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ORIGINAL ARTICLE

Screening of Yeasts for Selection of Potential Strains and Their Utilization for In Situ Microbial Detoxification (ISMD) of Sugarcane Bagasse Hemicellulosic Hydrolysate

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Abstract Many toxic compounds are produced and released in the hemicellulosic hydrolyzates during the acid pretreatment step, which are required for the disruption of the lignocelluloses matrix and sugars release. The conventional methods of detoxification i.e. overliming, activated charcoal, ion exchange or even membrane-based separations have the limitations in removal of these toxic inhibitors in fermentation process. Hence, it is imperative to explore biological methods to overcome the inhibitors by minimizing the filtration steps, sugar loss and chemical additions. In the present study we screened sixty-four strains of yeasts to select potential strains for detoxification of furfural, acetic acid, ferulic acid, 5-hydroxymethyl furfural (5-HMF) as carbon and energy source. Among these strains *Pichia occidentalis* M1, Y1′a, Y1′b and Y3′ showed a significant decrease in the toxic compounds but we selected two best yeast strains i.e. *P. occidentalis* Y1′a and *P. occidentalis* M1 for the further experiments with an aim to remove the fermentation inhibitors. The yeasts *P. occidentalis* Y1′a and *P. occidentalis* M1 were grown aerobically in sugarcane bagasse hemicellulose hydrolysate under submerged cultivation. For each yeast, a 2^2 full factorial design was performed considering the variables—pH (4.0 or 5.0) and agitation rate (100 or 300 rpm), and the percentage removal of HMF, furfural, acetic acid and phenols from hemicellulosic hydrolysates were responsive variables. After 96 h of biological treatment, *P. occidentalis* M1 and *P. occidentalis* Y1′a showed 42.89 and 46.04 % cumulative removal of inhibitors, respectively.

Keywords Microbial detoxification · Sugarcane bagasse · Dilute acid hydrolysis · Inhibitors

Introduction

Lignocellulosic materials are mainly composed of cellulose, hemicellulose and lignin [1, 2]. The carbohydrate fraction of lignocellulosic materials, if harnessed judiciously, can serve as an excellent building block for the production of renewable ethanol and other commodity chemicals via microbial fermentation. Production of fuel ethanol from lignocellulosic biomass so called second-generation (2G) ethanol is an important necessity due to the heavy usage of gasoline worldwide and environmental pollution. The 2G ethanol is completely renewable and is able to reduce atmospheric pollution due to the lower release of carbon dioxide, while offering several geo-political benefits [3]. Sugarcane bagasse (SB), a fibrous product after extraction of juice is an excellent carbohydrate source (~67 %) for 2G ethanol production [3].

Conventional process of ethanol production from lignocellulose biomass comprises of four major steps: pretreatment (responsible for the disruption of lignocellulosic matrix), enzymatic hydrolysis (leading to depolymerization of cellulose into glucose by the action of cellulolytic

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enzymes), the fermentation (which is the conversion of sugars to ethanol, generally carried out by yeast); distillation, rectification and dehydration steps (for separation and purification of the final product) [4]. The pretreatment of SB is an inevitable step to increase the accessibility of carbohydrate fraction towards enzymatic action [3]. Dilute acid hydrolysis of lignocellulosics is a fast and effective process but, it has bottlenecks, such as, by-product formation and non-selectivity [5]. Dilute sulfuric acid pretreatment precisely acts on hemicellulose releasing hemicellulosic monomers and releasing some undesired compounds like furans (5-hydroxymethylfurfural and hydroxymethylfurfural), phenolics, weak acids, and others. The dilute acid pretreatment is conducted at high temperature and pressure, and the reaction time takes for seconds or minutes [3]. Sulfuric acid (H_2SO_4) is mostly used catalyst, but other weak acids, such as: hydrochloric acid (HCl) and nitric acid (HNO_3) have also been used for the hemicellulose removal from variety of lignocellulosic materials [6].

Inhibitory compounds generated in lignocellulosic bioethanol production are main hurdle faced during the hydrolysis process. One of the main challenges associated with 2G ethanol production is to overcome the cumulative negative effect of cell wall derived inhibitors on the fermenting microorganisms during fermentation reactions [7–11]. Inhibitory compounds like acids, furans and phenolic compounds interferes the fermenting microorganisms and complete process by reducing ethanol yield and productivity during fermentation. These inhibitors negatively affect the performance of microorganisms in the fermentation process [5]. Concentration of these inhibitors produced depends on process conditions and raw materials used for hydrolysis. Furans and phenol monomers produced during hydrolysis of lignin are also major cofactors which inhibit or slow down the hydrolysis process. Generally, furans and phenolic compounds constrain growth and rate of ethanol production. Therefore, it is essential, to remove these inhibitors prior to fermentation in order to obtain the desired ethanol or any other metabolite. Detoxification process can be categorized into physical, chemical and biological methods. Biological methods certainly have unique advantages. Further, to consolidate the detoxification and ethanol or xylitol fermentation so called simultaneous detoxification and fermentation (SDF) is also possible by the appropriate use of microorganisms in a single vessel [11].

