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Influence of di-rhamnolipids on the enzymatic hydrolysis of steam-pretreated eucalyptus wastes

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ABSTRACT

In this present work, rhamnolipids produced from *Pseudomonas aeruginosa* TGC07 were evaluated as biosurfactants for enzymatic hydrolysis of steam pretreated (not delignified) eucalyptus wastes comparatively to the synthetic surfactant Triton X-100. Enzymatic hydrolyses were performed at different substrate loadings (1%, 5% or 10% w/w) using commercial enzymes (Cellic® CTec1) at different dosages (14 or 28 FPU/$g_{cellulose}$). The di-rhamnolipids were characterized by NMR spectroscopy. Cellulose conversions higher than 60% were achieved after 72 h runtime with the use of di-rhamnolipids at 5% solids, i.e., significantly superior to the conversions obtained from experiments performed without use of any surfactant, and similar to the performance of synthetic Triton X-100 at 5% solids. These results evidence that the use of natural renewable di-rhamnolipids as biosurfactants emerges as an interesting environmentally-friendly alternative to replace synthetic surfactant Triton X-100 as surfactant, aiming to increase production yields of cellulosic sugars by enhancing enzymatic hydrolysis of lignocellulosic biomasses such as steam pretreated eucalyptus wastes.

1. Introduction

Eucalyptus is a fast-growing plant and is used as raw material for the production of cellulose paper (Sarto and Sansigolo, 2010). During the processing of eucalyptus, there is the generation of small pieces of wood, called chips, which are used as solid fuel to supply thermal energy in the boilers (Silva et al., 2015). The eucalyptus chips present 42–45% of cellulose, 27–30% of hemicellulose, 20–28% of lignin and 3–5% of extractive and non-extractive (Rencoret et al., 2007; Klock et al, 2005). Therefore, the eucalyptus chips present potential for its reuse as a source of lignocellulose (Lin et al., 2017; Nonaka et al., 2013). The enzymatic hydrolysis of lignocellulosic biomasses such as eucalyptus wood has been pointed out as an interesting alternative for production of fermentable sugars (Araújo et al., 2017; Mcintosh et al., 2016).

Recent studies have reported that the utilization of surfactants can significantly enhance the enzymatic digestibility of the holocellulose (cellulose and hemicelluloses) fraction present in the lignocellulosic biomasses, particularly due to their interactions with lignin, preventing undesirable adsorption of the cellulases onto the surface of this material, optimizing the efficiency of the enzymes as saccharification agents and increasing total sugar recovery (Lin et al., 2017; Nonaka et al., 2013). However, the synthetic-based surfactants that are commonly employed as hydrolysis inducers, such as Triton X-100, are not biodegradable, as well as present toxicity to the environment (Tu and Saddler, 2010).

On the other hand, biosurfactants have attracted the attention due to their specificity, biodegradability and biocompatibility. Specifically, the rhamnolipids, biosurfactants produced by *Pseudomonas aeruginosa* have been pointed out as potential additives for enzymatic hydrolysis of lignocellulosic biomasses (Hou et al., 2017; Zhou et al., 2015; Feng et al., 2013; Wang et al., 2011). Beyond presenting elevated biodegradability, excellent surface activity and renewable properties, rhamnolipids can increase the transcellular transport of organic compounds in the plasma membrane, such as glucose, helping their permeation through the microbial cell surface, increasing ethanol production, and therefore there is no need for removal of such species from the hydrolysate after the saccharification phase (Zheng et al., 2019; Jiang et al., 2013; Zhong et al., 2008).

Due to this effect, the addition of rhamnolipids in enzymatic hydrolysis has been presented as an alternative to the delignification treatments of biomasses (chemical process commonly used to increase

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the yield of the enzymatic digestibility of lignocellulosic biomasses) (Araújo et al., 2017; Zhang et al., 2015). Hence, based on this perspective, the present study aimed to investigate the influence of rhamnolipids as biosurfactant for enzymatic hydrolysis of non-delignified eucalyptus chips, as well as to evaluate its potential comparatively to Triton X -100.

2. Materials and methods

2.1. Microorganism

Pseudomonas aeruginosa TGC07 was isolated from the sandy soil in the central area next to the pumps from a gas station in the city of João Pessoa-PB, Brazil (7°10′54″S - 34°54′01″W). This strain was preserved in 10% glycerol (v/v) at −20 °C.

2.2. Rhamnolipids production

To obtain the inoculum, the strain grown in Tryptone Soya Agar was added to a 250 mL Erlenmeyer flask, which contained 50 mL of Nutrient Agar, incubated in a rotary shaker at 30 °C and 150 rpm for 24 h. Then, 1.25 mL of the suspension of cells was transferred to another 250 mL Erlenmeyer flask, containing 25 mL of following culture medium (Patel and Desai, 1977; Bezerra, 2017): 1.42 g/L K_2HPO_4, 50 mg/L $MgSO_4.7H_2O$, 30 mg/L $FeSO_4.7H_2O$, 21 mg/L $ZnCl_2$, 10 mg/L $CaCl_2$, 1.54 mg/L $MnSO_4.H_2O$, 41 µg/L $CoCl_2.6H_2O$, 93 µg/L $CuSO_4.5H_2O$ e 25 µg/L $Na_2MoO_4.2H_2O$. Commercial glycerol and $NaNO_3$ concentrations were 40 and 4 g/L, respectively, in both culture media. Both cultivations were carried out for 120 h at the same temperature and with the agitation of the inoculum.

