Bioresource Technology 101 (2010) 4491–4498

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Fed-batch cultivation of *Arthrospira* (*Spirulina*) *platensis*: Potassium nitrate and ammonium chloride as simultaneous nitrogen sources

M.S. Rodrigues [a], L.S. Ferreira [a], A. Converti [b], S. Sato [a], J.C.M. Carvalho [a,*]

[a] Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, B-16, 05508-900 São Paulo, Brazil
[b] Department of Chemical and Process Engineering "G.B. Bonino", University of Genoa, Via Opera Pia, 15, 16145 Genoa, Italy

ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form 10 January 2010
Accepted 14 January 2010
Available online 12 February 2010

Keywords:
Arthrospira (*Spirulina*) *platensis*
Nitrogen source
Fed-batch process
Kinetic parameters

ABSTRACT

Arthrospira platensis was cultivated in minitanks at 13 klux, using a mixture of KNO_3 and NH_4Cl as nitrogen source. Fed-batch daily supply of NH_4Cl at exponentially-increasing feeding rate allowed preventing ammonia toxicity and nitrogen deficiency, providing high maximum cell concentration (X_m) and high-quality biomass (21.85 mg chlorophyll g cells^{-1}; 20.5% lipids; 49.8% proteins). A central composite design combined to response surface methodology was utilized to determine the relationships between responses (X_m, cell productivity and nitrogen-to-cell conversion factor) and independent variables (KNO_3 and NH_4Cl concentrations). Under optimum conditions (15.5 mM KNO_3; 14.1 mM NH_4Cl), X_m was 4327 mg L^{-1}, a value almost coincident with that obtained with only 25.4 mM KNO_3, but more than twice that obtained with 21.5 mM NH_4Cl. A 30%-reduction of culture medium cost can be estimated when compared to KNO_3-batch runs, thus behaving as a cheap alternative for the commercial production of this cyanobacterium.

1. Introduction

Cyanobacteria are microorganisms that have a bacterial morphological structure but the photosynthetic system of algae (Ashby and Houmard, 2006), whose cultivation can be a profitable process to obtain proteins for both human and animal feeding (Dallaire et al., 2007). *Arthrospira* (*Spirulina*) *platensis* is reported to reach very high protein content under favorable conditions (up to 74% of dry biomass). Moreover, it offers the possibility of obtaining pigments such as chlorophyll, carotenoids and phycocyanins, phycobiliproteins, polyunsaturated fatty acids, essential amino acids, as well as many vitamins and minerals (Colla et al., 2007; Hongsthong et al., 2007; Patil et al., 2008).

Among cyanobacteria, *A. platensis* is preferred for biomass production mainly because of its high cell growth rate, easy process control and biomass recovery, ability to grow on alkaline and high-salt media, reduced risks of contamination and flexibility and resistance to adverse or suboptimal conditions. For these reasons, there is an increasing interest on *A. platensis* cultivation, and its large-scale production is under consideration worldwide. According to Spolaore et al. (2006), this genus of cyanobacteria is currently cultivated mainly in the USA, China and Taiwan, but also in Japan, India, Israel and Thailand. *A. platensis* is commercialized as powder, pills and bars to be used as food integrator, but it also finds application as dye in the cosmetic, pharmaceutical and food industries. Recent researches show that *A. platensis* proteins are able to stabilize emulsions, foams and dispersions (Chronakis et al., 2000), whereas biomass itself has great potential as matrix for the production of selenium- and iodine-containing pharmaceuticals (Mosulishvili et al., 2002). Furthermore, *A. platensis* application to waste treatment has been widely studied, with particular concern to the removal of nutrients (Converti et al., 2006) and heavy metals (Li et al., 2006).

Both the kind and the quantity of the nitrogen source in the culture medium can influence the growth and/or the composition of *Arthrospira* sp. biomass (Colla et al., 2007). The conventional nitrogen source is nitrate, for it has been demonstrated to ensure the highest biomass yields (Costa et al., 2001). Nevertheless, the search for cheaper nitrogen sources such as ammonium salts and urea is particularly attractive from the economic viewpoint. Ammonium salts could be used as alternative nitrogen sources, because the cell incorporates ammonia directly into the nitrogen metabolism. However, low levels of ammonium salts in batch runs under alkaline conditions proved to limit the growth, with consequent decrease in biomass yield when compared with nitrate. On the other hand, growth inhibition or even cell death can take place when using these nitrogen sources at high concentrations, owing to ammonia toxicity (Carvalho et al., 2004). Because of its flexibility in supplying nutrients during the run, the fed-batch operation is

* Corresponding author. Tel.: +55 11 30913886; fax: +55 11 38156386.
E-mail address: jcmdcarv@usp.br (J.C.M. Carvalho).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.01.054

particularly suited to processes where nutrient levels must be adequately controlled, such as those in which ammonia sources are applied. The fed-batch addition of ammonium salts or urea was shown to ensure adequate cell growth (Bezerra et al., 2008; Soletto et al., 2005) and high-quality biomass, characterized by high levels of pigments, mainly chlorophyll (Rangel-Yagui et al., 2004).

The success of *A. platensis* large-scale production depends on the development of effective and economic cultures; up to 25% of the total production cost of cyanobacteria is related to the medium (Belay, 1997). The nitrogen source is important, for it is the second most abundant element in *Arthrospira* sp. biomass. Aside the high acquisition costs, cultures developed with KNO_3 generate effluents with high salinity that require highly priced treatments. Ammonium salts, such as NH_4Cl, are readily assimilated by the cyanobacterium (Carvalho et al., 2004), reduce the chances of culture contamination by other microorganisms (Borowitzka, 1999), and are essentially cheap.

