Spirulina Production in Different Sources of Nitrogen

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Abstract

The Cyanobacteria *Spirulina* is an attractive target for its pigments, proteins, vitamins and other high-value cell components. Also, it can be easily and cheaply harvested by filtration from the cultivation medium. In this study a simple protocol was developed for *Spirulina* production by using different types of nitrogen in ammonium (urea and $(NH_4)_2SO_4$) and nitrate (KNO_3, $NaNO_3$) forms in combination with NPK fertilizer. Results demonstrated high amount of nitrogen in both forms inhibited *Spirulina* growth. Ammonium showed a stronger inhibitory role than nitrate in biomass production while increased phycocyanin content. Best phycocyanin content occurred in high ammonium or low nitrate concentration. In media based on 1% Urmia lake salt and 1 g/L NPK, a combination of low concentration (0.1 -0.5 g/L) of urea and $(NH4)_2SO_4$ obtained best results in biomass production. 1.2 g/L biomass during 14 days without any carbon source can be compared with Zarrouk/2 medium. This composition can be used economically for *Spirulina* production since little amount of cheap material make the possibility of *Spirulina* production.

Keywords: Ammonium, Fertilizer, Nitrogen, NPK, *Spirulina*

Introduction

The current environmental conditions deteriorations, mental and physical stress, changes in the diet have been serious risk factors for the humans, increased the death rate and fatal diseases. These are the obvious reasons why new progressive trends are being extensively developed in modern medicine, pharmacology and biotechnology and more effective harmless medicaments are being sought for to treat and prevent various diseases. One of the trends in biotechnology is associated with blue green microalgae *Spirulina platensis* which have been widely employed as food and feed additives in agriculture, food industry, pharmaceuticals and perfume industry, (Saranraj and Sivasakthi, 2014).

Microalgae are a diverse group of microorganisms that have different morphological, physiological and genetic traits that have the potential to offer a variety of different biologically active metabolites like proteins, lipids, carbohydrates, carotenoids or vitamins for health, nutrient rich food and feed additives, cosmetics and for energy production (Priyadarshani and Rath, 2012).

In general, microalgae is able to provide a

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great variety of secondary metabolites, which do not happen in other organisms. The fundamental advantage of using microalgae for industrial production of valuable food ingredients depends on the fact that, for the majority of the species, cultivation is easy and growth is fast (El Baky et al., 2015).

The cyanobacteria *Spirulina* is widely commercialized as nutritional supplement for humans and as animal feed additives. *Spirulina* was approved by the Food Drug Administration (FDA) by the issuance of a generally recognized as safe (GRAS) certificate. *Spirulina* can be legally marketed as a food or food supplement without risk to human health (Costa and de Morais, 2013). It could be considered a luxury health food and a panacea for malnutrition since it is an excellent content of proteins (Colla et al., 2007), polyunsaturated fatty acids (PUFA) (Sajilata et al., 2008), pigments (Madhyastha and Vatsala, 2007), vitamins and phenolic (Ogbonda et al., 2007). Moreover, phycobiliproteins as a special group of pigments that are water-soluble occur only in cyanobacteria and Rhodophyta act as photosynthetic accessory pigments. C-phycocyanin (C-PC) is a blue pigment of phycobiliproteins with strong antioxidative and anti-inflammatory activities. Now a days, *Spirulina is* considered as the major source of phycocyanin with 20% of its dry weight (Benedetti et al., 2006). *Spirulina* is easy to culture and harvest in large- scale. Hence, these desired microalgae can be employed for commercial interest. Therefore, in this project, various types of nitrogen sources (urea, KNO_3, $NaNO_3$, $(NH_4)_2SO_4$) in composition with fertilizer (NPK) were studied to improve the biomass production and reduce the costs.

Materials and methods

Spirulina was obtained from algae collection of research institute of applied science of ACECR, Tehran, Iran. *Spirulina* was cultivated at the Zarrouk medium and used as inoculum in the experiments. Various culture media were prepared according to various nitro-

Table 1. Different combination of nitrogen sources in media

No.	Urea*	NPK*	KNO_3*	$NaNO_3$*
1	1.5	1.0	-	-
2	1.5	2.5	-	-
3	1.5	5.0	-	-
4	1.5	1.0	1.0	-
5	1.5	1.0	2.5	-
6	1.5	1.0	5.0	-
7	1.5	1.0	1.0	1.0
8	1.5	1.0	-	1.0
9	1.5	1.0	-	2.5
10	1.5	1.0	-	5.0
11	1.0	1.0	-	-
12	0.5	1.0	-	-
13	0.1	1.0	-	-
14	Zarrouk/2			

1-14 Different combinations on nitrogen

* g/l

gen sources $(NH_4)_2SO_4$, urea, KNO_3, $NaNO_3$ and NPK fertilizer (with 12:12:36 ratio) and followed the growth process for 2 weeks. In addition, C-phycocyanin content (C-PC) was evaluated on the 10th day growth (Wyman and Fay, 1986). The phycocyanin content (C-PC) was calculated according to the following equation:

C-PC= (A620 – 0.474 * A652)/ 5.34

where A is the absorbance of the substance at 620 and 652 nm.