2.3. Biosurfactants isolation and purification

After concentration, the biosurfactant was applied to a width 30 cm and diameter of 2 cm column containing 33 g of silica gel 60 (0.063–0.2 nm) eluted with $CHCl_3$/MeOH mixture with gradual increasing of the MeOH content (95:5–0:100). Subsequently, the fractions obtained were examined by TLC, using $CHCl_3$:MeOH (80:20, v/v) as mobile phase and sulfuric anisaldehyde as a spraying reagent. Fraction eluted in $CHCl_3$:MeOH 50:50 (v/v) consisted of rhamnolipid was analyzed by NMR spectroscopy.

2.4. Eucalyptus wastes

Eucalyptus grandis chips pretreated by steam explosion at 200 °C for 10 min in a 200-L STEX reactor (America Biomass Technologies, Brazil) were kindly provided by AmericaBio (Porto Alegre, Brazil). The contents of major biomass components such as cellulose/glucan, hemicelluloses/xylan, lignin and total ashes were performed using the methodology validated by Gouveia et al. (2009), which was based on the method published by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008).

2.5. Analytical methods

2.5.1. Cell concentration

Biomass concentration was determined as dry weight of solids at 80 °C during 24 h, after filtration in a Millipore (0.22 mm) membrane.

2.5.2. Rhamnose concentration

The rhamnose concentration was assayed by colorimetric method using thioglycolic acid (Rahman et al., 2002). The mixture containing one milliliter of cell-free culture broth and 4.5 mL of dilute sulfuric acid (6:1 v/v) was mixed thoroughly and heated at 100 °C. After 10 min the mixture was cooled down to room temperature. A volume of 0.1 mL of freshly prepared 3% solution of thioglycolic acid was added to the mixture, which was incubated in darkness for three hours. Absorbance was measured at 400 and 430 nm in a spectrophotometer (700 Plus, Femto). The rhamnose concentration was calculated using a calibration curve prepared using different concentrations of L-rhamnose (Sigma). The correlation coefficients (R^2) of the calibration curves were higher than 0.998.

2.5.3. Emulsification index

The emulsification index (E24) of supernatant samples was determined by adding 2 mL of commercial soybean oil to 2 mL of culture supernatant, mixing with a vortex for 1 min, and leaving to stand for 24 h. The emulsifying activity was calculated using the following Eq. (1).

$$E24(\%) = H_E / H_S * 100 \quad (1)$$

H_E is the height of the emulsified region and H_S is the total height of the solution. To verify the stability of the emulsion, the emulsification indices were determined for 120 h (E48, E72, E96 and E120).

2.5.4. High performance liquid chromatography

Glucose and glycerol concentrations were determined by high performance liquid chromatography (HPLC) in a Shimadzu system equipped with a quaternary pump coupled with a degasser, an oven for controlling column temperature, set at 40 °C, and a refractive index detector. The software used for data acquisition was LC Solutions, manufactured by Shimadzu Corporation (Kyoto, Japan). A 300 mm x 7.8 mm ionic exchange column (Aminex® HPX-87H, Bio-Rad, USA), with 9 mm particle size, was used. The mobile phase used was ultrapure water acidified with isocratic elution of H_2SO_4 5 mM at a flow rate of 0.6 mL/min. The correlation coefficients (R^2) of the calibration curves were higher than 0.999.

2.5.5. Nuclear magnetic resonance analysis

^1H and ^{13}C 1D and 2D NMR spectra were recorded on a Varian/AGILENT UNMRS spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C. All spectra were measure at 300 K. Sample was dissolved in $CDCl_3$ and transferred to a 5 mm NMR tube. The chemical shifts were given on the δ (ppm) scale, referenced to residual $CHCl_3$ signal (δ_H 7.24 and δ_C 77.23 ppm). The experiments were performed using the standard AGILENT pulse sequences. For ^1H NMR spectrum s2pul pulse program was used, 32 scans, 2.55 s acquisition time, 1.0 s interscan delay, 16 K complex data points; for ^{13}C s2pul pulse program was used, 1016 scans, 1.21 s acquisition time, 1.0 s interscan delay, 32 K complex data points; for the 2D ^1H-^1H COSY spectrum gCOSY pulse program was used, 8 scans, 0.15 s acquisition time, 1.0 s interscan delay, 481 complex data points in F2 and 128 in F1; and for ^1H-^{13}C HSQC spectrum gHSQC-EDITED pulse program was applied, 4 scans, 0.15 s acquisition time, 1.0 s interscan delay, 481 complex data points in F2 and 128 in F1. All spectra were zero-filled prior to Fourier transformation. Spectra were processed and analyzed using the MNova 10.0 software [Mestrelab Research SL, Santiago de Compostela, Spain.]

2.6. Enzymatic hydrolysis

Enzymatic hydrolyses were performed using 250 mL erlenmeyers, varying substrate loadings (1%; 5% or 10% w/w) and enzyme loadings of commercial enzymes Cellic® CTec1 (14 or 28 FPU/$g_{cellulose}$). The activity of cellulase was 100 FPU/mL. The amount of rhamnolipids was determined with basis on their critical mycelar concentration, i.e., 100 mg/L. The flasks containing the substrate (pretreated eucalyptus) were sterilized at 121 °C por 15 min prior to the addition of the biosurfactants and enzymes. The volumes of de mixtures were completed to 50 mL using sterilized sodium citrate buffer (pH 4.8 at 50 mM). The erlenmeyers were placed in a rotary shaker (incubator) at 50 °C e 150 RPM for 72 h. The concentration of the glucose (monomer) produced during the enzymatic hydrolysis were determined by High-Performance

Liquid Chromatography (HPLC). Similar experiments were performed in absence of surfactants and using Triton X-100 (100 mg/L), aiming to evaluate the performance of the rhamnolipids during the enzymatic hydrolysis, as well as its potential as inducer comparatively to the synthetic surfactant. The initial rates of enzymatic hydrolysis, expressed as g/L.h, were determined from the angular coefficient of the line adjusted to experimental glucose concentration as a function of reaction time, considering only the linear region of glucose formation. The enzymatic digestibility was calculated as the ratio of glucose in the enzymatic hydrolysis per 100 g of potential glucose in the water-insoluble solid fraction.

