**From N2 to cyanophycin: high-value compound production through biological nitrogen fixation in continuous systems**

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**1. Introduction**

Diazotrophic cyanobacteria have the ability to fix atmospheric nitrogen in their biomass during photosynthetic growth, so they could be a viable alternative to traditional and high energy demanding production processes, such as ammonia production [1–3]. However, photosynthetic cyanobacteria catalyse the reaction of nitrogen fixation at ambient temperature and pressure, driven by sunlight energy [4,5]. Cyanophycin is non-protein, non-ribosomally produced amino acid copolymer, composed of equimolar amounts of aspartic acid and arginine, which serves a temporary nitrogen reserve compound. The industrial application of cyanophycin is not still consolidated, but it is a promising starting point for the synthesis of many important chemicals. Industrial application focuses on its chemical derivatives, as cyanophycin can be chemically converted to poly-aspartic acid (PASP) and arginine by β-hydrolytic cleavage [6]. In this work, two diazotrophic cyanobacteria of the genus *Anabaena* were phototrophycally cultivated in continuous system under N2 fixing conditions to assess the possible stable production of cyanophycin. Indeed, *Anabaena* sp. was already cultivated in a continuous cultivation system diazotrophically, obtaining remarkable biomass productivities [7]. The effect of operating variables in continuous system as the inlet phosphorus concentration, the incident light intensity, the residence time, and the nitrogen availability, on biomass and cyanophycin productivities were investigated. In this way, it is possible to produce biomass having specific composition and constant quality over time, obtaining high productivities and, at the same time, reducing the costs associated with the process, thus developing a system compatible with large-scale production.

**2. Methods**

*Anabaena* sp. PCC 7122 (*Anabaena cylindrica*) and *Nostoc* sp. PCC 7120 were purchased from Pasteur Culture of Cyanobacteria in France. Cyanobacteria were maintained phototrophycally in diazotrophic conditions at a constant temperature of 24±1°C in the BG11 medium [8], modified to remove all nitrogen compounds present. Continuous experiments were carried out in vertical flat-plate photobioreactors, irradiated by a white LED lamp. The mixing was ensured by both a stirring magnet placed at the bottom of the reactor and the bubbling of 1 L h-1 of an air-CO2 (5% v/v) mixture. Microalgae growth was monitored daily through optical density measurement. At steady state, dry cell weight was measured, and biomass composition was characterized in terms of phosphorus, nitrogen and cyanophycin internal quotas, following the protocols reported in Trentin et al. (2021) [9].

The effect of the inlet phosphorus concentration on cyanophycin productivity was investigated with both the cyanobacterial species. The residence time and the incident light intensity were kept constant, according to previous literature on cyanophycin production in continuous system [9] and on continuous cultivation of diazotrophic cyanobacteria [7]. Then, a second set of experiments was carried out with *Nostoc* sp. PCC 7120 to test the effect of the incident light intensity, the residence time, and the nitrogen availability. Each variable was varied keeping the other at a constant value, to see their effect individually. As for nitrogen solubility, it was assessed how the pH could influence N2 solubility in the cultivation medium. Thus, sensitivity analysis on N2 solubility was carried out using Aspen Plus™ process simulator (V12.1).

**3. Results and discussion**

The effect of different inlet phosphorus concentrations on the growth of two diazotrophic species was addressed to identify which growth condition allows to obtain a higher cyanophycin productivity. For both species, the biomass concentration decreased at a decreasing inlet P concentration, whereas the cyanophycin quota increased, even if a species specificity was identified. (Figure 1).



**Figure 1**. Steady state biomass concentration (*cx*), cyanophycin concentration (*cCGP*), biomass productivity (*Px*) as function of the inlet phosphorus concentration (*cP*) obtained with *Anabaena cylindrica* (panel A) and with *Nostoc* sp. PCC 7120 (panel B). Error bars represent the standard deviation of at least 4 samples for each steady state (n≥4). Statistical analysis was conducted separately for each category of data. Data that do not share a letter are significantly different. Lines are just eye guides.

As for nitrogen fixation, *Nostoc* sp. PCC 7120 was more efficient in all conditions. These preliminary results confirmed that in a continuous system the amount of phosphorus fed at the inlet was a fundamental variable when studying cyanophycin production, but also suggested that other operating variables (incident light intensity, residence time, nitrogen availability) can influence its accumulation and productivity in a continuous system. Thus, the subsequent studies were carried out with *Nostoc* sp. PCC 7120, because in the preliminary experiments was more efficient both in biomass, nitrogen and cyanophycin productivity. In particular, a greater availability of nitrogen did not affect the internal quota of cyanophycin, but rather the growth of biomass. However, as a results, cyanophycin concentration and productivity increased. Overall, it was observed that the production of cyanophycin was strictly dependent on the concentration of phosphorus present in the culture medium: only decreasing the P quota, it was possible to measure a higher amount of cyanophycin in the biomass.

**4. Conclusions**

In this work, cyanophycin production by diazotrophic cyanobacteria (*Anabaena cylindrica* and *Nostoc* sp. PCC 7120) during photosynthetic cultivation was investigated in continuous experiments, with different inlet phosphorus concentration. The effect of other operating variables on biomass and cyanophycin accumulation was addressed, showing that P limitation is the main variable affecting the cyanophycin accumulation, and the internal quota of P in the biomass is the trigger for cyanophycin accumulation.

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