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Biopolymer Production from Microalgae in Different Culture Setting

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ABSTRACT

In modern days, the most prominent materials used in many aspects of human life are polymers. Without polymer it is impossible to see the advancement of nowadays technologies. The key source of polymeric content is fossil fuels, which are constantly decreasing. Therefore, it is utmost important to discover another alternative resource, especially from biological sources for the sustainability production of polymers. Biopolymers are polymers from renewable materials that are produce by plants, animals, bacteria, fungi and algae. In certain aspects, algae are one of the most promising species in the production of biopolymer. Microalgae that are left abandoned in the environment ultimately become waste material. With the use of their biomass, the wasted microalgae may provide a function. This research was carried out to determine the possible use of *Spirulina sp.* and *Chlorella sp*. microalgae strains for the biosynthesis of polyhydroxyalkonoate (PHAs) and study the effect of different culture setting toward the production of PHAs. In this study, the algae were cultivated in the lab scale for the selection of the best microalgae growth. The selected microalgae were then proceeded to be upscaled in the bottle culture and raceway pond system. The microalgae biomass was harvested during the stationary phase and the extraction of PHA was performed. The extracted PHAs were analysed using FTIR analysis in order to examine the purity, properties and composition of polymer produced for future used.

1. Introduction

In today's industrial driven world, the global demand of plastic-based material keeps increasing and adding more problem to the current waste management operation. Plastics are the most common materials in our modern economy because they are inexpensive, lightweight and adaptable [20]. It is reported that only 14 % of all plastic packaging and plastic based material is collected for recycling leaving more than eight million tons of used plastics waste discharged into the ocean. Nearly half of the plastic waste produced involve plastics that are made from non-renewable petrochemicals which is driven from fossil oil, natural gas and coal [6]. These types of plastics have large impact on the environment as they are usually non-biodegradable and lead to large accumulation of plastic waste. Degradable polymers such as bio-plastics have been recognised as an alternative to conventional oil-based plastics and their production has increased considerably in recent decades [8]. In order to reduce the use of non-renewable energy in the industry, the need for "green materials" has grown considerably in the recent years. The principle of green material lies on

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the factors of sustainability, industrial ecology, eco-efficiency and green chemistry [2]. Bioplastics are one of the green materials that is derived from renewable sources such as agricultural resources and biomass feedstock which comply with the principle of materials that are eco-efficient and sustainable.

The aim of this project is to create a bioplastic material based on cultivation of microalgae. Attempts will be made to remove synthesized polymers from the microalgae to accomplish this goal. Algae are photosynthetic organisms that can tolerate a wide range of temperatures, salinities, pH values, different light intensities and can grow in a range of aquatic habitats, including lakes, pounds, rivers, oceans and even wastewater either alone or in symbiosis with other organisms.

In recent research, it is said that microalgae can produce a wide range of bio products, including polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds and antioxidants which is a fundamental raw material in the industry. As microalgae naturally generate polymers, they may potentially be used in the development of bioplastics. The generation of polymer specifically, PHAs interest are currently being used in the production of plastics. PHAs is a valuable carbon-neutral polymer that are produced from carbon sources by microorganisms in natural habitats [18]. For this particular project there will also be an attempt to study the effect of different nitrogen concentration on both biomass and lipid production for the chosen microalgae and construct a plastic material following successful extraction of the PHAs. PHAs have normal thermoplastic properties that is suitable to create a plastic film without alternation. However, the properties of extracted PHAs depend on the composition of the PHA, the purity of the extracted material, molecular weight and the volume of PHA extracted. With this project, the possibility of extracting polymers from microalgae in different cultivation condition to create a bioplastic material will be investigated. If the results are encouraging and a reliable plastic material is made, further analysis into the optimum nitrogen content for microalgae in the production of PHAs components may lead to a reliable source of bioplastics.

Since the supplies for oil-based plastics are extracted from non-renewable resources that are eventually too expensive to recover, green materials have been recognised as a significant renewable option for a more sustainable life cycle for plastic products that could be generated in the manufacture of plastic using green materials. Microalgae have been found as a potential to develop a microalgae-based bioplastic material [5].

PHAs are the biopolymer of choice if we look for a substitute of petroleum-based non-biodegradable plastics. Microbial production of PHAs as carbon reserves has been studied for decades and PHAs are gaining attention for a wide range of applications in various fields. Still, their uneconomical production is the major concern largely attributed to high cost of organic substrates for PHA producing heterotrophic bacteria. Therefore, microalgae or cyanobacteria, being photoautotrophic, prove to have an edge over heterotrophic bacterium. They have minimal metabolic requirements, such as inorganic nutrients like the use of carbon dioxide, nitrogen and phosphorus. They can also survive under adverse environmental conditions. PHA production under photoautotrophic conditions has been reported from various strains of microalgae making it possible to be the next biopolymer sources.

Table 1
Classification of biopolymer according to degradability

Biodegradable			*Non-biodegradable*	
Biobased			*Fossil based*	*Biobased*
Plants	*Microorganism*	*Animals*		
Cellulose and its derivatives (polysaccharide)	PHAs (e.g., P3HB, P4HB, PHBHV, P3HBHHx)	Chitin (polysaccharide)	Poly(alkylene dicarbo-xylate)s (e.g., PBA, PBS, PBSA, PBSE, PEA, PES, PESE, PESA, PPF, PPS, PTA, PTMS, PTSE, PTT)	PE (LDPE, HDPE), PP, PVC
Lignin	PHF	Chitosan (polysaccharide)	PGA	PET, PTT
Starch and its derivatives (monosaccharide)	Bacterial cellulose	Hyaluronan (polysaccharide)	PCL	PU
Alginate (polysaccharide)	Hyaluronan (polysaccharide)	Casein (protein)	PVOH	PC
Lipids (triglycerides)	Xanthan (polysaccharide)	Whey (protein)	POE	Poly(ether-ester)s
Wheat, corn, pea, potato, soy, potato (protein)	Curdlan (polysaccharide)	Collagen (protein)	Polyanhydrides	Polyamides (PA 11, PA 410, PA 610, PA 1010, PA 1012)
Gums (e.g., *cis*-1, 4-)	Pullulan (polysaccharide)	Albumin (protein) polyisoprene	PPHOS	Polyester amides
Carrageenan	Silk (protein)	Keratin, PFF (protein)		Unsaturated polyesters
PLA (from starch or sugar cane)		Leather (protein)		Epoxy
				Phenolic resins

Abbreviations: HDPE, high density polyethylene; LDPE, low density polyethylene; P3HB, poly(3-hydroxybutyrate); P3HBHHX, poly(3-hydroxybutyrate-*co*-3- hydroxyhexanoate); P4HB, poly(4-hydroxybutyrate; PBA, poly(butylene adipate); PBS, poly(butylene succinate); PBSA, poly(butylene succinate-*co*-adipate); PBSE, poly(butylene sebacate); PC, polycarbonate; PCL, poly(ε-caprolactone); PE, polyethylene; PEA, poly(ethylene adipate); PES, poly(ethylene succinate); PESA, poly(ethylene succinate-*co*-adipate); PESE, poly(ethylene sebacate); PET, poly(ethylene terephthalate); PFF, poultry feather fiber; PGA, poly(glycolic acid) or polyglycolide; PHA, polyhydroxyalkanoate; PHBHV, poly(3 hydroxybutyrate-*co*-3-hydro-xyvalerate); PHF, polyhydroxy fatty acid; PHH, poly(3-hydroxyhexanoate); PLA, poly(lactic acid) or polylactide; POE, poly(ortho ester); PP, polypropylene; PPF, poly(propylene fumarate); PPHOS, polyphosphazenes; PPS, poly(propylene succinate); PTA, poly(tetramethylene adipate); PTMS, poly(tetramethylene succinate); PTSE, poly(tetramethylene sebacate); PTT, poly(trimethylene terephthalate); PVC, poly(vinyl chloride); PVOH, poly(vinyl alcohol); PU, polyurethane.