2.7. Statistical analysis

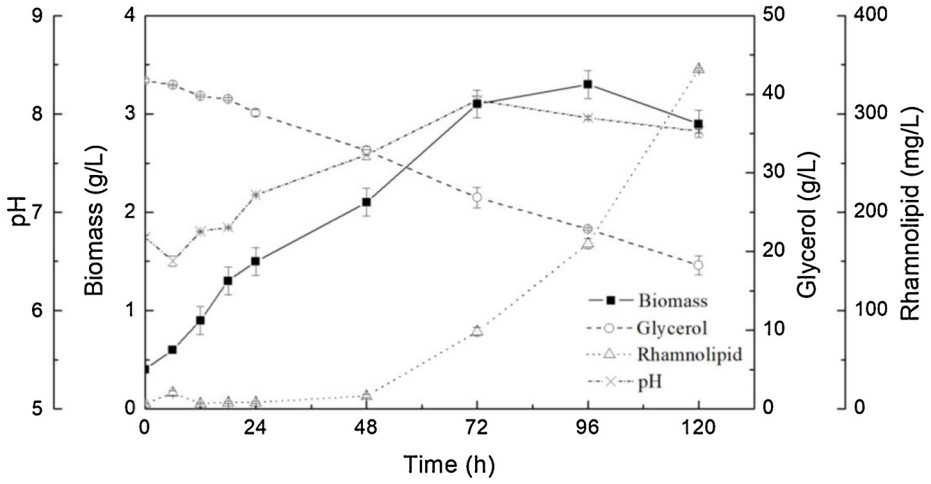
PAST software 3.14 for Windows was used to determine the Tukey test ($p \leq 0.05$).

3. Results and discussion

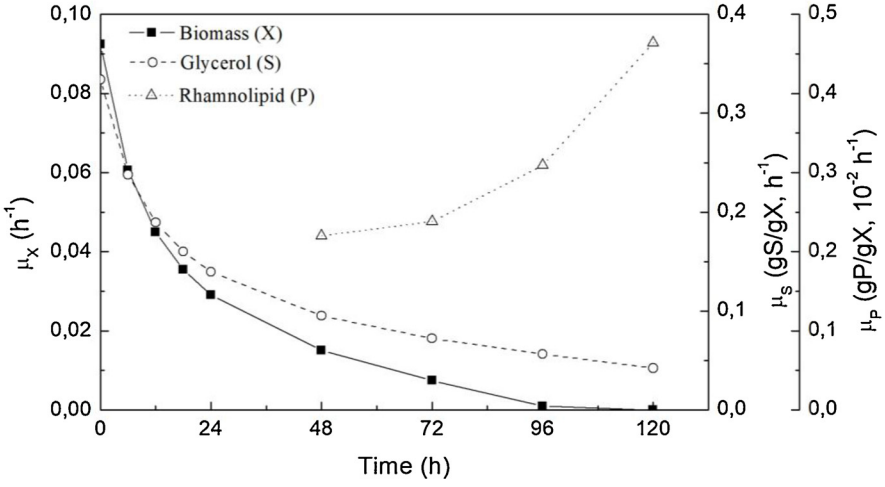
3.1. Production of biosurfactants

Fig. 1a shows the concentrations of biomass, glycerol, rhamnolipids and value of the pH during the cultivation. Fig. 1b shown the specific growth rates, substrate consumption and the formation of biosurfactant during cultivation of *Pseudomonas aeruginosa* TGC07. The rhamnolipids were expressed in terms of rhamnose.

There was observed an exponential growth during the first 24 h, but no relevant biosurfactants production. After 48 h the growth rate decreased and occurred a significant increase in the formation of the product. The synthesis of rhamnolipids was not associated to the growth, it was a behavior typical of secondary metabolites. Partovi et al. (2013), Thavasi et al. (2011) and Rahman et al. (2010) have observed similar behavior in the production of rhamnolipids by other strains of *Pseudomonas aeruginosa*. The pH increased reaching values close to 8.0 with 120 h. This increase was attributed to denitrification under micro aerobic conditions of nitrate to ammonia by *Pseudomonas aeruginosa*, which reacts with water to produce ammonium hydroxide, raising the pH of the cultivation (Onwosi and Odibo, 2012; Zhu and Rock, 2008; Sousa et al., 2014). The ratio of carbon and nitrogen (C/N) sources in 24 $g–g^{-1}$ and denitrification of nitrate provided a nitrogen limiting condition. This resulted in a total consumption of glycerol of approximately 55%. Sousa et al. (2014) observed that the ratios C/N of 86–12 in cultures of *P. aeruginosa* using glycerol and $NaNO_3$ as carbon and nitrogen sources, respectively, resulted in a total glycerol consumption of 37–51%.



a.



b.

Fig. 1. The concentrations of biomass, glycerol, rhamnolipid and value of the pH during the cultivation of *Pseudomonas aeruginosa* TGC07 (a) and specific rates of growth of biomass (X), consumption of glycerol (S) and production of rhamnolipid (P) (b).

