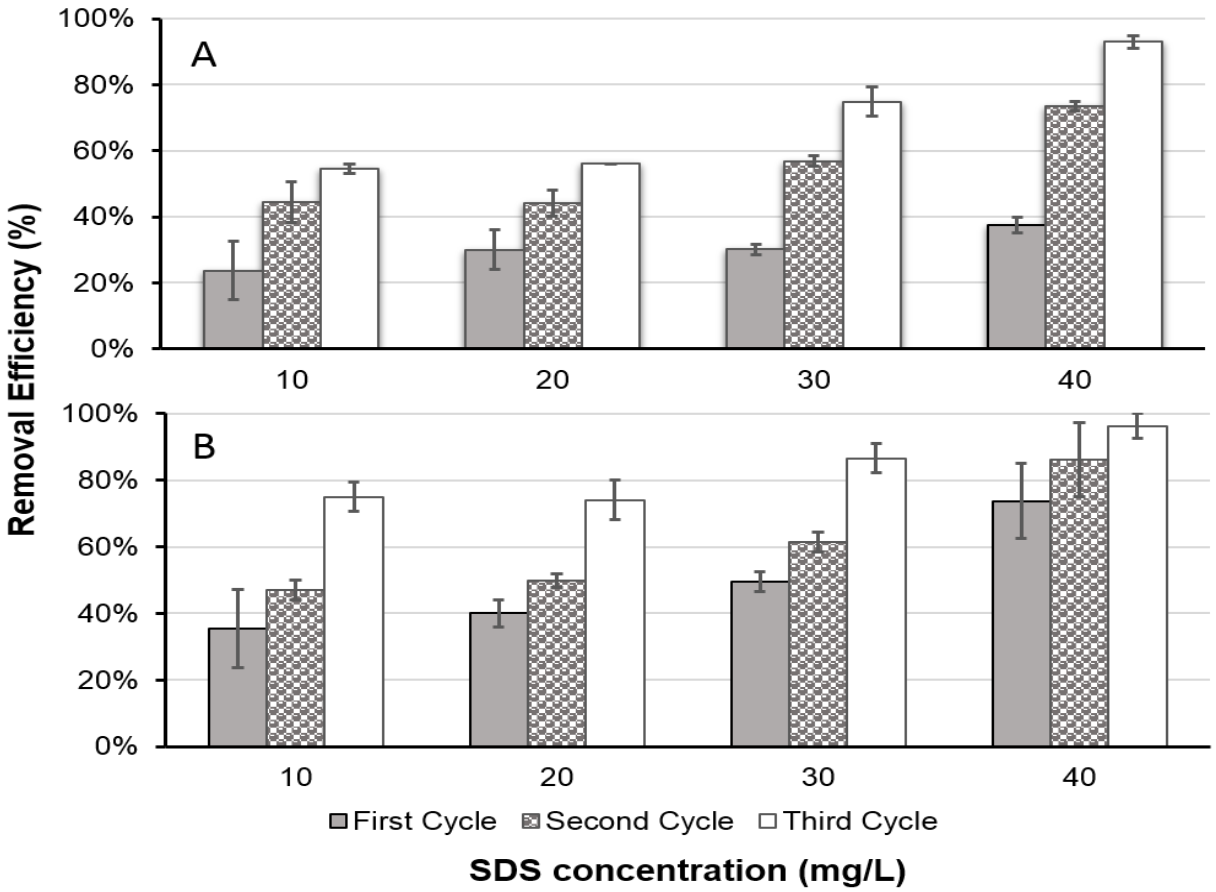


Figure. 3.4: Removal efficiency of *T. pyriformis* in a mixed algae−ciliates culture with different SDS concentrations as part of a multistage ciliates removal and SDS reuse strategy, at two air flow rates: A) 1 L min^{-1}, and B) 2 L min^{-1}. Mean ± St. Dev.

It was hypothesised that an anionic surfactant such as SDS could aid the removal of *T. pyriformis* from algae cultures (Fig 3.5) as their cell membrane is usually covered with Ca^{2+} and K^{+} ions (Munk and Rosenberg, 1969; Coleman *et al.*, 1972; Kim *et al.*, 2011). Therefore, in theory, the ionic interaction between the anionic SDS and the ciliates should increase the hydrophobicity of the ciliates, thus enhancing their removal potential. SDS was also chosen as

it is a relatively poor collector for microalgae (Chen *et al.*, 1998); therefore in theory, the ciliates would be removed leaving the microalgae *in situ*. This was found to be the case, and whereas some microalgae were incidentally collected in the foamate (determine via direct haemocytometer cell count of the algae cells within the foamate in the collection cup) the number of cells was very low (RE < 2%).

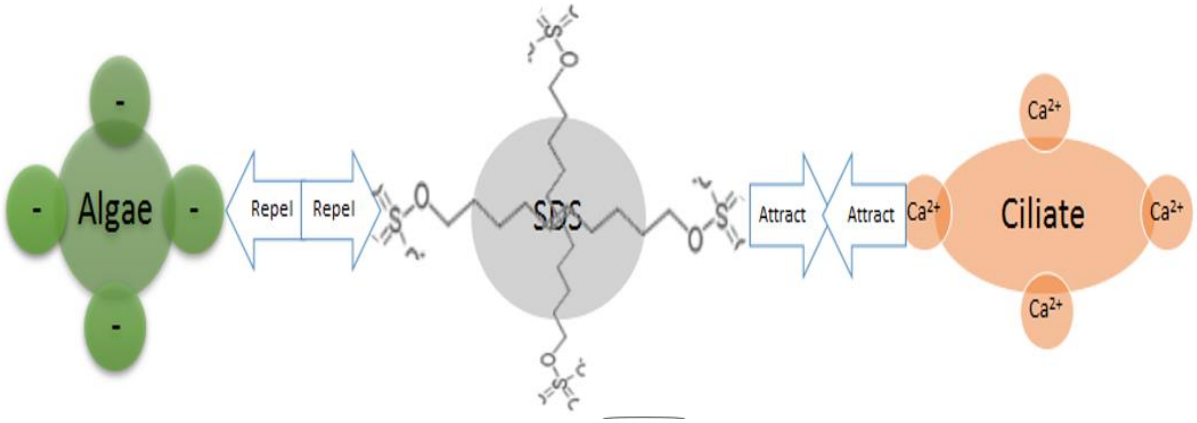


Figure. 3.5: Hypothesised microalgae, surfactant and ciliates interaction within a liquid medium

3.3.3 Effect of SDS on ciliates

The gradual increase in SDS concentration beyond 40 mg L^{-1} had a clear lysing effect on the ciliates with time, with both concentration and time being significant factors (concentration: F = 5.4, p = 0.002; time: F = 3.51, p = 0.01). SDS concentrations from 44 mg L^{-1} and above completely lysed the ciliates within six hours (Fig. 3.6). The highest effect was at 52 mg L^{-1} with lysis within two hours. However, at SDS concentrations of 40 mg L^{-1} and below, the ciliates population quickly recovered and dominated the culture within 48 – 72 h.

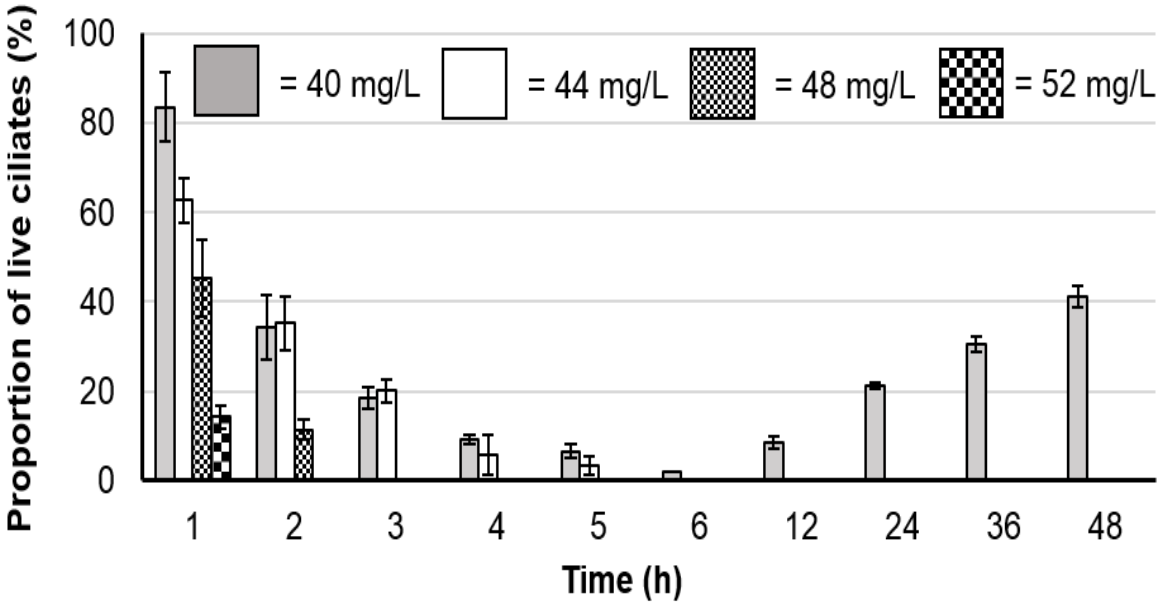


Figure. 3.6: Survival and population regrowth of *T. pyriformis* following exposure to a range of SDS concentrations. All ciliates had died within six hours with the exception of the 40 mg /L treatment. Number of viable ciliates left after each hour was determined using heamocytometer and the value was compared with the initial cell counts. Mean ± St. Dev.

3.3.4 Multistage ciliates control using high SDS concentration and foam column

Based on this knowledge of the susceptibility of *T. pyriformis* to SDS, a 50 mg L^{-1} concentration was used to treat four one litre batch algae cultures. Following treatment, the algae cultures grew ciliates free, increasing to 6.28×10^8 from 1.44×10^8 cells mL^{-1} within seven days. The microalgae were found to be free of cell damage. Ciliates control using a multistage SDS recovery and reuse strategy proved to be faster (15 h 30 min) and offer an economic advantage over other ciliates chemical control measures (Table 3.1). For instance, Ashraf et al. (Ashraf *et al.*, 2011) used 60 g L^{-1} sodium chloride and 80 mg L^{-1} quinine to control ciliates in outdoor microalgae cultures over a 24 hour period. Similarly, some of the chemicals used to control ciliates such as sodium chloride and oxytetracycline can also negatively affect the microalgae (Ashraf *et al.*, 2011). In a separate experiment conducted to determine the effect of SDS on *C. vulgaris* it was discovered that up to a 100 mg L^{-1} exposure had no physical effect on the microalgae. The exposed algae were successfully recultured and normal growth maintained; possibly due to the *C. vulgaris* cell wall being more resistant to SDS than the cell membrane of *T. pyriformis*.

Table 3. 1: Comparison of chemical methods for controlling ciliates and their associated costs (Ashraf *et al.*, 2011). Costs (US$) were derived from the mean value of the bulk chemical price. Currency rates correct as of 05/04/2017.

Chemical treatment	**Dose L^{-1}**	**Cost L^{-1}**
Falcon killer powder (pyrethroid 0.5 % w/w)	4 g, no effect on either ciliates or algae	
Oxytetracycline (liquid)	3 mL killed both ciliates and algae after 24 h	
Sodium chloride	60 g killed ciliates after 24 h 90 g killed both ciliates and algae after 24 h	$ 0.250
Quinine sulfate	80 g killed ciliates after 3 h while both ciliates and algae died after 24 h	$ 0.016
SDS (present work)	12.5 mg killed ciliates in 15 h 30 min	$ 0.0025

3.4 Summary

Some of the methods of dealing with ciliates contamination include chemical additives such as quinine (Moreno-Garrido and Cañavate, 2000), filtration techniques and manipulating environmental factors such as pH, light and air (Wang *et al.*, 2013). However, these approaches have drawbacks in terms of high energy consumption, maintenance cost as well as possible side

effects from the chemicals. Our approach to overcome such constraints was to use SDS-aided foam flotation to remove and eradicate the ciliates and subsequently recover the SDS for reuse.

The foam column approach, if optimised and scaled to work with photobioreactors and open pond culture systems (as demonstrated in the minerals extraction industries (Fuerstenau *et al.*, 2007; Schramm and Mikula, 2012)), could offer advantages for removing biological contaminants with similar characteristics to ciliates, particularly those with fragile cell membranes, whilst retaining the majority of the algae cells in the culture. It would also be important to test the approach on cultures with a much lower infestation intensity to validate it as a potential routine control measure. The lab scale system demonstrated here, may be particularly useful for laboratory-scale end-users and/or the maintenance of stock cultures. Testing on a range of microalgae with differing cell wall properties should be the next step in development.

Centrifugation is costly and energy intensive, thus using foam column to concentrate and harvest the microalgae could substantially save energy and cost for biocomposites fabrication. In the next chapter, we developed a system of immobilising cleaned microalgae on filter paper for carbon sequestration which require that, the suspended microalgae be centrifuged prior to application on the filter paper. Coward *et al.* (2013) and Alkarawi *et al.* (2018) were able to use foam column to concentrate and harvest microalgae using surfactant.

Chapter 4

Attached algae cultivation for carbon dioxide sequestration and improved lipid production

Abstract

The use of microalgae for carbon dioxide sequestration and as a feedstock for biodiesel production has been a topic of active research since the late 1950s. It has not been adopted as a technology due to the difficulties in growing and harvesting the microalgae and the excessive cost of the fuel produced via this route compared to fossil fuel. This research work focuses on a novel idea of an attached microalgae growth method to cultivate freshwater (*C. vulgaris*) and marine (*D. salina* and *N. oculata*) microalgae on filter paper substrates to sequester carbon dioxide and use the biomass (lipid) to produce biofuel. Here we demonstrate the concept of nourishing microalgae cells attached to filter paper via capillary action. Initial results indicate a good survivability of the immobilised cells with limited nutrients for 8 weeks. The average cumulative CO_2 fixation of 3.44 ± 0.16, 2.96 ± 0.53 and 2.02 ± 0.14 mmol g^{-1} day^{-1} for the attached cultivation for *C. vulgaris*, *D. salina* and *N. oculata* respectively were higher than the suspension control which was recorded as 0.924 ± 0.2, 0.41 ± 0.27 and 0.48 ± 0.11 mmol g^{-1} day^{-1} using 5% CO_2/air mixture in that order. The lipid content of *C. vulgaris*, and *N. oculata* also increased from 6.53%, to 17.19% and 15.93% to 23.97% respectively, while *D. salina* lipid content dropped from 10.07% to ~2% at the end of 8 weeks. This demonstrates the superiority of the attached algae cultivation method for both CO_2 sequestration and high lipid accumulation by the microalgae as compared to the traditional suspension cultivation.

4.1 Introduction

Population growth which comes with massive industrialisation and the indiscriminate use of fossil fuel to power our industries, automobiles and homes has increased the amount of carbon dioxide in the atmosphere. This surpassed the rate at which natural plants and other carbon absorbing entities are absorbing the atmospheric CO_2. Researchers are working to find workable solutions to reduce carbon emissions and reverse the current trend. There is renewed hope in the use of biological carbon capture by microalgae due to their high CO_2 consumption rate compared to terrestrial plants.

It is also predicted that most of the world's oil reserves will be depleted by the year 2050, assuming no new discoveries (Chen *et al.*, 2011). There is a pressing need to find alternative energy sources to avoid a potential future energy crisis. Roughly 10% of global energy supply currently comes from renewable sources, including bioenergy (Demirbas, 2009). The use of microalgae for CO_2 capture directly from flue gas and conversion to useful products e.g. biofuels is under investigation. Biofuel includes biomethanol, bioethanol, biobutanol, biomethane, biohydrogen, biodiesel, etc (Owolabi *et al.*, 2012). Biodiesel is the most promising candidate as it is free from aromatic compounds and contains no sulphur, thus significantly reducing emissions of partially burnt hydrocarbons, carbon monoxide and other particulate emissions. Currently, microalgae biofuel production remains at laboratory and pilot scales, with expansion inhibited by costs associated with energy consumption, land, water and nutrient requirements, to mention but a few. Combining CO_2 capture and biofuel production from microalgae could provide the incentive for large scale microalgae cultivation and biofuel production. Besides biofuel, there are other valuable products such as nutraceuticals, cosmetics and animal feeds that could be derived from microalgae.

The two dominant algae cultivation systems are open ponds and photobioreactors (PBR). However, there are many hurdles in cultivating microalgae via these methods, feeding into high capital and operating costs. Open pond cultivation is very water intensive. Similarly, water is sometimes required to cool photobioreactors in hot climates. Marine and brackish waters could be used to grow microalgae, but this may require further treatment which further adds to energy consumption and cost (Darzins *et al.*, 2010). Savings could be made by reusing water; however, this comes with a greater risk of contamination. Additional cost will also be incurred for water transportation, plumbing and pumping if there is no water source near the cultivation site (Lundquist *et al.*, 2010). Other factors to be considered include the production and use of plastic liners, and water treatment and disposal (Murphy and Allen, 2011).

Immobilisation techniques, wherein algae are grown on a supporting material and the cells are nourished by limited use of medium, could reduce overall water dependency. However, this is an untested technology. Here, we investigate the culturing of microalgae species with limited water and nutrient supply for an extended period compared to the traditional suspension cultivation. Three unicellular algae species (*C. vulgaris*, *D. salina* and *N. oculata*) were immobilised on filter paper and nourished via capillary for 8 weeks. CO_2 was fixed by the immobilised cells at the bioreactor gas headspace. The three algae species were evaluated for

CO_2 consumption on 48 h basis while biomass growth and lipid accumulation, were determined biweekly.

4.2 Material and methods

4.2.1 Algae strain, growth medium and cultivation conditions

Chlorella vulgaris (freshwater) was maintained in BG11 medium, containing (per litre): 1.5 g $NaNO_3$, 0.036 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.04 g K_2HPO_4, and 0.02 g Na_2CO_3. *Nannochloropsis oculata* and *Dunaliella salina* (marine) were grown in F/2 enriched seawater [made by adding 35g L^{-1} NaCl in deionise water and passing through filters (reverse osmosis) to remove particles], containing (per litre) 0.075 g $NaNO_3$, 0.00565 g $NaH_2PO_4.2H_2O$ while 1 ml of the following stock was added (4.16 g Na_2-EDTA, 3.15 g $FeCl_3.6H_2O$, 0.01 g $CuSO_4.5H_2O$, 0.022 g $ZnSO_4.7H_2O$, 0.01 g $CoCl_2.6H_2O$, 0.18 g $MnCl_2.4H_2O$ and 0.006 g $Na_2MoO_4.2H_2O$. All cultures were grown in 10 L polycarbonate carboys (Nalgene), at 18±2 °C, a 16/8 h light/dark photoperiod with 2,500 lux of illumination provided by daylight-type fluorescent lamps, and constantly aerated with HEPA filtered air using an aquarium pump (KOI AIR 50 Blagdon).

4.2.2 Microalgae cell immobilisation on filter paper

Microalgae were immobilised on filter paper by centrifuging 50 ml of 7-10 days old microalgae culture at 4,000 x *g* for 10 min, with the exception of *D. salina* that was centrifuged for 2 min. The cell paste was re-suspended in 200 µl of medium and thereafter 70 µl was pipetted on to a 2×6 cm pre-weighed autoclaved Sartorius filter paper (Sartorius FT-3-307-110 grade 3 S/h). The cells were carefully pipetted on one end of the filter paper leaving 1 cm to the top (Fig. 4.1). The filter paper strip was then inserted into an autoclaved glass bottle (97 mm height, 15.2 mm diameter with a total volume of 12 ml) containing 2 ml of the respective growth medium which travelled through the paper by capillary action. Strips were maintained under the light and temperature conditions described above with the bottle kept 30 cm from the lights and the algae cells facing the light source. One end of the bottle was covered with a symptomatic plastic cap through which CO_2 was introduced and collected. The tubes were flushed with 5% CO_2 which was replaced every 48 hours after CO_2 sampling. CO_2 was measured using a Geotech G100 handheld CO_2 analyser (Geotech UK).

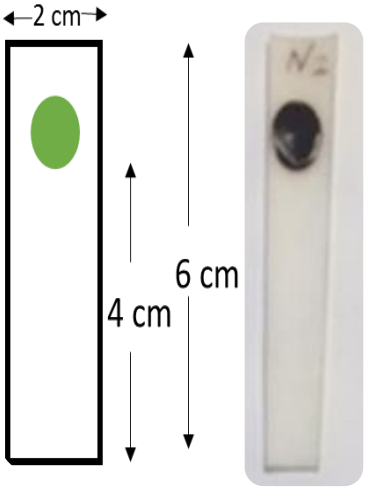


Figure 4.1: Diagram and image of microalgae (N. oculata) immobilised on filter paper.

Conventional suspension cultures were used as positive controls. The cells (2 ml suspension) were grown in the same type of glass bottle but without filter paper. A negative control comprising only the growth medium was used to determine the CO_2 solubility of the respective medium. A summary of the experimental procedure is depicted in Fig 4.2.

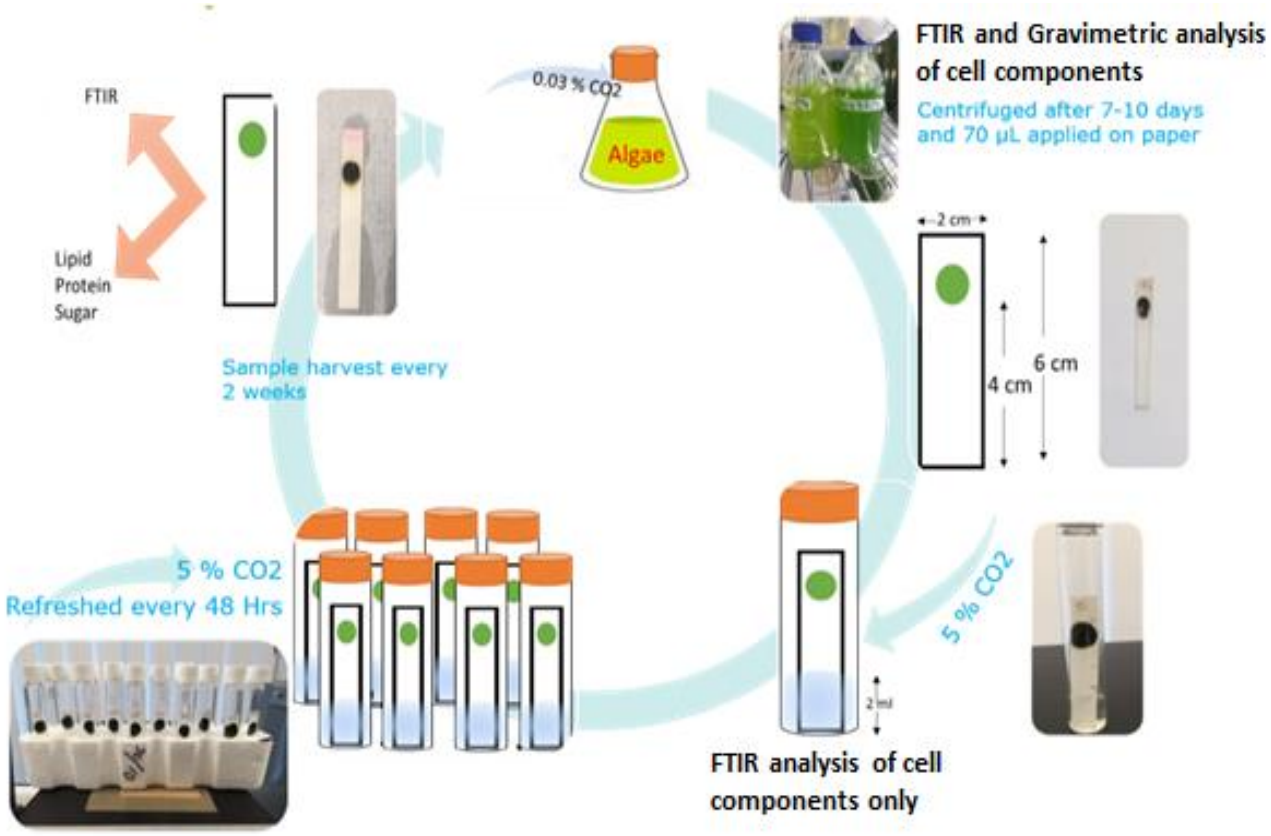


Figure 4.2: Experimental procedure for microalgae immobilisation on filter paper for CO_2 mitigation. Growth condition: 18±2 °C, a 16/8 h light/dark photoperiod with 2,500 lux of illumination provided by daylight-type fluorescent lamps, and constantly aerated with 5% CO_2.

4.2.3 Cell growth and biomass determination

Cell growth was monitored every 48 h for 2 months by chlorophyll *a* fluorescence using a microplate reader (FLUOstar OPTIMA, BMG LABTECH) at the excitation and emission wavelengths of 430-10 and 690-12 nm respectively. Data were analysed using the OPTIMA software. Samples were taken and freeze-dried every 2 weeks for further analysis. At the end of 8 weeks the stored samples were oven dried and weighed to calculate cell mass and compared with the chlorophyll *a* data. Biomass was determined by subtracting the mass of the filter paper strip from the final mass of the paper plus microalgae. The mass gain over time was obtained by subtracting the initial cell mass from the final biomass. For the marine species, there was a substantial amount of salt attached to the filter paper. The salt content was determine by cutting out the spot where the algae was localised and the weight of the remaining paper determined before and after soaking for 24 h and washing with deionised water and dried again. The salt quantity was subtracted from the algae/paper weight to obtain the final algae weight.

4.2.4 Lipid, protein and carbohydrate analysis: Gravimetric/wet quantification method

FTIR spectroscopy was used to determine the macromolecular content (lipid, carbohydrate and protein) of the algae biomass, and validated using gravimetric/wet analysis.

Each microalgae species was grown in suspension (10 L container) for 1, 2 and 4 weeks. At the end of each period 2 L was centrifuged at 4000 x *g* for 10 min and the pellet washed, oven dried and weighed. Total lipid was determined gravimetrically using the Bligh and Dyer (1959) method while protein and carbohydrate were determined by spectrophotometry as described in Lowry *et al.* (1951) and Kochert (1978) methods respectively, with calibration curves (Appendix 1a) obtained from bovine serum albumin (BSA) and D-glucose respectively. The calibration equations are shown in equations 4.1 and 4.2, where x and y represent the content of the analyte in the biomass (mg) and the integrated area under the curve respectively. Aliquot sample was also taken after each test for the FTIR analysis as described below.

Protein standard curve equation $\mathbf{y = 0.0021x + 0.1055}$ $R^2 = 0.9524$-------**4.1**

Glucose standard curve equation $\mathbf{y = 0.0041x + 0.061}$ $R^2 = 0.9891$-------**4.2**

4.2.5 Lipid, protein and carbohydrate analysis: FTIR method

Lipid, protein and carbohydrate content of the immobilised biomass (including samples taken from the wet method) were derived based on their respective FTIR absorption spectra as indicated in Fig. 4.3. A calibration curve (Appendix 1b) using BSA, glucose and glycerol

tripalmitate as standard for protein, carbohydrates and lipid was obtained and subsequently used to quantify the immobilised microalgae macromolecular components.

FTIR spectra (Fig. 4.3) were obtained at room temperature, using a Cary 630 FTIR (Agilent Technologies), equipped with a Diamond Attenuated Total Reflectance (ATR) detector. Biomass samples were analysed in duplicate. Characteristic peak areas at 2800–3000 cm^{-1}, 1500–1700 cm^{-1} and 1000–1200 cm^{-1}, were used to determine the values of lipid, protein and carbohydrate respectively. Data acquisition and spectral analysis was performed using Agilent MicroLab PC software. Additional analysis was carried out using OriginPro graphing and analysis 2017, V 94E. The percentage of each macromolecular content was calculated assuming that the biomass consisted only of lipid, protein and carbohydrate as indicated in equations 4.3-5 below;

$$\%\ Lipid = \frac{total\ integral\ of\ lipid\ \ band}{total\ biomass\ integral\ band} x\ 100 - - - - \mathbf{4.3}$$

$$\%\ Protein = \frac{total\ integral\ of\ protein\ band}{total\ biomass\ integral\ band} x\ 100 - - - - \mathbf{4.4}$$

$$\%\ Carbohydrate = \frac{total\ integral\ of\ carbohydrate\ band}{total\ biomass\ integral\ band} x\ 100 - - - - \mathbf{4.5}$$

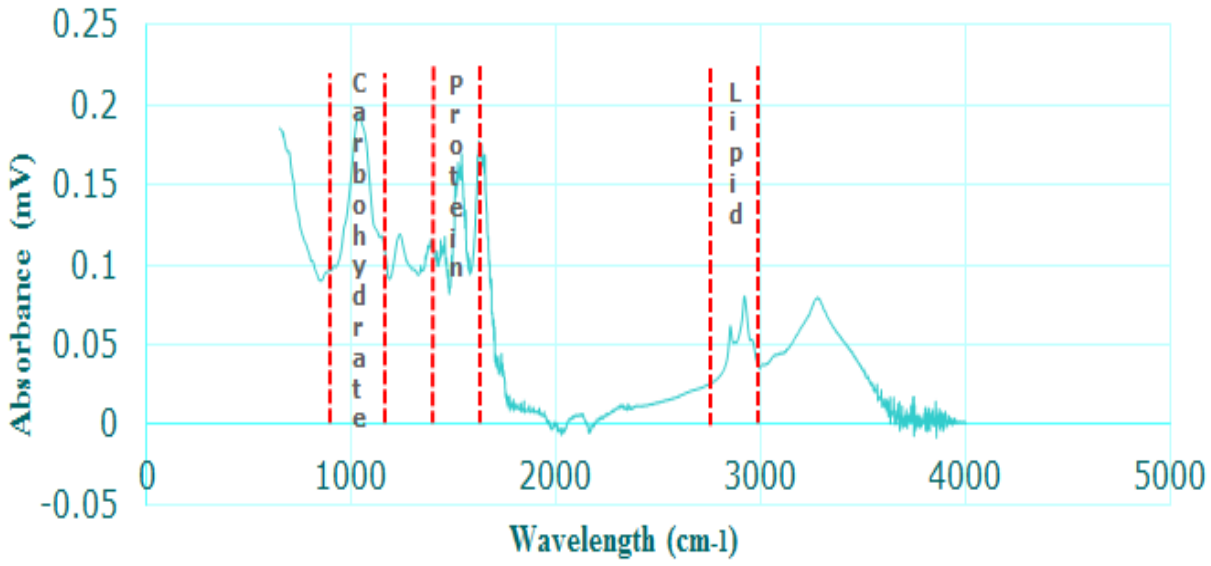


Figure 4.3: Absorbance peaks and wavelength limits for the identification and quantification of lipid, protein and carbohydrate, wavelength values are those suggested by Mayers et al. (2013) and Grube et al. (1999).

The symmetric C–H stretching vibration of $–CH_2–$ groups, found in lipid acyl chains and used to quantitatively determine lipid content is represented in the FTIR spectra at band 2,854 cm^{-1} (Fig. 4.3). A baseline correction based on two points between 2,800 and 3,000 cm^{-1} was done. Subsequently, a full integration was done over the range of C–H stretching vibrations (2,800–3,000 cm^{-1}) which stand for both $–CH_2–$ and $–CH_3$ vibrational bands. Similarly, the ester

stretching band appearing around 1,740 cm^{-1} and the phospholipid stretching band around 1,235 cm^{-1} were also included for lipid estimation after baseline correction.

The characteristic band of N–H of Amide I and C=O of Amide II belonging to protein groups appear around 1540 and 1650 respectively (Giordano *et al.*, 2001). Integration around those peak areas (Fig. 4.3) was used to estimate the amount of proteins contained in the algae biomass.

Carbohydrates content was obtained from the integrated band area between 1,185 and 950 cm^{-1}. Grube *et al.* (1999), used a similar range with a peak around 1,080 cm^{-1}. There is also the contribution from C–O stretching vibrations which appears around the 1150 cm^{-1} band. Carbohydrates content of algae biomass usually shows a large variation, however, this does not stop a semi-quantitative analysis of the biomass macromolecules, thus we used the integral band of 900 -1200 cm^{-1} (Fig. 4.3) to estimate the amount of carbohydrates content within the algae biomass.

4.2.6 Carbon dioxide reactivity measurement

The microalgae were fed with a 5% CO_2/air mixture. CO_2 was measured using a Geotech G100 handheld CO_2 analyser (Geotech UK). The CO_2 analyser was initially calibrated using GC (Appendix A1.3). The immobilised cell's CO_2 consumption rate was determine by subtracting the effluent CO_2 values from the influent CO_2 (5%v/v), and the percentage efficiency was calculated using equation 4.6. The specific CO_2 consumption (mmol/g) based on the weight of the immobilised cells and the fixation rates was also determined using the ideal gas equation 4.7.

$$Efficiency\ (\%) = \frac{Influent\ CO2 - Effluent\ CO2}{Influent\ CO2}\ x\ 100 \quad ----\mathbf{4.6}$$

$$(pV = nRT) \quad -----\ 4.7$$

Where: $p = Pressure\ in\ Pascal\ (Pa)$

$V = Volume\ in\ m^3$

$n = Number\ of\ moles$

$R = the\ gas\ constatnt, 8.31\ JK^{-1}mol^{-1}$

4.2.7 Statistical analysis

Experiments were repeated with triplicate number of samples (n=3 of samples) and the average values with their standard deviation are reported. One-way analysis of variance (ANOVA) Mann-Whitney and Krustal-Wallis test was used to compare mean values and statistical

significance using SPSS statistical software. In all instances, comparison which shows a p value of <0.05 were considered statistically significant.

4.3. Results and Discussion

4.3.1 Microalgae growth behaviour under attachment

The chloroplast, which houses the chlorophyll, is vital for photosynthesis. Therefore, the constituents of chlorophyll and the efficiency of the photosynthetic mechanism are important indicators upon which algal cell growth and adaptation to its environment can be investigated. Chlorophyll measurements increased steadily for all species over the first 2 weeks, peaking at day 10 for the marine algae and day 12 for *C. vulgaris*. Subsequently, chlorophyll intensity began to decline (Fig. 4.4). By week 3, chlorophyll intensity of the marine algae increased slightly then began a steady decline until week 8. *D. salina* had the lowest chlorophyll intensity. After 8 weeks, almost all the cells remained on the surface of the filter paper apart from *D. salina* which showed a significant decline in cell numbers (Fig. 4.5 A). The week 3 increase may be a result of new cells forming on top of the biomass which could not be sustained due to limited nutrient availability as well as the barriers formed by the old cells and the filter paper.

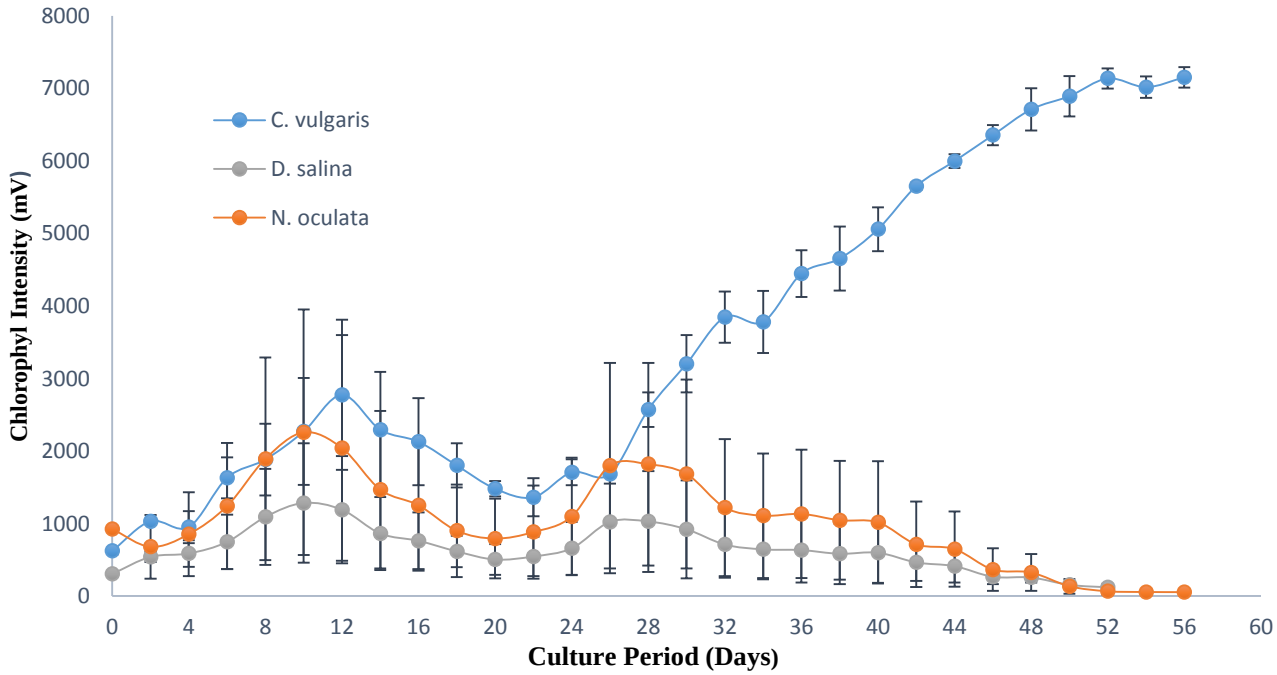


Figure 4.4: Chlorophyll *a* intensity of *C. vulgaris, D. salina* and *N. oculata* taken every 48 h for 8 weeks, Mean ± SD (n=3).

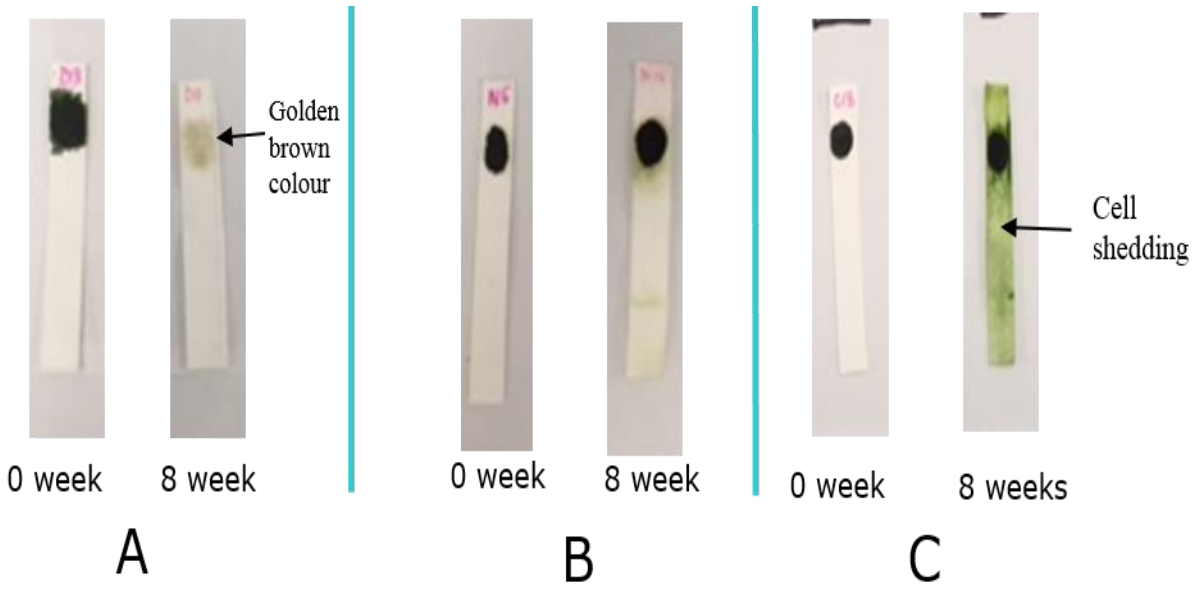


Figure 4.5: Images of (A) *D. salina*, (B) *N. oculata* and (C) *C. vulgaris* immobilised on filter paper at the beginning and end of the 8 week trial. Notice the discolouration of *D. salina* cells and the cell shedding of *C. vulgaris*.

Cells exposed to strong light under nutrient depleted conditions can experience photoinhibition which could result in a significant decline in chlorophyll intensity (Shariati and Hadi, 2011). Dipak (2005), cultivated *D. salina* under nitrogen depleted conditions and reported similar observations of low chlorophyll content. However, the *C. vulgaris* chlorophyll intensity rose sharply after week 3 and continued to week 8, likely due to the release and subsequent spread of cells along the filter paper as indicated in (Fig. 4.5 C); termed cell shedding in this work. Unlike the marine algae, *C. vulgaris* does not produce extracellular polymeric substances (EPS) which help bind cells together, thus there was limited affinity between the cells and the filter paper.

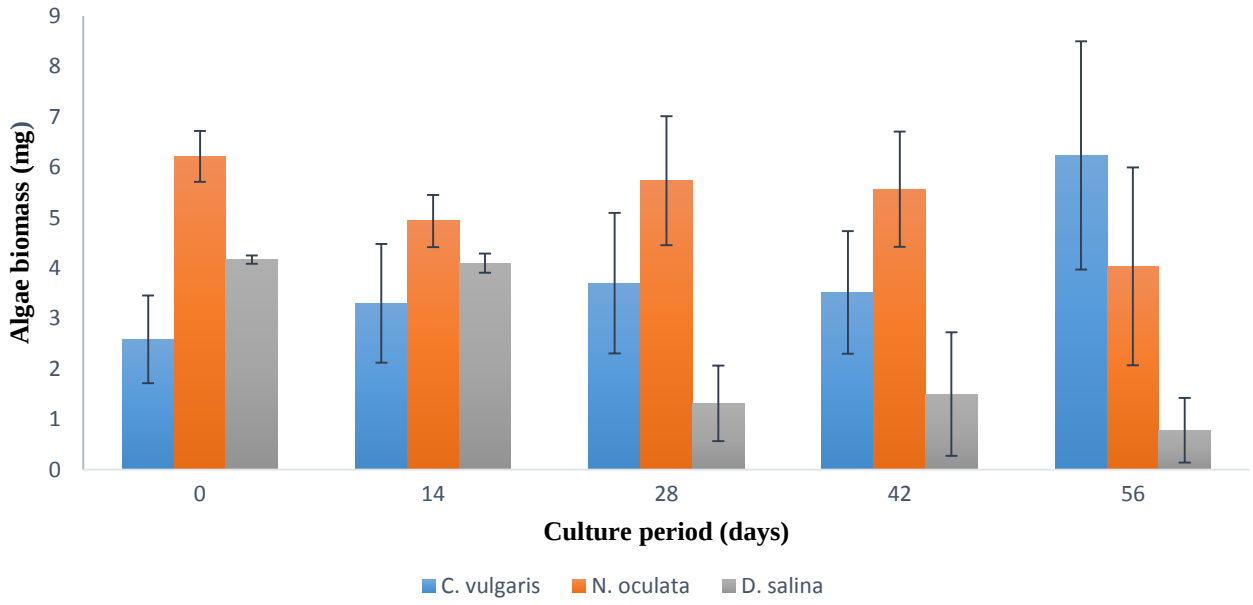


Figure 4.6: Growth of *D. salina*, *N. oculata* and *C. vulgaris* immobilised on filter paper for 8 weeks, showing decline in *D. salina* biomass and an increase of biomass for *C. vulgaris* after the 6th week, Mean ± SD (n=3).

Figure 4.6 shows the net biomass change on a two-weekly basis. There was no significant ($P > 0.05$) changes in the biomass concentration of *C. vulgaris* up to the 6th week, however, at the 8th week the biomass content increased significantly (ANOVA, $F=7.58$, $DF= 4, 25$, $P< 0.05$). The total biomass yield at the end of week 8 (6.23 mg DW) is twice the mass of the initial immobilised algae weight (2.58 mg DW), which can be attributed to cell shedding along the paper matrix. This highest value (6.23 mg DW) is still lower than what was reported in the literature for *C. vulgaris* suspension cultivation (doubling its biomass within 24 h). Biłos *et al.* (2016), reported a 25 times biomass increase of *C. vulgaris* within 72 h. This clearly demonstrate the effect of immobilisation on the cell division and subsequent growth. Regarding the possible sedimentation of metals from the F/2 medium, the negative control indicated that a substantial amount of salt residue is retained on the filter paper. Therefore, the measured yield was from the net biomass production, although this value is not absolute as some salt will still be left behind where the algae is directly immobilised on the filter paper.