Notwithstanding the above advantages, the use of ammonium salts as the sole nitrogen source can lead to nitrogen-deficient cultures. Within this context, the simultaneous use of two nitrogen sources can provide a way to avoid the above difficulties encountered in *A. platensis* cultures with only one nitrogen source; whereas KNO_3 ensures high biomass yields, NH_4Cl allows reduction of production costs.

In order to assure the success of such association, in this study KNO_3 and NH_4Cl were added through batch and fed-batch processes, respectively. The simultaneous use of different processes for two distinct nitrogen sources, as far as we are aware, was not yet applied to the cultivation of any photosynthetic microorganism, being this the main novelty of the present work. Then, the aim of this study was to evaluate *A. platensis* cultivation performed with both KNO_3 and NH_4Cl, and to investigate the influence of these nitrogen sources on cell concentration, cell productivity and nitrogen-to-cell conversion factor, as well as chlorophyll, lipids and proteins contents.

2. Methods

2.1. Microorganism and inoculum preparation

The strain *A.* (*Spirulina*) *platensis* (Nordstedt) Gomont was obtained from the University of Texas Culture Collection. Two different culture media were prepared in distilled water. A standard medium containing 2.57 g L^{-1} of KNO_3 as sole nitrogen source (Bezerra et al., 2008) was used to maintain the microorganism and to prepare the inoculum, whereas the same medium, but containing variable amounts of both KNO_3 and NH_4Cl, was used in the fed-batch runs.

A. platensis was grown in Erlenmeyer flasks containing 200 mL of standard medium on a rotary shaker at 100 rpm, 30 °C and 6.0 klux (72 μmol photons m^{-2} s^{-1}). The resulting suspension was harvested during the exponential growth phase, filtered and washed thrice with physiological solution (0.9% NaCl) to completely remove adsorbed salts, including KNO_3. The cells were then suspended again in the same standard medium without any nitrogen source and used to inoculate the cultivation tanks. The starting cell concentration was 50 mg L^{-1} dry weight. For experimentation, both KNO_3 and NH_4Cl were added later in form of water solution at variable concentration.

2.2. Experiments and cultivation conditions

Experiments were carried out in 68 cm-long mini-tanks (Belay, 1997) made of PVC foils, each having total area of 0.123 m^2. The cultivation volume was 5 L, which was maintained through the daily addition of distilled water to replace water lost by evaporation. The starting pH value was 9.5 ± 0.1. Paddle wheels ensured culture mixing at 18 rpm. Temperature was maintained at 30 °C by submersible electronic thermostats (Aristos, São Paulo, Brazil) and light intensity was regulated at 13 klux (156 μmol photons m^{-2} s^{-1}) (Bezerra et al., 2008) by fluorescent lamps and a light meter (Li-Cor 250A, Lincoln, NE, USA).

The cultivation conditions are summarized in Table 1. Runs 1–14 were performed according to the selected factorial design (see later), each containing different amounts of KNO_3 and NH_4Cl. Confirmation runs C_1–C_5 were carried out under conditions estimated by the regression model to maximize X_m and P_X. Standard runs S_N and S_A, each containing usual amounts of KNO_3 (Bezerra et al., 2008) and NH_4Cl (Carvalho et al., 2004), respectively, were performed so as to have a comparison basis.

2.3. Nitrogen source addition

The addition of total amount of KNO_3 (m_{T1}) (Table 1) was made entirely at the beginning of the runs ($t = 0$).

In order to avoid any inhibitory effect by ammonia, NH_4Cl was added by daily pulse-feeding at exponentially-increasing flow rates according to the experimental schedule (Table 1) and to the equation:

$$m_{t2} = m_i e^{kt} \quad (1)$$

where m_i and m_{t2} are the amounts of NH_4Cl added per unit reactor volume at the start and until the instant "t", respectively. The initial concentration of NH_4Cl (m_i) was 1.7 mM (Carvalho et al., 2004) and the total feeding time (T) was 17 days (Bezerra et al., 2008).

When $t = T$, then $m_{t2} = m_{T2}$, being m_{T2} the total amount of NH_4Cl added in the reactor. The choice of this flow rate pattern was suggested by the intention of supporting an exponential growth of biomass, according to Carvalho et al. (2004).

2.4. Analytical methods

Dry cell mass concentration was determined by optical density measurement at 560 nm using a calibration curve. The pH was determined by a potentiometer (Mettler Toledo 2100E, São Paulo, Brazil). The concentration of total ammonia was also determined by a potentiometer (Orion 710-A, Beverly, MA, USA), but using a selective ammonia ion electrode (Orion 95-12), after preliminary pH adjustment to 13.0 with NaOH 1.5 M (Carvalho et al., 2004). Calibration curves were prepared every 2 days, as instructed by the manufacturer of the equipment. Concentration of total carbonate was determined by titration. Nitrate was determined according to Vogel (2002). All last three analyses were performed on medium samples free of cells. The concentration of chlorophyll "a" was determined on cells samples taken on the day corresponding to the maximum cell concentration. For this, it was carried out a methanol-mediated extraction of the biomass pigment, the optical density measurement being done at 665 nm (Vonshak, 1997) using a calibration curve.

After harvesting at the end of cultivation, recovered cells were centrifuged, washed with distilled water in order to remove all adsorbed salts, and dried at 55 °C for 12 h. The lipids and proteins contents of biomass were analyzed in powdered samples, according to Olguín et al. (2001) and the Association of Official Analytical Chemistry (2007), respectively.