Production of xylitol and carotenoids from switchgrass and *Eucalyptus globulus* hydrolysates obtained by intensified steam explosion pretreatment

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A B S T R A C T

This study investigated the fermentability of hemicellulosic hydrolysates obtained by intensified steam explosion pretreatment of switchgrass (*Panicum virgatum*) and *Eucalyptus globulus*. Xylitol and carotenoids were the high-value molecules produced by fermentation. The intensified pretreatment allowed to process a large amount of biomass per unit of time and resulted in hydrolysates containing high amount of sugars, among of which, a significant fraction was in the form of oligomers (eucalyptus hydrolysate: 25.0 g/L of oligomers and 50.6 g/L of monomeric sugars; switchgrass hydrolysate: 18.9 g/L of oligomers and 39.6 g/L of monomeric sugars). To be used as fermentation media, a post-hydrolysis step was applied to increase the content of monomeric sugars in the hydrolysates. Then, a detoxification process was carried out to reduce the concentration of inhibitors present. Two evolved yeasts were used for fermentation: *Kluyveromyces marxianus* for xylitol production, and *Rhodosporidium toruloides* for carotenoids production. Results revealed that the hydrolysates produced by intensified steam explosion of switchgrass and eucalyptus present good fermentability and can be used to produce valuable compounds such as xylitol, after detoxification. *K. marxianus* presented better tolerance to inhibitory compounds still present in the detoxified hydrolysates (acetic acid up to 3.94 g/L and phenolic compounds up to 2.28 g/L) than *R. toruloides*, which favored the production of xylitol. Finally, the intensified pretreatment was found to be a potential strategy to obtain hydrolysates with high concentration of sugars, reducing the need of concentration in a subsequent step. Moreover, the detoxification strategy applied in this study allowed to recover valuable compounds from the hydrolysates, offering extra value to a biorefinery. Altogether, the findings of this study contribute to the advancement of a technology for valorization of hemicellulosic hydrolysates.

1. Introduction

Lignocellulosic biomass is an attractive feedstock for use on the production of valuable compounds, providing environmental, economic, and social benefits (Dragone et al., 2020). With an estimated production of 181,5 billion tons per year, lignocellulose is the most abundant biomass on Earth and, of them, 8.2 billion tons are used (Dahmen et al., 2019). Using lignocellulosic biomass as a feedstock to produce second generation biofuels, for instance, avoids utilization of petroleum, thereby reducing greenhouse gas emissions (Zhao et al., 2021; Mussatto and Dragone, 2016). In addition, an efficient utilization of lignocellulosic biomass for the production of different products in a biorefinery perspective increases the profitability and opens new opportunities for the biobased industry (Dragone et al., 2020).

Cellulose, hemicellulose, and lignin are the main components of lignocellulosic biomass structure. Deconstruction of this structure is necessary to obtain monomeric sugars, which can later be converted into fuels and chemicals by fermentation process. Several pretreatment processes have been proposed for biomass deconstruction, among of which, steam explosion has been the most used in industrial-scale to produce cellulosic ethanol as it is very efficient to disrupt the material structure exposing the cellulose fibers to enzymatic hydrolysis in the subsequent step (Duque et al., 2016; Mussatto and Dragone, 2016).

During the biomass pretreatment, a hemicellulosic hydrolysate is also generated. Hemicellulosic hydrolysates contain a mixture of pentose and hexose sugars and can be used to produce valuable compounds such as food ingredients, chemicals, and materials contributing to an effective use of all biomass fractions, adding value to biorefineries (Mussatto and Dragone, 2016). From the different compounds that can be produced from pentose sugars, xylitol and carotenoids are high-value

molecules with relevant properties and numerous industrial applications (Yamakawa et al., 2020; Liu et al., 2021a). So, the production of these compounds open good opportunities for valorization of hemicellulosic hydrolysates. Xylitol is a food sweetener with similar sweetening power than sucrose but with around 40 % less caloric value. In addition, xylitol can be consumed by diabetic people and can also be used to prevent dental caries, otitis, osteoporosis, and respiratory infections (Mussatto, 2012). Commercially, xylitol is produced by chemical synthesis through catalytic hydrogenation of xylose, a process that requires high energy demand (Delgado Arcaño et al., 2020). The bioconversion of xylose into xylitol by yeasts is seen as an attractive alternative to the chemical synthesis and has gained increased interest in the last years due to the potential advantages in both energetic and environmental aspects (Cortez et al., 2016; Dasgupta et al., 2017; Mussatto, 2012). Carotenoids are lipid-soluble natural pigments responsible for the yellow, red, orange, and purple colors (Leong et al., 2018). Besides acting as high-quality food color additives, they are also vitamin A precursors (de la Seña et al., 2014). Furthermore, carotenoids are largely used in the pharmaceutical industry as anticarcinogenic compounds (Frengova and Beshkova, 2009) and to reduce the risk of diseases such as coronary heart, fatty liver, neurodegenerative as Alzheimer and diabetes associated with obesity and hypertension (Clugston, 2020; Bhatt and Patel, 2020). Carotenoids can be produced by oleaginous yeasts, which are able to metabolize hexose and pentose sugars (Cabral et al., 2011; Liu et al., 2020; Mussagy et al., 2021).

Eucalyptus globulus and switchgrass are interesting feedstocks for utilization in biorefineries due to their composition and large availability. *Eucalyptus* spp. is the most planted hardwood tree of the world thanks to its fast growth and high quality wood (Elli et al., 2020). It is a hardwood rich in cellulose (Cebreiros et al., 2017; Guigou et al., 2019) and is one of the main feedstocks used in the pulp and paper industries, particularly in South America (Uruguay, Brazil and Chile) being, therefore, largely available. Cellulose content in eucalyptus range from 46 % to 55 % in a dry weight basis, hemicellulose 12%–23%, and lignin 22%–31%, these values being dependent of a series of factors such as age, geographical localization, growth and soil conditions (Mussatto and Dragone, 2016; Costa et al., 2016). The hemicellulose fraction of eucalyptus is a xylan backbone highly branched with acetyl groups (Sixta, 2006), while the lignin has extra linear linkages due to the presence of additional methoxy groups on aromatic rings (Erfani Jazi et al., 2019). Switchgrass (*Panicum virgatum*) is a perennial grass that also has interesting characteristics such as fast growth, high volume of production per area, longevity, low cost of production, and ability to grow in lands unsuitable for food or crops production (Lindsey et al., 2013). Additionally, it also has a high content of hemicellulose (xylan structure) in the composition, which may constitute up to 30 % in a dry weight basis (Yan et al., 2010), while the lignin content usually vary between 19 % and 29 % (Larnaudie et al., 2021).

