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Influence of cohabiting bacteria on carbohydrate accumulations in *Chlorella vulgaris* CCAP 211/21A

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By

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Dedication

To my great parents whom encourage me to love continuous learning, my wonderful husband Salman for his infinite love and support, my lovely little girl Kayan who is my optimism source in this life.

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List of Abbreviations

CO_2	Carbon dioxide.
GHGs	Greenhouse gases such as carbon dioxide, nitrous oxide and methane.
f/2	Algae growth medium which contains initial concentration of 0.88 mM nitrate and 0.036 mM phosphate.
R2A	A rich growth medium used for growth of bacteria (Reasoner's 2A agar).
CFU	Colony formation unit.
v/v	Volume per volume.
ABE	Acetone–butanol–ethanol.
g l^{-1}	Gram per litre.
CCAP	The Culture Collection of Algae and Protozoa, a microalgae culture collection located in Oban, UK.
2f	A medium modified from *f/2* which its nitrate and phosphate concentration has been increased by a factor of 4.
f/4	A medium modified from *f/2* which its nitrate and phosphate concentration has been decreased by a factor of 2.
LB	A rich growth medium used for growth of bacteria (Luria broth).
OD	Optical density.
CDW	Cell dry weight.
µl	Microlitre – unit for measuring.

mg	Milligram - unit for measuring
DIN	Dissolved inorganic nitrogen.
DIP	Dissolved inorganic phosphorous.
PCR	Polymerase chain reaction
mM	Millimolar - unit for measuring
SEM	Scanning electron microscope

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Abstract

Microalgae are considered to be an ideal developmental platform for capture of CO_2 from the atmosphere to produce biomass with potential to be of value as feedstock for conversion to biofuels and bioproducts with low negative impact on the environment. Carbohydrate is an energy storage component in algae cells which can be converted to several bioproducts, including biofuels. Therefore, increasing the content and productivity of carbohydrates from microalgae becomes a significantly impactful component to consider in developing algae as a sustainable feedstock. Co-culture is one approach that has been used to increase lipids and carbohydrates. The primary hypothesis we wished to study is if we can use cohabiting bacteria associated with algae as a biotic stressor to increase carbohydrate accumulation in microalgae cells, and the specific questions we addressed are: (1) whether the concentration of bacteria introduced and the specific time point of introduction of bacteria has a role to play in increasing carbohydrate accumulations, and (2) is there a difference in influence between single and multiple bacterial types? In this project, *Chlorella vulgaris* CCAP 211/21A, a halotolerant microalga that has been shown to accumulate high carbohydrates was investigated. Cultivation of non-axenic *C. vulgaris* under nutrient replete and deplete conditions was studied aiming to increase carbohydrate accumulation. Nutrient limitations resulted in a carbohydrate yield of 47% DCW, which is 74% higher than that found in replete medium. Three cohabiting bacterial species were isolated from all tested conditions. These were identified by 16S rRNA sequence to belong to *Halomonas* 2sp. and *Muricauda* sp. Distribution of the bacterial population was influenced by nutrient depletion/repletion in algae cultures, *Halomonas* sp. WSR2 was the dominant isolate under all tested conditions. All three isolates were studied in isolation to characterise the optimal conditions for bacterial growth. Different media that contain different nutrient concentrations were tested; *f/2*+R2A was found to be a suitable medium to grow all

isolates. Moreover, the bacterial isolates were cultivated in a range of pH and temperatures. It was found that both *Halomonas* sp. grew optimally at pH 7.5 and 30°C whilst the optimal conditions for growth of *Muricauda* sp. was at pH 8.5 and a temperature of 25°C. The dominant isolate, *Halomonas* sp. WSR2, was cultivated with axenic *C. vulgaris* in co-cultures in different ratios and different inoculation time during algal cultivation. Two inoculum concentrations (1 CFU/ml and 10^4 CFU/ml) of *Halomonas* sp. WSR2 were introduced separately into the algae culture during the start of cultivation. This resulted in doubling of algal maximum specific growth rate and a 99% increase in fold change of carbohydrate yield, for a bacterial concentration of 10^4 CFU/ml, compared to control axenic cultures. Introducing the same concentration of bacteria on day 2 of algae cultivation (beginning of stationary phase) resulted in a 82% increase in fold change of carbohydrate yield. However, 175% increase in maximum carbohydrate productivity could be achieved with the addition of 1 CFU/ml. In addition, mixed bacterial species (*Halomonas* sp. WSR2: *Halomonas* sp. WS1: Muricauda sp. WSR) have also been cultivated with algae in two different ratios at the start of cultivation that both showed a doubling of growth rate of the algae. We conclude that introducing single type of bacteria on the lag phase (day 0) or at the beginning of stationary phase (day 2) in high concentration (10^4 CFU/ml) resulted in high carbohydrate yield whilst adding small concentration (1 CFU/ml) on day 2 achieved high carbohydrate productivity compared to control. These findings could increase chances for using co-culture of *C. vulgaris* with *Halomonas* sp. on a large scale for biofuels and bioproducts production.

Chapter 1 Introduction

1.1 Background

Global warming and climate change are matters of concern that are attributed to increasing levels of CO_2 in the atmosphere. There is now a trend around the world to replace fossil fuels with renewable and sustainable sources of energy to mitigate CO_2 emissions in order to protect the environment and reduce the energy cost for using renewable sources, as fossil fuel is limited. Renewable energy sources including solar, hydroelectric, wind, geothermal and biomass power could contribute to a decrease in CO_2 and other greenhouse gas (GHG) emissions, reduce negative environmental impact, secure renewable energy sources and achieve environmental sustainability. Microalgae and another phototrophic microorganisms provide an ideal platform that can be developed to mitigate CO_2 biologically through photosynthesis and to produce biomass energy that can be converted into biofuels and bioproducts. Therefore, biofuel is a promising replacement energy source that could eventually account for 40% of the world's total energy consumption and reduce GHG emissions from the transport sector, which is responsible for about a quarter of such emissions in the European Union (Andersson and Börjesson, 2021).

Despite efforts to replace fuel-dependent vehicles with battery operated ones, heavy duty vehicles for terrestrial, air and marine transport still rely heavily on the availability of fuel. Thus, biofuels could be an efficient replacement for fossil fuels in order to meet energy demand and mitigate the problem of CO_2 emissions from fossil fuels if they can be produced through net carbon neutral or carbon negative processes (Khan et al., 2018; Xie et al., 2016; Ullah et al., 2014). Since the final decades of the twentieth century, there has been considerable interest in the production and use of liquid biofuels such as bioethanol and biodiesel. Both of these

have made their way to the pumps and are currently being used, albeit in a blended form with fossil fuels (e.g., E5 and B15 in Europe). Biobutanol has also been investigated as an alternative biofuel but has not made the commercial leap to the extent that bioethanol has, due to challenges with process economics (Abo et al., 2019).

Algal biomass plays a significant role in protecting the environment by recycling CO_2. It absorbs CO_2 and stores it in a carbon form (as sugar, starch or oil) that could be treated and converted into biofuels. It would burn in vehicles to release CO_2, in turn releasing O_2 into the atmosphere (Szulczyk, 2010; Adeniyi et al., 2018; Bušić et al., 2018). Microalgae represent a third-generation feedstock that produces different types of biofuels such as bioethanol, biodiesel and biobutanol. They are a sustainable biomass source for biofuel production and are environmentally friendly due to their ability to convert CO_2 into polysaccharides and lipids via photosynthesis. Applying microalgae as a renewable feedstock would be superior to other feedstocks for biofuel production because they are able to synthesise large amounts of lipids and carbohydrates in a short period of time and without competitive land use. They can also be a renewable source cultivated under controlled conditions for the required purpose with more control on the desired profiles (Ho et al., 2013; Jiang et al., 2017; Bušić et al., 2018). In addition, the lack of lignin in the microalgal cell wall composition would enable more cost-effective pre-treatment options than for lignocellulosic biomass. For these reasons, there is currently increased attention towards algal biomass cultivation for the third generation of biofuels (Murphy et al., 2013). This project therefore studies *Chlorella vulgaris* as a energy-rich feedstock for maintaining a sustainable society with feasible solutions for mitigating CO_2 and other gas emissions.

Microalgae-sourced carbohydrate is a high-energy fermentable sugar with varying amounts of content from different species of microalgae, depending on the culture and environmental conditions (Markou et al., 2012; Rodionova et al., 2017; Cho et al., 2020). The cost of microalgae cultivation is still rising, resulting in increased substrate cost for large-scale production. Since this is a challenge for using microalgae for biofuel production, many studies have focused on applying different strategies to decrease the cultivation cost (Kumsiri et al., 2021). This project aims to address this gap by increasing carbohydrate content and productivity in *Chlorella vulgaris* cells to reduce the cost of large-scale production. Of the many approaches used for enhancing carbohydrate accumulation in algae cells, nutrient manipulation is one way to promote carbohydrate yield in them (Yeong et al., 2018). In addition, the co-culture system is another strategy to enhance the growth of algae and increase carbohydrate accumulation, although its application is still limited in biofuel production. Many factors can affect the co-culture, including inoculation ratios, inoculation time and type of partner. For example, carbohydrate content was found to increase to 78% of starch accumulation after 72 hours in *Chlorella* sp. cells when grown in co-culture with *Azospirillum brazilense* as a growth-promoting partner (Wang et al., 2017). Microalgae cultivation in co-culture has been identified as an attractive research method for controlling contamination from microalgae cultivation and for improving the production economics for biochemical components that contribute to biofuel production on a large scale (Shokrkar et al., 2017). Several examples of helpful interactions between algae and bacteria have been noted, including improving the growth of algae, protecting the algae, supporting cellular differentiation, exchanging nutrients and providing vitamins that have a key role as enzyme co-factors in cells (Helliwell et al., 2018). Furthermore, algae and bacteria cultivation describe a symbiotic relationship between the two: algae provide O_2 through the process of photosynthesis to

bacteria for mineralizing the organic nutrients, and the bacteria produce CO_2 through bacterial respiration that is used to support algae photosynthesis (Fang et al., 2017).

This project studies the influence of cohabiting bacteria on the growth of co-cultured microalgae *Chlorella vulgaris* CCAP 211/21A, and its carbohydrate content and productivity.

Chapter 2 Literature review

2.1 CO_2 problem

Increasing atmospheric CO_2 influences environmental security, as it results in climate change and global warming. Thus, many attempts have been applied to mitigate CO_2, the best solution being using a process that captures and uses CO_2. Microalgae and another auto-phototrophic organisms, such as cyanobacteria and plants, provide a promising platform for capturing CO_2 from the atmosphere and using it through photosynthesis to produce biomass containing valuable molecules that can be converted into biofuels and other bioproducts with fewer negative environmental impacts. In particular, microalgae are superior to other phototrophic organisms because of their distinct advantages, the most prominent of which is that microalgae can capture a large amount of atmospheric CO_2 that is 10-50% greater than that of terrestrial plants (3-6% of CO_2) because of their rapid growth rate and their presence in different marine and fresh environments such as sea, lakes, soil (Wang et al., 2008; Cheah et al., 2015). For example, Doucha et al., 2005 reported that when *Chlorella* sp. cultivated in a $55m^2$ culture outdoor photobioreactor, it contributed to reduce 10−50% CO_2 emissions that released of flue gas (flue gas decarbonization).

2.2 Concerns over fossil fuels usage

Rapid population growth and civilization development has led to increased demand for energy sources that are mainly produced from fossil fuels. Demand for fossil fuels is expected to reach 84% of energy demand in 2030, although they comprise a limited, non-renewable and rapidly consumed resource that is expected to be deplete by 2050 (Quintana et al., 2011; Bhagea et al., 2019; Gunathilake et al., 2019; Abdelkareem et al., 2020). The major drawback when using fossil fuels is that the burning (for heating, transport, industry and electricity), which is a major

contributor to global air pollution as a result of increasing GHGs and CO_2 concentration in the atmosphere, which is directly related to increased global warming. A 1% rise in the consumption of fossil fuels would lead to an increase in CO_2 emissions of 0.404 percentage points (Rafindadi et al., 2014). Rising GHG emissions may affect human health and threaten environmental development due to their impact on climatic change and environmental degradation, leading to growing concern about natural disasters that may occur in the future and influence, for example, global warming, droughts and floods (Naik et al., 2010; Perera, 2018; Solaymani, 2019; Yin et al., 2020). The US Energy Information Administration expects that energy-related CO_2 emissions will increase to 45 billion tons in 2040 (Vo et al., 2020).

Consumption of conventional fuels represented about 80% of primary energy globally in 2019, while the total consumption of natural gas, oil and coal as primary energy has been shown to amount to 23.9%, 32.6% and 27.2% respectively (Rezania et al., 2020; Vo et al., 2020). For example, India is considered the fourth largest energy consumer behind Russia, China and the USA, and the consumption of imported crude oil is estimated to account for about 80% of India's energy (Ramachandra and Hebbale, 2020). However, human activities over the last 150 years have contributed to an approximately 25% increase in CO_2 concentration in the atmosphere, especially from the energy and transportation sectors, which are the main sources of GHGs emissions (El-Dalatony et al., 2017; Cuellar-Bermudeza et al., 2014). The transport sector, which accounts for 29% of global total energy consumption and 65% of global consumption of oil products, is one of the biggest contributors to energy consumption and air pollution (Solaymani, 2019). Several studies have reported that ambient air pollution is the main cause of adverse health outcomes in humans, killing about 3.7 million people in 2012, according to the World Health Organization (Norhidayah and Najmuddin, 2018).

Renewable energy development has received considerable recent attention. Increasing the usage of renewable energy has come under significant focus around the world; indeed, renewables should account for two-thirds of the total energy in the world by 2050, as mentioned by the International Renewable Energy Agency (Xu et al., 2019). Renewable energy sources, including algal biomass, can contribute to reducing CO_2 emissions more efficiently compared with conventional fuels, preserve environmental sustainability and are more cost-effective (Mata et al., 2010; Phwan et al., 2018). Production of microalgae-based biofuels was initiated by the US Aquatic Species Program, which launched many renewable energy programs after the oil crisis in the early 1970s (Musa et al., 2019). For this reason, this project focuses on enhancing the use of microalgae as a renewable substrate for biofuel production that may ensure environmental sustainability with less emissions.

The electrification of vehicles has contributed to reduce GHG emissions in the transport sector. However, it is expected that production of battery electric vehicles will increase to account for 50% of global vehicles in 2050 (Kosai et al., 2022). Battery electric vehicles (BEVs) are a type of vehicle that produces no emissions. There are some manufacturing limitations with BEVs, such as there being a lack of minerals for batteries, leading to mining processes that significantly affect human toxicity. In addition, the manufacturing of batteries for BEVs can contribute to increasing GHGs by 31- 46%. Moreover, the cost of batteries and lack of widespread charging stations are a marketing challenge (Wenig et al., 2019; Andersson and Börjesson, 2021). Another challenge with BEVs is that most developing countries require time to market them and put them to use, because of which, their opportunities are limited. There is still increasing demand for biofuels produced for airplanes and the aviation sector, which contributes to global CO_2 emissions to the tune of about 2% (Lim et al., 2021). Therefore, efforts are being made in many countries to use renewable and sustainable energy sources in an economically and environmentally friendly way.

2.3 Bioenergy as a renewable energy source

Nuclear energy and fossil fuels such as natural gas, oil and coal, as non-renewable energy sources, represent the largest share of energy production. Therefore, many countries are moving progressively towards using renewable and sustainable energy resources to reduce their use of fossil fuels. Nowadays, international energy consumption is met by about 20% of sustainable and renewable energy sources (Ervural et al., 2018). Renewable energy comes from different natural sources and is replenished continuously. There are various types of renewable energy resources, such as solar energy, wind, biomass, hydro, geothermal, waves and tidal energy, that have an infinite supply or can regenerate quickly through natural processes. Thus, such resources can be environmentally more sustainable and are available worldwide. Another benefit is that developing countries can access these resources indefinitely and at a fixed cost (Alrikabi, 2014; Musa et al., 2019). Presently, there is great interest in developing renewable energy sources that originate from natural processes, especially in industrialised countries. As of 2006, 18% of total international energy consumption was from sustainable resources (Quintana et al., 2011). The development of such resources has many advantages, including increased energy production, decreased pollution and environmental sustainability. Clean energy sources such as these also have less of an adverse impact on ecosystems (Das and Mohanty, 2018). Developing sustainable energy systems that increase energy security, reduce the cost of energy and lower CO_2 emissions is considered to be an energy trilemma (Ebhota and Jen, 2020).

Besides bioenergy production from wind, solar and nuclear power, it can be also come from plants, eukaryotic microalgae and cyanobacteria, which are all organic, and materials such as food crops, forestry and biomass residues including animal waste, municipal solid waste and agricultural residues (Kolesinska et al., 2019; Senthil and Lee, 2020). An advantage of using

biomass as an energy source is that it can be applied for a variety of purposes (e.g. heating, cooking or transport); it is renewable, abundant and can be used to generate diverse energy vectors. Biomass is also a natural and abundant resource for reducing carbon and can be used in place of fossil fuels as a source of energy (Khan et al., 2015; Wang et al., 2020). The total annual amount of generated biomass is around 100 billion tons on land and 50 billion tons at sea (Abdeshahian et al., 2010). Bioenergy production can be generated from biological sources using different biomass that can be converted directly into liquid or gas fuels or to other valuable chemical products through conversion processes such as anaerobic digestion, fermentation, pyrolysis, combustion and gasification. Bioenergy is seen to be environmentally friendly, as it does not have an impact on climate change due to its carbon neutrality, sustainable resource use and ability to reduce GHG emissions (David et al., 2019; Rahpeyma and Raheb, 2019; Getachew et al., 2020).

2.4 Biofuel production from biomass as an alternative source of energy

In the 21st century, biotechnological efforts have focused on producing biofuels and bioproducts as industrial feedstocks from renewable biomass sources (Abdeshahian et al., 2010). Biomass is seen one of the largest feedstock sources of sustainable energy, and it is said that it will contribute between 15% and 50% of the world's primary energy consumption by 2050. Globally, it is considered the fourth largest form of energy after coal, oil and gas, comprising 10% of primary energy supplies (Naqvi et al., 2018; Uzoejinwa et al., 2018; Getachew et al., 2020). The production of biomass-based biofuels as an alternative source of energy will be essential because of the ever-increasing cost of fossil fuels, the growing global population, the limited availability of biofuels and the need to reduce GHG emissions.

A biofuel is defined as a liquid fuel that is derived from renewable biological resources and is used mainly in the transportation sector. Biofuels have the potential to reduce global demand

for fossil fuels, which may lead to a healthier environment by reducing GHG emissions; thus, they can decrease global warming (Abdeshahian et al., 2010; Sharma et al., 2020). Biofuels including bioethanol, biodiesel, biobutanol and biogas are promising worldwide alternative energy sources that are renewable and sustainable and are expected to grow rapidly in the future, since they are based on biological resources. The most common biofuels are biodiesel and bioethanol, which accounted for 62% and 17.5% of biofuel consumption in the EU in 2018 (Shah et al., 2018; Ashani et al., 2020; Puricelli et al., 2021). Table 2.1 shows the properties of different types of biofuel compared with gasoline. Biofuels are usually naturally sustainable, accessible, available from renewable sources and are expected to increase in share to account for 25% of production by 2024 (Bórawski et al., 2019; Rezania et al., 2020). The European Union intended to increase biofuel production to replace 10% of transport fuel consumption by 2020, while global energy consumption is expected to increase by around 60% by 2030 (Saito, 2010; Stattman et al., 2018).

Biomass-based energy is an attractive approach for reducing GHG emissions and replacing petroleum fuel with biofuels with similar features. The big difference between biofuels and petroleum fuel is that biofuels have a high oxygen content (from 10% to 45% of oxygen level), whereas petroleum fuel does not contain any oxygen; thus, there are differences in the chemical properties associated with their storage, transportation and combustion (Bórawski et al., 2019). Non-toxicity, renewability and biological degradability are among the advantages of using biofuels. Consequently, biomass is considered a better source of alternative energy because it can be extracted from renewable sources, thereby contributing to economic development and environmental stability (Hossain et al., 2008). Moreover, biofuel production can create local employment opportunities while decreasing CO_2 emissions (Garcìa et al., 2011; Narchonai et al., 2020). Since the CO_2 released during biomass combustion is equal to that consumed during the photosynthesis process, it can be considered to be a carbon-neutral process.

Table 2.1 Properties of biofuels compared with gasoline (Kasmuri et al., 2017; Kolesinska et al., 2019; Pugazhendhi et al., 2019; Iliev, 2021)

Property	Methanol	Ethanol	Butanol	Gasoline
Chemical formula	CH_3OH	C_2H_5OH	C_4H_9OH	C_8H_{15}
Carbon content wt.%	38	52	65	86
Volumetric energy content MJ/m^3	15871	21291	26795	31746
Energy density (MJ/l)	16	20	29.2	32
Research octane number	112	111	96	96.5
Boiling temperature (at 1 bar)	65	79	118	25-215
Vapour pressure (at 20°C)	0.13	0.059	0.064	0.25-0.45
Flash point (°C)	12	14	35	-45
Specific CO_2 emissions (g/MJ)	68.44	70.99	71.9	73.95

2.5 Feedstock for biofuel production

First-generation biofuels are originally produced from edible plants such as wheat, sugarcane, corn, rapeseed and soybean that contain high energy molecules (sugars and oils) via biochemical or thermochemical conversion processes. Although first-generation based bioethanol production is widely practised globally from different crops such as corn (U.S.), wheat and sugar beet (EU), sugarcane (Brazil) and sugarcane molasses (India), the significant negative impact of using this feedstock on food security include competition with food demand

and for resources including demand for arable land (Gasparatos et al., 2013; Lin and Lu, 2021; Rodionova et al., 2021).

The second-generation biofuel feedstocks are derived from different forms of lignocellulosic biomass (non-food crops), including bagasse, straw and the woody tissues of crop plants, which are provided continuously from agricultural waste or domestic residue (Rodionova et al., 2021). Lignocellulosic biomass is composed of complex structures (lignin, cellulose, and hemicellulose). Although the presence of carbohydrates in cellulose and hemicellulose layers (such as xylan and glucan) can support biofuels production, the conversion of the polymeric components to the monomers presents the significant limitation because of those structures are strongly linked as well as the presence of lignin which is difficult to transform lignocellulosic biomass into biofuel easily without the pre-treatment process (Gunasekaran et al., 2021). However, the key benefit of using these feedstocks is that they can be in plenty, do not compete with food supply, or require arable land.

Microalgae represent the third generation of biofuel feedstock and have the advantage of being able to grow fast (typical biomass production rates compared to plants) in a short period of time without competing for land use or food cultures. They are easy to cultivate in large quantities due to their high growth rates, and they have low impact on the environment and biodiversity (Jiang et al., 2017; Phwan et al., 2018; Getachew et al., 2020). An additional advantage of microalgae is that they have a large content of lipids, carbohydrates and proteins as high-value components. The storage carbohydrates and lipids can be converted into biofuels, whilst proteins can be converted to rich feed supplements (e.g. animal feed) (Chia et al., 2022). All these features, especially the high biochemical molecule content (carbohydrate, lipid and protein) in microalgal cells, amount to a strong argument for microalgae to be a suitable

candidate for biofuel production (Siddiki et al., 2022). The advantages and disadvantages of the different feedstock generations are summarised in Table 2.2.

Table 2.2 Different sources of feedstock generation for biofuel production (Alam et al., 2015; Aro, 2016; Jiang et al., 2017; Salama et al., 2018)

Feedstock	**Examples**	**Advantages**	**Disadvantages**
Edible food crops (**First generation**)	Corn, potatoes and sugarcane	Abundant source, in current practice	Have a negative effect on food supply around the world
Lignocellulosic biomass (**Second generation**)	Non-edible materials such as straw and forest wood	Abundant and inexpensive non-edible materials	Will require extensive land use. Separation of lignin demands advanced technologies and is expensive
Algae (**Third generation**)	Macroalgae and microalgae	Capturing CO_2 and a high accumulated amount of carbohydrates and lipids in short time following cultivation; will not be competing as much as terrestrial plants for arable land use or water.	High harvesting costs, low biomass concentration and low bio component content

2.5.1 Algae feedstock for biofuel production

Algae (macro/microalgae) are viewed as a potential sustainable feedstock source for biofuel production, the most promising solution for reducing CO_2 emissions and environmental problems (Sudhakar et al., 2018). Algae are photosynthetic micro-organisms that are cultivated using carbon dioxide and light to produce carbohydrate, lipids, proteins and pigments that can be turned into high-value products such as biofuels, biofertilizer, feed additives, food supplements, bioplastics, cosmetics and bioactive products (Chisti, 2007; Banerjee et al., 2020). Algae accumulate different types of carbohydrates, such as glycogen in cyanobacteria, starch in green algae, floridean starch in red algae and chrysolaminarin in diatoms. The most common monosaccharides in algal cells are glucose, xylose, mannose and rhamnose (Markou et al., 2012). Algae-based carbohydrates can be fermented and converted by conversions (thermochemical and biochemical) via different pathways to bio-alcohol fuel and other value products (González-Gloria et al., 2021; Nassef et al., 2021) (Fig. 2.1). At the bioenergy production level, the energy produced by macroalgae from the gasification process has been estimated to be 11,000 MJ/t dry algae, while microalgae have produced 9500 MJ/t of biomass, according to a Life Cycle Assessment (Chen et al., 2015).

Macroalgae were first examined as a natural and renewable feedstock in 1973. This feedstock is rich in carbohydrates but low in lipids; it has polysaccharide content of around 25-60%, which can differ according to season, geographical location and many ecological factors (Kumar et al., 2016; González-Gloria et al., 2021). The most common macroalgae species used for biofuel production are *Ulva*, *Laminaria*, *Sargassum*, *Gracilaria* and *Gelidium* (González-Gloria et al., 2021). The challenge with using macroalgae for biofuel production is that they accumulate complex polysaccharides such as cellulose, glucans, galactan, mannitol and laminarin that microbes fail to metabolize, leading them to be less cost-effective for biofuel

production compared with microalgae-based carbohydrates (Øverland et al., 2019). Therefore, microalgae have received significant attention as a potentially sustainable source of biofuel production because of the simpler processes required for their cultivation and their ability to produce up to 20 times more oil than that produced from food crops, which is estimated to be about 10,000 L/hectare/year of annual production, and it is easier to convert microalgae into biofuels than it is with macroalgae (Chowdhury and Loganathan, 2019). In addition, microalgae are a rich source of valuable molecules such as carbohydrates, lipids and proteins; most microalgae species, including *Chlorella*, *Scenedesmus* and *Chlamydomonas* accumulate high levels of carbohydrates in their cells (up to 60%) that can be converted into biofuels (Kumar et al., 2016; Ruiz et al., 2020; Brar et al., 2021). *Chlorella vulgaris* was selected as an attractive species for this project because of its high carbohydrate content that can be converted easily into biofuels.

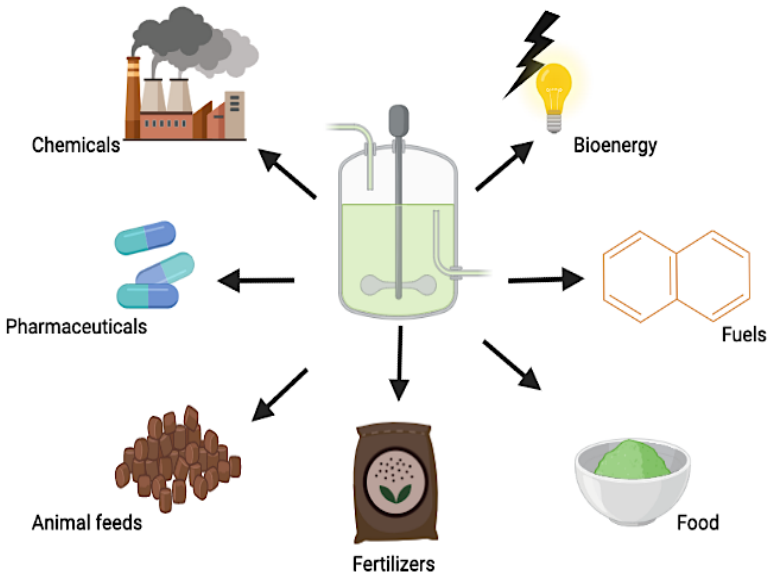

Figure 2.1 Applications of microalgae biomass.

2.5.2 Microalgae biomass as a feedstock

There has been considerable recent interest in the production of biofuels from microalgae, particularly green unicellular microalgae, as a clean and renewable source of energy that can supply natural, clean and environmentally sustainable fuels (Fig. 2.2), in contrast to fossil fuels (Pittman et al., 2011; Mata et al., 2010; Da Maia et al., 2020). Microalgae constitute a large group of species and thrive in both terrestrial and aquatic (marine, brackish and freshwater) ecosystems under a broad range of environmental conditions due to their unicellular or simple multicellular structure. Microalgae contribute to about 40% of global carbon fixed annually through photosynthesis process, which can contribute to reducing GHG emissions (Pires et al., 2012; Aratboni et al., 2019; Ma et al., 2020).

Compared with other renewable feedstocks, microalgae biomass possesses many significant benefits, including global availability, easy processing, low-cost equipment requirement, long storage periods and the consumption of organic waste materials, which in turn facilitates improved waste management. Moreover, only minor pre-treatment is needed due to the minimal presence of hemicellulose content. Microalgae consume CO_2 through photosynthesis, which reduces GHG emissions, and they can grow in both seawater, including brackish and coastal water, and wastewater, such as industrial and domestic wastewater, consequently reducing the consumption of freshwater needed for cultivation (Quintana et al., 2011; Chen et al., 2013; Yuan et al., 2020).