The experiments were carried out in three steps as follow: initially, in order to select industrial culture media, we used distilled water, 1% Urmia lake salt solution and breeding fish effluent in combination with 1 g/L NPK, 0.32 g/L KNO_3 and 2.5 – 5.0 g/L urea. In the next step, various nitrogen sources with different concentrations were combined in 1% Urmia lake salt as follows to determine the type of nitrogen and their amounts for *Spirulina* growth. Different combination compared to find the best medium near to Zarrouk/2 medium production (Table 1).

Finally, new source of nitrogen ($(NH_4)_2SO_4$) helped urea to provide ammonium in media. Different amounts of NPK (2.5 and 0.5 g/L) in combination with urea (0.1 and 0.5 g/L) and $(NH_4)_2SO_4$ (0.5 and 2 g/L) in 10 treatments (numbers 15 to 24) were evaluated (Table 2). The concentration of 0.5 g/L of KNO_3 and 1% Urmia lake salt was considered in all experiments.

Experiments were carried out in 3 liters containers included 1 liter media, 29 + 1 temperature, 8/16 photoperiod and permanent central aeration system. Zarrouk/2 medium (half of the total Zarrouk composition) was also used for all tests as an indicator to compare biomass production. For each treatment 30 ml of inoculum added to 1 L of media and the final pH adjusted to 9.2 ±0.3. The cultivation volume was 1 liter, which was maintained through the daily addition of distilled water to replace water loss by evaporation.

Results

Spirulina didn't grow in media based on distilled water and breeding fish effluent while 1% Urmia lake salt solution demonstrated *Spirulina* growth. Therefore, suitable substrate for growth of desired microalgae was considered as 1% sea salt. Among the various types of nitrogen sources in Table 1, which were mostly prepared on the basis of 1.5 g/L urea and 1 g/L NPK, the result showed a negative effect of $NaNO_3$ and KNO_3 on growth in different concentrations, somehow after one week, all the samples were wasted, but treatments containing different levels of NPK and urea were able to grow for up to 2 weeks (Fig. 1). The amount of urea in the media is a very important factor because a significant reduction for urea from 1.5 to 0.1 g/L has led to enhance in growth. Lower amount of NPK (with higher amount of nitrate than ammonium) increased growth; inhibition effect of nitrate observed after 10 days where 1 g/L NPK produce more biomass (1.2 g/L) than 2.5 g/L NPK (1.03 g/L). Reducing the amount of nitrogen in the environment (0.1 g/L urea, 1.0 g/L NPK) has resulted in the highest biomass production to 1.2 g/L. Although low total nitrogen levels have been

Table 2. Ammonium combination in media

No.	NPK*	Urea*	$(NH_4)_2SO_4$*
15	2.5	0.1	2.0
16	2.5	0.1	0.5
17	2.5	0.5	2.0
18	2.5	0.5	0.5
19	5.0	0.1	2.0
20	5.0	0.1	0.5
21	5.0	0.5	2.0
22	5.0	0.5	0.5
23	1.0	0.1	0.5
24		Zarrouk/2	

15-24 Different combination of ammonia
* g/l

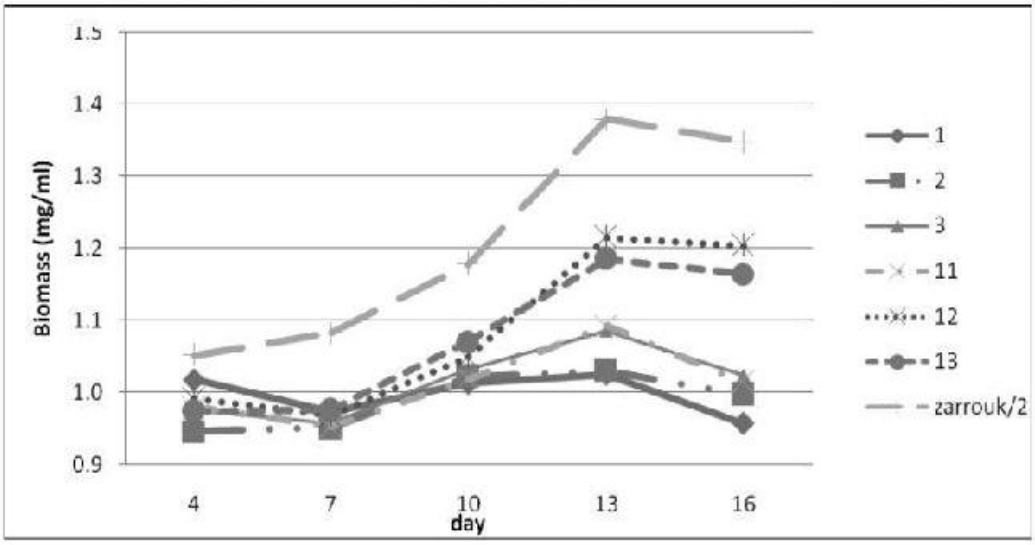

Fig. 1. *Spirulina* biomass production in different nitrogen sources.