Based on the above, the present work evaluated the fermentability of hemicellulosic hydrolysates obtained by intensified steam explosion pretreatment of switchgrass (*Panicum virgatum*) and *Eucalyptus globulus,* in order to find a potential opportunity for valorization of such aqueous stream generated after pretreatment of the biomass during the process for the production of cellulosic ethanol. More specifically, efforts were done to produce hemicellulosic hydrolysates containing high concentration of sugars through the intensification of the steam explosion pretreatment. This is an innovative approach tested in the present study, which was applied with the aim of obtaining hydrolysates rich in sugars, avoiding the need to perform a subsequent step of concentration to increase the sugars content before fermentation. Two evolved yeasts with ability to consume pentose and hexose sugars were used to evaluate the potential of the hydrolysates to be used as fermentation medium. Xylitol and carotenoids were the high-value compounds produced by fermentation, as their production could represent two possible high-value applications for the hemicellulosic hydrolysates. The need of submitting the hydrolysates to post-hydrolysis and detoxification steps before use as

fermentation medium was also evaluated. Finally, the potential impacts and perspectives related to the used of the eucalyptus and switchgrass hydrolysates for the production of valuable compounds were discussed.

2. Materials and methods

The overall methodology used in this study is summarized in Fig. 1. Briefly, hemicellulosic hydrolysates were produced by intensified steam explosion of two different raw materials: Switchgrass and *Eucalyptus globulus*. The composition of the hydrolysates was analyzed and a step of post-hydrolysis (acid hydrolysis) was performed to increase the concentration of sugar monomers in the hydrolysates. The presence of compounds that inhibit the yeasts' metabolism was detected in the hydrolysates and then, a detoxification process was applied to reduce the concentration of such inhibitory compounds in the hydrolysates. Fermentation experiments were performed using two evolved yeast strains with increased tolerance to biomass-derived inhibitors in order to evaluate the possibility to produce xylitol and carotenoids from the hydrolysates. Fermentation experiments were performed in hydrolysate-based media and also in complex media simulating the composition of the hydrolysates (with and without inhibitors) for comparison.

2.1. Feedstock

Switchgrass used in this study was provided by the Uruguayan state-owned company ANCAP. The material was harvested at the Agricultural Experimental Station Mario Cassinoni, department of Paysandú, in September 2016. *Eucalyptus globulus* chips were provided by the Uruguayan company Chipper. Both feedstocks were dried at 40 °C by forced convection oven until 10 % moisture, and then milled to an average particle size of 1 cm. Chemical compositional of the raw materials was determined according to NREL's standard procedure (Sluiter et al., 2012).

2.2. Steam explosion pretreatment

Switchgrass and *Eucalyptus globulus* biomasses were pretreated in a semi-continuous pre-pilot reactor installed at the Technological Laboratory of Uruguay. The conditions of temperature (200 °C) and residence time (10 min) used for pretreatment were based on a previous optimization study (Bonfiglio et al., 2019). In this equipment, the biomass enters the reactor continuously by means of an "infinite screw". There are two forces applied to the biomass: the pressure of the "infinite screw" pressing the mass into the reactor constantly, and the opposite steam pressure trying to escape the reactor. Therefore, higher pressure inside the reactor increases the torque of the screw, which if it is too high, the equipment automatically shuts down. In this study, intensification of the steam explosion pretreatment was done by increasing the high torque limit of the equipment, which made possible to process around 15 % more biomass per unit of time (up to 11.5 kg/h) than the previous study. Additionally, this change promoted a slightly greater "extrusion" effect to the biomass before entering in the reactor vessel, which acted as an additional pretreatment, improving the biomass deconstruction during the steam explosion step.

After pretreatment, the resulting solid material, composed mainly by cellulose and lignin, was separated by filtration using a press filter. The liquid fraction (hemicellulosic hydrolysate) was filtered through a fabric filter to remove remaining solid particles, and analyzed to determine the contents of oligosaccharides, monomeric sugars, carboxylic acids, furans, and phenolic compounds. The hemicellulosic hydrolysate was submitted to a post-hydrolysis process to break the oligomers into monomers using 4% (w/w) sulfuric acid in autoclave at 121 °C for 60 min (Sluiter et al., 2008).
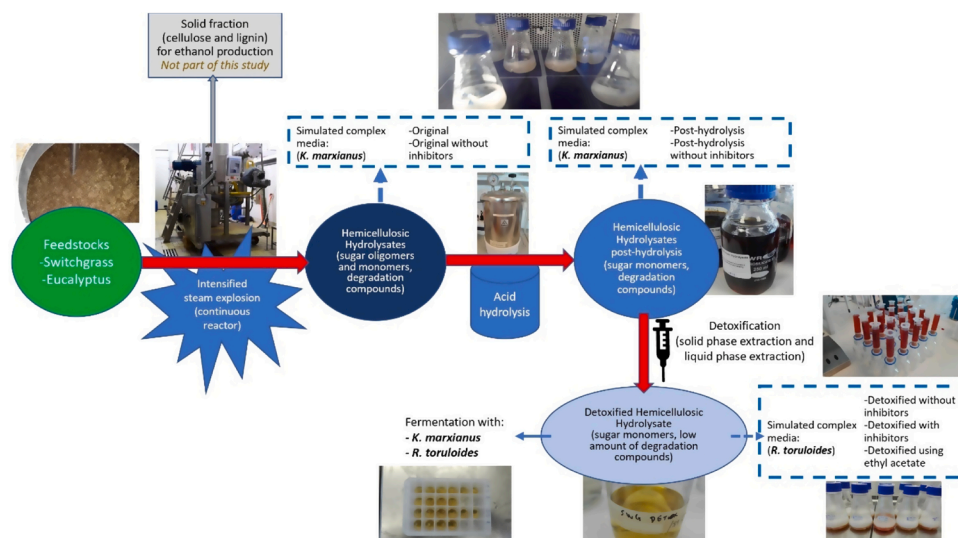
Fig. 1. Schematic representation of the overall methodology used in the present study.

2.3. Microorganisms and inoculum

Two evolved yeasts of the research group "Biomass Conversion and Bioprocess Technology" (Technical University of Denmark), with improved tolerance against toxic compounds present in biomass hydrolysates, were used in the fermentation experiments. The evolved strains were obtained by adaptive laboratory evolution of the wild-type strains of *Kluyveromyces marxianus* NRRL Y-6373 (unpublished data) and *Rhodosporidium toruloides* NRRL Y-1091 (Liu et al., 2021b). Inoculum of both evolved strains were prepared by transferring 1 mL of frozen stock cultures preserved at – 80 °C with 30 % glycerol, to the medium composed by (in g/L): yeast extract, 3.0; malt extract, 3.0; peptone, 5.0; and xylose, 20.0. *K. marxianus* inoculum was incubated in 250-mL baffled shaken flask using 50 mL of medium, at 40 °C, 250 rpm for 24 h. *R. toruloides* was incubated at the same manner, but at 30 °C. Afterwards, the cells were recovered by centrifugation and washed twice with 0.9 % NaCl solution.

