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C-phycocyanin from *Arthrospira maxima* LJGR1: production, extraction and protection

[1]Antonio-Marcos E., [2]Hernández-Vázquez L., [3]Olguín EJ., [4]Monroy O. & [5]Navarro-Ocaña A.
[1,2,4]Biological and Health Sciences Division, Universidad Autónoma Metropolitana; Av. San Rafael Atlixco 186, Col.Vicentina, 09340 Iztapalapa, Cd de México.
luna.gad@gmail.com, monroy@xanum.uam.mx
[3]Environmental Biotechnology Group. Institute of Ecology.Antigua a Coatepec 351, Congregación El Haya, 91070 Xalapa, Veracruz.
[5]Facultad de Química, Universidad Nacional Autonoma de Mexico. Av. Universidad 3000, CircuitoExterior, Ciudad Universitaria 04510 Coyoacán, Cd. de México.
arturono@unam.mx
1st

ABSTRACT

C-phycocyanin (C-PC) overproduced by *Arthrospira maxima* LJGR1 under high urea and salt concentrations was extracted by different methods and evaluated as an antioxidant activity. *A. maxima* was grown in flat plate photobioreactors, using modified Zarrouk medium with 624 mg L^{-1} urea and 15 g L^{-1} NaCl to produce 11 mg C-PC $L^{-1}d^{-1}$. Agitation, sonication at 35 and 45°C and enzymatic lysis of the cell wall were tried. The C-PC yields, purity and inhibition of the oxidant radicals (DPPH & ABTS) obtained by the best extraction methods were 215 mg C-PC g^{-1}, 0.74, 92% and 29%; when agitation and 221, 0.75, 87% and 27% using sonication at 45°C. Chia mucilage and amaranth flour coatings best preserved C-PC antioxidant activity from temperature variations and up to six days shelf life at 20°C. An activation energy of 160 kJ mol^{-1} has been obtained with chia mucilage, and the least color degradation rate ($r\Delta E^*$) of 4.2 d^{-1} with amaranth flour.

Indexing terms/Keywords

Keywords: C-phycocyanin, agitation, sonication, amaranth, chia.

Academic Discipline And Sub-Disciplines

Biotechnology

SUBJECT CLASSIFICATION

Antioxidant compounds, production and coating

TYPE (METHOD/APPROACH)

Experimental

INTRODUCTION

Arthrospira maxima LJGR1 is isolated from the alkaline and saline waters (pH 10.2-10.6, 0.36 and 3.5% NaCl) of the Texcoco Lake in Mexico. This strain has been identified, morphologically and genetically, as *Spirulina maxima* or *S. geitleri* (Ciferri *et al*., 1983; Dadheech *et al*., 2010). The genus *Arthrospira* produces bioactive compounds as C-phycocyanin (C-PC), β-carotene (βC) and γ-linolenic acid (γLA) which have numerous applications especially in the health area. However it is mainly characterized by the production of C-PC; around 195 mg C-PC g^{-1}, the highest concentration (Chaiklahan *et al*., 2010; Hifney *et al*., 2013; Öztürk-Ürek & Tarhan, 2012). The C-PC is a blue phycobiliprotein that is exclusively synthesized by microalgae, predominantly *Arthrospira*. This pigment is composed of a protein and a no-proteic portions, the latter being a chromophore called Phycocyanobilin (PBC). C-PC purity is evaluated based on the absorbance ratio A_{620}/A_{280}, which correspond to C-PC and total protein, respectively (Fernández-Rojas *et al*., 2014).

