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Effect of different growth conditions on certain biochemical parameters of different cyanobacterial strains

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ABSTRACT

Aims: Variation in the traditional growth medium conditions to enhance the production of lipids, carbohydrates, protein and the free amino acids content of three cyanobacterial species.
Methodology and Results: Three species of cyanobacteria *(Anabaena laxa*, *Anabaena fertilissim*a and *Nostoc muscorum*) were collected from the culture collection of Soils, Water and Environment Research Institute, Agriculture Research Center, Giza, Egypt, to investigate their biochemical composition under different growth conditions, using $BG11_0$ (nitrogen free) as growth medium. These conditions were represented by control medium, static glucose medium with (1%, w/v), aerated medium (aerated by bubbling technique depending on CO_2 normally existed in air with a concentration of 0.03%), molasses medium (0.7%, v/v) and aerated medium enriched with glucose (1%, w/v). Lipid content, total carbohydrates, soluble proteins and free amino acids were determined at the previous conditions. Glucose at 0.7% (w/v) was the most favorable for lipid production in *A. laxa,* where it exhibited the highest lipid content (427 µg/g fresh wt.). Increasing molasses concentration up to 0.7% (v/v) produced an increase in lipid contents of the tested cyanobacterial strains. The highest lipid content of both *N. muscorum* (366.2 µg/g fresh wt.) and *A. laxa* (357.4 µg/g fresh wt.) were recorded at molasses concentrations of 0.1 and 0.7% (v/v), respectively. *A. laxa* expressed high significant values for both proteins (31.6 µg/mL) and free amino acids (40.5 mg/g dry wt.) after 6 days of incubation period under aerated enriched glucose condition (1%, w/v). Also, at the same growth conditions, *A. fertilissima* exhibited high significant values for carbohydrates at 4th day (876.8 mg/g dry wt.).
Conclusion, significance and impact of study: Aerated enriched glucose medium (1%, w/v) was the best growth medium condition used in the present study.

Keywords: Cyanobacteria; mixotrophy; autotrophy; biochemical parameters

INTRODUCTION

Algae sequester significant quantity of carbon from atmosphere and industrial gases and were very efficient in utilizing the nutrients from industrial effluents and municipal wastewater. Therefore, cultivation of algal biomass provide dual benefit; production of biofuels and save our environment from air and water pollution (Singh and Olsen, 2011). Mixotrophic culture was a potential mode for mass production of microalgae and cyanobacteria by using heterotrophic capability of the photosynthetic microorganisms (Marquez *et al*., 1995; Chen, 1997). It was expected that mixotrophic growth can achieve high cell densities and synthesize light-induced products such as photosynthetic pigments and was especially suitable for the production of high value bioactive compounds, fine chemicals and pharmaceuticals. Cyanobacteria possess several advantages as organisms for bio-industrial processes, including simple input requirements, tolerance of marginal agricultural environments, rapid genetics, and carbon-neutral applications that could be leveraged to address global climate change concerns (Ducat *et al*., 2011). Some microalgae and cyanobacteria, which were regarded as obligate photoautotrophs, can utilize organic carbon compounds for their growth (Droop, 1974; Tuchman, 1996). *Nostoc spp*. assayed can grow mixotrophically by using; glucose, sucrose, and sugarcane molasses as organic substrates, and a greater production of biomass and phycobiliproteins can be reached when compared with the autotrophic growth (Borsari *et al*., 2007). Most bio-industrial processes rely on microorganisms metabolizing carbohydrate compounds to generate a diverse array of valuable chemicals, such as amino acids, vitamins and organic acids (Ducat *et al*., 2011). Sugarcane molasses (by-product of sucrose production) is the most inexpensive raw material widely used for fermentation in the world. Egypt has more than 250-300 thousand feds planted with sugar cane. Moreover waste molasses hydrolysate was

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confirmed as a sole source of full nutrients to totally replace glucose-based medium in support of rapid growth and high oil yield from algae. Also, the quality of biodiesel from waste molasses-fed algae was probably comparable to that from glucose-fed ones (Yan *et al*., 2011). Inexpensive *Spirulina* reactors that are capable of producing 10 folds of the biomass of soybeans, while utilizing equivalent amounts of water (Habib *et al*., 2008). This study aimed to investigate the capability of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum* to grow in mixotrophic culture, testing glucose and molasses sugarcane as organic carbon substrates.