The growth rate of the marine species as measured gravimetrically showed an initial decline for *N. oculata* in week 2 (Fig. 4.6). This is unexceptional as algae often require a latency period in order to adjust to a new environment. In general, there was no significant difference (ANOVA, $F=1.36$, $DF= 4, 25$, $P= 0.278$) in the biomass accumulation of *N. oculata* across the cultivation period. *D. salina* had bleached cells (golden brown colour) and suffered a significant decline (ANOVA, $F=5.627$, $DF= 4, 25$, $P= 0.002$) in its biomass weight over the 8 weeks cultivation period. To avoid undergoing cell division under unfavourable growth conditions β-carotenoids were likely produced, accumulated and stored as an extra photosynthetic product. This phenomenon stops cell division to sustain the cells, thus accounting for the limited growth (Fig. 4.6). This phenomenoum was also reported by Shariati and Hadi (2011). Similarly, unpublished work conducted by Oscar Bernal at the North Carolina State University, also suggests a photo-bleaching phenomenon when *Synechoccus* was grown under constant light illumination but the cells recover their chlorophyll pigmentation upon transfer to fresh culture medium. This shows that photo-bleaching does not necessarily kill the cells but their metabolic activities would be reduced. Additionally, photoinhibition of the underlying cells could also have contributed to the slow growth, especially in the marine algae. In general, the immobilisation method sustained the cells for longer than the suspension method, despite the algae biomass not significantly increasing over time (compared to suspension cultivation), although some CO_2 reactivity was maintained. The suspension control exhibited reduced metabolic activity compared to the attached cells as evident in their lower CO_2 reactivity (Fig 4.7). There was no effort to measure the growth pattern of the control as initially we are only interested in the CO_2 reactivity.

4.3.2. Carbon dioxide fixation of the attached algae system

CO_2 fixation rate is directly proportional to light utilisation efficiency and microalgae cell density. The CO_2 removal efficiency was determined as the difference in the CO_2 concentration of the incoming and outgoing effluents normalised to the abiotic (negative) control (Figs 4.7 and 4.8). The abiotic control recorded some CO_2 dissolution (0.009 mg/mL) within the liquid medium for a period of 6 days, thereafter no significant amount of CO_2 was dissolved in the medium or within the paper matrix. This could be due to CO_2 saturation and reaching equilibrium thus no further CO_2 could be dissolved as evident in the slight decrease in the growth medium pH from 7.0 to 6.3 ± 0.2 at the end of 2 months trial. There was an exponential increase ($P< 0.05$) from 2.42 – 113.36 mmol g^{-1} (min recorded by *N. oculata*) in cumulative CO_2 fixation by all immobilised species of microalgae for the eight weeks cultivation compared to the control. The CO_2 fixation of *D. salina* started declining after the 4th week, but maintained a minimal daily fixation until the 8th week. A 2-sample Mann-Whitney Test conducted between the suspension and attached cultivation for each algae species showed a clear improvement ($P< 0.05$) in the mean CO_2 fixation of the suspension cultures 0.924 ± 0.2, 0.41 ± 0.27 and 0.48 ± 0.1 mmol g^{-1} day^{-1} (Fig. 4.7), versus the immobilised 3.44 ± 0.16, 2.96 ± 0.53 and 2.02 ± 0.14 mmol g^{-1} day^{-1} (Fig. 4.8) for *C. vulgaris*, *D. salina* and *N. oculata* respectively, both in terms of the amount of CO_2 consumed and the duration of the cultivation period.

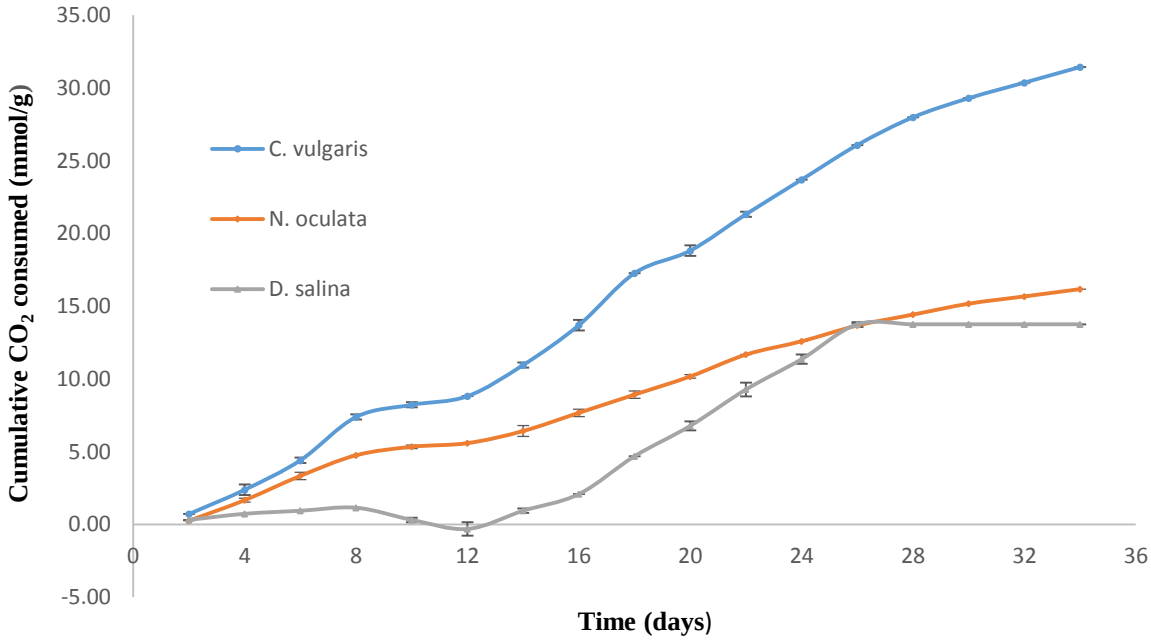


Figure 4.7: Biotic control showing cumulative CO_2 consumption with daily average of 0.924 ± 0.2, 0.41 ± 0.27 and 0.48 ± 0.11 (mmol/g of dry algae biomass) for *C. vulgaris*, *D. salina* and *N. oculata* respectively. The cell were grown in a suspension culture, Mean ± SD (n=3).

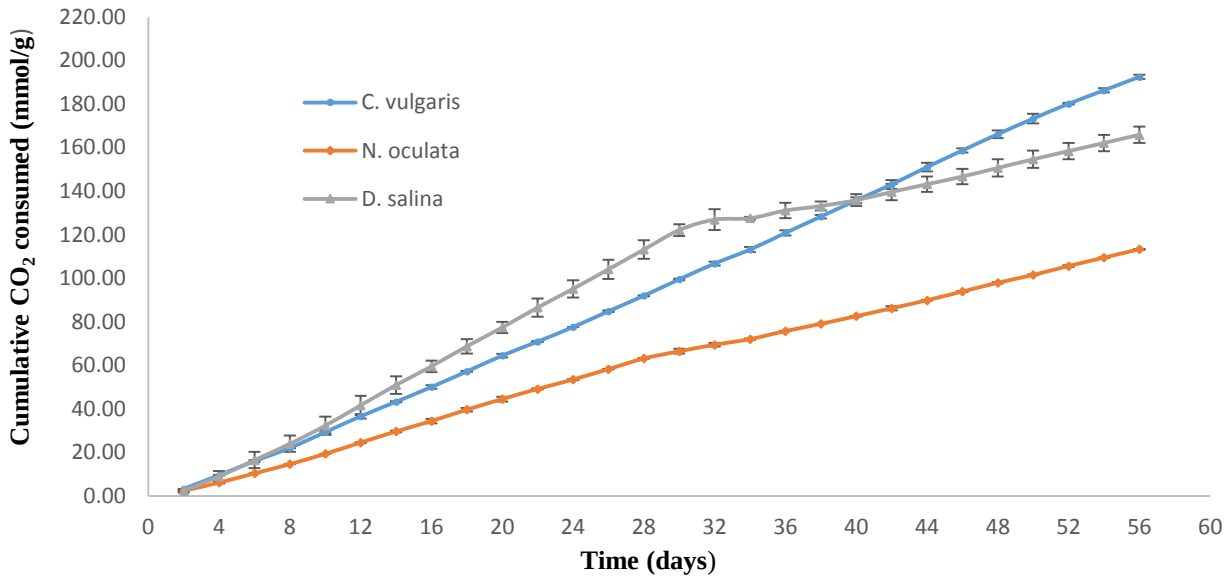


Figure 4.8: Cumulative CO_2 consumption of immobilised algae with daily average of 3.44 ± 0.16, 2.96 ± 0.53 and 2.02 ± 0.14 (mmol/g of dry algae biomass) for *C. vulgaris*, *D. salina* and *N. oculata* respectively. The cell were immobilised on filter paper, Mean ± SD (n=3).

The efficiency of CO_2 utilisation in a photobioreactor depends on (1) the algal species, (2) the initial CO_2 concentration, (3) the design of the photobioreactor, and (4) the operating parameters (Andrade and Costa, 2007; de Morais and Costa, 2007). There was a significant difference among the three algae species grown in the traditional suspension cultivation (Krustal-Wallis test, K= 11.06, DF =2 P= 0.004) and also the immobilised (Krustal-Wallis test, K= 23.27, DF= 2, P< 0.05) in their respective CO_2 fixation efficiency. At 5% CO_2 concentration, *C. vulgaris* immobilised on paper possessed a cumulative CO_2 consumption of 192.50 mmol g^{-1} and a daily average of 3.44 ± 0.16 mmol g^{-1} day^{-1} in our photobioreactor (Fig. 4.8), this is similar with the value (3.43 mmol g^{-1} day^{-1}) reported by (Adamczyk *et al.*, 2016) when they cultivated *C. vulgaris* using 4% inlet CO_2 concentration (direct comparison between immobilised and suspended algae is difficult due to cell growth in the suspension culture). Immobilised *D. salina* and *N. oculata* both had a cumulative CO_2 consumption of 165.90 and 113.36 mmol g^{-1} respectively, while their daily averages were 2.96 ± 0.53 and 2.02 ± 0.14 mmol g^{-1} day^{-1} respectively (Fig.4.8).

Of the tested species, *C. vulgaris* deserves special attention. Many *C. vulgaris* strains can fix up to 75% of CO_2 when the inlet gas CO_2 concentration is below 1% (Vance and Spalding, 2005); this CO_2 uptake value is indirectly proportional to CO_2 concentration of the inlet stream. The underlying issue faced by most researchers has been the CO_2 mass transfer limitation within the culture medium. According to our results, the average CO_2 reactivity of a suspension culture of *C. vulgaris* with 5% air/CO_2 was 0.924 ± 0.2 mmol g^{-1} day^{-1} (Fig. 4.7). When compared with

the attached cultivated system (3.44 ± 0.16 mmol g^{-1} day^{-1}), the CO_2 reactivity increased about four-fold. The marine species also demonstrated a marked increase when immobilised on paper, *D. salina* and *N. oculata* CO_2 average reactivity increased to 2.96 ± 0.53 and 2.02 ± 0.14 mmol g^{-1} day^{-1} when immobilised, which is a seven-fold increase for *D. salina* and four-fold increase for *N. oculata*. CO_2 injected into the photobioreactor bottles will have dissolved in the medium forming HCO_3^- which is then together with the nutrient transported to the immobilised algae cell through the paper matrix via capillary action. The excess CO_2 in the gaseous phase also gets into the immobilised cells via passive diffusion (leaked into the cell), get converted into HCO_3^- within the cell cytoplasm and converted to simple sugar through the Calvin Cycle, as supported by Mangan and Brenner (2014). The introduction of the second dimension (gaseous phase) to the CO_2 uptake and the elimination of the CO_2 mass transfer limitation in water could be responsible for the improved CO_2 fixation rate recorded in our experiment. Gosse *et al.* (2010), reported similar observation with a higher reactivity of *Rhodopseudomonas palustris* immobilised using latex binders when maintained in the gaseous phase of a bioreactor compared to the suspension cultivation system.

In higher plants and microalgae there is a direct proportional relationship between chlorophyll content and photosynthetic efficiency and CO_2 fixation up to certain level (Terry and Ulrich, 1974). Figures 4.4 and 4.6 shows a continuous excitation of chlorophyll a content (with period of decline 12-24 days) and biomass growth of *C. vulgaris* which also translate to continuous CO_2 fixation (Fig. 4.8) throughout the cultivation duration. The chlorophyll content increase significantly after a month due to cell shedding as explained earlier without a commensurate increase in the CO_2 fixation of the immobilised cell. This could be explain due to the CO_2 rate limiting property of the RuBisCO catalysed carboxylation of ribulose-1,5-bisphosphate. Conversely, there was significant decline in the chlorophyll and biomass content of *D. salina* and (Figures 4.4 and 4.6), which does not correspond with the marginal increase in the CO_2 fixations of the immobilised cells up to 32 days, Fig. 4.8. The production of β-carotenoids (yellowish pigment) by *D. salina* cells as result of the immobilisation and the limited nutrient supply could be responsible for the slow cell division (biomass) and low chlorophyll content recorded, while maintaining certain level of photosynthetic activity with moderate CO_2 fixation.

The positive controls suffered population crashes after one month. The increase in cell density and subsequent nutrient reduction likely contributed to the eventual decline, whereas the immobilised cells maintained metabolic activity due to the limited cell division and longer survival period with the available nutrients. In general, there were noticeable differences in CO_2 fixation rates between the three algae species and across the two culturing (suspension versus

attached) methods as reported earlier and summarised in Table 4.1.

Table 4. 1: Mean CO_2 consumption rates by 1 g of dry weight microalgae biomass per day, Mean ± SD.

Microalgae	**Mean CO_2 consumption (mmol g^{-1} day^{-1})**	
	Suspension cultivation	**Paper attached cultivation**
C. vulgaris	0.924 ± 0.2	3.44 ± 0.16
D. salina	0.41 ± 0.27	2.96 ± 0.53
N. oculata	0.48 ± 0.11	2.02 ± 0.14

Even though algae can produce some CO_2 overnight, as occurs with other plants; the net CO_2 uptake was still positive, which makes it an interesting candidate for carbon capture.

4.3.3 Carbon dioxide and liquid mass transfer within microbial filter paper matrix

Due to its high porosity, filter paper allow for a greater transfer of nutrients from the liquid medium up to the immobilised microalgae cells via capillary. Extensive work was done on microbial paper mass transfer improvement and limitation by Oscar Bernal at the North Carolina State University. Oscar opined that, surface modification could improve cell retention in microbial papers with increase in cell loading, alter porosity and improved mass transfer properties. Similarly, it was proved that the microalgae/filter paper gluing phase does not constitute a mass transfer or light transmission barrier, while the cells are fixed in place with their photosynthetic apparatus intact. This make us also assumed a higher nutrient and bicarbonate mass transfer within the filter paper to the immobilise cells at the photobioreactor gas headspace.

It appears that in non-photosynthetic systems diffusion mass transfer limitations and water activity are the key factors that limit long-term biodegradation activity of biofilters for air-pollution control applications. These findings encourage us to envision other potential environmental applications such as high-intensity gas-phase COx absorbers based on coated microbial paper that could simultaneously sequestrate greenhouse effect atmospheric gases and produce second generation biofuels from non-food sources.

4.3.4. Algae biomass macromolecular component analysis

Due to the small quantity of biomass immobilised on the filter paper, it was not possible to accurately determine macromolecular content using traditional gravimetric/wet analysis methods. Several studies have demonstrated the effectiveness of FTIR spectroscopy to determine algae biomass components (Mayers *et al.*, 2013; Meng *et al.*, 2014; Wagner *et al.*, 2014). The lipid, protein and carbohydrates content was thus determined using FTIR.

4.3.5. Validation of the FTIR method using gravimetric/wet algae biomass analysis

The macromolecular contents of suspension cultures as determined by the wet/gravimetrical method and their corresponding FTIR values are contained in Table 4.2.

Table 4. 2: Algae macromolecular values as determine by wet/gravimetric and FTIR methods, (Mean ± SD).

Macromolecules (% DW)	Carbohydrate			Lipid			Protein		
Cultivation period (weeks)	**1**	**2**	**4**	**1**	**2**	**4**	**1**	**2**	**4**
C. vulgaris									
Wet/gravimetric	29.84	38.46	33.29	16.00	11.25	12.25	54.16	50.29	54.46
FTIR	36.32	40.63	36.58	13.14	13.36	14.53	50.54	46.01	48.89
D. salina									
Wet/gravimetric	43.83	43.13	44.40	12.00	11.75	12.00	44.17	45.12	43.60
FTIR	41.63	44.38	45.16	12.04	13.56	14.86	46.33	42.06	39.98
N. oculata									
Wet/gravimetric	26.00	27.21	26.52	20.50	30.77	28.25	53.50	42.02	45.23
FTIR	30.41	34.39	35.53	15.77	18.10	16.20	53.82	47.50	48.27

The gravimetric/wet analysis and the FTIR (with some slight deviations) did not produce matching figures but did show a pattern that could be used to justify using the FTIR method for subsequent analyses. The dissolution of chlorophyll *a* by solvents could have possible interference on the final results for the traditional extraction method. The gravimetric lipid content of *C. vulgaris, D. salina* and *N. oculata* were in the range of 11-16%, 11-12% and 20-31% respectively, while the corresponding FTIR values were 13-15%, 12-15% and 15-18%. The *N. oculata* gravimetric values were slightly higher than the FTIR, possibly due to chlorophyll and other impurities dissolved in methanol during extraction as indicated by the dark green colour observed in the crude lipid after solvent evaporation; similar observations were reported by Cooney *et al.* (2009) and Laurens and Wolfrum (2011).

4.3.6. Macromolecular content of the algae biomass immobilised on filter paper

Biomass content can show some disparity depending on its species and origin; it is therefore necessary to separately discuss the individual algae biomass analysis. The suspension control macromolecular analysis for the three microalgae species is shown in Fig. 4.9. The cultivation was only conducted for a period of 5 weeks due to culture decline beyond this period. There was no marked changes in the protein content of *C. vulgaris* (ANOVA, $F= 4.52$, $df = 2, 6, 8$, $P= 0.063$) over the course of the cultivation period (0-4 weeks), however, 2nd and 4th weeks lipid (ANOVA, $F= 35.87$, $df = 2, 6, 8$, $P< 0.05$) and carbohydrate (ANOVA, $F= 31.88$, $df = 2, 6, 8$, $P= 0.001$) contents significantly differed from week 0 as shown in Fig 4.9A. There were no significant changes in macromolecular component of *D. salina* ($P> 0.05$) between the 2nd

and 4th week while they significantly differed from the initial week with P values < 0.05 (Fig 4.9B). A similar trend can be seen in Fig 4.9C with *N. oculata* were the only visible difference (P< 0.05) is with lipid and protein content of the 0 week when compared to the rest of the cultivation period.

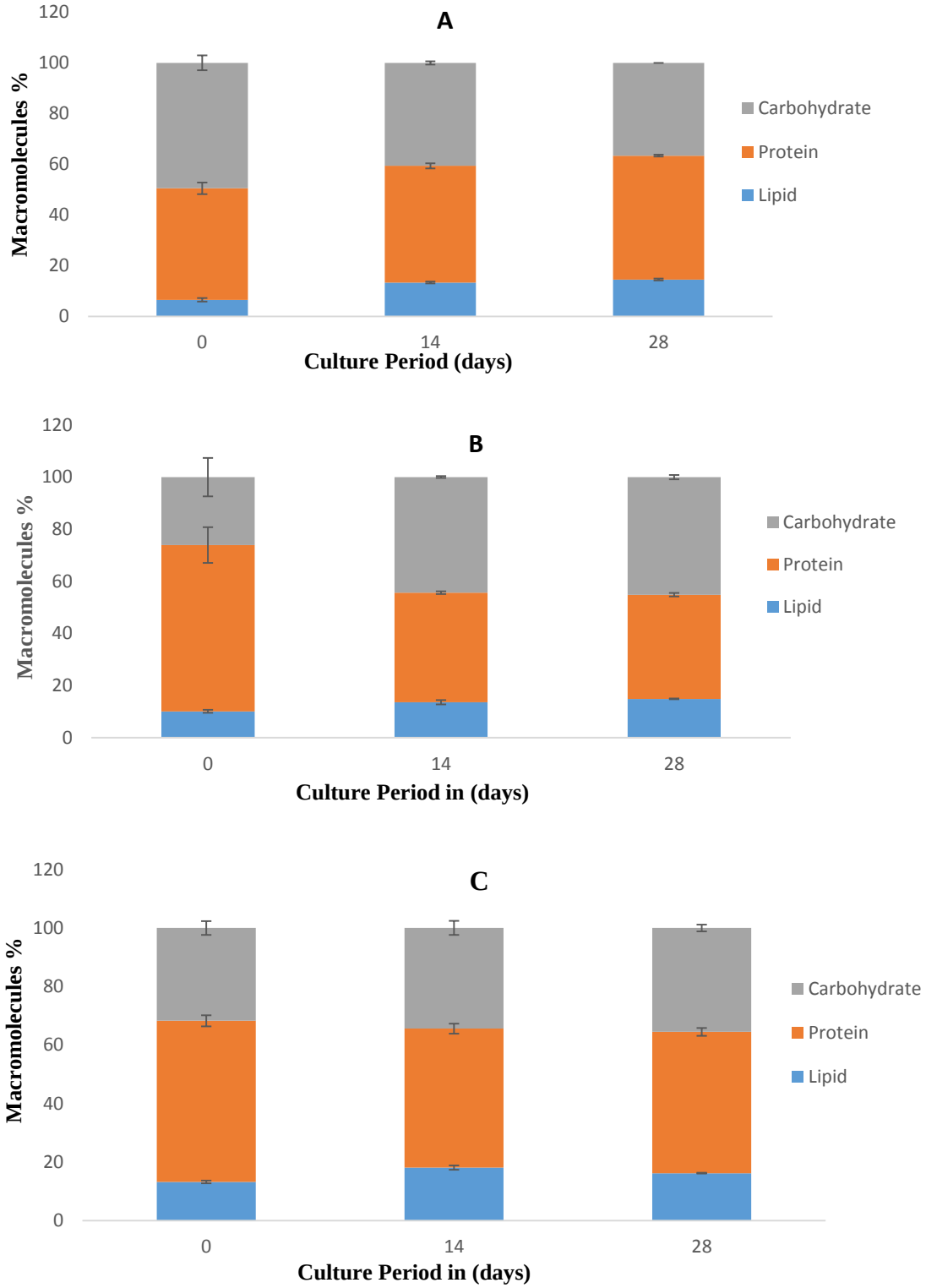


Figure 4.9: Lipid, protein and carbohydrates content determined using FTIR for **A**, *C. vulgaris*, **B**, *D. salina*, and **C**, *N. oculata* cultivated for 28 days in suspension cultivation, Mean ± SD (n=3).

4.3.6.1. Chlorella vulgaris

The lipid content increased steadily (Krustal-Wallis, K= 12.9, df= 4, P= 0.012) from week 0 peaking at week 6 (Fig. 4.10). Total lipid increased 3-fold from 6.53 to 17.19% within 8 weeks; this showed a similar trend with the suspension cultures (Fig. 4.9) where lipid content increased from to 6.53% to 14.53% within 4 weeks, however, this could not be sustained further. The higher lipid content for immobilised cultivation could be attributed to stress caused by the immobilisation technique. This is also suggested by the cell's slow or no division and thus storing more lipid. The protein content remained the same after week 2 but significantly decreased (Krustal-Wallis, K= 12.83, df= 4, P=0.012) during week 6 when lipid content was highest (Fig. 4.10), contrary to the control were protein increase for the 4 weeks was only marginal (Fig. 4.9). The carbohydrates content also showed a significant decrease (Krustal-Wallis, K= 13.23, df= 4, P= 0.01) throughout the cultivation period apart from week 6, increasing to 24.41% from 6.8%. The carbohydrate content for the control was indirectly proportional to the increase in both lipid and protein contents.

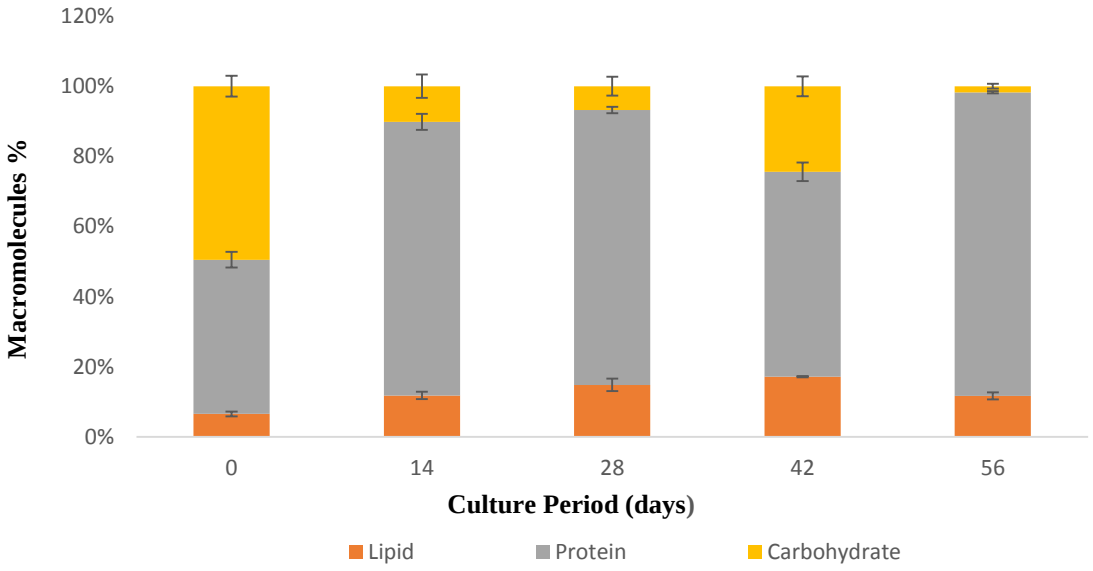


Figure 4.10: Lipid, protein and carbohydrates content determined using FTIR for *C. vulgaris*, cultivated for eight weeks on a filter paper, Mean ± SD (n=3).

4.3.6.2. Dunaliella salina

The total *D. salina* biomass present on the filter paper after eight weeks had dropped to less than 1 mg, comprising mostly protein. The percentage lipid dropped significantly (Krustal-Wallis, K= 12.49, df= 4, P=0.014) from 10.07% to less than 2% by week 8 as indicated in Fig. 4.11. The protein content also fluctuated throughout the cultivation period (Krustal-Wallis, K= 12.63, df= 4, P=0.013) reaching its peak at week 4. The carbohydrate content also show a

major variation along the cultivation period (Krustal-Wallis, K= 12.1, df= 4, P=0.017) where it gradually decreased from week 0 (26.11%), reaching its lowest at week 4 (5.17%) with a subsequent increase to week 8 (37.93%). The lipid and carbohydrates content of the suspension control respectively increased from 10.07% to 14.86% and 26.11% to 45.16%, while protein decreased from 63.82% to 39.98% within the 4 week cultivation period (Fig. 4.9).

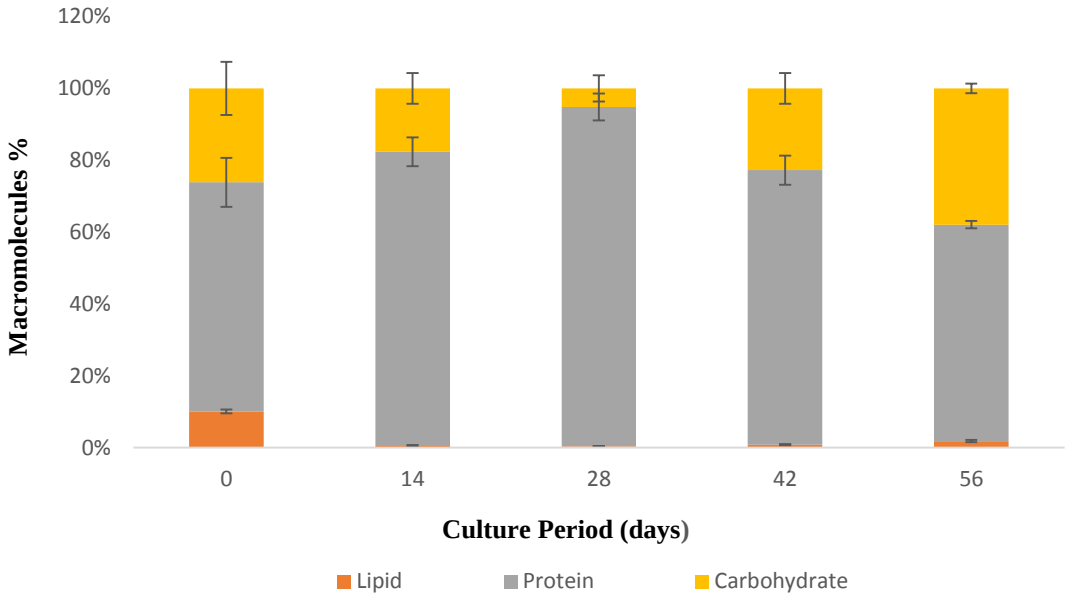


Figure 4.11: Lipid, protein and carbohydrates content determined using FTIR for D. salina, cultivated for eight weeks on a filter paper, Mean ± SD (n=3).

4.3.6.3. Nannochloropsis oculata

Lipid content increased considerably (Krustal-Wallis, K= 12.9, df= 4, P= 0.012) within the first two weeks, and was maintained throughout the cultivation period except for week 8 where it dropped to 15.93% from its peak of 23.97% in week 2 (Fig 4.12). Increased CO_2 concentration was reported to have a significant impact on lipid production in *N. oculata* (Hsueh *et al.*, 2009). Thus, the lipid increase may be assumed to be a result of changing CO_2 concentration from atmospheric levels in suspension culture (the inoculum) to transfer to 5% CO_2 within the bottles. Protein content also decreased marginally (Krustal-Wallis, K= 11.5, df= 4, P=0.021) after the first two weeks and remained stable (47% - 50%) for the next four weeks. The decrease in protein content was a result of the increase in the lipid production as was the case with *C. vulgaris*. There was no significant difference (Krustal-Wallis, K= 9.23, df= 4, P>0.05) in the carbohydrates content for the whole cultivation period apart from a marginal decrease at the 8th week. The suspension control shows a highest lipid value of 18.1 at the 2nd week of cultivation while protein decrease with an increase in carbohydrates value (Fig. 4.9).

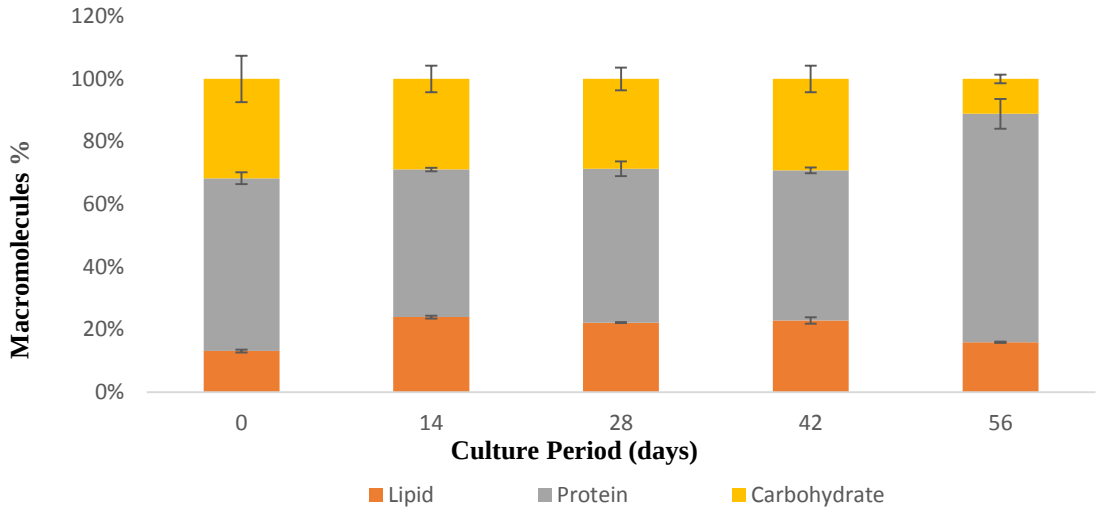


Figure 4.12: Lipid, protein and carbohydrates content determined using FTIR for *N. oculata*, cultivated for eight weeks on a filter paper, Mean ± SD (n=3).

Despite poor adhesion strength *C. vulgaris* survived the immobilisation with high CO_2 fixation and an optimal increase in lipid content. Table 4.3 summarises the performance of the three algae immobilised on filter paper.

Table 4. 3: Summary of the relative performance of the three microalgae species immobilised on filter paper.

Microalgae	Survivability	Adhesion strength	CO_2 fixation	Biomass	Total lipid % DW
C. vulgaris	Good	Poor	High	High	Average
D. salina	Poor	Good	Average	Low	Low
N. oculata	Good	Good	Low	Average	High

4.4. Summary

We have demonstrated the ability to grow algae on a supporting material (paper) to efficiently capture CO_2 and improve lipid content. This could significantly reduce land and water requirements associated with microalgae cultivation for carbon capture and biofuel production. There is now the hope of greater flexibility in siting algae cultivation systems around CO_2 emitting sources. Stronger and more flexible substrates are needed to replace the paper if this experiment is to be tested at a bigger scale. Additionally, cell release (especially *C. vulgaris*) has to be curtailed to avoid significant biomass growth that will plug reactors and cause culture crash. There is literature reporting the use of commercial binders for cell immobilisation (Gosse *et al.*, 2012). In this regard a screening process could be designed for both potential microbes, substrates and suitable nontoxic binders to engineer a living biocomposite for carbon capture and utilisation. A breakthrough in genetic engineering and progress in the interface between engineering and molecular biology may be exploited for microbial immobilisation on flexible substrates using binder.

Chapter 5

Microalgae biocomposite development: Materials testing, toxicity and adhesion screening protocols

Acknowledgment

Mr Adam Wallace and Miss Pichaya In-na helped in the initial screening for toxicity and adhesion test of microalgae immobilised on paper and cotton substrates, whilst visiting North Carolina State University.

Abstract

A screening protocol which took into account toxicity and adhesion strength of binders (organic and inorganic) on different species of freshwater and marine microalgae using 3 types of biodegradable substrates (paper, cotton and loofah) was developed. Simple visual screening and cell counts was employed for toxicity tests, while adhesion strength of binders tested used well plates and a shaker. Drawdown, pipetting and equilibrium coating methods were respectively employed to fabricate biocomposites from paper, cotton and loofah. Coating formulation was determined using binder/cell solid contents while deposition and drying of biocomposites was done under a controlled environment. Film formation and desiccation tests were conducted on the paper substrates to determine the optimum parameters for drying. SEM images showed the relationship between the binders, immobilised cells and the substrates. This is a potential tool toward screening of biocomposite components (binders, substrates and microbes) before undertaking a major experimental work which could save time and resources.

5.1 Introduction

Photobioreactors are an efficient means to grow microalgae, however they are primarily based on suspension culture practices. The use of cell immobilisation is a promising, yet under explored alternative to suspension culture. Encapsulating algae in hydrogels (i.e. forming a biocomposite) may intensify the culture process and significantly reduce water consumption; however, hydrogel biocomposites are often fragile with short operational lifetimes and may release the immobilised cells due to outgrowth and structural failure. Additionally, hydrogels tend to shrink if subjected to desiccation which may incapacitate or kill the immobilised organism. Hydrogels also have reduced gas mass transfer efficiencies due to the extra layer surrounding the cells. For instance, *Raphidocelis subcapitata* growth was 34.42% higher in suspension culture than when immobilised in alginate; this was attributed to the mass transfer

limitation caused by the $CaCl_2$ crosslinked Na-alginate barrier (Benasla and Hausler, 2018). Therefore, the idea of growing microalgae using hydrogels for industrial applications remains at a laboratory scale of development.

Chapter 4 dealt with microalgae immobilisation on filter paper. Some challenges were observed during the immobilisation process, including a lack of strong adhesion between the paper and the cells, especially using *C vulgaris*. There were also difficulties in attaining uniform coating thickness using the pipetting coating method. This prompted the need to find a better immobilisation method, and potentially the need to adopt an alternative to a paper-based substrate. An in-depth knowledge of porous substrates, coating technology and microalgae physiology (before, during, and after immobilisation) is a prerequisite for the design and development of robust biocomposites (Groboillot et al., 1994), especially if biocomposite functional longevity is deemed important. In the current Chapter, a series of biocomposite development trials are detailed covering a range of candidate substrates together with their methods for formation. Subsequently, through a collaboration with the Flickinger laboratory (NC State University), a comprehensive screening protocol was applied to a range of algae species, substrates (i.e. paper, non-woven cotton sheet and natural loofah sponge) and both organic and inorganic binders with the intention of developing a shortlist of functional biocomposites to be tested for long term CO_2 reactivity.

5.2. Material and methods

5.2.1 Microalgae specie, media, growth conditions and wet cell pellets preparation

Freshwater microalgae *Chlamydomonas reinhardtii CC125* and *Chlorella vulgaris* were grown aerobically in 1-l baffled Erlenmeyer flasks containing 250 ml TAP and BG-11 medium respectively, with constant stirring at 28 °C. Illumination was provided by cool white fluorescent light with a 12/12 light/dark cycle. The pH of both media was adjusted to 7.0. TAP contains the following per L; 1 M trisbase 20 ml, phosphate buffer (II) 1.0 ml, solution A (NH_4Cl = 20 g, $MgSO_4$-$7H_2O$ = 5 g, $CaCl_2$-$2H_2O$ = 2.5 g per 500 ml) 10.0 ml, Hutner's trace elements 1.0 ml, glacial acetic acid 1.0 ml. Marine microalgae used for this screening experiment were *Dunaliella salina*, *Nannochloropsis oculata* and *Tetraselmis suecica* (see Chapter 4 for culture conditions). All cultures were monitored for bacterial contamination by microscopic examination, and cell numbers were determined by direct cell counts with a haemocytometer.

5.2.2 Microalgae mixed with alginate and shredded paper

Shredded white office paper was soaked with tap water for 24 h and ground to smaller particle sizes using a portable table top electric grinder. The paper was sterilised by oven drying overnight at 105 ^{o}C. Alginate (3%) was prepared by dissolving 3 g of alginate power in 100 ml of deionised water. A 200 ml volume of 7-10 days old *C. vulgaris* culture was concentrated to 40 ml by centrifugation at 4,000 x *g* for 10 min. A flat paper biocomposite was made by mixing 2.5 g of dry paper with 25 ml of BG-11 growth medium and 20 ml of 3% alginate solution. To this, 40 ml of concentrated microalgae was added and thoroughly mixed. Approximately 10 ml of the final mixture was poured into a mould and hardened using 2% $CaCl_2$ solution. The final biocomposite was left to dry at room temperature for 24 h. A similar protocol was followed to produce another biocomposite but without the shredded paper support (Fig. 5.1).

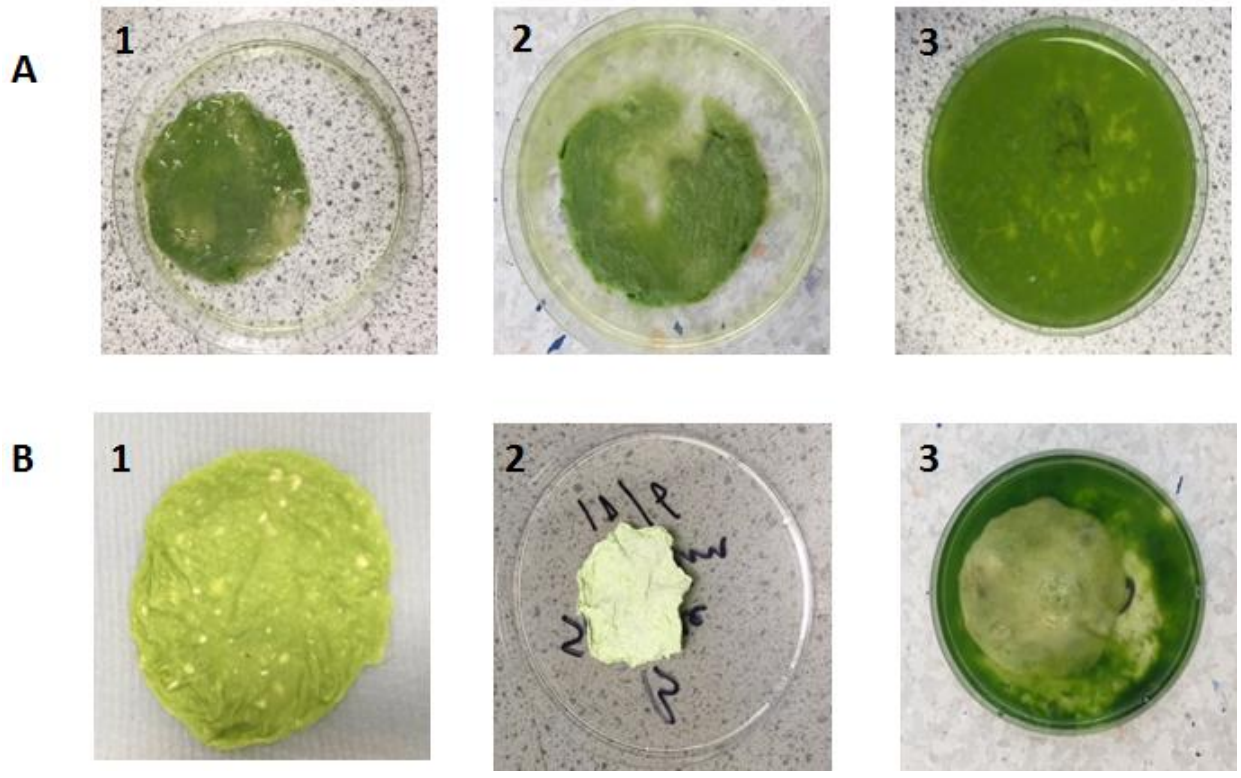


Figure 5.1: *Chlorella vulgaris* biocomposite fabrication using: **A** alginate and **B** alginate supported with paper. Showing the hydration and desiccation steps **1** immediately after biocomposite formation, **2** after 48 h at room temperature/pressure, and **3** upon rehydration with growth medium which caused the release of the cells.

5.2.3 Algae mixed with calcium carbonate with supercritical drying using CO_2

A slurry of *C. vulgaris* was mixed with 3% sodium alginate and 2% calcium carbonate solution for 2 min until homogenous. The mixture was poured into a small Petri dish and placed in a 0.5 L autoclave for gelation. The autoclave was pressurised using 99% CO_2 at 5 Mpa at room temperature for 12 h. The pressure was released at 0.02 Mpa min^{-1}. The bio-hydrogel formed (Fig. 5.2) was allow to stand at room temperature until bubble formation had ceased and then

washed stepwise using an ethanol-water mixture before drying using the same autoclave and 99% CO_2 at 10 Mpa at 40 ^oC.

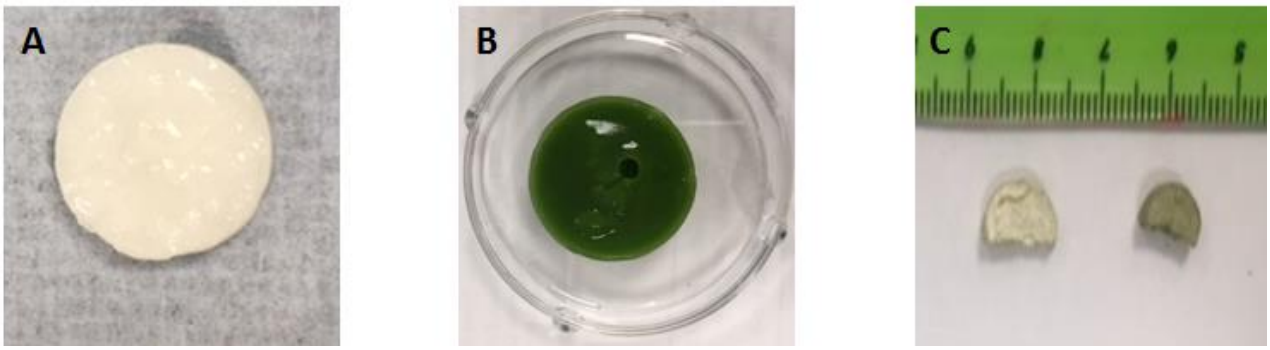


Figure 5.2: Bio-hydrogel formed using: **A**. alginate, **B**. *C. vulgaris*, and **C**. dried hydrogel after desiccation.

