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Growth of Microalgae for Simultaneous Treatment of Industrial Wastewater and Biodiesel Production

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GROWTH OF MICROALGAE FOR SIMULTANEOUS TREATMENT OF INDUSTRIAL WASTEWATER AND BIODIESEL PRODUCTION

Mohammed Ahmad Abujayyab

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemical Engineering

Under the Supervision of Professor Sulaiman Al-Zuhair

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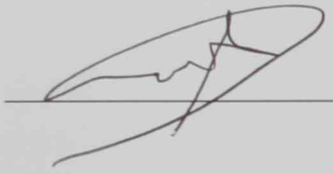
Declaration of Original Work

I, Mohammed Ahmad Abujayyab, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Growth of Microalgae for Simultaneous Treatment of Industrial Wastewater and Biodiesel Production*", 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions.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.

Student's Signature:  Date: 30-01-2020

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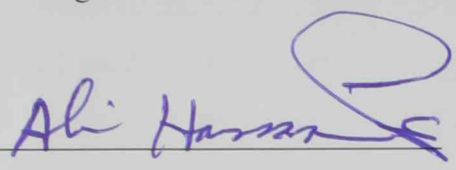
Signature  Date 30/11/2020

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Abstract

The treatment of industrial wastewater contaminated with phenols is among the main challenges in the chemical, and particularly the petrochemical industry. Phenols, which present in considerable amounts in industrial wastewater, are toxic even at low concentrations. Therefore, it is essential to reduce their concentrations in water to harmless levels before being discharged. On the other hand, the dependence on the conventional fossil sources of energy, which is non-renewable, is not sustainable and can cause harmful environmental impacts. Therefore, in this work, the dual utilization of microalgae as a cheap and efficient means to remove phenolic compounds commonly found in refinery wastewater and as a sustainable source for oils for biodiesel production has been proposed. Most conventional bacteria used in the treatment of phenolics are pathogenic and the collected biomass after treatment is considered a secondary waste that does not have an evident commercial value. Microalgae, however, are promising, sustainable and renewable sources of oils that can be used for biodiesel production. In addition, they contain important compounds, such as proteins and pigments, which have large applications in the food and pharmaceutical industries. Combining the production of these valuable products with wastewater treatment renders the cultivation of microalgae very attractive and economically feasible. In this work, two freshwater microalgae stains, namely *Chorella sp.* and *Tetraselmis sp.*, were used to remove several phenolic compounds commonly found in refinery wastewater (phenol, 4-nitrophenol and 2,4-dinitrophenol). These compounds were selected since they are very toxic and may cause serious threats for the ecosystem. In addition, the removal of 4-nitrophenol and 2,4-dinitrophenol is challenging, and there is no previous study conducted their removal using microalgae. The effect of initial concentrations of the phenols on the biomass growth and phenolic removal were evaluated and used to develop kinetics models to describe the system. The optimum removal results were obtained at concentration of 250 ppm of phenol, and 350 ppm for both 4-nitrophenol and 2,4-nitrophenol. The microalgae were then grown in a pilot scale open pond, and the biomass was harvested, and oils was extracted from it. The extracted oils were then converted to biodiesel using homemade heterogeneous alkaline catalyst. A parametric study was carried out to determine the effects of main reaction parameters, namely

catalyst amount, methanol:oil molar ratio and reaction temperature, on the conversion after 4 h. The results showed that increasing temperature at all methanol:oil molar ratios generally resulted in decrease the FAME conversion, except at catalyst load of 7%. The experimental results were used to determine a statistical second order interactive model that was used to determine the significance of all factors and to optimize the process. The optimum conditions were found to be a catalyst wt% of 7.7, methanol:oil molar ratio of 12 and temperature of 45^{o}C, at which the conversion after 4 h was 30.4%. The results were also used to determine a kinetic model's parameters. The significance of this work is to use a sustainable source to treat wastewater from phenols with cheap cost, and then to use the grown biomass to produce biodiesel. While kinetics studies of microalgae growth in water containing phenol are available in literature, as far as the investigators of this work know, this is the first work to present a kinetics study on the treatment of nitrophenols.

Keywords: Microalgae, phenols, degradation, biodiesel, transesterification.

Title and Abstract (in Arabic)

تنمية الطحالب الدقيقة لمعالجة مياه الصرف الصناعية ولإنتاج الديزل الحيوي

الملخص

تعتبر معالجة المياه الناتجة عن الصناعة والملوثة بالفينولات من أهم الصعوبات في الصناعات الكيميائية وخصوصاً في صناعات البتروكيمياويات. الفينولات، التي تظهر بشكل واضح في المياه الملوثة الناتجة عن الصناعة، سامة حتى في حال ظهورها بتراكيز قليلة. لذلك، من المهم تقليل تركيزها في المياه حتى تصبح بمستوى غير ضار قبل التخلص منها. في الجانب الآخر، يعتبر الاعتماد على مصادر الوقود الاحفوري التقليدية للطاقة، التي هي غير متجددة، غير مستدام ويمكن أن يسبب تأثيرات بيئية ضارة. لذلك، في هذا العمل، استخدام الطحالب الدقيقة كمصدر رخيص وفعال للتخلص من الفينولات التي تظهر غالباً في مياه مصافي البترول وكمصدر متجدد للزيوت لإنتاج الديزل الحيوي. الكثير من البكتيريا المستخدمة في معالجة الفينولات مسببة للأمراض، والكتلة الحيوية المجمعة بعد المعالجة تعتبر مصدر نفايات ثانوي لا يملك قيمة اقتصادية. الطحالب الدقيقة، في المقابل، تعتبر مصدر واعد، متجدد ومستدام للزيوت التي يمكن استخدامها في انتاج الديزل الحيوي. بالإضافة إلى ذلك، تمتلك الطحالب الدقيقة مركبات مهمة مثل البروتين والأصباغ التي تستخدم في مجالات كبيرة مثل الصناعات الغذائية والدوائية. يعتبر الدمج بين انتاج منتجات ذات قيمة عالية مع استطاعة معالجة المياه الملوثة يجعل تنمية الطحالب الدقيقة جاذباً جداً وممكناً اقتصادياً. في هذا العمل، تم استخدام نوعين من الطحالب الدقيقة وهما كلوريلا وتيترازيلميس لإزالة الفينولات الموجودة بشكل كبير في مياه مصافي البترول الملوثة (الفينول، 4-نيتروفينول و 2،4-نيتروفينول). تم اختيار هذه المركبات لأنها سامة وتشكل تهديداً للنظام الحيوي. بالإضافة إلى ذلك، تشكل إزالة 4-نيتروفينول و2،4-نيتروفينول تحدياً، وكذلك لم تتم دراسة إزالتها باستخدام الطحالب الدقيقة. تمت دراسة وتقييم تأثير التركيز المبدئي للفينولات في نمو الكتلة الحيوية وفي إزالة الفينولات ومن ثم تم تطوير المعادلات التي يمكن استخدامها في وصف نظام النتائج. تم التوصل إلى أن أعلى معدل إزالة للفينول كان عند تركيز 250 جزء في المليون، و 350 جزء في المليون ل 4-نيتروفينول و 2،4-نيتروفينول. ومن ثم تم تنمية الطحالب الدقيقة في نطاق تجريبي في بركة مفتوحة ومن ثم تم جمع الكتلة الحيوية، واستخراج الزيوت منها. تم تحويل الزيوت بعد ذلك إلى ديزل حيوي باستخدام محفز قلوي غير متجانسة مصنوع ذاتياً. تم عمل دراسة للعوامل المؤثرة في انتاج الديزل الحيوي بهدف تحديد تأثيرها، وهي كمية المحفز، نسبة المولية للميثانول إلى الزيت ودرجة حرارة التفاعل الذي استمر مدة 4 ساعات. تشير النتائج إلى أن زيادة درجة حرارة التفاعل في كل النسب

المولية للميثانول إلى الزيت تسبب نقص في نسبة الناتج، ما عدا عند تركيز المحفز المساوي ل 7%. تم استخدام النتائج المخبرية في تحديد نموذج تفاعلي إحصائي من الدرجة الثانية لتحديد العوامل المهمة المؤثرة في العملية، وأفضل الحالات التي تعطي أفضل النتائج. تم تحديد أفضل الحالات عند تركيز المحفز المساوي ل 7.7%، النسبة المولية للميثانول إلى الزيت تساوي 12 وعند درجة حرارة 45 درجة سيليزية، وذلك بعد 4 ساعات من التفاعل الذي نتج منه 30.4%. تم استخدام النتائج أيضاً في تحديد قيم الثوابت المستخدمة في المعادلات الواصفة للنتائج. تأتي أهمية هذا العمل في استخدام مصدر مستدام لمعالجة المياه الملوثة من الفينولات بتكلفة رخيصة ومن ثم استخدام الكتلة الحيوية التي تم تنميتها في انتاج الديزل الحيوي. كما أن المعادلات الواصفة لمعالجة المياه من النيتروفينول هي الأولى التي تم تطبيقات والتي لا توجد في أي عمل سابق. تضمن هذا العمل اندماجاً بين عملية المعالجة وإنتاج الديزل الحيوي.

مفاهيم البحث الرئيسية: الطحالب الدقيقة، الفينولات، الانحلال، الديزل الحيوي، الأسترة.

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Dedication

To my beloved parents and Lamis

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List of Abbreviations

BBM	Bold Basal Medium
BET surface area	Brunauer-Emmett-Teller analysis of surface area
BJH pore size	Determining pore size distribution based on the Kelvin equation
DOC	Dissolved organic carbon
FAME	Fatty acids methyl esters
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRP	Photo bioreactor
NER	Net energy ratio
PBR	Horseradish peroxidase
Ppm	Part per million
SEM	Scanning electron microscope
USAB	Up flow anaerobic sludge blanket reactor
XRD	X-ray diffractometer

Chapter 1: Introduction

1.1 Overview

Phenols are widely used in industrial processes such as petroleum refineries, resin plants and coking operations, and then released in wastewaters due their solubility in water. Phenol and its derivatives are toxic even at low concentrations and considered serious threats to the purity and quality of water. Pesticides and drugs are also considered of the main sources of phenol and its derivatives that are related to the environment through industrial sewage and landfill leachate (Das et al., 2015). Due to the toxicity of phenol and its derivatives, it is very important to reduce them to harmless levels excluding their negative effects on the environment. Several attempts using conventional methods such as adsorption or membrane cells were done to remove or to reduce the concentration of phenols in wastewater, however, the use of these conventional methods is either energy intensive or expensive. According to that, biological treatment of wastewater was performed including the use of bacteria. Although of the good results obtained while using bacteria, however, many used types were pathogenic and the collected biomass after wastewater treatment were not viable. Microalgae were used as a cheap and safe way to remove phenols from wastewater. In addition, the collected biomass after the treatment can be used for biodiesel production.

The main objectives of this work are to study the efficiency of removing different phenolic compounds (phenol, 4-nitropheno and 2,4-dinitrophenol) using two different stains of microalgae (*Chorella sp.* and *Tetraselmis sp.*), the corresponding growth of these strains and the kinetics of phenols removal. In addition, to use the collected biomass to produce biodiesel and determine the optimum conditions of the reaction in addition to the kinetics of the reaction.

1.2 Statement of the problem

The treatment of industrial wastewater contaminated with phenols is among the main challenges in the chemical, and particularly the petrochemical industry. Phenols are toxic even at low concentrations, and thus it is important to remove phenols or even to reduce their concentrations in wastewater to harmless levels before being discharged. Removing phenols using conventional methods is either energy intensive or expensive. Biological treatment of wastewater with phenols was considered as promising to remove phenols. Most studies on phenols degradation have been carried out using bacteria (Hirooka et al., 2002). Despite their high efficiency in degrading phenols, the grown bacteria do not have any obvious value, and above that, their release has harmful environmental impact. The release of these bacteria could cause diseases in plants and depletion of fish stocks and may also have the potential to cause diseases to humans. Therefore, it would be advantageous to use other types of microorganisms, which are less harmful, if they show comparable performance. The produced biomass in this case can be readily used to produce lipids that can be used for biodiesel production.

1.3 Relevant literature

1.3.1 Phenols

1.3.1.1 Phenols types and structure

Phenols are compounds that have aromatic ring with hydroxyl group, C_6H_5O. They are named according to the parent compound, as shown the ones in figure 1.

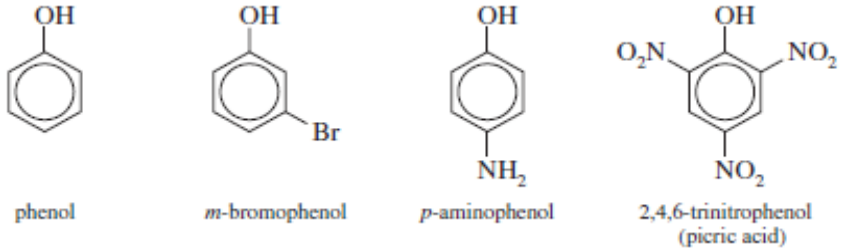

Figure 1: Different examples of phenolic compounds

Many natural compounds contain phenolic groups in their structure such as resorcinol and catechol, which are shown in figure 2.

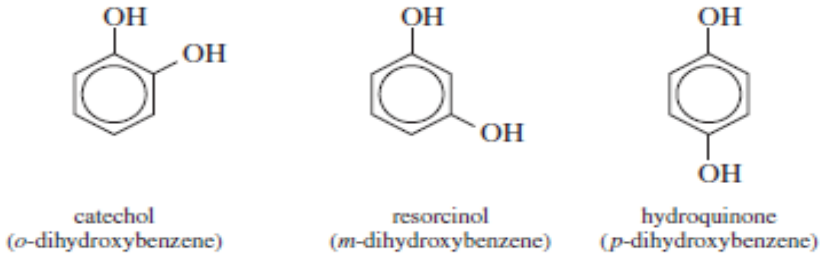

Figure 2: Natural compounds that includes phenolic group

At room temperature phenol is solid in its pure state and its melting point is 41^{o}C. It is a colorless, crystalline substance of characteristic odor. It is soluble in water and organic solvents (Michalowicz and Duda, 2006). It is a weak acid, more acidic than water and alcohols, but less than acetic acid or carbonic acid. Phenol can be also used for the production of aniline, pesticides, dyes, explosives and cresols. Generally, phenols are toxic especially for microorganisms. Therefore, they are widely used as disinfectants and antiseptics. This feature, however, makes the bio removal of phenols a challenging process. Phenols can be obtained commercially from coal tar. The process starts with reacting propene with benzene in order to form Cumene, which is then oxidized in the presence of air to cumene hydroperoxide. By treating this product with sulfuric acid, phenol and acetone are obtained (Hein et al., 2011). Phenol can also be obtained by toluene oxidation, happens between chlorobenzene and sodium

hydroxide (Basha et al., 2010).

1.3.1.1.1 Chlorophenol

Chlorophenols are the largest group of phenols and the most widespread. They are formed in the environment by chlorination of mono and polyaromatic compounds that present in soil and water. The process of chlorophenols synthesis is catalyzed by chloroperoxidases that are found in microorganisms and plants in the presence of inorganic chlorine and hydrogen peroxide (Adeola, 2018).

1.3.1.1.2 Catechol

Catechol is aromatic alcohol compound that has hydroxyl residues on the first and on the second positions of carbon. It is soluble in water and in organic solvents. It is formed in the catalytic hydrolysis process of 2-chlorophenol at high temperature or as a result of phenol and benzoic acid hydroxylation reactions (Krab, 2002). It is used in photography, rubber, drug synthesis and synthetic material production (Schweigert et al., 2001).

1.3.1.1.3 Nitrophenol

Nitrophenols is related by the anthropogenic activities and natural processes. 2-Nitrophenol and 4-nitrophenol, are formed as a result of the reaction of phenol and nitrite ions in water under the influence of UV irradiation in a wide range of pH (Kimura et al., 2013). Nitrophenols can also be formed by the reaction of phenol, hydroxyl radical and nitrite ions lead to form 2-nitrophenol (Harrison et al., 2005). Nitrophenols in the atmosphere are usually found in low concentrations of ng/L (Flox et al., 2005).

1.3.1.2 Harmful effects of phenols

Phenol was one of the first compounds described in the List of Priority Pollutants by the US Environmental Protection Agency (Bruce et al., 1987). Phenol and its derivatives are used as main components of dyes, drugs and polymers. The existence of phenols in the environment is related to chemical industries, petrol industries, degradation of pesticides, and the generation of industrial sewages. Phenols are toxic and this toxicity comes from the organic radicals' generation. There are three main classes of phenols in commercial applications, which are nitrophenols, chlorophenols and alkyl phenols such as cresols. These compounds occur in wastewater, soil, groundwater and air. For example, pentachlorophenol can be produced as a result of incineration of municipal wastes. Then, the produced pentachlorophenol may react with fly ashes at high temperature, 250 to 350^{o}C, and form dioxins which is considered as one of the most toxicological chemicals. Nitrophenols, chlorophenols and alkyl phenols show a relatively high degree of toxicity (Adeola, 2018).

Toxicity influence of organic compounds such as phenols depends on many factors. The diffusion of phenols into cells' membrane is significantly affected by the hydrophobicity of the compound. Increasing in the hydrophobicity of the compound increase the rate of diffusion and penetration through the cell's membrane and thus increasing the toxicity of xenobiotics (Bhatnagar and Minocha, 2006). The toxicity of phenol is the reactivity of the compound with the biomolecules is another main factor, which is related to the easiness of free electrons denotation by phenol from the oxidized substrate (Adeola, 2018).

1.3.1.3 Presence of phenols in wastewater

Environmental pollution is one of the main issues facing humanity, which increased exponentially during the past years. Toxic organics are one of the main pollutants that have direct effects on the environment. Such chemicals may cause a damage on kidney, central nervous system, and respiratory system. In the United States for example, around 580,000 people occupationally are exposed to phenol influence (Bruce et al., 1987). According to Agency for Toxic Substance and Disease Registry in U.S, phenol is classified as the 45th rank as a hazardous substance that should be treated before entering the environment. The removal of such substances or even the decreasing of its contamination to safe levels is considering a challenging issue (Kujawski et al., 2004). Phenol is one of the most important raw materials that are used for production of variety of resins, including polycarbonate, polyamide and epoxy resins. For this reason, organic pollutant including phenols could be found in wastewater that results from chemical industries (Villegas et al., 2016). The concentration of phenol in surface water are varied, for example, in natural water it is between 0.01-2.0 µg/L, in river water that is polluted with petrol processing sewage it may increase to be 40 mg/L. It is expected that the concentration of phenol in air is very low, around 1 ng/m^3 but becomes much higher near factories which impregnate wood and its value may reach 9.7 µg/m^3. Phenol is present also with much lower concentrations in food such as in honey with a concentration of 5 µg/kg (Michalowicz and Duda, 2006). The concentrations of phenols in surface water are different according to the source, for example, the concentration of phenols in the natural waters is between 0.01-2.0 µg/L (Michalowics and Duda, 2004). The concentrations of chlorophenols in oceanic waters are varied in a range of 5-10 ng/L, whereas the highest noted concentrations for river waters are found in a range of 2-2000 µg/L. Phenols also

present in drinking water as a results of substitution of organic matter with chlorine atoms that are derived from inorganic chlorine oxidants (Delfino and Dube, 1976). Chlorophenols also present in the environment as a result of using and degradation of organic compounds such as growth regulators, pesticides and phenolic biocides (Brain et al., 1996).

1.3.1.4 Conventional methods of phenols removal

Several technologies were developed to remove phenols from wastewater. These methods include electro coagulation, advanced oxidation process, thermal decomposition and adsorption. Most of these methods either suffer from low efficiency, or have another disadvantage (Jiang et al., 2003). Electrocoagulation is one of the electrochemical processes of water treatment where electrochemical cells, sacrificial anode, are used to dose the polluted water with active coagulant. The nature and delivery rate of coagulant affect the separation process by its speciation and the removal path (Holt et al., 1999). Electrocoagulation has the ability to remove different pollutants including organic compounds, dyes and heavy metals (Abu Zaid et al., 1998). A research on removal of phenol using electrocoagulation was conducted in which the cell was used with horizontally oriented aluminum cathode aluminum screen anode. Removal of phenol was achieved due to the effects of both adsorption and sweep coagulation. It has been shown that at solution pH of 7 and high current density, 97% of phenol was removed after two hours. The maximum rate of phenol removal was 30 mg/L. Furthermore, the rate of electrocoagulation increases as the phenol concentration decreases. The study did not include removal of phenol with higher concentration, which are commonly found in the industrial wastewater. This method is considered expensive, and there are some environmental concerns regarding the

neutral salty effluents that may be resulted after treatment (Abdelwahab et.al, 2009). The advantages of using electrocoagulation is that it is simple, easy to operate and able to remove different pollutants with high efficiency, but the sacrificial electrodes should be regularly replaced because they are dissolved with time due to the presence of pollutants. In addition to that, high conductivity of wastewater suspense and much electricity are required (Kulkarni and Kaware, 2013). The advanced oxidation process is considered as a highly water treatment technology. It has been developed to treat drinking and wastewater by laying it in stages of pre or post treatment to reduce the recalcitrance of pollutants (Oller et al., 2011). It is based on the generation of reactive species, which are used to degrade the organic pollutants (Oliver et al., 2000). The main disadvantage of using advanced oxidation process lies in the high cost of reagents such as hydrogen peroxide and ozone, or the cost of energy and light sources such as ultraviolet light which is used to enhance the degradation process (Esplugas et al., 2002).

Thermal decomposition can be used to remove phenol at high concentration (15,000 mg/L) from wastewater. However, this process not economical and can only be used for small scales due to its high energy demand. Absorption is another process that has been used to removal phenol from wastewater, however, it has relatively high cost and cannot be used with high concentrated phenols (Jiang et al., 2003). Bioremediation of phenols is the preferred technique, but it is time consuming and usually incomplete (Kujawski et al., 2004).

Adsorption is one of the popular methods in removing pollutants from wastewater. A substance which is used to adsorb the pollutant is called adsorbent. Activated carbon, bentonites, ash and ion exchange resins are examples of adsorbent that are used to remove phenols (Al-Asheh et al., 2003). An example of removing

phenol using adsorption is the work of Radovic et al., (2000). The adsorption of phenol from aqueous solution by Filtrasorb-400 was examined. 80% of adsorption equilibrium capacity was reached in the first hours, which the adsorption process took three weeks. High phenol removal percentages can be achieved by adsorption according to the type of adsorbent but the disadvantage of using adsorption is the high cost of the process and in some cases, the adsorbent cost (Al-Asheh et al., 2003).

1.3.1.5 Bioprocess of phenols removal

One of the safest and cheapest methods for removing phenol is bioremediation, using microorganisms (Chakraborty et al., 2010). Generally, it is difficult to remove phenol with a concentration above 200 mg/L by biological methods. In addition, at a concentration of phenol above 3000 mg/L, the microorganisms are completely deactivated. Degradation of phenol with different initial concentrations from 50 to 2000 mg/L has been performed using several types of reactors such as fluidized bed reactor, shake flask and continuous stirred tank bioreactor. The degradation of phenol was carried out in these types of reactors using variety strains of bacteria and fungi (Nair et al., 2008).

Biodegradation of phenol in wastewater has been carried out either anaerobically or aerobically. Anaerobic process is the preferred process because it saves the energy needed for aeration, converts pollutants to methane, and produces less sludge. It is mostly performed using upflow anaerobic sludge blanket reactor, which can be used under mesophilic or ambient temperatures (Fang et al., 2006). However, the applications of anaerobic process are limited for wastewater treatment that is resulted from food and agricultural industries, where most pollutants are readily biodegradable (Mu’azu et al., 2017).

