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Arthrospira maxima OF15 biomass cultivation at laboratory and pilot scale from sugarcane vinasse for potential biological new peptides production

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Abstract

An environmental friendly process was developed to produce *Arthrospira maxima's* biomass from sugarcane vinasse, which was generated in a bioethanol production chain, at laboratory and pilot scale. Peptides fractions were than obtained from enzymatically hydrolyzed biomass. High microalgae biomass productivities were reached (0.150 g $L^{-1}day^{-1}$) coupled with a significant reduction of BOD and COD (89.2 and 81%, respectively). Three peptide fractions were obtained from microalgae biomass through single or sequential enzymatic hydrolysis. Antioxidant, antimicrobial, anti-inflammatory, and/or anti-collagenase activities of biopetides' fractions were observed. The PHS showed multi-biological activities. The three peptides fractions could be potential candidates for different applications in pharmaceutical, cosmetic and food industry.

Keywords: peptides, sugarcane liquid residue, microalgae, antioxidant, antimicrobial, anti-inflammatory.

1. Introduction

In the process of sugarcane transformation into bioethanol, the liquid waste, denominated vinasse, is generated in large volumes. This effluent is originated in the distillation stage of bioethanol production from sugarcane fermentation (Fig. 1) and it is usually seen as the effluent with the greatest environmental impact. Vinasses's physico-chemical composition is variable (Table 1) and depends on the nature and composition of the raw material, the system used in the preparation of the must, the type of must, the fermentation method, the variety of yeast and type of distillation apparatus (Christofoletti et al., 2013). It presents a high organic content, with macronutrients such as carbon, nitrogen, phosphorus and potassium (Santana et al., 2017). Generally, sugarcane vinasse has a dark color, strong odor and a water content of approximately 93%.

Fig.1

According to Leme and Seabra (2017) for each liter of ethanol produced, around 10 to 15 L of effluent are generated. Consequently, between 2014 and 2015, approximately 336 million m^3 of sugarcane vinasse were generated in Brazil. The effluent was mainly used in processes of fertigation without prior treatment. Nonetheless, there is an increasing concern about the environmental impacts that can be generated with the application of effluents to the soil, such as salinization, contamination of aquifers, depletion in the concentration of oxygen in the soil, acidification, contamination with nitrates, chlorides and metals such as lead and zinc (Fuess and Garcia, 2014). According to Plaza (1999), fertilization with sugarcane vinasse partially replaces the use of chemical fertilizers, but it greatly increases the content of Ca, K, P and N in the soil (Table 1).

Table 1

The cyanobacteria *Arthrospira* (synonymie *Spirulina)* has been used by humans as food since ancient times due to its high protein content (43-70%), which can be hydrolyzed into biopeptides (Yu et al., 2016). It has been experimentally proven to have some activities in health conditions, such as anti-inflammatory, antioxidant, antiviral, anti-bacterial, hypertensive, immunomodulatory, anticancer (Jang and Park, 2016; Ovando et al., 2018). Furthermore, the pharmaceutical industry has shown a great interest in *Arthrospira* for its nutritional and biotechnological properties, as well as its GRAS (Generally Regarded as Safe) status by the FDA (Food and Drug Administration) (Oliveira et al., 2013).

The importance of novel bioactive compounds significantly increased in the last years. Bioactive peptides (BP) are specific protein fragments, they have a positive impact on body functions and may influence health (Singh et al., 2014). BP can be obtained from diverse raw materials, such as plants, macroalgae, microalgae, seafood, and fungi (Hayes, 2013).

Recently, there has been great interest in the use and evaluation of peptides that show biological activities. Antioxidant peptides are important, because of their protective effect in lessening the severity of diseases; considering that in our body oxidative stress can cause serious damage to proteins, lipids, and DNA by subtracting electrons (Nurdiani et al., 2016). These peptides act by preventing binding of other molecules to oxygen, and by the inhibition of free-radicals action (Kang et al., 2011). On the other hand, the antioxidant peptides can present aminoacids with nucleophilic sulfur-containing side chains (Cys and Met) or aromatic side chains (His, Tyr and Met), which can easily donate hydrogen atoms (Hayes, 2013). Also, iron-chelating peptides have action in the metabolic pathways of autoxidation mechanisms and have the capacity to increase non-heme iron absorption and bioavaility in the body (Wu et al., 2012).

These peptides may present a structure that contains Met, Gln, Lys and Arg (de Castro and Sato, 2015).

The antimicrobial peptides (AMPs) are of enormous interest because of their inhibitory activity against several pathogens and their ability as stimulators of the human immune system. AMPs are known as host defense peptides due to their innate presence in the immune system in animals, insect, plants, and humans with the role of defending them against the diversity of bacterial, fungal, viral, and other pathogenic agents (Zhang and Gallo, 2016). Furthermore, it is known that AMPs have the capacity to play other important roles in such processes as angiogenesis, attraction of leukocytes, inflammation, and inhibition of cell proliferation (Phoenix et al., 2013). The AMPs present common characteristics such as high proportion of hydrophobic residues like Leucine (Leu), Isoleucine (Ile), Valine (Val), Phenylalanine (Phe), and Tryptophan (Trp) (Haney and Hancock, 2014).

In the same way, anti-inflammatory peptides have received much attention due to their potential in the therapeutic treatment for several health problems (cancer, aging, allergy, asthma, autoimmune diseases, and coeliac disease) (Vo et al., 2013). These peptides act in the inhibition of hyaluronidase enzyme and prevent hydrolysis of hyaluronic acid, helping in the regeneration, proliferation, and repair of tissues. Besides, they can increase elasticity and decrease the loss of moisture on the skin (Prado et al., 2016). On the other hand, the anti-collagenase peptides prevent degradation of the extracellular matrix (ECM) by blocking the action of collagenase (Chattuwatthana and Okello, 2015). These peptides can present a sequence, which resembles the cleavage site in native collagen. Their structure can be composed of hydrophobic residues such as Leu, Ile, Val, and Phe (Aureli et al., 2008). Thus, the objective of this study is the generation of bioactive peptides from pilot scale production of *Arthrospira maxima* (syn *Spirulina maxima)*

with the valorization and decrease of environmental impact of sugarcane vinasse. Biopeptides were produced through a one-step or sequential biomass enzymatic hydrolysis with further separation and purification, and *in vitro* bioactivity tests to evaluate antioxidant, antimicrobial, anti-inflammatory, and/or anti-collagenase biological activities.

2. Material and methods

The main steps of biomass peptides production and analyses are presented in the following schema (Fig. 2).

Fig.2

2.1 Materials

All solvents used were of analytical grade. 1,1-diphenyl-2- picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (Ferrozine), Iron(II) chloride, Disodium ethylenediaminetetraacetate dihydrate (EDTA-Na_2), Subtilisin A from *Bacillus licheniformis* (EC 3.4.21.62), Pepsin from porcine gastric mucosa (EC 3.4.23.1), hyaluronidase from bovine testes (EC 3.2.1.35) and a Collagenase Activity Colorimetric Assay Kit were all purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Analysis of sugarcane vinasse composition

Sugarcane vinasse, from *Central Energética Moreno Açúcar e Álcool – Luis Antônio-S,* Brazil, was analyzed for its physical and chemical parameters before and after biomass cultivation. Chemical oxygen demand (COD), Biological oxygen demand (BOD) and nitrogen

concentration were determined. COD and BOD were analyzed through spectrophotometry, and the total organic nitrogen was evaluated using the Kjeldahl method according to the standard method of the American Public Health Association (APHA, 1989). Total nitrogen was evaluated by the elemental analysis performed in a CHNS Vario Macro analyser (Elementar, Germany).

Nitrate/nitrite and phosphate were analyzed spectrophotometrically by the standard tests for effluent analysis from AOAC (Horwitz, William, Latimer, 2005) for Nitrate/Nitrite (AOAC 935.48).

