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Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate

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HIGHLIGHTS

- *Candida shehatae* HM 52.2 is newly isolated; it was never used in bioprocess before.
- Rice hull was hydrolysed in order to be used as substrate for bioethanol production.
- Co-cultures of *C. shehatae* and *S. cerevisiae* were used to ferment rice hull hydrolysate RHH.
- Experiments were run in shaker and further scaled-up to bioreactor.
- The results showed near-theoretical yields of ethanol in the co-culture.

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ABSTRACT

The ability of *Candida shehatae*, *Saccharomyces cerevisiae*, or the combination of these two yeasts in converting the mixed sugar composition of rice hull hydrolysate (RHH) as substrate for ethanol production is presented. In shake flask experiments, co-cultures showed ethanol yields ($Y_{P/S}$) of 0.42 and 0.51 in synthetic medium simulating the sugar composition of RHH and in RHH, respectively, with both glucose and xylose being completely depleted, while pure cultures of *C. shehatae* produced slightly lower ethanol yields (0.40). Experiments were scaled-up to bioreactors, in which anaerobiosis and oxygen limitation conditions were tested. Bioreactor co-cultures produced similar ethanol yields in both conditions (0.50–0.51) in synthetic medium, while in RHH, yields of 0.48 and 0.44 were obtained, respectively. The results showed near-theoretical yields of ethanol. Results suggest the feasibility of co-cultures of *C. shehatae*, a newly isolated strain, and *S. cerevisiae* in RHH as substrate for second-generation ethanol production.

1. Introduction

Agriculture residues, forest products and other lignocellulosic biomass are the most abundant and low cost renewable resources for ethanol and energy production. Since biomass-derived energy is part of the global carbon cycle, the use of fuel ethanol can significantly reduce the net carbon dioxide emissions if technology develops to a point where bioethanol could economically replaces fossil fuels (Li et al., 2009). In a typical bioconversion process to produce second-generation bioethanol, the hemicellulose is chained-down to pentoses (predominantly xylose), while cellulose is converted to hexoses, basically glucose, by hydrolyses pretreatments (Chen et al., 2012). Rice hull (RH) is one of the almost abundant lignocellulosic waste materials in the world, accounting for more than 120 million metric tons generated per year (Yu et al., 2009). Although rice hull finds utilization as fuel in industrial boilers, its high content in ashes and huge amounts produced represents serious technological and environmental concerns. Therefore, it could be postulated its use as substrate for ethanogenic fermentation.

Ideally, ethanol production from lignocellulosic hydrolysates would require that microorganisms ferment both hexoses and the pentoses in the presence of the inhibitory compounds produced during hydrolysis. These inhibitory compounds are weak acids, such as acetic acid, furaldehydes and phenolic compounds, mainly furfural and hydroxymethylfurfural, resulting from several

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complex reactions during the physicochemical treatment of biomass (Mussatto and Roberto, 2006). Therefore, fermentation processes would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. *Saccharomyces cerevisiae*, which is used for industrial ethanol production, has several advantages due to its high ethanol productivity, high tolerance to ethanol and high inhibitor tolerance. However, it cannot utilize xylose, the predominant pentose sugar of biomass hydrolysates (Matsushika et al., 2009). On the other hand, yeasts like *Scheffersomyces* (*Pichia*) *stipitis*, *Pachysolen tannophilus* (Fu and Peiris, 2008), and *Meyerozyma* (*Candida*) *guilliermondii* (Mussatto et al., 2005), have been reported as xylose converters, but showing low tolerance to inhibitors; requiring a small and well-controlled supply of oxygen for maximal ethanol production; and being sensitive to ethanol (Matsushika et al., 2009).

One possibility to circumvent these problems is the use of co-cultures of different yeasts, capable of both hexoses and pentoses metabolisms. Successful co-culturing methods have been described to improve the efficiency of lignocellulosic biomass fermentation by *Spathaspora arborariae* and *S. cerevisiae*; immobilized *Zymomonas mobilis* and free-cell *S. stipitis*; among others (Cunha-Pereira et al., 2011; Fu and Peiris, 2008).