By modifying the nitrogen and carbon sources, and the type and intensity of light the C-PC yields can be improved (Chaiklahan *et al*., 2010; Ajayan *et al*., 2012). Also the NaCl concentration can increase the cell protein. For example with 0.22 M NaCl more protein was obtained than with 0.6 M NaCl (Ravelonandro *et al*., 2011). Ammonium is how the cell assimilates nitrogen into glutamate, it is energetically preferred to NO_3^-, as it only needs one reductant and one ATP while the latter requires nine reductants and one ATP. Urea, another N-source, hydrolyzed by urease present in *Arthrospira* can be important in C-PC accumulation (Ajayan *et al*., 2012; Oliver *et al*., 2012). Another factor that influences the natural bioactive compounds performance is the type of extraction: supercritical CO_2, microwave and ultrasound-assisted extraction (UAE). Which reduce the extraction time and the amount of solvent and yield a higher extraction ratio (Ruen-ngam *et al*., 2011). The enzymatic extraction can be a feasible tool for the release of cellular components and sometimes the antioxidant properties are better preserved (Pasquet *et al*., 2011). Experiments *in vivo* and *in vitro* have determined that C-PC obtained from *Arthrospira*, has the ability to decrease the effects of different reactive oxygen species (Bertolin *et al*., 2011). Antioxidants can deactivate a free radical or reduce an oxidant by donating an electron and forming an antioxidant radical cation, followed by a reversible deprotonation (Tan & Shahidi, 2014). The most commonly used *in vitro* methods to determine the antioxidant capacity towards the chromogenic substances of the radical, whose loss depends

on the kind of antioxidant and its concentration (Kuskoski *et al*., 2005), are the ABTS (2,2′-azinobis-[3-ethyl-benzthiazoline-6-sulfonic acid]) and the DPPH (2,2-diphenyl-1-picrylhidrazyl).

The use of C-PC is limited in the food industry due to protein precipitation or blue color loss, which drastically decreases between 50-60°C. Chaiklahan *et al*., (2012) demonstrated that 47°C is the critical temperature for stability of this pigment and the best color clarity and solubility occurs at pH 5.5-6, while C-PC is irreversibly denatured at 65°C. In order to achieve pigment protection, various coating materials have been used; these are mainly polysaccharides such as maltodextrin, alginate, chitosan and pectin. Maltodextrin is the most broadly used, particularly to protect β-carotene and betalain (Ge *et al*., 2009; Gandía-Herrero et al., 2013; Donhowe *et al*., 2014), and it is the most commonly found in food industry (Botrel *et al*., 2014). Alginate and chitosan have been used in CF encapsulation (Yan *et al*., 2014). Other coatings like the Mexican nopal cactus mucilage, chia mucilage, amaranth flour and inulin are seldom used. However, recently it has been used as a coating material inulin proteins and mucilage chia has great potential in the food industry as a coating. It has also been determined that amaranth flour has barrier properties of steam as a result of the amylose and amylopectin and their natural interactions with lipids (Tapia-Blácido *et al*., 2011; Segura-Campos *et al*., 2014; Botrel *et al*., 2014). Coating materials can effectively slow down spoilage and increase pigment and bioactive compound lifespan (Lacroix & Dang, 2014). Furthermore, they may improve the optic properties, and the thermal and mechanical stability of active compounds (Mishra *et al*., 2008).

1. MATERIAL AND METHODS

1.1 Samples of *Arthrospira*.

The *A. maxima* LJGR1 strain was obtained from the Algae Continental Laboratory collection at the Facultad de Ciencias, UNAM. It was cultivated at the Institute of Ecology with modified Zarrouk medium with 10.4 mM of urea as a nitrogen source and 0.26 M NaCl at 30±2°C. In acrylic flat plate photobioreactors (50 x 30 x 5 cm^3) agitated by air diffusers and with a light (photon) intensity of 108 μmol m^{-2} s^{-1} during 14 days, from an initial biomass concentration (X_o) of 0.06 a 0.08 g L^{-1}. The biomass concentration was determined by the method used by Bhattacharya *et al.* (2005) taking 10 mL from the agitated culture, every 2 days.

1.2 Extraction, centrifugation and quantification of C-PC

To determine the phosphate buffer concentration that would yield the highest amount of C-PC extracted by agitation, several concentrations were tried (at 0.025, 0.05, 0.1 and 0.2 M at pH 6.5). The highest yield was obtained with the 0.1 M solution so therefore in all subsequent extractions, 0.01 gramos of *Arthrospira* biomass was suspended in 1 mL of 0.1 M phosphate buffer at pH 6.5. The suspensions were centrifuged at 4800 g for 10 min (Chaiklahan *et al*., 2011) and supernatant was quantified by spectrophotometry at 620 nm.

