Fuel 318 (2022) 123489

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

Valorization of *Pinus taeda* hemicellulosic hydrolysate for the production of value-added compounds in an ethanol biorefinery

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ARTICLE INFO

Keywords:
Biorefinery
Hemicellulosic hydrolysate
Inhibitory compounds
Value-added products
Lactic acid

ABSTRACT

Production of cellulosic ethanol from lignocellulosic biomass leads to the generation of a hemicellulosic hydrolysate during the feedstock pretreatment. This hydrolysate is rich in sugars, but also contains inhibitory compounds (mainly acetic acid and phenolic compounds) in concentrations that may be toxic to microbial growth. Currently, this side-stream of the cellulosic ethanol production process is processed as a waste, due to the lack of feasible alternatives for tackling the complexity of wood hemicellulosic hydrolysate. Thus, this work evaluated the ability of six microorganisms to metabolize the raw and detoxified hemicellulosic hydrolysate produced from *Pinus taeda* for the production of lactic acid, ethanol, xylitol, single-cell protein, lipids and carotenoids, with the aim of selecting a potential alternative for valorization of this side stream generated during the production of cellulosic ethanol contributing to the implementation of a sustainable advanced biorefinery. The tested microorganisms included a lactic acid bacterium, *Bacillus coagulans*; a probiotic bacterium, *Lactobacillus salivarius*; two oleaginous yeasts, *Rhodosporidium toruloides* and *Saitoella coloradoensis*; a thermotolerant yeast, *Kluyveromyces marxianus*; and a methylotrophic yeast, *Hansenula polymorpha*. *L. salivarius*, *K. marxianus*, and *H. polymorpha* showed promising ability to metabolize the partially detoxified hydrolysate (composed of (g/L): mannose, 29.27; glucose, 17.25; galactose, 6.18; xylose, 4.94; arabinose, 1.23; acetic acid, 7.99; formic acid, 4.86; levulinic acid, 4.04; 5-hydroxymethylfurfural, 0.74; total phenolic compounds, 0.40). On the other hand, the oleaginous yeasts and *B. coagulans* presented high sensitivity to the inhibitory compounds. *L. salivarius* produced lactic acid with high yield (1.1 g/g), which was limited by product inhibition. *K. marxianus* produced xylitol at 0.37 g/g xylose and ethanol at 0.19 g/g hexoses. Finally, *H. polymorpha* converted hexoses and acetic acid into single-cell protein with yield of 0.27 g/g. The production of lactic acid by *L. salivarius* proved to be a promising alternative for valorization of *Pinus* hemicellulosic hydrolysate in an ethanol biorefinery.

1. Introduction

Pinus taeda is a lignocellulosic biomass produced in large amount in Uruguay, being used mainly in the solid wood industry. However, this industry discards a large part of the wood, and these residues are usually burned for energy generation [1–3]. This is not the best solution for a number of reasons ranging from the cost of electricity production to the contamination of the wood with different compounds that can affect the boilers used for biomass combustion [2,4]. Taking advantage of the main components of the lignocellulosic residues to produce different valuable products would be a better approach. The major polymers present in the composition of Pine wood (cellulose, hemicellulose, and lignin) could be used in a biorefinery, for example, to produce energy, biofuels, and other added-value compounds [5–7].

The production of cellulosic ethanol is based on the pretreatment of the lignocellulosic biomass and subsequent enzymatic hydrolysis of the cellulose fraction to obtain glucose, which is then converted into ethanol by fermentation. During the biomass pretreatment, a liquid fraction (hemicellulosic hydrolysate) is generated, which represents an important side-stream of the cellulosic ethanol process. For the development of advanced biorefineries, the utilization of all side-streams should be considered in order to get maximum value from biomass, while minimizing the generation of pollutants or their simple disposal by burning [8]. However, the utilization of hemicellulosic hydrolysates in

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https://doi.org/10.1016/j.fuel.2022.123489
Received 3 October 2021; Received in revised form 27 December 2021; Accepted 1 February 2022
Available online 24 February 2022

Table 1
Microorganisms, cultivation conditions and target products applied for bioconversion of Pinus hemicellulosic hydrolysate.

Microorganism	Type	Hydrolysate	Conditions	Target product
Bacillus coagulans	Lactic bacterium	Raw hydrolysate Partially detoxified hydrolysate	30–60 °C pH 6.0	Lactic acid
Lactobacillus salivarius	Probiotic bacterium	Raw hydrolysate Partially detoxified hydrolysate Diluted partially detoxified hydrolysate	35 °C pH 6.0	Lactic acid
Rhodosporidium toruloides	Oleaginous yeast	Partially detoxified hydrolysate Detoxified hydrolysate Diluted detoxified hydrolysate	25 °C pH 4.8	Lipids and carotenoids
Saitoella coloradoensis	Oleaginous yeast	Partially detoxified hydrolysate Diluted detoxified hydrolysate	25 °C pH 4.8	Lipids and carotenoids
Kluyveromyces marxianus	Thermotolerant yeast	Partially detoxified hydrolysate Detoxified hydrolysate	40 °C pH 5.8	Ethanol and xylitol
Hansenula polymorpha	Methylotrophic yeast	Raw hydrolysate Partially detoxified hydrolysate	37 °C pH 5.8	Single-cell protein

bioprocesses is a challenge due to the presence of some compounds that act as inhibitors for most wild-type or non-engineered microorganisms [9]. Although toxic to microorganisms, those so-called inhibitors are also valuable chemicals. Acetic acid, for example, can be converted into vinyl acetate monomer, which can be used as chemical building block [10]. Formic acid and levulinic acid are two important chemicals for the catalyst industry and can be used as fuel additives [11,12]. Even mannose, which is not an inhibitory compound, but the most abundant sugar in *Pinus taeda* hemicellulosic hydrolysate, is a natural bioactive monosaccharide with potential uses as food supplement [13]. Nevertheless, the recovery of these compounds from hemicellulosic hydrolysates is not always an economically feasible process, since their concentration in such media is usually not high enough to justify the costs related to separation and purification.

The hemicellulosic hydrolysate produced from *Pinus taeda* is a challenging substrate for use in bioprocesses due to the diversity of sugars present along with inhibitory compounds. In addition, due to the characteristics of the hemicellulosic hydrolysate, a process of conditioning (post-hydrolysis and detoxification) is usually necessary for an improved microbial cultivation. Since the capital investments and the operation costs are expensive, an appropriate choice of the product to be produced from this hydrolysate is crucial to contribute to the biorefinery investment return. The best scenario for an efficient utilization of the hemicellulosic hydrolysate by microorganisms is to apply a detoxification process for complete removal of inhibitory compounds. However, detoxification to completely eliminate inhibitors also significantly increases the operating costs and leads to the loss of sugars from the medium. To overcome this problem, finding the most tolerant and robust microorganism is the key to well-design a reasonable and feasible detoxification process.

In the present study, lactic acid, xylitol, ethanol, microbial lipids, carotenoids, and single-cell protein were selected as potential high value products to be produced from *Pinus taeda* hemicellulosic hydrolysate. Lactic acid is an important organic acid used in the food, cosmetic, pharmaceutical, and chemical industries. The highlighted importance of lactic acid is the production of poly lactic acid polymers [14], which can be used as bio-based plastics. The global market demand of lactic acid is projected to reach 1,960 kt in 2025 representing USD 9.8 billion [15]. Xylitol is a sugar alcohol largely used in food, pharmaceutical, and dental industries due to its health-related properties [16,17]. With a number of recent applications also in different areas, the xylitol demand has significantly increased in the last years, reaching a global market of USD 921 million in 2020 [17]. Ethanol is a well-recognized biofuel and an attractive alcohol for the production of several chemicals [18]. Microbial lipids are a greener alternative for the production of biodiesel, also acting on energy security [19] and have many applications in the food area. Carotenoids are natural pigments with valuable pharmaceutical properties [20]; while single-cell protein has been used for animal nutrition and is also considered a promising alternative to attend the world demand of protein for human nutrition [21]. Yeast is a good source of single-cell protein production and has been used for this purpose for a long time [22].

This study evaluated the ability of six microorganisms to convert the hemicellulosic hydrolysate of *Pinus taeda* for the production of lactic acid, ethanol, xylitol, single-cell protein, lipids and carotenoids, with the aim of selecting a potential alternative for valorization of the side-stream generated during the production of cellulosic ethanol contributing to the implementation of a sustainable advanced biorefinery. Assays were performed using raw and detoxified hydrolysates and at the end, the most promising product to be produced from this hydrolysate, with potential to be incorporated in an ethanol biorefinery was selected.

2. Material and methods

2.1. Hemicellulosic hydrolysate

Pinus taeda softwood biomass was provided by the National Agricultural Research Institute of Uruguay (INIA). The material was obtained by commercial thinning of a plantation located in the northeast of Uruguay.

