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Remediation of Crude Petroleum Oil-Water Emulsions Using Microalgae

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REMEDIATION OF CRUDE PETROLEUM OIL-WATER EMULSIONS USING MICROALGAE

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Water Resources

Under the Supervision of Professor Sulaiman Al-Zuhair

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Declaration of Original Work

I, Mohamed Shafi, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Remediation of Crude Petroleum Oil-water Emulsions Using Microalgae*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Sulaiman Al-Zuhair, in the College of Engineering at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Crude petroleum oil spills are among the most important organic contaminations, which result from uncontrolled releases and spillages during transportation or storage. The separated oils on that accumulate on top of the water can be removed by various conventional skimming methods. However, the emulsified portions that remain within the water phase are more difficult to remove and pose significant threats to the environment and could tamper the tertiary treatment in a wastewater treatment plant. Biological treatment, using bacteria, have proven to be an effective method in the removal of the emulsified oils. However, the biomass produced in this case does not have any significant remunerative value, and in most cases the used bacteria are pathogenic. In this work, microalgae have been proposed to be used, instead of bacteria, to combine the emulsified oil remediation with the microalgae potential as biofuel feedstock, which enhances the economic and environmental benefit of the process. A freshwater strain of *Chlorella vulgaris* was grown in water containing different concentrations of emulsified crude oil, up to 275 mg/L, at different temperatures. To enhance the removal of the emulsified oils, chemotrophic cultivation conditions was applied keeping the emulsified oils as a sole carbon source. The degradation was monitored by measuring the total organic carbon in the water. The specific growth rate of the microalgae at each initial oil concentration was determined and the results were fitted to a modified Monod kinetics model that takes specific interfacial area as the influential substrate, rather than the actual concentration. The microalgae growth was found to increase with the increase in temperature, in tested range, with μ_{max} increasing from 1.17-1.48 day^{-1} as the temperature increased from 30°C and 40°C, and the activation energy was found to be 19.05 kJ/mol. However, the increase in the microalgae growth with temperature did not result increase on the oils removal and the yield of oil removal per biomass growth was found to decrease with the increase in temperature.

Keywords: Crude oil-water emulsion, bioremediation, microalgae, *Chlorella sp.*, specific interfacial area, kinetics model.

Title and Abstract (in Arabic)

استخدام الطحالب الدقيقة لمعالجة مستحلبات النفط والماء النفطي الخام

الملخص

تعد انسكابات النفط الخام من أهم الملوثات العضوية التي تنتج عن الإطلاقات والانسكابات الغير منضبطة أثناء النقل أو التخزين. يمكن إزالة الزيوت المنفصلة التي تتراكم فوق الماء بطرق تقليدية مختلفة. ومع ذلك ، فإن الأجزاء المستحلبة التي تبقى في طور الماء تكون أكثر صعوبة في الإزالة وتشكل تهديدات كبيرة على البيئة ويمكن أن تدخل في مرحلة العلاج الثالث في محطة معالجة مياه الصرف الصحي. أثبت العلاج البيولوجي ، باستخدام البكتيريا ، أنه طريقة فعالة في إزالة الزيوت المستحلبة. ومع ذلك ، فإن الكتلة الحيوية المنتجة في هذه الحالة ليس لها أي قيمة مجزية كبيرة ، وفي معظم الحالات تكون البكتيريا المستخدمة مسببة للأمراض. في هذا العمل ، تم اقتراح استخدام الطحالب الدقيقة ، بدلاً من البكتيريا ، لدمج معالجة الزيت المستحلب مع الطحالب الدقيقة كمواد وسيطة للوقود الحيوي ، مما يعزز الفائدة الاقتصادية والبيئية للعملية. تم زرع سلالة من المياه العذبة من *الكلوريلا* الشائع في المياه التي تحتوي على تركيزات مختلفة من النفط الخام المستحلب ، تصل إلى 275 ملغم/لتر ، في درجات حرارة مختلفة. لتعزيز إزالة الزيوت المستحلبة ، تم تطبيق شروط الزراعة الكيميائية للحفاظ على الزيوت المستحلبة كمصدر وحيد للكربون. تم رصد التحلل بقياس إجمالي الكربون العضوي في الماء. تم تحديد معدل النمو المحدد للطحالب الدقيقة في كل تركيز زيت أولي وتم تركيب النتائج على نموذج Monod الحركي المعدل الذي يأخذ منطقة بينية محددة كركيزة مؤثرة ، بدلاً من التركيز الفعلي. وجد أن نمو الطحالب الدقيقة يزداد مع زيادة درجة الحرارة ، في النطاق الاختباري ، مع زيادة μ_{max} من 1.17 إلى 1.48 في اليوم. حيث زادت درجة الحرارة من 30 و 40 درجة مئوية ، ووجدت طاقة التنشيط بمقدار 19.05 كيلوجول/مول. ومع ذلك ، فإن الزيادة في نمو الطحالب الدقيقة مع درجة الحرارة لم ينتج عنها زيادة في إزالة الزيوت ووجد أن محصول إزالة الزيت لكل نمو للكتلة الحيوية ينخفض مع زيادة درجة الحرارة.

مفاهيم البحث الرئيسية: مستحلب النفط الخام والمياه، المعالجة الحيوية، الطحالب الدقيقة، *أنواع الكلوريلا*، منطقة بينية محددة، نموذج حركية.

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Dedication

To my beloved parents and family

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List of Abbreviations

BBM	Bold's Basal Medium
BTU	British Thermal Unit
DIA	Dynamic Image Analysis
DLS	Dynamic Light Scattering
FOG	Free Oil and Gas
LD	Laser Diffraction
PBR	Photo-Bio Reactor
PIB	Poly Iso-Butylene
TOC	Total Organic Carbon

Chapter 1: Introduction

In the last 30 years, the problem of crude petroleum oil spills and their effects has gained increased attention. Over the last decade, spillage from oil refineries in the US averaged over 12,000 barrels annually. This value is 19% lower than that reported in the decade before. The past decade also witnessed a 27% reduction in spillage per barrel of oil throughput as compared to the preceding year. Between 1974 and 1994, there were 175 major oil spills worldwide. The cost just to clean-up the contaminated site reached as high as 20-200$ per liter in USA and Canada, depending on the location and type of oil spilled (Fingas, 2012). Integrated strategies must be worked out to reduce socio-economic and environmental impact from oil spills.

Oil spill is the accidental release of petroleum crude oil into the environment by vehicle, vessel or pipeline. It happens due to human negligence and is considered a major form of pollution. Oil spills may result in pollution over large areas and present serious environmental hazards. They can prove fatal for plant, animal and human life. The substance is so toxic that it can cause massive loss of species that live in the sea. The harmful effects of oils spillage can be classified as environmental and economical.

Wastewater containing emulsified crude oil is one of the considered a major environmental concern. As the annual worldwide use of petroleum-based products and vegetable oils are approximated to exceed 100 and 92 million tons respectively (Abdullah et al., 2010), oily wastewater concentration generated from related industrial activities could reach as high as 40,000 mg/L (Sincero et al., 2002). Water

contaminated with oil emulsions can have adverse effects in the environment and economy.

Environmental Effects: The animal life in water or near the shores are the ones most affected by the spill. In most cases, the oil simply chokes the animals to death. Others that live face a number of other problems. The oil works its way into the fur and plumage of the animals. As a result, both birds and mammals find it harder to float in the water or regulate their body temperatures.

Economic Effects: The second major effect of the oil spill is seen on the economy. Whereas valuable crude oil is lost, it effects the amount of petroleum available for use. In addition, the process of cleaning the oil spill requires significant amount of financing. The fishing industry would also be significantly affected, and other activities, such as sailing, swimming, rafting, could not be performed.

The sources of the spill are many and can be released by tankers on land or by drilling rigs and offshore oil platforms in water. What is common in all oil spills is that they require long time to clean up. Cleaning up of oil spill is no easy task, and various factors need to be considered before carrying out operations. Two most important factors are the amount of oil spilled and temperature of water, which are the two factors considered in this work. Despite the increase in public attention towards oil spills in the last decades, numerous incidents are still happening.

Accidental oil spillage does not only increase the hydrocarbon load in the wastewater, but also phosphorous and nitrogen. The use of micro-algae for wastewater treatment has proven to be a cost-effective process. High concentration of nutrients and a variety of trace elements present inculcates the metabolism and

microalgae growth. It has been proven that wastewater rich in nitrogen and phosphorous serve as better medium for microalgae growth (Wang, Min, et al., 2010). The produced biomass contains appreciable amounts of lipids that can be used for biofuel production.

Chapter 2: Literature Review

2.1 Oil Emulsions

2.1.1 Sources and occurrence

Oil and grease are frequently present in many wastewaters and create enormous problems. It is present in domestic wastewaters due to household activities such as cooking or cleaning. Major domestic discharges are usually controlled at the point source itself by the installation of grease traps. However, the content still ranges between 50-150 ppm (Tchobanoglous et al., 1991) and are in the emulsified form. The stormwater also contains emulsified oil due to the leached automobile hydrocarbons from parking lots and gas stations.

The major source of oil and grease is from industrial wastewaters and it goes over 2000 ppm (Patterson, 1985). The major contributor to industrial wastewater is petroleum industries associated with oil refineries. The meat and food processing industries also discharge wastewaters containing natural fats derived from animal processing. Certain processes in the leather industry also produce oily wastewater in large quantity. Automotive and machining industries generate wastewater containing lubricants and other specialty fluids. Wastewaters generated from textile industries also contains oils and greases from scouring, de-sizing and finishing operations. The various oil mills such as vegetable oil mills, olive oil mills and palm oil mills also contribute largely to the industrial wastewaters though they all have an in-house effluent treatment plant. The removal of separated layer of oil and grease is relatively easy, whereas the emulsified portion of the oil in the wastewater is intricate and more

difficult to remove. The oil removal methods should be effective and allows feasibly recycling of the wastewater.

2.1.2 Properties and stabilization

Oil appears in wastewater in a number of different forms including mechanically emulsified oil, chemically emulsified oil and free oil and grease (FOG). These are a class of pollutants with low affinity to water. The droplets size of FOG are usually in the range of 150 μm (Pintor et al., 2016). However, these dispersions are unstable, and under normal condition, FOG rises rapidly to the surface of the water tank. As mentioned earlier, this type could be removed by a skimmer or by an overflow weir. The stabilized traces still present in the wastewater are the most challenging part.

Mechanically emulsified oil in water are formed by vigorous shear that can result from the wastewater traveling through a pump, wastewater splashing into a tank or any other force that break and disperse larger oil droplets. The droplets size range of mechanically emulsified oil in water are usually from 20-150 μm (Pintor et al., 2016). Electrical charges and other weak forces stabilize the coating of suspended solids. Mechanically generated emulsions are not stable. In fact, the mechanical emulsion separates substantially in just a minute or two.

Chemically emulsified oil in water occurs when surfactants are present. These chemicals have oleophilic end hydrophilic ends and act as a coupling agent between the oil and water phases. The oleophilic end of the chain gets attached to the oil droplet, while the hydrophilic end remains in the water. If the droplets are less than 20 mm in size (Pintor et al., 2016), chemical stable emulsions are produced rendering the color of the wastewater to be milky white. The surfactants act as a stabilizer

preventing the emulsion from separating. It keeps the immiscible layers intact by increasing the kinetic stability of the mixture and lowering the surface tension between liquids or between a solid and a liquid. The surfactants prevent the droplet size from getting large enough for components to be able to separate based on density.

2.1.3 Environmental impact

The crude oil spills and the associated emulsified oil pose alarming threat to the environment. Immediate adverse health conditions can be observed within the local communities that suffer from these pollutions, including nausea, headaches and skin irritations. Oil compounds have the ability for bioaccumulation as they are transferable along the food chain via oil-contaminated marine food (Aguilera et al., 2010). Acute effects and psychological symptoms of crude oil spill have been thoroughly presented in literature (Aguilera et al., 2010). The oily wastewater had deleterious impact on human health, especially to those who dealt with the crude oil without personal protective equipment. Even after the clean-up phase, dermatological problems occur within the beach-workers not wearing adequate protective clothing. Long term bare-handed contact lead to an increased risk of developing skin tumors in the volunteers due to the carcinogenic properties of the crude oil. An increase in the urinary volatile organic compounds and polycyclic aromatic hydrocarbons metabolite levels in oil spill cleanup volunteers have been reported after the cleanup (Ha et al., 2012).

