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The effect of salinity on growth, biochemical composition and fatty acid profile of *Spirulina* (*Arthrospira platensis*) grown in sheltered outdoor conditions in Oman

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Abstract

Salinization has been known as the main reason for crop failure in Oman. Since saline water is detrimental for crops production, it can be used in commercial Spirulina cultivation. Under sheltered outdoor condition in Oman, this study presents the investigation of different salinity concentrations (5, 15, 25 and 35 psu) on the growth, biochemical composition and fatty acid profile of *Arthrospira platensis*. Lipid productions were significantly higher in *A. platensis* grown in 35 psu (14.635±0.087% dry weight) whereas significantly lower in 15 psu (12.090±0.090% dry weight). Next, protein content was significantly lower in 5 psu (41.678±0.452% dry weight) while others were not significantly different. Besides that, carbohydrate content was significantly higher in 15 psu (18.656±0.098% dry weight) while significantly lower in 5 psu (12.261±0.104% dry weight). Furthermore, the fatty acid compositions were comparable between *A. platensis* grown in the different salinity concentrations. The variations in saturated fatty acids (SAFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) between different salinity are 56.26% to 58.42% 5.67% to 6.51% and 35.70% to 37.80% respectively. The major fatty acids acquired with the cultivation of *A. platensis* were Palmitic acid (C16:0, 54.78 – 57.25%), Palmitoleic acid (C16:1, 2.47 – 2.74%), Heptadecanoic acid (C17:0, 0.14 – 0.23%), Stearic acid (C18:0, 0.96 – 1.27%), Oleic acid (C18:1n9, 3.19 – 3.77%), Linoleic acid (C18:2n6, 23.84 – 24.94%), Y-linolenic acid (C18:3n6, 11.55 – 12.81%) and Eicosadienoic acid (C20:2n6, 0.22 – 0.31%). Adaptation process is one of the crucial aspects which could reduce the deviation in production of biochemical compounds by *A. platensis* grown in different salinity stress.

Keywords: *Arthrospira platensis*, Fatty acids composition, Biochemical analyses, Salinity, Sheltered outdoor

Introduction

Arthrospira platensis is a photosynthetic filamentous alkalophilic microorganism that has been widely investigated for having commercial importance as a source of single cell protein (Anupama, 2000), vitamins, minerals, abundance of essential amino acids and fatty acids (Bezerra *et al*., 2008; Pandey *et al*., 2010; Gami *et al*., 2011). This cyanobacterium has been acknowledged as a "wonderful health food'' due to its wide spectrum of complete organic nutrients, super antioxidant and secondary metabolites which are thought to be responsible for nutraceuticals, pharmaceutical and therapeutic properties of *A. platensis* (Colla *et al*., 2004; Ahmadzade-Nia *et al*., 2011).

Although *A. platensis* is valued for its high nutritional values (Belay *et al*., 1993) especially protein (Cifferi, 1983; Cohen, 1997), other component such as fatty acids especially gamma linolenic acid (GLA) claimed to have positive medical and dietary effects (Diraman *et al*., 2009). The beneficial effects of these fatty acids such as polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and GLA on human health have been clinically proven by reducing the risk of cardio-circulatory diseases (Tokusoglu and Unal, 2003) rheumatoid arthritis (Zurier *et al*., 1996), cancer, hyperlipidemia (Leaf and Weber, 1988) and atherosclerosis. Thus, numerous studies have been done previously in order to optimize these valuable essential fatty acids using different species (Otles and Pire, 2001) and strains (Muhling *et al*., 2005) of *A. platensis*.

These previous investigations shown that fatty acids composition of *A. platensis* are influenced by variable environmental conditions (light intensity, temperature, and salinity), nutrients availability (nitrogen, phosphorus and carbon) and growth phases (Vonshak *et al.,* 1996; Rafigul *et al.,* 2003; Koru and Cirik *et al*., 2006; Ayachi *et al*., 2007; Ronda and Lele, 2008; Yilmaz *et al*., 2010; Kim *et al*., 2013). But, most of the reported findings were done in laboratory condition with suboptimal light intensity and narrow range of temperature (Colla *et al*., 2004; Chaiklahan *et al*., 2007; Muhling *et al*., 2005) which were consistent throughout the experiment. Although these findings are commendable and replicable due to the fixed and standardized growth parameters

but, they are not applicable in ambient outdoor conditions where various environmental factors could affect the growth of culture (Naqqiuddin *et al*., 2014).