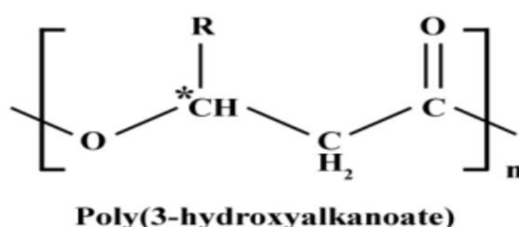
1.1 Polyhydroxyalkanoates (PHAs)

The general definition of a polymer is a macromolecule that is composed of a long chain of several repeating units, known as monomers [11]. Polymers are formed by polymerization of monomers and can either be one type that is homopolymers or of composed of several types that is copolymers. All polymers have unique and diverse properties because each polymer molecule is made of many parts of monomers that vary in their sequence and the length of polymer chains. Generally, it is possible to trace the origins of polymers from either natural or synthetic materials.

Naturally, different species of bacteria such as *Spirulina sp*. and *Chlorella sp*. synthesize PHAs as their intracellular compound for energy and carbon storage. In harsh conditions, such as under conditions of limiting nutrients the synthesize PHAs will be degraded and used by bacteria as a source of carbon and energy [12]. Where there is an excess supply of carbon and nutrients are limited, PHA production will occurs in the microorganism system [17]. PHA in bacteria can be observed, as granules contained in the cytoplasm. The first PHA discovered in *Bacillus megaterium* in the form of poly (3-hydroxybutyrate) (PHB) in 1925 by the French scientist Lemoigne [4]. PHAs are natural polyesters of 3-, 4-, 5-, and 6-hydroxyalknoic acids thus having a trait of thermoplastics. In recent discoveries, more than 90 generals of both Gram-positive and Gram-negative bacteria have been identified as PHAs producers under both aerobic and anaerobic conditions.

1.1.1 Chemical structure of PHAs

The structure of PHA is diverse and very dependent on the carbon source available and the species of microorganism involved. Generally, the majority of synthesize PHA consist of (R)-3-hydroxy fatty acid monomers linked together by ester bonds. The R-configuration is mainly because of their chirality and stereo-specificity of the enzymes involved in biosynthesis process. The length of PHAs depends on the carbon atoms present in the HA monomer unit. PHAs are grouped into four types based on the number carbon atoms present in each HA monomer unit that is the short chain length PHA, medium chain length PHA, long chain length PHA and the co-polymers consisting of short chain and medium chain length PHAs [1].



R group	Carbon no.	PHA polymer
methyl	C_4	Poly(3-hydroxybutyrate)
ethyl	C_5	Poly(3-hydroxyvalerate)
propyl	C_6	Poly(3-hydroxyhexanoate)
butyl	C_7	Poly(3-hydroxyheptanoate)
pentyl	C_8	Poly(3-hydroxyoctanoate)
hexyl	C_9	Poly(3-hydroxynonanoate)
heptyl	C_{10}	Poly(3-hydroxydecanoate)
octyl	C_{11}	Poly(3-hydroxyundecanoate)
nonyl	C_{12}	Poly(3-hydroxydodecanoate)
decyl	C_{13}	Poly(3-hydroxytridecanoate)
undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)

Fig. 1. General structure of PHAs [1]

1.1.2 Properties of PHA

Since PHAs have different structural variations in monomers, different properties and chemical composition are shown for homo and copolymers. However, the core properties of a polymer are the same for all PHA polymers thus making them efficient compared to conventional plastics and other bioplastics. PHAs have good resistance to hydrolytic attack making them insoluble in water and sink in water which facilitates anaerobic biodegradation in sediments. PHA also shows a resistant towards UV. In addition, they are biocompatible and biodegradable which means that it can naturally undergoes degradation in soils and behave as piezoelectric materials. However, the biodegradation of PHAs depend on the type and composition of the polymer, surrounding conditions and the type of microorganisms it came from due to the fact different that microorganisms produce different PHA-*depolymerases* to degrade certain PHAs. PHAs generally are soluble in chloroform and other chlorinated solvents. Their glass transition temperature varies from −50-4°C and have a melting temperature ranging from 40-180°C. Other properties such as thermodegradation temperature,

tensile strength, Young's modulus, water vapor and oxygen transmission rate vary according to the type of polymer produced and the composition of monomeric unit.

1.1.3 PHAs biosynthethic pathways

For the biosynthetic pathway of the PHA production there are three well-known and established pathways as in Figure 2. In each of the pathways the monomer composition is closely related to the carbon source in the process. The best and well known among the PHA biosynthetic pathways used by *C. Necator* is Pathway I. In this pathway, 3HB monomers are generated by the condensation of two acetyl-CoA molecules from the tricarboxylic acid (TCA) cycle and form acetoacetyl-CoA by the help *ketothiolase* enzyme. *Acetoacetyl CoA reductase* react with acetoacetyl-CoA to form 3-hydroxybutyryl-CoA. Finally, the *PHA synthase* enzyme catalyses the polymerization *via* esterification of 3 hydroxybutyryl-CoA into poly (3-hydroxybutyrate) (P (3HB)).

Pathways that involved in fatty acid metabolism generate different hydroxyalkanoate monomers and were utilized in PHA biosynthesis. Pathway II generates substrates by the oxidation process of fatty acids that can be polymerised by the PHA synthases of Pseudomonads belonging to the ribosomal RNA-homology group I such as *Pseudomonas aeruginosa*. In *A. Caviae*, the oxidation intermediate, trans-2-enoyl-CoA is converted to (R)-hydroxyacyl-CoA by a (R)-specific *enoyl-CoA hydratase*. Pathway III is of significant interest because it helps generate monomersfor PHA synthesis from structurally unrelated and simple, inexpensive carbon sources such as glucose, sucrose and fructose. The (R)-3-hydroxyacyl intermediates from the fatty acid biosynthetic pathway are converted from their acyl carrier protein (ACP) form to the CoA form by the enzyme *acyl-ACP-CoA transacylase* (encodedby phaG) [3].

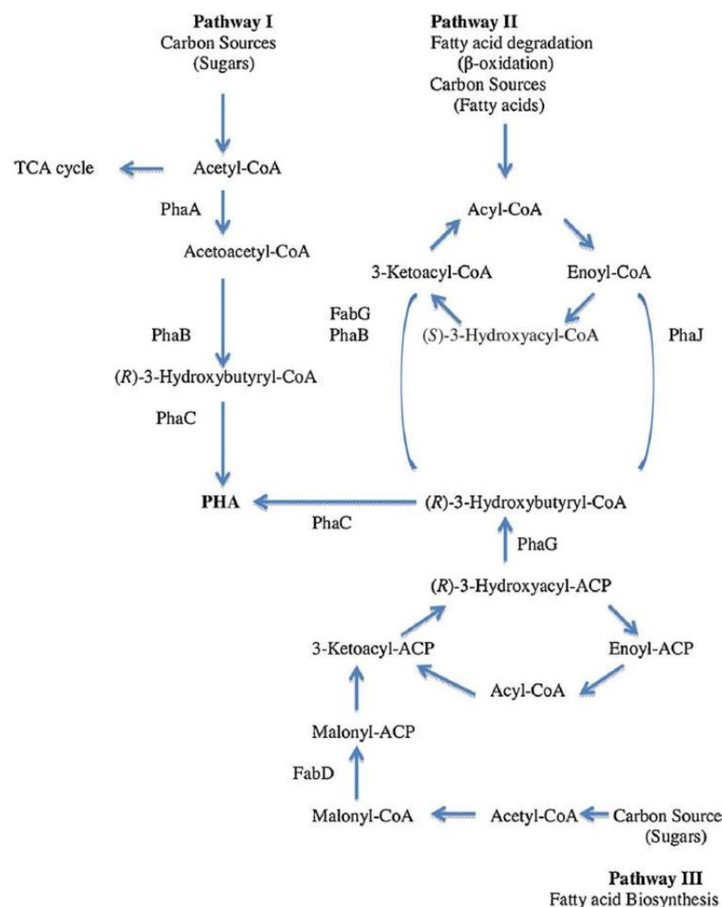


Fig. 2. PHA Biosynthetic Pathway [3]

