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RISK ASSESSMENT OF *SPIRULINA PLATENSIS* AS A SOURCE OF FOOD AND FEED ADDITIVES

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ABSTRACT

Egyptian isolate of *Spirulina platensis* was cultivated in closed and open systems using modified BG-11 medium. Regardless the cultivation conditions, protein represented the highest component (50.6 - 57.7%) followed by carbohydrates (12.4 - 18-4%), ash (10.7%), total lipids (7.3-8.1%), moisture (7.1%) and crude fiber (4.1-5.7%). The chemical composition of *Spirulina* in closed system characterized by its high content of protein, subsequently amino acids and lipids. Although, results of the risk assessment of *Spirulina* from both systems, performed during this study, indicated that this product is safe for food or feed consumption. It was found that *Spirulina* from open system was more subjected to contamination by several microorganisms and heavy metals. It was concluded that biogenic amines should be taken into account as potential hazards notably with mis-handled product.

Keywords: *Spirulina platensis*, food additives, risk assessment, biogenic amines, heavy metals.

INTRODUCTION

Food safety has occupied a central stage in the past few years to eliminate the outbreaks of many pathogenic organisms particularly in fresh products. Hygiene problems are associated with the production of conventional foods and also occurred in algae like *Spirulina*. Commonly, *Spirulina* is marketed as a dry product and it is reported that drying process may contribute to decrease the microbiological load which is a part of an overall good manufacturing practice. The major areas of concern for safety are microbiological contamination, heavy metals content, pesticides residues, extraneous matter and cyanobacterial toxins (Belay, 1997; FDA, 1998 and Belay, 2008).

A potential problem of *Spirulina* production in open system is the hazard of water contamination with pathogenic organisms. Handling of the product during processing, harvesting and drying, can also result in microbial contamination. The final microbial load of the product will therefore depend on how carefully the culture and product are handled at the various stages of production (Belay, 2008). Only good manufacturing practices and direct analysis of microbial flora as well as concentration in each lot of product can guarantee the safety of the product. The final product should meet microbiological standards set by the various national and international standards (Dillon and Phan, 1993 and Belay, 2008).

Standard plate counts and confirmed coliform densities are used in the food industry to monitor and inspect mal-handling of food products during processing (FDA, 1998). Analysis of hundreds of *Spirulina* samples from modern commercial farms in Thailand, Japan, Taiwan, and Mexico show that coliforms are rarely present (Jassby, 1988), indicating the good sanitary conditions of growth, harvest, drying and packaging.

As in other agricultural products, lead, mercury, cadmium, and arsenic are potential contaminants in algal products since they are components of industrial pollution and occur in trace amounts in certain agricultural fertilizers. It is known that certain microalgae are effective accumulators of heavy metals (Lacquerbe *et al*., 1970). The production of high quality *Spirulina* therefore requires the use of high-grade nutrients and routine analysis of heavy metals in the culture medium and the product. This is particularly important in situations where food-grade *Spirulina* is produced from open-ponds or natural lakes (Johnson and Shubert, 1989 and Belay, 2008).

There is no report of any cyanobacterial toxins above specified limits in *Spirulina* species and their discovery is considered very unlikely to occur during monoculture of *Spirulina* in properly controlled and managed systems (Belay, 2008). It is true that some cyanobacteria can produce hepatotoxins or neurotoxins, like *Microcystis aeruginosa* produces microcystins, which are potent hepatotoxins and probable tumor promoters (Fiore *et al*., 2009 and Gomaa *et al*., 2010). Also, *Oscillatoria brevis* and *Aphanizomenon flos-aquae* were reported to produce cyanobacterial neurotoxins anatoxin-a and anatoxin-a (s) (Carmichael, 1997 and Gilroy *et al*., 2000). Despite the fact that *Spirulina platensis* is not

considered a toxigenic cyanobacteria species in controlled systems, it can periodically invaded by toxic cyanobacteria species in natural lakes (Carmichael, 1997 and Chamorro *et al*., 2008).

Several worldwide studies have been tested the presence of natural toxins and heavy metals in *Spirulina* samples. (Becker and Venkataraman, 1984; Belay, 2008 and FDA, 2011). However, none of them evaluate the risk assessment of *Spirulina* produced in Egypt. Also, no studies have been tested the probability of biogenic amines occurrence in *Spirulina*. So, the aim of the present study is to compare between *Spirulina platensis* biomass chemical composition from open and closed systems in Egypt as a quality parameter and evaluate risks of the potential hazards in biomass from both systems.

MATERIALS AND METHODS

MICROORGANISM

Pure strain of *Spirulina platensis* was obtained from Marine Toxins lab., National Research Centre, Egypt (Marrez *et al*., 2013).

