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Production and characterization of alginate beads for growth of immobilized *Desmodesmus subspicatus* and its potential to remove potassium, carbon and nitrogen from sugarcane vinasse

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ABSTRACT

Among the main industrial wastewaters, sugarcane vinasse figures as an actual environmental concern, due to its polluting potential and large volumes available, about 10–15 L per liter of ethanol. Microalgae immobilization in biopolymeric matrices, such as alginate, represents a promising strategy for wastewater treatment aiming the removal of nutrients. Alginate beads for immobilization of Chlorophyceae microalgae *D. subspicatus* were prepared with different biopolymer and crosslinking agent concentrations. Alginate beads showed good stability in vinasse probably owned to wastewater high calcium content. The growth of immobilized and free microalgae in sugarcane vinasse (bulk phase) and its potential to remove potassium, carbon and nitrogen from this wastewater were evaluated. Immobilized *D. subspicatus* showed maximum specific growth rate (μmax) of 0.009 h^{-1} for alginate beads (2%) crosslinked with 5% $CaCl_2$, with 38, 27 and 28% of carbon, nitrogen and potassium removal. Free microalgae cultivation exhibit two μmax of approximately 0.01 h^{-1} (between 0-24 h and 48–72 h) and maximum carbon, nitrogen and potassium removals of 45, 49 and 8%, respectively. Potassium adsorption by the biopolymer matrix was significant, with maximum removals (37 and 35%) obtained by blank and immobilized microalgae alginate beads (2%) crosslinked with 10% $CaCl_2$. Results indicate interesting perspectives for the use of *D. subspicatus* immobilized in alginate for removal of nutrients from sugarcane vinasse.

1. Introduction

Brazil is currently the world's largest sugarcane producer, with an estimated total ethanol production of approximately 30.2 billion liters of ethanol in 2019/20 (CONAB, 2019). Vinasse is the main wastewater from the fermentation–distillation process, available in large volumes (about 10–15 L per liter of ethanol, depending on the distillery equipment). Concerning its highly polluting character, with high concentrations of organic matter and low pH, many alternatives for the use and treatment of vinasse have been proposed, such as recycling of vinasse in fermentation, fertigation, concentration by evaporation, microbial growth medium, energy production, and raw material to produce livestock and poultry feed (Campanhol et al., 2019; Christofoletti et al., 2013). Among these alternatives, fertigation is the most commonly used. However, fertigation is currently being questioned due to its effects on the soil and on groundwater, caused by the leaching of nutrients, such as potassium.

The use of sugarcane vinasse as a growth medium for microalgae biomass production was first proposed by Oliveira and Caceres (1986), who used diluted vinasse for cultivation of *Chlorella vulgaris*. Other recent study investigated the ability of *Desmodesmus subspicatus* to remove nitrogen and chemical oxygen demand (COD) from vinasse with microalgal biomass production (de Mattos and Bastos, 2015). *D. subspicatus* is a genus of Chlorophyceae microalgae naturally found in freshwater, especially in nutrient-rich environments. They have been cultivated as free and immobilized cells in industrial, domestic and artificial wastewater showing high cell viability, tolerance to pH and temperature variations, and similar specific growth rates (da Silva et al., 2017; Tsarenko et al., 2011).

Immobilization of microalgae has been considered for several biotechnological applications over the last years. Currently, immobi-

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lized microalgae, in a wide variety of matrices, are employed for biomass and metabolite production, bioremediation of wastewaters, specifically, in the biocapture of nutrients and heavy metals. Immobilization of cells brings some advantages such as facilitating cultivation and easy biomass harvesting, enhancing cell tolerance to unfavorable environmental conditions (e.g. extreme temperature, acidity, toxicants) and allowing continuous system operations (de-Bashan and Bashan, 2010; Eroglu et al., 2015).

Several immobilization techniques are available, including adsorption, confinement in liquid–liquid emulsions, capture on semipermeable membranes, covalent coupling, and gel entrapment (Mallick, 2002). This latter is the most commonly used method and consists of capturing the cells in a three-dimensional gel matrix, based on either natural (agar, cellulose, alginate, pectin) or synthetic (polyacrylamide, polyurethane, polyvinyl, polypropylene) polymers (de-Bashan and Bashan, 2010; Hameed and Ebrahim, 2007; Moreno-Garrido, 2008). Although synthetic polymers are reported to be more stable in wastewater samples, natural polymers allow higher nutrient/product diffusion rates and can reduce the environmental impact (de-Bashan and Bashan, 2010; Leenen et al., 1996). Besides, in the case of vinasse treatment or other nutrient rich wastewater, beads could be recovered after cultivation, dried and applied as soil fertilizer enhancing process sustainability (Bettani et al., 2019; Rajendran et al., 2018).

Regardless the polymer, the material should be hydrophilic to allow wastewater diffusion into the bead. For microalgae, gel entrapment in natural polysaccharide matrices is the most widely applied immobilization technique. The characteristics and useful selection criteria of polymers used for cell immobilization in wastewater treatment is well described in literature (Leenen et al., 1996).

Sodium alginate is commonly used to produce immobilization matrices by entrapment of microalgae and other microbial cells due to its ability to form gel beads in the presence of multivalent cations, such as Ca^{+2}. Alginate is an anionic polysaccharide extracted from brown algae, composed of β-D-mannuronic (M) acids and α-L-guluronic (G) acid residues covalently linked in varying proportions and sequential arrangements, depending on the alginate source. Calcium induced gelation in alginate results from specific and strong interactions between calcium ions and guluronate blocks. In this process, described as the "egg-box" model, the solvent is confined in the interstices of a three-dimensional network linked by junction zones that involve cooperative association of extended segments of the polymer chains (Voo et al., 2011).