In-vitro studies on Erika oil spill showed that the consumption of oil-contaminated marine food could cause detrimental effects on human health. Some examples are the induction of metabolic enzymes involved in carcinogenic processes and genotoxic

damage in consumers (Lemiere et al., 2005). Physical and chemical alteration of the natural habitats cause the disintegration of the marine ecosystem. As high levels of hydrocarbons are present on the surface, lethal toxic effects on the flora and fauna can occur. In addition, the emulsified oil layer limits the oxygen penetration into the bulk of the water, which is detrimental to the survival of aerobic aquatic organisms. The seabirds, mammals and their nesting sites are also affected by the deposition of oily layers.

2.1.4 Relevance to the UAE

The most important water-related challenges in the United Arab Emirates are the depletion of aquifers, saline-water intrusion and water quality degradation due to the activities associated with the oil industries and modern agriculture. To meet the ever-increasing demands for water, the United Arab Emirates mostly rely on non-conventional water resources such as the desalinated water and treated-sewage water. The existing non-conventional water resources include 475M m^3/year of desalinated water and 150M m^3/year of reclaimed water (Rizk & Alsharhan, 2003). A perceptive management of the available water resources in the United Arab Emirates can lead to water conservation and maintenance of better-quality water.

Membrane fouling remains a major challenge in the tertiary stage as it is linked to the total organic load of a wastewater (Henderson et al., 2011). Fouled membranes require chemical cleaning and ultimately result in water production loss, integrity loss, poorer water quality and a shorter membrane life, imposing a large economic weight on membrane plant operation (Beyer et al., 2017). The main contributor to organic fouling is the emulsified oil present in the wastewater. Hence, contending the

emulsified oil from wastewater helps in reducing maintenance cost, which ultimately curb down the unit cost of producing reclaimed water.

2.2 Oil Emulsions Remediation Methods

Different methods have been adopted for cleaning up oil spills and emulsified oils. These methods are classified as physical, chemical and biological.

2.2.1 Physical methods

Some of the commonly used methods for entangling the separated layers are oil booms, skimmers and in certain cases burning in-situ. Materials such as hay, peat moss, straw or vermiculite are used for adsorbing the spilt oil at lower radius of impact. Oil droplets can be removed from water by introducing small bubbles of air into the water. The bubbles attach themselves to the oil droplets and float them up to the surface where they can be skimmed off. In this case, the air bubbles act as oil scavengers.

Typical systems in use are dissolved air flotation and induced air flotation. In a dissolved air flotation, pressurized water supersaturated with air is used, which releases bubbles in size of 30-120 μm that float the flocs to the surface. In an induced air flotation system mechanical agitation however, articulate and entrain air into the water resulting in bubbles up to 1000 μm in size that bring the oil/solid flocs to the surface as froth. In such methods removal of froth floating on the surface is a tiresome process.

Organoclays/anthracite clay are commonly employed to remove emulsified oil from water by adsorption at concentrations of 60 ppm or less. The rate of removal using

organoclay is 7 times higher than that of activated carbon (Alther, 2002). However, oils and greases and other natural organic matters clog the pores of the adsorbent reducing its effectiveness significantly. This problem is more significant with granulated activated carbon filters, which are strongly fouled by the emulsified oil. Therefore, organoclay filters are unused prior to passing the water through the activated carbon filer, to avoid clogging the latter filter, and keep it available for adsorbing other contaminants. Disposal of the spent adsorbents are secondary pollutants that need to be dealt with. One way is to use the spent adsorbents in cement kilns. Spent organoclay has as much as 18,000 BTU as it contains the adsorbed oil. Landfills, bioremediation through land farming, cement encapsulation or incineration are other options for the spent adsorbents (Alther, 1995). Regeneration is possible only if large volumes of adsorbents are used.

When the oil droplets are larger than 0.015 cm, gravity separators are used to remove them. This is usually done in a settling tank and requires long time and is used only for mechanically emulsified oils.

2.2.2 Chemical methods

Oil spills can be treated chemically using chemical sorbents, dispersants or elastomizers. The chemical methods can be effectively used to contain the oil and prevent further spreading. Dispersants aid the natural breakdown of oil components and allows the oil to chemically bond with water by increasing the surface area of each molecule. This ensures that the slick does not travel over the surface of the water and is easier to degrade by microbes. Recently, experts have been using compounds like ‘Elastol’, which is basically poly iso-butylene (PIB) in a white powdered form, to confine oil spills. This compound gelatinizes or solidifies the oil

on the water surface and thus keeps it from spreading or escaping. The gelatin is easy to retrieve, and this makes the process highly efficient. It is a quick action method, with typical reaction times of 15-40 minutes. While PIB is non-toxic and commonly found in foodstuffs, the gelatin may pose a risk of entangling or suffocating the aquatic animals.

The addition of salts, polymers, bentonite-based powders and pH adjustment are the most successful methods for breaking oil emulsions chemically. When the pH is reduced below 3.5 using H_2SO_4 for example, the surfactants break-up. Coagulants and flocculants then may be added to remove the oil; certain polymers are also used for their effectiveness but are not economical below 330 ppm. $FeCl_3$, $AlSO_4$ and $AlCl_3$ are commonly used coagulants to break the oil water emulsion because they lower the pH and aid in the coalescence of oil droplets by adding ionic strength and high charge. The salts can then be removed by activated carbon, after the pH is raised back to 8. Cationic coagulant may also be used to neutralize the charges on the oil droplet. A polymer may also be required to floc the coagulated oils and achieve the desired separation. Generally, organic emulsion breakers produce much smaller amounts of sludge compared to inorganic coagulants. However, surfactants and solids accumulate in the rag layer which are the main problems faced in such processes. This method can be successful in preventing the transport of degreasing solution beyond the rinse stage, but the procedure involved is expensive both in equipment and operating costs. The oils and surfactants are only separated but isn’t eliminated. Elimination and disposal of the sludge, and its complexities has to be considered as well when deciding which method to use.

2.2.3 Biological methods

Bioremediation refers to the use of microorganisms, such as bacteria and fungi to degrade petroleum products, by metabolizing and breaking them into simpler and non-toxic molecules (mostly fatty acids and CO_2). Adequate nutrients are required for the microbes to be able to grow rapidly. All the biodegradable organic matter will be effectively depleted in a biological treatment process. To maintain high biological activity, a pH of about 9 and a temperature between 35-45°C are recommended. Seawater polluted with emulsified mineral oil was effectively treated in an aerobic biofiltering process by bacteria. Two fungal strains, namely *Mucor rouxii* and *Absidia coerulea* were also tested for the biodegradation of emulsified mineral oil from water (Srinivasan & Viraraghavan, 2010). At 77.2 mg oil per gm of biomass, *M. rouxii* showed a better performance than *A. coerulea*, with a removal efficiency of 77–93% at a pH of 5. Despite being effective for the bio-removal of the emulsified oils, the produced bacterial or fungal biomass, at the end of the process did not have any evident economical value.

2.3 Microalgae Characteristics and Classification

Microalgae is the primary producer of organic matter in aquatic environments by CO_2 fixation during photosynthesis. They are found almost in all water bodies and are able to flourish in terrestrial environments, even in harsh conditions. Microalgae are unicellular eukaryotic organisms, some of which form colonies resulting in green colonies that are visible to naked eye. Cyanobacteria, also known as blue-green algae as they are oxygenic photosynthetic bacteria, contain blue-green and green pigments. Most microalgae are in micrometer size, for instance, *C. vulgaris* is a green microalgae with spherical shape, 2 to 10 μm in diameter (Harrison et al., 1990). The

Figure 1 below shows 100 times magnified microscopic image of *C. vulgaris* which has a cell diameter of 10 µm (Fayad et al., 2017).

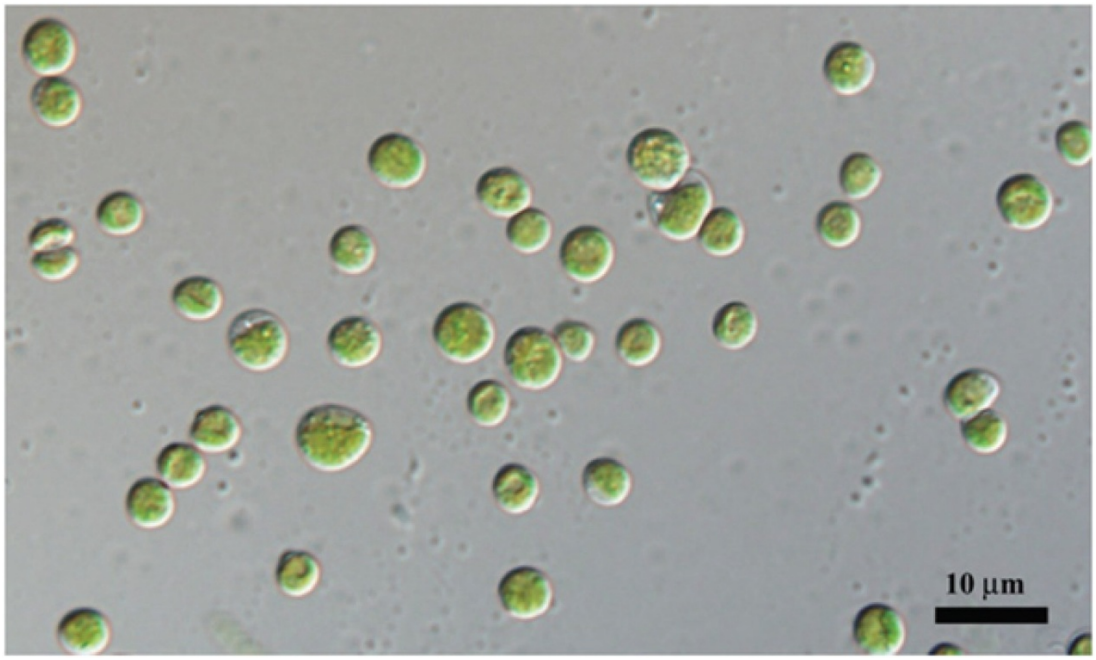

Figure 1: Microscopic view (100x magnification) of *Chlorella vulgaris*

Microalgae have been classified in terms of various parameters such as size, shape, pigments and cell wall composition. On the basis of the cell morphology, microalgae can be spherical, rod-shaped, spindle-shaped or club-shaped. They may be present in groups like filamentous clusters or as an individual strand that are either branched or unbranched. All microalgae contain chlorophyll 'a', but some contain other types of chlorophylls. Each chlorophyll is competent in absorbing a certain range of light wavelengths. Based on the presence of the different types of photosynthetic pigments, microalgae can be classified into different taxonomic groups (Rittmann & McCarty, 2001). Table 1 summarizes the various types of microalgae and their classification according to the different pigments present in their cells (Deglint et al., 2018).

Table 1: Microalgae Classification based on pigments present

Phylum	Common Name	Pigments			
		Chloro-phylls	Phycobilins	Carotene	Xanthophylls
Chlorophyta	Green Algae	a, b	N/A	α, β, γ	Prasinoxanthin Lutein
Cryptophyta	Cryptomonad	a, c	Phycoerythrin-545 R-Phycocyanin	α, β, ε	Alloxanthin
Cyanophyta	Cyanobacteria	a, b	C-Phycoerythrin C-Phycocyanin Allophycocyanin	β	Myxoxanthin Zeaxanthin
Euglenzoa	Euglenoids	a, b	N/A	β, γ	Diadionoxanthin
Ochrophyta	Diatoms	a, c	N/A	α, β, ε	Fucoxanthin Violaxanthin
Charophyta	N/A	a, b	N/A	α, β, γ	Lutein Neoxanthin Violaxanthin
Rhodophyta	Red Algae	a	B-Phycoerythrin R-Phycoerythrin R-Phycocyanin Allophycocyanin	α, β	Lutein

2.4 Extensive Production of Microalgae

Based on the exposure of the algae to the surrounding environment, the large-scale algae cultivation systems can be classified into three groups according to their design: open systems, closed systems and hybrid systems.

