SIMULTANEOUS EXTRACTION OF PHYCOBILIPROTEINS AND CARBONIC ANHYDRASE FROM *Spirulina platensis* LEB-52

LUISA SALA[1], CAROLINE COSTA MORAES[2], SUSANA JULIANO KALIL[3]

ABSTRACT

C-phycocyanin (C-PC), allophycocyanin (APC) and carbonic anhydrase (CA) are intracellular bioproducts produced by *Spirulina platensis* LEB-52. In order to extract them, a cell rupture step is required. The influence of extraction time on ultrasound-assisted extraction (UAE) was evaluated at two biomass concentrations (0.2 and 23 g/L). At biomass concentration of 0.2 g/L, 12-min UAE was sufficient to simultaneously obtain phycobiliproteins (C-PC and APC) and esterase-expressed CA. Enzyme activity of hydratase-expressed CA in cyanobacteria is very low. Therefore, to determine the activity of hydratase-expressed CA, biomass concentration of 23 g/L and 8-min extraction time were needed. UAE is an efficient method of rapid determination of phycobiliproteins in wet biomass, even though it is not a selective method, which extracts an array of contaminants from biomass.

KEYWORDS: C-PHYCOCYANIN. ALLOPHYCOCYANIN. MICROALGAE. ULTRASOUND-ASSISTED EXTRACTION. HYDRATASE.

EXTRAÇÃO SIMULTÂNEA DE FICOBILIPROTEINAS E ANIRASE CARBÔNICA DE *Spirulina platensis* LEB-52

RESUMO

C-ficocianina (C-FC), aloficocianina (AFC) e anidrase carbônica (AC) são bioprodutos intracelulares produzidos pela *Spirulina platensis* LEB-52, portanto, para as suas obtenções é necessária uma etapa de ruptura celular. A influência do tempo durante a extração assistida por ultrassom (EAU) foi avaliada em duas concentrações de biomassa (0,2 e 23 g/L). Com concentração de biomassa de 0,2 g/L e o tempo de ultrassom de 12 min foi suficiente para obter simultaneamente as ficobiliproteínas (C-FC e AFC) e AC expressa em termos de esterase. A atividade enzimática de AC expressa em termos de hidratase nas cianobactérias é muito baixa, portanto, para determinar a atividade de AC expressa em termos de hidratase, a concentração celular foi 23 g/L e o tempo de ultrassom foi de 8 min. A EAU não é um método de extração seletivo para as ficobiliproteínas, extraindo uma gama de compostos contaminantes, no entanto é um método eficiente para a rápida determinação de ficobiliproteínas na biomassa.

PALAVRAS-CHAVE: C-FICOCIANINA. ALOFICOCIANINA. MICROALGA. EXTRAÇÃO ASSISTIDA POR ULTRASSOM. HIDRATASE.

[1]Discente – Universidade Federal do Rio Grande (FURG)/EQA/PPG-ECA – e-mail: luisasalaea@gmail.com
[2]Docente – Universidade Federal do Pampa (UNIPAMPA) – e-mail: caroline.moraes@unipampa.edu.br
[3]Docente – Universidade Federal do Rio Grande (FURG)/EQA/PPG-ECA – e-mail: dqmsjk@furg.br

1. INTRODUCTION

Much interest has recently been focused on the biotechnological potential of microalgae, mainly due to the identification of several substances, such as polyunsaturated fatty acids [48], carotenoids [29], enzymes [38], phycobiliproteins [32,38] and polysaccharides [55], synthesized by these organisms. Both phycobiliproteins C-phycocyanin (C-PC) and allophycocyanin (APC), besides the enzyme carbonic anhydrase (CA) can be simultaneously obtained from microalga biomass.

Phycobiliproteins are accessory photosynthetic pigments that can be used as natural dyes to replace synthetic dyes in food and cosmetic industries [2,21]. Studies have shown that C-PC exhibits antioxidant [54], anti-inflammatory [45], hepatoprotective [35] and neuroprotective [40] properties. In addition, C-PC has an anticancer effect, which has already been demonstrated in a variety of cells, such as carcinogenic lung [25], breast [44] and pancreas [26] ones. APC exhibits antioxidant [12] and antienterovirus [50] properties.