3.2. Characterization of biosurfactant

The emulsification index expresses the emulsifying activity of the surfactants. According to Willumsen and Karlson (1997) and Müller et al. (2012), a method to determine a good emulsifying agent is to assess the ability of the surfactant to form an emulsion with hydrocarbons having a height equal to or greater than 50% of the total height of the blend for 24 h.

Due to the low production of rhamnolipids in the first 24 h of culture, samples from time 0 to 24 h did not show emulsion, and consequently, their emulsification index were zero. However, from the 48 h of culture, samples from the cell free broth provided E24 emulsification rates between 55 and 65%, showing that the rhamnolipids produced in this study are good emulsifying agents. It was also observed that even with different concentrations of rhamnolipids, in terms of rhamnose, there were no significant differences between E24 emulsification indices. Thus, below 80 mg/L in terms of rhamnose, the unpurified rhamnolipids did not present emulsifying activity, however from this concentration; the rhamnolipids presented a high emulsification capacity, which remained constant with the increase of the concentration of the rhamnolipids the samples. The emulsification index E48, E72, E96 and E120 were determined in order to follow the stability of the emulsions in the period of 120 h, which are presented in Fig. 2.

The sample with 48 h of culture presented a variation between its E24 and E120 indices of 42.85%. However, samples with 72, 96 and 120 h of culture did not show significant differences between their emulsification indices, indicating that their emulsions remained stable for 120 h. Thus, the emulsions formed tended to remain stable with a greater amount of biosurfactant in the sample.

The purified fraction of TGC07 strain was analyzed by analytical TLC using $CHCl_3$:MeOH:H_3CCOOH (65:15:2, v/v/v) as mobile phase. Essentially a major component with Rf 0.51 were observed. Raza et al. (2009) observed in TLC analysis of rhamnolipids produced by *P. aeruginosa* EBN-8 Rf values of 0.52–0.73, eluted on silica gel with a solution of $CHCl_3$:MeOH:H_3CCOOH (65:15:2, v/v/v), being the first band related to the di-rhamnolipid and the second to the mono-rhamnolipid. Therefore, the TGC07 strain produced predominantly only one homologue of the rhamnolipids, which was related to the di-rhamnolipid.

The structure of the di-rhamnolipid produced by *P. aeruginosa* TGC07 was confirmed by 1D and 2D ^1H and ^{13}C NMR analysis, including those from ^1H, ^{13}C, ^1H- ^1H COSY and ^1H-^{13}C HSQC experiments (See SM) and by comparison of their spectroscopic data with those found in the literature (Varjani and Upasani, 2016). ^{13}C NMR spectrum showed signals at δ 171.69 and δ 173.98 assigned to ester and carboxylic groups, respectively, characteristics of rhamnolipid (Raza et al., 2009; Monteiro et al., 2007). The signals from δ 22 to 42 ppm were attributed to the methylene groups of the lipid, and from δ 14 to 18 ppm, were consistent with the presence of methyl groups, suggesting two fatty acids chains linked through an ester bond. 2D ^1H-^{13}C HMQC experiment showed correlations at δ 4.9/94.34 and δ 4.9/102.17, attributed to the presence of two anomeric carbons, confirmed two rhamnose moiety. The spectroscopic data of the di-rhamnolipid produced by TGC07 strain were comparable to previous reports (Varjani and Upasani, 2016; Monteiro et al., 2007) and the results were in accordance with the structure.

3.3. Addition of surfactants in the enzymatic hydrolysis

Fig. 3 shows the glucose concentration during hydrolysis with and without addition of surfactants, by varying the enzyme load (14 and 28 FPU/g) and the substrate load (1% w/w - Fig. 3a; 5% w/w - Fig. 3b; 10% w/w - Fig. 3c). Initial rates of enzymatic hydrolysis of eucalyptus chip with the enzyme load of 14 and 28 FPU/g for conditions with and without addition of surfactants are shown in Fig. 4.

In relation to saccharification with 1% of eucalyptus and 14 FPU/g of enzymatic loading, it was observed that the glucose concentrations were equivalent, leading to initial velocities without significant differences. However, at 28 FPU/g the rhamnolipids provided an increase in glucose concentration of 29 and 64% on the condition with the synthetic surfactant and in the absence of surfactants, respectively. In the hydrolysis of 5% biomass, with two enzyme loads, the glucose concentrations with synthetic surfactant and biosurfactant showed similar profiles. There was a variation of less than 5% between the results with