2.3 Optimization of *Arthrospira maxima* OF15 biomass production

Arthrospira maxima OF15 inoculum was prepared in 125 mL of vinasse using 250 mL flasks with forced aeration (0.5 vvm) during 5 days at room temperature. The effect of medium dilution (10, 30 and 50%) on *Arthrospira maxima* biomass production was investigated. An assay with Zarrouk medium was also conducted as a control. Batch culture experiments were carried out in 2 L Erlenmeyer flasks with 1.8 L of working volume and constant air flow 0.5 vvm, at 30°C with 2500 Lux and photoperiod of 12 h. The illumination consisted of 32W fluorescent lights, with a photon flux density (PFD) of 31 µmol photons.m^{-2}.s^{-1} and a photoperiod of 12 h (light:dark). For open cultures, conducted from september to march, in Ribeirão Preto-Sp, Brazil (21.29ºS, 47.75ºW), where the average irradiation was 5.57kWh.m^{-2}.day^{-1}, or 1061 µmol photons.m^{-2}.s^{-1} for an average day length of 12.75h and an average pluviometric index of 170 mm/month. Cultures, with an initial cellular concentration of 0.20 g/L, were conducted during 15 days, without additional control of pH. Biomass concentration (productivity and growth), pH, lipid´s concentration, DBO and DQO were evaluated.

2.4 *Arthrospira maxima* OF15 pilot scale biomass production

Arthrospira maxima OF15 biomass was produced at pilot scale in Ouro Fino Agribusiness, Ribeirão Preto, São Paulo, Brazil. Inoculum was prepared in 250 mL flasks with forced aeration (0.5 vvm, volume of air per volume of medium, or 125 mL min^{-1}) during 5 days at room temperature. The microalgal cultures were then successively scaled up to 2.5 L (Erlenmeyer flasks, 0.5 vvm of air), 25 L (carboys, 0.5 vvm of air), 250 L (mini-raceway tanks, agitated by paddle wheels to 20 cm s^{-1} of superficial velocity) up to a 14 m^3 raceway bioreactor (12 x 3 x 0.4 m, also agitated to 20 cm s^{-1} using paddle wheels). The medium for all steps used vinasse diluted with water at 30% v/v and pH adjusted to 9.5-10 with $CaCO_3$. Biomass production was carried out for 15 days at 30°C in small scale, at pilot scale (raceway bioreactors) in environmental conditions with temperature that varied between 21 to 31°C.

After cultivation, biomass was separated using a filter press. The fresh paste was placed in plastic bags, for transportation and extruded to 50 x 70 cm trays and sun dried for 12 h, then transported to a drying chamber for another 12 h at 60°C. Dried biomass was used in the next steps for protein extraction and further enzymatic hydrolysis.

2.5. Proximate composition of *A. maxima* biomass

Analyses of *A. maxima* OF15 biomass were performed to determine its proximate composition. Analyses of total protein, ashes, carbohydrate and lipids composition were carried out according to the methods described by the Association of Official Analytical Chemists (Horwitz, William, Latimer, 2005). Total protein was estimated by the Khjeldhal method (digestion by $H_2SO_4/CuSO_4$ followed by distillation and titration of the evolved NH_3, AOAC

984.13), ashes were determined by calcination at 600°C for 2h (AOAC 942.05), lipids were determined by exhaustive extraction with ethyl ether, followed by drying and weighing the residue (AOAC 920.39), and carbohydrates were estimated by difference.

2.6. Protein extraction from *Arthrospira maxima*

Soluble protein extraction was performed as described previously (Wang and Zhang, 2016), with modifications. *A. maxima* OF15 powder (100 g) was suspended in 1 L pure water. The suspension was frozen at −20°C for 4 h and thawed at 37°C, with a total of 4 freeze– thaw cycles. After homogenization (2800 x *g* 30 s, 11000 x *g* 1 min, 2800 x *g* 30 s), the mixture was ultrasonicated under 160 W power for 25 min (every 10 s with 13 s interval) in an ice bath. Afterwards the extract solution was centrifuged at 10,000 x *g* and 4ºC for 15 min. The protein content of the supernatant was determined by Bradford protein assay.

2.7. Enzymatic hydrolysis of *A. maxima* biomass protein

The protein extract of *A. maxima* OF15 was diluted to 3% in 0.1 M citrate phosphate buffer in pH 7 and pH 3. Enzymatic hydrolysis was carried out with endopeptidases (pepsin and subtilisin A under specific conditions, individually or both sequentially (Fig. 3). The first hydrolysate was prepared using subtilisin A at an enzyme/substrate (E/S) ratio of 4% w/w, 60ºC, pH 7 and during 5 h. The second hydrolysate was obtained using pepsin at an enzyme to substrate ratio (E/S) of 3% w/w, 37ºC, pH 2 and during 5 h. The third hydrolysate was prepared using both enzymes sequentially. In this case, the protein solution was first treated using subtilisin A under the above conditions during 4 h. After inactivation at 85ºC, the solution was

then hydrolyzed by pepsin, under the conditions described above, during 3 h. The reactions were stopped by heating the solution in a boiling water bath for 10 min. The obtained hydrolysates were centrifuged at 6000 x *g* for 10 min. The liquid fraction was conserved and stored at -18ºC for further analyses.

Fig. 3

2.6 Determination of the degree of hydrolysis

The method used to determine the degree of hydrolysis (DH) was performed as described by Hoyle and Merritt, 1994. The three hydrolysates obtained before were evaluated: 1 mL aliquots were denatured by the addition of 9 mL of 6.25% (w/v) trichloroacetic acid (TCA) solution and left to settle for 10 min. The solution was then centrifuged for 5 min at 3000 x *g* and the precipitate removed. The soluble proteins content from the supernatant was determined using the Bradford (1976) method. DH was calculated as shown in Eq. 1:

$$DH\ (\%) = \left(\frac{PSt_i - PSt_0}{Ptotal}\right) x\ 100 \qquad \text{(Eq. 1)}$$

Where PSt_o, corresponds to the amount of soluble protein in TCA 6.25% w/v before enzymatic hydrolysis; PSt_i, is the protein soluble after enzymatic hydrolysis and P_{total} is the total amount of protein in the sample.

2.7 Purification of peptides by ultrafiltration

Peptides obtained from enzymatic hydrolysis were purified by ultrafiltration in a Vivaflow 200 Sartorius (tangential filtration) system. First the hydrolysate was microfiltrated using a 0.22 µm membrane. Then, it was ultrafiltrated using a membrane of 10 kDa molecular weight cut off (MWCO). The permeate fraction containing molecules below 10 kDa was collected and stored at – 80ºC, before lyophilization.

2.8 Peptide lyophilization and quantification

Peptide fractions were lyophilized (Freeze Dryer, ModulyoD, Thermo Scientific, USA) during 24 h at -45ºC to a pressure of 50 mbar. The lyophilized peptide fraction (<10 kDa) of *A. maxima* was solubilized in ultrapure water at a concentration of 1 mg mL^{-1}. Peptide concentration was determined using the Micro BCA Protein Assay Kit (Thermo Fisher) .

2.9 Mass spectrophotometry analysis

Peptide extracts from *A. maxima* were analyzed by MALDI-TOF in the Unit of Protein Chemistry and Mass Spectrometry (Uniprote-MS) of the Federal university of Rio Gande do Sul. Samples were prepared according to Udeshi et al. (2013) and Villén and Gygi (2008).