CA is a metalloenzyme that catalyzes the reversible reaction of CO_2 hydration in bicarbonate ions (HCO_3^-) and protons (H^+) [3]. It plays an important role in several processes, such as pH homeostasis, respiratory changes, photosynthesis and ion transport [15], since it is widely distributed in plant and animal tissues, besides microorganisms, including cyanobacteria [16,34]. CA can be applied to CO_2 enzyme capture and can contribute to reduce the amount of the gas released to the atmosphere. Anthropogenic CO_2 is considered the major contributor to the increase in the greenhouse effect whereas the remainder is attributed to the increase in concentrations of methane, nitrous oxide and fluorinated gases [6]. However, the extraction of CA from microalgae has still been little investigated, mainly regarding this use [24,36,37].

Phycobiliproteins and CA can be simultaneously obtained from cyanobacteria, such as *Spirulina*. To monitor the production of these bioproducts in microalga cultivation, an efficient and rapid cell rupture technique, which requires small sample volume and low biomass concentrations, must be used. During cell rupture, the cell wall must be ruptured so as to enable intracellular contents to be released into the medium, but its biological activities must be maintained [52]. Several factors, such as extraction time, solvent and biomass concentration, influence cell rupture and the

extraction of bioproducts from cyanobacteria [32,51]. Among different techniques of cell rupture, ultrasonic-assisted extraction (UAE), which is based on the cavitation phenomenon, has been widely used to extract several intracellular compounds from microalgae, such as carotenoids [9,13], chlorophyll [13,39], phenolic compounds [39], lipids [8,43,47], proteins [28], C-phycocyanin [1,38], allophycocyanin and carbonic anhydrase [38].

ORES, AMARANTE and KALIL [38] monitored the production of C-PC, APC and esterase-expressed CA from *Spirulina* sp. LEB18 and *Synechococcus nidulans*, with biomass concentration of 0.2 g/L for the extraction of bioproducts. In addition, these authors tested different methods of cell rupture to simultaneously extract C-PC, APC and esterase-expressed CA from *Spirulina* sp. LEB 18. Among the methods under investigation, UAE with a frequency of 20 kHz and 10 min extraction time (with biomass concentration of 5 g/L) provided high extraction yields of the bioproducts, which were 25.5 U/g for esterase-expressed CA, 90.0 mg/g and 70.0 mg/g for C-PC and APC, respectively. However, the influence of extraction time was not evaluated. This study aimed to define the conditions of UAE to simultaneously extract C-phycocyanin, allophycocyanin and carbonic anhydrase from *Spirulina platensis* LEB-52.

2. MATERIAL AND METHODS

2.1. Microorganism, culture medium and cultivation

The cyanobacterium *Spirulina platensis* LEB-52 was used for extracting C-PC, APC and CA simultaneously. For inoculum and cultivation, Zarrouk's medium was used [57]. It was diluted 20% (v/v) with distilled water [37]. Cultivation was carried out in agreement with conditions proposed by Ores [37], in a 2-L Erlenmeyer flask (1.8 L useful volume) with the addition of 20% (v/v) of inoculum. Cyanobacteria were incubated at 25°C and aeration was performed by sterile air constant injection (0.5 vvm). Illuminance was promoted by fluorescent lamps (40.5 µE/m^2/s) with photoperiod fixed at 12 h light and 12 h dark. After 30 d, biomass was recovered by centrifugation (4757 ×g, 30 min, 4°C).

2.2. Influence of extraction time on UAE

UAE of phycobiliproteins and CA was performed in agreement with ORES, AMARANTE and KALIL [38]. Wet biomass was resuspended in tris-HCl buffer (20

mmol/L and pH 8.3) with a final concentration of 0.2 g/L and 23 g/L. Cell suspension (45 mL) was exposed to UAE by the Sonic Ruptor 250 homogenizer (Omni International Inc., USA) with a frequency of 20 kHz (60 W power). During UAE, samples were maintained at 4°C and aliquots were withdrawn at different time periods (4, 6, 8, 10, 12 and 14 min). Afterward, suspensions were centrifuged (4757 ×g, 20 min, 4°C) and the supernatant was used for determining C-PC and APC concentrations, esterase- and/or hydratase-expressed CA enzyme activity and total soluble protein. Results were expressed in terms of extraction yield of C-PC and APC (mg/g of biomass) and CA (U/g of biomass). Experiments were carried out in triplicate.