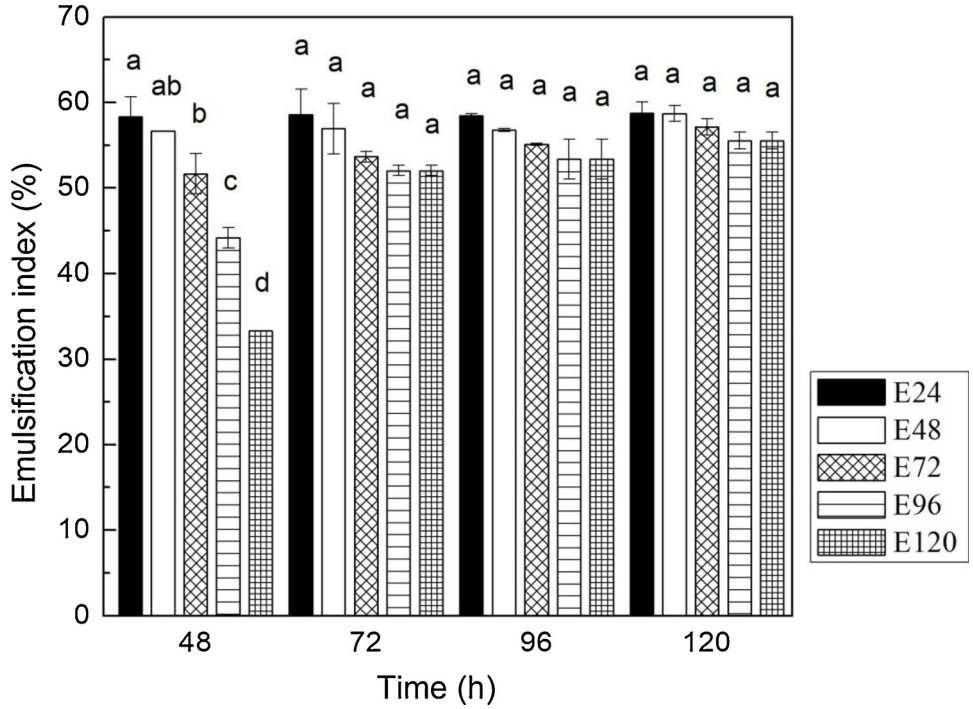
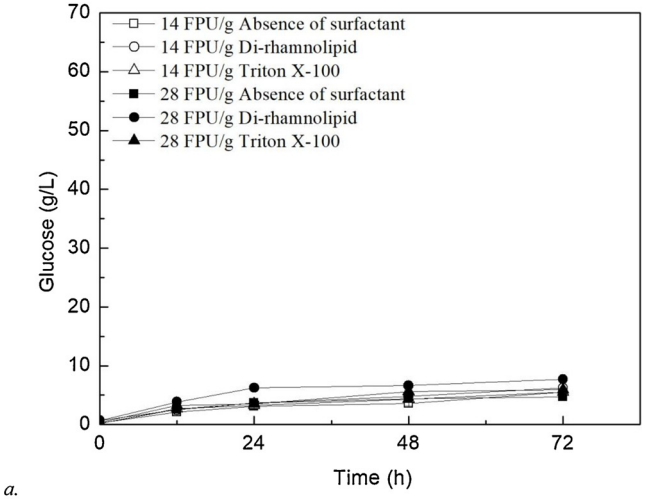
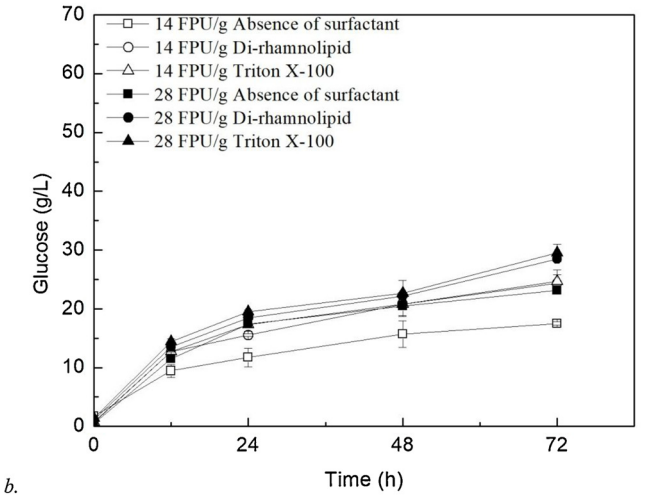


Fig. 2. Emulsification Index E24, E48, E72, E96 and E120 from the cell free samples during the cultivation of *Pseudomonas aeruginosa* TGC07. Different letters or set of lower case letters demonstrate significant difference ($p \leq 0.05$) between the emulsification indexes at each culture time.



a.



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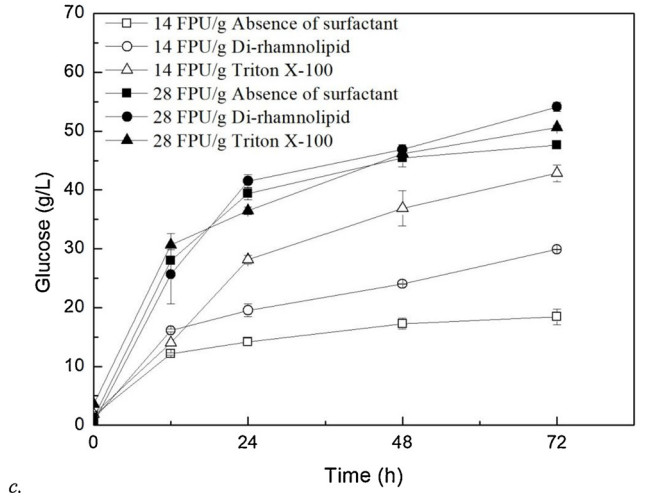
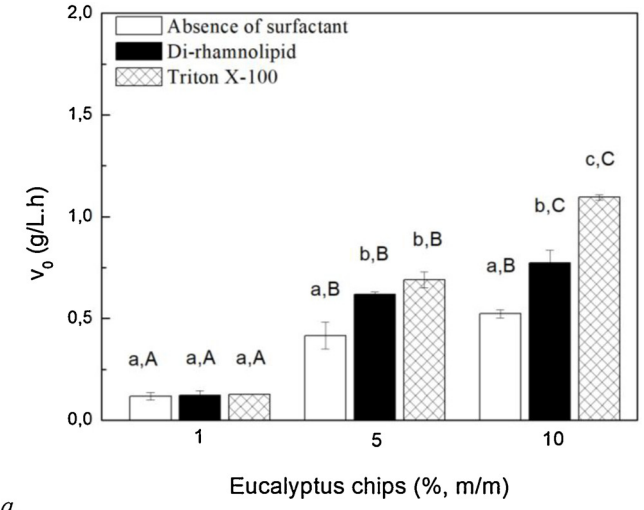
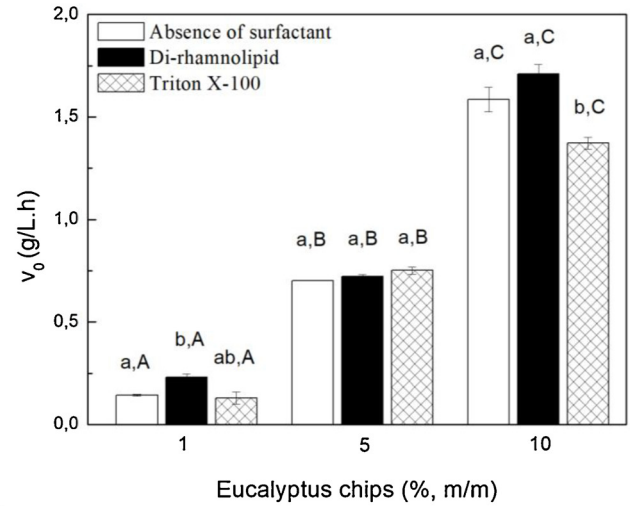