This study was aimed to develop a biological detoxification method for sugarcane bagasse hemicellulosic hydrolysates. For this, the yeasts *Pichia occidentalis* Y1′a and *P. occidentalis* M1 [12] were grown in concentrated sugarcane bagasse hemicellulosic hydrolysate. For both the strains of yeast, a 2^2 full factorial design was carried out. The variables, pH (4.0 or 5.0) and agitation rate (100 rpm or 300 rpm), were evaluated and the percentage removal of HMF, furfural, acetic acid and phenols after 96-h of biological treatment were responsive variables.

Materials and Methods

Yeast Screening

Sixty-four strains of yeasts were screened to search for potential strains for detoxification of furfural, acetic acid, ferulic acid, HMF as carbon and energy source. Of these, only two strains of *Picchia occidentalis* were selected for detoxification (ISMD) of sugarcane bagasse hemicellulosic hydrolysate.

The growth medium for yeasts was composed of 6.7 g/l of yeast nitrogen base (YNB), 18.0 g/l agar and with different concentrations of individual (furfural, 0.2820 g/l; 5-HMF, 0.0149 g/l; total phenolics, 3.13 g/l) and combined toxic compounds (furfural, 0.2820 g/l + 5-HMF, 0.0149 g/l + total phenolics, 3.13 g/l). The medium containing the toxic compounds (hydroxymethyl furfural, furfural, acetic acid, ferulic acid and syringaldehyde) was prepared for the screening of the yeasts.

Preparation of Sugarcane Bagasse Hemicellulosic Hydrolysates

The sugarcane bagasse was hydrolyzed in vertical rotary drum reactor of 50 L capacity at 121 °C for 10 min with H_2SO_4 (98 %) at 1:10 solid/liquid ratio (100 mg of H_2SO_4 g^{-1} dry sugarcane bagasse). The hydrolyzed hemicellulose was recovered after filtration and subsequently concentrated at 70 °C under vacuum to obtain a fivefold increase in the xylose content.

The pH of the hemicellulosic hydrolysate was adjusted with 2 M NaOH according to the values of design experiment. It was filtered and subsequently autoclaved at 110 °C for 15 min. Then, hydrolysate was centrifuged under aseptic conditions at $2600 \times g$ for 20 min to remove the suspended solids, in order to use the hydrolysates for biological detoxification experiments.

Inoculum Preparation

Pichia occidentalis Y1′a (Genbank accession number: KP033405) and *P. occidentalis* M1 (Genbank accession number: KP033404) were obtained from stock strains of Social Insects Study Center, Rio Claro Biosciences Institute, São Paulo State University (UNESP—Universidade Estadual Paulista, Rio Claro, São Paulo State, Brazil). Both the strains were identified according to the standard

methods described by Melo et al. [8]. All the strains were maintained at 5 °C in 2 % Sabouraud Agar Medium. The medium used for inoculum preparation contained 10 g/l of yeast extract, 20 g/l of peptone and 20 g/l of glucose. For inoculum preparation, loopful of cultures were transferred to 250 ml Erlenmeyer flasks containing 100 ml of YPX medium (10.0 g/l yeast extract, 20.0 g/l peptone, 30.0 g/l xylose, pH 6.0). The flasks were incubated at 30 °C, 200 rpm for 24 h. After 24 h of incubation, the cells were recovered by centrifugation (2000×*g*, 20 min) at room temperature, washed, centrifuged again and suspended in sterile distilled water to obtain an initial concentration of 0.5 g/l. Erlenmeyer flasks (500 ml) each containing 200 ml of medium closed with cotton plugs were incubated in rotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 200 rpm for 24 h at 30 °C.

Medium and Detoxification Conditions

The concentrated sugarcane bagasse hemicellulosic hydrolysate was supplemented with 6.7 g/l of YNB (Sigma-Aldrich, USA). Fifty milliliter of medium was taken in Erlenmeyer flasks (125 ml capacity), and inoculated with 0.5 g/l of cells. The biological detoxification was conducted at different agitation at 30 °C for 96 h on rotary shaker (New Brunswick Scientific—Edison, NJ, USA). Assays were performed in triplicates. Aliquots of 50 ml samples were taken at times 0, 24, 48, 72 and 96 h for the quantification of D-glucose, D-xylose, D-arabinose, HMF, furfural, phenols and acetic acid, and pH and cell growth determination.