Anaerobic treatment of wastewater has been developed in high-rate reactors that are used in processes such as in up flow anaerobic sludge blanket reactor (UASB) and anaerobic filter. UASB is most commonly used for treatment of wastewater (Fang et al., 1996). Anaerobic biological treatment at low temperature were performed for phenols treatment but this technology requires a specific applied loading of phenol. The higher loading limit was not investigated (Collins et al., 2005).

Biological treatment for wastewater and effluents that include chlorinated organic compounds such as chlorophenol often include the biofilm reactor utilization (Jonge et al., 1996). It was found that using granular activated carbon biofilm reactors combine two important features which are the capacity of the adsorption and the niches for bacterial colonization that are protected from the force of the fluid as a result of irregular shape of the granular activated carbon (Christensen and Characklis, 1990). The varies functional groups that are on the surface enhance the attachment of microorganisms (Weber et al., 1979).As a results of that, biomass is active even at very low concentrations of the organic chemicals and that enables the biomass to be less sensitive to the toxic and inhibitory materials that may be presented there (Lee et al., 1994).

Using halophilic or halotolerant microorganisms for phenols degradation was suggested to be attractive option for industrial wastewaters treatment that have high concentrations of salts (43.5%). Halophilic and halotolerant microorganisms have the ability to degrade phenol by either mineralizing or transforming it in the presence of osmotic stress (Oren et al., 1992).

The aerobic catabolism pathway of many aromatic compounds has been investigated using different microorganisms and for varies natural and xenobiotic compounds (D'Annibale et al., 2004). Selected strains of bacteria, used to degrade

phenols, were first isolated under low oxygen conditions and grown using phenol as a sole source of carbon (Chakraborty et al., 2010). One of the main disadvantages of biological treatment of wastewater is that the microorganisms used there cannot survive at high phenol concentrations. For example, it has been reported that at phenol concentrations higher than 3000 mg/L inhibition of the growth of the microorganism is applied. Moreover, it has been reported that some strains of bacteria cannot survive in wastewater that contains more than 5% salts (Jiang et al., 2003). Several approaches were used to overcome the inhibition of microorganism by high concentration of phenol. These approaches include cell immobilization, stepwise increase in the phenol concentration, or genetic manipulations (Zidkova et al., 2013).

Bacillus cereus, a strain of bacteria, was used for phenols degradation by Das et al., (2014). It was primarily isolated for the treatment of Chlorophenols and phenol. It was found that it can grow in a phenol solution with an initial concentration of 500 m and it can degrade up to 98.4% of phenol after 60 hours.

In another study, *Ochromonas Danica* was grown on phenol as main source of carbon with a concentration up to 4 mM. The removal of phenol was detected using DOC, dissolved organic carbon, analysis, which showed that the rate of phenol-carbon removal is similar to the rate of disappearance of the phenol using HPLC (Semple and Cain, 1997). Another study using bacteria for phenol degradation was done by Jiang et al., (2007). The selected strain in the study was *Alcaligenes faecalis*. It was investigated that the phenol-degrading potential of *Alcaligenes faecalis* is strongly related to the physiological phases of inoculum. 1600 mg /L of phenol was completely degraded within 76 hours.

The ability of some strains of fungi to degrade phenols was studied. In one study by Denizli et al., (2004) the ability of the fungus Pleurotus sajor caju to degrade

phenols from aqueous solutions was investigated. It was found that the adsorptions rate of phenol was 0.95 mmol/g. In addition, the adsorption rate of phenol increased with increasing pH. It has been concluded that using Pleurotus sajor caju was better that using polymers for adsorption of phenol due to the low cost of Pleurotus sajor caju but there were no valuable products after using it. Despite the good results achieved using bacteria or fungi, the produced biomass after the degradation of phenol does not have any commercial value (Hirooka et al., 2002). Above that, most of the effective bacteria are pathogenic (Ryan et al., 2007).

Enzymes are proteins that are considered as biological catalysts in all types of living. They are known for their ability to increase the reaction rates without affecting the reaction equilibrium. They catalyze specific types of reactions mostly under moderate conditions of temperature, pH and ionic strength (Demarche et al., 2011). Enzyme-based processes were used in removing targeted compound efficiently. These processes were first proposed by very efficient in removing targeted compounds. Several studies reported that peroxidases are the most widely enzymes that are used for phenol polymerization. In addition, it has been reported that purified horseradish peroxidase (HRP) was used to remove 30 different aromatics amines and phenols (Cooper and Nicell, 1996). It has been investigated that soybean seed hulls are rich source of peroxide and they are cheap and provide renewable source of peroxide (Wilberg et al., 2002). In addition, radish roots can be used for phenols degradation from wastewater (Naghibi et al., 2003). The pathway of phenol degradation starts with catalyzing the oxidation of phenol using peroxide, then forming free radicals. These free radical then go under spontaneous polymerization.

Pradeep et al., (2012) investigated a process for phenol removal including polymerization of phenol in the presence of HRP. It was found that phenol conversion using this technique was more than 90%.

There are several advantages of using enzymes-based treatment such as the following (Duran and Esposito, 2000):

- Process control is simple and predictable.
- Operation under varies conditions of temperature, pH and salinity and over wide range of substrate concentrations.
- No acclimatization is required.
- Fast reaction.

However, although enzymes-based treatment seems to be promising technology, it has several disadvantages compared to other biological methods of treatment. These disadvantages including the following (Ruggaber et al., 2006):

- May lose their reactivity after interacting with pollutants and could become completely inactive, and it is difficult to maintain their concentrations.
- Not able to adapt themselves to conditions outside the allowed range.

1.3.2 Microalgae

1.3.2.1 Microalgae structure

Microalgae are simple micro-organisms that are mainly aquatic, unicellular and microscopic. There are two main algae population, phytoplankton and filamentous. Biologists have classified microalgae in several classes, which are distinguished by their life cycle, cellular structure, and pigmentation. The most important classes of microalgae are the diatoms (Bacillariophyceae), the golden algae (Chrysophyceae), the green algae (Chlorophyceae) and the blue green algae

(Cyanophyceae). Diatoms are considered as the main and dominant form in phytoplankton (Demirbas, 2010).

Microalgae lack the structures of the complex cell that are found in other higher plants. They can be grown in diverse environments, like freshwater, sea water and saline water. Most microalgae species are photoautotrophic by which they convert solar energy to chemical forms by photosynthesis (Slade and Bauen, 2013). Microalgae contain fatty acids and lipids as in membrane components, source of energy, metabolites and storage products. Table 1 represents the percentage of each component in different species of microalgae.

Table 1: Chemical composition of different species of microalgae

Microalgae species	Protein	Nucleic acid	Lipids	Carbohydrates
Anabaena cylindrica	43-56	-	4-7	25-30
Synechoccus sp.	63	5	11	15
Spirulina maxima	60-71	3-4.5	6-7	13-16
Spirulina platensis	46-63	2-5	4-9	8-14
Porphyridium cruentum	28-39	-	9-14	40-57
Tetraselmis maculata	52	-	3	15
Prymnesium parvum	28-45	1-2	22-28	25-33
Euglena gracilis	39-61	-	14-20	14-18
Dunalienlla salina	57	-	6	32
Dunalienlla bioculata	49	-	8	4
Spirogyu sp.	6-20	-	11-21	33-64
Chlorella pyrenoidosa	57	-	2	26
Chlorella vulgaris	51-58	4-5	14-22	12-17
Chamydomonas rheinhardii	48	-	21	17
Scenedesmus dimorphus	8-18	-	16-40	21-52
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus abliquus	50-56	3-6	12-14	10-17

Microalgae contain proteins, lipids, carbohydrates and nucleic acids in different proportions and these percentages vary according to the type of microalgae. Some microalgae strains contain fatty acids with percentages up to 40% of their overall

mass. Microalgae are fast growing photosynthesizing organisms. They are able to complete a growing cycle within few days.

It can be seen from Table 1 that lipids, are varied in a percentage between 2% and 40%. Some microalgae can produce up 50% of their weight of bio oil. The oil content in some species of microalgae is presented in Table 2 (Demirbas and Demirbas, 2011).

Table 2: Oil content of different microalgae strains

Microalgae species	Oil content (wt% of dry basis)
Tetraselmis sueica	15-23
Schizochytrium sp.	50-77
Phaeodactylum tricornutum	20-30
Nitzschia sp.	45-47
Neochloris oleoabundans	35-54
Nannochloropsis sp.	31-68
Nannochloris sp.	20-35
Monallanthus salina	>20
Isochrysis sp.	25-33
Dunaliella primolecta	23
Cylindeotheca sp.	16-37
Crypthecodinium cohnii	20
Chllrella sp.	28-32
Botryococcus braunii	25-75

Fatty acids come in two forms, which are saturated and unsaturated fatty acids. Saturated fats are derived from animal products such as dairy and meat. Most vegetables oils are considered unsaturated. The overall fuel properties are determined by the properties of individual fatty esters that forms biofuel. Generally, microalgae produce polyunsaturated fats, which cause a decreasing in stability of produced biofuel. However, polyunsaturated fatty acids have lower melting point compared to monounsaturated fatty acids and thus the produced fuel will have better properties in cold weather (Demirbas and Demirbas, 2011).

1.3.2.2 Microalgae significance and application

Microalgae are very promising source of biodiesel. The fatty acid and lipids percentages microalgae with different culture conditions. In some cases, and by stress factors such as imposition of nitrogen starvation, then the lipids content in microalgae can be enhanced. There is no one specific strain of microalgae that can be said to be the best one in terms of oil yield. However, green algae and diatoms were considered to be the most promising. The optimum temperature required for growing most species of microalgae is between 293 and 303 K. If the temperature of growth medium is out of the range, then this may cause the death or damage of the cells (Demirbas and Demirbas, 2011).

1.3.2.2.1 Biodiesel production using microalgae

Microalgae are considered as a potential source of renewable energy through producing biofuels. Thermochemical processes are suitable to produce bio-oil and gas, and biochemical processes are suitable to produce biodiesel, bio hydrogen and bio ethanol (Demirbas, 2010).

Using fossil fuel as a source of energy have shown several drawbacks, and thus it is necessary to enhance the development of alternative sources of energy that are renewable and effective. There were many attempts to obtain renewable energy source for biofuel production. One of these attempts is by using food crops (first generation) and forest residues (second generation) as a source of biofuel but the concern was raised over the food competition and the use of arable lands. The third-generation source of biofuel was derived from microalgae and this source was considered as a promising and viable alternative source of energy that can overcome the main

disadvantages of using the first and the second generation of biofuel (Pinedo et al., 2015).

Biodiesel can be defined as the mono-alkyl esters of animals' fats for vegetable oils. It can be produced by transesterification of the fats or the parent oil in order to achieve a viscosity that is close to petroleum diesel viscosity. The chemical reaction of converting oil to biodiesel (fatty esters) is called transesterification reaction, shown in the following equation:

$$\text{Triglyceride} + 3\text{Methanol} \overset{catalyst}{\Longleftrightarrow} \text{Glycerine} + 3 \text{ methyl esters} \quad (1)$$

In many cases, an excess amount of methanol is used in order to force the reaction to the right side of the chemical equation, then the remaining amount of methanol in recovered in order to reuse it. The heating value of biodiesel derived from microalgae is about 41 MJ/kg compared to the heating value of petroleum diesel which is 42.7 MJ/kg (Demirbas and Demirbas, 2011).

The production of biodiesel, which is based on using microalgae as a feedstock, is considered as an attractive alternative of biofuel. Using of microalgae shows several benefits such as it is renewable feedstock, has high growth rate, and it can survive in harsh environments. Microalgae with their lipids can provide several different options of producing biofuels. An example of that is the production of bioethanol by fermentation, biodiesel by transesterification, and methane by anaerobic digestion. Chisti (2008) stated that renewable sources such as microalgae are needed in order to replace petroleum fuels by biofuel and the biodiesel that is produced from microalgae is the only one that has this potential (Pinedo et al., 2015). The yearly productivity and oil content of algae is greater than other crops. It can be seen from Table 3 that

microalgae can yield 100,000 liters of oil per hectare, which is more than 200 times of the amount of oil that can be produced by soybean (Demirbas and Demirbas, 2011).

Table 3: Comparison in oil yield in microalgae and other crops

Plant source	Oil yield (L oil/ha year)	Seed oil content (% oil by wt in biomass)	Land use (m^2 year/kg biodiesel)	Biodiesel productivity (kg biodiesel/ha year)
Corn/Maize	172	44	66	152
Hemp	363	33	31	321
Soybean	636	18	18	562
Jatropha	741	28	15	656
Camelina	915	42	12	809
Canola/Rapeseed	974	41	12	862
Sunflower	1070	40	11	946
Castor	1307	48	9	1156
Palm oil	5366	36	2	4747
Microalgae (low oil content)	58700	30	0.2	51927
Microalgae (medium oil content)	97800	50	0.1	86515
Microalgae (high oil content)	136900	70	0.1	121104

According to Table 3, the oil contents are similar between microalgae and seed plants, but there are significant differences in the overall biomass productivity and thus in the term of land use, it can be noticed that microalgae are clearly advantageous.

The type of microalgae that are used usually in biodiesel production are aquatic unicellular green algae. This type of microalgae is a photosynthetic eukaryote that are characterized by its high growth rates and also high population densities. Under suitable conditions, it can double its biomass in less than 24 h. It can contain huge lipid contents, which is over 50% (Demirbas and Demirbas, 2011).

There are three well-known processes of extracting oil from microalgae, which are solvent extraction with hexane, press/expeller and supercritical fluid extraction.

The simplest process is to use a press in order to extract a large percentage of oil (more than 70%). Oil can be extracted from microalgae using chemical solvents and the most popular chemical used in this process is hexane due to its cheap cost. Using supercritical fluid extraction is more efficient method. It is a selective process, so it can provide the product with high purity and extract much higher percentage of oil than other processes. In this process, carbon dioxide is liquefied under pressure and then heated to a point, where it has the properties of both, gas and liquid. Then it can act as a solvent for oil extraction process. After extracting process, the remaining biomass can be used as a source of high protein, which gives the process a further value and also reduce the remaining wastes (Demirbas and Demirbas, 2011). The cost of extracting biofuels from microalgae is considered higher than the cost of petroleum diesel production due to the process technology used in extracting biofuels (Pinedo et al., 2015).

Table 4 shows the advantages and the disadvantage of biodiesel that is derived from algal oil.

Table 4: The advantages and disadvantage of biodiesel derived from algal oil

Advantages	Disadvantages
- Microalgae have high growth rates and can yield much higher amount of oil compared to other crops. - Algal biofuel is not toxic, highly bio-degradable and contains no sulfur. - Using algal biodiesel that contains high levels of polyunsaturates is suitable for cold weather	- May lead to unstable biodiesel due to the high percentage of polyunsaturates - Relatively new technology - High overall cost

1.3.2.2.2 Other important applications

Another important application of using microalgae is in the industry of Human nutrition (Yamaguchi, 1997). Human nutrition derived from Microalgae are nowadays marketed in different forms such as tablets, liquids, pastas, gums, snack foods and capsules (Liang et al., 2004). Table 5 represents the compositions of different human food (Becker, 2004).

Table 5: The composition of different human food

Commodity	Lipid	Carbohydrate	Protein
Bakers' yeast	1	38	39
Meat	34	1	43
Milk	28	38	26
Rice	2	77	8
Soybean	20	30	37

Due to their diverse chemical properties, microalgae can act as a nutritional supplement or as a source of natural food (Apt and Behrens, 1999). The commercial applications of microalgae for human nutrition are dominated by Arthrosporic, *Chlorella* sp, D. saline and Aphanizomenon flos-aquae. and Arthrospira strains, which are frequently used for human nutrition due to their high nutritive value and high protein content (Rangel et al., 2004). In addition, it has various possible health-promoting effects such as the alleviation of hyperlipidemia, protection against renal failure and suppression of hypertension (Vilchez et al., 1997). The largest plant for Arthrosporic production is owned by Earthrise Farms and located in Calipatria, USA. Their Arthrosporic-based products are distributed in over 20 countries. More than 50% of the overall world production of Arthrosporic is used as feed supplement. (Yamaguchi, 1997). In addition, Chlorella sp. was also used as food additive and for human nutrition. The most important substance appears in Chlorella sp. is β-1,3-glucan, which is a free radical scavenger and a reducer of blood lipids (Spolaore et al.,

2006). Various health-promoting effects have been clarified by Chlorella sp., such as efficacy on gastric ulcers, and prevention against atherosclerosis (Gouveia, 1996). D. salina is used due to its β-carotene content, which can reach 14% of its dry weight. It offers Dunaliella powder as functional food and an ingredient of dietary supplements (Metting, 1996). Finally, according to many research studies, A. flosaquae is used alone or in combination with other nutraceuticals and natural food products. It promotes human nutrient and good overall health (Benedetti et al., 2004). In addition to human nutrient, microalgae are one of the major sources of animal nutrition. In reality, around 30% of the world algal production is sold for animal feed applications (Becker, 2004). β-carotene, astaxanthin, lutein, lycopene and bixin are the main component derived from microalgae for Carotenoids. The most important uses of these component are as natural food colorants, as additive for animal feed and they have some applications in cosmetics (DelCampo et al., 2000). Some carotenoids can act as provitamin A and be converted into vitamin A (García et al., 2005). Microalgal carotenoids are in competition with the synthetic pigments. Although the synthetic pigments are cheaper than the microalgal carotenoids, but microalgal carotenoids sully natural isomers in their natural ratio. Many studies showed that the natural isomer of β-carotene is much better than the synthetic forms (Guerin et al., 2006). It was found that the green halophilic flagellate D. salina is the most suitable strain for the big production of β-carotene because it can produce β-carotene up to 14% of its dry weight (Metting, 1996). It can be cultivated in open ponds under extreme conditions which it grows in such as low availability of nitrogen, hypersaline, and high levels of solar radiation (Leon et al., 2003).

1.3.2.3 Microalgae production

There are three popular methods for photoautotrophic algae cultivation, which are raceway pond systems, closed pond systems and photo bioreactors (PBRs). The conventional raceway pond (Figure 3) consists of a closed loop oval channel, with around 0.25 to 0.4 m depth. The channel is opened to the air. There is also a paddle wheel, which is used to circulate that water in order to prevent sedimentation and keep the microalgae cells circulation. Raceway pond systems are cheaper compared to other cultivation methods but there is a need for large areas in order to establish these systems. On the other hand, in the culture medium in PBRs is enclosed with an array of tubes where the microalgae move in a circulated path from a central reservoir. PBRs include better controlling system for the algae environment but the cost of PBRs is much higher than raceway pond and there is a demand of more energy (Slade and Bauen, 2013). Closed system (Figure 4) is a cultivation system, which is more expensive than open system and shows several significant operating challenges such as it cannot be scaled-up beyond a hundred square meters for an individual unit, and that causes gas exchange limitation (Demirbas, 2010).

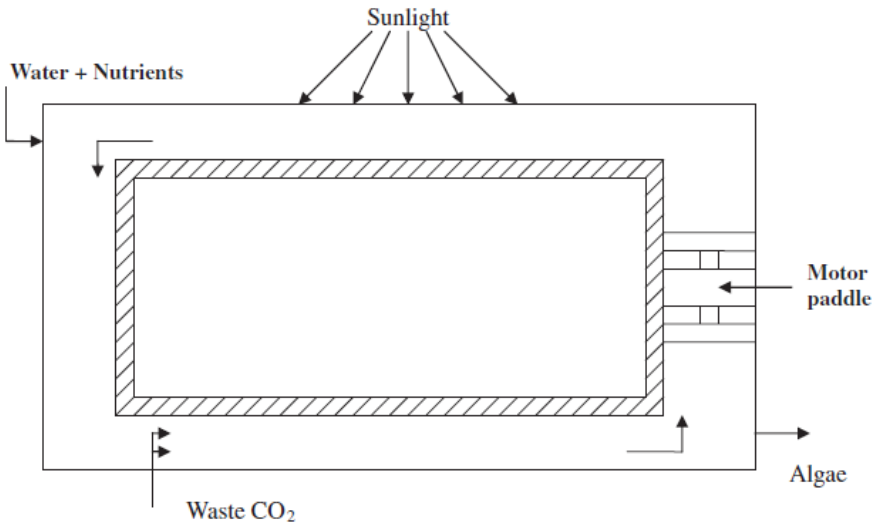

Figure 3: Open system of microalgae cultivation

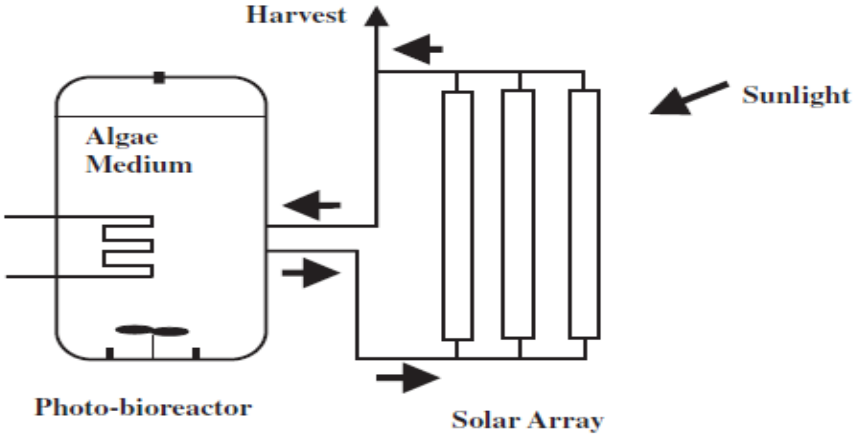

Figure 4: Close system of microalgae cultivation

Furthermore, both cultivation systems are usually compared in terms of energy or what is called the net energy ratio (NER) which can be defined as the summation of all required energies for cultivation, harvesting and drying, dived by the energy content value of the dried biomass. Most of raceway systems have a value of NER less than 1, which means that these processes produce an amount of energies more than what they consumed, whereas the NER values for PBRs are greater than 1. Several experiments showed that the best performance of PBR can be achieved using flat-plate system, due to the benefit of using a large surface area and the low amount of oxygen that build-up (Slade and Bauen, 2013). Table 6 shows a comparison between the previous three methods.

Table 6: A comparison between microalgae harvesting methods

The raceway pond (open bond)	Photobioreactor	Closed system
Opened to the air Cheaper compared to other cultivation methods There is a need for large areas for establishment	Includes better controlling system for the algae environment Much higher cost than raceway pond Energy intensive	It is more expensive than open system Cannot be scaled-up beyond a hundred square meters for each individual unit of growth. Gas exchange limitation

Cultivation process of algae biomass has four important advantages compared to other sources. First, algae biomass can be produced at high volumes and it yields much higher percentage of oil. Second, it can be cultivated on different environment (fresh water, sea water, wastewater and marginal land). Third, the produced algae oil has limited market competition. Fourth, innovations allow algae production to become more productive and able to be grown on resources that are considered as waste (Demirbas and Demirbas, 2011).

The growth rate of microalgae is affected by chemical, physical and biological factors, for example, chemical factors include the availability of carbon dioxide and nutrients, physical factors include temperature and light, and biological factors include virus infections and grazing by animals. In addition to that, there is operational factors that affect chemical, physical and biological factors. They basically concern the design of the bioreactor (Larsdotter, 2006). Water, carbon dioxide and the necessary nutrients for microalgae should be provided in a controlled way. Sunlight is received by algae either directly as in open ponds, or through light fibers or tubes that are connected to sunlight collectors (Demirbas, 2010).