2.4. Fermentation using simulated complex media

Fermentation experiments using complex media simulating the composition of the hydrolysates were performed with the aim of comparing the ability of the strains to grow in presence and absence of inhibitors. Simulated media were composed by cellobiose, glucose, xylose, arabinose, formic acid, acetic acid, 5-hydroxymethylfurfural (5-HMF), furfural, vanillin, vanillic acid, coumaric acid, syringaldehyde, 4-hydroxybenzaldehyde, and syringic acid, in concentrations similar to those found in the hemicellulosic hydrolysates.

For the experiments with *K. marxianus*, the media were supplemented with (g/L): yeast extract, 3.0; malt extract, 1.5; $(NH_4)_2HPO_4$, 5.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; and trace elements (mg/L): EDTA, 15; $ZnSO_4.7H_2O$, 4.5; $CoCl_2.6H_2O$, 0.3; $MnCl_2.4H_2O$, 0.84; $CuSO_4.5H_2O$, 0.3; $FeSO_4.7H_2O$, 3.0; $NaMoO_4.2H_2O$, 0.4; H_3BO_3, 1.0; and KI, 0.1. Four different complex media were formulated to simulate the composition of the raw hydrolysate 1) without inhibitors, and 2) with inhibitors; and of the post-hydrolysis hydrolysate 3) without inhibitors, and 4) with inhibitors. Fermentations were performed in 250-mL baffled shaker flasks using 100 mL of medium at 40 °C, 250 rpm, initial cell concentration of 1 g/L. Assays were performed in duplicate.

For the experiments with *R. toruloides*, the media were formulated with C/N ratio of 100 taking into account glucose, xylose, and arabinose as carbon sources. NH_4Cl (0.7−0.8 g/L) was the nitrogen source used. Additionally, yeast extract without nitrogen and ammonium sulfate was

used by 10 % of total monomeric sugars concentration. Three different complex media were formulated to simulate the composition of 1) Detoxified hydrolysate without inhibitors; 2) Detoxified hydrolysate with formic and acetic acids (2.00 g/L and 1.48 g/L, respectively, for simulated switchgrass hydrolysate; 2.15 g/L and 3.94 g/L, respectively, for simulated eucalyptus hydrolysate), and 3) Detoxified hydrolysate with ethyl acetate (2.0 g/L). Fermentations were performed in 250-mL baffled shaker flasks using 100 mL of medium at 30 °C, 250 rpm, initial cell concentration of 1 g/L. Assays were performed in duplicate.

2.5. Hydrolysate detoxification

A detoxification process was designed and applied with the aim of promoting a sequential removal of phenolic compounds and organic acids from the hydrolysates. The detoxification process was composed of two sequential steps; the first step corresponded to a solid-phase extraction using polystyrene-divinylbenzene resin membrane to retain phenolic compounds, and the second step corresponded to a liquid-liquid extraction with ethyl acetate to remove organic acids.

For the process, the hydrolysate obtained after post-hydrolysis was centrifuged to remove the black precipitate particles (humin). Then, the pH of the hydrolyzed was adjusted to 2.5 with NaOH pellets. A resin membrane column Chromabond® HR-X (Macherey-Nagel, Germany) of 15 mL and 500 mg of sorbent was first conditioned with 10 mL of methanol followed by 15 mL of deionized water. Then, 10 mL of hydrolysate were eluted through the column. Phenolic compounds retained in the column were recovered by using 10 mL of 5 % (v/v) ethanol followed by 20 mL of absolute ethanol. The partially detoxified hydrolysate had the pH adjusted again to 2.5 (using NaOH pellets) to be used for liquid-liquid extraction. In this step, 50 mL of hydrolysate and 200 mL of ethyl acetate were mixed in 250-mL baffled shaken flasks with closed lid for solvent, and incubated at 23 °C, 250 rpm for 40 min. After that, the mixture was transferred to a 500-mL funnel separator and left for 20 min for phase separation. The aqueous phase was collected and submitted again to the same liquid-liquid extraction process. The detoxified hydrolysate obtained at the end of the second extraction process was used for fermentation experiments.

2.6. Fermentation of detoxified hydrolysates

To be used as fermentation medium, the hydrolysates had the pH adjusted with calcium carbonate to 5.5 and 4.6, for *K. marxianus* and *R. toruloides,* respectively. Assays were carried out with and without

supplementation of the hydrolysates with nutrients (salts and extracts). In supplemented media, nutrients were added in the hydrolysates in the same amount used for simulated complex media preparation (item 2.4).

Fermentation experiments were performed in 24-wells microtiter plates (Enzyscreen, Netherlands) using 3 mL of hydrolysate per well. The plates were kept in an orbital shaking incubator with 50 mm amplitude, at 300 rpm (New Brunswick, USA). Sampling was done by completely removing the volume of the well followed by weighting to estimate the water evaporation. Control experiments to estimate the water evaporation during the fermentation were performed with non-inoculated medium. The water evaporation was estimated, and the value was used to adjust the volume of the fermentation wells using sterile deionized water (carbon dioxide formation was not considered). Fermentations were carried out at 40 °C and 30 °C for *K. marxianus* and *R. toruloides,* respectively. Assays were performed in duplicate.

2.7. Analytical methods

The concentration of carbohydrates, organic acids, furfural, 5-HMF, and phenolic compounds in the hydrolysates was determined by high-performance liquid chromatography (HPLC). Cellobiose, glucose, xylose, arabinose, acetic acid, formic acid, furfural, and 5-HMF were quantified using a Dionex Ultimate 3000 high-performance liquid chromatography UHPLC + Focused system (Dionex Softron GmbH, Germany) with a Bio-Rad Aminex® column HPX-87H (300 mm × 7.8 mm) at 60 °C, and 5.0 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. Sugars and acids were detected using a Shodex RI-101 refractive index detector, whereas 5-HMF and furfural were detected using UV measurement at 254 nm. Vanillin, vanillic acid, coumaric acid, 4-hydroxybenzaldehyde, syringaldehyde, and syringic acid were determined using a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germany) equipped with a Zorbax eclipse plus C18 column, eluted with a gradient method with mobile phase 0.05 % acetic acid in water and acetonitrile, column oven at 30 °C, 1 mL/min, UV detection. The content of oligomers in the hydrolysates was calculated as the difference between the concentration of monomeric sugars in the post-hydrolysate and in the original hydrolysate. Total phenolic compounds were quantified by colorimetric method using gallic acid as standard (Ballesteros et al., 2014). Nitrogen in the detoxified hydrolysates was quantified as primary amino nitrogen using the assay kit PANOPA (Megazyme).