Analytical Methods

The concentrations of glucose, xylose, arabinose and acetic acid were determined by HPLC with a refraction index detector (Waters 410; Milford, MA, USA). The samples were diluted in a ratio of 1:10 and filtered through a Sep Pak C18 filter. Subsequently, samples were injected into the chromatograph, using the following conditions: column BIO-RAD Aminex HPX-87H (7.8 × 300 mm) (Bio-Rad, Hecules, CA, USA), a temperature of 45 °C, eluent: 0.005 M sulfuric acid, flow 0.6 ml/min in a sample volume of 20 µL. Furfural and 5-HMF was also determined by HPLC with UV detector (Waters 2487/USA). The samples were filtered through Schleicher and Schuell membrane, 0.45 µm and subsequently injected into the chromatograph using the following conditions: column Eclipse XDB-C18 5 µm (4.6 × 150 mm), a temperature of 25 °C, eluent: acetonitrile and water at a ratio of 1:8 with 1 % acetic acid and phosphoric acid, flow 0.9 ml/min, sample volume of 25 µl. Total concentration of phenolics was determined by method described by Gouveia et al. [13]. Cell growth was determined by measuring absorbance at 600 nm using a Bioespectro SP-220 spectrophotometer. Cell concentration was calculated based on the relationship of optical density and cell dry weight through a calibration curve. Statistical analysis of the experiments was performed using Statistica 8.

Table 1 Composition of sugarcane bagasse hemicellulosic hydrolysates after acid hydrolysis and concentrated fivefold by vacuum evaporation

Compounds	Hemicellulose hydrolysates before concentration (g/l)	Hemicellulose hydrolyzates After concentration (g/l)
Xylose	14.19	69.65
Glucose	1.52	7.32
Arabinose	1.43	7.08
Acetic acid	0.85	3.15
Furfural	0.2820	0.1680
5-HMF	0.0149	0.0378
Phenolics	3.13	8.87
pH	0.99	0.24

Results

Preparation of Sugarcane Bagasse Hemicellulosic Hydrolysates

After hydrolysis, hemicellulose hydrolysate was concentrated by vacuum concentration process to increase the concentration of sugar in hydrolysate. Table 1 shows the composition of hemicellulose hydrolysates before and after the vacuum concentration process. Xylose was the major

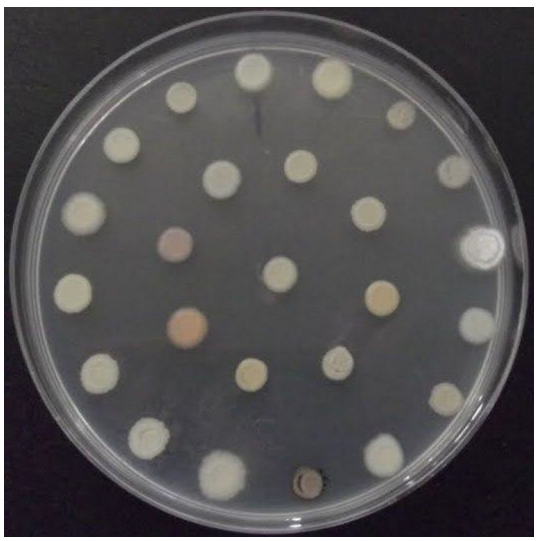

Fig. 1 Yeasts growth tested in positive control (glucose as carbon source), after 21 days of incubation at 25 °C

Table 2 Screening of different yeasts for their ability to utilize toxic compounds