The crosslinking of alginate to form gel beads for cell immobilization is usually obtained by external gelation with Ca^{+2}, also referred as "diffusion method" (Ma et al., 1994; Smidsrød and Skjak-Bræk, 1990). In this method, alginate-cell solution is dripped into a solution containing Ca^{+2}, such as a calcium chloride. The cations diffuse from the continuous phase into the interior of the alginate droplets and a gelled alginate matrix is formed (Paques et al., 2014). Ca^{+2} ions tend to crosslink the bead surface first, drawing the polymer chains closer to form a less permeable surface. This results in a matrix with a highly crosslinked surface and a less well crosslinked interior. Thus, one can conclude that there is an optimal amount of crosslinker that could be used to produce matrices with the desired characteristics (Chan et al., 2006).

The major advantages of microalgae immobilization in alginate matrices are related to its ease of process, non-toxicity, reversible nature, high retention of cell viability, transparency, and cost effective (de-Bashan and Bashan, 2010). However, alginate gels are chemically unstable in the presence of cation chelating agents such as phosphate, lactate or citrate and to cations such as sodium and magnesium, which can displace calcium and lead to bead disruption or dissolution. Removal of phosphate from the medium or presence of calcium tend to improve bead stability (Voo et al., 2011).

The physical properties of alginate gel beads, namely their mechanical strength, susceptibility to shrinkage, porosity and stability towards antigelling cations and chelating agents, are largely affected by the characteristics (molar mass and G/M blocks ratio) and concentration of the polymer and the type and concentration of the crosslinking agent (Martinsen et al., 1989; Moreira et al., 2006).

The aim of this study was to produce uniform and stable alginate beads, by varying the biopolymer and crosslinking solution concentrations, for *D. subspicatus* immobilization and evaluate its growth and potential to remove potassium, carbon and nitrogen from sugarcane vinasse.

2. Material and methods

2.1. Material

Medium viscosity sodium alginate, extracted from *Macrocystis pyrifera* seaweed (Sigma–Aldrich, St. Louis, MO, USA) with a M/G ratio of approximately 1.6 and an average molar mass (Mw) measured by gel permeation chromatography of 1.61×10^6 Da, and calcium chloride dihydrate, $CaCl_2{\cdot}2H_2O$, (Synth, Diadema, Brazil) were used as biopolymer and crosslinking agent, respectively. Vinasse was collected in a sugarcane processing industry located in the city of Araras (State of São Paulo, Brazil) directly from the distillation columns at 95 °C. It was homogenized, transferred to 2 L flasks and frozen stored at -20 °C until use. Vinasse was characterized according to its physical-chemical parameters (Table 1). pH, total soluble solids, total solids, phosphorous, potassium, calcium, magnesium, sulfate, organic matter, iron, copper, zinc and manganese were determined according to wastewater standard methods (APHA, 2005) and Total Organic Carbon (TOC) and Total Nitrogen (TN) were measured by TOC-LCPN (Shimadzu®, Tokyo, Japan).

2.2. Blank alginate beads preparation

Alginate solutions were prepared by dissolving alginate (1, 2 or 3% w/v) in deionized water at 25 °C. The system was maintained under constant stirring (1000 rpm) until complete dissolution and homogenization of the material (approximately 1 h). Aliquots of the polymeric solution (12.5 mL) were dropped into 50 mL of a gently stirred crosslinking solution ($CaCl_2$ 2, 5 or 10 g 100 mL^{-1}) using a peristaltic pump with a needle (BD Precision Glide™, 1.20 × 40 mm) attached to the tubing. The gel solution flow rate was adjusted to 10 mL min^{-1} and the distance from the needle tip to the surface of crosslinking solution was 10 cm. The beads were kept in the crosslinking solution at 4 °C for 3 h for stabilization, collected by filtration and rinsed with deionized water. The biopolymer and crosslinking concen-

Table 1
Physico-chemical characterization of vinasse.

Parameter	Result
pH	4.5
Total soluble solids (°Brix)	2.80
Total solids (g L^{-1})	26.80
Total nitrogen (mg L^{-1})	543.3
Total organic carbon (mg L^{-1})	7900
Phosphorous (mg L^{-1} P_2O_5)	85.0
Potassium (mg L^{-1} K_2O)	3923.3
Calcium (mg L^{-1} CaO)	680.0
Magnesium (mg L^{-1} MgO)	270.0
Sulfate (mg L^{-1} SO_4)	380.0
Organic matter (mg L^{-1})	13619.6
Fe (mg L^{-1})	18.3
Cu (mg L^{-1})	0.32
Zn (mg L^{-1})	0.47
Mn (mg L^{-1})	3.64

trations ranges were selected according to preliminary tests and literature data (Huang et al., 2011; Lee et al., 2013).