Light is one of the main requirements for algal biomass production especially in open pond systems, whereas closed systems require much less light than open ponds for algae growth. In the absence of nutrient limitation, photosynthesis in microalgae increases as the light intensity increases until the maximum growth rate of algae achieved (Macedo et al., 2002). After that, as the light intensity increases, then it could damage the light receptors in the algae and may lead to photo oxidation and that results in decreasing the rate of productivity in the algae (Richmond, 2004).

As mentioned before, the light energy is converted to chemical energy during the photosynthesis process in the microalgae. It was investigated by Oswald (1988)

that in outdoor ponds, more than 90 % of the total incident solar energy can be converted into heat while less than 10 % into chemical energy.

There are several methods used by microalgae to remain near the water surface in a way to get more light, decrease the specific gravity and minimize the sinking rate. These methods include selective accumulation of ions and fat accumulation (Fogg, 1975). In the dense cultures, the light availability may be decreased by algae due to the internal shading (Wood et al., 1999). In wastewater, the internal shading might be aggravated due to the high particulate matter contents. In order to avoid that, it is essential to have turbulence so all cells can be exposed to the light. Turbulence can be achieved using different ways, for example, by paddle wheels or air bubbling (Fontes et al., 1987). In addition, decreasing the depth of culture vessel can prevent the issue of light limitation (Borowitzka, 1998). However, even though light is one of the most important factors that affects the growth of microalgae, too much light may cause decreasing in the efficiency of photosynthesis and thus in the algal growth. This phenomenon is known as photo inhibition (Oliver and Ganf, 2000).

The rate of taking a specific nutrient by microalgae depends on the diffusion rate through the cell wall, the thickness of unstirred layer of water outside the cell and the difference between the inside and outside cell concentration. Thicker unstirred layer of water cause decreasing in the diffusion rates and in order to avoid that, turbulence is required (Borowitzka, 1998).

Water containing the required minerals and salts is another important requirement for algae production. Wastewater may already include high nutrient percentage specially nitrogen and phosphate salts. This may allow to produce algae production cheaply while treating wastewater simultaneously. An alternative source is saltwater that can be obtained from sea water or saline aquifer and thus the cost may

be cheaper. Each species of microalgae require specific source or type of water for better growth and nutrients must be supplied in most cases. The produced microalgal biomass contains around 50% of carbon from its dry weight and this amount is derived from carbon dioxide. As a result of that, for each 100 ton of produced algal biomass, then around 183 ton of carbon dioxide will be fixed (Demirbas, 2010). The organic carbon sources can be obtained by microalgae either chemo heterotrophically or photo heterotrophically (Borowitzka, 1998). In the case of chemo heterotrophically, the carbon source and the energy source can be obtained by the organic substrate. While in the photo heterotrophically the light act as energy source. In some strains of microalgae, such as the green Chlorella sp. and Scenedesmus sp., the carbon nutrition mode can be converted from autotrophy to heterotrophy when the source of carbon changed (Becker, 1994).

One of the important factors that affects the cost of microalgae production is the cost of water sully. Fresh water is needed to be added to raceway pond in order to compensate for the evaporation and it may be used in PBR for cooling purposes. It was suggested to use brackish water and seawater for algae cultivation. Using of brackish water will add more cost for pretreatment in order to remove inhibiting components, while it needs more energy. It was also suggested to use re-circulating water in order to reduce the consumption, but this may raise the risk of having more bacteria, viruses, and fungi that may affect or even destroy the algae cells, so the pretreatment is also needed in this case (Slade and Bauen, 2013).

Nitrogen is an important factor that affect regulating the lipid content in the algae cell (Brennan and Owende, 2010). It comprises more than 10 % of the biomass (Becker, 1994). It may exist in many forms, such as ammonium, nitrate, urea and nitrite (Oliver and Ganf, 2000). The existence of ammonium with concentrations

higher than 20 mg/L is not recommended due to ammonia toxicity (Borowitzka, 1998). When the nitrogen becomes the limiting factor of growth, then the liquid levels accumulated to more than 40% which reduced the algal growth (Tillett, 1988). Several strains of microalgae can take up nitrogen in excess of the immediate metabolic needs and then can use it later in case of nitrogen starvation (Larsdotter, 2006). In addition, the photosynthesis might be inhibited in case of presence of ammonia at high concentration at high pH value (Ogbonna et al., 2000). Furthermore, high pH values may lead to formation of calcium phosphates which causes precipitation of phosphate in the medium (Chevalier et al., 2000).

Phosphorus is one of the essential macro-nutrients for growth. It can be taken up by algae as inorganic orthophosphate. Orthophosphates can be provided to the microalgae by converting phosphates by phosphatases at the algal cell surface. Microalgae are able to store phosphorous within the algal cells in the form of polyphosphate and it can be used later in the absence of phosphorus for prolonged growth (Fogg, 1975) There are other macronutrients, such as Sulphur, calcium, potassium and magnesium and micronutrients, which are required by smaller quantities, such as manganese, iron, zinc, chloride, molybdenum, copper and nickel (Oliver and Ganf, 2000).

The rate of algal productivity increases as temperature increases until reaching the optimum temperature and then increasing the temperature above the optimum temperature cause reducing in the overall productivity by increasing the photorespiration (Pulz, 2001). The optimal temperature can be measured and assigned under the conditions, which provide the maximum growth rate of algae. These conditions include sufficient light and nutrients. The optimum temperature was measured for varies types of algae and it was found that it is often between 28 and

35°C (Soeder et al., 1985). The optimum temperature may have different values in case light conditions or nutrient or both are limiting (Harris, 1978). Further increase in the culture temperature leads to a rapid evaporation rate and thus decline in growth rate (Soeder, 1981). Using greenhouses is one of the suggested solutions to prevent high temperature in algal cultures (Borowitzka, 1998).

The pH value of the pond water could affect the algal growth and metabolism and the processes related to them such as the availability of nutrient ions and carbon dioxide for photosynthesis (Park and Craggs, 2010). It was found that the optimal pH value of many freshwater algae strains is around 8 (Kong et al., 2010). A pH value above the optimum value decreases the algal productivity, for example, the productivity of Chlorella sp. decreases by 22% when the pH value was increased above the optimum value which is 8 (Weissman and Goebel, 1988). The inorganic carbon that is used for the algal growth medium varies with pH value, for example, the addition of carbon dioxide results in decreasing in pH value as shown in figure 5 (Borowitzka, 1998).

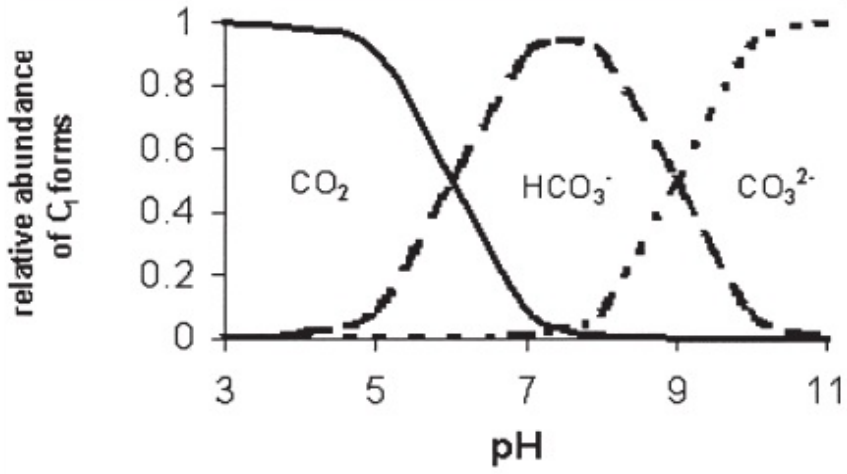

Figure 5: Effect of changing pH on the relative abundance of carbon forms

At higher pH values, more than 9, most of the inorganic carbon is in form of carbonate which cannot be assimilated by the algae (Borowitzka, 1998). High levels of dissolved

oxygen in excess levels of normal air saturation impacts the productivity of the algae, for example, in an experiment, the photosynthetic activity was reduced by 25% at 300% saturated dissolved oxygen. (Molina et al., 2001).

Some algal species have the ability of heterotrophic growth on organic sources of carbon (Neilson and Lewin, 1974). In this case, the algae cells can be encouraged to grow in the pollutants and the biodegradation of the organic pollutants can be investigated. Walker and others performed experiments using the achlorophyllous alga Prototheca zop¢i. This strain found to be able to degrade hydrocarbons that are in motor oils and crude oil. In the crude oil, it was found that 12 to 41% of the aromatic compounds and 38 to 60% of the saturated hydrocarbons were degraded. On the other hand, 10 to 26% of the aromatic compounds and 10 to 23% of the saturated hydrocarbons were degraded. These results concluded that the used algae strain is able to degrade different types of oil with varying percentages (Walker et al., 1975).

1.3.2.3 Microalgae harvesting

Harvesting process of algae form the tanks and then separating them form oil is an energy intensive and difficult process, due to the small size of microalgae (3 -30 µm in diameter). The conventional processes for algae harvesting include foal fractionation, ultrasonic separation, flocculation, and concentration through centrifugation and the harvesting process depends on several factors such as cell density, culture conditions, and the species itself. The harvesting cost contributes about 20-30% of the total cost of the production of algal biomass. Harvesting processes using chemical such as flocculation by ferric chloride and alum as flocculants are much more expensive for large scale of production (Demirbas, 2010).

At some stages of the lifecycle of several species of microalgae, they may produce toxins. The effects of producing toxins by microalgae can range from severe effects that may cause death, to chronic effects that can cause tissue changes over a period of time. The production of toxins may depend on the environmental conditions and thus the presence of toxins is difficult to be predicted. Algal toxins are considered, in some cases, as valuable products especially in toxicological and biomedical researches (Slade and Bauen, 2013)

1.3.2.4 Microalgae for phenol removal

Several studies were performed to degrade phenol using different strains of microalgae at specific conditions. For example, it has been proved that *Ankistrodesmus braunii* and *Scenedesmus quadricauda* have good capabilities to remove around 70% of phenol (Tikoo et al., 1997). The high concentration of phenol used in the medium however, affected some algal cells by increasing their sizes, increasing the oil droplets in the cytoplasm and causing anomalous shapes of the cells. Many strains of microalgae able to combine both heterotrophic and photo autotrophic conditions and they are called mixotrophs. In case of using microalgae that are sensitive to phenols, then the microalgae that are under mixotrophs conditions improve their capability to mineralize phenols or the toxicity is reversed upon photoheterotrophic growth of the culture by microalgae (Megharaj et al., 1992, Tikoo et al., 1997). This ability enables microalgae to live and grow under low conditions that are related to limitation of light or carbon dioxide (Pinto et al., 2002).

Generally, different strains of microalgae show different capabilities to remove phenol. Ochromonas sp. was found to be incapable of removing phenol from wastewater. Phenol was also found to be oxidized to catechol using different isolated

algae strains, such as *Volvox aureus*, *Nostoc Linckia* and *Oscillatoria rubescens* (El-Sheekh et al., 2012). However, the use of isolated strains is not practical, if the microalgae is to be used for the treatment of industrial wastewater.

In some studies, it was found that *Ochromonas Danica* can grow heterotrophically on phenol medium as a source of carbon, but this was limited to a concentration up to 4 mM. Its addition to that, the removal of phenol with no accumulating metabolites was accomplished using dissolved organic carbon. In some recent studied, it was found that three species of Chlorella sp. can degrade Pentachlorophenol and the mineralization of the chlorophenol was enhanced by glucose addition. The mechanisms and kinetics of phenol degradation and microalgae growth in phenolic medium were not studied (Semple et al., 1999)

Several strains of microalgae such as Chlorella sp., and Scenedesmus obliquus sp. were tested for phenols degradation. Phenols were dissolved in a medium that has a pH of 7 to 7.2, but no carbon source were added. It was found that many phenolic compounds can be degraded easily and converted to another isomer, such as converting 2,4-dimethylphenol to dimethyl benzenediol isomer using Chlorella sp. The rate of phenol degradation depends on the used biomass and the concentration of toxin. Complete degradation was achieved for some phenolic compound such as 2,4-dinitrophenol with a concentration of 190 mg/L and that was achieved *Scenedesmus* sp. where the adaptation period for this strain was 5 days. The degradation of other phenolic compounds can be achieved by the microalgae strains that show ability to survive at a higher concentration of toxin. The removal of phenols was accomplished by different strains of microalgae with light and dark and each strain show a different capability in these conditions. Figure (6) represents the effect of light and dark on

phenol degradation using Chlorella sp. It was found that phenol can be degraded easily by the previous mentioned strains of microalgae because illumination and presence of living cell which is necessary for phenol degradation process. As shown in figure 6, phenol was not degraded in the dark by using living cells of Chlorella sp. (Klekner and Kosaric, 1992).

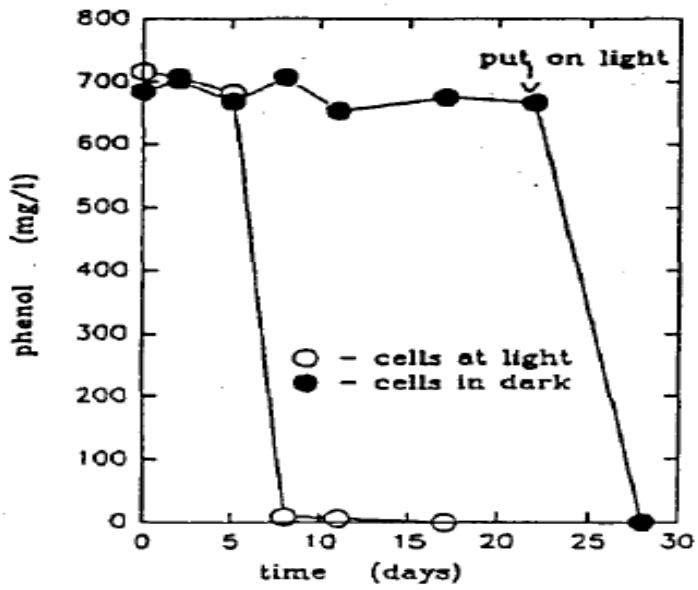

Figure 6: Phenols degradation by *Chlorella sp (*3.4 g/L) at light and in dark

Removal of phenol from wastewater was performed in laboratory scales by *Chlorella* sp. The resulted microalgae strain was obtained after about 95 days and the concentration of phenol was 500 mg/L. It was reported that the used microalgae strain could grow under 500 mg/L and 700 mg/L without major inhibition. Phenol with concentration of 500 mg/L was fully removed from wastewater using *Chlorella* sp.in 7 days and the initial cell density was 0.6 g/L. When the initial concentration of phenol was 400 mg/L, then 44% of growth inhibition for *Chlorella* VT-1 sp. were reported and around 100% of growth inhibition for C. *vulgaris* sp. were reported. It was suggested that high initial concentration of microalgae maybe helpful in order to allow

microalgae to toleration phenol. For example, Klekner and Kosaric (1992) reported that phenol with a concentration in a range of 700 mg/L to 810 mg/L was fully degraded using *Chlorella* sp. in less than 7 days and the initial cell density was between 3.4 g/L to 6.3 g/L which was considered as a high density and it causes that much nutrients were needed and the operating cost was high. Phenol with a high concentration may be degraded using low initial cell density if case of using a powerful strain of microalgae (Wang et al., 2016).

Jacobson and Alexander investigated another observation using non-axenic cultures of *Chlamydomonas*, where it was grown acetate in light and in the dark. It was found that the produced metabolite was 2-hydroxymuconic semialdehyde by dehalogenation 4-chloro-3,5-dinitrobenzoic acid. In addition, they found that bacteria were unable to do that (Jacobson and Alexander, 1981).

Another interesting observation was investigated by Jinqi and Houtian using *Chlorella vulgaris* sp. and C. *pyrenoidosa* sp. in azo dyes degradation. They found that specific types of dyes such as blackT and Eriochrome might be decolorized during the process of degradation and also, they are used as a source of carbon and nitrogen, but this depends on the chemical structure of the dye (Jinqi and Houtian, 1992). The use of microalgae in degradation of phenols has several advantages, for example, microalgae are renewable and abundant source of energy, the produced biomass is used to produce valuable products, the utilization is cheap (Oswald, 2003) and the growth process is easy (Tikoo et al., 1997).

1.3.3 Catalytic reaction of biodiesel production

The types of catalysts that can be used in the transesterification reaction to produce biodiesel are base catalysts, acid catalysts, and enzymes (Robles et al., 2009).

Base catalysis is fast, but it is limited by the content of the free fatty acids. Using of some kinds of base catalyst such as metallic potassium makes it dangerous to handle the reaction. However, using of metal alkoxides in methanol are better than using metal hydroxides as base catalysts. Small concentrations of Alkaline metal alkoxides are considered to be highly active and for short reaction time they can provide high yields up to 98%, however, presence of water will inhibit these catalysts from acting well (Schuchardt et al., 1998). Acid catalysts, such as sulfuric acid and hydrochloric acid, can be used in combination with base catalysts for high fatty acid that contains low-cost feedstock such as waste oil and that can be performed in two stages in which the free fatty acids are converted to methylester using acid catalyst in the first stage, then the left over triglycerides are converted to methyl esters using base catalysts in the second stage (Robles et al., 2009). Transesterification process based on using chemical catalysts is energy intensive and requires separation process of the catalysts from the product. The produced Alkaline water, in case of using base catalysts, needs remediation. The presence of free fatty acids and water ends with losing of the product due to saponification (Rawat et al., 2011). Another type of catalyst was introduced to be used in transesterification, which is enzymes. Enzymatic technology is implemented on industrial scale, for example, it is used in China with a capacity of 20,000 ton/year. The reaction rate when using enzymatic catalysts is lower than the one when using base catalysts. Table 7 provides a summary of the advantages and disadvantages of using chemical or enzymatic catalysis.

Table 7: Advantages and disadvantages of different types of transesterification

Transesterification type	Advantages	Disadvantages
Chemical catalysis	Large production scale High conversion of the production Produced methanol can be recycled Cheap cost of the process	High reaction temperature Energy intensive Need for methanol recycle installation
Enzymatic catalysis	Moderate conditions of the reaction Small amount of methanol is needed for the reaction	Chemicals exist in the reaction are poisonous to the enzyme Exist of wastewater that pollutes the environment
Supercritical fluid	Fast and safe Easy to control Environment-friendly	High production cost due to high pressure and high temperature needed Energy intensive

Table 8 provide a comparison between base catalysis, acid catalysis and enzymatic catalysis according to different parameters which are scale of application, rate of reaction, effect of alcohol, effect of free fatty acid content and effect on the environment (Robles et al., 2009; Rawat et al., 2011; Demirbas, 2009).

Table 8: Comparison between different catalysis types

Parameter	Base catalysis	Acid catalysis	Enzymatic catalysis
Scale of application	Widely used	Rarely used in industry because they are corrosive	May not be economically viable in large scale due to the high cost of enzyme production
Rate of reaction	Faster reaction	Slow reaction that needs to increase the pressure and temperature to enhance it	Slower than base catalysis
Effect of alcohol	The forward reaction rate increases with the addition of alcohol	Similar to base catalyst	High amount of methanol might deactivate large proportion of lipase

Table 8: Comparison between different catalysis types (cont'd)

Parameter	Base catalysis	Acid catalysis	Enzymatic catalysis
Effect of free fatty acid content	It is limited by FFA content. Oil feed should contain less than 2.0% FFA	Suitable with oils that contain high FFA contents.	Can be used with oils of high FFA contents
Effect on the environment	Not environmentally friendly since the treatment process of effluents consumes a lot of water	Similar to base catalysis	Separation and purification processes of biodiesel is easier than base catalysis

Base catalysis and acid catalysis transesterification are more preferable and most commonly used for biodiesel production since they are the more cost-effective processes compared to enzymatic catalysis (Zabeti et al., 2009). However, homogeneous catalysts cannot be regenerated or used, and this is one of their main disadvantages. In addition, the separation of homogenous catalysts from the final product is different and it requires more equipment which results in higher cost. Moreover, large amount of wastewater is produced after the process which needs more purification steps (Vicente et al., 2007). Furthermore, the biodiesel which is produced using alkali-catalyzed transesterification process should be separated and purified in order to fulfill the standard specifications of biodiesel (Stojkovic et al., 2014).

Promising technologies to overcome problems that are related to using homogenous catalysts in transesterification are based on using heterogeneous catalysts. Heterogeneous solid catalysts can be either basic or acidic. Basic catalysts include dolomites, hydroxides, neat metals, supported alkaline, hydrotalcites and others. Acidic catalysts include heteropoly acids, sulfonic ion-exchange resin, zeolites, sulfated metal oxides and others. Heterogeneous solid catalysts are environmentally friendly, can be reused with or without the need for regeneration, does not require

water washing, and can be used in continuous processes (Refaat, 2011). In addition to that, glycerol can be directly produced with high levels of purity with to be at least 98% (Melero et al., 2009). On the other hand, heterogeneous catalysis transesterification requires severe operating conditions including high pressure, high temperature and high molar ratio of alcohol-to-oil (6:1 to 40:1). The performance of heterogeneous catalysts is lower than the homogenous catalysts in transesterification. They might be deactivated over time due to poisoning and leaching (Refaat, 2011). Among heterogeneous alkaline catalysts, calcium oxide-based catalysts have been the most frequently used in transesterification studies (Boey et al., 2011). Calcium oxide is usually produced by thermal decomposition of some minerals such as calcite, limestone, or from other natural resources, such as eggshells, that contain calcium carbonate. The calcination temperature of the raw materials to produce calcium oxide depends on the type of these materials. For example, the calcination temperature of calcium hydroxide is between 693 and 923 K, however, the calcination temperature is over 1000 K for Calcite (Bilton et al., 2012). A study of transesterification of oil using a heterogeneous catalyst, namely CaO, was performed, and a conversion of 95% was achieved using methanol to oil ratio of 12:1 and 8% of catalyst based on the mass of oil and (Liu et al., 2008). Another study was performed using the same previous conditions and achieved 93% (Viola et al., 2012).

1.3.3.1 Kinetics of biodiesel reaction

Different models and kinetics of transesterification reaction have been studied using different types of catalysts. Table 9 summarized different kinetic models of transesterification reaction.

Table 9: Different kinetic models of transesterification reaction.

Model	Reaction order
One step, non-catalytic reversible reaction	First order
One step reversible reaction with different base catalysts	First order or third order according to the type of the catalyst
Three steps non-catalytic irreversible reaction	First order
Three steps reversible reaction using alkaline as catalyst	Second order

Different models and kinetics of transesterification reaction have been studied using different types of catalysts. In a non-catalytic system, the reaction was assumed to take place in a reversible one step, resulting in a first order model. Using different types of base catalysts and also assuming a reversible one step reaction, first or third order reaction was used depending on the type of catalyst. Dividing the reaction into three steps, with alkaline catalyst, resulted in a second order kinetics.

Marinković et al., (2018) also assumed a one-step reaction, to describe the transesterification in a batch and continuous systems. Although methoanolysis occur via three consecutive reactions, where FAME, glycerol, DAG and MAG are formed, the concentrations of MAG and DAG was assumed not exceed 1.5%. A homogeneous catalyst was used and hence, internal diffusion was totally eliminated. The model assumed a zero order for external TAG mass transfer limitation, which affected the overall rate of reaction in the initial stage, but as the concentration of TAG decreased with the reaction progress, the reaction order changed to a first order. The variations of FAME and TAG concentration with time would then be sigmoidal. By assuming an ideal mixing in the batch stirred reactors (BSR), a kinetics model in Eq (2) was presented as follow:

$$-r_A = -\frac{dc_A}{dt} = k_{app}\frac{c_A}{K+c_A}.(c_R^o + c_R) \quad (2)$$

Where, C_A is TAG concentration, C_R is FAME concentration calculated as $3(C_{Ao}-C_A)$, K and C_{Ro} are model parameters and k_{app} is the apparent rate constant of the reaction. The kinetic parameters were determined by fitting results from a batch reaction, as shown in Table 10 (Marinković et al., 2018).

