Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v41i1.39693

Screening of fungal strains with potentiality to hydrolyze microalgal biomass by Fourier Transform Infrared Spectroscopy (FTIR)

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ABSTRACT. The use of fungi is a promising alternative for harnessing biomass after lipid extraction of microalgae since this biomass may contain relevant levels of carbohydrates that can be converted into other compounds of interest, such as sugars. Fourier Transform Infrared Spectroscopy (FTIR) has proved to be an efficient and environmentally less impacting tool for the selection of microorganisms with biotechnological potential. This study aimed to apply FTIR for the selection of fungal strains with potential to hydrolyze the biomass of the microalgae *Desmodesmus subspicatus* and *Chlorella* sp after lipid extraction. Eleven fungal strains were screened for residual biomass hydrolysis and FTIR was applied followed by multivariate analysis for the selection of filamentous fungi. The highest cell density was 28.7 × 10^6 cells mL^{-1} for *Chlorella* sp. and 15.8 × 10^6 cells mL^{-1} for *D. subspicatus* and the values of total carbohydrates content were 23.1 and 16.9%, respectively. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were useful tools to screen fungal strains. After multivariate analysis, it was possible to observe that the fungi strains that presented the greatest ability to use microalgal biomass were *Penicillium* G12 due to the glucose and xylose sugars obtained after lipid extraction from *D. subspicatus* (with sugar yield of 9.4 and 6.6%, respectively) and *Trichoderma auricularis* for *Chlorella* sp. (with sugar yield of 12.9 and 9.6%, respectively). FTIR was successfully applied to screen fungal strains.

Keywords: *Chlorella* sp.; *Desmodesmus subspicatus*; fungi; infrared spectroscopy.

Received on September 26, 2017.
Accepted on April 16, 2018

Introduction

The constant increase in the demand for renewable energy and the concern for reducing environmental impacts are the primary reasons to intensify the search for new alternatives of renewable energy (Baicha et al., 2016). These efforts address the need for new technologies and reconcile environmental awareness on the use of waste materials for biofuel production (Singh & Gu, 2010; Daroch, Geng, & Wang, 2013). In this context, microalgae appear to be a promising alternative, considering the different applications that these micro-organisms can offer, such as bioremediation (Gressler et al., 2014; Raeesossadati, Ahmadzadeh, McHenry, & Moheimani, 2014), high value products (Koller, Muhr, & Braunegg 2014) and biofuel production (Özçimen, Gülyurt, & İnan, 2012; Hallenbeck, Grogger, Mraz, & Veverka, 2016; Milano et al., 2016; Chernova & Kiseleva, 2017).

The use of microalgae biomass as a feedstock for bioethanol production is becoming significant, particularly for such genera as *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, and *Spirulina*. (John, Anisha, Nampoothiri, & Pandey, 2011). These microorganisms have a high photosynthetic rate and considerable biodiversity and variability in biochemical composition, accumulating considerable amounts of lipids and carbohydrates (Brennan & Owende, 2010; Khan, Lee, Shin, & Kim, 2017). Besides that, microalgae present efficient assimilation of cellulose and have high concentrations of starch. These characteristics are very suitable for the production of biofuels, such as bioethanol (Silva & Bertucco, 2016).

Microalgae hydrolysis is a plausible method to disrupt cell wall and convert poly-carbohydrates (starch, cellulose) into fermentable mono-sugars. The starch present in microalgae is stored in chloroplasts while

cellulose, in the cell walls (Park et al., 2017). Nowadays, microalgae biomass is mainly applied to produce oil focusing on the development of biofuels, such as biodiesel (Song et al., 2016), or in animal and human nutrition (Esquivel□Hernández et al., 2016). One of the most common process for lipid extraction was developed by Bligh and Dyer (1959), which uses methanol and chloroform as solvents and ultrasound for cell disruption. However, after this process there is still a remaining biomass which can be hydrolysed to obtain other products of commercial interest since this biomass can contain high levels of carbohydrates (Markou, Angelidaki, & Georgakakis, 2012).