5.2.4 Using foam floatation to produce stable foam from algae/alginate/polystyrene beads in the presence of sodium dodecyl sulphate

Dry shredded paper (5 g) was mixed with 100 ml of deionised water and 5 ml of concentrated *C. vulgaris* slurry in the presence of 250 mg L^{-1} sodium dodecyl sulphate (SDS). The mixture was poured into a foam column rig to generate stable foam at 1 L min^{-1} air flow rate. A long glass tube with an opening at both ends was inserted into the entire length of the column to introduce 2% $CaCl_2$ solution to stabilise and harden the foam. The glass tube was gradually withdrawn (0.55 cm sec^{-1}) to the top while trickling the $CaCl_2$ solution throughout the length of the foam. The foam was set within the column for 5 h allowing excess $CaCl_2$ solution to drain before removing and drying at room temperature (Fig. 5.3). The biocomposite was supported with shredded paper pulp (25-75% w/w) and 10% w/w polystyrene (2% divinylbenzene copolymer) beads with average particles size of 50 ± 5 µm purchased from Fisher Scientific, UK.

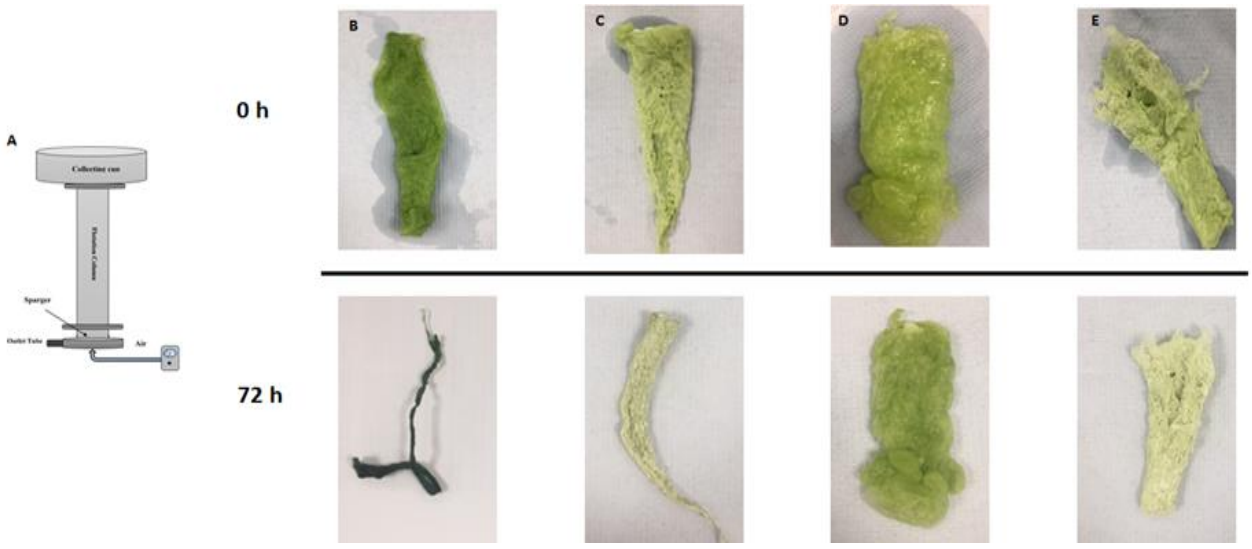


Figure 5.3: Fabrication of foam-based microalgae biocomposites using **A** foam column with different support materials **B**. alginate, **C**. alginate with paper, **D**. alginate with polystyrene beads, **E**. alginate with paper and polystyrene beads, , at ambiant temperature and pressure.

5.2.5 Microalgae immobilised using carboxymethyl cellulose and soy protein isolate

Carboxymethyl cellulose (CMC) and soy protein isolate (SPI) sheets were fabricated by casting and evaporation procedures (Su *et al.*, 2010), as follows; 10 g of CMC powder was dissolved in 100 ml of deionised water (solution A) under constant stirring and heating using a water bath at 50 ^{o}C for 30 min. SPI solution (solution B) was prepared by mixing 5 g of SPI with 100 ml of deionised water and the pH adjusted to 10 using 2.0 mol L^{-1} NaOH solution at 80 ^{o}C for 60 min. High pH promoted linkage formation and hydrolysis. A resin was formed by mixing solutions A and B with glycerol (0.5, 1 and 5%) added as a plasticiser and cryoprotectant. The pH was again adjusted to 10.0 at 80 ^{o}C. After vacuum defoaming and cooling to room temperature, concentrated *C. vulgaris* slurry was added to the mixture and known amounts of the mixture were poured into a Teflon mould to produce a biocomposite of uniform thickness. The biocomposite was dried in an oven at 30 ^{o}C for 6 h and kept at room temperature for another 24 h. A series of CMC/SPI blend biocomposites were prepared by modifying the CMC/SPI/glycerol weight ratios (Fig 5.4).

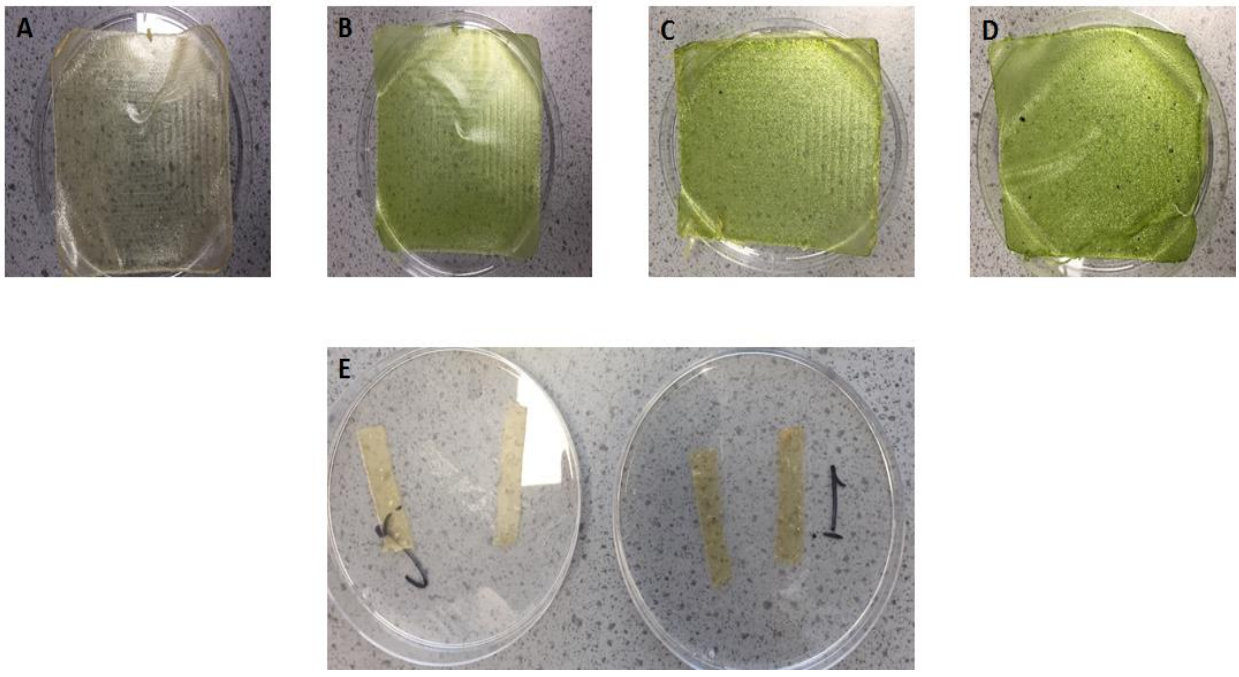


Figure 5.4: A. CMC/SPI biocomposites, prepared with glycerol ratios of **B**. 0.5%, **C**. 1% and **D**. 5% as plasticiser and osmoprotectant. **E**. Cut-out from samples **A**-**D** after 7 days showing decolouration of algae chlorophyll. The percentage of glycerol is in relation to the total weight of the CMC/SPI mixture, at ambiant temperature and pressure.

5.2.6 Algae immobilised on loofah sponge using alginate hardened with calcium chloride

A matured loofah sponge was washed, autoclaved and cut into smaller sizes (2.5 x 4.0 cm). Microalgae-alginate slurry was made by mixing concentrated *C. vulgaris* and 3% alginate

solution in a 1:1 ratio. Prepared loofah (Fig. 5.5A) was dipped into the algae-alginate slurry and subsequently set using 2% $CaCl_2$ solution (Fig. 5.5B). The biocomposite was transferred to a 1 L sample bag where CO_2 reactivity of the immobilised algae was measured. The biocomposite was maintained for 120 h by periodic sprinkling with BG-11 medium and injection with 5% CO_2.

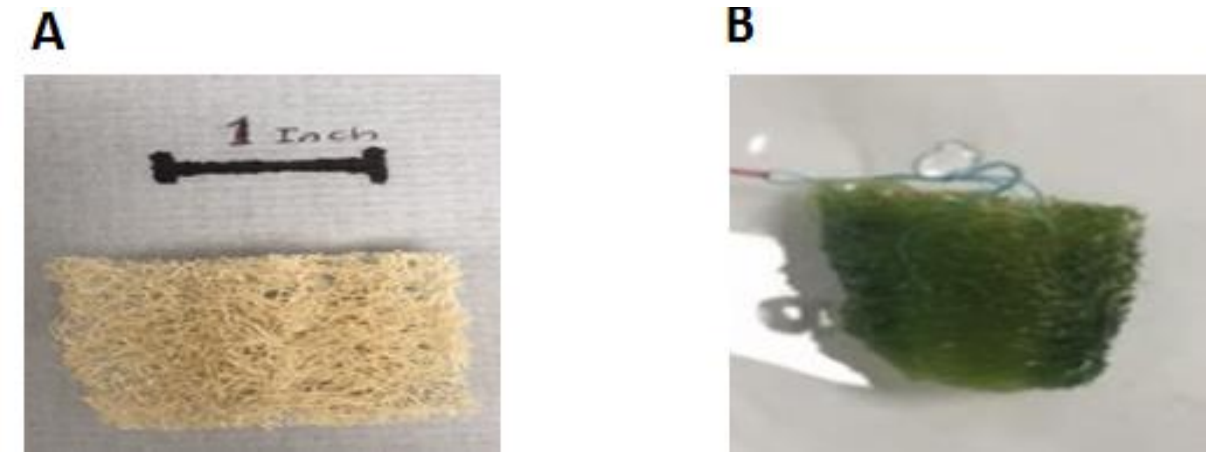


Figure 5. 5: A, Loofah sponge and **B**, cells of *C. vulgaris* immobilised on loofah sponge using sodium alginate , at ambiant temperature and pressure.

5.2.7 Algae immobilised on loofah sponge by direct immersion in algae culture

Washed and autoclaved loofah sponge was cut to a height of 5 cm. The cut loofah pieces were submerged in cultures of different microalgae species for 2 weeks (Fig. 5.6). The loofah was transferred into a 1 L sample bag and nourished using 5% CO_2 and growth medium while measuring CO_2 consumption.

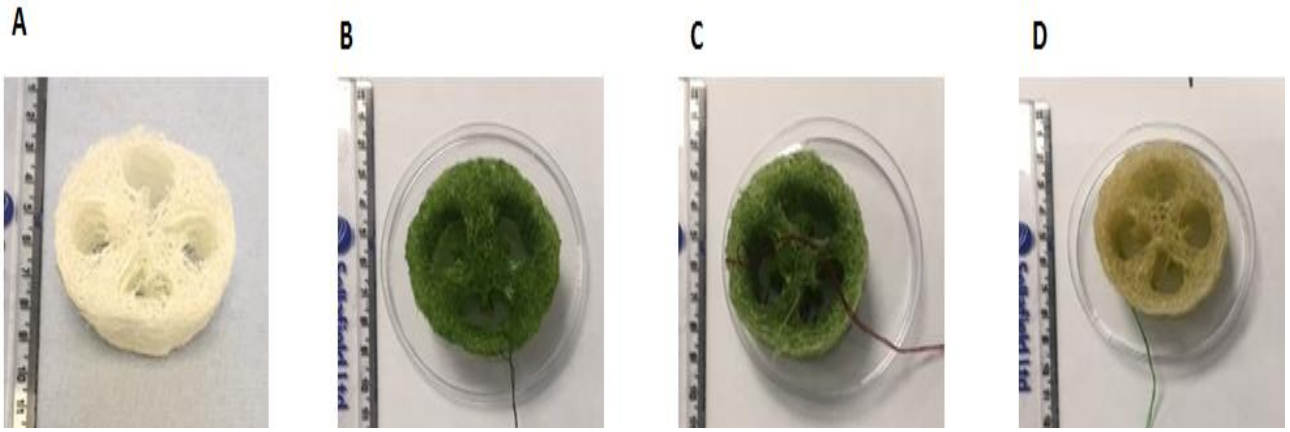


Figure 5.6: Images of **A**. loofah sponge immobilised with cells of **B**. *C. vulgaris*, **C**. *D. salina* and **D**. *N. oculata* after 2 weeks, at ambiant temperature and pressure.

5.2.8 Screening protocol: Substrates and binders

Three substrates (Fig. 5.7) were selected for this screening test: Whatman #1 chromatography paper (Whatman International Ltd.), woven cotton obtained locally from Thailand, and loofah

sponge from Nigeria and later sourced in the UK from a Primark store. Latex binders were obtained from the Flickinger latex binder library collection (mostly from Rohm and Haas Co., Philadelphia, PA), while organic materials with binding properties (pectin, alginate, carrageenan, carboxymethyl cellulose and soy protein isolate) were purchased from Sigma-Aldrich, UK with the exception of pectin that was also obtained from Flickinger binder library collection (commercial product sample by Kelco Company). There are two types of pectins used in the experiment, the brown coloured X-923-03 (pectin 1) and white coloured X920-02 (pectin 2). Information on their material origin and composition is limited by the manufactures.

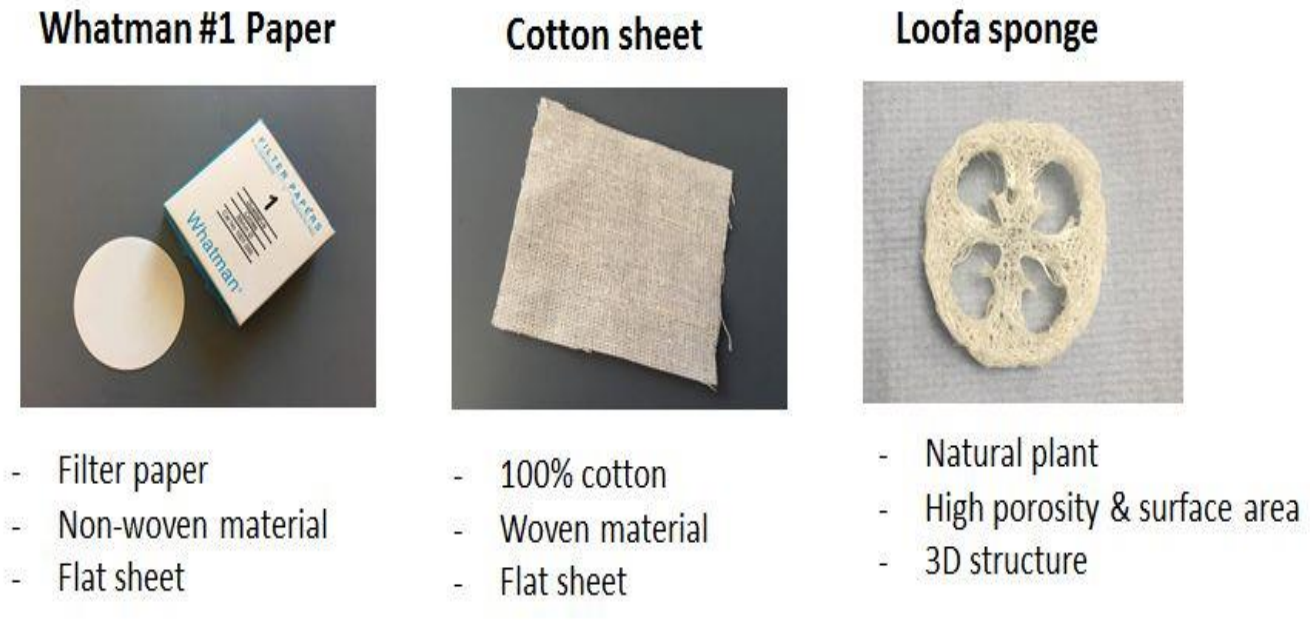


Figure 5.7: Substrate materials used for the cell/binder adhesion protocol with their characteristics.

5.2.9 Substrate pH determination

Paper, cotton sheet and loofa sponge were tested for pH values with TAP and BG-11 growth media. Small pieces of each substrate were placed in Eppendorf tubes with 1 ml of the respective medium or 1 ml of deionised water in triplicate. The samples were left to stand for 24 hrs to let the substrates react with the solution, then pH was measured using a probe.

5.2.10 Latex binder percentage solid determination and pH adjustment

Thirty five latex (aqueous) binders were screened. Ab-initio, 2 binders have negligible percent solid. A threshold of 5% total solids content (dry weight) was set for this protocol screening test. Total solids content was determined using a dry weight technique. Approximately 50 µl of each binder was placed in a pre-weighed 1.5 ml Eppendorf tube and oven dried overnight at 100 ℃. The percentage solid was determined by equation 5.1. pH of the binders was determined

using a pH probe, Orion 611 Benchtop pH/mV/ORP/BOD meter (Thermo Electron Corp. Beverly, MA). 1mL of binder was pipetted into a 1.5 mL Eppendorf tube and a glass probe inserted to measure the aqueous binder pH. Binders reaching the threshold were adjusted to pH 7 using 0.2M/1M ammonium hydroxide solution for acidic binders and 0.1M acetic acid for alkaline binders. pH adjusted binders that fell below the threshold (5%) were again screened out. The remaining binders progressed to the toxicity test.

$$\%\, Total\ solid = \frac{w2}{w1}(mg)x\ 100 - - - - - \mathbf{5.1}$$

Where: w1 = weight of binder before drying

w2 = weight of binder after drying

5.2.11 Toxicity test measurement using transparent 16-well plates: Visual screening for photo-bleaching of microalgae cells

Cell binder formulation (5% solid) was pH adjusted using acetic acid or ammonium hydroxide. To test the formulation, each binder was mixed with 2 ml of microalgae culture in duplicate wells of a 16-well plate, with biotic (microalgae with 2 ml growth medium) and abiotic (binder with 2 ml growth medium) controls (see Fig. 5.8). The well plates were placed under natural sunlight at 25 ^{o}C. Visual inspection (with photographs) and direct cell counts were conducted for 7 days to assess culture health relative to the controls.

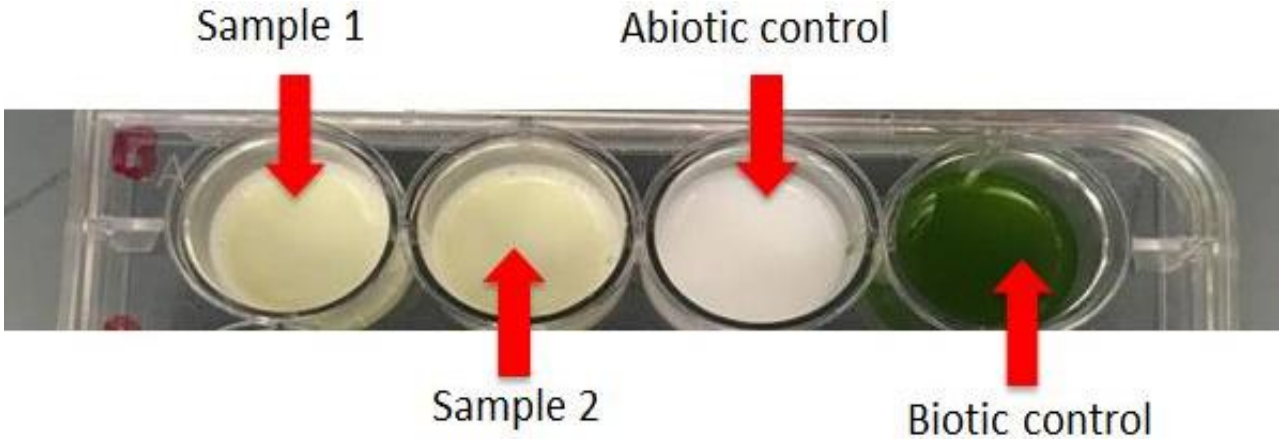


Figure 5.8: Arrangement of assay for the toxicity test. Sample replicates 1 and 2 contain the mixture of microalgae and binders, the abiotic control contains binders and 2 ml growth medium while the biotic control contains microalgae and 2 ml growth medium (ambiant temperature and pressure).

5.2.12 Chlorophyll a extraction and cell counts on non-toxic coatings

Binders that caused obvious photo-bleaching were screened out. For the remaining binders, chlorophyll *a* was extracted from 850 μL of the latex-cell mixture using 1.5 ml of 90% v/v ethanol solution. Extractions were performed over 30 minutes in the dark at room temperature

and tubes were centrifuged at 15000 x *g* for 10 min. The process was repeated three times and the supernatants were pooled into a single extract. Absorbance was measured at 480, 510, 665 and 750 nm using a Genesys 20 UV-Vis spectrophotometer (Thermo Electron Corporation, Marietta, OH). The amount of chlorophyll *a* (µg/L) extracted from each coating was calculated using equation 5.2 (Marker *et al.*, 1980). Cell count was conducted using a haemocytometer to rank the binders in order of viable cells.

$$chlorophyll\ a\ \left(\frac{\mu g}{l}\right) = \frac{[11.99\text{x}(A665-A750)s]}{Vp} \text{----------} \mathbf{5.2}$$

Where;
p = path length of cuvette, (cm)
s = solvent extract volume, (ml)
V = sample volume, (ml)
A665 = absorbance at 665 nm
A750 = absorbance at 750 nm

5.2.13 Latex binder formulation, coating and deposition techniques

All latex-cell formulations for both *C. reinhardtii* and *C. vulgaris* were prepared from wet cell pellets (WCP) by centrifuging 200 ml of a 5-7 days old culture in 250 ml plastic tubes in a Beckman J2-21 centrifuge for 7 min. The concentrated cell slurry was transferred to a 50 ml tube and centrifuged for another 5 min at 3,000 x *g*. Marine microalgae were centrifuged using a table top centrifuge at 4000 x *g* for 10 min while *D. salina* was centrifuged for only 2 min. However, the latex binders did not react favourably with natural seawater, as such artificial seawater containing 35 g L^{-1} of NaCl was prepared using deionised water. Hence, the artificial seawater was premixed with the latex binders before mixing with the WCP for the marine species.

The WCP were mixed with non-toxic pH adjusted acrylic latex emulsion or organic binder by vortexing (15 s) until homogenous slurries of cells were obtained at the desired concentration (5% total solids for paper and cotton substrates, and 1.25% total solids when loofah was used with *C. reinhardtii*). The quantity of latex binder/microalgae mixture, coating technique and quantity of coating formulation used for different substrates are given in Table 5.1.

Coatings for the paper were manually prepared by extruding 30 µl of formulation using a pipette tip (Finntip®250 Universal, Thermo Scientific, San Diego, CA) over a small area on one end of dry, sterile Whatmann #1 paper templates (4 cm by 6 cm) in triplicate. The coating mixture was placed in front of a Mayer rod (Wire No. 8) and the rod was manually pulled as depicted in figure 5.9. The pipette coating technique was chosen for the cotton sheet as the pores were too large for Mayer rod deposition. The cotton sheet was cut into squares (5 x 5 mm) and 10 µ l of

coating formulation was directly pipetted onto the surface. The formulations on both paper and cotton were left to dry in a dark control chamber (relative humidity = 50%, temperature = 30 ℃) for 24 hours. For loofa sponge, the samples are placed in a 12-well plate and 3 mL of the coating solution was added and left to dry in the control chamber for 48 h. The samples was turned over after 24 h to achieve uniform coating - this procedure is termed the equilibrium coating technique. Once all samples were dried, they were ready to be tested for their adhesion affinity.

Table 5. 1: Details of substrates for adhesion tests.

Substrate	WCP (g)	Amount of coating formulation used	Coating technique	Amount of medium used
Whatman#1 paper	0.085	30 µ L	Mayer rod deposition	350 µ L
Woven Cotton sheet	0.085	10 µ L	Extrusive/Pipetting	400 µ L
Loofa sponge	0.32	3 mL	Equilibrium coating	2 mL

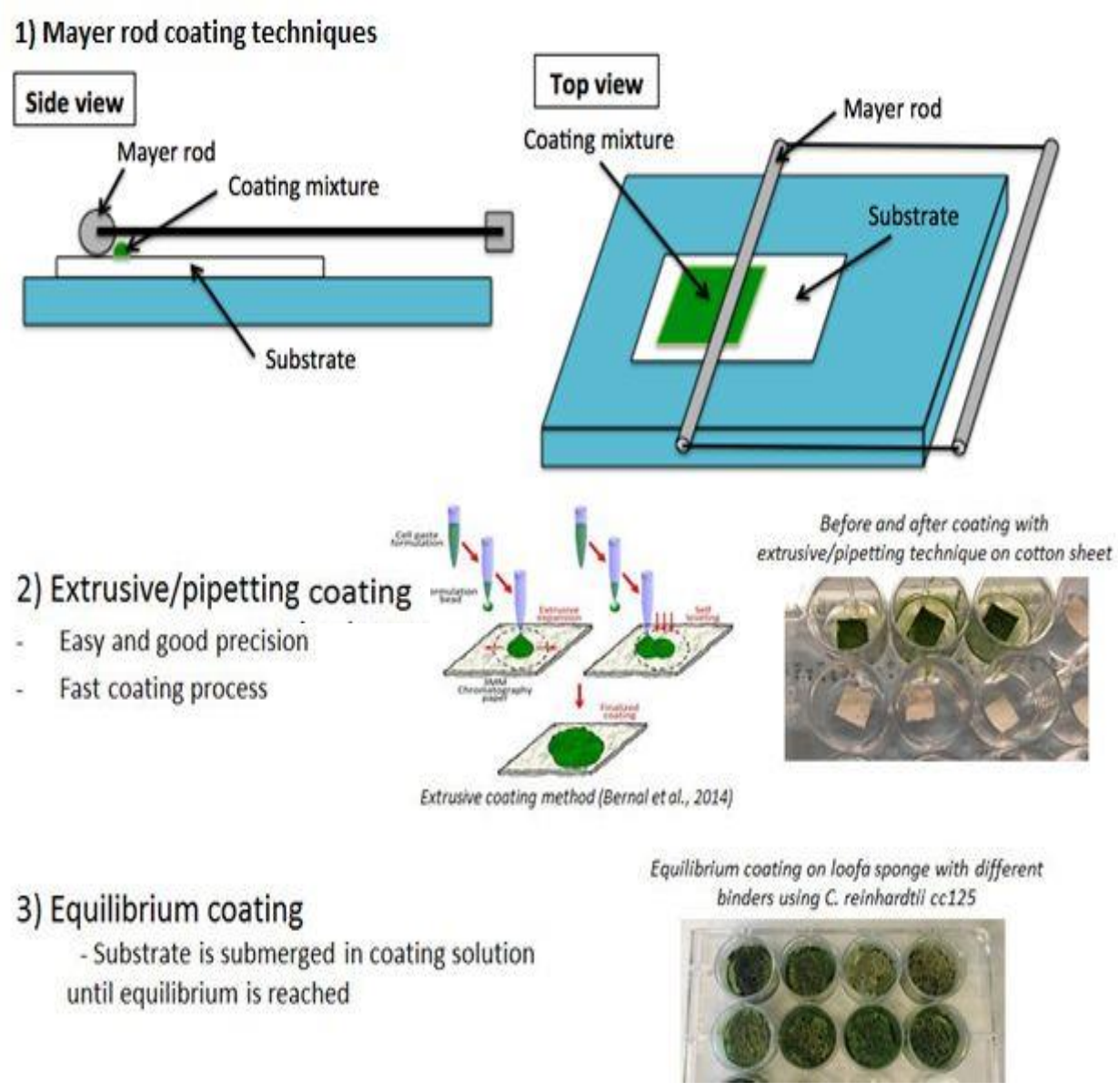


Figure 5.9: Schematic of Mayer rod, extrusive pipetting and equilibrium coating techniques used for paper, cotton and loofah sponge respectively, at ambiant temperature and pressure.

5.2.14 Binder adhesion test

The dried biocomposites were placed in 24-well plates and the corresponding media were added depending on the dimensions of the samples (see Table 5.1). The plates were then placed on a shaker (80 rpm) for 1, 24, 48 and 72 h. After each time interval the samples were transferred to fresh media. Cell counts were conducted for each time point using a haemocytometer to determine the number of detached cells. Assuming uniform coating and that all formulation made it to the paper, we estimate the total number of immobilised cells on each cut-out paper disc using equation 5.3. Number of cells released from cotton and loofah were determine gravimetrically using equation 5.4. Equation 5.5 was used to calculate the percent number of cells released from the biocomposites after the 72 h adhesion. Fig. 5.10 summarises the adhesion test procedure.

$$A1 = \frac{C}{B} \quad \mathbf{5.3}$$

$$A2 = D - E\ (mg) \quad \mathbf{5.4}$$

$$detached\ cells\ from\ biocomposites = \frac{F}{(A1\ or\ A2)}\ \% \quad \mathbf{5.5}$$

where:

A1 = number of cells immobilised on each cut out disc

B = number of cut out discs per paper biocomposite

C = amount of cells immobilised on paper

A2 = number of cells immobilised on loofah or cotton

D = biocomposite dry weight

E = weight of (loofah or cotton) + binder

F = Sum of all cells released (1-72 h)

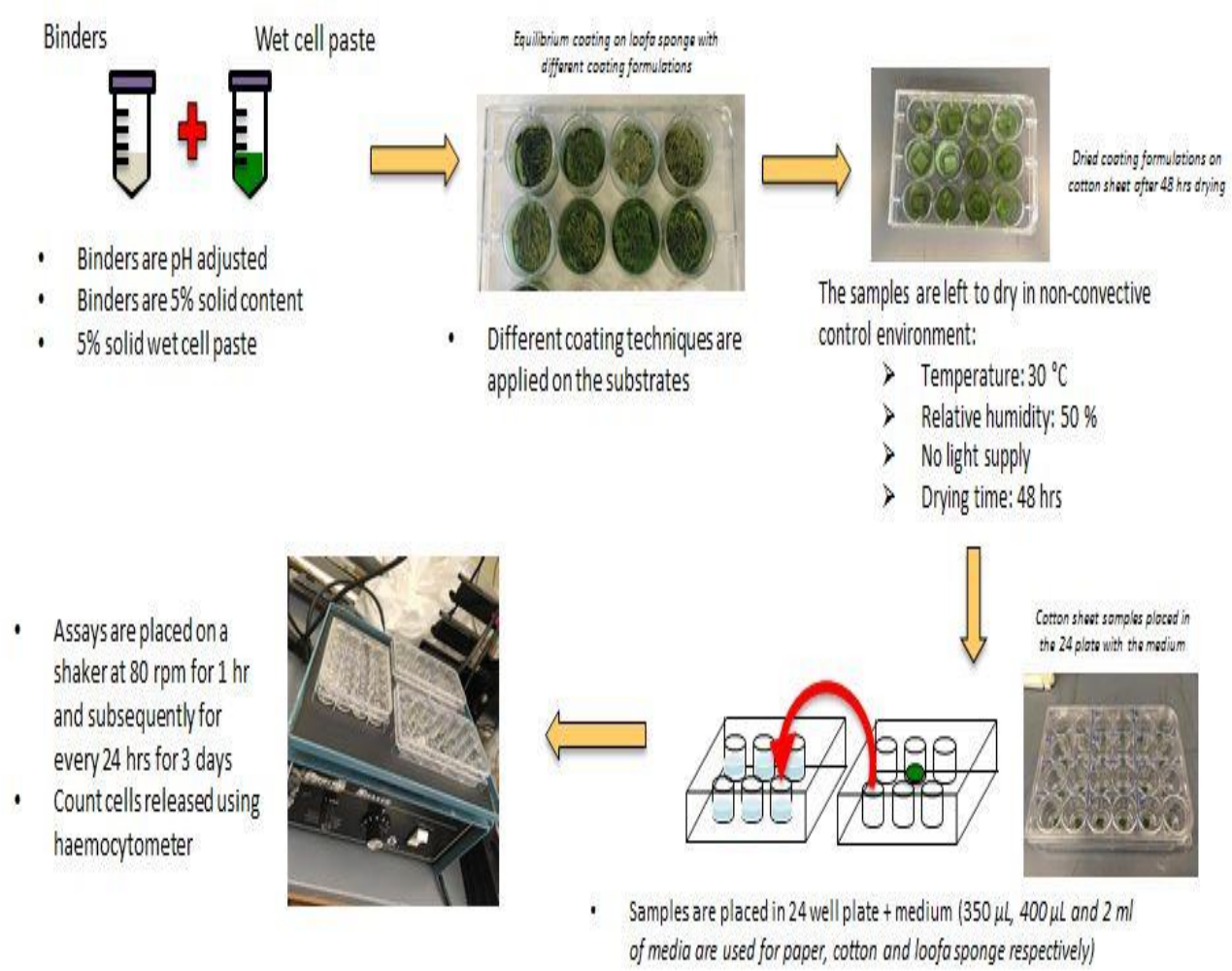


Figure 5.10: Schematic of binder adhesion test using well plates and shaker conducted for 1, 24, 48 and 72 hours.

5.2.15 Film formation and desiccation test on Whatman #1 paper containing biocoating

Due to an insufficient number of cells and time constraints while at NC State University, this test was only done on the paper substrate. The paper was washed with ethanol (70% v/v) and dried overnight at ambient temperature. After drying, the paper was pre-punched with a circular metal ring puncher (3/8 inches diameter) and attached to polyester to prevent it slipping off during the coating process. For the coating formulation, 5% solid w/v was used for both binder and WCP. Approximately 100 µL of pH adjusted coating mixture was used with the Mayer rod deposition technique to coat Whatman #1 filter paper. The rod was manually pulled down to uniformly deposit the coating on the substrate. Three pieces of pre-fabricated paper disc were removed from the coated paper at different drying intervals (0, 5, 15 mins and 1, 24 and 48 h) and placed into a 24-well plate filled with 350 μL of TAP media (Fig. 5.11). The plate was shaken at 80 rpm for 24 h. After, the number of cells washed off each coating was counted using a haemocytometer.

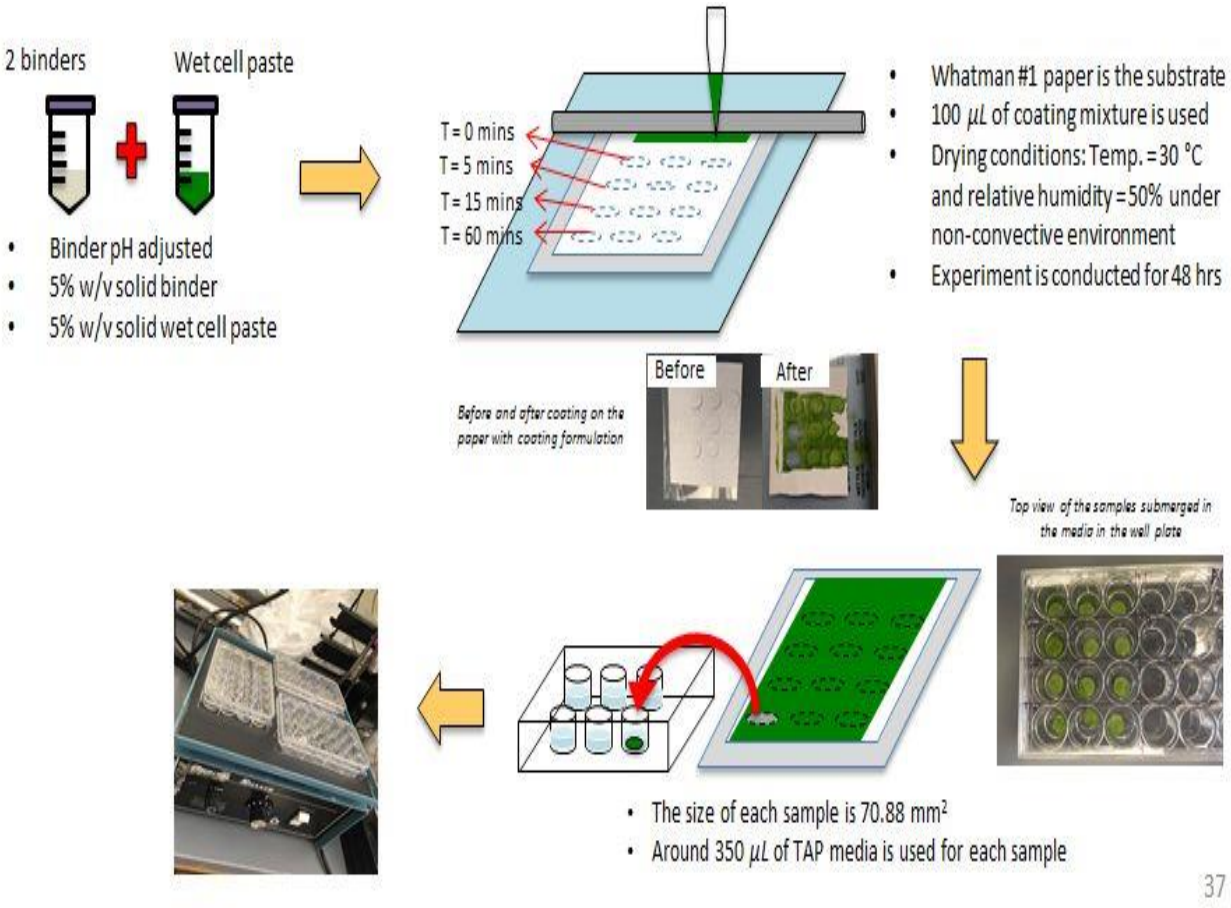


Figure 5.11: Film formation and timed desiccation test conducted on Whatman #1 paper and *C. reinhartii*.

5.2.16 Coating imaging and microstructure

Coating and substrate microstructures were obtained by scanning electron microscopy (SEM) using a table top Hitachi TM 3000 SEM equipped with a backscattered electrons system for digital image acquisition. All coatings were observed in two or more randomised locations using a 5 or 15kV accelerating voltage. Each location was imaged multiple times using sequential magnifications ranging from 15 to 30,000x to characterise surface topography, cell distribution and available pore space.

5.2.17 Statistical analysis

Experiments were repeated with triplicate number of samples (n=3 of samples) and the average values with their standard deviation are reported. One-way analysis of variance (ANOVA) and Krustal-Wallis test was used to compare mean values and statistical significance using SPSS statistical software. In all instances, comparison which shows a P value of <0.05 were considered statistically significant.

5.3 Result and discussion

5.3.1 Microalgae mixed with alginate and shredded paper

Initial data suggested successful preservation of *C. vulgaris* photoreactivity post-immobilisation, on paper. Fig. 5.1A1 shows a sodium alginate-algae biocomposite which, after daily rehydration with BG-11 medium, began to disintegrate (Fig. 5.1A2) and release the cells after 2 weeks (Fig. 5.1A3). Supporting the biocomposite with shredded paper (Fig. 5.1B1) gave it a strong support which could, to a certain extent, withstand shrinkage (Fig. 5.1B2). The dried paper was kept in darkness at room temperature for 2 months and thereafter rehydrated. Growth of the immobilised cells was monitored via chlorophyll *a* intensity for 10 days (Fig 5.12) which shows a significant increase (Krustal-Wallis, K= 14.52, df= 5, P= 0.01) in chlorophyll intensity from the second day of rehydration before stabilising at the sixth day. The binding affinity between the alginate/paper and the algae weakened and the cells were released despite the alginate/paper structure remaining intact (Fig. 5.1B3). CO_2 reactivity was not measured due to cell shedding.

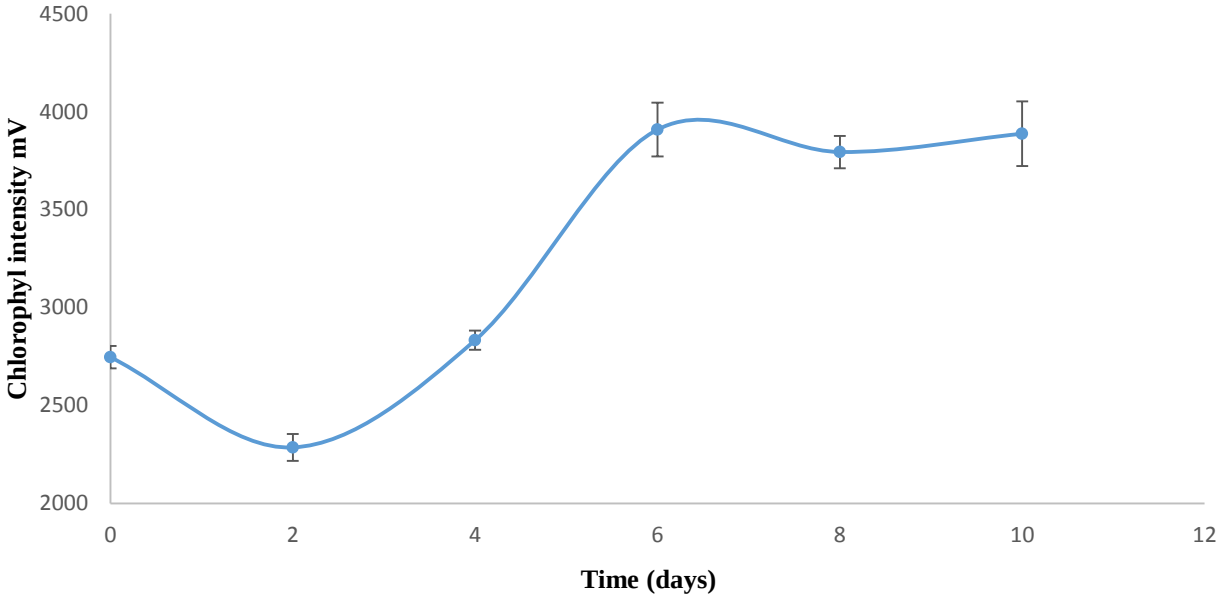


Figure 5.12: Chlorophyll *a* measurement of *C. vulgaris* immobilised within shredded paper using alginate rehydrated after 2 months, Mean ± SD, (n=3).

5.3.2 Algae mixed with calcium carbonate with supercritical drying using CO_2

Gurikov *et al.* (2014), reported the use of sodium alginate to produce an aerogel using gelation and supercritical drying via CO_2. Learning from their work and leveraging on the high pressure resistance of *C. vulgaris* to pressures up to 200 atm (Seckbach, 1971) we fabricate a biocomposite aerogel (Fig. 5.2A&B). Two major challenges were encountered: a) the inability to reach the required pressure for supercritical drying due to a lack of a suitable autoclave, and

b) the possibility of killing the algae cells during solvent extraction using ethanol. As a result, the final biocomposite did not exhibit the needed characteristics of porosity and stabilisation (Fig. 5.2C). However, this proof of concept can be applied if the above mentioned issues can be resolved and using a high pressure tolerant microalgae.