2.4.1 Open Raceway ponds

A raceway pond is an open field system for algae cultivation which has been extensively used since the 1950's. They are shallow artificial pond usually about 0.3 m deep to provide sufficient sunlight to allow photosynthesis by microalgae cells (Chisti, 2007). Rectangular grids divide the pond into different channels in the shape of an oval which resembles an automotive raceway circuit. A paddle wheel present in each rectangle propels the water flow continuously around the circuit. Generally,

commercial large-scale algae cultivation systems are open raceway ponds because they are relatively cheap. Many commercial producers of *Spirulina* sp. still use raceway ponds as their primary method of algae cultivation. Usually they are built near the wastewater treatment plants in order to easily access wastewater rich in nutrients.

However, there are certain disadvantages for open raceway ponds such as considerable water loss by evaporation as the ambient temperature increases. Major drawback is the requirement of large land areas for open ponds. Biomass productivity on a dry cell basis has been estimated to range from approximately 50 to 70 MT/ha/year when cultivated in high rate open raceway ponds (Sheehan et al., 1998). Figure 2 shows an aerial view of open raceway ponds (Gifuni et al., 2019)

Figure 2: Open raceway pond

2.4.2 Closed systems

Usually closed systems include flat plate reactors, tubular photobioreactors (PBRs) and bag systems (Wang, Li, et al., 2010). The cultivation is carried out through

closed tubular reactors, and it is particularly attractive for the robustness of the system and reducing the risk of contamination. This system has a higher productivity compared to open raceway systems, achieving a greater efficiency in the use and fixation of the CO_2 injected. This system allows maintaining suitable conditions for the growth of a specific strain, while at the same time obstructing the invasion by polluting organisms. The closed PBRs are quite expensive due to the construction, maintenance and operation costs of the system. Its use at industrial scale is justified by obtaining high value products, such as nutritional supplements and food for the aquaculture sector. In comparison to the open ponds, the closed systems minimize water evaporation and hence, increase photosynthesis efficiency due to culture conditions and operating parameters such as light intensity, temperature and CO_2 that can be controlled better in PBRs. However, the PBRs have certain drawbacks including overheating, oxygen accumulation, scale up difficulties and high capital and operating costs (Mata et al., 2010). Biomass productivity on a dry cell basis has been estimated to range from approximately 120-600 t/ha/year when grown in PBRs (Zittelli et al., 2013). Figure Figure *3* shows an example of tubular bioreactors (Alaswad et al., 2015)

Figure 3: Tubular photobioreactors

2.4.3 Two-Stage Hybrid cultivation system

Hybrid system combines both the open and closed systems in a twin-stage cultivation system. In the first stage, the PBR culture volume is rapidly grown to a high biomass concentration under nutrient-sufficient conditions. At least half of the lush grown cell culture is then transferred to the second stage, the open raceway ponds where the nutrients are limited for the accumulation of the lipid molecules from the harvested algae. The PBR is usually refilled with the nutrient's medium for the second cycle and thereon. In this way, the culture is exposed to minimal contamination before being fed to the large scale ponds (Chisti, 2007). This system would run continuously, with the photosynthetic activity assumed to be 8 hrs/day at maximum rate (Stephenson et al., 2010) and is operated as a batch process for the remaining time during the night. However, large scale applications of this particular approach have been limited due to the heavy initial cost incurred in the first stage.

2.4.4 Hetero-photoautotrophic two-stage cultivation

Certain algae species are photoautotrophic and grows in the presence of light, while others are heterotrophic which grows in the presence of an organic carbon. There are mixotrophic algae species such as *Nannochloropsis* sp. which can grow on dual culture when supplied with both organic and inorganic carbon under light/dark conditions (P. Das et al., 2011). A hetero-photoautotrophic two-stage cultivation process is an innovative way of sequentially growing microalgae heterotrophically and photo-autotrophically for an improved nutrient removal and augmented algal lipid accumulation in addition to CO_2 fixation for cost-effective and sustainable algae-based biofuel production (Zhou et al., 2012).

Heterotrophically cultivating algae is a novel approach to obtain high cell density biomass with high lipid content for higher quality biodiesel feedstock (Miao & Wu, 2006). The complete utilization of nutrients available in the wastewater is not possible to sustain continuous growth of the microalgae with the available amount of organic load in the wastewater. Hence, in such scenario a hetero-photoautotrophic microalgae cultivation could combat such hitches. The culture process is usually separated into two independent but sequential parts, a heterotrophic dominated stage using organic carbon in wastewater for maximal cell density and lipid accumulation and an autotrophic dominated stage to further remove nutrients in the recycled wastewater and accumulate lipid in the nitrogen-deficient environment (Zhou et al., 2012).

2.5 Factors affecting the growth of Microalgae

The major environmental factors that affect the growth of microalgae includes the nutrient concentration, growth temperature, light intensity, dissolved O_2 and CO_2, pH, solids retention time and the presence of toxic chemicals.

2.5.1 Nutrients

For a habitual algal growth, elements such as carbon, nitrogen and phosphorous are essential and mostly these elements are abundant in wastewater. Nitrogen can be an important factor controlling algal growth when other nutrients, such as phosphate, are abundant. If phosphate is not abundant it may limit algal growth rather than nitrogen. Nitrate and ammonia are the most common forms of nitrogen in aquatic systems. Though nitrate predominates in unpolluted waters, the assimilation of NO_3^- requires energy for the reduction of NO_3^- to NH_4^+. Ammonia is the preferred

nitrogen-containing nutrient for algae. Ammonia is excreted by animals and produced during decomposition of plants and animals, thus returning nitrogen to the aquatic system. Ammonium and nitrate are the critical inorganic nitrogen forms required for amino acid synthesis in algae. Availability of the nitrogen source affects cell content or composition of proteins, carbohydrates, pigments lipids and fatty acids (Harrison et al., 1990). For certain algae species, NH_4^+ at concentrations of 100-250 μM, may be inhibitory but mostly algae species tolerate concentrations as high as 1,000 μM (Ross, 1973).

Apart from carbon, nitrogen and phosphorus, both Fe and Mn are required for electron transport, energy transfer, nitrogen assimilation, oxygen metabolism and enzymatic processes, as well as for DNA, RNA and chlorophyll synthesis (Kuma & Matsunaga, 1995). Zinc is another important trace metal involved in algal growth. For optimal growth of *Isochrysis galbana*, Zn^{2+} at the concentrations of 10-100 μmol/L is essential but higher concentrations greater than 1,000 μmol/L leads to growth relegation (Sun & Wang, 2009). As such, microalgae feed on these essential nutrients indubitably and thus have the potential to naturally remove nitrogen, phosphorus and organics from wastewater, which helps in reducing the eutrophication in any water bodies.

2.5.2 Temperature

Temperature plays an inevitable role in algal growth entailing to find an optimal value for maximal growth for a specific species. Most commonly cultured species of algae tolerate temperatures between 16-27°C. The algal growth rates increase with increasing temperature up to a certain limit. For any micro algal species, the growth temperature reported with respect to various seasons ranged from 5-40°C. However,

the optimal temperature for the growth of major algae cultures generally ranges between 20–30°C (Singh & Singh, 2015), depending upon the composition of the culture medium, the species and the strain. Nonetheless, certain micro algal species has the ability to grow in temperatures range from 20-40°C, but the temperature affects their protein and carbohydrate levels. Temperature strongly influences the cellular chemical composition, uptake of nutrients, CO_2 fixation and the growth rates for every species of algae. For photosynthesis, dissociation of carbon-containing molecules is necessary which is temperature dependent. Temperature impacts respiration and photorespiration more strongly than photosynthesis (Necchi, 2004). However, if CO_2 or light is a limiting factor for photosynthesis, the influence of temperature can be insignificant. It was also found that, when the temperature was lower than 16°C, it demotes the algal growth. For a number of species temperature higher than 35°C may lead to micro algal cell damage or even death (Bitog et al., 2011). Under heat stress, Bajguz (2009) found that the algal protein content will be decreased and will produce abscisic acid, a stress hormone. This stress hormone is considered a key factor in controlling attributes such as gene expressions, growth, chlorophyll content, carotenoid, saccharides and lipid levels. It was reported that the lipids would increase from 5.9-14.7% when the temperature decreased from 30-25°C and at temperature over 38°C oleic acid, a mono-unsaturated omega-9 fatty acid production increased (Converti et al., 2009)

2.5.3 Light

Microalgae is an autotrophic organism which implies that the light intensity is an essential energy source as a controlling parameter in its growth and is required for photosynthetic activity. Low light intensities may limit photosynthetic activity and

thus biomass productivity. While certain algae species can become fairly adapted to different light intensities, the level of sensitivity to light spectrum varies with different species (Chisti, 2007). However, excessive light can lead to photoinhibition yielding toxic photocatalytic products including hydrogen peroxide, superoxide, $^1O_2$, OH^- and triplet chlorophyll causing damage to the photosystem II reaction center (Long et al., 1994). The light intensities for saturation and inhibition depend on the suitability of the environmental factors such as CO_2, temperature and nutrient level. Photosynthetically microalgae absorbs visible spectrum wavelength of solar radiation, which ranges from 400-700 nm (Červený et al., 2009). In laboratories, microalgal cultures like PBRs and culture tanks are either internally or externally illuminated with fluorescent lamps or other light distributors within the same wavelength range. For a successful photosynthesis, photons within wavelength range between 600-700 nm must be generated (Bitog et al., 2011). It is reported that both the CO_2 fixation rate and O_2 evolution rate of *C. vulgaris* were directly proportional to the light intensity less than 97.2 μmol/m^2/s (Fan et al., 2007). As soon as the intensity exceeded, the CO_2 fixation rate and O_2 evolution rate both increased slightly and then remained constant after 145.8 μmol/m^2/s. Thus, the light intensity value of 145.8 μmol/m^2/s was the saturation point, which should be the optimum value for *C. vulgaris* (Fan et al., 2007). Usually, the outermost layers in any microalgal growth culture may absorb too much light inhibiting the inner biomass growth. Subsequently, the inner layers of algae may be restricted from light due to self-shading phenomenon and hence the volumetric biomass productivity per unit of incident light will reduce. Hence, efficient light intensity throughout the microalgal growth culture should always be maintained essentially.

2.5.4 CO_2 and pH

During photosynthesis, microalgae consumes CO_2 as the sole source of carbon for cell growth. The CO_2 molecules diffuse to the microalgal cells and are then bio-fixed by photosynthesis, which in turn benefits in CO_2 sequestration and fostering the microalgal growth. Since algae has the ability to survive even on a high concentration of CO_2, greenhouse gases, NO_x and other pollutants in the atmosphere, there has been increasing focus on the use of microalgal culture technology for bio-fixation of CO_2 from flue gas (Ketife et al., 2017; Toledo-Cervantes et al., 2018). The amount of CO_2 needed for growth differs for various algal species based on the PBRs used. The effect of CO_2 level on the growth of microalgae varies between strain to strain. CO_2 fixation rate of various strains can be seen from Table 2 as reported by Sydney et al., (2010) and Hirata et al., (1996). In terms of maximum CO_2 tolerance, some species like *Synechocystis aquatiliscan* can survive even at very high CO_2 concentration and could fix up to 1500 mg.CO_2/L/day (Murakami & Ikenouchi, 1997), but in general, microalgae demands lower CO_2 concentration for their maximum growth.

Table 2: CO_2 fixation rate and amount of CO_2 used in Biomass generation.

Microalgae Species	CO_2 fixation rate (mg/L/day)	Percentage to Biomass (%)
Dunaliella tertiolecta	272.4	70.42
C. vulgaris	251.64	86.68
Spirulina platensis	318.61	80.40
Botryococcus braunii	496.98	87.96
Chlorella sp. UK001	31.8	4.3

On the basis of various studies carried out it can be concluded that microalgae are definitely going to be economical and a powerful tool for CO_2 mitigation. High concentrations of CO_2 decrease the water pH because the unutilized CO_2 will be converted to carbonic acid.

Contrariwise, using bicarbonate instead of gaseous CO_2 will result in pH increase producing alkaline media. Every microalga has its own optimal pH. The alkaliphilic microalgal species has an optimal pH at 8.5 – 9. Whereas, microalgae that are cultivated in an acidic environment (pH between 0 and 5.5) belong to the group of acidophiles and microalgae grown with an optimal pH between 5.5 and 8 belong to the group of the neutrophiles. It was found that, pH of around 8 seems most beneficial for maximum growth rate and lipid accumulation of *Nannochloropsis salina* (Bartley et al., 2014). β-Carotene was highly recovered in *Dunaliella bardawil* at pH 9. β-Carotene and vitamin E were greatly accumulated in *Chlorella ellipsoidea* cells grown at pH 6 and pH 10 respectively (Khalil et al., 2010). In general, pH values of 8 to 9 might be most conducive for increasing algae production and minimizing invading organisms. CO_2 addition seems more valuable to algae as an inorganic carbon source and not as an essential mechanism to reduce pH.