CULTIVATION AND PRODUCTION OF *SPIRULINA PLATENSIS*

The culture media used for cultivation of *S. platensis* in both closed and open systems was modified BG-11 medium (El-Sayed, 2004). It is composed of 0.53g urea (46.5%N); 0.004 g K_2HPO_4; 0.075 g MgSO4.$7H_2O$; 0.036 g $CaCl_2.2H_2O$; 0.006 g citric acid; 0.02 mg Na_2CO3; 0.001 g Na_2EDTA; 0.63 g ferric ammonium citrate and 1.0 ml trace elements (TE) in 1000 ml distilled water. TE (g/l) is combined of 2.86 g H_3BO_3; 1.81 g $MnCl_2.4H_2O$; 0.222 g $ZnSO_4.7H_2O$; 0.39 g $Na_2MoO_4.2H_2O$; 0.079g $CuSO_4.5H_2O$ and 0.0494 g $Co(NO3)_2.6H_2O$ in 1000 ml distilled water.

The optimum growth conditions for closed system production (30±2°C and light intensity of 4.5 Klux m^{-2} provided by fluorescent lamps) were applied for this study according to Rafiqual *et al*. (2005), Soundarandian and Vasanthi (2008), Hemlata and Fatma (2009) and Chauhan and Pathak (2010). *S. platensis* was cultivated in 500 ml Erlenmeyer flasks containing 250 ml of representative media using shaking incubator (MP-7552, cv-cc power supply, hsiHefer, San Francisco). Experiments were initiated with 10% (v/v) of inoculum.

Outdoor mass production scale within two ponds with a final capacity $30m^3$ of net cultivation volume with 0.3m depth and covered with plastic sheet. Sub culturing was performed within sequences and gradual volumes till 1200L plate photobioreactor. Outdoor culture was cultivated in July and August 2013 which recorded 23±1°C as a minimum temperature and 33±2°C as a maximum temperature (EMA, 2013). Harvesting method was done using continuous separating centrifuge apparatus (Westevalia Separator centrifuge 5000L h^{-1}) and drain water was recycled to the ponds. Biomass was overnight dried in oven at 50°C.

CHEMICAL COMPOSITION OF *SPIRULINA PLATENSIS*

The moisture content, total ash and crude fiber were determined according to AOAC (2000). Total carbohydrates were determined according to the Renol-Reaction method of Gerhardt *et al*. (1981).

Total protein was determined by the conventional Micro-Kjeldahl method (AOAC, 2000). Amino acid profile was carried out according to Bailey (1967) using Eppendrof-Germany LC3000 amino acid analyzer. The flow rate 0.2 ml/min, pressure of buffer from 0.0 to 50 bars, pressure of reagent from 0.0 to 105 bar, reaction temperature 123ºC were adjusted.

Total lipids were extracted from *S. platensis* dry weight according to the AOAC (2000) method using Soxhelet apparatus. Fatty acid methyl esters (FAMEs) of the total lipid were prepared by transesterification using 2% sulphuric acid in methanol (Christie, 1993).The fatty acid analysis was done by gas chromatography (Perkin Elmer Auto System XL) equipped with flame ionization detector and a DB5silica capillary column (60 m × 0.32mm i.d.).The oven temperature was maintained initially at 45°C and programmed to 60°C at a rate 1°C/min, then it programmed from 60°C to 240°C at a rate of 3°C/min. Helium was used as the carrier gas at flow rate 1 ml min^{-1}. The injector and the detector temperatures were set at 230°C and 250°C, respectively.

RISK EVALUATION

MICROBIAL CONTAMINATION

Microbial assays were performed for 10 g of *S. platensis* powder in both applied systems. Each sample was mixed with 90 ml of 0.1% sterile peptone water. Then it was shaked for 10 min and suitable dilution were made for the various assays.

Standard methods were followed to determine the bacterial plate counts using nutrient agar media (Merck, Darmstadt, Germany). The plates inoculated with the *Spirulina* powder samples were incubated at 37ºC for 24 h. Counts were expressed as colony forming unit *i.e*. cfu g^{-1} (APHA, 2005).

The most probable number (MPN) method was used to determine total and faecal coliforms in the *Spirulina* powder samples. Serial dilutions of 10^{-1} to 10^{-5} were prepared. One milliliter aliquots from each of the dilutions were inoculated into 5 ml of MacConkey Broth with inverted Durham tubes and incubated at 35ºC for total coliforms and 44ºC for faecal coliforms for 18-24 h. Tubes showing color change from purple to yellow and gas produced in the Durham tubes after 24 h were identified as positive for both total and faecal coliforms. Counts per Gram were calculated from Most Probable Number (MPN) tables (Tassew and Seifu, 2011).

Total yeast and molds were assayed on potato-dextrose agar medium, which composed of potato 200g, glucose 15g, agar 20g and 1000 ml distilled water, the pH was adjust at 7.0 (ATCC, 1984). The plates inoculated with the *Spirulina* powder samples were incubated at 25ºC for 5 days followed by counting.

Differential tests were used in the identification of the coliforms as *Escherichia coli*, *Enterobacter* and intermediate species (Speck, 1976). Standard methods were used for determining the coagulase positive

Staphyococcus aureus (ISO, 1999) and analysis of *Salmonella* was carried out using the method of Thatcher and Clerk (1968).

