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Approach to improve the productivity of bioactive compounds of the cyanobacterium *Anabaena oryza*e using factorial design

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ABSTRACT

Cyanobacteria are one of the richest sources of biomedical relevant compounds with extensive therapeutic pharmaceutical applications and are also known as producer of intracellular and extracellular metabolites with diverse biological activities. The genus *Anabaena sp.* is known to produce antimicrobial compounds, like phycocyanin and others. The goal of this study was to optimize the production of these bioactive compounds. The Plackett–Burman experimental design was used to screen and evaluate the important medium components that influence the production of bioactive compounds. In this present study, eight independent factors including $NaNO_3$, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2$, citric acid, ammonium ferric citrate, ethylene diamine tetraacetic acid disodium magnesium salt (EDTA-Na_2Mg) and Na_2CO_3 were surveyed and the effective variables for algal components production of *Anabaena oryzae* were determined using two-levels Plackett–Burman design. Results analysis showed that the best medium components were $NaNO_3$ (2.25 g l^{-1}); K_2HPO_4 (0.02 g l^{-1}); $MgSO_4$ (0.0375 g l^{-1}); $CaCl_2$ (0.018 g l^{-1}); citric acid (0.009 g l^{-1}); ammonium ferric citrate (0.009 g l^{-1}) and EDTA-Na_2 (0.0015 g l^{-1}) respectively. The total chlorophyll-a, carotenoids, phenol, tannic acid and flavonoid contents in crude extract of *Anabaena oryzae* were determined. They were 47.7, 4.11, 0.256, 1.046 and 1.83 μg/ml, respectively. The antioxidant capacity was 62.81%.

1. Introduction

Blue-green algae (Cyanobacteria) are group of Gram-negative photoautotrophic prokaryotes that contain a blue-green colored pigment (c-phycocyanin). Cyanobacteria have a fossil history from 3.3 to 3.5 billion years ago [1]. The cyanobacteria can be found in nearly all the imaginable habitats such as fresh and salty water, polluted waters of lakes, ponds, water tanks, soil, rocks and tree barks. Its morphological form varies from unicellular to filamentous or colonial forms [2]. Some cyanobacterial species are thermophilic and growing in thermal springs [3]. Some cyanobacteria are environmentally-friendly and have the ability to fix atmospheric nitrogen and solubilize phosphate [4]. Cyanobacteria are considered as a rich source of primary metabolites such as polysaccharides, proteins, fats, oils, vitamins, pigments and hydrocarbons [5]. They are also a rich source of many secondary metabolites like the antifungal cyclic peptides in *Tolypothrix byssoidea* [6,7], the antibacterial diterpenoids compounds in *Nostoc commune* [8], the algicidal phenolic compound 4,4′dihydroxybiphenyl and the indol alkaloid norharmane in *Nostoc insulare* and *Nodularia harveyana*, respectively [9]. There are a lot of intracellular and extracellular metabolites with diverse biological activities as antiviral [10], anticancer, anti-inflammatory, cytotoxic [11] and immunosuppressive agents. The production of extracellular antibiotic metabolites by marine microalgae and screening for other pharmacologically active compounds had been studied in past few decades [12]. In addition to that foregoing, there are many other important compounds with antioxidant activity like ascorbic acid, reduced glutathione, phenolic compounds and flavonoids [13]. Furthermore, photosynthetic pigments which consist of two classes, chlorophylls (α &β chlorophyll) and carotenoids. Carotenoids that contain oxygen known as xanthophylls like lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin and astaxanthin; while that are only hydrocarbons are known as carotenes like β-carotene and lycopene [14]. Sinha et al. [15] reported that the screening programs of cyanobac-

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terial biomass have led to discovery of new compounds with antimicrobial, antineoplastic and cytotoxic activities. The use of non-conventional microorganism in biotechnology had attracted much interest in recent years [16]. The conventional optimization of production factors is usually a time uncontrollable and labor-intensive process. On the contrary, statistically designed two level-factorial experiments, had been proved to be valuable tools for optimizing microbial culture conditions [17]. The objective of this study was to apply Response surface methodology (RSM) to evaluate the effects of the medium composition for attaining the highest bioactive compounds production by cyanobacterium *Anabaena oryzae*.