1.1.4 Application of PHAs

The PHA biopolymer has the same potential with synthetic plastics and capable to be processed for the food packaging industries as they are safe for food and can help to improve self-life. These type of bioplastics can also contribute to the decrease in carbon food print for packaging industries. There have been a series of report that PHB was used for heart valve tissue engineering. At present moment extensive research activities are still in progress to use PHAs polymers to construct a biodegradable scaffold to be use in the construction and engineering of living tissue heart valves and also be used as replacement for defective or diseased values. The biodegradability and biocompactibilty of PHA has been the main reason to be involved in modern medical care methods, such as drug delivery system. Micro and nanospheres of PHA are used as polymers and act as outer shell in which drugs are incorporated and released when the polymer coating starts to degrade in the body. The PHAs can also be used in surgical application for repairing damaged nerves as they are piezoelectric. The PHB had also been used as an alternative to the conventional nerve graft and showed promising results in rats regarding nerve regeneration and wound dressing.

1.2 Microalgae

Microalgae or microphytes, are a type of microscopic-scale algae that can grow in practically any atmosphere dependent on water. The size of the microalgae ranging from a few hundred micrometres, and unlike plant they do not grow leaves or roots. Microalgae usually generate oxygen through photosynthesis, just like other green plants. Microalgae also contain different compounds of pigment that are formed during development that can be distinguished by closed aromatic rings or long chains of carbon and their color are determined by the presence of pigments such as chlorophylls, carotenoids and phycobiliproteins that are responsible for the green, orange and red or blue colors, respectively. Among the 5 different species of microalgae reported to be high in PHA in this project, *Spirulina sp*. and *Chlorella sp.* was focused on an effort to create a bioplastic substance. Algae are also very common in aquatic ecosystems because of their role as the key producers of oxygen that perform photosynthesis. In recent years microalgae are also used for the benefit of mankind with the aid of biotechnology, in the production of food additives, source of fine chemicals and also the production of biodiesel. Several species of microalgae have been studied for these purposes, including *Spirulina sp.*, *Chlorella sp.* and *Dunaliella sp*. [15]. In addition, the production of microalgae on an industrial scale can be successfully accomplished due to the vast amounts of biomass that can be cultured in photobioreactors.

1.2.1 Chlorella sp.

Chlorella is a microalgae originates from the genus of green algae, which can be found in freshwater and can contains around 58 % protein by weight. Due to its dense cell walls, *Chlorella sp.* has a higher crack resistance and higher thermal stability compared to *Spirulina.* This species is often used in biomass– polymer blends due to the fact that after comparing between bioplastic production from 100 % microalgae biomass and blends containing additives and polymers, it was ruled out that blending is necessary for commercial applications. Many tests are conducted to measure product quality showed that when using *Chlorella*, a higher quality of bioplastic could be obtained compared to *Spirulina*. However, *Spirulina* has showed a better blending property when compared to *C. vulgaris* based on the physicochemical features of the material. A study was conducted to examine the effect of the compatibilizer ratio on the quality of produced PVA (polyvinyl alcohol)-C. vulgaris composites.

The study suggested that the best quality can be obtained with a compatibilizer that is maleic anhydride with a concentration of 6 % in the mixture.

1.2.2 Spirulina sp.

Spirulina, has been widely used as a protein source for many years in the food industry, the microalgae is also known for its adaptation potential to extreme environments. *Spirulina platensis* contains a high concentration of protein. Several studies have been conducted to study the potential of *Spirulina* for bioplastic production. Closely related to *Chlorella*, *Spirulina* also have a small cell size that makes both of the microalgae suitable for bioplastic blend production. Despite their similarities, due to the varying amino acid contents, *Chlorella* and *Spirulina* showed different behaviours and bioplastic properties while blending with PE. With the addition of 6 wt % of a compatibilizer into *S. platensis* and PVA mixture, a higher tensile strength bioplastic film compared to commercial plastic bags was produced.

It is also noted that the usage of a compatibilizer can also increase the elongation ability of the plastic and smoother surfaces can be observed. *S. platensis* was observed to be used as a reinforcement material in plasticized wheat. However, the microalgae biomass particles that have a particle diameter higher than 5 µm did not show efficient reinforcement ability and were not able to blend with other material compared to smaller particles. Adding glycerol at different concentrations in the rage of 15–30 % is another method to increase the flexibility of the plastic produced from *S. platensis* and addition of 30 % glycerol were observed to increase tensile strengths and lower elongations from the produced bioplastic when compared to commercial plastic bags. These results suggest that this type of bioplastic can be used for food packaging, pharmaceutical applications or cosmetics, where high elongation is not needed. After the plasticization or implementation of blending of *Spirulina* a more improved homogenous phase distribution and higher inter-surface adhesion were obtained. The addition of compatibilizer however, did not show an improvement in the mechanical properties of the produced bioplastic [18].

2. Methodology

The production and characterisation of the PHAs from *Spirulina sp.* and *Chlorella sp.* in different culture setting which is lab scale and mass production were researched. This study was divided into three parts and carried out accordingly. The first part was to cultivate and produce PHAs from *Spirulina sp.* and *Chlorella sp.* under standard lab scale in the respective culture medium. The first part include the preparation of the medium and the cultivation condition of the microalgae species. The second part of the research focused on the method used to extract and quantify the PHAs from the cultured microalgae. Lastly the final part was to examine the effect of different culture setting toward the biomass and PHA production in the selected microalgae.

2.1 Preparation of Culture Medium and Microalgae Cultivation

The medium that was used is Zarrouk's medium and AF6 Medium. AF6 medium was used to culture *Chlorella sp.* and Zarrouk's medium was prepared for cultivating *Spirulina sp.* The second species that is *Spirulina sp.* required relatively high pH values between 9.5 and 9.8 in order to grow, which effectively inhibits contamination by most algae in the culture [13]. Therefore, Zarrouk's medium was successfully served as the standard medium (SM) for *Spirulina* culture for many years [16]. The AF6 medium was prepared by adding 4 stocks solution in the table 2 into a 1000 mL of the

medium. The stock solution in the Table 2 was prepared by weighing the compounds and dissolving with distilled water based on formula. Then, by following the AF6 formula measurements pipette the amount of the stock solution in a 1 L Schott bottle and the bottle was added with distilled water until the medium achieved 1 L. The Zarrouk's medium was prepared by weighing each of the chemical listed in Table 3 and the chemical was mixed with 1000 mL of distilled water in the Schott bottle. Both of the medium was then covered and autoclaved to avoid contamination.

