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 435% and 221% increased, respectively.

**Keywords**

*Spirulina*  
Freshwater microalgae  
Starch  
Light wavelength  
Carbohydrate

**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 worldwide 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 Tomasselli 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
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 (MgSO₄•7H₂O, 0.2 g), calcium chloride dihydrate (CaCl₂•2H₂O, 0.04 g), iron (II) sulfate heptahydrate (FeSO₄•7H₂O, 0.01 g), disodium ethylenediaminetetraacetae dihydrate (Na₂EDTA•2H₂O, 0.08 g), dipotassium phosphate (K₂HPO₄•0.25 g), sodium nitrate (NaNO₃, 2.5 g), potassium sulfate (K₂SO₄, 1.00 g), sodium bicarbonate (NaHCO₃, 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 (H₃BO₃, 2.86 g), ammonium molybdate tetrahydrate ((NH₄)₆Mo₇O₂₄•4H₂O, 0.02 g), manganese (II) chloride tetrahydrate (MnCl₂•4H₂O, 1.8 g), copper (I) sulfate, (CuSO₄, 0.08 g) and zinc sulfate heptahydrate (ZnSO₄•7H₂O, 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 1 h dark and 1 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, 32°C 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 microalgal suspension was centrifuged at 6000 rpm for 10 min at 4°C 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.

\[
\text{% Carbohydrates} = \frac{(\text{Absorbence} - \text{intercept})/\text{Slope}}{\text{Sample weight (mg)/Total hydrolyze (mL)}} \times 100 - - - - (1)
\]

\[
\text{% Starch} = \Delta A \times F/W \times FV \times 0.9 - - - - - - - - (2)
\]

Where

\( \Delta A = \) Absorbance (reaction) read against the reagent blank

\( F = 100 \mu g \) 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).

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
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 CO2 concentration. At low CO2 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).

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<th>Table 1. <em>Spirulina</em> sp. starch analysis at three main growth phases</th>
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<td>Early of exponential phase</td>
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<td>Late stationary phase</td>
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*Data was reported as means of triplicates ± standard error.*

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<th>Table 2. Effect of aeration and non aeration on starch and carbohydrate production during cultivation of <em>Spirulina</em> sp.</th>
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<td>Parameter</td>
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*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.
On the other hand, red light has the highest carbohydrate content (32.97±2.09%), 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±2°C Spirulina sp. microalgae shows the lowest dried cell weight, starch content and carbohydrate content of 0.104±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 35°C. 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 32.4°C, in which the maximum biomass productivity is achieved.

Starch content produced during Spirulina sp. cultivated at 32°C (1.94±0.07%) was significantly different (P<0.05) compared to room temperature and 30°C. In addition, the highest starch yield was obtained at 32°C (0.664±0.037 mg/L). This result was significantly (P<0.05) higher compared to other temperatures used. In term of carbohydrate content, 34°C 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 38°C during photosynthesis and isotopes of carbon dioxide fixation (14C) resulted in significant decreased in 14 carbon isotope (14C) in starch. Besides that, Converti et al. (2009) study had reported that at 35°C, microalgae showed significant decreased in its growth rate and at higher temperature
such as 38°C will lead 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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**References**


Biomass 1: 20-32.