In general, acid/enzymatic hydrolysis is the most common method for biomass saccharification. Acid hydrolysis is commonly faster, easier and cheaper than other types of hydrolysis, however the conditions may lead to sugar decomposition into unwanted compounds that can cause inhibition of fermentation (Silva & Bertucco, 2017). Enzymes used to hydrolyze cellulose and hemicellulose are environmentally acceptable, however, the high costs of enzyme production and the cost of hydrolysis are negative aspects that warrant improvement (Shokrkar, Ebrahimi, & Zamani, 2017).

Microbial bioconversion has been studied as a promising alternative, since the cellulose saccharification can release simple sugars for subsequent yeast fermentation. Conversely, hemicellulose saccharification releases five-carbon sugars able to be fermented (Rasmussen, Shrestha, Khanal, Pometto, & van Leeuwen, 2010). Fungi are microorganisms capable of degrading these carbohydrates as carbon and energy sources through an extracellular enzymatic system that produces hydrolases, which are responsible for polysaccharide degradation (Skory, Freer, & Bothast, 1997).

Thus, fungi hydrolysis is a relevant alternative because these microorganisms have a rapid metabolism for transforming cellulose and hemicellulose into pentoses and hexoses (Yoon, Cha, Kim, & Kim, 2008; Rasmussen et al., 2010; Okamoto, Nitta, Maekawa, & Yanase, 2011; Lee et al., 2015). The fungal-mediated hydrolysis of microalgae biomass is a technique that merits further investigation, and efficient methods must be developed to screen these microorganisms. The development of a rapid, low cost technique with less environmental impact for the selection of microorganisms that hydrolyze raw materials for conversion into biofuels has garnered considerable research attention (Baum, Hansen, Meyer, & Mikkelsen, 2013; Girard, Deschênes, Tremblay, & Gagnon, 2013; Wang, Yuan, Ji, & Li, 2013; Dong, Yang, Zhu, Wang, & Yuan, 2013). Fourier Transform Infrared Spectroscopy (FTIR) can be successfully applied for this purpose, as this technique is able to monitor enzymatic reactions, as well as changes in the structure of molecules, in real time. If the molecular structure is modified during the reaction, the infrared spectrum is able to monitor this variation (Kansiz et al., 1999; Pacheco, Karmali, Serralheiro, & Haris, 2005; Kumar & Barth, 2010; Santos, Fraga, Kozakiewicz, & Lima, 2010).

Considering the potential to obtain sugars through microalgae, the aim of the present study was to apply FTIR for selection of fungi strains to hydrolyze *Desmodesmus subspicatus* and *Chlorella* sp. biomass.

Material and methods

Microalgae and fungi strains cultivation

The microalgae strain *D. subspicatus* was provided by the laboratory of Ecotoxicology of the University of Santa Cruz do Sul (UNISC), State Rio Grande do Sul, Brazil. The microalgae strain of *Chlorella* sp. was generously provided by the Federal University of Santa Catarina (UFSC), State Santa Catarina, Brazil. *D. subspicatus* and *Chlorella* sp. were cultivated in photobioreactors and 1 L Erlenmeyer flasks, respectively. The strains were acclimated to a solution of 3 g L^{-1} of N:P:K (18:6:18) fertilizer (Gressler et al., 2014), and CO_2 was supplied to the cultures through diaphragm pumps with a flow rate of 0.22 vvm. A solenoid valve was used to control the injection of CO_2 into the system, which was interspersed in periods of 3 min. every 2 hours. Continuous artificial illumination with fluorescent light was applied (2500 lux, 24 hours day^{-1}), and the temperature was maintained at 25°C. The cell density was monitored daily for each species through spectrophotoscopy at 682 nm, and the results were plotted as calibration curves. Experiments were conducted in triplicate.

Filamentous fungi strains examined in the present study were transferred from the Culture Collection of Microorganisms at Industrial Microbiology from UNISC. A total of 11 strains of filamentous fungi were grown on plates containing potato dextrose agar medium for 7 days at 25°C. The cultures were maintained in slants with Sabouraud agar medium.