All those studies are important for basic understanding on the biochemical composition of Spirulina but, it is equally important to know the biochemical composition of Spirulina grown in natural environment with diurnal, seasons, cloud cover and temperature variations. Besides that, studies on the effect of salinity on fatty acid profile of Spirulina cultured in outdoor conditions are also lacking but it is very important to be investigated due to increasing human population, climate change, desertification, rising of sea level and so on. Thus, this paper investigates the growth, biochemical composition and the fatty acid profile of Spirulina cultured in different salinity stress which were 5, 15, 25 and 35 psu under sheltered outdoor condition.

Materials and Methods

Experimental Design

The experiment was conducted in the Agricultural Experimental Station of Sultan Qaboos University, Muscat, Oman. Strain of *Arthrospira platensis* brought from Plant Physiology Lab, Biology Department, Faculty of Science, Universiti Putra Malaysia. The cultivation process was carried out in green house, covered by a polyethylene sheet with 60 - 70% of transparency. The stock cultures of the *A. platensis* were adapted to 4 different salinities (5, 15, 25 and 35 psu) in 100 L wooden tanks (areas of 100 x100 cm) with 10 cm depth which were lined with clear polythene sheets (Almahrouqi *et al*., 2015). After 3 cycles of acclimation phase, 5 % of *A. platensis* cultures were inoculated into Kosaric medium with different salinity level. Growth was measured daily through biomass dry weight following Sorokin (1973).

Kosaric medium was prepared (Tompkins *et al*., 1995) with minor modification using commercial fertilizer (g L^{-1}): 5.0 $NaHCO_3$, 0.25 NaCl, 0.1 $CaCl_2$, 0.2 $MgSO_4.7H_2O$, 0.221 Urea, 0.07 H_3PO_4, 0.242 KOH, 0.02 $FeSO_4.7H_2O$ and 0.5 mL/L of trace metals solution composed of following elements (g L^{-1}): 2.86 H_3BO_4, 1.81 $MnCl_2.4H_2O$, 0.22 $ZnSO_4.7H_2O$, 0.08 $CuSO_4.5H_2O$, 0.01 MoO_3, and 0.01 $COCl_2.6H_2O$ (Sukumaran *et al*., 2014a). Urea was added to culture medium by using fed-batch methods (pulse fertilization) (Danesi *et al*., 2002).

Each salinity treatment (5, 15, 25 and 35 psu) was prepared in triplicate. The proportion of common sea salts were dissolved slowly in prepared growth medium and adjusted to achieve required salinity level. Salinity was measured using salinometer (YSI Model 33, S-C-T Meter).

Biochemical analyses

Cultured *A. platensis* was harvested using Nylon filter cloth (25 µ pore size) and rinsed several times with freshwater to remove excess salt which might influence the analyses. Harvested samples were freeze dried and kept in freezer at -80°C prior to analyses.

Protein was extracted using 1N NaOH and been analyzed by adding alkaline copper solution and Folin-Ciocalteau reagent following Lowry *et al*. (1951). Protein standard was prepared using bovine serum albumin at different concentrations. The absorbance was measured through spectrophotometer (Hitachi U-1900) at a wavelength of 750 nm.

Lipid was extracted according to Bligh and Dyer (1959) and determined through sulfuric acid-charring method following Marsh and Weinstein (1966) using tripalmitin as standard. The absorbance was measured at 375 nm using spectrophotometer (Hitachi U-1900).

Carbohydrate analysis was conducted following the method of Kochart (1978). The freeze dried samples were hydrolyzed with 2N HCl and been analyzed by adding 1 mL of a 5% of phenolic solution and 5 mL of concentrated sulfuric acid. Standard was prepared using glucose. The absorbance was measured at 488 nm using a spectrophotometer (Hitachi U-1900).

Fatty acid determination

The FAME extracted from the lyophilized *A. platensis* biomass using conventional method (Araujo *et al*., 2008). Briefly, 4 mL of chloroform: methanol 2:1 (v/v) was added to 0.2 g of sample in a 10 mL Sovirell pyrex tube and followed by additional of 1 mL of Heneicosanoic acid (3mg/mL) (Chemika, 99%) as an internal standard. The vortex-mixed sample was left at -20 ˚C overnight. The sample was filtered on the next day and the solvent was removed with a rotary evaporator (Buchi R-215) at 40 ˚C. The residue was dissolved in 6 mL of diethylether, dried under stream of nitrogen and saponified in 1 mL of methanolic NaOH (0.5M). The mixture was vortex-mixed and heated for 15 min at 100 ˚C then cooled in water. Then, the transesterification process was performed by adding 2 mL of boron trifluoride (BF_3) (Acros organics) and heated for 5 min at 100 ˚C. After FAME synthesis, the tube was cooled in a cold tap water and 1 mL Hexane (Sigma-aldrich# 20872, > 95%) and 2 mL of ultra pure water (Elga system, pure lab option-Q) was added. Then, the vortex-mixed tube was centrifuged at 3000 rpm for 15 min, and the hexane layer was transferred to a vial for FAME analysis.

