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Chemical Constituents of Zarrouk's Medium Affect Growth, Pigments and Metabolites Productions of *Spirulina platensis*

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GROWTH (OD, Dw), pigments (chlorophyll a, carotenoids) and metabolites (proteins, carbohydrates, phenolics, flavonoids) were evaluated in different component concentrations of Zarrouk's medium (NaCl, $NaNO_3$, K_2HPO_4 and K_2SO_4) as well as some heavy metals (Zn, Cu, Se). The normal concentration of the tested components in Zarrouk's medium (control) induced maximum growth parameters, pigments and metabolite contents. Acetone extracts of the normal concentrations (1.0g/L NaCl, 2.5g/L $NaNO_3$, 0.5g/L K_2HPO_4 and 1g/L K_2SO_4) had the highest phenolic content. Ethanolic extracts of starved media (0.0g/L) of N or S, P (0.2g/L) as well as high salinity (4g/L) showed the greatest flavonoid production. Concerning heavy metals, Zn enhanced all the investigated parameters relative to the Cu, Se and control samples. In the absence of heavy metals, both acetone and ethanolic extracts revealed the greatest phenolic and flavonoid contents.

Keywords: Mass production, *Spirulina platensis*, Zarrouk's medium.

Introduction

The blue-green algae *Spirulina platensis* has been consumed since ancient times as a perfect food and has been frequently studied for many purposes, as a food supplement, a functional food medicine, for cosmetics, and for many other biomaterials (e.g. Hernadez et al., 2002; García et al., 2017). *Spirulina* is a photosynthetic, filamentous, spiral shaped, multicellular blue green microalgae. Since *Spirulina platensis* is easy to culture, harvest, and dry, it has become the most popular species in microalgal biotechnological studies (Belay, 2008; Mani et al., 2008). The basic biochemical constituents of *Spirulina* include proteins (55–70%), carbohydrates (15–25%), essential fatty acids (18%), minerals (potassium, calcium, iron, zinc, chromium, magnesium, manganese, and selenium), vitamins (B1, B2, B3, B6, B9, B12, C, D, and E) and photosynthetic pigments, like xanthophyll, beta-carotene, chlorophyll a, and phycocyanin. By providing these nutritional requirements, *Spirulina* can be considered a "wonder food" (Habib et al., 2008; Ravi et al., 2010). The biochemical composition of *Spirulina* depends upon the culture conditions and season of production (e.g. Phang et al., 2000, Habib et al., 2008; Parages et al., 2012).

Cultivation medium has a great impact on productivity of biomass and compounds of interest. For example, nitrogen concentration in the medium (Çelekli & Yavuzatmaca, 2009) (optimum at 2.5g/L) and also nitrogen source (Soletto et al., 2005) have a great effect on *Spirulina* productivity. Additionally, phosphate concentration, in the form of K_2HPO_4 (250mg/L), was found to optimize biomass production (Markou et al., 2012). Environmental stresses also affect growth and biopigment accumulation of microalgae, including nutrient availability, high pH, light, salinity and temperature (Pandey et al., 2011). Furthermore, studies indicate that the quantities of phenolic compounds increase when culture conditions altered, enhancing the antioxidant potential of *S. platensis* biomass so that it can be better exploited as a nutritional supplement (Colla et al., 2007).

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The objective of this study is to evaluate the influence of various chemical compositions for Zarrouk's medium on *Spirulina platensis* biochemical products.

Materials and Methods

Microorganism cultivation and growth conditions

Spirulina platensis was obtained from the Hydrobiology Lab, National Institute of Oceanography and Fisheries, Egypt. It was cultivated axenically by batch culture using Zarrouk's medium (Zarrouk, 1966). It was grown in Erlenmeyer flasks (1000 ml), each with flask containing 700mL of Zarrouk's medium, adjusted to different chemical stresses. The stress concentrations were: as follows: NaCl [(control 1.0g/L) - 0.0, 0.5, 2.0, 4.0 and 8.0g/L)], $NaNO_3$-[(control 2.5g/L), 0.0, 1.25, 5 and 10g/L]- K_2HPO_4 [(control 0.5g/L), 0.0, 0.2, 1.0 and 2.0g/L], K_2SO_4 [(control 1.0g/L), 0.0, 0.5, 2.0 and 4.0g/L]. In addition, some heavy metals [were studied ($ZnSO_4$, $CuSO_4$ and Na_2SeO_3 all at 1mg/L]. Each flask was inoculated with 70mL of a *Spirulina* pre-culture. The flasks were placed on shelves illuminated by fluorescent lamps to a light intensity of 26µE/m^2/s, and grown at pH10 and 30°C±1°C. At the end of the experimental period, growth, production of chlorophyll a, carotenoids, carbohydrates, proteins, flavonoids and phenols were measured.

Zarrouk's medium (Zarruok, 1966) was prepared as follows:

Solution I	g/L	Solution II (Trace elements)	g/L
$NaNO_3$	2.5	H_3BO_3	2.86
K_2HPO_4	0.5	$MnCl_2.7H_2O$	1.81
$CaCl_2.2H_2O$	0.04	$ZnSO_4.7H_2O$	0.22
$MgSO_4$ $7H_2$	0.2	$CuSO_4.$ $5H_2O$	0.08
NaCl	1.0	$Co(NO_3)2.6H_2O$	0.04
K_2SO_4	1.0	Na_2MoO_4	0.0177
$FeSO_4.7H_2$	0.01		
EDTA	0.08		
$NaHCO_3$	16.8		

Growth evaluation

Cyanobacterial growth was monitored using the optical density of the culture according to Fatma et al. (1994) at 750nm (OD750) and by determination of cellular dry weight (CDW). Biomass productivity was calculated according to APHA (2005).

Estimation of total carbohydrates

Total carbohydrates were quantitatively determined by the phenol sulfuric acid method as described by Kochert (1978) using glucose as a standard reference with concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000mg L^{-1} were prepared from a stock solution of 10g L^{-1} glucose in deionized water.

