THE EFFECTS OF NITROGEN DEFICIENCY ON THE PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) USING *SPIRULINA PLATENSIS*

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Abstract

The effects of nitrogen deficiency at the level of 0% (N-), 50% (N-), and 100% (N-) for the growth of *Spirulina platensis* (*Cyanophyceae*) for the production of Polyhydroxybutyrate (PHB) was investigated. Biomass and optic density of the cell under laboratory conditions were measured. *Spirulina platensis* cultures were grown in Zarrouk medium and kept at the constant room temperature of 25±2°C, illuminated with fluorescent lamps at Photosynthetic Active Radiation (PAR) level of 120 µmol m^{-2} s^{-1} with photoperiod of 24:0 (light:darkness, L:D) and aerated continuously. The experiment was carried out with three replications. The percentages of PHB measured were 1.13, 1.43, 2.61 and biomasses obtained were 2.30, 2.92, and 1.85 g/L for control (0%N-), 50% N(-) and 100% N(-), respectively for *S. platensis* biomass harvested. The highest PHB value and the lowest biomass were recorded from the culture treated at the level of 100% N(-).

Key words: *Spirulina platensis*, polyhydroxybutyrate, biomass, nitrogen deficiencies

Plastic packaging materials is one of the issues highlighted in the studies to increase awareness of environmental protection. It is clear that all kinds of prohibitions and restrictions cannot prevent the use of plastic as the packaging material as long as no other material can be used instead (Mercan and Beyatl, 2004). Plastic materials constitute a large portion of wastes. Plastic and other wastes are dumped to municipal waste disposal area. There has been increase in environmental problems with dump site, increasing cost of alternative methods such as incineration, various technical problems, conservation of energy resources, and demand for reuse of wastes as environmentally acceptable manner (Savaşç *et al*, 1998). The main problem is environmental pollution resulting from the disposal of polymeric materials, which may take many years to decompose. Recycling alone has proved to be insufficient to solve this problem (Falcone, 2004). Furthermore, the stages of collection, sorting, and converting waste materials to raw materials for reprocessing of waste plastics increase the cost. Indeed increasing and unknown environmental impacts lead to the growth in interest in the biodegradable plastic (Dave *et al*, 1996).

Bioplastics are types of plastics produced from biomass such as vegetable oil, corn starch, and pea starch etc. They are produced by many different types of bacteria and algae as food storage material (Falcone, 2004). Bacteria and algae in an environment having the deficiency of any of the elements such as carbon, nitrogen, phosphorus accumulates a polymer, which is called "polyhydroxybutyrate (PHB)", as an energy storage for use in the future in order to maintain viability in this tense environment. Poly-3-hydroxybutyrate is a linear polyester of D (-)-3-hydroxybutyric acid. PHB is the most common and best known types of polyhydroxyalkanoates (PHA) (Ediz, 2004). PHB is resistant to moisture, insoluble in water, also quite resistant to oxygen, resistant to UV (ultraviolet) and has optical purity.

Algal bioplastics are plastics derived from algal biomass or by using algal materials. The algal bioplastic are easily biodegradable than compared to commercial plastics. The direct algal biomass usage for algal bioplastics is responsible for biodegradation. Algae based plastics have been a recent trend in the era of bioplastics (Stevens, 2001). The various bioplastics that can be made from algae feedstock include Hybrid Plastics, Cellulose-Based Plastics, Poly-Lactic Acid (PLA), Bio-Polyethylene. Hybrid plastics are made by adding denatured algae biomass to petroleum based plastics like polyurethane and polyethylene as fillers. The PHB content of the algal biomass increase biodegradable property of plastic. It thus decreases the amount of petroleum used per unit of plastic (Maheswari and Ahilandeswari, 2011).