By agitation: with in an orbital shaker at 300 rpm for 4 h at room temperature.

By sonication: with a Cole-Parmer 8892 sonicator at a frequency of 40 kHz for 25 min (Oh *et al*., 2011 modified) at a fixed temperature. A set of different temperatures was tried to obtain the highest yield (5, 15, 25, 35, 45, 55 y 65°C).

By lysozyme: For lysozyme extraction Sekar & Chandramohan, (2008) report a concentration between 1 and 10 mg lysozyme mL^{-1}. Was used 1.5 mg of lysozyme was added to 1 mL of the suspension and was stirred for 2 h at 200 rpm at 37°C (Sekar & Chandramohan, 2008).

1.3 Antioxidant assays: ABTS and DPPH

On C-PC extracted by different, soniction at 35 & 45 °C, agitation and lysozyme.

$ABTS^{\bullet+}$ radical-scavenging assay: $ABTS^{\bullet+}$ radical cation was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and keeping the mixture in dark at room temperature for 16-24 h. The solution was diluted with water to an absorbance of 0.70 ± 0.02 at 754 nm. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) at 1, 10, 20 and 30 min. A Trolox calibration graph was prepared (Erkan *et al*., 2008).

$DPPH^{\bullet}$ radical-scavenging assay: The DPPH radical was formed by adding 1.1 mg of DPPH diluted in 10 mL of methanol. Extract concentrations which caused 50% of antioxidant activity of DPPH were expressed as IC_{50} (Erkan *et al*., 2008).

In both techniques, a volume of 100 μL of the radical solution was added to the plate wells and the same amount of extracts (0.22, 0.42, 0.62 and 0.82 mg C-PC mL^{-1}) was also added. Radicals were read by spectrophotometry (Epoch, with Gen 5 software) at a wavelength of 517 nm (DPPH) and 754 nm (ABTS).

1.4 Coating used:

To favor the antioxidant capacity of C-PC, different coatings were used: inulin powder (obtained from the juice of *Agave tequilana* Weber, sold as *e-nature)*, yellow dextrin (Drogueria Cosmopolita S.A. de C.V.), pectin from citrus peel (SIGMA), chia (*Salvia hispanica* L., Naturchiamex Products S.A. de C.V.), nopal (*Opuntia ficus indica*), amaranth (*Amaranthus hypochondriacus*) commercial flour and aloe (*Aloe barbadensis*) bought in a traditional market.

Colorimetric method for the stability evaluation of C-PC with different coatings. Coated samples of C-PC were dispersed at 10 mg mL^{-1} in a phosphate buffer solution, with a final concentration of 1 mg C-PC mL^{-1}. The color change

(ΔE*) was measured by the CIELAB method during 15 days at 20±2°C. The color model parameters (L*a*b*) were measured at 10° and primary illuminant D65 (Gandía-Herrero *et al*., 2013).

Thermal resistance method. Samples in relation to 1:10 C-PC- coating (w/w) were put in a water bath at 60, 70, 80 and 90°C for 30 min. Then it was centrifuged at 4800 g for 10 min (Chaiklahan *et al*., 2011) and supernatant was quantified by spectrophotometry at 620 nm.

1.5 Statistical analysis

Using the statistical package SPSS 15, at a significance level of P<0.05.

2. RESULTS AND DISCUSSION

2.1. Production of *A. maxima* and C-PC

One of the most important factors to obtain C-PC is the type and concentration of nitrogen source (Öztürk-Ürek & Tarhan, 2012; Ajayan *et al*., 2012); another factor is a concentration of 0.3 M NaCl (Hifney *et al*, 2013). In this paper, the *A. maxima* LJGR1 strain was cultured at 0.26 M NaCl and 10.4 mM urea and a biomass concentration of 1 g L^{-1}.