Estimation of pigment content (chlorophyll a and carotenoids)

A known volume (5mL) of *S. platensis* culture was centrifuged at 6000rpm for 10min. The supernatant was decanted and an equal volume of methanol was added to the pellet. The sample was then incubated in a water bath at 55°C for 15min, and subsequently centrifuged. Absorbance of the extract (A) was measured against blank of free methanol at 650, 665, and 452nm. Chlorophyll a and carotenoids were estimated as µg ml^{-1} of culture suspension using the following equations (MacKinney, 1941):

Chlorophyll a (µg ml^{-1})= 10.3 E665 - (0.918 E650) and Carotenoids (µg ml^{-1})= 4.2 E452 – (0.0246 chlorophyll a).

Estimation of total soluble protein content

After pigment extraction, residual cells were extracted using 1 N NaOH in a boiling water bath for 2hrs, as described by Payne & Stewart (1988). Protein concentration, as mg ml^{-1}, was determined according to Lowry et al. (1951) using bovine serum albumin as a standard reference with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 10mg of bovine serum albumin were prepared from the stock solution of 0.01g of bovine serum albumin dissolved in 100mL deionized water.

Preparation of Spirulina platensis extracts

About 0.2g of algae was soaked in 70% methanol (5mL) for 24hrs and then centrifuged. The residues were resoaked with 70% methanol (5mL) for 24hrs and then centrifuged. Other extractions were then repeated separately with 70% acetone and 70% ethanol. The filtrates were concentrated by vacuum until and then analyzed for total phenols and flavonoids. The extracts were diluted by 5mL of the same solvent used for each treatment.

Estimation of total flavonoid content

The aluminum chloride colorimetric method, as modified by Chang et al. (2002), was used to estimate flavonoid content. Different concentrations of quercetin of (10, 20, 30, 40, 50, 60, 70, 80, 90, and 10mg) were used to form a calibration curve. with concentrations of quercetin were prepared from the stock solution of 0.01g dissolved in 100mL in deionized water.

Estimation of total phenolic compounds

Total phenolics were estimated quantitatively according to Jindal & Singh (1975) using pyrogallol as a standard reference with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 10mg of pyrogallol were prepared from the stock solution of 0.01 g dissolved in 100mL in deionized water.

Statistical analyses

Results are presented as mean ± SD (standard deviation) for three replicates. All the data were subjected to one – way analysis of variance (ANOVA) using SPSS software, version 21. Test of significance was carried out using TUKY test at the significance level P≤ 0.05.

Results

Effect of $NaNO_3$ concentration on growth parameters and pigment production in S. platensis

The effect of different nitrogen concentrations (in the form of $NaNO_3$) on growth of *S. platensis* was recorded as OD at 750nm, at 3 day intervals, for 30 days of incubation and illustrated in Fig. 1. Generally, decrease or increase in $NaNO_3$concentration relative to standard Zarrouk's medium led to a reduction in growth. The optimum concentration of $NaNO_3$ for production of pigmented biomass was found to be 2.5g/L, which is the normal content in Zarrouk's medium. Table 1 records the maximum growth (0.048g/20mL), protein content (700mg/g), carbohydrates (93mg/g) on a dry weight bases, Chl. a content (3.1µg/mL) and carotenoids (1.5µg/mL).

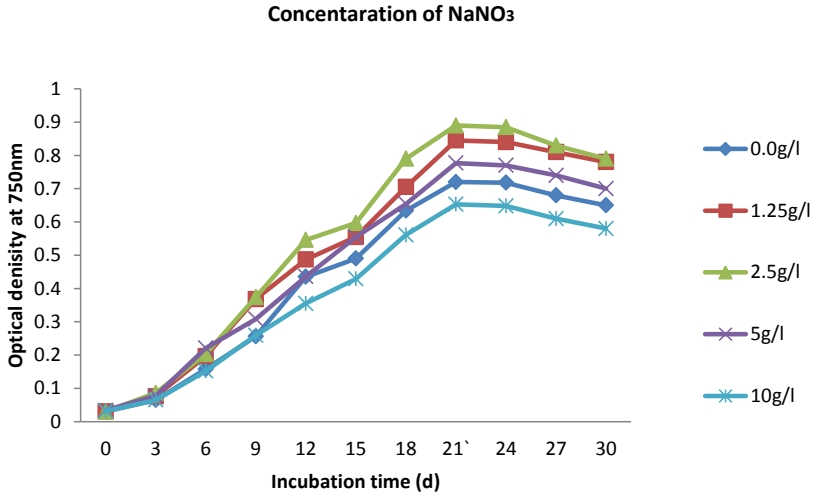

Fig. 1. Effect of $NaNO_3$ concentrations on growth of *S. platensis* during 30 days incubation

TABLE 1. Effect of $NaNO_3$ concentrations on growth, proteins, carbohydrates, chlorophyll *a* and carotenoids contents of *Spirulina platensis*.

$NaNO_3$	D. Wt. (g/20ml)	Proteins (mg/g d.wt)	Carbohydrates (mg/g d.wt)	Chlorophyll *a* (µg/ml)	Carotenoids (µg/ml)
0.0	0.028±0.0004^{e}	411±6^{e}	77±1.7^{c}	2.3±0.031^{d}	1.42±0.009^{c}
1.25	0.032±0.0003^{d}	687±5^{b}	84±0.6^{b}	2.9±0.022^{b}	1.29±0.012^{e}
2.5	0.045±0.0002^{a}	700±3^{a}	93±0.5^{a}	3.1±0.024^{a}	1.58±0.019^{a}
5	0.035±0.0002^{b}	575±5^{c}	82±1^{b}	3.0±0.022^{b}	1.49±0.015^{b}
10	0.033±0.0003^{c}	491±7^{d}	78±0.5^{c}	2.6±0.024^{c}	1.34±0.008^{d}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

The effect of nitrogen concentration on the cellular component of total phenolics and flavonoids in *Spirulina platensis* was investigated using three different extraction solvents (acetone, methanol and ethanol). The results showed that the highest phenolic contents were found in the acetone extract, in the control (0.52mg/g), and flavonoids were found in the ethanol extract (9.3mg/g), under nitrogen starvation conditions (0.00g/L) (Table 2).