Table 10: Parameters of the kinetic model of batch transesterification

Methanol:oil molar ratio	C_{Bo} (mol/L) *	C_{cat} (mol/L)	K (mol/L)	K_{app} (min^{-1})	C_R^o (mol/L)
7:1	0.063	5.69	0.200	0.0040	0.300
12:1	0.054	8.37	0.300	0.0049	0.271
17:1	0.048	10.39	0.308	0.0057	0.150

* 0.5% of the mass of oil

As shown in Table 10, the increase in catalyst concentration C_{cat} and initial methanol concentration C_{Bo} resulted in increasing in the value of K and k_{app} and decreasing in the value of C_{Ro}.

A kinetics model that takes both reactants and enzyme catalyst into consideration was proposed by Fabiano et al., (2012), as shown in Eqs (3) and (4)

$$\frac{dC_A}{dt} = -v_A\rho_0 C_{cat} k C_A C_B \quad (3)$$

$$\frac{dC_B}{dt} = -v_B\rho_0 C_{cat} k C_A C_B \quad (4)$$

Where, C_A and C_B are oil and alcohol molar concentrations, respectively, ρ_0 is the initial mass concentration of oil, C_{cat} is the catalyst concentration for oil unit mass, k is reaction rate constant, v_A and v_B are reactants coefficient of oil and alcohol, respectively. The term d was defined as follow:

$$\frac{C_A}{v_A} - \frac{C_B}{v_B} = \frac{C_{A0}}{v_A} - \frac{C_{B0}}{v_B} = d \quad (5)$$

Where, d is a constant that its sign indicates either lack or excess of reagents at the start of the reaction. When methanol is in excess, the sign of d would be negative (d <

0). By combining Eqs (3-5), a single expression in the concentration of oil can be written as given in Eq (6):

$$\frac{dC_A}{dt} = -v_A \rho_0 C_{cat} k C_A [v_B \left(\frac{C_A}{v_A} - d\right)] \quad (6)$$

This model was tested on the transesterification of waste cooking using different catalysts. Rate constants of sodium methylate and potassium hydroxide were reported to be 0.301 and 0.235 $\frac{m^3}{mol.s}$, respectively, which showed the superiority of the former catalyst.

Another kinetic model assumed transesterification reaction to be a second order reaction, as given in Eq (7):

$$-r_A = -\frac{dC_A}{dt} = k_1 C_A C_B - k_2 C_c C_D \quad (7)$$

Where, C_i is the concentration, and the subscripts A, B, C and D are for oil, methanol, methyl ester and glycerol, and k_1 and k_2 are the rate constants of the backward reaction. The reaction model can be expressed in terms of the conversion, as shown in Eq (8):

$$\frac{dx_A}{dt} = k_1 C_{A0} [(1 - x_A) \left(\frac{C_{B0}}{C_{A0}} - 3x_A\right) - \frac{3k_2}{k_1} x_A^2] \quad (8)$$

By defining M and K to be $\frac{C_{B0}}{C_{A0}}$ and $\frac{k_2}{k_1}$, respectively and integrating Eq (7), Eq (8) can be derived:

$$\frac{1}{2C_{A0}\sqrt{\Delta}} \ln \frac{6(1-K)x_A - M + 3 - \sqrt{\Delta}}{6(1-K)x_A - M + 3 + \sqrt{\Delta}} = K_t t + C \quad (9)$$

Where, K and Δ are equilibrium constants, expressed by Eqs (10) and (11)

$$K = \frac{(1-x_{Ae})(M-3x_{Ae})}{3x_{Ae}^2} \quad (10)$$

$$\Delta = (M+3)^2 - 12M(1-K) \quad (11)$$

Where, x_{Ae} is equilibrium degree of TG conversion and M is initial molar ratio of TG to methanol.

The rate constant is related to the temperature of the reaction by Arrhenius equation Eq (12):

$$\ln k = -\frac{E_a}{RT} + C \quad (12)$$

Where, E_a is the calcination energy, T is Absolute temperature, C is a constant and R is gas constant. Other thermodynamic parameters, such as heat of reaction, ΔH, and change of entropy of reaction, ΔS, Boltzmann constant, k_b and Planck constant, h. are related using Eyring-Polanyi equation (Eq 13):

$$\ln \frac{k}{T} = -\frac{\Delta H}{RT} + \ln \frac{k_b}{h} + \frac{\Delta S}{R} \quad (13)$$

The change of Gibbs free energy of reaction is related to ΔH and ΔS by Eq (14) (Yahida et al., 2018):

$$\Delta G = \Delta H - T\Delta S \quad (14)$$

1.4 Research Hypothesis

Microalgae, both marine and freshwater, are used to remove different phenols at different concentration levels from wastewater. This proves that microalgae can be used to treat industrial wastewater, contaminated with the tested phenols, whether it is in fresh or saline water bodies. The removal rates of the contaminated and the microalgae growth rates are determined and used to develop a kinetics model to describe the system, which can be used for scale-up and design of a treatment unit. In addition to the wastewater treatment, oils are extracted from harvested microalgae cells and used to produce biodiesel using heterogeneous alkaline catalyst.

The use of microalgae is proposed to remove different phenols from wastewater with a cheaper cost compared to conventional methods and lower energy demand. Above that, unlike other biological treatment techniques, the collected biomass, after removing phenols, are valuable, and can be used as a source for

biodiesel production. Biodiesel could be effectively produced from oil extracted from microalgae using heterogeneous alkaline catalyst.

Chapter 2: Methods

2.1 Research Design

2.1.1 Materials

Analytical grade phenols (phenol, 2,4-dinitrophenol, 4-nitrophenol) were purchased from BDH Chemicals, UK. All other chemicals used to prepare the medium were purchased from Sigma-Aldrich, USA. These types of phenols were used in this work since there is no published work about the removal of these compounds using microalgae and due to their toxicity and negative effects on the environment. Two strains of microalgae, namely freshwater, *Chlorella* sp. and marine *Tetraselmis* sp., were obtained from a local marine environment research center in Umm Al-Quwain, UAE. The freshwater strain was cultivated in the modified bold bassel medium (3N-BBM) and the marine strain was grown in Gaillard F/2 medium, as described by Taher et al., (2015). Calcium acetate and γ- alumina were purchased from Sigma Aldrich, USA. The used solvents including methanol, chloroform and n-hexane were purchased from fisher scientific, USA with high analytical degree. A standard solution of high purity FAMEs consisting of: 4% myristic acid (C14:0), 10% palmitic acid (C16:0), 6% stearic acid (C18:0), 35% oleic acid (C18:1), 36% linoleic acid (C18:2), 2% of arachidonic acid (C20:0), and behenic acid (C22:0) was obtained from Sigma–Aldrich, USA.

2.1.2 Experimental procedures

2.1.2.1 Phenols degradation and microalgae growth

Flasks containing 100 mL of medium with the different concentrations of phenol(s) in the range of 150-350 m were placed in shaking water bath (LabTech,

Daihan lab Tech Co. ltd., Korea) set at 25ºC and 70 rpm. The flasks were inoculated with initial concentrations of 150, 200, 250, 300 and 350 mg/L microalgae, and were covered with cotton to reduce evaporation and atmospheric carbon dioxide utilization by the microalgae to promote chemotropic growth. The culture was subjected to excessive lighting using strong white neon lights. Control experiment containing 100 mL of media with same concentrations of phenol(s), but without microalgae were carried out under the same conditions to account for the drop-in phenol(s) by means other than the bioactivity of the microalgae.

On a daily basis, aliquots of 3 mL were withdrawn and analyzed for their biomass and residual phenol(s) concentrations. The biomass dry weight concentration was determined using UV-spectrophotometer (UVe1800, Shimadzu, Japan) at 680 nm. The instrument was calibrated against different dilutions of cells suspensions of known dry weight analyses. Different calibrations were prepared for the two tested strains. After biomass measurement, the sample was centrifugation using an IEC-CL Multispeed centrifuge (Model No. 11210913, France) at 6,000 rpm for 5 minutes. The supernatants were then filtered using a 0.45-μm syringe filter and the residual phenol(s) were analyzed using HPLC (Prostar, Varian, USA) equipped with UV–visible detector operating at 280 nm and a C-18 column maintained at 45ºC. The analysis was performed using two mobile phases; the gradient profile started with mobile phase consists of Methanol: water (30:70%), the ratio of the methanol was increased until reaching 100%. The run time was about 30 min, with flow rate of 1 mL/min for the mobile phase mixture. The instrument was calibrated using samples of standard cresols of known concentrations. Each experiment was carried out in duplicates, and the presented results are the average of the two runs. The reproducibility of the results can measure from the standard deviation.

2.1.2.2 Oil extraction

Folch method (Amani et al., 2013) was used to extract the oil from the harvested biomass. In each batch, 12 g of harvested microalgae (*Chlorella* sp.) biomass which was oven dried until constant weight, were homogenized with 240 mL of chloroform and methanol mixture of 2:1 volume ratio. The cells were thoroughly agitated for 3 minutes with the solvent under a continuous ultra-sonication (Branson Sonifier 450, USA). The mixture was then kept on orbital shaker (Stuart Lab scale Orbital Shaker /SSL1) at 120 rpm for 20 min at 25oC. After that, 48 ml (20% volume ratio) of distilled water was added to the mixture and mixed thoroughly, then the mixture was centrifuged at 1000 rpm for 3 minutes to separate the biomass. The collected supernatant was centrifuged again at 2000 rpm for 5 min to separate the two layers and was then placed in a separation funnel. The upper aqueous layer was discarded and the lower chloroform layer containing lipids was placed in a pre-weighed dry beaker, and dried in the oven (ULE 400, Memmert Universal) until reaching a constant weight.

2.1.2.3 Catalyst preparation

The method to prepare the CaO/Al_2O_3 catalyst was that proposed by Zabeti et al., (2009). Briefly, 8 g of γ-alumina was heated at 300^{o}C in a muffle furnace (WiseTherm, Korea) for 3-4 hours to completely remove any moisture. Calcium acetate, with a mass ratio of 1:1 to γ-alumina was dissolved in 50 ml of distilled water. The solution was introduced to γ-alumina and the mixture was stirred for 4 hours. The obtained slurry was then heated at 100^{o}C overnight in order to ensure the removal of any moisture. The calcination of the catalyst was performed at 700^{o}C for 4 hours in the muffle furnace.

2.1.2.4 Transesterification experiment

In each run, 1 g of extracted oil was mixed with methanol and catalyst in a tightly closed vial. The vial was placed in water bath and shaken at a speed of 250 rpm. The tested parameters were: temperature (in the range of 45 to 65 °C), methanol to oil ratio (in the range of 1:12 to 1:24) and catalyst weight percent with respect to oil (in the range of 5 to 10 wt%), to evaluate the relationship between the response, namely the biodiesel yield, defined by Eq (14).

$$\text{Biodiesel yield} = \frac{m_{FAME}}{m_{oil}} \times 100\% \qquad (14)$$

The reaction duration was performed 4 hours, and at the end of the reaction, the produced biodiesel was collected in 5 ml of n-hexane. The 4 hours duration was determined in a preliminary test, which showed that the production rate remains constant within this period of time. The solvent was added 1 ml at a time, mixed thoroughly, and then centrifuged at 8,000 rpm for 2 minutes to separate the two layers. The total fatty acids methyl esters (FAMEs) produced were analyzed using Gas Chromatography (GC) (Shimadezo, GC-2010, and Japan), equipped with a flame ionization detector (FID) and a SP-2560 capillary column. Injected samples of 1 μL were filtered through a 0.45 μm pore size filter syringe. Helium was used as a carrier gas at a flow rate of 40 mL/min. The oven temperature was set to 195 °C. After an isothermal period of 4 min, the GC oven was heated at a rate of 5 °C min-1 to 240 °C, and held for 12 min. A split ratio of 30 was used with injector and detector temperatures at 240 °C and 260 °C, respectively. Prior to samples analysis, a FAMEs standard of known composition was injected and used to calibrate the instrument.

MiniTab 17 statistical software was used to fit the experimental results, shown in Table 12, to generate a second-order polynomial model relating the biodiesel yield,

Y, and the three key variables: catalyst wt%, x1, methanol:oil ratio, x2 and temperature, x3. Full factorial design was used to design the experiment with randomized order of experiment. It was used with the three previously mentioned factors and by considering a replicate in each experiment. The levels of uncoded factors in the experiment are shown in Table 11.

Table 11: Biodiesel experiment factors and levels

Operating parameters	Symbol	Levels		
		-1 (Low)	0 (Medium)	1 (High)
Catalyst wt%	x_1	5	7	10
Methnaol:oil molar ratio	x_2	12	18	24
Temperature, ^oC	x_3	45	55	65

Table 12: Full factorial arrangement and experimental results

Run Order	x_1	x_2	x_3	FAME yield%
1	0	1	0	19.62
2	-1	1	-1	0.33
3	-1	0	1	1.12
4	1	1	0	0.15
5	0	0	-1	15.07
6	-1	-1	1	0.74
7	1	-1	-1	30.85
8	0	0	0	28.33
9	-1	1	-1	0.35
10	1	-1	0	1.12
11	-1	1	1	0.30
12	0	0	1	27.00
13	1	1	-1	10.26
14	-1	0	0	0.89
15	0	1	1	9.41
16	1	1	-1	10.11
17	1	-1	-1	31.11
18	-1	0	-1	0.91
19	-1	0	-1	0.97
20	0	-1	1	26.74
21	-1	-1	1	0.74
22	1	1	0	0.17
23	-1	-1	-1	0.86

Table 12: Full factorial arrangement and experimental results (cont'd)

24	0	-1	1	26.58
25	-1	1	1	0.32
26	0	1	-1	15.53
27	1	0	-1	22.76
28	1	1	1	0.47
29	-1	1	0	0.37
30	0	1	0	19.43
31	-1	-1	0	0.80
32	1	1	1	0.47
33	1	0	0	0.48
34	-1	0	0	0.88
35	1	-1	1	3.64
36	0	-1	0	28.37
37	1	0	-1	22.59
38	1	-1	0	0.98
39	0	1	1	9.60
40	1	0	1	1.83
41	-1	0	1	1.13
42	1	-1	1	2.61
43	1	0	0	0.49
44	-1	-1	0	0.77
45	0	0	0	28.37
46	0	0	-1	14.24
47	-1	1	0	0.35
48	0	-1	0	28.33
49	0	0	1	27.17
50	0	-1	-1	30.77
51	0	1	-1	15.36
52	1	0	1	1.73
53	0	-1	-1	32.81
54	-1	-1	-1	2.35

To determine the significance of each factor, response surface analysis was used to obtain p-values for each factor and their interactions.

2.1.2.5 Catalyst characterization

2.1.2.5.1 SEM analysis

Scanning electron microscope, SEM (JCM-5000 NeoScope, Japan) was used to analyze the morphology of the prepared catalyst particles. Before performing the

analysis, a portion of the catalyst, before and after calcination, was coated with gold, since the catalyst is a nonconductive material and it cannot be analyzed by SEM. Coating was performed to increase the specimen conductivity and to prevent the build-up of charges with high voltage on the specimen. The coating was done using Auto Fine Coater (JFC-1600, Japan). After coating process, the samples were cleaned in order to remove unwanted deposits such as silt, dust and detritus. The samples were mounted on a holder that could be inserted into SEM.

2.1.2.5.2 Diffraction analysis

X-ray diffractometer (XRD system, Phillips- Holland) was used to analyze the crystallinity diffraction. The anode material was copper, and the divergence slit was fixed at a value of 1.52 mm. The scanning range angle was from 10 to 70 with a scan step size of 0.01. The number of scanned points was 4569 and the scanning type was continuous.

2.1.2.5.3 Porosity and surface area analysis

A gas physisorption instrument (TriStar II 3020 Analyzer, Japan) was used to measure the porosity and surface area of the catalyst. Since nitrogen was used for analysis, the thermal temperature was kept at 77.3 K. The analysis was conducted at 45 mV and 40 mA.

Chapter 3: Results and Discussion

3.1 Phenols degradation and microalgae growth

The concentrations of phenol in media containing *Chloerella sp*. and *Tetraselmis sp.* with an initial biomass concentration of 0.0925 and 0.091 mg/L respectively at 25°C and different initial concentrations of phenol, were plotted versus time, and results are shown in Figures 7 and 8, respectively. The corresponding biomass growth curves, represented as the logarithmic of concentration of the biomass over initial biomass concentration (X/X_o) are shown in Figures 9 and 10, for *Chloerella sp.* and *Tetraselmis sp*., respectively. Running a parallel experiment without microalgae, showed minimal drop in the concentration over the five days, which did not exceed 1.5% at the highest concentration of 350 mg/L. This proves that the drop on phenol was mainly due to the bioactivity of the microalgae. It should be noted that the data shown in Figures. 7 and 8 are average values of two runs.

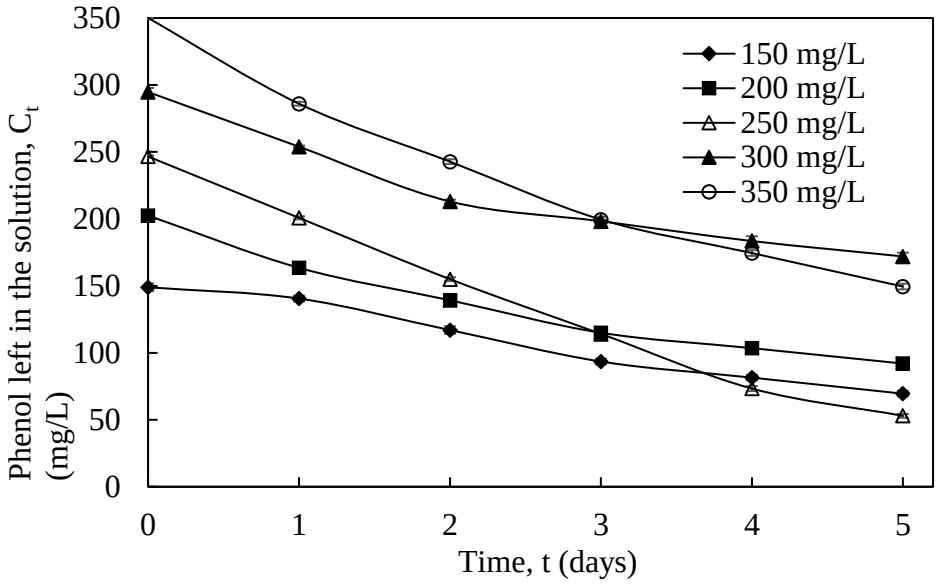

Figure 7: Changes in phenol concentrations in media containing Chorella sp. with an initial biomass concentration of 0.0925 mg/L at 25°C

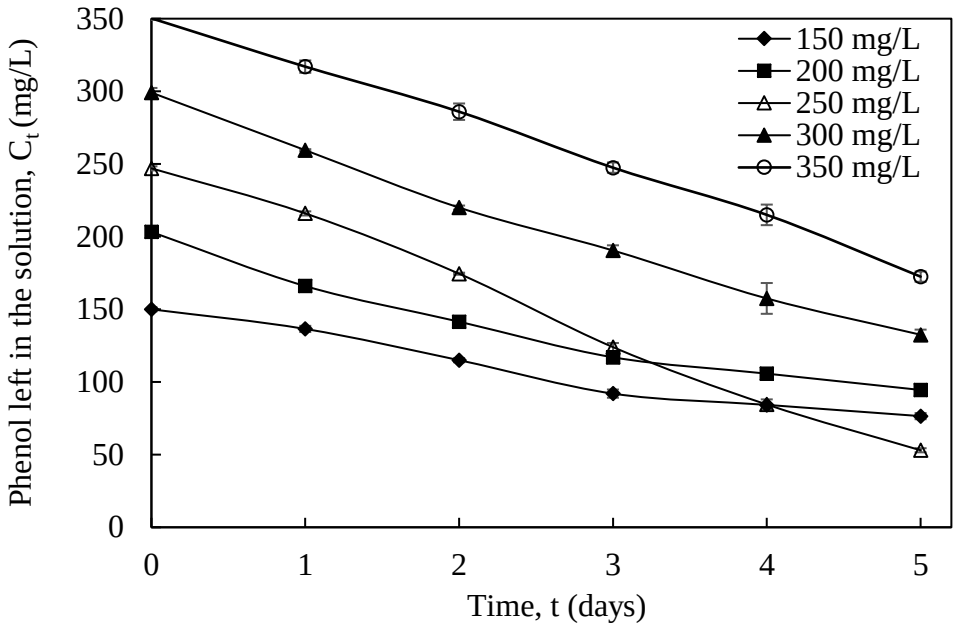

Figure 8: Changes in phenol concentrations in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.091 mg/L at 25ºC

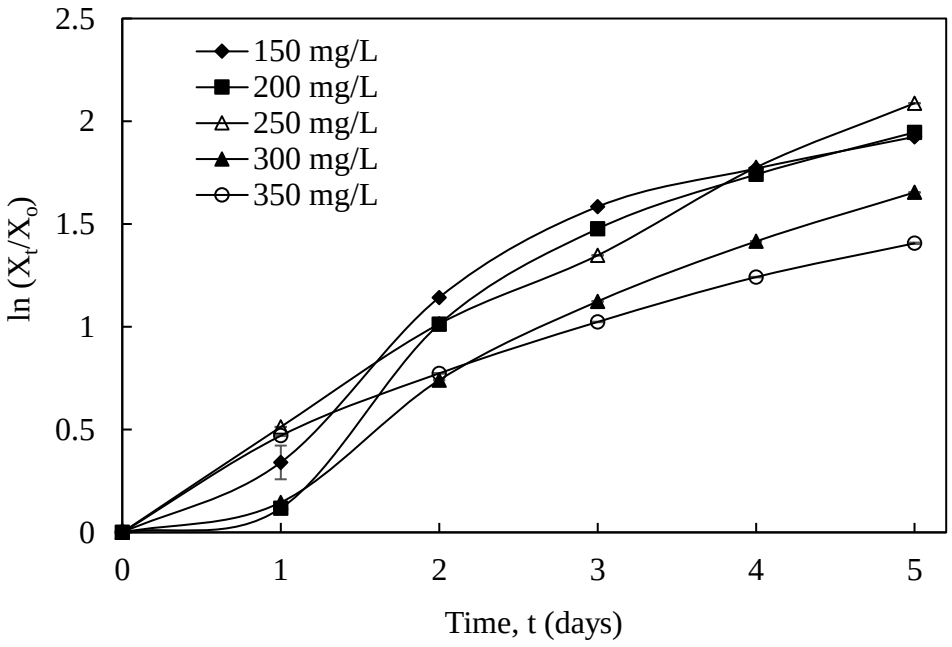

Figure 9: Changes in ln (X/X_o) in media containing *Chorella* sp. with an initial biomass concentration of 0.0925 mg/L at 25ºC and different initial phenol concentrations

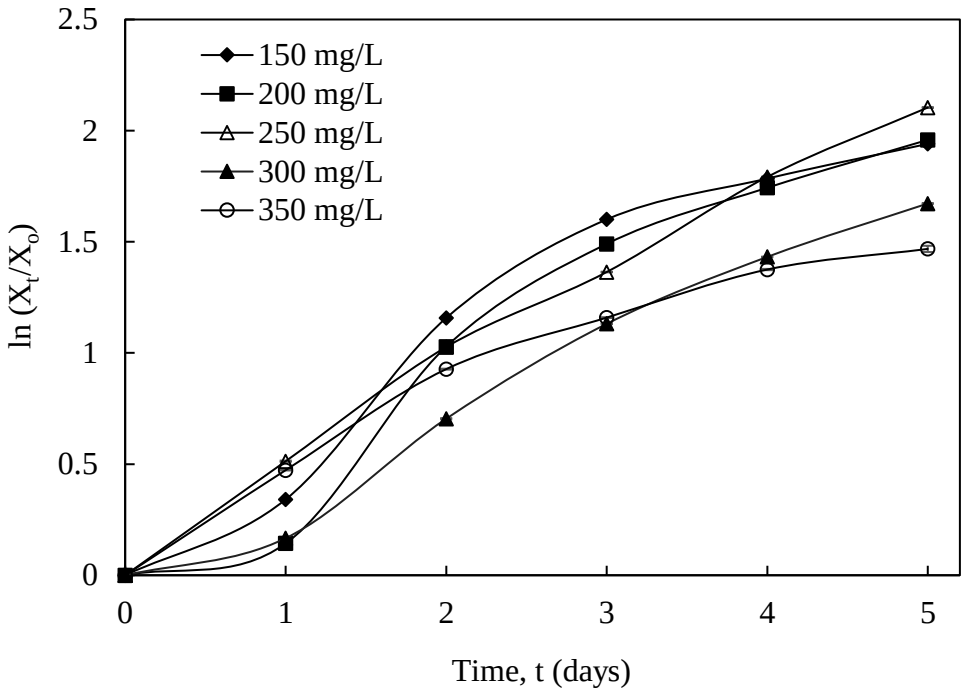

Figure 10: Changes in ln (X/X_o) in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.091 mg/L at 25ºC and different initial phenol concentrations

It was clearly seen from that both strains were capable of growing and removing phenol, but with different growth rates. The results in Figures 9 and 10 show that a longer lag phase was observed by both strains at low and high concentrations of phenol and becomes negligible at intermediate concentrations. This is mainly due to the lack of substrates at the low concentrations and the longer time needed to adopt at the high concentration.