2.4. Concentration and extraction yield of phycobiliproteins

C-phycocyanin (C-PC, Equation 1) and allophycocyanin (APC, Equation 2) concentrations (mg/mL) were calculated as described by BENNETT and BOGORAD [5]. Absorbance at 620 and 652 nm represent maximum absorptions of C-phycocyanin and allophycocyanin, respectively. They were performed by a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). Extraction yields (mg/g biomass) of C-PC (Equation 3) and APC (Equation 4) were calculated as proposed by SILVEIRA et al. [51], where BC is the biomass concentration (mg/mL).

$$\text{C-PC} = \frac{A_{620} - 0.474 \cdot A_{652}}{5.34} \quad (1)$$

$$\text{APC} = \frac{A_{652} - 0.208 \cdot A_{620}}{5.09} \quad (2)$$

$$\text{C-PC Yield} = \frac{\text{C-PC}}{\text{BC}} \cdot 1000 \quad (3)$$

$$\text{APC Yield} = \frac{\text{APC}}{\text{BC}} \cdot 1000 \quad (4)$$

2.5. Carbonic anhydrase activities

Enzyme activity of CA was monitored by the reactions: hydratase and/or esterase activities. The hydratase reaction (CO_2 hydration) was carried out in agreement with the electrometric methodology described by WILBUR and ANDERSON [56]. The reaction mixture consisted of 6 mL Tris-HCl buffer (20 mmol/L, pH 8.3), 0.4 mL enzyme solution and 4 mL deionized water solution with CO_2. The enzyme reaction was kept at 4°C. A Wilbur-Anderson enzyme activity unit (U) is defined as $[(t_0/t)-1]$,

where t_0 and t are the times for a pH change (8.0 to 7.0) without (t_0) and with the enzyme (t).

Esterase activity was performed in agreement with POCKER and STONE [41].The reaction mixture consisted of 1.8 mL Tris-SO_4 buffer (50 mM, pH 7.4), 0.2 mL enzyme extract and 1 mL 3 mM *p*-nitrophenyl acetate (*p*-NPA). After the addition of substrate, the increase in absorbance at 400 nm ($\varepsilon_{p\text{-nitrophenol}}$: 11.43 mL/cm/µmol) was recorded every 30 s for 4 min at room temperature by a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). A baseline measurement was obtained, as described above, except for the addition of the enzyme solution where it was replaced by water. One enzyme activity unit (U) is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute under assay conditions.

2.6. Biomass and protein concentrations

Biomass concentration was determined by absorbance at 670 nm [7] and conversion to dry biomass by a previously prepared standard curve. Total soluble protein concentration was analyzed as proposed by Lowry, Rosebrough [42], with BSA (bovine serum albumin) as standard.

2.7. Statistical analysis

All experiments were performed in triplicate and statistically analyzed by the analysis of variance (ANOVA), followed by the Tukey's or *t*- tests at 95% confidence level.

3. RESULTS AND DISCUSSION

The extraction process comprises two steps: cell rupture and extraction with either an aqueous solvent or an organic one. Both steps can occur concomitantly. Since both steps occur concomitantly in the case of UAE, in this paper, the term extraction comprises both steps.

A problem related to the use of the ultrasonic homogenizer with thermosensitive bioproducts, such as phycobiliproteins [11,49] and CA, is the increase in temperature. To avoid CA denaturation and intracellular significant losses of C-PC and APC, the extract was maintained at 4°C throughout the extraction process.

Bioproduct extraction was carried out at biomass concentration of 0.2 g/L (Figure 1), based on the study carried out by ORES, AMARANTE and KALIL [38].

They monitored and extracted CA, APC and C-PC throughout the cultivation of *Spirulina* sp. LEB18 and *Synechococcus nidulans*, with fixed biomass concentration of 0.2 g/L.

The esterase-expressed CA yield ranged from 13.7 to 15.7 U/g (Figure 1). It was not significantly influenced by the increase in extraction time. However, phycobiliprotein extraction yields were positively affected by the extraction time. C-PC and APC yields ranged from 86.4 to 132.8 mg/g and from 44.4 to 83.6 mg/g, respectively. The increase in the extraction time provided high phycobiliprotein concentrations (mg) per g cell. The highest extraction yields were obtained from 12 min on. Similar behavior was observed in β-carotene extraction by UAE from *S. platensis*, in which the extraction yield increased as extraction time increased [9,14].