Effect of NaCl concentration on growth parameters and pigment production of S. platensis

S. platensis was grown in Zarrouk's medium under different concentrations of NaCl (0.0, 0.5, 1.0, 2.0, 4.0 and 8.0g/L) and incubated under the previously mentioned optimal conditions. On day 21 of incubation, the growth parameters and pigments were quantified. Figure 2 shows the effect of NaCl concentration on the growth of *S. platensis* in which NaCl concentration present in the basal medium (1.0g/L), was used as a positive control, was proved to be the optimal concentration for growth and production of pigmented biomass (Table 3). In addition, *S. platensis* culture achieved maximum dry weight (0.047g/ 20mL), protein content (569mg/g), carbohydrates (41mg/g), Chl. a (2.5µg/mL) and carotenoids (1.4µg/ mL) at 1.0g/L of NaCl.

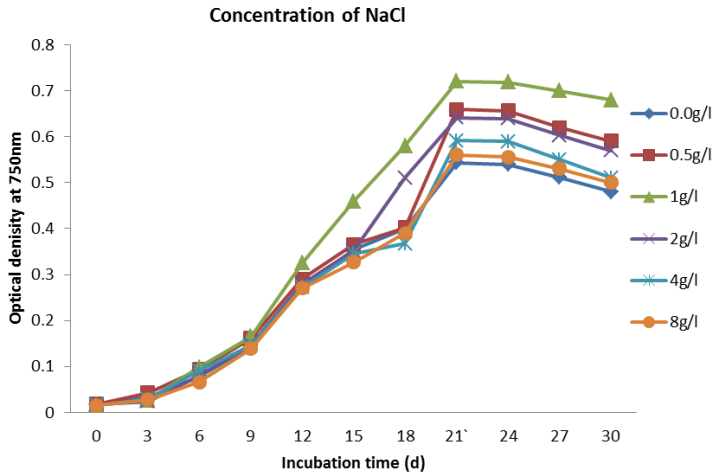

Fig. 2. Effect of NaCl concentrations on growth of *S. platensis* during 30 days incubation

TABLE 2. Effect of $NaNO_3$ concentrations on the phenolic and flavonoid contents of *Spirulina platensis*

	Phenolic (mg/g d.wt)			Flavonoid (mg/g d.wt)		
$NaNO_3$	Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol
0.0	0.33±0.0004^{e}	0.23±0.0004^{b}	0.16±0.0004b	4.3±0.019^{e}	6.2±0.007^{a}	9.3±0.025^{a}
1.25	0.37±0.0004^{b}	0.30±0.0004^{a}	0.13±0.0003c	6.7±0.038^{b}	4.1±0.026^{e}	6.7±0.019^{e}
2.5	0.52±0.0004^{a}	0.18±0.0006^{c}	0.17±0.0004a	7.0±0.033^{c}	5.7±0.012^{b}	7.6±0.045^{c}
5	0.24±0.0004^{e}	0.12±0.0006^{d}	0.09±0.0003d	5.2±0.019^{d}	4.4±0.012^{c}	7.0±0.043^{d}
10	0.25±0.0003^{d}	0.11±0.0006^{e}	0.08±0.0007e	4.8±0.026^{e}	4.3±0.036^{d}	7.7±0.037^{b}

- Each value is the mean of three readings ± standard deviation.
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05)

TABLE 3. Effect of NaCl concentrations on growth, proteins, carbohydrates, chlorophyll *a* and carotenoids contents of *Spirulina platensis*

NaCl	D. Wt. (g/20mL)	Proteins (mg/g d.wt)	Carbohydrates (mg/g d.wt)	Chlorophyll *a* (µg/ml)	Carotenoids (µg/mL)
0.0	0.033±0.0030^{c}	456±6^{bc}	31±3^{c}	1.0±0.014^{f}	0.75±0.008^{e}
0.5	0.038±0.0017^{b}	534±8^{a}	39±1^{ab}	1.5±0.016^{d}	1.20±0.008^{c}
1.0	0.047±0.0020^{a}	563±10^{a}	44±1^{a}	2.5±0.016^{a}	1.42±0.015^{a}
2.0	0.043±0.0026^{ab}	476±4^{b}	38±3^{b}	1.7±0.020^{b}	1.31±0.012^{b}
4.0	0.034±0.0015^{c}	450±10^{bc}	40±2^{ab}	1.6±0.013^{c}	1.28±0.010^{b}
8.0	0.033±0.0017^{c}	418±8^{c}	34±4^{bc}	1.4±0.011^{e}	0.94±0.013^{d}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

Total phenolic and flavonoid extracted with the different solvent 70 % (acetone, methanol and ethanol) of *S. platensis* are presented in Table 4. It demonstrated that the highest phenolic contents was found in acetone extracts (0.52mg/g), at 1.0g/L NaCl (control), maximum flavonoid content was recorded in ethanol extracts (11.9mg/g) at high salinity concentrations (4.0 and 8.0g/L of NaCl).

Effect of K_2SO_4 concentration on growth parameters and pigment production of S. platensis

Various concentrations of K_2SO_4 were tested for maximum growth and pigment formation, on day 21, of incubation. Figure 3 shows the effect of K_2SO_4 concentration on the growth of *S. platensis*. The optimum concentration of K_2SO_4 for production of maximum pigmented biomass was 1.0g/L, as presented in Table 5. The maximum measurements recorded were as follows: Growth (0.094g/20mL), proteins (495mg/g), carbohydrates (44mg/g dry weights), Chlorophyll a (3.0µg/mL) and carotenoids (1.3µg/mL).

The effect of various concentrations of K_2SO_4 on the total phenolic and flavonoid contents of *S. platensis* was measured by extraction using the solvents ,as presented in Table 6. The highest phenolic contents was found in 70% acetone extract (0.52mg/g), while the maximum flavonoid contents was obtained with ethanol extract (8.1mg/g), at 0.0g/L K_2SO_4 (sulfur starvation).

Effect of K_2HPO_4 concentration on growth parameters and pigment production in S. platensis

K_2HPO_4 is known to be the most favorable source of phosphorus for growth and synthesis of pigments in *S. platensis.* Therefore, various concentrations of K_2HPO_4 were tested for maximum growth and production of pigments. On day 21, the growth and production of pigments were determined.

