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Optimization of freshwater microalgae, *Arthrospira* sp. *(Spirulina)* for high starch production

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Abstract

Freshwater microalgae that contained high starch, carbohydrates and lipids contents are beneficial to human being in many industrial especially in food and food packing industries. There are a few freshwater microalgae that contained high starch and carbohydrates content such as *Chlorella vulgaris, Chlorella emersonii, Chlorella sorokiniana, Nannochloropsis salina* and *Spirulina* sp. *Spirulina* sp. is chosen in this research because of its strong adaptation to the environment changes, short life cycle and able to produce high intracellular starch. In this project, results have shown that microalgae at late exponential phase contained the highest starch and carbohydrate yield of 0.491±0.046 mg/L and 0.090±0.046 mg/L, respectively. Thus, the harvesting time for microalgae with high starch production and yield is phase dependent rather than time dependent. Under optimized cultivation conditions of aeration at 5 L/min, using white wavelength and 32ºC, *Spirulina* sp. produced the highest starch and carbohydrate yield of 0.664±0.03 mg/L, and 1.019±0.025 mg/L, respectively. In conclusion, compared to before optimization, starch and carbohydrate yield after optimizations were increased. In conclusion, compared to before optimization, starch and carbohydrate yield after optimization were 435% and 221% increased, respectively.

Introduction

Microalgae are autotrophic organisms which are commonly found in both marine and freshwater environments (Siti Syazwina, 2014). It can be existed in unicellular, chains or groups' species and can convert solar energy into organic compound mainly because of their simple cellular structures. Microalgae are currently being investigated intensively world-wide for its potential to replace the fossil fuels.

However, microalgae biotechnology has been developed for different commercial application (Harun *et al.*, 2010). As photosynthetic organism, microalgae contain chlorophyll that can be used for food and cosmetic purposes (Spolaore *et al*., 2006). As some species of microalgae produce bioactive compounds such as antioxidants, antibiotics and toxins (García-Casal *et al.*, 2009). Besides, microalgae are used as nutrient supplements for human consumption due to high in protein, vitamins and polysaccharides contents (Carballo-Cárdenas *et al.*, 2003). Some microalgae species contain high levels of carbohydrate or starch which have potential application in food industry.

Microalgae culture need to be maintained at the optimum condition to ensure high growth rate and biomass production (Siti Syazwina, 2014). Nutrient, light, temperature, salinity and pH are the major factors that can influence the overall biomass productivity and biochemical composition (Renaud et al., 2002; de Castro Araújo and Garcia, 2005). The biochemical composition of microalgae also varies with their growth rates, environmental conditions and growth phase cycles (Richmond, 1986). According to the study conducted by Tomaselli *et al.* (1988) high growth temperature can cause *Spirulina platensis* M2 strain significantly decreased in protein content (22%) but remarkably increased in lipids (43%) and carbohydrates contents (30%) at 40ºC.

Hence, choosing an appropriate experimental design for optimization of microalgae cultivation conditions and factors induce starch production and accumulation will help in increasing the productivity of microalgae biomass, starch and carbohydrate content. Therefore, the objectives of this paper were to determine the growth profile of microalgae (*Spirulina* sp.) in which starch accumulation is the most. In addition, microalgae cultivation conditions which affect starch and carbohydrate production were optimized.

Materials and Methods

Strain andinoculums preparation

Spirulina sp. culture was obtained from School

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of Industrial Technology, Universiti Sains Malaysia. The strain was maintained in 100 ml Zarrouk medium under irradiance of about 258 Lux (3.612 µmol photons $m^{-2}s^{-1}$), 12/12h (light/dark) regime and at room temperature (28±2°C). Inoculum was prepared by transferring stock culture into fresh media at a ratio of 1:9 (inoculum media). The inoculums size was standardized at OD 0.50-0.51 using spectrophotometer (Hach DR 2800, Australia) at the measurement wavelength of 680 nm.

Growth medium composition

The following chemical compositions of Zarrouk medium was dissolved in 1L of distilled water: sodium chloride (NaCl, 1.00g), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 0.2 g), calcium chloride dihydrate ($CaCl \cdot 2H_2O$, 0.04 g), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, 0.01 g), disodium ethylenediaminetetraacetate dihydrate ($Na_2EDTA \cdot 2H_2O$, 0.08 g), dipotassium phosphate (K_2HPO_4,0.25 g), sodium nitrate ($NaNO_3$, 2.5 g), potassium sulfate (K_2SO_4, 1.00 g), sodium bicarbonate ($NaHCO_3$, 16.8 g) and 1 ml of trace element that were prepared separately. The chemical compositions of trace element consisted of (g/L): boric acid (H_3BO_3, 2.86 g), ammonium molybdate tetrahydrate ($(NH_4)6Mo7O_{24}$, 0.02 g), manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, 1.8 g), copper (I) sulfate, (Cu_2SO_4, 0.08 g) and zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, 0.2 g) (Zarrouk, 1966).