Table 2
Procedure for standard AF-6 culture

Solution A		2 mL in 1L
$NaNO_3$	14 g	Stock in 200 mL
NH_4NO_3	2.2 g	
$MgSO_4.7H_2O$	3 g	
$CaCl_2.2H_2O$	1 g	
Fe-citrate	0.2 g	
Citric acid	0.2 g	
Solution B		1 mL in 1L
KH_2PO_4	1 g	Stock in 100 mL
K_2HPO_4	0.5 g	
PIV metals		1 mL in 1L
$FeCl_3.6H_2O$	98 mg	Stock in 100 mL
$MnCl_2.4H_2O$	18 mg	
$ZnSO_4.7H_2O$	11 mg	
$CoCl_2.6H_2O$	2 mg	
$Na_2MoO_4.2H_2O$	1.25 mg	
$Na_2.EDTA.2H_2O$	500 mg	
Vitamin Mix		1 mL in 1L
Biotin	0.2 mg	Stock in 100 mL
Thiamine HCL	1 mg	
Vitamin B6	0.1G	

Table 3
Procedure for standard Zarrouk's medium

Ingredients	Amount (g/l)
NaHCO3	16.8
NaNO3	2.5
NaCl	1.0
K2SO4	1.0
K2HPO4	0.5
MgSO4・7H2O	0.2
FeSO4・7H2O	0.01
CaCl2.2H2O	0.04
EDTA	0.08
Distilled water	1000 mL

For lab scale cultivation, 200 mL of Zarouk's medium and AF6 medium are used for the cultivation of *Spirulina sp*. and *Chlorella sp*. respectively. The inoculation rate of 10 % from the total volume of the medium was taken from the stock culture provided in the laboratory. The aeration rate for the lab scale culture system was set approximately 0.5 l/min using aquarium pump and 24 h light intensity of 150 µE $m^{-2}s^{-1}$ w was optimal for biomass production [23].

2.2 Growth Curve Determination

The growth curve of the respective microalgae in lab scale, bottle culture and raceway pond were determined using UV-vis spectroscopy for the cell optical density once every two days in the wavelength of 750 nm in order to prevent the absorption of light by cellular pigments such as chlorophyll and carotenoids and is treated as a pure light scattering measurement [24]. A graph for the optical density against 30 days was plot to determine the phase of the microalgae and comparing their respective growth rate. The underlying absorption theory is according to the Beer-Lambert law (1760) of spectrometry. The equation is as follows:

$$A = \varepsilon c l \tag{1}$$

Where:
A= absorbance (unitless), ε = molar absorptivity of compound or molecule in solution ($M^{-1}cm^{-1}$), c = concentration of solution (M), l = path length (usually 1 cm).

UV-vis spectroscopy data can be used to analyses both qualitative and quantitative information about the substance used in the process. For quantitative information on the substance, a calibration of the instrument using known concentrations of the component in concern of the solution with the same solvent as the unknown sample are required and an unknown sample would be necessary in order to analyses for quantitative information on the substance. Lastly, a calibration curve is required along with the concentration of the component in solution must be determined when analyzing a degradation research or reaction.

2.3 Upscaling Process

After comparing the growth curve between *Spirulina sp*. and *Chlorella sp*., microalgae with the best growth curve were selected for the upscaling process. The upscaling process comprises of two type of system which is the open system and the closed system. For the closed system a 20 L bottle culture that mimic a photobioreactor was used for the cultivation process. The conditioning for the bottle culture was about the same as the lab scale which is 20°C temperature but the lighting condition was double to 300 µE $m^{-2}s^{-1}$ w 24 h fluorescent luminescence due to lager volume used. The total volume for the culture medium is 15 L per bottle culture and the inoculation rate are 10 % which is 1.5 L of *Spirulina* stock culture. The open system used a 300 L raceway pond with the condition of 27-31°C temperature 100 L of tap water disinfected by sodium hypochlorite overnight and 100 mL inoculation for economical purposed. The growth rate, condition, biomass and PHA production for both system were recorded and analysed.

2.4 Optimization of Raceway Pond Condition

The optimization of the raceway pond culture was done by manipulating the medium and the inoculation rate. The initial setup for the raceway pond was using 100 L of disinfected tap water and 100 mL of stock culture for the inoculation process. To further improve the condition, 100 L of the disinfected water was added with 10 L of medium and the volume of the inoculation was also increase to 1 L stock culture with and additional agitation using the motor-based system.

2.5 Extraction of PHA from Microalgae
2.5.1 Freeze dry

Freeze drying often referred to as lyophilization or cryodesiccation, is a low-temperature dehydrating technique that requires freezing the product, decreasing pressure and then sublimating the ice [21]. This contrasts with the dehydration caused by the majority of conventional methods, which use heat to evaporate water from the sample [22]. For the first part of the extraction process, 50 mL of sample from lab scale, bottle culture and raceway pond are harvested at their stationary phase and centrifuged at 5000 rpm for 10 mins and the supernatant was discarded. The pellet was then stored in the freezer under -80°C overnight to undergo pre freezing process before entering the freeze dry machine for 24 h.

The freeze-drying process involve primary and secondary drying. Primary drying is the first process that happen as soon as the sample enter the machine and the vacuum pump start to eliminate the air inside the machine. The decreasing of the air from the vacuum suction will create a low-pressure environment that initiate the process of evaporative cooling on the sample which allow an energy in the form of heat to hasten the overall process. It is estimated that approximately 93 % of the water content inside the sample was sublimated out at the end of the primary drying process [7].

Secondary drying phase aim to drive off water molecules that are bound in the sample. In this stage to further remove the excess moisture from the sample, an additional heat is supplied to the drying process which in the end leaving only as little as 2 % of moisture content in the sample [7]. The main objective of freeze drying is to preserve the biological and chemical structure of a sample and making sure that the sample can be stored in longer term and ready to be used for testing. Freeze drying also important in determining the dry mass of the sample in an experiment.

2.5.2 PHA extraction

Algal species from respective culture system was harvested during the stationary phase of the growth curve with a same volume of 50 L per sample and were freeze dry. The initial weight in powder form after freeze-drying process were taken before the extraction process. By using an extraction tube, the dried biomass was diluted with 4 % sodium hypochlorite solution and keep under the temperature of 45°C in the water bath system for 30 mins. After that, the sample was centrifuged for 30 mins at 6000 rpm. The supernatant was discarded and the pellet obtained was diluted using hot chloroform with the temperature of 60°C and the solution was precipitated using cold methanol with the temperature of -20°C.The solution was then centrifuged for another 30 mins at 6000 rpm to obtain a pellet. Lastly the pellet obtained from the process was dissolved in hot chloroform and let dry in the oven at 60°C. The last product after the extraction process was then weight and recorded [10].

2.6 Analysis of Extracted Compound
2.6.1 Fourier-Transform Infrared Analysis (FTIR)

Following the last part of the experimental process for the extraction method, FTIR was conducted onto the sample collected to analyse the composition and type of compounds extracted. IR absorption spectroscopy was used in order to calculate the absorption of infrared light for this research process. A Microsoft Excel spreadsheet was also used to create and analyse the spectra

from the FTIR microscope. The interval of the wave number for the FTIR was set to 4000-600 cm^{-1} and the scan number was set to 16.