MATERIALS AND METHODS

Strains and growth medium

Anabaena laxa (Rabenhorst) Braun A., *Anabaena fertilissima* Rao C.B. and *Nostoc muscorum* Agardh C. were provided from the culture collection of Soils, Water and Environment Research Institute, Agriculture Research Center, Giza, Egypt. The culture growth medium was $BG11_0$ (nitrogen free) (Allen's and Stanier, 1968). The isolates were grown autotrophically and axenically in batch cultures under 28±2 ºC with continuous illumination at intensities of 2500 Lux.

Growth conditions

The investigated cyanobacterial strains were sub-cultured, separately, in 5 L Erlenmeyer bottles, containing 3 L $BG11_0$ medium (nitrogen free) inoculated with 30 mL of pre-cultured isolates during exponential phase. These strains were cultured under two different growth conditions; autotrophic and mixotrophic. The autotrophic conditions were represented by static and aerated control $BG11_0$ medium (aeration was provided by bubbling air at regular pressure, 200 mL/min and frequency 50Hz), that depended on CO_2 (0.03%) which exist normally in air. The mixotrophic growth conditions that depended on organic carbon sources (glucose and molasses of sugarcane) were created by adding glucose (1%, w/v) and molasses of sugarcane (0.7%, v/v) separately to the growth media under static condition (without aeration). The last one was formed by adding glucose (1%, w/v) to the growth medium under aerated condition.

Sampling and analysis

Samples of 250 mL every two days intervals from each of the experimental conditions were harvested by centrifugation at 4000 rpm for 15 min. The pellet was rinsed three times and re-suspended in sterilized distilled water to remove traces of growth medium (Roger and Burns, 1994). These samples were taken to determine carbohydrates, proteins and free amino-N acids. Carbohydrates were quantified as glucose by anthron technique according to Yemm and Willis (1954). The Total soluble proteins were quantitatively determined using the method described by Lowry *et al.* (1951). Estimation of free amino-N in a protein free sample using ninhydrine reagent, was suggested by Lee and Takahashi (1966). Total lipid content was determined by phosphovaniline method (Barnes and Blackstock, 1973).

Effect of different glucose and molasses concentrations on lipids

Our future work aimed to study the efficiency of a cyanobacterial strain to produce biofuel, so we were interested in studying the effect of different carbon sources on production of lipids. This experiment was carried out in order to study the effect of different glucose concentrations (0.1, 0.3, 0.5, 0.7 and 1%, w/v) and different molasses concentrations of sugarcane (0.1, 0.3, 0.5, 0.7 and 1%, v/v) on total lipid content at the stationary phase for each cyanobacterial species.

Statistical analysis

The obtained results were analyzed for statistical significant between control and treated groups by using one-way analysis of variance (ANOVA, SPSS 16.0.Ink program). Values were expressed as mean (±) standard deviation and values of $P \leq 0.05$ were statistically significant.

RESULTS

Table 1 showed that increasing glucose concentration up to 1% (w/v) led to the increase in lipid content of the all tested cyanobacterial strains. Where, *Anabaena laxa* recorded the highest lipid content (427.1 µg/g fresh wt.) in glucose medium condition (0.7%, w/v). Also, by increasing molasses concentration up to 0.7% (v/v) led to the increase in lipid content of the tested strains (Table 2). *N. muscorum* showed the highest significant difference of lipid content (366.2 µg/g fresh wt.) under 0.1% dose in comparable to the other tested cyanobacterial strains. No growth was recorded at levels higher than 0.1% (v/v) of molasses. *A. laxa* recorded high significant lipid values under 0.7% dose (357.4 µg/g fresh wt.). At high level of molasses (1%, v/v), *A. fertilissima* exhibited no growth, moreover total lipid content of *A. laxa* decreased markedly.