The biomass was pretreated by steam explosion in a semi-continuous pre-pilot reactor installed at the Technological Laboratory of Uruguay, at 200 °C for 10 min of residence time [23]. This was the first step of a global process for the production of cellulosic ethanol. The hemicellulosic hydrolysate generated during the pretreatment step (raw hydrolysate) was characterized in terms of sugars, organic acids, furan derivatives, and total phenolic compounds.

2.2. Detoxification process

A selective method was applied to remove phenolic compounds and organic acids from the hydrolysate, based on the study of Bonfiglio et al. [23] with some modifications. The detoxification process comprehended an acid post-hydrolysis, solid-phase extraction, and liquid–liquid extraction. The acid post-hydrolysis consisted of a reaction with sulfuric acid (4% w/w) in autoclave at 121 °C for 60 min [24]. The reaction was performed in 500-mL borosilicate bottles with a screw cap containing 200 mL of working volume. The acid post-hydrolysis hydrolysate was filtered in 0.45 μm polyethersulfone membrane to remove the precipitate (humin). Then, the pH was adjusted to 2.47 with NaOH pellets to remove phenolic compounds using a solid-phase extraction system in 35-mL cartridge with 10 g of silica-based sorbent [25]. The hydrolysate obtained after this step was called partially detoxified

Table 2
Characterization of Pinus hemicellulose hydrolysate before (raw) and after post-hydrolysis, partially detoxified and detoxified.

Analyte	Raw hydrolysate (g/L)	Post-hydrolysate (g/L)	Partially detoxified hydrolysate (g/L)	Detoxified hydrolysate (g/L)
Cellobiose	1.93 ± 0.03	n.d.	n.d.	n.d.
Glucose	7.24 ± 0.10	16.93 ± 0.20	17.25 ± 0.07	13.97 ± 1.76
Xylose	5.39 ± 0.39	4.50 ± 0.73	4.94 ± 0.37	4.97 ± 2.50
Galactose	4.14 ± 0.02	7.90 ± 1.21	6.18 ± 0.43	4.58 ± 0.85
Arabinose	1.64 ± 0.04	2.96 ± 0.16	1.23 ± 0.09	1.17 ± 0.04
Mannose	8.70 ± 1.87	29.82 ± 1.97	29.27 ± 1.55	24.86 ± 1.88
Formic acid	2.00 ± 0.15	4.96 ± 0.05	4.86 ± 0.04	1.15 ± 0.10
Acetic acid	5.41 ± 0.33	8.36 ± 0.20	7.99 ± 0.19	3.47 ± 0.03
Levulinic acid	n.d.	5.70 ± 0.01	4.04 ± 0.33	n.d.
5-HMF	3.62 ± 0.02	1.99 ± 0.01	0.74 ± 0.17	n.d.
Furfural	0.11 ± 0.01	0.15 ± 0.01	n.d.	n.d.
Total monomeric sugars	27.11 ± 2.42	62.11 ± 4.28	58.87 ± 0.50	49.55 ± 1.41
Total hexoses	20.08 ± 1.99	54.65 ± 3.38	52.70 ± 0.68	43.41 ± 1.50
Total pentoses	7.03 ± 0.43	7.46 ± 0.90	6.17 ± 0.23	6.14 ± 0.79
Total phenolic compounds	5.26 ± 0.24	4.31 ± 0.29	0.40 ± 0.04	0.27 ± 0.04
pH	3.28	0.44	–	–

n.d.: not detected.

Table 3
Summary of the best conditions of the experimental design tested for the production of lactic acid by *Bacillus coagulans* from raw Pinus hydrolysate.

Variables			**Response in 12 h**		**Response in 24 h**	
Temperature (°C)	**Hydrolysate (%)**	**Yeast extract (g/L)**	**Lactic acid (g/L)**	**$Y_{P/S}$ (g/g)**	**Lactic acid (g/L)**	**$Y_{P/S}$ (g/g)**
36	28	24	0.10 ± 0.02	0.01 ± 0.00	2.74 ± 0.15	0.14 ± 0.02
36	82	6	0.07 ± 0.28	0.02 ± 0.11	0.20 ± 0.22	0.11 ± 0.15
54	28	24	3.19 ± 0.14	0.13 ± 0.05	5.32 ± 0.21	0.19 ± 0.07
45	10	15	2.97 ± 0.09	0.10 ± 0.00	3.79 ± 0.00	0.12 ± 0.00
45	55	30	n.d.	n.d.	1.28 ± 0.93	0.20 ± 0.29

$Y_{P/S}$ = Substrate to product conversion yield, calculated as the ratio between product (lactic acid) formed and substrate (total carbohydrates) consumed.

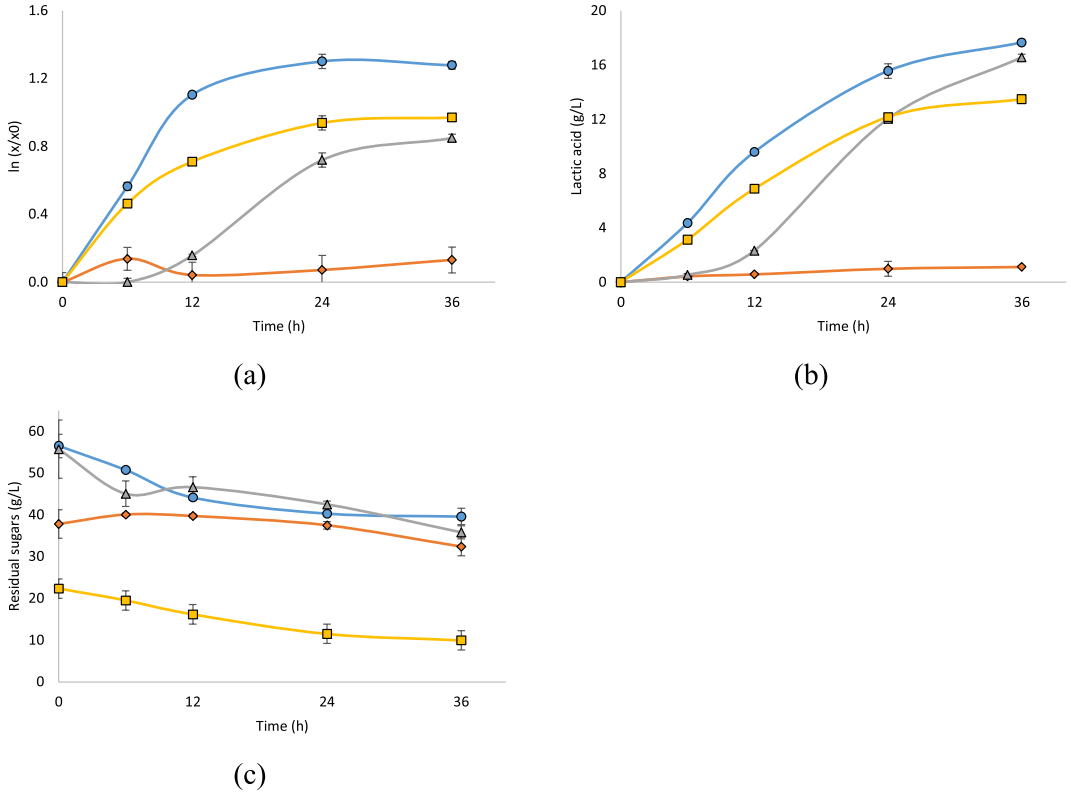


Fig. 1. Fermentation profile of *Lactobacillus salivarius* in different media (blue circle: control; orange square: raw hydrolysate; grey triangle: partially detoxified hydrolysate; yellow diamond: diluted partially detoxified hydrolysate): (a) Linearized growth; (b) Lactic acid production; (c) Residual sugar concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Performance of *Lactobacillus salivarius* cultivated in Pinus hydrolysates. Results for 36 h of fermentation.

Media	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	μmax (h^{-1})
Raw hydrolysate	0.02 ± 0.04	0.21 ± 0.24	0.003 ± 0.002
Partially detoxified hydrolysate	0.05 ± 0.02	0.83 ± 0.37	0.025 ± 0.002
Diluted partially detoxified hydrolysate	0.08 ± 0.01	1.09 ± 0.01	0.063 ± 0.003
Control	0.11 ± 0.03	1.05 ± 0.30	0.092 ± 0.001

$Y_{X/S}$ = substrate to cell conversion yield, calculated as the ratio between cell produced and substrate (total carbohydrates) consumed. $Y_{P/S}$ = Substrate to product conversion yield, calculated as the ratio between product (lactic acid) formed and substrate (total carbohydrates) consumed. μmax = maximum specific growth rate, calculated as the slope of linear region on an ln (X/X0) versus time plot, where X is the cell concentration per volume and X0 is the cell concentration at the initial time.

hydrolysate.