In this context, the aims of this research were to investigate the use of rice hull hydrolysate (RHH) as substrate for ethanol production and the kinetics of glucose, xylose and arabinose consumption by *Candida shehatae* and the co-cultures of *C. shehatae* and *S. cerevisiae* in the presence of the inhibitory compounds: acetic acid, furfural and hydroxymethylfurfural. The *C. shehatae* HM 52.2 strain has been recently isolated and never tested in bioprocesses before. Oxygen limited conditions were compared against anaerobiosis using synthetic medium and concentrate RHH in shaker and bioreactor cultivations following sugar consumption, cell growth, and ethanol productivity.

2. Methods

2.1. Chemical characterization of rice hull

Rice hull (RH) was obtained from a local rice mill (State of Rio Grande do Sul, Brazil, centroid geo-coordinates at 30°51′04″S and 51°48′44″W; 39 m above sea level) as dried material and processed without any further treatments before hydrolysis, which is described below. Unless otherwise stated, all chemicals used in this research were of analytical grade and purchased from Sigma–Aldrich (St. Louis, USA). For analytical characterization of RH, it was submitted to quantitative acid hydrolysis with 72% (mass fraction) sulphuric acid solution, in a solid–liquid proportion of 1:10. Monosaccharides and acetic acid liberated by hydrolysis were determined by HPLC in order to estimate (after corrections for stoichiometry and sugar decomposition) the contents of cellulose (as glucan), hemicelluloses (as xylan and arabinan), and acetyl groups. The acid soluble lignin was determined by UV-spectrophotometry (see Section 2.6.). Protein was determined as total nitrogen content by the Kjeldahl method, using the $N \times 6.25$ conversion factor. Ashes were determined by weight difference before and after incineration of the soybean hull sample in a muffle furnace at 600 °C for 4 h (Silva and Queiroz, 2005). The mineral content was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). All determinations were carried out in triplicate.

2.2. Microorganisms, cell maintenance, and materials

The strains used in this study were *S. cerevisiae* ICV D254 (Lalvin, Institut Coopératif du Vin, France), a commercial wild-type strain isolated from Syrah grapes from the Rhône Valley region, in France, used for wine fermentation, and *C. shehatae* HM 52.2, a recently isolated yeast strain from rotting wood, isolated as follows. Rotting wood samples were collected at the Private Natural Heritage Reserve of Bello & Kerida, an area of Atlantic Rain Forest ecosystem located in the city of Nova Friburgo, Rio de Janeiro, Brazil (centroid geo-coordinates at 22°17′14″S and 42°32′01″W; 858 m above sea level). The local climate in this ecological reserve is altitudinal tropical, with cold and dry winter and fresh and rainy summers, with annual mean temperatures around 16 °C. The samples were stored in sterile plastic bags and transported under refrigeration to the laboratory over a period of no more than 24 h. One gram of each wood sample was placed in flasks with 20 mL sterile xylan (yeast nitrogen base 0.67%, xylan 1%, chloramphenicol 0.02%; pH 5.0 ± 0.2) medium. The flasks were incubated at 25 °C on an incubator shaker (New Brunswick, USA) at 150 rpm for 3–10 days. When growth was detected, 0.5 mL of the cultures was then transferred separately to tubes containing 5 mL sterile xylan and the tubes were incubated as described above. One loopful of culture from each tube was streaked on yeast extract-malt extract agar (YM, glucose 1%, yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, agar 2% and chloramphenicol 0.02%) (Cadete et al., 2012). The yeast was identified based on the sequencing of the D1/D2 variable domains and internal transcribed spacer (ITS) of the large-subunit rRNA gene as described by Cadete et al. (2012). *C. shehatae* HM 52.2 was never tested in bioprocesses before. Yeasts were kept frozen at −20 °C in stock cultures of 20% (volume fraction) glycerol and 80% of culture medium containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, and glucose, 5.