2.10 DPPH Radical Scavenging -*in vitro* assay

The capacity of the peptides for sequestering the free radical 2,2-diphenyl-1-picryl-hidrazol (DPPH) was performed as described previously (Yu et al., 2016). For the preparation of the DPPH reagent, 4 mg DPPH (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 100 mL 95% methanol. For each peptide fraction, at concentrations of 2, 5, 10, 25, 50, 100 µg mL^{-1} were

used in the assay of each peptide fraction. Vitamin C (0.1 mg/mL) was used as a positive control. A 96-well microplate was used to determine the scavenging activity, where 100 µl of the samples or standard were mixed to 100 µL of DPPH reagent, and incubated for 30 min in the dark at room temperature. After this time the absorbance was measured in a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, USA) at 517 nm. The percentage of DPPH radical scavenging was calculated as shown in Eq. 2:

$$DPPH\ radical\ scavening\ (\%) = \left[\frac{A_0 - A_1}{A_0}\right] x\ 100 \qquad \text{(Eq. 2)}$$

Where A_0 is the absorbance of the control; A_1 is the absorbance of the sample.

2.11 ABTS radical scavenging- *in vitro* assay

The 2,2'-azinobis-3-etilbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay was performed as described previously (Lee et al., 2015). The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS (Sigma-Aldrich, St. Louis, MO, USA) with 88 µL of 140 mM potassium persulfate, and reacting for 16 h at room temperature in the dark. After this time, the ABTS reagent was diluted to 1:45 with ethanol (99%) until reaching an absorbance of 0.700, which was measured in the spectrophotometer at 734 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, St. Louis, MO, USA) was prepared from a stock solution (1 mM) in the concentration range of 2, 5, 10, 25, 50, 100 and 200 µM. Peptide fractions and the positive control (vitamin C) were tested at 2, 5, 10, 25, 50 and 100 µg mL^{-1}. For determining the scavenging activity, a 96-well microplate was used where 100 µl of

the samples or standard were mixed with 100 µL of ABTS reagent, in the dark at room temperature. The absorbance was measured in a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, USA) at 734 nm. The percentage of ABTS radical scavenging was calculated as shown in Eq. 3:

$$ABTS\ \ radical\ scavening\ (\%) = \left[\frac{A_0 - A_1}{A_0}\right] x\ 100 \qquad \text{(Eq. 3)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample.

2.12 Ferrous ion-chelating activity - *in vitro* assay

The ferrous ion-chelating activity was performed as described previously (Wang et al., 2009). Peptide fraction (100 µL) was added in the following concentrations: 1.25, 2.5,5,10 and 25 µg mL^{-1}, and mixed with 135 µL of distilled water and 5 µL of 2 mM $FeCl_2$ in the microplate. The reaction was initiated by the addition of 10 µL of 5 mM ferrozine, and incubated for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm with a PowerWave XS Microplate Spectrophotometer. Distilled water (100 µL) instead of sample was used as the control. For the blank, distilled water (10 µL) instead of ferrozine was used. EDTA-Na_2 was used as reference standards. All measurements were performed in triplicate. The ferrous ion-chelating activity was calculated as shown in Eq. 4:

$$\text{Ferrous ion-chelating activity } (\%) = \left[\ \frac{A_0 - (A_1 - A_2)}{A_0}\right]\ x\ 100 \qquad \text{(Eq. 4)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.13 Antimicrobial activity - *in vitro* assay

The determination of minimal inhibitory concentration (MIC) of a substance was carried out by the Broth microdilution method (EUCAST, 2003).

Minimum Inhibitory Concentration (MIC) Determination

This assay was carried out to determine the antimicrobial potential of the peptide fraction. A 96-well microplate assay was used to determine the MIC: 80 µL of Mueller Hinton broth (MHB) were added to the wells with 100 µL of peptides fractions at different concentrations (0.13, 0.63, 1.25, 6.25 mg mL^{-1}) and inoculated with 20 µL of each bacterial suspension (1.0x 10^7 UFC mL^{-1}) (*Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (ATCC 14028), *Escherichia coli* (ATCC 35218)). Chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control, and the culture medium without the peptide fraction was the negative control. The microplate was sealed and incubated at 37ºC for 24 h. After incubation, the absorbance was measured at 600 nm. The percentage of growth inhibition was calculated as shown in Eq. 7:

$$\text{Grow inhibition (\%)} = 1 - \left(\frac{A_c}{A_0}\right) x\ 100 \qquad \text{(Eq. 7)}$$

Where A_c is the absorbance of the sample, A_o is the absorbance of the control. Finally, the microplate was colored with 30 µl of resazurin indicator solution (0.1%), and incubated for 2 h.

Minimum Bactericidal Concentration (MBC) Determination

After MIC determination of the peptide fractions, an aliquot of 5 µL from all microplate assay wells was seeded in Mueller Hinton Agar (MHA) plates. The plates were then incubated at 37°C for 24 h. The MBC endpoint was defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the MHA plates.

2.14 Anti-inflammatory activity - *in vitro* assay

The anti-inflammatory activity was evaluated by the inhibition of the enzyme hyaluronidase (Type IV), as described previously (Prado et al., 2016), with few modifications. Briefly, the three lyophilized peptide isolates (<10 kDa) of *A. maxima* were used in different concentrations (3.3, 10, 33,100, 333 µg mL^{-1}). The propolis commercial fraction (Bitmel, São José do Rio Preto, SP) was used as positive control. 100 µL of peptide fraction or the positive control was added to 500 µl of the potassium salt of hyaluronic acid (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 5 min at 37ºC. Then, 350 units of the enzyme hyaluronidase type IV-S were added (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37ºC for 40 min. The reaction was inactivated by adding 10 mL of sodium hydroxide solution (4 N) and 100 µL of potassium tetraborate at 0.8 M, and incubated for 3 min at 100ºC. Afterwards, 3 mL of 4-dimethylaminobenzaldehyde (DMAB) were added to the tubes, mixed and transferred to a water bath for 20 min at 37ºC. Finally, the absorbance was measured in a spectrophotometer at 585 nm. The percentage of inhibition was calculated as shown in Eq. 5.

$$\text{Hyaluronidase inhibition activity (\%)} = \frac{A_m}{A_c}\ x\ 100 \qquad \text{(Eq. 5)}$$

Where: A_m is the absorbance of the sample after enzymatic reaction, and A_c corresponds to the absorbance of the control.

2.15 Collagenase inhibition - *in vitro* assay

This assay was performed according to the descriptive instructions supplied with the Collagenase Activity Colorimetric Assay Kit (Sigma). The principles of this assay are based on the enzyme-substrate interaction between collagenase from *Clostridium histolyticum* and the synthetic N-[3-(2- furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA). Peptide fractions (10 µL) were added in different concentrations (10, 25, 50, 75 µg/mL). Negative controls consisted of water and positive control consisted of 10-Phenanthroline. Absorbance was measured at 345 nm. All measurements were performed in triplicate. The collagenase inhibition activity was calculated as shown in Eq. 6:

$$\text{Collagenase inhibition activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] x\ 100 \qquad \text{(Eq. 6)}$$

Where A_0 was the absorbance of the control, A_1 was the absorbance of the sample.

2.16 Statistical analysis

Statistical analysis was performed using one-way and two-way ANOVA tests with the support of Graphpad Prism 305 software.

3 Results and discussion

3.1 Optimization of *Arthrospira maxima* biomass production

Arthrospira maxima OF15 biomass production was tested in different conditions using vinasse at three concentrations. It was observed that the microalgae showed some difficulties to adapt in the initial phases of cultivation. Best results were reached when vinasse was diluted at 30% with a biomass production of 3.015 g L^{-1} after 15 days of culture with a productivity of 0.201 g $L^{-1}day^{-1}$ at laboratory scale (Table 2). With Zarrouk medium, which is one of the reference mediums for microalgae of *Arthrospira* genus cultivation, the production was similar 3.090 g L^{-1} of culture with a productivity of 0.206 g $L^{-1}day^{-1}$. Increasing productivities at laboratory scale were attained when CO_2 was injected continuously in the media at different concentrations of 5, 10 and 15% with corresponding productivities of 0.206, 0.221 and 0.279 g $L^{-1}day^{-1}$, respectively. This fact happened possibly because the microalgae assimilation of CO_2, depends on the medium pH, and the balance of CO_2/CO_3^{2-}.