FTIR is an easy qualitative analysis that is rapid, economical and non-destructive technique. The technique is extremely useful to identify changes in organic, polymeric and sometimes inorganic material and can even identify the identity of materials. The basic principle of the technique is based on identification of functional groups within molecules that vibrate by stretching or bending in different ways when exposed to a specific wavelength of light. In order to generate an FTIR spectrum the vibrations and the intensity or percent transmission are plotted against the light frequency (cm^{-1}) to which the sample is exposed. Figure 3 shows the basic optical path for FTIR**.**

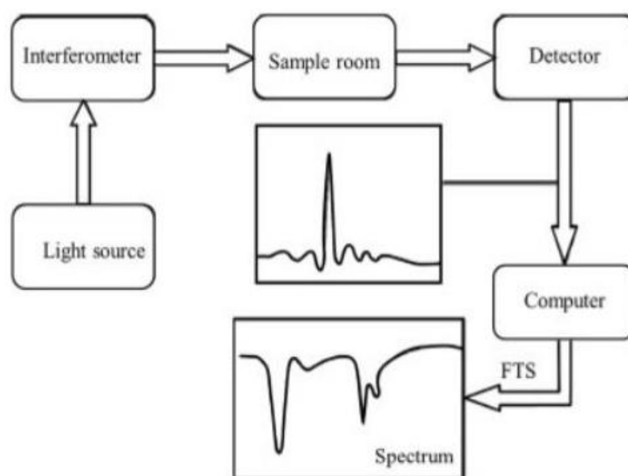


Fig. 3. Basic optical path of Fourier transform Infrared spectroscopy

2.6.2 Accumulation of PHAs

In terms of the initial mass of dry biomass used in each extraction, percentage yield of PHAs that were obtained after the extraction process were calculated using a gravimetric method, and expressed as g L^{-1} in accordance to equation below:

$$Y = \frac{mp}{mb} \times 100 \quad (2)$$

Where:
Y = accumulation of PHAs as a percentage, Mp = mass of PHAs (grams), Mb = mass of dry biomass used for the extraction (grams).

3. Result and Discussion

The growth rate, condition, biomass accumulation and properties of PHA in *Spirulina sp.* and *Chlorella sp.* under different cultures setting were researched. The results of the properties of PHA according to its monomer composition, FTIR and X-ray diffraction analysis is shown for each of the nitrogen concentration in the medium.

3.1 Cultivation Condition of Microalgae in Lab Scale

For the lab scale cultivation, pre-treatment for the apparatus such as 250 mL conical flask, plastic tube rubber stopper Zarouk's medium and AF6 medium are sterilized for 30 mins in the autoclave machine to avoid any contamination. An inoculation rate of 10 % from the total volume of 200 mL medium was taken from the stock culture provided in the laboratory and mixed together into the conical flask for cultivation process. A laminar flow system was used during the cultivation process as a measure to remove any unwanted contaminant. The aeration rate for the lab scale culture system was set approximately 0.5 L/min using aquarium pump and 24 h light intensity of 150 μE m^{-2} s^{-1} w was set for optimal biomass production [23].

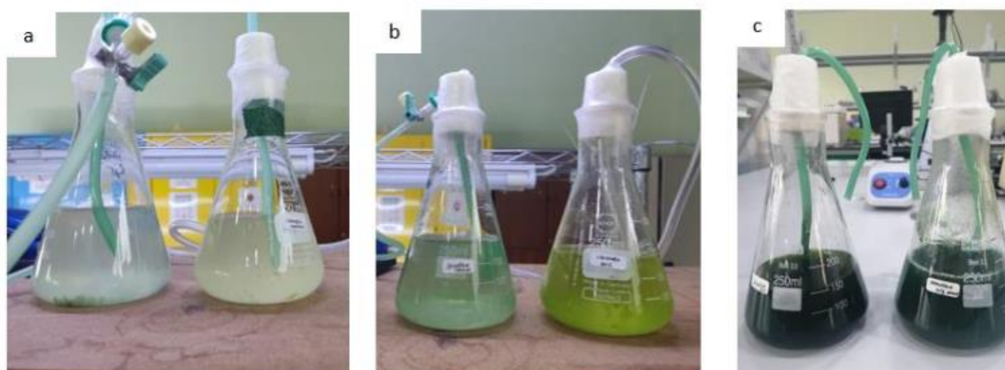


Fig. 4. Lab scale (a) Culture condition on day 0 (b) Culture condition on day 15 (c) Culture condition on day 30 of *Chlorella sp.* (right) and *Spirulina sp.* (left)

From the observation of the culture condition day 0 where the cultivation process started, the colour of the solution was seen as a pale grey colour as the medium over diluted the microalgae inoculation. On the 15th day of the cultivation process a light green colour started to convey and the intensity of the green colour are more prominent for the *Spirulina sp*. culture. At the end of day 30 *Spirulina sp*. culture in Zarouk's medium again shows a darker and more concentrated green colour compare to *Chlorella sp*. culture in AF6 medium thus concluding the higher growth rate of the microalgae species.

3.2 Cultivation of Microalgae in Bottle Culture and Raceway Pond

From the observation of the growth rate between *Spirulina sp*. and *Chlorella sp*., it was noted that the growth rate of the *Spirulina sp*. was higher compared to *Chlorella sp*. thus making it the best selection for the mass production. The mass production was focused on two system that is bottle culture and raceway pond.

Initially for the bottle culture system, the first step taken was to sterilized and disinfect the bottle culture using 70 % alcohol to eliminate any contamination in the system. The medium used for the cultivation is Zarouk's medium in the total of 15 L and the volume of inoculation of 1.5 L which is 10 % of the total medium used. The setting for light intensity is double from lab scale system which is 300 μE $m^{-2}s^{-1}$ w and the aeration system used a compact air pump at approximately 1 L/min.

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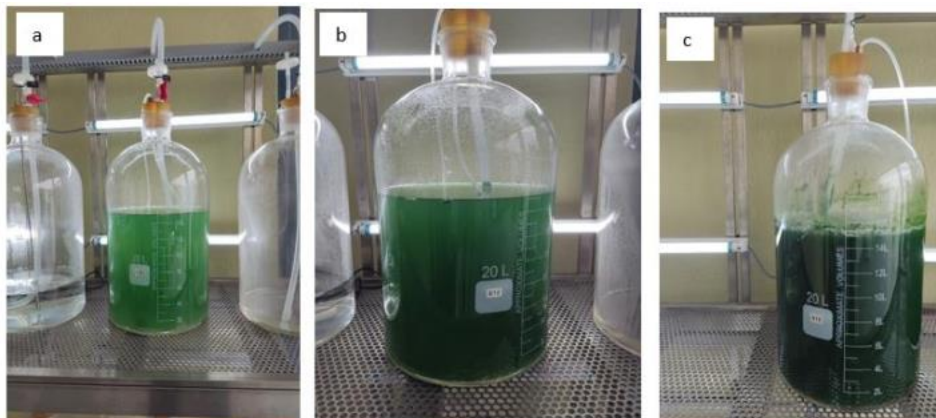

Fig. 5. Bottle culture (a) Culture condition on day 0 (b) Culture condition on day 15 (c) Culture condition on day 30 of *Spirulina sp.*

From the observation of the bottle culture condition on the day 0 where the cultivation process started, the colour of the solution was seen as light green colour insisting that the microalgae used for the inoculation process was in good condition. Following the 15th day of the cultivation process a much darker green colour started to convey showing a great increase in the biomass production of the microalgae. Finally at the end of day 30 the *Spirulina sp.* in bottle culture shows high intensity of biomass in the culture setting thus giving a concentrated dark green colour. It was also observed that there were a decreased in the culture medium due to high aeration rate and increase in temperature. The last sample was taken and the cultivation system was a success.

As for the raceway pond system, the system underwent the same pre-treatment which was the disinfection of the whole pond using 70 % alcohol and left with a cover overnight. The medium used in the first trial of raceway pond cultivation was 100 L tap water that have been disinfected with sodium hypochlorite and left covered overnight. The condition for the first raceway pond were 27-31°C temperature with no agitation in the raceway pond system. The optical density was recorded daily during the cultivation process.in the first 2 to 3 days of cultivation in the raceway pond, the condition of the pond shows no significant changes, however at day 10 the culture started to show browning in colour and there are some types of larvae suspected to be mosquitoes larvae exist in the pond system. The first cultivation is accepted to be unsuccessful due to low inoculation rate and high contamination occur in the system.