2.3. Inocula preparations

Inocula for all cultivations were prepared by cultivating the yeasts in synthetic medium according to compositions described below in 500 mL Erlenmeyer flasks filled with 150 mL of medium. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 30 °C for 24 h. Late exponential-phase cells were collected by centrifugation at 3000*g* for 10 min, the pellets were washed with sterile distilled water and resuspended directly into the medium to be used as inoculum (10% volume fraction) for the cultivations, always with cell concentrations of 1.0 OD (600 nm), corresponding to cell dry weights of 2.3 g L^{-1} of *C. shehatae* and 2.9 g L^{-1} of *S. cerevisiae*.

2.4. Media composition and cultivation conditions in orbital shaker

The microorganisms, either isolated or in consortium, were cultivated in synthetic medium and in rice hull hydrolysate (RHH). For shaker flasks cultivations the synthetic medium ($G_{20}X_{20}A_{10}$) had the following composition (in g L^{-1}): yeast extract, 3; peptone, 5; glucose, 20; xylose, 20; and arabinose 10; pH adjusted to 5 with 1 M HCl. Sugars were always autoclaved separately from yeast extract and peptone in order to avoid caramelization and other reactions. For the cultivations, RHH was obtained by the diluted acid hydrolysis of rice hull in autoclave (121 °C, 60 min, solid–liquid ratio of 1:10, 1% volume fraction of sulphuric acid). The liquid fraction was recovered by filtration and the pH was adjusted to 5 with solid drops of sodium hydroxide. The hydrolysate was vacuum-concentrated at 70 °C in order to increase its sugar and protein concentrations to the following final amounts (in g L^{-1}): glucose, 35; xylose, 13; arabinose, 4; and protein 5. The amount of toxic compounds (or inhibitors of microbial growth), formed during hydrolysis, in the final RHH was determined to be (in g L^{-1}): HMF, 0.07; furfural, 0.01; acetic acid, 1.6. Neither detoxification nor supplementation was made to the RHH. Cultures were carried out in 2 L Erlenmeyer flasks containing 450 mL of either

$G_{30}X_{15}A_5$ or RHH in an orbital shaker at 180 rpm, 30 °C for 108 and 240 h, simulating a oxygen limited condition (Cunha-Pereira et al., 2011). Samples were collected at stipulated points for determination of biomass by cell counting (CFU) or cell dry weight and quantification of sugars, xylitol, ethanol, and acetic acid. All experiments were conducted in triplicates.

2.5. Co-cultures in bioreactor

Experiments were carried out in fully equipped 2 L bioreactors (model Biostat B, Braun Biotech International, Germany) with RHH or synthetic medium ($G_{30}X_{15}A_5$) with the following composition (in g L^{-1}): yeast extract, 3; peptone, 5; glucose, 30; xylose, 15; and arabinose 5; pH adjusted to 5 with 1 M HCl. The different amounts of sugars in the synthetic medium used in the bioreactor were intended to better simulate the RHH composition. For each experiment, a 75 mL seed culture of each strain (OD = 1.0), totaling 150 mL of inoculum, was added into 1500 mL of medium. The pH of the cultures were controlled and maintained at 5 by automatically adding 1 M solutions of NaOH or HCl. The oxygen-controlled experiments were run using an aeration rate of 0.33 vvm, controlled by a needle valve and with a rotameter. Temperature and agitation speed were maintained at 30 °C and 180 rpm, respectively, in all bioreactor experiments. The total cultivation time was 228 h. Samples were collected at stipulated points for determination of biomass by either colony forming units (CFU) or cell dry weight (CDW) and the quantification of sugars, xylitol, and ethanol. All experiments were performed in duplicate.