For quantification of carotenoids, 1 mL of sample with cells was centrifuged (10,000 rpm, 5 min, 4−5 °C), washed twice with distilled water, resuspended in 1 mL of dimethyl sulfoxide (DMSO) and incubated in a Thermomixer at 30 °C for 15 min. Then, the sample was centrifuged (10,000 rpm, 5 min, 4−5 °C) and the extract recovered. This procedure was repeated until cells become white, and all the extracts were combined. The concentration of carotenoids was measured by absorbance at 450 nm against DMSO, using β-carotene dissolved in DMSO as standard (Liu et al., 2020).

During the fermentation course, total sugars were estimated by measurement of the total soluble solids expressed by °brix using an optical refractometer (Atago, Japan) and water as blank. Fermentation samples for HPLC analysis were centrifuged (9600 *g*, 5 min, 5 °C) using a Micro star 17 R (VWR, Denmark) centrifuge. Supernatants were kept in freezer at −20 °C until analysis. Cell concentration was determined by measurement of the optical density (OD) at 600 nm using a V-1200 spectrophotometer (VWR, Denmark), which was correlated with the concentration using a calibration curve prepared to each yeast. The calibration curves were validated for OD range of 0.1 to 0.8 (R^2 = 1.0). The maximum specific growth rate (μ_{max}, h^{-1}) was determined as the slope of linear region on an *ln* (X/X_0) versus time plot, where X (g/L) was the cell concentration at a specific time, and X_0 (g/L) was the cell concentration at the initial time (0 h).

3. Results and discussion

3.1. Feedstock composition and hydrolysate characterization

Table 1 presents the composition of switchgrass and *Eucalyptus globulus* biomasses used in the present study in terms of cellulose, hemicellulose, lignin, ash, protein, and extractives. Overall, the composition of eucalyptus wood was similar to values reported in the literature for cellulose, hemicellulose, and lignin; while for switchgrass the composition differed in terms of lignin content (literature reports 13.2–22.5 wt%), but was in the range that has been reported for cellulose and hemicellulose (Mussatto and Dragone, 2016).

Chemical composition of original hydrolysates produced from switchgrass and eucalyptus is shown in Table 2. Eucalyptus hydrolysate presented total monomeric sugars (50.61 ± 0.61 g/L) in higher concentration than switchgrass hydrolysate (39.57 ± 0.25 g/L), which is in agreement with the characterization of both feedstocks. Although most of the monomers corresponded to xylose, some glucose was also present in the hydrolysates. As reported in previous studies, the steam explosion pretreatment results in the disruption of the lignocellulosic matrix and cleavage of glycosidic bonds, therefore solubilizing monomers and oligomers mainly from hemicellulose, but also glucose and phenolic compounds from cellulose and lignin fractions respectively (Bonfiglio et al., 2019; Duque et al., 2016). The content of acetic acid in eucalyptus hydrolysate was also higher than in switchgrass hydrolysate, which can be explained by the fact that the eucalyptus xylan backbone is more branched with acetyl groups (Koch, 2008). The high amount of formic acid, 5-HMF, and furfural in the hydrolysates reveals that sugars degradation reactions occurred during pretreatment. The formation of these compounds could be minimized by optimizing the pretreatment conditions. However, this should be carefully evaluated since the use of a lower severity factor during the steam explosion pretreatment results in less formation of sugar degradation compounds, but also promotes less deconstruction of the lignocellulose structure, which will affect the subsequent access of the cellulose fibers to the cellulase enzymes during the subsequent step of enzymatic hydrolysis (Pielhop et al., 2016; Auxenfans et al., 2017). The content of phenolic compounds was also high in both hydrolysates, revealing that lignin depolymerization reactions also occurred during the biomass pretreatment. It is interesting to note the presence of different types of phenolic compounds in the hydrolysates according to the feedstock used (Table 2). For instance, coumaric acid and 4-hydroxybenzaldehyde (both derived from lignin precursor type p-hydroxyphenyl (H) units) were found in switchgrass hydrolysate, but not in eucalyptus hydrolysate. The main lignin precursor in *Eucalyptus* spp. is syringyl (S) unit, which explains the higher content of syringaldehyde in eucalyptus hydrolysate than in switchgrass hydrolysate. For vanillin and vanillic acid (both derived from the guaiacyl (G) unit), higher amounts were found in switchgrass hydrolysate, which was expected since it is a non-hardwood material.

Cellobiose was detected in both hydrolysates, which, together with the chromatogram profile of the samples, indicated the presence of oligomers in the media. A post-hydrolysis of the hydrolysates was then performed and confirmed the presence of oligomers in the original

Table 1

Composition of switchgrass (*Panicum virgatum*) and *Eucalyptus globulus* biomasses used in the present study.

Compound	Switchgrass (wt%)	Eucalyptus (wt%)
Cellulose	31.8 ± 0.8	47.9 ± 1.0
Hemicellulose	25.0 ± 1.2	16.0 ± 0.8
Lignin	31.2 ± 0.8	26.0 ± 0.8
Ash	3.2 ± 0.3	0.4 ± 0.1
Protein	1.8 ± 0.3	0.6 ± 0.3
Extractives	7.4 ± 1.1	3.1 ± 1.1
Water extractives	5.0 ± 0.4	2.6 ± 0.3
Ethanol extractives	2.4 ± 0.4	0.5 ± 0.2

Table 2

Chemical composition of switchgrass and *Eucalyptus globulus* hemicellulosic hydrolysates obtained by intensified steam explosion pretreatment.

| Compound | Concentration in the hydrolysate (g/L) | | | |
| | Original hydrolysate | | After post-hydrolysis | |
	Switchgrass	Eucalyptus	Switchgrass	Eucalyptus
Cellobiose	3.59 ± 0.08	11.89 ± 0.03	nd	nd
Glucose	5.30 ± 0.09	7.82 ± 0.10	14.96 ± 0.03	14.02 ± 0.17
Xylose	26.46 ± 0.10	36.01 ± 0.33	39.99 ± 0.01	58.06 ± 0.48
Arabinose	7.81 ± 0.06	6.78 ± 0.13	3.47 ± 0.16	3.50 ± 0.02
Formic acid	8.82 ± 0.12	5.93 ± 0.07	9.87 ± 0.07	6.96 ± 0.07
Acetic acid	15.39 ± 0.16	20.13 ± 0.09	18.44 ± 0.01	29.03 ± 0.35
5-HMF	3.08 ± 0.12	2.74 ± 0.09	1.75 ± 0.06	1.28 ± 0.09
Furfural	3.11 ± 0.28	3.42 ± 0.06	3.23 ± 0.59	4.82 ± 0.23
Total phenolic	9.12 ± 0.91	7.74 ± 0.64	5.91 ± 0.38	4.29 ± 0.98
Vanillin	0.23	0.08 ± 0.01	0.12 ± 0.01	0.05
Vanillic acid	0.15	0.03	0.14	0.05
Coumaric acid	0.15	nd	0.04	nd
Syringaldehyde	0.10	0.21	0.03	0.14 ± 0.01
Syringic acid	0.09	0.09	0.09	0.10
4-Hydroxybenzaldehyde	0.12	0.01	0.08	0.02

nd: not detected.