In the literature, there are reports of successful cultivation of microalgae strains using vinasse. However, the use of this effluent at high concentrations seems to inhibit algal growth. *Spirulina maxima* biomass dry weight productivities ranging from 0.240 to 1 g $L^{-1}day^{-1}$ were reported by Barrocal et al. (2010) using synthetic media supplemented with 1 to 7 g L^{-1} of beet vinasse. Ramirez et al. (2014) cultivated *Scenedesmus* sp. in synthetic media supplemented with sugarcane vinasse at a concentration of 50%, although they reached lower productivities of biomass dry weight (0.024 g $L^{-1}day^{-1}$). In complete study of the influence of different vinasse concentrations, Santana et al. (2017) cultivated *Micractinium* sp. Embrapa|LBA32 and *Chlamydomonas biconvexa* Embrapa|LBA40 strains (isolated from a sugarcane vinasse stabilization pond) in 50% diluted vinasse or 100% clarified vinasse obtaining average productivities of 0. 222 g $L^{-1}day^{-1}$ of biomass dry weight. In their work, dos Santos et al., (2016) cultivated *S. maxima* in sugarcane vinasse medium in a cyclic two-stage cultivation – CTSC –

with photoautotrophic condition (12 h, 70 μmol photons m^2 s^{-1}) followed by a heterotrophic condition during the dark phase of the photoperiod (12 h, 3.0% v/v vinasse). The CTSC strategy, separated by autotrophic rest periods of few days between CTSC cycles, shows an increase of biomass concentration between 0.495 g L^{-1} and 0.609 g L^{-1} at the 7th day of each cycle and high protein content (between 74.3 and 77.3% w/w).

In this work, the results obtained with the selected strain *Arthrospira maxima* OF15 in terms of biomass productivities was considered promising for the use in pilot scale using as culture media only vinasse diluted in water at 30% and $CaCO_3$ to increase the pH to 9-10.

3.2 A. maxima cultivation in sugarcane vinasse at pilot scale

Microalgae biomass production was carried out in a raceway bioreactor with filtered diluted vinasse at 30% (v/v). Biomass reached 2.25 g L^{-1} after 15 days (a productivity of 0.150 g $L^{-1}day^{-1}$, and average specific growth rate of 0.23 day^{-1}). These values are very promising when compared to those reported in the literature with the use of synthetic media at laboratory scale, and the final biomass is superior to other reports for vinasse use as culture medium. Barrocal et al. (2010) produced *Spirulina maxima* OF15 0.150 g $L^{-1}day^{-1}$ using Schlösser medium supplemented with beet vinasse (5 g/L). About 1 g.L^{-1} and 0.177 g $L^{-1}day^{-1}$ of biomass were produced by dos Santos et al. (2016). Santana et al. (2017) obtained *Chlamydomonas biconvexa* Embrapa|LBA40 biomass productivities of 0.182 g $L^{-1}day^{-1}$ and 0.222 g $L^{-1}day^{-1}$ with the use of vinasse medium (50%) and clarified vinasse (100%), respectively.

The developed process also reduces the environmental pollution impact of sugarcane vinasse with the decrease of total nitrogen from 144.4 to 55.5 mg.L^{-1}. Significant reduction of

BOD and COD were also observed, of 89.2% and 81%, respectively. Nitrate and phosphate contents were also significantly reduced in 100% and 50%, respectively (Table 2).

Table 2

3.1 *A. maxima* biomass proximate composition

The composition of *A. maxima* OF15 produced in pilot scale was defined: 57.04±0.031% (w/w) proteins, 5.65±0.276% (w/w) ash, 10.67±0.12% (w/w) carbohydrates and 11.2±0.36% (w/w) lipids. Biomass proximate composition was similar to that reported in the literature (Bills and Kung, 2014). As a protein-rich material (57% of protein), the biomass can be used as a source for new peptides production with functional and biological activities.

3.2 Production of peptide fractions

About 80% of proteins from *A. maxima* OF15 were fractionated through water extraction, freeze-thawing, homogenization, and ultrasonication. The protein isolates were subjected to single-step and two-step hydrolysis, under controlled conditions. Three enzymatic procedures, in a single or combined hydrolysis process, resulted in three different peptides' fractions: the first one was obtained by enzymatic hydrolysis with subtilisin A (PHA), the second was obtained by pepsin hydrolysis (PHP) and the third one was produced after the hydrolysis with both enzymes (PHS). The three fractions showed a degree of hydrolysis between 43-50% (Table 3), thus generating a large amount of peptides. According to previous research, the same enzymes have been used for peptides' production from protein extracts of *Navicula* sp., *Porphyra yezoensis*,

Chlorella vulgaris, whey and others, which showed antioxidant, anticancer, anti-microbial and ACE-inhibitory, among other activities (Kang et al., 2011).

Table 3

Peptides from different enzymatic hydrolysates (PHA, PHP and PHS) are shown in Table 5. They were analyzed using MALDI-TOF showing very different molecular masses. The single enzymatic hydrolysis using subtilisin A generated 4 peptides. The hydrolysis with pepsin resulted in the liberation of 15 peptides and the sequential hydrolysis (pepsin/subtilisin A) showed only one peptide (data not shown). Subsequently, the physicochemical properties of each peptide sequence were analyzed with a database (www.pepcalc.com) and the details are summarized (Table 4). These peptides presented a small molecular weight, and GRAVY values between -2 to +2, which can indicate that they have hydrophobic chains. According to previous research, peptides that have a low molecular weight and are hydrophobic can be absorbed into our body through two mechanisms, the paracellular pathway, and the transcellular pathway (Maestri et al., 2015). In addition, as it can be seen, most peptides present negative charges, which means that they can interact with the surfaces of the cellular membranes, and can be used as antimicrobial and anti-cancer agents (Andersson et al., 2016; Perumal and Pandey, 2013). For example the Microplusin from *Rhipicephalus* (*Boophilus*) *microplus* showed antimicrobial activity against Gram-positive bacteria (Silva et al., 2009). The PopuDef (anionic peptide) from *Polypedates puerensis* presented antimicrobial activity against Gram-positive and Gram-negative (Wei et al., 2015).

Table 4

3.3 Evaluation of peptides' fractions biological activities

DPPH radical scavenging

All peptides fractions exhibited the capacity of sequestering the free radical DPPH (Fig. 4A). PHA showed a percentage of sequestering of 78±0.44% at a concentration of 0.1 g mL^{-1}, PHS of 78±0.21% and PHP of 77±0.71%. The IC_{50} values for DPPH scavenging by three fractions are summarized in Table 5. PHS fraction demonstrated a strongest radical scavenging activity IC_{50} of 1.79 mg/ mL if compared with Vitamin C that presented only an IC_{50} value of 1.2 mg mL^{-1}. These results indicate that PHS contained peptides with a high potential DPPH scavenging activity. Previously, Yu et al., (2016) reported the *A. platensis* hydrolysate showed antioxidant activity 85.21 ± 1.59% at 10 mg mL^{-1}. Lisboa et al., (2016) reported an antioxidant activity between 48.5 and 73.2% at 2.5 mg mL^{-1} of *Spirulina* sp. LEB 18 hydrolysate. Compared to the literature, PHS fraction showed better antioxidant activity using lower concentration.