Analysis of lipids and carbohydrates in microalgae biomass

The electroflotation method was applied for the biomass separation of *D. subspicatus* and *Chlorella* sp. A power supply with direct current (Instrutherm, FA – 3003) was used. The electrode was constructed using 6 iron plates (100 x 60 mm), 3 mm thickness; the distance between the plates was 0.4 mm, and the current density was 1.0 mA cm^{-2} as shown in previous work (Baierle et al., 2015). The biomass dry weight was measured for each microalgae species.

Lipids were extracted from the microalgae biomass using the method of Bligh and Dyer (1959) using chloroform: methanol (2:1, v v^{-1}) as solvents and using ultrasound (Unique USC-1400) for cell disruption, carried out with 25 kHz and 4 cycles of 30 min. After extraction, the solvent was evaporated using a rotary evaporator (Quimis, Q344M), and the residual biomass was dried at 50°C. These residues were stored under refrigeration temperature for subsequent tests of fungal hydrolysis.

The carbohydrate content was determined in microalgae biomass after lipid extraction using the method described by Sluiter (2008). A portion of 300 mg of the residual biomass was weighed and placed into a pressure tube with 3 mL of 72% sulfuric acid. The tube was incubated for 60 min. in a water bath at 30°C, followed by the addition of 84 mL deionized water. The samples were then autoclaved at 121°C for 1 hour.

FTIR screening to evaluate the fungi ability to hydrolyze microalgae biomass

The dry biomass residue (20 mg) was placed in test tubes, and 2 mL deionized water was added. These mixtures were sterilized through autoclaving at 121°C for 20 min. The spores (approximately 1×10^8 spores mL^{-1}) of 11 fungal strains were inoculated, and the tubes were incubated at 30°C for 7 days. The fungal growth on microalgae biomass was monitored daily through visual evaluation and the fungi strains were identified as: (-) without micellar growth, (+) little micellar growth, and (++) high micellar growth.

The fungi mycelium was removed and aliquots of 100 µL of the supernatant from each culture were transferred to 1.5 mL microtubes containing 100 mg potassium bromide, lyophilized for 2 hours at 1.10^{-4} Torr (Labcomco) and ground with a pestle. The infrared spectrum was acquired in triplicate using a diffuse reflectance device (Pyke Technologies, Madison) coupled to a Fourier Transform Infrared Spectroscopy (FTIR; Perkin Elmer model 400) at a range of 4000 to 400 cm^{-1}, with 16 scans and a resolution of 4 cm^{-1}. The spectral data (intensity of absorbance for a given frequency associated with specific bands) were evaluated to check for the presence of characteristic bands that indicated that the hydrolysis step was effective with the selected fungal strains. The analysis was performed in order to observe the presence of FTIR bands that could reflects the potential of hydrolysis, such as the bands in approximately 3300 (O-H stretching vibrations), 733 (C=O stretching originating from esters or amides), 1100 (C-O-C indicating carbohydrates as saccharides and polysaccharides) and 1600 cm^{-1} (NH_2 deformation corresponding to proteins or amino acids).

Multivariate analysis using PCA and HCA to select fungi strains

The normalized average spectra were pre-processed through mean centering, and subsequently analyzed for a hierarchical cluster analysis and principal component analysis using PIROUETTE 3.1 software (Infometrix™). Fungi strains were chosen considering the results found through the analysis with FTIR and by the evaluation of the results obtained by PCA and HCA. The PCA was applied in the exploratory analysis to identify similarities and differences between different fungi strains species, after data compression. Hierarchical Cluster Analysis was applied to the auto-scaled data and the Euclidean distances generated a dendrogram for the samples.

Hydrolysis of microalgae biomass with selected fungal strains

For the assays, 1 g of residual biomass of both microalgae and 100 mL deionized water were added to 500 mL Erlenmeyer flasks and sterilized at 121°C for 20 min. The selected fungal strains were grown on Potato Dextrose Agar (PDA) at 25°C during 7 days. A saline solution containing the spores was analyzed on a Neubauer chamber and the inoculum was adjusted to 1×10^8 spores mL^{-1}, approximately. The samples were incubated for 7 days at 30°C in an orbital shaker (20 rpm). The hydrolysis reactions were performed in quintuplicate for all fungal strains selected.