media (Table 2). The content of oligomers in switchgrass and in eucalyptus hydrolysates corresponded to 18.9 g/L and 25.0 g/L, respectively. Chemical composition of the hydrolysates after post-hydrolysis reveals that oligomers were hydrolyzed into monomers, which is evidenced by the absence of cellobiose and increased amount of monomeric sugars, mainly xylose. The amount of acetic acid also increased after post-hydrolysis, more notably in the eucalyptus hydrolysate, which can be explained by the fact that the xylan backbone in hardwood is more branched with acetyl groups, as explained before. The total content of phenolic compounds in both hydrolysates decreased after post-hydrolysis, which could be related to the formation of a black precipitate called humin. Humins are formed as byproduct of glucose dehydration, and can also be derived from the conversion of 5-HMF (Hetzel et al., 2016); the later would explain the reduction of 5-HMF concentration observed in the hydrolysates after post-hydrolysis.

3.2. Fermentation of complex media simulating the hydrolysates by K. marxianus

Fermentation performance of *K. marxianus* in terms of maximum specific growth rate (μ_{max}) and sugars consumption (estimated by the difference between the initial and final values of °brix (Δ°brix)) is presented in Table 3. As can be seen, *K. marxianus* had similar behavior in both complex media simulating the hydrolysates without inhibitors, with μ_{max} of 0.184−0.201 h^{-1} and a Δ°brix of 2.65−3.00. The difference between the original and post-hydrolyzed media without inhibitors was the content of sugars; post-hydrolyzed hydrolysates presented higher amount of sugars, as shown in Table 2. These results reveal that the sugars concentration increase did not affect the yeast performance, which was able to grow and consume sugars similarly in both

conditions. However, in the presence of inhibitors, i.e., under conditions more similar to the real hydrolysates, the yeast was unable to grow or consume sugar. These results allowed to conclude that the amount of inhibitors present in the hydrolysates is harmful to *K. marxianus* and a detoxification process is necessary to overcome this barrier.

3.3. Detoxification process

Due to the high contents of acetic and formic acids and phenolic compounds in the hydrolysates after post-hydrolysis (Table 2), a detoxification process able to selectively recover these compounds from the hydrolysates was proposed with the ultimate goal of not only obtaining a suitable medium for fermentation, but also of getting additional value from the produced hydrolysates. Table 4 shows the composition of switchgrass and eucalyptus hydrolysates after each detoxification step: solid-phase extraction and liquid-liquid extraction. When compared to the original hydrolysates, the removal of organic acids corresponded to 73–91 %, and for total phenolic compounds corresponded to 81–88 %, with complete removal of 5-HMF and furfural. Additionally, phenolic compounds adsorbed into the resin membrane during solid-phase extraction were recovered by using 5 % (v/v) ethanol followed by absolute ethanol; while acetic acid and formic acids were recovered from the organic phase of liquid-liquid extraction by solvent evaporation. So, the detoxification strategy used in the present study was found as being an efficient method to selectively remove inhibitor compounds from hemicellulosic hydrolysates and could also contribute to improve the income of a biorefinery.

Table 3

Fermentation performance of *Kluyveromyces marxianus* in complex media simulating the hydrolysate's composition.

| Simulated hydrolysate condition | Simulated switchgrass | | Simulated eucalyptus | |
	μ_{max} (h^{-1})	Δ°brix	μ_{max} (h^{-1})	Δ°brix
Original hydrolysate without inhibitors	0.184 ± 0.002	2.85 ± 0.07	0.201 ± 0.001	3.00 ± 0.00
Original hydrolysate with inhibitors	–	–	–	–
Post-hydrolyzed without inhibitors	0.184 ± 0.004	2.75 ± 0.07	0.192 ± 0.003	2.65 ± 0.07
Post-hydrolyzed with inhibitors	–	–	–	–

Values after 48 h of fermentation.

Table 4

Chemical composition of detoxified hydrolysates after sequential solid-phase extraction and liquid-liquid extraction.

| Compound | Switchgrass hydrolysate (g/L) | | Eucalyptus hydrolysate (g/L) | |
	Solid-phase extraction	Liquid-liquid extraction	Solid-phase extraction	Liquid-liquid extraction
Cellobiose	nd	nd	nd	nd
Glucose	10.41 ± 0.09	11.70 ± 0.05	9.16 ± 0.0	9.89 ± 0.02
Xylose	27.88 ± 0.10	32.44 ± 0.05	38.50 ± 0.25	42.75 ± 0.15
Arabinose	2.24 ± 0.25	2.38 ± 0.21	1.90 ± 0.02	2.05 ± 0.31
Formic acid	9.18 ± 1.06	2.00 ± 0.26	5.43 ± 0.42	2.15 ± 0.0
Acetic acid	13.72 ± 0.73	1.48 ± 0.63	20.06 ± 0.89	3.94 ± 0.81
5-HMF	0.18 ± 0.0	nd	0.03 ± 0.0	nd
Furfural	0.03 ± 0.0	nd	0.03 ± 0.0	nd
Total phenolic	2.80 ± 0.03	2.28 ± 0.07	2.33 ± 0.02	1.29 ± 0.08

nd: not detected.

3.4. Fermentation of detoxified hydrolysates by K. marxianus

The detoxified hydrolysates obtained in the previous step were tested as fermentation medium for xylitol production by *K. marxianus*. Assays were carried out with and without nutrient supplementation to the hydrolysates. Fig. 2A and B show the results in hydrolysate supplemented with nutrients, and Fig. 2C and D show the results using non-supplemented hydrolysate. As can be seen, removal of toxic compounds and addition of nutrients benefited the sugars consumption rate, which were exhausted within 48 h, but favored the cell growth instead of the xylitol production. Since the second sample was taken after 48 h of cultivation and a fast sugar consumption rate was observed during this period, it is not possible to affirm that there was no xylitol formation in these experiments. However, when compared to the fermentation from non-supplemented media, it is clear that the nutritional supplementation deviated the yeast metabolism to higher cell formation instead of xylitol production.