2. Materials and methods

2.1. Culture preparation and isolation of micro-organism

Cyanobacterium *Anabaena oryzae* was isolated from the rice fields in Egypt. The soil samples were collected, air-dried, ground to pass a 2-mm sieve. Soil samples were transferred into Petri-dishes, wetted by sterilized distilled water and incubated under fluorescent illumination for three weeks. The formed mats were inculcated into modified (BG-11) medium [18], and the mat was re-cultured several times to new solid BG-11 plates and incubated under continuous illumination using white fluorescent light intensity of 2000–2500 lux for three weeks at 26 ± 2 °C, with regular microscopic examination.

Modified BG-11 medium was used in the present study and its final pH after autoclaving and cooling was 7.4.

2.2. Alga identification

Cyanobacterium *Anabaena oryzae* was identified according to Desikachary [19] and the samples were prepared according to the procedure described by Cronberg [20] using light microscope at 100× oil immersion lens.

2.3. Maintenance medium for alga

The *Anabaena oryzae* alga was maintained on BG-11 medium, 250 ml of algal culture was transferred to 500 ml Erlenmeyer flasks. The flasks were exposed to a light/dark cycle of 14/10 h, respectively at room temperature, left to grow and harvested after 2 weeks by centrifugation at 1000 rpm for 10 min.

2.4. Optimization of the bioactive compounds production based on multi-factorial experiments

The Plackett–Burman experimental design, [21] an efficient technique for optimization of the factors affecting active compounds' production was used to reflect the relative importance of various chemical factors on bioactive compounds production.

This model does not describe the interaction between the factors, but was used to screen and evaluate the important media components that influence the production of antibacterial compounds.

Eight independent variables (Table 1) were screened in thirteen runs organized according to the Plackett–Burman design matrix as indicated in Table 2. The studied factors were: $NaNO_3$, K_2HPO_4, $MgSO_4$, $CaCl_2$, citric acid, ammonium ferric citrate, ethylene diaminetetraacetic acid disodium magnesium salt (EDTA-Na_2Mg) and Na_2CO_3. For each variable, a high level (+) and low level (−) were tested, which represents two different nutrient concentrations. The trials were performed in triplicate using 250 ml Erlenmeyer flasks and final data was calculated as the mean of triplicate.

Table 1
Factors and coded levels examined as independent variables affecting bioactive components production by *Anabaena oryzae* and their levels in the Plackett–Burman design experiment.

Factor ($g\ l^{-1}$)	Symbol	Low level (−1)	High level (+1)
$NaNO_3$	A	0.75	2.25
K_2HPO_4	B	0.02	0.06
$MgSO_4$	C	0.0375	0.1125
$CaCl_2$	D	0.018	0.054
Citric acid	E	0.003	0.009
Ammonium ferric citrate	F	0.003	0.009
Ethylenediaminetetraacetic acid disodium magnesium salt (EDTA-Na_2Mg)	G	0.0005	0.0015
Na_2CO_3	H	0.01	0.03

2.5. Cell growth and determination of cell dry weight

The growth of *Anabaena oryzae* was determined by measuring the optical density of the algal suspension at wavelength 660 nm for 7 days using spectrophotometer (UV-200-RSLW scientific) [22]. The algal biomass can be determined by measuring its dry weight. The pellets were washed with distilled water, recentrifuged and kept for drying at 60 °C till constant weight [23].

2.6. Preparation of alga extract

One hundred mg of alga was soaked in 5 ml petroleum ether (60–80 °C) for 48 h and filtrated; the precipitate was soaked in dichloromethane for the same period and repeated for the last time in methanol.

2.7. Analysis of bioactive compounds

The methanol extracts of *Anabaena oryzae* were used for the tannin, flavonoids, total phenol content, antioxidant, ascorbic acid and pigments analyses.