Hydrolysate sugar determination

Sugar determination in the hydrolysates was conducted using an HPLC equipped with refractive index detector RID-20A, model LC-20AD from Shimadzu (USA). Software LabSolutions (Thermo Scientific, USA) was used for data acquisition and processing. The compounds from the sugar mixture (glucose and xylose) were separated in a Rezex RHM monosaccharide H^+ column (8% cross-linked sulfonated styrene-divinylbenzene, 300 × 7.8 mm) from Phenomenex. The analysis was performed with isocratic flow using ultrapure water. The total chromatographic run time was 20 min. The optimum flow rate was 0.8 mL min.$^{-1}$ and the injection volume was 20 µL. A standard solution at 10 mg mL^{-1} of each sugar was subjected to chromatographic analysis and the linearity was evaluated by the coefficient of determination (r^2) from the analytical curves containing a mixture of the selected sugars at the levels 0.1; 0.2; 0.3; 0.4 and 0.5 mg mL^{-1}.

Results and discussion

Microalgae and fungi cultivation

Microalgae cell density was evaluated as a function of days of cultivation, and *D. subspicatus* and *Chlorella* sp. presented similar growth, as shown in Figure 1. *Chlorella* sp. showed a slightly higher cell density (28.7×10^6 cells mL^{-1}) compared to *Desmodesmus subspicatus* (15.8×10^6 cells mL^{-1}) and the dry weight obtained was 0.36 and 0.28 g L^{-1}, respectively. Coefficients of determination were satisfactory since both microalgae presented values of $r^2 > 0.99$. The maximum growth was observed on the 10th day of culture for both microalgae.

A visual analysis for the ability of 11 filamentous fungal strains to hydrolyze microalgae biomass in submerged culture was performed. As listed in Table 1, we assessed the development of 11 fungal strains with different rates of mycelial growth, indicating the consumption of the substrate compared with a control sample (B).

Analysis of lipids and carbohydrates in microalgae biomass

After lipid extraction, the lipid content obtained in the separation of biomass through electroflotation was 6.07% for *D. subspicatus* and 4.72% for *Chlorella* sp. Mallick, Bagchi, Koley, and Singh (2016), indicate that microalgae lipids can exceed 50-60% of dry cell weight. On the other hand, Silva and Bertucco (2016) describes levels in a range of 8-15%. According to Schneider et al. (2012), the lipid content can range from 7 to 77%, depending on the species and growth conditions. Considering that lipids can be used in biodiesel production (Doan, Sivaloganathan, & Obbard, 2011), we applied the biomass residue to examine the hydrolytic capacity of fungal strains using FTIR.

The total carbohydrate content was obtained considering the amount of microalgae biomass after lipid extraction. As a result, *D. subspicatus* and *Chlorella* sp. presented 16.9 and 23.1% of total carbohydrates, respectively.

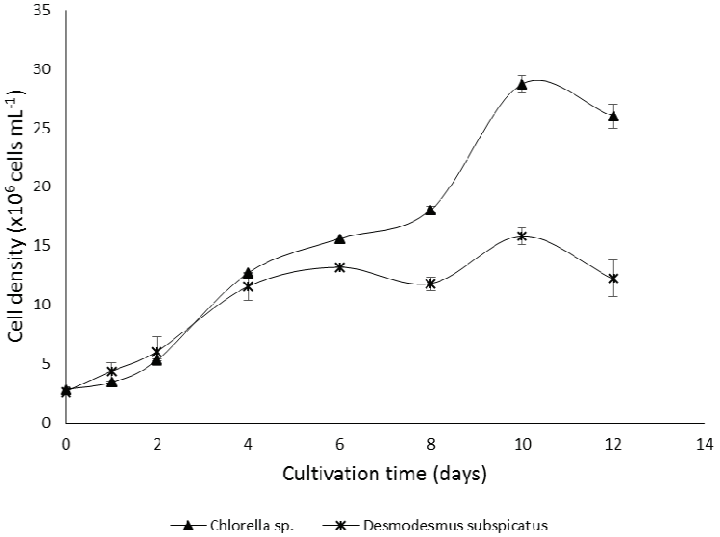

Figure 1. Growth curve of *Chlorella* sp. and *Desmodesmus subspicatus* in N:P:K (3 g L^{-1}) medium and CO_2 supply during 12 days.