	Strain	Species	Substrate where strain was obtained	Results											
				Acetic acid		Syringaldehyde		Ferulic acid		Furfural		5-hydroxymethylfurfural		Combination of compounds	
				1 g/L	5 g/L	0.2 g/L	0.7 g/L	8×10^{-2} g/L	0.4 g/L	1.8×10^{-3} g/L	1.6×10^{-2} g/L	4.0×10^{-3} g/L	2.0×10^{-2} g/L	1[a]	2[b]
1	PBM 63	*Candida metapsilosis* ATCC 96144	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
2	PBM 52	*Candida guilliermondii* TJY14a	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
3	TO 100	*Aureobasidium pullulans*	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
4	TO 182	*Aureobasidium* sp.	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
5	TO 178	*Aureobasidium pullulans*	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
6	TO 047	*Cryptococcus laurentii*	*Acromyrmex balzani*	+	−	−	−	−	−	−	−	−	−	−	−
7	PBM 44	*Pichia caribbica*	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
8	PBM 64	*Candida parapsilopsis*	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
9	PBM 30	*Pichia burtonii*	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
10	BR3-3BY	*Candida silvae*	*Vriesia* sp.	+	−	−	−	−	−	−	−	−	−	+	−
11	TO 050	*Rhodosporidium toruloides*	*Acromyrmex balzani*	+	−	−	−	−	+	−	−	−	−	+	−
12	TO 049	*Sporisorium elionuri*	*Acromyrmex balzani*	−	−	−	−	−	+	−	−	−	−	+	−
13	TO 217	*Trichosporon mycotoxinivorans*	*Acromyrmex balzani*	−	−	−	−	−	+	−	−	−	−	−	−
14	TO 023	*Cryptococcus laurentii*	*Acromyrmex balzani*	+	−	−	−	−	−	−	−	−	−	−	−
15	TO 018	*Rhodotorula mucilaginosa*	*Acromyrmex balzani*	+	−	−	−	−	+	−	−	−	−	−	−
16	SIA 24.1	*Sporobolomyces japonicus*	Fallen vegetation litter	−	−	−	−	−	−	−	−	−	−	−	−
17	TO 057	*Sporisorium elionuri*	*Acromyrmex balzani*	−	−	−	−	−	+	−	−	−	−	−	−
18	BR6-2AI	*Candida shehatae*	Bromeliad water	−	−	−	−	−	−	−	−	−	−	−	−
19	54	*Trichosporon chiarellii*	*Myrmicocrypta* sp.	−	−	−	−	−	−	−	−	−	−	−	−
20	CG8-8BY	*Candida shehatae*	Mushroom	−	−	−	−	−	−	−	−	−	−	−	−
21	PT1-1BASP	*Candida shehatae*	*Euterpe* sp.	−	−	−	−	−	−	−	−	−	−	−	−
22	BR6-2AY	*Candida shehatae*	Bromeliad water	−	−	−	−	−	−	−	−	−	−	−	−
23	N8a	*Meyerozyma guilliermondii*	–	+	−	−	−	−	−	−	−	−	−	+	−
24	N12a2	*Trichosporon asahii*	–	−	−	−	−	−	−	−	−	−	−	−	−
25	N4ap2	*Trichosporon asahii*	–	−	−	−	−	−	−	−	−	−	−	−	−

Table 2 continued

	Strain	Species	Substrate where strain was obtained	Results											
				Acetic acid		Syringaldehyde		Ferulic acid		Furfural		5-hydroxymethylfurfural		Combination of compounds	
				1 g/L	5 g/L	0.2 g/L	0.7 g/L	8×10^{-2} g/L	0.4 g/L	1.8×10^{-3} g/L	1.6×10^{-2} g/L	4.0×10^{-3} g/L	2.0×10^{-2} g/L	1[a]	2[b]
26	PI-II5	*Aureobasidium pullulans*	*Polybia ignobilis*	−	−	−	−	−	−	−	−	−	−	−	−
27	PI-II63	*Aureobasidium pullulans*	*Polybia ignobilis*	−	−	−	−	−	+	−	−	−	−	−	−
28	32	*Aureobasidium pullulans*	*Polybia ignobilis*	−	−	−	−	−	−	−	−	−	−	−	−
29	MP2-2CB	*Pseudozyma hubeiensis*	–	−	−	−	−	−	+	−	−	−	+	+	−
30	PBM 39	*Meira Arqovae* CBS 110053	*Atta laevigata*	+	−	−	−	−	−	−	−	−	−	+	−
31	A1 M-A12	*Pichia caribbica*	*Atta* sp.	+	−	−	−	−	−	−	−	−	−	+	−
32	PBM 3	*Aureobasidium pullulans*	*Atta laevigata*	−	−	−	−	−	−	−	−	−	−	−	−
33	FB1-1DASP	*Candida* sp.	Campanulaceae	−	−	−	−	−	−	−	−	−	−	−	−
34	H10-10AY	NI	*Hedychium coronarium koening*	−	−	−	−	−	−	−	−	−	−	−	−
35	HFLS-5BASP	*Candida tenuis*	*Hedychium coronarium koenig*	−	−	−	−	−	−	−	−	−	−	−	−
36	TO O11	*Cryptococcus mangaliensis*	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
37	TO 23	*Cryptococcus laurentii*	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
38	A1M-A7	*Exophiala* sp.	–	−	−	−	−	−	−	−	−	−	−	−	−
39	FB8	NI	–	+	−	−	−	−	−	−	−	−	−	+	−
40	Lj-3	*Issatchenkia occidentalis* CCTCC M 206097	Wastewater and sludge	+	+	−	−	+	−	−	−	−	−	+	−
41	S-7	*Issatchenkia orientalis* CCTCC M 206098	Wastewater and sludge	+	−	−	−	−	−	−	−	−	−	+	+
42	W7	*Lecythophora* sp.	*Polybia ignob ilis*	−	−	−	−	−	−	−	−	−	−	−	−
43	B22a2	*Candida azyma*	*Polybia ignob ilis*	−	−	−	−	−	−	−	−	−	−	−	−
44	S11	*Saccharomycopsis crataegensis*	*Polybia ignob ilis*	+	−	−	−	+	−	−	−	−	−	+	−
45	Mel 28	*Pichia caribbica*	*Polybia ignob ilis*	+	−	−	−	+	−	−	−	−	−	+	−
46	YS4	*Candida kofuensis*	*Polybia ignob ilis*	−	−	−	−	−	−	−	−	−	−	−	−
47	W13a1b	*Pichia guilliermondii*	*Polybia ignob ilis*	−	−	−	−	−	−	−	−	−	−	+	−
48	Mel 10	*Aureobasidium pullulans*	*Polybia ignob ilis*	−	−	−	−	−	−	−	−	−	−	−	−