2.6 Microalgae Application

2.6.1 Microalgae in biodiesel production

Microalgae have long been recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production. They are photosynthetic microorganisms able to fix carbon dioxide and convert it to organic

matter that can be transformed into biofuels, foods, feeds and high-valuable products. Many species of microalgae are known to have high contents of lipids, which could be converted to biofuel, mainly biodiesel, via the transesterification (Khalil et al., 2010). Due to their rapid growth and ability to accumulate oil in concentrations of up to 50% of their dry weight, together with the possibility of year-round production, microalgae oil yield is much higher than oilseed crops. Some algae species, such as *C. vulgaris,* have high lipid contents when grown in wastewater. The lipid productivity is also the highest for *C. vulgaris* at 204.91 mg/L/day. Studies show that *C. vulgaris* grown in an artificial medium resulted in 20-42% (dry basis) lipids with removal efficiencies of 97% for ammonium and 96% for total phosphorus (Johnson et al., 2013). The potential yield of oil from microalgae has been estimated to be approximately 40 tons/ha of oil on a large scale, which is significantly greater than 1.5 tons/ha of oil from rapeseed grown in the U.K (Rodolfi et al., 2009).

Using microalgae for biodiesel production offers many potential advantages like:

i. Broad spectrum of products including biodiesel, green diesel, gasoline, bioethanol, methane, bio-oil and biochar can be produced from microalgae.

ii. High oil yields in the range of 1,000–4,000 gallon/acre/year can be produced, which is significantly higher than that of soybeans and other oil crops.

iii. Utilization and sequestering of atmospheric CO_2.

iv. Growing in a wide variety of climates and water conditions.

v. Cultivation in non-arable land, and hence do not compete with traditional agriculture.

2.6.2 Other applications

Microalgae have a wide range of applications, owing to their physiological and biochemical characteristics. Useful components in a microalgae cell are the pigments/carotenoids, polyunsaturated fatty acids, vitamins and antioxidants. They contain up to 50-70% protein, over 30% lipids, around 8-14% carotene and a fairly high concentration of vitamins such as A, C, B_1, B_2, B_3, B_6, B_{12}, E, K, D, etc. Microalgae are considered important raw materials for amino acids, vitamins and production of pharmaceuticals, nutraceuticals, food supplements and natural food colorants. The cultivation of microalgae is a profitable business as it is a waste-less, ecologically pure, energy and resource saving process. Currently, microalgae are used in aquaculture feeds. Strains rich in pigments, such as *Dunaliella salina*, *Haematococcus pluvialis* and *Spirulina* sp. are used as a source of natural pigments for the culture of prawns, salmonid fish and ornamental fish.

Microalgae are rich in essential nutrients such as iodine, potassium, iron, magnesium and calcium making them a major source of food and nutritional supplement, especially in Asian countries (Becker, 2007). Microalgae are also used in cosmetics as thickening agents, water-binding agents and antioxidants (Mourelle et al., 2017). They are also found in sun protection and hair care products (Priyadarshani & Rath, 2012). Marine microalgae are important renewable sources of bioactive lipids of high polyunsaturated fatty acids contents which are effective in preventing or treating several diseases (Li et al., 2008). In addition, ß-carotene extracted from microalgae have been reported to be anti-carcinogenic and effective in controlling cholesterol (Priyadarshani & Rath, 2012). Microalgae are also employed in agriculture as biofertilizers and soil conditioners. Majority of cyanobacteria are capable of fixing

atmospheric N_2 and can play an important role in the maintenance and build-up of soil fertility (Song et al., 2005).

Above all these, the flexibility of microalgae to perform either photoautotrophic, heterotrophic or mixotrophic metabolism epitomize promising biological treatment systems for various sources of wastewater. The advantages and the rate of CO_2 fixation by various microalgae species and their respective biomass growth has already been discussed in Table 2. The microalgae can be used in wastewater treatment for a range of purposes, some of which are tabulated in Table 3.

Table 3: Applications of microalgae in wastewater treatment

Applications	Treatment reports	References
Assimilation of N and/or P	The ability of *C. vulgaris* in the nutrient removal efficiency have been reported as 86% for inorganic N and 78% for inorganic P.	(Lau et al., 1996)
Reduction of both COD and BOD	Elimination of BOD and COD was found to be 68.4% and 67.2% respectively in a domestic wastewater treatment plant.	(Colak & Kaya, 1988)
Disinfection of wastewater	High-rate sewage stabilization ponds have reported a reduction of 99% in total coliform counts, thus helps in disinfection of the sewage.	(Colak & Kaya, 1988)
Removal of heavy metals	Numerous species of algae are capable of sequestering significant quantities of toxic heavy metal ions such as Cu^{2+}, Pb^{2+}, Cr^{3+}, Ni^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Mo, Ca, Mg, Zn and Se from aqueous solutions by bioaccumulation within their cells.	(Abdel-Raouf et al., 2012)

2.7 Kinetics Modeling

2.7.1 Growth and substrate removal kinetics models

Kinetic models, which describe the activity of a microorganism on a particular substrate is crucial for understanding any biological process and for providing quantitative data required for the design and optimization of the process. A variety of mathematical models have been proposed to describe the dynamics of metabolism of compounds exposed to cultures of microorganisms. The effect of substrate concentration (S) on the specific growth rate (μ) of a population of microorganisms is the main information needed for generating the growth models. The most commonly used model to describe microbial growth kinetics is the empirical model originally proposed by Monod (Monod, 1942). The Monod model introduced the concept of a single growth-limiting substrate as shown in Equation (1):

$$\mu = \frac{\mu_{max}.S}{K_s + S} \qquad (1)$$

Where, μ and $\mu_{max.}$ are the specific and maximum growth rates respectively, S is the concentration of the single growth-limiting substrate and K_S is the substrate saturation constant (substrate concentration at half $\mu_{max.}$). Monod also related the yield coefficient ($Y_{X/S}$) to the specific biomass growth rate, μ and the specific rate of substrate utilization, q using the Equation (2) as:

$$Y_{X/S} = dX/dS = \mu/q \qquad (2)$$

Monod model however cannot describe substrate inhibit effect. In this case, Monod derivatives that provided corrections for substrate inhibition can be used to describe the growth-linked biodegradation kinetics by incorporating the inhibition constant,

K_i. Among the various substrate inhibition models, Haldane's model given by Equation (3) is the most widely used (Sokol, 1987). The kinetics follow simple Monod's model when the inhibition constant is infinitely large.

$$\mu = \frac{\mu_{max}.S}{(K_S + S + S^2/K_i)} \quad (3)$$

The Aiba growth inhibition model, given by Equation (4), depicts a decrease in specific growth rate with an increase in product concentration. This model includes an exponential term to account for the decrease in the specific growth rate:

$$\mu = \left[\frac{\mu_{max}.S}{(K_S + S)}\right] .e^{-S/K_i} \quad (4)$$

The exponential term found in the Equation (4) is added to consider the product inhibition (Aiba et al., 1968). However, it fails to give the critical value of inhibitory substrate/product concentration.

2.7.2 Thermodynamics analysis

The temperature of the culture medium is one of the ecological key factors in the growth of microalgae. Above and below the optimal temperature range, much higher activation energies are required for the growth of microalgae. Laboratory experiments and ecological observations have led to the distinction between psychrophilic, mesophilic and thermophilic microalgae exhibiting optimum growth temperatures below 20°C, between 20-35°C and above 35°C respectively. It is possible to describe the growth rate 'μ' solely as a function of temperature if the light intensity is constant by using the Arrhenius equation (Goldman et al., 1974) as given in the Equation (5):

$$\mu_{max} = A.e^{-E_a/RT} \quad (5)$$

Where, A is Arrhenius frequency factor (day^{-1}), E_a is the activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol.K) and T is the absolute temperature in K. It is widely used to describe the temperature effect on various biological processes, from enzyme catalysis to community activity.

2.8 Drop size distribution analysis

In dilute oil in water emulsions, the drops at equilibrium are isolated and generally spherical in shape. Droplet size distribution is one of the most important characteristics of emulsions (Sjöblom et al., 2003). Laser diffraction (LD), dynamic image analysis (DIA) and photon correlation spectroscopy (PCS) are the most common techniques used to characterize the droplets size distribution of an emulsion. LD measures particle size distributions by measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample. Large particles scatter light at small angles relative to the laser beam and small particles scatter light at large angles. The angular scattering intensity data is then analyzed to calculate the size of the particles responsible for creating the scattering pattern. DIA characterizes particles in motion by digitizing photographs of each particle and storing them in an image file. Each particle is measured in real time, while a software calculates morphological parameters based on the known size and location of the pixels in each image.

PCS is based on the dynamic light scattering (DLS) of a laser beam of a certain wavelength by particles or macro-molecules in a liquid medium. This was the method used in this work, which measures the diffusion of particles moving under

Brownian motion and converts this into size and a size distribution using the Stokes-Einstein relationship. It does this by illuminating the particles with a laser and analyzing the intensity fluctuations in the scattered light. Brownian motion is the movement of particles due to the random collision with the molecules of the liquid that surrounds the particle. An important feature of Brownian motion for DLS is that the small particles move quickly whereas the large particles move much slowly. The analysis is performed at a scattering angle of 90° using DLS, which is widely used to determine the size of Brownian nanoparticles in colloidal suspensions in the nano and submicron ranges. The scattered and diffracted laser beam has a distribution in frequency owing to the Doppler shift by Brownian motion of the particles. Although droplets in emulsions have a range of sizes, in a bell-shaped distribution versus their numbers, one single representative diameter is usually needed in modeling the droplet size versus concentration of the dispersed phase. The definitions of different average diameters of the drop size distribution are shown in Table 4 (Filella et al., 1997). Where, n_i is the number of drops of diameter d_i and I_i is the respective intensity of the drop. Among those average diameters, the volume-surface known as the Sauter mean drop diameter, d_{32} is the most commonly used to represent the average diameter of an emulsion (Princen et al., 1986; Weaire & Fu, 1988; Yoshimura & Prud'homme, 1988)

Table 4: Different drop size distribution average diameters

Type of average	Diameter definition
Intensity-average diameter, d_i	$\frac{\sum n_i I_i d_i}{\sum n_i I_i}$
Number-average diameter, d_n	$\frac{\sum n_i d_i}{\sum n_i}$
Volume-average diameter, d_v	$\frac{\sum n_i d_i^3}{\sum n_i d_i^2}$

2.9 Concluding Remarks

2.9.1 Problem statement

Crude petroleum oils found as emulsions in water have a number of negative impacts on the environment, human health and wastewater treatment processes. Emulsified crude oil wastewater contains toxic substances that are carcinogenic to humans and can inhibit the growth of plants and animals. It also reduces the efficiency of a wastewater treatment plant by blockages, pump failures and reduced oxygen for biological treatment. Low efficiency treatment produces a discharge that does not meet the effluent standards, resulting in an increased concentration of contaminants in receiving streams and local ecosystems. Emulsified crude oil might also kindle rusting of the pipelines, and gets stuck in pipes, grits and screens, leading to blockages that decreases the flow efficiency or even burst pipes (Alade et al., 2011).

2.9.2 Hypothesis and novelty statement

Besides their hazardous impact, the presence of emulsified oil in the water results in fouling, clogging and sludge deposits on the membrane surfaces of filtration processes in wastewater treatment plants. By using microalgae, the emulsified crude oil is eliminated, and the microalgae biomass thus generated has economic value. The harvested microalgae biomass has high lipid content and can thus be used as a feedstock for biodiesel production. In the treatment of the emulsion, the specific interfacial area is considered the significant factor, rather than the actual concentration, as the treatment is taking place at the interface. Therefore, a correlation describing the relationship between the concentration and the interfacial area of the emulsion was developed and used to determine kinetics model to describe

the process. To the best of the knowledge of the researchers of this project, this is the first work to investigate the removal of emulsified petroleum crude oil found in wastewater using microalgae.

