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Ethanol effect on batch and fed-batch *Arthrospira platensis* growth

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Abstract The ability of *Arthrospira platensis* to use ethanol as a carbon and energy source was investigated by batch process and fed-batch process. *A. platensis* was cultivated under the effect of a single addition (batch process) and a daily pulse feeding (fed-batch process) of pure ethanol, at different concentrations, to evaluate cell concentration (X) and specific growth rate (μ). A marked increase was observed in the cell concentration of *A. platensis* in runs with ethanol addition when compared to control cultures without ethanol addition. The fed-batch process using an ethanol concentration of 38 mg L^{-1} $days^{-1}$ reached the maximum cell concentration of 2,393 $\pm$ 241 mg L^{-1}, about 1.5-fold that obtained in the control culture. In all experiments, the maximum specific growth rate was observed in the early exponential phase of cell growth. In the fed-batch process, μ decreased more slowly than in the batch process and control culture, resulting in the highest final cell concentration. Ethanol can be used as a feasible carbon and energy source for *A. platensis* growth via a fed-batch process.

Keywords *Arthrospira platensis* · Ethanol · Fed-batch culture · Microbial growth · Mixothrophic culture

Introduction

The filamentous cyanobacterium *Arthrospira platensis* is an important organism for industrial applications and as a food supply [3]. *Arthrospira* sp. have been photoautotrophically cultivated in open ponds in which cell concentration was low. Culturing *A. platensis* in mixotrophic conditions could potentially yield a higher biomass concentration [5]. With recent improvements in closed systems for mass cultivation of microalgae, mixotrophic growth of cyanobacteria may become economically feasible in the near future.

Some authors have investigated the mixotrophic growth of *A. platensis* on different organic substrates, such as acetate [6], glucose, and propionate [17]. These results indicate that different carbon sources have a strong impact on *A. platensis* growth. Li et al. [16] estimated the cost of glucose to be about 80 % of the total medium cost for mixotrophic cultivation of *Chlorella* sp., which justifies the importance of evaluating low-cost organic carbon sources as substitutes for glucose. In order to reduce microalgal production costs, it is important to find cheap organic substrates or cost-free ones.

Matsudo et al. [18] and Carvalho et al. [4] showed that CO_2 released by alcoholic fermentation can be used for the production of photosynthetic microorganisms. This CO_2 can be piped directly from the fermenter to the microalgae or cyanobacterium bioreactor without any treatment, but in this case up to 1 % of the ethanol produced in the fermentation can be dragged by the exhaust gas to the photosynthetic microorganisms in cultivation [10, 18].

World demand for ethanol is expected to increase in response to anticipated economic growth, rising oil prices, and the mandates in many countries to replace fossil fuel with renewable energy sources. Brazil is in a good position to satisfy the demand: sugarcane-based ethanol is one of the most efficient sources of biofuel per hectare, with a yield in liters of ethanol per hectare that is almost double that of corn-based ethanol [25].

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Ethanol is an attractive raw carbon source for microbial production of a variety of biochemicals. *Crypthecodinium cohnii* has the capability to produce docosahexaenoic acid (DHA) when grown on ethanol as the sole carbon source [24], and *Euglena gracilis* has a higher production of vitamins when ethanol is added in the cultures [1].

The aim of this study was to investigate whether ethanol addition by batch process or fed-batch process is suitable as a carbon source for the production of *A. platensis* biomass.

Materials and methods

Cultivation conditions

Arthrospira platensis UTEX 1926 was obtained from the University of Texas Culture Collection (Austin, TX). The microorganism was maintained and cultivated in the mineral medium suggested by Schlösser [23]. The cultivations were carried out in 500-mL Erlenmeyer flasks containing 200 mL of the culture medium, either using inorganic carbon alone or organic/inorganic carbon mixture. Each culture was inoculated with an initial biomass concentration of 80 mg L^{-1}. The cultures were kept on a rotary shaker at 100 min^{-1}at 30 °C and continuous light intensity of 72 μmol photons m^{-2} s^{-1}. The cultivations were performed with either a single feeding of ethanol (batch reference tests) or a constant mass flow rate of ethanol once a day (fed-batch references tests) in the complete Schlösser medium. The ethanol concentration varied in a range from 9.5 to 155,648 mg L^{-1} for the batch cultures and 9.5 to 304 mg L^{-1} $days^{-1}$ for the fed-batch cultures (Table 1). The control culture was performed using only the standard culture medium proposed by Schlösser [23] without ethanol addition.