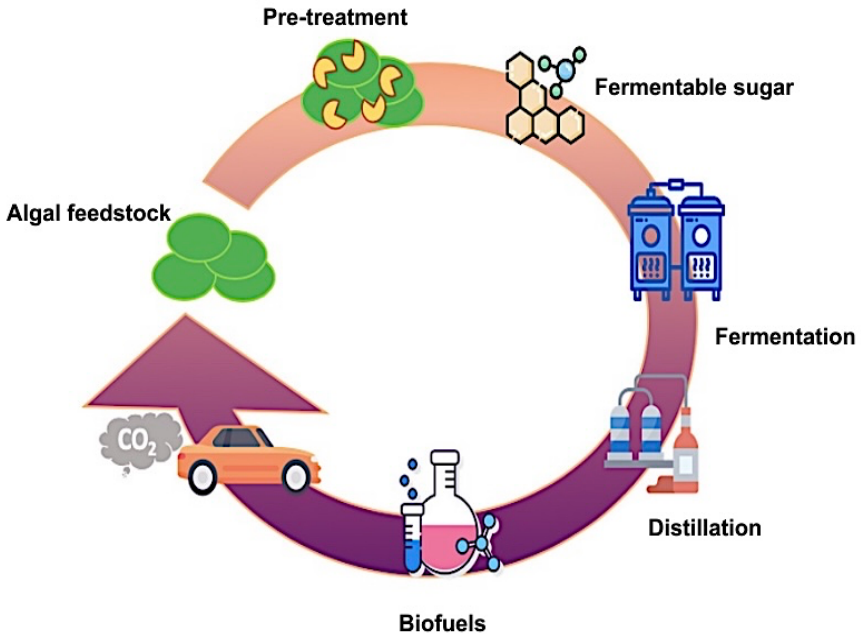

Figure 2.2 Algae as feedstock for biofuel production

Microalgae biomass can be used as a sustainable feedstock for biofuel production following the extraction of valuable chemical compounds that can be converted to either liquid or gas biofuels via different treatment processes (Fig. 2.3), including biodiesel from lipid transesterification, bioethanol via carbohydrate fermentation, biomethane by anaerobic assimilation of organic material, biohydrogen production from a fermentation or photosynthesis process and bioelectricity through cultivation in a microbial fuel cell (Jaiswal et al., 2020; Zielinski et al., 2020; Chia et al., 2022). The hydrolysis process is necessary to degrade carbohydrates into simple sugar, such as glucose, which is directly used for the fermentation process. As the pre-treatment of biomass is done earlier than the fermentation process to achieve the saccharification of the degraded algal biomass, the saccharification of the microalgae is much easier than the saccharification of the lignocellulosic biomass because

the microalgae cells do not contain lignin (Wang et al., 2016; Abo et al., 2019; Da Maia et al., 2020).

However, enzymatic and thermo-chemical hydrolysis are the most common methods used for releasing sugars from biomass (Jiang et al., 2019). The most common chemical pre-treatment method is dilute acid treatment, which uses sulphuric acid (0.1–3% v/v) in high temperatures of 120–130 °C for 15–120 min (Daroch et al., 2013; Wang et al., 2016). Microalgal cells could hydrolyse easily by dilute acid treatment to release carbohydrates with high sugar yield. This method is inexpensive and fast, but it produces inhibitors that may be toxic to a cell's growth (Jiang et al., 2019). Otherwise, enzymatic hydrolysis is a biological method that does not produce inhibitors. It is more efficient with lignocellulosic biomass, whilst enzymatic hydrolysis treatment of microalgae is not needed because microalgae cells' wall structure is completely different to that of plant cells; thus, it can be simply pre-treated. The enzymatic hydrolysis method is seen to be expensive, amounting to around 50% of the production cost, which decreases production economy (Jiang et al., 2019). In addition, external enzymes may be sources of contamination, and some fermenting-microorganisms may consume sugar immediately released by the hydrolysing enzymes (Dehghanzad et al., 2020). Moreover, it can be used for mechanical methods such as bead beating method with small glass beads, microwaves and ultra-sonication for algal biomass degradation that are usually combined with organic solvents such as chloroform, alcohols to decrease chemical use and promote the pre-treatment process (Khan et al., 2018).

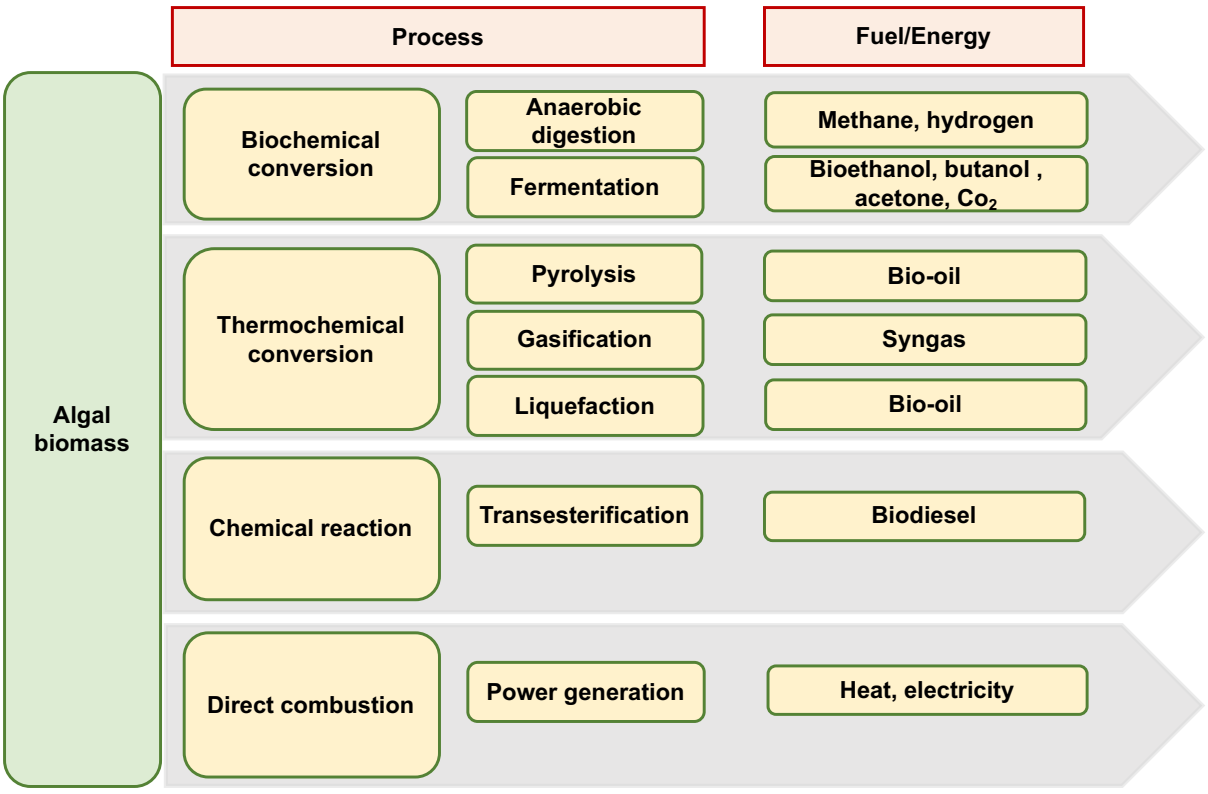

Figure 2.3 Bioenergy pathway produced from algal biomass as a sustainable feedstock.

2.5.2.1 Challenges and opportunities in alcohol production by microalgal feedstock

Feedstock complexity and the appearance of by-products can increase the cost of production and pose difficulty in terms of the purification and separation of biofuels (Shanmugam et al., 2018). The preference for biomass feedstock involves many industrial, economic and environmental factors, including raw material cost and availability. Thus, the high cost of substrate is a significant factor that affects the economic viability of large-scale alcohol fermentation. For example, some reports have demonstrated that substrate cost represents up to 79% of the cost of conventional ABE fermentation to produce solvents (Chen et al., 2013; Li et al., 2014). In addition, some microalgae contribute to increasing the production cost by producing low biomass yield, which affects economic industrial production.

The selection of a microalgae strain for the industrial-scale production of biofuels could contribute to increased production yield from algal biomass with high productivity of components under optimal conditions of growth (Tandon and Jin, 2017; Chia et al., 2022). For example, *Chlorella vulgaris* has been investigated as a potentially favourable feedstock for biofuel production because of its ability to accumulate more than 50% dry cell weight of carbohydrates in the biomass and has been viewed as a good substrate for biofuel production (Wang et al., 2016; Yeong et al., 2018; Phwan et al., 2018). Another solution to reduce the cost of microalgae feedstock is that of applying microalgal genetic engineering, and microbial metabolics also plays a key role in overcoming the limitations of biofuel production by increasing metabolic molecules. Moreover, symbiotic interactions in a co-culture of microalgae-bacteria could help enhance microalgal biomass and biochemical molecules. Several studies have noted that the co-culture of microalgae–bacteria not only promotes both microalgal and bacterial growth but may also modify the metabolism of the microalgae and bacteria to meet the other's requirement in terms of the availability of nutrients (Tandon and Jin, 2017; Yee et al., 2021). All these solutions can help either increase the biomass or carbohydrates and lipids, which could achieve high economic production. If there is interest in increasing biomass productivity and enhancing carbohydrate yield, what about increasing carbohydrate productivity on a large scale?

2.6 Microalgae-sourced carbohydrates

Among the components of microalgal cells are carbohydrates, as a result of photosynthesis. These may be either monosaccharides or polysaccharides, which are synthesised in chloroplast in eukaryotes or in cytosol in prokaryotes. Glucose is the most abundant monosaccharide carbohydrate, amounting to 67.8%, followed by galactose (16.9%) and mannose (9.1%) (Ravindran et al., 2016; Seon et al., 2020). The main sugars in *Chlorella vulgaris* are glucose

and galactose (Ma et al., 2020); however, the carbohydrates are either stored in the plastids as a reserve material (e.g. starch and glycogen) or are used in cell walls as a basic component (e.g. pectin and cellulose). Stored carbohydrates and cell wall polysaccharides of the microalgae can be converted into fermentable simple sugar for biofuel production. Microalgae-sourced carbohydrates are seen as a promising and renewable alternative substrate for replacing petroleum-based products across different applications, including the production of bioplastics, renewable energy and feed additives (Singh et al., 2019).

Microalgae have received greater attention because they are able to accumulate up to 60% carbohydrate in their cells, depending on strain type, growth mode and environmental conditions. However, several cultivation strategies have been developed to improve microalgal carbohydrate content; these include CO_2 supplementation, nitrogen starvation, pH shifts, temperature alterations and changes in light intensity (Table 2.3) (Chen et al., 2013; Singh et al., 2019).

Table 2.3 Different approaches for carbohydrate enhancement in different species of algae

Algae	Carbohydrate type	Carbohydrate content	Conditions for carbohydrate increase	Reference
Chlorella vulgaris	Starch	37–55%	Under nitrogen starvation	Chen et al. (2013)
Chlamydomonas reinhardtii UTEX 90	Starch	53%	Batch culture	Chen et al. (2013)
Scenedesmus obliquus	Glucose	46.65–51.8%	Under nitrogen starvation	Ho et al. (2012), Chen et al. (2013)
Scenedesmus obliquus CNW-N	Glucose	73–80%	High light intensity (180 μmol m^{-2} s^{-1}) and 2.5% CO_2 aeration	Ho et al. (2012)
Chlorella sp. AE10	Starch	76.89%	Under nitrogen starvation (1/8 N medium)	Yuan et al. (2018)
Chlorococcum sp. TISTR 8583	Starch	34.02%	Under nitrogen limitation	Rehman and Anal (2019)
Scenedesmus sp. CCNM 1077	-	35.91%	Salinity stress (400 mM NaCl)	Pancha et al. (2015)

2.6.1 Factors influencing the accumulation of carbohydrates

2.6.1.1 Abiotic factors

There are several factors that can affect microalgal carbohydrate content and algal growth, including environmental conditions (pH, temperature, CO_2, light intensity and the availability of nutrients), cultivation conditions and microalgal species (Juneja et al., 2013; Andreeva et al., 2021). Carbohydrate content also can be affected by cultivation time and culture conditions (Cheng et al., 2017).

2.6.1.1.1.1 pH

pH can significantly affect the algal growth and its biochemical composition because it determines CO_2 solubility and the availability of essential nutrients. In turn, the absorption and availability of some elements, such as carbon and iron, can be affected by the pH range. pH variation not only inhibits algal growth and alters the biochemical composition but can also cause cell death. However, depending on the type of algal strain, microalgae grow optimally within a specific pH range of between 7.5 and 8.5, which rises gradually in microalgae cultures during the day as a result of CO_2 consumption in the photosynthesis process (Juneja et al., 2013; Almutairi et al., 2020; Jaiswal et al., 2020).

2.6.1.1.2 Salinity

The accumulation of microalgal biomass is influenced by salinity, although numerous microalgae species can tolerate different salt concentrations. Otherwise, excess salinity influences biomass accumulation, which inhibits photosynthesis (Cheng and He, 2014). Changing the pH or salinity in an algal culture can cause changes in metabolite productivity

and cell physiology, which influences carbohydrates, lipids, proteins and pigments (Almutairi et al., 2020).

2.6.1.1.3 Nutrient availability

Microalgal cultures must include both macronutrients (phosphorus, nitrogen, sulphur and carbon) and micronutrients (iron, cobalt, copper and zinc) to ensure algae growth. The most important nutrients for the growth of microalgae and their metabolism are phosphorus and nitrogen; a deficiency in these macronutrients' availability may lead to reduced algal growth rates and biomass yields as well as cause morphological and physiological changes in microalgal cells. Limiting or neglecting macronutrients in the cultivation medium could change the metabolic pathways of microalgae and lead to a modification to their biomass composition (Markou et al. 2012). Thus, accumulation of biochemical composition (carbohydrate, lipids and protein), growth rate and the synthesis process in microalgae could be affected by changes in nitrogen concentration.

Nitrogen is a key element that can be an inherent component of proteins and the photosynthetic machinery; in consequence, its deficiency decreases the pigments of photosynthetics such as chlorophyll. However, nitrogen limitation is one of the most effective methods for enhancing carbohydrate accumulation in different green algae species because it leads to decreased protein production as a result of the excess energy absorbed being transferred to energetic reserves such as carbohydrates and lipids (Markou et al., 2012; De Farias Silva et al., 2018). For example, *Chlorella* sp. was shown to accumulate around 69% carbohydrate content in its cells under nitrogen and phosphorus deficiency (Da Maia et al., 2020). There are different sources of nitrogen, such as nitrite, nitrate, urea and ammonia, which can all be consumed by algae and affect their components. Some studies have shown how much nitrogen limitation influences carbohydrate/lipid enhancement, and the reduction of algal biomass productivity

can contribute to improving biofuel production by increasing the biochemical composition (Yao et al., 2012; Cheng and He, 2014; Zarrinmehr et al., 2020).

2.6.1.1.4 Light

The main source of energy for photoautotrophic organisms is light; thus, light intensity is one of the most significant factors that influence the photosynthesis process of microalgae, which affects the growth and productivity of algae. Furthermore, various light conditions (amount of light or intensity of light and dark cycle) play an important role in the productivity of algae and the chemical composition of cultivated microalgae. For example, high light intensity has been shown to achieve a threefold increase in carbohydrate accumulation in *Porphyridium* sp., while the carbohydrate content of *Spirulina maxima* was found to increase from 7–10% to reach 34% due to high light intensity. The protein content of *C. vulgaris* also increased with rising light intensity. On the other hand, when algae have been cultivated under stress conditions, such as by using very high or low light intensities, the growth rate and productivity of algae have been seen to reduce (Markou et al., 2012; Metsoviti et al., 2020; Yuan et al., 2020).

2.6.1.1.5 Temperature

Microalgae can grow within a broad temperature range between 15°C and 35°C, although the optimal temperature range for survival is limited to 20–30°C (Serra-Maia et al., 2016). However, temperature plays a key role in the availability of nutrients in the culture and their absorption by algal cells, which affects biomass productivity, carbohydrates, lipids, proteins and phenolic compounds of algae (Da Maia et al., 2020). On the biochemical composition level, for example, the carbohydrate content has been seen to increase in *Spirulina* species by 50% when the temperature increases from 25°C to 40°C (Cheng and He, 2014). Temperature can also negatively influence algal metabolism as a result of acting on the cell enzymatic

process. However, high temperature leads to protein degradation, which causes algal cell death (Serra-Maia et al., 2016; Jaiswal et al., 2020).

2.6.1.2 Biotic factors

The growth of microalgae and its chemical composition are influenced by microalgae associated with commensal bacteria and the existence or absence of other microbes. Co-culture is a significant factor that affects the growth/productivity of algae and influences natural ecosystems. Many studies have reported natural associations between microalgae and different microbes. Artificially, microalgae have been cultivated successfully with associated bacteria, fungi and yeast, aiming for an increase in the algal growth or the enhancement of biochemical compositions (Gonçalves et al., 2016; Das et al., 2022). In this project, we focus on the influence of co-cultivation of microalgae and bacteria for enhancing carbohydrate yield and productivity. However, bacteria associations have been shown to have both positive and negative impacts on algal growth and productivity. As this project's emphasis is on the positive side of bacterial association with microalgae, symbiotic interaction between microalgae and bacteria (in co-culture) not only enhances microalgal biomass but also increases the accumulation of valuable algal components and energy molecules that are used as substrates for biofuel production. As bacteria promote algal growth by providing microalgae with CO_2, vitamins and promotor factors as well as degrading large organic molecules to be much easier to absorb, microalgae in turn ensure that there is O_2 for the bacteria survive and offer to the organic molecules (Fuentes et al., 2016; Yao et al., 2019). The co-culture system is still developing and requires more detailed investigations on specific associations to develop underlying principles and conceptual frameworks that are useful in establishing strategies to enhance productivity.

In addition, it is known in the marine environment that viruses associated with algae and cyanobacteria infect specific species and strains (Day et al., 2012). Viruses have an important role in the marine environment and provide food by releasing and recycling the nutrients of their dead hosts. They can also reprogramme the metabolism of their living host, such as through the photosynthesis process, the metabolism pathway of central carbon, nitrogen and phosphorus and inserting resistance genes in stress conditions for survival supported by gene transfer/shift, which could affect the genetic development of their hosts and influence the chemical components in algal cells (D'Adamo et al., 2021). So far, there are not enough studies on viral contribution to algal genomes, but there is evidence that viruses may help their host to adapt to various environments (Nelson et al., 2021).

Moreover, some microalgae predators cause contamination of the algal culture leading to a decrease in microalgal productivity. For example, when a *Chlorella* culture was contaminated by predatory *Poterioochromonas malhamensis*, *Chlorella* growth was seen to be reduced from 4.0×10^8 cells mL^{-1} to 1.0×10^8 cells mL^{-1}, and the growth of *p. malhamensis* was shown to increase from 1.0×10^3 cells mL^{-1} to 1.1×10^6 cells mL^{-1} in three days (Wen et al., 2021). However, Hue et al. (2018) reported that marine microalgae have chemical defence mechanisms to prevent a predator's growth; *Phaeocystis* sp. is one example that produces dimethylsulfoniopropionate (DMSP) to inhibit this.

On the other hand, because of the stress of the abiotic and biotic factors, microalgae must continually modify their biological pathways, leading to an alteration to the gene expression (epigenetic regulation). Some studies have shown that epigenetic regulation can help unicellular algae to adapt positively and enhance their tolerance against stress, although there is not much information about the changes mechanisms of the environmental response, changes in microalgae at the epigenetic level have been confirmed. Epigenetics is defined as studying

changes in gene expression that do not include any modification in DNA sequences (Bacova et al., 2020).

2.7 Microbial co-culture

2.7.1 Natural consortia

In nature, around 99% of microorganisms are in microbial consortia form, which exists in all natural ecosystems, including in food, waste, soil, water and mammalian guts (Ren and Murray, 2019; Rosero-Chasoy et al., 2020). A co-culture is defined as a biological society in which two or more different microorganisms are growing with some degree of communication between them within natural or artificial media (Rosero-Chasoy et al., 2020). In nature, microbes live together in either a commensal or a cohabiting form, both of which have positive interactions. Microbial interaction plays a considerable role in nature but is usually neglected; nevertheless, it may enhance microbial productivity so that it can be more effective. Studying cohabiting microbes is one example of neglected consortia, whereby there is not enough information about their role and behaviour with partners.

One example of natural co-culture is the culturing of anaerobic fungi and methanogen (*Piromyces* and *Methanobrevibacter ruminantium*). *Piromyces* live in the herbivore rumen because of their ability to decompose plant biomass, which is responsible for degrading lignin and lignocellulose materials, as they have a wide spectrum of fibrolytic enzymes for effective lignocellulose degradation into monosaccharides, whereas methanogen microorganisms use H_2 and CO_2 resulting from the decomposing process (Wei et al., 2017). Co-culture (consortium) can also be carried out in vitro for many purposes, such as for new drug development, the production of high-value compounds, wastewater treatment, soil bioremediation and biofuel production (Table 2.4) (Rosero-Chasoy et al., 2020). The existing interactions between

microalgae and bacteria in natural communities could be a considerable cause for the failure of microalgae isolation in vitro. Algal isolation usually involves one or more of the associated bacteria species; thus, many cultures of algae preserve the symbiotic relationship between the isolated algae and their associated bacteria (Baggesen et al. 2014; Yao et al., 2019). However, some cells cannot grow efficiently in an artificial monoculture, but the existence of another microorganism population could enhance the success of cultivation (Goers et al., 2014). Compared with a pure culture, a co-culture plays a key role in improving the productivity and efficiency of a natural microbial co-culture. Thus, a designed artificial co-culture can regulate the behaviours of a multitude of species to perform complex functions (Ren and Murray, 2019). It is important to know that the nature and dynamic of interactions between partners sometimes may change over time in a long-term consortium as a result of the influence of some environmental factors (Ghosh et al., 2016).

2.7.2 Managed co-culture

Microbial co-cultures have been successful across a broad spectrum of biotechnological applications including food production, wastewater treatment, hydrolytic processes and biofuel production. The artificial co-culture can perform multiple functions to overcome the limitations of genetic paths in single cells (Ren and Murray, 2019). Artificial microbial consortia could carry out complex functions and afford the change of environment conditions more than monoculture (Jiang et al., 2018; Lu et al., 2020). The associations in the co-cultivation system may be symbiotic, such as commensalism and mutualism, which contribute to nutritional production such as of growth promoters and phytohormones. Otherwise, some associations in the co-culture system may be harmful, such as depredation and parasitism, which are excreted alkaloids, antibiotics and toxins causing inhibition of microbial growth (Rosero-Chasoy et al., 2020). Synthetic co-cultures are more productive than pure cultures because they use resources

efficiently and those that are over-yielding (Ridley et al., 2017). For example, a co-culture of a native bacterial consortium with exogenous *Bacillus subtilis* (ratio 2:1) for crude oil degradation showed that the co-culture would degrade the crude oil by 85.01% after seven days of incubation, compared with the native bacterial consortium, which degraded 71.32% of crude oil (Tao et al., 2017). Moreover, the cultivation of *Arthrobacter* sp. NB1, *Serratia* sp. NB2, and *Stenotrophomonas* sp. NB3 (ratios 4:4:5, respectively) could enhance the nitrobenzene degradation at 400 mg/l initial concentration, which achieved twice as much degradation as pure cultivation (Jin et al., 2012). Table 2.4 shows different purposes for applying artificial co-culture systems among various microorganisms.

Table 2.4 Different purposes for applying artificial co-culture system among various microorganisms

Microorganism	Partner	Purpose of applying a co-culture	Production achievement	Reference
Bacillus cereus (***bacteria***)	*Bacillus thuringiensis* (***bacteria***)	Increasing production of alpha amylase	Maximum enzyme production 44 U/ml/min using tryptone	Abdullah et al. (2018)
Trichoderma reesei ***(fungi)***	Lactic acid bacteria (***bacteria***)	Production of lactic acid from lignocellulosic	Maximum lactic acid 34.7 gl^{-1} of production using 5% (w/w) microcrystalline cellulose.	Shahab et al. (2018)
Saccharomyces cerevisiae (***yeast***)	*Oenococcus oeni* X (***bacteria***)	Malolactic fermentation economically	Increasing consumption of malic acid by 3.7 g l^{-1}	Nehme et al. (2010)
Rhodotorula pacifica ST3411 (***yeast***)	*Cryptococcus laurentii* strain ST3412 (***yeast***)	Degradation of edible oil	Highest degradation rate 79.4% ± 13.8% of salad oil degradation in 24 h	Sugimori (2009)

Clostridium phytofermentans (***bacteria***)	*Saccharomyces cerevisiae* (***yeast***)	Ethanol production	Maximum ethanol production 22 gl^{-1} in coculture produced from 100 gl^{-1} α-cellulose	Zuroff et al. (2013)
Chlorella vulgaris ***(microalgae)***	*Rhizobium* sp. ***(bacteria)***	Wastewater treatment	Achieved increase in biomass concentration (0.63 ± 0.03 gl^{-1}) with efficient wastewater treatment	Ferro et al. (2019)
Scenedesmus sp. ***(microalgae)***	*Aspergillus niger* ***(fungi)***	Harvesting algal biomass economically	Flocculation efficiency of algal biomass enhanced to reach 99.4% after 48 h.	Pei et al. (2021)
Chlorella ellipsoidea ***(microalgae)***	*Leptolyngbya tenuis* ***(cyanobacteria)***	Biodiesel production	Maximum lipids content was 41.43 ± 0.71% with increased algal biomass yield to 3.95 ± 0.13 gl^{-1}	Satpati and Pal (2021)

2.7.3 Microalgae-based co-culture

The presence of a co-culture between microalgae/macroalgae and bacteria has been observed in natural habitats, and the growth of unicellular microalgae with bacteria is quite common (Santos and Reis, 2014; Selvarajan et al., 2019). Algae are present alongside a large group of microorganisms including bacteria, cyanobacteria, fungi and archaea in both soil and aquatic (freshwater and marine) environments. Thus, a wide range of interactions between different microorganisms in the environment is expected. Several studies have indicated that microalgae and bacteria live together in complicated microbial communities, and those communities usually carry out synergistic interactions that do not occur while there is an absence of partners (Helliwell et al., 2018; Yao et al., 2019). In addition, microalgae were inoculated artificially with a single type of bacteria (Cho et al., 2015; Marticorena et al., 2020) or with multiple species of bacteria, as reported by Han et al. (2016).

Several examples of helpful interactions between algae and bacteria have been noticed, including improving the growth of algae as well as hormonal stimulation, protecting the algae of bactericidal agents, supporting cellular differentiation, exchanging nutrients and providing vitamins that have a key role as enzyme co-factors in a cell. However, an analysis of environmental samples indicated that over 50% of the studied microalgae species required cobalamin for growth, which they would obtain from co-existing bacteria and algae in several marine ecosystems (Helliwell et al., 2018). One example of using co-culture between microalgae and bacteria is that of wastewater treatment. Co-cultures of algae with bacteria are considered an environmentally friendly method for treating wastewater because of their ability to remove nitrogen and phosphorus efficiently, compared with treatment by monoculture. Table 2.5 shows the different purposes of applying co-cultures between microalgae species and other partners. This cultivation describes a symbiotic relationship between algae and bacteria; algae provide the O_2 through a photosynthesis process to bacteria for mineralising the organic

nutrients, and the bacteria produce CO_2 through bacterial respiration that is used to support algae photosynthesis (Fang et al., 2017; Zhang et al., 2020).

Table 2.5 Co-culture microalgae and bacteria for different purposes

Microalgae	Co-partners	Associated in	Purpose of association	Reference
Chlorella sp.	*A. brasilense* (**Bacteria**)	Co-culture	Increase algal biomass, cell size and pigment, lipid content	De-Bashan et al. (2002)
Chlorella sp.	*Paramecium bursaria* (**Virus**)	Co-culture	Enhancement of lipid extraction with cost-effective disruption	Sun and Zhou (2019)
Chlorella vulgaris	*A. brasilense* (**Bacteria**)	Co-culture	Enhancement of carbohydrates and starch content	Choix et al. (2012)
Chlorella vulgaris	*A. brasilense* (**Bacteria**)	Co-culture	Enhancement of microalgal growth and pigments level	Gonzalez and Bashan (2000)

Chlorella vulgaris	*Microbacterium, Bacillus* sp. (**Bacteria**)	Consortia	Enhancement of nutrients' removal efficiency and promotion of algae growth	Xu et al. (2020)
Chlorella vulgaris	*Rhizobium* sp. (**Fungi**)	Consortia	Increasing waste removal efficiency and increasing the algae biomass	Ferro et al. (2019)
Chlorella vulgaris	*Aspergillus niger* (**Fungi**)	Co-culture	Decrease harvest costs and 90% microalgae harvesting performance achieved	Zhang and Hu (2012); Gultom et al. (2014)
Chlorella vulgaris	*Bio-flocculant-producing bacteria (Rhizobium radiobacter*) (**Bacteria**)	Co-culture	Enhancement of microalgal harvesting and lipids production	Wang et al. (2015)

Chlorella sorokiniana	*A. brasilense Cd* and *Bacillus pumilus* (**Bacteria**)	Co-culture	Enhancement of microalgal growth and increase in carbohydrates, lipids and chlorophyll a	Amavizca et al. (2017)
Chlorella sorokiniana	*Methylococcus capsulatus* (**Bacteria**)	Co-culture	Recovering and recycling nutrients from industrial wastewater	Rasouli et al. (2018)
Chlorella sorokiniana	*Rhodotorula glutinis* (**Yeast**)	Co-culture	Increasing waste removal efficiency	Das et al. (2022)
Chlorella sorokiniana	*Exiguobacterium aurantiacum, Stenotrophomonas acidaminiphila Chryseobacterium scophthalmus*	Co-culture	Removing NH_4 and PO_4 of wastewater, increasing algae biomass and promoting production of chlorophyll a + b	Qi et al. (2018)

	(**Bacteria**)			
Chlorella minutissima	*Aspergillus awamori* (**Fungi**)	Co-culture	Enhancement of biomass and lipid production	Dash and Banerjee (2017)
Nannochloropsis sp.	Maritalea porphyrae, *Labrenzia aggregate* (**Bacteria**)	Consortia	Enhancement of microalgal growth and chlorophyll level	Lian et al. (2021)
Nannochloropsis sp.	*Halomonas aquamarine* (**Bacteria**)	Consortia	Enhancement of algal biomass and lipid production	Subasankari et al. (2020)
Chlamydomonas reinhardtii	*Azotobacter chroococcum* (**Bacteria**)	Co-culture	Increasing biomass and lipid yield	Xu et al. (2018)

Scenedesmus sp.	*A. brasilense* (**Bacteria**)	Co-culture	Increasing algae biomass and lipid productivity	Contreras-Angulo et al. (2019)
Scenedesmus sp.	*Candida pimensis* (**Yeast**)	Consortia	Enhancement of biomass and metabolic production for biofuels	Suastes-Rivas et al. (2019)
Scenedesmus sp.	*Phingomonas*, *Burkholderia cepacia*, *Pseudomonas* and *Pandoraea pnomenusa* (**Bacteria**)	Co-culture and consortia	Enhancement of aromatic hydrocarbon degradation of crude oil	Tang et al. (2010)
Tetradesmus obliquus	*Piscicocus intestinalis* (**Actinomycete**)	Co-culture	Enhancement of biomass, chlorophyll a and lipid production for biofuels	Kumsiri et al. (2021)

Characium sp.	*Pseudomonas composti* (**Bacteria**)	Consortia	Enhancement of lipid production and increasing algal biomass	Berthold et al. (2019)

2.7.3.1 Commensal bacteria in algal culture

Mutualism relationships among algae and other microorganisms have a significant impact in natural environments (Yee et al., 2021). Several marine algae allow some microbial microorganisms (bacteria, protozoa, fungi, diatoms and larval forms of marine invertebrates) to live on their surfaces, but their quantity and quality vary compared with free-living bacteria in the same aquatic ecosystem (Karthick and Mohanraju, 2018; De Mesquita et al., 2019). Synergic interactions between microalgae and bacteria could ensure nutrient availability and the biogeochemical cycles in the ecosystem; however, attachment sites of bacteria on microalgae surfaces are rich in a high concentration of nutrients, compared with surrounding environments (Zhang et al., 2020). However, commensal bacteria-associated marine algae have been shown to play a considerable role on the growth of algae, their morphological development and protecting them against infectious microbes (De Mesquita et al., 2019). Microalgae-associated bacteria have also been found to promote the health of microalgal cells and seaweeds in different ways, including degradation and remineralization of organic matter to bioavailable biological elements such as N and P, synthesis of vitamins, killing of pathogens and algal predators by secretion antibiotic (Samo et al., 2018; Selvarajan et al., 2019).