2.1.1. Analysis and quantification of C-phycocyanin using different extraction methods.

The three methods preferentially used for C-PC extraction are agitation, sonication and lysozyme cell cleavage all with phosphates buffer as solvent.

Extraction by sonication was optimized for temperature. The higher C-PC concentrations were obtained at 35 and 45°C, while at 55°C the C-PC concentration decreased 50-64% compared to that at 35°C (Figure 1).

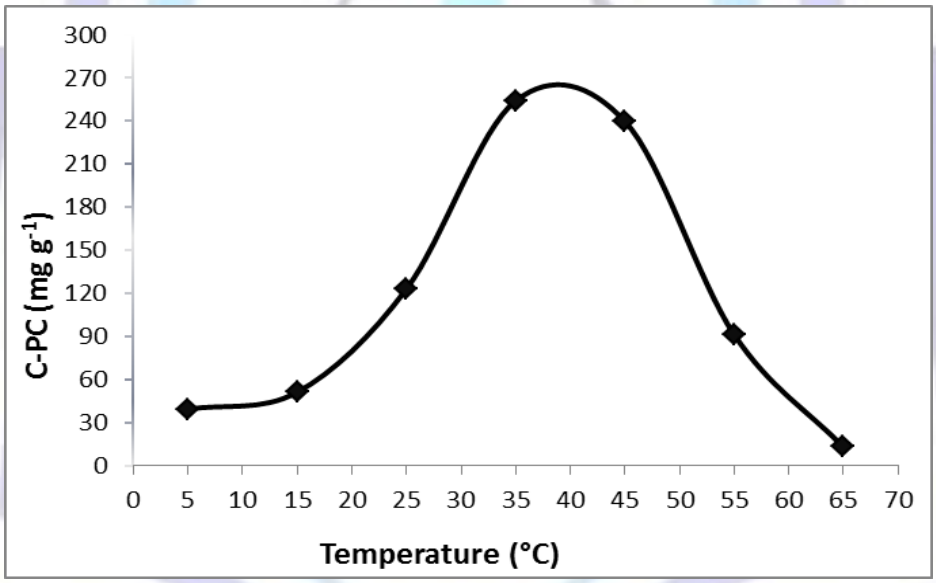

Figure 1. Quantification of C-PC at different temperatures (°C).

The best C-PC concentration obtained by enzymatic extraction was 281±2 mg C-PC g^{-1} but with a lower purity than that obtained by agitation and sonication at 45°C (Table 1). These yields are 13 to 32% higher than the best values previously reported (Chaiklahan *et al*., 2010; Öztürk-Ürek & Tarhan, 2012; Ajayan *et al*., 2012). Based on these results, it can be said that the C-PC yields and purity depend on the type of culture and the extraction method.

Table 1: C-PC concentration and purity.

Extraction method	Concentration (mg g^{-1})	Purity A_{620}/A_{280}*
Enzymatic	281±2[a]	0.29±0.001[c]
Sonication 45°C	221±13[b]	0.75±0.06[a]
Maceration	215±17[b]	0.74±0.025[a]
Sonication 35°C	187±13[c]	0.63±0.025[b]

n=3. Values with different superscripts indicate significant difference (P<0.05) among the extraction methods

2.1.2 In vitro evaluation of antioxidant capacity

On the other hand, it is known that the capacity of C-PC to trap reactive oxygen species is due to its chromophore structure, which is similar to bilirubin (Zheng *et al.*, 2013). However, other studies have shown that the apo-protein fractions affect the antioxidant function because they interact with the chromophore fractions (Zhou *et al.,* 2005). Because of this, the apo-protein fraction is likely to be the most affected and therefore the extraction method is important to keep the antioxidant activity.

The C-PC yields, purity and inhibition of the oxidant radicals (DPPH & ABTS) obtained by the best extraction methods were 92% and 29%; when agitation and 87% and 27% using sonication at 45°C (figure 2).