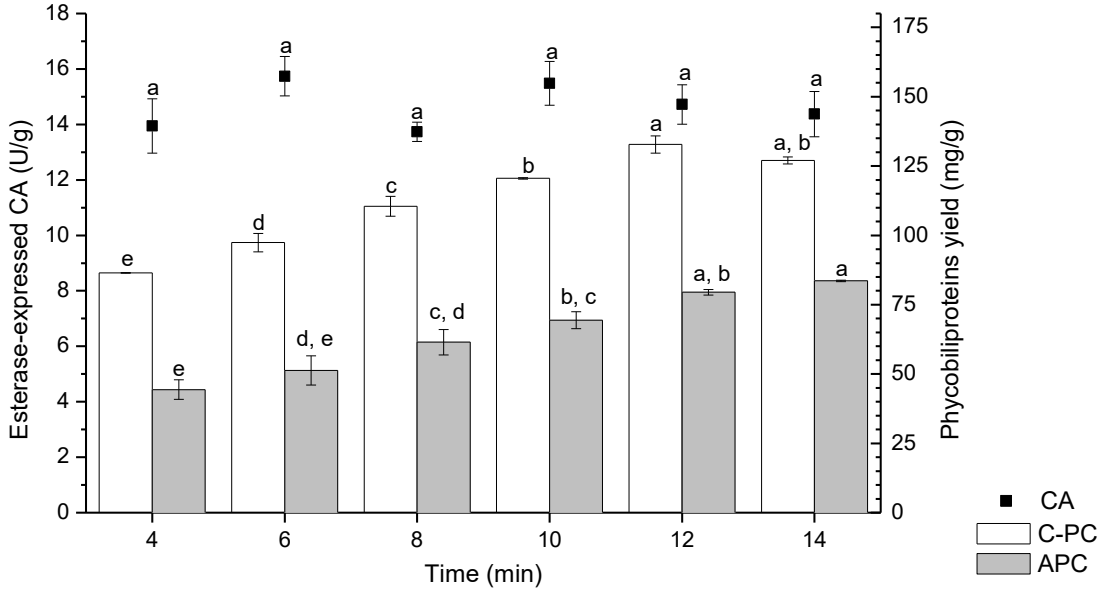

FIGURE 1. Esterase-expressed CA and phycobiliprotein extraction yields throughout UAE with biomass concentration of 0.2 g/L. Equal letters indicate that there is no significant difference among the means of each bioproduct (*Tukey's* test, $p>0.05$).

Different methods of cell rupture of *S. platensis* LEB-52 to obtain C-phycocyanin are reported, especially the ultrasonic bath with glass beads [33] and the treatment of drying, milling and freezing of biomass [32,51]. C-PC extraction yields of these methods range from 43.7 to 82.5 mg/g. C-PC has been extracted from *Spirulina* by

UAE [1,10,17,38,42]. PRABUTHAS et al. [42] studied extraction time, amplitude and volume and then applied UAE (50 W and 30 kHz) to obtain C-PC. The best condition (C-PC concentration of 0.2 mg/mL, purity of 0.62 and extraction yield of 90 mg/g) was obtained when 45.3 mL (biomass concentration of 2.2 g/L), time of 9.3 min and amplitude of 79.7% were used. In this study, C-PC concentration (12-min extraction time) was 0.028 mg/mL; it was below the one obtained by PRABUTHAS et al. [42]; however, extraction yield was 1.47-fold higher.

AFTARI et al. [1] evaluated the effect of time (1-9 min) and pH (5-9) on UAE (20 kHz and 100 W) of C-PC from *S. platensis*, followed by freezing and thawing. Both factors (time and pH) influenced the extraction and the best results were obtained with 7 min extraction time and pH of 6.0. C-PC concentration was 2.84 mg/mL whereas purity was 0.65. Regarding CA extraction, ORES [37] evaluated the influence of time extraction (1-10 min) on UAE of esterase-expressed CA from *Dunaniella tertiolecta* with biomass concentration of 0.1 g/L. The highest extraction yields were obtained from 3 min.