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PHB is a very common and widespread storage material in many micro-organisms. PHB is an environmentally degradable material. PHB belongs to a family of polyesters called polyhydroxyalkanoates or PHAs. The one striking difference between PHB and petroleum-based plastics is that PHB are biodegradable. When these bioplastics are disposed to the environments populated by organisms such as bacteria, fungi and algae, PHB are broken down to their essence-carbon dioxide and water-and recycled by the natural metabolic processes of these microbes (Lemoigne, 1927).

The purpose of this study was to investigate effects of nitrogen deficiency on the production of Polyhydroxybutyrate using *Spirulina platensis* (*Cyanophyceae*).

MATERIAL AND METHOD

Microalgae *S. platensis* was used in this study. The starter culture was obtained from Çukurova University, Faculty of Fisheries, Turkey. *S. platensis* cultures were kept at a constant room temperature of 25±2°C and illuminated with fluorescent lamps (TEKFEN, 36W, Daywhite) at PAR (Photosynthetically Active Radiation) level of 120 µmol m^{-2} s^{-1} measured by Delta Ohm PAR meter with photoperiod of 24:0 (light:darkness, L:D). The final pH of Zarrouk medium (*table 1*) was 7.0 after being autoclaved. *S. platensis* was grown in 1.5 L glass jar in a batch culture system with an initial biomass concentration of 0.60 gL^{-1} and the culture was continuously aerated.

Measurements of optic density were performed by spectrophotometer (Mecasys Optizen, UV/VIS) at 680 nm wavelengths to determine the daily growth of *S. platensis* microalgae. In order to determine the increase in dry weight, certain volume of microalgal culture was filtered by Whatman GF/C (0.45 µm). Then, the filtered microalgal biomass was dried in the oven at 105°C until the weight remained constant. All measurements were performed with three replications.

For PHB analysis, microalgae samples were collected in stationary phase. The separated biomass was dried for 1.5 hours at 65°C and it was pulverized. Powdered biomass was stored at -18°C for analysis. The amount of PHB was determined based on conversion of PHB to crotonic acid with acid and measurement of crotonic acid with a spectrophotometer by measuring absorbance at 235 nm (Bonartseva and Myshkina, 1985). Seven days later, the required microalgal biomasses and PHB were obtained.

Table 1

Preparation of Zarrouk Medium

Media for Control treatment, (0% (N-))	Macro nutrients (gL^{-1}): 16.8 $NaHCO_3$, 8.06 Na_2CO_3 , 1.00 K_2HPO_4 , **5.00 $NaNO_3$** , 2.00 K_2SO_4 , 2.00 NaCl , 0.40 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$, 0.02 $FeSO_4.7H_2O$, 0.16 $EDTANa_2$ Micro nutrients (gL^{-1}): 0.001 $ZnSO_4.7H_2O$, 0.002 $MnSO_4.7H_2O$, 0.01 H_3BO_3 , 0.001 $Na_2MoO_4.2H_2O$, 0.001 $Co(NO_3)_2.6H_2O$, 0.00005 $CuSO_4.5H_2O$, 0.7 $FeSO_4.7H_2O$, 0.8 $EDTANa_2$
Media with 50 % nitrogen deficiency, (50% (N-))	Macro nutrients (gL^{-1}): 16.8 $NaHCO_3$, 8.06 Na_2CO_3 , 1.00 K_2HPO_4 , **2.50 $NaNO_3$** , 2.00 K_2SO_4 , 2.00 NaCl , 0.40 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$, 0.02 $FeSO_4.7H_2O$, 0.16 $EDTANa_2$ Micro nutrients (gL^{-1}): 0.001 $ZnSO_4.7H_2O$, 0.002 $MnSO_4.7H_2O$, 0.01 H_3BO_3 , 0.001 $Na_2MoO_4.2H_2O$, 0.001 $Co(NO_3)_2.6H_2O$, 0.00005 $CuSO_4.5H_2O$, 0.7 $FeSO_4.7H_2O$, 0.8 $EDTANa_2$
Media with 100 % nitrogen deficiency, (100% N(-))	Macro nutrients (gL^{-1}): 16.8 $NaHCO_3$, 8.06 Na_2CO_3 , 1.00 K_2HPO_4 , **0.00 $NaNO_3$** , 2.00 K_2SO_4 , 2.00 NaCl , 0.40 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$, 0.02 $FeSO_4.7H_2O$, 0.16 $EDTANa_2$ Micro nutrients (gL^{-1}): 0.001 $ZnSO_4.7H_2O$, 0.002 $MnSO_4.7H_2O$, 0.01 H_3BO_3 , 0.001 $Na_2MoO_4.2H_2O$, 0.001 $Co(NO_3)_2.6H_2O$, 0.00005 $CuSO_4.5H_2O$, 0.7 $FeSO_4.7H_2O$, 0.8 $EDTANa_2$