Fig. 4

Table 5

ABTS radical scavenging

The antioxidant ability of peptides fractions to scavenge the blue-green colored $ABTS^{+}$ radical cation was measured according to the radical scavenging ability of Trolox and IC_{50} (Fig. 4B). As shown in Table 5, the results clearly indicate that PHS and PHA have a good reducing power with TEAC values of 540.7 and 465.7 µM of Trolox /g sample and IC_{50} of 8.6 and 9.5 µg

mL^{-1}, respectively. Lisboa et al., (2016) reported a total antioxidant capacity expressed as TEAC value of 248 µM of Trolox /g sample, using peptides of *Arthrospira* sp. Norzagaray-Valenzuela et al. (2017), reported the enzymatic hydrolysis of three species of microalgae, *Dunaliella tertiolecta*, *Tetraselims suecica* and *Nannochloropsis* sp. obtaining fractions with a TEAC value of 437.01±1.34 Trolox µM g^{-1} protein hydrolysate, 696.99±1.82 Trolox µM g^{-1} protein hydrolysate, and 519.44±4.46 Trolox µM g^{-1} protein hydrolysate, respectively. Comparing the peptide fractions PHS and PHA with those previously reported in the literature, it is possible to observe that they have a better total antioxidant capacity. This fact would be probably due to the presence of cysteine (Cys) and methionine (Met) in their structure, or aromatic side chains with amino acids histidine (His) and tyrosine (Tyr), which can easily donate hydrogen atoms. Furthermore, based on these results, it can be said that PHS and PHA fractions have the ability of scavenging free radicals, and to prevent oxidative damage to proteins, lipids, and DNA (Nurdiani et al., 2016).

Iron-chelating activity

Peptide fractions (PHP, PHS, and PHA) were assayed for their Fe^{2+}chelating activity at five different concentrations and their activity was compared with the chelating activity of the synthetic metal chelator EDTA. PHA fraction chelated more iron (97.3%) than PHP and PHS at 25 µg mL^{-1} and IC_{50} of 0.007 mg/mL (Table 6). The EDTA-Na_2 presented a chelating activity of 61% at 25 µg mL^{-1}. These values indicated that PHA was more efficient than the commercial chelator Na_2-EDTA. This results suggested that PHA fraction could be used as an agent to preserve foods with high lipid content, a catalyst of metal ions for reducing cell damage and a promoter in the absorption and bioavailability of non-heme iron in the body (Carrasco-Castilla et

al., 2012; Wu et al., 2012). Previously, Kim et al., (2014) reported that the peptide Threonine (Thr)-Aspartic acid (Asp)-Proline (Pro)- (Ile(Leu)-Alanine(Al)-Al-Cys-Ile(Leu), which was obtained from *Arthrospira* sp., showed 80% of iron-chelating activity. Wu et al., (2012) reported that hydrolysates from anchovy showed IC_{50} of 0.048 and 0.086 mg mL^{-1}. Comparing PHA fraction with those previously reported, it is possible to observe that it has a better chelating activity, suggesting that the peptides can present in their composition the following amino acids: Met, glutamine (Gln), lysine (Lys) and arginine (Arg) (de Castro and Sato, 2015).

Antimicrobial activity

The three peptide fractions were evaluated at different concentrations, from 0.13 to 6.25 mg/mL, for their antimicrobial activity against human pathogenic bacteria (*E. coli*, *S. typhi*, *B. subtilis* and *S. aureus*). The three peptide fractions (PHP, PHS, and PHA) showed antimicrobial activity (Table 6). The PHP displayed better values of MIC and IC_{50} values against *B. subtilis, S. aureus and S. typhi*. Additionally, this peptide fraction is unique in that it showed bactericidal action against all four pathogenic bacteria used in this experiment. PHS was the most efficient against *E. coli.* The PHP showed activity against both Gram positive and Gram negative bacteria, this fraction present a promising potential to be used as an antibiotic in the future. The research of new antimicrobial substances is important because these pathogens mutations increase resistance to existing drugs (Allen et al., 2014). The *Arthrospira* genre is renowned for having antibacterial activity through the production of phycocyanins and carotenoids, but the existence of antibacterial peptides was rarely reported (Ozdemir et al., 2004). Previously, Sun et al., (2016), reported antimicrobial activity against *E. coli* and *S. aureus* with a peptide from *A. platensis*, with MIC values of 8 and 16 mg mL^{-1}, respectively. Comparing our PHP fraction to

that reported in the literature, PHP exhibits a better antimicrobial activity against *E. coli* and *S. aureus*. In addition, with respect to *B. subtilis* and *S. typhi* A, there are no previous reports.

Table 6

Anti-inflammatory activity

For anti-inflammatory activity, the inhibition by PHA, PHP and PHS of hyaluronidase Type IV was evaluated. The three peptide fractions isolates showed anti-inflammatory activity. PHS showed the higher anti-inflammatory activity with 38.8± 1.1% at 333 µg mL^{-1} (Fig. 5) and lowest IC_{50} 0.92 mg mL^{-1}. Meanwhile, PHA and PHS showed values of inhibition <32% and IC_{50} 1.63 mg mL^{-1}, and 1.66 mg mL^{-1}, respectively. Furthermore, the control + (propolis) showed a IC_{50} 23.61 mg mL^{-1}. These results suggest that the peptides generated by the sequential action of both proteases (PHS) have a higher anti-inflammatory activity at lower doses than propolis ethanol fraction, in addition, to help maintain the elasticity in the skin and increased proliferation, tissue regeneration, and repair by inhibiting hyaluronic acid hydrolysis (Prado et al., 2016). Norzagaray-Valenzuela et al., (2017) reported that *Dunaliella tertiolecta* exhibited a IC_{50} of 5.542 mg mL^{-1} and *Tetraselmis suecica* showed a IC_{50} of 5.907 mg mL^{-1}. This research showed that PHS is a potent anti-inflammatory agent when compared with similar compounds reported in the literature. The anti-hyaluronidase activity of peptides from *Arthrospira* has not been reported in the scientific literature until the present date.

Fig. 5

Anti-collagenase activity

Collagen, which is the major component of the skin, is degraded by the enzyme collagenase provoking the aging of the skin. The peptide fractions (PHP, PHS, PHA) were assayed at different concentrations for their ability to inhibit collagenase. The anti-collagenase activity was compared with the synthetic inhibitor 10-Phenanthroline. PHS at 75 µg/mL showed the best anti-collagenase activity (92.5 %) and an IC_{50} of 32.5 µg mL^{-1} (Fig. 6), while PHP and PHA showed values <70% at the same concentration and IC_{50} values of 43.9 and 96.7 µg mL^{-1}, respectively. The 10-Phenanthroline presented an inhibition activity of 57.13±1.9% at 75 µg mL^{-1}. Based on these results, it can be said that PHS showed higher collagenase inhibitory activity compared with the synthetic inhibitor (10-Phenanthroline). It can also be suggested that it has the ability to prevent the cutting of collagen, delaying the process of pre-collagen fibers formation and the loss of strength, flexibility, and elasticity of the skin (Chattuwatthana and Okello, 2015). Combined enzymatic hydrolysis with both proteases is a potential alternative for producing bioactive peptides for anti-aging treatment. To our knowledge no existing scientific reports about the potentialities of peptides from *Arthrospira* that display anti-aging activity.

Fig. 6

Conclusions

This study presented the development of a highly advantageous process for sugarcane vinasse valorization together with significant reduction of its environmental impact. The microalgae *Arthrospira maxima* OF15 was cultivated in sugarcane vinasse with high biomass productivities at laboratory and pilot scale. A very performant enzymatic hydrolysis of microalgae biomass was conducted to generate biopeptides. Noticeable biological activities of three biopeptides' fractions (PHA, PHP and PHS) were observed in different *in vitro* test models. Antioxidant, chelating,

antimicrobial, antioxidant, anti-hyaluronidase and anti-collagenase activities were identified. Biopeptides' fractions from *A. maxima* OF15 are certainly potential candidates for pharmaceutic, cosmetic and food industry.