As expected, protein extraction yield (Figure 2) increased as extraction times increased, from 0.55 g/g in 4 min to 0.72 g/g in 14 min. The increase in extraction time enables other proteins, besides phycobiliproteins and enzymes, to be extracted. The highest protein extraction yields, i.e., 0.69 and 0.72 g/g, occurred at 12 and 14 min, respectively.

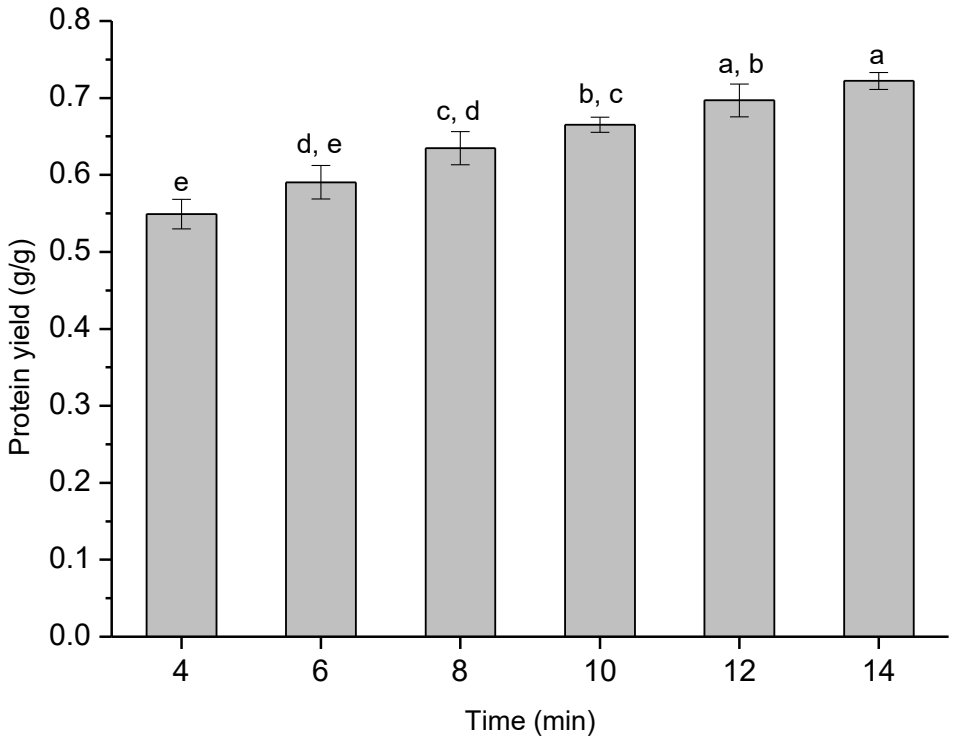

FIGURE 2. Protein extraction yield throughout UAE with biomass concentration of 0.2 g/L. Equal letters indicate that there is no significant difference among the means (*Tukey*'s test, $p>0.05$).

The extracted protein content is a very important factor in the purification process, since the higher the number of extracted contaminants, the higher the cost of purification, because more steps may be needed. In β-galactosidase enzyme extraction by UAE, the increase in enzyme activity (U/mL) and protein concentration increased as extraction time increased at a fixed biomass concentration (40 g/L) [23]

By comparison with other methods of cell disruption and extraction, UAE is an alternative method with a significant reduction in time (by comparison with drying, freezing and grinding biomass [32] and ultrasonic bath with glass beads for 40 min [33]) and with low cost (by comparison with extraction assisted by pulsed electric fields and high voltage electrical discharge). Therefore, it can be implemented in the food and pharmaceutical industries [4]. C-PC, APC and esterase-expressed CA can be obtained by UAE for 12 min, with extractions yields of 132.8 mg/g, 79.5 mg/g and 14.7 U/g, respectively, at biomass concentration of 0.2 g/L (Figure 1).

Hydratase-expressed CA activity in cyanobacteria is very low and not always detectable by the hydratase reaction [22]. In this study, as expected, hydratase activity

was not detected in the biomass extracted at concentration of 0.2 g/L. SOLTES-RAK, MULLIGAN and COLEMAN [53], KUPRIYANOVA et al. [22] and ORES [37] also found difficulties in determining hydratase-expressed CA activity in microalgae. As a result, in this study, biomass concentration was increased to 23 g/L, so as to detect the enzyme.