The most common marine bacteria are *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Achromobacter* and *Vibrio* sp., which are known as seawater bacteria (Hamidi et al., 2019). Chegukrishnamurthi et al. (2020) reported that some bacterial species, including *Pseudomonas*, *Rhizobium*, *Sphingomonas* and *Mesorhizobium*, are associated mainly with microalgae such as *Chlamydomonas reinhardtii*, *Chlorella*, *Scenedesmus* and *Botryococcus braunii*. Furthermore, some studies have reported the positive influence between macroalgae (seaweed) and their associated microbes, such as bacteria and yeast that play a considerable role as a promising strategy in bioenergy production, pharmaceutical and biotechnological applications (Singh et

al., 2015; Sasaki et al., 2018). The surface of a seaweed provides an appropriate substratum to host other microorganisms, as it releases various organic molecules that act as nutrients for microbial multiplication (Singh and Reddy, 2014). The most commonly associated bacteria with macroalgae are classified as *Bacteroidetes*, *Proteobacteria*, *Planctomycetes*, *Actinobacteria*, *Cyanobacteria* and *Verrucomicrobia* (Mancuso et al., 2016; Florez et al., 2017). Selvarajan et al. (2019) reported that the most common bacteria on the surfaces of red and brown macroalgae are from the *Vibrio* species that was isolated from Peter the Great Bay in the Japan Sea, while *Vibrio* and *Halomonas* are the most abundant bacteria isolated from four different surfaces of seaweed species in China. However, the density of macroalgae bacteria differs from 10^2 to 10^7 cells cm^{-2}, according to the season, type of macroalgae, and thallus section (De Mesquita et al., 2019).

On the other hand, eight cohabiting bacteria were isolated from non-axenic microalgal cultures of *Botryococcus braunii* and co-cultured with the algae at an exponential phase. One of them was *Rhizobium* sp., which influenced the algal growth positively, whilst *Acinetobacter* sp. had a negative impact on algal growth at 20°C (Rivas et al., 2010). Moreover, 43 bacterial species were isolated from 16 xenic microalgae cultures, which were classified into α-proteobacteria and γ-proteobacteria, Flavobacteria and Bacteroidetes as cohabiting bacteria. *Muricauda* sp. is one species that was tested with these 16 species and showed positive influence on growth (Han et al., 2016).

2.8 Co-culture system for strategies in algal applications

2.8.1 Co-culture of microalgae and bacteria for algae growth enhancement

The co-culture system is one approach that can be used to enhance microalgae cultivation and the harvesting process (Ravindran et al., 2016). Applying the co-culture strategy has been done

since the twentieth century as an effective approach for using complex substrates to produce high-value products by activating the bioactive pathways of microorganisms' consortia in the environment (Rosero-Chasoy et al., 2020). Nowadays, co-cultures of algae and bacteria are receiving great attention because of their high potential for phyco-remedial, enhancement of algal biomass productivity and reduction of the extraneous contaminating bacteria that degrade the culture (Ravindran et al., 2016; Ridley et al., 2017). Considering the enhancement of algal growth, a co-culture of *Scenedesmus* sp. with *Azospirillum brasilense* was shown to improve algae growth and prolong algal life span, *A. brasilense* has been considered a microalgae-growth-promoting bacterium (MGPB) that alters the metabolism, microalgal population density and cell size as a result of the ability of *A. brasilense* to produce several phytohormones (Contreras-Angulo et al., 2019). In addition, Cho et al. (2015) reported that the growth of *Chlorella vulgaris* as well as its productivity was ~1.3 times greater in non-xenic culture, compared with in axenic culture. Most studies about co-cultivation between microalgae and bacteria have focused on the influence of co-culture on algae growth as well as its productivity, and there were no reported data on bacterial growth.

However, interactions between algae and heterotrophic bacteria are among the most important factors affecting algal growth and survival in natural and artificial aquatic ecosystems, as bacteria serve in nutrient remineralizers and recyclers through their association with algal cells and microalgal-released organic matter (Samo et al., 2018). In addition, bacteria excrete micronutrients such as vitamin B_{12}, thiamine, phytohormones (gibberellins, cytokinins, abscisic acid and IAA) that could promote the growth and metabolism of microalgae (Zhang et al., 2020). A survey showed that 171 algae species out of 326 species needed exogenous vitamin B_{12} for growth, which is considered to be a key-factor in algal metabolism for vitamin B_{12}-dependent methionine synthesis, which can be obtained from vitamin-producer bacteria in an aqua environment as a result of symbiotic interaction between microalgae and bacteria

(Santos and Reis, 2014). In turn, algae can enhance the growth of bacteria by providing oxygen and releasing extracellular metabolites such as an extracellular polymeric substance that plays a key role in the association between bacteria and algae, leading to bacteria adhering into the algal cell surface. Thus, the consortium system can preserve their stability during cultivation for long periods, and microalgal growth improves because bacterial metabolism provides an appropriate microenvironment for microalgae (Han et al., 2016; Javed et al., 2019; Huo et al., 2020).

Otherwise, some bacteria have shown a negative impact on algal growth. For example, when *Pseudomonas aeruginosa* existed with several microalgal species, and algae growth could be inhibited by producing anti-algal substances from bacteria. Moreover, the association of *C. vulgaris* with its natural associative bacterium, *Phyllobacterium myrsinacearum*, led to metabolism change and caused algae death (De-Bashan et al., 2002). Equally, microalgae could decrease or inhibit the activity of bacteria by releasing antibiotics, increasing temperature and changing the pH of the culture (Ferro et al., 2019).

2.8.2 Co-culture of microalgae and yeast for algae growth enhancement

The co-culture of microalgae and yeast is also applied for different aims, including increased chemical production and feed aquaculture. This co-cultivation is better than using a pure culture because of its higher growth rate and improved biomass concentration with high-value products. The symbiotic interaction between microalgae and yeast, as known, could provide CO_2 for microalgae used in the photosynthesis process, and oxygen availability was supplied by microalgae for heterotrophic growth of yeast, which contributed to reducing the cost of microalgae production (Fig. 2.4) (Santos and Reis, 2014; Suastes-Rivas et al., 2019). The symbiotic relationship in the artificial co-culture, for example, between microalgae *Scenedesmus obliquus* and oleaginous yeast *Rhodotorula glutinis* for lipid production achieved

increasing algal biomass and improved lipid production to reach 40–50% of productivity, compared with the monoculture (Yen et al., 2015; Qin et al., 2019). In addition, co-cultures have demonstrated synergistic relationships between algae and fungi (e.g., *A. fumigatus*) that have additional benefits, including enhanced lipid yield and improved biomass production (Ravindran et al., 2016).

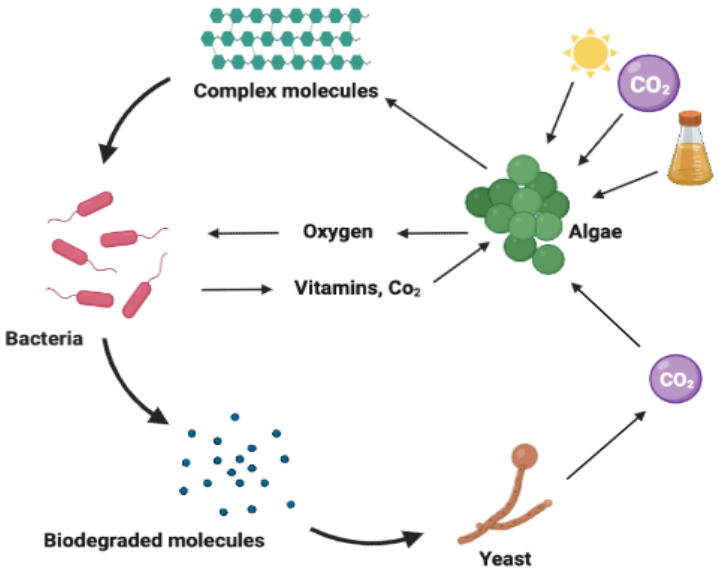

Figure 2.4 Summary of symbiotic relationship between microalgae-bacteria and microalgae-yeast. Microalgae produce organic molecules and oxygen (through the photosynthesis process), which are consumed by anaerobic bacteria and yeast, whereas they provide carbon dioxide (through respiration) to algae. Bacteria can degrade the complex molecules produced by algae into small molecules into the medium to be consumed by algae and yeast.

2.8.3 Co-culture for algal cell wall degradation

Microalgal cell walls are composed of glycoprotein and polysaccharide matrix to protect the cell against its environment, which can be degraded by several enzymes such as lysozyme, sulfatase, chitinase, laminarinase and pectinase. For example, lysozymes and chitinases are the

most common enzymes that can degrade the cell wall of *Chlorella* sp. (Gerken et al., 2013; He et al., 2016).

One approach to economically decompose the algal cell wall is by using co-culture with enzyme-producer bacteria. The bioconversion process is the most common method to degrade lignocellulosic biomass on an industrial scale, and it has recently been receiving more attention from researchers (Wei et al., 2017). The isolated marine bacteria *Flammeovirga yaeyamensis* could disrupt the cell wall of *C. vulgaris* efficiently within three days of co-culture as a result of enzymes produced by the bacteria. The enzymatic hydrolysis of the cellulose components in the algal cell wall and its release of sugars are considered to be one mechanism for disrupting a cell that has achieved a high efficiency of lipid extraction at 21.5% (Chen et al., 2013; Córdova et al., 2018). However, the ratio of inoculation size plays a significant role in the degradation process by a specific bacterial consortium (Tao et al., 2017).

2.8.4 Co-culture for the enhancement of microalgal biochemical components and production of biofuels

Symbiotic interaction of microalgae with other microorganisms such as bacteria, fungi and yeast not only contributes to increasing the biomass of microalgae but also enhances the accumulation of carbohydrate and lipid in algal biomass, which contributes to producing natural biofuels with low-cost production (Nath et al., 2019; Ray et al., 2022). The aim of this project is to increase carbohydrates in a co-culture *of Chlorella vulgaris* with its cohabiting bacteria that are used as substrate for cost-effective biofuel production.

The symbiotic relationship between various microorganisms provides a perfect solution for overcoming the limitations of biofuel production, including low feedstock biomass or low yield and productivity of carbohydrates/lipids, as well as reducing the production cost. Co-culture

between *Tetradesmus obliquus* and *Piscicocus intestinalis* has achieved 1.3 times higher biomass production and 1.55 times increased lipid productivity compared with monoculture, leading to increased biodiesel production (Kumsiri et al., 2021). In addition, consortia of *Chlorella sorokiniana* and *Methylococcus capsulatus* with bacteria achieved a ~2-fold increase in carbohydrates (Rasouli et al., 2018).

Similarly, applying co-culture during the fermentation stage has been found help to increase biofuel production. The cultivation of *Clostridium beijerinckii* and *S. cerevisiae* for butanol production increased the butanol concentration and productivity to reach 203% and 155%, respectively, compared with monoculture (Wu et al., 2019). In addition, a co-culture of *Clostridium butylicum* and *Bacillus subtilis*, which produces amylase, has been applied to produce amylase and consume oxygen in the fermenter, leading to it being cost effective ABE fermentation (Luo et al., 2015). This is beneficial for the fermentation process as compared with a monoculture because of the synergistic benefit of multiple metabolic pathways for all microorganisms involved in co-culture cultivation (Li et al., 2013). The inoculum concentrations of each microorganism will influence the outcome of the final co-culture, as well as timing (Padmaperuma et al., 2018).

2.9 Co-culture medium and factors affecting conditions

Isolated microorganisms from a symbiotic co-culture will grow in their original medium while in an artificial co-culture; the growth medium of the main partner is prepared by mixing media of A and B for growing both partners. Some supplement may be added to help partner B. For example, yeast extract and glucose have been supplemented to an algal medium to support yeast growth (Padmaperuma et al., 2018). As in the pure culture, nutrients, N/P ratio, pH, light intensity, salinity and carbon source availability will influence the growth of microorganisms in the co-culture system (Padmaperuma et al., 2018). The inoculation ratio and time of

inoculation are the most important factors in the co-culture; for example, growth of algae and bacteria were increased when the inoculation ratio was higher, compared with a low inoculation ratio (Han et al., 2016).

2.10 Conclusion

CO_2 and other GHG emissions that are released from fossil fuels and petroleum products threaten our environmental security. Consequently, a process that utilises carbon dioxide and replaces petroleum fuels with biofuels promises a future solution driving a clean environment with less toxic gases. Sustainable and renewable sources of feedstock could ensure sustainability and contribute to reducing the negative impact on the environment. Microalgae offer an ideal developmental platform that can enable utilisation of CO_2 and produce biomass that can be used as feedstock for generation biofuels with lower CO_2 emissions. An advantage of using microalgae as that the feedstock has the superiority of microalgae over food crops and lignocellulosic biomass because (a) of their fast growth, (b) they do not directly compete as a food source and (c) they lack lignin, leading to ease of pre-processing and cost-effective production, compared with the first and second feedstocks.

Microalgae-based carbohydrate is a rich-energy substrate that can be fermented and converted into biofuel, although cost-effective strategies to produce carbohydrates from microalgae as a source of fermentable sugars needs to be developed. Increasing microalgal carbohydrate yield and productivity will help produce biofuels economically. Several approaches have been applied to enhance the yield and productivity of carbohydrates, and the most common of these is nutrient manipulation, which influences the biochemical composition of the algae. In addition, co-culturing algae with other microorganisms has been shown to be useful for improving productivity in microalgae, which is a strategy that can be examined for improving carbohydrate productivity.

Managed co-culture between microalgae and bacteria has achieved great success for many purposes, such as the algae's growth enhancement, efficient nutrient removal from wastewater and biofuel production. Inoculation of single or multiple bacteria with algae affect the growth of algae and biochemical compositions. Considering algae's biochemical composition, bacteria play a great role in carbohydrate enhancement, as well as for lipids, as reported by several studies. Many factors could affect the co-culture, such as the time of introduction, initial bacterial load and size of the cultivation system, which may affect the abundance of the bacterial population. Therefore, this project studies the influence of managed co-cultures with cohabiting bacteria on the microalga *Chlorella vulgaris* CCAP 211/21A and investigates its impact on the yield and productivity of carbohydrates that can be converted to biofuels. A strain of *Chlorella vulgaris* that is known to accumulate carbohydrates was selected for this project as a rich-carbohydrate strain. A laboratory culture of *C. vulgaris* CCAP 211/21A with cohabiting bacteria will be grown at different nutrient conditions to study the changes in the bacterial population that can influence carbohydrate accumulation in the algae. This will be compared with the axenic strain and followed by a study of managed co-culture using the isolated bacteria in different concentrations and introduced at different times of algal growth. Two different compositions of a mixed bacterial population will also be examined, all with the aim of studying the influence of the changes on carbohydrate accumulation in *Chlorella*.

2.11 Project aim and objectives

The aim is to study the effectiveness of using a co-culture of algae with cohabiting bacteria as a strategy to enhance carbohydrate accumulation in a halo tolerant *C. vulgaris* CCAP 211/21A that is known to accumulate high levels of carbohydrates.

2.11.1 The specific objectives of this project are:

Objective 1 is the isolation and identification of cohabiting bacteria in a non-axenic culture of *Chlorella vulgaris* CCAP 211/21A. In addition, the objective would be to study the influence of relevant nutrient conditions on the bacterial load and species distribution. To achieve this, bacteria isolates were purified and identified by 16S rRNA sequencing. The distribution and the abundance of isolated species were detected at three points during algae cultivation grown under three nutrient conditions. This objective is addressed in Chapter 4.

Objective 2 is to study and characterise the behaviour of the cohabiting bacteria, in isolation, to optimise the isolation medium and identify optimal growth conditions of the cohabiting bacteria. To address this, different media with various concentrations of essential elements for growth were tested, the influence of the starting medium pH from 5.5 to 9.5 was studied, and the growth temperature from 10°C to 40°C was examined. This objective is discussed in Chapter 5.

Objective 3 is to study the effect of (a) bacterial concentration, (b) the time of inoculation of cohabiting bacteria in coculture and (c) the type of bacterial species/composition of bacterial mixture on algal growth and carbohydrate content and productivity. To realise this, the co-culture was studied in *f/2* medium with inoculation of (a) one of the cohabiting bacteria, at two different initial bacterial concentrations, introduced at two stages of algal growth and (b) a

mixture of cohabiting bacteria at two different ratios. The resultant algal growth, carbohydrate accumulation and changes in bacterial population were studied. This objective is covered in Chapter 6.

Chapter 3 Materials and methods

This chapter shows the general materials and methods that have been used for examination and investigation purposes. Details of the microorganisms used are provided, including their sources, growth and requirements for growth. Data are presented about each species cultivation method, including optical density, cell count, colony formation units (CFU), nutrient consumption assays and carbohydrate content measurement in algal cells.

3.1 Microorganism maintenance and cultivation

3.1.1 *Chlorella vulgaris* CCAP 211/21A

Chlorella vulgaris CCAP 211/21A was sourced from the Culture Collection of Algae and Protozoa (CCAP, UK). The culture was inoculated in log phase and cultivated in sterile *f/2* medium (Guillard, 1975) for 7 days. A sufficient volume of cells of algae culture stock was used; this was used in an exponential growth phase to give approximately an OD_{595} of 0.25 for starting the experiment.

All the cultures were grown in sterile Duran bottles (1L). The cultures were connected with a connector tube linked to a Luer lock at the valve gas to provide the culture with continuous air bubbling. This connection was also used to introduce 5% CO_2 for 1 hour or 100% CO_2 for 10 minutes daily as an additional carbon source for algae. The culture was also irradiated with continuous illumination at 200 μmol photons/m^2/s of light (LED fluorescent lamp). Magnetic stirrers placed below the flasks were used to mix the algae culture at room temperature (20°C ± 1).

3.1.1.1 Microalgae cultivation media

C. vulgaris was grown and maintained in *f/2* medium, which is suitable for all microalgal marine species. The stocks of *f/2* medium were prepared according to the protocol of Guillard and Ryther (1962) and the modifications of Guillard (1975). The medium consisted of (g/l): **major nutrient stock**: $NaNO_3$ 75 g; $NaH_2PO_4.H_2O$ 5 g; **trace metal stock**: $FeCl_3$ $.6H_2O$ 3.15 g; $Na_2EDTA.2H_2O$ 4.36 g; $CuSO_4$ $.5H_2O$ 9.8 g; Na_2MoO_4 $.2H_2O$ 6.3 g; $ZnSO_4$ $.7H_2O$ 22.0 g; $CoCl_2$ $.6H_2O$ 10.0 g; $MnCl_2$ $.4H_2O$ 180.0g; **vitamins:** thiamine HCl (B_1) 0.2 g; biotin 1.0 g; cyanocobalamin (B_{12}) 1.0 g. Each stock was added separately into 950 mL of dH_2O, which brought the final volume to 1 L. The stocks were then autoclaved, the vitamin was sterilized by filter and 1 ml of the major nutrient stocks, 1 ml of the trace metal stock and 33.5 g of Instant Ocean Salt (Aquarium Systems) that composed of different ions including chloride, sodium, sulphate, magnesium, potassium, calcium, carbonate, bromide, strontium, boron, fluoride, lithium, iodide (see appendix for details, Table 9.1) were added into the 950 ml dH_2O and then mixed well. Instant Ocean Salt was added into the medium to create an artificial marine medium. 1 ml of mixed vitamins was then added into a cool medium after medium autoclaving at 121°C for 15 mins.

In this project, all media were prepared without pH buffering which is adjusted with 1M of Hydrochloric acid (HCL); after autoclaving the *f/2* media, the pH of the media was found to be 8.4±2. In addition to cultivation of algae in *f/2,* algae was also cultivated in two different media (a) in a deplete medium, in which the nitrate and phosphorus concentrations were reduced to half (*f/4*) and (b) a replete medium (*2f*), in which the nitrate and phosphorus concentrations were increased four times compared to the *f/2* medium (concentration of nitrate: 800 µM and concentration of phosphorus: 36 µM).

3.1.2 *Halomonas* and *Muricauda* species (isolated from *C. vulgaris*)

Halomanas and *Muricauda* sp. were isolated from the *C. vulgaris* culture as cohabiting bacteria by serial dilution; 100 µl of algae culture was diluted in 900 µl of distilled water, then, 10 µl of diluted sample was transferred into petri dish that contain *f/2*+R2A medium and spreader by a sterile spreader. The plates have been incubated upturned at room temperature (20°C ± 1) for 7 days. After that, bacterial isolates were characterized in a wide range of different conditions in isolation.

First, pure bacterial cells were grown in 250 ml conical flasks containing 100 ml Luria broth (LB) medium plus salt that had been plugged with foam bungs after autoclaving at 121°C for 15 minutes. Flasks were shaken at 150 rpm and incubated at room temperature (20°C ± 1). Bacteria were grown on plates before they were suspended in broth medium.

3.1.2.1 Bacterial culture

Reasoner's 2A agar (R2A) was prepared as a rich medium for bacteria detection from algae culture by adding 18.12 g R2A agar into 950 ml of prepared normal *f/2* or deplete and replete *f/2* media to prepare the agar plate, then the volume was brought to 1000 ml after pH adjustment (pH 7.2 ± 0.2). The suspension was mixed well to dissolve the culture completely (Stark and McCoy, 1938; Reasoner and Geldreich, 1985). Before running any experiments of bacteria, the bacteria colonies that formed on agar plates at room temperature were resuspended in in 250 ml conical flasks containing 200 ml of LB medium plus salt and plugged with foam bungs for inoculation, the concentration of bacteria in this stock was estimated by plating and serial dilutions to be 10^5 CFU/ml which were used for running effect different media on bacterial growth experiments by transferring 1 ml of each isolate separately.

3.1.2.2 Estimation of bacterial growth

Colonies forming unit (CFU) was used in all bacterial experiments to estimate the bacterial growth in the culture by serially diluting. 100 µl of bacteria were diluted with 900 µl of sterile distilled water making different dilutions (10^3, 10^4, 10^5) and then 10-15 µl of the diluted samples from each dilution was plated by sterile spreaders on *f/2*+R2A agar plates. Serial dilution with multiple volumes was adopted to ensure countable colonies on each plate, and to get a statistically accurate number of counts. Plates were incubated at room temperature upturned with plate edges sealed by parafilm.

3.2 Harvesting algae for assays

From the culture, 5 ml was collected and centrifuged twice daily for 7 days at 4000 rpm for 10 minutes to harvest the pellets. Algal pellets were then resuspended in 1 ml distilled water and washed once and recentrifuged to remove residual salts. The pellets were stored at -20°C until the end of the experiment (7 days) and then transferred to -80°C for 24 hours before the pellets were dried at -110 °C overnight using a freeze-drier. The algal suspension was divided into two 2-ml samples to measure the amount of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) that was consumed by the algae, and 1 ml of the suspension was kept as a backup. In addition, 1 ml was taken for monitoring the algal growth rate and pH measurement. A pH meter (Mettler Toledo) was used to measure the pH of the algae culture

In addition, microscopic examination (oil lens, X100) was carried out, and photographs were taken with an Olympus microscope (BX51 model, Germany).

3.3 Monitoring of microorganism growth

There are different methods to determine microorganisms' growth, depending on their species. The optical density (OD), cell counting (CC) and dry cell weight (DCW) were used to measure

the algal density in the medium. The OD is a direct way to measure algae concentration, which was measured at 595 nm wavelength by spectrophotometer (SEPECTROstar NANO).

Cell count is a method that can determine the cell abundance of a diluted sample (diluting 100 µl of algae with 900 µl of water) by using the Neubauer counting chamber and calculating the cell averages in 4 large squares on the Neubauer Haemocytometer. A total of 10 µl was transferred to the Haemocytometer Chamber and counted under ×40 magnification using the counter. For the diluted sample:

Cell abundance (cells/ml) = (Average of cells in 4 quadrants / 4) × conversion factor (average × 10^4) × dilution factor.

Dry cell weight (DCW) was also used to monitor the algal density, and 5 ml of the algal biomass was harvested and washed and dried by lyophilization at -110°C for 24 hours in order to get dry pellets. Then, the biomass amount (mg) was calculated by the weight difference between the empty Eppendorf and the lyophilised Eppendorf.

The growth rate (µ) determination for all measurements (OD, CC and DCW) was calculated according to following formula:

$$\mu = \frac{ln\ (N2) - ln\ (N1)}{t2 - t1}$$

where N_1 is the optical density at the first time point (t_1) and N_2 is the optical density at the second time point (t_2).

The growth of bacterial cells was monitored by CFU on agar plates (section 3.1.2.1.3). The OD and the DCW methods are not specific to algae and may include estimation of bacteria, and the cell count method is not very accurate (hence the usage of all three metrics to measure algal growth).

3.4 Analytical methods

The consumption rate of major nutrients (N and P) by algae were estimated over 7 days. The biochemical assay was applied to estimate the carbohydrate content in the algae cells as intracellular molecules that were affected by changing parameters of cultivation.

3.4.1 Estimation of dissolved inorganic nitrate

To measure how much nitrate the algae consumed during cultivation, we determined the dissolved inorganic nitrate levels, using the method of Collos et al. (1989). In line with the specific protocol, we prepared $NaNO_3$ standards in the following concentrations: 500, 250, 125, 62.5, 31.25, 15.625 and 0 μM (Appendix, Fig. 9.1). A total of 2 ml of standards and samples was measured directedly in white quartz cuvettes at a wavelength of 220 nm, using nitrate-free medium as a control. Preparing different standard concentrations produced a plot of a standard concentration curve by linear relationship, which was applied to calculate the nitrate total of the standards and samples.

3.4.2 Estimation of dissolved inorganic phosphorus

An estimation of the amount of phosphorus consumed by the algae during cultivation was carried out using the method of Strickland and Parsons (1968). In line with the specific protocol, five phosphate standards were prepared using sodium dihydrogen in the following concentrations 20, 15, 10, 5, 2.5 and 0 μM (Appendix, Fig. 9.2). Mixed reagent was prepared (ammonium molybdate 1 ml, sulphuric acid 2.5 ml, ascorbic acid 1 ml and potassium antimolnyl tartrate 0.5 ml) and 100 μl was added to 1 ml of standards or samples. Phosphate was measured at 885 nm in plastic cuvettes. Phosphorus-free medium was used as a control.

3.4.3 Carbohydrates assay

Carbohydrates are an intracellular molecules that accumulates inside *Chlorella vulgaris* as a result of photosynthesis. In line with the specific protocol, using the method of Chen and Vaidyanathan (2013), eight carbohydrate standards were prepared using glucose in the following concentrations: 400, 200, 100, 80, 60, 40, 20 and 0 mg/ml (Appendix, Fig. 9.3) Spectrophotometry was used to measure the biochemical composition of the cultures. The following contains a brief description of the steps that were followed to prepare the samples for the carbohydrate assay:

1. Pellets with 5 ml of algal culture in triplicate were taken in 2 ml Eppendorf tubes, then 1 ml glass beads, 24.3 µl phosphate buffer (pH 7.4) and 1800 µl of R1 (25% methanol in 1N NaOH) (concentration buffer) were added. The pellets were destroyed by bead-beating for 30 min (2 min cool down after each 10 min) to release the biochemical components outside the cells.
2. Two volumes of 100 µl of supernatant (one volume for sample, one for control) were stored at -20°C.
3. 1200 µl pre-chilled 75% H_2SO_4 was added to the control, and 400 µl pre-chilled 75% H_2SO_4 and 800 µl anthrone reagent were added to the samples and standards.
4. The samples and controls were covered with aluminium foil and incubated at 100°C for 15 minutes. Standards, samples and controls were then measured at a 578 nm wavelength.

The sample's blank was distilled water, and the control's blank was 200 µl distilled water plus 1200 µl 75% H_2SO_4.

3.4.4 Identification of isolated bacteria

3.4.4.1 Morphological observation of isolated bacteria

Isolated bacterial strains were plated on R2A agar containing *f/2* medium and incubated at room temperature (20°C ± 1) for 7 days. Morphological shapes of colonies such as their surface, edge and colour were observed.

3.4.4.2 Identification with gram staining

Pure bacterial cells were transferred onto a clean glass slide and dyed with crystal violet as a primary stain. They were rinsed with water after 1 min and then dyed with iodine for 1 min before being rinsed again with water. The slides were rinsed with 95% alcohol as a means to decolorize and remove the stain from gram negative cells. Cells were then redyed with Safranin for 1 min as a counterstain that would colour the gram-negative cells red. Finally, the slides were washed with water and dried. A microscope was used to identify bacteria. Cells with dark purple indicated gram-positive bacteria, while red cells indicated negative gram bacteria.

3.4.4.3 DNA extraction and 16S gene amplification

Bacteria were grown in LB broth medium containing salt for 48 h. Of the bacteria, 2 ml was collected by centrifugating the Eppendorf tubes at 10,000 g for 1 min. Colonies' PCR 16S rRNA genes were carried out for isolated bacteria using universal as forward primer 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5' GGTTACCTTGTTACGACTT-3') as a reverse primer (Lane 1991; Turner et al., 1999). Phusion polymerase kit (NEB, Biolabs) was used to perform amplification of the target templates following the PCR kit manufacturer's guidelines. The total volume of PCR for all samples was 50 µl: 25 µl master mix, 2.5 µl each of the forward and reverse primers, 1 µl of 20 ng/µl g DNA and made up to 50 µl with nuclease-free water. The PCR was carried out in a thermocycler (Applied Biosystems, USA) applying the following conditions: the initial denaturing step at 98°C for 30 secs followed by 30 cycles

of the denaturing step at 98°C for 1 min, annealing at 58°C for 10 secs, and elongation at 55°C for 1 min. Lastly, final elongation was at 72°C for 5 min. The PCR products were cleaned using PCR clean up kit and analyzed by spectrophotometer (Thermo Scientific Nanodrop, 2000) to determine the concentration, and they were investigated by gel electrophoresis (MupidTM-one, Advance Co. Ltd, UK) using 1% agarose gel containing aliquot of Gel Red (6 µl/100ml) (Insight Biotechnology Limited).