c.

Fig. 3. Glucose concentrations during the enzymatic hydrolysis of 1 (a), 5 (b) and 10% (w/w) (c) eucalyptus chip using Cellic® CTec1 at 14 and 28 FPU/gcellulose containing Triton X 100, di-rhamnolipids and in the absence of surfactant.



a.



b.

Fig. 4. Initial rates of the enzymatic hydrolysis reaction of the eucalyptus chip at 1, 5 and 10% (w/w) using Cellic® CTec1 at 14 (a) and 28 FPU/gcellulose (b) with Triton X, di-rhamnolipids and in the absence of surfactant. Different capital and small letters demonstrate significant statistical differences ($p \leq 0.05$) between initial rates for a given substrate loading. Different capital letters demonstrate significant statistical differences ($p \leq 0.05$) between the initial velocities of each group.

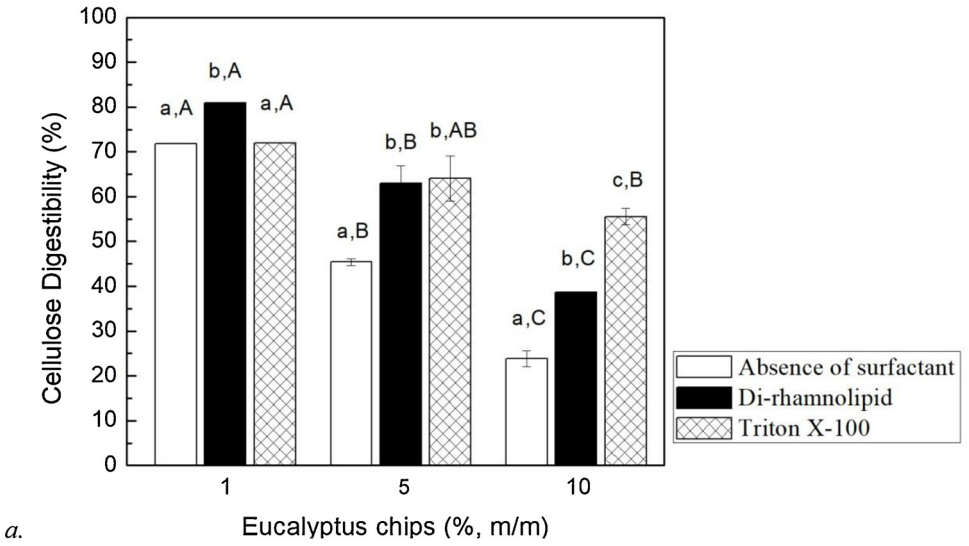
Triton and biosurfactant. These results were 41 and 27% higher than those without surfactant with 14 and 28 FPU/g, respectively.

In the 10% chip process with the lowest enzyme loading, Triton X-100 increased the glucose concentration by 43% in relation to biosurfactant. However, in the hydrolysis with biosurfactant the glucose concentration was 62% higher than that obtained without addition of surfactant. Moreover, with the higher enzymatic loading, the di-rhamnolipids showed a better performance in the enzymatic saccharification, resulting in a 7 and 14% increase in glucose concentration in the hydrolysis with Triton X-100 and in the absence of surfactant, respectively.

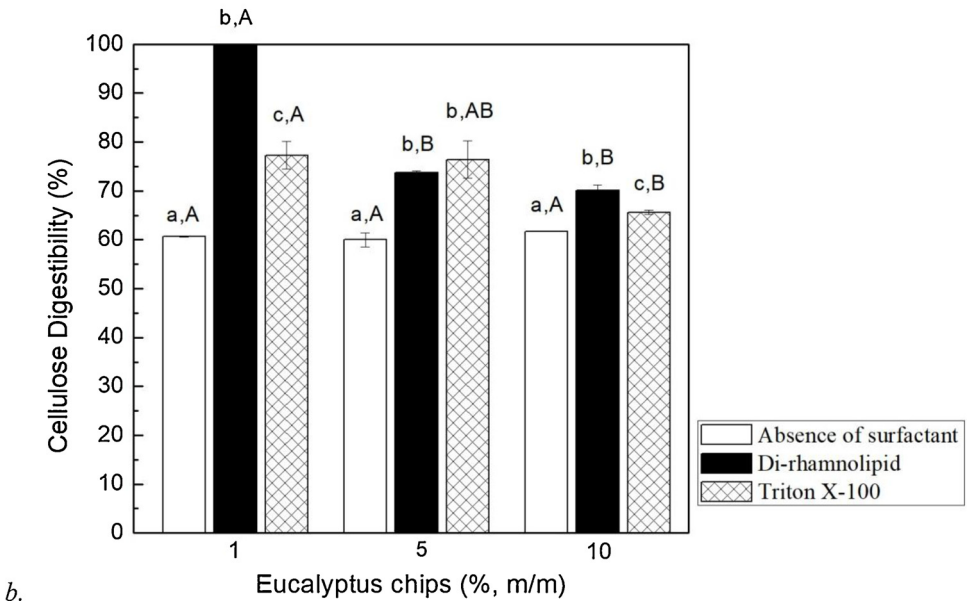
Fig. 5 presents the cellulose digestibility after 72 h enzymatic hydrolysis experiments performed in absence and presence of surfactants (rhamnolipids and Triton X-100) using different substrate and enzyme loadings.