Growth profile of Spirulina *sp.*

Spirulina sp. culture was cultivated using 2L of Erlenmeyer flask with 1.5L of working medium. Inoculum (10%, v/v) which was previously prepared was inoculated into the Zarrouk medium. Sample was incubated at room temperature, with aeration of 5 L/min using aquarium air pump and placed under illumination of white fluorescence lamps at 3000 Lux (42 µmol photons $m^{-2}s^{-1}$) with 12 h dark and 12 h light cycle. The growth rate of *Spirulina* sp. was determined by taking absorbance reading daily using Spectrophotometer at 680 nm. The *Spirulina* sp. culture was harvested at different growth phase (early exponential phase, late exponential phase and stationary phase) and the sample was analyzed for starch production. The growth phase which showed the highest starch production was used in future experiment unless otherwise stated. All the experiment was conducted in triplicates and the results obtained were presented as mean of triplicate results.

Factors affecting the production of starch and carbohydrate

Some factors that influence the production of starch and carbohydrate were examined and optimized which includes the effect of aeration, light/type wavelength and cultivation temperature. The optimum condition obtained from each experiments were used unless otherwise stated.

For the effect of aeration on starch and carbohydrate production, *Spirulina* sp. culture was grown in room temperature, without aeration and aeration at 5 L/min using aquarium air pump. The samples were placed under illumination of white fluorescence lamps at 3000 Lux (42 µmol photons $m^{-2}\ s^{-1}$) with 1 h dark and 1 h light cycle. For the effect of light type/wavelength, the light used were white light (390-700 nm), blue light (430-450 nm), red light (600-700 nm) and yellow light (560-590 nm). On the other hand, for the effect of cultivation temperature, the culture was incubated at room temperature, 30°C, 32ºC and 34°C. For samples cultivated at 30-34°C growth chamber (Hitec, Malaysia) was used.

Determination of biomass dry cell weight

To evaluate the biomass production, 1000 mL of microalgae suspension was centrifuged at 6000 rpm for 10 min at 4ºC to obtain the biomass pellet. The pellet was then thoroughly washed with sterile distilled water twice to remove salt before sending for freeze dry.

Determination of total carbohydrate and starch content

Total carbohydrates content was determined based on the method described by previous researchers (Grandy *et al.,* 2000; Laurentin and Edwards, 2003). A volume of 0.5 mL concentrated acetic acid was added to 10 mg dry algal sample before incubated at 85°C in water bath for 15 min. Then, 10 ml of acetone was added to the sample tube before centrifuged at 4000 rpm for 10 min. The supernatant was removed and for not colorless sample, 5 mL 4 M trifluoroacetic acid (TFA) was added and the sample was incubated in boiling water bath for 4h. Thereafter, the sample was top up with distilled water to a final volume of 10 mL. Hydrolyzed sample (20 µL) was transferred into ice bath and 0.9 mL sulfuric acid–phenol reagent was added before incubated in boiling water bath for 20 min. Thereafter, the sample was cooled down in ice bath before measured using spectrophotometer at 490 nm. Equation 1 was used to determine the carbohydrate content. On the other hand, starch content was determined accordingly to the method provided by Megazyme (Wicklow,

Ireland) accepted by AOAC Official Method 996.11 and AACC method 76-13.01. In the method, starch is hydrolyzed to glucose using amyloglucosidase and α-amylase (Megazyme, Ireland). Percentage of starch was calculated based on equation 2.

$$\%\ Carbohydrates = \frac{(Absorbance - intercept)/Slope}{Sample\ weight\ (mg)/Total\ hydrolyzate\ (mL)} x\ 100 - -(1)$$

$$\%\ Starch = \Delta A\ x\ F/W\ x\ FV\ x\ 0.9 - - - - - - - - -(2)$$

Where

ΔA = Absorbance (reaction) read against the reagent blank

F = 100 µg of D-glucose/absorbance for 100 µg of glucose (conversion from absorbance to µg)

FV = Final volume (10-100 mL)

W = Weight (mg) of sample used

Statistical analysis

The significance of difference between each test variable were determined using one way ANOVA analysis and Duncan's Multiple-Range Test, computed using SPSS version 22 software. All tests were done with a confidence interval of 95%.