NATURAL TOXINS

MYCOTOXINS

Sigma standards of aflatoxins (AFB1, B2, G1 and G2), ochratoxin A (OTA), fumonisin (FUM B1) and zearalenone (ZER) were used throughout the present study (Sigma, chemical company, USA).

Extraction, clean up and determination of AFs, OTA, FUM B1 and ZER were done according to AOAC (2007).

The HPLC system used for mycotoxins determination was Perkin-Elmer, series 200 system (USA), equipped with quaternary pump, fluorescence detector and a C18 column chromatography Phenomenex (250 x 4.6 mm, 5 µm). The mobile phase was water: methanol: acetonitrile (60:30:10) using as isocratic flow rate of 1.2ml min^{-1} at 360nm excitation and 440nm emission wave length and a 30 min run time for aflatoxins. For ochratoxin A analyses, acetonitrile: water: acetic acid (99:99:2) was isocratically used at flow rate of 1ml min^{-1} at 333nm excitation and 460nm emission wave length. The run time for samples was 20 min.

Regarding to fumonisin B1 analyses, the mobile phase methanol: sodium dihydrogen phosphate (0.1 M) (77:23) adjusted to pH 3.3 with phosphoric acid was used. At 335nm excitation and 440nm emission, isocratically samples run for 15min at 1ml min^{-1} flow rate. Fluorescence detector set at 274 nm excitation and 440nm emission wave length was used for zearalenone determination. The mobile phase acetonitrile: water: methanol (48:50:3) was isocratically used at flow rate 1ml min^{-1}according to (Zaied *et al*., 2012). Finally data of mycotoxins analysis were collected and integrated using Totalchrom Navigator Chromatography Manger Software.

CYANOTOXINS

Standard cyanobacterial toxin microcystin-LR used throughout the present study was obtained from Sigma, chemical company, USA. Determination of microcystin was carried out according to Amé *et al*. (2003). Twenty mg of dried cells of *S. platensis* were placed in Eppendrof tubes, extracted with 1.5 ml of 5% acetic acid and sonicated for 5 min using ultrasonic microtip probe of 400 Watt. The suspension was centrifuged at 4500 xg for 7 min. Supernatant was retained and the pellet re-extracted as before. Combined supernatants were centrifuged at 4500 for 20 min.

Extracted sample supernatant was applied to a C-18 solid phase extraction cartridge (strata C18, 500 mg/3ml, Phenomenex), which was previously conditioned with 10 ml methanol and 10 ml 5% acetic acid. The cartridge was washed 3 times with 10 ml of 10, 20 and 30% aqueous methanol and toxins were eluted with 10 ml of methanol HPLC grade. The elute was evaporated to dryness at 40˚C and resuspended in 200 µl of methanol prior HPLC analysis. The HPLC system used for microcystin-LR determination was Perkin-Elmer, series 200 system (USA), equipped with quaternary pump, UV diode array detector set at 238nm and a C18 column chromatography ODS Phenomenex (250 x 4.6 mm, 5 µm). Mobile phase gradient program at flow rate was 1 ml min^{-1} is mentioned in detailed in Table (1).

Table 1- HPLC mobile phase of microsystin-LR using gradient program

Time (min)	Solution (A) Acetonitrile with 0.05 % TFA	Solution (B) Water with 0.05 % TFA
0	30	70
5	35	65
15	70	30
17	100	0
19	30	70
22	30	70

TFA: Trifluoroacetic acid

BIOGENIC AMINES

Histamine, putrescine, cadaverine, tyramine, tryptamine and β-phenyl ethyl amine standards and Dansyl chloride (5-Dimethylaminonaphtalene -1- sulfonyl chloride) were obtained from Merck Company. All chemicals used were analytical grades. Biogenic amines were extracted and determined in tested samples according to Maijala and Eerola, (1993) and Ayesh *et al*. (2012).

In triplicate 25 g of homogenised dried *Spirulina platensis* biomass were blended with 125 ml of 5% trichloroacetic acid for 3 min using a warning blender. Filtration was achieved using filter paper Watman No. (1). Ten millilitres of the extracts was transferred into a culture tube with 4g NaCl and 1 ml of 50 % NaOH then shacked and extracted three times by 5 ml n-butanol / chloroform (1: 1 v/v) stoppered and shacked vigorously for 3.0 min. Mixture was centrifuged for 5.0 min. at 3000 rpm and the upper layer was transferred to 50 ml separating funnel using disposable Pasteur pipette. To the combined organic extracts (upper layer), 15 ml of n-heptane was added and extracted three times with 1.0 ml portions of 0.2N HC1, the HCl layers were collected in a glass stoppered tube. Solution was evaporated just to dryness using water bath at 95°C with aid of a gentle current of air.