The partially detoxified hydrolysate was then submitted to liquid–liquid extraction using ethyl acetate to remove organic acids (acetic, formic, and levulinic). The mixture of the liquid–liquid extraction was performed using 50 mL of partially detoxified hydrolysate at pH 2.47 [26] and 200 mL of ethyl acetate in a 500-mL shake flask with screw cap mounted with PTFE-faced liner, which was placed in an incubator at 25 °C, 250 rpm for 20 min. Afterwards, the mixture was transferred to a 500-mL separatory funnel and left for 20 min for phase separation. The hydrolysate obtained after this second step of detoxification was called detoxified hydrolysate.

At the end of the process, a mass balance was performed taking into account the volumes after/ before each step (acid post-hydrolysis, solid–liquid extraction, and liquid–liquid extraction) and considering the compound concentration (g/L) obtained by HPLC, in order to obtain the corresponding value in mass (g).

2.3. Microorganism, inoculum, and cultivation conditions

Table 1 summarizes the microorganisms, cultivation conditions, and target products tested for bioconversion of Pinus hemicellulosic hydrolysate. Two lactic acid bacteria, two oleaginous yeasts, a thermotolerant yeast, and a methylotrophic yeast were used in this study. The yeasts were selected based on previous works [20,23,27] in which they proved to be promising candidates for the conversion of lignocellulosic hydrolysates into value-added products. The selected bacteria are already known as being good candidates for the production of lactic acid.

To be used as cultivation media, the pH of the hydrolysates (raw, partially detoxified, and detoxified) was adjusted to 6.0 with NaOH pellets. The pH adjustment was done after the fermentation media preparation and before sterilization in 0.22 μm polyethersulfone membrane. Specific conditions were used to each microorganism/ fermentation process as described below.

2.3.1. Bacillus coagulans

The thermotolerant lactic bacteria *Bacillus coagulans* DSM 2314 was used for the production of lactic acid at high temperature (54 °C). The strain was obtained from Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany). Stock cultures were kept frozen at − 80 °C with 20% glycerol in tryptone soy medium composed of (g/L): casein peptone, 15.0; soy peptone, 5.0; and sodium chloride, 5.0. The stock culture was activated in 50-mL tube containing 25 mL of the same cultivation medium, incubated statically at 54 °C for 24 h. The inoculum was prepared similarly by transferring 1 mL of the activated culture to fresh medium. After 24 h of incubation, the cells were recovered by centrifugation using a Multifuge™X3 centrifuge (Thermo Scientific, USA) at 6000 rpm, 15 min, 4 °C. The cells were washed twice with saline solution (0.9% NaCl) to be inoculated in the fermentation medium.

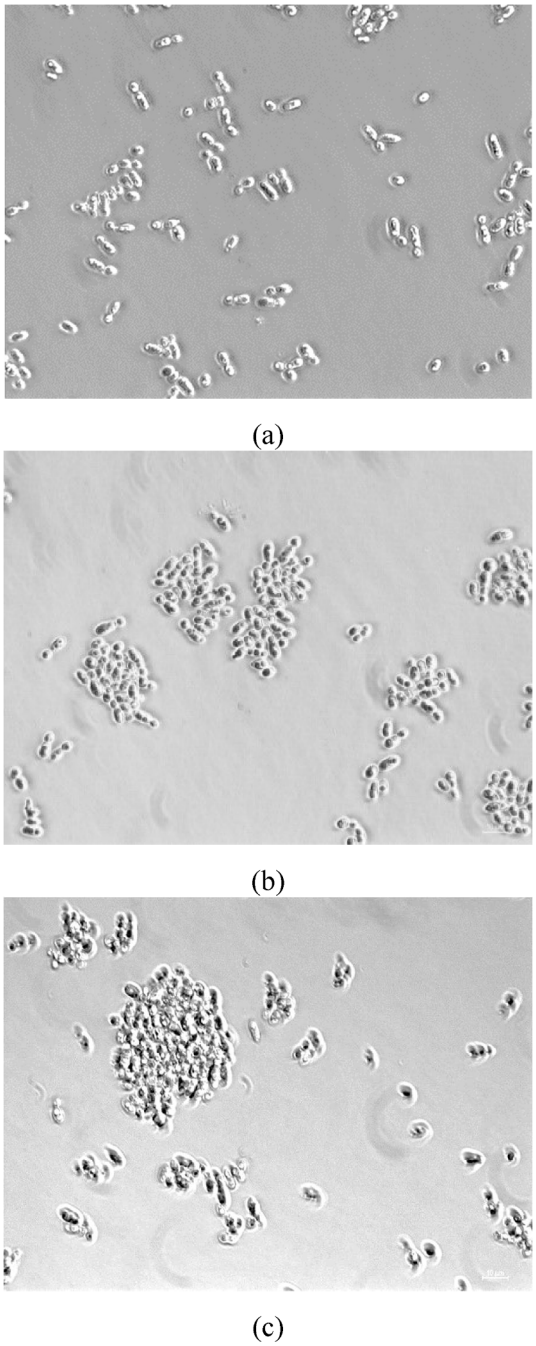


Fig. 2. Light microscope image of *Rhodosporidium toruloides* cells cultivated in different media: (a) Control medium; b) Partially detoxified hydrolysate; c) Detoxified hydrolysate. Images at 63-fold magnification.

The fermentation experiments were performed using the raw and partially detoxified hydrolysates. Assays in raw hydrolysate were carried out according to a 2^3 central composite design, with 5 levels (-1.68, −1, 0, +1, and + 1.68) to each independent variable, in order to investigate the effects of the temperature [30 °C (-1.68), 36 °C (-1), 45 °C (0), 54 °C (+1), 60 °C (1.68)], content of raw hydrolysate [10% (-1.68), 28% (-1), 55% (0), 88% (+1), 100% (1.68)], and yeast extract [0 g/L (-1.68), 6 g/L (-1), 15 g/L (0), 24 g/L (+1), 30 g/L (1.68)], on the production of lactic acid by the microorganism. The experiments were carried out statically in a bottom square polypropylene 24 deep well

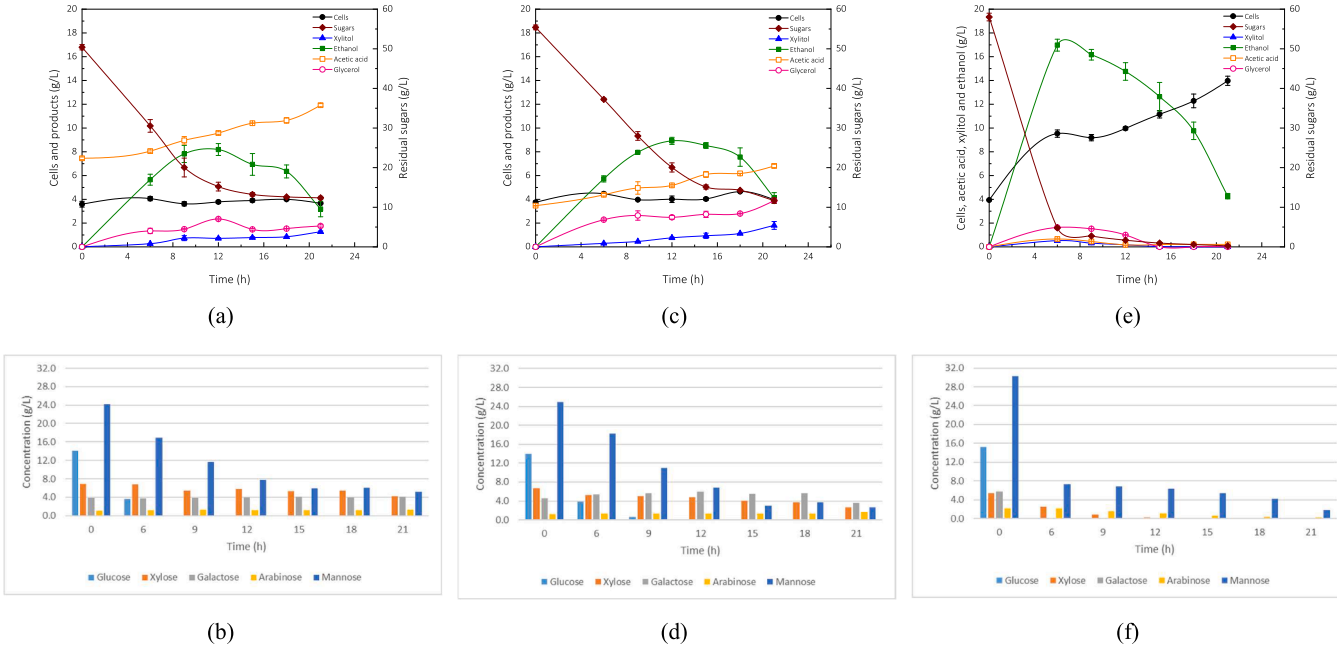


Fig. 3. Performance of *Kluyveromyces marxianus* cultivated in Partially detoxified hydrolysate: (a) Fermentation profile; (b) Residual sugar concentration; in Detoxified hydrolysate: (c) Fermentation profile; (d) Residual sugar concentration; and in the Control medium: (e) Fermentation profile; (f) Residual sugar concentration.