2.3. Immobilization of microalgae in alginate beads

Green microalgae (Chlorophytae) *D. subspicatus* from culture collection of LABMAC/CCA/UFSCar (Araras, Brazil) was maintained in BG11 media (30 mg L^{-1} K_2HPO_4, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 36 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 6 mg L^{-1} ferric ammonium citrate, 1 mg L^{-1} Na_2EDTA, 6 mg L^{-1} citric acid, 20 mg L^{-1} Na_2CO_3, 1.5 g L^{-1} $NaNO_3$, 72 mg L^{-1} NaCl, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.22 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.39 mg L^{-1} $NaMoO_4{\cdot}2H_2O$, 0.079 mg L^{-1} $CuSO_4{\cdot}5H_2O$, and 0.04 mg L^{-1} $CoCl_2{\cdot}6H_2O$) (Rippka et al., 1979). *D. subspicatus* inoculum was grown autotrophically in Flasks using BG11 media with pH 7.5, at 25 °C, aeration of 1VVM, $3\times g$ mechanic agitation, and photo flux of 45 μmol m^{-2} s^{-1} for at least seven days until 1.8 g L^{-1} (de Mattos and Bastos, 2015).

Approximately 500 mL of a stock suspension (1.8 g L^{-1}) of *D. subspicatus* was centrifuged at 1844 g for 20 min. The cell residue was resuspended in 50 mL deionized water prior to use. The diluted algal suspension was mixed with 350 mL alginate solution resulting in 400 mL of an alginate-microalgae solution (1, 2 or 3 g alginate 100 mL^{-1}). The beads with immobilized microalgae were produced as described above for blank alginate beads.

The immobilization efficiency (%IE) was calculated by the ratio of the viable cell count (Neubauer Chamber) in the alginate-microalgae solution to the count in the $CaCl_2$ solution after crosslinking.

2.4. Bead characterization

The blank and immobilized microalgae alginate beads were characterized according to the following methods. Visual observations of beads were performed considering aspects such as bead formation, homogeneity, presence of air bubbles and shape.

2.4.1. Average bead diameter

Ten beads of each formulation were measured at two different positions for average diameter calculation using software Image J®.

2.4.2. Chemical stability

The chemical stability of the beads was determined by exposing them to the BG11 medium, distilled water and vinasse. Thirty beads of each formulation were immersed in 10 mL of each liquid medium (in triplicate) and the number of intact beads was visually inspected and recorded daily for seven days (Voo et al., 2011). The flasks were kept at 25 °C in the dark.

2.4.3. Mechanical properties

The mechanical strength of single beads was evaluated by compression test using a TA.XT2 texturometer (Stable Micro System SMD, Surrey, United Kingdom) equipped with a load cell of 1 kg. Compression was performed until 70% of sample deformation at a compression speed of 0.05 mm s^{-1}, according to methodology adapted from Tomovic et al. (2015). The distance between the probe (P/0.5, radius cylinder, diameter 12.7 mm) and the flat plate was adjusted to 10 mm. The force required to perform 70% bead deformation was recorded. Ten beads of each formulation were evaluated and the average maximum force (N) was registered.

2.5. Cultivation of free and immobilized microalgae in vinasse

Selected bead formulations (according to the characterization tests) were chosen for microalgae cultivation experiments using vinasse as the growth medium. Vinasse was centrifuged in a refrigerated centrifuge (Solab® SL-701, Piracicaba, SP, Brazil), at 20 °C and 1844 g for 20 min, with pH adjusted to 7.6 and sterilized for 20 min/121 °C. A defined amount of immobilized cells (total beads produced with 12.5 mL of microalgae-alginate solution) were added to 125 mL Erlenmeyer flasks containing 25 mL vinasse and the flasks were kept under constant stirring (100 rpm) and 25 °C in the dark to allow the heterotrophic microalgal growth. Samples were taken at 0, 6, 12, 24, 48 and 72 h. The experiments were made in triplicate. Ten beads from each time interval were dissolved in 10 mL sodium citrate solution (3%) for viable cell counting in a Neubauer chamber. The maximum specific growth rate (μ_{max}) was estimated by the slope of the exponential phase in the semi-log curves, i.e. ln(X/Xo) vs. time, where Xo is the initial cell concentration and X is the cell concentration at experimental times. During cultivation, the average diameter of ten beads of each formulation were measured at two different positions for average calculation using the Software Image J®. Potassium content in vinasse was measured by flame photometry (DM-62 Digimed®, São Paulo, Brazil) (APHA, 2005), while TOC and TN were measured by TOC-LCPN (Shimadzu®, Tokyo, Japan).

Blank alginate beads (same selected formulations for microalgae immobilization), were produced and kept in the same cultivation conditions to evaluate the % potassium removal by the biopolymer.

Free microalgae cultivation was conducted in the same conditions. Microalgal stock suspension (1.8 mg L^{-1}) was concentrated as described in section 2.3 and resuspended direct in vinasse. Viable cell counting was measured at each time interval in the Neubauer chamber. Vinasse samples were centrifuged for 20 min at 20 °C and 1844 g before potassium, TOC and TN quantification.

2.6. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were applied to determine statistically significant differences ($p < 0.05$) among averages, using the Software Statistica V.1.1.5.

3. Results and discussion

3.1. Alginate beads preparation

Adequate conditions for bead formation (blank and with microalgae) were tested by varying the alginate (1, 2 and 3% w/v) and crosslinking solution ($CaCl_2$ 2, 5 and 10% w/v) concentrations. Regarding the biopolymer concentration, 2% alginate beads showed homogeneity, regular spherical shape and adequate texture. On the other hand, the lowest biopolymer concentration (1%) produced deformed, fragile-handled particles with droplet shape and smaller diameter, compared to the other concentrations tested, as shown in Fig. 1 for alginate beads with immobilized microalgae. In a previous study, Al-Hajry et al. (1999) obtained deformed 1% alginate particles compared to higher polymer concentrations, suggesting that at 1% the surface tension was not high enough to allow the formation of a perfect spherical alginate drop before impacting the calcium chloride solution. In addition, these authors reported stretch marks at 1% alginate bead surface, as observed in the present study. Bugarski et al. (1994) reported the formation of particles with a long and thin neck, when alginate concentration decreased from 1.5 to 0.8%. These authors observed a filament connecting the new drop to the tip of the needle, resulting in droplet-shaped particles. Alginate (3%) beads were homogenous and spherical, but beads were slightly flattened. Moreover, dripping 3% alginate solution was hindered due to its high viscosity, which would become impracticable for microalgae immobilization.