FTIR screening to evaluate the hydrolytic capacity of fungi strains on microalgae

After evaluating hydrolysis, the biomass of both microalgae was analyzed through infrared spectroscopy. The same profile was obtained for both microalgae, as illustrated in Figure 2.

The vibration frequency depends on the linkage power involved in the functional groups. Most of the absorption bands observed in the hydrolysates reflect the presence of various functional groups in the medium (Giordano et al., 2001). The biomass hydrolysis produced a complex mixture of compounds derived from proteins, lipids, nucleic acids, and carbohydrates, such as carboxylic acids, amino acids and oligosaccharides (Stehfest, Toepel, & Wilhelm, 2005), which are observed as an increasing O-H band area. Despite the difficulty in distinguishing certain bands, characteristic stretches of microalgae composition have been observed in other studies. The corresponding wavelengths and bands found in microalgae under study are reported in Table 2. The spectra recorded for both microalgae in this study were identified using data reported in previous studies.

Table 1. Selection of microorganisms for microalgal biomass hydrolysis.

Fungal strain	Abbreviation	Development	
		D. subspicatus	*Chlorella* sp
Control sample	B	-	-
A. fumigatus USP-174	AF	++	++
A. gigantus MI 2012 G12-10	AG	+	+
A. terreus 233-3	AT	++	++
A. niger USP 9	AN	+	+
Penicillium MI 2012 G1	PG1	+	+
Penicillium MI 2012 G4 A5	PG4	++	-
Penicillium MI 2012 G7	PG7	+	+
Penicillium MI 2012 G9	PG9	++	+
Penicillium MI G12 A	PG12	++	+
Trichoderma auriculares	TA	+	++
Trichoderma BAS 7A	TB	+	+

(-) no mycelial growth, (+) little mycelial growth, and (++) high mycelial growth.

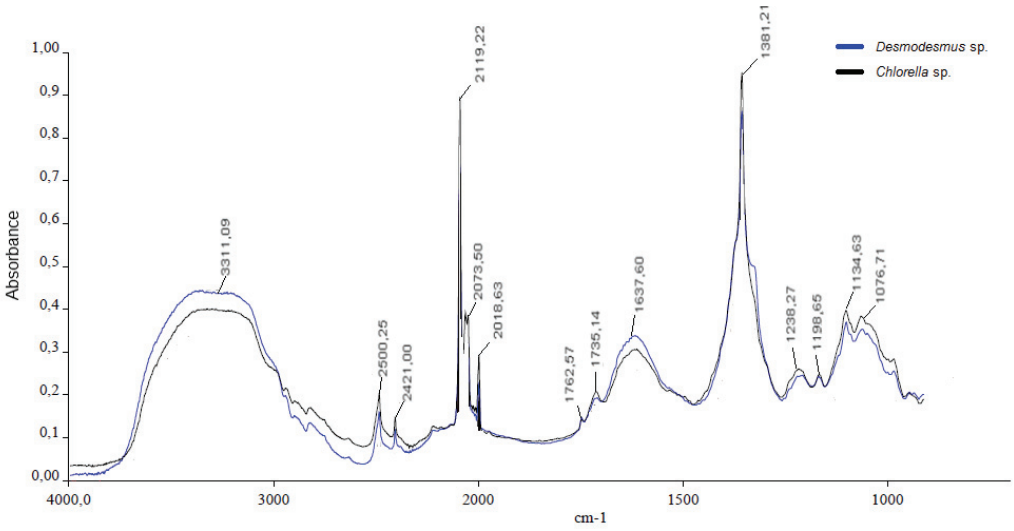

Figure 2. Spectrum profile obtained after hydrolysis of *Desmodesmus subspicatus* and *Chlorella* sp.

Table 2. Characteristics bands revealed in studies on microalgae biomass.