Chapter 3: Materials and Methods

3.1 Chemical and Microalgae Strains

Crude petroleum oil, collected from the Bu Hasa Oil Field (84 km northwest of Abu Dhabi Islands), was a kind gift from the Petroleum Engineering Department at UAE University. A freshwater strain, namely *Chlorella sp.*, was obtained from a culture grown in Bold's Basal Medium (BBM). The microalgae suspensions were prepared by growing the strains in BBM for two weeks, prior to be used in the experiment. The BBM medium consisted of 2.94 mM $NaNO_3$, 0.17 mM $CaCl_2.2H_2O$, 0.3 mM $MgSO_4.7H_2O$, 0.43 mM K_2HPO_4, 1.29 mM KH_2PO_4, 0.43 mM NaCl, 17.1 mM EDTA, 55.3 mM KOH, 0.179 mM $FeSO_4.7H_2O$, 18.5 mM H_3BO_3 and 1 ml/L of trace metal solution which consisted of 0.307 μM $ZnSO_4.7H_2O$, 7.28 μM $MnCl_2.4H_2O$, 0.307 μM MoO_3, 6.29 μM $CuSO_4.5H_2O$, 1.68 μM $Co(NO_3)_2.7H_2O$.

3.2 Crude Oil Emulsion Preparation

To prepare the stable crude oil emulsion, 12 mL of crude oil was mixed in a 1 L of culture medium containing 250 mg of Gum Arabic. The mixture was emulsified by mixing thoroughly using a blender (Electrolux, ESB7300SAR) for 15 min. The TOC reading of the emulsified solution was monitored for over a week to obtain a stable emulsion. The TOC of the stable emulsion of the highest oil content was 275 mg/L and was taken as the maximum initial oil concentration in subsequent tests. Several dilutions of the stabilized emulsion were prepared by adding known amounts of the medium and remixing in the blender.

3.3 Experimental Set-up

The experiment was set up in a 125 mL Erlenmeyer flask, filled with 90 mL emulsion solution of different dilutions, and inoculated with 10 mL of microalgae suspension. The inoculation suspension was prepared to bring the initial biomass concentration to 0.38 mg/L in the experimental mixture in all samples. A control experiment was run in parallel, consisting of 90 mL of the emulsion solution mixed with 10 mL of medium free of microalgae. The flasks were placed in a temperature-controlled water bath shaker (DAIHAN Scientific, Maxturdy-30, Korea) as shown in Figure 4 and incubated for five days at 100 rpm and 30°C.

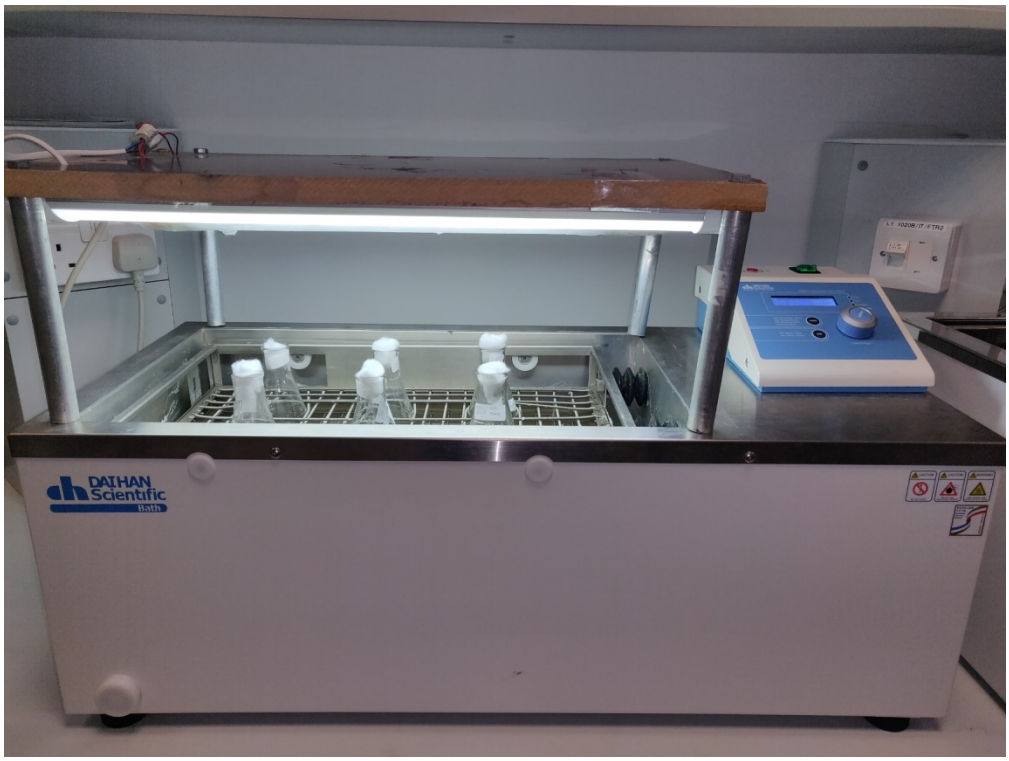

Figure 4: Incubator setup with water temperature control

No aeration was provided to the samples, to enhance the heterogeneous cultivation, and direct the microalgae to use the emulsified crude oil as the carbon source. On a

daily basis, 5 mL samples were collected, and analyzed for their TOC and biomass concentration. All the runs were duplicated, and the average has been shown; the error bars implies the range of standard deviation.

3.4 Analytical Methods

To minimize the evaporation effect, before withdrawing a sample at any day, the level of the mixture in the experiment was brought back to the level it was at when the sample was withdrawn on the previous day. The withdrawn aliquots (5 mL) were diluted with 10 mL distilled water in a centrifuge capped vial. The samples were then centrifuged at 6000 rpm for 5 minutes (Thermo Scientific, IEC CL31 Multispeed, USA), as shown in Figure 5 to separate the microalgae cells. The supernatant was taken for TOC measurement, whereas the separated cells, stuck on the walls of the centrifuge tube, were re-suspended in 10 mL distilled water using ultrasonicator (Qsonica, CL-188, USA) at 100 amplitudes for 1 minute. Chlorophyll a is present in microalgae and it absorbs the light with wavelength of 680 nm. Hence, the amount of absorbed light is proportional to the cell density or cell number in suspension by using a standard curve (Griffiths et al., 2011). The optical density of the re-suspended microalgae sample was measured at 680 nm using UV Spectrophotometer (Shimadzu UV-1800, Japan) as shown in Figure 6. The spectrophotometer was zeroed using the distilled water and calibrated against serial dilutions of microalgae suspension of known concentrations.

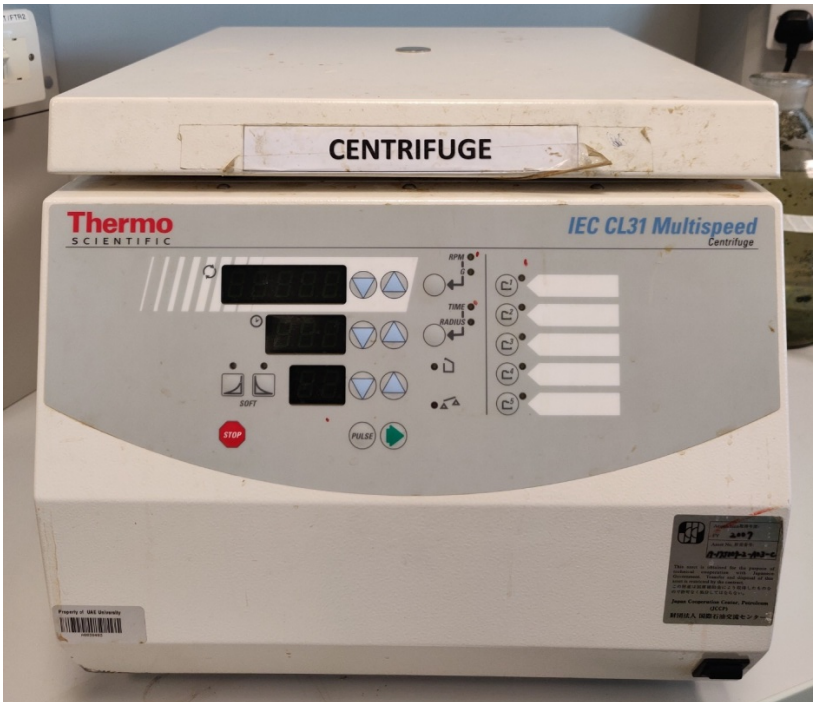

Figure 5: Centrifugation instrument

Spectrophotometry is a convenient indirect method to record the optical density value at a specific wavelength. Optical density is represented in terms of transmittance, which can be determined by Beer-Lambert Law of Absorbance. The Beer-Lambert law relates the concentration of a sample to the attenuation of light as it passes through the sample, so that it is capable of proportionally correlating the cell concentration with the optical density (Myers et al., 2013)

Figure 6: Spectrophotometer

The cells concentration in g/mL at any cultivation time was calculated from a pre-prepared calibration curve. The calibration curve was prepared by measuring the absorbance of several dilution of microalgae suspension of known concentration determined by the dry weight analysis (Zhu & Lee, 1997). The analysis was done by filtering the algal suspension using a pre-washed and dried Whatman filter paper, dried overnight at 105^{o}C in an oven (Memmert, Germany) until constant weight. Prior to measuring the concentration of any unknown sample, a blank was used to minimize deviation from the original readings.

The TOC concentration in the supernatant was measured using a TOC Analyzer (SHIMADZU, TOC-V_{CSH}, Japan). The instrument as shown in Figure 7 was programmed to take the best three readings out of five. The readings presented are the average of the best three readings. The instrument was initially calibrated in three

different ranges using potassium hydrogen phthalate (KHP) stock solution of 1000 ppm.

Figure 7: TOC instrument

Subsequent dilutions were made from 500 ppm to 10 ppm in order to cover the ranges from 0–50, 0–100 and 0–500 ppm. After the analysis of each sample, a blank solution was run to minimize the deviation of the proceeding samples. All the experiments were run in duplicates and the results presented in this work are the average values. To confirm the reproducibility of the data, the standard deviation of the two runs were determined and presented as error bars in the figures. The drop size distribution of the different dilutions of the crude oil emulsions were determined

using DLS particle size analyzer (Zetasizer - Nano ZS, Malvern, UK) as shown in the Figure 8.

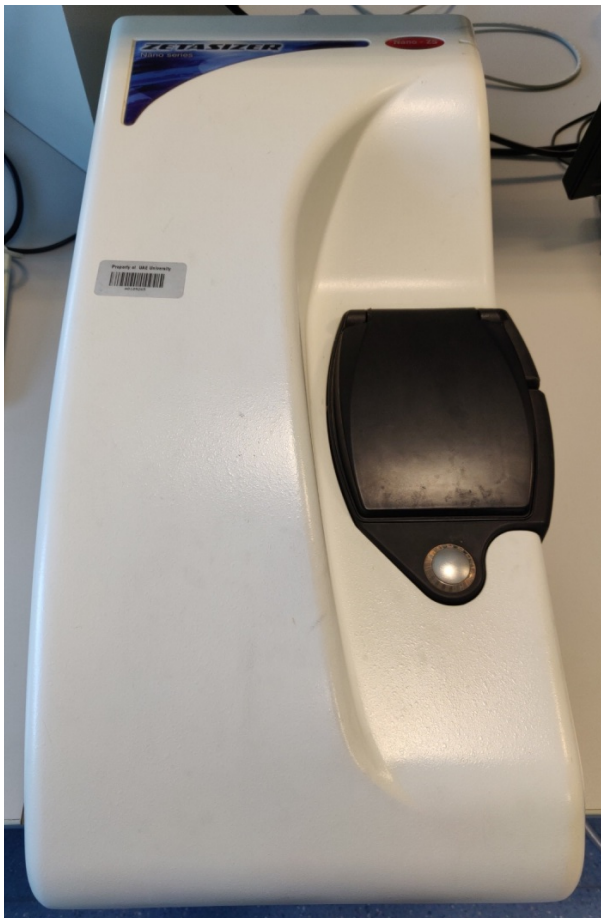

Figure 8: Drop size distribution measurement

The size measurements for each dilution were done in duplicates and the average was considered. A minimum sample volume of 12 µL was taken in the standard cell, which is approximately 10 mm from the bottom of the cell. After filling the cell, it was carefully inspected for any trapped bubbles. The cell was inserted into the cell holder, and the measurement sequence was completed, and the measurement was displayed. The intensity-based particle size distribution, volume-based particle size distribution and number-based particle size distribution for each dilutions of the emulsions were analyzed separately thereafter.