Data in Table 3 showed that the total carbohydrates of the tested cyanobacterial species in the mixotrophic growth media (static glucose, molasses treatments and aerated enriched glucose medium) greatly exceeded other autotrophic conditions (control, aerated medium). *A. laxa* reached its maximum value of carbohydrate content (609.2 mg/g dry wt.) after 4 days under static glucose medium which was about 2.8 folds of zero time. Aerated enriched glucose medium was in the second level, followed by molasses treatment. Carbohydrate content of *A. fertilissima* increased gradually during exponential phase to reach its maximum value during stationary phase then declined gradually. The maximum significant value of carbohydrate was about 2.9 folds of zero time that was achieved under aerated enriched glucose medium and

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followed by static glucose medium which was about 2.3 folds as zero time. While the minimum values were obtained at both air mixture and control media conditions.

Table 1: Effect of different glucose concentrations on total lipid contents of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*, measured as µg/g fresh weight at stationary phase for each species.

Microalgal	Control	0.1%	0.3%	0.5%	0.7%	1%	LSD
Anabaena laxa	162.9±7.4	234.0±6.8	252.3±5.6	289.8±5.4	427.1±23.5	364.7±6.7	9.2
Anabaena fertilissima	37.4±3.3	71.3±3.5	81.4±1.7	135.9±14.2	154.8±15.8	170.3±4.7	7.5
Nostoc muscorum	228.8±2.0	236.4±9.7	241.7±10.3	259.3±3.7	275.3±3.2	278.3±7.6	5.7
LSD	3.9	5.9	5.6	7.4	13.5	5.3	

LSD; the least significant difference at P≤0.05. Values are means of 3 replicates ± SD

Table 2: Effect of different molasses concentrations on total lipid contents of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*, measured as µg/g fresh weight at stationary phase for each species.

Microalgal	Control	0.1%	0.3%	0.5%	0.7%	1%	LSD
Anabaena laxa	168.9±7	167.3±1.9	232.5±3.3	283.2±7.7	357.4±11.1	292.6±2.8	5. 3
Anabaena fertilissima	52.4±7	67.8±1.7	94.7±8.9	95.0±2.6	109.9±5	0	4.3
Nostoc muscorum	224.1±10.2	366.2±10.8	0	0	0	0	5.0
LSD	6.7	5.2	4.5	3.9	5.8	1.3	

LSD; the least significant difference at P≤0.05. Values are means of 3 replicates ± SD

Similarly, *N. muscorum* exhibited more or less the same pattern of the previously mentioned cyanobacterial strains behaviors, where total carbohydrate content increased gradually with time and maximum values were obtained at stationary phase then the recorded values began to decline. Meanwhile, it showed no growth on molasses medium at concentration of 0.7% (v/v). The effect of different growth conditions on total soluble protein of *A. laxa*, *A. fertilissima* and *N. muscorum*, were recorded at different incubation periods.

The total soluble protein increased gradually during growth phase till it reached its maximum content in stationary phase for all growth conditions and for the three organisms except for *A. laxa* under molasses medium where the highest value was obtained at exponential phase (Table 4). *A. laxa* recorded high significant soluble protein under aerated enriched glucose medium after 6 days which was about 13.2 folds of zero time and followed by aerated medium treatment that exceeded 8.6 folds of zero time. While the minimum protein value was recorded under glucose medium. With regard to *A. fertilissima* the highest soluble protein value was under static glucose and followed by aerated medium to record about 12.2 and 8.2 folds of zero time, respectively. Regarding to *N. muscorum,* the highest soluble protein content was shown after 6 days for all growth media while, the highest one was in the aerated medium and exceeded 27.9 folds of zero time. The changes in total free amino acids content of *A. laxa*, *A. fertilissima* and *N. muscorum* in response to different growth conditions are presented in Table 5. *A. laxa* the maximum free amino acids 40.5 mg/g dry wt. which exceeded 3.26 folds of zero time at 6th day under aerated enriched glucose, followed by 18.2 mg/g dry wt. under static glucose medium at the same time interval and exceeded 1.4 folds of zero, followed by 13.5 mg/g dry wt. at 6th day in the control medium. *A. laxa* produced the minimum content of free amino acids under both aerated and molasses medium which exhibited gradual decline during incubation periods compared to zero time.