Table 1 Comparison of final cell concentration and maximum specific growth rate achieved by batch and fed-batch cultivations of *A. platensis*

	Ethanol concentration (mg L^{-1} $days^{-1}$)	Total ethanol concentration (mg L^{-1})	Ct[a]	X_m (mg L^{-1})[b]	μ_{max} ($days^{-1}$)[c]
Control	0	0	8	1,596 ± 129[A]	0.77 ± 0.14[D]
Batch	9.5	9.5	8	1,639 ± 173[A]	0.76 ± 0.04[D]
	19	19	8	1,677 ± 115[A]	0.79 ± 0.04[D]
	38	38	8	1,681 ± 113[A]	0.75 ± 0.01[D]
	76	76	8	1,587 ± 76[A]	0.78 ± 0.02[D]
	152	152	8	2,004 ± 236[B]	0.85 ± 0.01[D]
	304	304	8	2,046 ± 203[B]	0.84 ± 0.02[D]
	608	608	8	1,919 ± 23[B]	0.78 ± 0.04[D]
	1,216	1,216	8	1,866 ± 64[B]	0.74 ± 0.07[D]
	2,432	2,432	7	1,224 ± 113[C]	0.70 ± 0.02[D]
	4,864[d]	4,864	3	f	–
	9,728[d]	9,728	3	f	–
	19,456[d]	19,456	3	f	–
	38,912[e]	38,912	f	f	–
	77,824[e]	77,824	f	f	–
	155,648[e]	155,648	f	f	–
Fed-batch (daily addition)	9.5	76	8	1,568 ± 134[A]	0.74 ± 0.01[D]
	19	152	8	1,907 ± 275[A]	0.81 ± 0.02[D]
	38	304	8	2,397 ± 241[B]	0.84 ± 0.01[D]
	76	608	8	1,885 ± 130[A]	0.81 ± 0.05[D]
	152	912	6	1,656 ± 237[A]	0.80 ± 0.01[D]
	304	2,128	7	1,692 ± 238[A]	0.80 ± 0.02[D]

–, Not calculated

[A,B,C,D] Values with the same cap letters superscript are not significantly different according to the Tukey test ($p > 0.05$).

[a] Cultivation time

[b] Maximum cell concentration

[c] Maximum specific growth rate

[d] Cell death in the 2nd–3rd day

[e] Cell death in the first day

[f] Cell death

Ethanol concentration was chosen considering that 1 % of ethanol is released with CO_2 during alcoholic fermentation [10]. Then 19 mg L^{-1} $days^{-1}$ of ethanol is added to the culture together with the CO_2 necessary to produce 1 g L^{-1} $days^{-1}$ of *A. platensis* containing about 50 % carbon in the cell [9]. The lowest ethanol concentration was determined to be 9.5 mg L^{-1}, corresponding to half of 19 mg L^{-1}.

Analytical techniques

Cell concentration was determined daily by measuring the optical density of samples at 560 nm using a calibration curve [14]. Incident photon flux density of photosynthetically active radiation (400–700 nm) was measured by means of an Integrating Quantum/Radiometer/Photometer, model LI-190 SB (Li-Cor, Lincoln, NE, USA), equipped with an LI-190 SB quantum sensor cell. Ethanol concentration was determined by the micro-dichromate method [12]. The specific growth rate (μ) was calculated by the method of Leduy and Zajic [15].

Statistical analysis

All results are shown as mean values of three replicates with standard deviation (SD). Maximum cell concentration (X_m, mg L^{-1}) was evaluated by the analysis of variance (ANOVA, General Linear Model) and the Tukey test to compare the mean values at a significance level of 5 % ($p < 0.05$) using the MINITAB 15 statistical software.