TABLE 4. Effect of NaCl concentrations on the phenolic and flavonoid contents of *Spirulina platensis*.

	Phenolic (mg/g d.wt)			Flavonoid (mg/g d.wt)		
NaCl	**Acetone**	**Methanol**	**Ethanol**	**Acetone**	**Methanol**	**Ethanol**
0.0	0.25±0.0006^{b}	0.22±0.0006^{a}	0.37±0.0006^{a}	11.5±0.025^{a}	6.2±0.036^{c}	10.0±0.025^{b}
0.5	0.15±0.0004^{c}	0.13±0.018^{cd}	0.20±0.0004^{b}	10.2±0.025^{d}	5.7±0.019^{d}	7.7±0.025^{d}
1.0	0.52±0.0004^{a}	0.18±0.0006^{b}	0.17±0.0004^{d}	7.0±0.033^{f}	5.7±0.025^{d}	7.6±0.045^{e}
2.0	0.16±0.0016^{c}	0.14±0.0006^{c}	0.19±0.0008^{c}	11.0±0.031^{c}	8.8±0.025^{a}	9.3±0.031^{c}
4.0	0.13±0.0003^{d}	0.14±0.0004^{c}	0.15±0.0003^{e}	11.2±0.037^{b}	7.1±0.007^{b}	11.9±0.052^{a}
8.0	0.12±0.0007^{e}	0.11±0.0004^{d}	0.15±0.0003^{f}	10.0±0.025^{e}	4.2±0.043^{e}	10.0±0.050^{b}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

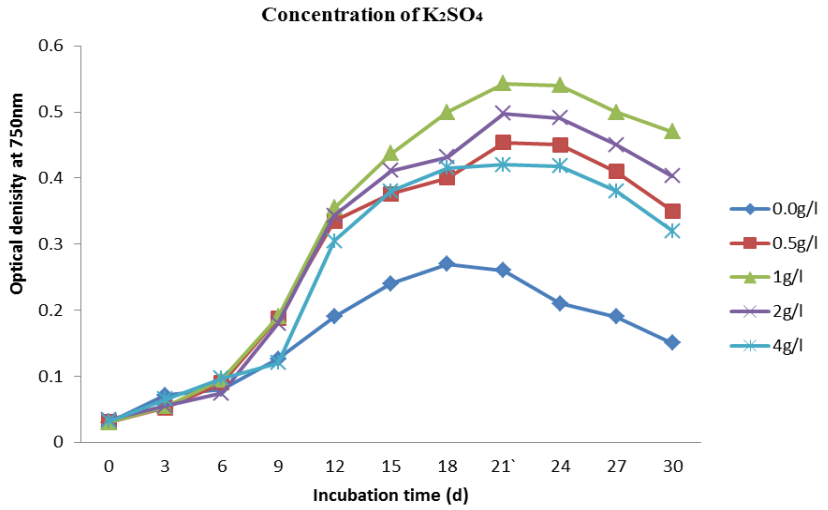

Fig. 3. Effect of K_2SO_4 concentrations on growth of *S. platensis* during 30 days incubation

TABLE 5. Effect of K_2SO_4 concentrations on growth, proteins, carbohydrates , chlorophyll *a* and carotenoids contents of *Spirulina platensis*

K_2SO_4	D. Wt. (g/20mL)	Proteins (mg/g d.wt)	Carbohydrates (mg/g d.wt)	Chlorophyll *a* (µg/mL)	Carotenoids (µg/mL)
0.0	0.044±0.0025^{d}	286±4^{d}	32±0.1^{c}	0.9±0.008^{e}	0.90±0.008^{c}
0.5	0.077±0.0015^{b}	404±3^{b}	34±0.3^{b}	3.3±0.025^{b}	1.09±0.008^{bc}
1.0	0.094±0.0040^{a}	495±7^{a}	44±1.6^{a}	3.7±0.016^{a}	1.29±0.0016^{a}
2.0	0.054±0.0020^{c}	371±5^{c}	21±0.7^{d}	3.1±0.014^{c}	1.20±0.012^{ab}
4.0	0.053±0.0026^{c}	101±3^{e}	8±0.5^{e}	3.0±0.015^{d}	1.00±0.014^{abc}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

TABLE 6. Effect of K_2SO_4 concentrations on phenolic and flavonoid contents of *S. platensis*

	Phenolic (mg/g d.wt)			Flavonoid (mg/g d.wt)		
K_2SO_4	Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol
0.0	0.090±0.0004^{e}	0.19±0.0004^{a}	0.15±0.0006^{e}	4.9±0.045^{d}	5.3±0.019^{c}	8.1±0.026^{a}
0.5	0.206±0.0006^{b}	0.15±0.0006^{c}	0.20±0.0004^{a}	6.7±0.025^{b}	4.9±0.019^{d}	7.7±0.025^{b}
1.0	0.520±0.0004^{a}	0.18±0.0004^{b}	0.17±0.0004^{c}	7.0±0.033^{a}	5.7±0.012^{a}	7.6±0.045^{b}
2.0	0.203±0.0006^{c}	0.12±0.0004^{d}	0.16±0.0004^{d}	6.4±0.031^{c}	5.4±0.019^{b}	7.6±0.037^{b}
4.0	0.201±0.0006^{d}	0.12±0.0004^{d}	0.18±0.0004^{b}	6.3±0.026^{c}	4.7±0.021^{e}	7.1±0.025^{c}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

Figure 4 and Table 7 showed the effect of phosphorus concentration on growth of *S. platensis*. The optimum concentration of K_2HPO_4 (0.5g/L) showed maximum growth parameters (dry weight and chl a contents) and metabolites (proteins and carbohydrates), while the greatest carotenoids content were recorded at concentration (2g/L).-

Effect of different solvents 70% (acetone, methanol and ethanol) on total phenolic and flavonoid contents of *S. platensis* extract were recorded in Table 8. The highest phenolic content was found in 70% acetone (0.52mg/g) ~~at~~ in control (0.5gL), while ~~as~~ flavonoid (10.9mg/g) were recorded at 0.2g/L K_2HPO_4.