Table 2 continued

	Strain	Species	Substrate where strain was obtained	Results											
				Acetic acid		Syringaldehyde		Ferulic acid		Furfural		5-hydroxymethylfurfural		Combination of compounds	
				1 g/L	5 g/L	0.2 g/L	0.7 g/L	8×10^{-2} g/L	0.4 g/L	1.8×10^{-3} g/L	1.6×10^{-2} g/L	4.0×10^{-3} g/L	2.0×10^{-2} g/L	1[a]	2[b]
49	Ml	*Issatchenkia occidentalis*	*Syzygium jambolanum*	+	+	−	−	−	−	−	−	−	−	+	+
50	Yl′a	*Issatchenkia occidentalis*	*Syzygium j amb olanum*	+	+	−	−	−	−	−	−	−	−	+	+
51	Yl′b	*Issatchenkia occidentalis*	*Syzygium jambolanum*	+	+	−	−	−	−	−	−	−	−	+	+
52	Y3′	*Issatchenkia occidentalis*	*Syzygium jambolanum*	+	+	−	−	−	−	−	−	−	−	+	+
53	JP26/06a	*Issatchenkia terricola*	*Syzygium jambolanum*	+	−	−	−	−	−	−	−	−	−	+	−
54	W14	*Pichia guilliermondii*	*Polybia ignobilis*	+	−	−	−	−	−	−	−	−	−	+	−
55	BD149	*Cryptococcus* sp. (in description)	Electrical wiring of lamp post	−	−	−	−	−	−	−	−	−	−	+	−
56	A2-1	*Cryptococcus* sp. (in description)	Electrical wiring of lamp post	+	−	−	−	−	−	−	−	−	−	+	−
57	A2-4	*Cryptococcus* sp. (in description)	Electrical wiring of lamp post	−	−	−	−	−	−	−	−	−	−	−	−
58	TO 622	NI	*Acromyrmex balzani*	+	−	−	−	+	+	−	−	−	−	−	−
59	TO 623	NI	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
60	TO 621	NI	*Acromyrmex balzani*	+	−	−	−	+	−	−	−	−	−	+	−
61	TO 443	NI	*Acromyrmex balzani*	+	−	−	−	−	−	−	−	−	−	−	−
62	TO 440	NI	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
63	TO 753	NI	*Acromyrmex balzani*	−	−	−	−	−	−	−	−	−	−	−	−
64	CBS 5813	NI	–	−	−	−	−	−	−	−	−	−	−	−	−

[a] Combination 1: Acetic acid (1 g/L) + Syringaldehyde (0.2 g/L) + ferulic acid (8×10^{-2} g/L) + furfural (1.8×10^{-3} g/L) + HMF (4.0×10^{-3} g/L)

[b] Combination 2: Acetic acid (5 g/L) + Syringaldehyde (0.7 g/L) + ferulic acid (0.40 g/L) + furfural (1.6×10^{-2} g/L) + HMF (2.0×10^{-2} g/L)

+ Microorganism growth in presenting the selected compound. − Microorganism no growth under the selected compound