2.8. Total phenolic content (TPC)

Total phenolic content was performed by Folin-Ciocalteu method [24]. Distilled water (3.16 ml) was mixed with the 40 µl of sample and then 200 µl of Folin Ciocalteu reagent was added. After 5 min, 600 µl of 20% sodium carbonate solution (Na_2CO_3) was added and solution mixed again. The solution was left at room temperature for 2 h. The color intensity was measured using spectrophotometer at wavelength 750 nm. The total phenol content was compared to with gallic acid standard curve according to the formula:

$$\text{TPC (mg/g)} = C \times V/g$$

where;

C = concentration of the gallic acid equivalent from standard curve (mg/mL),
V = volume of the extract used (ml),
g = weight of extract (g).

2.9. Pigments estimation

A known volume of culture was centrifuged at 8000 rpm for 10 minutes, after that the algal pellets were treated with known volume of methanol and kept in water bath for 30 min at 55 °C, then centrifuged again. The color of pellets must be white to ensure complete extraction of pigments. Absorbance was measured at 666, 653 and 470 nm. Calculations were made according to the for-

Table 2
Randomized Plackett–Burman experimental design for the eight tested BG-11 medium factors.

Trial no.	$NaNO_3$	K_2HPO_4	$MgSO_4$	$CaCl_2$	Citric acid	Ferric ammonium citrate	EDTA	Na_2CO_3
1	2.25	0.02	0.1125	0.018	0.003	0.003	0.0015	0.03
2	2.25	0.06	0.0375	0.054	0.003	0.003	0.0005	0.03
3	0.75	0.06	0.1125	0.018	0.009	0.003	0.0005	0.01
4	2.25	0.02	0.1125	0.054	0.003	0.009	0.0005	0.01
5	2.25	0.06	0.0375	0.054	0.009	0.003	0.0015	0.01
6	2.25	0.06	0.1125	0.018	0.009	0.009	0.0005	0.03
7	0.75	0.06	0.1125	0.054	0.003	0.009	0.0015	0.01
8	0.75	0.02	0.1125	0.054	0.009	0.003	0.0015	0.03
9	0.75	0.02	0.0375	0.054	0.009	0.009	0.0005	0.03
10	2.25	0.02	0.0375	0.018	0.009	0.009	0.0015	0.01
11	0.75	0.06	0.0375	0.018	0.003	0.009	0.0015	0.03
12	0.75	0.02	0.0375	0.018	0.003	0.003	0.0005	0.01
13	1.5	0.04	0.075	0.036	0.006	0.006	0.001	0.02

mula devised by Costache [25] for chlorophyll (a) and carotenoids estimation.

Chlorophyll (a) = 15.65 Ab 666-7.340 Ab653
Carotenoids = 1000 Ab 470-2.860 Chlorophyll (a)

2.10. Flavonoid content determination

The flavonoid content of *Anabaena oryzae* extract samples were determined according to the aluminum chloride method described by Barku [26]. One ml of sample solution of alga extract was added to 0.5 ml of distilled water. About 0.075 ml of 5% sodium nitrite solution was then added to the mixture followed by incubation for 6 min after which 0.15 ml of 10% aluminum chloride was added, shaked and left to stand for 6 min, 0.5 ml of 1 M sodium hydroxide was finally added and the mixture was diluted with 0.275 ml distilled water, shaked and left to stand for 15 min before absorbance reading, using the sample solution without coloration as reference solution. The absorbance of the reaction mixture was measured at 500 nm.

Flavonoid content was expressed as mg of quercetin equivalent (QE)/g dry weight.

2.11. Antioxidant capacity

Antioxidant activity of *Anabaena oryzae* extract was determined by the ability of extract to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH)radicals [27]. One hundred µl of test extract was mixed with 1 ml of methanolic solution of 0.1 mM DPPH. The mixture was vortexed and incubated at 37 °C for 30 min in dark. The absorbance of the samples was measured at 517 nm.

Lower absorbance of the reaction mixture indicated higher free radical scavenging activity which was calculated using the following equation:

$$\text{DPPH scavenging effect } (\%) = 100 \times (A^{\circ} - A_1)/(A^{\circ})$$

where: A° is the absorbance of the control reaction and A_1 is the absorbance of reaction mixture.

Percentage of the DPPH scavenging activity was plotted versus the different concentrations of sample extract used and the concentration causing a 50% reduction of DPPH absorbance was expressed as the half maximal inhibitory concentration (EC_{50}) value.