With respect to *A. fertilissima,* data in Table 5 showed that the free amino acids content increased gradually during growth phase and reached its highest value at stationary phase 35.3 mg/g dry wt. about 7.9 folds of zero time obtained under molasses at 6th day followed by 29.7 mg/g dry wt. and exceeded 6.6 folds of zero time was produced at 4th day under static glucose medium, *A. fertilissima* produced minimum content of free amino acids 7.1 mg/g dry wt. at 4th day under control medium. With regard to *N. muscorum*, as it appeared from Table 5, free amino acids increased gradually during growth phase till reached its highest values at stationary phase, for control, static glucose and aerated enriched glucose medium, while aerated medium exhibited gradual decrease during its growth phase compared to zero time. The highly significant free amino acids of *N. muscorum* was 15.9 mg/g dry wt. at 6th day and exceeded 4.7 folds of zero time under glucose medium followed by aerated enriched glucose medium 9.3 mg/g dry wt. at the same day and increased by 2.7 folds of zero time, followed by 6.3 mg dry wt. at 6th due to control condition.

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Table 3: Effect of different growth conditions on total carbohydrate of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*, measured as mg/g dry weight at different time periods.

Time period (days)	Growth medium					LSD
	Control	Static glucose	Aerated	Molasses	Aerated enriched glucose	
	Anabaena laxa					
0	213.4±2.5	213.4±2.5	213.4±2.5	213.4±2.5	213.4±2.5	2.1
2	309.5±2.4	507.5±13.4	265.7±11	401.7±5.1	312.8±15.8	8.9
4	324.7±6.4	609.2±2.3	295.1±4.9	370.8±8.1	596.4±19.4	8.3
6	324.8±3.5	536.1±2.9	244.5±10	321.3±5.2	572.6±7.3	5.2
LSD	3.3	5.8	6.5	4.6	10.7	
	Anabaena fertilissima					
0	299.2±1.5	299.2±1.5	299.2±1.5	299.2±1.5	299.2±1.5	1.3
2	414.3±14.2	604.2±12.8	329.3±6.1	390.9±14.8	678.6±63.4	24.9
4	415.3±19.0	715.8±18.6	400.0±11.4	493.8±27.7	876.8±15.62	15.7
6	353.5±3.3	593.5±2.2	369.8±9.2	610.6±14.3	824.6±68.7	25.9
LSD	9.8	9.3	6.5	14.2	38.7	
	Nostoc muscorum					
0	343.7±6.0	343.7±6.0	343.7±6.0	343.7±6.0	343.7±6.0	5.0
2	420.6±11.1	341.5±11.5	305.7±4.6	0	528.5±29.9	12.5
4	443.4±9.5	393.6±0.8	306.2±1.4	0	560.8±12.8	5.9
6	365.4±4	452.4±3.5	337.5±6.4	0	831.2±55.4	20.5
LSD	6.7	5.5	4.1	2.5	26.4	

LSD; the least significant difference at P≤0.05
Values are means of 3 replicates ± SD

Table 4: Effect of different growth conditions on total soluble proteins of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*, measured as µg/mL of algal suspension at different time periods.

Time period (days)	Growth medium					LSD
	Control	Static glucose	Aerated	Molasses	Aerated enriched glucose	
	Anabaena laxa					
0	2.38±0.25	2.38±0.25	2.38±0.25	2.38±0.25	2.38±0.25	0.21
2	3.41±0.67	1.83±0.11	3.54±0.11	8.01±0.19	10.35±1.5	0.61
4	4.72±0.22	3.04±0.19	5.14±0.62	6.45±0.41	25.87±1.48	0.62
6	8.06±0.38	2.13±0.36	20.53±0.19	3.14±0.29	31.57±4.04	1.5
LSD	0.35	0.20	0.29	0.25	1.9	
	Anabaena fertilissima					
0	0.99±0.17	0.99±0.17	0.99±0.17	0.99±0.17	0.99±0.17	0.14
2	1.83±0.08	3.76±0.22	1.02±0.11	3.98±0.35	1.79±0.16	0.17
4	2.97±0.48	12.08±0.33	2.13±0.30	3.17±0.47	3.26±0.50	0.35
6	2.57±0.40	8.85±0.04	8.18±0.85	5.64±0.29	4.88±0.48	0.40
LSD	0.27	0.18	0.38	0.28	0.30	
	Nostoc muscorum					
0	0.20±0.08	0.20±0.08	0.20±0.08	0.20±0.08	0.20±0.08	0.07
2	1.04±0.19	1.29±0.11	0.11±0.08	0	1.11±0.61	0.24
4	1.66±0.33	3.49±0.67	1.34±0	0	2.74±1.72	0.67
6	1.98±0.47	5.59±0.15	5.91±0.33	0	4.28±2.27	0.86
LSD	0.25	0.29	0.15	0.04	1.2	