Fermentation of detoxified hydrolysates without addition of nutrients resulted in longer fermentation time, but also in higher production of xylitol and lower cell growth. The maximum concentration of xylitol obtained from switchgrass hydrolysate was 13.71 ± 0.19 g/L that corresponded to 0.48 ± 0.04 g xylitol/g xylose; while the concentration of xylitol from eucalyptus hydrolysate achieved 28.07 ± 1.70 g/L and corresponded to 0.68 ± 0.10 g xylitol/g xylose. These results suggest that the higher amount of sugars and possibly the lower content of phenolic compounds present in detoxified eucalyptus hydrolysate (Table 4) influenced the yeast metabolism resulting in higher xylitol production. High concentration of sugars reduce the level of free dissolved oxygen in the yeast membrane interface. Xylitol formation is favored under physiological stress conditions, in particular under anaerobic or oxygen-limited conditions, which cause NADH accumulation and then inhibition of NAD-linked xylitol dehydrogenase (Goli et al., 2012).

Besides the higher sugar content, the higher nitrogen content in eucalyptus hydrolysate may also have positively influenced the yeast performance to produce xylitol. Nitrogen, measured as primary amino nitrogen, corresponded to 18 ± 4 mg/L in switchgrass hydrolysate and 46 ± 5 mg/L in eucalyptus hydrolysate. However, the fermentation results suggest a possible lack of essential elements in the hydrolysates due to the long fermentation time observed. Essential elements are important for the formation of the enzymes in the metabolic pathway. For example, yeast extract contain biotin, a vitamin that is essential for yeast metabolism towards fermentation (Lee et al., 1988; Parrondo et al., 2009) as well as potassium, magnesium and other metals that play important role in the enzyme activity, homeostasis and cell division among others (Udeh, 2013). A correct balance of nutrients is very important to favor the yeast metabolism for the synthesis of the product of interest.

Although a significant production of xylitol was achieved from both detoxified and non-supplemented hydrolysates (0.48 ± 0.04 g xylitol/g xylose from switchgrass hydrolysate and 0.68 ± 0.10 g xylitol/g xylose from eucalyptus hydrolysate), there is a room for improvement of the xylitol yield since the maximum theoretical value of xylitol production from xylose corresponds to 0.917 g/g (Barbosa et al., 1988). However, these results compare well with studies performed in larger scale experiments. Zhang et al. (2021), for example, produced xylitol with an yield of 0.32 g/g from a medium containing 300 g/L of xylose using a 5-L bioreactor, while Prabhu et al. (2020) obtained a xylitol yield of 0.67
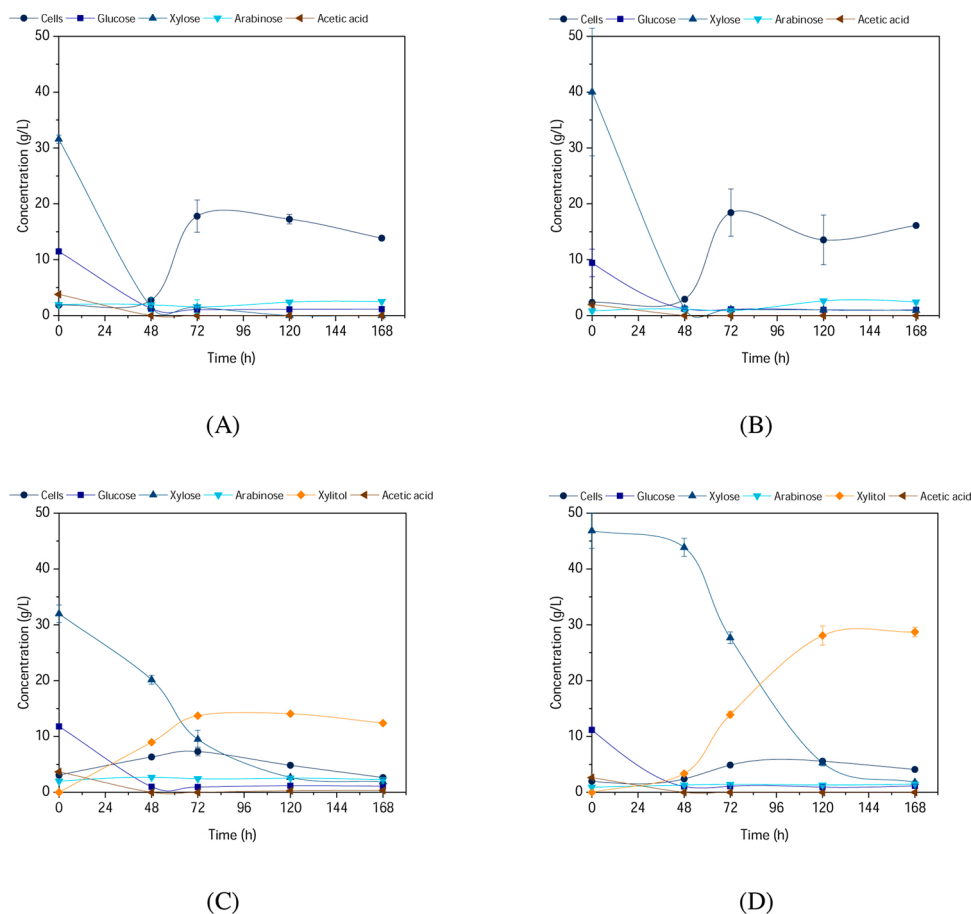

Fig. 2. Fermentation of detoxified hydrolysates with (A and B) and without (C and D) nutrient supplementation by *Kluyveromyces marxianus*. (A,C) Switchgrass hydrolysate; (B,D) *Eucalyptus globulus* hydrolysate.

g/g from pure xylose and 0.54 g/g from sugarcane bagasse hydrolysate using a 2.5 L bioreactor. It is worth noting that small-scale cultivations, in the microliter and milliliter range, have gained increased importance in recent years in modern biotechnological process development, playing an essential role in high-throughput screening experiments as well as for the production of specific compounds, phenotypic tests, mechanistic studies, among others (Lattermann and Büchs, 2015; de Mello et al., 2019). Scalability and correlation with lab scale stirred tank bioreactors has also been reported (Garrigós-Martínez et al., 2021; Hemmerich et al., 2021); however, aeration is a critical parameter. In the case of the present study, establishing the optimum conditions of dissolved oxygen and an appropriate balance of essentials nutrients to be added to the hydrolysate, for example, could help to reach higher production yields. Anyway, the results demonstrate that the hemicellulosic hydrolysates produced by intensified steam explosion of switchgrass and eucalyptus present good fermentability and can be used to produce xylitol after detoxification.