The concentration of the $CaCl_2$ solution, in the same biopolymer concentration, did not alter the visual aspect of the beads, except for the 3% alginate bead crosslinked with 10% $CaCl_2$ which showed non-

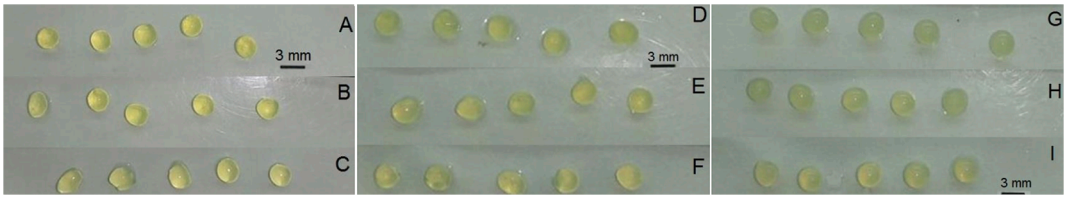

Fig. 1. Visual aspect of alginate beads with immobilized microalgae produced with different alginate and crosslinking agent ($CaCl_2$) concentrations. Alginate 1% (A - 2%, B - 5%, C - 10% $CaCl_2$), alginate 2% (D - 2%, E - 5%, F - 10% $CaCl_2$) and alginate 3% (G - 2%, H - 5%, I - 10% $CaCl_2$).

spherical shape. The presence of microalgae also did not affect the shape of the beads.

Considering cell immobilization applications, aiming to obtain metabolic products or the removal of pollutants, the size of the spherical particle controls the rate of diffusion of substrates and products. In general, smaller particles lead to higher throughput or productivity of the process because the diffusion limitation increases with particle size, thereby limiting cellular metabolism. The sphericity of alginate particles has a remarkable effect on their mechanical and chemical stability. Non-spherical shape of the particles has been reported to reduce gel strength compared to spherical particles (Lee et al., 2013). The average diameters of the alginate beads, with and without microalgae, are presented in Table 2. As observed, the increase in the biopolymer concentration tends to increase the size of the bead. Beads with immobilized microalgae were not significantly different in size.

Table 2

Average diameter of blank alginate beads and with microalgae produced with different alginate concentrations (1%, 2% and 3% w/v) and different crosslinking solution concentrations (2%, 5% and 10% w/v).

Alginate (w/v)	$CaCl_2$ (w/v)	Average diameter (cm)	
		Blank beads	Beads with microalgae
1%	2%	$0.231 \pm 0.004^{a,A}$	$0.256 \pm 0.011^{a,A}$
	5%	$0.240 \pm 0.012^{a,b,A}$	$0.258 \pm 0.022^{a,A}$
	10%	$0.261 \pm 0.008^{b,A}$	$0.278 \pm 0.033^{a,b,A}$
2%	2%	$0.304 \pm 0.013^{c,A}$	$0.306 \pm 0.019^{b,c,A}$
	5%	$0.301 \pm 0.007^{c,A}$	$0.308 \pm 0.020^{c,A}$
	10%	$0.302 \pm 0.019^{c,A}$	$0.308 \pm 0.018^{c,A}$
3%	2%	$0.320 \pm 0.010^{c,d,A}$	$0.328 \pm 0.019^{d,A}$
	5%	$0.319 \pm 0.004^{c,d,A}$	$0.324 \pm 0.014^{d,A}$
	10%	$0.342 \pm 0.018^{d,e,A}$	$0.313 \pm 0.022^{d,A}$

Average ± standard deviation of 10 experimental determinations. Same capital letter in the same line and same lower-case letter in the same column indicate no significant difference ($p < 0.05$) among averages by the Tukey test.

3.1.1. Chemical stability

The chemical stability tests, which consisted of counting the remaining beads over seven days of immersion in 10 mL of different solutions (distilled water, BG11 medium and vinasse) at 25 °C relative to the number of beads initially added (30 beads), indicated that for all conditions tested there was no dissolution of the beads, i.e., recovery was 100% in all cases. Despite this, it was noticed by tactile perception that beads immersed in BG11 medium and distilled water were slightly more fragile than beads immersed in vinasse at the end of the incubation period. The presence of chelating agents and no crosslinking cations, such as sodium, present at higher concentrations in BG11 medium, may affect the structure of the polymer matrix, weakening the calcium-alginate electrostatic interactions and increasing the chain mobility (Voo et al., 2011). On the other hand, it is important to mention that vinasse is rich in calcium, which would help to strength the polymer structure and hence bead stability. Leenen et al. (1996) observed that Ca–Ba-alginate beads were completely dissolved in a few days in domestic wastewater, but the same beads showed only discrete dissolution, for as long as 120 days, when added to a medium with Ca^{+2} (2 mmol L^{-1}) as a counterion independent of the Na^{+} concentration.