Table 5
Performance of *Kluyveromyces marxianus* cultivated in partially detoxified and detoxified Pinus hydrolysates, and in control medium. Results for 12 h of fermentation.

Yield (g/g substrate)	Partially detoxified hydrolysate	Detoxified hydrolysate	Control medium
Cell	–	–	0.08 ± 0.0
Ethanol	0.19 ± 0.01	0.22 ± 0.01	0.26 ± 0.01
Xylitol	0.37 ± 0.02	0.28 ± 0.03	0.06 ± 0.03
Glycerol	0.06 ± 0.0	0.06 ± 0.0	0.02 ± 0.0
Acetic acid	0.03 ± 0.0	0.03 ± 0.0	0.01 ± 0.0

plate, in duplicate. The media prepared with raw hydrolysate required addition of yeast extract (as a nutrient) and a synthetic solution of sugars (to maintain the same initial sugar concentration in all the experiments), final pH adjustment to 6.0, and sterilization in 0.22 μm polyethersulfone membrane. The initial cell concentration used in these experiments was correspondent to 0.02 ± 0.01 units of OD (optical density).

The partially detoxified hydrolysate was tested statically in 50-mL tube using 10 mL of medium at 54 °C. The fermentation medium was prepared by supplementing the partially detoxified hydrolysate with nutrients, final pH adjustment, and membrane sterilization. The following nutrients were added to the medium (g/L): K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.10; $MnSO_4{\cdot}4H_2O$, 0.05; sodium acetate trihydrate, 5.0; ammonium citrate dibasic, 2.79; yeast extract, 4.0; meat extract, 8.0; and peptone, 10.0. The initial cell concentration used in these experiments was correspondent to 0.20 ± 0.01 units of OD.

For both sets of experiments with raw and partially detoxified hydrolysates, control experiments were incubated together using the same inoculum load and using complex synthetic media formulated with tryptone soy medium and MRS broth, with the same amount of sugars (arabinose, galactose, glucose, mannose, and xylose) present in the hydrolysates.

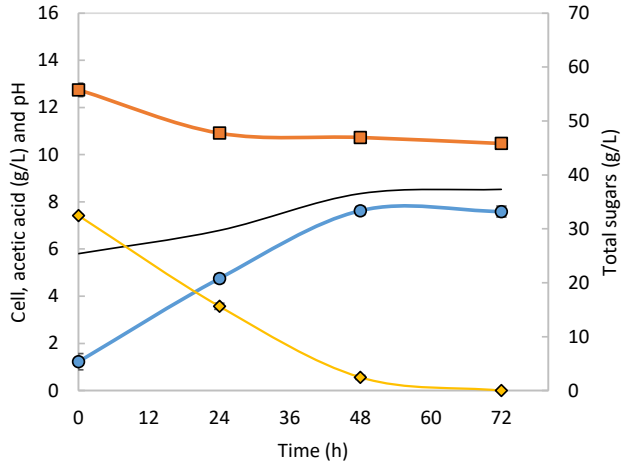
2.3.2. *Lactobacillus salivarius*

The probiotic bacteria *Lactobacillus salivarius* ATCC 11742 was used for lactic acid production at 35 °C. The strain was obtained from the culture collection of Fundação André Tosello (Campinas, Brazil). Stock cultures were kept frozen at − 80 °C with 20% glycerol in MRS broth composed of (g/L): glucose, 20; K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.10; $MnSO_4{\cdot}4H_2O$, 0.05; sodium acetate trihydrate, 5.0; ammonium citrate dibasic, 2.79; yeast extract, 4.0; meat extract, 8.0; peptone, 10.0. The stock culture was activated in 50-mL tube containing 10 mL of MRS broth, incubated statically at 35 °C for 24 h. Then, an aliquot of 200 μL was transferred to 10 mL fresh MRS broth in a 50-mL tube and incubated in the same manner as in the activation step. After 24 h, the cells were recovered by centrifugation using a Multifuge™X3 centrifuge (Thermo Scientific, USA) at 6000 rpm, 15 min, 4 °C. The cells were washed twice with saline solution (0.9% NaCl) to be inoculated in the fermentation medium.

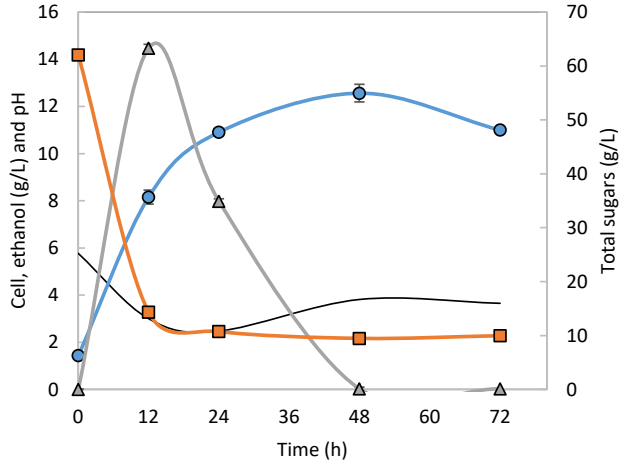
The fermentation experiments were performed using the raw, partially detoxified, and diluted partially detoxified hydrolysates. Additionally, a control experiment was performed using defined medium containing sugars combined with the same nutrients found in MRS broth (in g/L: K_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.10; $MnSO_4{\cdot}4H_2O$, 0.05; sodium acetate trihydrate, 5.0; ammonium citrate dibasic, 2.79; yeast extract, 4.0; meat extract, 8.0; peptone, 10.0). The sugar content in the control experiment corresponded to (g/L): mannose, 30.0; glucose, 18.0; galactose, 6.2; xylose, 5.5; and arabinose, 2.0. Fermentation media based on hydrolysates were prepared by adding MRS broth nutrients, adjusting the final pH to 6.0, and filter-sterilizing in 0.22 μm polyethersulfone membrane. The initial cell concentration used in these experiments was correspondent to 0.72 ± 0.09 units of OD. All experiments were performed statically at 35 °C in 12-mL tubes containing 2 mL of media, in duplicate.

2.3.3. *Rhodosporidium toruloides*

An evolved strain of the oleaginous yeast *Rhodosporidium toruloides* with improved tolerance to toxic compounds, obtained by adaptive laboratory evolution in wheat straw hydrolysate [28] was used in this study to produce lipids and carotenoids. The stock culture was activated in 250-mL baffled shake flasks with 50 mL of medium, at 25 °C, 250 rpm, for 72 h. The composition of the activation medium corresponded to (g/L): glucose, 50; $(NH_4)_2SO_4$, 1.0; and yeast nitrogen base without amino



(a)



(b)

Fig. 4. Fermentation profile of *Hansenula polymorpha* (orange square: total sugars, blue cycle: cell, yellow diamond: acetic acid, grey triangle: ethanol, black line: pH) cultivated in (a) Partially detoxified hydrolysate; b) Control medium.

acids and ammonium sulfate, 1.7 [20].

The inoculum was prepared at the same conditions used for activation by transferring 1 mL of activated culture to new fresh medium. The same medium was used for inoculum cultivation except that glucose was replaced by the following mixture of sugars (in g/L): mannose, 30.0; glucose, 18.0; galactose, 6.2; xylose, 5.5, and arabinose, 2.0. Fermentation media were prepared with the partially detoxified and detoxified hydrolysates, supplemented with 1 g/L of $(NH_4)_2SO_4$ and 1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate. Then, the pH of the media was adjusted at 4.8 and filter-sterilized. A control assay was prepared similarly as for the inoculum cultivation. The initial cell concentration used in the experiments was correspondent to 1.74 ± 0.51 units of OD. All experiments were performed in 50-mL shake flasks with working volume of 10 mL, at 25 °C, and 250 rpm.