5.3.3 Using foam floatation to produce stable foam from algae/alginate/polystyrene beads in the presence of sodium dodecyl sulphate

Foam floatation (Fig. 5.3A) was used to generate a stable foam with sufficient porosity for cell immobilisation. However, alginate biocomposites need to be hydrated to keep the cells metabolically active whereas desiccation could cause cell damage due to pressure exerted on the cells by the collapsing structure (Fig. 5.3B). We hypothesised that incorporating a supporting structure into the algae-alginate biocomposite mix could help maintain the structural integrity during and after desiccation. Fig. 5.3C-E shows the effect of various supporting materials incorporated using a foam column. Shredded paper support maintained the structural integrity (Fig. 5.3C) but lacked enough porosity to allow for nutrient and air diffusion. Incorporating polystyrene beads (Fig. 5.3D) provided greater porosity compared with alginate and paper-alginate support structures, but could not maintain a rigid structure upon hydration. Blending paper and polystyrene beads (Fig. 5.3E) provided a better opportunity to create a sustainable biocomposite but the rate of cell release was high as the alginate lost its adhesion strength with frequent hydration. Incorporating different percent of paper pulp (25 -75% w/w) into the algae-alginate mixture increased the biocomposite strength but reduced its porosity (Fig. 5.13B-E).

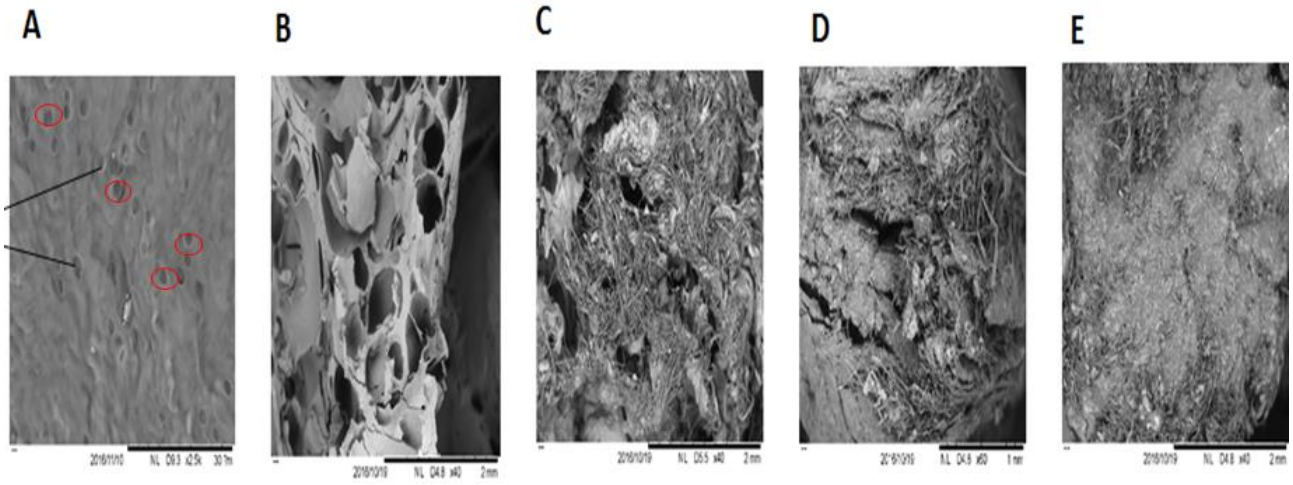


Figure 5.13: Biocomposite SEM images, **A**. top view of alginate biocomposite showing cells of *C. vulgaris* (in red circles) and its cross sectional area, **B**. alginate with 0% paper, **C**. alginate with 25% paper, **D**. alginate with 50% paper, and **E**. alginate with 75% paper.

5.3.4 Microalgae immobilised using carboxymethyl cellulose and soy protein isolate

The idea of using CMC/SPI to develop microalgae biocomposites was derived from the work of Wang and Su (2014). The fabrication process using various percent mixes of glycerol was successful (Fig. 5.4). Producing the biocomposite without glycerol caused the structure to crack upon drying (Fig. 5.4A), while glycerol addition (at different concentrations between 0.5-5%) made the film more flexible (Fig 5.4B-D). To unfold the protein molecules in the SPI and to achieve excellent crosslinking the solution pH was raised to 10, which could partly be responsible for the observed cell death within one week (Fig. 5.4E). The strength and durability of the biocomposite upon hydration was not tested after the biocomposite failed a toxicity test.

5.3.5 Algae immobilised on loofah sponge using alginate hardened with calcium chloride

There was a gradual increase in CO_2 uptake for *C. vulgaris* immobilised on loofah using alginate Fig. 5.14 with BG-11 hydration performing better than without. The daily medium addition had a significant impact (Krustal-Wallis, K= 7.08, df= 1, P= 0.008) on CO_2 reactivity. Where no medium was added, the cells were maintained by taking their nutrient from the medium trapped within the alginate matrix during initial immobilisation. The major setbacks to this immobilisation procedure included: a) the weakening of the biocomposite with time and the pressure exerted by the growing cells which contributed to bursting the structure and cell release, and b) without hydration the alginate soon began to lose water and shrink thereby exerting internal pressure on the immobilised cells and incapacitating them in the process.

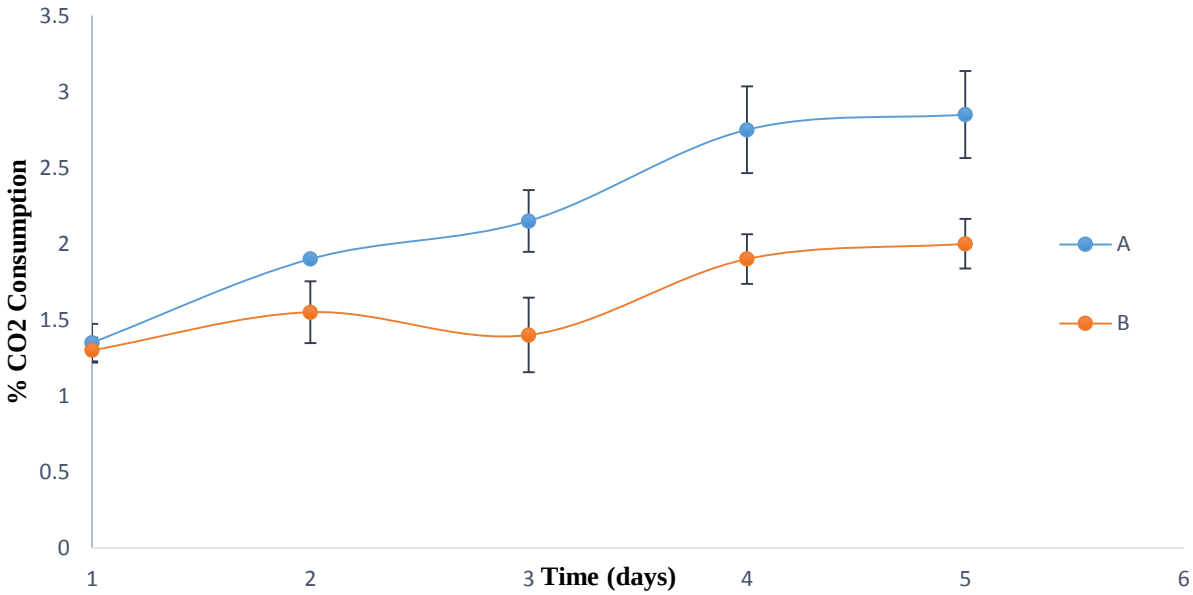


Figure 5.14: Daily CO_2 consumption (5 days) of *C. vulgaris* immobilised on loofah sponge using alginate, **A**. with medium added daily, **B**. without added medium, Mean ± SD, (n=3).

5.3.6 Algae immobilised on loofah sponge by direct immersion in algae culture

Direct cell deposition was achieved when loofah sponge (Fig. 5.5A) was immersed into microalgae culture, with free floating cells attaching to the loofah strand (Fig. 5.5B&C). Adhesion with *N. oculata* was negligible after 2 weeks (Fig. 5.5D) which could be a result of the hardened tough cell wall and their smaller size compared to *C. vulgaris* and *D. salina*; as such, no CO_2 reactivity was attempted on the loofah *N. oculata* matrix. *C. vulgaris* had a significantly higher CO_2 reactivity (Krustal-Wallis, K= 8.36, DF= 1, P= 0.004) compared with *D. salina* (Fig. 5.15), similarly, the CO_2 consumption of freely attached *C. vulgaris* was higher than those immobilised using alginate where there was no mass transfer limitation. It was difficult to directly compare between the two procedures as there was no control of the number of cells that were freely attached on the loofah. A setback of this procedure is the limited amount of medium retained on the loofah compared to the alginate immobilised method. The only way to sustain the cells for longer is by constant medium addition which subsequently washed away the attached cells.

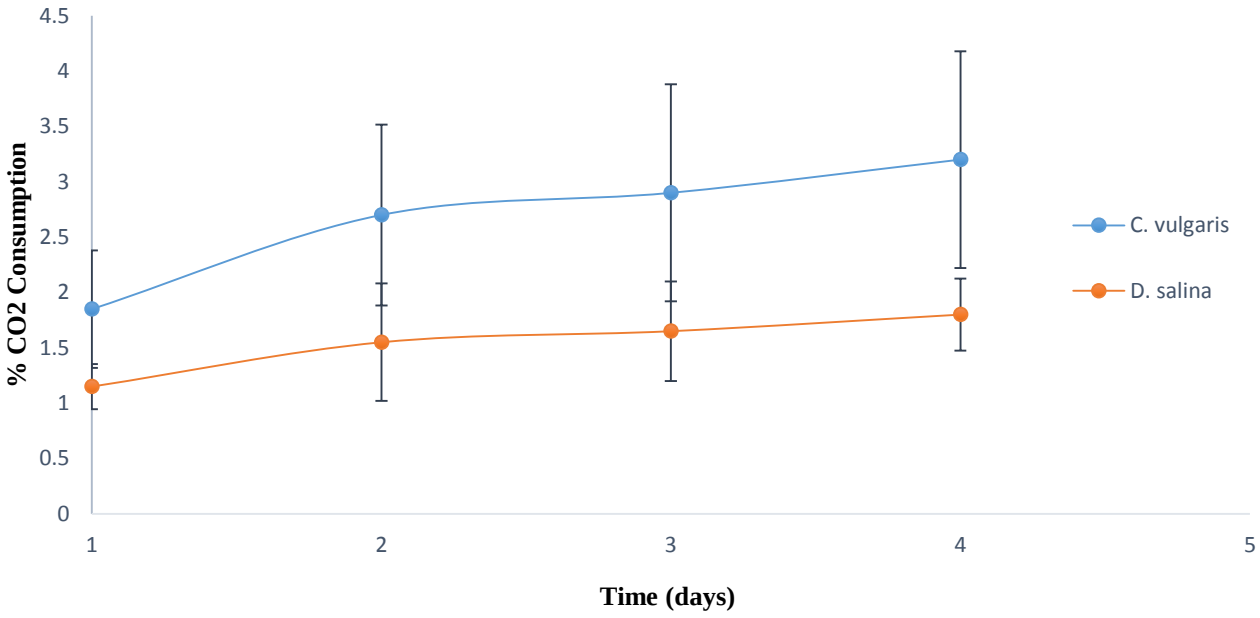


Figure 5.15: Carbon dioxide reactivity (4 days) of *C. vulgaris* and *D. salina* immobilised by direct dipping inside the respective culture medium for 2 weeks, Mean ±SD.

5.3.7 Substrates and binders: Substrate

Three different materials were selected for this protocol; Whatman #1 paper, woven cotton textile, and loofah sponge. Whatman #1 paper has a flat structure and is non-woven, making it a suitable substrate for the drawdown coating technique. Paper has previously been used as a support material to immobilise microbes (Gosse *et al.*, 2010; Gosse *et al.*, 2012). The cotton textile is made from pure untreated cotton which has a limited 2D structure and higher porosity

than the filter paper. The cotton does not retain liquid on its surface so the use of the drawdown coating technique was impractical, therefore direct pipetting was employed. Prasad *et al.* (2016), used cotton to immobilise *C. vulgaris* and *C. reinhardtii*, which maintained their photosynthetic ability even after 18 months of immobilisation.

The loofah sponge is from the mature fruit of the *Luffa aegyptiaca* plant and comprises fiber (55%) and carbohydrate (40%) (Saeed and Iqbal, 2013). Uncoated loofah has an open-pore structure with a broad distribution of cellulose fibres that offer high porosity for gas and nutrient distribution (Fig 5.16). This surface topography combined with the microporous structure of the loofah including abundant O–H, C–H, and C=O groups (Qi *et al.*, 2016), is responsible for its high moisture retention property. Loofah is a 3D structured material with higher porosity and surface area compared to either the paper and or cotton, as such, only the equilibrium coating technique is suitable for binder/cell deposition. Loofah had previously been used to immobilise both plant and animal cells (Akhtar *et al.*, 2003; Akhtar *et al.*, 2004; Akhtar *et al.*, 2008; Saeed and Iqbal, 2013).

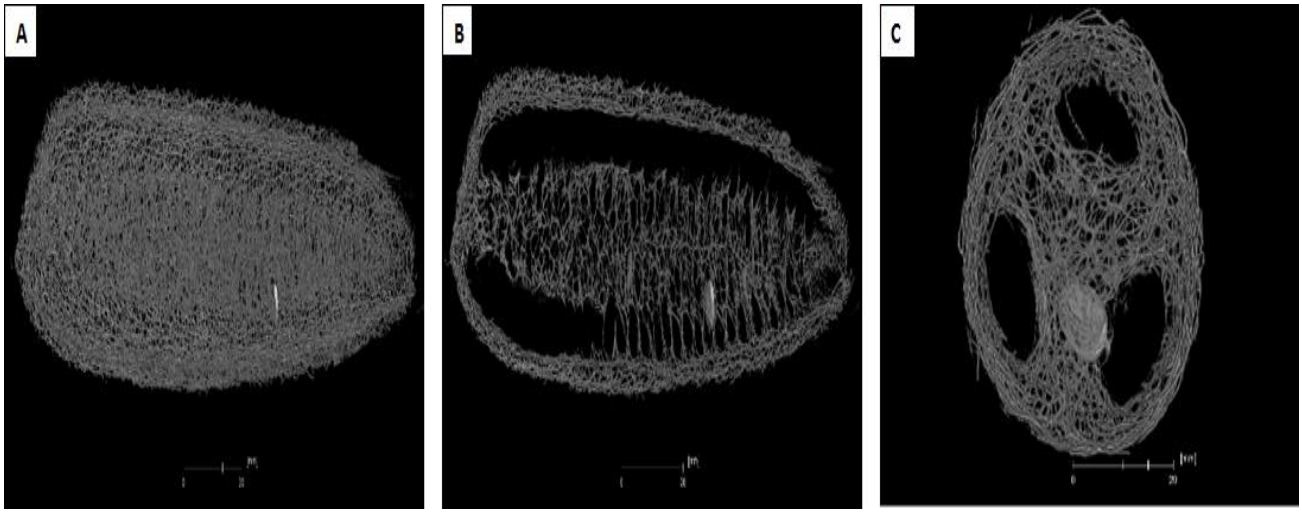


Figure 5.16: CT scan of a loofah sponge: **A** lateral, **B** transverse, and **C** cross sectional views, demonstrating the porosity of the sponge (courtesy of the Shared Material Instrumental Facility, Duke University NC).

5.3.8 Binders

Two groups of binders were used in this experiment; organic **(**alginate, carrageenan, CMC/SPI mixture, pectin) and inorganic latex binders. Where available, some of the physical and chemical components of these binders are shown in Table 5.2. After the initial percent solid screening a total of 33 latex and 4 organic binders were screened, however, only 22 latex and 2 organic binders were used for the protocol (Table 5.2), binders were levelled and coded for easy

identification. Note that the organic binders were all in a solid state so there was no requirement for percent solid determination.

Table 5. 2: Summary of latex binders screened for adhesion, showing their main composition and percent solid content.

Binder Name	Binder Code	Main component	% Solid	Additional component	Additional component %
Primal P-376 LO Emulsion	A	Acrylic polymer	49-51	Aqua ammonia	0.1 Max
Rhoplex SF-3122	B	Acrylic polymer	60-61	Aqua ammonia	0.2 Max
Emulsion E-3131	H	Acrylic polymer	45-48	Aqua ammonia	0.2 Max
ROPAQUE HP-1055 Emulsion	L	Styrene/acrylic copolymer	26-27	Aqua ammonia	0.1 Max
Ropaque	AB	Styrene/acrylic copolymer	29-31	Residual monomers	<500 PPM
Rhoplex SG -10M	AD	Butyl Acrylate/ Methyl methacrylate	51.30		
Acrysol RM-8W	O	Polyurethane resin	17-18	Enzymatically modified starch	3-5
Acrysol RM-12W	Q	Polyurethane resin	14-16	Enzymatically modified starch	3-5
Acrysol SCT-275	S	Polyurethane resin	17-18	Diethylene glycol monobutyl ether	20-21
PD-0413	U		80.37		
PN-3415-W	V		46.55		
Primal P-554 Emulsion	W	Acrylic polymer	46-48	Formaldehyde	<0.1
Genflo 8045	Y		57.08		
Rhoplex SG-10	AG	Polymer of (Butyl Acrylate/ Methyl methacrylate)	49-51	Aqua ammonia	<=0.2
Baycusan C-1003 polyester polyurethane dispersion	AH	Polyurethane resin	52.6	N/A	N/A
HB 3691 M	Z	Polyester polyurethane	48.42	N/A	N/A
Rhoplex SF-3122	AA		65.19		
Baycusan C-1000	AL	Polyester polyurethane	40	N/A	N/A
Baycusan C-1004	AI	Polyester polyurethane	40	N/A	N/A
Baymedix CH-120 polyurethane resin dispersion	AJ	Polyurethane polymer	34.7	N/A	N/A
Nuplex Setaqua 6776	AK	Acrylic polymer	42.77	N/A	N/A
Rhoplex SF-012	AO	Acrylic polymer	43-44	Aqua ammonia	<=0.1

5.3.9 Substrate pH determination

Substrate pH is important as algae must maintain a neutral or near neutral internal pH; the greater the pH difference the greater the metabolic cost to the cell in order to achieve the target internal pH. The loofa pH (pH 3.5) was more acidic than both paper (pH 5.9) and cotton (pH

5.5) due to the presence of strongly (carboxylic) and moderately acidic (lactonic, enolic and carboxylic) groups on the loofah (Saeed and Iqbal, 2013). Growth media were adjusted to pH 7, however the pH was significantly reduced (ANOVA, F= 155.04, DF= 6, P< 0.05) by the acidic nature of the loofah to 4.53 and 6.28 for BG-11 and TAP respectively. There were no significant decreases in media pH with cotton (ANOVA, F= 332.33, DF= 6, P< 0.05) or paper (ANOVA, F= 152.07, DF= 6, P< 0.05) as indicated in Fig. 5.17. There was no attempt to modify the substrate pH before the adhesion screening process.

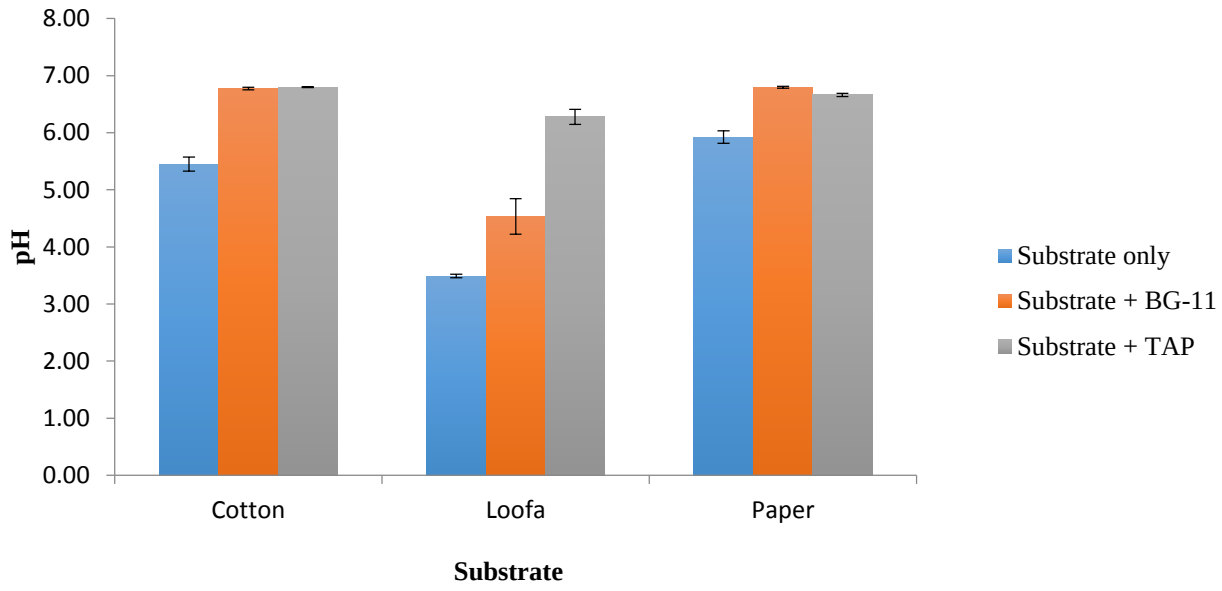


Figure 5.17: pH values of cotton, loofah and Whatman paper in BG-11 and TAP media, (Courtesy: Pichaya In-na)

5.3.10 Latex binder percentage solid determination

The binder screening criteria required the percent solids content of binders to be greater than 10% after dilution from pH adjustment as it is the solids content of the aqueous binder solution that fixes the algae cells to the substrate upon drying. Five binders failed to meet the 10% criteria and were eliminated (Rhopaque OP-96, ACM 7179, JP 1192, Rovace 9900 vinyl acetate and Baycusan C-1001), leaving 28 available for pH adjustment and the contact toxicity assay. After pH adjustment, 6 more binders fall below the 10 % solid threshold, leaving 22 binders.

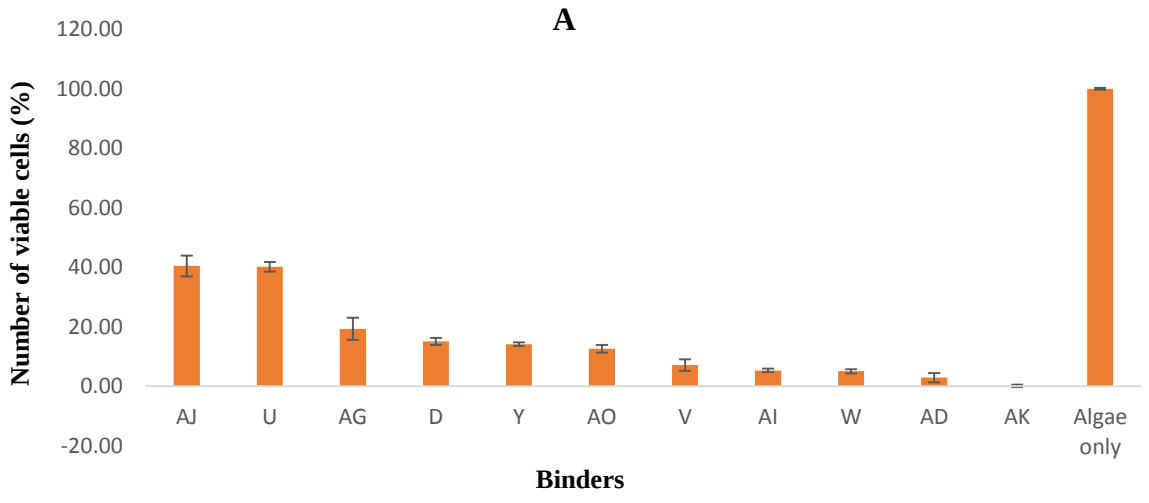
5.3.11 Contact toxicity test measurement

Chlorophyceae (green algae) produce their food via photosynthesis, utilising chlorophyll *a* (which has a deep green colour) as the main light capturing pigment. Colour fading is a useful visual indicator of poor cellular health and as such was used to parameterise the 7-day binder toxicity assay prior to direct cell counts. Twenty two latex binders and four organic binders

were screened using five microalgae species with different physiological characteristics; however, only three species were tested to the end (*C. vulgaris*, *D. salina* and *N. oculata*). *C. vulgaris* is a freshwater chlorophyte with a moderately hard cell wall; it is easy to cultivate and can thrive in diverse environmental conditions. *D. salina* and *N. oculata* are marine; *D. salina* is larger in size and has no cell wall, *N. oculata* is very small and has a very tough cell wall. *C. reinhardtii* was not screened with the organic binders due to its poor initial CO_2 reactivity as observed with the latex binders (data not presented).

5.3.12 Freshwater microalgae species contact toxicity result

Half of the 22 binders caused pigment bleaching in *C. reinhardtii*, and no viable cells were observed. Percentage cell viability for the remaining 11 non-toxic binders which is less than half of the control (ANOVA, F= 89.04, DF= 11, P< 0.05) is reported in Fig. 5.18A. Thirteen binders were non-toxic to *C. vulgaris* (Fig. 5.18B), however their viable cell numbers were significantly lower than the control (ANOVA, F= 50.87, DF= 13, P< 0.05) with the exception of Binder **U** (P> 0.05) which has similar numbers. Apart from binders **W** and **AJ**, all binders that supported growth in *C. reinhardtii* were also safe for *C. vulgaris*. *C. vulgaris* has a tougher cell wall than *C. reinhardtii* and could be more resistant to the chemical environment of the latex binders. The limited available information on the binders from the manufacturers made it difficult to explore the nature of the toxicity any further. Experiments on binder particle size, surface charge and zeta potential were conducted (data not presented) to see if there were any trends in the toxicity profile, but the data were inconclusive. All binders that supported cell growth above 10 and 20% for *C. reinhardtii* and *C. vulgaris* respectively, were selected for adhesion testing.



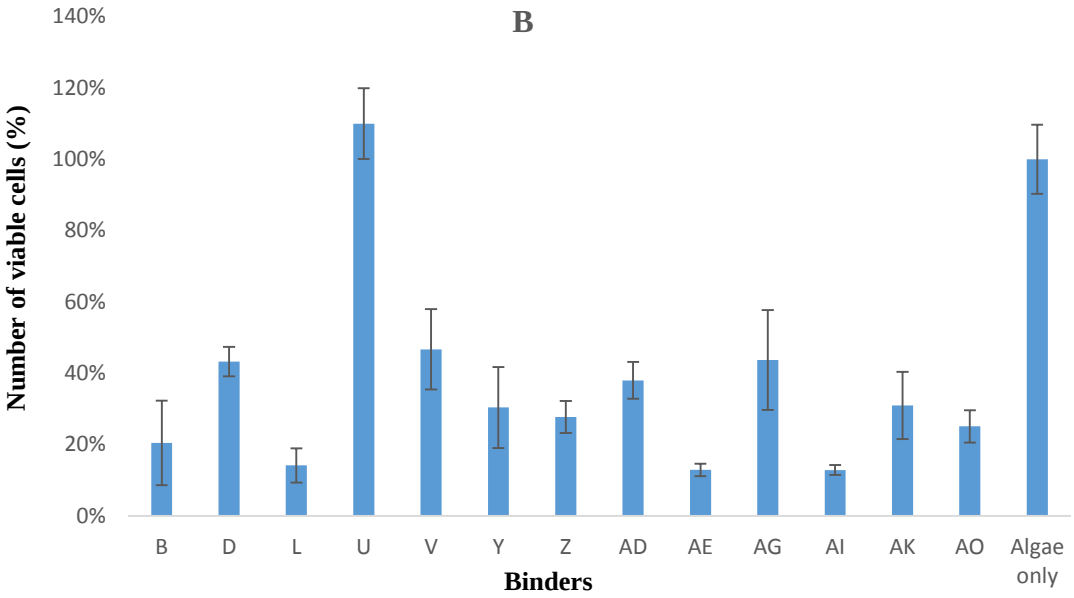


Figure 5.18: Percentage of viable freshwater microalgae cells remaining in culture after 7 days contact toxicity test with latex binders: **A**. *Chlamydomonas reinhardtii* and **B**. *Chlorella vulgaris*, Mean ±SD.

Only *C. vulgaris* was tested for toxicity with the organic binders (Fig. 5.19). There was no marked difference (ANOVA, F= 26.48, DF= 5, P> 0.05) in cell viability of alginate, carrageenan and pectin 2 compared with the control. Only pectin 1 had a significantly lower cell density (P< 0.05) in respect to the control. In general, all binders bar the CMC/SPI mixture supported *C. vulgaris* cell growth. The non-toxic nature of these binders is unsurprising as they are commonly used for microalgae encapsulation (Moreno-Garrido *et al.*, 2005; Moreno-Garrido, 2008; de-Bashan and Bashan, 2010). Additional chemical components within the binding gel could be responsible for the higher cell growth.

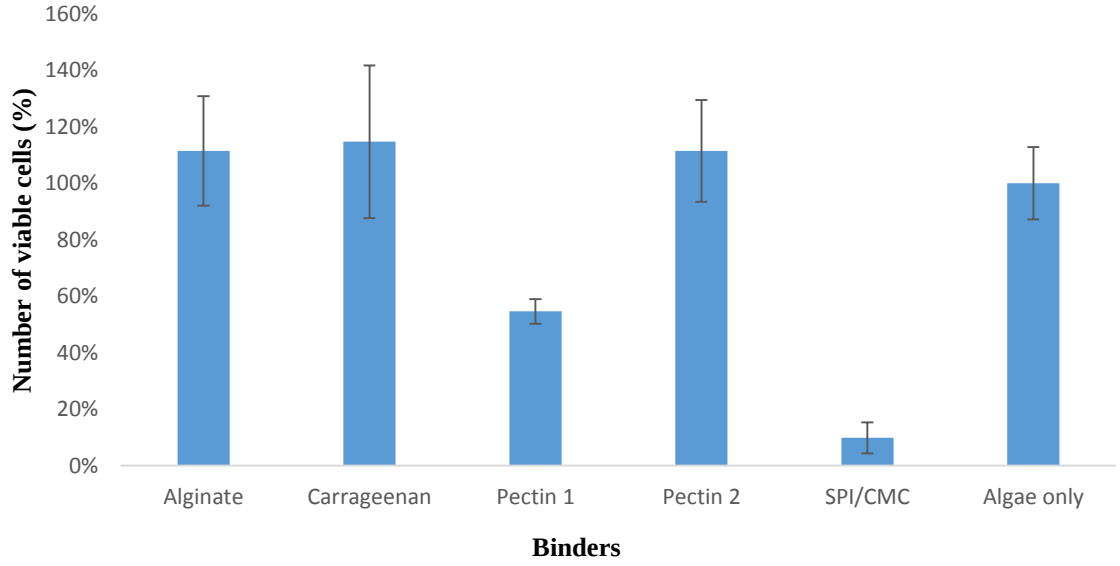
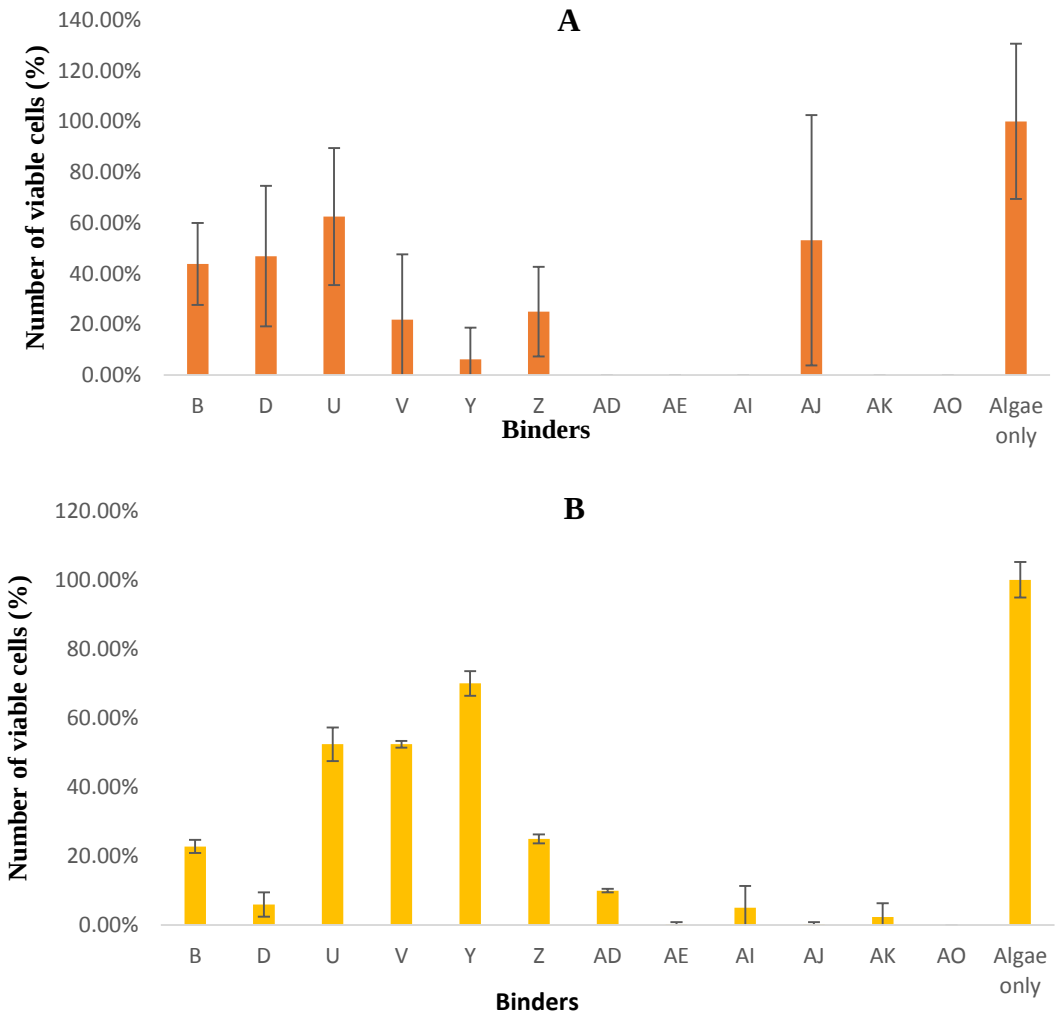


Figure 5.19: Percentage of viable *C. vulgaris* cells remaining in culture after 7 days contact toxicity test with organic binders, Mean ±SD.

5.3.13 Marine microalgae species contact toxicity result

Seven latexes supported growth of *D. salina* (Fig. 5.20A) with noticeable difference (ANOVA, F= 4.26, DF= 7, P= 0.003) compared to the control with the exception of Binder AJ (P= 0.3). Eleven binders also maintained the cells of *N. oculata* (Fig. 5.20B) and when compared with the control the cell numbers significantly dropped (ANOVA, F= 288.49, DF= 9, P< 0.05) to not more than 60%. Binders V and Y did have similar cell viability numbers (P> 0.05). Nine binders supported the growth of *T. suecica* (Fig. 5.20C) with noticeable difference (ANOVA, F= 5.69, DF= 9, P< 0.05) in viable cell numbers compared to the control, with the exception of Binders U, V and AJ (P> 0.05). All 7 binders that supported *D. salina* also supported *N. oculata* and *T. suecica*. Due to our previous experience with alginate immobilisation using marine species, no further screening tests were conducted using the organic binders.



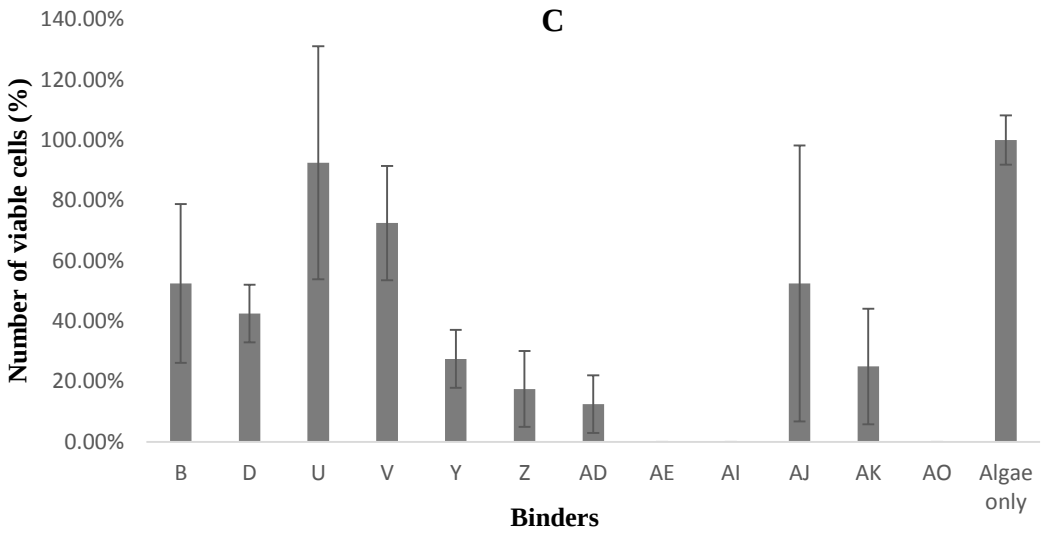


Figure 5.20: Percentage of viable marine microalgae cells remaining in culture after 7 days contact toxicity test with latex binders: **A**. *D. salina,* **B**. *N. oculata* and **C**. *T. suecica*, Mean ±SD.

5.3.14 Binder formulation, coating and deposition screening techniques

Binders that passed the toxicity screening were coated onto the paper, cotton and loofah. The coating techniques employed differed by substrate (paper = drawdown, cotton = direct pipetting, loofah = equilibrium). A 5% w/w binder solids content was used for all substrates while 1.25% w/w solids content was used for the loofah (due to limited amount of cells and binders available) with *C. reinhardtii*. The drawdown technique spread the cells with uniform thickness and dried faster compared to other coating techniques. Fig. 5.21 shows the SEM images of: A. loofah, B. loofah coated with binder **AJ** (good in adhesion), and C. loofah coated with binder **V** (poor in adhesion). Upon drying, some of the binders became brittle and broke off from the substrates (Fig 5.21C) before the adhesion test was conducted. This was taken into account when comparing the adhesion test for the binders.

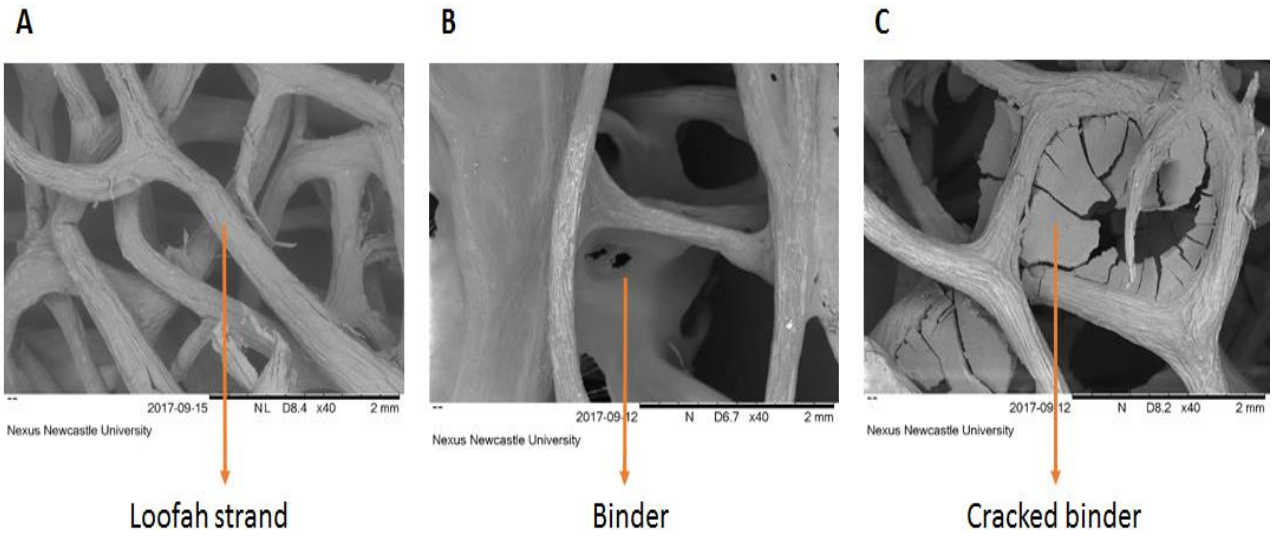


Figure 5.21: SEM of **A**. loofah sponge matrix immobilised with **B**. good latex binder and **C**. bad/cracked binder.

Fig. 5.22 shows contrasting binders used to immobilise *C. reinhardtii* on cotton. Before immobilisation, the cotton fibres can be clearly seen (Fig. 5.22A) with the gaps between the woven yarns. Fig. 5.22B shows that a good binder (i.e. **AO**) smoothly filled in the gaps, while a poor binder (i.e. **D**) formed many rough lumps and cracks (Fig. 5.22C). These images support the adhesion results whereby binders that were brittle and cracked were unable to properly entrap the cells.

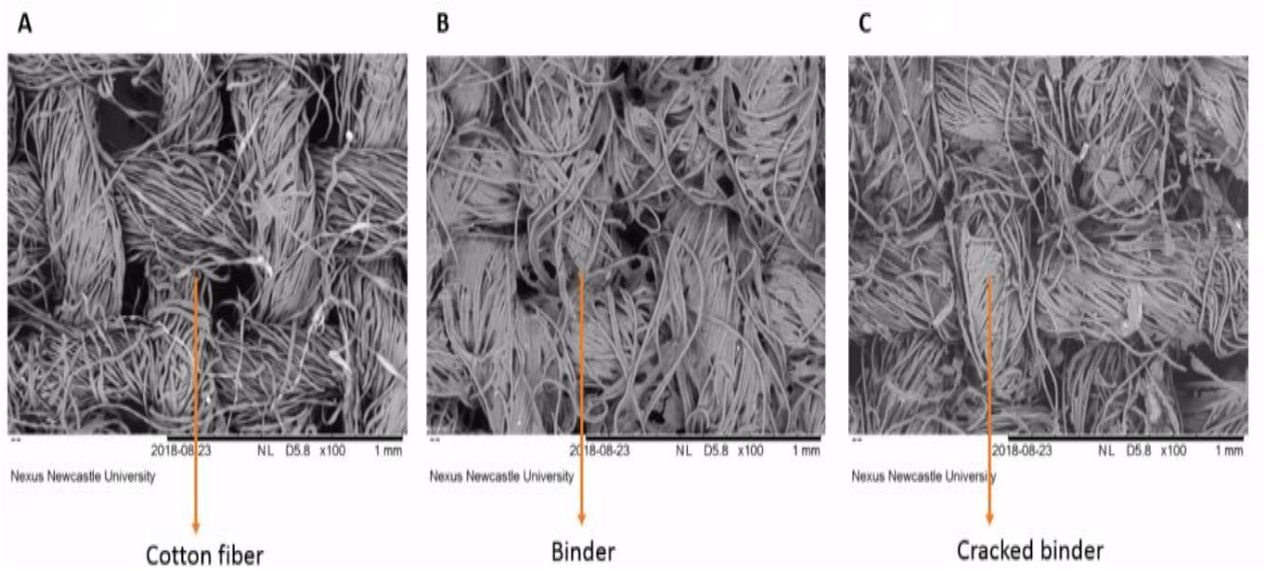


Figure 5.22: SEM of **A**. cotton sheet immobilised with **B**. good latex binder (i.e. AO) and **C**. bad/cracked binder, (*Courtesy: Pichaya In-na*).

5.3.15 Binder adhesion test on substrates in the presence of microalgae cells

Upon drying, the biocomposites were subjected to adhesion tests in their respective media (TAP, BG-11 and F/2). Cell counts and mass balance were used to determine the number of cells removed after 1, 24, 48 and 72 h. Fig. 5.23 shows *C. reinhardtii* and *C. vulgaris* immobilised on loofah.