Wavelength (cm^{-1})	Indentification of the bands	References
~ 3311	Water; *v**(O-H);	Tan, Balasubramanian, Das, Obbard, and Chew (2013)
~ 2500-2421	ND	-
~ 2073-2018	ND	-
~ 1762	Lipids; *v** (C = O)	Duygu et al. (2012); Tan et al. (2013)
~ 1735		
~ 1637	Amides from protein; *v**(C = O)	Tan et al. (2013)
~ 1381	Chlorophyll	Neault and Tajmir-Riahi (1999)
~ 1238	Phosphodiester; v_{as}^{**}(> p = O). Nucleic acids	Duygu et al. (2012)
~ 1198-1134	Carbohydrates; *v**(C-O-C) of saccharides and polysaccharides	Tan et al. (2013); Jiang, Yoshida, and Quigg (2012)
~ 1076	*v**Si-O of silicate	Jiang et al. (2012)

In the present study, similarities between the microalgae compositions were observed after comparing the spectral profiles, highlighting the bands indicative of sugars (saccharides and polysaccharides), proteins and lipids. Indeed, spectroscopy is widely used for microalgae analysis, because this technique facilitates the characterization and quantification of the compounds present in the samples. Considering the main bands obtained through infrared spectroscopy (Table 2), the multivariate analysis was performed.

Multivariate analysis using PCA and HCA to select fungal strains

In this study, the analytical methods used were Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) (Tan, Aziz, & Aroua, 2013; Liu, Mukherjee, Hawkes, & Wilkinson, 2013). After hydrolysis, the main components of the microalgae *D. subspicatus* and *Chlorella* sp. were assessed (Table 3).

The results showed that the first component described 99.22% of the data obtained for *D. subspicatus*. The analysis of the principal components of *Chlorella* sp. biomass showed a similar patterns of biomass uptake, where 99.5% of the data can be described using a single component.

PCA score plots were obtained for both microalgae to distinguish common parameters through an assessment of the similar components between samples. In this case, we used PCA analysis (PC1 x PC3) to demonstrate the grouping based on similarities between fungal strains as shown in Figure 3. In *Desmodesmus subspicatus,* the formation of three major distinct groups on the score graph was observed in Figure 3(1). In *Chlorella* sp., a better separation was achieved compared to *D. subspicatus*, with the distinction of two groups, as shown in Figure 3(2). This separation indicated that more fungal strains are useful for hydrolysis, considering that only one strain grouped with the control sample.

Subsequently, the HCA of the *D. subspicatus* and *Chlorella* sp. biomass was performed (Figure 4) to check for a profile consistent with the PCA results and determine which fungal strains were most favorable for the hydrolysis step. HCA was run in order to emphasize the natural clusters and similar patterns between the samples. The obtained dendrogram and the distance between the samples were performed to verify the similarity of fungal strains.

Table 3. Principal components obtained for *Desmodesmus subspicatus* and *Chlorella* sp.

PC	*Desmodesmus subspicatus*		*Chlorella* sp.	
	%	Cumulative	%	Cumulative
1	99.30	99.22	99.50	99.50
2	0.41	99.63	0.20	99.70
3	0.29	99.86	0.14	99.84