The hydratase- and esterase-expressed CA yields were influenced by extraction time (Figure 3) at biomass concentration of 23 g/L. The esterase-expressed CA yield in 8 min (8.9 U/g) was statistically equal to the ones at 10 and 14 min (9.5 to 10.0 U/g). The behavior of esterase-expressed CA yields at 0.2 g/L and 23 g/L (biomass concentration) were different. It may be due to the high concentration of biomass, so, additional time is required to break up a larger number of cells and achieve the maximum yield of CA extraction. In addition, the increase in biomass concentration (from 0.2 to 23 g/L) affected the efficiency of extraction of esterase-expressed CA (Figures 1 and 3) and, as a result, the yield of the process decreased. For example, when the fixed extraction time was 8 min, the extraction yield decreased about 35% after biomass concentration was increased from 0.2 to 23 g/L. Similar behavior was observed when the enzyme β-galactosidase from *Kluyveromyces marxianus* CCT7082 was extracted by an ultrasonic bath, in which the extraction yield of the enzyme decreased when biomass concentration was increased from 20 to 50 g/L [31].

The hydratase-expressed CA enzyme activity, especially at initial times (4 and 6 min) (Figure 3), had high standard deviations, probably related to heterogeneous extraction [31]. Thus, both times were excluded from the statistical analysis. At extraction times of 8 and 10 min, the hydratase-expressed CA yield is not statistically different, i.e., 140.9 and 124.1 U/g, respectively. It decreased after 10-min extraction. This drop can be attributed to the instability of the enzyme when subjected to long times of UAE. Thus, 8 min is ideal for CA extraction at biomass concentration of 23 g/L; its activity may be determined by both reactions, esterase and hydratase.

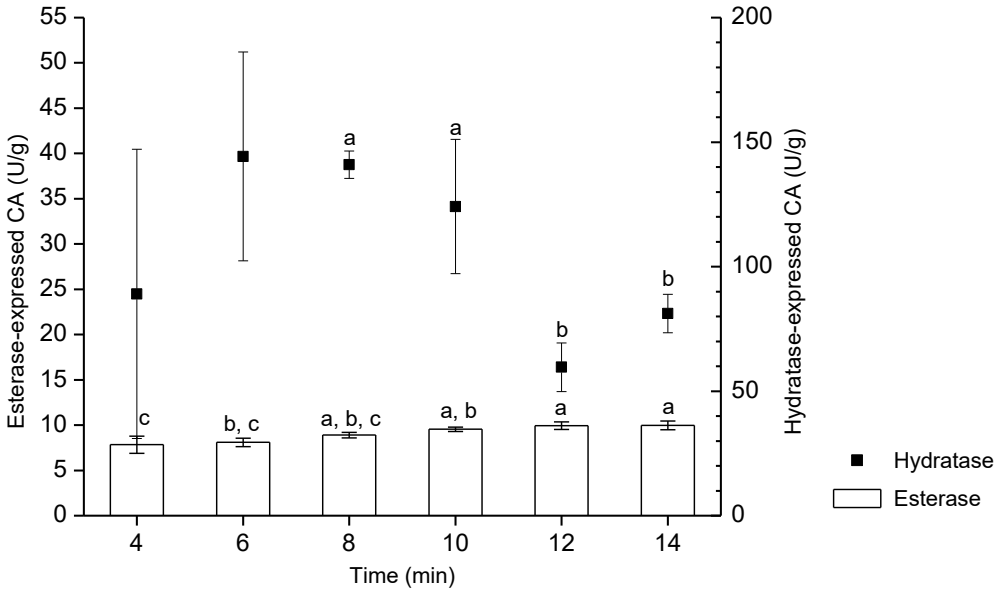

FIGURE 3. Esterase- and hydratase-expressed CA yields throughout UAE at biomass concentration of 23 g/L. Equal letters indicate that there is no significant difference among the means of each bioproduct (*Tukey*'s test, $p>0.05$).

The only report of hydratase-expressed CA determination from *S. platensis* found in the literature is the study which was carried out by KOMAROVA et al. [20]. The enzyme was extracted by ultrasound for 6 min. However, activity values cannot be compared because, in their study, these values are shown in different units.