An 18 µl sample of DNA was mixed with 3 µl of 6 × gel loading dye purple (NEB, USA) and loaded onto agarose gel. Then 5µl of 1 kb DNA ladder (GeneRuler 1kb Plus Ladder, ThermoFisher) was loaded onto agarose gel. Electrophoresis was carried out at 80 V for 45 min. DNA bands were visualized using a UV lamp (transilluminator ChemiDoc-lt2, UVP, UK), and images were taken (Appendix, Fig. 9.4). The positive samples were cleaned up using a QIAquick PCR purification kit (Qiagen Inc., CA). Following analysis, the samples were sent to Eurofins Genomics, UK for Sanger's sequencing.

DNA concentration was determined by Nanodrop spectrophotometer, and the absorbance ratio was at A260 nm. A total of 1 µl of bacterial DNA was used to measure DNA quantity.

However, all sequences have been submitted to GenBank for getting accession number for all bacterial isolates. Nucleotide BLAST on NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for bacterial sequence identification and getting bacterial species name. A phylogenetic tree was established using the Neighbour-Joining method and 500 bootstrap analysis performed by MEGA 11. The evolutionary distance between microbial families was estimated by Tamura-Nei model.

3.5 Statistical analysis

All algal and bacterial experiments were carried out in triplicate runs, and so were the biological replicates. Data are shown as mean and standard error about the mean. A one-way analysis of variance (ANOVA) test was applied to assess the statistical significance of the

influence of the conditions on the various outcomes. In some instances, a two-way ANOVA was carried out when two factors were considered, and a t-test with unequal variance was employed to assess statistical significance for comparing two sets of data. Where p values are reported, these are for the t-test, except for in cases where ANOVA is mentioned. All statistical analyses were carried out by Microsoft Excel (Version 16.41)

Chapter 4 *Chlorella vulgaris* CCAP 211/21A growth, carbohydrate enrichment and identification of commensal bacteria in the algae culture

***Part of this chapter has been presented in conference**

Wasayf and Seetharaman (2021). Enhancement of carbohydrate production in *Chlorella vulgaris* bioprocess using nutrient modification. ChemEngDayUK 2021, 7-8 April, Bradford.

4.1 Introduction

Microalgae biomass has been identified as a sustainable feedstock for a clean future for biofuels and bioenergy production (Ellis et al., 2012; Singh et al., 2019). Microalgae are photosynthetic microorganisms that have the ability to capture solar energy and CO_2 and convert them into biochemical molecules (carbohydrates and lipids), which can have valuable applications for human use without affecting food security and damaging the environment (Yao et al., 2019; Getachew et al., 2020). The high contents of energy components in the algal biomass, such as lipids and carbohydrates, are the most attractive characteristics for their use as a biofuel producer (Yeong et al., 2018; Ma et al., 2020). Therefore, it is necessary to select a suitable microalgal strain that can accumulate a high content of carbohydrates/lipids. *Chlorella vulgaris* is one example of microalgae that has a high growth rate along with accumulating high carbohydrate yield, which accounts for 43.4% of their cells (Biswas et al., 2017; Yeong et al., 2018; Ma et al., 2020). Several strategies have been adopted to increase the carbohydrate content among microalgae species, such as genetic modification of microalgae, changing of culture conditions and co-culture systems with other microorganisms (Suastes-Rivas et al.,

2020). One of the most common methods for enhancing carbohydrate accumulation in microalgae species is nitrogen limitation (de Farias Silva and Sforza, 2016).

In addition, cultivation systems (photoautotrophic, heterotrophic and mixotrophic) can affect microalgal growth and carbohydrate accumulation among species. A photoautotrophic system is an attractive cultivation mode because it utilises CO_2 from the ecosystem, leading to a reduction in greenhouse gas emissions. However, this system has low microalgal biomass yield contrary to a mixotrophic system, which increases biomass yield and carbohydrate accumulation (Singh et al., 2019). Microalgae are facing challenges to become effective, large-scale economies, but they offer a conceptually attractive framework to be developed as sustainable feedstock for commercial-scale productions. Despite its higher cost of production compared to conventional fuel production, biofuel production is growing worldwide (Quintana et al., 2011; Chen et al., 2013; Yuan et al., 2020). There is still a great interest in developing feedstock for biofuel production. In addition to biodiesel, algae can be cultivated to accumulate fermentable carbohydrates, which can then be processed and fermented into biofuels.

In the natural environment, microalgae can cohabit with a wide range of microbes in many forms, including commensalism, mutualism and parasitism. Symbiotic relationships between microalgae and other microbes significantly influence natural ecosystems; generally, microalgae can produce oxygen during photosynthesis, which can be used by bacteria to improve the respiration process and increase the consumption of organic molecules (Commault et al., 2017; Yee et al., 2021). As is known, the presence of bacteria in the algae culture is considered contamination, but recently, the perception has changed; algae–bacteria interactions have become a promising technique for biotechnology applications **(**Yao et al., 2019). The industrial cultivation of algae has different challenges; nevertheless, microalgae have recently received great interest in the industrial field due to their applicability in different areas, such as the nutraceutical industry, biopharmaceuticals and renewable energy production. Industrial

cultivation can be developed by implementing communities between different species in bioreactors. For example, synergic cultures between algae and bacteria are a great strategy to eliminate the contamination of undesirable bacteria in aquaculture media according to the competitive exclusion principle between microorganisms (Krug et al., 2020). Several bacterial species were noticed in different phases of *C. vulgaris* growth. At least 43 bacterial strains were detected, with 16 axenic microalgal strains identified in classes α-proteobacteria, γ-proteobacteria, Bacteroidetes and Flavobacteria. The variety of bacterial species associated with microalgae indicates the potential interaction between bacteria and particular microalgae. These bacterial populations were similar to those of marine ecosystems (Han et al., 2016).

This chapter analyses the increase in carbohydrate accumulation in non-axenic *Chlorella vulgaris* CCAP by nutrient modification. We also try to develop further understanding of this strain with respect to cohabiting bacteria and study the behaviour of the bacterial population under nutrient changes towards developing strategies for enhancing the carbohydrate content and productivity in *C. vulgaris*.

4.2 Experimental Design

A non axenic culture of *Chlorella vulgaris* CCAP 211/21A, obtained after long-term cultivation (2 months) in the lab, was used. This was cultivated in *f/2* medium as a control. In addition, It was cultivated in deplete medium (*f/4*) and replete medium (*2f*) to elicit different carbohydrate accumulation profiles in the algal cells. In addition, the cohabiting bacterial population was isolated and its distribution studied in all tested media.

4.3 Results and Discussion

4.3.1 Algal growth and biomass productivity in three culture media

The non-axenic algal cultivation in the three media showed different growth densities (Fig. 4.1). During the microalgae cultivation under different nutrient concentrations, a continuous

growth of the microalgae was observed in all three media (Fig. 4.2). The results of all growth measurements (OD, cell count and DCW) showed that increasing the nutrient concentrations in the medium led to an increase in algal growth (Fig. 4.2A–C). The algal biomass was the highest in the replete medium (*2f)* (Fig. 4.2D). In addition, over 7 days of cultivation, the highest the median growth rate over the cultivation period was observed in the replete medium (*2f*) medium as it contained sufficiently high amounts of the major essential elements (3200 µM nitrogen, 1800 µM phosphate and other elements) for algal growth (Fig. 4.2E), while biomass loss was observed in the deplete medium (*f/4*) because the nutrients were not sufficient for algal growth. Approximately four-fold increases in nitrate and phosphor concentrations led to an increase of 78% in the median growth rate (CDW basis), while a 50% reduction in the nutrient concentration resulted in a 34% decrease in the median growth rate (CDW basis) ($p < 0.05$). Biomass productivity also showed a similar trend (Fig. 4.2F). However, the increasing cell count of the algae began to differ after 24 h among the media containing different nutrient concentrations. The availability of nitrate from 400 µM in the deplete medium (*f/4*) to 3200 µM in the replete medium (*2f*) resulted in an increase in the microalgae biomass (19.5×10^{-3} to 37×10^{-3} cells/ml) after 7 days of cultivation. A two-fold increase was observed in the cell count in the replete medium (*2f*) compared to the deplete medium (*f/4*) after 7 days of cultivation ($p < 0.01$). In addition, the final DCW increased by two-fold in the medium that contained high initial nitrate levels (*2f*) compared to the deplete condition (*f/4*) ($p < 0.05$).

Nitrogen is an essential element in microalgal culture for growth; thus, its deficiency can inhibit the growth and division of microalgae cells, leading to decreased algal biomass productivity (Wong et al., 2017; Araujo et al., 2020; Zarrinmehr et al., 2020). It is not only the presence of a nitrogen source that is important to maintain high cell density and increase growth rate availability but the availability of high phosphorus concentration in the culture can also

promote algal biomass productivity (Mujtaba et al., 2012; Chandra and Ghosh, 2019). Compared to the replete condition, the absence of nitrogen or phosphorus negatively influenced biomass productivity by 15-20%, while phosphorus limitation alone in freshwater *C. vulgaris* culture resulted in a 92% decrease in biomass productivity (Shen et al., 2015). Both nitrogen and phosphorus deficiencies in the medium affect the growth of algae and its biochemical composition; however, nitrogen deficiency has higher influence than phosphorous deficiency (Li et al., 2019). Moreover, nitrogen or phosphorus alone cannot sufficiently stimulate algal growth; both need to be used together in algae cultivation. Mayers et al. (2018) reported that the maximum biomass productivity of *Chlorella salina* was reduced by 15% between nutrient replete and nitrogen-deplete conditions. Nutrient enrichment played a significant role in increasing algal biomass. The change in algae biomass during the stationary growth phase depends on the phosphorus-to-nitrogen ratio in the medium (Zhoua et al., 2011). Moreover, nitrogen deficiency not only affects algal growth and biomass concentration negatively but also affects the synthesis of chlorophyll, which negatively impacts the photosynthetic process in microalgae (Li et al., 2019; Cho et al., 2020). Different nitrogen sources are used in the algae culture. Feng et al. (2020) reported that the presence of $NaNO_3$ and urea as a nitrogen source in the culture can help algae achieve high biomass and μmax. Urea contains high nitrogen content, which is one of the reasons for the high microalgal growth rate.

However, CO_2 concentration also has another influence on algal growth: algal productivity is increased in cultures connected with high CO_2 compared to those connected with air during the algal culture cycle (Tanadul et al., 2014). For example, supplying 5% CO_2 to algal culture represents the most suitable carbon source for the growth of *Chlorella sorokiniana,* as reflected by the high biomass productivity observed during the log phase in the batch-mode cultivation system (Kumar et al., 2013; Mohsenpour and Willoughby, 2016). Supplying algal culture by

2% CO_2 resulted in a significant increase in algal growth as compared to the culture connected with air alone; the growth rates of *Chlorella salina* and *Picochlorum oklahomensis* were increased by two-fold compared to the control (Mayers et al., 2018).

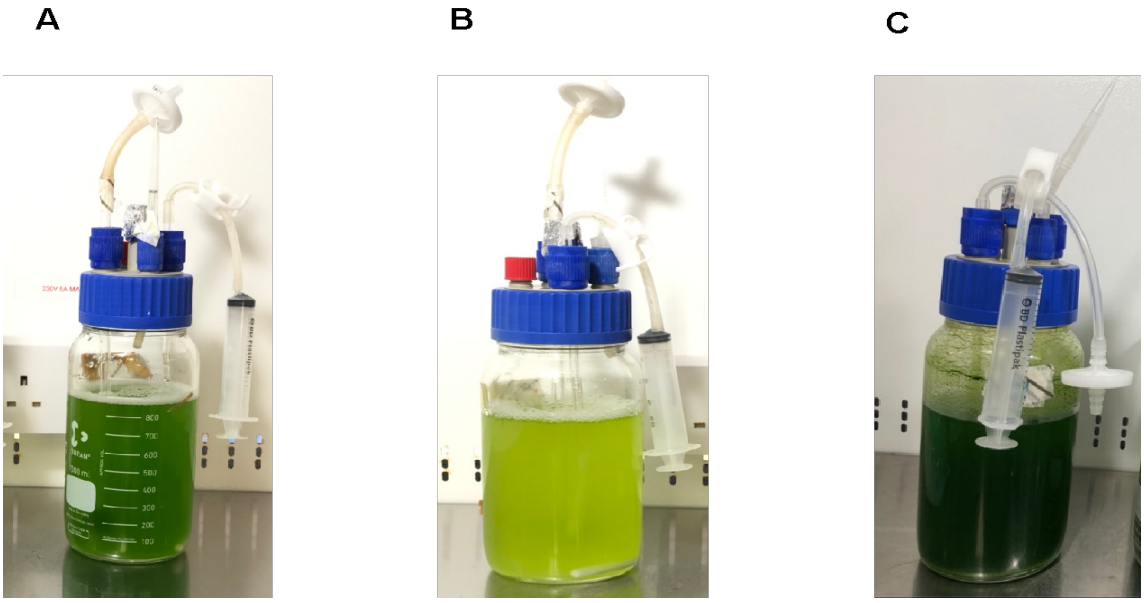

Figure 4.1 *Chlorella vulgaris* growth after 3 days of cultivation in three different media, **A**: *f/2*; **B**: deplete medium (*f/4*) and **C**: replete medium (*2f*) incubated at room temperature.

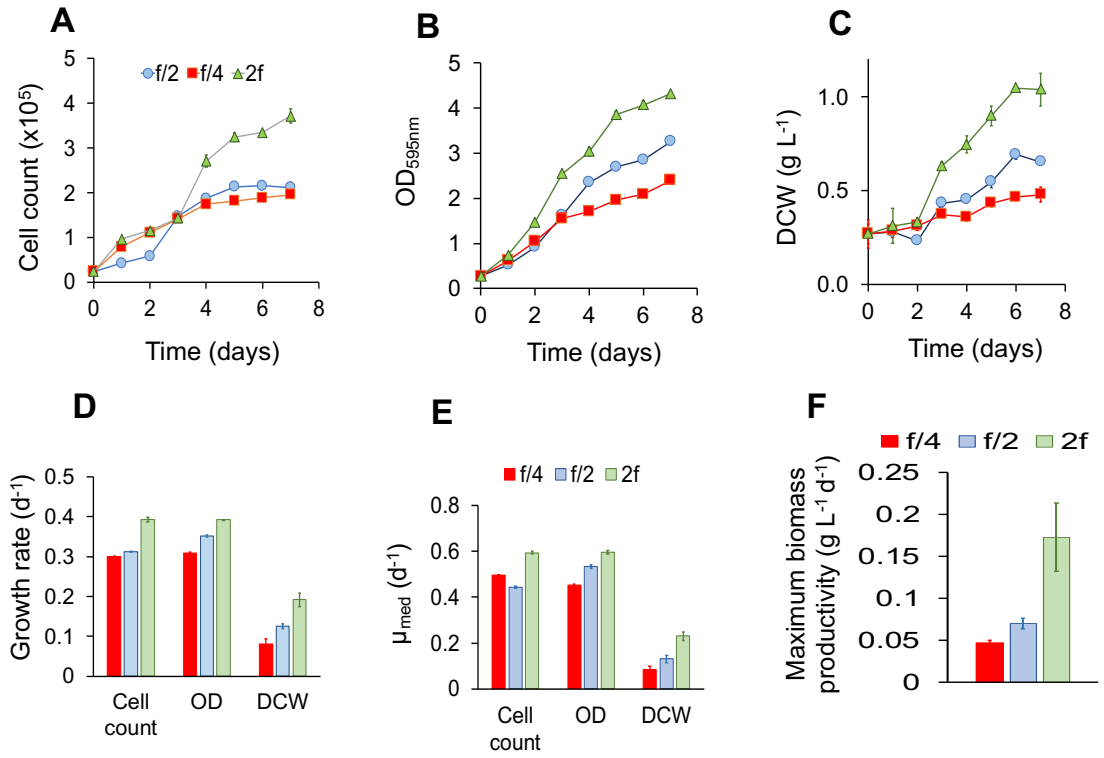

Figure 4.2 Growth of *C. vulgaris* in *f/2*, deplete (*f/4*) and replete *(2f)* media cultured in batch mode for 7 days at room temperature under continuous light and supplied with 5% CO_2: (**A**) cell count, (**B**) OD_{595nm}, (**C**) DCW of algae, (**D**) growth rate on day 7 of cultivation, (**E**) median growth rate of algae over the cultivation period and (**F**) maximum biomass productivity.

4.3.2 pH profile

Culture pH is a factor that influences microalgal growth. Most microalgae can grow in wide ranges of pH (6 - 8.76). Some microalgae can tolerate wide ranges of pH; for example, *C. vulgaris* exhibits a high growth rate and maximum biomass productivity at pH ranging between 9 and 10 (Khan et al., 2018).

In this study, the initial pH of the algal cultivation was approximately 8.61, 8.07 and 8.49 in *f/2*, *f/4* and *2f*, respectively. The pH was increased in *f/2* and *f/4* media during the first two days of cultivation, then decreased gradually from day 3 until the end of cultivation on day 7 to be between 8.2 and 8.7 (Fig. 4.3); a similar result was reported by Zhoua et al. (2011). pH measurement increased in the *2f* medium over time to reach 9.78 on day 7 of cultivation. When

the growth of *C. vulgaris* started to increase dramatically from day 3, the pH level was approximately 8.40−9.18 in all media. Although the optimal pH range for marine microalgae is approximately 8.1−8.3 (Goldman et al., 1982), Bartley et al. (2014) found that the highest growth rate of marine *Nannochloropsis salina* was higher at pH 8 and 9. Therefore, algal growth is associated with culture pH.

Meanwhile, the presence of inorganic carbon, such as CO_2, in the culture can influence the pH range in the culture, as Hernández-García et al. (2019) reported that the availability of inorganic carbon increased the culture pH from 8 to 10 during microalgal growth. The carbon sources in microalgae cultures are essential to preserving algal growth but increasing the CO_2 concentration in the culture can lead to a reduction in pH, resulting in a decrease in the algal growth rate. The supply of CO_2 in a microalgae culture not only affects the growth but also the biochemical composition of algae (Kumar and Saramma, 2018; Moraes et al., 2020).

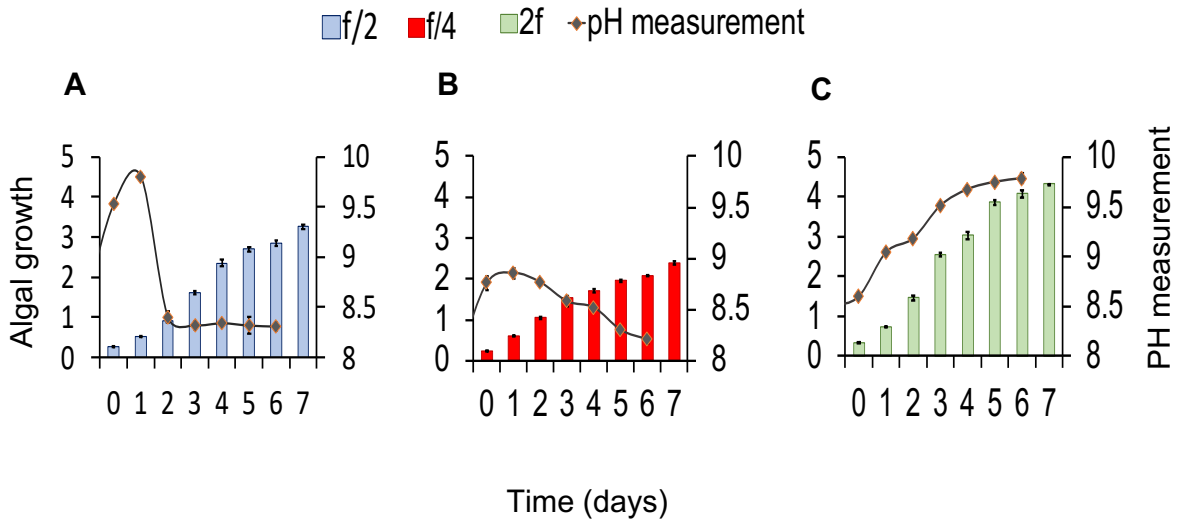

Figure 4.3 pH profile during cultivation of *C. vulgaris* in three different media. ***A:*** *f/2;* ***B:*** *f/4* and ***C:*** *2f.*

4.3.3 Nitrate depletion in the media

In this study, when starting algae cultivation in the *f/2*, *f/4* and *2f*, the initial nitrate concentrations were approximately 800, 400 and 3000 µM, respectively. The nitrate concentration decreased with algal growth over time and was consumed completely from *f/2* and *f/4* in 4 days of cultivation; however, its complete consumption from *2f* took longer (6 days) (Fig. 4.4A). The highest nitrate uptake rate was noticed in the replete medium (*2f*), as shown in Fig. 4.4C. When the algae started the log phase of growth (on day 3), nitrate consumption was 59.67% and 70.75%, respectively, higher in the deplete (*f/4*) and replete (*2f*) media compared to the control in *f/2* (16.59%) (Fig. 4.4B).

However, as a result of the rapid growth of microalgae, 79.3% - 93.5% of nitrate added to the medium is consumed (Li et al., 2019). The lower nitrate uptake by microalgae in the culture negatively impacts the growth rate and cell number of microalgae (Araujo et al., 2020).

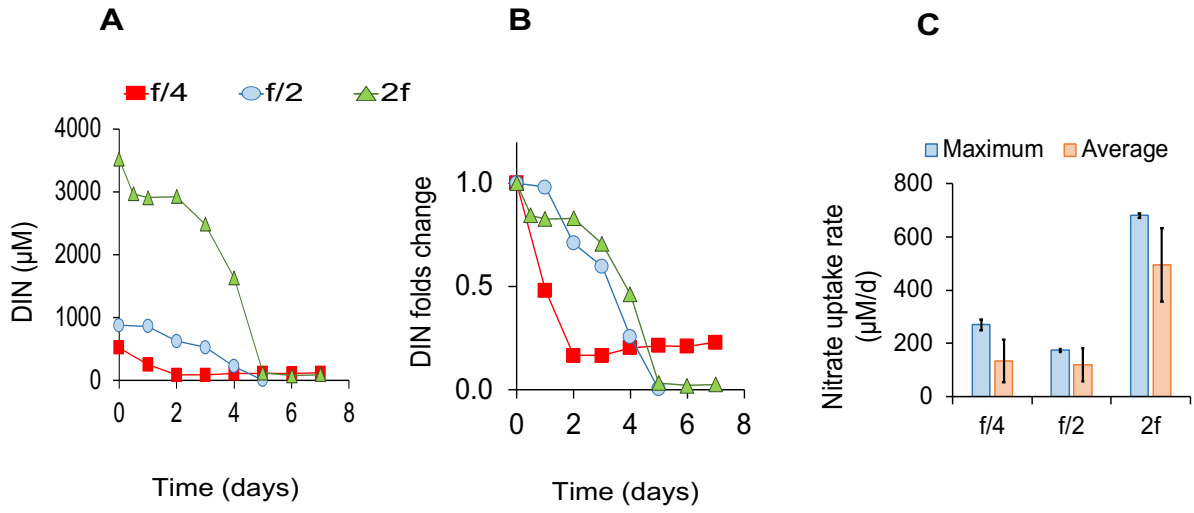

Figure 4.4 Consumption of DIN profile by *C. vulgaris* in three different media (*f/2*, *f/4* and *2f*) within 7 days of cultivation.

4.3.4 Phosphate depletion in the media

In this study, the initial phosphate concentrations in the cultures were approximately 23, 12 and 36 µM, respectively, at the beginning of cultivation of *C. vulgaris* in the *f/2*, *f/4* and *2f* media. In all three media, phosphate was consumed significantly after three days of growth (Fig. 4.5A, B) as the algae began the log phase and started growing faster. The maximum phosphate uptake was observed in the *2f* medium (Fig. 4.5C), which was 2.4-fold compared to that observed in *f/4* ($p > 0.05$). A similar result was reported by de Lourdes et al. (2017), who noticed that *C. vulgaris* consumed nitrogen and phosphorous completely from the culture after three days of cultivation in real wastewater. However, Paes et al. (2016) noticed that the algae growth rate can be increased with a carbon source supply if high concentrations of nutrients are available.

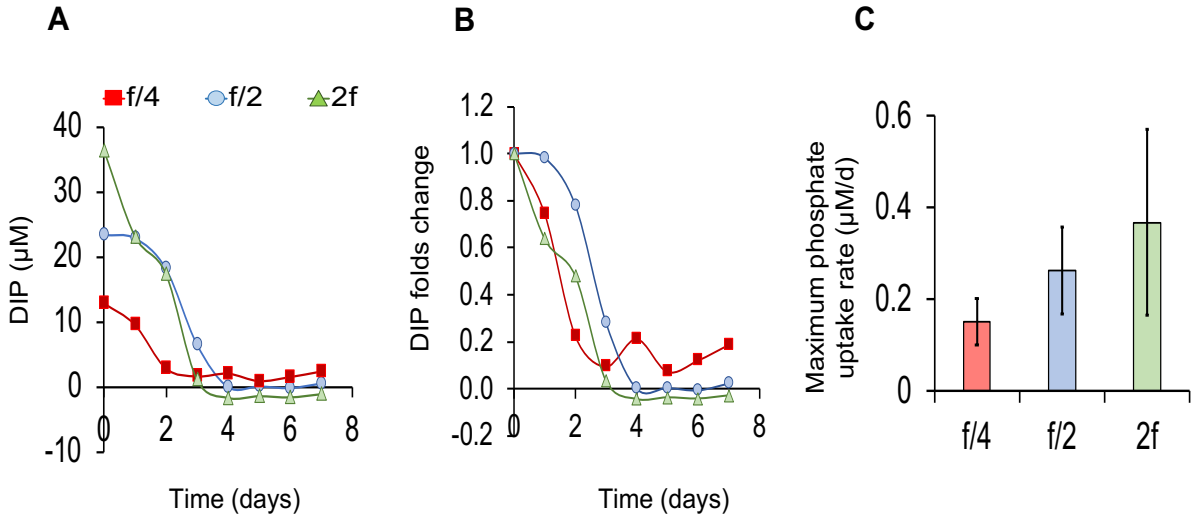

Figure 4.5 Consumption of DIP profile by *C. vulgaris* in three different media (*f/2*, *f/4* and *2f*) within 7 days of cultivation.

4.3.5 Carbohydrate accumulation in the algal cells

Carbohydrate accumulation was monitored in *C. vulgaris* cells in the three media (*f/2*, *f/4* and *2f*) within 7 days of cultivation. In all three media, carbohydrate yield increased gradually to reach the accumulation peak on day 3 of cultivation in the stationary phase of the growth and then declined (Fig. 4.6A). Carbohydrate yield in the algal cells was highest (47% CDW) on day 3 under the *f/4* condition, compared to the highest yield of 27% DCW observed under the *2f* condition. An 8-fold reduction in nitrogen and phosphorus supply resulted in a 75% increase in carbohydrate yield ($p < 0.01$). However, when considering maximum carbohydrate productivity, halving the N and P supply (*f/4*) resulted in decreasing the productivity of the control (*f/2*) to 75%, whereas a 4-fold increase in N and P supply (*2f*) led to a 1.6-fold increase in carbohydrate productivity ($p < 0.05$). The maximum carbohydrate productivity was reduced by 53% in the deplete condition (*f/4*) compared to the replete condition (*2f*) (Fig. 4.6B).

One of the most common approaches for increasing carbohydrate accumulation in microalgae is nitrogen limitation, which converts fixed carbon from Calvin's cycle to produce lipids and carbohydrates instead of nitrogen-based products (proteins) (De Farias Silva and Sforza, 2016). The source of nitrogen and its concentration in the microalgal culture significantly influence the biochemical composition of microalgae (Singh et al., 2019), and the biochemical composition of algal biomass can be modified by changing the growth conditions of algae (Brennan and Owende, 2010).

According to Gonçalves et al. (2019), nitrogen deficiency in the algae culture negatively affects the algal growth rate and protein synthesis, while it can contribute to increased reserve compounds, such as carbohydrates and lipids. Therefore, nitrogen deficiency is a factor that can enhance carbohydrate accumulation in *Chlorella* sp. For example, *C. vulgaris* can accumulate up to 12%–55% of carbohydrates of CDW under nitrogen depletion (Safi et al., 2014). Some studies have also reported that phosphorous deficiency in the medium encourages

up to 55% increase in carbohydrate accumulation in *C. vulgaris* biomass as well as lipids in algae cells (Paes et al., 2016; Samiee-Zafarghandi et al., 2018). Nordin et al. (2020) reported that the carbohydrate accumulation in *C. vulgaris* increased rapidly under nitrogen limitation to reach the peak on day 4 (42.3%), and then decreased drastically. However, several studies have reported that carbohydrate accumulation is affected by the phase of algae growth; Samiee-Zafarghandi et al. (2018) noticed that carbohydrate accumulation decreased during the logarithmic phase of algae, and Paes et al. (2016) reported that carbohydrate content increased during the stationary growth phase but doubled in the nitrogen medium in the same growth phase.

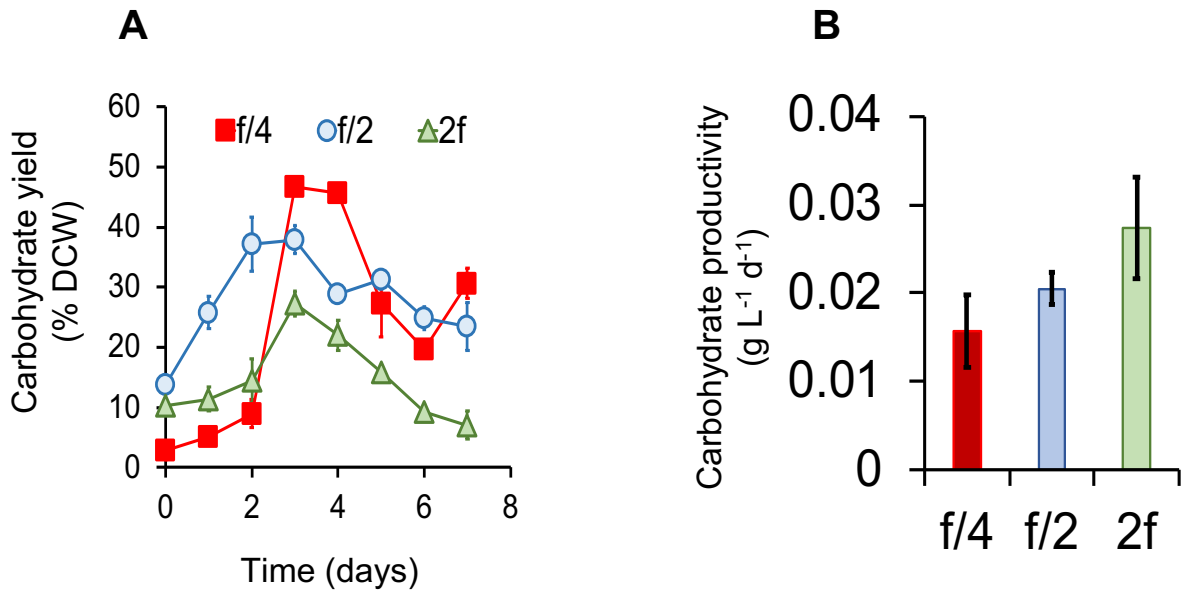

Figure 4.6 Time profiles of **A**: carbohydrate yield (%CDW) of *C. vulgaris* in *f/2*, *f/4* and *2f* media for 7 days of cultivation; **B**: maximum carbohydrate productivity over the cultivation period.