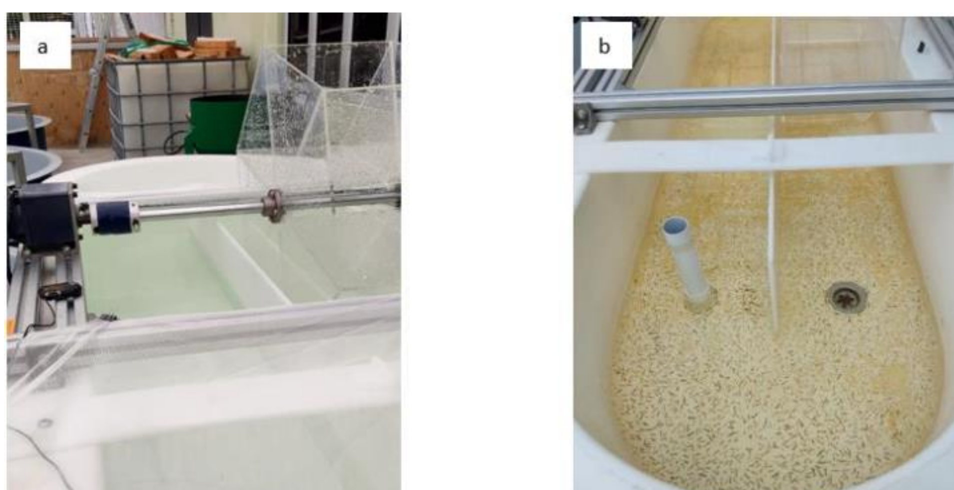

Fig. 6. Raceway pond culture (a) Culture condition on day 0 (b) Culture condition on day 15 of *Spirulina sp.*

After the first experiment, some modifications were made to optimize the culture condition in the raceway pond system. Condition modification includes the addition of Zarouk's medium into the disinfected tap water with a dilution factor of 1:10 that is 100 L of tap water to 10 L of Zarouk's medium for additional nutrient. Another factor that changes the system is the addition of motor-based agitation from 9 am-4 pm, 5 times a week. The same procedure was carried out to monitor the growth of the microalgae in the pond by measuring the optical density at 750 nm daily [24]. From the observation, for the first 3 days of cultivation, there were no changes in the colour and concentration of the culture solution. However, during the seventh day of the culture a slight change of the colour in the pond was observed as the colour change slightly yellowish. Unfortunately, on the 15th day of the experimentation, due to the two-day stoppage of the agitation motor, a biofilm was form in the raceway pond. Upon further inspection under the microscope, the biofilm form shows an unknown species and some levels of contamination have occurred. According to theory, the ability of microalgae to produce biofilms in unfavourable circumstances allows them to preserve their structural integrity and levels of proliferation. The cultivation for the system was stopped and the microalgae was harvested at the end of day 15.

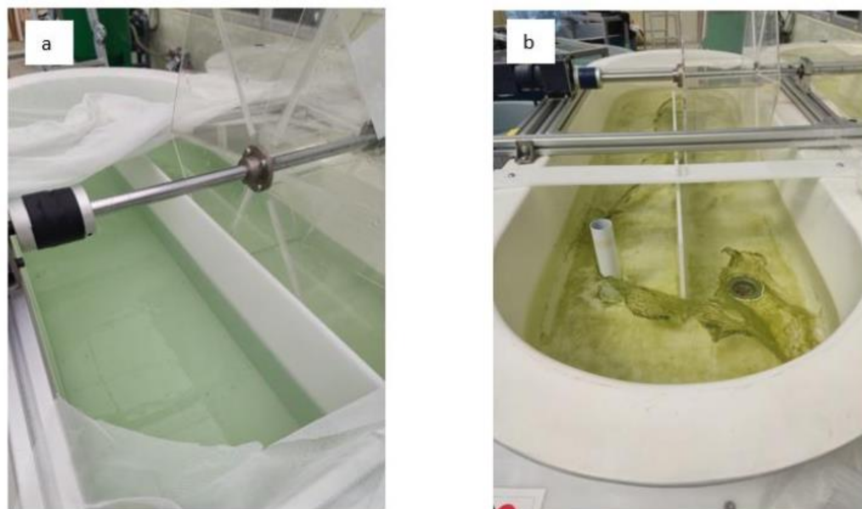

Fig. 7. Raceway pond culture (a) Culture condition on day 0 (b) Culture condition on day 15 of *Spirulina sp.*

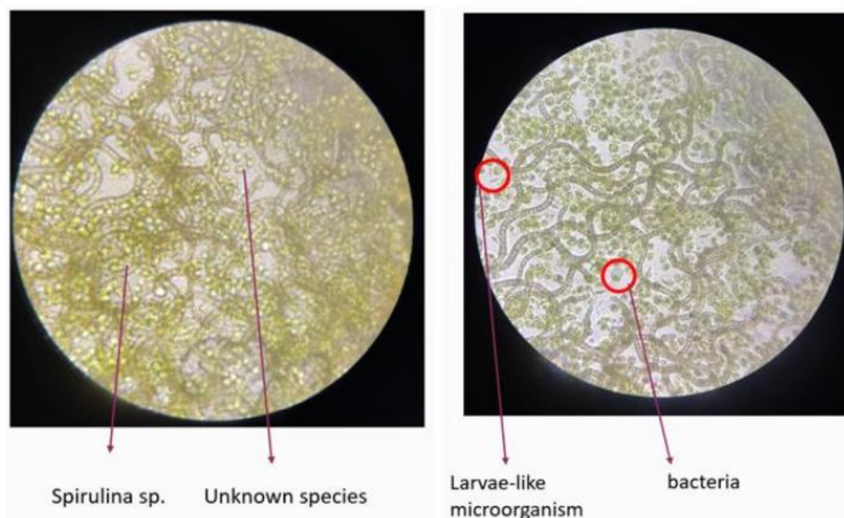

Fig. 8. Raceway pond sample of biofilm under microscope viewed at 100x magnification

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3.3 Determination of Growth Curve for the Culture System

Determination for the growth curve of the respective microalgae in lab scale, bottle culture and raceway pond were determined using UV-vis spectroscopy for the cell optical density once every two days. In order to prevent the absorption of light by cellular pigments such as chlorophyll and carotenoids the measurement was taken in the wavelength of 750 nm [24]. The absorbance for each sample was tabulated and a graph for the optical density against 30 days was plot to determine the phase of the microalgae and comparing their respective growth rate.

For the first part of the experimentation, which is in the lab scale, the optical density for *Spirulina sp.* and *Chlorella sp.* was taken simultaneously once every two days for over a period of 30 days. The data for the absorbance are as in the Table 4 below. Following the data, a graph for the growth curve was plotted as in Figure 9. Based on the same figure, the growth curve of *Spirulina sp.* shows minimal difference between the three main stages that is lag phase, exponential phase and stationary phase. However, by following the trend of the graph, from the starting day of the inoculation process to the 5th day of cultivation the microalgae were slowly adapting to the new condition and having slight increase in the optical density thus making it the lag phase. As for exponential phase in the bottle culture system, it was observed that in day 7 to day 30 of the cultivation process. The longer exponential phase in the bottle culture system was due to the larger growth space and higher nutrient supplement in the culture medium.