Results and discussion

Effects of ethanol on cell concentration

The effect of different ethanol concentrations using batch process on *A. platensis* growth is shown in Fig. 1 and Table 1. Ethanol concentrations in the batch process up to 76 mg L^{-1} did not influence the *A. platensis* growth, providing X_m values (1,587–1,681 mg L^{-1}) close to control cultures (1,596 ± 129 mg L^{-1}) (Table 1). These results indicated that a single feeding of ethanol concentration in the range from 9.5 to 76 mg L^{-1} did not influence the *A. platensis* growth and is not appropriate for high-cell-density cultivation of this cyanobacterium on ethanol.

When ethanol concentration increases up to the range of 152–1,216 mg L^{-1}, the X_m values increase 24 % when compared to control cultures (Fig. 1), enabling the achievement of the highest X_m values of all batch cultures. Over ethanol concentrations of 1,216 mg L^{-1}, a decrease of X_m was observed with an increase of ethanol concentration, reaching an inhibitory ethanol concentration of 4,864 mg L^{-1},

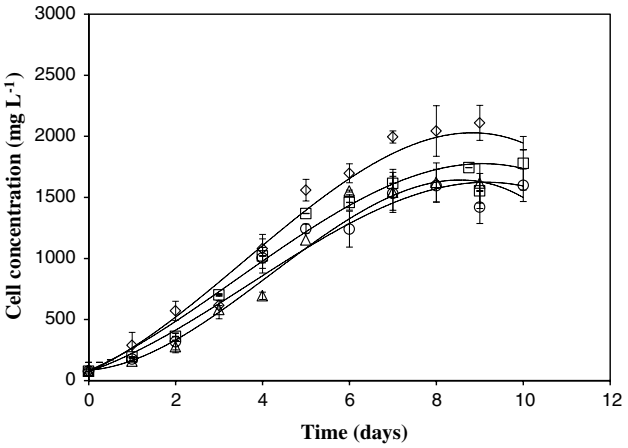

Fig. 1 Growth of *A. platensis* under batch process with different ethanol concentrations (mg L^{-1}): *circle* 0; *triangle* 9.5; *square* 38, *diamond* 304. The values presented correspond to an average of three experiments with standard deviation.

which showed cell death because of evident ethanol inhibition. With ethanol concentrations above 38,912 mg $L^{-1,}$ no growth was observed just after ethanol addition. It is evident that ethanol concentrations significantly influenced X_m values, whereas no appreciable effect was observed related to the type of processes.

In the Batch process, all ethanol is added in the beginning of cultivation, and high ethanol concentration can damage the cell, as observed in the culture using ethanol concentrations from 4,864 mg L^{-1}. Therefore, to investigate the potential of ethanol fed-batch process for the production of *A. platensis* biomass, ethanol in concentrations ranging from 9.5 to 304 mg L^{-1} was added daily until the cells entered the stationary phase of growth (Table 1).

Ethanol fed-batch cultivations of *A. platensis* were performed with six different pulse feedings. The high X_m was about 2,397 ± 241 mg $L^{-1,}$ obtained at a daily pulse feeding of 38 mg L^{-1}, corresponding to values 16.4 and 33.4 % higher than those obtained in the best conditions of batch process culture and in the control culture, respectively (Table 1). It showed that the cell concentration increased with ethanol addition via fed-batch process. The ethanol added in small quantities is done to support higher microalgal biomass and simultaneously prevent excessive bacterial growth, which would be the outcome if the ethanol substrates were added in large quantities [21].