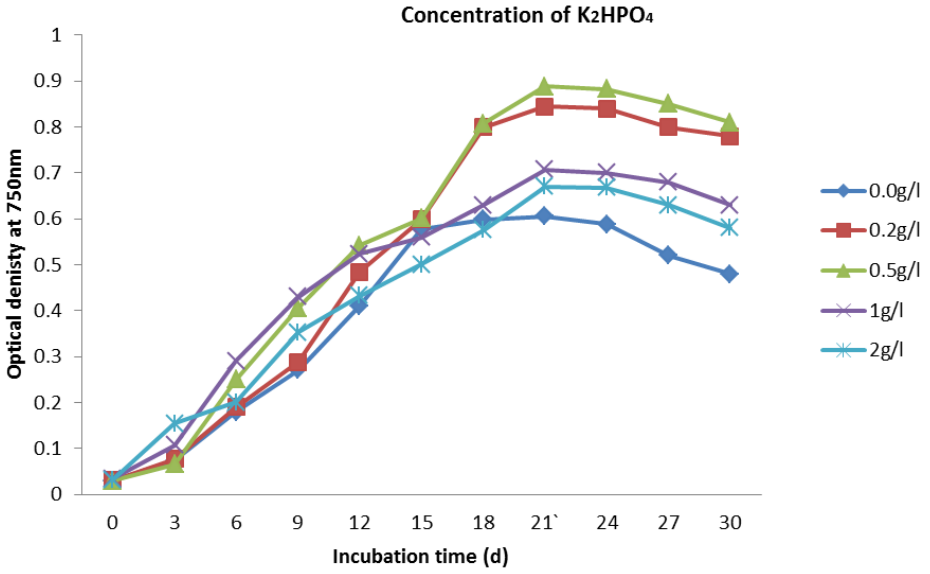

Fig. 4. Effect of K_2HPO_4 concentrations on growth of *S .platensis* during 30 days incubation

TABLE 7. Effect of different concentrations of K_2HPO_4 on the growth, proteins, carbohydrates, chlorophyll a and carotenoids contents of *Spirulina platensis*.

K_2HPO_4	D. Wt. (g/20ml)	Proteins (mg/g d.wt)	Carbohydrates (mg/g d.wt)	Chlorophyll *a* (µg/ml)	Carotenoids (µg/ml)
0.0	0.047±0.0003^{c}	401±5^{c}	68±0.6^{c}	0.9±0.015^{d}	1.5±0.008^{d}
0.2	0.054±0.0023^{ab}	428±3^{b}	67±0.7^{c}	2.8±0.036^{c}	1.83±0.009^{c}
0.5	0.058±0.0060^{a}	512±9^{a}	83±1.0^{a}	3.2±0.032^{a}	1.84±0.004^{c}
1.0	0.052±0.0046^{bc}	442±8^{b}	77±1.0^{b}	3.1±0.030^{b}	1.9±0.013^{b}
2.0	0.049±0.0024^{bc}	333±4^{d}	65±0.6^{d}	3.1±0.029^{b}	2.2±0.011^{a}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

TABLE 8. Effect of K_2HPO_4 concentrations on phenolic and flavonoid contents of *Spirulina platensis*.

	Phenolic (mg/g d.wt)			Flavonoid (mg/g d.wt)		
K_2HPO_4	Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol
0.0	0.145±0.0004^{b}	0.163±0.0015^{b}	0.158±0.0003^{d}	7.5±0.025^{d}	4.9±0.012^{e}	9.0±0.026^{a}
0.2	0.130±0.0004^{d}	0.161±0.0004^{b}	0.185±0.0004^{a}	10.9±0.025^{a}	7.1±0.012^{a}	8.1±0.025^{d}
0.5	0.520±0.0004^{a}	0.180±0.0006^{a}	0.172±0.0004^{c}	7.0±0.033^{e}	5.7±0.012^{c}	7.6±0.045^{e}
1.0	0.139±0.0009^{c}	0.166±0.0009^{b}	0.179±0.0007^{b}	9.3±0.025^{b}	6.2±0.019^{b}	8.7±0.019^{c}
2.0	0.110±0.0007^{c}	0.130±0.0003^{c}	0.179±0.0004^{b}	8.1±0.019^{c}	5.1±0.033^{d}	8.8±0.025^{b}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

Effect of heavy metals on growth parameters and pigment production of S. platensis

Heavy metals (Zn, Cu and Se) at 1mg/L were tested for maximum growth and formation of pigments. Zn concentration was increased from the control (0.0mg/L) to 1mg/L maximum growth parameters (dry weight 0.085g/ 20mL, Chl. a content 3.7µg/mL and carotenoids 2.4µg/ mL) as well as protein content (572mg/g), carbohydrate content (66mg/g dry weights) (Fig. 5, Table 9). The results showed in Table 10 that the highest phenolic contents were found in acetone extract (0.52mg/g) in control (absence of all heavy metals) while flavonoids were found in ethanol extract (7.7mg/g) at presence of selenium (1mg/L).

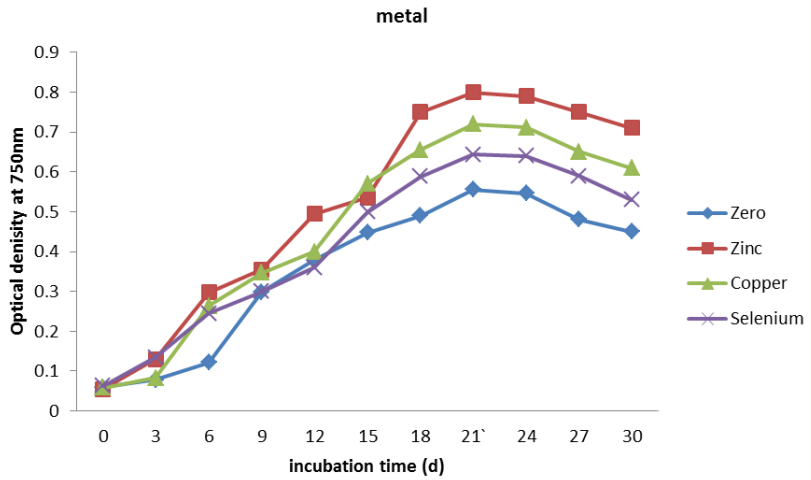

Fig. 5. Effect of some heavy metals, Zn, Cu, and Se, on growth of *S. platensis* during 30 days of incubation