LSD; the least significant difference at P≤0.05
Values are means of 3 replicates ± SD

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Table 5: Effect of different growth conditions on free amino acids of *Anabaena laxa*, *Anabaena fertilissima* and *Nostoc muscorum*, measured as mg/g dry weight at different time periods.

Time period (days)	Growth medium					LSD
	Control	Static glucose	Aerated	Molasses	Aerated enriched glucose	
	Anabaena laxa					
0	12.40±0.19	12.4±0.19	12.4±0.19	12.4±0.19	12.4±0.19	0.16
2	13.1±0.16	15.02±0.16	7.16±0.20	9.75±0.92	20.92±3.68	1.39
4	13.21±1.24	15.45±1.33	3.05±0.45	7.05±0.02	23.77±1.66	0.92
6	13.53±0.38	18.22±0.70	3.13±0.07	8.19±1.62	40.49±4.78	1.86
LSD	0.54	0.62	0.22	0.77	2.6	
	Anabaena fertilissima					
0	4.45±0.09	4.45±0.09	4.45±0.09	4.45±0.09	4.45±0.099	0.08
2	3.14±0.36	26.66±0.70	13.68±0.36	28.07±1.0	3.71±0.30	0.49
4	7.08±1.91	29.71±0.10	17.39±0.63	28.85±1.0	7.67±2.77	1.3
6	4.77±1.17	27.46±0.38	16.31±1.82	35.32±0.34	13.61±3.31	1.46
LSD	0.93	0.33	0.80	0.59	1.77	
	Nostoc muscorum					
0	3.34±0.10	3.34±0.10	3.34±0.10	3.34±0.10	3.34±0.10	0.08
2	5.00±1.67	9.99±1.35	2.26±0.20	0	5.54±0.48	0.81
4	5.40±0.39	14.17±0.53	2.27±0.16	0	6.80±0.93	0.42
6	6.32±0.03	15.87±1.51	2.09±0.25	0	9.30±1.45	0.77
LSD	0.70	0.86	0.16	0.04	0.73	

LSD; the least significant difference at P≤0.05
Values are means of 3 replicates ± SD