3.5. Fermentation of simulated complex media by R. toruloides

Complex media simulating the composition of detoxified switchgrass and eucalyptus hydrolysates (Table 4) were formulated and used for cultivation of *R. toruloides*. Overall, the strain exhibited a slow growth rate when cultivated in such media, which could be attributed to the higher content of xylose than glucose in the media. In this metabolic pathway, the induction of a xylose entry system is necessary, which is repressed by the presence of glucose (Ounine et al., 1985). In media without inhibitors, the yeast growth (Fig. 3A and 3C) and total sugars

consumed (Fig. 3B and D) were similar independent of the hydrolysate used, which suggest that the content of sugars and the difference in the xylose-to-glucose ratios among the hydrolysates (about 2.7 in switchgrass hydrolysate, and 4.3 in eucalyptus hydrolysate), did not affect the strain performance to grow. After 144 h, a carotenoid production of 7.96 ± 0.64 mg/L was observed in complex medium simulating eucalyptus hydrolysate without inhibitors, and 8.29 ± 0.50 mg/L in complex medium simulating switchgrass hydrolysate without inhibitors. Such values are in close agreement with a previous study on the production of carotenoids by this same yeast strain (Liu et al., 2020).

On the other hand, interesting results were observed in simulated media containing inhibitors. In medium simulating switchgrass hydrolysate containing inhibitors (2.0 g/L of formic acid and 1.48 g/L of acetic acid), the yeast growth was favored; while in medium simulating eucalyptus hydrolysate with inhibitors (2.93 g/L of formic acid and 3.94 g/L of acetic acid), the yeast growth was completely inhibited (Fig. 3A and C). In fact, the strain of *R. toruloides* used in this study was previously evolved by adaptive laboratory evolution to better tolerate inhibitors present in wheat straw hydrolysates (which contained 1.5 g/L of acetic acid and 1.5 g/L of total phenolic compounds) (Liu et al., 2021b). The metabolism of oleaginous yeasts requires a constant supply of acetyl-CoA and malonyl CoA. Therefore, low amount of acetate contributes to the TCA cycle stimulating the carotenoid and fatty acids synthesis (Beopoulos et al., 2011; Xue et al., 2018). However, high amount of acetate may strongly affect the yeast performance. This would explain the inhibition of the yeast growth observed in simulated eucalyptus hydrolysate. It is worth highlighting that acetic acid has an inhibitory effect above certain concentrations (depending on yeast and
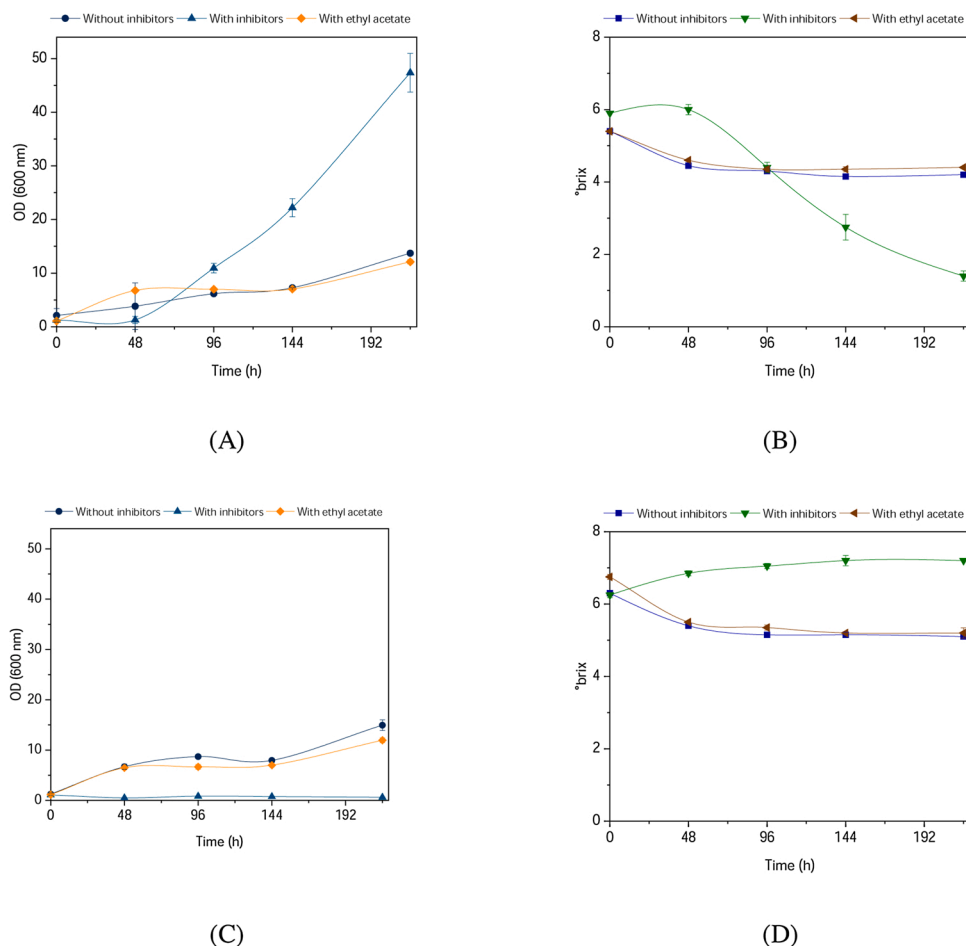
(A)

(B)

(C)

(D)

Fig. 3. Growth (A,C) and estimated sugars consumption (B,D) during the cultivation of *Rhodosporidium toruloides* in complex medium simulating switchgrass hydrolysate (A,B) and *Eucalyptus globulus* hydrolysate (C,D).

evolution), but it can also be used as carbon source by the yeasts when present in low concentration (Chen et al., 2009; Liu et al., 2020). Concerning the experiments with ethyl acetate, the presence of 2.0 g/L in the media slightly affected the yeast growth in both simulated hydrolysates. However, when cultivated in medium simulating switchgrass with ethyl acetate, the yeast produced 7.81 ± 0.60 mg/L of carotenoids after 144 h, which was very similar to the value obtained in the simulated media without inhibitors. These results reveal that the detoxification step used in the present study employing ethyl acetate as extraction solvent, would not be a problem for subsequent cultivation of *R. toruloides* in the hydrolysate, as after detoxification, a small amount of ethyl acetate dilutes in the hydrolysate.