Chapter 4: Results and Discussion

4.1 Biomass Production and TOC Drop

Four samples with different predetermined TOC concentration were prepared in 125 mL flasks. The flasks were inoculated with the same initial biomass concentration 0.381 ppm and were monitored on a daily basis at two different temperatures of 30^{o}C and 40^{o}C, to determine the TOC and biomass concentrations. Parallel set of flasks were prepared as blanks, in the same way but in the absence of microalgae. After an adaptation period, required for the microalgae to adapt to the new environment, the solutions started to get greener, indicating growth in microalgae using the emulsified crude oil as the sole carbon source. The drop in TOC was recorded on a daily basis for both the blanks and the main experiment. The results in Figure 9 show the TOC concentration C, versus time for the blank and the sample with microalgae at an initial concentration of 222 mg/L. Since the stability of the emulsion increased with dilution, the control experiment was done using the emulsion solution of the highest concentration, considered at each testing temperature. It can be clearly seen that the drop in TOC in the blank experiment, in absence of microalgae, was insignificant, as compared to that with the microalgae, in which almost 80% of the emulsified crude oil was removed within the first five days. The results prove that the drop of the TOC within the experimental duration, was mainly due to the biological degradation by the microalgae.

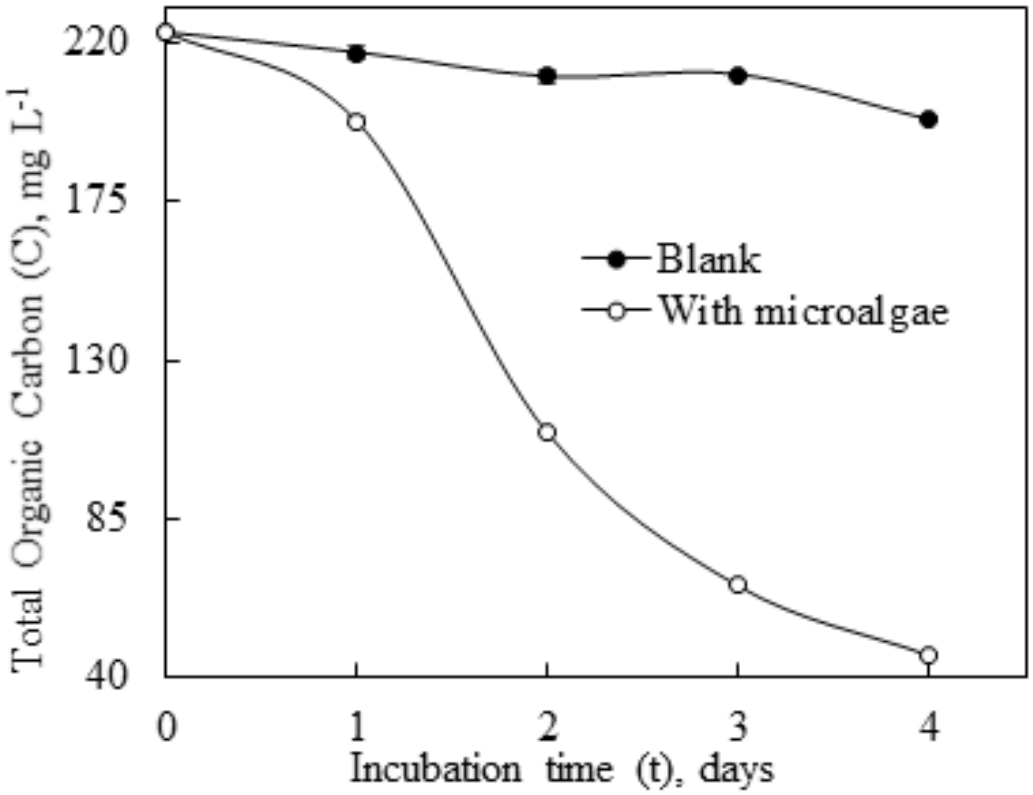

Figure 9: Changes of oil concentration at 30°C with time when microalgae was used compared to the blank at highest initial oil concentration C_o, of 222 mg/L

The same experiment was repeated using different initial crude oil concentrations, C_o, and temperatures. The relative concentrations with respect to the respective initial concentrations were plotted versus time at two temperatures, namely 30°C and 40°C, as shown in Figure 10.

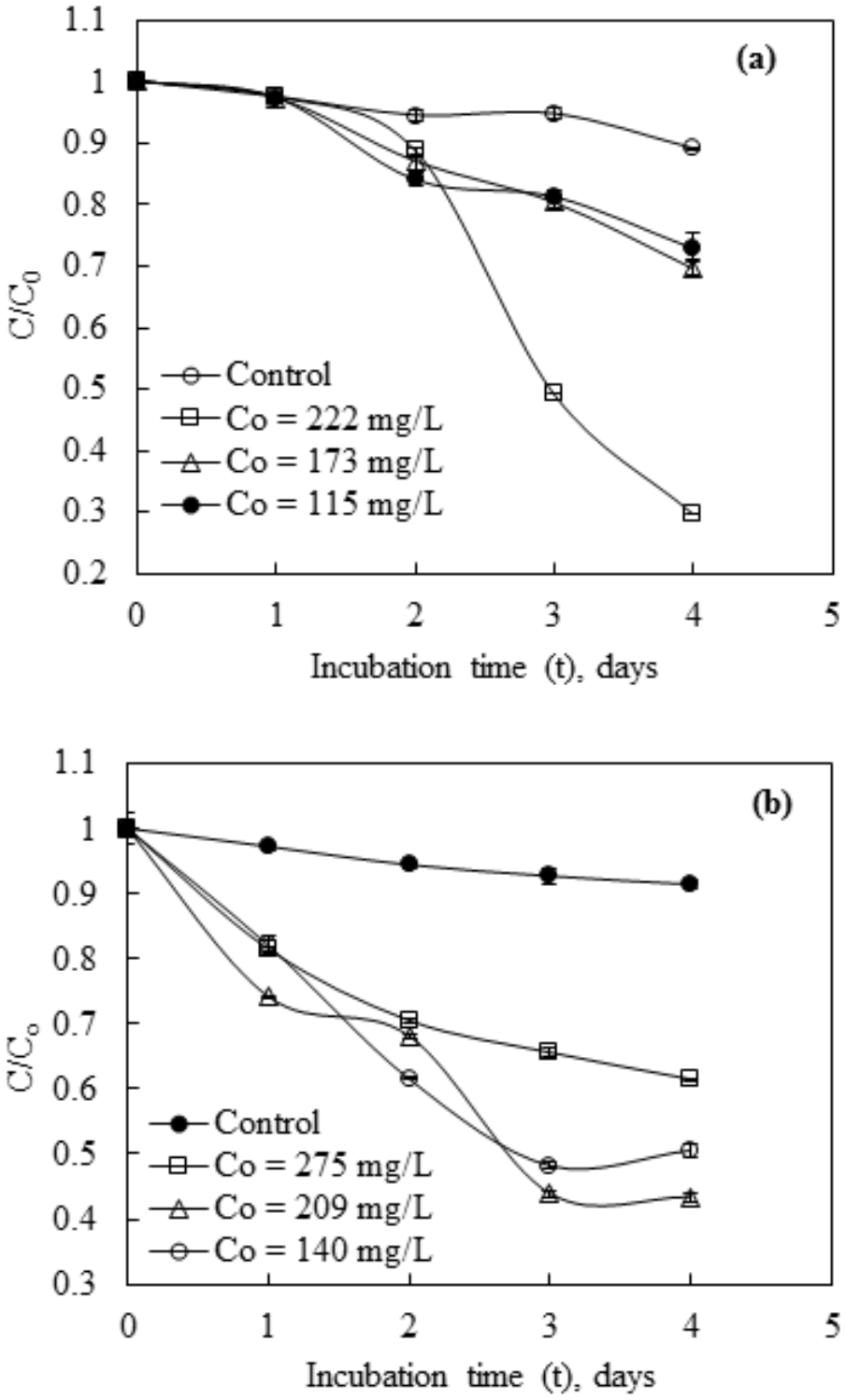

Figure 10: Changes of relative concentration with time at different initial oil concentration C_o, and temperatures of (a) 30°C and (b) 40°C

It can be seen from the Figure 10 that at 30°C there was a delay in the oil drop-rate in the first day, which was not observed at 40°C. This is because the culture

temperature did not differ from that of the nutrient medium, from which it was inoculated. Hence the cells were able to continue growing depending on the nutrients from the growth medium, before shifting to utilize the abundant carbon sources from the crude oil as substrate. Whereas at 40^{o}C, the microalgae had to start immediately utilizing the carbon sources as soon as it was administered in the new environment at harsher conditions. It was also found that the drop in TOC increased with the increase in crude oil content. This is because the specific interfacial area is higher at higher concentration, and hence, more substrate is available for the microalgae. At 40^{o}C, the observed trend was slightly different, wherein a lag was encountered at initial concentration of 275 mg/L. In both cases however, 60-70% removal of the initial TOC content was removed within five days. The overall TOC removal rate was higher at 40^{o}C for all dilutions, which suggests a higher biological activity of *Chlorella sp.* at the higher temperature. The same maximum biological activity was also observed at 40^{o}C for *Chlorella sp.* grown autotrophically using CO_2 as the sole carbon source in the presence of light (Sorokin & Krauss, 1962).

The drop in the TOC cannot be definitely confirmed to be due to the biological activity of the microalgae. Adsorption on the surface of the cells could also contribute to the drop. Having said that, the growth of the microalgae would benefit both removal mechanisms. The more biomass results in increasing the biological activity and increase the available surface for adsorption. To be certain about the respective contribution of both mechanisms, more investigation would be needed. One way to do so, is by carrying out the experiment with dead cells.

The results shown in Figure 9 and Figure 10 are the averages of runs done in duplicates at the same conditions, with the standard deviation shown as error bar.

The lines connecting the data points were drawn to show the trend. The biomass concentration was also monitored and presented for the two temperatures in Figure 11, as ln (X/X_o) versus time, where X and X_o are the biomass concentrations at any time and at time zero, respectively. The values shown in Figure 11 are the averages of runs done in duplicates at the same conditions, with the standard deviation shown as error bar. The lines connecting the data points were drawn to show the trend.

The lag phase, the period of time required for the biomass to adapt to the new environment, was almost negligible at 30°C, but becomes more obvious at 40°C, especially at the higher concentrations of oil. This is due to the harsher environment that the microalgae were subjected to at these conditions. At lower concentrations, the lag phase was shorter, even at the higher temperature. Once adapted to the environment, exponential phase triggered. When *Chlorella sp.* was examined for phenol degradation in a standard Fog's medium, a similar lag phase of one day was also observed. It was also found that the lag phase was lower at lower phenol concentration of 25 mg/L (Das et al., 2015).

The results in Figure 10 were used to determine the initial rate of drop in oil concentration, determined from the slope of the TOC concentration at time equal zero, and the rate versus the initial oil concentration is shown in Figure 12(a). Assuming there is negligible maintenance requirement for the cells, the specific growth rate was determined from the slope of the growth curves, shown in Figure 11, in the exponential growth phase, and the specific growth rate, μ_{max}, at each initial oil concentration is shown in Figure 12(b).