3.1.2. Mechanical properties

Compressive resistance of the beads usually increases with increasing concentration of the biopolymer and the crosslinking agent used (de-Bashan and Bashan, 2010). The results in Fig. 2 demonstrate that the compression force significantly increased by increasing the biopolymer concentration from 1 to 3%. $CaCl_2$ crosslinked beads prepared with an alginate from the same source and with similar M/G ratio as the one used in our study, Kaygusuz et al. (2016) observed that mechanical properties of the beads increased by a factor of approximately 5–6 by increasing the alginate concentration form 1–4% (w/v) attributed to a densification of biopolymeric matrix. At higher concentrations, the number of crosslinking points increases and alginate

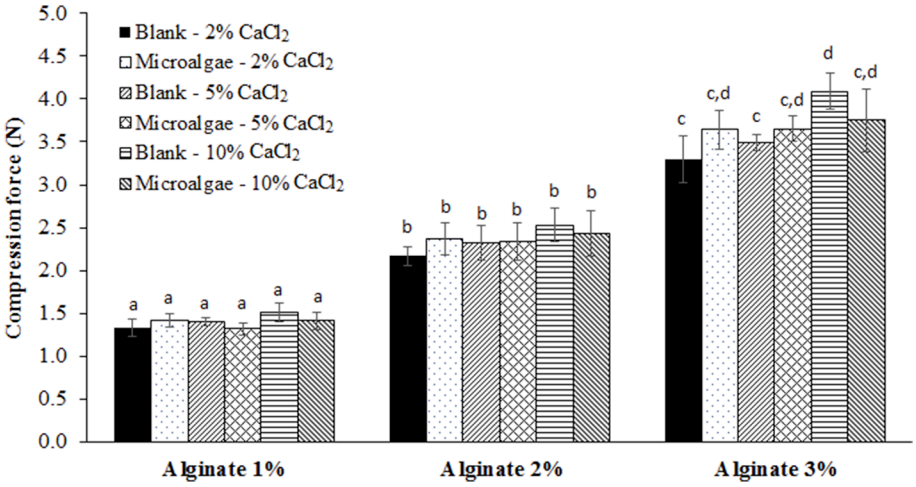

Fig. 2. Mechanical strength of blank alginate beads and beads with microalgae produced with different alginate concentrations (1%, 2% and 3% w/v) and different crosslinking solution concentrations (2%, 5% and 10% $CaCl_2$). ($n = 10$, different letters indicate significant differences ($p < 0.05$) by Tukey's test.

chains themselves can form aggregates via hydrogen bonding decreasing chain mobility and therefore increasing mechanical resistance. The same trend is reported by Kaklamani et al. (2014) for $CaCl_2$ crosslinked hydrogels formed by alginate solutions in the range of 2.5–5% (w/v). However, the increase in the crosslinker concentration from 2 to 10% did not significantly alter bead resistance, expect for blank 3% alginate beads crosslinked with 10% $CaCl_2$, which showed higher compression force compared to other crosslinker concentrations. Kaygusuz et al. (2016) also noticed only slight differences in the mechanical resistance of alginate beads by increasing the crosslinking agent ($CaCl_2$) concentration from 2 to 5% (w/v). For alginates, the gel forming ability depends on the G-blocks binding divalent cations. The ionic interaction between alginate chains and calcium ions leads to the formation of a three-dimensional polymer network. Generally, an increase in Ca^{+2} concentration increases the gel network tightening. However, it has been reported that when there are sufficient Ca^{+2} to fill up the cavities formed by G-blocks of polymer chains no further changes are observed. Typical values for low G alginates are approximately 0.1 mol L^{-1} Ca^{+2} (Klokk and Melvik, 2002; Lee et al., 2013). Higher concentrations of $CaCl_2$ tend to form more spherical beads and homogeneous network, besides rapid bead entrapment and stabilization is attained as a result of accelerated gelation kinetics (Lee et al., 2013). The presence of microalgae caused no significant effect in this parameter.

3.2. Microalgae immobilization and cultivation in vinasse

Since alginate (2%) produced stable spherical beads, in all $CaCl_2$ concentration, by a simple and fast crosslinking procedure, they were chosen to immobilize *D. subspicatus* and evaluate its growth in sugarcane vinasse. As the crosslinking degree can have an impact in the diffusion behavior inside the polymer matrix, the effect of different $CaCl_2$ concentrations (2, 5 and 10%) were tested in terms of normalized bead diameter, microalgae growth, and TOC, TN and potassium removal from vinasse.

Immobilization of *D. subspicatus* in 2% alginate beads was successfully achieved with % IE higher than 97%, for all crosslinking conditions. The externally crosslinked calcium alginate beads are more suitable for cell encapsulation, displaying higher immobilization efficiency, compared to internally crosslinked alginate beads. The diffusion of Ca^{+2} ions from the crosslinking solution to the droplet surface forms a less permeable surface structure (Chan et al., 2006).