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Figure Captions

Fig. 1. Simplified process of bioethanol production and vinasse generation

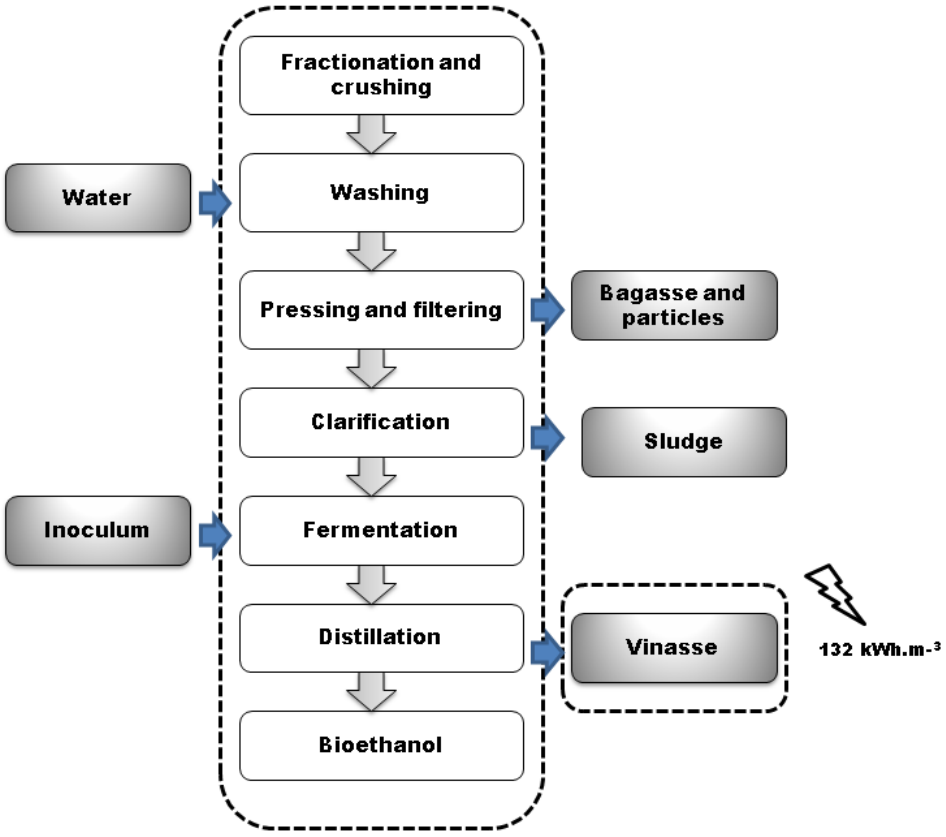
Fig. 2. Steps of *Arthrospira maxima* OF15 biomass and peptides' production from sugarcane vinasse

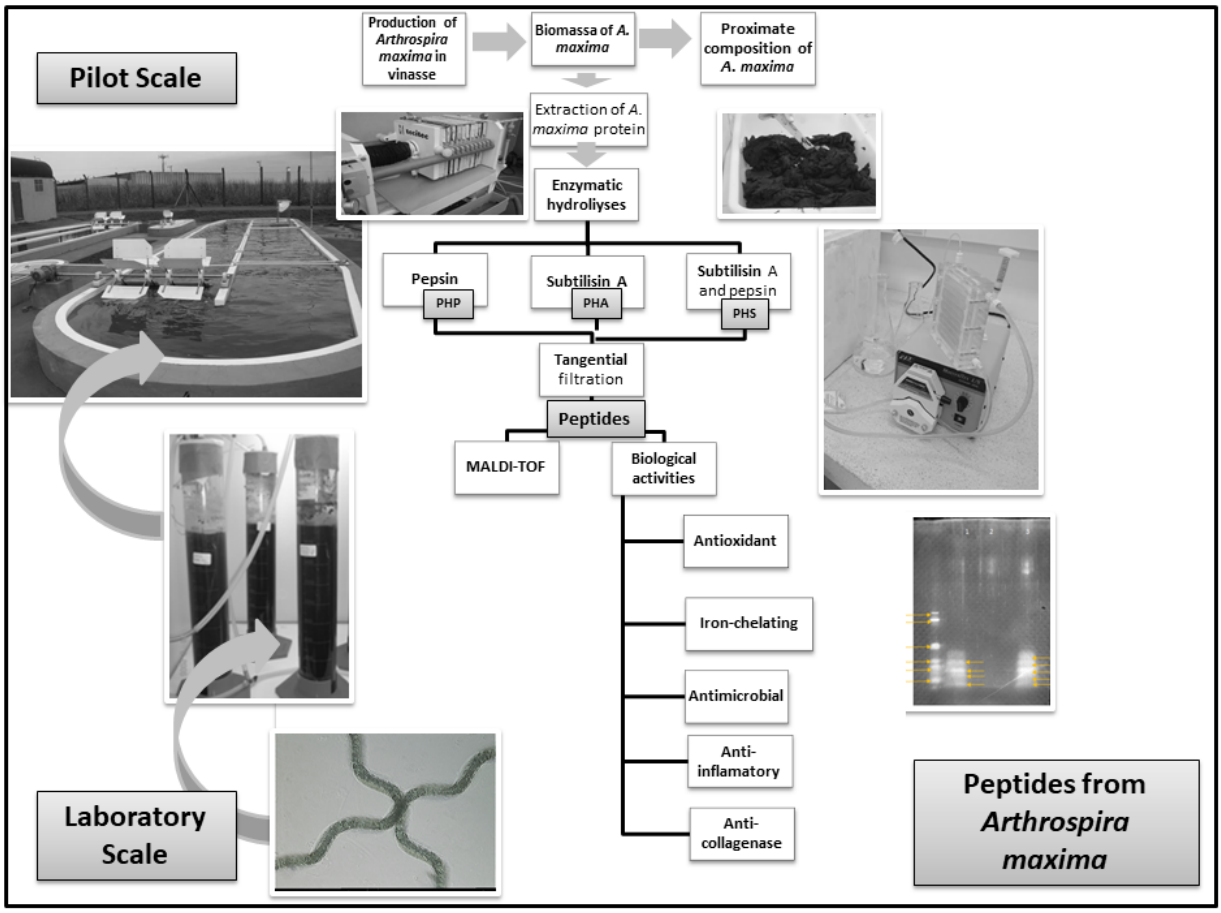
Fig. 3. Enzymatic hydrolysis of *Arthrospira maxima* OF15 proteins

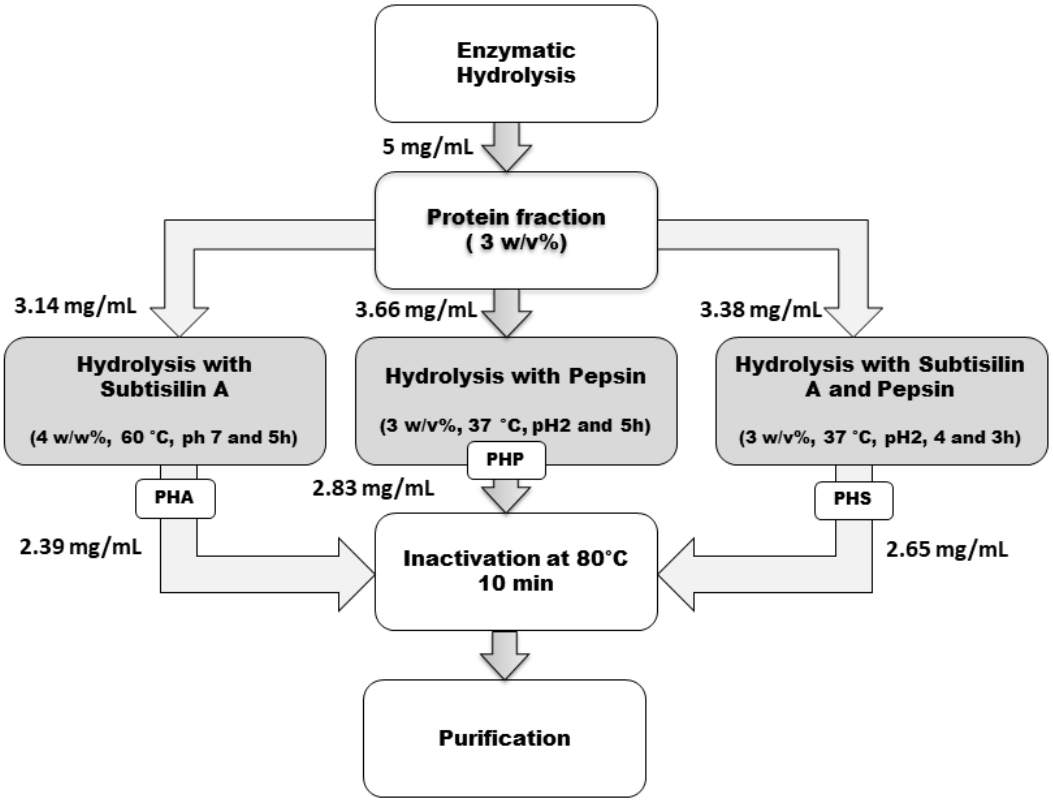
Fig 4. PHA, PHP, PHS peptide fractions biological activity essays: (A) Percentage of radical scavenging using DPPH assay; (B) Percentage of radical scavenging using ABTS assay