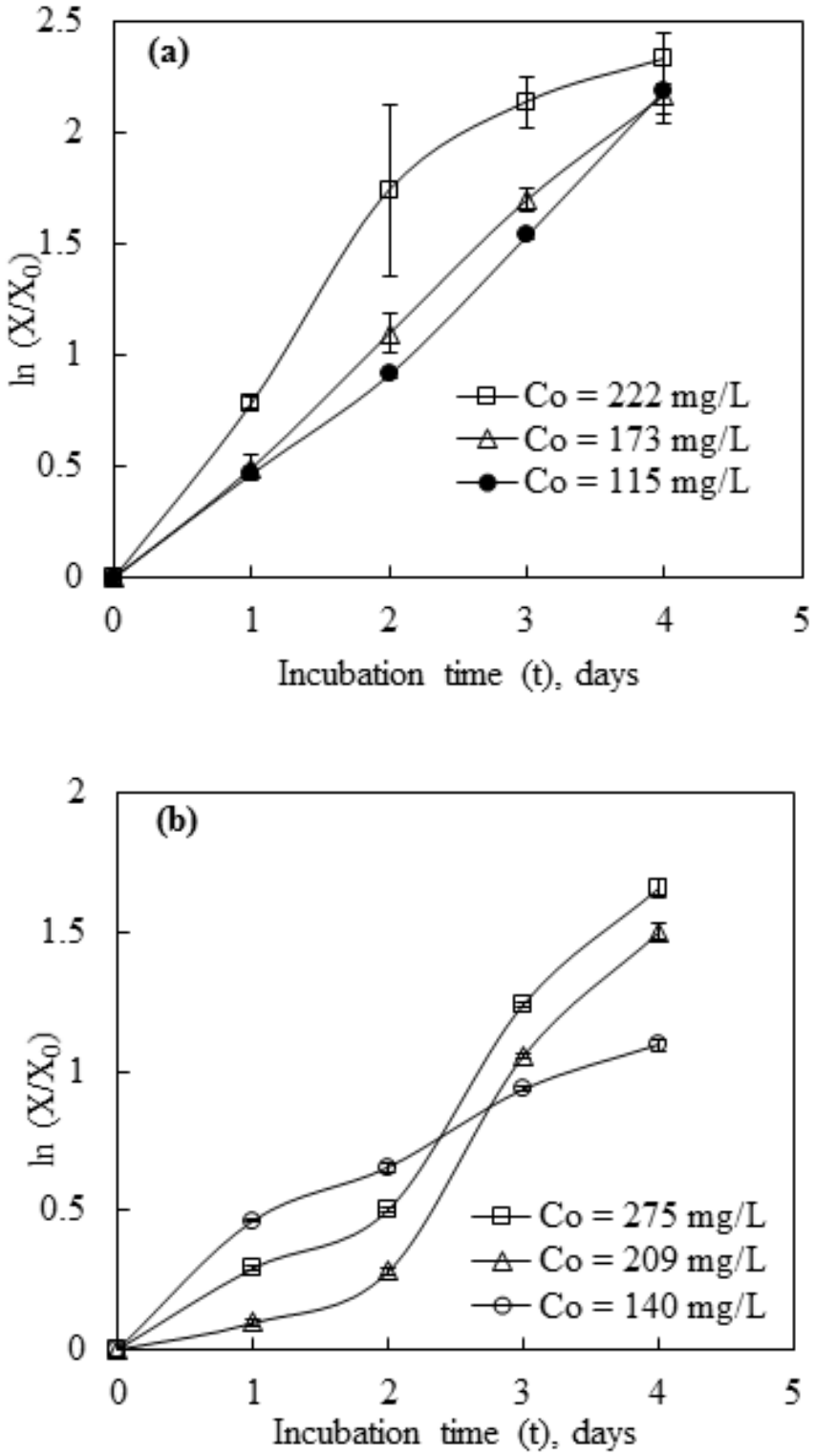

Figure 11: Changes of ln (X/X_o) with time at different initial oil concentration C_o, and temperatures of (a) 30℃ and (b) 40℃

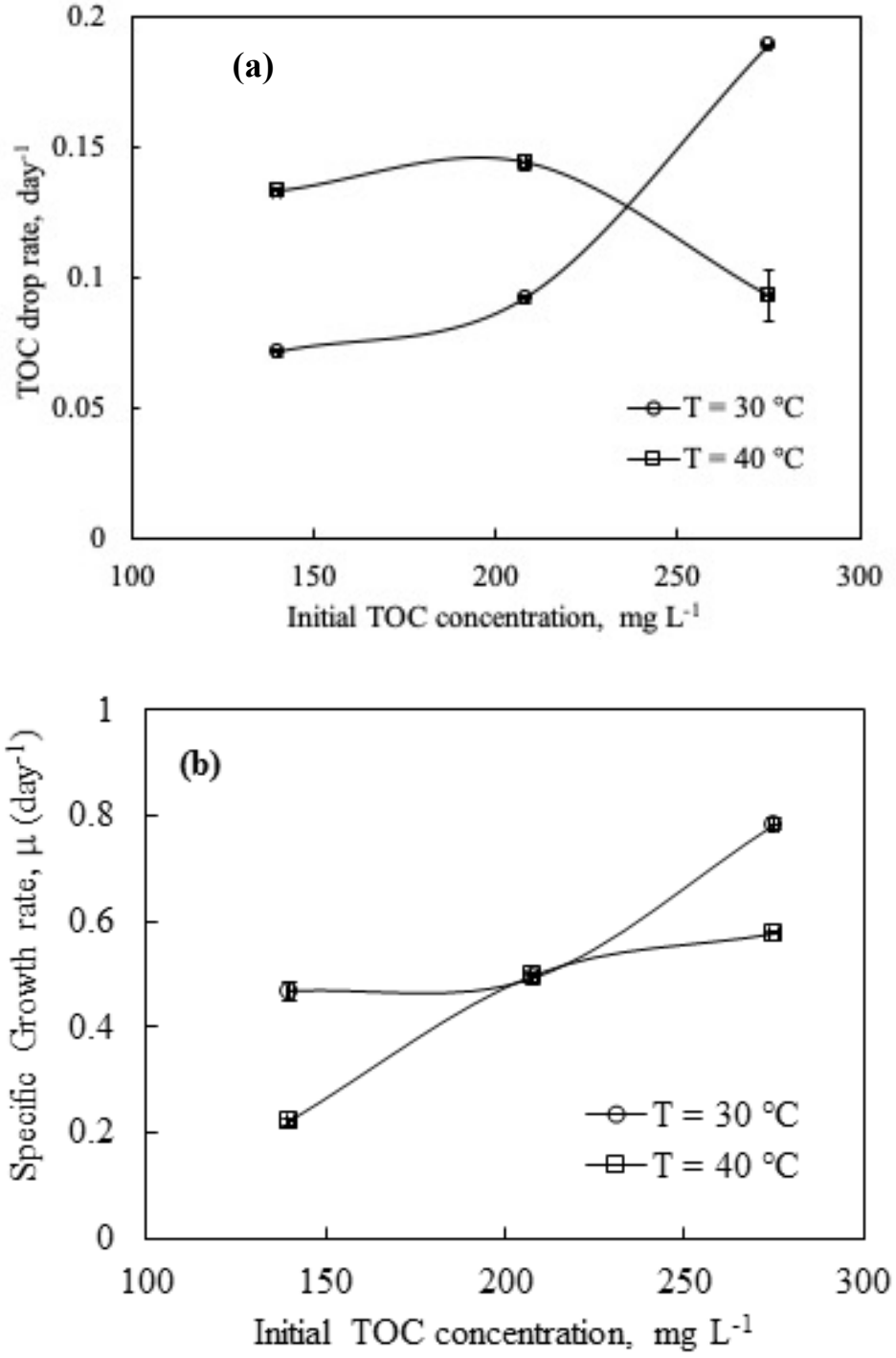

Figure 12: Effect of initial oil concentration C_o, on (a) oil removal rate and (b) microalgae specific growth rate at different temperatures

It was found that the specific growth rate increased with the increase in the initial concentration of the emulsified crude oil. Whereas there is a decrease in oil drop rate at higher temperature at higher concentration of oil. This is due to the lag perceived

by the microalgae in coping up with the harsh conditions at higher temperatures with the increase in the concentration.

The effect of temperature on the oil drop rate and the specific growth rate were opposite. The oil drop rate decreased at the higher temperature, 40^{o}C. However, the significance of the temperature effect on the drop rate increased as the initial concentration increased. The temperature effect on the specific growth rate was opposite i.e., higher growth rates were observed at the lower temperature, 30^{o}C. At the higher temperature, majority of the utilized substrate were used for the maintenance of the cells and not for growth. These results are in agreement with those found when *C. vulgaris* was cultivated autotrophically using CO_2 as the sole substrate in the presence of light in temperatures between 18-25°C (Serra-Maia et al., 2016), wherein the specific growth rate was higher at the lower temperature. It was also shown that the sensitivity of the specific growth rate towards temperature increased with the increase in temperature, which also agrees with the results found in the current study.

4.2 Kinetics and Thermodynamics Results

The growth kinetics of *C. vulgaris* in crude oil emulsion was studied. As no substrate inhibition was observed in the results obtained in this work, the growth pattern is sufficiently represented using the Monod model Equation (6).

$$\mu = \frac{\mu_{max}.C_{oil}}{K_s + C_{oil}} \quad (6)$$

Where, C_{oil} is the oil concentration, μ_{max} , is the maximum specific growth rate and Ks is the Monod kinetic constant. However, due to the heterogeneous nature of the

oil in water emulsion, the available substrate to the microalgae does not depend on the bulk concentration of the oil, but rather on the interface area between the aqueous phase containing the microalgae and the oil phase. Therefore, the Monod kinetics Equation (6) was modified to replace the specific interfacial area, a_t, instead of the bulk concentration, C_{oil}, as shown in Equation (7)

$$\mu = \frac{\mu_{max} . a_t}{K_s + a_t} \tag{7}$$

To relate the specific interfacial area to the oil concentration, the oil drop distribution was measured at different oil concentrations, and the results are shown in Figure 13.

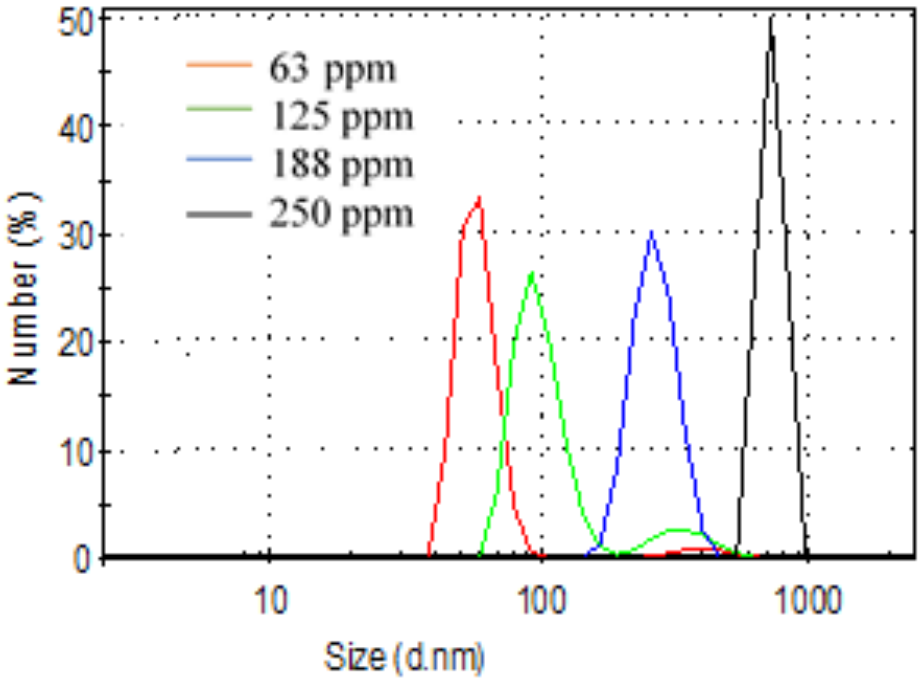

Figure 13: Oil drop size distribution of emulsions with different oil concentrations