Strains 40—Lj-3 *Issatchenkia occidentalis* CCTCC M 206097 e 41—S-7 *Issatchenkia orientalis* CCTCC M 206098 were used as positive control [22]

constituent in the hydrolysate (14.19 g/l) showing the maximum depolymerisation of hemicellulosic fraction from sugarcane bagasse. Also, glucose has been released from the hemicellulose or/and cellulose fractions. However, most of the cellulose remained in substrate and is not hydrolyzed by dilute acid hydrolysis. Fermentation inhibitors i.e. acetic acid, furfural, 5-HMF were also produced in hydrolysate. The non-concentrated acid hydrolysate also contained furfural (0.2820 g/l), 5-HMF (0.0149 g/l) and total phenolics (3.13 g/l). The vacuum evaporated acid hydrolysate represents almost fivefold increase of each component present in native hydrolysate except acetic acid, furfural and total phenolics.

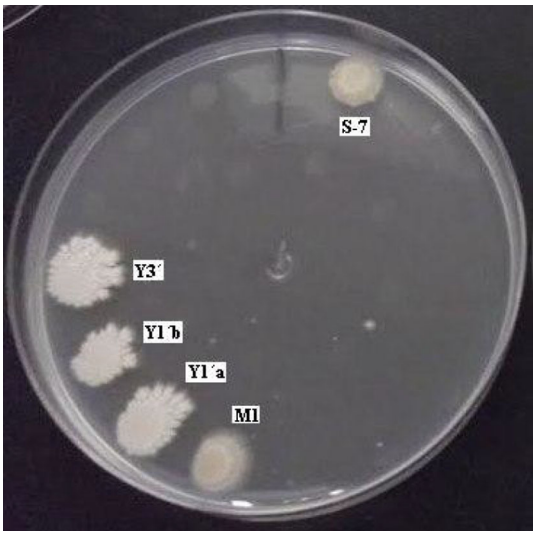

Fig. 2 Yeast strains *M1 Y1′a*, *Y1′b*, and *Y3′* and *Pichia occidentalis* S-7, CCTCC M2006098 used as standard growth observed in the combination with higher concentrations of the toxic compounds tested

Screening of Yeast for Detoxification

We have screened sixty-four yeast strains for their potential to use as detoxification of compounds present in hemicellulose hydrolysates (Fig. 1; Table 2). Among these, *P. occidentalis* M1, Y1′a, Y1′b and Y3′ showed a significant decrease in the combination of toxic compounds acetic acid, syring aldehyde, ferulic acid, furfural and HMF in synthetic culture medium (Fig. 2). Out of these tested strains, we selected two yeast strains i.e. *P. occidentalis* M1 and Y1′a for further experiments with an aim to remove the fermentation inhibitors from sugarcane bagasse hemicellulosic hydrolysates. A 2^2 full factorial design with three repetitions in central point was carried out considering two process variables-pH and agitation. Cumulative inhibitors removal (%) refers to the difference between the initial and final concentrations of 5-HMF, furfural, phenols and acetic acid concentrations from hemicellulose hydrolysates after vacuum concentration process. Tables 3 and 4 show the initial and final concentrations in g/l of toxic compounds present in hemicellulosic hydrolyzate before and after the biological treatment step employing the strains of *Picchia occidentalis* M1 and Y1′a all experiments.

According to Pareto diagram, agitation (X2) showed significant reduction of inhibitors (90 %). However, pH

Table 3 Initial and final concentrations of toxic compounds in hemicellulose hydrolyzate treated biologically by *Picchia occidentalis* M1 (g/L)

Exp.	Concentration	HMF (g/L)	Furfural (g/L)	Acetic acid (g/L)	Total phenols (g/L)	Total concentration (g/L)[a]	Removed amount of toxic compounds (%)[b]
1	Initial	0.0364	0.1402	1.3	6.373	7.85	19.43
	Final	0.0297	0.1256	0.91	5.26	6.325	
2	Initial	0.0324	0.0153	1.47	6.04	7.557	22.87
	Final	0.002	0.0043	1.2	4.623	5.829	
3	Initial	0.0371	0.1135	1.12	6.51	7.781	36.29
	Final	0.0005	0.0065	0	4.95	4.957	
4	Initial	0.032	0.0925	1.42	6.41	7.954	42.89
	Final	0.0017	0.0007	0	4.54	4.542	
5	Initial	0.0341	0.1359	2.06	5.31	7.538	39.52
	Final	0.002	0.007	0	4.55	4.559	
6	Initial	0.0341	0.1284	2.04	5.356	7.562	39.98
	Final	0.002	0.007	0	4.53	4.539	
7	Initial	0.0335	0.1229	2.09	5.27	7.521	39.01
	Final	0.002	0.005	0	4.58	4.587	