Figure 2 shows that a progressive increase in ethanol concentrations in the fed-batch process from 9.5 to 38 mg L^{-1} led to an increase in maximum biomass concentration (X_m) after 8 days from 1,568 ± 134 to 2,393 ± 241 mg L^{-1}. A further increase in ethanol concentration to 76 mg L^{-1} led to decrease in X_m value to 1,885 ± 130 mg L^{-1}, likely because from 76 mg L^{-1}, the ethanol concentration

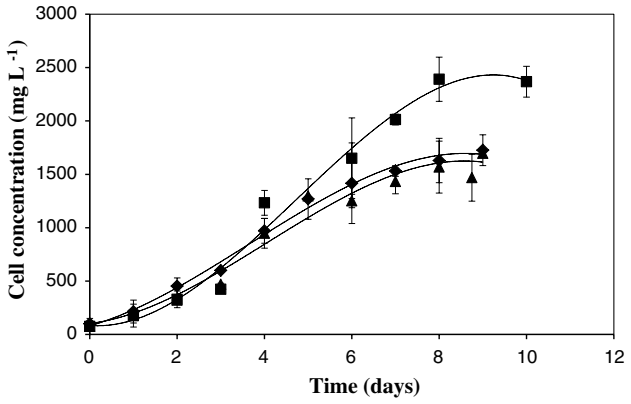

Fig. 2 Growth of *A. platensis* under fed-batch process with different ethanol concentrations (mg L^{-1}): *filled triangle* 9.5; *filled square* 38; *filled diamond* 304. The values presented correspond to an average of three experiments with standard deviation. The control culture data, without ethanol addition, is the same as in Fig. 1

was toxic to cell growth (Table 1). Menzyanova and Bozhkov [19] related that ethanol at a concentration of 0.1 % is highly toxic to *Spirulina platensis* metabolism. On the other hand, in the present study ethanol concentrations up to 1,216 mg L^{-1} (0.15 %) provided an increase in the cell concentration.

Ethanol fed-batch process cultivation has been recognized as a technique for biomass yield improvement in microbial cultivations. In this work, the growth of *A. platensis* was enhanced by the addition of ethanol by a fed-batch process, which confirms that *A. platensis* can consume ethanol as a carbon and energy source. In the cell, alcohol dehydrogenase oxidizes ethanol to acetaldehyde, which is in turn further oxidized to acetate by the acetaldehyde dehydrogenase enzyme [2]. Acetate is converted to acetyl-CoA by Acetyl-CoA synthetase [20], which enters the tricarboxylic acid cycle or the glyoxylate cycle, where the isocitrate lyase activity, a key enzyme in acetate assimilation, could not be usually detected in autotrophic cells [20]. Ono et al. [20] observed that ethanol as the sole carbon source resulted in increases in alcohol dehydrogenase and the two glyoxylate cycle enzymes, indicating that ethanol is assimilated by alcohol dehydrogenase and the glyoxylate cycle in *Euglena gracilis* cells. The results of the present study showed that cell growth in the presence of ethanol, either in batch or fed-batch processes, was higher than in the control cultures.

Comparison between ethanol and other carbon sources

Mixotrophic microalgae cultivation is poorly studied, and the available examples are quite limited. Although microalgae can grow with various organic carbon sources, glucose is preferred because of its ease of handling, accessibility, and safety [1, 11] and because it possesses more energy content per mol compared with other substrates. However, the high cost of glucose may limit its use in industrial production, and therefore, other carbon sources with lower costs, such as ethanol, have to be evaluated. In this study, the highest X_m value in ethanol-grown fed-batch cultures (about 2,393 mg L^{-1}) was higher than that reported for cultures grown on glucose (1,300 mg L^{-1}) [21] or sodium acetate (1,650 mg L^{-1}) [7]. Therefore, far less ethanol is required in comparison with acetate or glucose in order to produce a similar amount of biomass. This, together with the lower (tax-free) price of ethanol compared to that of acetate and glucose, results in better carbon-source economy. Yet another advantage is that ethanol is less corrosive than acetate, which may reduce capital investment and maintenance costs for cultivation equipment [24]. Under mixotrophic conditions some microalgae are known to grow rapidly and to have a growth rate higher than that observed in photoautotrophic conditions. Chen et al. [5], growing *Spirulina platensis* under mixotrophic conditions, reported a productivity 2.4-fold higher than that of photoautotrophic cultures.