A normal Gaussian distribution of the oil droplets was observed for all concentrations of the crude oil, with the size span increasing with the increase in oil concentration. As explained earlier, one single representative diameter is needed in

modeling the droplet size versus concentration of the dispersed phase. The most commonly used average diameter is the volume average, also known as the Sauter diameter, which was shown in Table *4*. The results shown in Figure 13 were used to calculate the Sauter mean diameter at each oil concentration, and was used to determine the specific interfacial area using Equation (8):

$$a_t = \frac{6\varphi}{d_{32}} \tag{8}$$

Where, φ is the volume fraction of the oil in the mixture and d_{32} is the Sauter mean drop diameter. By substituting the volume fraction with the concentration of the oil in the emulsion Equation (9) was developed:

$$a_t = \frac{(6\times10^{-6}).C_{oil}}{SG_{oil}.d_{32}} \tag{9}$$

Where, C_{oil} is the concentration of the crude oil (in ppm) and SG_{oil} is the specific gravity of the oil. The specific interfacial area was then drawn versus the crude oil concentration, as shown in Figure 14. Although the average oil drops were smaller at the lower concentrations of oil, as shown in Figure 13, the specific interfacial area increased with the increase in the crude oil concentration. The results in Figure 14 were used to determine the polynomial to relate the specific interfacial area to the oil concentration, as given in Equation (10)

$$a_t = 0.092C_{oil}^2 + 8.72C_{oil} \tag{10}$$

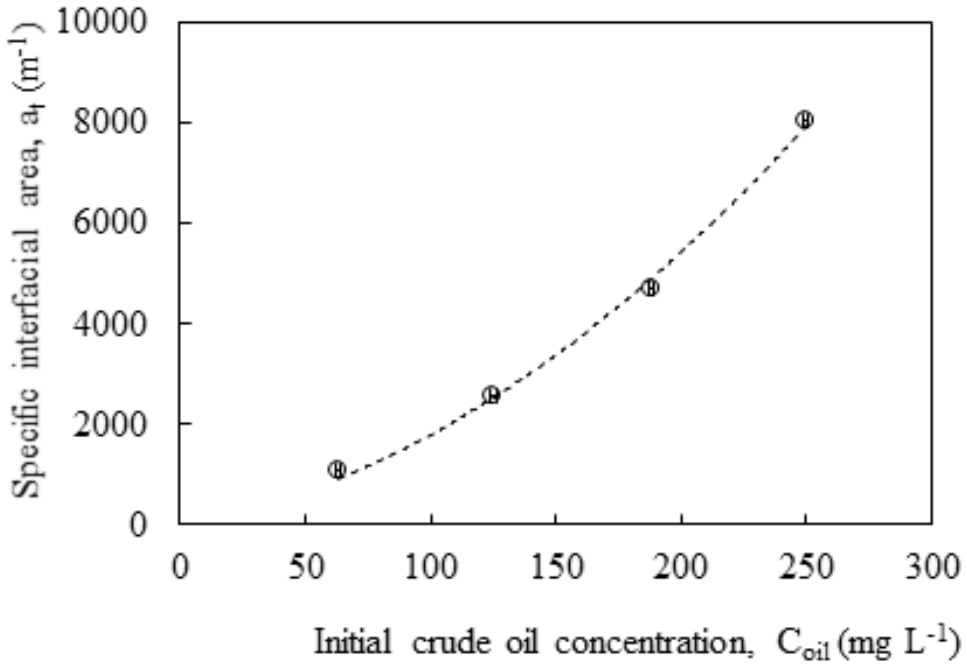

Figure 14: Specific interfacial area versus crude oil concentration

The experimental results shown in Figure 12(b) were then fitted to Monod model using Excel solver with an objective function, O.F. given by Equation (11).

$$O.F = \sum_{i=1}^{n} (\mu_{Exp} - \mu_{Pred})^2 \quad (11)$$

Where, μ_{Exp} and μ_{Pred} are the experimental and predicted specific growth rate, respectively and n is the number of data points. The estimated values of the kinetic model at the two tested temperatures are shown in Table 5. The goodness of the fit is measured by the coefficient of determination and from comparison between the experimental results and the model prediction shown in Figure 15.

Table 5: Estimated parametric values of Monod growth kinetics

Temperature	$\mu_{max.}$ (day^{-1})	K_s (ppm)	Coefficient of Determination (R^2)
30°C	1.1667	5,577.32	0.737
40°C	1.4853	13,806.25	0.917

The determined maximum specific growth rate found in this study was close to that found for *C. vulgaris* cultivated for various applications. For example, the maximum specific growth rate in a synthetic medium containing piggery waste was 1.20 day^{-1} at temperatures ranging from 27-32^{o}C during the day and from 21-25^{o}C during the night (Travieso et al., 2006). When the strain was cultivated in Kolkwitz medium (KTM-A) for biological sequestration of CO_2, the maximum specific growth rate was 1.547 day^{-1} at 25^{o}C (Concas et al., 2012). The maximum specific growth rate of *C. vulgaris* at 20^{o}C was also in the range 1.2–2.4 day^{-1} when grown in a microfluidic culture containing BG11 medium under different nitrate stress conditions (Saad et al., 2019).

The biomass production per substrate consumption yield coefficient, $Y_{X/S}$, was also determined at the two tested temperatures, and the results are shown in Figure 16. It is evident from the figure that the yield of biomass varied with temperature. It was found that at 40^{o}C, the yield was independent on the initial oil concentration as the biomass yield per substrate consumed was lower at higher temperature. This suggests that at 40^{o}C, the portion of the substrate decomposition used for cell maintenance was high and that used for biomass growth was lower. This agrees with the results shown in Figure 12, in which less biomass growth was needed to achieve high removal of oil. However, at 30^{o}C there was a clear drop in the yield with the increase in the initial oil concentration, which suggests that a significant portion of the substrate consumption went for biomass maintenance, not for biomass growth. Similar drop in the biomass yield with the increase in the initial phosphorous concentration was also observed in the growth of *Scenedesmus obliquus* cultivated in a mineral medium with phosphorus concentrations of up to 372 μM and temperature at 20^{o}C (Martínez et al., 1999).

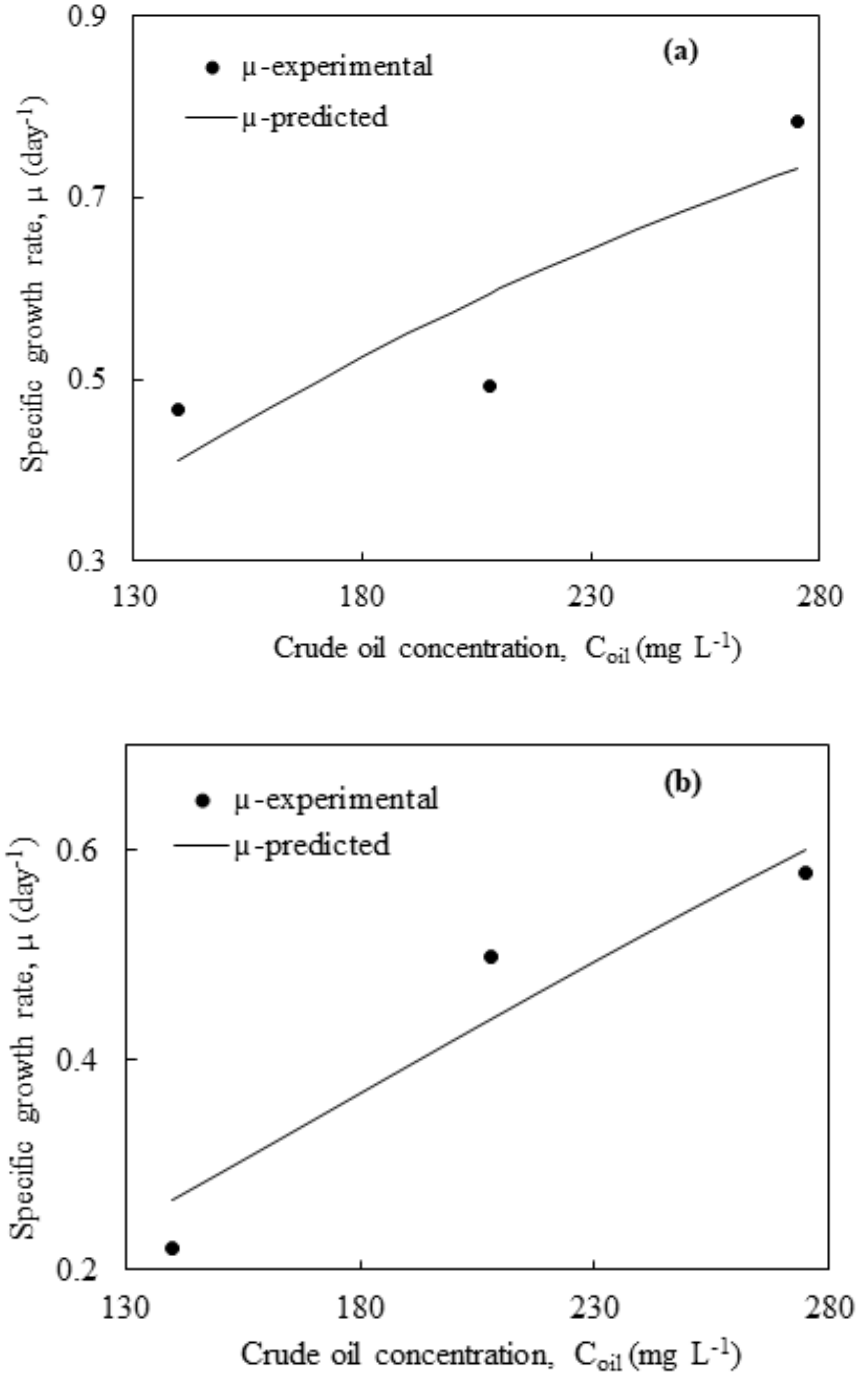

Figure 15: Comparison between the experimental specific growth rate and the model predictions at (a) 30°C and (b) 40°C

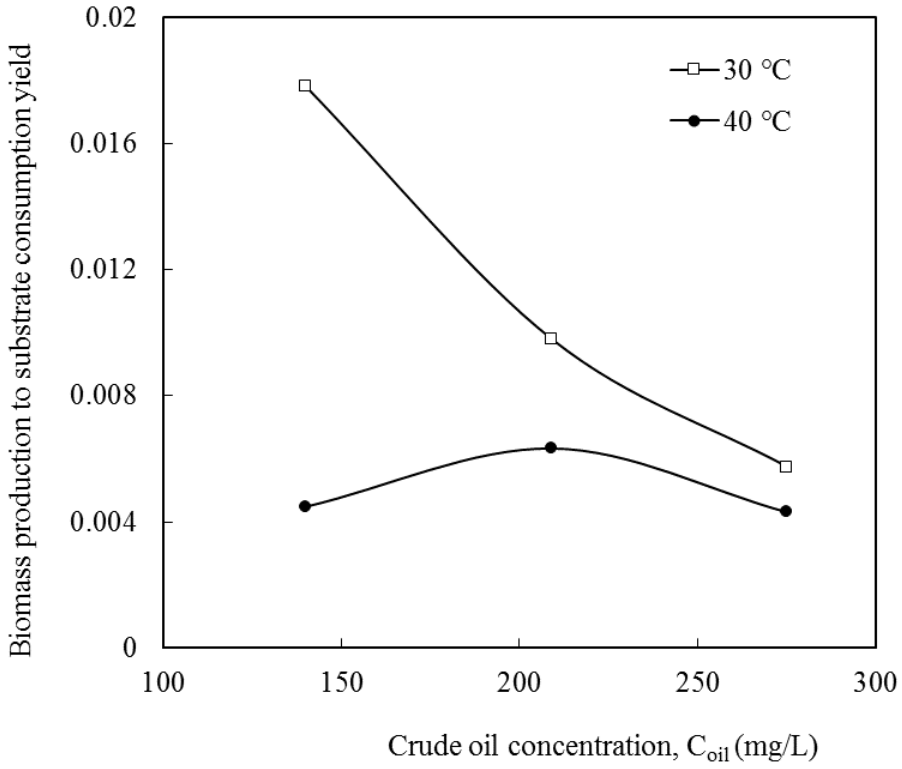

Figure 16: Yield versus crude oil concentration

The results of the effect of temperature on the growth rate were used to determine the thermodynamics properties of the system. As the determination of the growth of microalgae at the two temperatures 30°C and 40°C was done using a constant light intensity, it was possible to describe the growth rate μ, solely as a function of temperature using the Arrhenius Equation(5) (Goldman & Carpenter, 1974). The activation energy was determined to be 19.05 kJ/mol, which is within the range of 12.6-84 kJ/mol, reported for heterotrophic microorganisms grown in similar temperature range. Above and below the normal temperature range, much higher activation energies were reported (Precht, 1973). The growth of green microalgae, such as *S. obliquus*, grown axenically in a mineral N8 medium was found to have activation energies within a range of 34.82-83.35 kJ/mol (Soeder et al., 2014).

Chapter 5: Conclusion

C. vulgaris was successfully cultivated in crude petroleum oil emulsion under a batch condition using BBM as culture medium. The effects of temperature and oils concentration on the specific growth and oil drop rates and biomass/substrate yield were determined. Modified Monod model, in which the specific interfacial area was used instead of the substrate concentration, to describe the growth kinetics. Lag phases were observed at high temperature at initial concentration of the emulsified crude oil and tended to reduce as the temperature and concentration reduced. The yield of biomass versus oil consumption was higher and more sensitive to temperature at the lower temperature. The successful results found in this work could pave the way for the utilization of microalgae in the removal of emulsified oil, which could further be coupled with biodiesel production.

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