The increment of substrate loading resulted in lower cellulose digestibilities at reduced enzyme loadings, for experiments performed in absence of surfactant. Conversely, the increment of substrate loading resulted in similar results when the enzyme loading was doubled. Hence, it was evidenced that, regardless of substrate loading, it is necessary to increase the enzyme loading in order to achieve cellulose digestibilities greater than 60% when surfactants are not used.

With respect to the experiments performed with use of surfactants, it was evidenced that, regardless of substrate or enzyme loadings, the



a.



b.

Fig. 5. Cellulose digestibility after 72 h of enzymatic hydrolysis of steam pretreated eucalyptus chips. Solids: 1, 5 and 10% w/w. Enzyme (Cellic® CTec1) load: 14 (a) and 28 FPU/gcellulose (b), using surfactants (Triton X-100 or di-rhamnolipids) or in absence of surfactant. Different capital and small letters demonstrate significant statistical differences ($p \leq 0.05$) between cellulose digestibilities for a given substrate loading. Different capital letters.

addition of biosurfactants (di-rhamnolipids) resulted in superior glucan digestibility levels. For perspective, it was possible to achieve enzymatic digestibility of glucan greater than 60% (at 5% substrate load) without need to double the enzyme amounts when surfactants were added to the reaction medium. Specifically, the increment of enzyme loading (at 5% substrate loading) using surfactants resulted in enzymatic digestibilities 17.5% superior to the previous ones obtained in experiments performed without use such species.

The use of synthetic surfactant (Triton X-100) also increased the cellulose digestibility comparatively with the hydrolysis experiments performed without surfactant. The highest increment was evidenced for substrate loadings of 10% w/w at lower enzyme dosages. Conversely, for experiments performed with biosurfactants at similar substrate loading (10% w/w) it was necessary to double the enzyme dosages in order to achieve glucan digestibility levels superior to 60%.

The presence of surfactant agents improved the enzymatic digestibility of the steam pretreated eucalyptus chips. Particularly, the di-rhamnolipids presented suitable performance with regard to the enzymatic hydrolysis of the steam pretreated eucalyptus chips, comparatively to the synthetic-based Triton X-100. Therefore such natural-based di-rhamnolipids can be pointed out as a potential alternative to be used for the enzymatic saccharification of the cellulose fraction of the steam pretreated eucalyptus.

According to Lin et al. (2017) and Zhou et al. (2015), the differences between the structure of Triton-X (hydrophilic portion constituted by a polyethylene oxide chain combined with a hydrophobic region constituted by tetra-methyl-butyl-phenil groups) and the structure of the di-rhamnolipids (hydrophilic region constituted by rhamnose units coupled with a hydrophobic portion constituted by alkyl polysaccharides plus two fatty acids chains linked by esther bindings) might result in different effects over the performance of the hydrolysis of the eucalyptus. Hence, the interactions involving the fatty acids-based chain from the di-rhamnolipids and the hydrophobic portion of the LCC (Lignin-Carbohydrate Complex) might have been particularly efficient to prevent irreversible adsorption of cellulases onto the cellulignin (LCC) surface, comparatively to the action of non-polar regions of Triton X-100. As result, the accessibility of the enzymes to the cellulose regions of the substrate, and subsequent hydrolysis, have been

significantly enhanced by the action of the di-rhamnolipid-based surfactant.

Non-delignified biomasses present higher resistance to enzymatic hydrolysis, promoting the loss of activity by unproductive adsorption of cellulases to the lignin surface (Lin et al., 2017). Therefore, to increase the yield of the hydrolysis of lignocellulosic biomasses, generally the lignin must be removed or modified (Linder et al., 1995).

Not-delignified steam pretreated *Eucalyptus grandis* chips contained 69.47 ± 1.56% cellulose/glucan, 2.19 ± 0.01% hemicellulose/xylan, 36.61 ± 4.11% lignin and 1.42 ± 0.04% of ashes. Cellulose conversions obtained in the present study using 10% w/w and 28 FPU/g were compared to results gathered from literature related to the conversion of pre-treated and delignified eucalyptus biomasses.

Cebreiros et al. (2018) in the enzymatic hydrolysis of *Eucalyptus grandis* delignified with 16% (w/w) and 25 FPU/g, obtained a conversion 58.6% lower than the conversion obtained with substrate loads and enzymes, 10% w/w and 28 FPU/g, respectively, and with addition of biosurfactant. Sun et al. (2014) and Park et al. (2012) using delignified eucalyptus loads at 2 and 5% (w/w) and 17 and 30 FPU/g, respectively, obtained cellulose conversions 6% lower than the conversion obtained in the present work with non-delignified 10% eucalyptus (w/w) and 28 FPU/g containing di-rhamnolipid. The same behavior was verified when comparing this result with Lima et al. (2013) which used non-delignified eucalyptus at 5% (w/w) and 25 FPU/g. Romaní et al. (2011), using non-delignified eucalyptus at 25% (w/w) and 10 FPU/g, obtained conversion 40% lower than the conversion obtained in the present work with non-delignified 10% eucalyptus (w/w) and 28 FPU/g containing di-rhamnolipid.