2.3.4. *Saitoella coloradoensis*

Saitoella coloradoensis NRRL YB-2330 was another oleaginous yeast tested for the production of lipids. This strain was kindly provided by ARS Culture Collection (Peoria-IL, USA). Stock cultures were preserved at − 80 °C with 20% glycerol in YPD medium composed of (g/L): glucose, 20.0; bacteriological peptone, 20.0; and yeast extract, 10. The activation of the stock culture, inoculum preparation, and fermentation conditions were performed as described in Section 2.3.3. Fermentation media were prepared using the partially detoxified hydrolysate and detoxified hydrolysates, as well as in a control medium simulating the sugar content present in the hydrolysates without inhibitors.

2.3.5. *Kluyveromyces marxianus*

An evolved strain of the thermotolerant yeast *Kluyveromyces marxianus* obtained by adaptive laboratory evolution in our laboratory (unpublished data) was used for the production of ethanol and xylitol at 40 °C. Stock cultures were maintained at − 80 °C with 20% glycerol in YM medium composed of (g/L): xylose, 20; malt extract, 3.0; yeast extract, 3.0; and peptone, 5.0. The same medium was used for cell activation in a 250-mL baffled shake flask with 50 mL working volume, at 40 °C, 250 rpm, for 24 h. Afterwards, an aliquot of 1 mL of activated cells was transferred to the inoculum medium composed of (g/L): mannose, 10.0; glucose, 6.0; galactose, 2.07; xylose, 1.83; arabinose, 0.67; yeast extract, 3.0; $(NH_4)_2HPO_4$, 5.0; KH_2PO_4, 3.0; $MgSO_4{\cdot}7H_2O$, 0.24; and trace elements (mg/L): EDTA, 15; $ZnSO_4{\cdot}7H_2O$, 4.5; $CoCl_2{\cdot}6H_2O$, 0.3; $MnCl_2{\cdot}4H_2O$, 0.84; $CuSO_4{\cdot}5H_2O$, 0.3; $FeSO_4{\cdot}7H_2O$, 3.0; $NaMoO_4{\cdot}2H_2O$, 0.4; H_3BO_3, 1.0; and KI, 0.1.

The inoculum was incubated similarly to the activation step. After 24 h, the cells were recovered by centrifugation using a Multifuge™X3 centrifuge (Thermo Scientific, USA) at 8000 rpm, 10 min, 4 °C. The cells were washed twice with saline solution (0.9% NaCl) to be inoculated in the fermentation medium. The initial cell concentration in the fermentations was 4 g/L. The fermentation media were prepared with the partially detoxified and detoxified hydrolysates, supplemented with the same nutrients used for inoculum cultivation (except sugars), pH adjusted to 5.8. The fermentation experiments were performed in a bottom square polypropylene 24 deep well plate, in duplicate, at 40 °C, 250 rpm, for up to 24 h. Assays without nutrient supplementation to the hydrolysate, as well as a control experiment (using chemically defined medium) were also performed. The composition of the control experiment was the same used for inoculum cultivation, but with a higher amount of sugars (in g/L): mannose, 30.0; glucose, 18.0; galactose, 6.20; xylose, 5.5, and arabinose, 2.0.

2.3.6. *Hansenula polymorpha*

The methylotrophic yeast *Hansenula polymorpha* CBS 4732 was used for the production of single-cell protein as this yeast has shown some resistance to acetic acid and formic acids [27]. The stock culture, cell activation, inoculum preparation, fermentation, and control experiments were performed under the same conditions described in section *2.3.5*, but at 37 °C, using raw and partially detoxified hydrolysates as fermentation media, and with an initial cell concentration of 1.0 g/L.

2.4. *Evaluation and comparison of microbial performance*

Two criteria were adopted in this study to rank the best candidates for cultivation in Pinus hemicellulosic hydrolysate to produce value-added compounds. The first criterion was based on the performance of the strain in control medium without inhibitors. The second criterion was based on the performance of the strain when cultivated in Pinus hydrolysate. For each criterion, five questions were used to score it, as presented below.

First criterion - performance in control media without inhibitors:

I. Did the strain consume all the sugars?
II. Did the strain show good growth yield?
III. Did the strain produce the target product?
IV. Did the strain produce the target product preferably?
V. Was the culture stable / simple inoculum preparation?

Second criterion - performance in Pinus hemicellulosic hydrolysate:

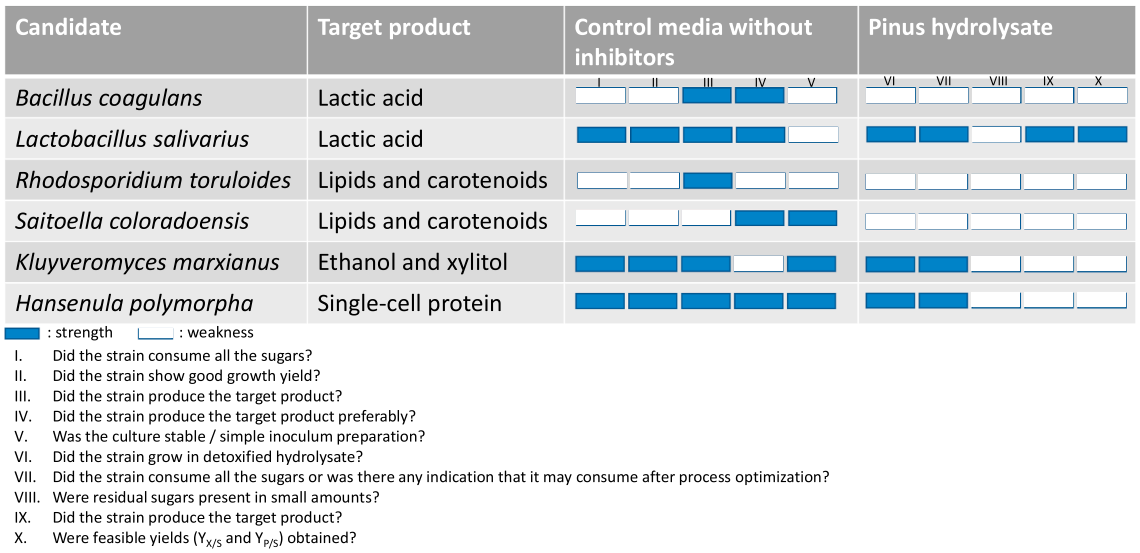


Fig. 5. Assessment chart to rank the best microorganisms for cultivation in Pinus hydrolysate to produce value-added products.

VI. Did the strain grow in detoxified hydrolysate?
VII. Did the strain consume all the sugars or was there any indication that it may consume after process optimization?
VIII. Were residual sugars present in small amounts?
IX. Did the strain produce the target product?
X. Were feasible yields ($Y_{X/S}$ and $Y_{P/S}$) obtained?

2.5. Analytical methods

Cell growth was monitored during the course of the experiments by optical density (OD) at 660 nm (Synergy spectrophotometer, Biotek) using a polystyrene plate (96-microWell, Nunc) with appropriate dilution in deionized water. Blanks were prepared at the same dilution with centrifuged samples (supernatant only). Cell morphology was observed using a LEICA DM 4000B microscope with a camera LEICA DFC300 FX.

Quantification of organic acids, carboxylic acids, 5-hydroxymethylfurfural (5-HMF) and furfural was carried out by 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, using a Shodex RI-101 refractive index detector and a UV–Vis at 254 nm for 5-HMF and furfural, 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min, and sample injection of 20 μL. Quantification of carbohydrates was analyzed in the same system, but using an Aminex HPX-87P column (Bio-Rad) at 55 °C, a Shodex RI-101 refractive index detector, deionized water as mobile phase in a flow rate of 0.5 mL/min, and 10 μL of injection.

The content of oligomers in the hydrolysates was estimated 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 [29].

The substrate to cell conversion yield ($Y_{X/S}$) was calculated as the ratio between cell produced and substrate (total carbohydrates) consumed. The maximum specific growth rate (μ_{max}) was determined as the slope of linear region on an ln (X/X_0) versus time plot, where X was the cell concentration per volume and X_0 was the cell concentration at the initial time. Substrate to product conversion yield ($Y_{P/S}$) was calculated as the ratio between product formed and substrate (total carbohydrates) consumed.

3. Results and discussion

3.1. Hemicellulosic hydrolysate

The characterization of *Pinus taeda* hemicellulosic hydrolysate is shown in Table 2. The total monomeric sugars in raw hydrolysate corresponded to approximately 27 g/L distributed into hexoses and pentoses. Additionally, significant amount of acetic acid (5.41 g/L), and phenolic compounds (5.26 g/L) were also present in the hydrolysate, which can probably harm the microbial growth. The presence of cellobiose, as well as some unidentified peaks in the HPLC chromatogram suggested the presence of oligomers in raw hydrolysate, which was confirmed after post-hydrolysis. The content of oligomers in raw hydrolysate was estimated in 33.29 ± 6.70 g/L, while monomers corresponded to 27.11 ± 2.42 g/L.