[a] "Total concentration" (g/L) refers to the sum of the concentrations of HMF, furfural, acetic acid and total phenols

[b] "Removed amount of toxic compounds" (%) refers to the difference between initial concentration and final total concentration (g/L)

Table 4 Initial and final concentrations of toxic compounds in hemicellulose hydrolyzate treated biologically by *Picchia occidentalis* Y1′a (g/L)

Exp.	Concentration	HMF (g/L)	Furfural (g/L)	Acetic acid (g/L)	Total phenols (g/L)	Total concentration (g/L)[a]	Removed amount of toxic compounds (%)[b]
1	Initial	0.037	0.1443	1.02	6.495	7.696	17.69
	Final	0.035	0.0293	0.92	5.35	6.334	
2	Initial	0.0322	0.0575	1.57	6.02	7.679	20.5
	Final	0.0018	0.0035	1.33	4.77	6.105	
3	Initial	0.037	0.1283	2.13	6.52	8.815	46.04
	Final	0.0009	0.0051	0	4.75	4.755	
4	Initial	0.0317	0.1002	1.43	5.9	7.462	38.97
	Final	0.0015	0.002	0	4.55	4.553	
5	Initial	0.034	0.1394	2.02	5.69	7.882	43.08
	Final	0.0016	0.005	0	4.48	4.486	
6	Initial	0.0347	0.1415	2.04	5.63	7.842	42.79
	Final	0.0016	0.0049	0	4.48	4.486	
7	Initial	0.034	0.1374	1.9	5.7	7.769	42.41
	Final	0.0015	0.0031	0	4.47	4.474	

[a] "Total concentration" (g/L) refers to the sum of the concentrations of HMF, furfural, acetic acid and total phenols

[b] "Removed amount of toxic compounds" (%) refers to the difference between initial concentration and final total concentration (g/L)

(X1) and the interaction factor (X1 and X2) showed no significant removal of inhibitors for both strains of *P. occidentalis*. The agitation variable (X2) acted positively in the removal of toxic compounds (Fig. 3). Experiment 4 (pH 5.0, 300 rpm) demonstrated the most significant cumulative removal (42.89 %) of toxic compounds by *P. occidentalis* M1 (Table 5) allowing the removal of 94.77 % of 0.032 g/l 5-HMF, 99.19 % of 0.0925 g/l furfural, 29.17 % of 6.41 g/l phenolics and 100 % of 1.42 g/l acetic acid. On the other hand, experiment 3 (pH 4.0 and 300 rpm) showed the most significant cumulative removal (42.89 %) of toxic compounds by *P. occidentalis* Y1′a (46.04 %), i.e. 97.68 % of 0.037 g/l HMF, 96.06 % of 0.128 g/l furfural, 27.15 % of 6.52 g/l phenol and 100 % of 2.13 g/l acetic acid.

Discussions

Xylose and arabinose are the monomeric pentose sugars from hydrolysis of the hemicellulose fraction [9, 14]. Also, glucose produced in hydrolysate is from hemicellulose or/and cellulose fractions [15]. Acetic acid is derived from the hydrolysis of the acetyl groups linked to the monomers of this polymer [9]. Xylose sugar has the highest concentration in the hemicellulosic hydrolysates due to the considerable amount of xylan present in hemicellulose [15, 16]. After vacuum evaporation of hydrolysate, acetic acid, furfural and total phenolic compounds might have evaporated or eliminated during the filtration of concentrated hydrolysates, therefore did not show the fivefold increase in concentarion. The pH of the concentrated hydrolysate was slightly decreased. These results are in well accordance with the studies of Martiniano et al. [17] and Milessi et al. [18].

Vacuum concentration process is essential to increase the concentration of sugars in the lignocellulosic hydrolysates [19]. The concentration of xylose, glucose and arabinose increased in accordance with the concentration factor employed (fivefold). However, acetic acid, 5-HMF, furfural and phenolics concentrations did not show the same behavior. The concentration of acetic acid was increased, but not according to concentration factor used, which may be related, in part, to the volatility of it. Phenolics and 5-HMF concentrations showed the same behavior, suggesting a partial volatilization and/or degradation of 5-HMF [20]. Also, Furfural concentration might be reduced due to its volatile nature. Our results showed resemblance with the report of Parajó et al. [19] and Carvalho et al. [20]. Detoxification results of hdrolysates by four strains i.e. *P. occidentalis* M1, Y1′a, Y1′b and Y3′ corroborates with the work of Fonesca et al. [21].