Results and Discussions

Growth profile and starch analysis at different phase

The growth profile of *Spirulina* sp. microalgae was basically differentiated into four phases; lag phase or early exponential phase (t_1); log phase or late exponential phase (t_2); stationary phase (t_3); and death phase (t_4) (Figure 1). The chemical composition of algal growth medium can affect the growth rate and biomass of microalgae (Blair *et al.*, 2014). Hence, the chemical composition of growth media in this experiment was maintained constant to prevent the dissimilarities between the growth profiles of *Spirulina* sp. From Figure 1, it can be seen that early exponential phase was occurred between 0 to 4 days (t_1); t_2 indicated the late exponential phase which was occurred between 4 to 14 days. On the other hand, stationary phase started from 14 to 15 days (t_3) while death phase occurred from 15 to 18 days (t_4).

In term of starch production, late exponential phase of *Spirulina* sp. produced the highest starch (2.20±0.020%) which are significantly different ($P<0.05$) compared to late stationary phase due to higher growth rate during exponential phase (Table 1). Besides that, the yield of starch is relatively high (0.491±0.046 mg/L) in late exponential phase compared to other phase of growth profile of *Spirulina* sp. The results obtained shown that in order to obtain the highest starch production, *Spirulina* sp. should be harvested at the late exponential phase. This findings are also in agreement with previous researcher findings who obtained the highest intracellular starch content at late exponential phase of microalgae cultivation (Dragone *et al.*, 2011; Blair *et al.*, 2014).

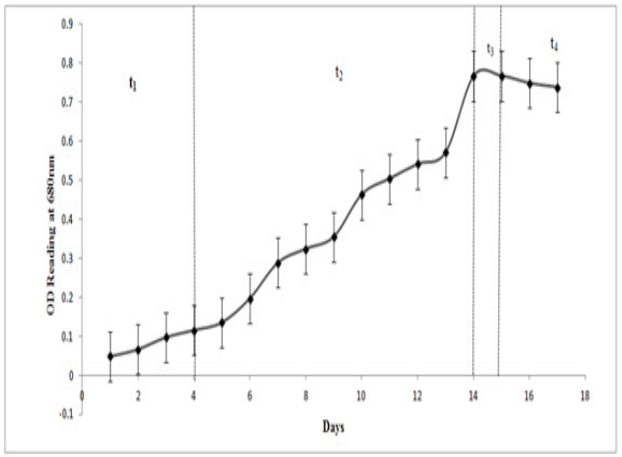

Figure 1. Growth profile of *Spirulina* sp. t_1= early of exponential phase; t_2= late exponential phase; t_3= late stationary phase; t_4= death phase. Arrow bar indicates means with standard error of triplicates.

Effect of aeration on starch and carbohydrates production

The total dried cell weight harvested from the aerated (0.105±0.003 g/L) *Spirulina* sp. culture after 14 days was lower compared to the total dried cell weight from non-aerated (0.223±0.020 g/L) sample (Table 2). This probably may due to the strong agitation rate provided during the cultivation process which causes hydrodynamic stress to the growth of *Spirulina* sp. According to Camacho *et al.* (2001), direct air sparging on microalgae culture in bubble columns and airlift photo bioreactors can caused cell damaged towards microalgae. The bursting of small air bubbles at the surface of the culture can cause cell damaged towards the microalgae. On the other hand, in term of starch content of aerated *Spirulina* sp. culture has the lower percentage of starch content (1.18±0.062%) and yield (0.1242±0.009 mg/L) when compared to non-aerated culture. However, aerated culture produced higher percentage of carbohydrate and yield which are 2.97±0.970% and 0.3166±0.110 mg/L, respectively.

In addition, the aeration rate need to be controlled in a reasonable range in order to prevent it from creating shear stress on the microalgae culture (Zheng *et al.*, 2013), which are in agreement with current research findings. High aeration rate provided by the air pump could possible increase the level of CO_2 in the *Spirulina* sp. culture. Besides

Table 1. *Spirulina* sp. starch analysis at three main growth phases

Phases	% Starch	Yield (mg/L)
Early of exponential phase	2.16 ± 0.087	0.0632 ± 0.008
Late exponential phase	2.20 ± 0.020	0.491 ± 0.046
Late stationary phase	0.60 ± 0.078	0.490 ± 0.000

*Data was reported as means of triplicates ± standard error.