Effects of ethanol on specific growth rate

The values of μ_{max} were always obtained at the beginning of the cultivations and did not vary appreciably among the runs ($0.70 \leq \mu_{max} \leq 0.85$ $days^{-1}$). During this period, there was, in fact, high availability of nutrients and light energy to the cell because of the low cell concentration. After 1 day of cultivation, μ progressively decreased with time due to the increased cell concentration and consequent reduction of nutrient and energy availability for the cell. This may have been caused by always maintening the cells under extreme conditions of pH, light limitation and/or lack of available nutrients. Consistent with these findings, Vonshak et al. [26] observed that μ was inversely proportional to biomass concentration. During the beginning of the cultivation, autotrophic activity prevailed due to the high light energy available and the lower ethanol concentration, resulting in a value of μ_{max} that was similar in all the cultivations. This result may be supported by the data reported by Yang et al. [27] who found that light was the major source for ATP production in the early phase of mixotrophic cultivation. With increasing cell concentration, light energy available for the cells decreased, so the microorganism starts consuming the organic carbon (ethanol) present, with cell growth favored in the culture with higher ethanol concentration added, that is the fed-batch culture. This result is consistent with the observation of Zhang et al. [28], in which the μ_{max} was reached more slowly for batch culture than fed-batch culture in *A.*

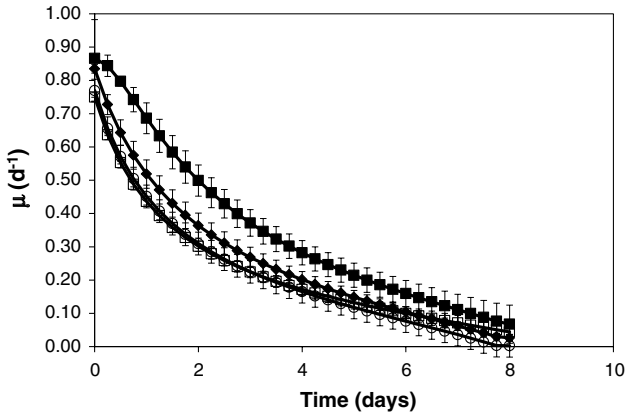

Fig. 3 Specific growth rate (μ) of *A. platensis* at different conditions: *circle* 0, without ethanol; *square* batch with *Ec* = 38 mg L^{-1}; *filled square* fed-batch process with *Ec* = 38 mg L^{-1} $days^{-1}$; *filled diamond* batch process with *Ec* = 304 mg L^{-1}. The values presented correspond to an average of three experiments.

platensis mixotrophic growth using glucose. Rym et al. [22] suggest that the increased growth rate of *A. platensis* is due to the synergistic effect of photosynthesis and glucose oxidation. The first phase of mixotrophic cultures was identified as mainly photoautotrophic, while the second was photoheterotrophic.

The highest maximum specific growth rate (0.85 $days^{-1}$) was strongly higher than 0.49 $days^{-1}$ using 2,500 mg L^{-1} of glucose [13] 0.52 $days^{-1}$ using 246 mg L^{-1} of acetate [6].

Although the same profile was obtained in all the cultivations, in the fed-batch process μ decreased more slowly than in the batch process and control cultures (Fig. 3). This behavior resulted in the highest final cell concentration in the fed-batch cultivations (Table 1). The specific growth rates and biomass concentrations increase, apparently due to a synergistic effect of light and the organic substrate [8]. Although this study clearly demonstrates the potential of ethanol as a carbon source for large-scale production of *A. platensis*, several aspects of the process can be further optimized.

Conclusion

This study shows that ethanol can be an interesting carbon and energy source for *A. platensis.* This provides extra opportunities for this photosynthetic microorganism in the advantage of using both organic and inorganic carbon sources to obtain high cell concentrations. Cultivations using ethanol provided a higher cell concentration when compared with control cultures without ethanol addition. Similar values of maximum specific growth rate (μ_{max}) were found at the beginning of all the cultivations, but the specific growth rate decreased more slowly in the fed-batch process, which led to higher values of final cell concentration.

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