Hou-Rui et al. [22] also, used *P. occidentalis* CCTCC M 206097 to remove fermentation inhibitors from sugarcane bagasse hemicellulosic hydrolysates and confirmed that this strain was able to remove 100 % of 2.0 g/l acetic acid and 0.02 g/l furfural. Likewise, Fonseca et al. [21] employed the same yeast strain *P. occidentalis* CCTCC M

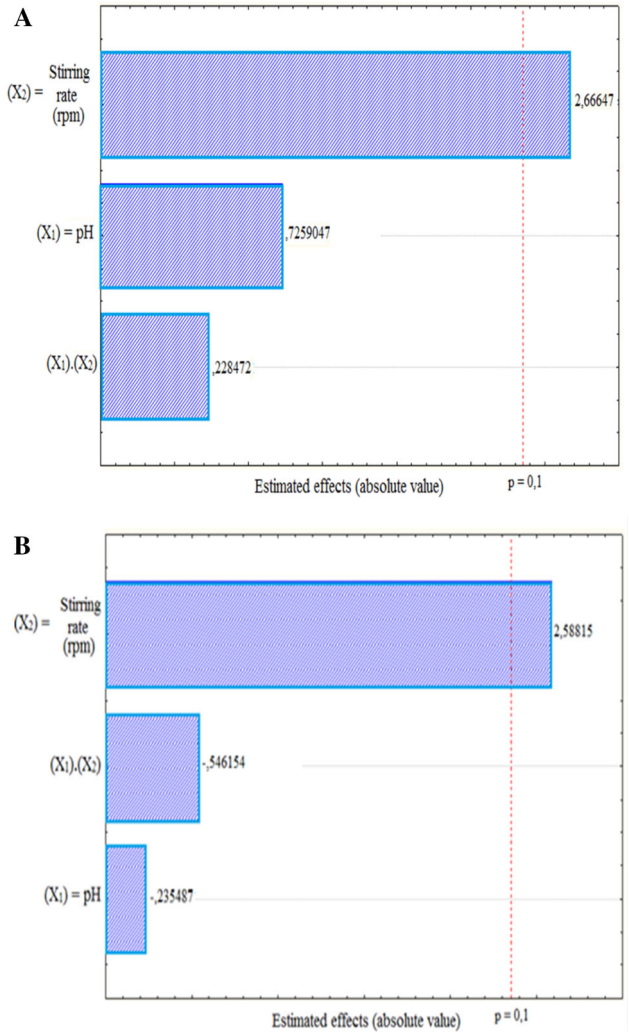

Fig. 3 Pareto diagram representing the estimated effects of the variables and their interactions to a confidence level of 90 % ($p = 0.1$) for removal of toxic compounds by *Pichia occidentalis* strains M1 (**a**) and Y1′a (**b**)

Table 5 Experimental matrix of full factorial 2^2 design of experiments with the variables' values and the cumulative inhibitors removal (%) in each experiment by *Pichia occidentalis* M1 and *P. occidentalis* Y1′a

Experiment	Real levels		Cumulative inhibitors removal (%)	
	X_1 (pH)	X_2 (rpm)	*P. occidentalis* M1	*P. occidentalis* Y1′a
1	4.0	100	19.43	17.69
2	5.0	100	22.87	20.50
3	4.0	300	36.29	46.04
4	5.0	300	42.89	38.97
5	4.5	200	39.52	43.08
6	4.5	200	39.98	42.79
7	4.5	200	39.01	42.41

206097 and reported 6.1 % removal of 3.3 g/l acetic acid, 100 % of 0.02 g/l of HMF and 100 % of 0.16 g/l furfural, after 72 h of biological detoxification. These results showed that *P. occidentalis* is a promising candidate for the in situ removal of toxic compounds from hemicellulosic hydrolysate. It is important to note that 5-HMF and furfural removal are not only related to the type of yeast used in the process, but also the kind of lignocelullosic material used to obtain the hemicellulosic hydrolysates. The different hemicellulosic hydrolysates provide different percentages removal by *P. occidentalis* [21].

This study demonstrates the potential of the yeasts for removal of inhibitors under the experimental conditions, pH (4.0 or 5.0) and agitation rate (100 rpm or 300 rpm), employing microbial detoxification approach. *P. occidentalis* M1 and *P. occidentalis* Y1′a exhibited the significant cumulative removal of inhibitors by 42.89 and 46.04 %, respectively. The major advantage of inhibitors removal by biological treatment is the cost-effectiveness and the minimization of filtration steps. Whereas, other detoxification methods required substantial investments. Also, biological treatment is an eco-friendly, economically-viable and can be used for the industrial applications.

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Compliance with Ethical Standards

Conflict of interest The authors also declare that they have no conflict of interest in the publication.

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