Figure 1 **Cultivation phase in stock culture**

RESULTS AND DISCUSSIONS

The experiment lasted for 7 days. Optic density, biomass, and pH were measured during the experiment every day while PHB was measured at the end of experiment. In this study, *S. platensis* was cultivated at three different media (0% N(-), 50% N(-), and 100% N(-)) to investigate effects of the level nitrogen deficiency on the production of PHB. Optic density as a function of time is given in *figure 2* for all treatments. Result showed that all optic densities are in increasing trend with time. The drop occurred at day 5 for 100% N(-) treatment could be due to error in the measurement of optic density.

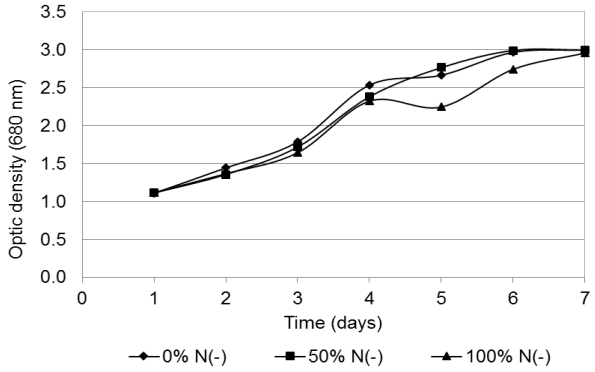

Figure 2 **Optic density curve depending on time**

Biomasses changing with time are given in *figure 3* for all treatments. Results showed that all biomasses are in increasing trend with time. The stationary phase of biomass production started at the fifth day of the experiment. The highest biomass concentration was achieved for the treatment of 0% N(-).

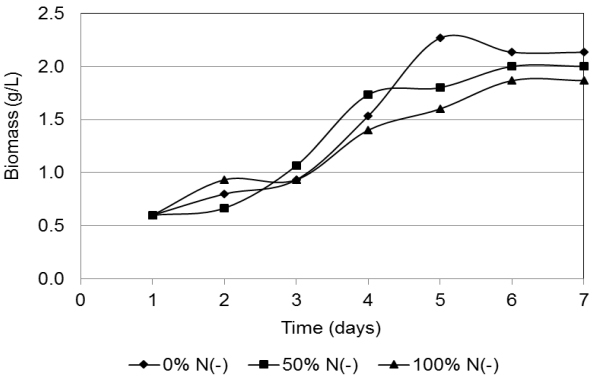

Figure 3 **Biomass curve depending on time**

pH as a function of time is given in *figure 4* for all treatments. Results showed that all pH values are in increasing trend with time. The highest pH value was obtained from the treatment of 0% N(-).