Similar results using different concentrations of 4-nitrophenol and 2,4-nitrophenol with *Chloerella sp.* and *Tetraselmis sp*., are shown in Figures 11-14. The corresponding growth results of *Chloerella sp.* and *Tetraselmis sp*. are shown in figures 15-18.

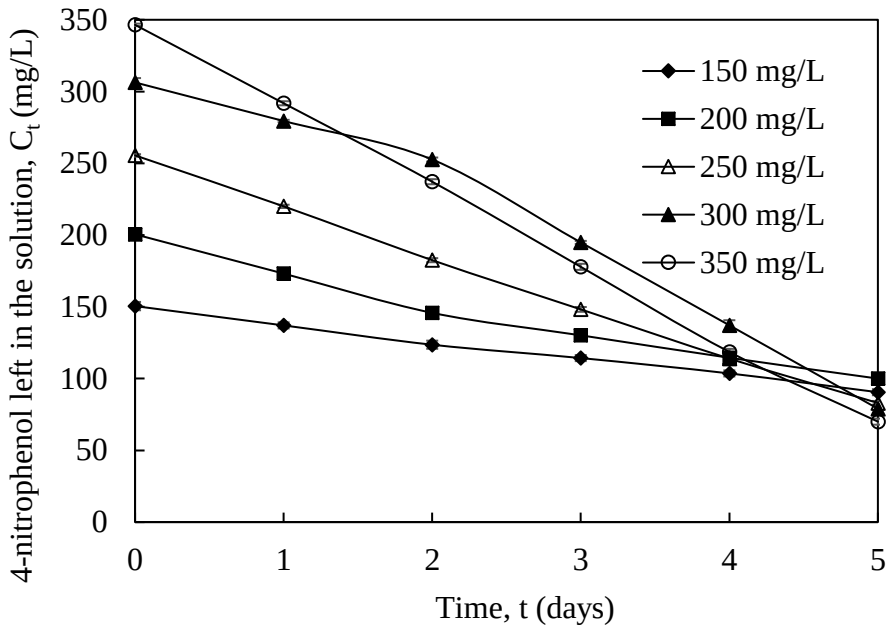

Figure 11: Changes in 4-nitrophenol concentration in media containing *Chlorella* sp. with an initial biomass concentration of 0.0355 mg/L at 25°C

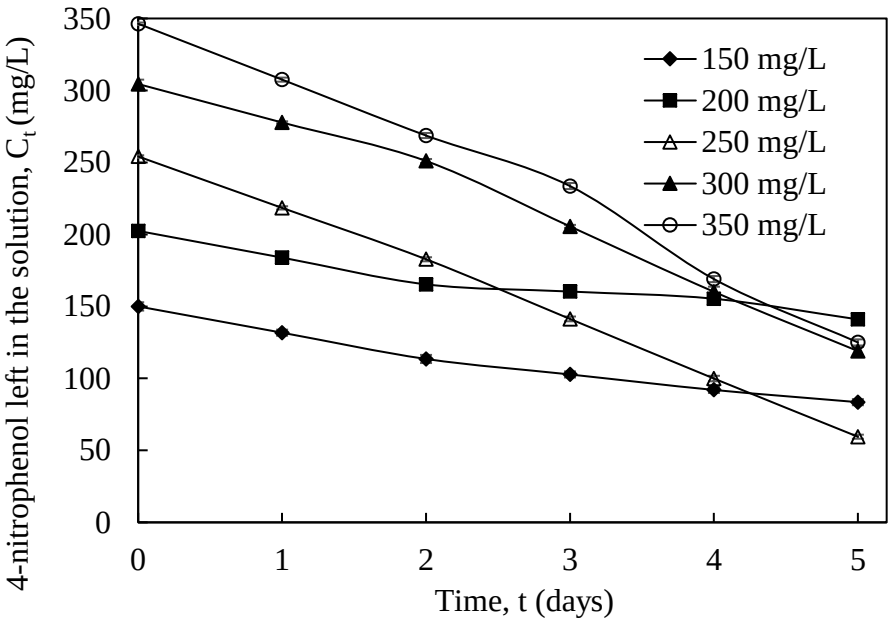

Figure 12: Changes in 4-nitrophenol concentration in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.0125 mg/L at 25°C

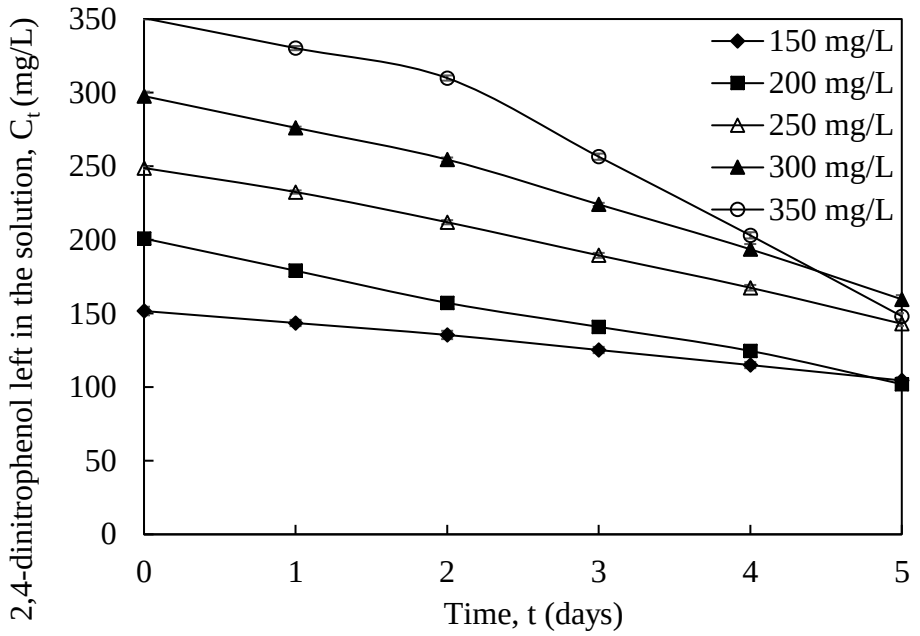

Figure 13: Changes in 2,4-dinotrophenol concentration in media containing *Chlorella* sp. with an initial biomass concentration of 0.0355 mg/L at 25ºC

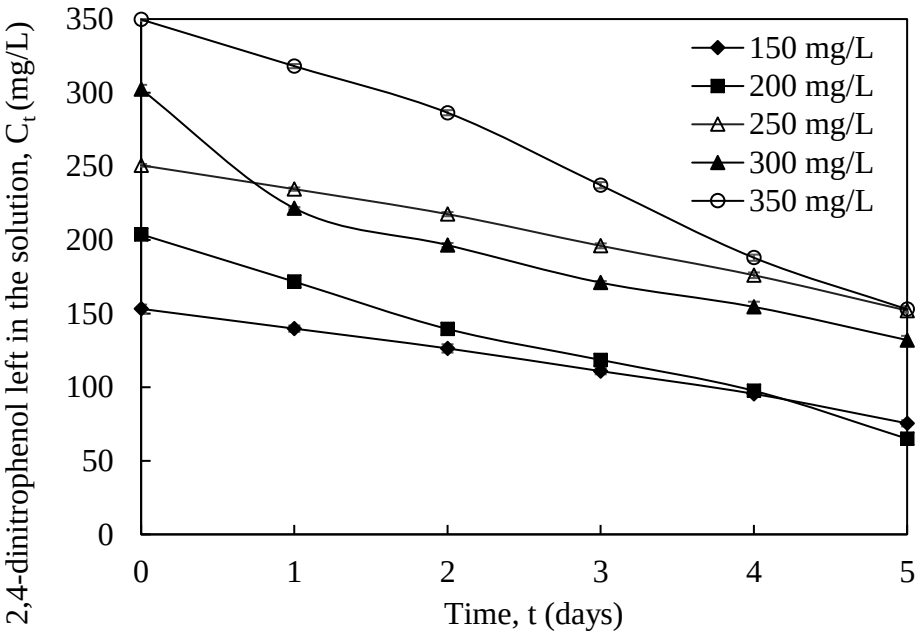

Figure 14: Changes in 2,4-dinotrophenol concentration in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.0125 mg/L at 25ºC

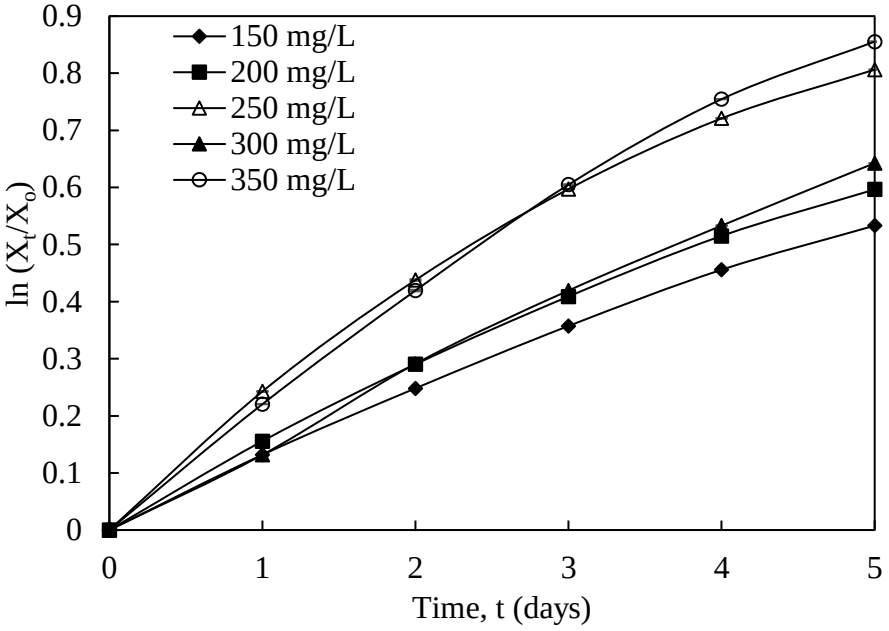

Figure 15: Changes in ln (X/X_o) in media containing *Chlorella* sp. with an initial biomass concentration of 0.0125 mg/L at 25^{o}C and different initial concentrations of 4-nitrophenol

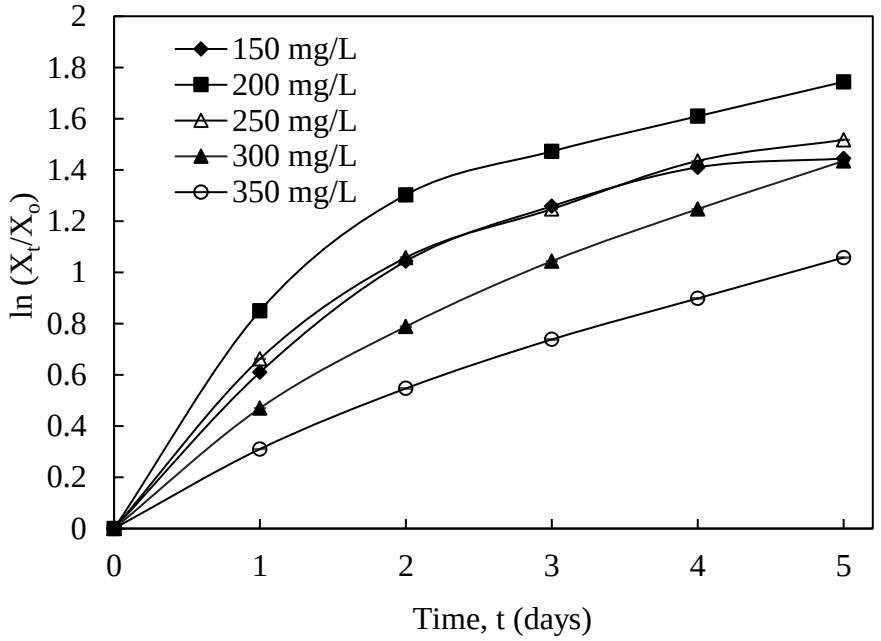

Figure 16: Changes in ln (X/X_o) in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.0140 mg/L at 25^{o}C and different initial concentrations of 4-nitrophenol

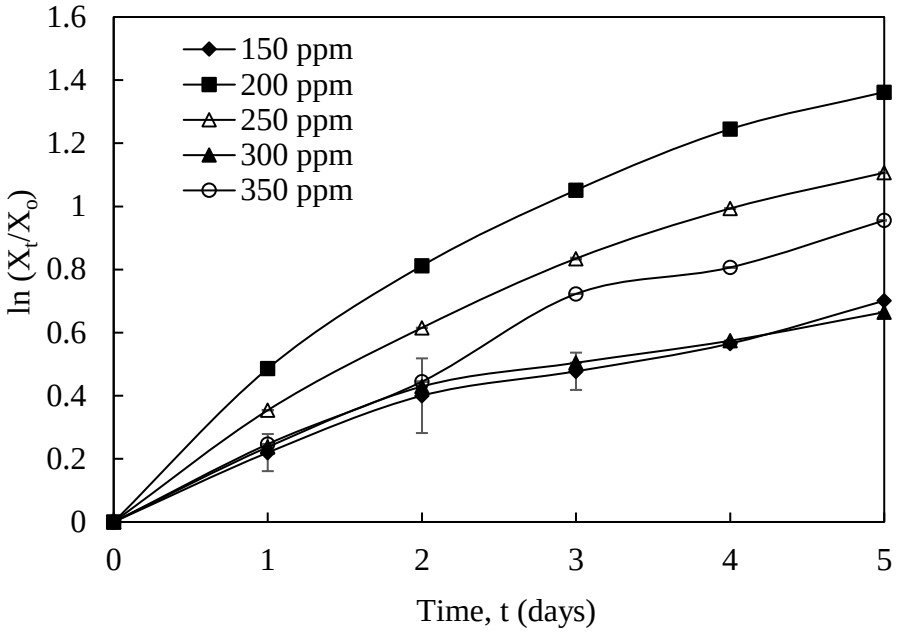

Figure 17: Changes in ln (X/X_o) in media containing *Chlorella* sp. with an initial biomass concentration of 0.0125 mg/L at 25ºC and different initial concentrations of 2,4-dinitrophenol

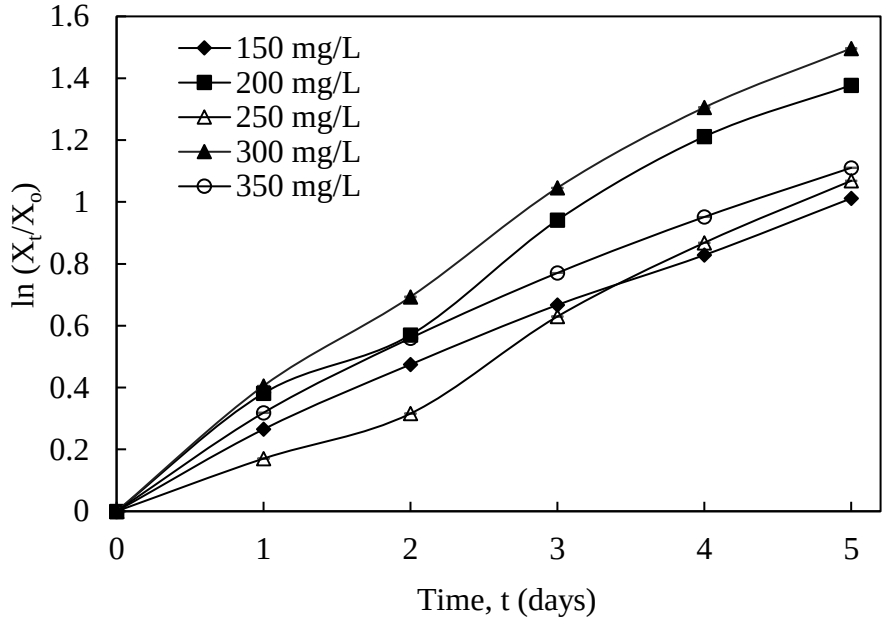

Figure 18: Changes in ln (X/X_o) in media containing *Tetraselmis* sp. with an initial biomass concentration of 0.0140 mg/L at 25ºC and different initial concentrations of 2,4-dinitrophenol

The biodegradation rates of all phenols were determined from the slope of the straight line in the initial part of the degradation. The specific growth rate was

determined from the slope of the exponential part of the logarithmic of X/X_o vs time, after the lag period (Figures 19-24).

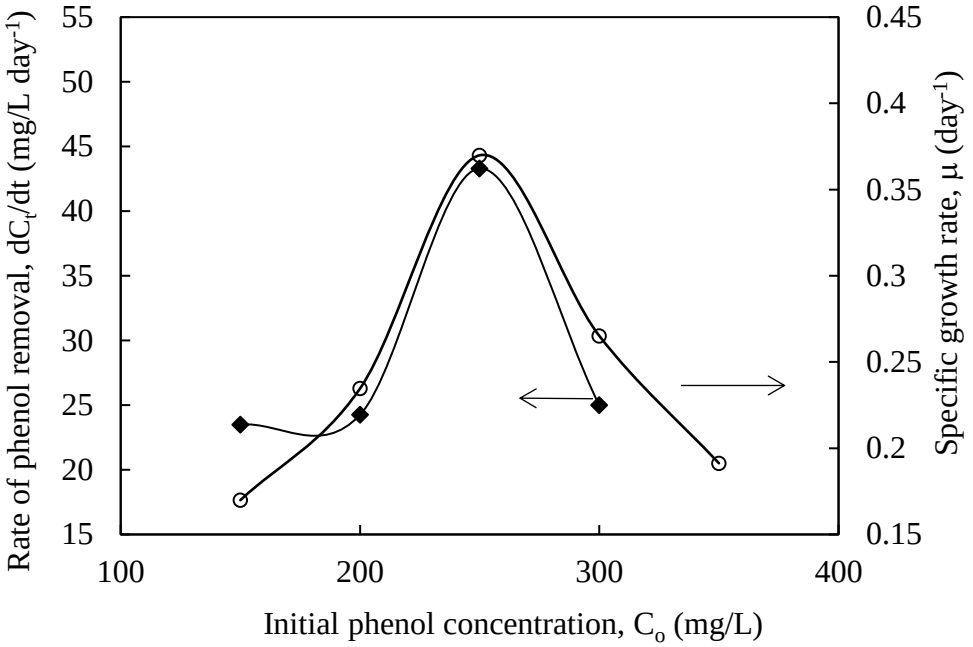

Figure 19: Phenol degradation rate and specific growth rate of *Chlorella* sp. at initial biomass concentrations of 0.0925 mg/L at 25ºC and different initial phenol concentration

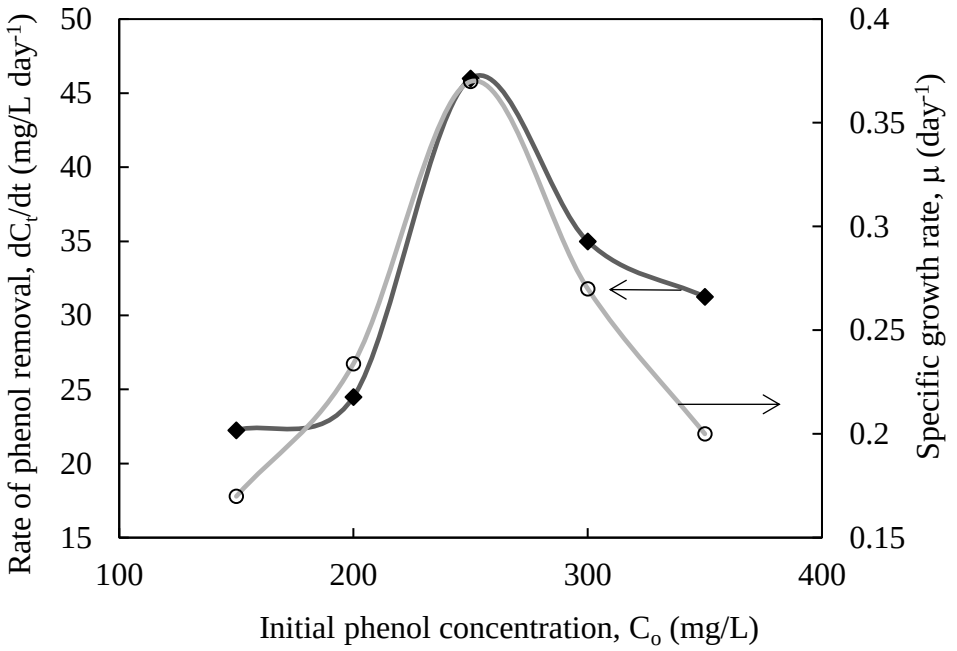

Figure 20: Phenol degradation rate and specific growth rate of *Tetraselmis* sp. at initial biomass concentrations of 0.0921 mg/L at 25ºC and different initial phenol concentration