4.3.6 Characterisation of cohabiting bacteria

4.3.6.1 Morphological characterisation

The serial dilutions on agar plates (R2A agar with *f/2*, *f/4* and *2f* media separately) that were grown at room temperature (20±1°C) showed the presence of at least three different species of bacteria at all isolated points with various colony sizes (Fig. 4.7). At all isolation points, the bacteria grew faster on 10^1 and 10^2 plates as their concentration was higher in these dilutions. Small white colonies appeared after two days on 10^1 and 10^2 plates and after three days on 10^3 and 10^4 plates. Later, large white colonies appeared after three days on 10^1 and 10^2 plates and after four days on 10^3 and 10^4 plates. Furthermore, small yellow colonies appeared after six days on 10^1 and 10^2 plates and after seven days on 10^3 and 10^4 plates. The dilutions of 10^3 and 10^4 plates were the best to count the forming colonies (30–300). However, the cultivation of bacteria in the nutrient agar and R2A broth without any salt did not show any bacterial growth, which confirms that the bacterial species is halophilic.

The small, white and yellow colonies had non-serrated (smooth) edges and raised elevation, whereas the larger, white colonies had irregular edges and no elevation. All colonies were clear, glistening and smooth. In addition, light microscopy performed using Gram stain showed that all species were Gram-negative rod-shaped.

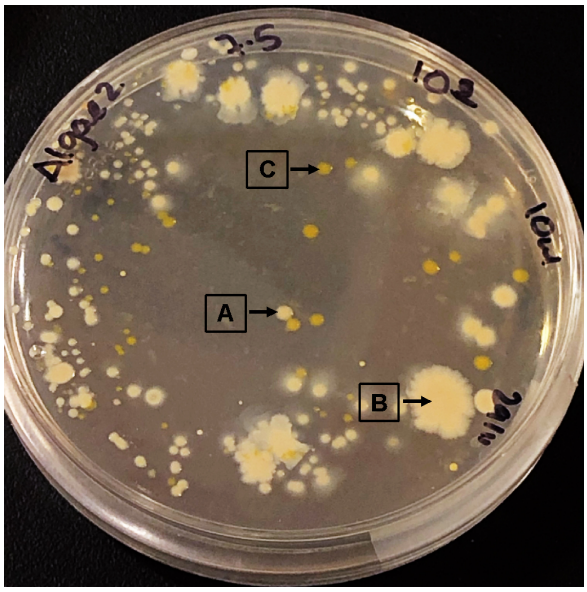

Figure 4.7 Three species of co-habiting bacteria isolated from *C. vulgaris* culture after 7 days of cultivation on *f/2*+R2A agar plate incubated at room temperature.

4.3.6.2 Molecular identification

Molecular typing was performed using 16S rRNA, which indicated that two of the isolates (small and large colonies) belonged to the *Halomonas* species (Accession numbers OM666636.1 and OM665417.1), while the third (yellow colony) belonged to the *Muricauda* sp. (Accession number OM666632.1), with homologies of 100% and 99.43%, respectively (Fig. 4.8). The percentage identity ranging between 95% and 98% for different species would allow the identification of genus only; to determine the bacterial species, special methods need to be applied between these species. If the percentage identity is less than 95%, a new strain or unavailability of sequence for alignment can be discovered (Barghouthi, 2011; Huo et al., 2020). Although *Halomonas* sp. is generally associated with *Dunaliella* cultivations (Keshtacher-Liebson et al., 1995), it has also been shown to be co-cultivated with *Chlorella* sp. (Zhang et al., 2020). *Muricauda* sp. has been isolated from algal cultivations and is useful

in developing co-cultures with different microalgae species (Han et al., 2016). The bacteria of classes Gamaproteobacteria (to which *Halomonas* belongs) and Flavobacteria (to which *Muricauda* belongs) are associated in industrial algae cultivations (Fulbright et al., 2018) and are the dominant bacterial types associated with microalgae in an aquatic karst ecosystem (Yan et al., 2020).

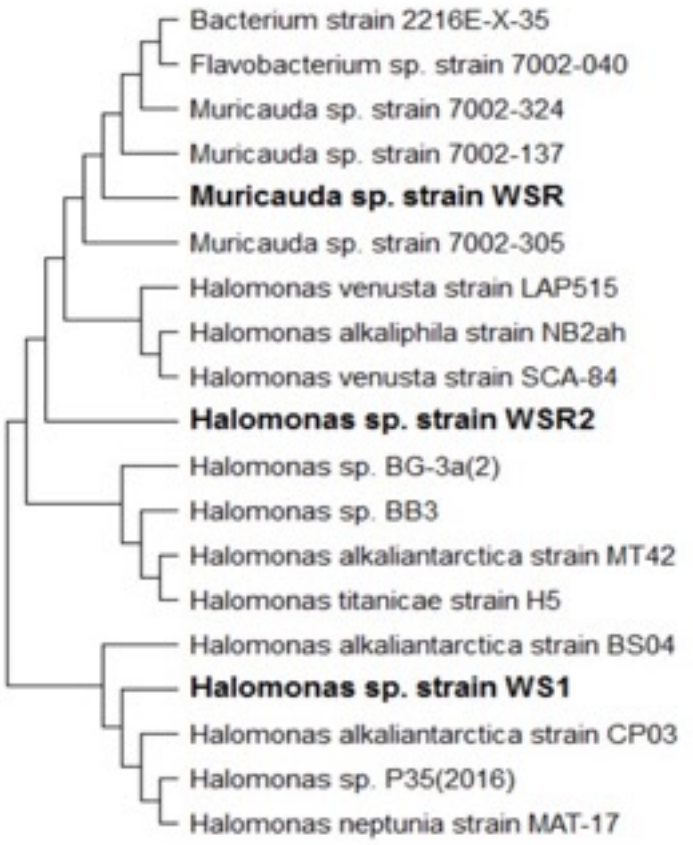

Figure 4.8 Phylogenetic tree of co-habiting bacteria and closely related species based on Internal Transcribed Spacers (ITS) sequences of ribosomal DNA. Distance was estimated with the Tamura-Nei model, and branch support was assessed with 500 bootstraps using Mega 11 software. Small colonies (*Halomonas* sp. WSR2); large colonies (*Halomonas* sp. WS1) and yellow colonies (*Muricauda* sp. WSR).

4.3.7 Abundance of co-habiting bacteria under different nutrient concentrations

The abundance and distribution of bacteria associated with algae were influenced by nutrient availability/depletion in the algae culture, in the three media (*f/2*, *f/4* and *2f*) at the three time points (0, 3 and 7) of algae cultivation, as shown in Fig. 4.9. The numbers of colonies formed in *f/2* and *2f* media were higher than those formed in *f/4*. In all three media, the total colony count of the bacteria increased over time as compared to the first day of isolation. This might result from the algae providing the essential nutrients for bacterial growth. Under all conditions, *Halomonas* sp. WSR2 (small colonies) dominated the bacterial population. *Muricauda* sp. WSR (yellow colonies) appeared on all days in *f/2* but only on day 7 in deplete (*f/4*) and day 3 in replete (*2f*) cultures. This species appears to be sensitive to changes in the nutrient supply, as movement to both replete (*2f*) and deplete conditions (*f/4*) from the control condition (*f/2*) resulted in lower cell counts or a complete absence. *Halomonas* sp. WS1 (large colonies) showed a similar trend of reduced numbers in replete (*2f*) and deplete (*f/4*) conditions, compared to *f/2*. *Halomonas* sp. WSR2 (small colonies), in contrast, showed an increase in cell counts in both deplete (*f/4*) and replete (*2f*) conditions compared to the control (*f/2)*. Changes in nutrient availability significantly perturbed the co-habiting bacterial composition of the algal culture ($p < 0.001$ on ANOVA).

However, the total bacterial count increased when the nutrient supply regime was changed. A more uniform bacterial composition was observed throughout the *f/2* culture compared to the *f/4* and *2f* cultures, and the increase in total bacterial load was also higher in *f/4* and *2f* compared to *f/2*. Symbiotic associations are known to exist between bacteria and microalgae, where vitamins from bacteria are provided for the algae in exchange for organic carbon (Croft et al., 2005; Kazamia et al., 2012). Increased bacterial population over the cultivation period in co-

cultures has been frequently observed (Qu et al., 2014). Furthermore, environmental changes influence composition and behaviour in microbial communities (Nguyen et al., 2021), and changes in bacterial composition of co-cultures between algae and associated bacteria exposed to different nutrient regimes are known to occur (Tait et al., 2019). Changes in community compositions in bacteria associated with microalgae in response to nitrogen variations in the culture are known (Wang et al., 2021), as are designed nitrogen source variations affecting bacterial composition in algae–bacterial consortia (Poddar et al., 2018). We conclude that a shift in nutrient supply (both nitrogen depletion and repletion) reduces the diversity of the co-habiting bacterial population, despite increased bacterial growth in the co-cultures.

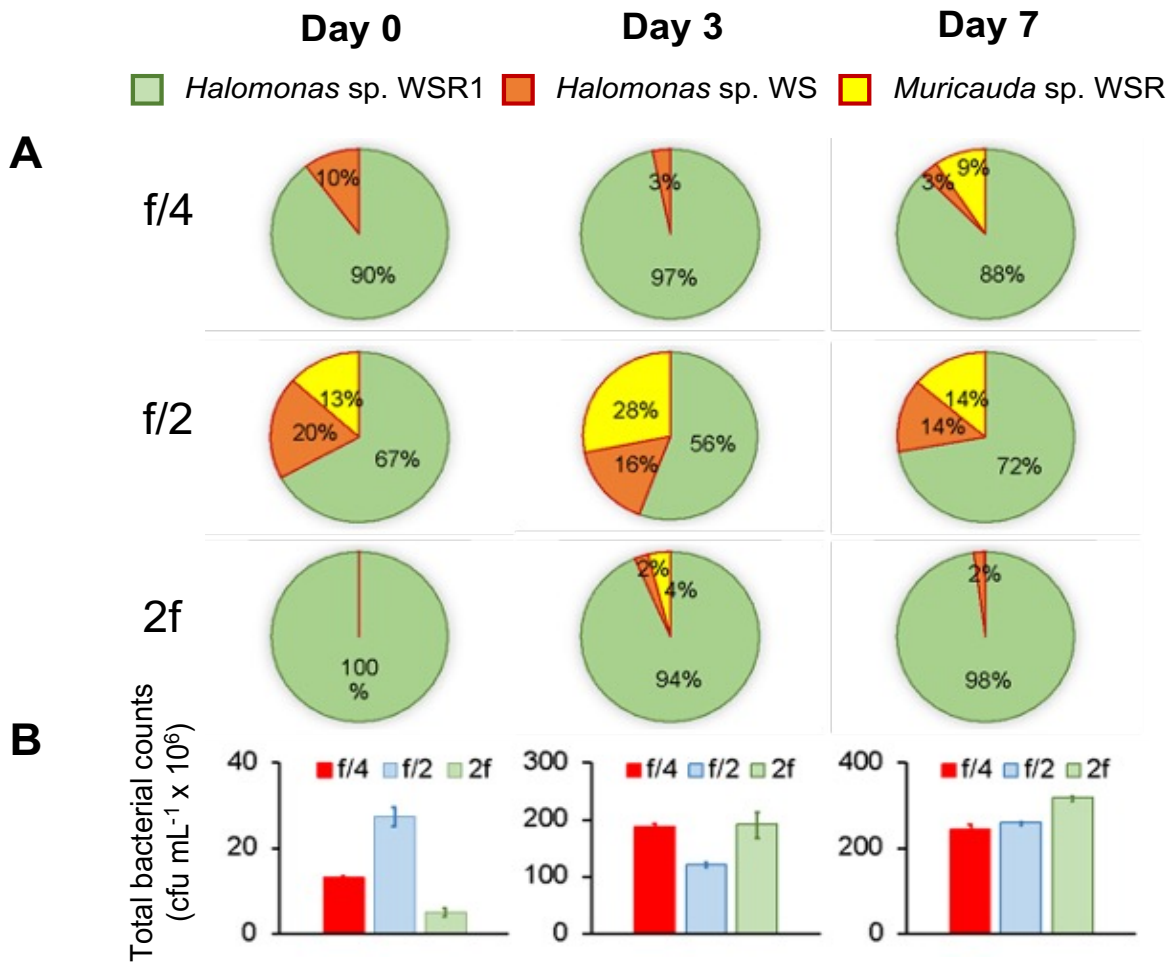

Figure 4.9 Abundance and distribution of three species of co-habiting bacteria at three isolation points during microalgae cultivation in *f/2*, *f/4* and *2f* media.

4.3.8 Conclusion

Three different media (*f/2*, *f/4* and *2f*) containing different concentrations of nitrate and phosphate were tested with an aim to enhance the carbohydrate content in *C. vulgaris* cells. The highest carbohydrate content (47% CDW) was observed in the deplete medium (*f/4*) on day 3 of cultivation. The carbohydrate yield was higher (74%) in the deplete medium than the replete medium (*2f*), while a higher carbohydrate productivity was observed in the replete medium (*2f*) as compared to the *f/4* medium (74% increase). Nutrient depletion/repletion did

not only affect carbohydrate accumulation but also algal growth. Although the carbohydrate content was the highest in the deplete medium, the algal growth was the lowest in this medium. Increasing the nutrient concentration in the culture (*2f*) resulted in increasing the productivity of algal biomass.

At least three cultivable cohabiting bacteria (belonging to *Halomonas* and *Muricauda* sp.) were isolated from the *C. vulgaris* culture for developing co-cultures between algae and bacteria. The bacterial population existed at different growth phases of algae cultivation and increased over time with the microalgae, which indicates a synergistic relationship between bacteria and algae. The total colony count was higher during the log and stationary phases of algal growth. In addition, changes in nutrient supply influenced bacterial abundance and distribution in the algae culture. Nutrient supply in the algae culture not only influenced algal growth but also affected cohabiting bacterial abundance. Three isolates were observed at all isolation points in *f/2*, but only one of these species was dominant when the nutrient concentrations were increased (*2f*) or decreased (*f/4*). *Halomonas* sp. WSR2 was the dominant species under different nutrient conditions.

Chapter 5 Characterisation of bacterial population cohabiting with *Chlorella vulgaris* CCAP 21/211

5.1 Introduction

An increasing number of studies have indicated that different species of bacteria can be found within the consortium of the algal culture, which can enhance algal growth and are affected by different factors, such as nutrient availability and growth phase of algae. In contrast, limited studies have explored the microbes isolated from algae cultures individually. Thus, in this chapter, we will study cohabiting bacteria with *C. vulgaris* under different conditions, aiming for optimal growth conditions.

It is well known that *Halomonas* sp. is a Gram-negative halophilic bacteria belonging to *Halomonadaceae*; it commonly grows in saline areas, such as sea, marshes, ocean and salt lakes and can survive in a wide range of temperatures and alkaline pH. *Halomonas* sp. was applied effectively on an industrial scale for bioproduct production because of its unique characteristics (Mormile, 1999; Chen et al., 2018; Ye and Chen, 2021) as well as its ability of high exopolysaccharide accumulation (Mukherjee et al., 2019). Many studies have reported that *Halomonas* sp. significantly influences the enhancement of microalgal growth and increased lipid production (Subasankari et al., 2020).

Muricauda sp. is Gram-negative, rod-shaped, aerobic bacteria; their colonies are characterised with yellow colour, belonging to the *Flavobacteriaceae* family. They commonly grow in saline environments and tolerant wide ranges of pH (6.0–9.0) and temperature (16–40°C) (Wang et al., 2017; Kim et al., 2020). *Muricauda* sp. interacts positively in the co-culture with microalgae, which contributes to the enhancement of algal growth, as reported by Han et al.

(2016), although only a few studies have reported the use of *Muricauda* sp. for industrial purposes and co-cultivation with algae.

In this chapter, we aim to characterise the isolated bacteria with respect to their growth and cultivation conditions to specifically identify the conditions in which the bacteria can survive, as well as those in which the bacteria cannot survive.

5.2 Experimental Design

The bacterial stocks after 48 h (10^5 CFU/ml for each isolate) were used to run all experiments. 1 ml of each stock was transferred separately into 250 ml conical flasks with foam bungs containing 200 ml of medium in triplicate. All flasks were shaken at 150 rpm on a tray shaker and incubated at room temperature (20±1°C) (unless a specification mentioned). The formed bacterial colonies were counted after 5 days of incubation at room temperature (20±1°C) (unless a specification mentioned).

5.2.1 Effect different media on bacterial growth

The growth of bacterial isolates was monitored in different media containing different sources of essential nutrients for 72 h: (a) *f/2*, (b) R2A broth, (c) *f*/2 + Glucose (*f/2*+ G), (d) *f*/2+ Glucose+ Ammonium chloride (*f/2*+ G+ N), (e) *f*/2 + Glucose+ Ammonium chloride+ Yeast extract (*f/2*+ G+ N+ Y), (f) *f/2*+ R2A (1:1) and (g) R2A broth+ salt (33.5 g/l of instant ocean salt). Glucose, ammonium chloride, and yeast extract were added at a final concentration of 0.5 g/l each in the respective medium they were present. However, *f/2*+ R2A medium was used as a favourable medium to determine the optimal pH and temperature conditions in different bacterial experiments (pH and temperature).

5.2.2 Effect different initial pH on bacterial growth

200 ml of *f/2*+R2A in 250 ml Erlenmeyer flasks with foam bungs were adjusted at different initial pH values (5.5–10) to investigate the behaviour of bacteria. The pH was adjusted aseptically in a laminar flow chamber by adding either sterile 1M HCL or 1M NaOH into sterile *f/*2+R2A medium to create acidic or alkaline culture that was monitored by a pH meter (Mettler Toledo).

5.2.3 Effect different temperature on bacterial growth

Triplicates of 250 ml Erlenmeyer flasks containing 200 ml of *f/2*+R2A medium with foam bungs for testing the bacterial growth in varied ranges of temperature (10–40°C). Each triplicate flask was incubated in incubators under different temperatures for 48 and 96 h for *Halomonas* sp. and *Muricauda* sp., respectively. The plates were incubated at the temperature at which the experiment was conducted and then counted after 5 days of incubation.

However, in all bacterial experiments, daily readings were taken to observe the bacterial growth and characterise the bacteria under different conditions using the CFU method. The pH changes of the culture were also monitored daily.

5.3 Results and Discussion

5.3.1 Determination of bacterial growth in different media

In this experiment, the CFU method (CFU/ml) was applied to monitor the growth of cohabiting bacterial isolates (Fig. 5.1A–C). The bacterial growth was detected in different media; according to the results, no growth was observed in the *f/2* medium (control) because it does not contain the carbon source required for bacterial growth. Although the R2A medium is rich in fundamental elements for growth, the growth was not observed due to lack of salt. The bacterial isolates are halophilic bacteria that need salt and sufficient nutrients for their growth.

Little growth was noticed in *f/2*+G (containing carbon source) and *f/2*+GN (containing carbon and simple N sources). The bacterial growth in *f/2*+GNY was better than that in *f/2*+G and *f/2*+GN ($p < 0.001$ for *Halomonas* sp. and $p < 0.05$ for *Maricauda* sp.), as it contains complex carbon and nitrogen sources, which are important for bacterial growth. The *f/2*+R2A medium was the best growth medium for all bacteria isolated from marine *C. vulgaris* CCAP 21/211, followed by R2A+salt, although the growth profile indicated that the growth in R2A +salt was slower than that in *f/2*+R2A (Fig. 5.1A–C). Both R2A broth and *f/2* contain sufficient concentrations of nitrogen and phosphorous, which can promote bacterial growth as well as salt availability.

The *f/2*+R2A medium was selected as a rich medium for bacterial growth for subsequent experiments under different conditions. However, the maximum growth rate of different species of bacteria was observed in *f/2*+R2A, *f/2*+GNY and R2A+salt ($p > 0.05$) for *Halomonas* sp. WSR2, while *Halomonas* sp. WS1 and *Maricauda* sp. WSR recorded a higher growth rate in *f/2*+R2A ($p < 0.05$ for *Halomonas* sp. WS1 and $p < 0.001$ for and *Maricauda* sp. WSR) (Fig. 5.1D). The growth of both *Halomonas* sp. peaked within 48 h, while *Muricauda* sp. took longer to reach the peak (72 h). The growth of all three bacterial species was synchronised with algal growth, as these individual growth rates are quite low for bacteria. This result agrees with previously reported results (e.g. Han et al., 2016; Tait et al., 2019).

Halomonas sp. is a halophilic bacteria growing in saline environments, such as sea, oceans, salt marshland and lakes or brackish water, that does not grow on artificial media without salt addition (Ye and Chen, 2021). Different media enriched with essential nutrients containing salt have been used for *Halomonas* sp. growth. Bibi et al. (2021) tested four media (MA, ½ R2A, ½ TSA and ½ NA) to grow *Halomonas*; ½ R2A and ½ TSA showed a high number of bacterial colonies due to the presence of enough essential nutrients for growth. The growth of 34% of *Marinobacter* and *Halomonas* strains was observed in the R2A medium with 10% NaCl

concentration (Nosalova et al., 2022). Moreover, several studies have cultivated *Halomonas* species in the minimal MM63+salt medium (Shivanand et al., 2013; Stiller et al., 2018; Hobmeier et al., 2020). Furthermore, several studies have indicated that the LB+salt medium can be used to grow *Halomonas* sp. as an enriched medium (Ren et al., 2018; Wang et al., 2019; Liu et al., 2020; Hobmeier et al., 2022).

However, no studies have reported the cultivation of *Muricauda* sp. in R2A broth for growth. Several studies (Le Chevanton et al., 2013; Yang et al., 2013; Tang et al., 2015; Wang et al., 2017) reported that marine broth (MB), an artificial medium, can be used as a rich medium to grow *Muricauda* sp. optimall

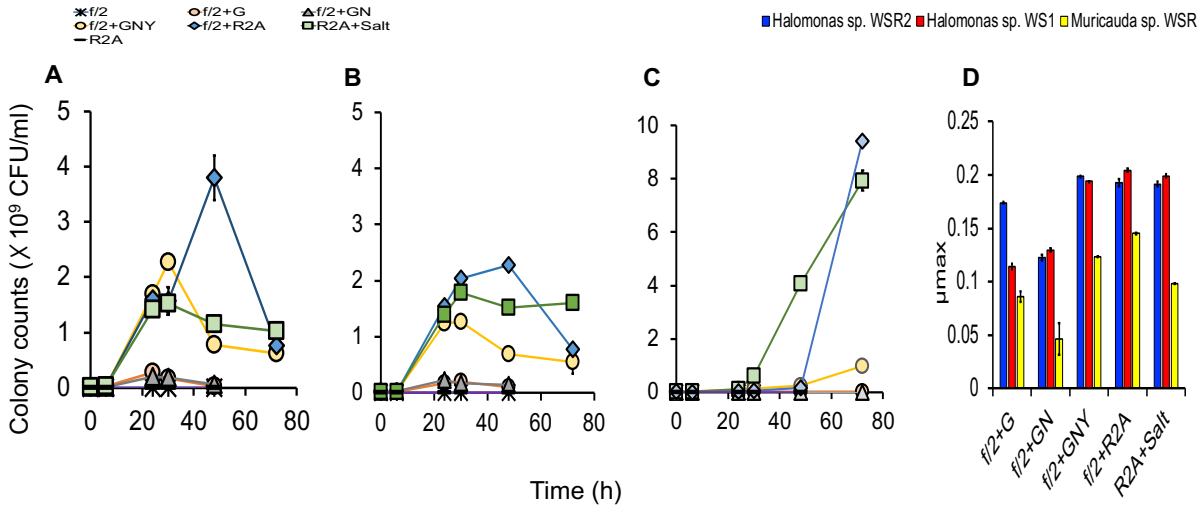

Figure 5.1 Growth of bacterial isolates in different media **A:** *Halomonas* WSR2 sp., **B**: *Halomonas* WS1 sp., **C**: *Muricauda* WSR sp. and **D**: µmax of bacterial growth in different media with shaking at 150 rpm and incubation at room temperature.

Bacterial growth causes pH changes over time. In the *Halomonas* sp. culture (Fig. 5.2A and B), the pH decreased slowly within 24 h of starting cultivation, and then increased to approximately 8.0 as the growth was higher at this pH in different media. However, when the growth was higher in *f/2*+R2A at 48 h, the pH was approximately 7.8–7.90. A similar result

was reported by Donio et al. (2013); they monitored the highest growth of *Halomonas* sp. BS4 in the pH range of 6.0–8.0 when cultivated in the nutrient broth medium containing salt. The pH decreased from 7.0 to 5.6 during the growth of *Halomonas* because of the production of organic acids as a result of glucose metabolism or the release of bioflocculants, as described by Mabinya et al. (2011).

Meanwhile, the pH of *Muricauda* cultures changed to range between 6.8 and 7.71 in different media after 72 h of cultivation as the growth reached its peak (Fig. 5.2C).

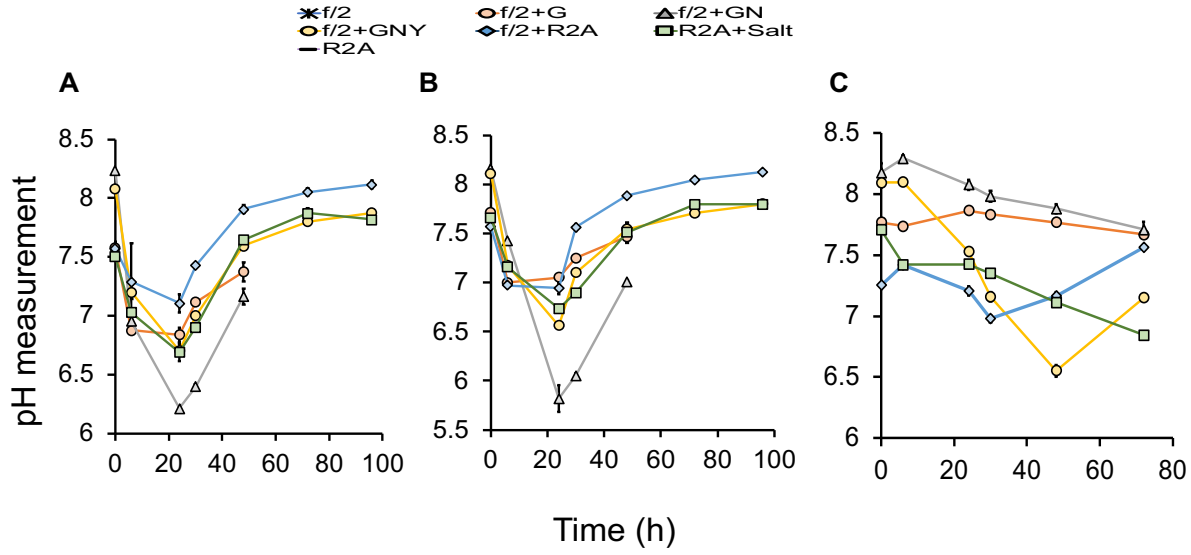

Figure 5.2 pH profile of bacterial isolates in different growth media **A:** *Halomonas* WSR2 sp., **B**: *Halomonas* WS1 sp. and **C**: *Muricauda* WSR sp. with shaking at 150 rpm for 72 h at room temperature.

5.3.2 Bacterial growth in a wide range of initial pH

The *f/2*+R2A medium is considered the best medium for the growth of bacterial isolates. In this study, the results of the CFU method showed that both *Halomonas* species could grow in a wide range of starting initial pH (5.5–10.0), while *Muricauda* sp. grew in the pH range 5.5–9.5 for 72 h, with an optimal pH range of 6.5–7.5 for the growth of both *Halomonas* species and 8.5 for *Muricauda* (Fig. 5.3A and B). There was no statistically significant difference

between the growth of both *Halomonas* sp. in the starting pH range of between 6.5 and 7.5 ($p > 0.05$). However, little growth of *Halomonas* sp. WSR2 and *Halomonas* sp. WS1 was observed in acidic and alkaline ranges compared to the growth in the pH range of 6.5–9.5. In addition, there was little growth of *Muricauda* sp. WSR at pH 5.5 and no growth at pH 10.0 (Fig. 5.3C). A statistically significant difference in the growth rate was noticed under the initial pH range on ANOVA ($p < 0.05$ for *Halomonas* sp. WSR2, $p < 0.001$ for *Halomonas* sp. WS and $p < 0.01$ for *Muricauda* sp. WSR1).

Similar to our results, Shivanand et al. (2013) observed that *Halomonas* sp. grew in pH range 7.0–10.0, with optimal growth at pH 7.1. In contrast, Mormile et al. (1999) recorded the highest growth rate of *Halomonas campisalis* sp. in the alkaline range (pH 9.5) when tested in a wide range of pH from 6.0 to 11.0. Additionally, Alquier et al. (2013) reported that the optimal pH of *Halomonas desiderata* is 9.7. However, several studies have reported that *Muricauda* can grow optimally at pH 7.0–7.5, while no growth can be observed at pH 5.0 in the MA medium containing 2%–3% NaCl (Lee et al., 2012; Zhang et al., 2018; Zhao et al., 2022). Wang et al. (2017) reported that the optimal pH for maximum growth of *Muricauda lutea* is 7.0, while Huntemann et al. (2012) reported that *Muricauda* can grow well within the pH range of 6.0–8.0, with optimal growth at 6.5–7.5.