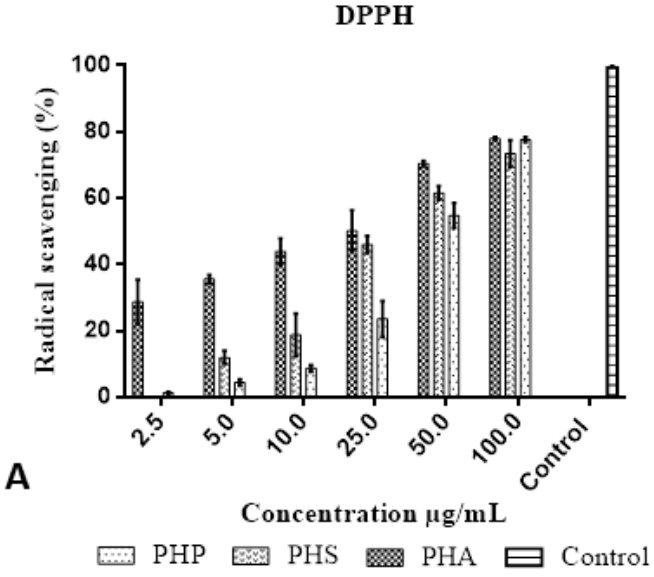
Fig. 5. Percentage of inhibition of hyaluronidase enzyme by PHS, PHA and PHP at different concentrations

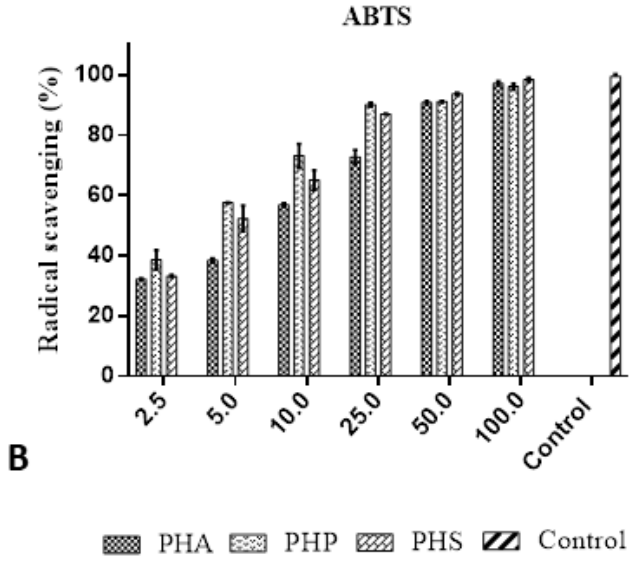
Fig. 6. Percentage of inhibition of collagenase enzyme by PHP, PHA and PHS peptide fractions at different concentrations (10, 20, 50 and 75 $\mu g\ mL^{-1}$) ($p=<0.0001$)

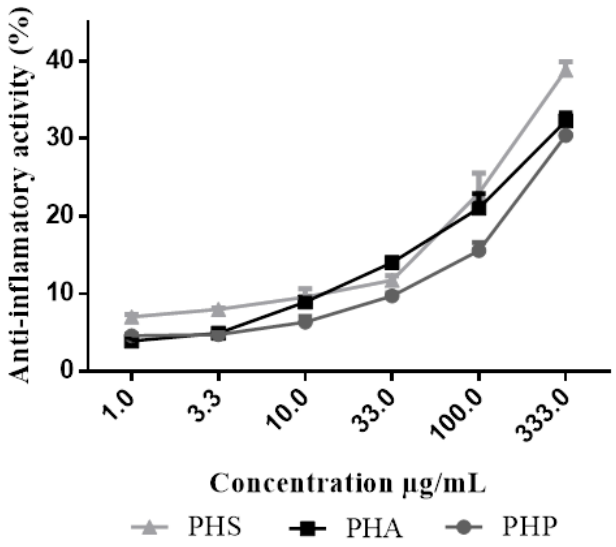












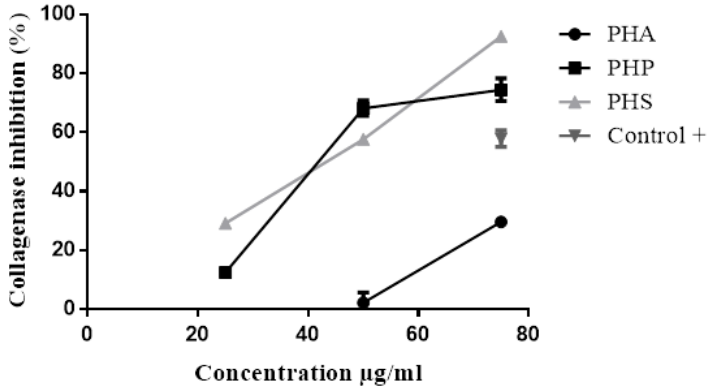


Table 1. Average physico-chemical composition of sugarcane vinasse generated from bioethanol producing chain

Parameters	Cortez et al., 1992	Robles-González et al., 2012		Nitayavardhana et al., 2013		Average
Nitrogen mg L^{-1}	1025	660	843	—	—	842.66
Phosphate mg L^{-1}	195	290	850	28.58	31.2	278.95
Magnesium mg L^{-1}	970	—	—	376.7	426.9	591.2
Calcium mg L^{-1}	2815	—	—	559.83	1166.5	1513.7
Sulfate (mg L^{-1})	6400	308	947	—	—	2551.6
Potassium (mg L^{-1})	3740	—	—	1734.83	4451.1	3308.6
COD (mg O_2 L^{-1})	65,000	56,230	60,560	64,480	42,990	57,852
BOD_5 (mg O_2 L^{-1})	25,000	26,500	22,000	—	—	24,500
pH	4.6	3.7	3.6	4.25	4.15	4.06

Table 2. Sugarcane vinasse composition before and after *Arthrospira maxima* OF15 biomass cultivation at pilot scale

	Sugarcane vinasse composition	
	Before cultivation	After cultivation
Biomass (mg L^{-1})	0.20	2.25
BOD (mg L^{-1})	7,642.8	785.1
COD (mg L^{-1})	24,605.3	4,788.0
Total nitrogen (mg L^{-1})	144.4	55.5
pH	8.6	7.5
Nitrate	20.0	0.0
Phosphate	10.0	5.0

Table 3. Characterization of *Arthrospira maxima* OF15 protein hydrolysates

Peptide fraction	Degree of hydrolysis (%)	Concentration (µg mL^{-1})
PHA	49.5	2395.6
PHP	43	2831.5
PHS	43.3	2651.1