The dansylated derivatives of the extracted amines were formed by dissolving the sample residue with 0.5 ml of saturated $NaHCO_3$ solution, then 1 ml of dansyl chloride solution (500 mg/100 ml acetone) was added ,and the mixture was incubated at 55ºC for 45 min. The dasylamines were extracted using diethyl ether then

evaporated under gentle stream of nitrogen (Sultan, 2004 and Ayesh, 2012). The obtained dry film was dissolved in 1ml methanol, then 10 ul injected in HPLC, under the following condition (Table 2):

Table 2. Mobile phase solvents of biogenic amines consists of solvent A (0.02 N acetic acid), solvent B (Methanol) and solvent C (Acetonitrile) which were used in gradient program as follow

Time (min.)	Flow rate ml/min	Solvent		
		A%	B%	C%
0	1	60	20	20
10	1	20	40	40
15	1	15	35	50
20	1	60	20	20
25	1	60	20	20

HEAVY METALS

Cadmium (Cd), nickel (Ni), manganese (Mn), lead (Pb), zinc (Zn), copper (Cu) iron (Fe) were determined according to the method described in AOAC (2000).The sample was ashed at 450 - 500°C using muffle furnace until the sample was completely combusted. The obtained ash was dissolved in 1 ml HCl concentration at crucible walls. Dissolved samples were transferred to a 50 ml volumetric flask and de-ionized water was added to complete volume. The solution was filtered through ashless filter paper Whatman No. 42 and stored in a refrigerator until determination by Atomic Absorption Units (GBC 932 AA).

STATISTICAL ANALYSIS

Statistical significance was measured using Statistica Version 9 (State Soft, Tulsa, Okla., USA).

RESULTS AND DISCUSSION

The maintenance of the quality and safety of *Spirulina* product depends on how the entire process from cultivation system to powder production is controlled. *Spirulina* powder is manufactured in accordance with current good manufacturing practices to assure that the food products are safe for consumption and have been prepared, packed and held under sanitary conditions. Quality control for *Spirulina* as a food includes microbiological standard tests, chemical composition and test for heavy metals, mycotoxins and biogenic amines.

CHEMICAL COMPOSITION OF *SPIRULINA PLATENSIS*

Consistent chemical and physical properties are considered remarkable aspects of high quality production of *Spirulina*. Although, seasonal and annual variations in product quality are observed in open system production. The yield of *Spirulina* biomass in closed system was higher (4.79 g l^{-1}) than that in open system (1.84 g l^{-1}). Consistency of biochemical composition is observed as well (Belay and Ota, 1994). In the present study chemical composition of *S. platensis* grown in open ponds and closed systems were determined (Fig. 1). No significant difference ($p<0.05$) was noticed in the moisture content of both cases. The mean values are in the range of general recommendation (<10%) for a quality (Albert *et al*., 2012). Also, ash contents in *S. platensis* cultivated from either closed or open system recorded statistically the same value (around 10.7%). Becker and Venkataraman (1982) reported that ash content in *Spirulina* ranged between 6 to 15%. Also, Albert *et al* (2012) found that ash content of *S. platensis* from different sites in Chad was ranged from 7.8 to 7.96%. Observations recorded by Habib *et al.* (2008) can explain why the ash content did not affect by changing system. They reported that bioaccumulation of minerals in *Spirulina* can be changed by culturing on different media, at different temperatures, pH and salinity.

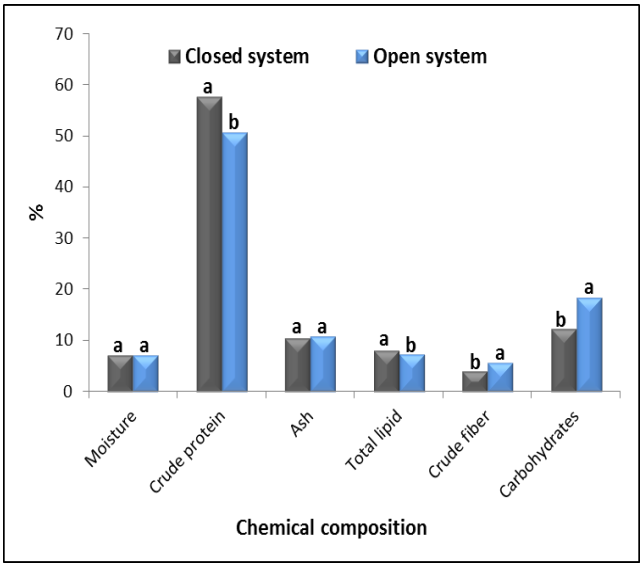

Fig. 1- Chemical composition of *Spirulina platensis* grown in open and closed systems

The protein content in *S. platensis* of closed system (57.67%) was significantly higher ($p<0.05$) than open ponds (50.62%). These results are in agreement with Alvarenga *et al*. (2011) and Albert *et al*. (2012) who reported that protein content ranged from 50.2 and 58.6% in *S. platensis* from open system. In contrast, Mbaïguinan *et al*. (2006) determined high amount of protein (69.2%) from *Spirulina* in open system. Also, Soni *et al*. (2012) indicated that *S. platensis* protein in outdoor conditions (56.5%) was higher than under laboratory conditions. Zeng and Vonshak (1998) reported that cells under stress conditions, including environmental stress, have a lower protein synthesis capacity. This fact can probably explain lower protein contents found in biomasses grown in open ponds as temperature variation between day and night are found.