Based on these comparisons, it was found that addition of the di-rhamnolipids have promoted the enzymatic hydrolysis of non-delignified eucalyptus. This has led to an increase in eucalyptus hydrolysis efficiency indicating that the use of di-rhamnolipids as surfactant may be a promising alternative to delignification treatments.

It is emphasized that in these studies were used different species of eucalyptus, which have different compositions and structures of the lignin itself (SGPHB - Syringil, guayacyl and p-hydroxyphenyl - ratio and distribution) as well as for the whole LCC (Lignin-Carbohydrate Complex). Hence, each treatment and different severity levels (Log Ro) cause specific modifications in both chemical composition and physical structure of a given lignocellulosic biomass material. Therefore, studies of enzymatic hydrolysis with diverse eucalyptus species submitted to different treatments with and without surfactants are certainly required to be performed in future works and investigations.

3.4. Economic benefit in 2G ethanol production

The use of rhamnolipids as surfactant might represent relevant cost reductions with regard to the production of 2 G ethanol from eucalyptus wastes. As shown in Fig. 6, the use of rhamnolipids during the enzymatic hydrolysis of steam pretreated eucalyptus chips resulted in an overall 2 G Ethanol production cost approximately 5.0% lower when compared to the one obtained for the process conducted without any surfactant. Basically, major cost reductions (approximately 21%) can be attributed to the pretreatment phase (see Table 1), particularly as result of the elimination of the organosolv delignification step, which has significant impact over the operational Formula Cost, as well as due to significant lower capital costs (CAPEX) obtained for the process baseline performed with the use of surfactant. It is also noteworthy that, as mentioned by Zhong et al. (2008) and Jiang et al. (2013), the presence of rhamnolipids in the enzymatic hydrolysates might significantly enhance the ethanol yields during the fermentation step, most probably due the fact that such surfactants promote the transport of the sugars, particularly glucose, through the membrane of the cells, thus favoring their permeation and absorption by the surface of the yeast.

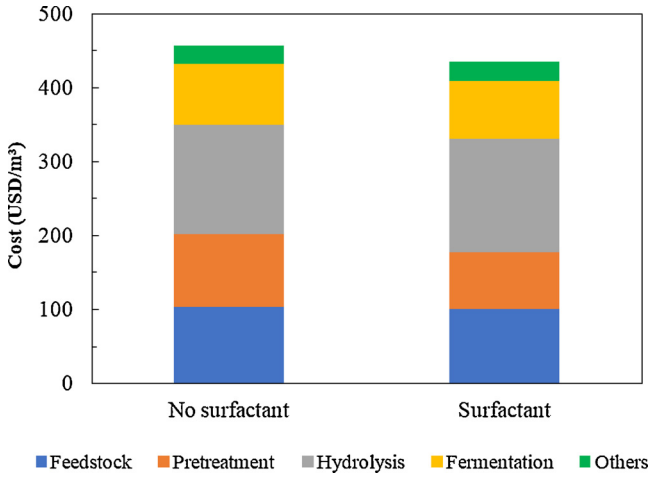


Fig. 6. Overall Production Cost of 2 G ethanol from eucalyptus wastes with no surfactant and with use of rhamnolipids as surfactant. (USD/m³ ethanol). Feedstock cost: 22 USD/ton. Pretreatment routes: steam treatment + organosolv delignification (no surfactant); steam pretreatment only (with surfactant). Ethanol yield (C6 basis, with surfactant): 215 L/ton dry biomass. Enzyme cost: 5.50 USD/kg (70 mg protein/g product, not purified). Surfactant cost: 3.50 USD/kg (not purified).

Table 1
2G ethanol production cost (USD/m³).

	No surfactant	Surfactant	Reduction (%)
Feedstock[a]	104	100	3.6
Pretreatment	98	77	21.4
Hydrolysis	148	154	−4.1
Saccharification[b]	**246**	**231**	**6.1**
Fermentation	83	79	4.8
Others	25	25	0,0
Total	**458**	**435**	**5.0**

[a] Moisture content: 30%.
[b] Enzyme dosage: 4 mg protein/$g_{cellulose}$, Total Solids (TS) content: 15%. Fed-batch enzymatic hydrolysis. 50 °C, pH 5.0, 48 h reaction time. Saccharification encompasses pretreatment and enzymatic hydrolysis steps.

4. Conclusions

Pseudomonas aeruginosa TGC07 produces a di-rhamnolipid, which can be pointed out as an effective emulsifying agent. The emulsions produced tend to remain stable when higher amounts of the di-rhamnolipids are employed in the medium, regardless the fact that the concentration of the biosurfactants are not linearly correlated to their emulsifying activity. It is noteworthy that biosurfactants such as di-rhamnolipids emerge as environmentally-friendly alternatives for production of cellulosic sugars from eucalyptus wastes, and possibly from other lignocellulosic biomasses, via enzymatic hydrolysis. Additionally, the use of rhamnolipids as surfactants for saccharification of eucalyptus wastes via enzymatic hydrolysis can be pointed out as potential alternative to chemical delignification, thus resulting in more environmentally-friendly processes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111835.

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