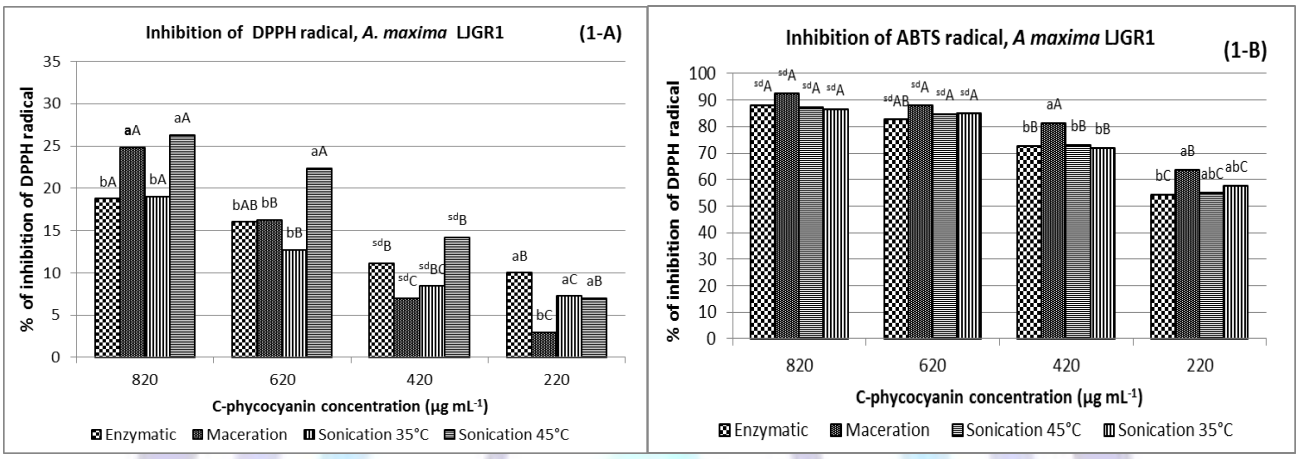

Figure 2. DPPH (A) and ABTS (B) radicals inhibition of C-PC obtained from: (1) *A. maxima* LJGR1 for 30 minutes.

Letters with significant difference (P<0.05) (lowercase letters significant difference of different extraction techniques and uppercase letters significant difference inside concentrations of (C-PC). ^{sd} without significant difference.

C-PC from sonication and agitation inhibited the DPPH$^{\bullet}$ radical by 50% (IC_{50}) with 1.5±0.18 mg C-PC mL^{-1} (figure 3A). IC_{50} of 0.08 and 1.75 mg C-PC mL^{-1} with purities of 1.5 and 0.58 have been obtained (Gantar *et al*., 2012 and Thangam *et al*., 2013 respectively). Chen & Wong (2008) reported IC_{50} = 0.7 mg mL^{-1} DPPH$^{\bullet}$ radical inhibitions. As shown, purity is determinant in the DPPH$^{\bullet}$ radical inhibition. Inhibition of the ABTS radical (as TEAC) is dependant on the extraction method where the sonication at 45°C yields the best results (figure 3B) obatining an initial TEAC concetration of 40 µg mL^{-1} and an increase rate of 0.73 µg mL^{-1} min^{-1}. A trolox equivalent of 219±2.1 µM g^{-1} of C-FC is obtained via sonication at 45°C. This is more than what is reported for other fruits such as pomegranate, soursop and olives, where 81±2, 40.6±0.1 and 23.6±0.1 µM g^{-1}, respectively, were obtained (Fu *et al*., 2012). 108.5±2.2 µM g^{-1} of *A. platensis* SAG 85.79 biomass has been obtained, where a correlation of antioxidant activity with phycobiliprotein was observed, more than with this cyanobacteria's carotenoids (Tarko *et al*., 2012). Another work which made use of myofibrillar proteins from carp's (Cyprinus carpio L.) duodenum achieved similar trolox equivalent concentrations to the ones attained in the present work. They obtained 232±4.5 µM g^{-1} with in-vitro experiments and 173±5.7 µM g^{-1} with ex-vivo experiments (Borawska *et al*., 2016).