able to stimulate growth as well as increase in phycocyanin levels there were some cases which along with an increase in the amount of nitrogen in the media (1.5-2.5 g/L urea or 1.0 g NPK), the production of phycocyanin also increased.

Results of the last experiment showed combination of urea and NPK could be increase the production of *Spirulina* and phycocyanin simultaneously. In the final experiment, the combination of two recent salts with $(NH_4)_2SO_4$ exhibited that the amount of nitrogen in the media played a significant role in the biomass production of *Spirulina*. The highest growth rate of microalgae occurs in the combination of 1.0 to 2.5 g/L NPK, 0.1 to 5.0 g/L urea, and 0.5 g/L $(NH4)_2SO_4$, which produced even higher biomass than Zarrouk/2 medium (Figure 2). The amount of phycocyanin in low nitrogen treatments was high and the maximum amount of phycocyanin was observed in treatment of 0.1 g/L NPK along with 0.1 g/L urea and 0.5 g/L $(NH_4)_2SO_4$.

Discussion

The results of the first part of the experiment showed that 1.0 g/L NPK, 0.32 g/L KNO_3 along with 2.5 g/L urea could be effective for *Spirulina* cultivation with 1% of Urmia lake salt solution while combination of above mixture with breeding fish effluent treatment and distilled water showed no growth. Stimulation of *Spirulina* growth in the composition of 1% salt of Urmia lake, in comparison with distilled water, due to the presence of mineral elements, even in low amounts of trace element in media but excess amount of ammonium in breeding fish effluent (Hargreaves and Tucker, 2004) severely inhibited the *Spirulina* growth.

Several investigations report inhibitory effect of ammonium in different ranges (2 mM till 15 mM) from various source of nitrogen like urea, $(NH_4)_2SO_4$, $(NH_4)Cl$, $(NH_4)NO_3$ (Costa et al., 2001; Danesi et al., 2002; Sassano et al., 2007; Rodrigues et al., 2010). The second experiment showed that in intervals less than 2.5 g/L urea (0.1 to 1.5 g/L), better growth could be seen but not more than Zarrouk/2. Zarrouk/2 contains nitrogen (1.25 g/L sodium nitrate) and 8g/L $NaHCO_3$ while we didn't include any carbon source except air aeration in our treatments. So the biomass production in our media (1 g/L NPK, 0.1 g/L urea and 0.5 g/L $(NH_4)_2SO_4$) can be considerable.

In the third experiment, increasing the nitrate concentration (NPK) up to 5.0 g/L inhibited the *Spirulina* growth. Therefore, *Spirulina* concentration less than 2.5 g/L could keep on growing for up to 2 weeks. Decreasing the nitrate concentration in NPK up to 1.0 g/L could increase the level of *Spirulina* growth even more than Zarrouk/2, but less amount of $(NH_4)_2SO_4$(0.5 g/L) and urea (0.1 g/L) should be included in media composition. As mentioned before, due to the lack of carbon source in desired mixture, the *Spirulina* growth was significant.

The usage of commercially available fertilizer and chemicals in the market for inorganic nutrition of plants is also economical for large-scale production of algae and could be considered as an appropriate alternative

for algal composition media in lab. Chemical fertilizers with various formulations and with the abbreviation NPK, which indicate the ratio of presence of nitrogen (N), phosphate (P), potassium (K) and sometimes magnesium (Mg) are easily available and can be used not only to resolve the requirement of three basic elements of alga cultivation, but also is a suitable basis for cultivating algae as a cultivation medium in combination with sea salt (in offshore areas) or seawater (in coastal areas). Supplementary usage of other nitrogen sources can be used to optimize growth based on the availability of ammonium and nitrate sources.