The lipid content was significantly ($p<0.05$) influenced by both cultivation systems 8.13% in closed system and 7.33% in open system. Lower values than that of the present study obtained by Mbaïguinan *et al*. (2006) who found that lipid content ranged from 4.6 to 5.5% in open ponds. Whereas, close values was observed 5.6 to 7% in open system (Fox, 1996). These results were in contrary with Tanticharoen *et al*. (1994) who found that lipid content of *Spirulina* cultures grown outdoors (8.2%) was shown to be higher than that of laboratory cultivated cultures (5.9%).

Crude fiber content of *S. platensis* differed significantly ($p < 0.05$) from 4.11% in closed system and 5.68% in open system. Albert *et al*. (2012) revealed that the fiber content in *Spirulina* varied from 4 to 7 % in open ponds, which is close to the results of the present study. However, Moreiro *et al*. (2013) reported that crude fiber content in *S. platensis* was just 0.5% which considered much lower than 8 to 10% that reported by Habib *et al*. (2008), 8.4% by Mbaïguinan *et al*. (2006) and 6.15% by Alvarenga *et al*. (2011).

The carbohydrates content in *S. platensis* varied from 12.42% in closed system and 18.39% in open system. These results are similar to those obtained by Fox (1996) with values between 13 and 16.5% and with the percentage of 17.07 that reported in *Spirulina* from Kanem Lake Chad (Mbaïguinan *et al*., 2006). Also, Soni *et al*. (2012) reported that carbohydrates content in *S. platensis* from outdoor culture (14.5%) was higher than those obtained from laboratory conditions (14.2%).

AMINO ACIDS CONTENT IN CLOSED SYSTEM AND OPEN PONDS

Amino acids concentration of *S. platensis* (mg $100g^{-1}$) grown in open ponds and closed system are shown in Table 3. In general, the amino acids in dried *Spirulina* biomass harvested from closed system were higher than those of open system. It is shown that the essential amino acid contents in both closed system and open ponds were much higher than the minimum values reported by FAO/WHO (Volkmann *et al*., 2008).

Obgonda *et al*. (2007) studied the influence of temperature on protein content and amino acid compositions of *S. platensis* at 25, 30, 35 and 40°C. They found that highest amount of protein and amino acids were obtained at temperature of 30°C. This may be the reason of higher protein and amino acid content in closed system than open ponds. Also, Ciferri (1983) reported that low temperatures reduced amino acids content in *S. maxima*.

Table 3-Amino acids composition of *Spirulina platensis* in open and closed systems after 30 day of cultivation

Amino acid	Amino acids concentration $mg.g^{-1}$ (Mean±S.E)		
	Closed system[a]	Open system	FAO/WHO[b]
Essential amino acids			
Isoleucine	$14.16±0.98^{A}$	$9.29±0.93^{B}$	2.80
Leucine	$29.42±1.27^{A}$	$23.48±0.81^{B}$	6.60
Lysine	$19.10±1.01^{A}$	$12.01±0.71^{B}$	5.80
Methionine	$5.31±0.81^{A}$	$2.51±0.19^{B}$	2.50
Phenylalanine	$23.78±1.21^{A}$	$17.28±0.78^{B}$	6.30
Threonine	$13.59±0.87^{A}$	$10.55±0.72^{B}$	3.40
Valine	$18.40±1.41^{A}$	$13.46±0.82^{B}$	3.50
Histidine	$13.46±1.19^{A}$	$11.87±0.88^{B}$	1.90
Non-essential amino acids			
Cystin	$3.30±0.41^{A}$	$2.70±0.17^{B}$	
Alanine	$33.81±1.21^{A}$	$30.71±0.72^{B}$	
Aspartic	$36.69±1.09^{A}$	$31.24±0.54^{B}$	
Serine	$18.43±1.14^{A}$	$11.61±0.74^{B}$	
Glycine	$15.06±1.04^{A}$	$10.29±0.77^{B}$	
Glutamic acid	$47.03±1.12^{A}$	$39.18±1.17^{B}$	
Arginine	$44.91±1.02^{A}$	$42.91±1.02^{B}$	
Tyrosine	$19.74±1.03^{A}$	$17.15±0.78^{B}$	
Proline	$14.88±0.34^{A}$	$12.95±0.34^{B}$	
Ammonia	$54.91±1.12^{A}$	$46.65±0.81^{B}$	

(n=3), p < 0.001, a. Marrez *et al* (2014), b. recommendation for children of 2-5 year, according to FAO/WHO (Volkmann *et al*., 2008).