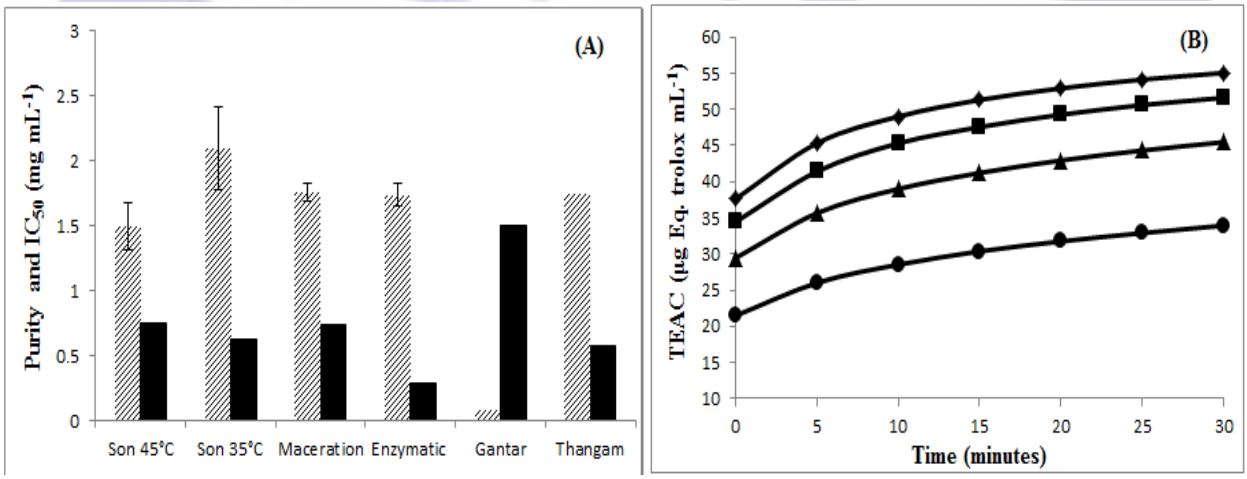

Figure 3. C-PC antioxidant inhibition with different extraction methods. A) As TEAC and B) Purity and IC_{50} for DPPH radical. Son= sonication, ■=purity and ▒ IC_{50}. ♦= sonication 45°C ■= maceration, ▲= sonication 35°C, ●= enzymatic.

2.1.3. Evaluation of heat resistance and color at different coating treatments

C-PC degradation is temperature-dependent, its activity being completely lost (93%) at 65°C but when a coating is introduced this dependency is reduced (figure 4A) showing a protection against temperature rises. For example, with an aloe mucilage coating, at 60°C and higher temperatures, allows 65% C-PC activity loss while the chia mucilage coating only allows 18% and at 90°C it losses 48%. This behavior suggests a change in the activation energy (Ea) for the C-PC degradation reactions (figure 4B); where those C-PC+Coating systems with higher Ea are more affected by temperature changes than those with lower Ea, suggesting that the chia mucilage (Ea= 160 kJ mol^{-1}) is the best coating against temperature changes. This is important since C-PC can retain its color property because its proteins do not degrade under the coating protection.