Acid post-hydrolysis released a significant amount of monomeric sugars from oligomers, increasing the content of monomeric sugars to 62 g/L. However, this process also increased the amount of acetic and formic acids in the medium, besides promoting the formation of levulinic acid. Formic acid and levulinic acid are formed by the degradation of hexoses in a non-stoichiometric reaction [30]. In addition, a black precipitate (humin) was also formed during the acid post-hydrolysis, which corresponded to 0.51% (w/v). Humins can be formed as a by-product of glucose dehydration, can be derived from the conversion of HMF [31], or can be originated from xylose [32], which would explain the decrease of xylose and 5-HMF concentrations after post-hydrolysis.

A volume loss of 2% occurred after acid post-hydrolysis process due to the formation of precipitates, as previously explained, and also because the filtration to remove the precipitate also led to the retention of some liquid in the membrane. A mass balance and comparison between the raw and after post-hydrolysis hydrolysates revealed mass loss of cellobiose, xylose, and 5-HMF. Cellobiose was completely hydrolyzed into glucose, xylose was possibly converted into furfural and humin, 5-HMF was converted into humin, and probably acetic acid was oxidized by SO_4^- [33]. These results indicate that the conditions to be used for acid post-hydrolysis should be optimized in order to avoid loss of sugars. Reducing the temperature, the reaction time or changing the amount of mineral acid [34] could help solving this problem. By optimizing the post-hydrolysis conditions, the total amount of hexoses in the present study could be increased by more than 30% if degradation reactions are avoided.

Concerning the distribution of sugars, mannose was the most abundant hexose (29.82 ± 1.97 g/L) followed by glucose (16.93 ± 0.20 g/L)

and galactose (7.90 ± 1.21 g/L). Pentoses accounted for 7.45 ± 0.90 g/L only. This result is in agreement with the type of wood used in this study. Hemicellulose in softwoods like Pinus is mainly composed by galactoglucomannan that consists of a linear β-1,4-linked D-glucopyranose and D-mannopyranose backbone with α-1,6-linked D-galactopyranose residues as single side chain substituents [34].

3.2. Detoxification process

The contents of organic acids and phenolic compounds in Pinus post-hydrolysate can significantly impair microbial growth [9]. The selective detoxification process applied in this study removed preferentially phenolic compounds by solid-phase extraction and organic acids by liquid–liquid extraction (Table 2). Solid-phase extraction removed 91% of the initial phenolic compounds, but with 14% loss of volume (compared to its initial value); whereas liquid–liquid extraction removed 70% of the initial organic acids with 13% loss of volume (compared to its initial value). Nevertheless, the two steps of detoxification process did not remove all inhibitory compounds present in the hydrolysate. Detoxified hydrolysate still contained 3.47 g/L of acetic acid, 1.15 g/L of formic acid, and 270 mg/L of total phenolic compounds.

3.3. Hydrolysate fermentation with Bacillus coagulans

Only a few conditions, 5 out of the 17 assays of the experimental design using raw hydrolysate, led to the production of lactic acid by *B. coagulans* after 12 h or 24 h of cultivation (Table 3). The most notable production of lactic acid (5.32 g/L with yield of 0.19 g/g) occurred at the condition of 54 °C, using 28% of hydrolysate, and 24 g/L of yeast extract supplementation. Such condition minimized the effect of inhibitors and provided essentials nutrients for the bacteria growth. At the same conditions, but decreasing the temperature to 36 °C, lower production of lactic acid was observed, especially after 12 h of fermentation. Overall, these results demonstrated that high temperature, dilution of the hydrolysate, and high supplementation with yeast extract favored the production of lactic acid, but still, the results were far when compared to the theoretical conversion yield of 1.0 g/g. It is worth highlighting that the experiments with non-diluted partially detoxified hydrolysate, which were performed with an even higher initial cell concentration (0.20 ± 0.01 units of OD) than the experiments with raw hydrolysate (0.02 ± 0.01 units of OD), did not succeed too, revealing that the removal of the majority of phenolic compounds was insufficient to promote an efficient microbial growth. Indeed, carboxylic acids and 5-HMF, which were still present in high amount in partially detoxified hydrolysate (Table 2), have been reported as being highly toxic for *B. coagulans* [35].

3.4. Hydrolysate fermentation with Lactobacillus salivarius

Production of lactic acid by *L. salivarius* was tested in different media including raw, partially detoxified, and diluted partially detoxified hydrolysate. Raw hydrolysate presented the highest content of furan derivatives, while the partially detoxified hydrolysate contained reduced amount of furan derivatives (furfural and 5-HMF) as show in Table 2. Diluted partially detoxified hydrolysate contained the lowest amount of inhibitors (acetic acid, 4.22 g/L; formic acid, 1.97 g/L; levulinic acid, 1.45 g/L; and 5-HMF, 0.13 g/L) and 22.32 g/L of total sugars. As can be seen in Fig. 1**a**, the exponential growth in diluted partially detoxified hydrolysate followed the same tendency of the control medium, while a lag phase of 6 h was observed from partially detoxified hydrolysate, and no significant growth occurred in raw hydrolysate.

Lactic acid production was associated to the microbial growth in which the course of the production followed the growth profile (Fig. 1**b**). It was interesting noting that the final product titer obtained in partially detoxified hydrolysate, reached almost the same value of the control experiment, a synthetic media without inhibitory compounds. Although an initial lag phase was observed in partially detoxified hydrolysate, after entering in the exponential phase, the production of lactic acid occurred efficiently, reaching a high value comparable to that observed in medium without inhibitors. Similar titer was not obtained from diluted partially detoxified hydrolysate due to the lower amount of sugars present in the medium. The sugar concentration profile exhibited in Fig. 1**c** shows the amount of sugars used for lactic acid production. Among the sugars, glucose was preferentially consumed in all the media - control, partially detoxified hydrolysate, and diluted partially detoxified hydrolysate (data not shown). After glucose exhaustion, xylose and galactose were consumed concomitantly. Mannose consumption started from 12 h in the diluted detoxified hydrolysate, which coincided with glucose, xylose, and galactose exhaustion. In the raw hydrolysate, glucose, mannose, xylose, and galactose were slowly consumed at the same time. Arabinose was not consumed in any condition. It is worth highlighting that sugars were consumed until lactic acid reached a titer of approx. 12–16 g/L, which probably caused inhibition of the microbial metabolism. Other authors have also reported inhibition of *L. salivarius* by lactic acid concentrations higher than 10 g/L, which could be related to the availability of amino acids [36].

To better compare the performance of the different systems, the fermentation parameters were calculated. Table 4 summarizes the parameters calculated for 36 h of fermentation. The cell yield ($Y_{X/S}$) and the specific growth rate (μ) were inversely proportional to the amount of inhibitors present in the hydrolysate, i.e., the lowest cell yield and μ were due to the presence of high level of inhibitors in the medium. Less pronounced effect of the inhibitors was observed for lactic acid yield ($Y_{P/S}$) in the experiments using partially detoxified hydrolysate, diluted or not (near to 1.0 g/g). The lactic acid yield was calculated taking into account the sum of all monomeric sugars, hexoses and pentoses.

3.5. Hydrolysate fermentation with Rhodosporidium toruloides and Saitoella coloradoensis

The oleaginous yeast *R. toruloides* demonstrated high sensitivity to the inhibitors present in partially detoxified and detoxified hydrolysates. The yeast morphology inhibition pattern was noticeable in hydrolysate media revealing the presence of pronounced cell flocculation in contrast to the distributed cells observed in the control experiment without inhibitors (Fig. 2). This yeast did not show promising results when cultivated in Pinus hemicellulosic hydrolysates, even in the detoxified form, suggesting that a more pronounced removal of inhibitors is necessary to improve the strain's performance to grow and produce lipids and carotenoids. Other authors have also observed a high sensitivity of *R. toruloides* to the inhibitors present in lignocellulosic biomass hydrolysates [23,37]. To overcome this issue, adaptive laboratory evolution of the yeast in hydrolysate medium containing inhibitors has been proposed as a promising alternative to improve the strain's performance from biomass hydrolysates [28].

The other oleaginous yeast, *S. coloradoensis,* showed similar performance as *R. toruloides* with absence of growth in hydrolysates and non-viable cells after 48 h of cultivation. However, this yeast exhibited an interesting sugars metabolism pattern in the control experiment with concomitant consumption of glucose, mannose, and xylose, which is a desirable characteristic for bioconversion of hydrolysates. In summary, the results obtained with both strains reinforce the idea that a more significant removal of toxic compounds is needed for an efficient utilization of Pinus hemicellulosic hydrolysate by oleaginous yeasts.