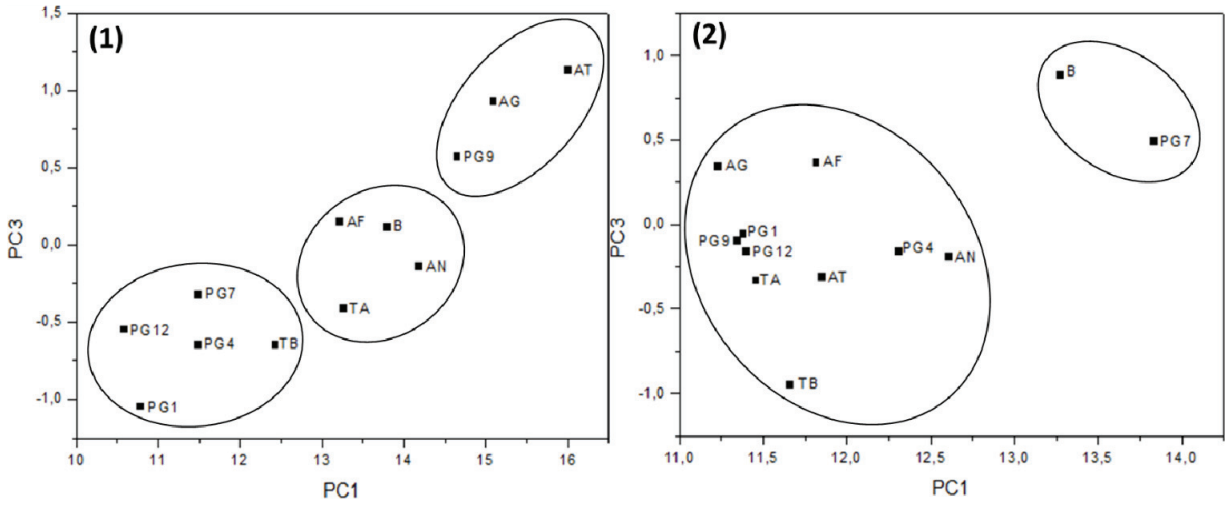

Figure 3. Plots of PCA scores (PC1 x PC3) of (1) *Desmodesmus subspicatus* and (2) *Chlorella* sp., showing the best groups (not inserted in B group) of fungal strains to be selected for the hydrolysis step. B (control sample), AF (A. *fumigatus* USP-174), AG (A. *gigantus* MI 2012 G12-10), AT (A. *terreus* 233-3), AN (A. *niger* USP 9), PG1 (*Penicillium* MI 2012 G1), PG4 (*Penicillium* MI 2012 G4 A5), PG7 (*Penicillium* MI 2012 G7), PG9 (*Penicillium* MI 2012 G9), PG12 (*Penicillium* MI G12 A), TA (*Trichoderma auriculares)*.

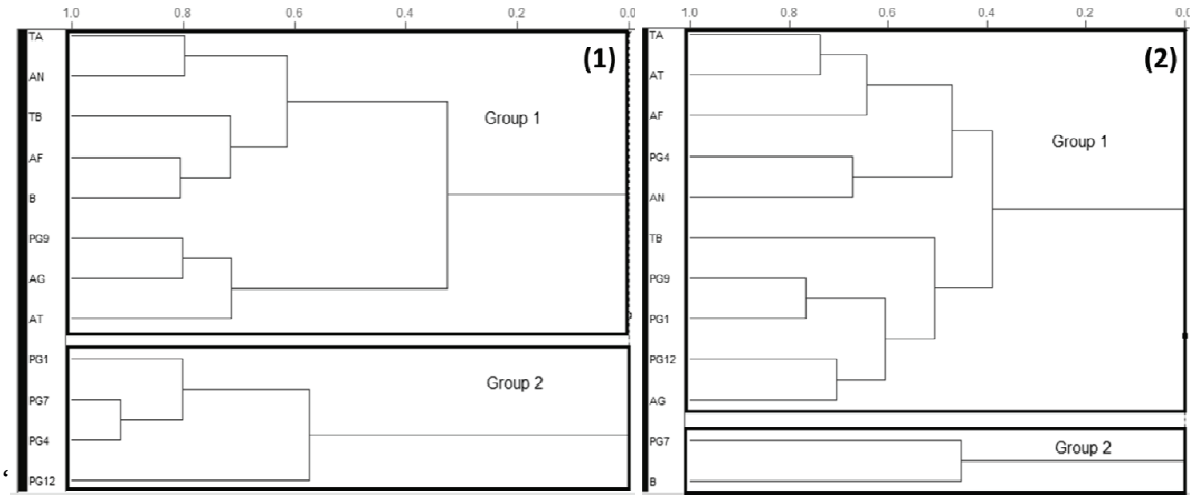

Figure 4. Dendrogram generated from the analysis of (A) *Desmodesmus subspicatus* and (B) *Chlorella* sp. biomass hydrolyzed with different fungal strains showing two main groups. B (control sample), AF (A. *fumigatus* USP-174), AG (A. *gigantus* MI 2012 G12-10), AT (A. *terreus* 233-3), AN (A. *niger* USP 9), PG1 (*Penicillium* MI 2012 G1), PG4 (*Penicillium* MI 2012 G4 A5), PG7 (*Penicillium* MI 2012 G7), PG9 (*Penicillium* MI 2012 G9), PG12 (*Penicillium* MI G12 A), TA (*Trichoderma auriculares)*.