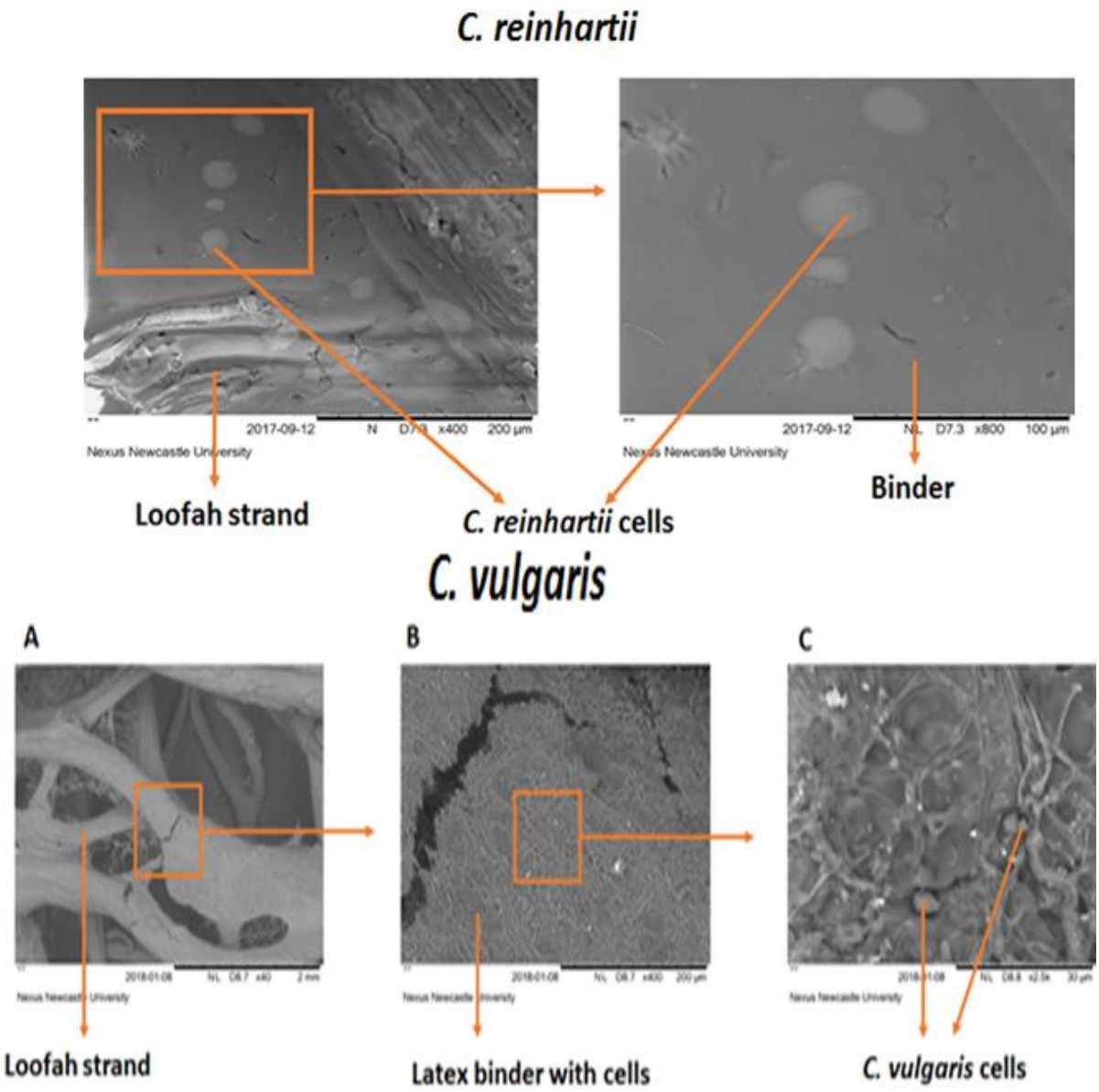
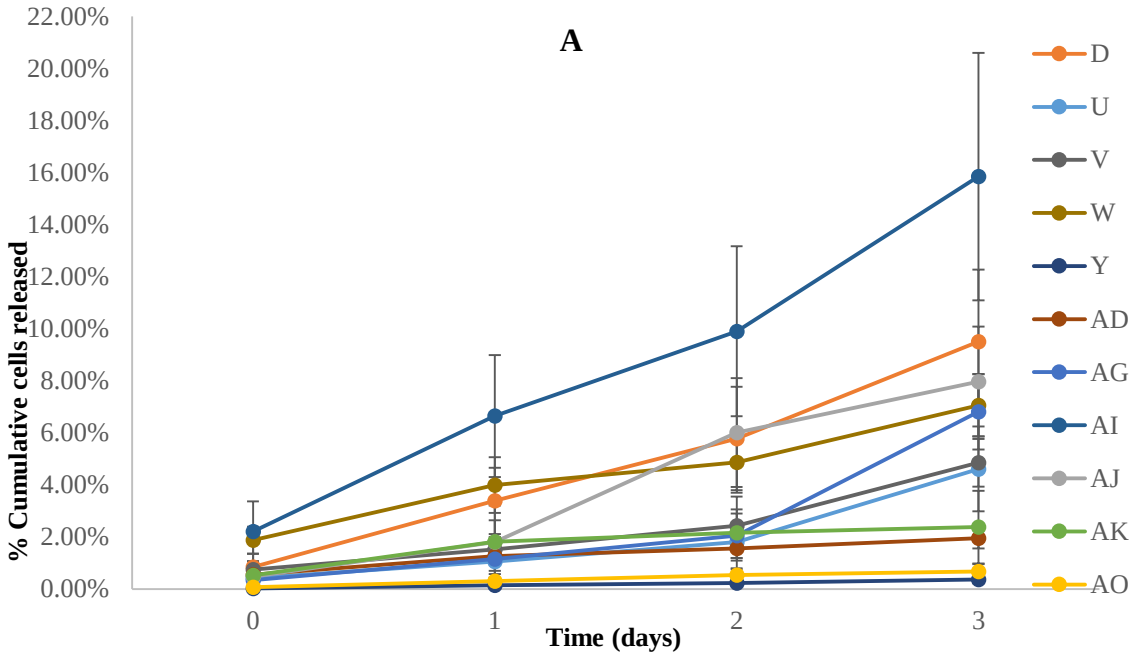


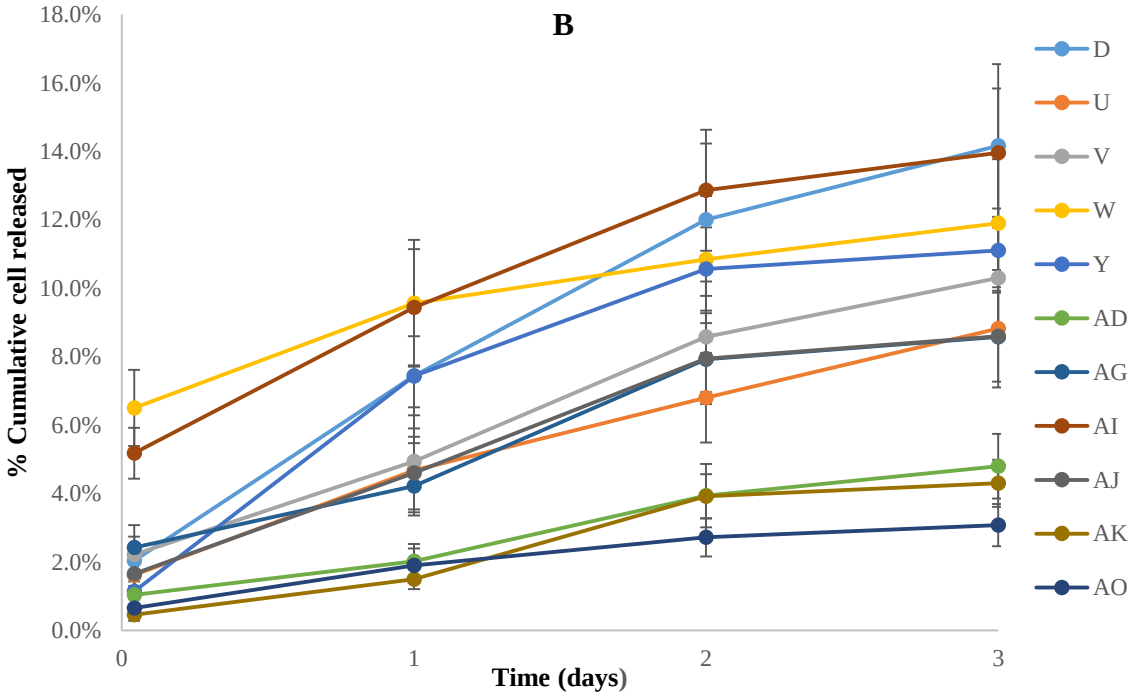
Figure 5.23: Top: Immobilised *C. reinhardtii* with latex binder on loofah. **Bottom**: Immobilised *C. vulgaris* with latex binder on loofah. Loofah strand (**A**), binder with *C. vulgaris* (**B**), binder-cell composite showing cells of *C. vulgaris* (C).

5.3.16 Adhesion test on freshwater microalgae with latex binders

The cumulative number of cells released into the media when compared against each other over time shows a significant difference for *C. reinhardtii* (ANOVA, paper, F= 4.29, DF= 10, P= 0.001, cotton, F= 2.95, DF= 10, P= 0.009 and loofah, F= 7.45, DF= 10, P< 0.05) and *C. vulgaris* (Krustal-Wallis, paper K= 21.96, DF= 10, P= 0.015, cotton, K= 36.37, DF= 10, P< 0.05, and loofah K= 25.37, DF= 10, P= 0.005,) as shown in Figures 5.24 and 5.25 respectively. A release threshold of 5% was set, above which binders were deemed ineffective for cell retention. Six paper, three cotton and six loofah *C. reinhardtii* combinations met the threshold compared with seven, eight and nine for *C. vulgaris*. The best performing binders were then selected for subsequent CO_2 reactivity tests. Binders **AO** and **AD** gave good adhesion for all substrates

using *C. reinhardtii*, while binder **AK** was also good for cotton and loofah. Binders **AE**, **AJ** and **AK** were better in adhesion for both cotton and loofah with *C. vulgaris*, while binder **AK** performed well for all substrates. Loofah had the poorest cell retention because it had a high cell loading due to its 3D nature. In all cases, there were significant differences in terms of numbers of cells released between the selected binders and others ($P< 0.05$) which strongly informed our decision for screening to the next stage of CO_2 reactivity test.





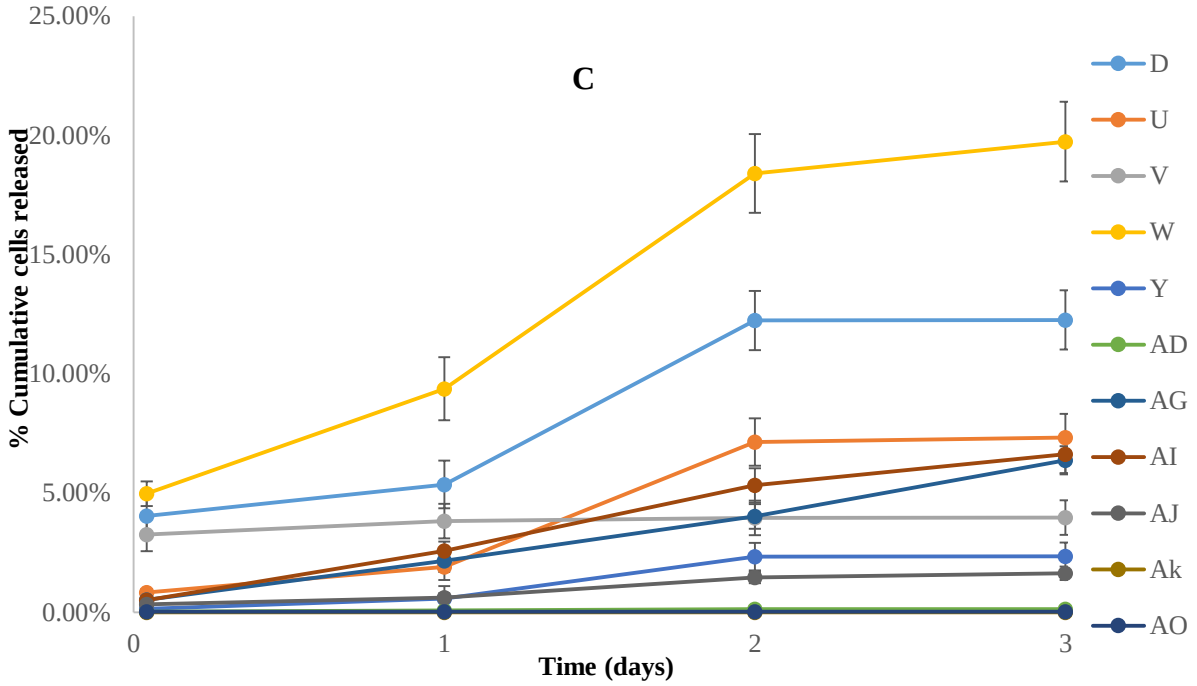
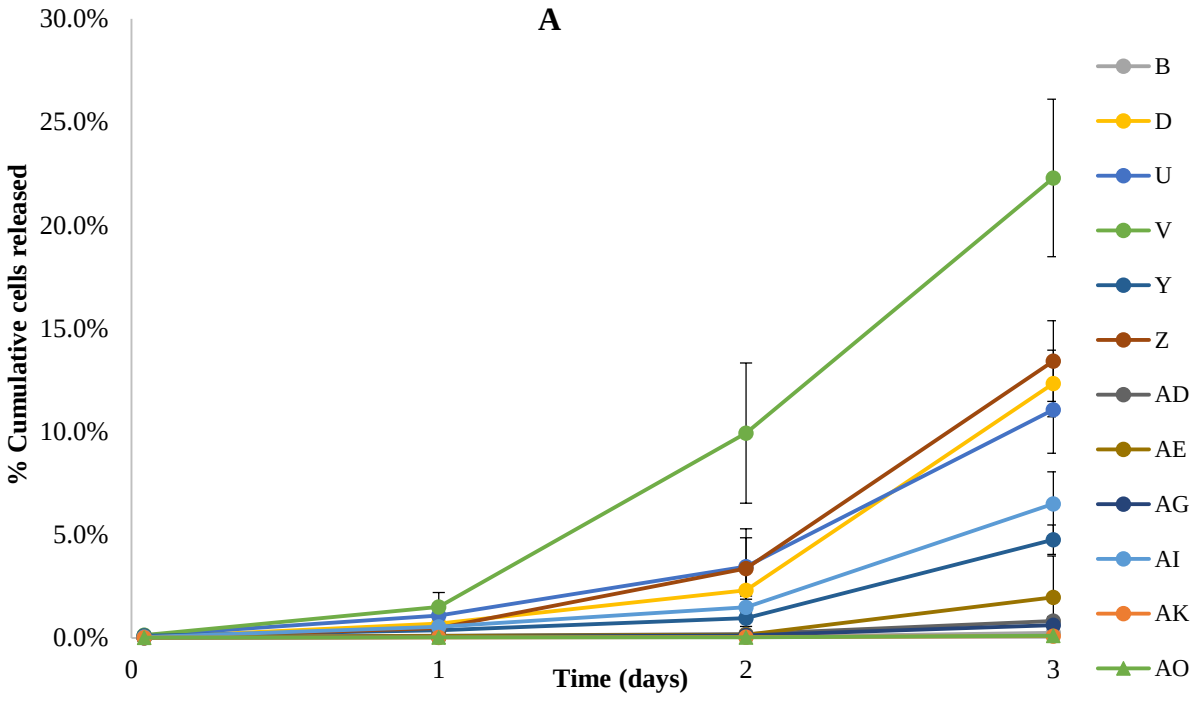


Figure 5.24: Cells of C. reinhardtii released over time from coating formulations for A, Whatman #1 paper, B, cotton and C, loofah with latex binders, Mean ±SD, (Courtesy: Adam Wallace and Pichaya In-na).



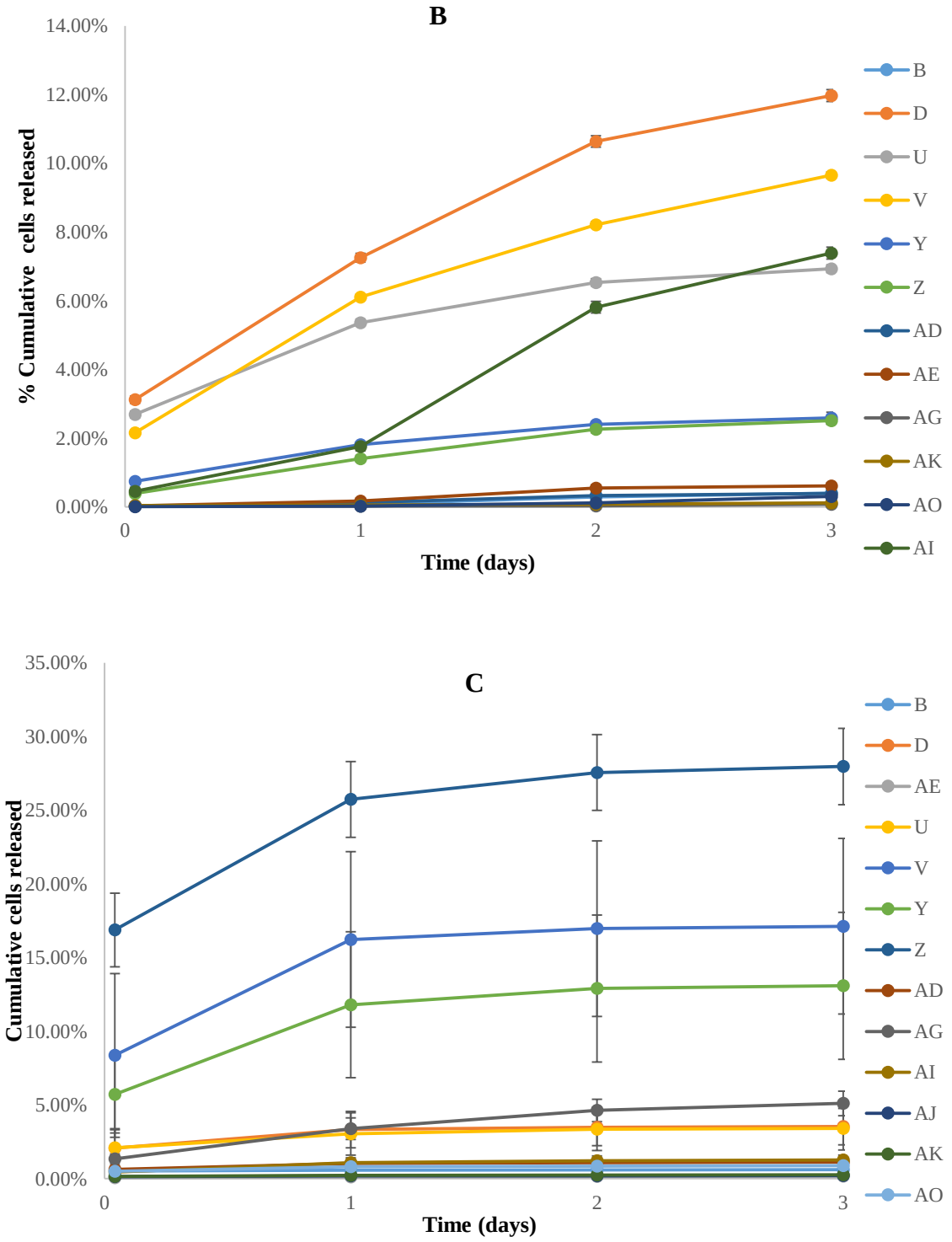


Figure 5.25: Cells of *C. vulgaris* released over time from coating formulations for **A**, Whatman #1 paper, **B**, cotton and **C**, loofah with latex binders, Mean ±SD, (Courtesy: Adam Wallace and Pichaya In-na).

In terms of the coating method, the drawdown technique ensured a uniform coating of the cell/binder mixture on the paper, giving a large surface area for good single or double layer adhesion. The structural complexity of the loofah meant that the equilibrium coating method caused some of the cells to attach to each other without necessarily touching the substrate; hence the initial high cell release (Fig 5.25C). After shedding the excess coating layers, there was little

or no further cell shedding as the remaining cells were effectively attached to the loofah strand by the binder.

5.3.17 Adhesion test on freshwater microalgae with organic binders

Chlorella vulgaris was the only microalgae tested for adhesion using organic binders. With cotton, alginate and pectin 1 had the strongest adhesion (Fig. 5.26A) releasing less than 5% of total immobilised cells which significantly differed (P= 0.02) from k-carrageenan and pectin 2. All binders had strong adhesion with loofah except pectin 2 which contrasts from the rest (P= 0.02). k-carrageenan was effective on loofah only, with pectin 2 ineffective for both substrates. This may be due to differences in surface charge, mechanical strength and chemical structures of the organic binders, which should be further investigated. This test was not conducted for paper as Newcastle University did not have the drawdown coating equipment.

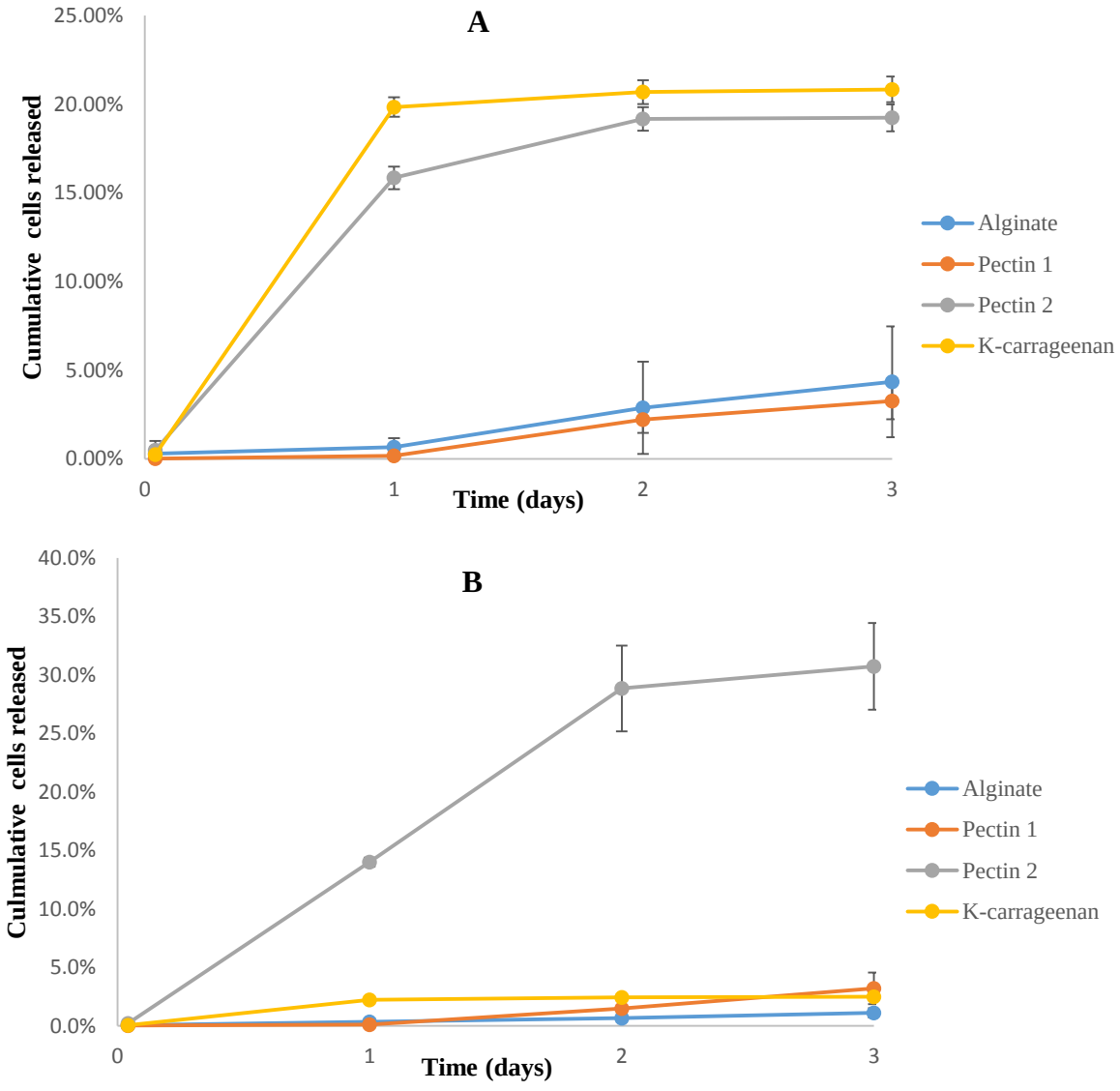
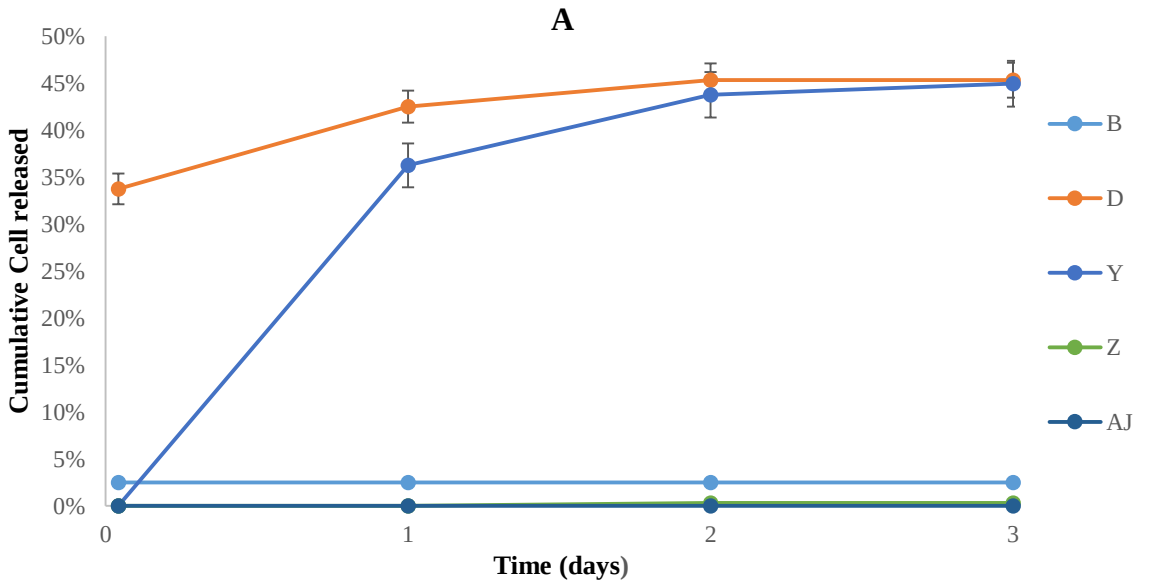
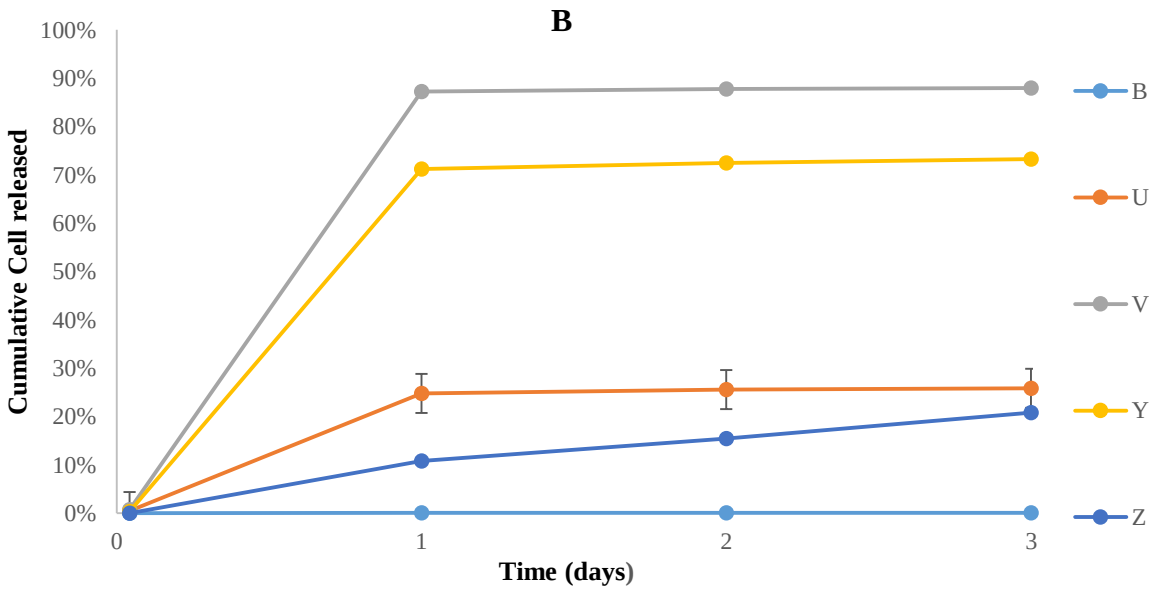


Figure 5.26: *Chlorella vulgaris* cells released over time for coating formulations on: **A**, cotton and **B**, loofah with organic binders, Mean ±SD.

5.3.18 Adhesion test on marine microalgae with latex binders

On loofah, binders **B**, **Z** and **AJ** performed best with *D. salina* with statistically significant differences (Krustal-Wallis, K= 8.26, DF= 4, P= 0.004) compared with the remaining binders **D** and **Y** (Fig. 5.27A). Binders **B, Z** and **U** were relatively good (P< 0.05) with *N. oculata* when compared with binders **V** and **Y** (Fig. 5.27B). Binders **AJ**, **AK** and **B** also show strong adhesion for *T. suecica* (Fig. 5.27C), when compared with binder **D** (P< 0.05). The adhesion strength of the marine species was weaker compared with the freshwater microalgae, this may be a seawater effect on the binders. Most of the cells were trapped within the binder between the spaces created by the loofah sponge matrix (Fig. 5.28). In the case of *D. salina* the space occupied by cells which came off the matrix are shown; a similar phenomenon was observed by Lyngberg *et al.* (1999b).





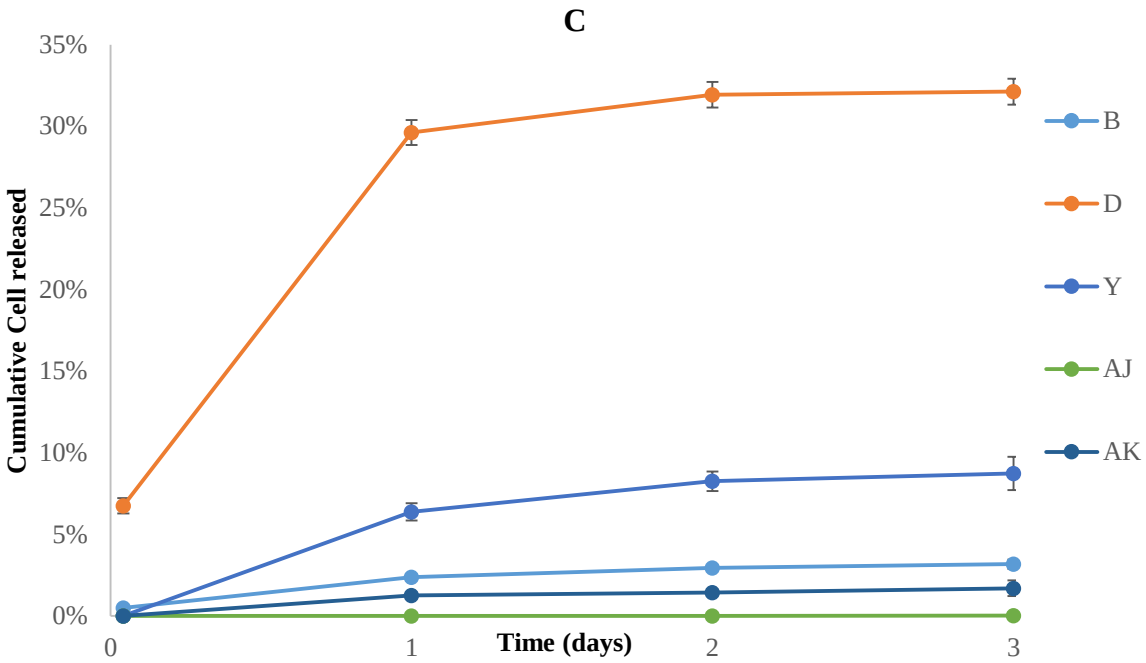


Figure 5.27: Cell release over time for: **A.** *D. salina*, **B.** *N. oculata*, and **C.** *T. suecica* coating formulations on loofah with latex binders, Mean ±SD.

It should however be noted that, all the immobilization with marine microalgae was done using artificial sea water prepared using 35 g/L NaCl and deionized water. Mixing natural sea water with latex binders produced a turbid or sometime gelled mixture (depending on the type of binder used) which makes it impossible for deposition onto a substrates. Limited knowledge of the latex binder composition (not available from the manufacturers) also make it difficult to further ascertain the possible interference with the sea water. A similar phenomenon was reported by Moreno-Garrido *et al.* (2005) when they tried mixing alginate with marine microalgae. This is a result of the substitution of divalent cations in the beads by Na cations present in the seawater and leads to structural destruction.

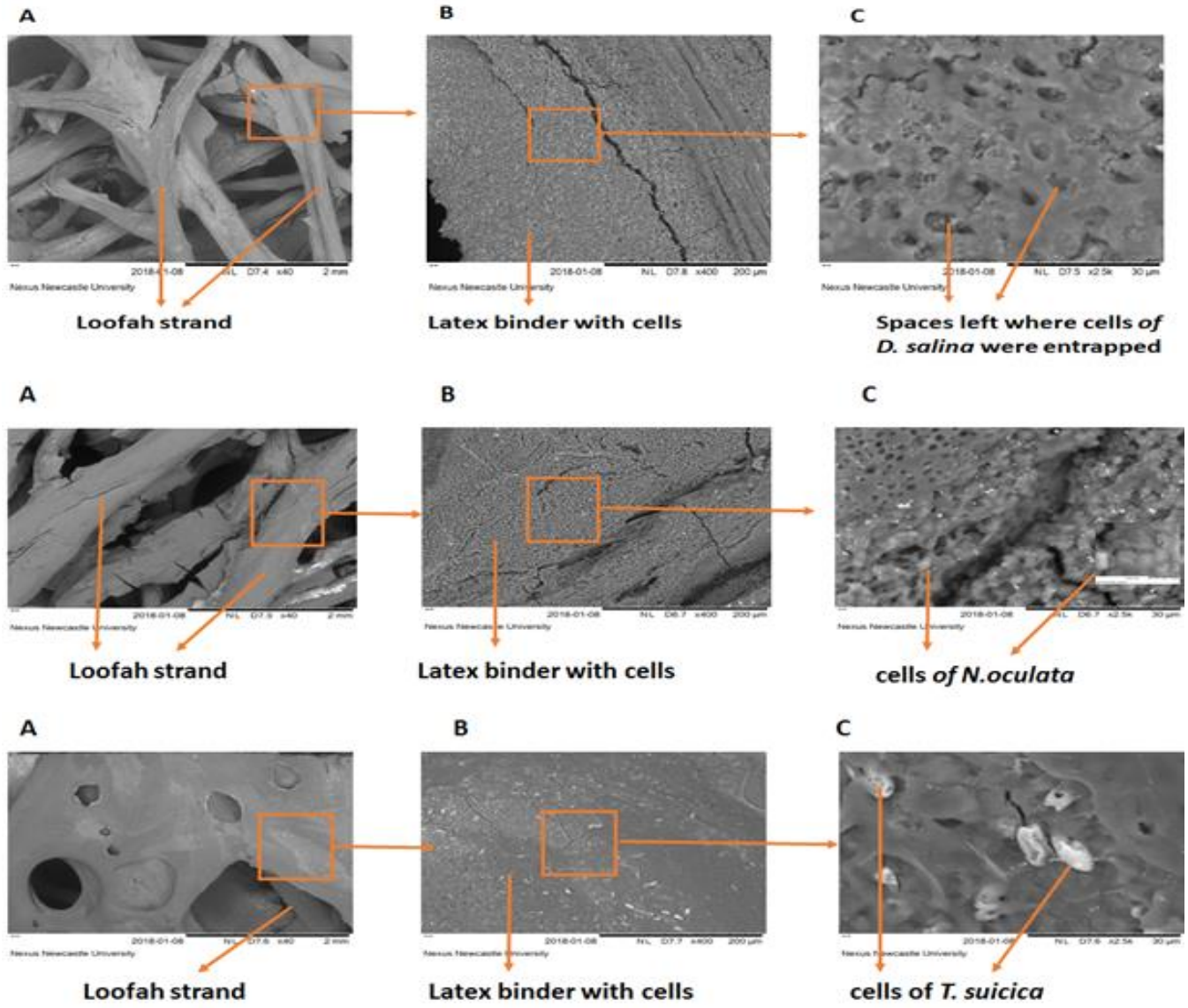


Figure 5.28: Loofah covered with cell binder mixture **A**, binder with the microalgae cells **B**, High resolution SEM showing microalgae cells immobilised within the binder **C**.

5.3.19 Film formation and desiccation test

A timed desiccation test was conducted on paper coated with *C. reinhardtii* using the best binders (**AO** and **AD**; Fig. 5.29). A similar method was employed by Flickinger *et al.* (2007) to determine microbe viability when immobilised on polyester. The chlorophyll *a* colour increased as the drying time increased. Most of the cells were washed off at t=0 due to insufficient time for the binder to dry effectively and entrap the cells before being submerged. The longer it takes to dry the better the adhesion (Fig 5.29). Fig. 5.30 shows the number of cell released from the coating, it is again visible from the bar chart that t = 0 min released more cells compared with t = 60 min. The graph also indicates that after 60 min of drying time the number of cells released into the medium was negligible. It is therefore concluded that, 1 h drying time is enough for effective adhesion using this technique. A CO_2 reactivity measurement was set

up to test the effect of the drying time on the cell viability, however after 24 h there was no CO_2 consumption from any of the samples, this needs to be further investigated.

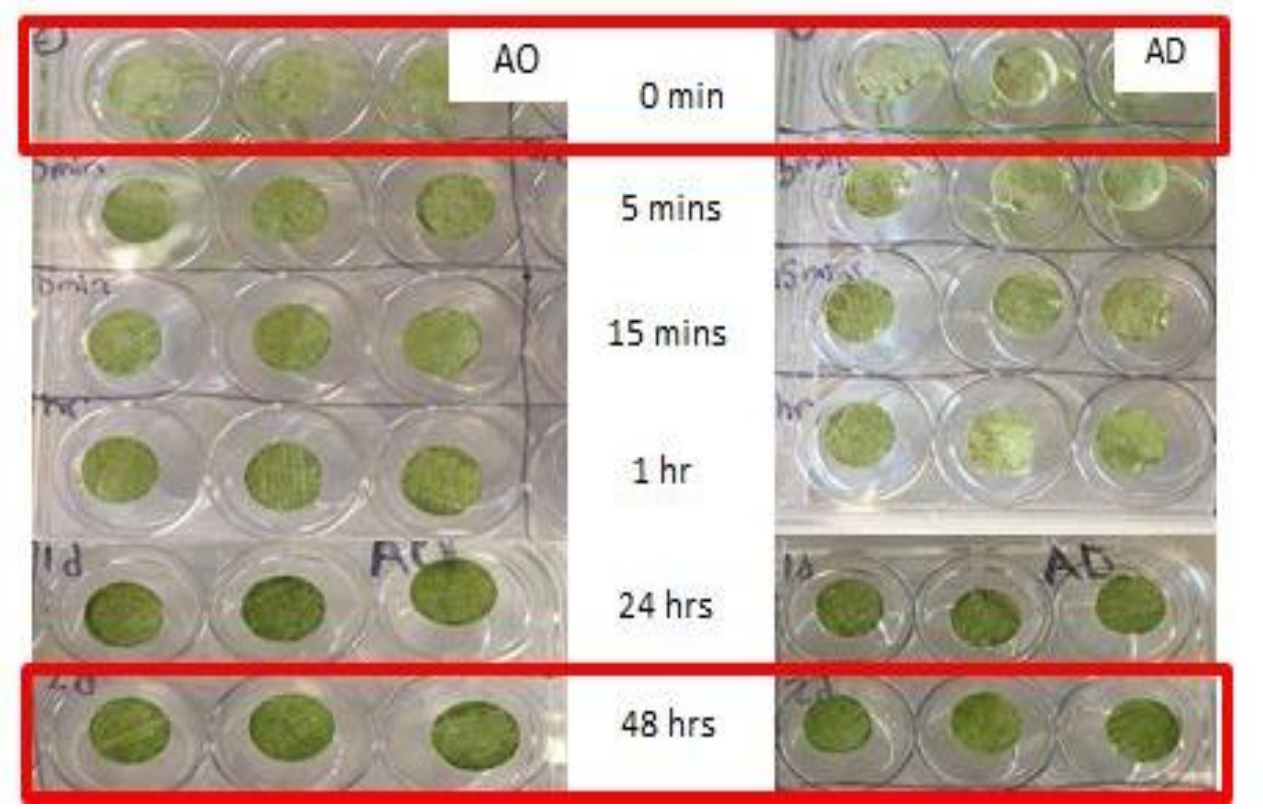


Figure 5.29: The effect of drying time on adhesion strength for *C. reinhardtii* in binders AO and AD on Whatmann paper after drying for various periods and subsequent 24 h adhesion test.

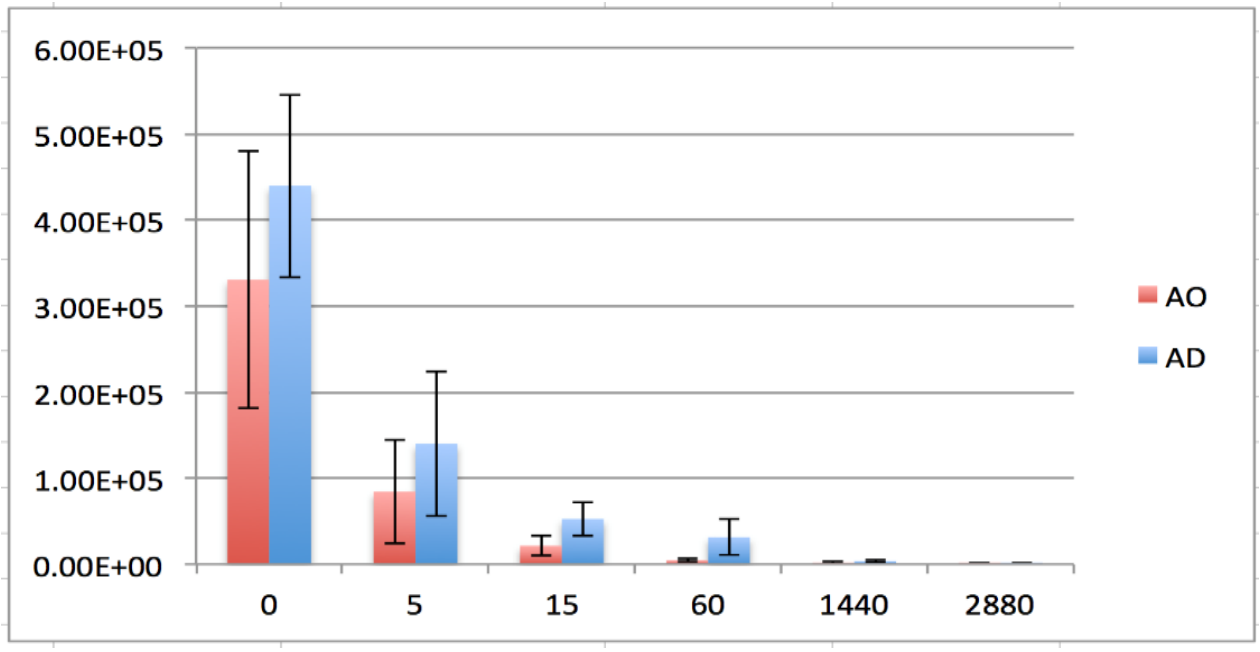


Figure 5.30: No. of cells released vs film formation time in minutes for binders AO and AD on Whatman #1 paper with C. reinhardtii, Mean ±SD, (Courtesy: Adam Wallace).