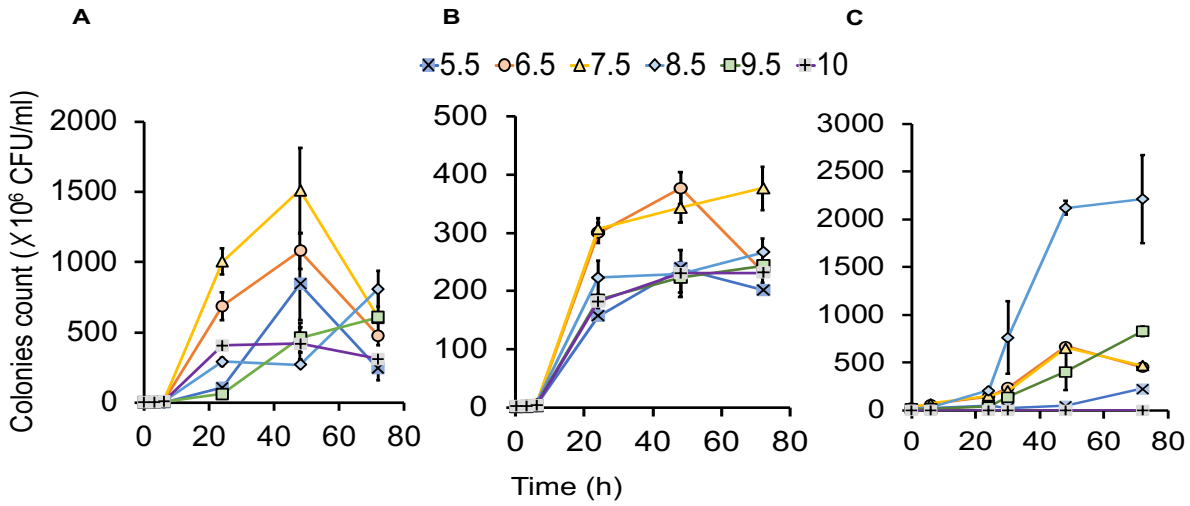

Figure 5.3 Growth profile of bacteria isolates (CFU/ml) **A:** *Halomonas* WSR2 sp., **B**: *Halomonas* WS1 sp. and **C**: *Muricauda* WSR sp. in the *f/2*+R2A medium in different initial pH ranges (5.5–10) for 72 h with shaking at 150 rpm at room temperature.

In the experiments conducted to determine the influence of pH on bacterial growth (Fig. 5.4A-C), bacteria began growing at different pH ranges. In both *Halomonas* cultures, changes in pH were recorded between 24 and 48 h to reach 6.75–7.6 during 48 h; after 48 h, all different pH values increased over time. In *Muricauda* cultures, significant changes in pH (6.0–7.24) were observed at 48 h. After 48 h, the pH rose to 7.14–7.51 as the bacterial growth peaked (log phase). There was no statistically significant difference in the pH change after 48 h under all tested pH values for all three species ($p > 0.05$).

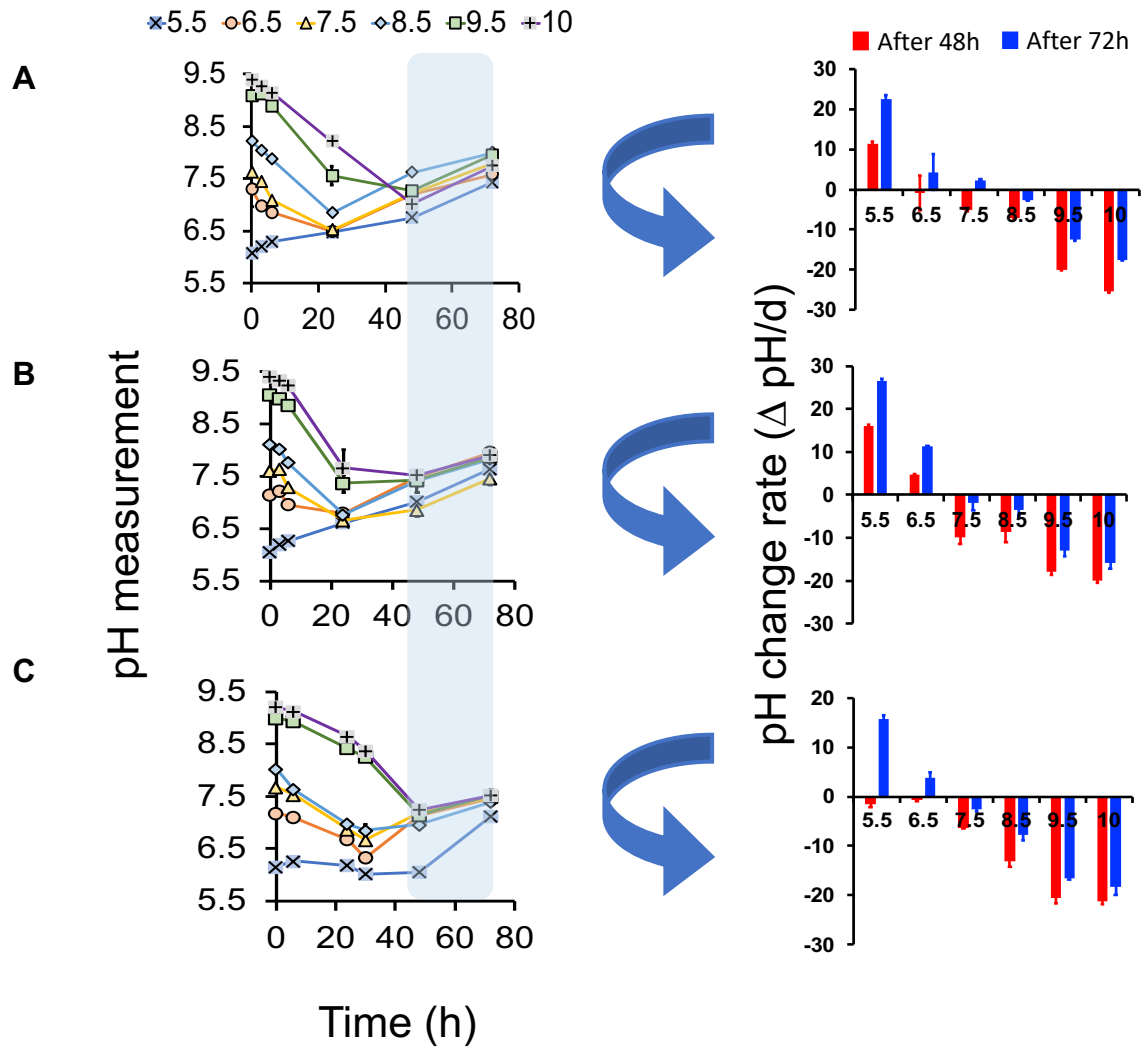

Figure 5.4 pH profile of bacterial growth **A:** *Halomonas* WSR2 sp., **B**: *Halomonas* WS1 sp. and **C**: *Muricauda* WSR sp. at different initial pH ranges for 72 h at room temperature and shaken at 150 rpm.

5.3.3 Bacterial growth at different ranges of temperature

Bacterial isolates were grown in the *f/2*+R2A medium for 48 h at different temperature ranges (10–40°C), and the growth was estimated using the CFU method. All three isolates for both *Halomonas* sp. (15–35°C) and *Muricauda* sp. (20–35°C) could survive in a wide range of temperatures. The optimal temperature for growth of bacterial isolates was observed at 30°C for both *Halomonas* sp. and at 25°C for *Muricauda* sp. (Fig. 5.5A-C). In both *Halomonas*

cultures, little growth was observed at 10°C and 40°C. Little growth of *Muricauda* sp. was observed at temperatures between 15°C and 20°C and no growth was detected at 10°C. A statistically significant difference in the growth rate was noticed under the tested temperatures range on ANOVA ($p > 0.05$ for all three species).

Halomonas sp. can be isolated from different marine environments that grow in a wide range of optimum temperatures for growth and have high tolerance to pH and salinity. Our results are in line with those obtained by Shivanand et al. (2013) and Delabary et al. (2020): *Halomonas* sp. growth was examined at a wide range of temperature (5–30°C), and *Halomonas* grew at all temperatures, achieving a high growth rate at 30°C. Additionally, the optimal temperature for *Halomonas campisalis* growth was 30°C when tested in a wide range of temperatures between 4°C and 50°C (Mormile et al., 1999).

A similar observation was reported by Zhang et al. (2018), where the optimal growth of *Muricauda* was recorded between 25 °C and 37 °C when it was tested at a wide range of temperatures (10–41°C). Huntemann et al. (2012) and Kim et al. (2020) also observed that the highest growth of *Muricauda* was achieved at 20–30°C. Yoon et al. (2005) reported that the maximum growth of *Muricauda* was achieved at 30–37°C and that no growth was detected at 4°C when tested at a wide range of temperatures (10–44°C). Zhao et al. (2022) observed that the optimal temperature of *Muricauda* growth ranged from 28°C to 32°C.

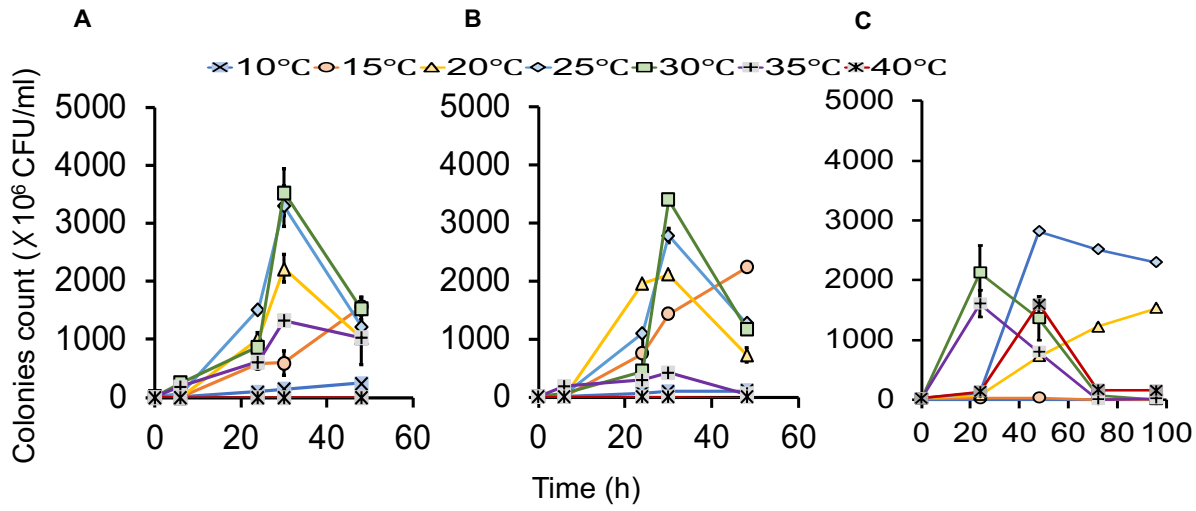

Figure 5.5 Growth profile of bacteria isolates (CFU/ml) **A:** *Halomonas* WSR2 sp., **B**: *Halomonas* WS1 sp. and **C**: *Muricauda* WSR sp. in *f/2*+R2A medium in different ranges of temperatures (10–40°C) for 48 h with shaking at 150 rpm.

The pH changes of all incubated cultures decreased sharply after 6 h of incubation, resulting in bacterial growth. Then, changes began increasing slowly at temperatures 10, 15, 20 and 40°C compared to a faster pH increase observed at temperatures within 25–35°C.

The pH reached approximately 8.0 after 48 h when the growth of *Halomonas* peaked under optimum growth temperatures ranging from 30°C to 35°C (Fig. 5.7). In the *Muricauda* sp. culture, the pH decreased during the first 24 h of incubation, and then started increasing under all tested conditions. The pH increase was faster at high temperatures (25–40°C), as the growth was higher than that at low temperatures (15–20°C) (Fig. 5.8).

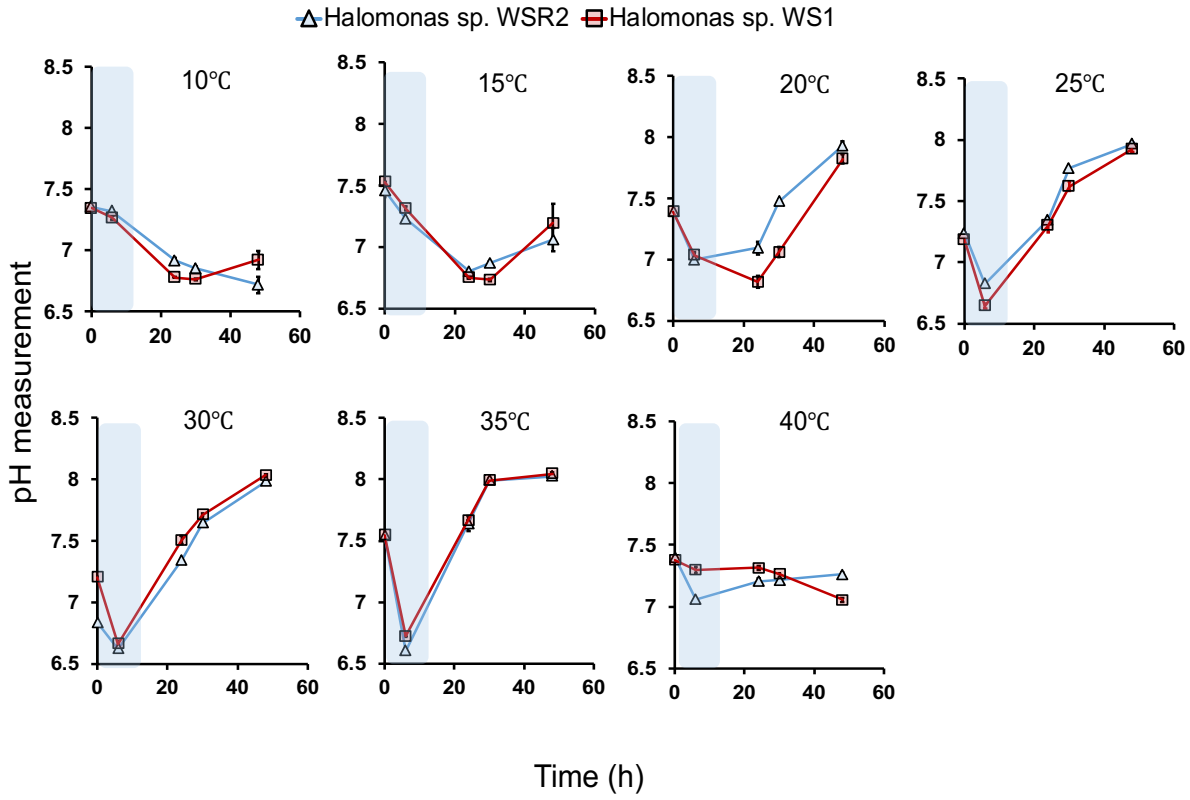

Figure 5.6 pH profile of bacterial growth of both *Halomonas* sp. at different temperatures (10–40°C) for 48 h with shaking at 150 rpm.

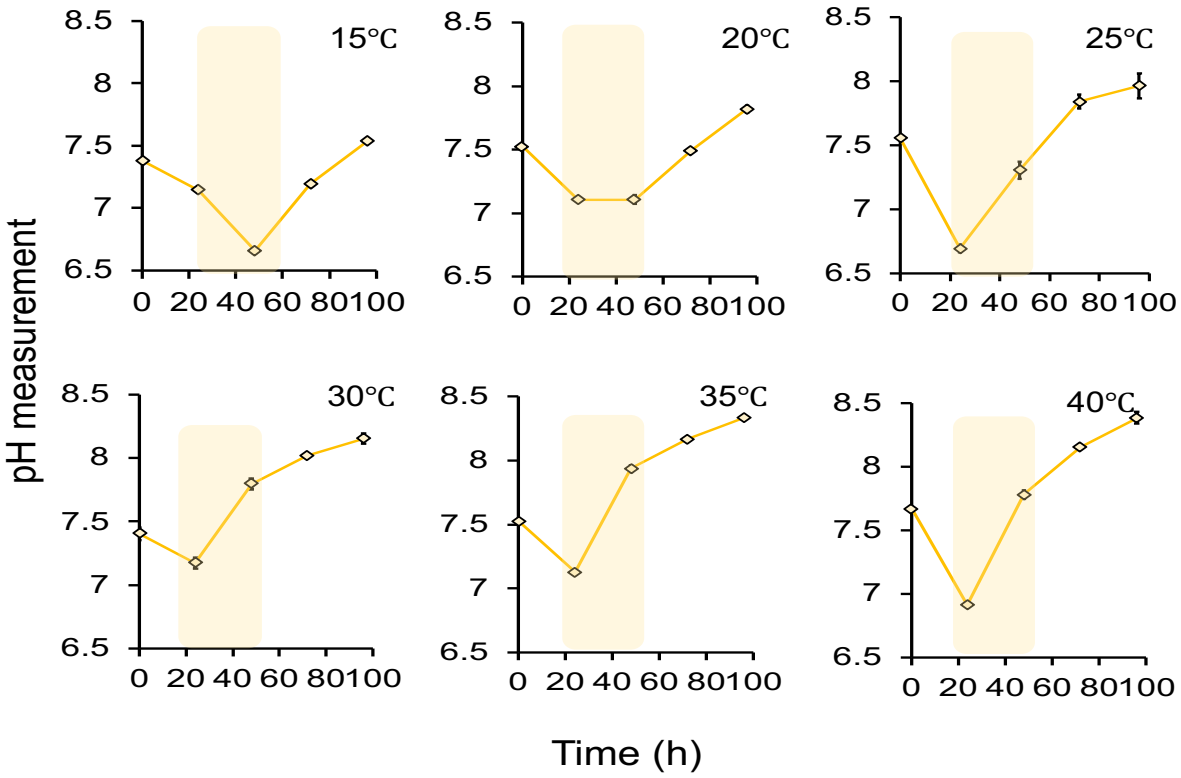

Figure 5.7 pH profile of *Muricauda* sp. WSR growth at different temperatures (15–40°C) for 72 h with shaking at 150 rpm.

5.4 Conclusion

In the assessment of different media that included different sources of carbon and nitrate, the *f/2*+R2A medium containing salt was found to be the most favourable medium for achieving the highest growth rates of all three isolated bacteria. Consequently, this medium was selected to examine bacterial growth under wide ranges of pH (5.5–10) and temperature (10–40°C). The optimal range of pH for the growth of both *Halomonas* sp. was 6.5–7.5, while *Muricauda* sp. grew optimally at 8.5. All three bacterial isolates achieved a high growth rate at 30°C and 25°C as an optimal temperature for growth of both *Halomonas* species and *Muricauda* sp. WSR1, respectively. We conclude that all three bacteria were not grown in extreme conditions of pH and temperature.

Chapter 6 Co-culture between *Chlorella vulgaris* CCAP and its cohabiting bacteria for promoting carbohydrate accumulation

6.1 Introduction

In natural communities, most cyanobacteria and microalgae are observed in combination with different aerobic or anaerobic microorganisms. It is well known that an association between microalgae and bacteria can promote or decline algal growth (Subashchandrabose et al., 2011). The phycosphere is an extracellular secretion area of algae that is rich in organic molecules and provides nutrients for a wide range of microorganisms that are associated with microalgae. Meanwhile, phycosphere bacteria can secrete a range of antibiotics that inhibit the growth of other microorganisms and protect algal growth. The interaction between microalgae and other microbes can be commensalism, mutualism or parasitism. Considering helpful interactions, microalgae growth can be promoted by bacterial metabolites, such as organic and non-organic components, or by bacterial secretions, such as growth-promoting compounds, including vitamins and trace metals. In turn, microalgae can supply bacteria with carbon and nitrogen from secreted extracellular products, such as polysaccharides and proteoglycans (Liang et al., 2014; Yao et al., 2019). However, for negative interactions between microalgae and bacteria, microalgae can release bactericide compounds and antibiotics that negatively affect the growth of associated bacteria, while bacteria can produce algaecide compounds that inhibit algal growth (Liu et al., 2020).

Specific bacterial species have positive associations with microalgae as microalgae growth-promoter bacteria in the co-culture. As reported, *Muricauda* sp., *Alteromonas* sp. and *Roseobacter* sp. promote algae growth when cultured with *Lobomonas rostrata*, *Dunaliella* sp. and *Phaeodactylum tricornutum*, respectively (Liu et al., 2020). In addition, the co-culture of

microalgae and bacteria not only promotes algal growth but also increases biochemical components. For example, the co-cultivation between *Scenedesmus obliquus* and *Acidovorax facilis* increases microalgal biomass by 3.5%–24.8% and enhances lipid accumulation in microalgae cells (Wang et al., 2015).

Many studies have reported that bacteria can enhance microalgal growth through symbiotic interactions with algae. According to González-González and de-Bashan (2021), some positive associations between microalgae and bacteria result in an increase in biomass productivity, growth rate and cell size. Considering the association of *Chlorella* with bacteria, there are many bacteria, such as *Flavobacterium*, *Azospirillum*, *Rhizobium*, *Sphingomonas* and *Hyphomonas*, that have a positive impact on the growth of *Chlorella* and increase in lipid production (Tait et al., 2019). *Azospirillum brasilense* is also a microalgae growth-promoting bacteria that plays a key role in the growth promotion of *Chlorella* sp. by fixing nitrogen; assimilating NO_2, NO_3 and NH_4 and producing indole-3-acetic acid (IAA) (Liu et al., 2020).

Many recent studies have applied natural and artificial co-cultures of microalgae and bacteria for different applications, such as wastewater treatment (Huo et al., 2020), increasing algal biomass (Subasankari et al., 2020) and biofuel production (Contreras-Angulo et al., 2019). Although co-cultured bacteria have both positive and negative influences on microalgae cultivations, some of which are known to influence microalgal carbohydrate productivity (Amavizca et al., 2017; Lopez et al., 2019; Marticorena et al., 2020; Biswas et al., 2021), the field is still nascent, requiring more detailed investigations on specific associations to develop conceptual frameworks and underlying principles that can be useful in establishing strategies to improve productivity. The co-cultivation of different microorganisms is influenced by several factors, such as number of shared partners, culture volume and time of co-culture (González-González and de-Bashan, 2021); one of the most influential factors in algae–bacteria associations that affects algal growth is the inoculum ratio. Therefore, bacterial cell

density in the algae culture plays a significant role in algal growth (Huo et al., 2020). Table 6.1 shows the inoculum ratios applied in the co-culture between microalgae and bacteria for different purposes.

This chapter aims to explore introduction of cohabiting bacteria into axenic culture of *C. vulgaris* towards achieving positive results on carbohydrate yield and productivity as well as the growth of *Chlorella vulgaris*, with an aim to increase the substrate for economic biofuel production. A high-yielding strain of *Chlorella vulgaris* (CCAP 211/21A) that showed a high propensity to accumulate carbohydrates in preference to lipids was reported earlier (Slocombe et al., 2021). Co-culture (between *C. vulgaris* and *Halomonas*) and mixed- culture (*Halomonas*/ *Muricauda* with *C. vulgaris*) with different inoculum ratios introduced at different growth phases of *C. vulgaris* have been investigated. We believe this to be a first attempt of this type with marine *Chlorella vulgaris* CCAP, whose findings will enable the development of a conceptual framework that can be used to establish practically scalable strategies for a sustainable production of feedstock with high fermentable sugar content.

Table 6.1 Inoculation ratio of microalgae and bacteria in artificial co-culture

Algae sp.	Partner sp.	Inoculum ratio	Bacteria was introduced	Purpose of cultivation	Reference
C. vulgaris (**freshwater**)	*Rhizobium* sp.	1:1	-	Wastewater treatment	Ferro et al., 2019
Chlorella sp. (**freshwater**)	*Activated sludge*	3:1	-	Nutrient removal	Nguyen et al., 2020
Scenedesmus sp. (**freshwater**)	*Azospirillum brasilense*	8 ml of 8×10^6 algae 2 ml of 1×10^9 bacteria	Day 0	Biofuels production	Contreras-Angulo et al., 2019
Nannochloropsis sp. (**marine water**)	*Halomonas aquamarina*	100:1	Day 0	Lipids production	Subasankari et al., 2020
C. vulgaris (**freshwater**)	*Activated sludge*	1:1	-	Increased algal biomass	Feng et al., 2020
C. vulgaris (**freshwater**)	*Mesorhizobium sangaii*	40:1	-	Biodiesel production	Wei et al., 2020
C. ellipsoidea (**freshwater**)	*Brevundimonas* sp.	1:1	-	Increase algal biomass	Park et al., 2008
C. vulgaris (**freshwater**)	*Stenotrophomonas maltophilia*	2:1	-	Biodiesel production	Xue et al., 2018

6.2 Experimental Design

6.2.1 Co-culture of *C. vulgaris* and *Halomonas* sp. WSR2

Halomonas sp. WSR2 were grown in 200 ml of the *f/2*+R2A medium for 48 h (~42×10^5), and the cells were harvested at 4000 rpm for 10 min. Two inoculum bacterial concentration, 1 and 10^4 CFU/ml of bacterial cells (approximate algae:bacteria ratios of 1000:1, 1:10) were introduced into a fresh *f/2* medium containing approximately 1.8×10^3 cells/ml of an axenic *C. vulgaris* on day 0 of algal cultivation (at the beginning of lag phase of algae growth). The same inoculum ratios (1 and 10^4 CFU/ml) of *Halomonas sp. WSR2* were also introduced into the algal culture in a separate experiment on day 2 of algal cultivation (at the beginning of stationary phase of algae growth).

The algal pellets were centrifuged at 4000 rpm for 5 min and then transferred into 1L of the fresh *f/2* medium. Co-cultivation was conducted for 7 days at room temperature (20±1°C) with continuous light intensity at 200 μmol photons/m^2/s. Continuous air flow was supplied, and 100% CO_2 was provided for 10 min daily.

6.2.2 Mixed culture of *C. vulgaris* with three bacterial isolates

100 ml of different inoculation ratios of *Halomonas* sp. WSR2, *Halomonas* sp. WS1(~42×10^5) and *Muricauda* sp. WSR (~80×10^5) were carried out in two different experiments. Mixed ratios composed of 5:75:20 ml (case1) and 5:20:75 ml (case2) of *Halomonas* sp. WSR2, *Halomonas* sp. WS1 and *Muricauda* sp. WSR, respectively, were used in two separate experiments. Combinations of bacteria (case 1 and case 2) were introduced into a fresh *f/2* medium containing approximately 1.8×10^3 cells/ml of an axenic *C. vulgaris* on day 0 of algal cultivation. The experimental setup was the same as that used for the co-culture experiments.

6.2.3 Scanning electron microscopy (SEM)

The samples were processed using an electron microscope unit according to Cuo and Tong's (2014) protocol. The samples were fixed on glass slide with glutaraldehyde (1.5%) and kept at 4°C for 24h. Then, the samples were washed with ultra-pure water and dehydrated by 25- 100% Ethanol. The fixed samples were coated with gold and observed under SEM (TESCAN Vega 3 LMU, Tescan UK Cambridge England). Scanning Electron Microscope was at an accelerating voltage of 15Kv.

6.3 Results and Discussion

6.3.1 Algal growth influenced by introducing different concentrations of single/mixed bacterial species

When 1 and 10^4 CFU/ml of *Halomonas* sp. WSR2 were introduced into the algae culture on day 0 (Fig. 6.1B) and day 2 (Fig. 6.1C) of algal cultivation, respectively, continuous algal growth was observed during 7 days of cultivation by all three growth measurements (OD, cell count and DCW); the growth increased rapidly after 2 days. Going forward, cell density was used to compare changes, as it was a more reliable metric. The introduction of *Halomonas* sp. WSR2, as the sole bacterial type, showed a statistically significant increase in median specific growth rate (cell density basis), for inoculations at day 0 ($p<0.01$ for 1 CFU/mL and $p<0.05$ for 10^4 CFU/mL), and no statistically significant difference for day 2 inoculations at both concentrations compared to the control (Fig. 6.1A). Of the two inoculation time points, day 0 inoculations resulted in higher median specific growth rate compared to day 2, for an initial inoculum of 10^4 CFU/mL. The maximum fold change in biomass increased by approximately 515% when *Halomonas* sp. WSR2 was introduced on day 0 (cell density basis), and the final biomass reduced by 20% when bacteria were added on day 2 (compared to the inoculation on day 0). Introducing a small concentration (1 CFU/ml) of single bacterial type to the algae

culture increased the final biomass by 34%, while the introduction of a high concentration (10^4 CFU/ml) yielded the highest increase (by 45%) in the final biomass on day 7 (cell density basis).

On the other hand, for mixed cultures in two ratios, when 10^4 CFU/ml of bacterial combinations were introduced into the algae culture on day 0, continuous algal growth was observed during 7 days of cultivation for all three growth measurements (OD, cell count and DCW) as the growth increased rapidly after 2 days (Fig. 6.2B). Mixed cultures in both cases 1 and 2 doubled the growth rate of algae ($p < 0.0001$ for case 1 and $p < 0.001$ for case 2 on median growth rate, cell count basis), which indicates the significant role played by multiple bacteria inoculation in the algae culture compared to the control (Fig. 6.2C). A higher algal growth was observed in case 1 than in case 2, because of the rapid growth of *Halomonas* sp. which represented a higher proportion (80%) in case 1, compared to case 2 (25%), which was dominated by *Muricauda* sp. WSR (75%). The maximum fold changes were observed through the mixed culture in case 1 by 72% and 87% increase in case 2, compared to the control. The final biomass of algal cell density can be seen to be higher by 72% in case 1 and 87% in case 2, compared to the control (Fig. 6.2B).

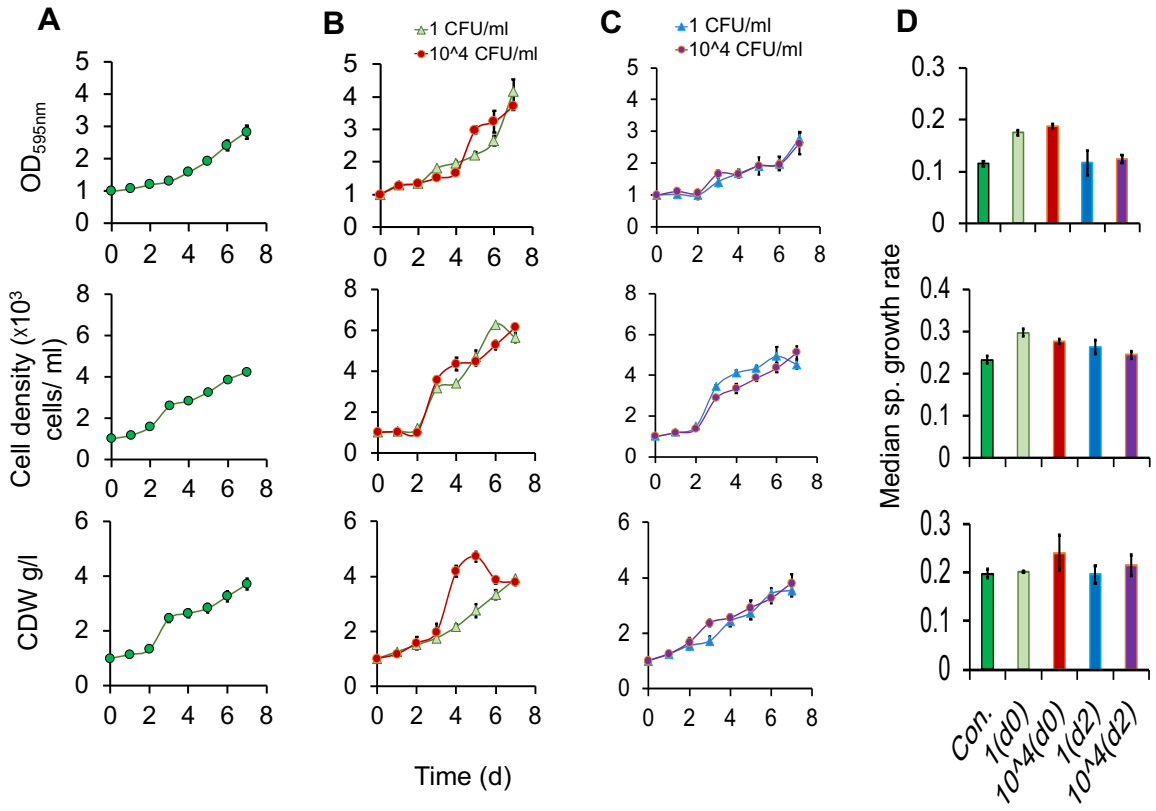

Figure 6.1 A: Growth of axenic *C. vulgaris* in batch mode culture for 7 day control, **B**: co-culture with *Halomonas* sp. WSR2 at different concentrations (1 and 10^4 CFU /ml) introducedon day 0 of algae cultivation, **C**: on day 2 of algae cultivation and **D**: maximum growth rates of algae.