Normalized average diameter of the beads (crosslinked with 2, 5 and 10% $CaCl_2$) during the 72 h of cultivation in vinasse are shown in Fig. 3. After 24 h, the diameter of the beads crosslinked with 2% $CaCl_2$ showed a decreasing profile, indicating a possible bead dissolution. On the other hand, beads crosslinked with $CaCl_2$ 5% exhibited swelling after 24 h and a tendency to decrease in diameter after 48 h. The diameter of the beads crosslinked with 10% $CaCl_2$ showed an increasing profile, obtaining a maximum diameter in 48 h with a slight decrease after that period. It is important to note that beads crosslinked with $CaCl_2$ 10% underwent less matrix swelling compared to the ones crosslinked with $CaCl_2$ 5%. This can be explained by the diffusive effect on the bead. Diffusivity in the particle can be affected by the structure of the polymer such as pore size, polymer chain flexibility and density, and these factors are dependent on the concentration of the polymer and the crosslinking agent. The increase in Ca^{+2} concentration in the crosslinker solution tends to decrease the degree of swelling of the polymer matrix, reflecting the lower increase in bead volume (Bierhalz et al., 2014).

Microalgae growth immobilized in the alginate beads, as well as for free cell cultivation, are shown in Fig. 4. Growth profile in the alginate beads crosslinked with 5% $CaCl_2$ showed a decrease at 12 h with subsequent growth. Immobilized microalgae in beads crosslinked with 2% $CaCl_2$ showed a maximum in 6 h, remaining practically constant until the end of the experiment. This may be related to the smaller diameter of the particle and possible bead wear. Cells immobilized in 10% $CaCl_2$ crosslinked beads, showed maximum growth at 12 h followed by cell death, possibly caused by a limitation of nutrient diffusion inside the bead. Maximum specific growth rates (μ_{max}) for immobilized *D. subspicatus* in vinasse were in the order of 0.001 h^{-1} (t_g = 693 h), 0.009 h^{-1} (t_g = 77 h) and 0.002 h^{-1} (t_g = 346 h) for alginate beads (2%) crosslinked with $CaCl_2$ 2, 5 and 10% (w/v), respectively. Free microalgae cultivation showed a constant growth rate until 48 h. After that period, there was a slight decrease indicating a possible contamination of the medium (de Mattos and Bastos, 2015). Despite being considered as a great disadvantage of microalgae heterotrophic cultivation, contamination is usually not quantified in terms of growth kinetics (Perez-Garcia et al., 2011). Since vinasse has more than a single assimilable carbon source to heterotrophic microalgae, such as glucose, glycerol and organic compounds with high molecular weight, diauxic growth could have happened (Baroukh and Bernard, 2016), as shown by growth recovery at 72 h. So, two maximum specific growth rates of approximately 0.01 h^{-1} (tg = 66 h) were estimated, one between 0-24 h and the other between 48-72 h. The effect of two different carbon sources was better perceived for free mi-

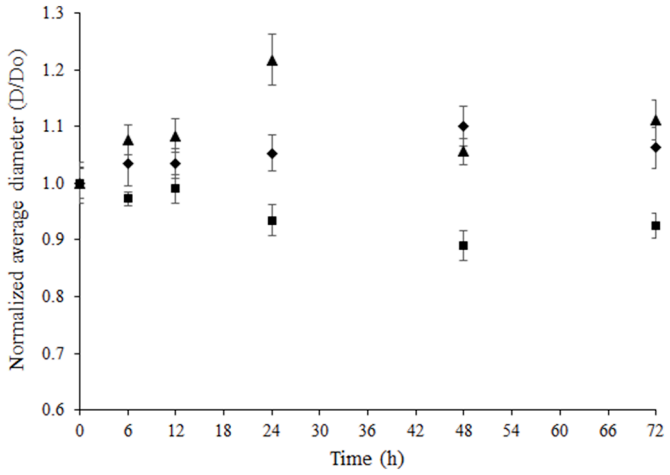

Fig. 3. Normalized average diameter of alginate beads with immobilized microalgae during cultivation in vinasse at 25 °C and 100 rpm. Alginate beads (2% w/v) crosslinked with 2% $CaCl_2$ (■), 5% $CaCl_2$ (▲) and 10% $CaCl_2$ (♦).

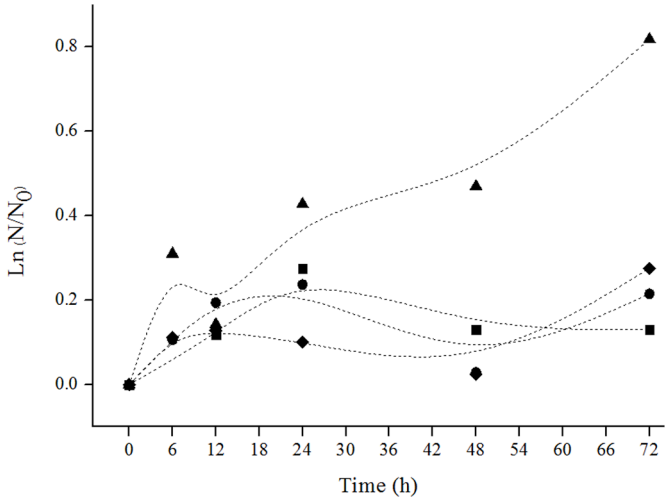

Fig. 4. Growth profile of immobilized *D. subspicatus* during cultivation in vinasse at 25 °C and 100 rpm. Alginate beads (2% w/v) with immobilized microalgae crosslinked with 2% $CaCl_2$ (■), 5% $CaCl_2$ (▲), 10% $CaCl_2$ (♦) and free microalgae (●).

croalgae since, in this case, the interaction between substrates and cells is not mass transfer limited. Under optimized cultivation conditions of aeration and mixing, da Silva et al. (2017) reported higher μ_{max} (0.04 h^{-1}) for free cells of *D. subspicatus* in vinasse at 25 °C in a batch reactor. Lower specific growth rates are expected compared to free cells possibly due to limitation of nutrients and oxygen diffusion through the immobilization matrix (de-Bashan and Bashan, 2010).