Table 4. Characterization of peptide fractions' physicochemical properties

Sample	Code	Mass (Da)	Isoelectric point (pI)	Net charge (z)	Extinction coefficient ($M^{-1}*cm^{-1}$)	Grand average of hydropathy (GRAVY)
PHA	SMA1	996.4247	6.4	0	1490	-1.38
	SMA2	1628.6866	3.93	-1	1490	0.973
	SMA3	1597.7098	4.01	-1	1490	-1.228
	SMA4	1365.5810	4.08	-1	Nd	-1.95
PHP	SMP1	2524.1089	2.56	-7	11000	-0.37
	SMP2	1424.6696	4.01	-1	Nd	-0.28
	SMP3	1273.5525	5.19	-1	Nd	-0.175
	SMP4	2015.8913	6.25	0	1490	-1.3
	SMP5	2773.2644	3.63	-2	1490	-0.07
	SMP6	1395.6245	3.79	-2	1491	-1.45
	SMP7	3614.6116	3.13	-8	12490	-0.73
	SMP8	3469.5550	3.45	-7	1490	-1.54
	SMP9	1859.7904	4	-1	0	-1.1
	SMP10	3726.6921	3.63	-7	0	-1.66
	SMP11	1142.5122	5.19	-1	1490	-0.36
	SMP12	2125.9916	4.21	-1	0	-0.98
	SMP13	1535.6499	3.62	-2	0	-1.5
	SMP14	1404.6096	3.63	-2	1490	-1.78
	SMP15	1859.7904	4	-1		-1.1
PHS	SMS1	1843.8898	4	-2	5500	0.39

PHA – Peptide fraction - with subtilisin A; PHP – Peptide fraction - with pepsin; PHS Peptide fraction – with both enzymes

Table 5. Antioxidant and iron-chelating activities of different peptide fractions from *Arthrospira maxima* OF15

	Scavenging of DPPH radical scavenging	**Scavenging of $ABTS^{+}$ radical scavenging**		**Fe^{2*} Chelating activity**	
Samples	IC_{50} (µg mL^{-1})	IC_{50} (µg mL^{-1})	TEAC (Trolox µM g^{-1} sample)	IC_{50} (µg mL^{-1})	(%) 25 (µg mL^{-1})
PHA	21.25 [a]	9.5 [b]	465.7 [b]	6.98 [c]	97.3 ± 0.4
PHP	34.63 [a]	15.63 [b]	282.2 [b]	724.7 [c]	<30
PHS	17.93 [a]	8.6 [b]	540.7 [b]	492.2 [c]	<30
Vitamin C	11.97	6.1	Nd	nd	Nd
Trolox	nd*	44.11	Nd	nd	Nd
EDTA-NA_2	Nd	nd	Nd	14.31	61±1.3

[a] Analysis of variance showed p=0.006 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS).

[b] Analysis of variance showed p=0.0113 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS).

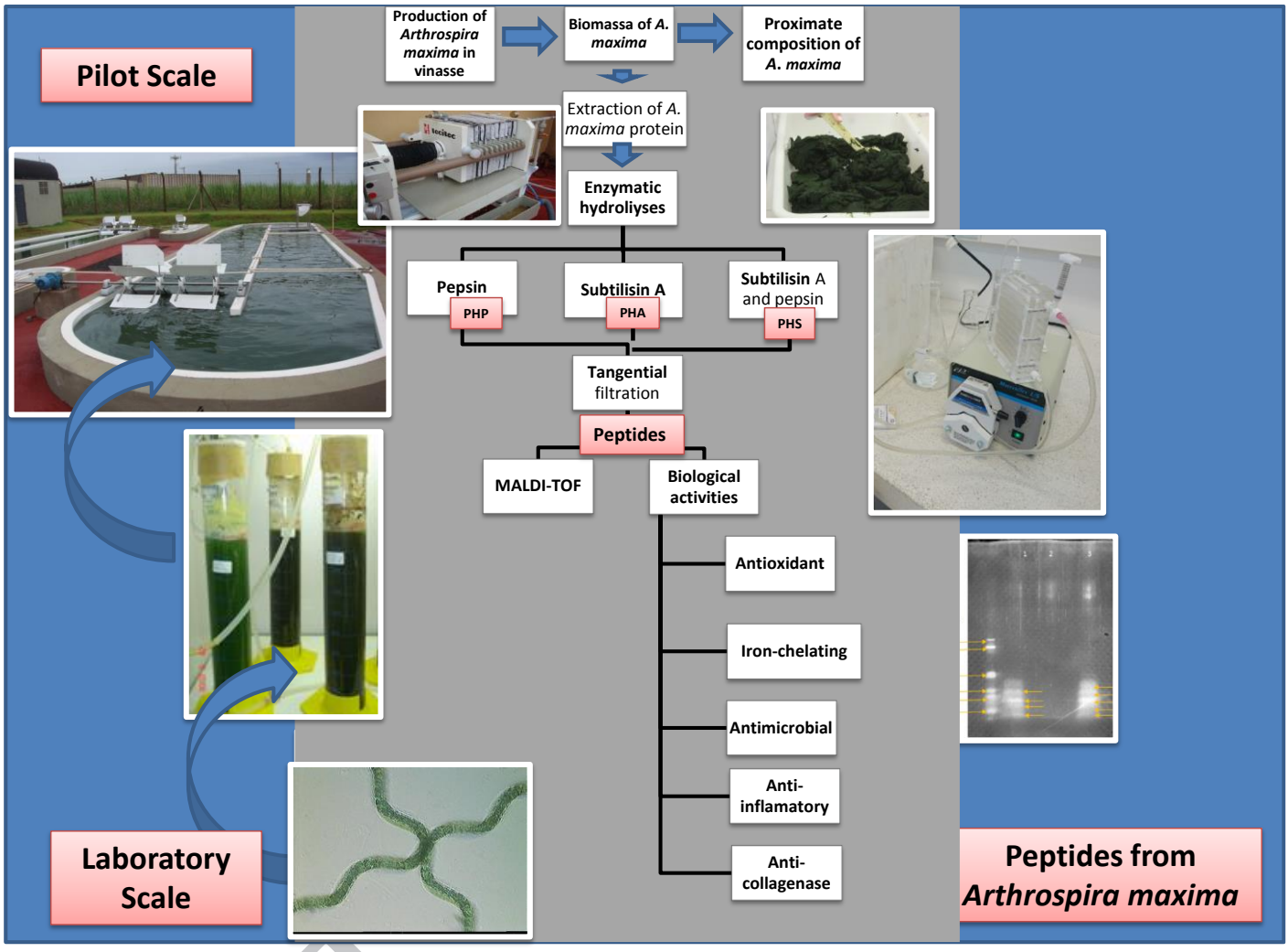
[c] Analysis of variance showed p=<0.0001 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS)

*nd=not determinate

Table 6. Antimicrobial activity of peptides from *Arthrospira maxima* determined by MIC, MBC and IC_{50}

Sample	**Peptides fraction of hydrolysis with subtilisin A (PHA)**			**Peptides fraction of hydrolysis with Pepsin (PHP)**			**Peptides fraction of hydrolysis with both enzymes (PHS)**		
Bacteria	IC_{50} (μg mL^{-1})	MIC (mg mL^{-1})	MBC (mg mL^{-1})	IC_{50} (mg mL^{-1})	MIC (mg mL^{-1})	MBC (mg mL^{-1})	IC_{50} (mg mL^{-1})	MIC (mg mL^{-1})	MBC (mg mL^{-1})
B. subtilis	2.17	6.25	>6.25	0.34	0.63	0.63	1.25	1.25	6.25
S. aureus	1.62	6.25	>6.25	0.62	0.63	0.63	0.88	1.25	>6.25
S. typhi	7.15	>6.25	>6.25	0.99	1.25	1.25	1.22	1.25	6.25
E. coli	11.89	>6.25	>6.25	0.94	1.25	1.25	0.79	1.25	6.25

Analysis of variance showed p = <0.0001 (significant).



- High titers of *Arthrospira maxima* biomass cultivated at Laboratory and pilot scale

- Microalgae produced using sugarcane vinasse with BOD and COD reduction

- Peptide fractions obtained through biomass enzymatic hydrolysis

- Antioxidant, antimicrobial, anti-inflammatory, and anti-collagenase properties