2.12. Vanillin – HCL assay

Sample (0.2 g) of dried *Anabaena oryzae* biomass was extracted with 10 ml of methanol for 24 h at 30 °C. One ml of the resulting extract was reacted with 5 ml of vanillin reagent 50:50 (mixture of 1% vanillin:8% HCl in methanol) for 20 min at 30 °C, and absorbance was measured at 500 nm. For the control sample, vanillin reagent was replaced with 5 ml of 4% HCl in methanol. Blank value was subtracted from experimental value to give adjusted data.

Tannic acid standard curve from 0.0–1.0 mg/ml was used in calculating tannin levels [28].

2.13. Ascorbic acid content

Ascorbic acid content of *Anabaena oryzae* was estimated according to the method of Loeffler and Ponting [29]. Ten ml of 2,6-dichlorophenol indophenol dye solution was added to 5 ml of 100 mg of dried *Anabaena oryzae* biomass then was extracted by 6% HPO_3, vortexed well and the red color was measured within 15–20 second at 518 nm.

Ascorbic acid standard curve was used in calculating mg of ascorbic acid per g of dry sample according to the formula:

$$\text{Mg of ascorbic acid per g of dry sample} = \frac{\text{Ascorbic acid content} \times \text{volume made up} \times 100}{\text{ml of solution taken for estimation} \times 1000 \times \text{weight of sample taken (0.1 g)}}$$

2.14. Statistical analysis

Statistical were carried by using Minitab 16 software.

3. Results and discussion

In the present study screening of basic medium compositions influencing active constituents production by *Anabaena oryzae* were analyzed by Plackett–Burman experimental design under shaking conditions.

The data shown in (Table 3) revealed a wide variation in total phenol concentrations. The maximum phenol production was

Table 3
Randomized Plackett–Burman experimental design for the eight tested BG-11 medium factors affecting bioactive components production by *Anabaena oryzae*.

Trial no.	A	B	C	D	E	F	G	H	Total Phenol (mg/ml)	Chlorophyll-a (μg/ml)	Carotenoid (μg/ml)	Flavonoid (μg/ml)	Antioxidant capacity (%)	Tannic acid (mg/ml)	Ascorbic acid (mg/ml)
1	+	–	+	–	–	–	+	+	0.215	03.4	0.94	0.91	23.44	0.166	2.51
2	+	+	–	+	–	–	–	+	0.222	08.3	1.26	1.45	17.41	0.173	4.35
3	–	+	+	–	+	–	–	–	0.215	03.5	0.52	1.44	18.54	0.045	3.26
4	+	–	+	+	–	+	–	–	0.212	02.7	0.46	1.35	17.56	0.466	2.49
5	+	+	–	+	+	–	+	–	0.195	02.2	1.66	1.15	12.40	0.385	3.46
6	+	+	+	–	+	+	–	+	0.163	01.7	0.54	0.75	17.80	0.285	4.48
7	–	+	+	+	–	+	+	–	0.125	03.8	0.04	0.16	12.87	0.003	3.37
8	–	–	+	+	+	–	+	+	0.097	06.7	0.56	1.71	26.73	0.664	4.70
9	–	–	–	+	+	+	–	+	0.184	27.8	0.74	0.95	07.47	0.656	4.29
10	+	–	–	–	+	+	+	–	0.256	47.7	4.11	1.83	62.81	1.046	2.95
11	–	+	–	–	–	+	+	+	0.164	13.5	1.86	0.72	16.79	0.563	3.88
12	–	–	–	–	–	–	–	–	0.103	12.5	0.65	0.46	20.63	0.366	4.82
13	0	0	0	0	0	0	0	0	0.115	12.7	1.65	0.61	23.39	0.711	2.45

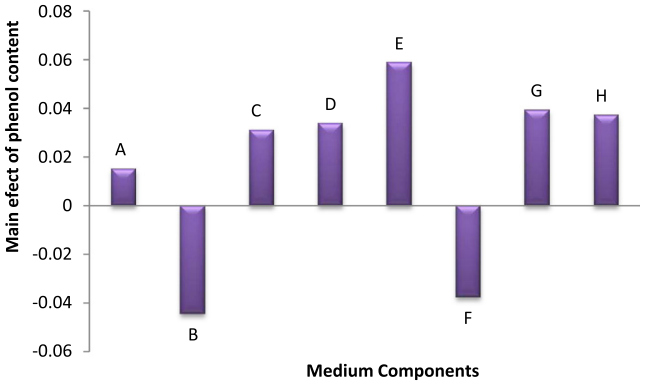

Fig. 1. The main effects of different medium variables on the total phenol content of *Anabaena oryzae*.