3.6. Hydrolysate fermentation with Kluyveromyces marxianus

The thermotolerant yeast *K. marxianus* is a versatile non-conventional yeast able to consume both, hexose and pentose sugars. When cultivated in partially detoxified and detoxified hydrolysates, ethanol and xylitol were produced together with cells, acetic acid and

glycerol (Fig. 3**a,c**). Ethanol is formed by the conversion of glucose with a simultaneous side reaction producing glycerol and acetic acid depending on the fermentation conditions. Acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase; while glycerol is formed due to the oxygen imbalance [38]. In fact, aeration plays a key role in the sugars metabolism by this yeast. In addition, inhibitors may cause stress that lead to the expression of secondary metabolites such as aroma compounds [39].

The fermentation profile in both hydrolysates was similar with practically absence of cell growth and the same preference of sugars consumption (Fig. 3**b,d**). Glucose was the preferred carbon source followed by mannose. Xylose and galactose were consumed at a low rate. The sugars consumption rates in partially detoxified hydrolysate within 6 h corresponded to (g/h): glucose 1.74, mannose 0.22, galactose 0.2, and xylose 0.1. The values in detoxified hydrolysate were slightly higher for glucose (1.69), mannose (1.10), and xylose (0.24). In the control medium, glucose was completely depleted within 6 h and the consumption rate for other sugars corresponded to 3.83 for mannose, 0.96 for galactose, 0.48 for xylose, and 0.01 for arabinose. Interestingly, mannose was not completely consumed in hydrolysates neither in the control medium (Fig. 3**f**), while arabinose consumption only started after xylose depletion. In addition, acetic acid and glycerol production was very low in medium without inhibitors (Fig. 3**e**).

Table 5 summarizes the fermentation yields obtained for 12 h of cultivation. As can be seen, except for xylitol, the product yields were similar for both hydrolysates, while the xylitol yield was higher (0.37 g/g) from partially detoxified hydrolysate. The control experiment gave the highest ethanol yield (0.26 g/g), but the lowest yields for the other fermentation products.

3.7. Hydrolysate fermentation with Hansenula polymorpha

H. polymorpha is another interesting non-conventional yeast for cultivation in lignocellulosic hydrolysates due to its ability to consume different types of sugar. However, when cultivated in raw Pinus hydrolysate, no growth was observed, probably due to the toxicity of the medium. On the other hand, cultivation of the yeast in partially detoxified hydrolysate resulted in significant growth until 48 h of cultivation (Fig. 4**a**), which occurred concomitantly with the consumption of acetic acid. In fact, a significant consumption of acetic acid was observed by this yeast, faster than the consumption of sugars, which resulted in a pH increase from 5.8 up to 8.35 in 48 h.

The performance of the yeast in control medium was better than in hydrolysate with faster growth, higher single-cell accumulation and consumption of all sugars, except arabinose (Fig. 4**b**). The pH profile was different in this case since the medium did not contain acetic acid in the composition, and a significant formation of ethanol was observed.

To better compare the performance of the yeast in the different media, the cell yield ($Y_{X/S}$) was calculated. In hydrolysate medium, the cell yield calculated taking into account the total sugars consumed resulted in a high value of 0.41 $\pm$ 0.05 g/g, but probably the yeast metabolized acetic acid into cells too. So, when including the acetic acid consumed in the calculation, the cell yield corresponded to 0.27 g/g. For the control experiment, a lower cell yield (0.12 g/g) was observed, but ethanol was also produced with a yield of 0.27 g/g. These results allow concluding that *H. polymorpha* can be used for single-cell protein production from Pinus hemicellulosic hydrolysate being also able to metabolize acetic acid for such purpose. However, other inhibitory compounds present in the hydrolysate negatively affect the yeast performance to grow. An alternative to solve this issue could be by applying a co-cultivation strategy for simultaneous product formation and hydrolysate detoxification [40], where *H. polymorpha* would be used for single-cell protein production and another microorganism would consume the inhibitory compounds present in the hydrolysate, promoting an *in-situ* detoxification. At the end, both cell mass could be recovered and used as single-cell protein.

3.8. Evaluation of microorganism performance

Fig. 5 summarizes the scores obtained for each criterion used to compare the different microorganisms cultivated in Pinus hemicellulosic hydrolysate. In this figure, colored bars mean a strength response while blank bars mean weakness. The best candidates scored in this study for cultivation in Pinus hydrolysate were *L. salivarius*, *K. marxianus*, and *H. polymorpha,* among of which, cultivation of *L. salivarius* for the production of lactic acid was considered the most promising alternative for valorization of this hydrolysate. In the case of *K. marxianus*, metabolic engineering for expression of xylitol from hexoses, and also to silence the gene of xylitol uptake could be useful strategies to maximize the xylitol production. However, if ethanol is the target product, process optimization can be applied to maximize the ethanol formation. Finally, the production of single-cell protein by *H. polymorpha* could be improved by defining the optimal growth conditions (pH, aeration, nutrients, and temperature). In addition, the application of a co-cultivation strategy could help to alleviate the toxicity of the inhibitory compounds allowing to obtain even more cell mass at the end of the cultivation.

4. Conclusion

Pinus taeda hemicellulosic hydrolysate obtained by steam explosion can be used as fermentation medium for the production of value-added compounds contributing to advance the wood biomass biorefineries. However, post-hydrolysis and detoxification processes are necessary to maximize the sugars yield and turn the hydrolysate suitable for microbial growth. By-products generated from both process steps could also be recovered as additional valuable compounds for incorporation in a biorefinery, such as humin, which is formed as a precipitate and has application as fertilizer. The best microbial candidates for cultivation in *Pinus taeda* hemicellulosic hydrolysate were the bacterium *Lactobacillus salivarius* for the production of lactic acid, followed by the yeasts *Kluyveromyces marxianus* for the production of ethanol and xylitol, and *Hansenula polymorpha* for the production of single-cell protein. Since all these products are high-value compounds, their incorporation in an ethanol biorefinery can contribute to minimize the generation of wastes and improve the revenues of the overall biomass processing.

CRediT authorship contribution statement

Celina K. Yamakawa: Methodology, Formal analysis, Data curation, Writing – original draft. **Ilaria D'Imperio:** Methodology. **Fernando Bonfiglio:** Methodology, Funding acquisition. **Solange I. Mussatto:** Conceptualization, Supervision, Resources, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Novo Nordisk Foundation (NNF), Denmark (grant number NNF20SA0066233), and Agencia Nacional de Investigación e Innovación (ANII), Uruguay (grant number FSE_1_2017_1_144465).

References

[1] Rachid-Casnati C, Resquin F, Carrasco-Letelier L. Availability and environmental performance of wood for a second-generation biorefinery. Forests 2021;12:1609. https://doi.org/10.3390/f12111609.

[2] Xavier L, Barrenengoa M, Dieste A, Amilivia A, Palombo V, Sabag M, et al. Valorization of Pinus taeda bark: source of phenolic compounds, tannins and fuel. Eur J Wood Prod 2021;79(5):1067–85. https://doi.org/10.1007/s00107-021-01703-4.

[3] Morales Olmos V. "Análisis de la cadena de valor forestal", serie Estudios y Perspectivas-Oficina de la CEPAL en Montevideo, N° 52 (LC/TS.2021/113-LC/MVD/TS.2021/2), Santiago, Comisión Económica para América Latina y el Caribe (CEPAL), 2021.

[4] Dieste A, Clavijo L, Torres AI, Barbe S, Oyarbide I, Bruno L, et al. Lignin from *Eucalyptus* spp. kraft black liquor as biofuel. Energy Fuels 2016;30(12):10494–8. https://doi.org/10.1021/acs.energyfuels.6b02086.

[5] Mendes CVT, Moreira R, Portugal A, Carvalho MGVS. Biorefining of *Pinus pinaster* stump wood for ethanol production and lignin recovery. Chem Eng Technol 2021;44(6):1043–50. https://doi.org/10.1002/ceat.v44.610.1002/ceat.202000447.

[6] López M, Vila C, Santos V, Parajó JC. Manufacture of platform chemicals from pine wood polysaccharides in media containing acidic ionic liquids. Polymers 2020;12:1215. https://doi.org/10.3390/polym12061215.

[7] Safari A, Karimi K, Shafiei M. Dilute alkali pretreatment of softwood pine: A biorefinery approach. Bioresour Technol 2017;234:67–76. https://doi.org/10.1016/j.biortech.2017.03.030.

[8] Yamakawa CK, Qin F, Mussatto SI. Advances and opportunities in biomass conversion technologies and biorefineries for the development of bio-based economy. Biomass Bioenerg 2018;119:54–60. https://doi.org/10.1016/j.biombioe.2018.09.007.

[9] Mussatto SI, Roberto IC. Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: A review. Bioresour Technol 2004;93:1–10. https://doi.org/10.1016/j.biortech.2003.10.005.