TABLE 9. Effect of heavy metals (Zn, Cu and Se) on growth, proteins, carbohydrates, chlorophyll a and carotenoids contents of *Spirulina platensis*

Heavy metals	D. Wt. (g/20mL)	Proteins (mg/g d.wt)	Carbohydrates (mg/g d.wt)	Chlorophyll *a* (µg/mL)	Carotenoids (µg/mL)
0.0	0.059±0.0032^{d}	363±2^{c}	35±0.2^{c}	1.6±0.038^{d}	0.7±0.006^{d}
Zn	0.085±0.0003^{a}	572±2^{a}	66±0.2^{a}	3.7±0.028^{a}	2.4±0.015^{a}
Cu	0.079±0.0004^{b}	452±4^{b}	57±0.2^{b}	3.0±0.047^{b}	2.1±0.016^{b}
Se	0.068±0.0003^{c}	397±4^{bc}	57±0.5^{b}	2.0±0.010^{c}	1.9±0.009^{c}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

TABLE 10. Effect of heavy metal (Zn, Cu and Se) on the phenolic and flavonoid contents of *Spirulina platensis*

	Phenolic (mg/g d.wt)			Flavonoid (mg/g d.wt)		
Heavy metals	Acetone	Methanol	Ethanol	Acetone	Methanol	Ethanol
0.0	0.52±0.0006^{a}	0.181±0.0004^{a}	0.17±0.0004^{b}	7.0±0.033^{a}	5.7±0.012^{b}	7.6±0.045^{b}
Zn	0.22±0.0004^{c}	0.110±0.0010^{d}	0.11±0.0006^{d}	5.5±0.019^{c}	4.8±0.012^{c}	7.2±0.031^{c}
Cu	0.19±0.0006^{d}	0.142±0.0004^{c}	0.14±0.0003^{c}	6.5±0.025^{b}	6.6±0.043^{a}	5.0±0.025^{d}
Se	0.39±0.0010^{b}	0.148±0.0003^{b}	0.34±0.0004^{a}	5.4±0.025^{d}	5.8±0.028^{b}	7.7±0.031^{a}

- Each value is the mean of three readings ± standard deviation .
- Values with the same small letter in the same column showed insignificant difference (at P≤0.05).

Discussion

In the experiments presented,the growth of *S. platensis* began to decline after 21 days of incubation (growth curve in Figure. 1). Previous studies measured biomass after 15 and 25 days, and none of them used further time points to follow biomass to depletion (Kumar et al., 2011). Delrue et al. (2017) found that the optimum culture conditions even after 21 days of batch culture.

Reduced $NaNO_3$ in Zarrouk's medium led to a remarkable decrease in *S. platensis* growth parameters, protein and carbohydrate, contents in agreement with the results obtained by Abo-Shady et al. (1992), Fatma et al. (1994), Hamza (2007) and El-Shouny et al. (2015).

NaCl present in the basal medium (1.0g/L), as a positive control, was shown to be the optimal concentration for growth and production of pigments .Our results were in agreement with Hamza (2007). Abo-Shady et al. (1992) reported that the highest values of biomass and protein for *S. platensis*, grown in Zarrouk's medium, are attained at 0.8% (w/v) NaCl. Fatma et al. (1994) used the 0.1% NaCl initially present in Zarrouk's medium for maximum production of biomass, protein and β-carotene by four strains of *Spirulina* species. Sujatha & Najarajan (2013) concluded that carotenoid content of *Spirulina* increases at high saline levels and they attributed that to excessive formation of free radicals under stress, so carotenoids over production by *Spirulina fipardler* to protect cells from the harmful effect of ROS and continue its growth. El-Shouny et al. (2015) reported that decreasing NaCl by 50 % below the control showed significant increase of biomass productivity. A decrease or increase of salt concentration also increased lipid productivity. Additionally, carotenoids and carbohydrates increased with increasing salinity (defense system).

Sulfur is an essential element for amino acid and lipid synthesis (Romano et al., 2000), and changes in its concentration in cyanobacteria medium have not previously been well investigated. The present study showed that reduction of sulfur in the growth medium led to an insignificant decrease in *S. platensis* growth and biomass productivity. However, protein production decreased as sulfur was depleted due to the lack of sulfur containing amino acids and proteins. Our results were in agreement with those of El-Shouny et al. (2015).

Phosphorus is an essential macronutrient for plants and algae, as it is required for growth and production of energy molecules. Lowering the phosphorus concentration led to a reduction in biomass and protein production as well as increased carbohydrate and carotenoid production. Our results agreed with those reported by El-Shouny et al. (2015) who show that phosphorus limitation decreases protein content of the microalga *Chaetoceros muelleri*. Kilham et al. (1997) also demonstrate that phosphorus starvation reduces protein content in algal cells. Phadwal & Singh (2003) show that increase of β-carotene accumulation in *Dunaliella salina* was obtained with phosphorus starvation. In phosphorus starvation an increased carbohydrate content of some algal cells occurred (Kilham et al., 1997) due to alteration of metabolic pathways to produce hydrocarbons.

Heavy metals are trace elements required for organisms in very low concentrations, and are toxic when exceeding physiological requirements (Banvalvi, 2011). In the present study, biomass production of *S. platensis* was evaluated in response to different heavy metals (Zn, Cu and Se of 1mg/L) for growth and pigment synthesis. The optimum of heavy metal concentration for the production of pigmented biomass was Zn at 1mg/L. Soeprobowati & Hariyati (2014) show that high growth of *S. platensis* was achived with 1mg/L Cu^{2+} due to improvement enzymatic process, while it was inhibited at both 3mg/L and 5mg/L which were toxic. Mohy El-Din (2017) showed that the population growth of *S. platensis* was high in the concentration of 0.5mg/L Cu^{2+} whereas other concentrations (1 and 3mg/L) cause a clear reduction in the growth of *Spirulina platensis.* On the other hand, Nalimova et al. (2005) showed that growth of *S. platensis* reached its maximum at 2mg/L Zn and was inhibited with 8mg/L which was toxic.