Table 2. Effect of aeration and non aeration on starch and carbohydrate production during cultivation of *Spirulina* sp.

Parameter	Starch contents			Carbohydrates contents	
	Dry Weight (g/L)	Percentage (%)	Yield (mg/L)	Percentage (%)	Yield (mg/L)
Aeration	0.105±0.003	1.18±0.062	0.124±0.009	2.97±0.970	0.317±0.110
Without aeration	0.223±0.020	2.20±0.020	0.491±0.046	0.40±0.222	0.090±0.046

Note: Data presented are means with standard error of triplicates. The experiment was carried out at the indicated aeration rate, at room temperature using white light with 12 h dark and 12 h light cycle.

that, CO2 concentration in microalgae culture can affect the physicochemical properties and content of starch (Izumo et al., 2007), in which according to Zheng *et al.* (2013), the intracellular starch content decreased with increasing CO_2 concentration. At low CO_2 condition, there is 2.5 fold increased in starch per cell and 2.5 fold increased in diameter of starch granule. Hence, aeration is more favourable for carbohydrate accumulation in *Spirulina* sp. rather than starch accumulation. According to Fábregas *et al.* (1995), aeration rate can increase carbohydrate cellular contents and proteins concentration in microalgae. Optimization for another two parameters proceeded with aeration, even though non-aerated produced higher yield. This is being done, to avoid sedimentation of *Spirulina* sp. on the bottom of flask and to provide continuous mixing (Richmond and Grobbelaar, 1986).

Effect of different light wavelength

In this experiment, different wavelength of light such as white wavelength (390-700 nm), blue wavelength (430-450 nm), red wavelength (600-700 nm) and yellow wavelength (560-590 nm) were used. Microalgae absorbed different types of wavelength depending on the species (Blair *et al.,* 2014). The intensity of each wavelength of light in this experiment were keep constant at approximately 3000 Lux because different light intensities could affect the microalgae cell growth, biomass, lipid production and starch production (Pandey and Tiwari, 2010; Pandey *et al*., 2011; Blair *et al.*, 2014;). The results obtained indicated that *Spirulina* sp. grown well with white light illumination with the highest dried cell weight of 0.536±0.087 g/L with the least dried cell weight (0.086±0.016 g/L) detected using blue light (Table 3). Although the growth was different but no significantly different ($P>0.05$) in term of starch produced which was in the range of 1.543-2.207%.

Blue light shows the highest starch content which was 2.21±0.134% compared to white wavelength 1.88±0.043%. Based on the previous study, blue wavelength was absorbed well by chlorophyll and suitable for starch production (Lawlor, 1987). Lawlor (1987) stated that chlorophyll a and chlorophyll b absorbed red wavelength (600-700 nm) and blue wavelength (430-450 nm) more strongly. Study also showed that blue wavelength improved the efficiency of photosynthesis and increased sugar production in microalgae (You and Barnett, 2004). Furthermore, study conducted by Fernandes *et al.* (2010) regarding the attenuation of light by fresh water microalgae in photo bioreactor (PBR) verified that blue wavelength and red wavelength has the highest attenuation of light. This is due to chlorophyll a absorb this two spectrum of light more actively (Yun and Park, 2001). Results obtained from this study showed that although blue light gave the highest starch production but white wavelength would be the most favorable for cellular production because starch yield (1.000±0.147 mg/L) was significantly higher ($P<0.05$) when compared to others tested wavelength. This due to shorter wavelength for blue wavelength contained high energy and inhibit the growth of microalgae (You and Barnett, 2004).

Table 3. Effect of different light type/wavelength on starch and carbohydrate production during cultivation of *Spirulina* sp.

Parameter		Starch contents		Carbohydrate contents	
	Dry weight (g/L)	Percentage (%)	Yield (mg/L)	Percentage (%)	Yield (mg/L)
White	0.536±0.087	1.877±0.043^{a}	1.000±0.147^{b}	26.56±7.929^{A}	15.611±6.151^{B}
Blue	0.086±0.016	2.207±0.134^{a}	0.187±0.028^{a}	26.94±7.659^{A}	2.475±1.041^{A}
Red	0.219±0.089	2.080±0.224^{a}	0.492±0.211^{a}	32.97±2.098^{A}	7.015±2.893^{AB}
Yellow	0.189±0.033	1.543±0.294^{a}	0.272±0.007^{a}	15.47±3.531^{A}	3.149±1.059^{A}

Note: Data presented are means with standard error of triplicates. The experiment was carried out at the indicated light wavelength with 5 L/min of aeration rate, at room temperature and 12 h dark and 12 h light cycle. Means with the same letter in the same column indicated no significantly difference at 5% level of probability by Duncan's Multiple-Range Test.