GC-MS analysis was performed on a Perkin Elmer Clarus 600 GC System, fitted with a SP-2560 *Supelco* capillary column (100 m × 0.250 mm i.d. × 0.2μm film thickness) coupled to a Perkin Elmer Clarus 600C MS. Ultra-high purity helium (99.9999%) from air products was used as carrier gas at a constant flow of 1.0 ml/min. The injection, transfer line and ion source temperatures were 250, 250 and 250 ℃, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The injected sample volume was 1 µl with a split ratio of 50:1. The oven temperature program was 80° C (held for 5 minutes) and accelerated at a rate of 4° C / min to 240 ^{o} C and held for 15 minutes. The unknown compounds were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition) and further confirmed with Supelco 37 component FAME mixture (cat.# 47885-U).

Statistical analysis

One-way Analysis of variance (ANOVA) and Tukey-honest significant difference (HSD) test with SPSS 21.0 were used to determine any significant difference in growth (dry weight), biochemical composition and fatty acid composition of *A. platensis* grown under different salinity stress.

Results and Discussion

At the end of culture period on day 11, dry biomass of group 1 were (5 and 15 psu) and (25 and 35 psu) for group 2 respectively. There are two distinct biomass (g L^{-1}) dry weight pattern; group 1: 5 and 15 psu groups which had significantly higher dry weight ($p < 0.05$) compared to group 2: treated with 25 and 35 psu and no significant difference ($p > 0.05$) within group (5 and 15 psu) and (25 and 35 psu) (**Fig 1**).

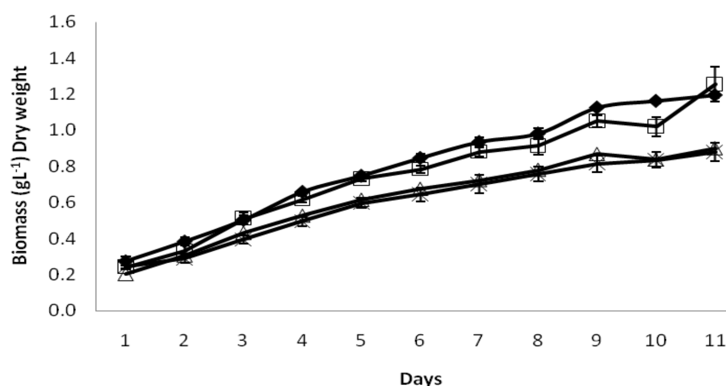

Figure 1 **Dry weight (g L^{-1}) at different salinity: 5 psu; (◆), 15 psu; (□), 25 psu; (△), 35 psu; (×). Values are presented as Mean ± SE (n = 3).**

As much as the growth is affected by salinity, protein, lipid and carbohydrates are also affected. Protein (% dry weight) is significantly higher ($p < 0.05$) at 15, 25 and 35 psu than at 5 psu. Lipid (% dry weight) was highly significant in 35 psu ($p < 0.05$) followed by 5 and 25 psu while 15 psu contained the lowest. Carbohydrate (% dry weight) was highest significantly in 15 psu ($p < 0.05$) followed by 25 and 35 psu while the lowest was at 5 psu (**Table 1**).

Table 1 Biochemical composition of *A. platensis* (% dry weight) in different salinity concentration (5, 15, 25 and 35 psu).

Salinity (psu)	Protein (% dry weight)	Lipid (% dry weight)	Carbohydrate (% dry weight)
5	41.678±0.452[b]	12.684±0.070[b]	12.261±0.104[c]
15	48.091±0.288[a]	12.090±0.090[c]	18.656±0.098[a]
25	46.998±0.499[a]	13.018±0.092[b]	16.457±0.094[b]
35	46.424±0.315[a]	14.635±0.087[a]	16.449±0.108[b]

* Each value is presented as Mean ± SE (n = 3). Means within each row with different letter (a-c) differs significantly ($p < 0.05$).

Results of prominent fatty acids in *Arthrospira platensis* in different salinities are shown in **Table 2**. Palmitic acid ME (C16:0) comprises the bulk of fatty acids (54.78-57.25%) in all the salinity tested without any significant different among them ($p > 0.05$). The Palmitoleic acid ME (C16:1) (% dry weight) was highest ($p < 0.05$) than others at 15 psu. The amount (% dry weight) of Heptadecanoic acid ME (C17:0) at 5psu was significantly higher ($p < 0.05$) than in other concentrations (15 and 35 psu) but 25 psu is not significantly different ($p > 0.05$) to the rest of salinity tested.