The health benefit of *Spirulina* has been partially attributed to its richness in flavonoids and diverse phenolic compounds, which interact synergistically together as potent antioxidants (Miranda et al., 1998; Kulshreshtha et al., 2008). The present study, recorded the highest phenolic and flavonoids contents in *S. platensi* when extracting with acetone. This result was in agreement with Salamatullah (2014) who test the total phenolic and flavonoids with different solvent extracts (acetone, methanol and ethanol), and shows the highest phenolic contents in acetone and highest flavonoid with methanol.

Conclusion

The variation in in the chemical composition of Zarrouk's medium plays an important role in the growth and biochemical parameters of *S. platens*is. High yield of dry matter, proteins, carbohydrates, chlorophyll a, carotenoids and phenolics were observed when the alga was grown in control Zarrouk's medium .Flavonoid accumulation varied with both the chemical constituents of the medium and the type of solvent used for extraction.

Conflicts of interest: No conflicts of interest have been declared.

Authors contribution: All authors contributed equally. All authors read and approved the final manuscript.

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References

Abo-Shady, A.M., Abu El- Souod, S.M., ElShanshoury, A.R., Mahmoud, Y.A.G. (1992) Factors affecting the productivity of *Spirlina plalensis*. *Delta Journal of Science, Tanta University, Egypt*, **16**(1), 212-228.

APHA (2005) "*Standard Methods for Examination of Water and Wastewater*", 21st edition.

Banvalvi, G. (2011) "*Cellular Effects of Heavy Metals*". Springer. London, 364p.

Belay, A. (2008) *Spirulina* (*Arthrospira*) production and quality assurance. In: "*Spirulina in Human Nutrition and Health*". Gershwin, E. and Belay, A. (Eds.), pp. 1-23. CRC Press, Taylor & France Group, Boca Raton, London, New York.

Çelekli, A., Yavuzatmaca, M. (2009) Predictive modeling of biomass production by *Spirulina platensis* as function of nitrate and NaCl concentrations. *Bioresource Technology*, **100**(5), 1847–1851.

Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C. (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug analysis*, **10**(3), 178-182.

Colla, L.M., Furlong, E.B., Costa, J.A.V. (2007)

Antioxidant properties of *Spirulina* (*Arthospira*) *platensis* cultivated under different temperatures and nitrogen regimes. *Brazilian Archives of Biology and Technology*, **50**(1), 161-167.

Delrue, F., Alaux, E., Moudjaoui, L., Gaignard, C., Fleury, G., Perilhou, A., Richaud, P., Petitjean, M., Sassi, J. (2017) Optimization of *Arthrospira platensis* (*Spirulina*) Growth: From Laboratory Scale to Pilot Scale. *Fermentation,* **3**(4), 59.

El-Shouny, W., Sharaf, M., Abomohra, A., Abo-Eleneen, M. (2015) Production enhancement of some valuable compounds of *Arthrospira platensis*. *Journal of Basic and Environmental Sciences,* **2**, 74–83.

Fatma, T., Sarada, R., Venkataraman, L.V. (1994) Evaluation of selected strains of *Spirulina* for their constituents. *Journal of the Phycological Society,* **33**(1-2), 89-97.

García, J.L., Vicente, M., Galán, B. (2017) Microalgae, old sustainable food and fashion nutraceuticals. *Microbial Biotechnology*, **10**(5), 1017–1024.

Habib, M.A.B., Parvin, M., Huntington, C., Hasan, R. (2008) Fisheries and aquaculture circular (A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals). Food and Agriculture Organization of the United Nations No. 1034: 33.

Hamza, W.T. (2007) Studies on pigments from some species of the cyanobacterium *Arthrospoira* and the yeast *Rhodotorula*. *Mc. Thesis.* Faculty of Science Tanta University.

Hernadez- Corona A.C., Nieves, I., Meckes, M., Chamorro, G., Barron, B.L. (2002) Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. *Antiviral Research,* **56**(3), 279–285.

Jindal, K.K., Singh, R.N. (1975) Phenolic content in male and female *Carica papaya*: A possible physiological marker for sex identification of vegetable seedlings. *Physiologia Plantarum*, **33**(1), 104-107.

Kilham, S., Kreeger, D., Goulden, C., Lynn, S. (1997) Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. *Freshwater Biology,* **38**(3), 591-596.

Kochert, G. (1978) Carbohydrate determination by the phenol-sulphoric acid method. In: "*Hand Book of Physiological and Biochemical Methods*". Cambridge University press, Cambridge.

Kulshreshtha, A., Zacharia, A.J., Jarouliya, U., Bhadauriya, P., Prasad, G.B.K.S., Bisen, P.S. (2008) *Spirulina* in health care management. *Current Pharmaceutical Biotechnology*, **9**(5), 400-405.

Kumar, V., Bhatnagar, A.K., Srivastava, J.N. (2011) Antibacterial activity of crude extracts of *Spirulina platensis* and its structural elucidation of bioactive compound. *Journal of Medicinal Plants Research,* **5**(32), 7043-7048.

Lowry, O.M., Rosebrough, N.J., Farr, L.A., Randall, R.J. (1951) Protein measurements with the folin phenol reagent. *Journal of Biological Chemistry,* **193**, 265-257.

Mani, V., Iyer, M., Dhruy, A., Mani, U., Sharma S. (2008) Therapeutic utility *Spirulina*. In: "*Spiurlina Human Nutrition and Health*", Gershwin, M.E. and Belay, A. (Eds.), pp.71-100. CRC Press, Boca Raton.

Markou, G., Chatzipavlidis, I., Georgakakis, D. (2012) Effects of phosphorus concentration and light intensity on the biomass composition of *Arthrospira* (*Spirulina*) *platensis*. *World Journal of Microbiology & Biotechnology*, **28**, 2661–2670.