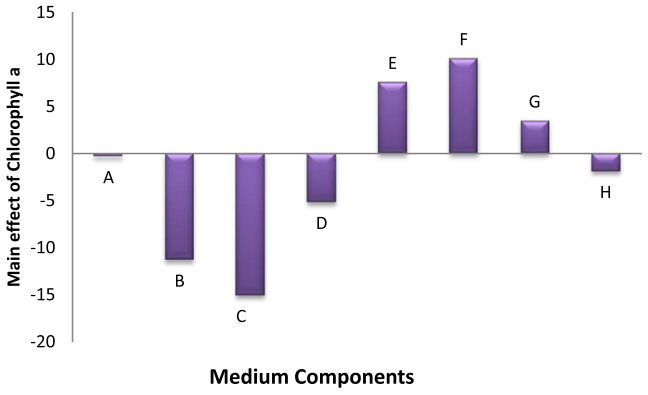

Fig. 2. The main effects of different medium variables on the Chlorophyll content of *Anabaena oryzae*.

found in experimental trial no. 10 (0.256 mg/ml), whereas minimum concentration of phenol was in trial no. 8 (0.097 mg/ml).

Statistical results of eight tested medium variables in case of the phenol production revealed that K_2HPO_4 and ferric ammonium citrate had a negative effect, other variables had a positive effect and in general there was no significant effect between medium variables on phenol production by *Anabaena oryzae* at $P \leq 0.05$ (Fig. 1).

In case of chlorophyll (a) and carotenoid pigments production, maximum concentration of chlorophyll (a) was found in trial no. 10 (47.7 μg/ml) and minimum concentration was found in experimental trial no. 6 (1.7 μg/ml), while maximum carotenoid pigment production also was present in trial 10 (4.11 μg/ml). Statistical results of eight tested medium variables in case of the chlorophyll (a) revealed that ferric ammonium citrate and citric acid had a significant positive effect, while in carotenoid pigment production, $NaNO_3$, citric acid and ferric ammonium citrate had a significant positive effect. The results revealed that $MgSO_4$, K_2HPO_4 and $CaCL_2$ variables had a significant negative effect in chlorophyll (a) production while $MgSO_4$ and $CaCL_2$ had that effect in production of carotenoid, respectively (Figs. 2 and 3).

The highest record of total flavonoid content was observed in trial no. 10 (1.83 μg/ml). (Fig. 4) shows that both $NaNO_3$ and citric acid variables had a significant positive effect on the production of flavonoids.

All experimental trials have a scavenging activity toward the DPPH reagent; however the trial no. 10 exhibited the highest scavenging activity (62.81%). It was found that among the eight investigated medium variables, EDTA, citric acid and $NaNO_3$ had positive main effects on antioxidant capacity (Fig. 5); while the K_2HPO_4 and

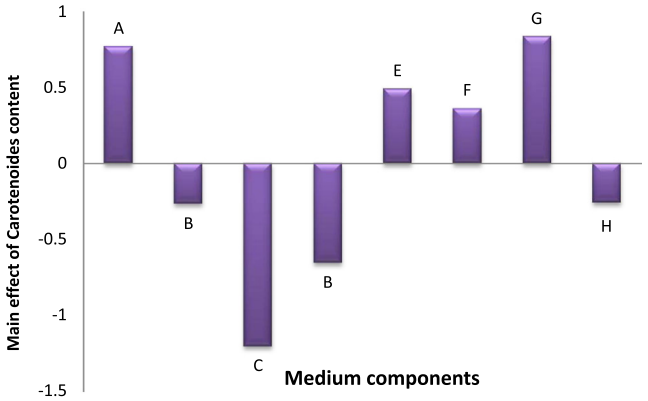

Fig. 3. The main effects of different medium variables on Carotenoied content of *Anabaena oryzae*.