R. toruloides is an oleaginous yeast with ability to produce lipids and carotenoids (Liu et al., 2021a). This yeast is a natural producer of β-carotene, γ-carotene, torulene, and torularhodin, which are valuable compounds for the chemical, pharmaceutical, feed, and cosmetic industries (Qi et al., 2020; Lin et al., 2017). In this study, the yeast cells became orange-red color even in the experiments where cell growth was not observed. However, cells cultivated in simulated hydrolysate without inhibitors presented a light orange color (Fig. 4A), while a stronger orange-red color was observed in simulated hydrolysate with inhibitors (Fig. 4B). According to the probable carotenoids present in the fermented hydrolysate and the observation of the color, it could be suggested that there was a prevalence of γ-carotene (Liu et al., 2021a; Buzzini et al., 2007). Nevertheless, an associated production of lipids in the simulated media without inhibitors could explain the lighter color compared to the media with inhibitors, as carotenoids are formed in higher amount under stress conditions (Elfeky et al., 2019; Liu et al., 2021b).

3.6. Fermentation of detoxified hydrolysates by R. toruloides

Finally, fermentation experiments were performed using the real detoxified hydrolysates produced from switchgrass and eucalyptus, but the yeast was unable to grow in these media. These results revealed that, although the detoxification step removed high amount of inhibitory compounds, the remaining amount present in the hydrolysates was still very toxic to *R. toruloides*. Phenolic compounds in the hydrolysate can penetrate the membrane damaging the cellular membrane, creating

DNA mutation or increasing the level of reactive oxygen species resulting in cytoskeleton damage and cell death (Wang et al., 2018; Chandel et al., 2013). Although acetic acid can also act as an inhibitor to *R. toruloides*, it may not have been the main problem in the case of the present study, as previously discussed. In this sense, further detoxication strategies or strain adaptation/engineering should be done to improve the yeast performance to grow and produce carotenoids from such complex media. In the line of the latter strategy, considering the protective role of carotenoids against different stress conditions such as exposure to light, a mutagenesis using UV radiation could be an alternative to improve the resistance of the yeast and its production of carotenoids, for example (Avalos and Carmen Limón, 2014; Sridhar et al., 2002; (Liu et al., 2021b).

4. Conclusion and final remarks

Intensification of steam explosion pretreatment was demonstrated to be an interesting strategy to process more biomass per unit of time, producing hemicellulosic hydrolysates with high concentration of sugars, suitable for use as fermentation medium. In addition, by producing hydrolysates with high sugars content during pretreatment, the subsequent step of concentration, which is usually performed to have enough sugars for fermentation purposes, can be eliminated or at least minimized. This may lead to less energy expenses for the overall hydrolysate bioconversion process.

The intensification strategy used in the present study was tested with two different raw materials, switchgrass and *Eucalyptus globulus*, and, in both cases, resulted in hydrolysates containing high concentration of sugars and also high concentration of inhibitor compounds. The formation of toxic compounds can potentially be minimized by optimizing the pretreatment conditions. However, the detoxification strategy here applied, which allowed to selectively recover phenolic compounds and acetic/formic acids, can also be considered a relevant way to get more value from the hydrolysate while contributes to improve its fermentability. At the end, this strategy of detoxification can help to improve the economic feasibility of ethanol biorefineries, where a valuable product can be produced by fermentation of hemicellulosic hydrolysates, and acetic acid and phenolic compounds can also be recovered from the hydrolysates as extra value-added byproducts. This approach can
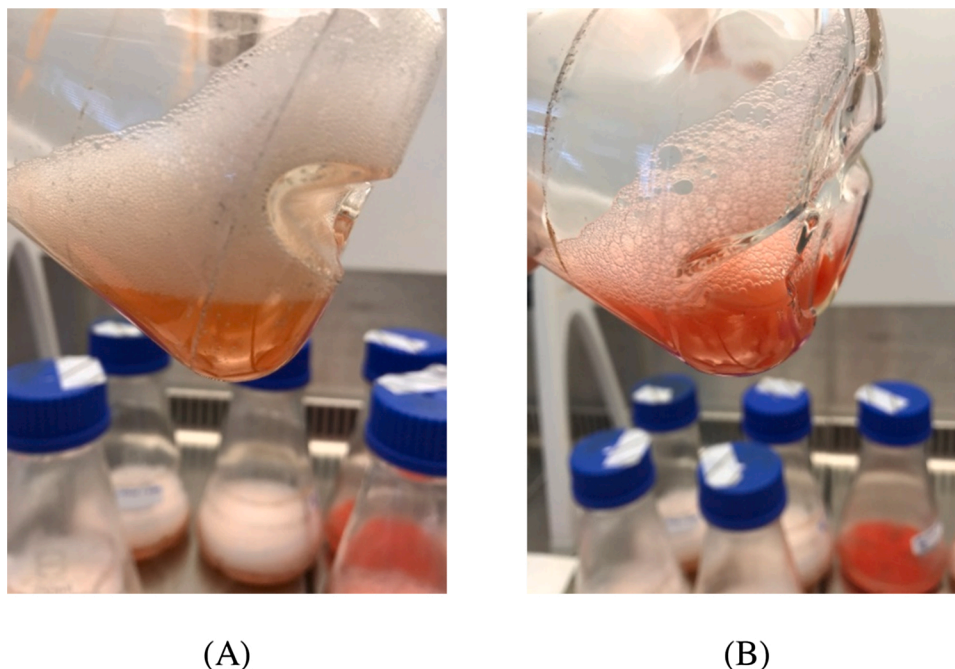
(A)

(B)

Fig. 4. Cells of *Rhodosporidium toruloides* cultivated in complex medium simulating *Eucalyptus globulus* hydrolysate without inhibitors (A) and with inhibitors (B).

positively impact the sustainability of the overall process, contributing not only economically, but also to the environment and to the society by minimizing the generation of wastes and by producing chemicals through routes that avoid the use of fossil resources.

Hemicellulosic hydrolysates obtained by intensified steam explosion of switchgrass and *Eucalyptus globulus* were efficiently used as fermentation medium after detoxification, especially for xylitol production by *K. marxianus*. In the case of carotenoids, promising results were obtained from complex media simulating the hydrolysates, which suggest that the production of carotenoids from these hydrolysates can be a feasible process. However, further studies are required to minimize the toxicity of the remaining inhibitory compounds to *R. toruloides*. In summary, it can be concluded that, by selecting appropriate microbial strains, the hydrolysates can be used as a rich source of sugars and nutrients to produce added-value compounds by fermentation.

CRediT authorship contribution statement

Fernando Bonfiglio: Methodology, Formal analysis, Funding acquisition, Writing - original draft. **Matías Cagno:** Methodology, Formal analysis. **Celina K. Yamakawa:** Methodology, Formal analysis, Data curation, Writing - original draft. **Solange I. Mussatto:** Conceptualization, Supervision, Resources, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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