Table 4
Absorbance data of *Spirulina sp*. and *Chlorella sp.* in lab scale

Date	Day	*Spirullina sp.*	*Chlorella sp.*
		K x ABS	K x ABS
2 Feb	0	0.1375	0.1661
4 Feb	3	0.1619	0.1764
7 Feb	6	0.1887	0.2086
10 Feb	9	0.2024	0.2218
14 Feb	12	0.5265	0.4783
17 Feb	16	0.7908	0.6903
21 Feb	20	0.9272	0.7902
24 Feb	23	1.0317	0.823
28 Feb	27	1.0493	0.8308
3 Mac	30	1.0517	0.8296

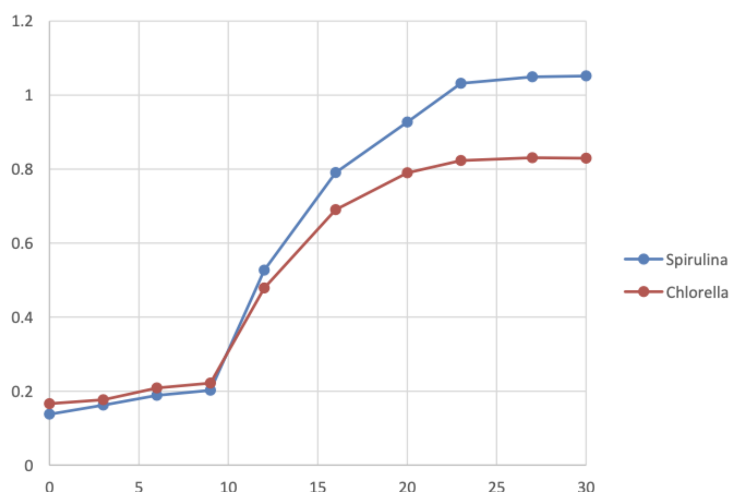

Fig. 9. Graph of the absorbance against time for lab scale cultivation of microalgae

From the above graph, the first 9 days of the cultivation known as the lag phase. In this phase the biomass produce are in constant rate as the inoculum was still adapting to the condition and culture media used in the system [10]. Subsequently at day 10 until day 15th, the culture was in the exponential phase where the microalgae began to actively perform cell division thus exponentially increase the culture's biomass. Lastly at the 23rd day of the cultivation process the microalgae enter the stationary phase where the rate of the cell division was equal to the cell death and taken into account of the nutrients depletion in the medium. Furthermore, by comparing the growth curve between the two microalgae species, *Spirulina sp*. has a slower start in the lag phase but have a higher percentage of cell division in the exponential phase showing prominent growth compared to *Chlorella sp.* From the graph analysis, *Spirulina sp.* was selected as the best species for the continuation of the mass production system due to its higher growth rate with a peak of 1.0317 K x Abs in the exponential region.

In the second part of the experimentation which is the upscaling process, the same procedure was taken to determine the growth rate of the microalgae in the culture system. The data for the optical density of the raceway pond and the bottle culture are as in the Tables 5 and 6 below.

Table 5
Absorbance data of *Spirulina sp.* in raceway pond

Date	Days	K x ABS
24/5/2022	0	0.00865
26/5/2022	3	0.0963
30/5/2022	6	0.1032
1/6/2022	9	0.1124
3/6/2022	12	0.1515

Table 6
Absorbance data of *Spirulina sp.* in bottle culture

Date	Days	K x ABS
11 Apr	0	0.196
13 Apr	3	0.235
15 Apr	5	0.29
18 Apr	7	0.382
20 Apr	10	0.466
22 Apr	12	0.4951
25 Apr	15	0.522
27 Apr	17	0.59
9 May	20	0.642
13 May	22	0.735
16 May	25	0.804
18 May	27	0.905
20 May	30	0.967
23 May	32	0.996
26 May	35	1.038
30 May	37	1.041

The comparison between the mass production in the bottle culture and raceway pond is as in Figure 10 with lab scale as the referral state or optimum state. From the graph, it was noted that the lag phase in the lab scale was longer compare to the bottle culture system. The lag phase in the lab scale cultivation was around 9 days and the lag phase in the bottle culture was around 5 days. The main reason for this difference may due to the adaptation process of the first inoculation from a storage state to a new and fresh growing medium. In addition the duration of the exponential phase

was longer than the lab scale cultivation process. According to a study, the amount of biomass produce is highly dependent to the microorganism's cellular metabolism and its growth conditions [19].

The least growth for *Spirulina sp*. was in the open raceway pond where the lag phase extended to 9 days and the growth of microalgae started to show some improvement on day 10 before the formation of biofilm on day 15. As for the slow growth of microalgae in raceway pond apart from contamination, it can be explained due to the low concentration of nutrient such as nitrogen that is essential nutrient in the constitution of proteins, nucleic acids and also plays an important role for the maintenance of the life of all organisms in the biosphere [14].

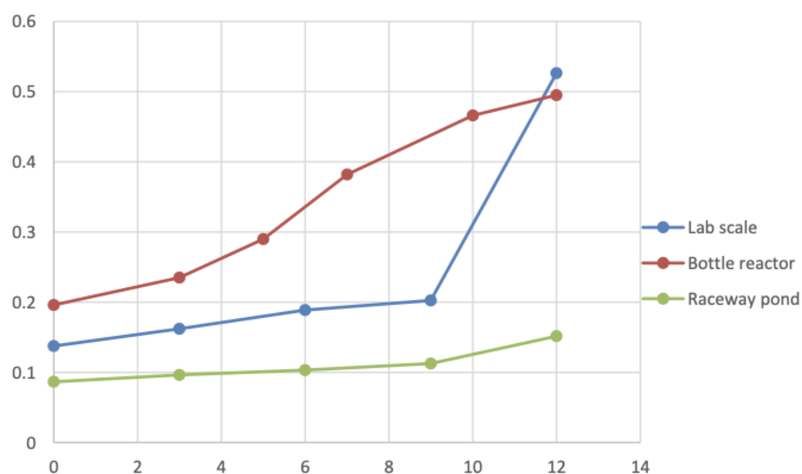

Fig. 10. Absorbance of lab scale, bottle culture and raceway pond against time for *Spirulina sp.*

3.4 PHA Extraction and Quantification

The extraction of PHAs was performed with some modification. 50 L of sample was collected, centrifuged and the pellet obtained was freeze dried. The initial weight in powder form after freeze-drying process were taken before the extraction process. In the extraction tube, the dried powdered biomass was diluted with 10 % instead of 4 % sodium hypochlorite solution and incubated under the temperature of 45°C in water bath for 30 mins. After that, the sample was centrifuged for 30 mins at 6000 rpm. the pellet obtained was diluted using hot chloroform with the temperature of 60°C followed by the precipitation using cold methanol with the temperature of -20°C and was then centrifuged for another 30 mins at 6000 rpm to obtain a pellet. Lastly the pellet obtained from the process was dissolved in hot chloroform and let dry in the oven at 60°C. The last product after the extraction process was then weight and recorded [9]. The reason for using 10 % of sodium hypochlorite is due to the unsuccessful attempt in breaking down the cell structure in the first process when using only 4 % of sodium hypochlorite. Figures 11 and 12 below shows the difference in the product obtained when using 4 % and 10 % sodium hypochlorite.

Fig. 11. Final product using 4 % sodium hypochlorite

Fig. 12. Final product using 10 % sodium hypochlorite

The weight of the dry sample obtained before the extraction process for every 50 mL sample and after the extraction process was recorded along with the calculation of the percentage yield of PHA using gravimetric method as in the Table 7 below.