Table 2 Results of fatty acids composition (% dry weight) in different salinity concentration of *Arthrospira platensis* (5, 15, 25 and 35psu)

Name	**Fatty acid (% dry weight)**			
	5 psu	**15 psu**	**25 psu**	**35 psu**
Palmitic acid ME (C16:0)	54.78±1.06[a]	56.66±0.80[a]	57.25±0.54[a]	55.20±1.09[a]
Palmitoleic acid ME (C16:1)	2.47±0.03[b]	2.74±0.03[a]	2.48±0.07[b]	2.56±0.02[b]
Heptadecanoic acid ME (C17:0)	0.23±0.02[a]	0.17±0.01[b]	0.18±0.01[ab]	0.14±0.00[b]
Stearic acid ME C18:0)	1.24±0.06[a]	0.96±0.04[b]	0.99±0.06[b]	1.27±0.07[a]
Oleic acid ME (C18:1n9)	3.47±0.05[a]	3.77±0.15[a]	3.19±0.19[a]	3.72±0.14[a]
Linoleic acid ME (C18:2n6)	24.94±0.14[a]	23.84±0.54[a]	24.01±0.22[a]	24.08±0.17[a]
γ -linolenic acid ME (C18:3n6)	12.63±1.01[a]	11.55±0.32[a]	11.67±0.36[a]	12.81±0.94[a]
Eicosadienoic acid ME (C20:2n6)	0.23±0.05[a]	0.31±0.03[a]	0.23±0.02[a]	0.22±0.03[a]
∑SAFA	56.26±0.99[a]	57.79±0.83[a]	58.42±0.60[a]	56.60±1.08[a]
∑MUFA	5.94±0.08[ab]	6.51±0.16[a]	5.67±0.26[b]	6.28±0.16[ab]
∑PUFA	37.80±1.00[a]	35.70±0.82[a]	35.91±0.57[a]	37.11±1.07[a]
Saturated:Unsaturated	1:0.78	1:0.71	1:0.73	1:0.77

* Each value is presented as Mean ± SE (n = 4). Means within each row with different letter (a-b) differs significantly ($p < 0.05$).

Amount (% dry weight) of Stearic acid ME (C18:0) achieved in (5 and 35 psu) were significantly higher ($p < 0.05$) than group (15 and 25 psu). MUFAs (% dry weight) are significantly high ($p < 0.05$) with 15 psu than 25 psu but not significantly different ($p > 0.05$) to 5 and 35 psu. Oleic acid ME (C18:1n9), Linoleic acid ME (C18:2n6), γ -linolenic acid ME (C18:3n6) and Eicosadienoic acid ME (C20:2n6) were not significantly different ($p > 0.05$) in all the salinity tested.

As mentioned earlier, most finding reported are done in laboratory and probably without sufficient acclimatization that showed profound effects on growth (Zeng and Vonshak, 1998; Dhiab *et al*., 2007), biochemical composition (Mary Leema *et al*., 2010; Sukumaran *et al*., 2014b) and fatty acid profile (Yilmaz *et al*., 2010) of Spirulina (*Arthrospira platensis*). During acclimatization, Spirulina probably undergoes genetic and physiological adjustment to optimally survive in a particular salinity. According to Kebede *et al*. (1997), Spirulina can tolerate extreme salinity level and was spotted continuously growing even beyond salt concentration of 88 g L^{-1}. However, the optimum salinity having highest growth of Spirulina was observed at 13 g L^{-1} of salinity concentration (Ravelonandro *et al*., 2011). At high salinity, energy was utilized to maintain optimal osmotic balance thus affecting the growth of microalgae. In this study, 5 and 15 psu seemed to be optimal for the growth of Spirulina while 15 psu showed high protein level and carbohydrates. Despite having Spirulina grown under sheltered outdoor conditions where light varies from 100 - 1800 μmol$m^{-2}s^{-1}$ and temperature fluctuate from 15°C to 38°C, the growth (dry weight biomass), biochemical compositions and fatty acids profiles are at par with many studies reported previously (Diraman *et al*., 2009; Yilmaz *et al*., 2010). Although Spirulina is traditionally cultivated in freshwater but this finding shown that Spirulina can be successfully cultivated in area where water salinity is high.

Conclusion

This study conclude that with sufficient acclimatization, Spirulina can be grown in salinity ranges from 5 to 35 psu without large differences of dry weight biomass, biochemical composition and fatty acid profile.

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