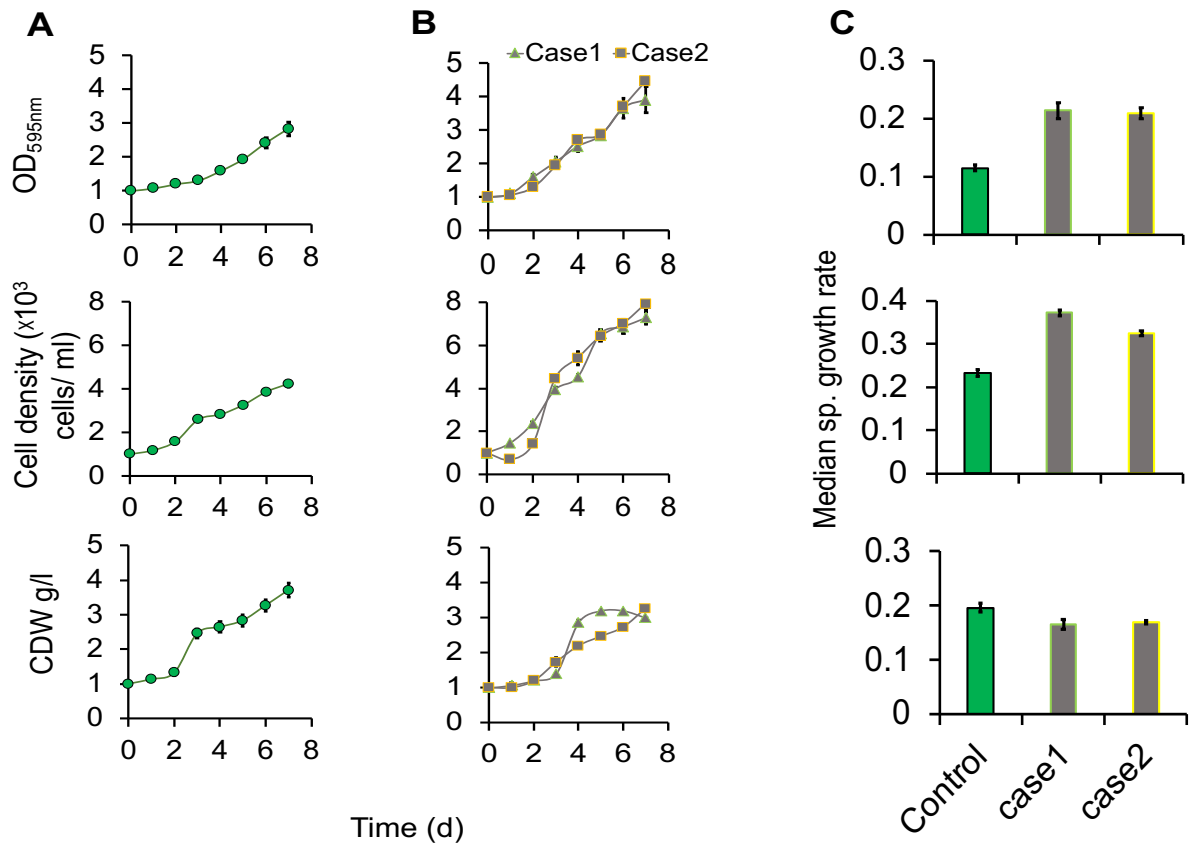

Figure 6.2 A: Growth of axenic *C. vulgaris* in batch mode culture for 7 day control, **B**: co-culture with different combinations of bacteria (10^4 CFU /ml) introduced on day 0 of algae cultivation and **C**: maximum growth rates of algae.

Many studies have reported that co-culture plays a significant role in enhancing algal growth. For example, separate co-cultures between *C. vulgaris* and four strains of bacteria (*Hyphomonas*, *Flavobacterium*, *Sphingomonas* and *Rhizobium*) isolated from the algal culture led to 100% increase in microalgae cell density compared to the monoculture of *C. vulgaris* (Cho et al., 2015). Moreover, the co-cultivation of *Muricauda* sp. with different microalgae *Tetraselmis chuii*, *Nannochloropsis gaditana* and *Cylindrotheca fusiformis* individually under mixotrophic conditions for 33 days resulted in enhanced growth of *T. chuii* and *C. fusiformis* but deteriorated the growth of *N. gaditana* (Han et al., 2016).

According to our results, *Halomonas* sp. plays a significant role in enhancing algal growth. In line with our results, it has been noted that growth of *Nannochloropsis oceanic*a (a marine

species) increased by 17% when the culture was inoculated with *Halomonas aquamarine* (100 algae: 1 bacteria) (Subasankari et al. (2020)). Introducing single bacteria (*A. facilis*) into the microalgae *Scenedesmus obliquus* culture has been shown to increase the maximum algal biomass by 25% compared to the monoculture, while introducing multi-bacteria into the culture resulted in a 2% reduction in maximum algae biomass (Wang et al., 2015). In addition, Xu et al. (2020) reported that the co-cultivation of two bacterial species *Microbacterium* and *Bacillus* with fresh *Chlorella vulgaris* in a ratio of 10:1 (bacteria:algae) led to a 48% increase in the algae growth rate compared to the monoculture. Marticorena et al. (2020) observed that indoor and outdoor co-cultivation between five bacterial species with microalgae *Muriellopsis* sp. for 20 days increased the biomass concentration by 22% and 27%, respectively, compared to the control.

In this study, the symbiosis between *C. vulgaris* and *Halomonas* sp. in a co-culture and that of *C. vulgaris* with *Halomonas* and *Muricauda* sp. was found to enhance algal growth, as shown by the microbial association in Fig. 6.3. SEM was carried out on the axenic algal cells and co-cultures. Figure 6.3 shows a close physical contact between *C. vulgaris* cells and different bacterial species (*Halomonas* sp. and *Muricauda* sp.), which confirms the microbial co-cultures in our study. As reported by Ferro et al. (2019), a microbial interaction in the co-culture between *C. vulgaris* and *Rhizobium* indicated that the gas exchange or release promoting molecules at the cell surface resulted in positive interactions between them. There are three types of interactions between microalgae and associated bacteria: gene transfer, nutrient exchange and signal transduction. Of these, the most important and well-known is nutrient exchange (González-González and de-Bashan, 2021). The exchange of metabolites, such as essential nutrients, growth hormones and vitamins, is important for the survival of each microorganism of the microalgae–bacteria consortium in marine environments (Perera et al., 2021).

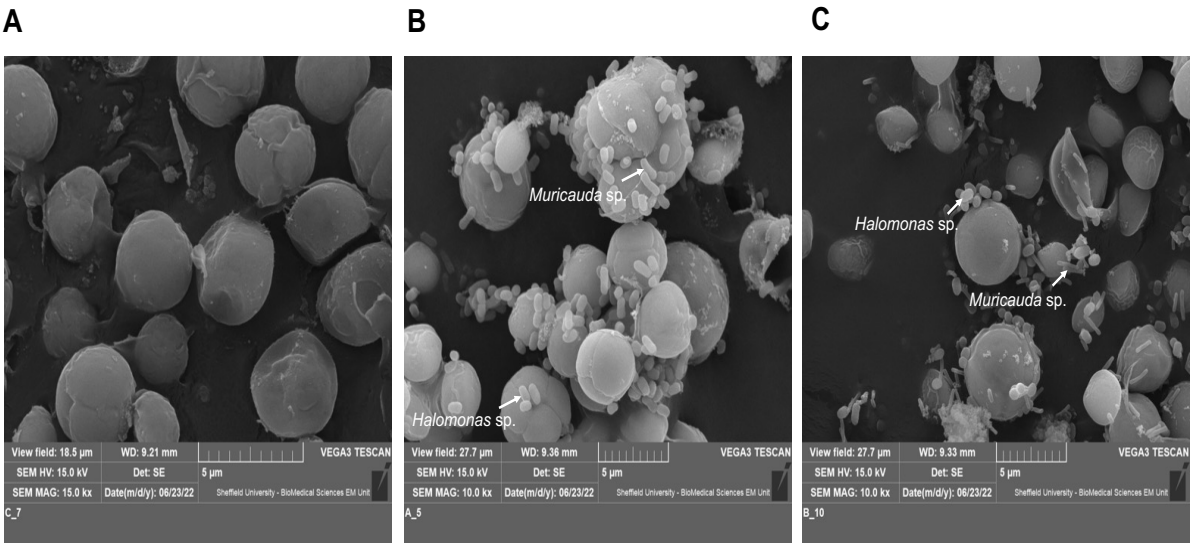

Figure 6.3 SEM images of **A:** axenic *C. vulgaris* cells, **B:** microalgae–bacteria association in case 1 and **C**: microalgae–bacteria association in case 2.

6.3.2 pH profile through co-cultivation

The initial pH in the *f/2* medium (Fig. 6.4A–D) was approximately 8.3–8.5, which increased over time and resulted in algae growth. The following pH profile was observed: When the algae culture was inoculated with a single bacteria (B) or with mixed bacterial species (D) on day 0, the pH peaked (~9.1 to 9.3) on day 4 of algae cultivation, and then decreased sharply. A different pH trend was observed when a single type of bacteria was introduced on day 2 of algae cultivation; the pH increased directly on day 2 to reach the peak (~9.05 to 9.2), and then remained steady. The pH range of 8.8–9.3 in the culture helps algae growth to increase over time. There was no significant difference between pH changes in the control and co-culture ($p > 0.05$ on ANOVA).

In line with our results, Daliry et al. (2017) reported that although *C. vulgaris* can grow in a wide variety of pH conditions, pH 9–10 provides the best growth rate and biomass productivity.

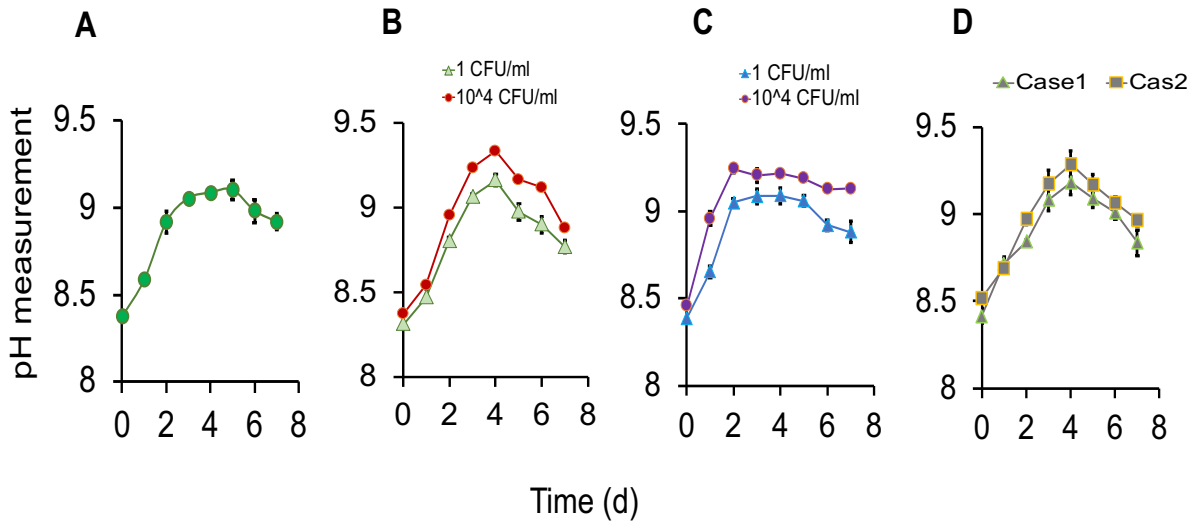

Figure 6.4 pH profile in **A**: axenic *C. vulgaris* culture, **B**: co-culture of *C. vulgaris* with single bacteria type on day 0, **C**: on day 2 and **D**: co-culture of *C. vulgaris* with combination bacteria on day 0.

6.3.3 Consumption of nitrate and phosphate concentrations under different co-culture systems

Under all situations (Fig. 6.5A–D), the initial nitrate concentration in the *f/2* medium was 880 µM. The nitrate consumption profile decreased over time as a result of algal growth. The nitrate available in all media was consumed completely within 4 days. The nitrate consumption profiles of the co-culture experiments were similar to the consumption profile of the algae in the control medium, which indicates that, in all co-culture experiments, nitrate was consumed by microalgae and not used by bacteria. Figure 6.5E–H shows the fold-change profile of nitrogen concentration in the control medium and co-culture media.

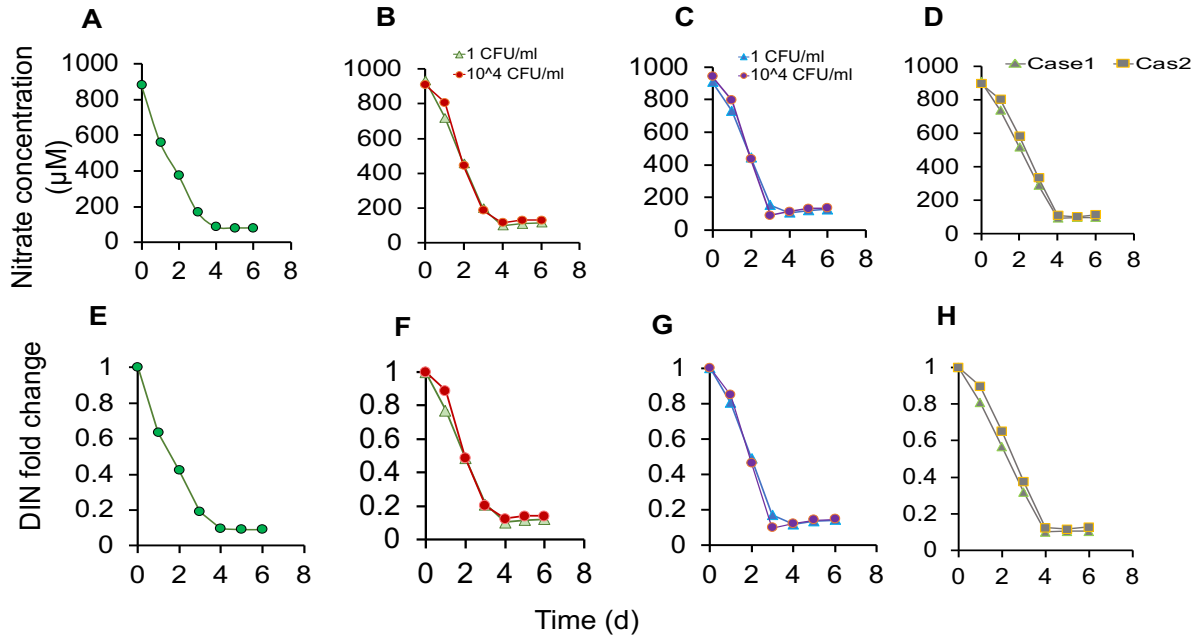

Figure 6.5 Consumption of dissolved inorganic nitrate in **A**: axenic *C. vulgaris* culture, **B**: co-culture of *C. vulgaris* with single bacteria type on day 0, **C**: on day 2 and **D**: co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR), fold change of dissolved inorganic nitrate in **E**: axenic *C. vulgaris* culture, **F**: co-culture of *C. vulgaris* with single bacteria type on day 0, **G**: on day 2 and **H**: co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR).

Figure 6.6 (A–D) shows the initial phosphate concentration in the *f/2* medium, which was approximately 20–25 µM. The phosphate profile decreased over time and was completely consumed from the media after 2 days because of algal growth. The phosphate consumption in all co-culture experiments was similar to the consumption by algae in the control medium, which indicates that, in all co-culture experiments, phosphate was consumed by microalgae and not used by bacteria. Figure 6.6 (E–H) shows the fold-change profile of phosphate concentration in the control medium and co-culture media. Our results indicated that the available phosphate was consumed only by *C. vulgaris*, as bacteria use phosphorus for growth.

Our results confirmed that bacteria did not apparently contribute to nitrate and phosphate consumption, although many studies (Delgadillo-Mirquez et al., 2016; Tang et al., 2018; Wang et al., 2020; Carvalho et al., 2021) have reported the significant role of co-culture in nitrogen and phosphate removal from wastewater; bacteria can use nitrogen in NH_4 form and a simple form of phosphorus. Haberkorn et al. (2020) reported that when *Chlorella vulgaris* was co-cultured with *Sphingopyxis* sp. and *Pseudomonas* sp., the available ammonium was quickly consumed within the first 72 h of cultivation.

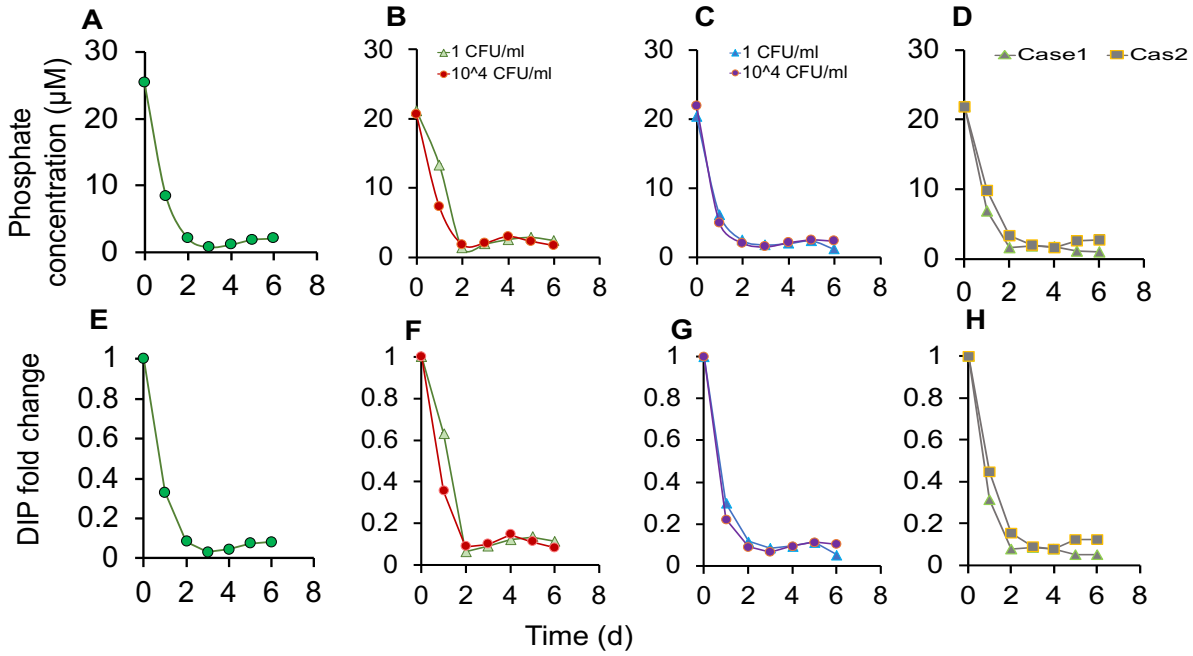

Figure 6.6 Consumption of dissolved inorganic phosphate in **A**: axenic *C. vulgaris* culture, **B**: co-culture of *C. vulgaris* with single bacteria type on day 0, **C**: on day 2 and **D**: co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR), fold change of dissolved inorganic phosphate in **E**: axenic *C. vulgaris* culture, **F**: co-culture of *C. vulgaris* with single bacteria type on day 0, **G**: on day 2 and **H**: co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR).

6.3.4 Influence of different co-culture systems on increased carbohydrate content and productivity

The co-cultivation between *C. vulgaris* and *Halomonas* sp. WSR2 at different concentrations (1 and 10^4 CFU/ml) on day 0 of algae cultivation doubled the carbohydrate content in algae cells on day 4 (for 1 CFU/ml) and day 7 (for 10^4 CFU/ml) of cultivation (Fig. 6.7B), where higher maximum carbohydrate yield was observed under inoculation with higher initial bacterial concentrations on both days (Fig. 6.7E) compared to the control. Introducing 1 CFU/ml of *Halomonas* sp. WSR2 on day 0 resulted in a 65% increase in carbohydrate yield fold change ($p < 0.01$), while introducing a higher bacterial concentration (10^4 CFU/ml) achieved an even higher carbohydrate yield fold change increased by 99% ($p < 0.05$) compared to the control. In addition, introducing 10^4 CFU/ml of *Halomonas* sp. WSR2 on day 2 of *C. vulgaris* cultivation doubled the carbohydrate content of algae cells on day 5 of cultivation (Fig. 6.7C) ($p < 0.05$ compared to control), while the addition of 1 CFU/ml of bacteria on day 2 increased the carbohydrate yield by one fold. The highest carbohydrate yield was observed when *C. vulgaris* was co-cultured with 10^4 CFU/ml of *Halomonas* sp. WSR2 on day 0 (99%) and day 2 (82%) of algae cultivation. Introducing *Halomonas* sp. WSR2 into the algae culture on day 2 not only enhanced the carbohydrate accumulation (with the addition of 10^4 CFU/ml) but also increased fold change in carbohydrate productivity by 175%, compared to the control ($p<0.05$), for 1 CFU/ml) (Fig. 6.8E). In contrast, the co-cultivation of *C. vulgaris* with a mixture of bacterial species (*Halomonas* and *Muricauda* sp.) in different ratios did not influence the yield and productivity of carbohydrates in cases 1 and 2 (Figs. 6.7D and 6.8D, respectively). The maximum fold changes in case 1 and case 2 were 23% and 28% reduction in carbohydrate content, respectively. There was no significant difference in carbohydrate yield between case 1 and case 2. A comparison between single (*Halomonas* WSR2) and mixed bacterial population (case 1 and 2) introduced at day 0, at an initial bacterial concentration of

10^4 CFU/ml, showed that there was a statistically significant difference in carbohydrate yield ($p < 0.01$), whilst there was no significant difference in carbohydrate productivity ($p > 0.05$), for both the mixed cases compared to single bacterial introduction.

It is well known that the co-culture between microalgae and bacteria enhances the growth of microalgae and its biochemical compositions; however, most studies have focused on increased lipid content in algal cells rather than the carbohydrate content. Rasouli et al. (2018) found that the co-cultivation between *Chlorella sorokiniana* and *Methylococcus capsulatus* resulted in 32% DCW increase in carbohydrate content and 34% DCW increase in lipid content.

Similar to our results obtained for the mixed culture, Marticorena et al. (2020) observed that the inoculation of microalgae *Muriellopsis* sp. with five bacterial species enhanced the carbohydrate productivity by 30% when cultured indoor and by 23% when cultured outdoor.

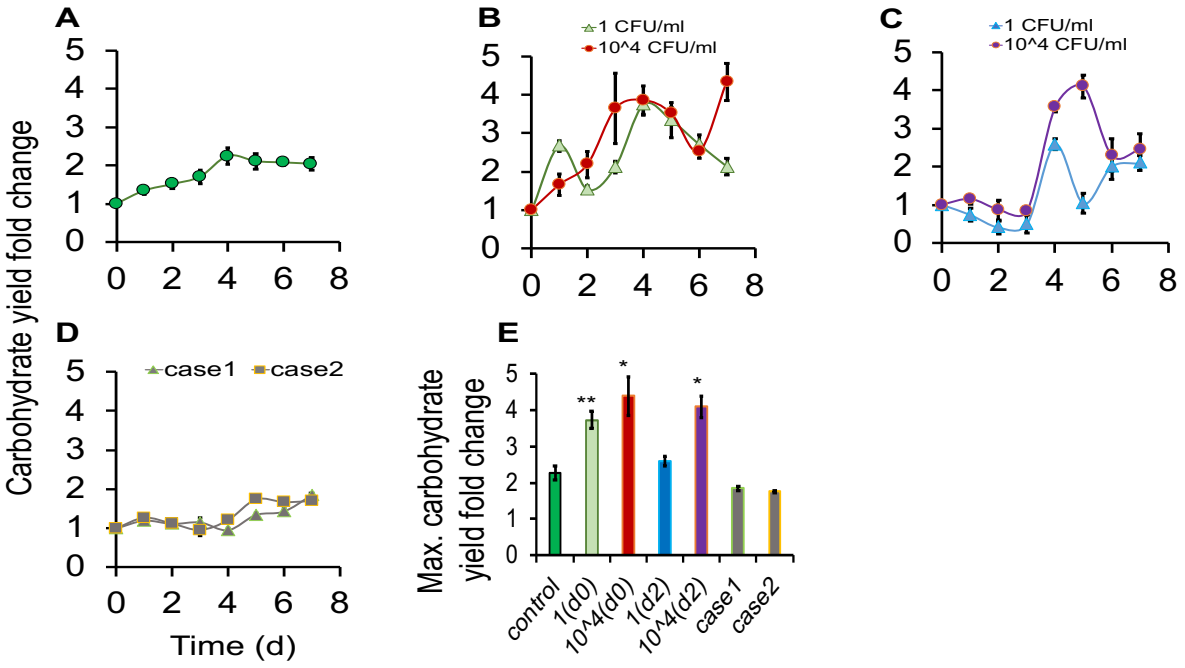

Figure 6.7 Profiles of carbohydrate content in algal cells of **A:** axenic *C. vulgaris*, **B:** co-culture of *C vulgaris* with single bacteria type on day 0, **C:** on day 2, **D:** co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR) and **E:** maximum fold change of carbohydrate yield, showing significant difference in maximum carbohydrate yield when compared to the control.

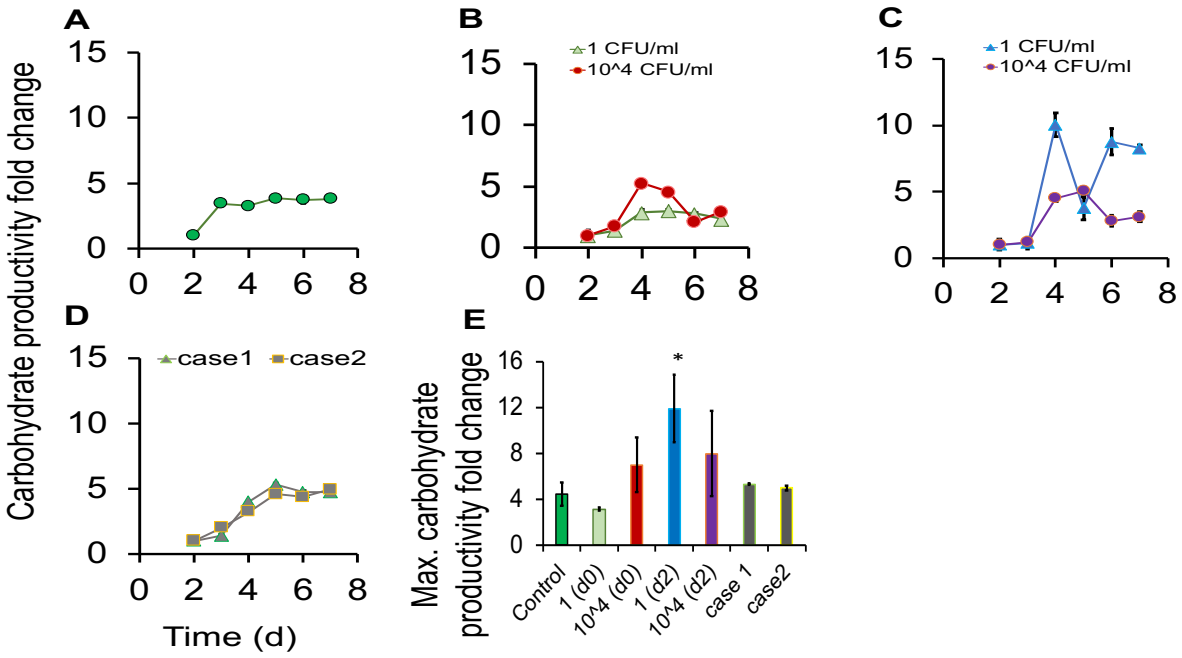

Figure 6.8 Profiles of carbohydrate productivity in algal cells of **A:** axenic *C. vulgaris*, **B:** co-culture of *C vulgaris* with single bacteria type on day 0, **C:** on day 2, **D:** co-culture of *C. vulgaris* with combination bacteria on day 0 in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR) and **E:** maximum fold change of carbohydrate productivity, showing significant difference in maximum carbohydrate productivity when compared to the control.

6.3.5 Inoculation with single/mixed bacterial species changes the dynamics of bacterial density

Changes in bacterial densities of the *Halomonas* sp. WSR2 (1 and 10^4 CFU/ml) introduced on day 0 were monitored at three different isolation points (0, 3 and 7 days) of algae cultivation. Figure 6.9A shows that bacterial density increased over time of cultivation. The total colony count (CFU/ml) was significantly higher when the culture was inoculated with 1 CFU/ml of bacteria ($p < 0.01$), while no significant difference in the total bacterial count was observed with 10^4 CFU/ml inoculation of bacteria ($p > 0.1$). The average bacterial count increased by 2400 times on day 7 when the culture was inoculated with a small concentration of bacteria

and by only 1.9 times when the culture was inoculated with 10^4 CFU/ml of bacteria. At the three isolation points of algae cultivation, the growth of the added *Halomonas* sp. WSR2 (1 and 10^4 CFU/ml) introduced on day 2 (Fig. 6.9B) shows little increase in bacterial density with the higher bacterial inoculum (10^4 CFU/ml). The increase in fold change of bacterial counts was higher for the day 0 introduction compared to the day 2 introduction, for both the inocula (1 and 10^4 CFU/ml). Moreover, the average fold change on day 7 of algae cultivation was higher for bacterial inoculation on day 0 (~780% increase) than for bacterial inoculation on day 2 (41% increase) with the bacterial inoculum of 1 and 10^4 CFU/ml, respectively ($p < 0.001$ for 1 CFU/ml and $p < 0.01$ for 10^4 CFU/ml on day 7).