FATTY ACIDS PROFILE OF *SPIRULINA PLATENSIS* IN OPEN AND CLOSED SYSTEM

Fatty acids profiles of *S. platensis* grown in open and closed system are listed in Table 4. In general, very close values of fatty acids were obtained when compared between both systems. The total percentage of unsaturated fatty acids ranged from 15.43% in *S. platensis* from open system and 15.86% from closed system, while the saturated fatty acids ranged from 84.14% and 84.59% in closed and open system, respectively. The major fatty acids were palmitic acid and myristic acid that represented about 45% of total fatty acids.

The main concern in the present study was the occurrence of polyunsaturated fatty acids (PUFA) in *S. platensis* which have a significant nutritional importance which is effective in lowering plasma cholesterol,

Table 4-Fatty acids profile of *Spirulina platensis* in open and closed system after 30 day of cultivation

Fatty acid	% Fatty acid in dried *Spirulina platensis* (Mean±S.E)	
	Closed system*	Open system
C10:0	8.02±0.06^{A}	6.85±0.25^{B}
C12:0	14.96±0.14^{A}	13.95±0.17^{B}
C14:0	22.68±0.04^{A}	21.92±0.14^{B}
C16:0	22.19±0.11^{B}	24.98±0.52^{A}
C17:0	12.07±0.06^{A}	12.17±0.08^{A}
C18:0	4.23±0.03^{B}	4.72±0.06^{A}
C18:1	3.8±0.04^{A}	3.33±0.05^{B}
C18:2	2.31±0.05^{A}	1.72±0.12^{B}
C18:3	2.37±0.05^{A}	2.47±0.08^{A}
$C_{20}H_{30}O_2$	5.06±0.02^{A}	4.56±0.07^{B}
$C_{20}H_{32}O_2$	2.32±.02^{B}	3.35±0.11^{A}

*** Marrez *et al* (2014), n=3, different letters are significantly different (p < 0.01).**

Common name of C10:0, Capric acid; C12:0, Lauric acid; C14:0, Myristic acid; C16:0, Palmitic acid; C17:0, Margaric acid; C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid; C18:3, γ- Linolenic acid; $C_{20}H_{30}O_2$, Abietic acid; $C_{20}H_{32}O_2$, Arachidonic acid.

stimulating prostaglandins and has been used as a dietary supplement for the treatment of various chronic health problems (Kay, 1991). Close percentage of γ- Linolenic acid (Gamma Linolenic Acid, GLA) 2.4% was recorded in *Spirulina* from both systems, while the highest percentage of arachidonic acid 3.35% was recorded in *Spirulina* from open ponds.

In this respect, Cohen *et al*. (1997) reported that cultivation of *Spirulina* at 30–35°C achive the best yield of GLA acid. However, Hirano *et al*. (1990), Tanticharoen *et al*. (1994) and Colla *et al*. (2008) reported that GLA yield depends on dark and light cycles indoor or outdoor cultivation and harvest time of the culture. Moreover, it is conceivable that highest GLA contents will be obtained by harvesting at the end of the dark period. Also, Mühling *et al*. (2005) studied the temperature influence on *S. platensis* M2 and determined the fatty acids contents of *S. platensis* at the different temperatures; they observed that the lowering temperature led to the decrease of the C16:0 content. Quoc and Dubacq (1997) observed an increase in the level of C18:2 with the lowering of the temperature.

HEAVY METALS

The contents of 8 heavy metals content (mg100g^{-1}dry matter) in *S. platensis* growth in both open and closed systems are represented in Table 5. Generally, the content of studied metals in *Spirulina* from open system was significantly higher than those determined in closed system. This means that in addition to the composition of the medium as a source of metals, *Spirulina* can be contaminated from the outer environment.

Iron in *Spirulina* recorded the highest amount among other metals (12.4 and 47.0 mg100g^{-1} in closed and open systems, respectively). Whereas, other metals recorded less than 10 mg100g^{-1} in either closed or open systems. These findings agree with Koru *et al*. (2008) as they reported that the concentration of iron in *Spirulina* from open ponds was 53.6 mg100g^{-1}. Also, the iron value 87.4 mg100g^{-1} in *Spirulina* was reported by Belay (2008). The content of heavy metals in *Spirulina* collected from natural lake was completely different than those cultivated from synthetic media either from closed and open systems. Albert *et al*. (2012) found that the concentrations of zinc, copper, iron and magnesium in *Spirulina* from lake in Chad were 216, 250, 789 and 900 mg100g^{-1}, respectively. They explained that natural lake can be contaminated by agricultural fertilizers and industrial wastes in addition to the nature of soil which originally contains high amount of metals that can release into lake water and consequently to algae.

The metals nickel, lead and cadmium were determined as they can consider as a parameter of contamination occurrence. Among these 3 metals, just lead was found in *Spirulina* from open system (0.07 mg100g^{-1}). This result revealed that *Spirulina* products in the present study can be considered as a safe product for consumption. Ortega-Calvo *et al*. (1993) performed a survey on commercial *Spirulina* products from different places in Spain regarding its content with Ni, Pb and Cd. They found that lead content ranged from 0.21 to 0.92 mg100g^{-1}, nickel valued from 0.39and 0.77 mg100g^{-1} and cadmium ranged from 0.01 and 0.02 mg100g^{-1}.