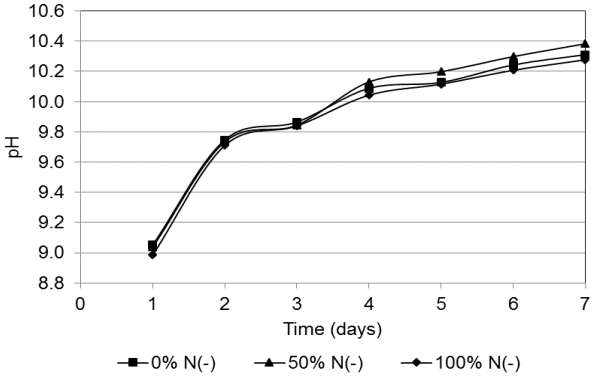

Figure 4 **pH curve depending on time**

Figure 5 shows biomass as a function of optic density for all treatments. Linear relationship between optic density and biomass was determined with R^2 of 0.90, 0.92, and 0.94 for 0% N(-), 50% N(-), and 100% N(-), respectively.

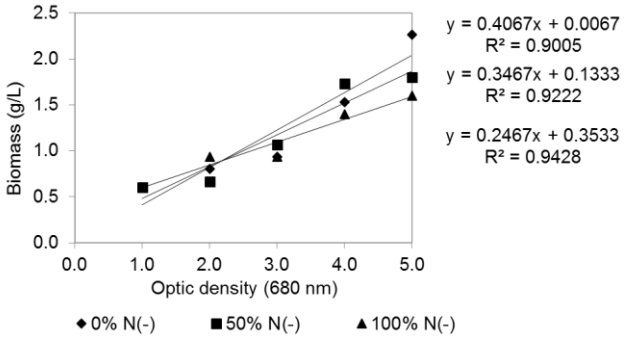

Figure 5 **The relationship between biomass and optic density**

Final optic density, biomasses, and PHB level obtained from *S. platensis* grown in the media with different nitrogen deficiency are given in *table 2*. As given in *table 2*, the maximum biomass was found for 0% N(-) treatment with 2.13 gL^{-1}. In contrast to, the lowest biomass (1.87 gL^{-1}) was obtained from the treatment of 100% N (-). Differences in microalgal biomass due to environmental stress conditions with nutrient limitation could be led by reduction of cell division as time progressed (Sharman *et al*, 2012). At the same time, the amount of PHB was found to be directly affected by the nutritional stress. Therefore, it could be stated that an inverse relationship between the microalgal biomass density and PHB. While the lowest biomass was found as 1.87 gL^{-1} for the highest PHB production (0.048 gL^{-1}) for the treatment of 100% N(-), the lowest PHB (0.026 gL^{-1}) was obtained from the treatment of 0% N(-) with the highest biomass production of 2.13 gL^{-1}.

Table 2

Final optic density, biomass, and PHB level obtained from *S. platensis* grown in the media with different nitrogen deficiency

Nutrient Media	Optic density (680nm)	Biomass (gL^{-1})	PHB (gL^{-1})
0% N(-),	3.00	2.13	0.026
50% N(-),	3.00	2.00	0.042
100% N(-)	3.00	1.87	0.048

The relationship between biomass and PHB is given in *figure 6*. Results revealed that an inverse relationship between the microalgal biomass density and PHB determined.

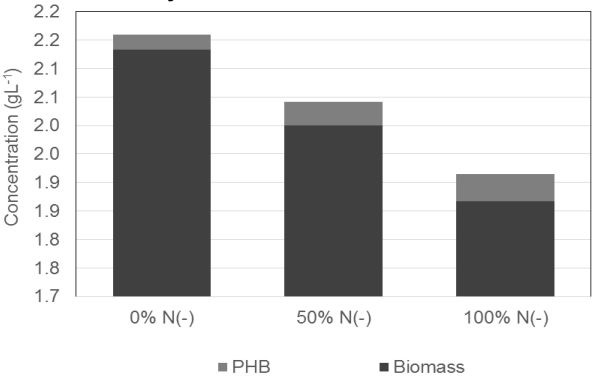

Figure 6 **The relationship between biomass and PHB**

CONCLUSION

Microalgal biomass exposed to environmental stress conditions with nutrient limitation (100% nitrogen deficiency) produced approximately two times more PHB than control treatment with no nutrient limitations.

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