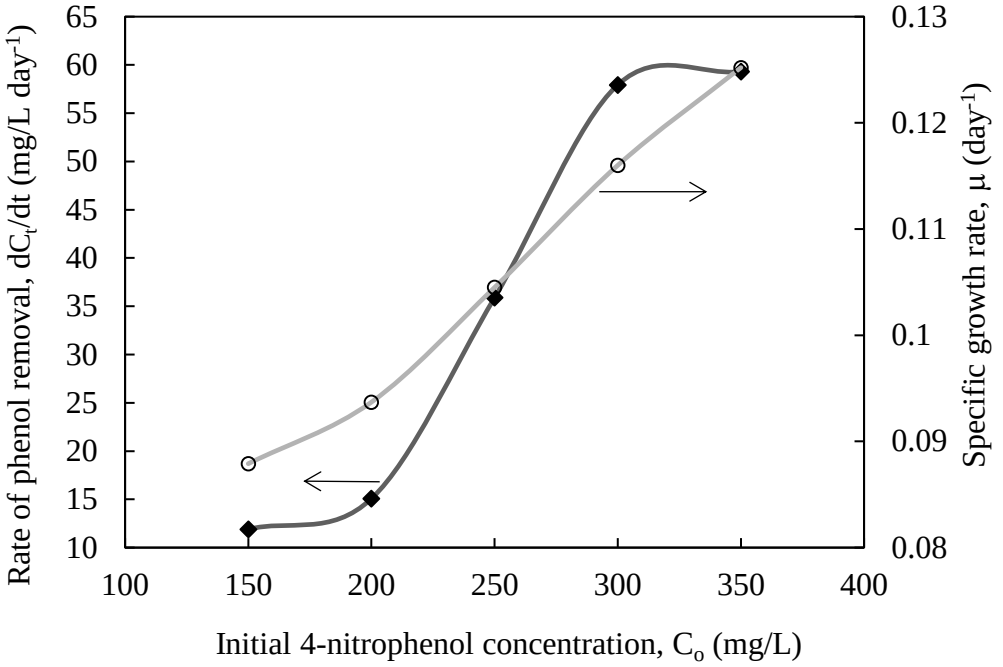

Figure 21: 4-Nitrophenol degradation rate and specific growth rate of *Chlorella* sp. at initial biomass concertation of 0.0355 mg/L at 25^{o}C and different initial 4-nitrophenol concentrations

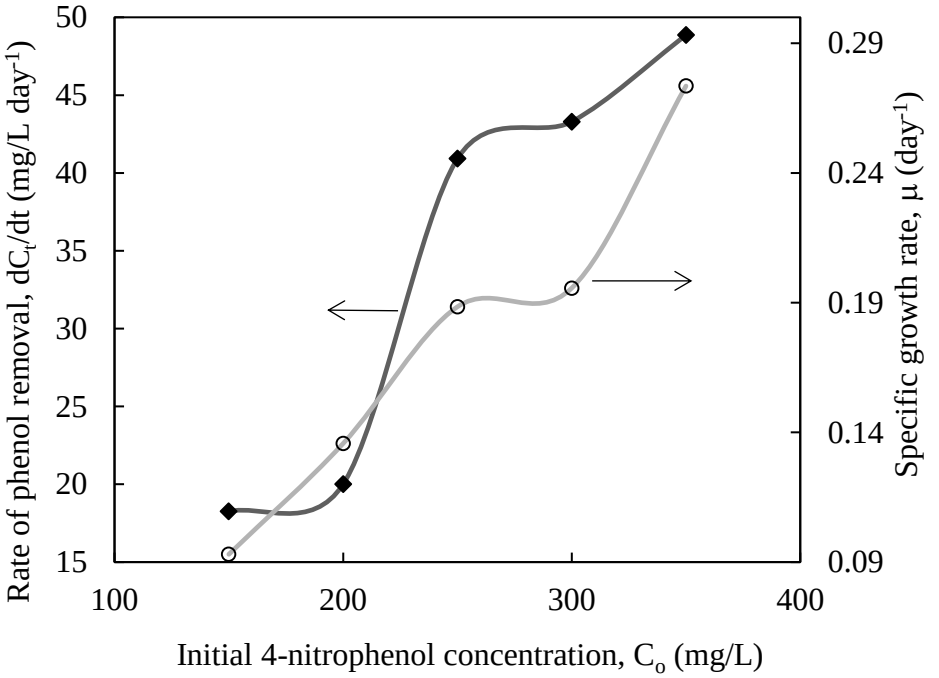

Figure 22: 4-Nitrophenol degradation rate and specific growth rate of *Tetraselmis* sp., at initial biomass concertation of 0.0125 mg/L at 25^{o}C and different initial 4-nitrophenol concentrations

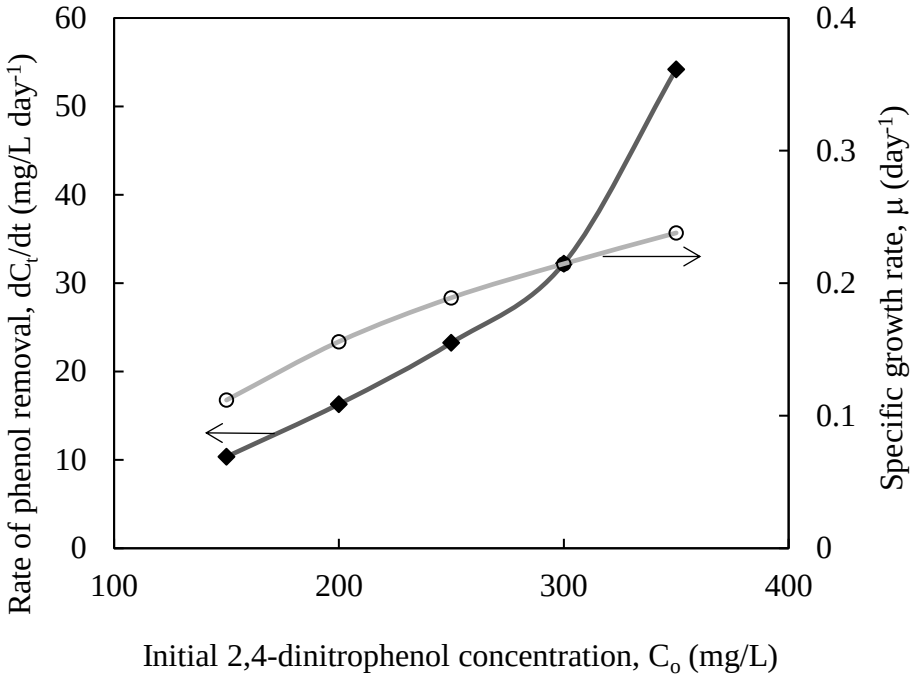

Figure 23: 2,4-Dinitrophenol degradation rate and specific growth rate of *Chlorella* sp. at initial biomass concentrations of 0.0125 mg/L at 25^{o}C and different initial 2,4-dinitrophenol concentrations

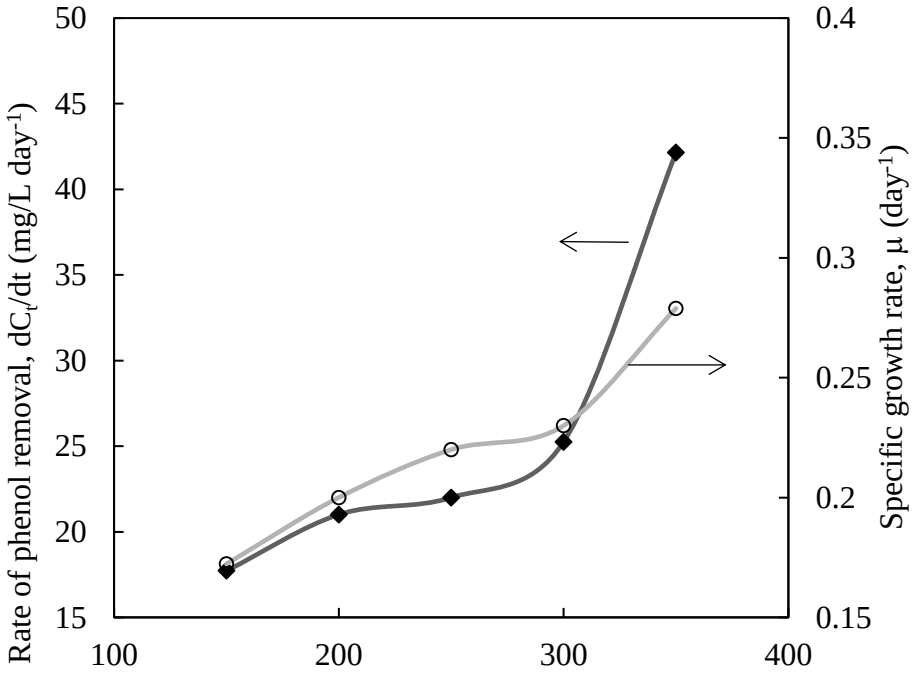

Figure 24: 2,4-Dinitrophenol degradation rate and specific growth rate of *Tetraselmis* sp., at initial biomass concentrations 0.0140 mg/L at 25^{o}C and different initial 2,4-dinitrophenol concentrations

As expected, it was found that both rates (biodegradation and specific growth) had similar trends. When the growth rate increased, the biodegradation rate increased and vice versa. It was found that the growth rate of both *Chlorella* sp. and *Tetraselmis* sp. increased with increasing the initial concentration of Phenol, up to 250 mg/L, at which the optimum growth rates of 0.3699 day^{-1} were reached for the two strains. The growth sharply dropped after that, due to substrate inhibition. Similar behavior was observed for the phenol degradation, but the optimum drops were 43.3 and 46 mg/L. day for the two strains, respectively. These results agree with previous study done suing the *Chlorella sp.* for phenol degradation (Al-Zuhair et al., 2016). However, the maximum biodegradation and growth rates were obtained at an initial phenol concentration of 200 mg/L. The optimum drop rate was 54.5 mg/L. day, which were very close, to the ones found in this work. The maximum growth rate found in the previous study was 0.25 day^{-1}, which slightly lower than the ones found in this work. The lower growth rate was mainly due to the limited light intensity used in the previous study, which suggested the enhancement of heterotrophic growth. Similar trend was also observed using *C. pyrenoidosa* (Das et al., 2015), but the highest specific rate was reached at 125 m. The maximum specific growth rate was 0.65 day, which was slightly higher than the ones found in this work.

The concentration of both *Chlorella* sp. and *Tetraselmis* sp. increased with increasing the initial concentration of initial concentration of 4-nitrophenol till reaching the maximum among other values with growth rates of 0.1252 and 0.2735 day^{-1} respectively at an initial concentration of 4-nitrophenol of 350 mg/L. day. Similar behavior was observed for the 4-nitrophenol degradation, but the optimum drops were 59.297 and 48.85 mg/L /day for the two strains, respectively.

Similar trends were found when using 2,4-nitrophenol in which the concentration of both *Chlorella* sp. and *Tetraselmis* sp. increased with increasing the initial concentration of initial concentration of 2,4-nitrophenol till reaching the maximum among other values with growth rates of 0.2379 and 0.2789 day^{-1} respectively at an initial concentration of 4-nitrophenol of 350 mg/L. day. Similar behavior was observed for the 4-nitrophenol degradation, but the optimum drops were 54.206 and 42.148 mg/L /day for the two strains, respectively.

The results shown in Figures 19-24, were used to determine the parameters of three growth models that incorporate limiting substrate-inhibition kinetics. The models used were Haldane, Aiba and Andrews, given in Eqs. (15) to (17), respectively

$$\mu = \frac{\mu_m C_o}{K_S + C_o + C_o^2/K_i} \quad (15)$$

$$\mu = \frac{\mu_m C_o}{K_S + C_o} \exp\left(-C_o/K_i\right) \quad (16)$$

$$\mu = \frac{\mu_m}{\left[\left(K_S/C_o\right)+1\right]\cdot\left[1+\left(C_o/K_i\right)\right]} \quad (17)$$

Where, μ and μ_m are the specific and maximum specific growth rates (day^{-1}), respectively, C_o is the initial substrate concentration (mg/L), and K_s and K_i are the substrate and inhibition constants (mg/L), respectively. These models were selected in this study because they were used in previous studies and gave good fits as mentioned below. The selected models are the ones that incorporate limiting kinetics of substrate inhibition. The estimated values for the model kinetic parameters were determined by fitting non-linear regression model, using Excel solver with an objective function (O.F.) given by Eq. (18), and shown in Table 13.

$$O.F. = \sum_i^n \left(\mu_{exp} - \mu_{pred.}\right)^2 \quad (18)$$

Where, μ_{exp} and μ_{pred} are the experimental and model predicted specific growth rates, respectively, and n is the number of points used.

Table 13: Estimated value of microalgae growth kinetic parameters in phenol(s) containing media

Strain	Substrate	μ_m (day^{-1})	K_S (mg/L)	K_i (mg/L)	R^2	Ref.
Haldane Model						
Chlorella sp.	Phenol	1.15	400	168.99	0.57	This work
Tetraselmis sp.		0.51	170	170	0.36	This work
Chlorella sp.		0.43	145.58	177.51	-	Al-Zuhair et al., 2016
C. pyrenoidosa		0.55	89.99	100.24	-	Das et al., 2015
Chlorella sp.	4-Nitrophenol	0.43	581.68	415.7	0.92	This work
Tetraselmis sp.		0.28	170	170	0.92	This work
Chlorella sp.	2,4-Dinitrophenol	1.58	1857	1634.5	0.99	This work
Tetraselmis sp.		0.52	170	169	0.76	This work
Aiba Model						
Chlorella sp.	Phenol	0.85	282.34	500	0.75	This work
Tetraselmis sp.		1.39	280	459.56	0.74	This work
Chlorella sp.		0.45	85.24	291.97	-	Al-Zuhair et al., 2016
C. pyrenoidosa		0.71	58.13	200.40	-	Das et al., 2015
Chlorella sp.	4-Nitrophenol	0.49	578.08	833.28	0.92	This work
Tetraselmis sp.		0.79	570.58	820.57	0.89	This work
Chlorella sp.	2,4-Dinitrophenol	1.53	1730.2	5170.5	0.99	This work
Tetraselmis sp.		0.76	490.8	1971.2	0.91	This work
Andrews Model						
Chlorella sp.	Phenol	1.55	230	246.08	0.72	This work
Tetraselmis sp.		1.06	160	365.21	0.71	This work
Chlorella sp.		0.63	150.79.39	141.82	-	Al-Zuhair et al., 2016
Chlorella sp.	4-Nitrophenol	0.56	661.1	604.36	0.94	This work
Tetraselmis sp.		0.69	630.57	640.52	0.91	This work
Chlorella sp.	2,4-Dinitrophenol	1.76	1983.2	3752.9	0.99	This work
Tetraselmis sp.		1.21	789.3	856.7	0.91	This work

A variation was observed in the values of μ_m, K_S and K_i found in this work, as compared to those found in previous work done on phenol, namely Al-Zuhair et al., (2016) and Das et al., (2015). This was expected when compared to the results reported by Das et al., (2015), as a different strain was used there. However, when it comes to the work by Al-Zuhair et al., (2016), in which a similar strain, *Chlorella sp.*, was used, the discrepancy could be due to the different experimental conditions, initial concentration of phenol and initial prediction of the parameters. The values were

relatively higher in this work compared to the values of the previous work. It was not possible to compare the results for 4-nitrophenol and 2,4-dinitrophenol with previous studies done on microalgae, because none of them included any kinetics studies.

In general, the goodness of the fittings of the three models were relatively similar. However, from the values of the coefficient of determination, R^2, it was found that in phenol and 2,4-dinitrophenol media, the Aiba model best fit the experimental data for both *Chlorella* sp. and *Tetraselmis* sp. In 4-nitrophenol medium however, the models that presented the experimental results of *Chlorella* sp. and *Tetraselmis* sp were Andrews model and Haldane model, respectively.

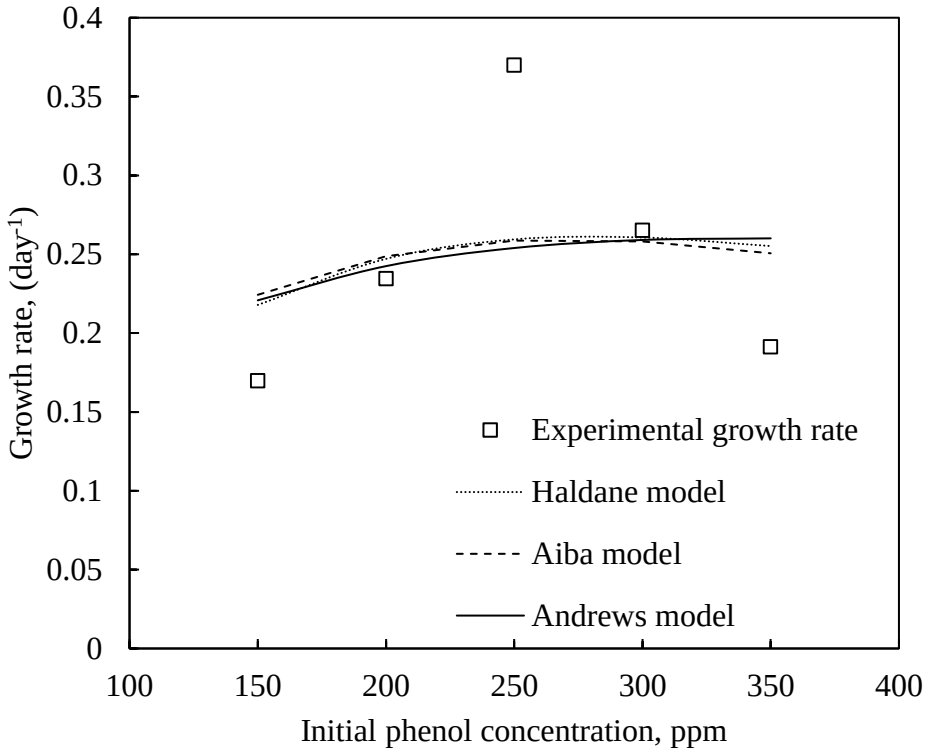

Figure 25: Growth rates of microalgae at different phenol concentrations using *Chlorella* sp.

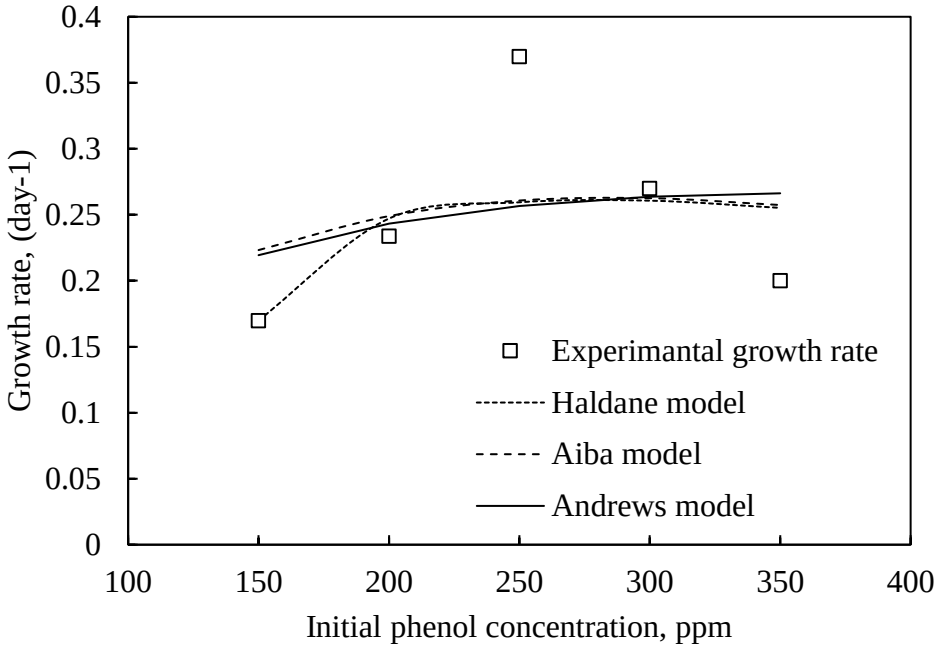

Figure 26: Growth rates of microalgae at different phenol concentrations *Tetraselmis* sp.

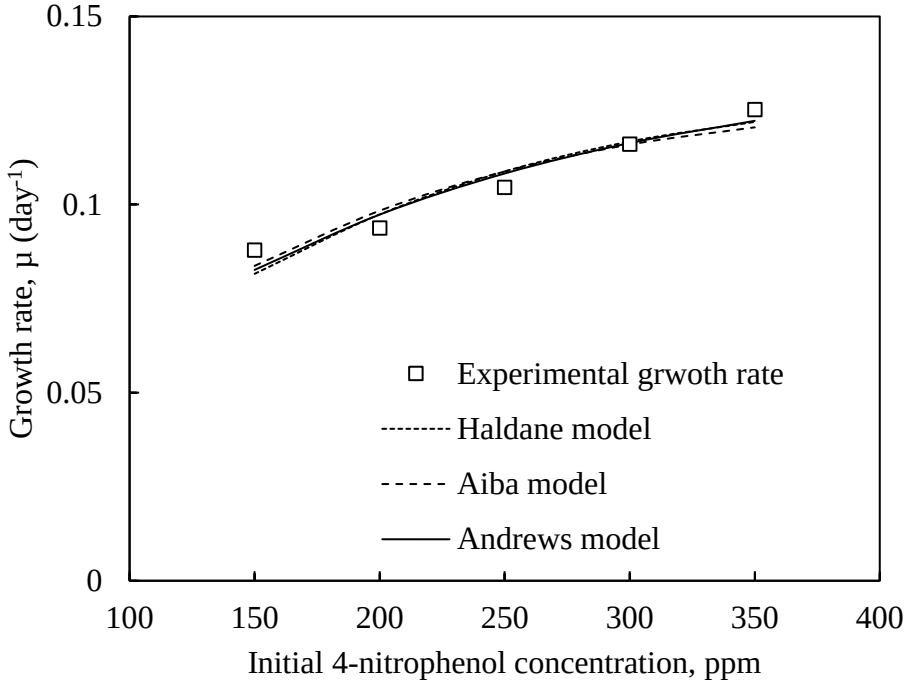

Figure 27: Growth rates of microalgae at different 4-nitrophenol concentrations using *Chlorella sp.*

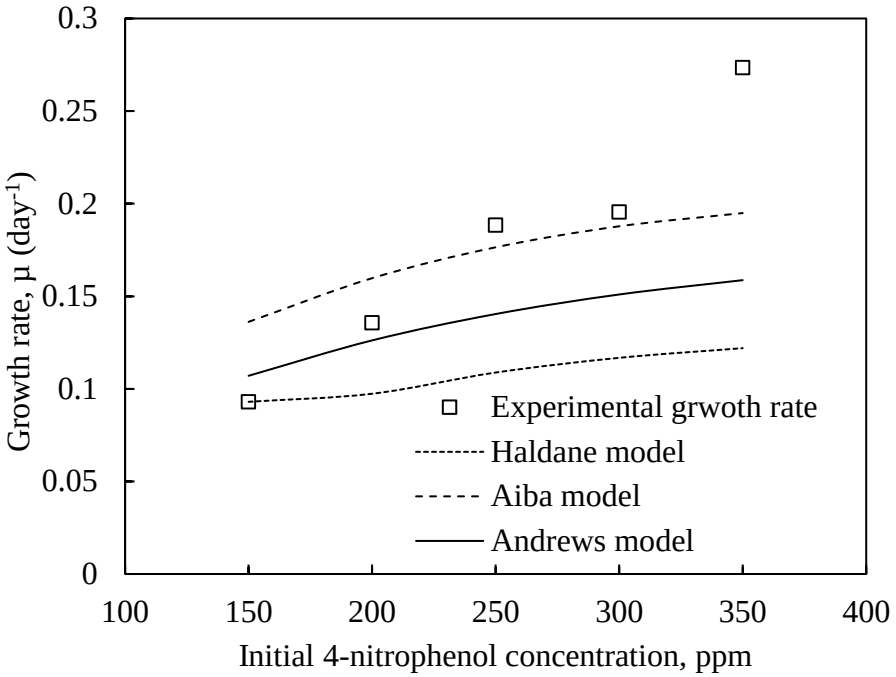

Figure 28: Growth rates of microalgae at different 4-nitrophenol concentrations using *Tetraselmis sp.*