In addition, for the mixed bacterial inoculum cases (case 1 and 2), the total colony counts (CFU/ml) at the three isolation points increased over time of cultivation (Fig. 6.10), achieving higher growth on day 7 in both cases; case 1 showed increased bacterial numbers than case 2 on day 7 ($p < 0.01$) because of the fast growth rate of *Halomonas* sp., which was dominant. There was no significant difference between the two cases ($p > 0.05$). Figure 6.11 shows the distribution of the bacterial population in case 1 and case 2, over the three isolation time points. In both cases, it can be seen that *Halomonas* sp. WSR2 disappeared whilst *Muricauda* sp. WSR increased in dominance.

Although limited studies have reported bacterial density through co-culture between microalgae and bacteria, some studies have shown that bacterial growth increases over time during algae cultivation, which confirms that microalgae provide bacteria with important nutrients for growth. Cho et al. (2015) indicated that the bacterial colony count increased over time through co-culturing with *C. vulgaris* tested at three isolation points (10, 15 and 25) of algae cultivation with inoculation (algae 10: bacteria 1). A drastic increase in the total colony count of *Flavobacterium* and *Rhizobium* was observed in the late phase of algal growth (on

day 25), although an increase in the total colony counts of *Hyphomonas* and *Sphingomonas* was slow in the same growth phase of algae. Liang et al. (2014) indicated that the bacterial growth rate rises over the time of cultivation because the product of organic materials during the logarithmic phase of *C. vulgaris* is low, whereas the content of organic materials increases rapidly from the stationary phase to the decline phase. In the first days of algae cultivation, the bacteria do not interact with algae to provide energy and carbon sources, because the medium contains high concentrations of organic nutrients (Huo et al., 2020).

However, several reports have indicated that the composition of algae-associated bacterial species is affected by not only nutrient availability but also the growth phase of algae. Changing the nutrient concentration in the medium can lead to converting the interaction from a mutualistic relationship to a competitive relationship between microorganisms (Tait et al., 2019). In the present study, single introduced into algae culture on day 0 or day 2, and mixed bacteria introduced in day 0 showed increased growth over time to achieve higher colonie numbers on day 7 with concomitant increase in algal growth, which can confirm a positive interaction between *C. vulgaris* and its cohabiting bacteria (*Halomonas* and *Muricauda* sp.). An increase in bacterial density in algae co-culture may indicate the presence of organic molecules released by algae into the medium. The inoculation with mixed bacteria showed lower bacterial increase than single bacterial increase possibly due to competition between the bacteria in the mixed inoculum and the more dominant strain (*Halomonas* sp. WS1 or *Muricauda* sp.) being less effective than the chosen single species (*Halomonas* sp. WSR2).

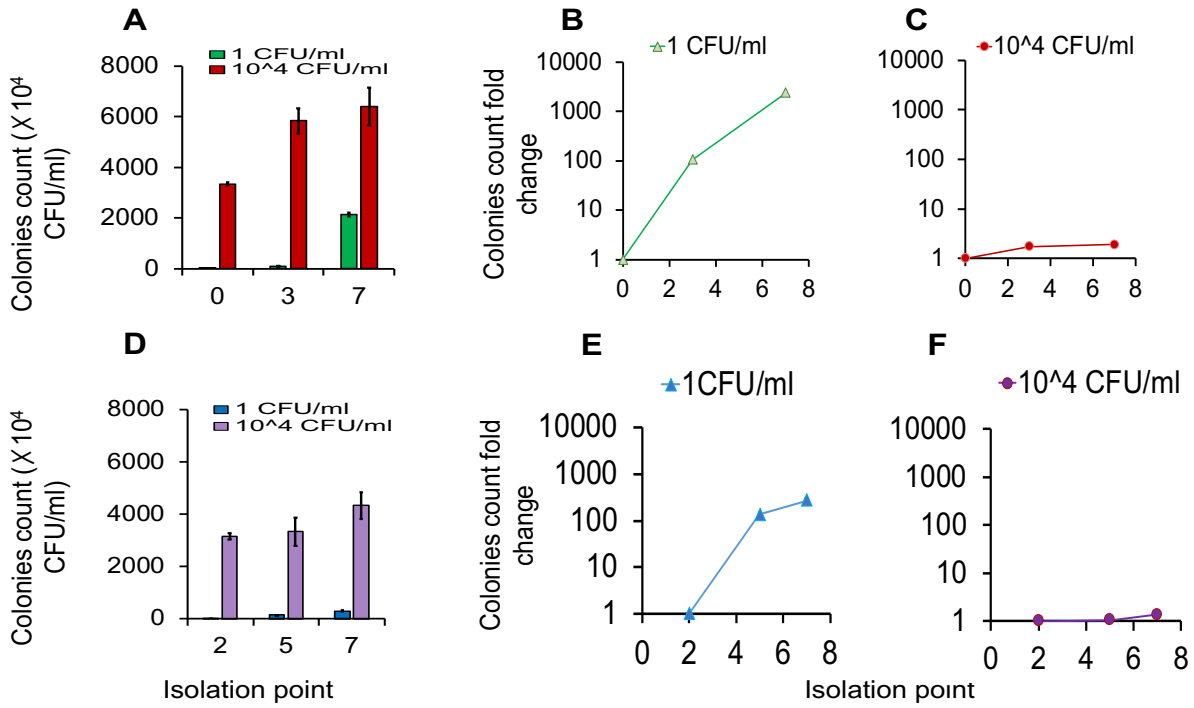

Figure 6.9 Total bacterial density (CFU/ml) of different bacterial concentrations and individual bacteria count introduced into the algae culture on **A:** day 0, **B:** total bacterial increase when the culture inoculated on day 0 with small bacterial concentration 1CFU/ml, **C**: total bacterial increase when the culture inoculated on day 0 with high bacterial concentration 10^4 CFU/ml, **D**: total bacterial density (CFU/ml) of different bacterial concentrations and individual bacteria count introduced into the algae culture on day 2 of algae cultivation, **E**: total bacterial increase when the culture inoculated on day 2 with small bacterial concentration 1CFU/ml, **F**: total bacterial increase when the culture inoculated on day 2 with high bacterial concentration 10^4 CFU/ml.

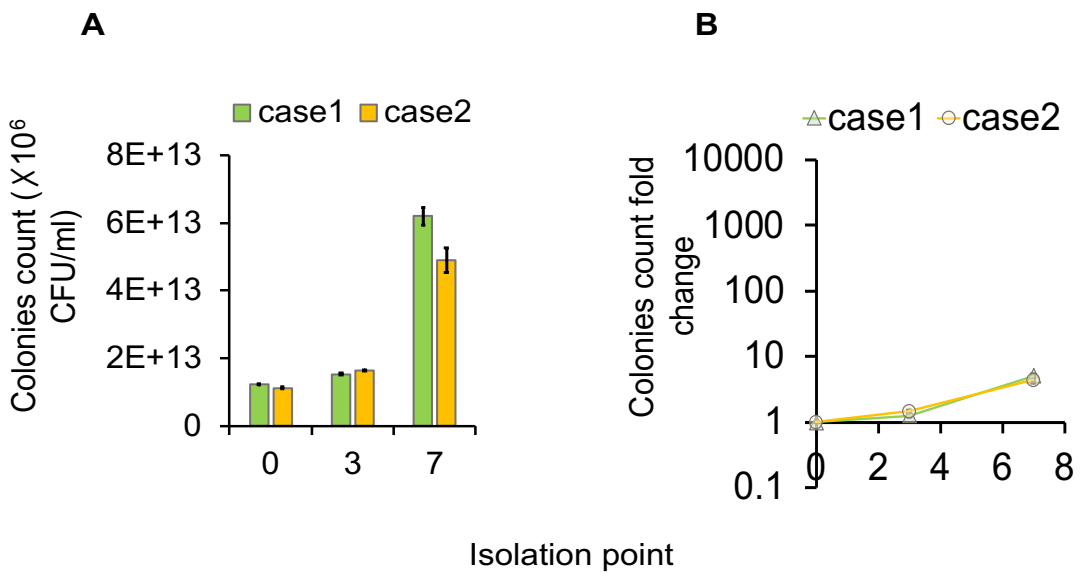

Figure 6.10 A: Total bacterial density (CFU/ml) of different concentrations of combination bacteria and **B:** individual bacterial count introduced into algae culture on day 0 of algae cultivation in two different cases, case 1 (more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR).

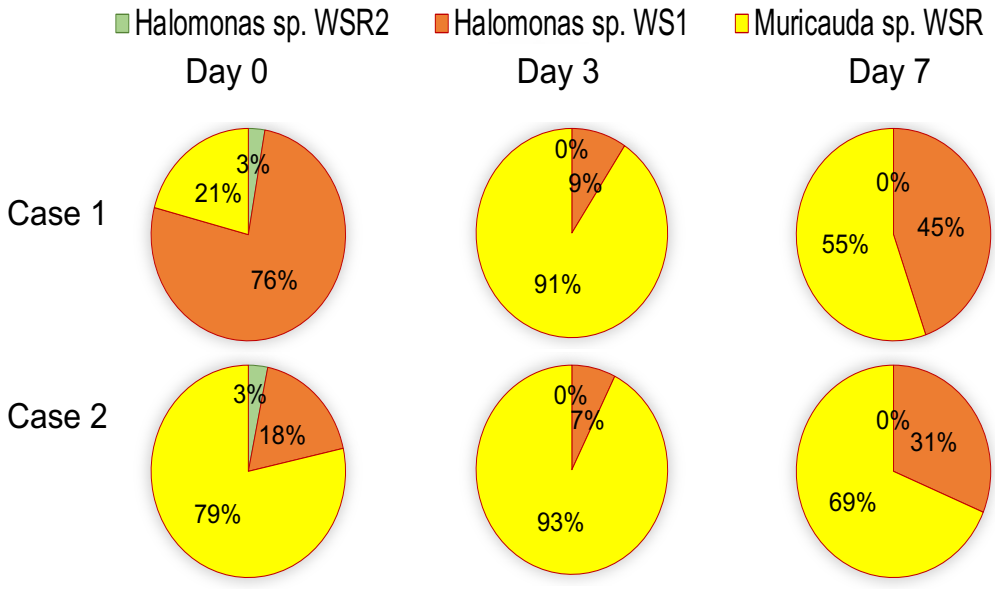

Figure 6.11 Distribution of introduced bacterial species in case 1(more *Halomonas* sp. WS1) and case 2 (more *Muricauda* sp. WSR).

6.4 Conclusion

The current study shows a successful symbiotic relationship in co-cultivation between *C. vulgaris* and *Halomonas* and the mixed culture with combinations of bacterial species (two species of *Halomonas* and *Muricauda* sp.) for enhancing the microalgae biomass and carbohydrate content and productivity as valuable energy molecules. The interaction between microalgae and its associated microbes is considered a significant tool to increase the microalgae biomass and its productivity economically. The co-cultivation of *C. vulgaris* with a single bacteria type or mixed bacterial species on day 0 of algae cultivation doubled the algal growth rate. In addition, the highest carbohydrate content was achieved under inoculation algal culture with a high concentration (10^4 CFU/ml) of single bacterial type, while the highest carbohydrate productivity was observed when a small concentration (1 CFU/ml) of the single bacteria type was introduced on day 2 of algae cultivation. In addition, changes in bacteria density varied under different inoculation ratios. Introducing a small concentration of single bacteria either on day 0 or day 2 of cultivation increased the average number of colonies dramatically, while no significant increase was observed when introducing a high concentration of single bacteria (10^4 CFU/ml). Moreover, the increase in colony count in the mixed culture (case 1 and case 2) was limited. We can conclude that the co-culture with single bacterial types achieved our aim of increasing carbohydrate content and productivity as well as enhancing the algal biomass.

Chapter 7 Conclusions and future works

7.1 General discussion and conclusion

Using microalgae as an alternative feedstock for biofuels and bioproducts production could help in environmental security by mitigating CO_2 emissions, which is also a promising source for industrial production because it contains different valuable molecules such as carbohydrates, lipids and pigments. For this reason, *Chlorella vulgaris* CCAP 211/21A, as a strain identified to be rich in carbohydrate content (Slocombe et al., 2021), has been used in this project as a model candidate to study carbohydrate accumulations. Chapter 1 focused on increasing carbohydrate accumulation under different concentrations of nitrate and phosphate. Growing *C. vulgaris* under a nutrient deficient condition had achieved high carbohydrate accumulation as expected. However, nitrogen concentrations play a key role in microalgae cultivation which may influence the growth rate of algae and synthesis of biochemical molecules such as carbohydrate, protein and lipids (Zarrinmehr et al., 2020). Nutrient limitation not only plays a role on growth of algae and its biochemical components but also influences the contribution and distribution of cohabiting bacteria, as reported in Chapter1. Tait et al., 2019 also reported similar results about changes in bacterial composition through microbial co-culture between algae and bacteria that cultivated under different nutrient cultures. Three cohabiting bacteria were isolated, purified and identified genetically to belong to *Halomonas* sp. and *Muricauda* sp.

Chapter 2 highlighted the behavior of isolated bacteria from laboratory *C. vulgaris* culture where each species was studied separately under different conditions. Different media, a wide range of pH and temperatures were tested for all three bacterial isolates. *f*/2 +R2A medium was the optimal medium for all species. Several studies reported that for *Halomonas* growth but

there are no reported studies for growing *Muricauda* sp. in R2A medium. *Halomonas* sp. were grown optimally in pH 7.5 and at 30ºC similar observation was noticed by Shivanand et al. (2013) and Delabary et al. (2020). The highest growth rates of *Muricauda* sp. were observed at pH 8.5 and 25ºC, similar results found by Yoon et al. 2005; Huntemann et al. 2012; Zhang et al. 2018 and Kim et al. 2020).

The goal of chapter 3 was to study the influence of cohabiting bacteria on microalgal biomass and carbohydrate content by co-culture between *C. vulgaris* and its cohabiting bacteria. Microalgae are a promising feedstock for biofuels production. Therefore, different studies have been performed to increase the microalgae efficiency of the culture and enhance their metabolites products (Han et al., 2016). Co-culture system is a promising method to increase the growth of microalgae and enhance metabolite production along with change of culture conditions (carbon sources, nutrient concentrations, temperatures and light intensity) and metabolic engineering (González-González and de-Bashan, 2021). However, the unialgal culture may contain natural consortium between microalgae and its commensal bacteria. Interactions between microalgae and bacteria can be either competitive or cooperative (Subashchandrabose et al., 2011).

In this project, we found that introducing single or multi- bacterial isolates into the algal culture on day 0 of cultivation had a great influence on the algae growth with doubling growth rates, compared to introducing the bacteria on day 2. The interaction of co-culture between *C. vulgaris* and *Halomonas* sp. WSR2 and mixed culture between *C. vulgaris* and *Halomonas* sp. WSR2, *Halomonas* sp. WS1 and *Muricauda* sp. WSR in different ratios have been shown to influence the algal growth as well as the bacterial densities in the cultures, which confirm the positive interactions between them. Moreover, inoculating the algae culture by a single type of

bacterium (10^4 CFU/ml of *Halomonas* sp. WSR2) either on day 0 or day 2 of algal cultivation resulted in increasing carbohydrate content in the cells by 99% and 82% when bacteria introduced into the culture on day 0 and day 2, respectively. Several studies reported that co-cultivation of *Halomonas* sp. with different green microalgae enhanced the growth of algae and biochemical components (Subasankari et al., 2020). In addition, Han et al., 2016 reported that when *Muricauda* sp. co-cultured with different green microalgae *Tetraselmis chuii*, and *Cylindrotheca fusiformis* separately, algal growth increased than the growth in mono-culture.

Cohabiting bacteria in *Chlorella* culture, both from non-axenic and managed cultivations, influenced carbohydrate yield and productivity. The mixed culture dominated by *Halomonas* WSR2 from the non-axenic culture (chapter 4 data) showed up to a 3-fold increase in carbohydrate yield compared to the managed mixed cultures, dominated by the other two bacterial species (chapter 6 data). However, a higher increase in carbohydrate productivity is noted for the mixed cultures dominated by the other two bacterial species, compared to that dominated by *Halomonas* WSR2 (Fig. 7.1). The dominating influence of *Halomonas* WSR2 can be seen more clearly when this was the single species introduced in managed cocultures (Fig. 7.1). Figure 7.2 shows the change in bacterial distribution over the cultivation period for the three mixed culture scenarios examined in this thesis. This shows that *Muricauda* sp. WSR takes a dominant role when *Halomonas* sp. WSR2 is added to a lower extent in the inoculum, but this is not the case when the latter is present to a larger extent in the inoculum. We conclude that *Halomonas* sp. WSR2 had a greater influence on carbohydrate production as reported in chapter 6 (with single inoculation).

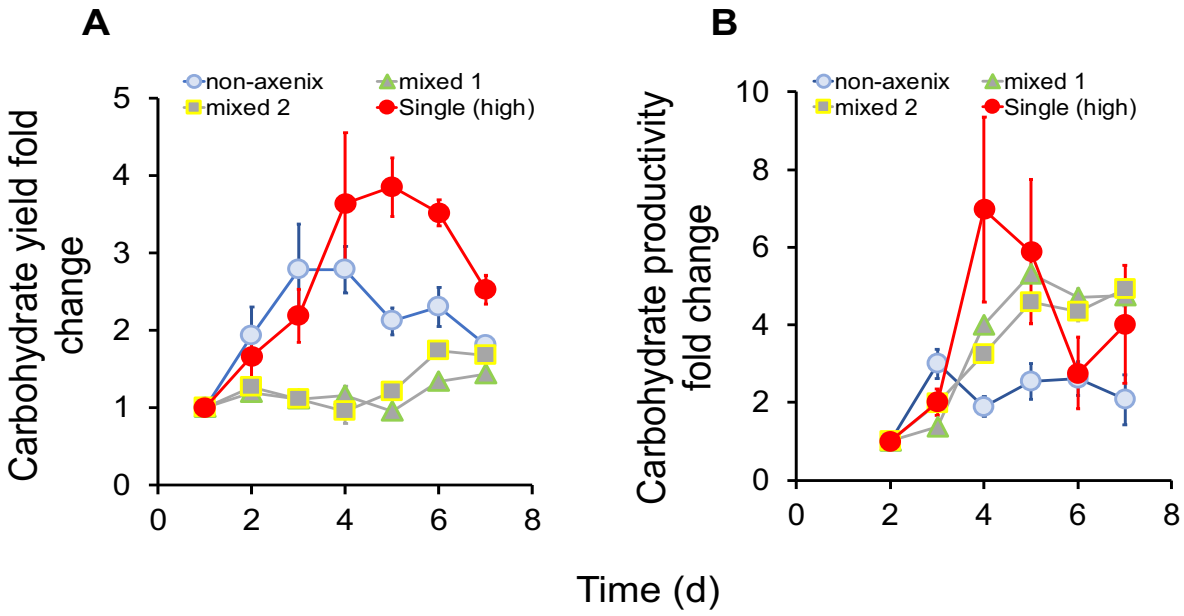

Figure 7.1 A comparison between carbohydrate yield and productivity in non-axenic and managed co-cultures (mixed and single bacterial inoculation).

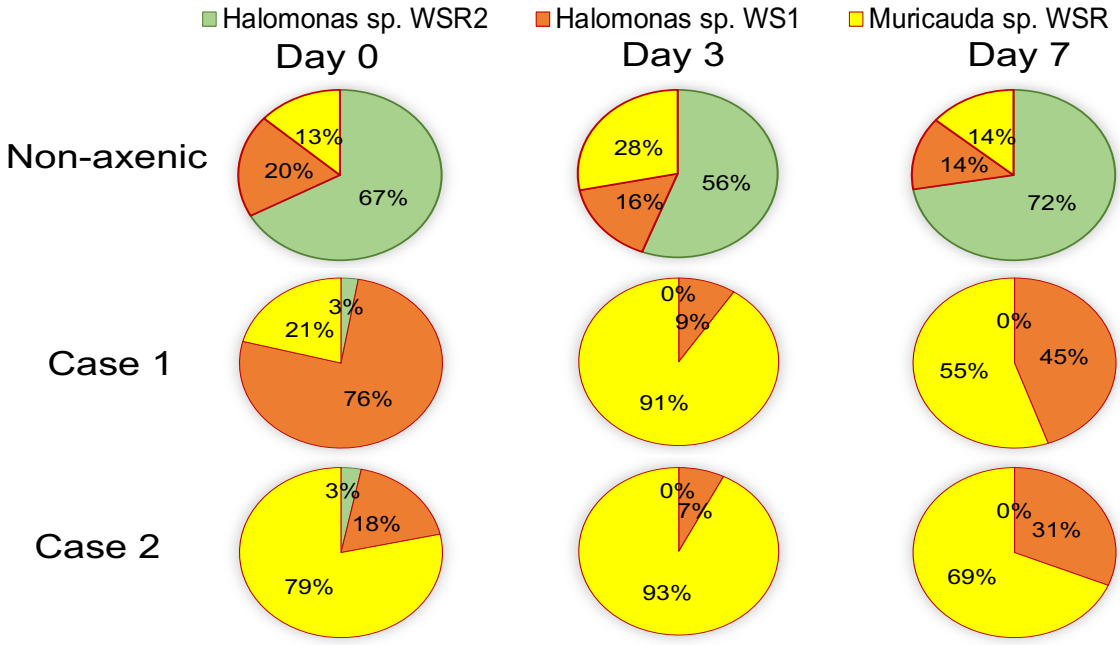

Figure 7.2 The change in bacterial distribution over the cultivation period for the three mixed culture scenarios.

These findings can be developed to find use in the industrial field for increasing carbohydrate production from algae cells by an economic approach (co-culture). Carbohydrates, as a sustainable substrate, can be converted into several products; one of them is biofuels that can be environmentally friendly.

7.2 Future work

Many benefits of co-culture have been achieved such as increasing algal biomass, carbohydrate content and reducing contamination risk. Increasing carbohydrate content by co-culture inspires to be an applicable method for different purposes in the industrial field. In our project, we achieved high growth of algae with high carbohydrate content and productivities although there were some influence aspects neglected.

Co-cultivation between *C. vulgaris* and *Muricauda* sp. can be studied toward carbohydrates enhancement in different concentrations. Besides, applying different bacterial species for inoculation, studying the environmental factors such as nutrient availability, pH, temperatures etc. as well as introducing time and culture time influence factors which could contribute to increase co-culture achievements. Moreover, studying morphological changes of algal cells and size as well as bacterial colonies is recommended. In addition, studying released substances from bacteria is important to understand the relationship between *C. vulgaris* and its co-partner bacteria in the co-culture.

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Chapter 9 Appendix

Appendix chapter 3

Determination of dissolved inorganic nitrate (DIN)

For preparation standard curve, prepare 10 $NaNO_3$ standards from 750 µM solution which were diluted with media free nitrate. Triplicates of each standard were measured in white quartz cuvette at 220nm, and a blank is deionized water. Plotting standard nitrate concentration and the average of absorbance to calculate the calibration curve by linear regression.

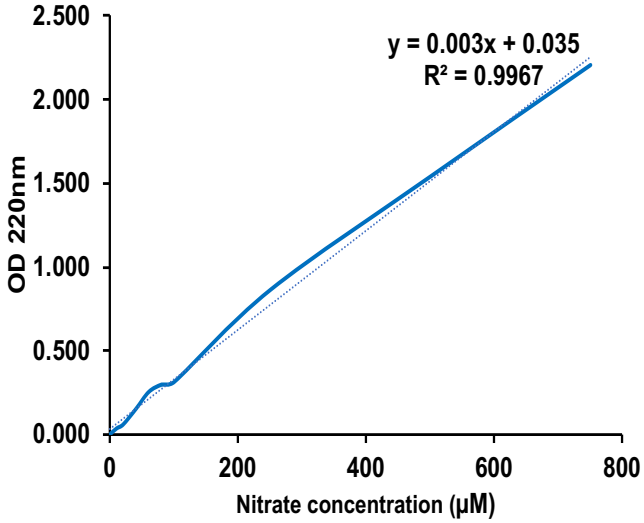

Figure 9.1: A calibration curve using nitrate solution as a standard.

Determination of dissolved inorganic phosphate (DIP)

For preparation standard curve, prepare 6 $NaH_2PO_4*H_2O$ standards from 36 µM solution which were diluted with water. 1 ml of triplicates of each standard were transferred into plastic cuvette and added 100 µl of mixed reagents (1 ml of Ammonium molybdate: 2.5 ml of Sulfuric acid: 1 ml of Ascorbic acid: 0.5ml Potassium antimolnyl tartrate). The samples were measured after half an hour at wavelength 885 nm; blank is deionized water. Plotting standard nitrate

concentration and the average of absorbance to calculate the calibration curve by linear regression.

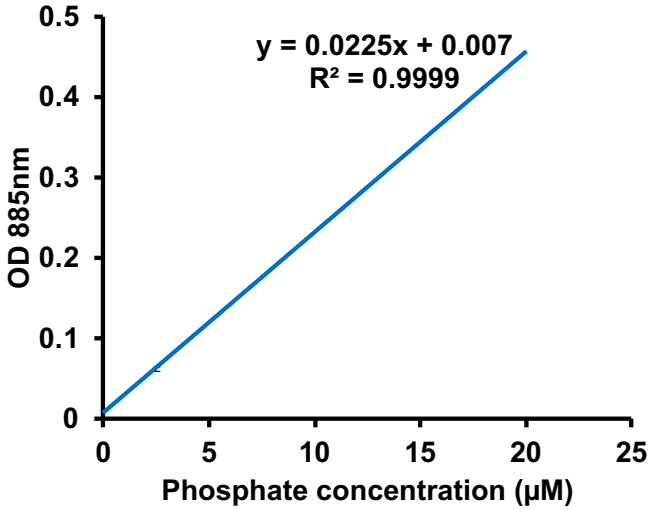

Figure 9.2: A calibration curve using phosphate solution as a standard.

Carbohydrate assay

For preparation standard curve, 0.08g of glucose was dissolved in 100 ml water and diluted with water to prepare 8 different concentration of glucose standards (0-400). 200 µl of triplicates of each standard was taken and added 400 µl 75% H_2SO_4 + 800 µl Anthrone. The samples were incubated at 100 °C for 15 minutes; then, transferred 1 ml into plastic cuvette and measured at 578nm. Bland was deionized water. Plotting standard glucose concentration and the average of absorbance to calculate the calibration curve by linear regression.

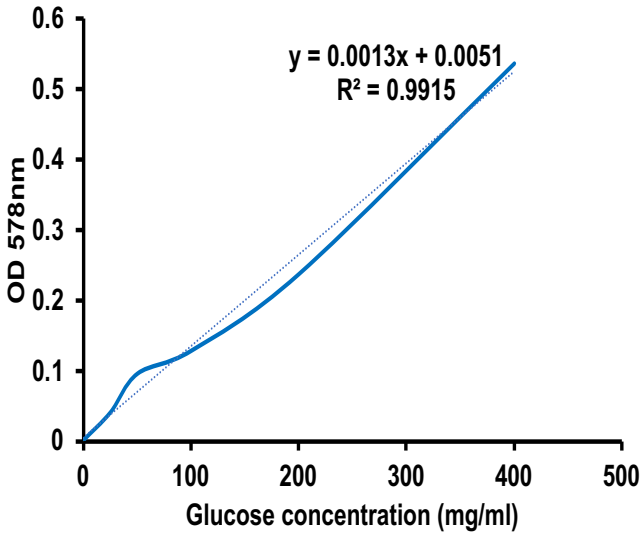

Figure 9.3: A calibration curve using glucose solution as a standard.

Identification of isolated bacteria from *Chlorella* culture

Samples A, B and C were loaded into agarose gel wells that connected to electric field. the DNA fragments moved to toward the positive electrode. Short fragments move faster than longer fragments that will remain near the top. However, DNA fragments are in the range of 400 bp, the DNA fragments separate according to the size and isolated.

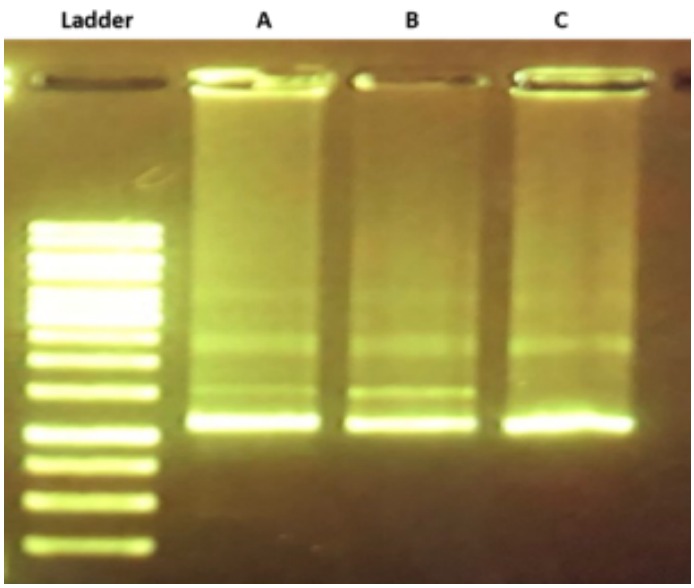

Figure 9.4 Agrose gel electrophoresis of isolated genomic DNA on a 1% (w/v) agarose gel, **A and B**: *Halomonas* sp, and **C**: *Muricauda* sp.

Table 9.1: Composition of Instant Ocean Salt

Ion	Concentration (mg/L)	Ion	Concentration (mg/L)
Chloride	19251	Manganese	Trace (<0.01)
Sodium	10757	Molybdenum	Trace (<0.01)
Sulfate	2659	Cobalt	Trace (<0.05)
Magnesium	1317	Vanadium	Trace (<0.04)
Pottasium	402	Selenium	Trace

Calcium	398	Fluorine	Trace (<0.05)
Carbonate/ Bicarbonate	192	Lead	Trace (<0.005)
Strontium	8.6	Arsenic	Trace (<0.0002)
Boron	5.6	Cadmium	(<0.02)
Bromide	2.3	Chromium	Trace (<0.0006)
Iodide	0.22	Aluminum	Trace (<0.04)
Lithium	0.18	Tin	Trace
Copper & Iron	Trace (<0.03)	Antimony	Trace
Nickle	Trace (<0.04)	Rubidium	Trace
Zinc	Trace (<0.02)	barium	Trace (<0.05)