Protein yield at biomass concentration of 23 g/L (Figure 4) increased (from 0.065 to 0.094 g/g) as extraction time increased (from 4 to 14 min). The highest extraction yields were obtained from 12 min on, i.e., 0.089 g/g. However, the protein extraction yield was different at both biomass concentrations. The highest yields of protein extraction (0.69 and 0.72 g/g, Figure 2) were obtained at the lowest biomass concentration (0.2 g/L), a fact that may have occurred because the efficiency of cellular disruption is affected when the biomass concentration is high (23 g/L).

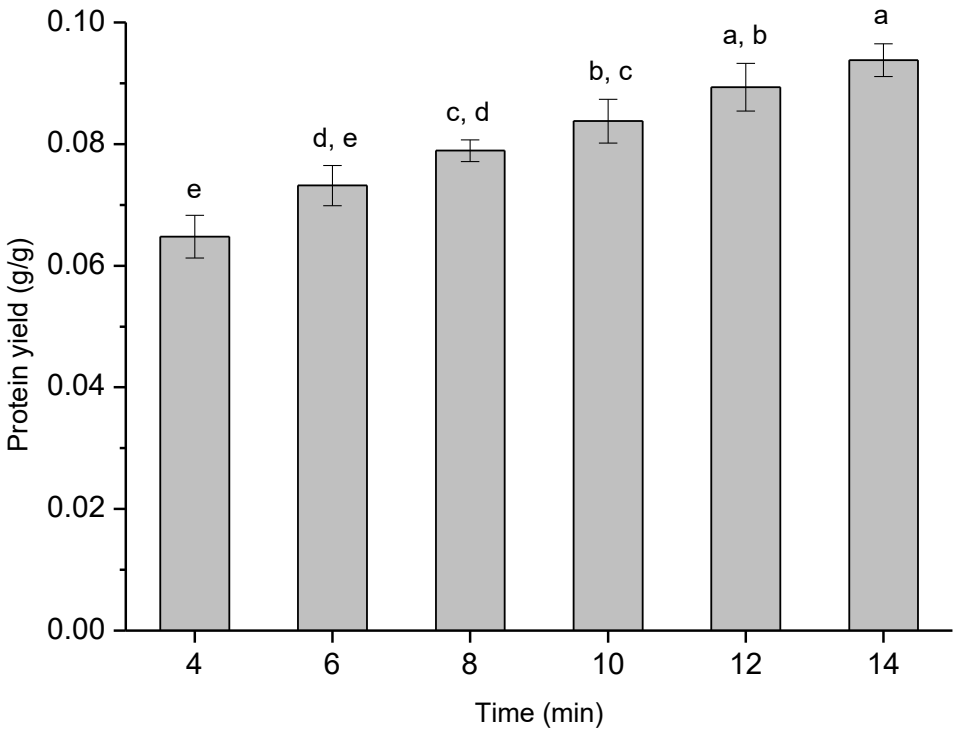

FIGURE 4. Protein extraction yield throughout UAE at biomass concentration of 23 g/L. Equal letters indicate that there is no significant difference among the means (*Tukey*'s test, $p>0.05$).

Many studies of C-phycocyanin extraction can be found in the literature [1,29,30,32,33,38,42,51], while studies of CA and APC extraction are still scarce [36,38]. UAE was efficient for simultaneous extraction of bioproducts (C-PC, APC and CA) from the microalga *S. platensis*. These results enable the production of phycobiliproteins and esterase-expressed CA to be monitored throughout microalga cultivation at low biomass concentrations (0.2 g/L) and 12-min extraction time. However, when the determination of hydratase-expressed CA is aimed at, UAE should be used for 8 min and biomass concentration should be 23 g/L.

UAE was seen to be efficient to disintegrate cells, as reported by KALIL et al. [18]. In addition, UAE is not selective to extract C-phycocyanin and allophycocyanin (Figure 5), since it extracts other compounds at the same time. It should be highlighted that the extract containing bioproducts was green. This color was also observed by GERDE et al. [13] and SAFI et al. [46] in microalga extracts which extracted lipids and proteins, respectively, from different microalgae by UAE. These authors attributed this phenomenon to chlorophyll-*a*, a hydrophobic pigment which is released during cell

extraction and remains even after the centrifugation process. Besides extraction, UAE has other applications, such as the homogenization of solids in liquid medium and emulsification of immiscible phases [27]. Therefore, the formation of micellar structures of chlorophyll in the aqueous medium may have occurred [46] or some cell fragments containing chlorophyll may have been reduced in size by UAE. They did not precipitate in the pellet after centrifugation [13].