Table 5- Heavy metals concentration (mg100g^{-1} DW) of *Spirulina platensis* in open and closed systems

Mineral	Mineral concentration (Mean±S.E)	
	Closed system	Open system
Mg	6.2±0.06^{B}	7.9±0.11^{A}
Mn	0.72±0.04^{B}	1.39±0.17^{A}
Zn	2.6±0.21^{A}	1.27±0.14^{B}
Cu	5.1±0.66^{B}	8.97±1.35^{A}
Fe	12.4±0.16^{B}	47.01±1.5^{A}
Ni	ND	ND
Pb	ND	0.07±0.01
Cd	ND	ND

n=3, different letters are significantly different (p< 0.01), ND: not detected.

MICROBIOLOGICAL ANALYSIS

Microbial monitoring of *Spirulina* powder is necessary, since it is likely to affect the quality and safety of the final product. The results of microbiological analysis (Table 6) showed that the levels of bacterial contamination

in closed system ($1.2x10^3$ cfu g^{-1}) were smaller than that for dried *Spirulina* from open system ($1.4x10^4$ cfu g^{-1}). Also, total yeast and molds in closed system ($1.8x10^2$ cfu g^{-1}) were lower than that in *Spirulina* from open system ($1.1x10^2$ cfu g^{-1}). Coliforms groups and pathogenic bacteria, *E. coli*, *Enterobacteria*, *Salmonella* and *Staphylococcus* were not found in dried *Spirulina* from both open and closed cultures systems. All obtained data of the microbial contents were within the limits established by EU and WHO. Regulations of standard plate count differed from country to another within a very narrow range. Vonshak (1997) reported that the standard plate count limits for dried *Spirulina* are < $0.1x10^6$ cfu g^{-1} in France, <10 $x10^6$ cfu g^{-1} in Sweden, < $0.05x10^6$ cfu g^{-1} in Japan and < 1 x 10^6 cfu g^{-1} for *Spirulina* produced by Earthrise Farms, USA. Also, he reported that the drying process would probably decrease the level of contamination.

Jittanoonta *et al*. (1999) found that microbial contamination was within the safety ranges since solar drying might provide sufficiently high temperature to destroy the pathogens. Also, they reported that total bacterial count was 1.2 x 10^4 cfu g^{-1} and molds 1.9 x 10^2 cfu g^{-1}.

Likewise, Vermorel *et al*. (1975) revealed that the microflora associated with *Spirulina* crops is generally non pathogenic. The high alkalinity of *Spirulina* environment (pH 8.5 -11.0) was an excellent barrier against contamination, whether by bacteria, fungi or algae. Further certain substances secreted by or present in *Spirulina* have a bactericidal or at least bacteriostatic effect. Similarly, Jacquet (1975) and Mbaïguinan *et al*. (2006) reported that Bacteriological analyses of *Spirulina* produced industrially in Mexico or the United States confirm the complete absence of pathogens such as *Salmonella*, *Shigella* and Staphylococci.

Mahadevaswamy and Venkatraman (1987) indicated that microbial contamination of dried *S. platensis* was raised in outdoor open ponds when compared to indoor cultures. In both indoor and outdoor cultures, coliform group, *Salmonella* and *Staphylococcus* were not found. Belay (1997) reported that microbiological analyses confirmed that dry powder passed the necessary sanitary control regulations to qualify the product as food for human consumption.

The cultivation of microalgae in large open ponds will invite contamination of the harvested product by other microorganisms. Subsequent handling of the product during harvest, drying and packaging could also result in microbial contamination. The final microbial load of the product will therefore depend on how carefully the culture and product are handled at various stages (Belay, 2008).

Table 6- Microbial contamination (cfu g^{-1}) of dried *Spirulina platensis* in open and closed systems and related regulatory standards

Microorganisms	Closed system	Open system	EU*	WHO**
Total plate count	1.2 x 10^3	1.4 x 10^4	1.0 x 10^5	1.0 x 10^5
Total yeast & mold	1.1 x 10^2	1.8 x 10^2	1.0 x 10^4	1.0 x 10^3
Total coliforms	Negative	Negative	No data	No data
Fecal coliforms	Negative	Negative	No data	No data
E. coli	Negative	Negative	Absent in 1g	10 cfu/g
Enterobacteria	Negative	Negative	1000 cfu/g	No data
Salmonella	Negative	Negative	Absent in 10g	Negative
Staphylococcus	Negative	Negative	Absent in 1g	No data

*****EU, European Union Pharmacopeia (Kneifel *et al*. 2002), **WHO, World Health Organization (Belay, 2008).

NATURAL TOXINS

The mycotoxins aflatoxins, ochratoxin A, fumonisin B1, zeralenone and cyanobacterial toxins, microcystin-LR, were determined in dried *S. platensis* (Table 7). No mycotoxins were presented in *Spirulina* from open and closed system with the exception of aflatoxins (0.2 µg kg^{-1}).