Total organic carbon and nitrogen profiles in the vinasse are shown in Fig. 5A and B. A similar trend was observed regardless of the crosslinker concentration used. Removals of approximately 33, 38 and 29% for carbon and 31, 27 and 34% for nitrogen, after 72 h, were obtained for experiments with 2, 5 and 10% of $CaCl_2$ crosslinked beads, respectively. Although microalgae specific growth rate was higher in the experiment using beads crosslinked with 5% $CaCl_2$, this similarity in the carbon and nitrogen profiles may indicate that these compounds were also incorporated by the polymer matrices, not necessarily consumed and consequently not generating biomass and products via metabolic pathways. In terms of wastewater biological treatment, this would be a favorable situation, i.e., carbon and nitrogen removal without considerable biomass growth. For free microalgae, as a consequence of cell growth, carbon and nitrogen removal (Fig. 5A and B) were 45% and 49%, respectively, after 72 h.

Potassium concentration during cultivation of immobilized *D. subspicatus* in vinasse is shown in Fig. 5C. When alginate beads crosslinked with 10% $CaCl_2$ were used as support matrix, a removal of 35% of the initial potassium concentration was achieved, while the removal using 2% and 5% $CaCl_2$ alginate beads, were 25 and 28%, respectively. It has been reported that some polymers are able to adsorb nutrients, which could enhance the efficiency of immobilized cells in wastewater treatment (Khorasani and Shojaosadati, 2019; Mallick and Ray, 1994; Wang et al., 2016). The structure of alginate gel matrix is ruled by the kinetics of its formation, which in turn affects diffusion of solutes in the beads. During the formation of cross-linked alginate beads, diffusion of Ca into the alginate droplet and the self-diffusion of alginate molecules towards an inward moving calcium-induced gelling zone could lead to a high alginate concentration at the Ca^{+2}-alginate interface (Chan et al., 2006; Paques et al., 2014; Skjak-Bræk et al., 1989). So, in the higher $CaCl_2$ concentration (10%), a denser Ca-alginate interface would be formed enhancing potassium uptake by the polymer. In addition, a lower degree of crosslinking may be associated with a higher porosity of the matrix facilitating the diffusion of nutrients through the matrix. In the case of alginate beads crosslinked with 2% $CaCl_2$, a slight increase in the potassium concentration (in bulk solution) is observed after 24 h, which could indicate polymer destabilization, confirmed by the reduction of the bead diameter, especially for the beads crosslinked with 2% $CaCl_2$. For free microalgae the maximum potassium removal was 8% after 72 h, indicating a significant adsorption by the biopolymer. Potassium uptake is most likely to have occur as a function of chemical equilibrium rather than metabolic consumption by cells. Berry et al. (2003) reported potassium accumulation by cyanobacteria *Anabaena* and *Anacystisin* in photoautotrophic growth at 25 °C with luminosities of up to 15 nmol m^{-2} s^{-1}. According to these authors, halophilic cyanobacteria show different mechanisms of ions transfer depending on potassium availability. At low potassium environments, active transport is required. Considering the high potassium concentration in vinasse, passive transport probably occurred due to concentration gradient. Although the importance of transport and accumulation of ions for microalgae and cyanobacteria cultivation, with potential applications on wastewater treatment, few researches evince these aspects (Alahari et al., 2001;