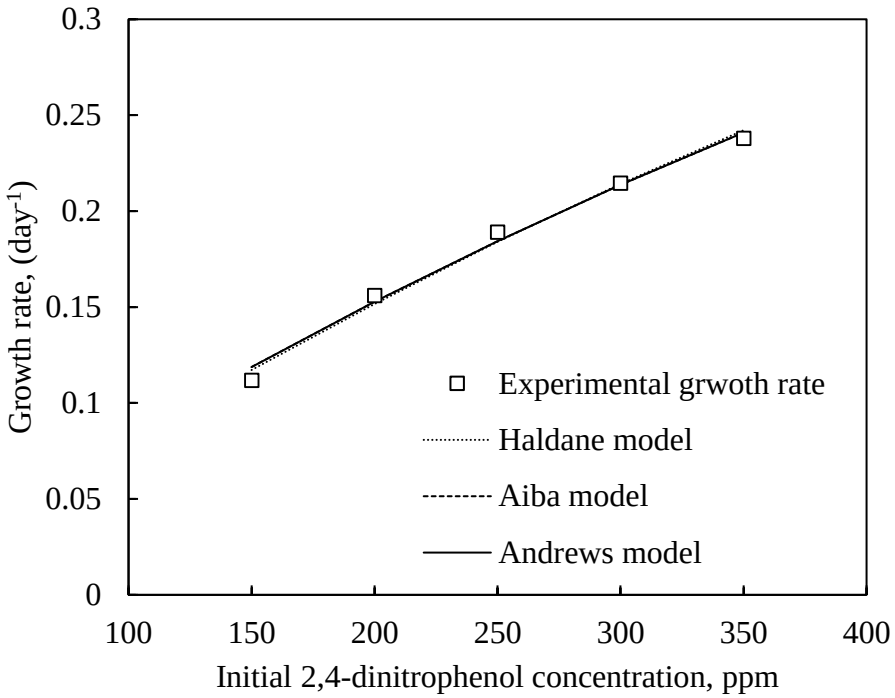

Figure 29: Growth rates of microalgae at different 2,4-dinitrophenol concentrations using *Chlorella sp.*

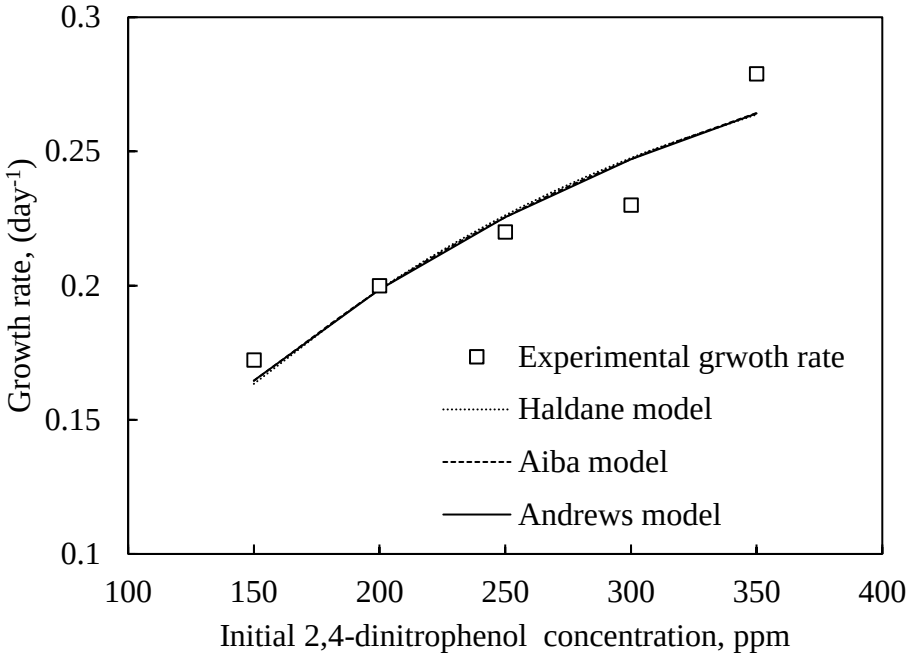

Figure 30: Growth rates of microalgae at different 2,4-dinitrophenol concentrations using *Tetraselmis sp.*

The goodness of the fitting can also be seen in Figures 25-30, which show the comparisons between the experimental data and models predictions. For phenol removal using *Chlorella* sp., the experimental values of microalgae growth rate were close to those obtained using Aiba model. The microalgae growth rate values for Haldane and Andrews models were similar to each other, but different than those of the Aiba model. Similar trends can be observed when *Tetraselmis* sp was used. For 4-nitrophenol and 2,4-dinitrophenol removals, the experimental values of microalgae growth rate and the ones predicted by the three models were very close to, except for *Tetraselmis sp.*

3.2 Catalyst characterization

To assess the effect of calcination on the catalyst structure, the morphology of the catalyst particles before and after calcination were analyzed using SEM, as shown in Figures 31 and 32, respectively.

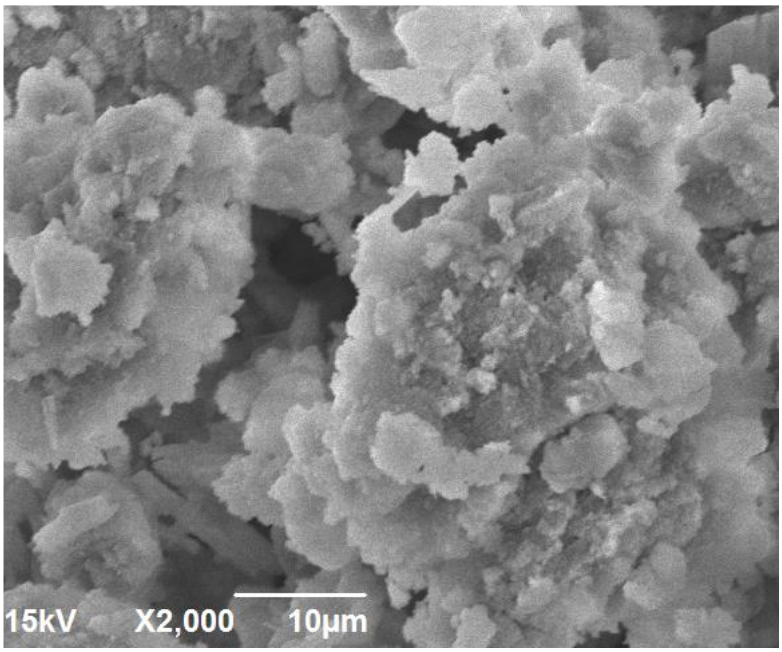

Figure 31: Catalyst morphology before calcination

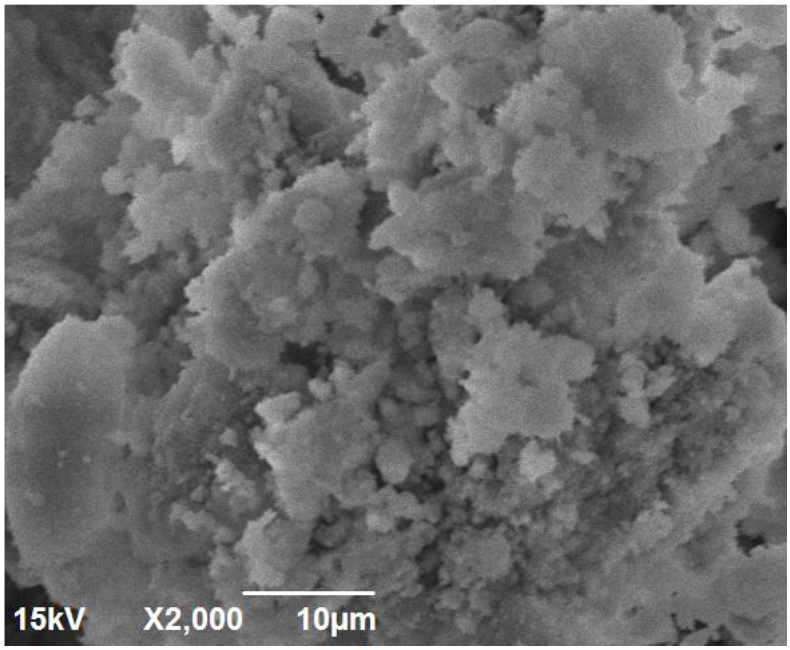

Figure 32: Catalyst morphology after calcination

The images show that there was no significant difference in the morphologies of the catalyst before and after calcination, which proves that the structure of the catalyst is not affected by the exposure to the high temperature of calcination. The images show that the catalyst before and after calcination is porous with a spongy appearance. To

further investigate the effect of calcination on the catalyst structure, the XRD of the catalyst before and after calcination has been analyzed, as shown in Figures 33 and 34, respectively.

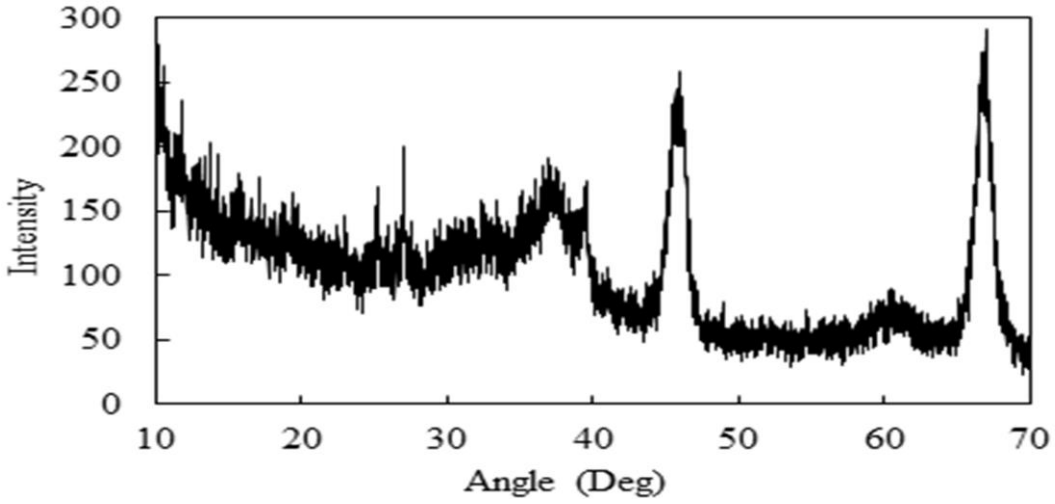

Figure 33: Catalyst intensity before calcination

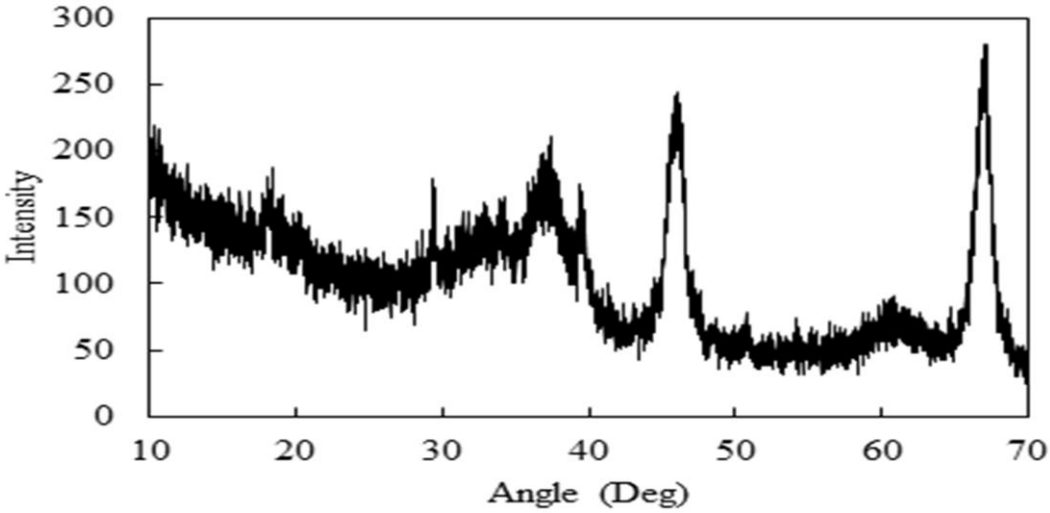

Figure 34: Catalyst intensity after calcination

The results also showed that there were no major differences between the intensity of the catalyst at different angles for the samples before and after calcination. The highest intensities before and after calcination were located at angles 66.7^{o} and 47^{o} respectively. The XRD analysis of γ-alumina only was performed before and after exposing γ-alumina to high temperatures (same as calcination temperature performed

with the catalyst). It was difficult to do that with calcium acetate since it burned and turned to black color at high temperatures of calcination. Figures 35 and 36 show the γ-alumina intensity before and after exposing to a high temperature.

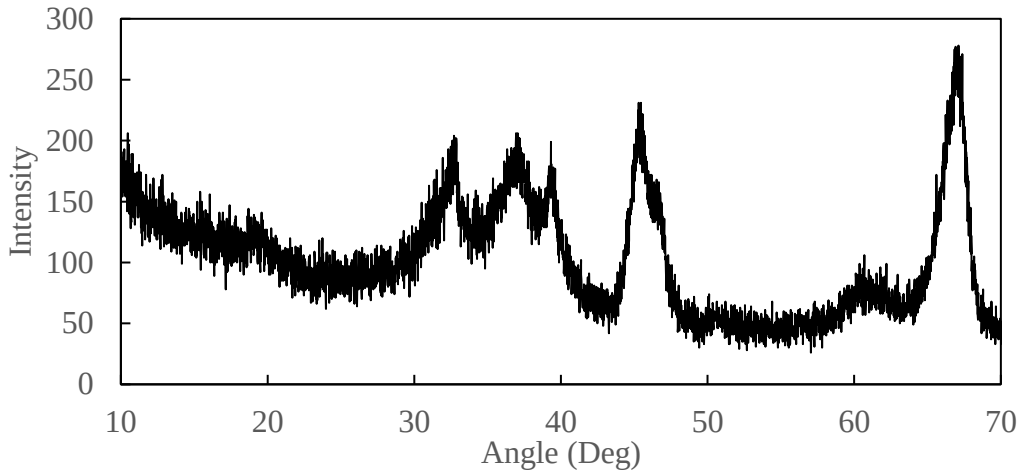

Figure 35: γ-alumina intensity before exposing to calcination temperature

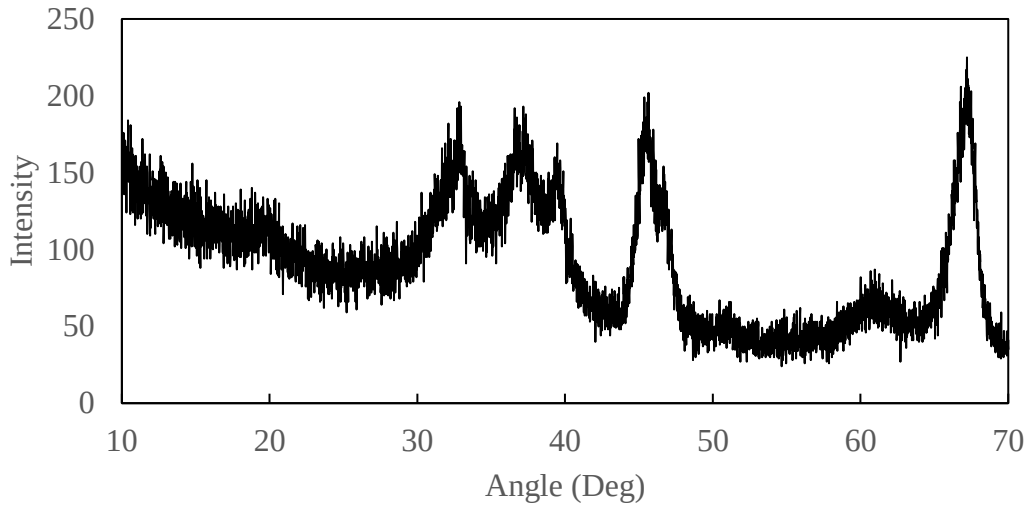

Figure 36: γ-alumina intensity after exposing to calcination temperature

As shown in figures 35 and 36, there are not major differences in the intensity of γ-alumina before and after exposing to a high temperature. Comparing these two figures with the catalyst intensity before and after calcination shown in figures 33 and 34, it can be noticed that the addition of calcium acetate is clear at angles 47º and 67º, which

was the same before and after calcination, and that gives an evidence that the catalyst contains both, γ-alumina and calcium acetate.

Besides assessing the effect of calcination on the morphology and crystallinity of the catalyst, it was also important to investigate the effect on pore size distribution. This is extremely important, because the catalytic activity depends strongly on the available surface area. Table 14 shows the main measurements of the catalyst before and after calcination.

Table 14: Surface area and porosity analyses of the catalyst before and after calcination

Properties	Before calcination	After calcination
BET surface area (m^2/g)	74.16	78.59
BJH pore size (cm^3/g)	0.361	0.415

As shown in Table 15, after catalyst calcination, the BET surface area and pore size slightly increased. The pore diameter after calcination is attributed to the centering, which may have taken place during the exposure to the excessive high temperature of calcination. The results agree with those reported earlier, in with the pore size of the catalyst increased with the increase of calcination temperature (Marinkovic et al., 2018), when using the same type of catalyst used in the previous experiments. The surface area of the catalyst in Marinkovic et al., (2018) work after calcination is 89.4 (m^2/g), which is close to the value got in this work. In addition, the pore size of the catalyst in Marinkovic et al., (2018) work after calcination is 0.455 (cm^3/g), which is also similar to the value got in this work. Nevertheless, the effect was not significant, and the pore sizes could be assumed to be mainly reserved.

The isotherm graphs, obtained by drawing the values of quantity adsorbed at different relative pressure values of the catalyst before and after calcination are shown in Figures 37 and 38, respectively.

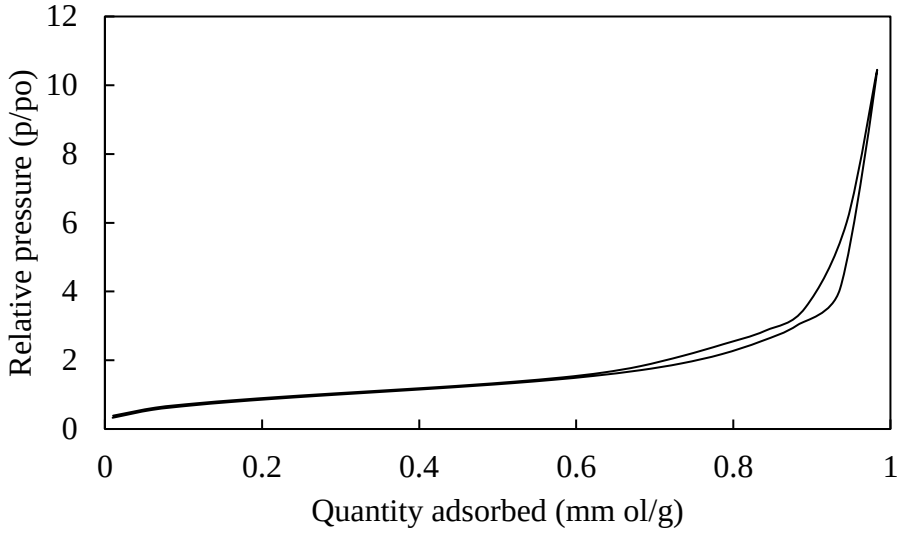

Figure 37: Adsorbed quantity at different relative pressures for the catalyst before calcination

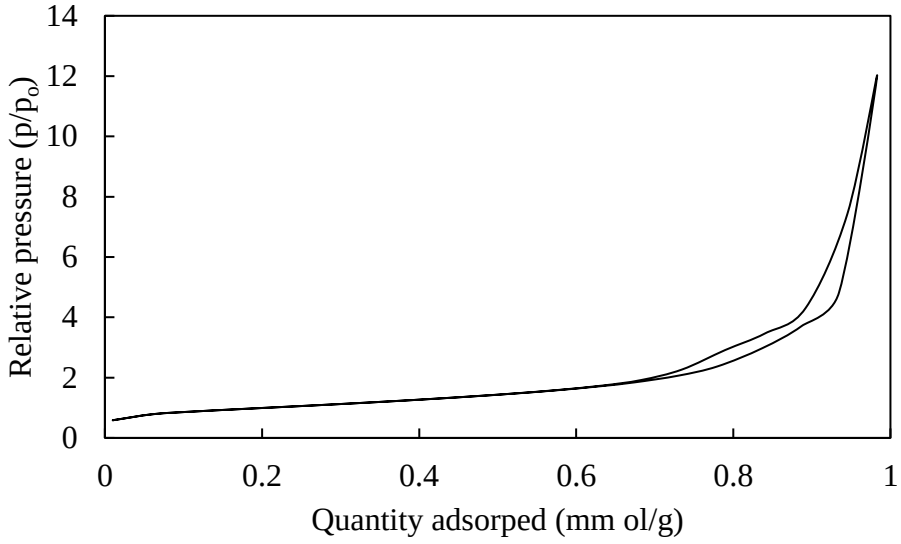

Figure 38: Adsorbed quantity at different relative pressures for the catalyst after calcination

The lower lines present the adsorption process, whereas the higher ones present the desorption process. The insignificant difference for both active and inactive catalyst, suggests that there are minimal mesopores. The difference between both lines represents the mesoporosity, which started to appear at and after a relative pressure of 2. There is low uptake in the high relative pressure, till reaching a value of 2, in which

there is a sharp increase in the uptake after this value of the relative pressure, and that can be noticed in both samples, before and after calcination. According to the isotherm classification, the catalyst is considered as IV type from IUPAC classification, that shows adsorption and desorption pathway with relative pressure where this type indicates the mesopores dominance with negligible amount of micropores. IV type is with pores in the range from 1.5 nm to 100 nm. As the pressure increases, increased uptake of adsorbate is shown as pores become filled.

3.3 Biodiesel experiment

The effects of catalyst wt%, methanol to oil ratio and temperature on the biodiesel production yield, after 4 h of reaction, were tested to optimize the process. The generated table for coded coefficients was used to analyze the significance of each variable (Table 15). If the p-value is less than 5%, then the factor or interaction is considered significant, as illustrated in the Pareto chart, shown in Figure 39.

Table 15: Coded coefficients for variable and interactions in the experiment

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	21.45	2.02	10.63	0.000	
x_1	3.546	0.934	3.80	0.000	1.00
x_2	-3.821	0.934	-4.09	0.000	1.00
x_3	-3.213	0.934	-3.44	0.001	1.00
x_1*x_1	-18.04	1.62	-11.15	0.000	1.00
x_2*x_2	-0.81	1.62	-0.50	0.620	1.00
x_3*x_3	2.19	1.62	1.36	0.182	1.00
x_1*x_2	-1.85	1.14	-1.62	0.113	1.00
x_1*x_3	-4.81	1.14	-4.21	0.000	1.00
x_2*x_3	1.51	1.14	1.32	0.193	1.00

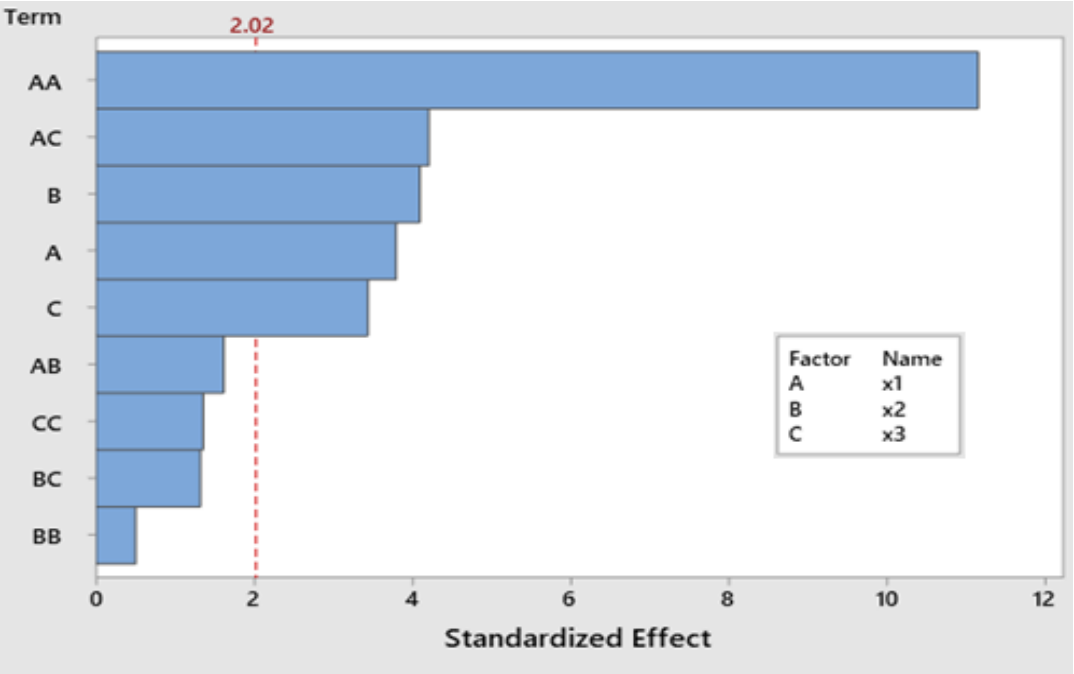

Figure 39: Pareto chart for the standardized effects (response is FAME yield %, α = 0.05)

It was found that the linear effects of the three factors were all significant. All higher order terms were insignificant, except x_1^2 and $x_1 x_3$. The results were used to fit the interactive regression model, given by Eq (19).