[10] Dragone G, Kerssemakers AAJ, Driessen JLSP, Yamakawa CK, Brumano LP, Mussatto SI. Innovation and strategic orientations for the development of advanced biorefineries. Bioresour Technol 2020;302:122847. https://doi.org/10.1016/j.biortech.2020.122847.

[11] Lopes ES, Leal Silva JF, Rivera EC, Gomes AP, Lopes MS, Maciel Filho R, et al. Challenges to levulinic acid and humins valuation in the sugarcane bagasse biorefinery concept. Bioenergy Res 2020;13(3):757–74. https://doi.org/10.1007/s12155-020-10124-9.

[12] LealSilva JF, Grekin R, Mariano AP, MacielFilho R. Making levulinic acid and ethyl levulinate economically viable: a worldwide technoeconomic and environmental assessment of possible routes. Energy Technol 2018;6(4):613–39. https://doi.org/10.1002/ente.v6.410.1002/ente.201700594.

[13] Hu X, Shi Y, Zhang P, Miao M, Zhang T, Jiang B. d-Mannose: properties, production, and applications: an overview. Compr Rev Food Sci Food Saf 2016;15:773–85. https://doi.org/10.1111/1541-4337.12211.

[14] Taskila S, Ojamo H. The current status and future expectations in industrial production of lactic acid by lactic acid bacteria. In: Kongo M, editor. Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes. IntechOpen, Rijeka; 2013. https://doi.org/10.5772/51282.

[15] Alves de Oliveira R, Komesu A, Vaz Rossell CE, Maciel FR. Challenges and opportunities in lactic acid bioprocess design—From economic to production aspects. Biochem Eng J 2018;133:219–39. https://doi.org/10.1016/j.bej.2018.03.003.

[16] Mussatto SI. In: D-Xylitol. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 309–23. https://doi.org/10.1007/978-3-642-31887-0_14.

[17] Queiroz SS, Jofre FM, Mussatto SI, Felipe MDGA. Scaling up xylitol bioproduction: Challenges to achieve a profitable bioprocess. Renew Sustain Energy Rev 2022;154:111789. https://doi.org/10.1016/j.rser.2021.111789.

[18] Dagle RA, Winkelman AD, Ramasamy KK, Lebarbier Dagle V, Weber RS. Ethanol as a renewable building block for fuels and chemicals. Ind Eng Chem Res 2020;59(11):4843–53. https://doi.org/10.1021/acs.iecr.9b05729.

[19] Shields-Menard SA, Amirsadeghi M, French WT, Boopathy R. A review on microbial lipids as a potential biofuel. Bioresour Technol 2018;259:451–60. https://doi.org/10.1016/j.biortech.2018.03.080.

[20] Liu Z, Feist AM, Dragone G, Mussatto SI. Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. J Clean Prod 2020;249:119308. https://doi.org/10.1016/j.jclepro.2019.119308.

[21] Ritala A, Häkkinen ST, Toivari M, Wiebe MG. Single cell protein-state-of-the-art, industrial landscape and patents 2001–2016. Front Microbiol 2017;8:2009. https://doi.org/10.3389/fmicb.2017.02009.

[22] Bratosin BC, Darjan S, Vodnar DC. Single Cell Protein: A potential substitute in human and animal nutrition. Sustainability 2021;13:9284. https://doi.org/10.3390/su13169284.

[23] Bonfiglio F, Cagno M, Yamakawa CK, Mussatto SI. Production of xylitol and carotenoids from switchgrass and *Eucalyptus globulus* hydrolysates obtained by intensified steam explosion pretreatment. Ind Crops Prod 2021;170:113800. https://doi.org/10.1016/j.indcrop.2021.113800.

[24] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of sugars, byproducts, and degradation products in liquid fraction. Technical report, NREL/TP-510-42623, 2008. Available at https://www.nrel.gov/docs/gen/fy08/42623.pdf (accessed November 10, 2021).

[25] McDonald PD. A sample preparation primer and guide to solid phase extraction methods development. Waters 2001:1–83. Available at https://www.waters.com/webassets/cms/library/docs/wa20300.pdf 9accessed November 10, 2021).

[26] Cebreiros F, Guigou MD, Cabrera MN. Integrated forest biorefineries: Recovery of acetic acid as a by-product from eucalyptus wood hemicellulosic hydrolysates by solvent extraction. Ind Crops Prod 2017;109:101–8. https://doi.org/10.1016/j.indcrop.2017.08.012.

[27] Yamakawa CK, Kastell L, Mahler MR, Martinez JL, Mussatto SI. Exploiting new biorefinery models using non-conventional yeasts and their implications for sustainability. Bioresour Technol 2020;309:123374. https://doi.org/10.1016/j.biortech.2020.123374.

[28] Liu Z, Radi M, Mohamed ETT, Feist AM, Dragone G, Mussatto SI. Adaptive laboratory evolution of *Rhodosporidium toruloides* to inhibitors derived from lignocellulosic biomass and genetic variations behind evolution. Bioresour Technol 2021;333:125171. https://doi.org/10.1016/j.biortech.2021.125171.

[29] Ballesteros LF, Teixeira JA, Mussatto SI. Selection of the solvent and extraction conditions for maximum recovery of antioxidant phenolic compounds from coffee silverskin. Food Bioprocess Technol 2014;7(5):1322–32. https://doi.org/10.1007/s11947-013-1115-7.

[30] Flannelly T, Lopes M, Kupiainen L, Dooley S, Leahy JJ. Non-stoichiometric formation of formic and levulinic acids from the hydrolysis of biomass derived hexose carbohydrates. RSC Adv 2016;6(7):5797–804. https://doi.org/10.1039/C5RA25172A.

[31] Hetzel J, Patil SKR, Lund CRF. Humin formation pathways. In: Schlaf M, Zhang Z, editors. Reaction Pathways and Mechanisms in Thermocatalytic Biomass Conversion II. Green Chemistry and Sustainable Technology. Singapore: Springer; 2016. p. 171–202. https://doi.org/10.1007/978-981-287-769-7_5.

[32] Wang S, Lin H, Zhao Y, Chen J, Zhou J. Structural characterization and pyrolysis behavior of humin by-products from the acid-catalyzed conversion of C6 and C5 carbohydrates. J Anal Appl Pyrol 2016;118:259–66. https://doi.org/10.1016/j.jaap.2016.02.009.

[33] Criquet J, Leitner NKV. Degradation of acetic acid with sulfate radical generated by persulfate ions photolysis. Chemosphere 2009;77(2):194–200. https://doi.org/10.1016/j.chemosphere.2009.07.040.

[34] Marzialetti T, Valenzuela Olarte MB, Sievers C, Hoskins TJC, Agrawal PK, Jones CW. Dilute acid hydrolysis of loblolly pine: A comprehensive approach. Ind Eng Chem Res 2008;47(19):7131–40. https://doi.org/10.1021/ie800455f.

[35] Ali Abdel-Rahman M, El-Din Hassan S, Fouda A, Radwan AA, Barghoth MG, Desouky SG. Evaluating the effect of lignocellulose-derived microbial inhibitors on the growth and lactic acid production by *Bacillus coagulans* Azu-10 2021. Fermentation 2021;7:17. https://doi.org/10.3390/fermentation7010017.

[36] Vasala A, Panula J, Neubauer P. Efficient lactic acid production from high salt containing dairy by-products by *Lactobacillus salivarius* ssp. *salicinius* with pre-treatment by proteolytic microorganisms. J Biotechnol 2005;117(4):421–31. https://doi.org/10.1016/j.jbiotec.2005.02.010.

[37] Liu Z, Fels M, Dragone G, Mussatto SI. Effects of inhibitory compounds derived from lignocellulosic biomass on the growth of the wild-type and evolved oleaginous yeast *Rhodosporidium toruloides*. Ind Crops Prod 2021;170:113799. https://doi.org/10.1016/j.indcrop.2021.113799.

[38] Rapin J-D, Marison IW, von Stockar U, Reilly PJ. Glycerol production by yeast fermentation of whey permeate. Enzyme Microb Technol 1994;16(2):143–50. https://doi.org/10.1016/0141-0229(94)90077-9.

[39] Yamakawa CK, Mussatto SI. Bioconversion of wheat straw into value-added products by evolved *Kluyveromyces marxianus* strain. Book of abstracts summaries - EUBCE 2018 - 26th European Biomass Conference and Exhibition. Copenhagen, Denmark, 2018, p. 178.

[40] Mussatto SI, Yamakawa CK, van der Maas L, Dragone G. New trends in bioprocesses for lignocellulosic biomass and CO2 utilization. Renew Sustain Energy Rev 2021;152:111620. https://doi.org/10.1016/j.rser.2021.111620.