5.3.20 Microalgae toxicity and adhesion decision matrix

A decision matrix based on toxicity and adhesion data was used to identify the most suitable binders for subsequent CO_2 fixation trials (Fig. 5.31). The toxicity score was weighted slightly higher than the adhesion score because it is more important to keep the cells alive rather than maintaining them on the substrates. Most binders were on the extreme end of either toxicity or adhesion scoring. The decision matrix gives a clear picture of the performance of each binder in regard to toxicity and adhesion making it easy for selection.

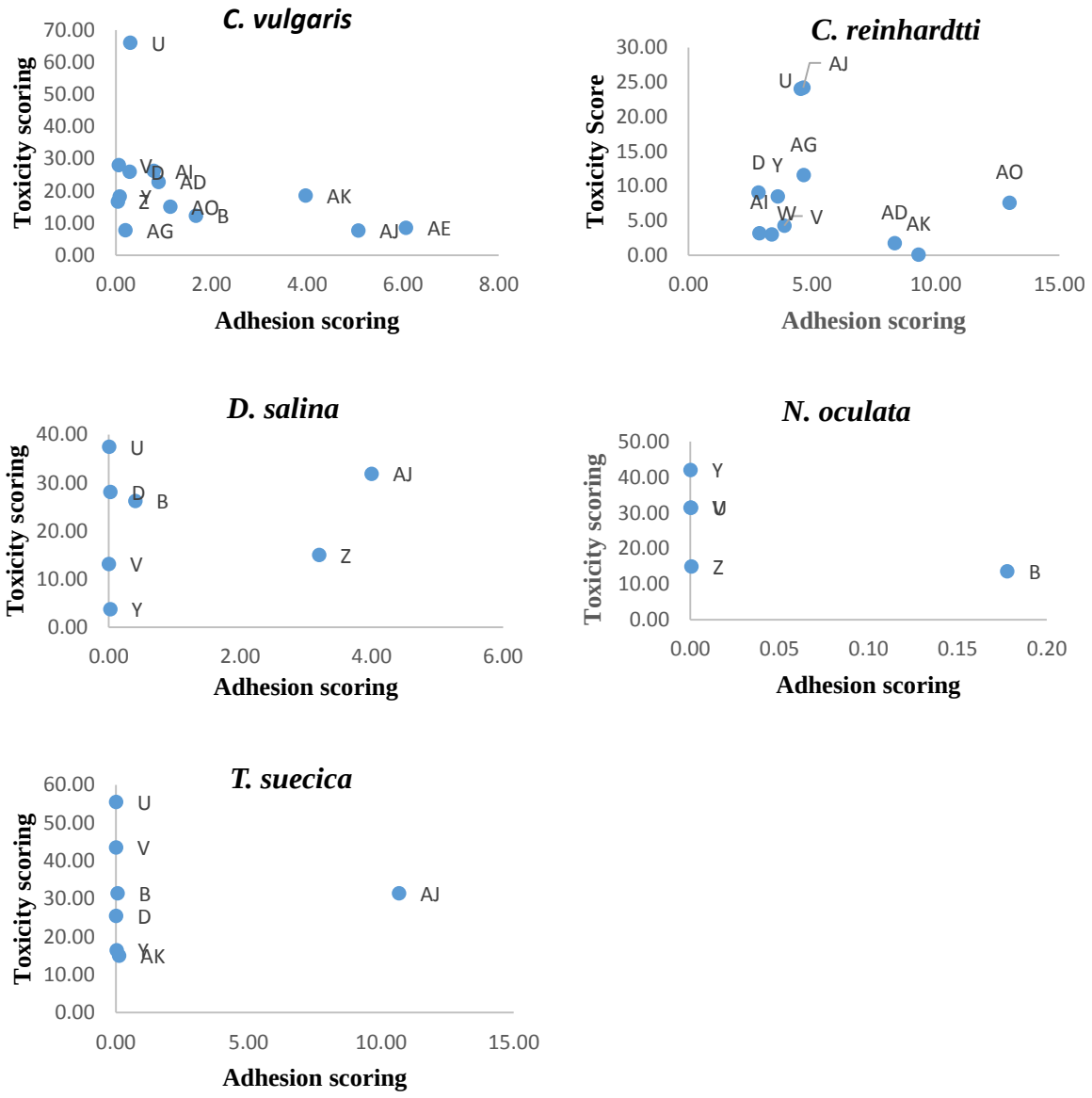


Figure 5.31: Adhesion and toxicity matrices of microalgae cells immobilised with latex binders, which show at a glance the best combination necessary for biocomposite fabrication. The best combination are those binders at the right top corner which are less toxic binders with good adhesive property.

Figure. 5.32 summarises the entire screening protocol used to produce a biocomposite that can withstand desiccation and remain viable, starting with visual observation and cell counts to

determine toxicity, followed by substrate screening based on porosity. Compatible solutes and cryoprotectants can also be used to enhance the final emulsion property, though this was not used in this screening protocol. Coating is conducted under defined environmental parameters (temperature and relative humidity) to preserve the microbes. Coating and adhesion strength are a function of substrate and microbe physical and chemical properties. The best coating formulation is determined by the number of adhered microbes and their metabolic activities after desiccation and upon rehydration.

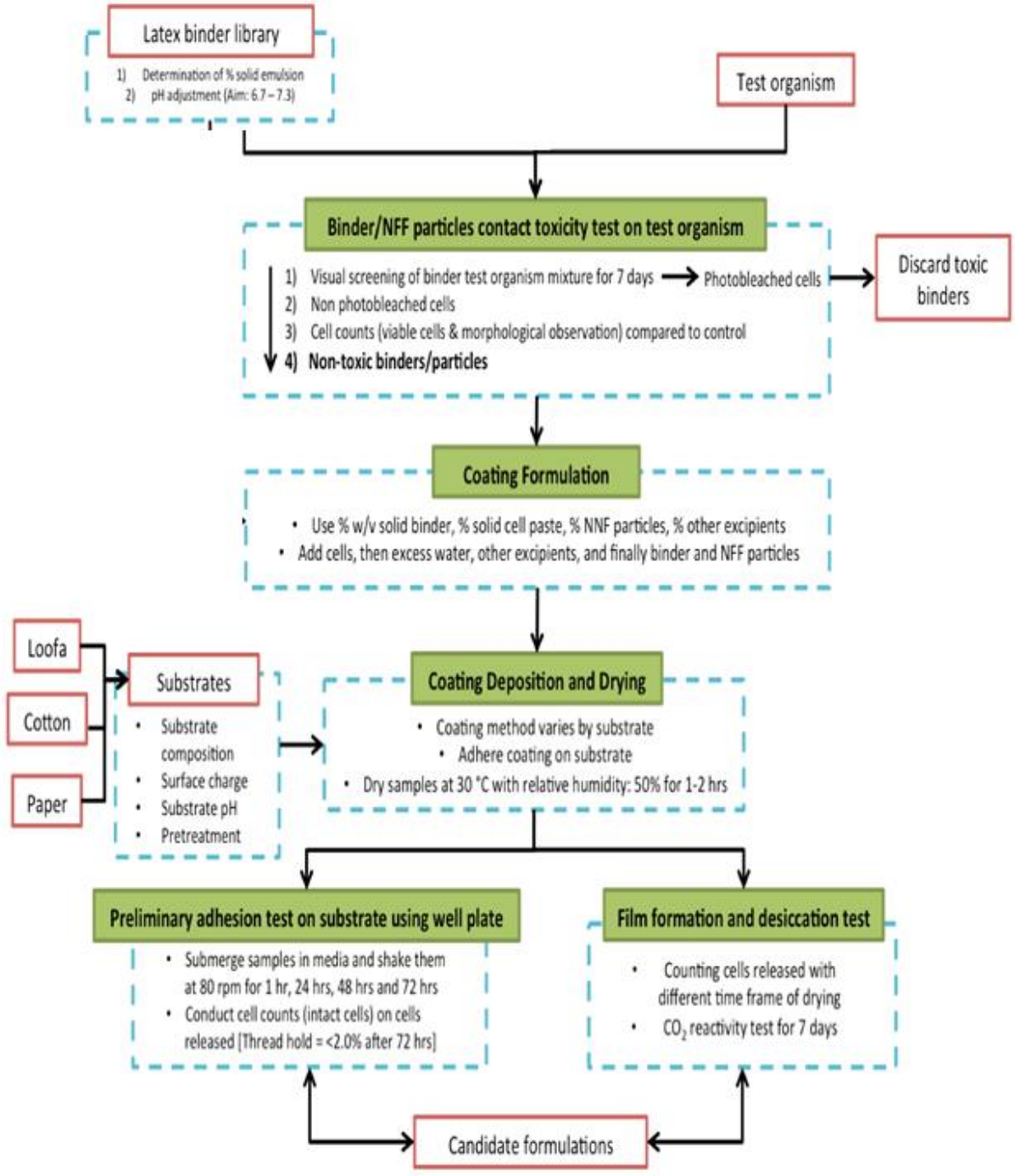


Figure 5.32: Screening protocol for the binder/cell adhesion test to produce viable biocomposites that can withstand desiccation, paper and cotton screening was done by Adam Wallace and Warm In-NA.

5.4 Summary

Although the simple toxicity and adhesion methods described herein require little more than cells, a nontoxic latex binder, and support materials, the method has many potential applications. Microorganisms can now be incorporated into various support materials and survive film formation and drying processes that were hitherto unrealistic. With this simple and fast screening technique, it is now possible to determine the molecular mechanism of cell preservation, viability and metabolic function as a function of desiccation stress, which can be used to evaluate how the coating and drying process can affect cell metabolism and the rate of CO_2 fixation. Thus, this screening tool is a handy method that can be employed to identify microbes for subsequent downstream processing such as immobilisation for continuous carbon sequestration by microalgae. Based on its high porosity (90 porous), high surface area to volume ratio, biodegradability, hydrophobicity, good mechanical strength accessibility and affordability, loofah sponge was selected as the substrates material for microalgae immobilisation to fabricate biocomposites for semi-continuous carbon sequestration. As such paper and cotton substrates were not further evaluated beyond this point.

Chapter 6

Continuous carbon capture by microalgae-loofah biocomposite with associated lipid yield

Abstract

Here we developed an experimental procedure to determine the CO_2 reactivity of three microalgae species (*C. vulgaris*, *D. salina* and *N. oculata*) immobilised on loofah sponge using different non-toxic latex binders that were initially screened for adhesion and toxicity. The experiment was run for 8 weeks in batch mode. The best binder and cells combinations were subsequently selected and scaled up for a continuous CO_2 reactivity for 6 weeks. *C. vulgaris* and *D. salina* daily average CO_2 fixation was improved from 0.924 ± 0.2 and 0.41 ± 0.27 in suspended cultivation system to 3.93 ± 0.31 and 5.69 ± 0.43 mmol/g day^{-1} respectively when immobilised on loofah using latex binders. The immobilised cells' lipid content also increased from 14.53 ± 0.4 and 14.86 ± 0.22 to 69.38% and 66.22% biomass dry weight for *C. vulgaris* and *D. salina* respectively. This intensification process translated to a reduction in land and water usage when compared to open pond algae cultivation or traditional suspension photobioreactors. This novel research work has the potential to substantially reduce the cost associated with biological carbon capture and biofuel production using microalgae when compared with open ponds and photobioreactors.

6.1 Introduction

In Chapter 4 we grew freshwater and marine microalgae on filter paper for 8 weeks with continuous CO_2 fixation, with the cells exhibiting higher CO_2 consumption compared with the equivalent suspension cultures. However, the absence of appreciable extracellular polymeric substances (EPS) (which bind the cells to the support material) meant that the cells could not be confined to the paper and they could be washed off when adding growth medium to the biocomposite. This led us to develop the idea of immobilising cells on a strong and highly porous material (i.e. loofah) using latex binders as a substitute EPS. Chapter 5 detailed an extensive screening programme based on paper, cotton and loofah substrates with a range of organic and inorganic binders. A combination of loofah with selected latex binders appeared most suitable for subsequent CO_2 fixation tests. Due to its high surface area to volume ration

the loofah sponge is our prepared choice of material compared to both paper and cotton materials.

Loofah sponge contains 4.2% protein, 1.08% lipid, 1.04% ash, 37.81% carbohydrate and 55.78% fibre (60% cellulose, 30% hemicellulose, and 10% lignin). A matured loofa fruit is cylindrical, 20–25 cm long, 6–10 cm across with high porosity (79– 93%) and low density (0.02–0.04 g/cm^3) (Saeed and Iqbal, 2013). Loofah sponge is used extensively in many areas for household cleaning, packaging, insulation and water filtration (Qi *et al.*, 2016). Loofah has also been reported in cell immobilisation (animal and higher plants) using alginates and carrageenan (Saeed and Iqbal, 2013) for bioremoval and biodegradation of toxic metals, dyes, and chemicals. There are also reports of using latex binders to immobilised living cells on solid supports such as paper for CO_2 remediation and hydrogen production (Gosse *et al.*, 2010). To our knowledge, loofah and latex have yet to be applied in tandem for cell immobilisation. The principle was to create a strong biocomposite, supporting nano-porous gaps between binder and cell to allow for gas and nutrient circulation. This biocomposite should be robust in terms of desiccation and rehydration tolerance. Here we report the use and combination of nano-porosity created by the binder cell interaction and macro-porosity created by the loofah strands to enhance gas and nutrient circulation within the biocomposite. Additionally, we report a high increase in the amount of total lipid accumulated by the immobilised cells, which could in theory be utilised for biodiesel production.

6.2 Materials and method

6.2.1 Microalgae species and culture media

For culturing details for the microalgae species used in this experiment (*Chlorella vulgaris, Nannochloropsis oculata, Dunaliella salina* and *Tetraselmis suecica*) please refer to Section 4.2.1.

6.2.2 Loofah sponge preparation for cell immobilisation

Loofah (Fig. 6.1) (average length = 100 ± 5 mm, diameter = 59.54 ± 1.58 mm, wall thickness = 9.0 ± 0.63 mm) was purchased from Primark, UK and soaked in hot tap water for 24h, washed thoroughly, and placed in distilled water for 1 h which was changed 3–4 times. The sponge was placed on a wire mesh to dry at room temperature and sterilised by autoclaving at 121 ^{o}C and 1.06 kg/cm^2 pressure for 15 min. The loofah size determination as reported above was determined by the bioreactor bottle volume which is a 100 mL.

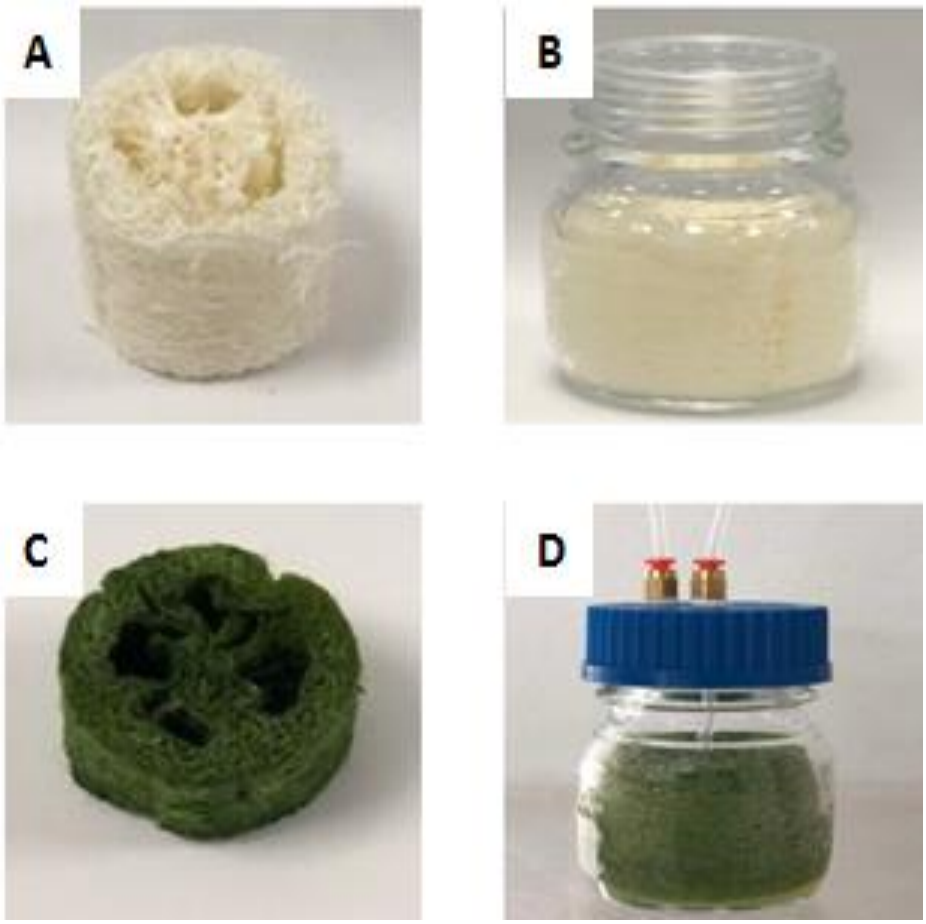


Figure 6.1: A, Loofah sponge, **B**, Loofah sponge inside a glass bottle **C**, Loofah sponge with immobilised microalgae cells *Chlorella vulgaris* using latex binders (biocomposites) **D**, Loofah biocomposites inside reactor bottle without liquid medium, water consumption of each bioreactor bottle is approximately 1 mL day^{-1}, which was supplied every 72 hr via portable 1L bottle sprinkler.

6.2.3 Cell immobilisation and binder screening for batch CO_2 reactivity testing

Loofah was cut into longitudinal sections (length = 60 mm, width = 20 mm) and weighed. Cell immobilisation was done by centrifuging 50 ml of 7-10 days old microalgae culture at 4,000 x *g* for 10 min, apart from *D. salina* that was centrifuged for 2 min. The cell paste was re-suspended in a 5% solids content binder formulation (artificial sea water 35 g L^{-1} $NaCl_2$ was used for the marine algae immobilisation) and thereafter 70 µl (approx. 3.5 mg algae biomass) was applied to the loofah. The cells were carefully spread on one end of the sponge (Fig. 6.2) and left to dry for 6 h at room temperature. The biocomposite was then inserted into a 12 mL autoclaved glass bottle containing 2 mL of the respective microalgae growth medium. The bottles were flushed with 5% CO_2 and kept in the aquarium under similar conditions as described in Section 4.2.1. The CO_2 was refreshed every 48 h and the percentage reduction was determined using a Geotech G100 handheld CO_2 analyser (Geotech UK). The quantity of CO_2 fixed by the immobilised cells was then calculated based on the total immobilised algae mass. The difference in weight of the loofa strips, before and after algal immobilisation, was taken as

the weight of immobilised algal biomass once binder density was taken into account. A diagrammatic summary of the immobilisation procedure is shown in Fig. 6.2.

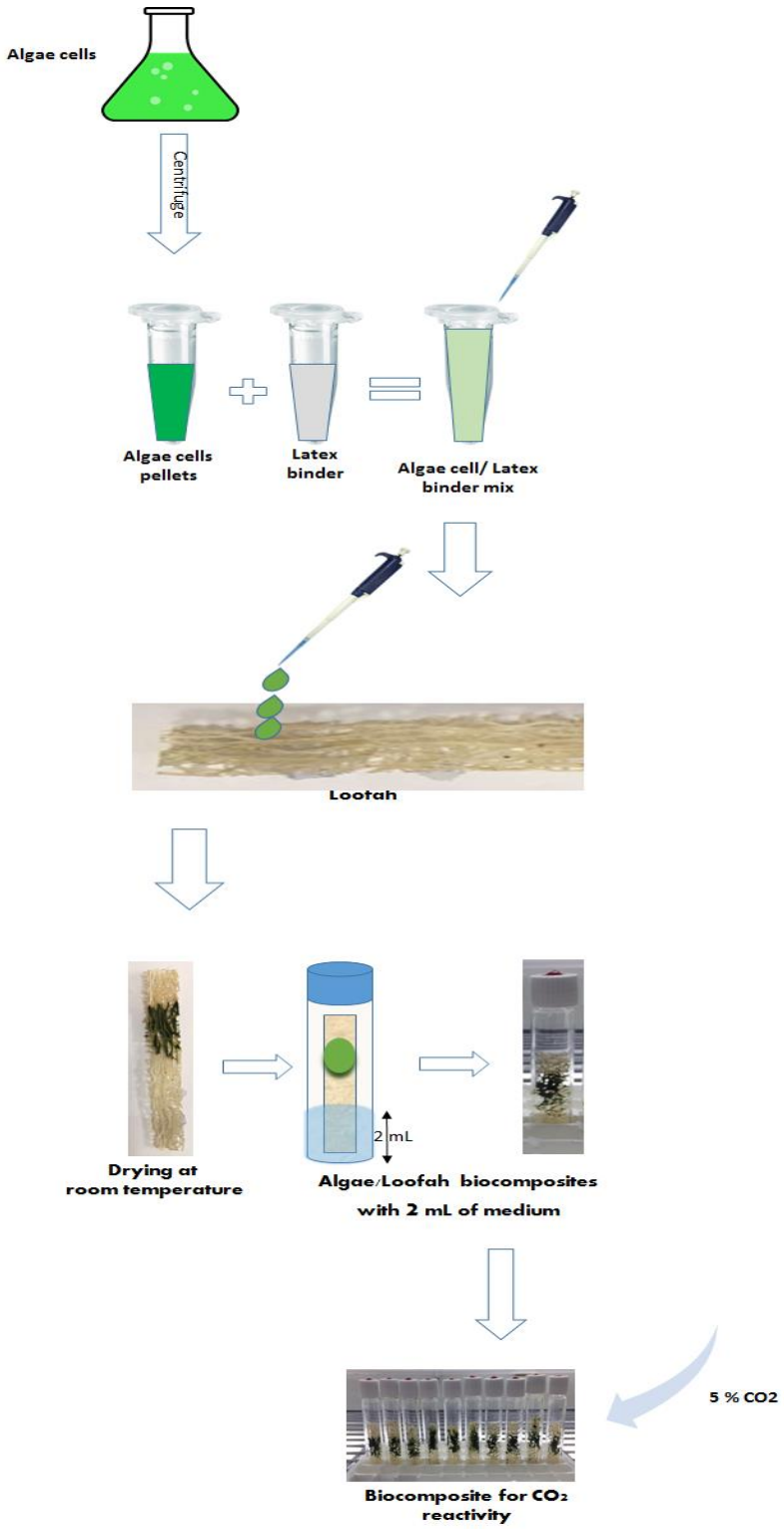


Figure 6.2: Immobilisation of microalgae on loofah sponge using latex binders. Drying help bind the immobilsed cells, binder and the loofah substrate together to avoid cells leaching into the liquid medium, while keeping the immobilised cells viable. Growth condition: 18±2 °C, a 16/8 h light/dark photoperiod with 2,500 lux of illumination provided by daylight-type fluorescent lamps, and constantly aerated with 5% CO_2. 2 mL of water (medium) was used throught the experiment. Biocomposites drying time -6 hrs at room temperature and pressure.

6.2.4 Cell immobilisation and adhesion procedure for continuous CO_2 reactivity testing

Loofah sponge were prepared as described in Section 6.2.2. The sponge was inserted into a 100 mL glass beaker. A slurry of 1 g wet cell paste microalgae was prepared using 1 ml of pH adjusted latex binders and vortexed until homogeneous. BG11 medium (50 ml for *C. vulgaris*) or artificial sea water (for marine microalgae) was added to the algae-latex slurry and mixed thoroughly. The mixture was then carefully poured into the beaker containing the loofah. To dry the biocomposite and to avoid cells settling at the bottom of the beaker, the freshly prepared loofah biocomposite was shaken at 250 RPM at 30 ^{o}C for 48-60 hr. After drying, the biocomposites were removed, placed on a wire mesh and sprinkled with medium to maintain the cells until ready for CO_2 fixation testing. Abiotic controls with and without binders were also prepared. A summary of the procedure is given in Fig. 6.3. The biocomposites photobioreactor (100 mL bottle) was sprinkled with 5 mL of medium every 72 hrs to moist the biocomposites and provide the much needed nutrient to sustain the immobilised cells. After 72 hrs the left over water was collected measured to determine the rate of water absorption and the circle begins all over. This ensure that, the biocomposites is always filled with high gas proportion (similar to a hairy plant root culture bioreactor) as against the traditional bioreactor which contains water. A similar photobioreactor was reported by Stiles and Liu (2013).

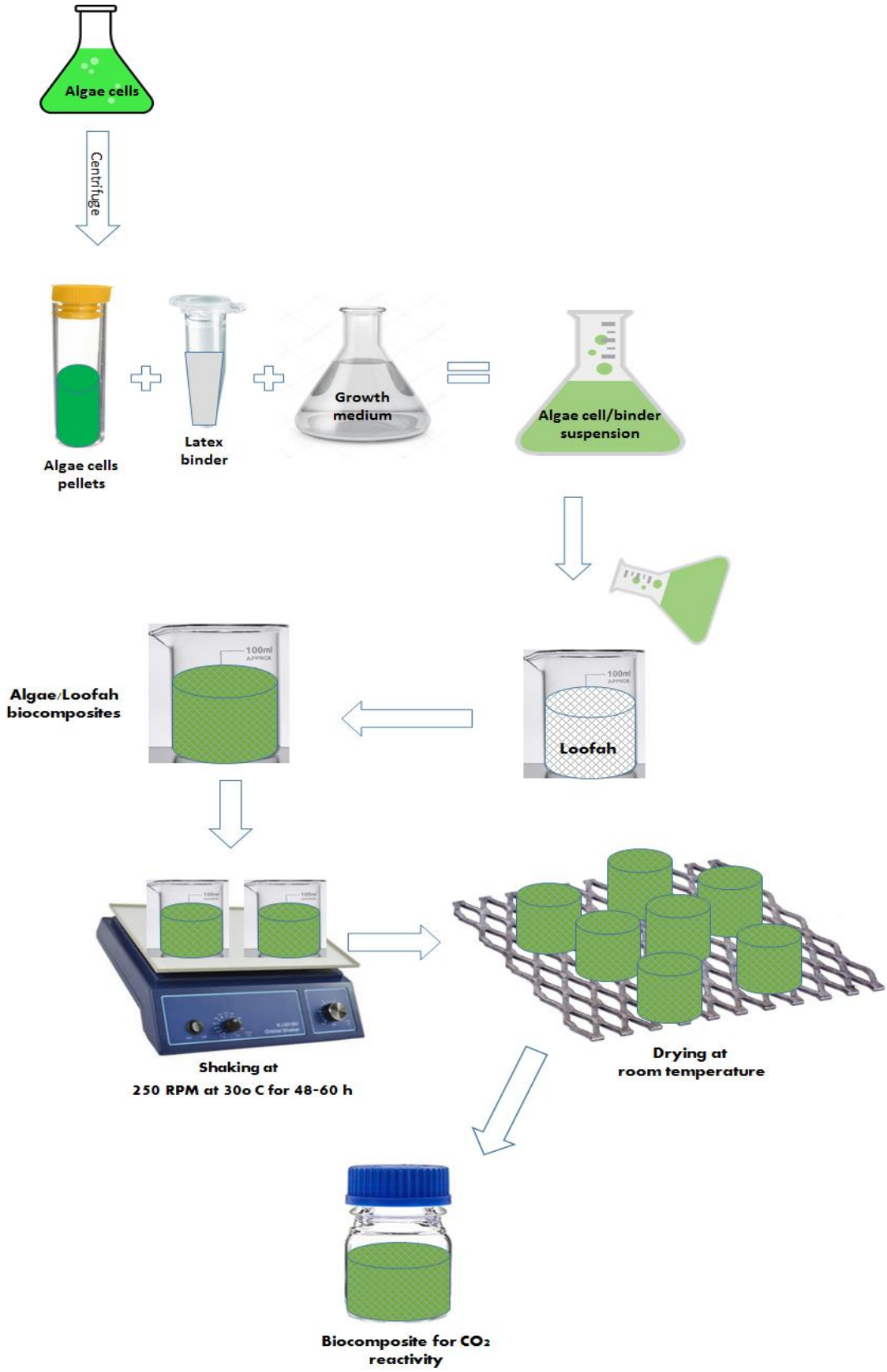


Figure 6.3: Schematic sequence of microalgae biocomposite formation using loofah sponge and latex binders. The biocomposites was maintained within the bioreactor gas headspace with limited water sprinkled only to moist the biocomposites. Growth condition: 18±2 °C, a 16/8 h light/dark photoperiod with 2,500 lux of illumination provided by daylight-type fluorescent lamps, and constantly aerated with 5% CO_2

6.2.5 Photobioreactor design, construction and setup

A bioreactor was constructed by the Newcastle University School of Engineering workshop using a polypropylene tube (length = 91.5, diameter = 63.0, wall thickness = 5, internal diameter = 53.0 mm) attached to 100 ml Duran glass bottles (Merck, UK). The tube was sealed at one end and 1/4" PTFE tube was connected from the other end to a 5% CO_2 cylinder. Gas flow was regulated via a Cole-Parmer gas mass flow controller. CO_2 was distributed to each glass bottle via 1/4" PTFE tube connected to the main polypropylene tube; a needle valve was connected to regulate the gas flow to the bottles from the main tube. CO_2 was passed into the biocomposite containing bottles and collected using 1L sampling bags (Merck, UK). Fig. 6.4 shows the schematic diagram and picture of the rig setup. The experiment ran for 6 weeks using the same culture room conditions as described in Section 4.2.1. Water consumption of each bioreactor bottle is approximately 1 mL day^{-1}, which was supplied every 72 hr via portable 1L bottle sprinkler. Excess water was collected from the bottle every 3 days to measure the actual volume of medium used by the cells and lost due to evaporation.

Figure 6.4: Photograph of the microalgae biocomposites rig for continuous CO_2 fixation trials. Growth condition: 18±2 °C, a 16/8 h light/dark photoperiod with 2,500 lux of illumination provided by daylight-type fluorescent lamps, and constantly aerated with 5% $CO_{2.}$, water consumption of each bioreactor bottle is approximately 1 mL day^{-1}, which was supplied every 72 hr via portable 1L bottle sprinkler.

6.2.6 Inlet and outlet CO_2 gas analysis

A plastic hypodermic syringe was used to collect 30 mL of CO_2 from each sampling bag. CO_2 consumption was quantified using a Geotech G100 CO_2 analyser every 24 h and converted to moles per gram of immobilised cells (dry weight) using equation 6.1, assuming all experiments were conducted under standard temperature and pressure;

$$nCO2 = C\ x\ V - - - - - 6.1$$

Where:

C = CO_2 mole fraction in the gas
V = volume of gas used

6.2.7 Biocomposite imaging and microstructure

Biocomposite microstructure was obtained by scanning electron microscopy (SEM) using a table top Hitachi TM 3000 SEM equipped with a backscattered electrons system for digital image acquisition. All coatings were observed in two or more randomised locations using a 5 or 15 kV accelerating voltage. Each location was imaged multiple times using sequential magnifications ranging from 15x to 30,000x to characterise surface topography, cell distribution and available pore space.

6.2.8 Total lipid extraction of the immobilised microalgae cells

The percent lipid content of the immobilised algae cells was determined using a modified gravimetric method described in Chapter 4. The amount of solvent used was increased to 40 mL due to the size of the biocomposites. Lipid extraction was also conducted on blank loofah and loofah immobilised with binder.

6.2.9. Statistical analysis

All experiments were repeated, with triplicate number of samples (n=3 of samples) and the average values with their standard deviation are reported. One-way analysis of variance (ANOVA) and Krustal-Wallis tests were used to compare mean values and statistical significance using SPSS statistical software. In all instances, comparison which shows a p value of <0.05 were considered statistically significant.

6.3 Results and Discussion

6.3.1 Carbon dioxide reactivity of the immobilised cells using the pipetting/batch method

There are many reports indicating that microbes immobilised by nano-porous adhesive latex coatings offer superior CO_2 fixation or hydrogen production potential compared with the equivalent suspension culture (on a biomass basis) (Gosse *et al.*, 2010; Piskorska *et al.*, 2013; Bernal *et al.*, 2014; Bernal *et al.*, 2017). We recorded a substantial increase in CO_2 reactivity of both freshwater and marine microalgae after immobilisation with porous latex coatings on loofah, with the biocomposites demonstrating up to three times faster CO_2 fixation rates.

Seven latexes that supported *C. vulgaris* growth were tested for CO_2 reactivity over eight weeks with a limited nutrient supply using a batch system. There were significant differences (Krustal-Wallis, K= 52.04, df= 7, P< 0.05) between the mean CO_2 consumptions of the respective biocomposites, with some of them failing after a few weeks. Binders **B** (Rhoplex SF-3122), **AD** (Rhoplex SG -10M) and **AI** (Baycusan C-1004) supported cell growth for the full eight weeks period and, in that order, had the maximum cumulative CO_2 fixation (Fig. 6.5). The mean daily CO_2 fixation of the three selected binders (**B** = 2.52 ± 0.46, **AD** = 1.73 ± 0.21, **AI** = 1.17 ± 0.58 mmol/g) though statistically different from each other (Krustal-Wallis, K= 16.92, df= 2, P< 0.05) represented the highest CO_2 fixation rates for the cultivation period. There was no cell release during the experiment, either into the medium or onto the loofah substrates, suggesting that the cells remained immobilised to the loofah-latex matrix. However, the cumulative CO_2 consumption (**B** = 140.95, **AD** = 96.90 and **AI** = 65.26 mmol/g) was lower than our previously reported CO_2 consumption on paper without the binder which was 192.50 mmol/g (Chapter 4.3.2). The high CO_2 reactivity reported (Chapter 4.3) for the paper immobilised *C. vulgaris* cells corresponded with biomass increase (2.58 g to 6.23g) and cell shedding due to poor binding affinity of the cells (Chapter 4.3.1). There was a significant improvement in CO_2 fixation rates (Krustal-Wallis, K= 15.13, df= 1, P< 0.05) by the selected binders (Binder AI, which has the lowest CO_2 fixation of the best binders) when compared with the suspension culture in the initial five weeks of cultivation. It was not immediately clear what advantage the top three binders conferred regarding *C. vulgaris* CO_2 fixation. Binders **B** and **AI** were from the same manufacturer and contained a quantity of ammonium hydroxide (NH_4OH), which potentially could have been utilised by the algae as an additional nitrogen source.

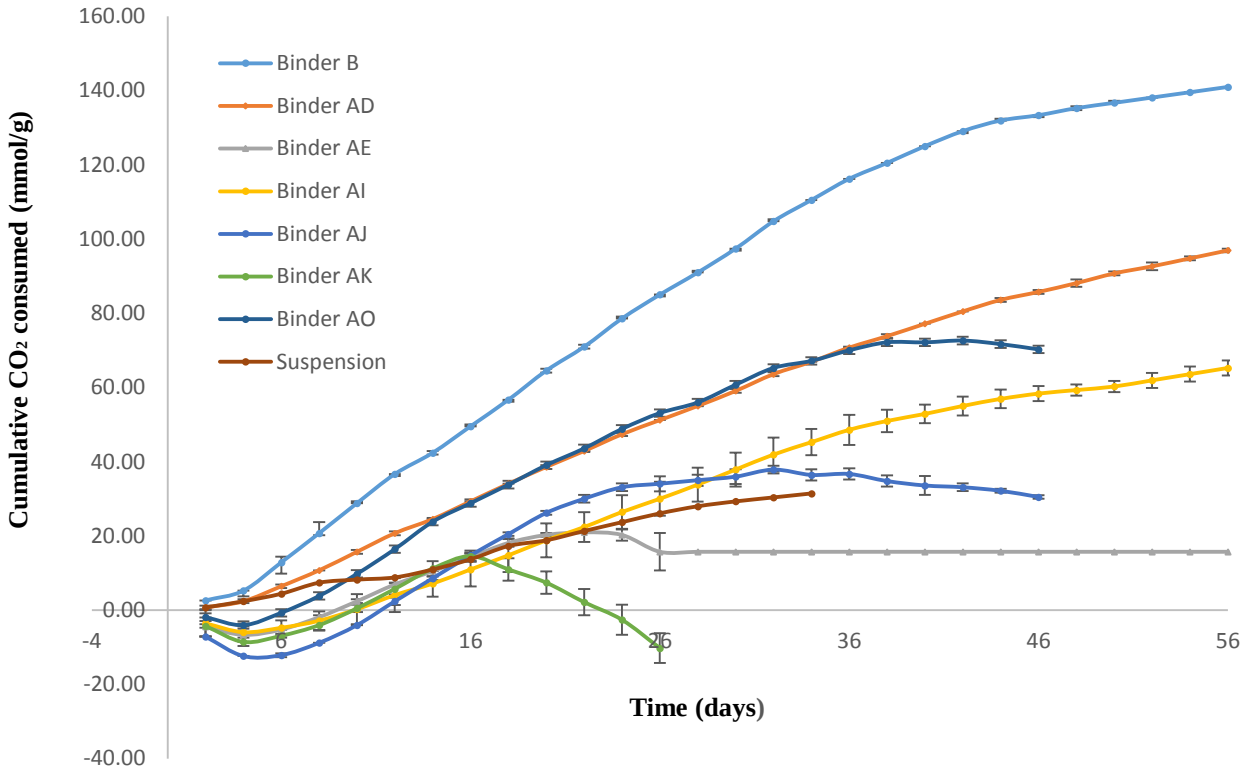


Figure 6.5: Cumulative CO_2 fixation of *C. vulgaris* immobilised on loofah sponge using latex binders in a batch system. The cells were sustained using 2 mL of medium for 8 weeks, Mean ± SD.

Only three binders were used for the marine algae. When immobilised with *D. salina,* binders **Z** (HB 3691 M) and **AJ** (Baymedix CH-120 polyurethane resin dispersion) had similar cumulative CO_2 fixation (165.24 and 167.38 mmol/g) (ANOVA, F= 9.76, P > 0.05) with daily averages of 2.95 and 2.99 mmol g^{-1} day^{-1} followed by binder **B** (109.67 mmol/g, 1.96 mmol g^{-1} day^{-1}) which significantly differed (ANOVA, F= 9.76, P< 0.05) from Binders **Z** and **AJ** (Fig. 6.6). These values were similar to those of the immobilised *D. salina* cells on filter paper 2.96 mmol g^{-1} day^{-1} (165.9 mmol/g). The five weeks cumulative CO_2 fixation of the immobilised cells Binder **Z**, 2.63 mmol g^{-1} day^{-1} (cumulative, 92.14 mmol/g) was a six times order of magnitude greater than the suspended culture 0.39 mmol g^{-1} day^{-1} (13.75 mmol/g). Summarily, the mean CO_2 reactivity of the immobilised cells (Binders **B, Z** and **AJ**) significantly differ (Krustal-Wallis, K= 33.32, df= 1, P< 0.05) from the suspension control.

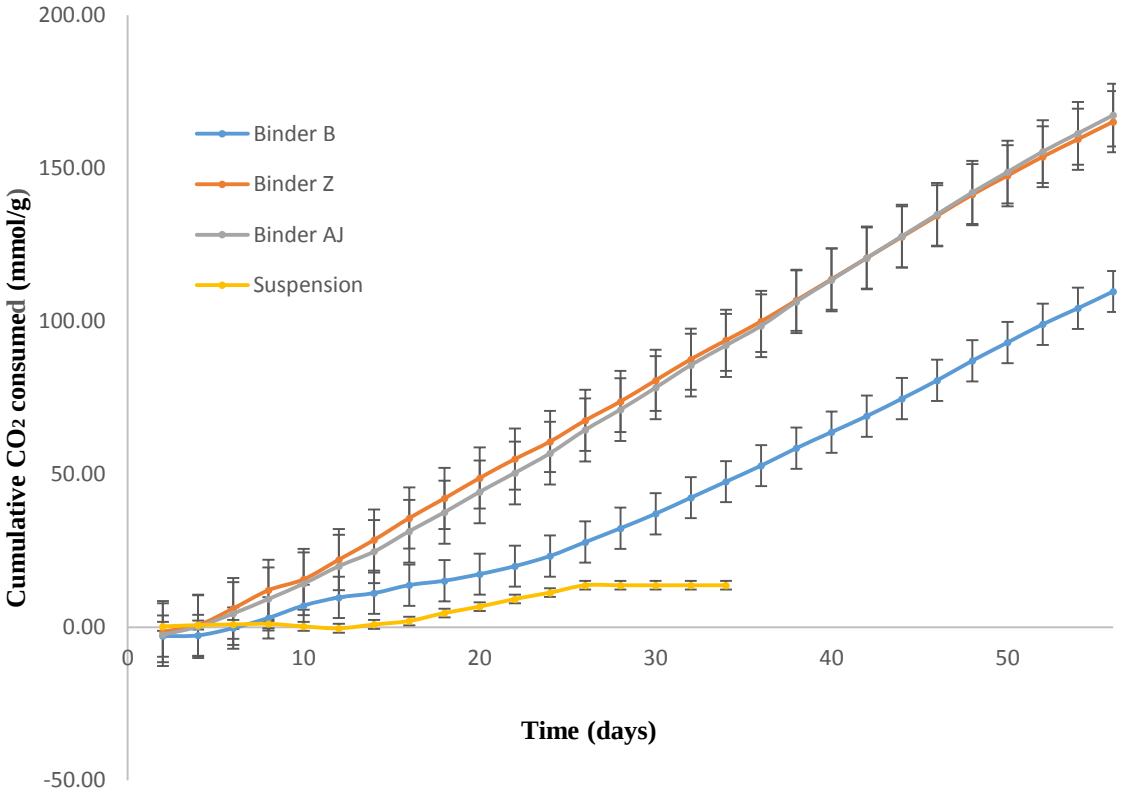


Figure 6.6 Cumulative CO2 fixation for D. salina immobilised on loofah sponge using latex binders in a batch system. The cells were sustained using 2 mL of medium for 8 weeks, Mean ± SD.

Nannochloropsis oculata immobilised using binders **B** (Rhoplex SF-3122), **U** (PD-0413) and **Z** (HB 3691 M) showed no significant variation (ANOVA, F= 0.007, P > 0.05) at cumulative CO_2 reactivity of 150.62, 151.90 and 150.95 mmol g^{-1}, respectively (Fig. 6.7). Comparing the two cultivation methods, the mean CO_2 fixation of each of the three binders (**B, U** and **Z**) for five weeks was significantly higher (P < 0.05) than the suspension. For instance, the five weeks daily CO_2 fixation values for binder **B** 2.66 mmol $g^{-1}day^{-1}$ (cumulative, 93.24 mmol g^{-1}) were higher than the suspension culture 0.46 mmol $g^{-1}day^{-1}$ (cumulative, 16.17 mmol/g). Data for *N. oculata* attached to paper 2.02 mmol $g^{-1}day^{-1}$ (cumulative, 113.36 mmol g^{-1}) were lower than the loofah immobilised cells 2.71 mmol $g^{-1}day^{-1}$ (Binder U, cumulative, 151.90 mmol g^{-1}). Therefore, the marine microalgae were able to fix more CO_2 on the loofah than on the filter paper, contrary to what was observed with *C. vulgaris*. The only drawback to *N. oculata* CO_2 fixation on the loofah was the high degree of cell release to the medium (Chapter 5, Section 3.18).