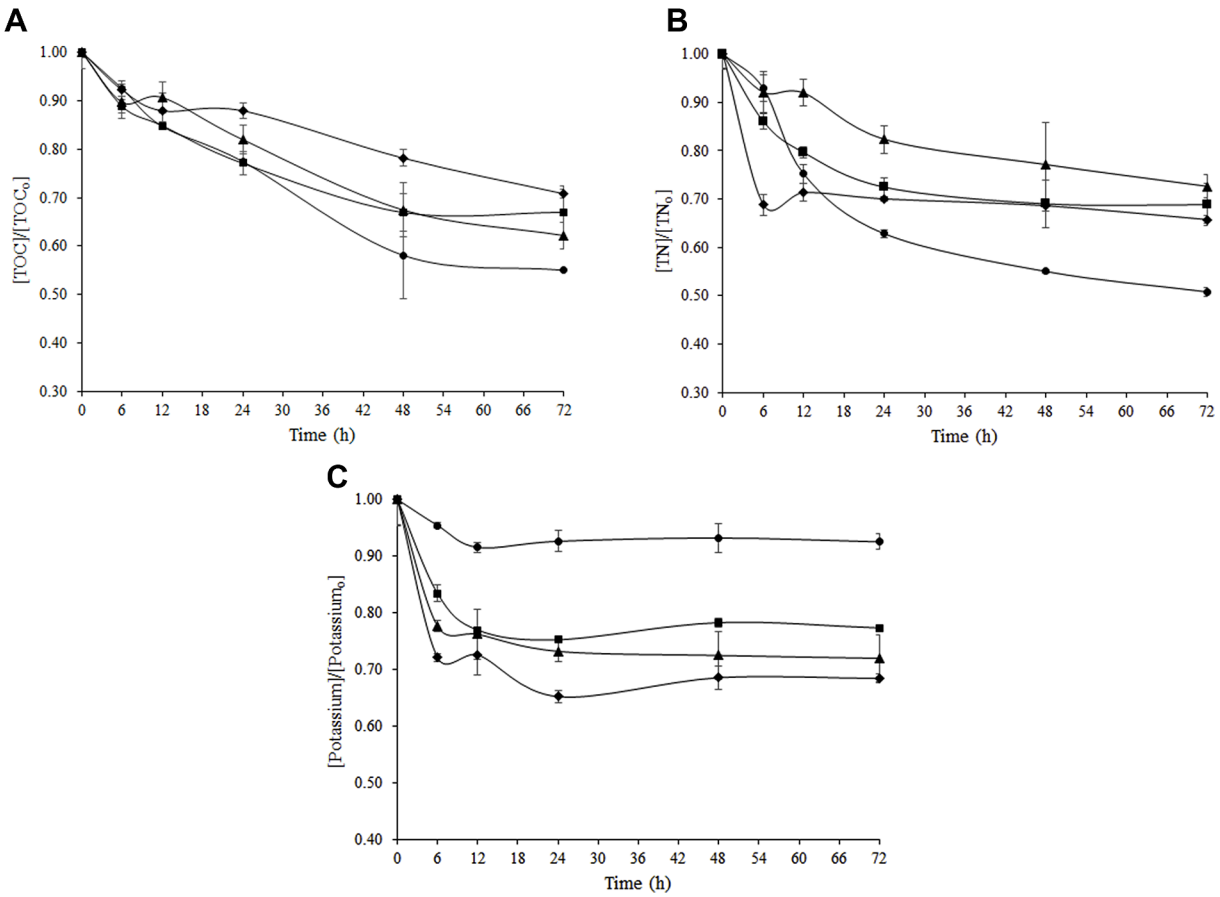

Fig. 5. Total organic carbon (A), total nitrogen (B) and potassium (C) concentration in vinasse during cultivation at 25 °C and 100 rpm. Alginate beads (2% w/v) with immobilized microalgae crosslinked with 2% $CaCl_2$ (■), 5% $CaCl_2$ (▲), 10% $CaCl_2$ (♦), and free microalgae (●).

Berry et al., 2003; Reed et al., 1981). For microalgae, absorption is the main mechanism for nutrient removal, with a direct relationship between specific growth rate and nutrient removal, as for potassium (Xin et al., 2010). Other nutrients, such as nitrogen and phosphorous can be used for cell growth and efficient removal from wastewaters with adequate N/P ratios. This is evidenced by carbon and nitrogen uptake, which were higher for free microalgae compared to immobilized cells (Fig. 5A and B), possible due to lower diffusional restrictions and therefore higher growth rates.

Since potassium is one of the most important nutrients in vinasse composition, not readily removed biologically, potassium removal by blank alginate beads (without microalgae) were evaluated (Fig. 6). In contact with vinasse, blank alginate beads showed, in general, a slightly lower % potassium removal when compared to beads containing microalgae cells (Fig. 5C). Blank alginate beads crosslinked with 2, 5 and 10% $CaCl_2$ promoted maximum potassium removal from vinasse of 17, 27 and 37%, respectively, showing the same trend observed for alginate beads with microalgae, i.e. greatest removal in the first hour of contact with the wastewater. It is important to note that there may have been a restriction on the diffusion of nutrients in the beads crosslinked with higher $CaCl_2$ concentration, exhibiting similar potassium removal for the treatment with blank and microalgae-containing beads.

4. Conclusions

Stable, uniform and spherical alginate beads for immobilization of microalgae *D. subspicatus* were successfully produced. Regarding the biopolymer concentration range tested, 2% alginate leads to the more suitable polymeric matrices, in terms of bead characteristics and process practicability. Bead diameter and mechanical strength increased by increasing the alginate concentration. The presence of microalgae did not significantly alter bead properties. All beads produced showed good chemical stability (100% bead recovery) after 7 days of contact with different liquid media (water, BG11 growth medium and vinasse). Immobilized *D. subspicatus* in alginate (2%) beads crosslinked with different $CaCl_2$ concentrations (2, 5 and 10%) were able to grow and remove significant amounts of potassium, carbon and nitrogen from vinasse. The results indicate good perspectives for the use of these immobilized microalgae for removal of nutrients from vinasse.

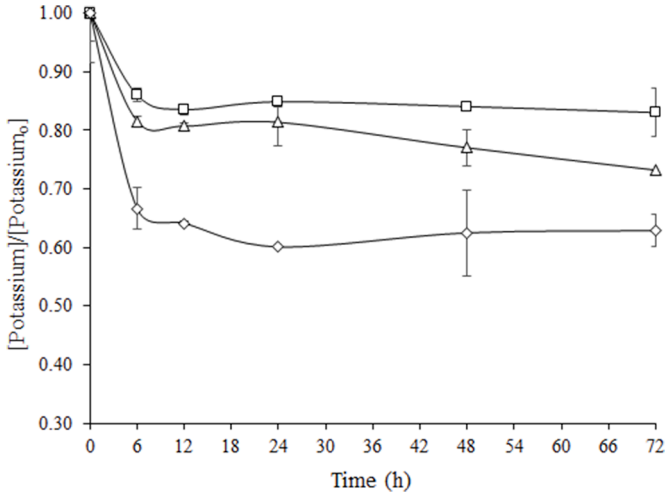

Fig. 6. Potassium concentration in vinasse under cultivation conditions at 25 °C and 100 rpm and presence of blank (without microalgae) alginate beads (2% w/v) crosslinked with 2% $CaCl_2$ (□), 5% $CaCl_2$ (Δ) and 10% $CaCl_2$ (◇).

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