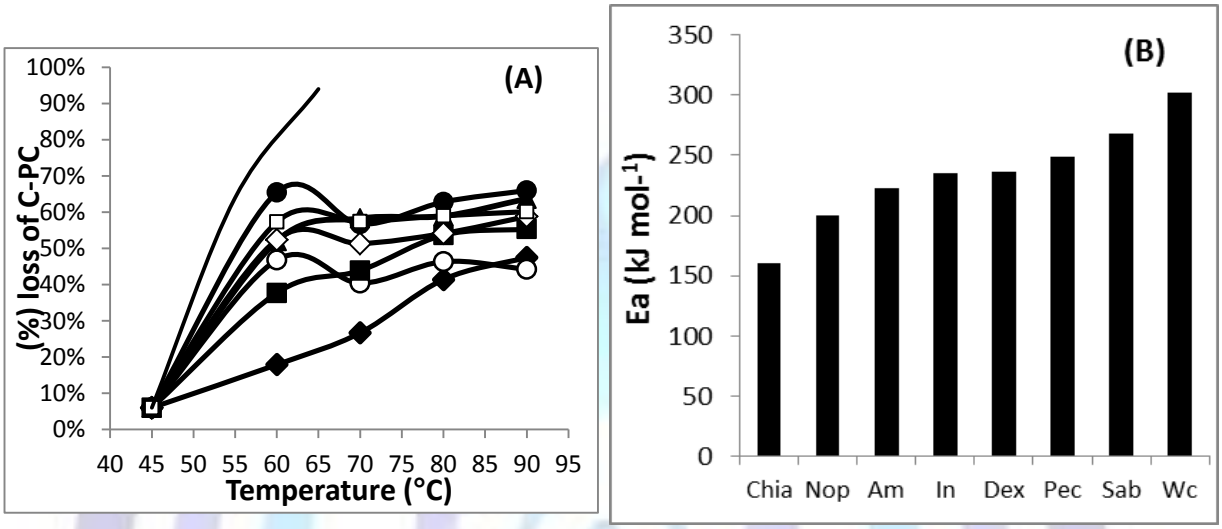

Figure 4. (A) Loss of C-PC without and with different C-PC coatings; (B) Activation energy of C-PC degradation with different coatings. ■= nopal (nop), □= pectin (pec), ▲= inulin (In), ◊= dextrin (Dex), ○= amaranth (Am), ●= aloe (Sab), ♦=chia and — = without coating (Wc).

Other experiments have reported a 30-38% degradation of CF when a 20-40% glucose was added (Chaiklahan *et al*., 2012), and a 20% degradation of CF with a pH of 5-6 and using 30% sorbitol as coating (Antelo *et al*., 2008) at a temperature of 59-62°C. Other experiments, which employ a saturated fructose solution at 80°C for 30 minutes obtain a 40% degradation of CF (Martelli *et al*., 2014). Considering both reports it can be affirmed that the chia coating at 60°C has a similar percentage to those obtained with sorbitol, and it is greater than that achieved with glucose. The percentage acquired using chia coating at 80°C is similar to the one obtained with fructose. The C-PC with different coatings changes color through time (figure 5). All coatings preserve C-PC color for three days, amaranth, nopal and inulin can keep color up to six days, thereafter amaranth losses color. The coating materials in which color degradation (ΔE*) of C-PC was higher were pectin and the mucilages from *nopal* cactus, *chia* and *aloe* obtained a ΔE* 105, 96, 107 and 108, respectively (table 2). The lowest color degradation was obtained with amaranth (ΔE* 60) with at a rate of ($r_{\Delta E^*}$) 4.2 day^{-1}.

Table 2. Color parameters (CIELAB) of C-PC de *A. maxima* LJGR1 with different coated

	Initial					15 days				
	Amaranto	**Nopal**	**Inulin**	**Chia**	**Dex**	**Amaranto**	**Nopal**	**Inulin**	**Chia**	**Dex**
L*	98.9±0.5	99.9±0.13	100.5±0.0	97±1.3	99.8±0.2	100.6±0.2	102±0.01	101.7±0.2	99±0.5	101.5±0.1
a*	-3.2±0.4	-1.7±0.04	-2.54±0.0	-3±0.5	-3.3±0	-1.2±0.14	-0.45±0.01	-0.6±0.02	-0.6±0	-0.73±0.1
b*	-2.9±0.5	-0.8±0.08	-2.5±0	-1.8±0.5	-2.2±0.6	0.39±0.16	1.3±0.058	0.83±0.28	2.5±0.3	1.6±0.06
C*	4.3±0.6	1.9±0.06	3.54±0.07	3.5±0.7	4±0.6	1.3±0.08	1.4±0.05	1.05±0.16	2.6±0.3	1.8±0.05
h°	222±2	205±1.7	224±0.64	211±5	213.7±5	161.9±8.8	109.3±1.3	127±16	104±2.3	114±3
					ΔE*	60	96	97	107	100