The HCA analysis was useful to demonstrate that the fungi strains that were grouped together presented similar patterns of biomass uptake. The groups of fungi strains that resemble with the control sample (water and microalgae biomass, without fungi strains) were considered unsuitable to the further tests of hydrolysis. The dendrogram arrangement of *Desmodesmus subspicatus* in Figure 4(1) showed the formation of two distinct groups: Group 1, comprising the control and other fungal strains considered less favorable to interact with microalgal biomass; and Group 2, comprising only *Penicillium* strains, grouped according to similarities in the components present in this genus and showing a suitable profile for pretreatment considering the distance to the control sample.

For *Chlorella* sp., the HCA analysis in Figure 4(2) showed the distinction of two main groups: Group 1, containing most of the fungal strains capable of hydrolyzing biomass; and Group 2, containing only one fungal strain (*Penicillium* MI 2012 G7) and the control sample which, in this case, would not be useful for hydrolysis.

Hydrolysate sugars determination

To demonstrate the ability of the fungi examined and the efficiency of FTIR for screening, we selected two fungal strains from the dendrogram analysis. The *Penicillium* G12 strain was selected for the hydrolysis of *D. subspicatus,* and *T. auriculares*, for *Chlorella* sp.

The HPLC analysis was successfully applied and results demonstrated that analytical curves presented good linearity with $r^2 \geqslant 0.99$ for the evaluated sugars. The concentration of glucose and xylose per gram of biomass after lipid extraction and hydrolysis with fungal strains obtained by *D. subspicatus* was 1.9 and 1.3 mg mL^{-1}, which represents a yield of 9.4 and 6.6%, respectively. For *Chlorella* sp., the concentration of glucose and xylose was 2.6 and 1.9 mg mL^{-1}, which represents a yield of 12.9 and 9.6%, respectively.

The determination of sugars from raw materials demonstrated that pretreatment is crucial to improve the release of sugars, as this treatment effectively degrades complex carbohydrates (Sun & Cheng, 2002; Harun, Danquah, & Forde, 2010). In the present study, pretreatment was not performed, considering that several studies have revealed other alternatives for the release of sugars; for example, the addition of enzymes (Aikawa et al., 2013).

The use of different microorganisms for hydrolysis of biomass has primarily been evaluated at a low cost. The hydrolytic potential of microalgae biomass was identified in a group of fungi examined in the present study. Nevertheless, additional studies using commercial enzymes are required to optimize the hydrolysis conditions of these competitive strains with respect to time and rate of biotransformation.

The development of an easy and rapid method to select hydrolytic fungal strains is the main highlight of our research. FTIR followed by multivariate analysis was successfully applied to screen these microorganisms, distinguishing groups based on hydrolytic capacities.

Conclusion

FTIR followed by multivariate analysis was an efficient tool for screening fungi strains and could be considered environmentally friendly and a suitable alternative, since chemicals or commercial enzymes were not necessary for the hydrolysis step. *Penicillium* G12 and *Trichoderma auriculares* showed efficient performances to hydrolyze microalgal biomass. The full harnessing of microalgae biomass could be achieved, since the extracted lipids can be reserved for biodiesel production, while the remaining biomass can be used to obtain bioethanol. The application of FTIR for fungal strain selection to hydrolyze microalgae biomass of *Chlorella* sp. and *Desmodesmus subspicatus* to obtain sugars was successfully achieved.

Acknowledgements

The authors thank Capes Finance Code 001 and AES – Uruguaiana for scholarships to P.D.G, M.P.S, T.R.B and PNPD/CAPES – n. 1266911. Also to the Center for Research in Processes and Products Oleochemical and Biotechnological (MCTIC-01.0144.00/2010). This work was also financially supported by CNPq (09652/2016-2), Fapergs for the DOCFIX Scholarship and Productivity in research CNPq (306178/2012-5).

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