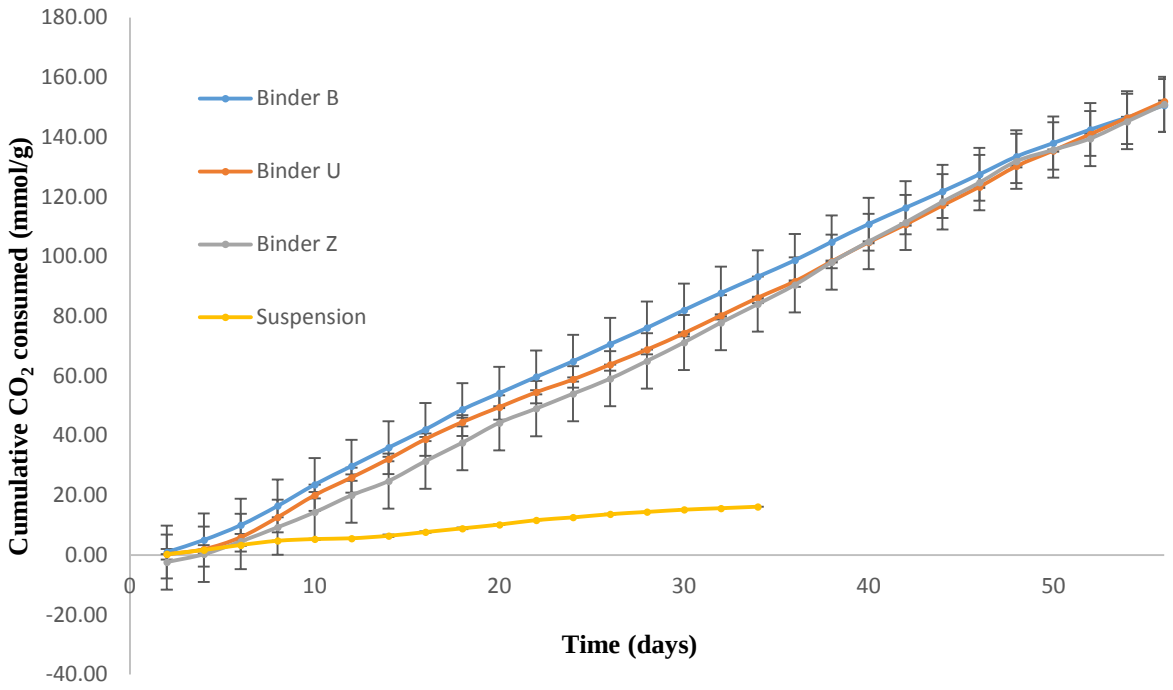


Figure 6.7 Cumulative CO2 fixation for N. oculata immobilised on loofah sponge using latex binders in a batch system. The cells were sustained using 2 mL of medium for 8 weeks, Mean ± SD.

The daily CO_2 fixation for *T. suecica* immobilised using latexes **B, AJ** and **AK** were 1.96, 0.51 and 2.40 mmol $g^{-1}day^{-1}$ with corresponding cumulative values of 109.76, 28.57 and 134.52 mmol/g respectively (Fig. 6.8). This shows a significant variation (Krustal-Wallis, K= 27.55, df= 2, P< 0.05) in the way the binders influenced the CO_2 fixation of the immobilised cells. There were no equivalent experiments conducted using filter paper or suspension cultures. Nonetheless, it is evident that *T. suecica* had a lower CO_2 fixation efficiency than *D. salina* and *N. oculata* and was subsequently screened out ahead of the semi-continuous CO_2 fixation test.

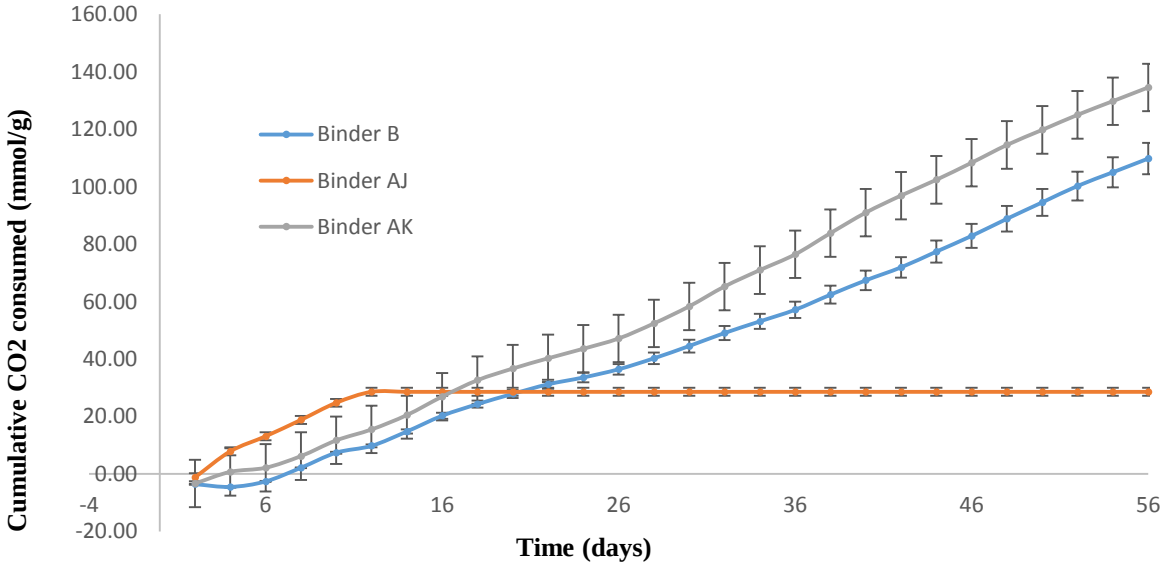


Figure 6.8: Cumulative CO2 fixation for T. suecica immobilised on loofah sponge using latex binders. The cells were sustained using 2 mL of medium for 8 weeks, Mean ± SD.

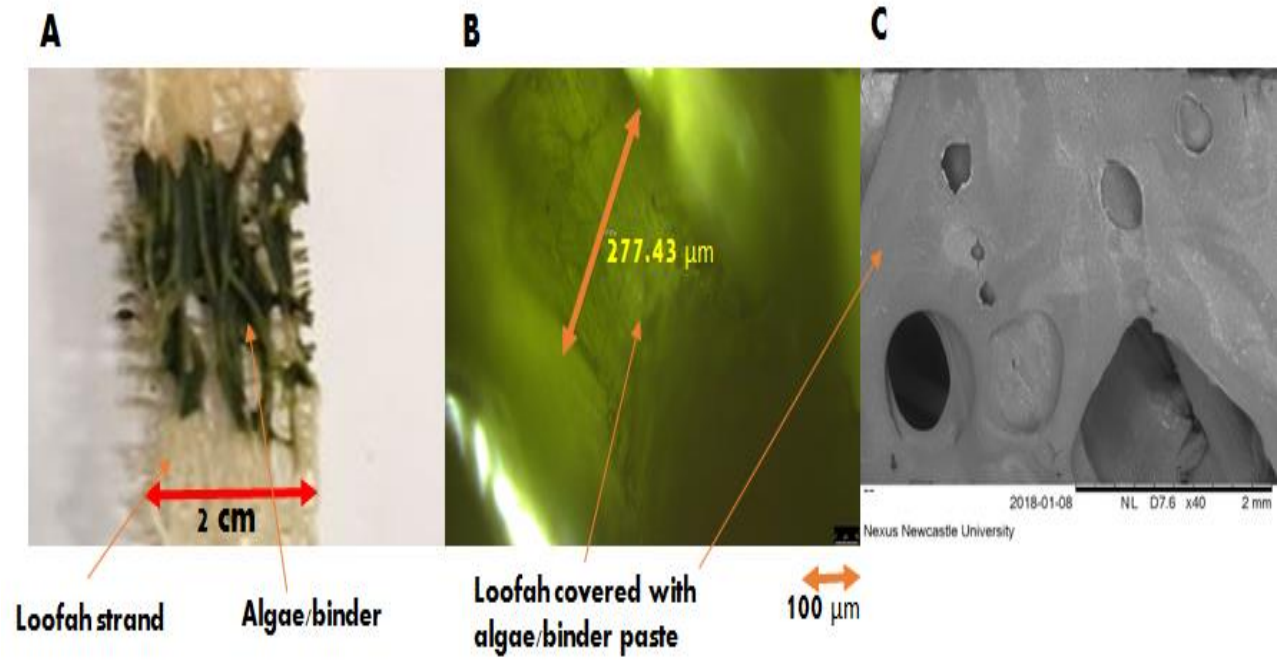


Figure 6.9: Microalgae immobilised on loofah: **A** cell/latex mixture spread on the sponge strip covering most of the spaces, **B** and **C** microscopic and SEM images showing the loofah strand covered with the cell/latex mixture, which reduces the extent of CO_2, light and mediumn penetration within the biocomposites.

As discussed in Chapter 4, the increase in CO_2 fixation by the immobilised microalgae was likely due to improved mass transfer within the liquid medium, driven by direct cell/gas contact within the gas headspace. The loofah surface (Fig. 6.9A) had a heterogeneous distribution of cell/binder formulation, which was caused by the manual extrusive pipetting technique. Additionally, the direct pipetting coating technique will have caused some of the loofah pores to be covered by the cell/binder mixture upon drying (Fig. 6.9B-C).

The pipetting technique is dependent on the self-labelling of the latex-cell formulation before film formation which can be modified by changing the emulsion composition and immobilisation techniques as reported by Gosse *et al.* (2012). Biocomposite coating uniformity can be improved using other coating methods (i.e. spin coating, roll printing, spraying, convective sedimentation assembly, ink-jet printing and shaking techniques), that accurately control the thinness and distribution of the cells on the substrate (Gosse *et al.*, 2012). Cells can also span a portion of the available macropores on the surface of the loofah where neighbouring cells are linked by latex particles between the loofah fibres (Fig. 6.10). In this case, additional photoreactive surface area is created by partially filling the void spaces of the loofah with reactive cells while preserving the matrix porosity to allow for gas absorption and liquid hydration. A better immobilisation technique (the equilibrium/shaking method) supporting uniform cell-binder coating was thus developed. Drying time for both methods (6 hrs for the

pipetting and 48- 60 hrs for the equilibrium/shaking) is also crucial in having a good adhesion. Biocomposites drying below these time periods result in immobilised cell leakage during medium replenishment. The drying time was determined from the cell adhesion, film formation and desiccation test discussed in Chapter 5.

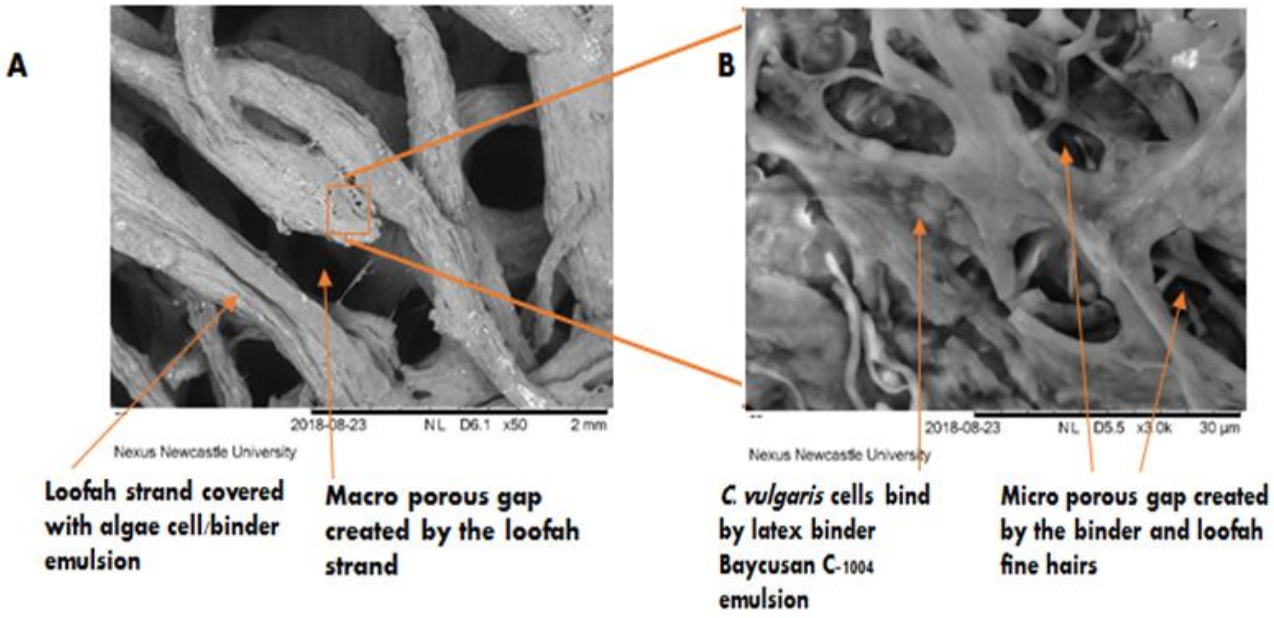


Figure 6.10 SEM images of *C. vulgaris* immobilised on loofah using the latex binder Baycusan C-1004. Macro- and microporous gaps which aid in gas, water and nutrient circulation can be seen in both images.

6.3.2 Semi-continuous carbon dioxide reactivity of loofah biocomposite fabricated using the equilibrium coating method

For the semi-continuous trial the process was scaled up utilising a higher cell loading with larger sections of loofah. Binder selection was restricted due to binder availability as we ran out of binders **Z** and **AJ** which are not commercially available. The experimental design was modified from the batch trial as more void spaces were created by the continuous shaking of the algae/binder mixture while drying the biocomposite thereby helping with efficient CO_2 and nutrient circulation within the biocomposites. This was not possible with the direct pipette application. The hydrophilic nature of the loofah due to the abundant O–H, C–H, and C=O groups (Qi *et al.*, 2016), helps in moisture retention within the loofah structure which help in sustaining the attached cells.

The highest CO_2 fixation with *C. vulgaris* came from binder **AD** (Rhoplex SG-10M; 165.25 mmol/g) followed by binders **B** (Rhoplex SF-3122; 150.37 mmol/g) and **AI** (Baycusan C-1004; 132.41 mmol/g) (Fig. 6.11). There was no significant difference between the three binders (ANOVA, F= 2.08, DF= 2, P= 0.13) in terms of their ability to sequester CO_2, while

they both differed from the suspension control (P< 0.05) when analysed up to 5 weeks. Fig. 6.12 shows SEM images of *C. vulgaris* attached to the loofah after 6 weeks of immobilisation.

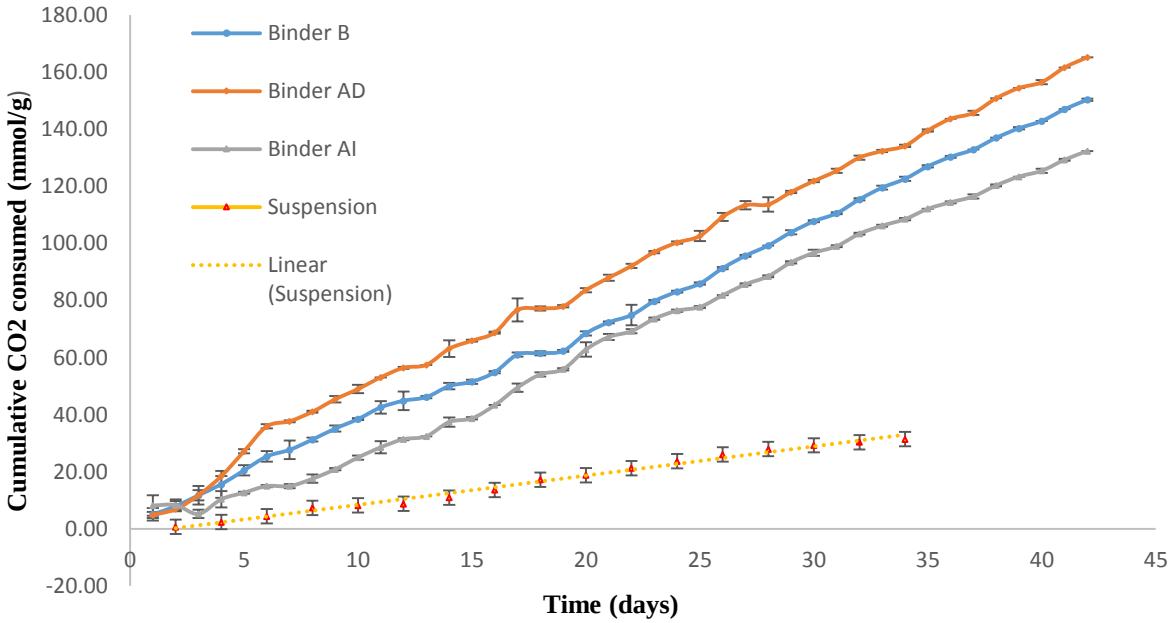


Figure 6.11: Cumulative carbon dioxide fixation for C. vulgaris immobilised on loofah sponge using latex binders in a semi-continuous mode. The cells were sustained using 1 mL of medium refreshed every 24 h for 6 weeks, Mean ± SD.

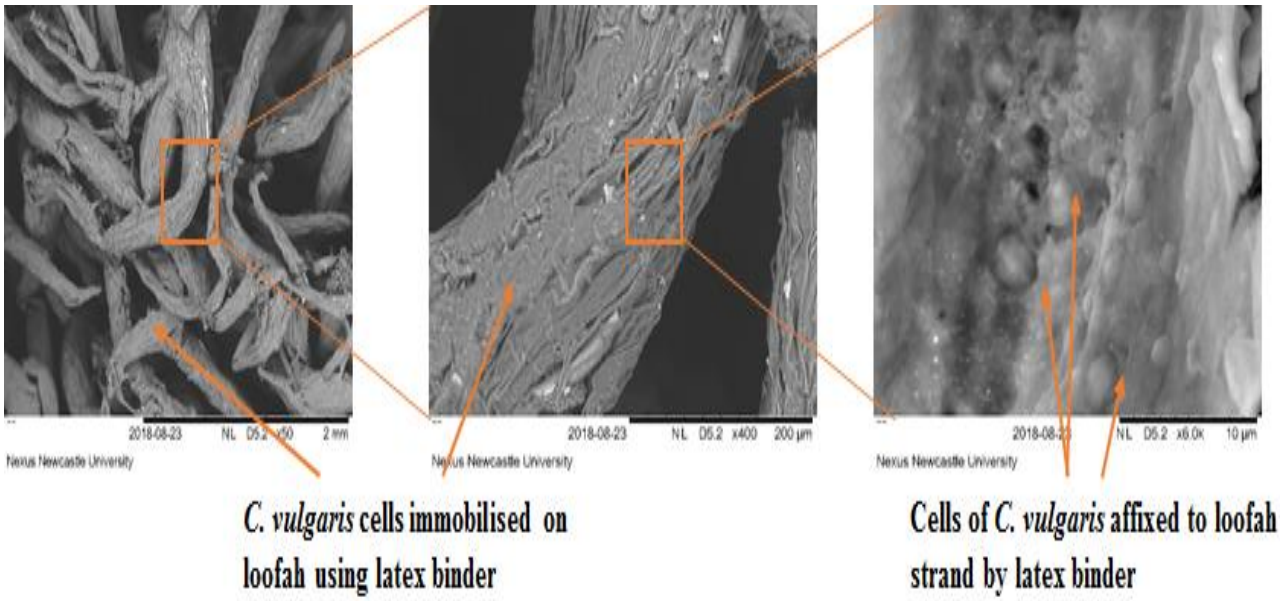


Figure 6.12: SEM images of *C. vulgaris* attached to the loofah after 6 weeks of immobilisation.

Similar patterns were observed with *D. salina* (Krustal-Wallis, K= 41.29, df= 2, P< 0.05), and *N. oculata* (Krustal-Wallis, K= 37.42, df= 2, P< 0.05) where they both significantly differed in their respective CO_2 fixation when analysed against the suspension control. The highest cumulative CO_2 fixation by *D. salina* was with binder **AJ** (Baymedix CH-120 polyurethane resin dispersion; 239.15 mmol/g) which was the highest value recorded for the experiment (Fig. 6.13). The daily cumulative CO_2 fixation for *D. salina* at five weeks (5.69 mmol/g day^{-1}) was

15 times higher than the corresponding suspension culture (0.40 mmol/g day^{-1}) during the same period. *N. oculata* fixed 162.43 mmol/g CO_2 when immobilised with binder U (Fig. 6.14). However, there was a poor adhesive mechanism between *N. oculata* and the binders as reported in Chapter 5. Therefore, the CO_2 fixation readings from *N. oculata* will have included cells deposited at the bottom of the glass bottle, and should be treated with caution.

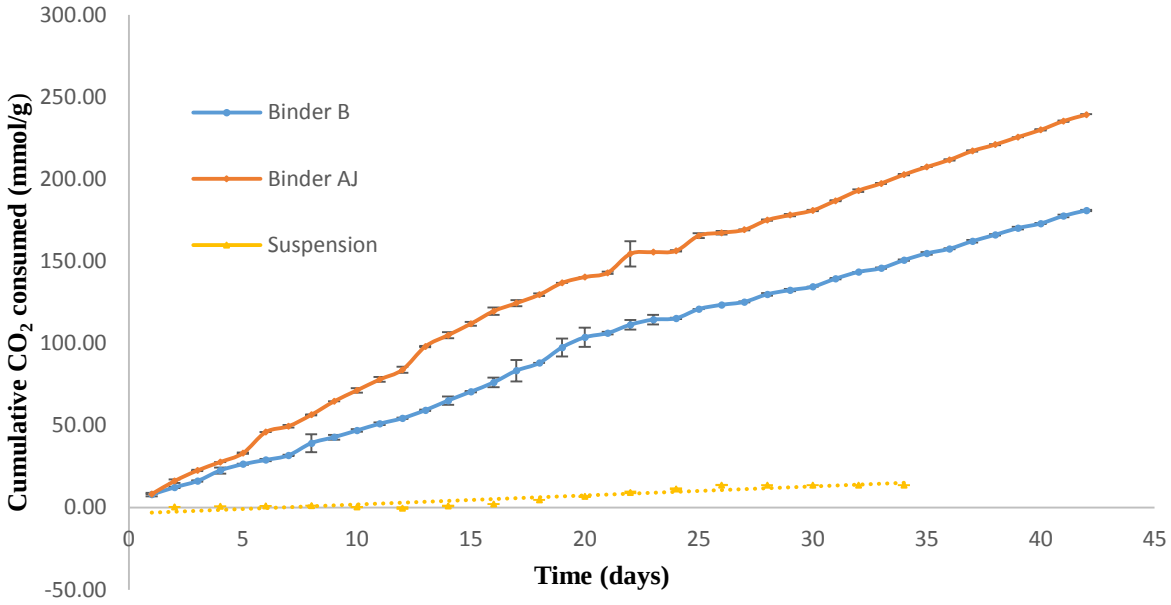


Figure 6.13 Cumulative carbon dioxide fixation for *D. salina* immobilised on loofah using latex binders in a semi-continuous mode. The cells were sustained using 1 mL of medium refreshed every 24 h for 6 weeks, Mean ± SD.

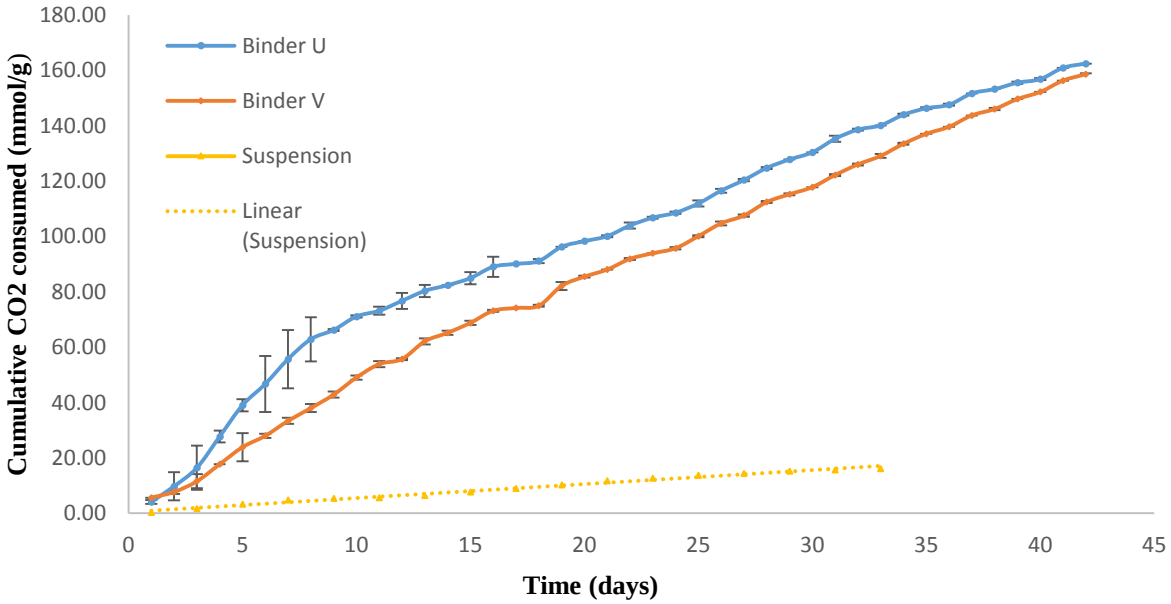


Figure 6.14 Cumulative carbon dioxide fixation for *N. oculata* immobilised on loofah using latex binders in a semi-continuous mode. The cells were sustained using 1 mL of medium refreshed every 24 h for 6 weeks, Mean ± SD.

The overall cumulative CO_2 fixation rate could have been diminished by insufficient light reaching the biocomposite from the top and sides due to the geometrical arrangement of the bottle in relation to the bulk polypropylene tube gas distributor. Cell death (decomposition and release of CO_2) as a result of poor light penetration could also contribute to the amount of CO_2 released within the biocomposite.

The conclusion that can be drawn from the mean CO_2 consumption (Table 6.1) of the three algae species (top binders selected), is that the different culturing (suspension versus immobilisation) methods had an influence on the cumulative CO_2 fixation. Similarly, the immobilisation technique (on paper without binder, on loofah with binder using pipette or equilibrium coating) greatly increased the CO_2 capture efficiency of the three microalgae species tested in that order, with the exception of *C. vulgaris* on the paper coating method where cell outgrowth and increase in biomass (Chapter 4.3.1) resulted in increased CO_2 fixation. The superiority of the loofah immobilisation (batch) over paper could be as a result of the loofah's high porosity compare to the paper which aids in better light penetration and CO_2 diffusion, also the near uniform distribution of light and CO_2 gas round the immobilised cell in contrast to one-sided cell immobilisation on the paper further assists in CO_2 fixation. The equilibrium coating could also be responsible for the improved CO_2 fixation recorded for the semi-continuous CO_2 reactivity. The coating method produces higher porosity with the loofah biocomposite compare to the pipetting technique. Frequent flushing of medium (every three days) also help in higher CO_2 fixation compared to the batch system that was nourished via capillary. Maintenance of the immobilised microalgae cells within the bioreactor gas space similar to the hairy plant root culture bioreactor could also be responsible for the higher CO_2 fixation rates of the cells. The promotion of active gas diffusion directly into the algae cell cytoplasm in the form of CO_2 rather than dissolved HCO_3^- also helped in faster CO_2 accumulation around the cell carbon concentration areas. This can be related to the higher CO_2 fixation of *D. salina* with no cell wall when compared with either *C. vulgaris* or *N. oculata* with cell wall (Table 6.1).

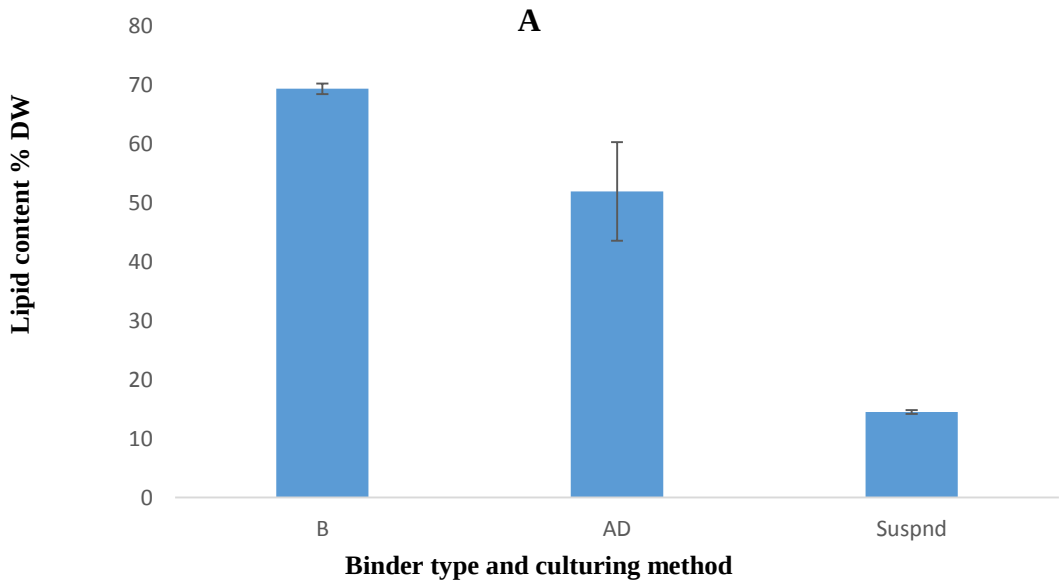
Table 6. 1: Mean cumulative CO_2 fixation of microalgae immobilised on paper/loofah using binders and the suspension culture method ± SD.

Microalgae	**Mean CO_2 consumption (mmol g^{-1} day^{-1})**			
	Suspension cultivation	**Paper batch cultivation**	**Loofah batch cultivation**	**Loofah semi-cont'd cultivation**
C. vulgaris	0.924 ± 0.2	3.44 ± 0.16	2.52 ± 0.46	3.93 ± 0.31
D. salina	0.41 ± 0.27	2.96 ± 0.53	2.99 ± 0.37	5.69 ± 0.43
N. oculata	0.48 ± 0.11	2.02 ± 0.14	2.71 ± 0.28	3.87 ± 038

6.3.3 Biocomposite lipid synthesis

At the end of the 6 weeks immobilisation experiment, the lipid content of *C. vulgaris* was 69.38 ± 1.27 % and 51.96 ± 11.84 % DW for binders **B** and **AD** respectively (Fig. 6.15), and 36.1 ± 4.72 % and 66.22 ± 6.02 % for *D. salina* with binders **D** and **AJ** respectively. The immobilisation significantly increased the lipid contents of both *C. vulgaris* (ANOVA. F= 33.28, DF= 2, P= 0.009) and *D. salina* (ANOVA. F= 68.14, DF= 2, P= 0.003) when compared to their respective control. Lipid extraction was not conducted on *N. oculata* due to substantial loss of cell from the biocomposite during the experiment. These figures compare favourably with literature values. The reported lipid content of *C. vulgaris* ranges from 14-53% of dry weight (DW) biomass (Becker, 1994b; Demirbas, 2009; Araujo *et al.*, 2011; Huang and Su, 2014), whereas the suspension control yielded between 9-17% at stationary phase. For *D. salina*, the reported lipid content is around 16-44% DW (Tornabene *et al.*, 1980; Weldy and Huesemann, 2007). The high lipid content of binder **AJ** corresponded with the higher CO_2 fixation of the binder.

Immobilisation increased the lipid content for *C. vulgaris* and *D. salina*, likely due to decreased cell division rates that supports lipid accumulation, raising hopes of converting algae biomass to biofuel after CO_2 fixation (assuming an efficient method of separating the cells from the biocomposites). Separating the cells from the biocomposite could also mean than the loofah could be reused for another round of immobilisation, thereby saving costs.



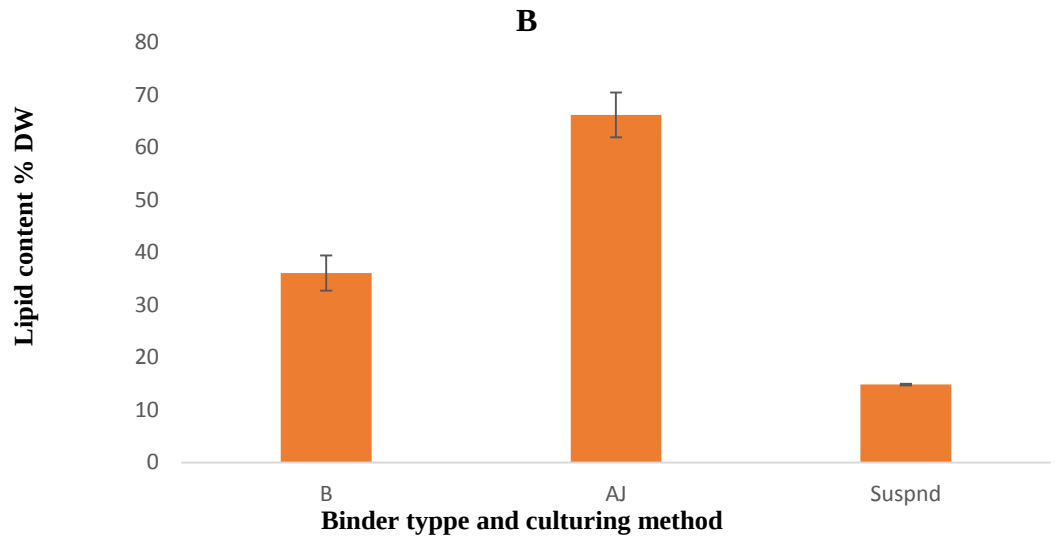


Figure 6.15: Total lipid content (% dry weight) of **A**, *C. vulgaris* and **B**, *D. salina* immobilised on loofah after 6 weeks of CO_2 reactivity, compared with the suspension control, Mean ± SD.

Table 6. 2: Summary of cumulative CO_2 fixation and lipid content of three species of microalgae immobilised on loofah sponge using latex binders after 6 weeks of continuous CO_2 reactivity, Mean ± SD.

Microalgae	Cumulative CO_2 fixation (mmol/g)	Lipid content (% DW)
C. vulgaris	165.25 ± 4.62	69.38 ± 1.27 %
D. salina	239.15 ± 11.29	66.22 ± 6.02 %
N. oculata	162.43 ± 39.26	-

6.3.4 Economic benefit of CO_2 mitigation using immobilised algae technology

It has been suggested that microalgae have the potential to sequester CO_2 emitted from power generating facilities, however the practicalities of this process are notoriously challenging. Miniaturising the growth technology and intensifying the CO_2 fixation process while optimising lipid production could offer a practical solution. This experiment demonstrates the feasibility of utilising inexpensive materials to construct and develop a simple microalgae bioreactor that could be co-located near a CO_2 emission point. The harvested cells could theoretically be converted to biofuel while keeping the cost of production lower than current methods due to limited use of water, land and nutrients. However, there are limitations that need to be addressed before this technology can be deployed.

6.3.5 Experimental limitations

While cell immobilisation and binding affinity of latex binders with *C. vulgaris* and *D. salina* are good, the same cannot be said for *N. oculata* which suffered from extensive cell release

after rehydration. Therefore, not all microalgae can be immobilised using this technique. However, the adhesion screening protocol developed in Chapter 5 could be used to further screen cells, substrates and binders.

Equal light distribution is critical for healthy cell growth and metabolism in algae culturing systems. The bioreactor design was done in such a way that some parts of the biocomposite received more light than others due to the opaque plastic tube used to distribute the gas to the bioreactor bottles. Similarly, the blue lids of the Duran bottles will not allow light penetration to the top of the biocomposite (Fig. 6.16). Additionally, latex-entrapped cells in deeper layers of the loofah matrix may be light limited, which could also lower the cell's photosynthetic capacity. This would decrease the efficiency of the whole process and needs to be addressed. The use of a transparent gas distributor and bottle cover could allow the passage of light from the side and top of the biocomposite which could eliminate this problem.

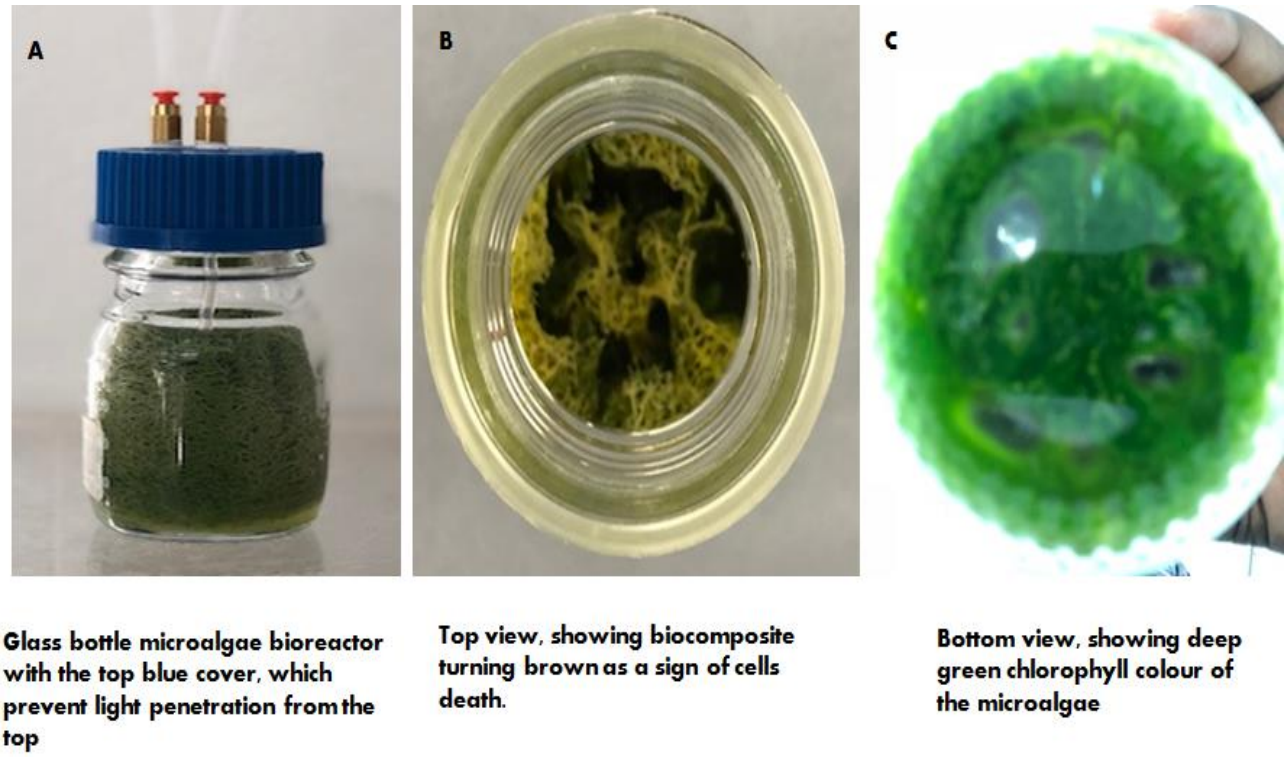


Figure 6.16: Images of **A**, microalgae biocomposite within a 100 mL glass bioreactor **B**, top view showing the pale green chlorophyll colour due to poor light penetration **C**, the bottom of the bottle bioreactor showing deep green chlorophyll colour due to good light penetration.

The amount of immobilised cells (1 g) on the loofah was insufficient to fix the entire 5% CO_2. At most, only 30-40 % of the inlet CO_2 was utilised. Arranging 2-3 biocomposite bottles in series could fix most, if not all of the 5% CO_2 from the inlet stream. Conversely, reducing the inlet CO_2 concentration to 1-2% could also improve efficiency.

Additionally, loofah sponge has some limitation as regards to cell immobilisation and flexibility in applications. The circular shape of the sponge means it is difficult to use it within a flat panel bioreactor. Cutting the loofah sponge beyond certain dimension (0.5x0.5 cm) greatly reduced its cohesion. Limited light diffusion at the centre of the sponge was also experienced when the sizes goes beyond certain level say 2x2 cm.

6.4 Economic assessment of using loofah biocomposites for carbon capture from power plant

This work is the first attempt to investigate the possibility of integrating microalgae biocomposites to sequester CO_2 from a power plant. Chapter 6 has demonstrated the feasibility of carbon capture using immobilised microalgae biocomposites for an extended period. Part of this chapter will look at the possibility of integrating microalgae biocomposites into a hypothetical power plant for carbon capture. The system will be compared with other hypothetical microalgae cultivation techniques (raceway and PBR) to see their respective economic performances. Other carbon capture technologies such as MEA will also be compared with the current work in the light of government regulations and incentives for carbon capture.

The most prevalent and efficient power generating plant in operation uses natural gas as the main source of fuel, as such this technoeconomic analysis will make use of a purported 430 MWe plant using natural gas with performances and attributes reported in Table 7.1. The data was adapted from the work of Rezvani *et al.* (2016), where they used Artificial Neural Network (ANN) modelling to extrapolate data from different work conducted in the field of microalgae cultivation. Technoeconomic analysis with and without microalgae CO_2 capture will be conducted, including the sale of algae biomass for fuel or high value products. The results will be compared with other CCS methods.

One of the advantages offered by microalgae CO_2 mitigation compared to other CCS methods is the minimum drop in efficiency due to lack of CO_2 compression, energy for solvent regeneration or long distance transportation to a dumping site - which can be up to 12% in some cases (Rezvani *et al.*, 2016). The highest energy consumption in microalgae cultivation is in mixing, pumping and harvesting that are significantly reduced in this current work due to the immobilisation process. Thus, the current work is less energy intensive compared to either raceway or PBR.

Table 6. 3: The characteristics of the natural gas combined cycle power plants for the integration of microalgae based CO_2 bio-fixation. Adapted from (Rezvani *et al.*, 2016).

Parameters	**Values**
Power output [MW]	430.0
Net efficiency [%]	58.29
CO_2 release [kg/s]	41.45
Total plant cost [million £]	304
Specific invest. – net [£/kW]	720
Fixed Operation &Maintenance cost [£/kW_{gross}]	23
Variable O&M cost [£/MWh]	0.5
Fuel cost [£/GJ]	6
Annual operating time (hrs/a)	7000
BESP [£/MWh]	53
Contingencies + owner's cost [%]	15
Discounted cash flow [%]	8%

6.4.1 Key assumptions

We ignore the cultivation cost of the algae that is used to fabricate the biocomposite but assume a value of £400/tonne of algae biomass, which is added to the final cost of the biocomposites. The number of days for the power plant operation is 292 days (7,000 h), with average CO_2 fixation rate of 40% (5% inlet CO_2 concentration). Microalgae photosynthetic efficiency of 4% and radiation 2,200 lux (the PBR uses 28 MJ m^{-2} d^{-1}) is assumed. Price for construction of loofah PBR is estimated at £10 m^2 (assuming £70 m^2 for normal PBR reported in the literature, and our work reduced the footprint x7 due to minimal water usage). Natural gas price at £6/GJ. No cost is assumed for drying/immobilisation of microalgae as biocomposite will dry under natural sunlight (30 ^oC). Operating cost of £100/t ± £30 for sensitivity analysis is also assumed. We calculate our investment cost from equation 7.1, which was adapted from Rezvani *et al.* (2016). Summary of all the assumptions made are contained in Table 7.2. We also assumed that the amount of algae biomass immobilised on loofah (1g wet cell paste) will occupy 7 times less space using a normal PBR.

$$l\,mc = \left(\frac{Mpp}{Xm}.\left(\frac{SCl}{dR} + SCR\right)\right).(\rho + \omega + 1)\text{----------}(7.1)$$

Where:
lmc= total investment for the microalgae cultivation,
Xm= CO_2 fixation rate (kg $CO_2\,m^{-3}d^{-1}$),
Mpp= CO_2 flow rate from the power plant to algae (kg $CO_2\,d^{-1}$),
SCl= specific cost of land (£/m^2),

SCR= specific cost of the reactor or pond for microalgae cultivation,
ρ = contingency ratio in decimals,
ω = owner's cost ratio in decimals,
dR= equivalent cultivation depth (m).

Table 6. 4: The parameters assumed for microalgae cultivation and CO_2 fixation using a raceway, conventional PBR and loofah biocomposites PBR, Data extrapolated from (Rezvani *et al.*, 2016).