DISCUSSION

In this investigation, we observed that increasing glucose concentrations in the cyanobacterial medium up to 1% (w/v) led to a gradual increase in total lipid content for *Anabaena fertilissima* and *Nostoc muscorum*. This observation coincided with Liang *et al*. (2009) who mentioned that maximum biomass density of *Chlorella vulgaris* was 2 g/L and lipid productivity (54 mg/L/day) were obtained when cells grown with 1% (w/v) glucose within 6 days. In addition, Bhatnagar *et al*. (2010) reported that lipid accumulation in *C. minutissima* cells was maximum (14.9%) under light incubated glucose-supplemented conditions and minimum (5.4%) in BG 11 medium without glucose. Matsuka *et al*. (1969) reported that glucose was mainly incorporated into lipids (particularly fatty acids) to optimize growth and production of desired chemicals from microalgae, it was essential to supply the right carbon source. So enriched glucose medium gives the highest lipid content rather than the other media. This explained why carbon sources assimilated through different metabolic pathways that mixotrophically under different culture conditions. In this context, Chu *et al*. (1995) studied the effect of different carbon sources on lipid production by *Ankistrodesmus convolutes* and concluded that stationary phase was the best growth phase for lipid production. In addition, Borowitzka (1994) established that lipid material are influence the biochemical composition of the investigated cyanobacterial strains. *A. laxa, A. fertilissima* and *N. muscorum* were grown photoautotrophically or secondary metabolites and thus are usually most abundant in stationary phase of growth. The present data showed that glucose (1%, w/v) caused reduction of total lipid contents of *A. laxa* compared to concentration of 0.7% (w/v). This might be due to the inhibitory effect of glucose concentration. Liang *et al*. (2009) mentioned that, under mixotrophic growth conditions, glucose at 1% and 2% (w/v) improved cell growth significantly compared with those at 5% and 10% (w/v). On the other hand, increasing molasses concentrations in the cyanobacterial medium up to 0.7% (w/v) led to a gradual increase in total lipid content for *A. laxa* and *A. fertilissima*. In accordance with our results, Yan *et al*. (2011) reported that increasing molasses led to increasing lipid content. Waste molasses hydrolysate was confirmed as a sole source of full nutrients to totally replace glucose-based medium in support of rapid growth and high oil yield from algae under optimized conditions. This was due to waste molasses containing both organic carbons and other nutrients as vitamins, trace elements and many other kinds of ingredients that consist mainly of 48% sugars (Crueger and Crueger, 1993; Yan *et al*., 2011). Our results revealed that *N. muscorum* was very sensitive to high molasses concentrations. It exhibited no growth on greater than 1% (v/v) of molasses medium. This might be attributed to the high osmotic pressure of sugars in raw molasses which suppress *Nostoc* growth. In accordance with Yan *et al*. (2011) who reported that *C. protothecoides* could not directly use molasses as organic carbon source because raw waste molasses contains 36.24% of sucrose. In addition, raw waste molasses contains colloidal and other impurities which might inhibit algal cell growth (Lee *et al*., 1999).

Static glucose medium 1% (w/v), molasses medium 0.7% (v/v) and aerated enriched glucose medium 1% (w/v) exhibited the highest values of both soluble and insoluble sugars for the experimental cyanobacterial

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species specially at early stationary phase or at stationary phase compared to control and aerated medium. Becker (1994) concluded that most studies on the biochemical production of algae and their analysis were carried out in stationary phase of growth period. These finding may be due to the dual benefit of both the presence of exogenous glucose sugar plus the fixed carbon dioxide which is present normally in air and the agitation effect caused by bubbling which increased the amounts of oxygen present in the growth medium that enhanced mass production and consequently led to high carbohydrate content. These finding are in accordance with Chu *et al.* (1995), but only at glucose levels of 0.25 and 0.5% (w/v). Also, Liang *et al*. (2009) reported that bubbling air into *C. vulgaris* culture exerted positive effect on cell growth.

The present study revealed that *A. laxa* exhibited the highest significant protein production under aerated enriched glucose medium at 6^{th} day compared to *A. fertilissima* and *N. muscorum*. It seemed that this medium condition was favorable for protein production. This was attributed to the high content of exogenous carbon source (glucose present in the medium) plus the fixed nitrogen gas which constitutes about 70% of air presented to growth medium via bubbling technique. Nitrogen fixation was a normal biochemical process that takes place in heterocystous filamentous microalgae as the medium $BG11_0$ was free of nitrogen. So, both carbon and nitrogen sources were available in algal media with large proportions and consequently channeled to protein or free amino acids synthesis.

The obtained results showed that any changes in growth condition led to a significant effect on total free amino acids content. High proportion of free amino acids were obtained under aerated enriched glucose medium for *A. laxa.* This finding was due to the richness of this medium with both exogenous carbon source and fixation of nitrogen into ammonium containing compounds as described before. The utilization of molasses medium exerted a pronounced effect on total free amino acids of *A. fertilissima,* this was due to the luxury of molasses with both organic and nitrogenous compounds. This finding was in accordance with Teclu *et al*. (2009) and Yan *et al*. (2011) who reported that waste molasses itself contains nitrogenous compounds (2-6%), which was apparently sufficient in support of algal growth.

CONCLUSION

The investigated cyanobacterial strains showed different response against the type of medium nutrition, where they succeeded to grow under both autotrophic and mixotrophic growth conditions. Aerated enriched glucose medium (1%, w/v) was the best growth medium condition used in the present study. The highest lipid content, total carbohydrates, total soluble proteins and total free amino acids were achieved under the medium incorporated with glucose.

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