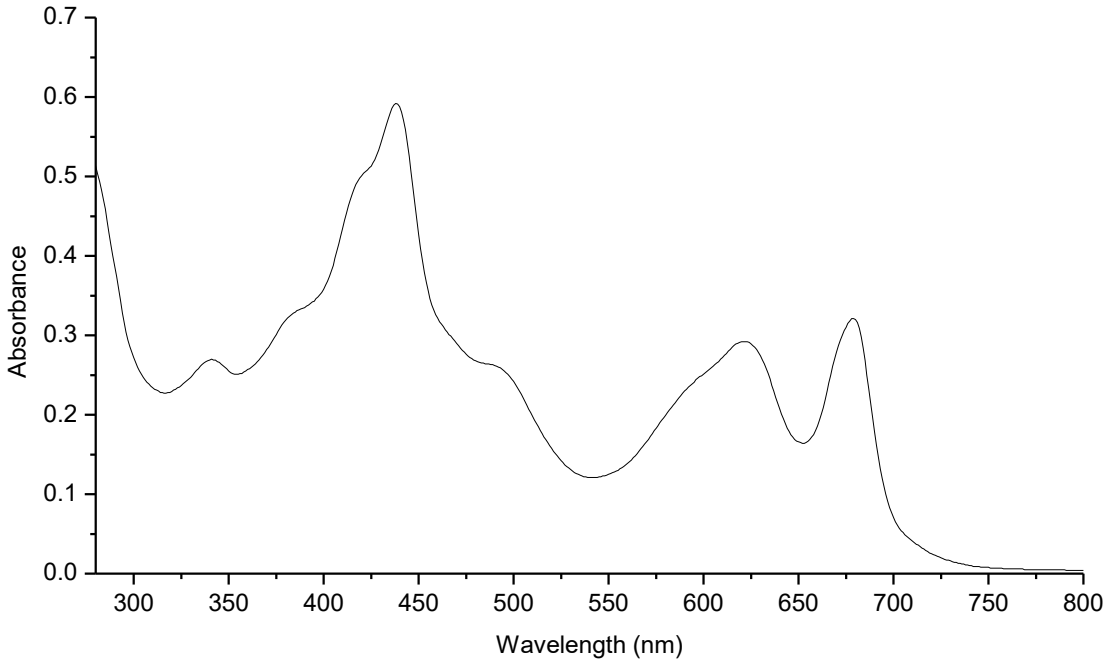

FIGURE 5. Absorption spectrum of the extract with phycobiliproteins and carbonic anhydrase after 12-min UAE (biomass concentration of 0.2 g/L).

Chlorophyll-*a* from *Spirulina platensis* shows maximum absorption at the wavelength of 680 nm [19]. A peak with maximum absorption may be perceived at the wavelength of 679 nm (Figure 5), which is very close to the maximum absorption of chlorophyll-*a*.

In the absorption spectrum (Figure 5), several absorption peaks, besides C-phycocyanin (λ_{max}=620 nm) and allophycocyanin (λ_{max}=652 nm), can be observed. Carotenoids, mostly hydrophobic, exhibit maximum absorption in the range from 410 to 510 nm. Carotenoids were similar to chlorophyll, since the absorption spectrum has several peaks ranging from 410 to 510 nm.

4. CONCLUSION

Both phycobiliproteins C-phycocyanin and allophycocyanin and the enzyme carbonic anhydrase were simultaneously extracted from *S. platensis* LEB-52 by UAE. At biomass concentration of 0.2 g/L, 12-min UAE was sufficient to obtain phycobiliproteins and esterase-expressed CA. To determine hydratase-expressed CA, biomass concentration of 23 g/L and 8-min extraction time were needed. UAE was efficient to disintegrate cells; however, it was not selective to extract phycobiliproteins, since it led to concomitant extraction of other compounds.

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