Mackinney, G. (1941) Absorption of light by chlorophyll solution. *The Journal of Biological Chemistry*, **140**, 315: 322.

Miranda, M.S., Cintra, R.G., Barros, S.B.M., Mancini-Filho, J. (1998) Antioxidant activity of the microalga *Spirulina maxima*. *Brazilian Journal of Medical and Biological Research*, **31**(8), 1075-1079.

Mohy El-Din, S.M. (2017) Effect of copper and lead on growth and some metabolic activities of cyanobacterium *Spirulina platensis* (Nordstedt). *Egyptian Journal of Botany,* **57**(3), 445- 456.

Nalimova, A.A., Popova, V.V., Tsoglin, L.N., Pronina, N.A. (2005) The effects of copper and zinc on *Spirulina platensis* growth and heavy metal accumulation in its cells. *Russian Cournal of Plant Physiology,* **52**(2), 229-234.

Pandey, J.P., Tiwari, A., Singh, S., Tiwari, D. (2011) Potential of different light intensities on the productivity of *Spirulina maxima*. *Journal of Algal Biomass Utilization,* **2**, 9–14.

Parages, M.L., Rico, R.M., Abdala-Diaz, R.T., Chabrillon, M., Sotiroudis, T.G., Jimenez, C. (2012) Acidic polysaccharides of *Arthrospira* (*Spirulina*) *platensis* induce the synthesis of TNF-alpha in RAW macrophages. *Journal of Applied Phycology,* **24**(6), 1537–1546.

Payne, J.K., Stewart, J.R. (1988) The chemical composition of the thallus wall of *Characiosiphon rivularis* (Characiosiphonaceae, Chlorophyta). *Phycologia,* **27**(1), 43-49.

Phadwal, K., Singh, P.K (2003) Effect of nutrient depletion on beta-carotene and glycerol accumulation in two strains of *Dunaliella* sp. *Bioresource Technology,* **90**(1), 55-58.

Phang, S.M., Miah, M.S., Yeoh, B.G., Hashim, M.A. (2000) *Spirulina* cultivation in digested sago starch factory wastewater. *Journal of Applied Phycology,* **12**, 395-400.

Ravi, M., De, S.L., Azharuddin, S., Paul, S.F. (2010) The beneficial effects of *Spirulina* focusing on its immunomodulatory and antioxidant properties. *Nutrition and Dietary Supplements,* **2**, 73–83.

Romano, I., Bellitti , M.R., Nicolaus, B., Lama, L., Manca, M.C., Pagnotta, E., Gambacorta, A. (2000) Lipid profile: A useful chemotaxonomic marker for classification of a new cyanobacterium in *Spirulina* genus. *Phytochemistry,* **54**(3), 289-294.

Salamatullah, A. (2014) Characterization of extraction methods to recover phenolic rich antioxidants from blue green algae (*Spirulina*) using response surface approaches. *Masters Thesis,* University of Nebraska – Lincoln.

Soeprobowati, T.R., Hariyati, R. (2014) Phycoremediation of Pb^{+2}, Cd^{+2}, Cu^{+2}, and Cr^{+3} by *Spirulina platensis* (Gomont) Geitler. *American Journal of Bio-Science*, **2**(4), 165-170.

Soletto, D., Binaghi, L., Lodi, A., Carvalho, J.C.M., Converti, A. (2005) Batch and fed-batch cultivations of *Spirulina platensis* using ammonium sulphate and urea as nitrogen sources. *Aquaculture,* **243**(1-4), 217–224.

Sujatha, K., Nagarajan, P. (2013) Optimization of growth conditions for carotenoid production from *Spirulina platensis* (Geitler). *International Journal of Current Microbiology and Applied Sciences,* **2**(10), 325-328.

Wells, M.L., Potin, P., Craigie, J.S., Raven, J.A., Merchant, S.S., Helliwell, K.E., Smith, A.G., Camire, M.E., Brawley, S.H. (2017) Algae as nutritional and functional food sources: Revisiting our understanding. *Journal of Applied Phycology,* **29**(2), 949–982.

Zarrouk, C. (1966). Contribution à l' étude d'une Cyanophycée, influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de" *Spirulina maxima*"(Setch et Gardner) Geitler *(Doctoral dissertation)*.

تاثير التركيب الكميائي لبيئة زاروك على النمو والأصباغ والعمليات الحيوية لسبيرولينا بلاتنسيس

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تم تقيم النمو والكتلة الحيوية الجافة، الأصباغ (كلوروفيل أ و الكاروتينات) والعمليات الإيضية (البروتينات، الكربوهيدرات، الفينولات، الفلافونويدات) بتركيزات مختلفة من مكونات بيئة زاروك (كلوريد الصوديوم، نترات الصوديوم، فوسفات ثنائي البوتاسيوم وكبريتات البوتاسيوم) وبعض المعادن الثقيلة مثل (الزنك، النحاس، السلينيوم) تم الحصول على أعلى قيمة في النتائج في تركيب بيئة زاروك الأساسية للنمو والأصباغ والعمليات الإيضية. بالإضافة إلى مستخلص الأسيتون مع الفينولات. اظهرت النتائج أعلى إنتاجية من التركيزات الطبيعية في بيئة زاروك (1جرام/لتر كلوريد الصوديوم، 2.5جرام/لتر نترات الصوديوم، 0.5جرام/لتر فوسفات ثنائي البوتاسيوم و1جرام/لتر كبريتات البوتاسيوم) في حين أن مستخلص الإيثانول في غياب كلا من النتروجين والكبريت وعند تركيز 0.2 جرام/لتر من الفوسفات وكذالك الملوحة العالية 4جرام/لتر أظهرت أعلى إنتاجية للفلافونويدات. فيما يتعلق بالمعادن الثقيلة أظهر الزنك أعلى إنتاجية من النحاس والسيلينوم وبيئة زاروك الأساسية. في غياب المعادن الثقيلة أظهر كلا من مستخلص الأسيتون والإيثانول أعلى إنتاجية للفينولات والفلافونويداتز.