Parameters	Raceway pond	PBR	Biocomposite PBR
***S–V* ratio [m^{-1}]**	4	40	40
Solar radiation [MJ m^{-2} d^{-1}]	20	20	20
Photosynthetic efficiency [%]	4	4	4
CO_2 fixation rate [% of CO_2 output]	40	40	40
Specific cultivation cost [£/m^3]	120-250	(1500–3000)	(1150–2150)
Specific operating cost [£/dt]	100 (±30)	100 (±30)	40 (±15)
Specific energy consumption [W/m^3]	4	40	10
Contingency + owner's cost [%]	30	30	30
Discounted cash flow [%]	8	8	8

6.4.2 The economic assessment for using algae biomass for biofuel

The capital cost of the respective algae cultivation (raceway, photobioreactor and biocomposites PBR) were derived from the investment estimates and assumptions reported above. A summary of the cultivation requirements is reported in Table 7.3. The reported CO_2 emission of 430 MWe plant is 41.45 kg CO_2 sec^{-1} which will require 16.3 ha of land to build a loofah biocomposite reactor based on our current work to sequester the whole CO_2 emitted. Conversely, 1,100 and 114 ha is required to sequester the same amount using the raceway pond or conventional PBR respectively. In a Natural Gas Combine Circle power plant, the energy requirement for CCS (absorption) methods is usually 17-40% higher than what is required for microalgae cultivation at solar radiation of between 12 – 28 MJ m^{-2} d^{-1} (EBTF, 2011). But the CO_2 fixation is lower when compare to the CSS. However, in this current work, the intensification procedure applied could also increase the comparative rates of CO_2 intake to be at par with non-algae carbon sequestration methods. With the recent increase in EU carbon emission tax (£16/t of emitted CO_2) (Vaughan, 2018), this technology could not have come at a better time.

Table 6. 5: Analysis of land, water and microalgae biomass utilisation for carbon capture using raceway, PBR and biocomposites at a photosynthetic efficiency of 4% and CO2 fixation rate of 40%. Values for the biocomposites were extrapolated from 6 weeks biocomposite experiment and from the data used by Rezvani et al. (2016).

Cultivation Type [a]	Land (ha)	Water Req (million L/yr)	Microalgae utilised ($kg\ m^{-3}\ yr^{-1}$)	Radiation ($MJ\ m^{-2}\ d^{-1}$)	$S–V$ m^{-1}	CO_2 fixation ($kg\ m^{-3}\ d^{-1}$)	Annual CO_2 fixation (t/a)	Annual CO_2 fixation (tonnes $ha^{-1}\ yr^{-1}$)	Reference
Raceway pond	1,100	2.84	64.24	28	4	0.40	41,832	38.03	Rezvani et al. (2016)
Photobioreactor	114	0.29	639.5	28	40	4.02	418,314	3,669.42	Rezvani et al. (2016)
Loofah Biocomposites	16.3	0.03	0.12 [b]	28	40	21.8	2,268,469	139,170	This work

*a. **Assumptions**:*

- *All biocomposites last for 6 weeks*
- *Biocomposite is replaced every 6 weeks which is 8 time per annum.*
- *Assuming plat operating days of 292.*
- *Water consumption requirement for Raceway and PBR was derived from work of Rezvani et al. (2016), while that of the biocomposites was calculated from the current work.*
- *Other assumptions are mentioned in Section 7.1*

b. This significant reduction in algae biomass has to do with resting nature of the microalgae cell as they are not dividing due to the immobilisation unlike the raceway and PBR cultivation systems, which record significant increase in biomass.

The economic analysis takes into account selling the algae biomass for either biofuel or HVP as well as carbon avoidance credit receive from the government. All economic comparison are made using a reference NGCC power plant without any carbon capture. In the raceway cultivation scenario, the microalgae biomass had to be sold at the price of £366/t to have a breakeven selling price (£230-£380 could be a good selling price for biofuel). A CO_2 avoidance of around £33/t CO_2 is assumed and can significantly improve the economics of the process. In the case of a normal photobioreactor, the algae biomass price should approach the range of (£450-£800 /t) to be competitive with the base case scenario. Adding the CO_2 avoidance cost of around £67/t CO_2, will increase the economics of the process and make the investment worthwhile. With intensification of the PBR via the biocomposites development route, we tend to see improvement in the overall process ranging from low land/water requirement and significant reduction in energy usage due to lack of mixing, frequent harvesting and drying. A fivefold improvement is generally assumed based on the CO_2 fixation calculated and compared with the normal PBR (Table 7.3). The fivefold improvement assumption could mean that, even without selling the algae biomass (which in our case is lower than raceway and PBR due to non-growing cells) we could have a breakeven investment similar to those that could be realised

by the power plant without carbon capture. Selling the attached biomass for high value product (such as β-carotene, with a selling prices above £400/kg) could add additional income to the whole business model.

6.4.3 Using microalgae biomass as high value product

To make microalgae carbon capture and utilisation a reality, the overall cost related to land areas, water usage and energy has to be reduced. At the moment algae cultivation for CO_2 sequestration, be it open pond or PBR, is more expensive than the current carbon capture technology such as the physical and chemical absorption methods. However, the cost can be improved if algae biomass can be further utilised or sold as a high value commodity (pharmaceutical, nutraceutical and food supplement). Chisti (2007), suggests that for economic production of biofuel, microalgae biomass has to be sold below £300/t. It is difficult to achieve this number in our current work due to lack of high biomass productivity, but it can be sold for other high value uses that could significantly increase the biomass price. Current market price of product derived from microalgae biomass exceeds those for the biofuel, for example the price of astaxanthin is £2000/kg and £80,000/kg for the low and nutraceutical grade respectively (Lorenz and Cysewski, 2000; Olaizola, 2003). The price of β-carotene is around £700/kg and significant amount (up to 10 % DW) of this product can be produce by *D. salina,* which happens to have the highest CO_2 mitigation among the microalgae tested in this study. These prices are higher than the estimates used for biofuel biomass in this analysis.

There is a promising economic potential of using the current work to remove CO_2 not only from point emission sources but from the ambient environment. There are several start up and pilot scale projects directed toward carbon capture from the atmosphere. A recent report by Carbon Engineering, suggested removing CO_2 from the atmosphere at around £76/t as against the current average of £460 using their pilot plant located in British Columbia (Keith *et al.*, 2018). Similar work is being tested by Climeworks, Zurich, where CO_2 is pulled out from the ambient air (Fig 7.1), concentrated and sold at £460/t with a capacity to capture 900 t of CO_2 per annum (Matt, 2018). Learning from these (and with almost 99% CO_2 capture from the atmosphere), a microalgae biocomposite could be fabricated and arranged in a transparent material to capture CO_2 from the ambient environment. The captured CO_2 could be locked into the loofah fabric and converted to bio-plastic for long-term storage (Kestur, 2010; Masmoudi *et al.*, 2016; Saffian *et al.*, 2016).

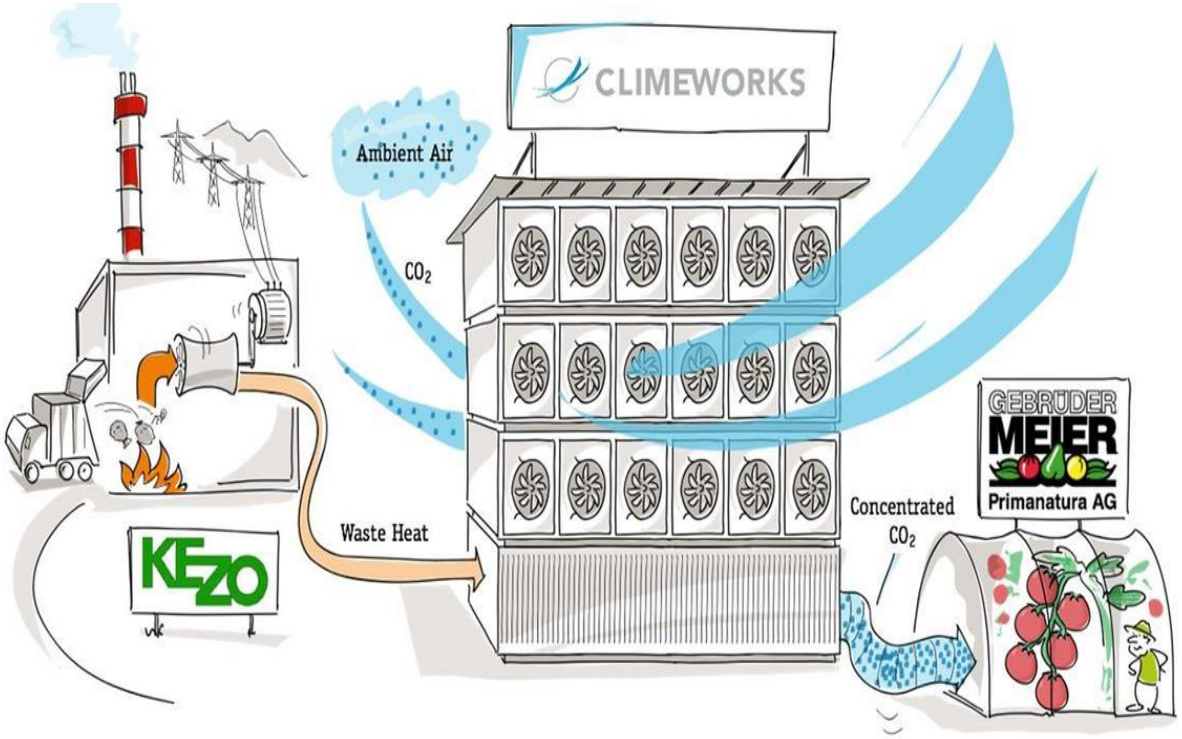


Figure 6.17: Integration of direct air capture system to use waste heat from power plant and release concentrated CO_2 for use by plant in green house (Climework System).

6.5 Summary

The cost, photo-efficiency, and active half-life of a biological CO_2 sequestration option will ultimately be the determining factor for any method that will be employed for CO_2 abatement in the long run. Additional value added products that could be derived from the process will further lower the overall cost of CO_2 mitigation. Here, we report stable CO_2 fixation by macro and nano-porous latex coatings of different species of fresh and marine microalgae immobilised onto loofah sponge for a period of six weeks. Cells remained photo-reactive over this time without a requirement for growth. Data from the continuous CO_2 reactivity indicated higher CO_2 consumption by the immobilised cells showing a superior coating technique compared to the batch pipette procedure.

Loofah sponge is an inexpensive, flexible, highly porous substrate, supporting latex coatings of photoreactive microalgae in the gas-phase to function as biosolar absorbers or biomimetic leaves, absorbing CO_2 and producing O_2. We coated three different microalgae strains with diverse cell wall characteristics and demonstrated that this simple method is capable of intensifying up to 15 fold the specific CO_2 fixation compared to suspension cultivation. This level of photoreactivity encourages further development of methods to concentrate and coat multiple organisms together to fabricate model highly reactive leaf-like biocomposites with a flexible microfluidic vascular system.

The high lipid content reported for the immobilised cells also gives impetus to further investigate the physiological changes that take place after latex entrapment onto the loofah surface. This coating method can be used to fabricate multi-organism coatings that harvest light in a broader region of the light spectrum, or to fabricate inexpensive cellular composite devices that require little more than water and light to sustain long-term photoreactivity with a subsequent release and harvesting of high-value products that could offset some financial burdens.

Loofah-based devices have been reported for water filtration and heavy metal removal (Saeed and Iqbal, 2013). A loofah-latex matrix that does not block the pores may further improve use of loofah as a cost-effective nonwoven porous material to support cellular biocomposites. We envisage the use of this versatile inexpensive material as the next step towards the creation of durable cellular composite material with flexible applications as intensified cellular biosensors, biocatalysts and highly reactive gas or liquid biofilters. The open pore structure presents a wide range of possibilities for fabricating nonwoven materials that combine photoreactive microorganisms with a porous substrate that provides structural support, a pore network for nutrient and product transport, waste removal, and also functions as a separation device for products secreted by the cells.

Biocomposites are emerging and promising technologies (Gosse *et al.*, 2012). To our knowledge, there has not been any attempt to immobilise microorganisms on loofah using binders, which support and maintain cells for long periods of time while maintaining CO_2 reactivity. This adds to the list of loofah functionality. There are coatings and inks that contain enzymes, biocides or other photoreactive biomolecules, and microcrystalline cellulose but are not photosynthetic compliant (Gosse *et al.*, 2012). Only intact cells have these functions in the context of a biomimetic matrix built around the cells and custom-tailored to intensify their reactivity and CO_2 fixation potentials.

Chapter 7

Conclusion and future direction

7.1 Thesis summary

In this research the fabrication and utilisation of biocomposite for improved CO_2 fixation and lipid production by microalgae was demonstrated. The major conclusion that can be drawn from this work is that, process intensification and miniaturisation of an algae cultivation system can overcome the challenges of land and water requirement for open pond and photobioreactor algae cultivation as well as improving mass transfer within the culture medium. These aforementioned challenges were among the issues responsible for slow deployment of biological carbon capture methods as an alternative to the current chemical and geological systems.

A full literature review was conducted into the current methods of carbon mitigation strategies employed at different levels to understand the methods used and their setbacks. Traditional algae cultivation and lipid extraction was also looked into with the aim of finding a better and more efficient system that will not only improve the economics but also total lipid yield. Gaps within the research field were noted which form the basis of the microalgae immobilisation using support matrixes to improve CO_2 absorption. Prior to that, chemical decontamination processes were employed to separate ciliates from microalgae cultures using the foam column. Thereafter, microalgae cell immobilisation on filter paper was done to assess the viability of living cells within the gas phase of a bioreactor. Lesson learnt from the paper immobilisation prompted the need for a screening procedure for binders and substrate that later dovetailed into production of biocomposite material using loofah for carbon capture and utilisation. The promising conclusions gained from this research are summarised into four main sections based on the thesis chapters as follows.

7.1.1 Chemical decontamination of algae culture using foam column

This chapter extensively looked at the use and employment of foam floatation to remove grazers and predators attacking and destroying microalgae cultures with the aid of surfactants that could be connected to either open pond or closed photobioreactor system. Hypothesising from the interaction between anionic and cationic particles to attract each other, we proposed that anionic SDS could be used to collect and recover ciliates which were covered by positively charged cations due to the presence of Ca^{2+} and K^{+} ions. This is the first work conducted in this field

where chemicals were used, recovered via foam floatation and reused to kill ciliates within microalgae cultures without destroying the algae cells. High concentration of SDS sufficient to lyse and kill ciliates but safe for the treated microalgae culture was employed for *C. vulgaris* cultures. The effect of SDS concentration on the ciliates and the effect of air flow rates on the recovery and reuse of the SDS was investigated. When compared with other chemical control methods of microalgae decontamination this current work offers cost saving benefits and can be integrated into both open pond and close photobiorector designs.

7.1.2 Microalgae deposition and maintenance on a filter paper

The hope of using microalgae in carbon capture and utilisation cannot be realised without a practical solution to reduce land requirement and water usage in microalgae cultivation. Another pertinent issue that needs urgent solutions is the mass transfer within algae culture medium. Photobioreactors and hydrogels fall short of addressing these problems. In this chapter, we demonstrated the possibility of growing and maintaining different species of microalgae on filter paper for an extended period of time compared to the suspended culture cultivation. Three parameters were employed in determining the efficiency of microalgae immobilisation on a solid substrate (filter paper), they include; the chlorophyll excitation measurement, cell growth and carbon dioxide assimilation. These parameters evaluation were also extended to the traditional suspension microalgae cultivation system.

The three species of algae (*C. vulgaris, D. salina* and *N. oculata*) used for the immobilisation procedure showed relatively higher carbon fixation compared to the suspended culture. The improved carbon capture by the cells was attributed to mass transfer improvement at the gas phase of the bioreactor where the gas had direct contact with the cells. There was also a slight increase in cell numbers for *C. vulgaris* due to cell outgrowth. *N. oculata* remained stable while *D. salina* lost most of its biomass. These values were also corroborated with the chlorophyll excitation measurement which shows a similar pattern with the cell mass to a certain extent.

Accumulation of lipid was also observed in two of the immobilised microalgae (*C. vulgaris* and *N. oculate*), while *D. salina* had significant reduction in lipid content when compared with the suspension cultivation system. While *C. vulgaris* has a relatively moderate CO_2 consumption *D. salina* has higher carbon assimilation in respect to their immobilised cells. The low lipid content of *D. salina* was somewhat compensated with the production of high level of B-carotenoid, which sustain the cells under unfavourable cultivation condition. High lipid

accumulated by *N. oculate* and *C. vulgaris* could be harnessed and converted to other useful products such as biofuel via transesterification process.

Thus, we demonstrated the extended cultivation of algae on the gas phase of a bioreactor with improve carbon capture, high lipid production and substantial reduction in water usage, land requirement and cost for nutrients. Additionally, we were able to extent the cultivation period of the immobilised cells as compared to the suspension cultivation by almost 4 weeks. However, the promising results obtained from this work was marred by the lack of strong adhesion of the immobilised cells with the filter paper and possibility of cell outgrowth which could lead to fouling and blockage of reactors. This drawback necessitated the search for a solution to the problem.

7.1.3 Screening protocol for binder toxicity and adhesion on microalgae

The challenges experienced during algae immobilisation on filter paper gave rise to a screening protocol that was applied to microbes for effective immobilisation and desiccation tolerance. Thus, Chapter 5 deals exclusively with the screening of different types of support materials (paper, cotton and loofah), binders (organic and inorganic) and microalgae (freshwater and marine) species. The result from the screening exercise was applied to produce a highly reactive biocomposite that can be used for improved carbon capture.

Four organic (alginate, carrageenan, pectin and carboxymethyl cellulose) and inorganic (acrylic latex emulsion) binders were initially screened for their toxic effect of the cells of freshwater microalgae (*C. reinhartii* and *C. vulgaris*) and marine algae species (*D. salina, N. oculata* and *T. suecica*). Visual inspection and cell counts were used to eliminate toxic binders after 7 days of cultivation under natural sunlight, with suspended culture as a control. The result indicates a sustainable preservation of freshwater algae with the binders as compared with marine species. *D. salina* had the lowest growth with the binders due to its lack of cell wall. Safe binders were subsequently used for adhesion tests on the selected solid substrates.

Three substrates (paper, cotton and loofah) were chosen based on their design, texture and porosity. The paper is a flat open surface material suitable for the drawdown coating technique which can ensure uniform cell deposition over a wide area but lacks rigidity. The cotton is similar to the paper but less densely packed than paper, which is suitable for the pipetting coating technique. Loofah is a tough, rigid, 3D structured material good for equilibrium coating,

has the ability for higher cell loading and enough porosity of liquid and gas diffusion. All the substrates are environmentally friendly and biodegradable.

Finally, an adhesion test was done to rank non-toxic binders based on their adhesion affinity with the substrates and with the immobilised cells. Drawdown, pipetting and equilibrium coating techniques were used to immobilise algae cells on paper, cotton and loofah respectively. The formed biocomposites were dried under control environments and the binder ranking was done by counting the number of cells freed from the substrates after 1, 24, 48 and 72 h shaking test at 80RPM. Poor adhesion was observed with marine microalgae possibly due to the presence high salt concentration. Artificial sea water was thus premixed with the non-toxic binders to improve the adhesion strength of the marine algae biocomposite.

SEM images were obtained to see the distribution of the immobilised cells on the substrates. Viability of cell on all the tested substrates was also attributed to the generated porosity created by the substrate/binder/cell interaction as visualised by SEM and microscope. Cell loading and binder volume also plays a critical role in determining the biocomposites porosity which in turn help in improved cell viability as a result of CO_2/light and nutrient diffusion

A simple and fast screening protocol was thus developed which can be applied easily to screen microbes for adhesion and biocomposites fabrication with the aim of saving time and resources. Durable biocomposites can only be derived from strong supporting material which is safe for algae immobilisation and is biodegradable. Thus, loofah was chosen for subsequent CO_2 reactivity tests after the screening protocol test.

7.1.4 Carbon sequestration using microalgae biocomposites

Harnessing and integrating the results that was examined and discussed in Chapters 4 and 5, we designed a photobioreactor rig using microalgae biocomposites made from simple inexpensive materials that could continuously sequester CO_2 from an incoming gas containing 5% CO_2/air mixture. The biocomposites was sustained in the gas phase of the reactor with a minimal amount of medium. The materials used for the biocomposites fabrication were sourced locally keeping cost and availability in mind. The three major composition of the biocomposites are the microalgae, attached onto loofah sponge, using latex binders. The algae carbon fixation was greatly improved using this cultivation technique compared to the tradition cultivation method of open pond or photobioreactor where the algae cells are grown in suspension liquid medium.

Similarly, high lipid accumulation was achieved after 6 weeks of cultivation which can be extracted and converted to biofuel.

Intensifying the cultivation process resulted in the use of reduced space and low water requirement in comparison to the same amount of algae biomass grown in the traditional systems. Nutrient requirement for medium preparation and use would also be reduce. These reduction could be translated to a reduction in production and maintenance costs and a reduced algae culturing footprint. Land requirement for open ponds is usually not available near CO_2 emitting sources could then be bypassed via this improved method with higher carbon fixation efficiency. Offsetting some of the costs associated with the set up and maintenance of the system could be reduced by leveraging on the high lipid content of the immobilised cells which can be extracted and converted to biofuel via transesterification.

7.2 Challenges and future direction

7.2.1. Challenges

There are challenges encountered during these research work which if addressed can further add value and benefit from the processes employed. Some of the limitation had to do with the foam column ciliate separation from algae culture and the others from the continuous carbon sequestration process.

7.2.1.1 The foam column separation

Some limitations were observed during the ciliates harvesting via foam floatation and SDS. SDS does not foam with seawater due to the presence of metals like Na. Finding a suitable surfactant that can be used for both freshwater and marine algae would make the process more encompassing. Additionally, only *C. vulgaris* which has a moderate cell wall and *T. pyriformis* ciliates were tested with the foam column - there is a need to test other freshwater microalgae species and different types of grazers to add to the data obtained by our work.

7.3.1.2 Decreasing biocomposite photosynthetic efficiency due to limited light penetration

The blue top cover of the bioreactor hindered light penetration reaching the top part of the biocomposites. This caused the cells to photo-bleach and lose their photosynthetic capacity. Cell death may occur after some time with associated release of CO_2 which will add to the outlet CO_2 concentration. The polypropylene tube also cast shadows at the side of the bioreactor close

to it, thus the bottle needs to be constantly rotated to avoid cell shading. The rotating would also mean not only one side it constantly exposed to high light irradiation that will cause photo-inhibition. Addressing these issues by having a transparent reactor cover for light penetration and adding a source of light that can ensure uniform light distribution would enhance the immobilised cell metabolic activities and increase CO_2 fixation efficiency.

7.2.1.3 Environmental concerns due to use of latex binders

Even though the entire constituents of the binders used in the research work was not disclosed, the general believe is that latex binders are not biodegradable and could be toxic when used in high concentration, which could lead to environmental concerns. The major constituent of commercial latex binders is acrylic or vinyl acrylic that is derived from acrylic or methylacrylic acids. Acrylic and methylacrylic acids are safe at low concentration while constant high exposure can cause pulmonary oedema. Before this work is put into pilot or commercial scale production, the total latex constituent must be known and if it contains any harmful VOCs the binders should be substituted with a safer replacement.

7.2.2 Future direction

7.2.2.1. Improved rig design

At the end of the 6 weeks carbon capture experiment, two reactors began a slow contamination which could be attributed to frequent opening of the bottles for medium refreshment. Designing a sealed reactor (Fig 7.2), that sustains the cells via periodic liquid flow will limit the chances of microbial contamination. Future biocomposite rig design will take into account the nature of the material used for the rig construction, which would be transparent all round with source of light equidistance from each biocomposites unit. This concept will eliminate light shading and subsequent cell death. Another improvement would be to have a rig that allows for a continues flow of CO2 and medium without the need for opening the encasement. Figure 7.2 clearly shows a proposed design which would allow for CO_2/ medium injection from one end of the rig and collected from the other end. This would minimise the biocomposites exposure to the environmental elements and thus reduced chances of contamination. This proposed design would also allow for both CO_2 and medium to be recycle and reused until exhausted.

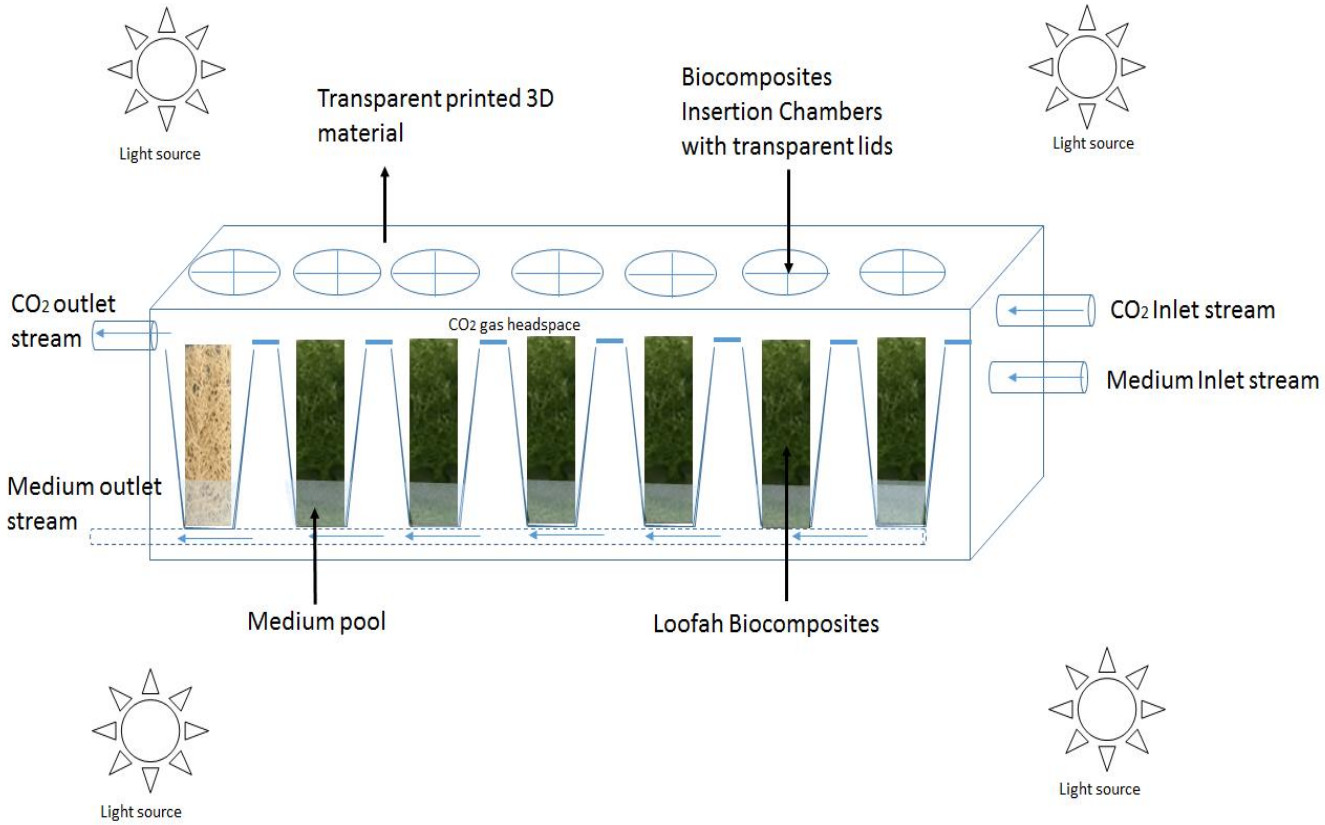


Figure 7.2: Proposed design for microalgae biocomposites rig for continues carbon capture and utilisation. Strip of microalgae biocomposite made from loofah sponge would be arranged to maximise solar light harvesting from all directions, with periodic CO_2/liquid flow from one direction and collected from another. This enhanced designed would eliminate the bioreactor bottles used in this experiment which requires frequent opening for media replenishment.

7.2.2.2. Substituting latex with sustainable, environmentally friendly binders.

Environmentally friendly biolatex binders and cyanobacteria are currently been screened by Pichaya (Warm) In-Na as a possible substitute for the current non-biolatex binders used in this research work. Promising results are been obtained for both adhesion and CO_2 reactivity with loofah immobilised cells. A breakthrough in this screening work will further push the acceptability of this CO_2 biomitigation concept by an environmentally friendly biocomposite material. Additionally, a proposed continuous flow bioreactor is being designed by Dr Jonathan Lee to address the light and medium distribution challenges earlier mentioned.

7.2.2.3. BET surface area measurement

One of the promising features for the loofah sponge when compared to other 3D materials such as polyurethane is its high surface area to volume ration, (up to 70 %) which could accommodate high cell loading. During the course of this research work loofah mass was used to determine the amount of immobilised algae cell within the biocomposites. This method was however not so efficient due to the loofah/ algae cell weight difference that could be prone to

scale error. Employing the Brunauer, Emmett and Teller (BET) surface measurement to obtain the loofah sponge surface area per gram could have been a better a way of quantifying and measuring the amount of immobilised microalgae cells in relation to the loofah surface area, weight and or volume. BET analysis is the best and idealised method of measuring porous materials (such as the loofah sponge) surface area using Nitrogen adsorption isotherm. The basic principle had to do with physical adsorption of gaseous molecules on a solid substrate. The gases employed for BET analysis had to be inert to the solid material been measured and Nitrogen is the most common gases employed for the procedure. BET analysis is usually conducted at the boiling temperature of N_2 (77 k). Other gases that could be used for BET analysis include steam, argon and carbon dioxide. BET has been employed for the measurement of various materials surface areas such as cement, activated carbon and catalysis, thus can be employed to measure the loofah sponge surface area.

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Appendix 1

1.1 Calibration curves wet quantification method for microalgae macromolecular components.

Bovine serum albumin (BSA) and glucose were used as the standard to obtain calibration curves for protein and carbohydrates respectively for the traditional quantification method for microalgae. Figures A1.1a, A1.1b were the calibration curves used for the quantification of microalgae protein and carbohydrates respectively.

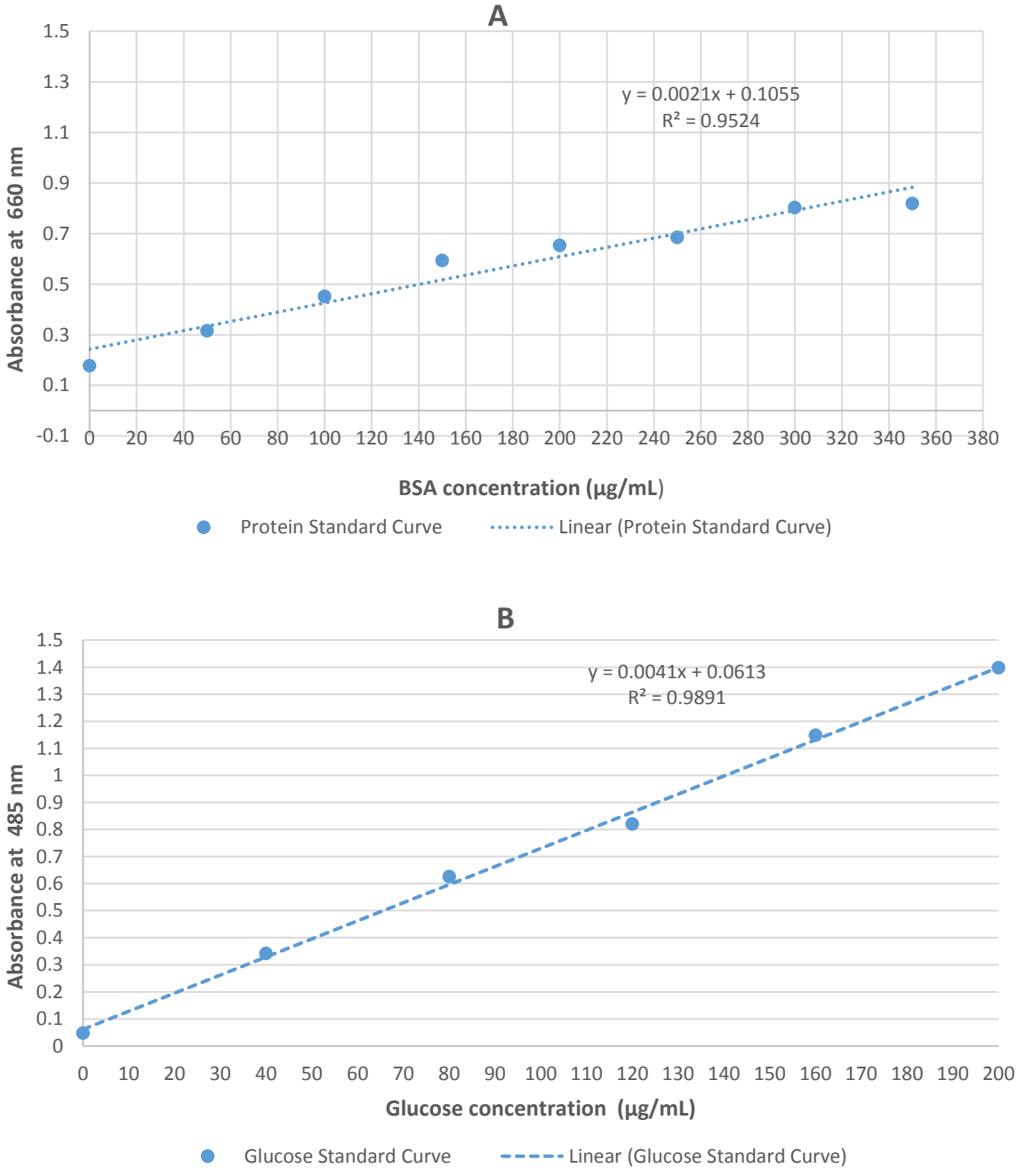


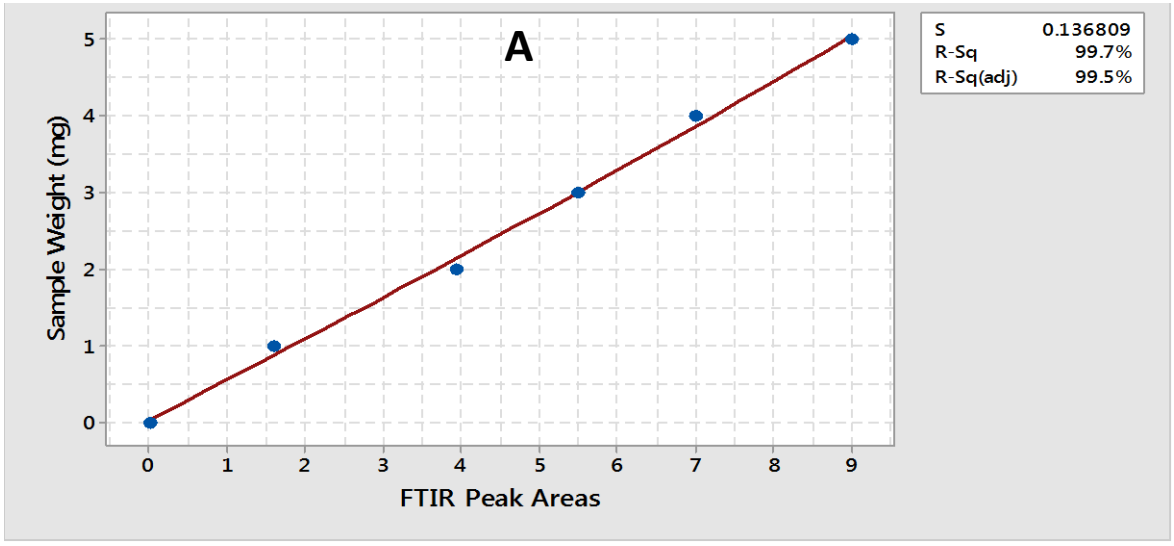
Figure A1.1: Correlation of the **A**, BSA concentration vs absorbance at 660 nm and **B**, glucose concentration vs absorbance at 485 nm for protein and carbohydrate quantification respectively.

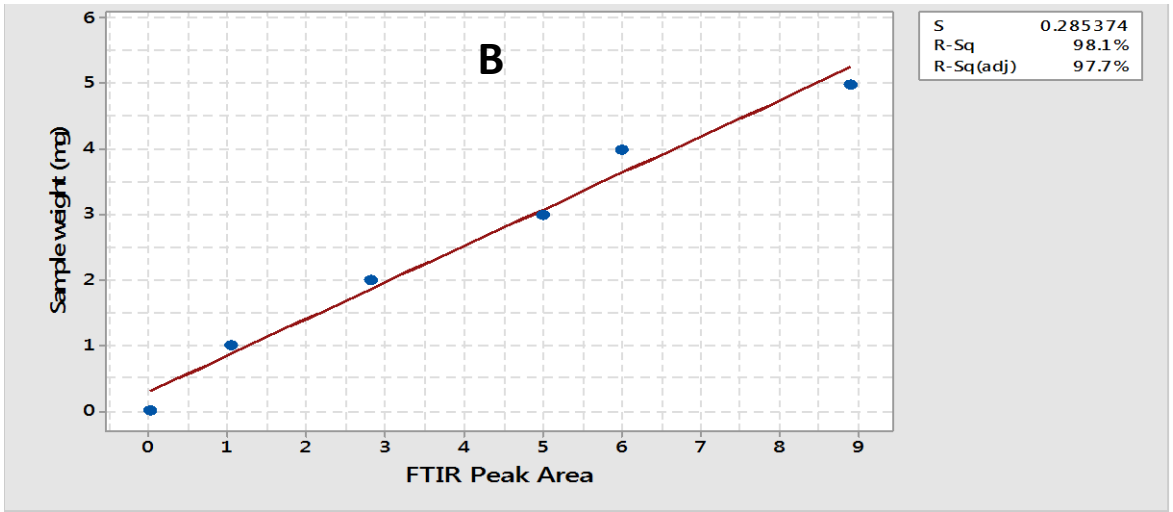
1.2 Calibration curves FTIR quantification method for microalgae macromolecular components.

Glycerol tripalmitate, glucose and BSA were used as standard for lipid, carbohydrates and protein quantification to obtain a calibration curve.

Series of samples from known weight of glycerol tripalmitate, yielded the calibration equation (Fig A1.2a) $y = 0.04 + 0.52x + 0.0042x^2$ (correlation coefficient, r = 0.982). The calibration line (Fig A1.2b), $y = 0.29 + 0.56x$ (r = 0.986) was used to determine the amount of carbohydrate present in the algae biomass. The calibration line (Fig A1.2c), $y = 0.498 + 3.59x$ (r = 0.94) was used to determine the amount of protein present in the algae biomass.

A t-test statistical analysis conducted on the regression coefficient indicates that the abscissa did not considerably deviate from zero (P>0.05). Thus, it was ignored in the above equation. In the above equation, x and y represent the content of the analyte in the biomass (mg) and the integrated area under the band respectively.





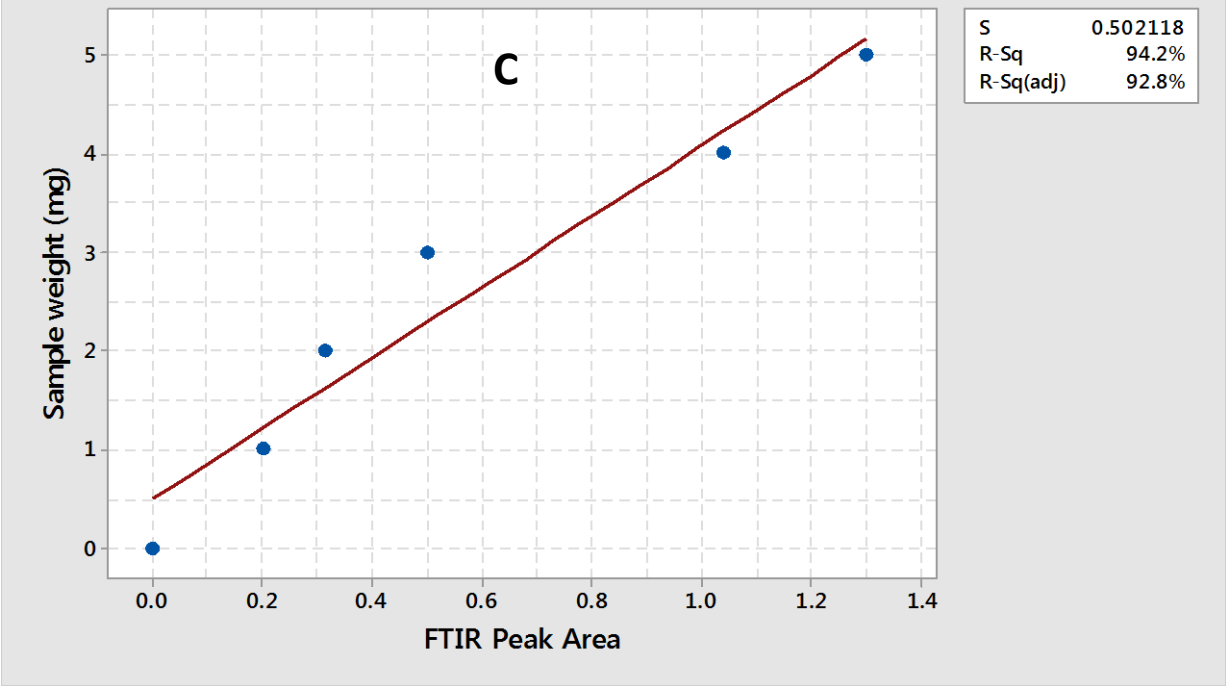


Figure A1.2: Correlation of the **A**, glycerol tripalmitate weight vs FTIR integrated area between (2800-3000 cm^{-1}), **B**, glucose weight vs FTIR integrated area between (900-1200 cm^{-1}) and **C**, BSA weight vs FTIR integrated area between (1540- 1650 cm^{-1}) for lipid, carbohydrate and protein quantification respectively.

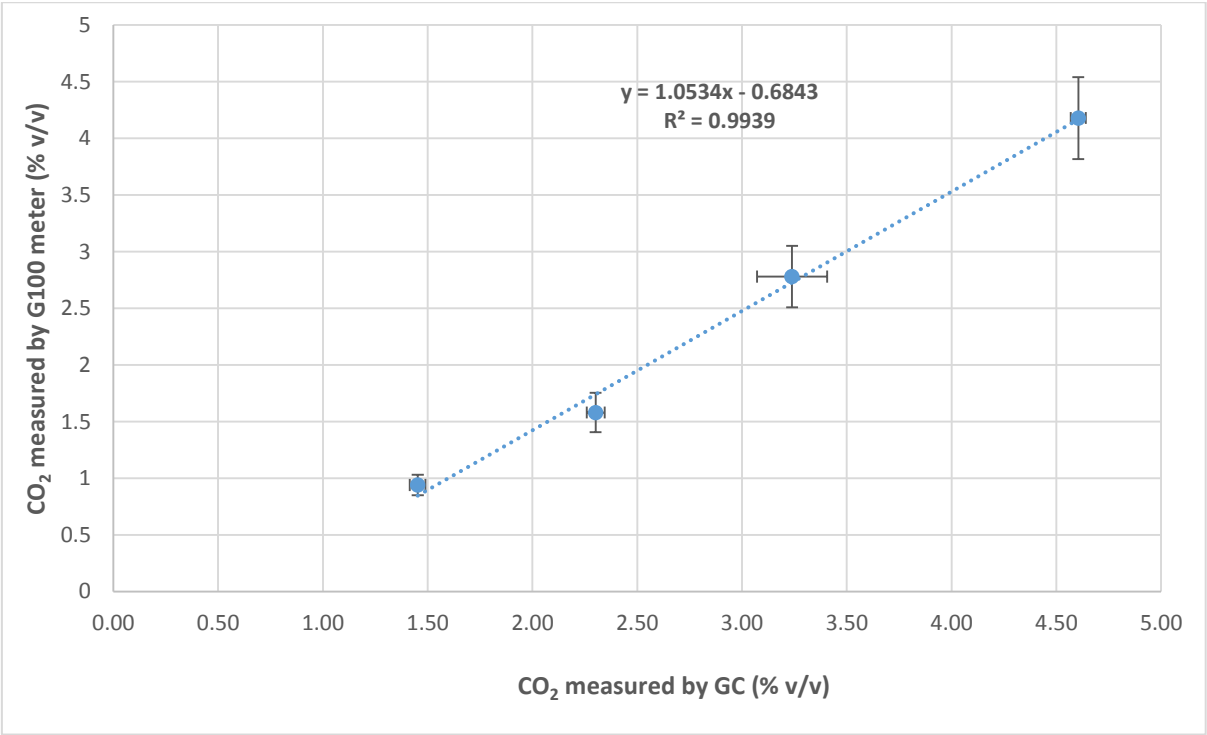


Figure A1.3: Correlation of CO_2 measurement of G100 handheld analyser vs GC.