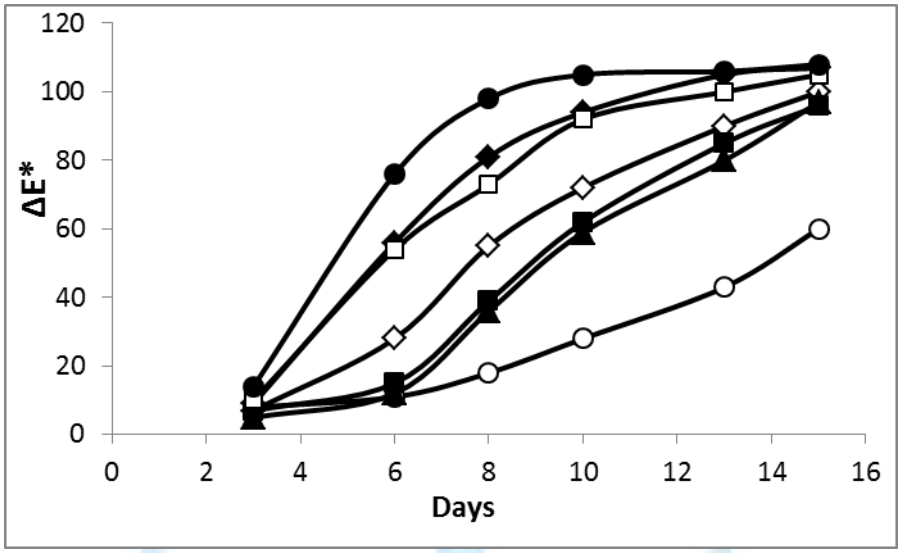

Figure 5. Color change (ΔE*) with time of C–PC with different coatings. ■= *nopal,* □= pectin, ▲= inulin, ◊= dextrin, ○= amaranth, ●= aloe and ♦=chia.

4. CONCLUSIONS

When *Arthrospira maxima* LJGR1 was cultivated in a flat plate photobioreactor using high concentrations of urea (10.6 mM) and NaCL (0.26 M), an overproduction of 221±13 mg g^{-1} of C-FC with a purity of 0.75±0.06 was achieved. This was done with the sonication extraction method at 45°C. The antioxidant capacity of this extract was evaluated with DPPH and ABTS methods, thus obtaining an IC50 of 1.5 mg C-FC mL^{-1} and 55 µg mL^{-1} of trolox equivalent or 219± µM g^{-1} of C-FC. The trolox equivalent concentration is higher than the one reported for various fruits such as pomegranate, soursop and olives, obtaining concentrations ranging from 24±0.1 µM g^{-1} to 81±2 µM g^{-1}. The concentration is also similar to the one achieved using carp's myofibrillar proteins.

From these materials used, one can conclude that chia protects C-PC from temperature degradation ranging between 60-90°C, getting an 18% CF loss at 60°C compared to an 80% loss when no coating is used. All coatings preserve C-PC color for three days; amaranth, nopal and inulin can keep color for up to six days, therefore amaranth loses color at a rate of (rΔE*) 4.2 day-1, nopal at 7.4 day-1, and inulin at 7.6 day-1, with a ΔE* of 18, 36 y 39, repectively, at day 8.

ACKNOWLEDGMENTS

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Author' biography with Photo

Emma Gabriela Antonio Marcos

Miss Emma Gabriela Antonio Marcos has a bachelor's degree in Food Engineering, a master's degree in Biotechnology and is presently doing her doctorate in Biotechnology. Her research interests are in food technologies for native people in Oaxaca where she was born in 1983.

She has attended several conferences to present oral (2) and poster (6) contributions which have published in the respective proceedings. She has in preparation one more paper on the use of *Arthrospira maxima* as a biofactory for antioxidant substances.

She have been lecturing food technology in both Iberoamerican University and Metropolitan Autonomous University in Mexico City where she also is working on the antioxidant capacity of *Salvia* and sweet potato extracts.