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \sum_{j=1}^{i-1} \beta_{ij} x_i x_j \quad (19)$$

Where, Y is the response (i.e., FAME yield%) and β_i and β_{ij} are the coefficients estimated from regression for the linear and quadratic terms, respectively. The overall equation with the estimated coefficients is shown Eq (20).

$$Y = 21.45 + 3.55x_1 - 3.82x_2 - 3.21x_3 - 18.04x_1^2 - 0.81x_2^2 + 2.19x_3^2 - 1.85x_1x_2 - 4.81x_1x_3 + 1.51x_2x_3 \quad (20)$$

A simplified form of the general model equation (Eq 20), with all insignificant terms eliminated in shown in Eq (21).

$$Y = 21.45 + 3.55\, x_1 - 3.82\, x_2 - 3.21\, x_3 - 18.04\, x_1^2 - 4.81\, x_1 x_3 \quad (21)$$

The assumption that the errors are normally distributed must be satisfied before statistically analyzing the experimental data. If this assumption was valid, the statistical procedures would then be an exact test of the hypothesis been made to test the effect of the factors on the response variable. The adequacy of the model has been investigated by examining the residuals, which are defined as the differences between the experimental values and the fitted value as per the model equation. As shown in the normal probability plot in Figure 40, the p-value was 0.431, which means to agree that the residuals are normally distributed, even if some of the blue points did not fall on the straight line, which indicates differences between observed and the fitted values, presented by the diagonal.

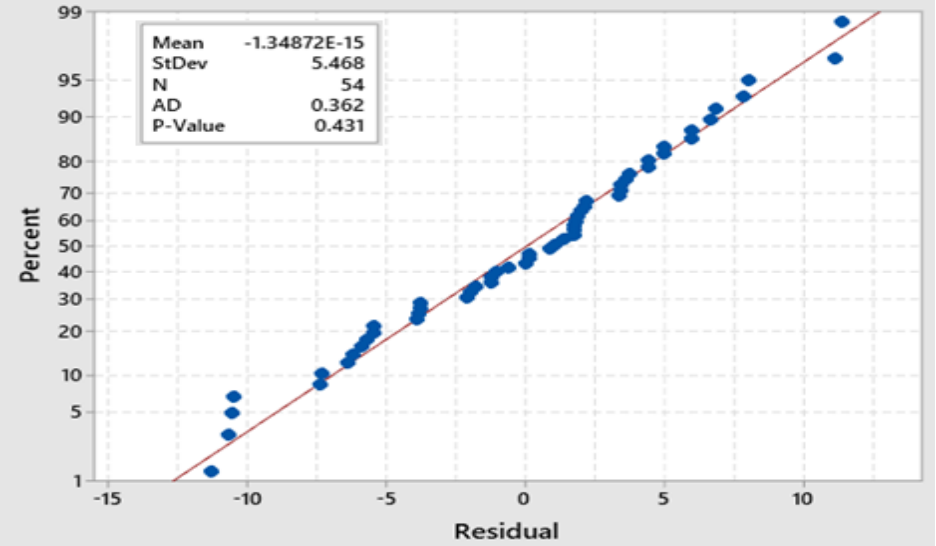

Figure 40: Normal probability plot of residuals

The plot of the residuals versus fitted value, shown in Figure 41, reveals no obvious pattern, which suggests a constant variance of the residuals. It also means that the predicted values of the dependent variable (i.e., production yield) by the regression model (Eq 21) was consistent across all the experimental values. Figure 42 shows the residuals versus the observations order, which clearly indicates that the residuals were randomly distributed around the zero line, which suggest that there is no correlation

between the residuals in case of observations order and thus, the residuals are independent.

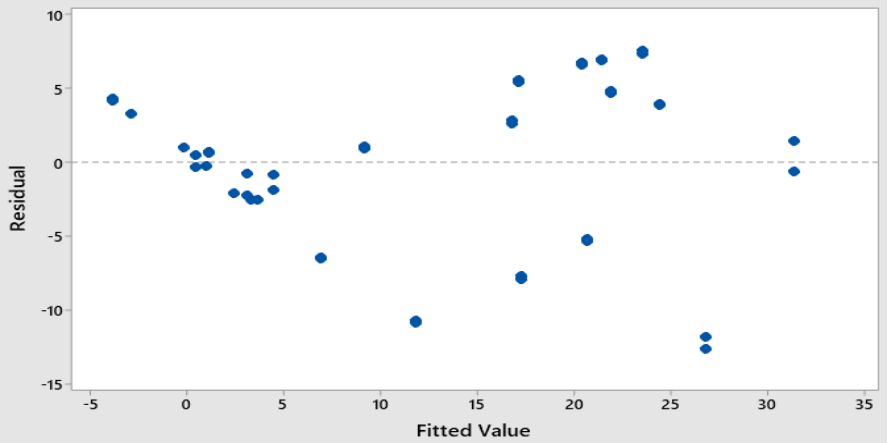

Figure 41: Residual versus fitted value

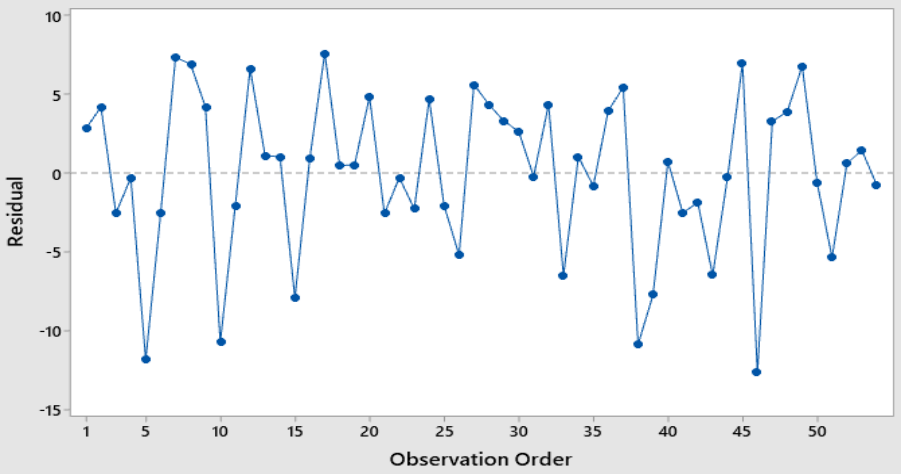

Figure 42: Residual versus observations order

The optimum conditions to achieve to the highest yield of FAME was obtained using response optimizer to be x_1=0.23, x_2=-1, x_3=-1. At these optimum conditions, the highest biodiesel yield was found to be 30.4%. The optimum conditions represent a catalyst wt% of 7.7, methanol:oil molar ratio of 12 and temperature of 45^{o}C. The optimum conditions were expected, since it was found previously that the increase in methanol:oil ratio and temperature decrease the FAME yield%.

The profile of FAME with respect to microalgae growth were determined. At optimum conditions, the FAME profile was determined as shown in Table 16.

Table 16: FAME profile at optimum conditions

Compound	Percentage, %
Methyl Palmitate	0.03
Methyl Palmitoleate	27.22
Methyl Stearate	0.51
Methyl Oleate	57.93
Methyl Linoleate	7.52
Methyl Arachidate	0.51
Methly Eicosenoate	0.62
Methyl Eicosadienoate	1.20
Methyl Behenate	0.07
Methyl Erucate	0.18
Methyl Decosadiconate	0.16
Methly Lignocetrate	3.69
Methyl Nervonate	0.37

The effect of changing catalyst wt%, methanol:oil molar ratio and temperature on FAME yield% is illustrated in response surface plots as shown in Figures 43-45.

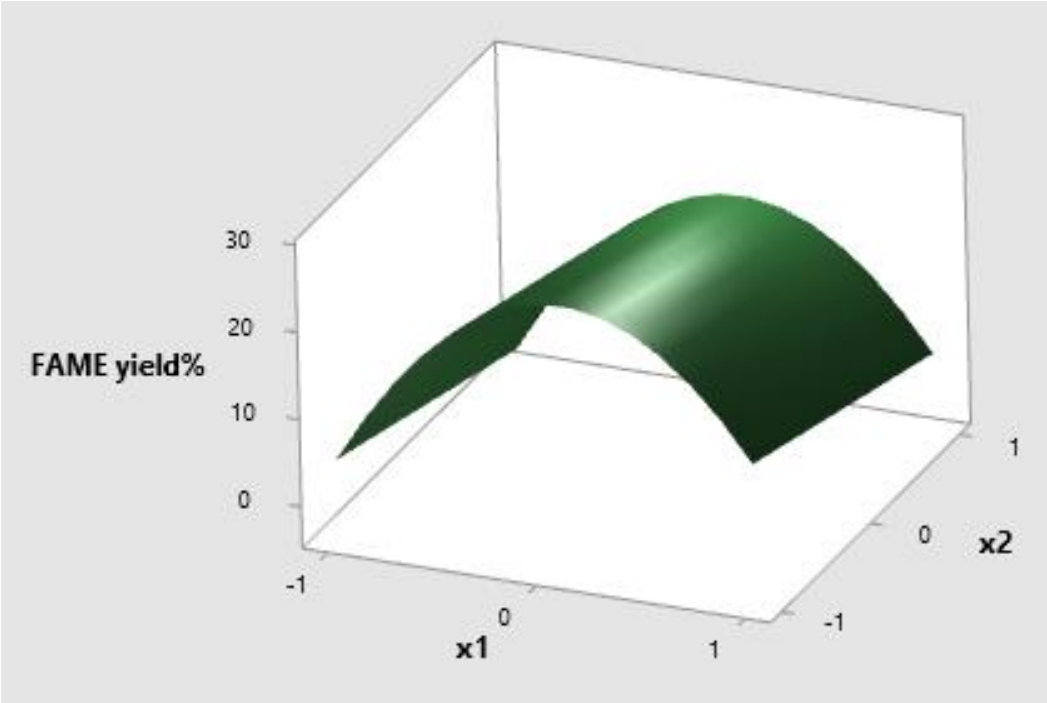

Figure 43: Surface plot showing the effect of catalyst and methanol to oil molar ratio at a constant temperature of 55ºC

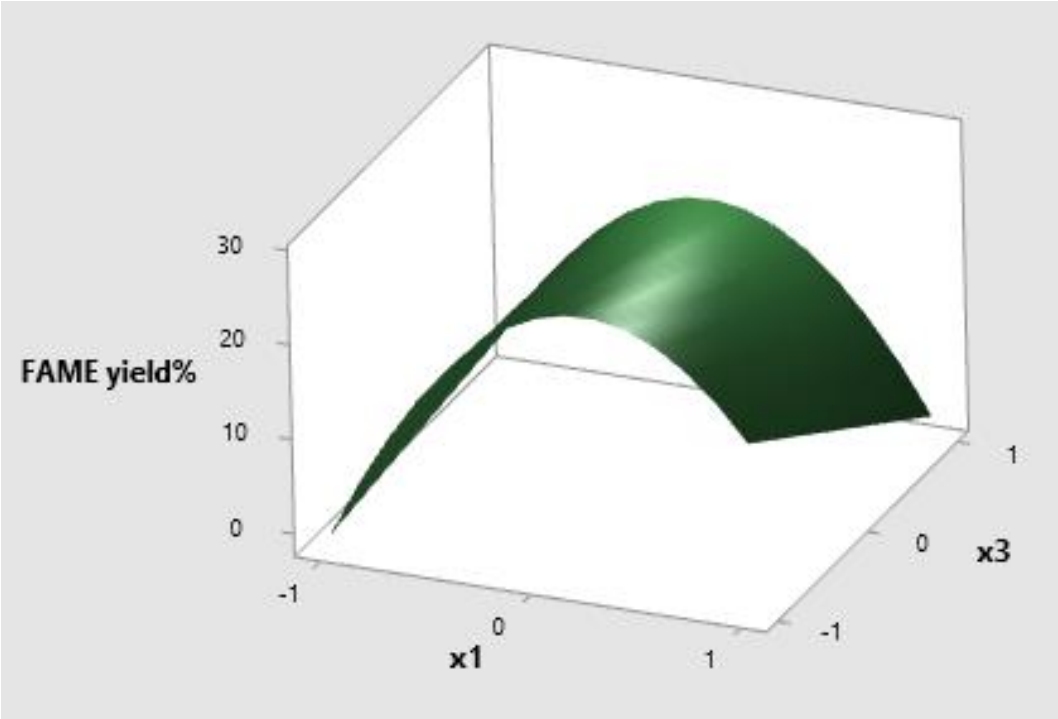

Figure 44: Surface plot showing the effect of catalyst wt% and temperature ratio at a constant methanol:oil ratio of 18

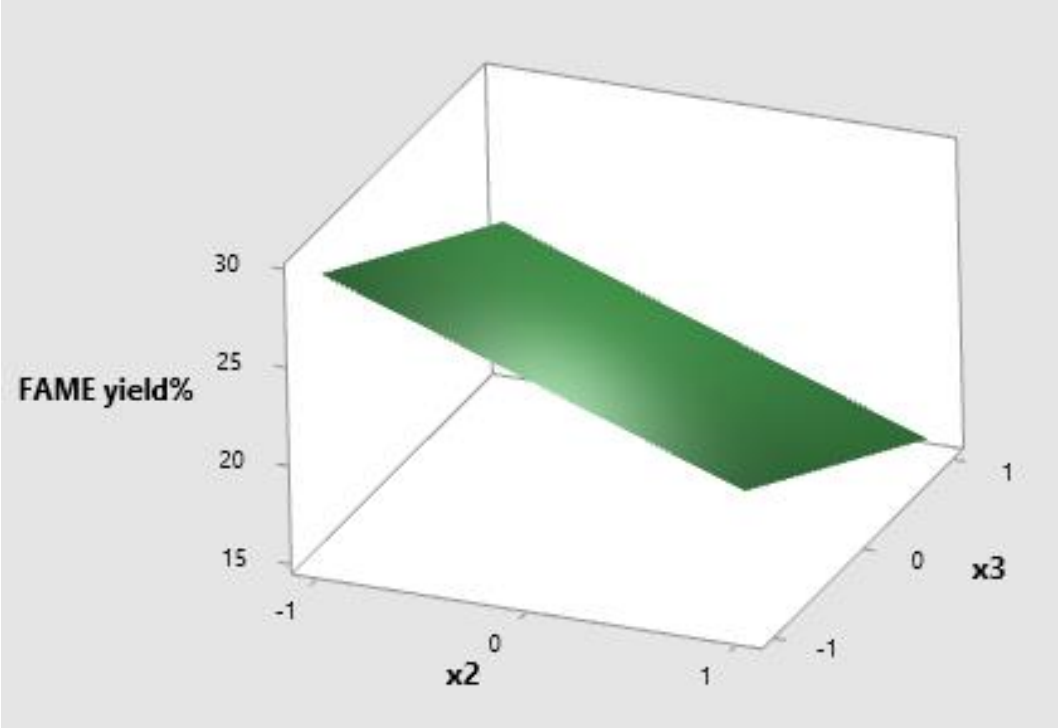

Figure 45: Surface plot showing the effect of temperature and methanol to oil molar ratio at a constant catalyst loading of 7%

It was found that the increase in temperature at all methanol:oil molar ratios generally resulted in decrease the FAME yield, except at catalyst load of 7%, where the effect of temperature tended to diminish. The reason could be due to the increase in the solubility of glycerol in the reaction mixture with excess amounts of methanol, which negatively affects the reaction rate by pushing the reaction backward on one hand and blocking the catalyst from the reactants on another (Rashid et al., 2008). High methanol:oil molar ratio (about 6:1), may cause difficulties in separating glycerol and biodiesel after the reaction. When performing transesterification using a solid catalyst, the formation of three phases (oil, solid catalyst and alcohol) restricts the contact between reactants, which reduces the FAME yield. According to that, the determination of the initial concentration of alcohol is important to keep a compromise between the diffusion rate by forming two phases of fluid and shifting the reaction toward FAME production (Encinar et al., 2010).

In the present work, the yield was found to drop with the increase in methanol:oil ratio for all temperatures and catalyst load. The increase in molar ratio of methonal:oil, usually results in driving the transesterification reaction forward (Hu et al., 2011). However, excess amounts of methanol increase the solubility of glycerol in the reaction mixture (Meher et al., 2006; Omar and Amin, 2011), which otherwise would be separated from the reaction mixture. The increase in glycerol solubility also results in increasing the viscosity of the system (Encinar et al., 2007). In addition, the highest FAME yield was obtained at catalyst load of 7 wt%.

A higher amount of catalyst had a negative effect on the yield. This unusual effect could be due to the lack of good mixing in the system, which resulted in the settling of catalyst in the bottom of the tubes when larger amounts were used.

The model given by Eq (2) was used to describe the rate of the transesterification reaction. It was assumed that the reaction rate be linear within the considered initial 4 h of reaction. Therefore, the rate was determined as the amount produced after 4 h over 4, and considered as initial rate of reaction, and the initial TAG concentration, C_{ao} was used instead of the concentration at any time, C_a. As the rate constants are temperature dependent, the terms in Eq (1) were calculated separately at each tested temperature.

Least square regression model was used to determine the rate constants, with Excel solver used to determine the constants that result in a minimum objective function given in Eq (22)

$$OF = \Sigma (R_{exp} - R_{pred})^2 \quad (22)$$

Where, OF represents the objective function and R_{exp} and R_{pred} are the experimental and the predicted initial rates of reaction. Tables 17 and 18 show the estimated kinetics parameters at different temperatures, and a comparison between the experimental reaction rate and the ones obtained by model, respectively.

Table 17: Kinetics parameters of transesterification reaction

Temperature (°C)	k_{app} (min^{-1})	K (mole/L)	C_r^0 (mole/L)
45	0.06	200	0.01
55	0.02	412	0.012
65	0.01	190	0.03

Table 18: Experimental and predicted reaction rate at different conditions

Temperature	Catalyst %	Methanol:oil	FAME yield%	Standard deviation	R_{exp} (mmole/L.h)	Standard deviation	R_{pred} (mmole/L.h)
45	5	12	1.60	1.05	0.11	0.26	5.55
		18	2.60	0.04	0.15	0.01	3.80
		24	3.60	0.01	0.18	0.00	2.76
	7	12	4.60	1.44	0.33	0.36	7.77
		18	5.60	0.12	0.33	0.03	5.31
		24	6.60	0.12	0.33	0.03	3.86
	10	12	7.60	0.19	0.54	0.05	11.08
		18	8.60	0.12	0.51	0.03	7.58
		24	10.19	0.11	0.51	0.03	5.50

Table 18: Experimental and predicted reaction rate at different conditions (cont'd)

Temperature	Catalyst %	Methanol:oil	FAME yield%	Standard deviation	R_{exp} (mmole/L.h)	Standard deviation	R_{pred} (mmole/L.h)
55	5	12	0.79	0.02	0.06	0.00	1.08
		18	0.88	0.00	0.05	0.00	0.74
		24	0.36	0.01	0.02	0.00	0.54
	7	12	28.35	0.03	2.02	0.01	1.50
		18	28.35	0.03	1.67	0.01	1.03
		24	19.53	0.13	0.98	0.03	0.75
	10	12	1.05	0.10	0.07	0.03	2.16
		18	0.49	0.01	0.03	0.00	1.48
		24	0.37	0.01	0.02	0.00	1.07
65	5	12	0.74	0.00	0.05	0.00	2.92
		18	0.31	0.06	0.02	0.02	2.00
		24	0.31	0.01	0.02	0.00	1.45
	7	12	26.66	0.11	1.90	0.03	4.06
		18	27.08	0.12	1.59	0.03	2.77
		24	9.50	0.13	0.48	0.03	2.03
	10	12	3.12	0.01	0.22	0.00	5.84
		18	1.78	0.02	0.10	0.01	4.00
		24	0.47	0.01	0.02	0.00	2.91

In order to obtain calcination energy using Arrhenius equation (Eq 12), the values of natural logarithmic of rate constant were plotted versus 1/T. The obtained value of the linear slope was used to calculate the calcination energy. The calcination energy for Marinković et al., (2018) model is 80.2 KJ/mole. Increasing temperature of the reaction resulted in slightly increase in the values of rate constant, which can be explained according to the increase in the kinetic energy of particles that makes them move faster that before. Similar trend is shown with the values of C_r^0, which represents the initial available active catalyst surface. On the other hand, the values of K, which represents the affinity of TAG for the active sites of the catalyst, increases as temperature increases till 55°C, and then it decreases. The results of the model didn't show the effect of temperature on the calculated constants since it was based only on one reaction temperature, which is not the case in the experiments of this work. According to that, it is difficult to compare the trends in this work and Marinković et al., (2018) work.

Chapter 4: Conclusion

To sum up, this work focuses on using microalgae as a cheap and safe way for phenol removal and biodiesel production. The main objectives of this thesis are to examine the ability of different stains (*Chorella sp.* and *Tetraselmis sp.*) to remove different types of phenols (phenol, 4-nitrophenol and 2,4-dinitrophenol) with different concentrations, and then to use the collected biomass for biodiesel production. The optimum conditions of transesterification reaction to produce biodiesel from biomass was determined. It was found that the increase in temperature at all methanol:oil molar ratios generally resulted in decrease the FAME yield, except at catalyst load of 7 %. The highest biodiesel yield was found to be 30.4% at a catalyst wt% of 7.7, methanol:oil molar ratio of 12 and temperature of 45^{o}C.

It was found that using microalgae to remove phenols is a safe and cheap way compared to other conventional methods, since this way is doesn't require a lot of energy and it doesn't cause any negative impacts. In addition, the collected biomass after phenols removal is a valuable source of biodiesel. This work indicates that microalgae is a valuable source of biodiesel production with many advantages compared to other sources.

Future researches should focus on enhancing the ability of microalgae to remove other hydrocarbons rather than phenols only from industrial wastewater and studying the factors that could affecting that. In addition, it is necessary to look into the possible ways that could enhance the content of fatty acid in microalgae in order to high more yield of biodiesel and reducing the overall cost of biodiesel production using microalgae. The main limitations of this work include the difficulties of extracting oil from small amounts of microalgae.

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