Table 4. Effect of different cultivation temperature on starch and carbohydrate production during cultivation of *Spirulina* sp.

Parameter		Starch contents		Carbohydrate contents	
	Dry weight (g/L)	Percentage (%)	Yield (mg/L)	Percentage (%)	Yield (mg/L)
Room Temperature	0.105±0.003	1.18±0.062^{a}	0.124±0.009^{a}	2.97±0.970^{A}	0.317±0.110^{B}
30 °C	0.259±0.009	1.42±0.076^{a}	0.367±0.008^{a}	3.87±1.002^{A}	1.013±0.293^{A}
32 °C	0.341±0.006	1.94±0.079^{b}	0.664±0.037^{b}	2.97±0.067^{A}	1.019±0.025^{A}
34 °C	0.156±0.006	2.007±0.081^{b}	0.312±0.012^{a}	6.21±0.320^{A}	0.323±0.054^{B}

Note: Data presented are means with standard error of triplicates. The experiment was carried out at the indicated temperature, with 5 L/min of aeration rate, using red light with 12 h dark and 12 h light cycle. Means with the same letter in the same column indicated no significantly difference at 5% level of probability by Duncan's Multiple-Range Test.

On the other hand, red light has the highest carbohydrate content (32.97±2.098%), but statistical analysis showed no significantly different (P>0.05). The overall carbohydrate yield by white light are significantly different (P<0.05) compared with blue and yellow light. Yellow wavelength showed the lowest starch content and carbohydrates content are mainly due to weak penetration of yellow wavelength by the chlorophyll molecules. In summary, white light is the most suitable light for the production of starch and carbohydrate because the yields were the highest among the different tested light.

Effect of temperature on starch and carbohydrate production

At room temperature 28±2ºC *Spirulina* sp. microalgae shows the lowest dried cell weight, starch content and carbohydrate content of 0.1048±0.0032 g/L, 1.18±0.062% and 2.97±0.970%, respectively (Table 4). This have comply with the study according to Ogawa *et al.* (1972) who showed that the optimum temperature of *Spirulina* sp. lies between 30 to 35ºC. Among the tested temperatures, 32°C is the optimum temperature for *Spirulina* sp. growth with the highest dried cell weight (0.341±0.006 g/L), starch yield (0.664±0.037 mg/L) and carbohydrate yield (1.019±0.025 mg/L). According to the study conducted by Mayo (1997), the maximum growth rate for microalgae, 0.50 was obtained at optimum temperature of 32.4ºC, in which the maximum biomass productivity is achieved.

Starch content produced during *Spirulina* sp. cultivated at 32ºC (1.94±0.079%) was significantly different (P<0.05) compared to room temperature and 30ºC. In addition, the highest starch yield was obtained at 32ºC (0.664±0.037 mg/L). This result was significantly (P<0.05) higher compared to other temperatures used. In term of carbohydrate content, 34ºC showed the highest content (6.21±0.320%). However, no significant different when compared to other tested temperatures (P>0.05). On the other hand, the carbohydrate yield was low at 34°C compare with 30°C and 32°C. Similar results have been obtained from Tomaselli *et al.* (1988) in which at higher cultivation temperature, *Spirulina* sp. showed significant increased in lipids (43%) and carbohydrates content (30%). Nakamura and Miyachi (1982) studied have showed that increasing temperature from 20 to 38ºC during photosynthesis and isotopes of carbon dioxide fixation (^{14}C) resulted in significant decreased in 14 carbon isotope (^{14}C) in starch. Besides that, Converti *et al.* (2009) study had reported that at 35ºC, microalgae showed significant decreased in its growth rate and at higher temperature

such as 38°C will led to growth inhibition and death of the cells. In conclusion, 32°C was the most suitable temperature to cultivate *Spirulina* sp. for high starch and carbohydrate yield with the highest biomass content.

Conclusion

In can be concluded that high intracellular starch content of *Spirulina* sp. was detected during late exponential phase. Thus, harvesting process should be done on this stage. Besides that, high aeration rate are not able to increase the growth rate and intracellular starch content in *Spirulina* sp. but inversely caused damaged to the *Spirulina* sp. Hence, adequate control of aeration can be used to increase the intracellular starch, biomass and starch yield. On the other hand, cultivation of *Spirulina* sp. using white light at 32°C gave the highest dried cell weight, starch and carbohydrate yield.

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