Table 7
Biomass and PHA quantification

Culture setting	Weight of dry biomass	Weight of product after extraction	Percentage yield (%)
Lab scale	0.04	0.016	40
Bottle culture	0.08	0.06	75
Raceway pond	0.02	0.002	10

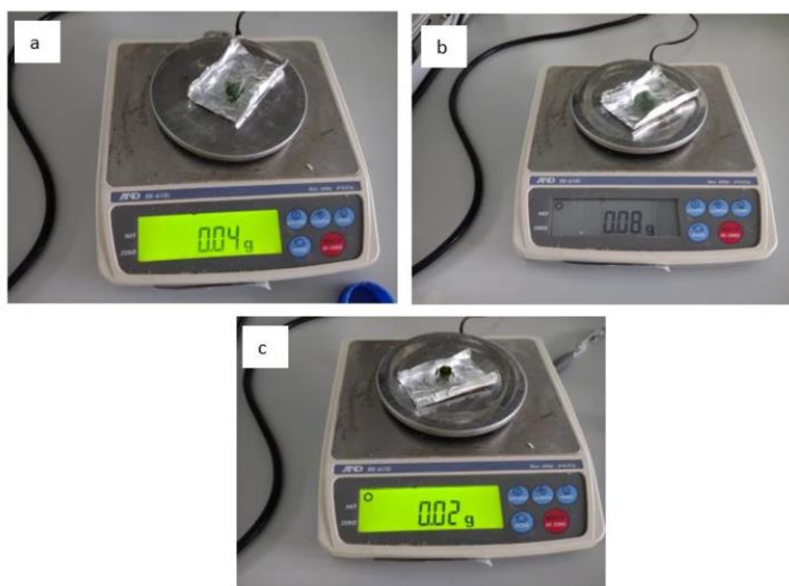

Fig. 13. Dry mass of 50 mL sample after freeze dry (a) Lab scale (b) Bottle culture and (c) Raceway pond

Fig. 14. Highest mass of product obtained after extraction

From the result of the biomass and gravimetric analysis of PHA production for each culture setting, it can be observed that the highest biomass and percentage yield of PHA was from the sample in the bottle culture system followed by lab scale and lastly raceway pond. In addition to accumulating high biomass with the high supply of nutrient during cultivation and optimum conditioning, the microalgae also increased the accumulation of PHAs that is linked with the higher lipid production in the species. For the raceway pond sample the result of low percentage yield was due uncontrollable condition such as inconsistent lighting and temperature that affect the growth rate of the *Spirulina sp*. in the system. Other factors such as unavoidable contamination also leads to unsuccessful attempt in the cultivation process and low percentage of both biomass and PHA production.

3.5 FTIR Analysis

For this analysis, it was noted that the FTIR spectrum of PHA samples collected were in correlation to the study by Costa *et al*., [9] as shown in Figure 15 and indicates a correlation between the chemical structure of PHA overall. From the study the main bands observed in the PHA spectrum were related to the axial deformation of the ester carbonyl group (C=O) at the wavelength of 1710–1750 cm^{-1} and from the results of the PHA extracted from *Spirulina sp*. the closest peak in the range was at the wavelength of 1748 cm^{-1}. The formation of the C-O-C groups were also expected to appear in the spectral region ranging from 1260 to 1300 cm^{-1} in the crystalline phase. In the graph the closest peak in that range was at the 1298 cm^{-1}. Furthermore, the symmetric angular deformation in the plane of the methyl groups (CH_3) can be observed at the bands close to 1380 cm^{-1}. Lastly the vibration of the ester group carbonyl (C-C) was observed at the bands close to 980 cm^{-1} which in this case at the 978 cm^{-1} [25].

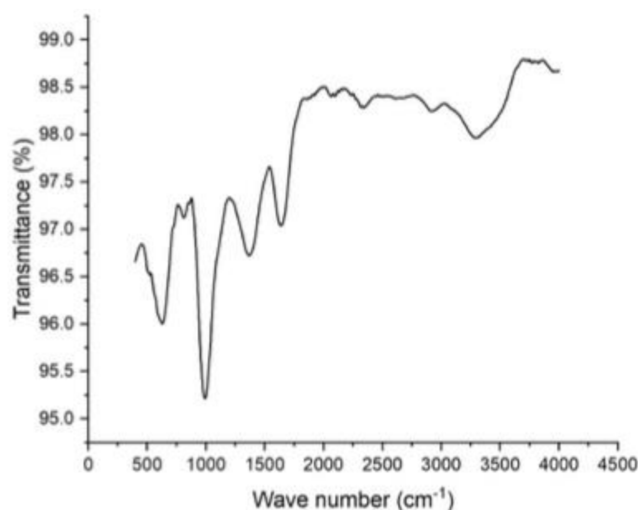

Fig. 15. FTIR spectra of PHA produced by *Spirulina sp*.

As for the *Chlorella sp.*, the result of FTIR analysis was referred to the study conducted by Pinho *et al*., [14]. The FTIR spectrum of the microalgae shows the characteristic vibrations at closest to 1740 cm^{-1} which is the minor peak of 1736 cm^{-1} in correspond to the stretch of the C=O ester bonds. From the spectrum at wavelength of 1450 cm^{-1} to 1375 cm^{-1} the characteristic corresponding to the C–H of the aliphatic chains [14]. The functional group of ester group carbonyl (C-C) was also observed at the bands close to 980 cm^{-1} [25].

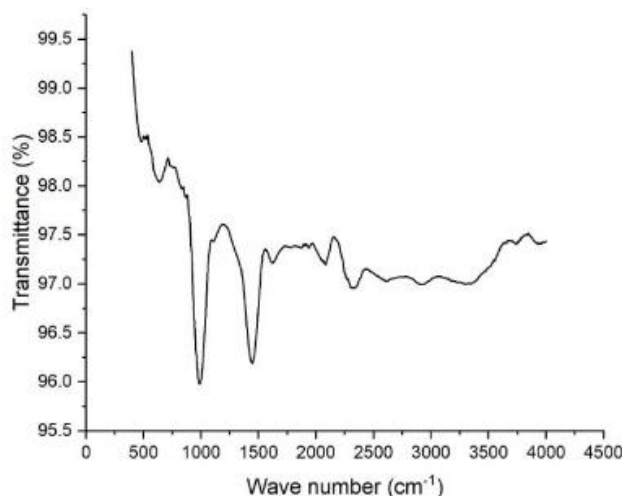

Fig. 17. FTIR spectra of PHA produced by *Chlorella sp*.

Typically, the chemical composition of PHAs comes from all the functional groups stated in the analysis, thus confirming that the extraction method has resulted in PHAs as major compounds. However, among the samples collected, different levels of band strength were observed between the species. This indicates different levels of PHAs purity among the samples and can be further confirmed using the monomeric composition analysis [11].

5. Conclusions

This research mainly focused on the growth rate of *Spirulina sp.* and *Chlorella sp.* and their PHAs production under different cultivation setting and conditions toward the production of PHA which is

a viable source for the production of bioplastics. Initially, both of the microalgae species were cultured using standard medium which is AF6 medium for *Chlorella sp.* and Zarouk's medium for *Spirulina sp.* in the lab scale cultivation system. After the cultivation process occur, a set of data related to their optical density by using UV-vis spectroscopy were taken up to 30 days of cultivation. The data was then used to plot the growth curve of each microalgae in their respective medium and the microalgae with the highest growth rate was chosen for the upscaling process. After all of the sample from different culture setting that is in lab scale, bottle culture and raceway pond have reached their stationary phase, a final sample with a volume of 50 mL was taken in order to extract the PHAs produce by the microalgae in different culture setting and condition. The extracted PHAs will be further analyzed using the FTIR in order to analyze the composition and properties of the extracted PHAs produced by both microalgae species.

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