Nitrogen in various forms (ammonium or nitrate) along with other chemicals such as phosphate, sodium bicarbonate (as a carbon source), irradiance, etc. can play an important role in the growth of algae or the production of algal metabolites. Rodriguez et al. (2011) reported that the application of two sources of nitrogen (nitrate and ammonium) in the development of *Spirulina* showed more positive effects compared to separate use of nitrogen sources. The obtained results also showed urea and $(NH_4)_2SO_4$ in NPK-containing medium exhibited significant effect on growth, compared to the presence of KNO_3 or $NaNO_3$ salts. Some reports also indicate 25% nitrogen reduction in the Zarrouk medium and show no change in the final biomass (Colla et al., 2007; El-Baky et al., 2008). Castro et al. (2015) were able to have the highest amount of biomass (3.27 g/L) in concentration of 1.25-2.5 g/L sodium nitrate (Castro et al., 2015). Danesi et al. (2002) also suggested using urea instead of potassium nitrate. Urea, which contains two ammonium groups, was able to stimulate *Spirulina's* growth by less energy consumption in compare with other nitrogen sources. Also, it has a cheaper price (Castro et al., 2015).

High concentrations of nitrogen (either in the form of nitrates or ammonium) aren't always the reason of growth increasing (Castro et al., 2015; Gupta et al., 2017) and its inhibitory effects against *Spirulina* growth were observed. The inhibitory effects of ammonium have been reported with regard to the decomposition of urea in alkaline conditions in compare with nitrate consumption in *Spirulina* culture medium (Danesi et al., 2011; Rodriguez et al., 2011; Cruz-Martínez et al., 2015).

Our results demonstrated the concentration of 1.0 g/L nitrate in NPK and 0.5 g/L KNO_3 was stimulated and higher concentration was inhibited the growth. Costa et al. (2001) showed that $NaNO_3$ stimulated algae growth up to 50 mM and has reported the toxicity effect of $(NH_4)NO_3$ and urea above 10mM concentration.

Gupta et al. (2017) reported that higher amount of nitrogen (up to 100 mM $NaNO_3$) decreases biomass production while maximum C-phycocyanin produces in 40mM followed by 60, 80 and 100 mM $NaNO_3$. In second experiment, C-phycocyanin amount increased in higher amount of urea; while in the third experiment we preferred to use less amount of nitrogen to reduce inhibition effect. However, in new composition, higher value of C-phycocyanin obtained in media

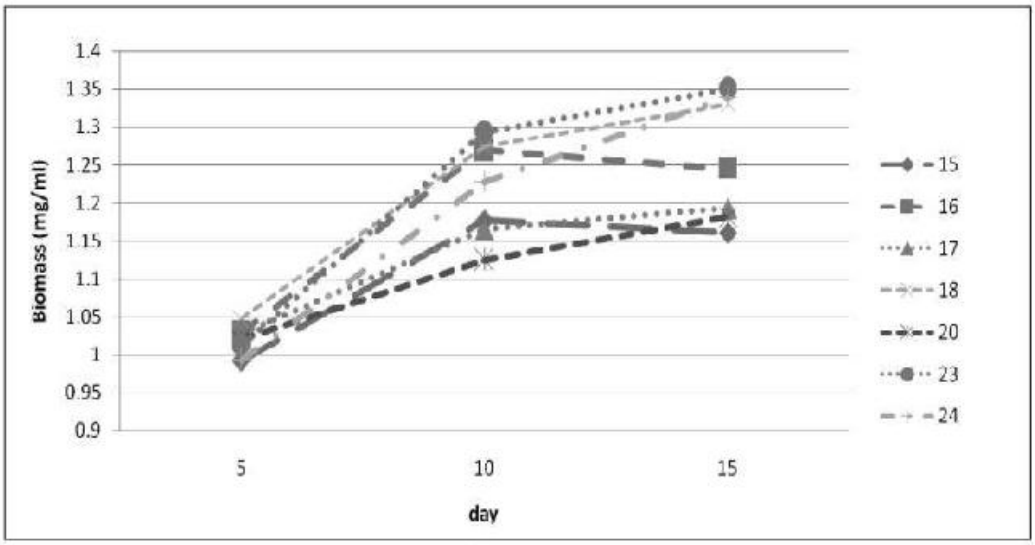

Fig. 2. Ammonium sulfate combination's effect on *Spirulina* production.

with lower nitrogen content.

According to the critical important role of nitrogen in the microalgae growth and quick consumption of ammonium by microalgae, it is suggested that ammonium could be added continuously in several steps to prevent its accumulative effect at the early phase of growth (Rodriguez et al., 2011). Urea is rapidly converted into ammonium and is evaporated; therefore, it could be suggested to feed algae at intervals and dividing nitrogen sources in the growth period to prevent ammonium shortage (Danesi et al., 2002).

Using NPK fertilizers, in combination with urea and $(NH_4)_2SO_4$ in 1% Urmia salt allowed to produce *Spirulina* microalgae with acceptable results similar to Zarrouk/2 medium. The results of this study can be used in wastewater treatment with different sources of nitrogen along with generating microalgae biomass.

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