$CaCL_2$ variables had negative main effects. The differences between both positive and negative variables were significant on antioxidant capacityat $P \leq 0.05$.

Also, in case of tannin production, the highest tannin content measured at the end of the experimental period was 1.046 mg/ml in trial no. 10, whereas the lowest content was 0.003 mg/ml in the trial no. 3. Statistical results of eight tested medium variables in tannin production revealed that K_2HPO_4 and $MgSO_4$ variables had a significant negative effect; and citric acid, ferric ammonium citrate and EDTA had a positive effect, respectively (Fig. 6).

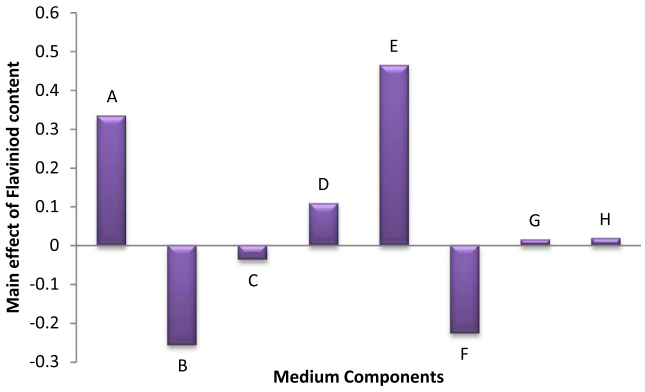

Fig. 4. The main effects of different medium variables on the total flavonoid production of *Anabaena oryzae.*

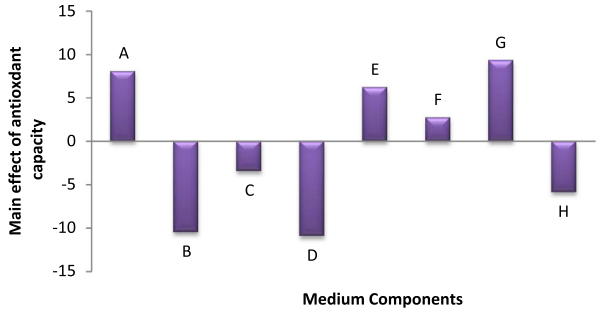

Fig. 5. The main effects of different medium variables on the antioxidant capacity of *Anabaena oryzae.*

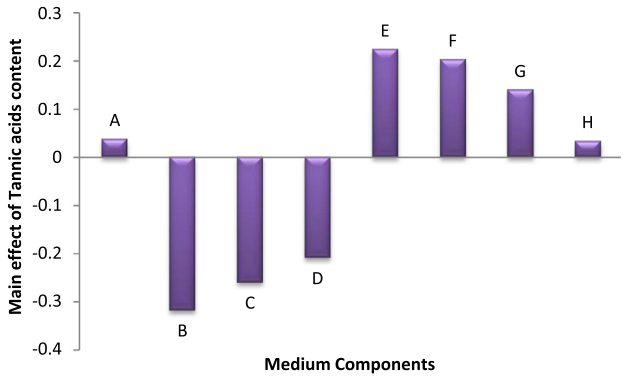

Fig. 6. The main effects of different medium variables on the tannic acid production of *Anabaena oryzae.*

The maximum concentration of ascorbic acid was 4.82 mg/ml in trial no. 12, from the statistical results represented in (Fig. 7). The main effect of the eight different factors in the Plackett -Burman experimental design, the *p*-value and the significant of the different factors that affect ascorbic acid production showed that, $NaNO_3$, $MgSO_4$ and EDTA have negative main effects on the ascorbic acid content, while the other factors have positive main effects on the ascorbic acid content. Statistical analysis also estimated that $NaNO_3$, $MgSO_4$, EDTA and Na_2CO_3 were significant variables on production of ascorbic acid by *Anabaena oryzae* at $P \leq 0.05$.

Among the eight nutrient components used in this study, K_2HPO_4, $MgSO_4$ and citric acid had contributed to a large extent for most bioactive compounds production.