Jacquet (1975) confirmed this finding who reported that the conservation of dried *Spirulina* preparations seems to pose no problem because the product appears to be fully resistant to molds. Thus, *Aspergillus flavus* and aflatoxins, secreted by this fungus, have never been detected in batches of *Spirulina*. Aldridge (1996) tested some *Spirulina* samples for aflatoxin, ochratoxin A, sterigmatocystin, citrinin, patulin, penicillin acid, zearalenone, diacetoxyscirpenol and thrichothecene. None of these compounds could be detected in *Spirulina*. In addition, Jittanoonta *et al*. (1999) reported that no aflatoxins in *spirulina* which cultivated in open ponds in Thailand and dried by solar energy.

Habib *et al*. (2008) found that no microcystins was detected in *Spirulina* which collected from open pond in Canada. While, Gilroy *et al*. (2000) found that MC-LR 15 *Spirulina* samples (dietary supplements) were detectable but below the regulatory level (1 µg g^{-1}) established by the FDA for microcystins in blue-green algae products. Similar findings were scored by Kay (1991), Carmichael (1994) and Hanrikson (2010) when they reported that *Spirulina* does not normally contain microcystins but contamination of outdoor culture by other cyanobacteria such as *Anbaenopsis circularis* and *Microcystis aeruginosa* is possible.

BIOGENIC AMINES

Figure (2) illustrate the concentrations of the most common biogenic amines which can be considered as a health hazards. In general, the content of this amines in *Spirulina* grown in closed system were significantly lower than of those in open system. This result can be attributed to the potential contamination by bacteria in open system. These bacteria can convert the amino acids to amines by bacterial decarboxylase enzymes.

There was no available work determined biogenic amines in *Spirulina* as parameter of risk assessment.

Putrescine represented the highest amount (21.9 and 20.7 mg kg^{-1}) in *Spirulina* from both open and closed system, respectively, whereas, tryptamine was absent. According to European and Egyptian specification of amines, the

Table 7- Mycotoxins and cyanotoxins (µg kg^{-1}) in dried *Spirulina platensis* from open and closed systems and related regulatory standard

Parameter	Mycotoxins (µg kg^{-1})				Cyanotoxins (µg kg^{-1})
	Afs	OTA	FB1	ZER	MC-LR
Open system	0.2	ND	ND	ND	ND
Closed system	ND	ND	ND	ND	ND
EU	20	30	400	35	1
WHO	20	30	200	40	1

Afs: Aflatoxins, OTA: Ochratoxin-A, FB1: Fumonisin- B1, ZER: Zearalenone, MC-LR: Microcystin-LR, ND: Not Detected.

permissible limit of histamine in food should not exceed 50 mg kg^{-1} and 200 mg kg^{-1} for total biogenic amines.

In our study the occurrence of biogenic amines represented no problem as its content of histamine was less than 2 ppm and less than 35 ppm for total biogenic amines. Likewise, maximum daily intake (30g) of *Spirulina* was recommended in order to have a safety margin which decrease the potential influent on health (Becker, 1980).

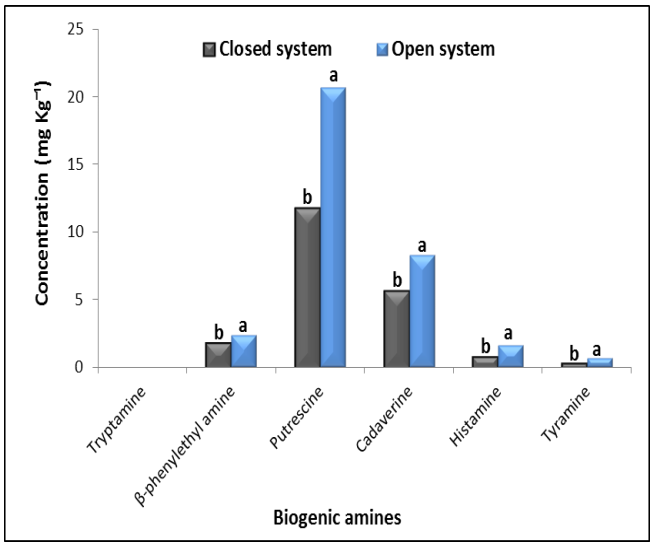

Fig. 2. Biogenic amines in *Spirulina platensis* grown in open and closed systems

CONCLUSION

During this study, it was found that *Spirulina platensis* cultivated in both systems were rich in various nutrients. The results showed that it could be used safely either for human nutrition or animal feed. Closed system is the promising for *Spirulina* cultivation by means of contamination level. Finally, Egyptian product of *Spirulina*, like other worldwide products, can be exported and hopefully become one of the Egyptian income sources.

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