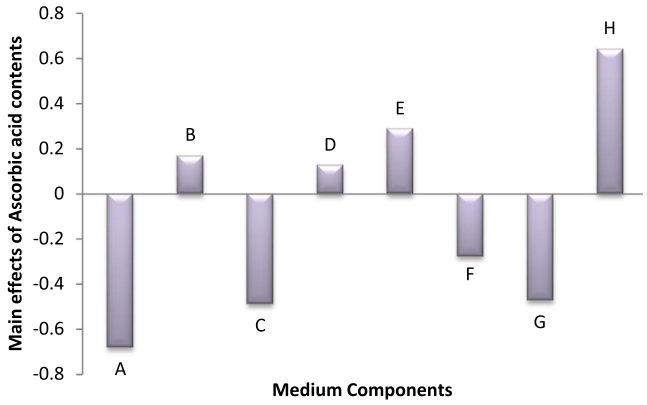

Fig. 7. The main effects of different medium variables on the ascorbic acid production of *Anabaena oryzae.*Medium components: $NaNo_3$(A), K_2HPO_4(B), $MgSO_4$(C), $CaCl_2$(D), Citric acid(E), Ammonium ferric citrate(F), EDTA-Na_2Mg(G) and Na_2CO_3(H).

The chlorophyll (a) and carotenoid pigments, antioxidant capacity, flavonoid content and tannin content yields were increased in the presence of high concentration of citric acid and low concentrations of K_2HPO_4 and $MgSO_4$.

$NaNO_3$ was effective in higher level in increasing concentration of carotenoid pigments, flavonoid content and antioxidant capacity; while ferric ammonium citrate was effective in case of chlorophyll (a), carotenoids and tannic acid production. On the other hand the $CaCl_2$ was the most important medium variable in lower level in increasing of chlorophyll (a), carotenoid pigments and percentage of antioxidant activity.

The Plackett–Burman experimental design has been used to evaluate the components of BG-11 medium, affecting the production of active constituents like the phycocyanin. $MgSO_4$ and ferric ammonium citrate were found to be the effective components affecting the phycocyanin production [30]. Bloor and England [31], studied the increasing of nitrate from its base level of 8.8 mM to 26.4 mM which increased the antibiotic production by *Nostoc muscorum.* However, increasing the concentration of iron from its base level of 5 mM to15 mM caused a dramatic decrease in antibiotic production. Similar results obtained by Ohta et al. [32] who reported that the increase in magnesium and phosphate concentrations cause increase in antibiotic production from *Chlorococcum* strain HS-101, whereas, the antibiotic production from *Chlorococcum* strain HS-101 increased by 1.8- fold in the optimized BG-11 medium than in the standard BG-11 and also the antibiotic production from the alga *Dunaliella primolecta* increased by 2.3-fold in the optimized BG-11 medium. Yin et al. [33] stated that changes in phosphate, nitrate, calcium, irradiance and temperature all caused quantitative, but not qualitative, changes in toxin composition produced by the Cyanobacterium *Lyngbya wollei* where, lower phosphate, nitrate and higher calcium levels gave rise to higher biomass and toxicity. Lower calcium or phosphate deficient medium and high nitrate or phosphate caused a large decrease in dry weight and toxicity. The results obtained from Plackett–Burman experimental design stated that the highest main effect and *t*-value obtained by NaCl was significant variable within seven independent variable, so the effect of NaCl on the antifungal activity of *Spirulina platensis* is the highest and positive [34].

4. Conclusion

Blue-green algae *Anabaena oryzae* was used in this work for bioactive compounds production using modified blue-green med-

ium (BG-11). Eight independent factors including $NaNO_3$, K_2HPO_4, $MgSO_4$, $CaCl_2$, citric acid, ammonium ferric citrate, ethylene diamine tetra acetic acid disodium magnesium salt (EDTA-Na_2Mg) and Na_2CO_3 surveyed considered as more and effective variables for the production of chlorophyll (a) and carotenoid pigments, phenols, flavonoids, tannins. Also increased the antioxidant capacity of the algal extract. The Plackett–Burman experimental design, an efficient technique for optimization of the factors affecting active constituent's production was used to reflect the relative importance of various chemical factors on bioactive compounds production.

Conflict of interest

The authors declare that they have no conflict of interest.

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