2.6. Analytical methods

Hydrolysed samples were analyzed by HPLC. Glucose, xylose, arabinose, and acetic acid concentrations were determined with a refractive index (RI) (Shimadzu) detector and a Bio-Rad HPX-87H (300 × 7.8 mm) column at 45 °C, using 0.005 M sulphuric acid as eluent, flow rate of 0.6 mL/min and sample volumes of 20 μL. Furfural and hydroxymethylfurfural were determined with a UV detector (at 276 nm) and a Nucleosil C18 5-μm pore size (250 × 4.6 mm) column at room temperature, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, flow rate of 1.1 mL min^{-1} and sample volumes of 20 μL. Samples were centrifuged, washed twice with cold distilled water and dried up in pre-weighted plastic tubes at 80 °C to a constant weight (Schimer-Michel et al., 2008), Alternatively, biomass was estimated as viable cells, using CFU (colony forming units) plated in yeast morphology agar (YMA) medium. Soluble lignin (SL) was estimated by UV spectrophotometry at 280 nm. The pH of hydrolyzed samples were raised to 12 with 6 M NaOH and this solution was diluted with distilled water in order to obtain an absorbance reading not exceeding 1 unit of absorbance.

The osmotic pressure of RHH was measured by placing 30 μL samples into the chamber of an osmometer (VAPRO 5520).

2.7. Kinetic parameters calculation

The yields of ethanol production ($Y_{P/S}$, g g^{-1}) was defined as the ratio between the amount of ethanol produced and total sugars consumed present in medium up to the moment xylitol started to appear in the medium; for xylitol, conversion yields ($Y_{X/X}$, g g^{-1}) calculation was the ratio between xylitol produced and xylose consumed.

3. Results and discussion

3.1. Rice hull composition

The chemical composition of rice hull varies depending on the processing technology, plant genetics, soil, and growth conditions, among other factors. The composition (% mass fraction, dry weight) of the rice hull used in this work was determined to be: cellobiose, 0.4; glucose, 34.1 xylose 12.7; arabinose 1.3; acetic acid 1.3; HMF 0.3; furfural 0.9; insoluble lignin 21.9; soluble lignin 6.1; extractives 3.1; ashes 15.9; proteins (N × 6.45) 2.0. This composition is rich in sugars to be fermented into ethanol, but also contain high quantities of lignin (28%) and – in contrast with other agro residues – ashes (15.7%). Other raw materials such as brewers spent grain, sugarcane bagasse and wheat straw have around 28%, 24% and 24% lignin, respectively (Mussatto and Roberto, 2006; Laser et al., 2002; Mielenz et al., 2009). One of the possible problems caused by the high lignin content of lignocellulosic residues is related to the pre-treatment of dilute acid hydrolysis, which can result in the appearance of phenolic compounds, from the partial degradation of the polymer (Almeida et al., 2007). The broad composition of RH is shown in Table 1, compared to other lignocellulosic materials. The content of fermentable sugars in RH exceeds 45%, matching the values found for brewers spent grain (46%), and soybean hull (48%) suggesting that RH is a very promising substrate for the bioconversions. The composition of RH sugars found in this work is similar with data reported by other authors, the differences among values being explained by the natural variations of plant origin, classification, and processing technologies.

3.2. Shake flask cultures of C. shehatae and its co-cultures with S. cerevisiae in $G_{20}X_{20}A_{10}$ and RHH

The kinetics of *C. shehatae* HM 52.2 cultivation in $G_{20}X_{20}A_{10}$ and in RHH is shown in Fig. 1A and B, respectively, while the kinetic parameters (in comparison with bioreactor cultivations) are shown in Table 2. *C. shehatae* HM 52.2 was able to metabolize both glucose and xylose, showing that this is yeast has the enzymes for xylose transport and metabolism, but is carbon catabolite repressed (CCR) in the presence of glucose (Fig. 1A). Somewhat contrasting, (Kastner et al., 1999) reported that glucose did not completely repress xylose utilization by *C. shehatae* strain ATCC 22984, since both glucose and xylose were simultaneously consumed during the fermentations. In general, glucose, mannose, and xylose share the same, unspecific, transporters and the active transport systems are repressed by both glucose and high substrate concentrations in *S. stipitis* and *C. shehatae* (Gírio et al., 2010).

The low production of xylitol in RHH could be explained by the presence of furanic toxics in the medium, the osmotic pressure or the combination of these factors. Furfural can work as external electron acceptors regenerating NAD^+, a cofactor of xylitol dehydrogenase that converts xylitol to xylulose, which is finally fermented to ethanol (Wahlbom and Hahn-Hägerdal, 2002), rerouting the metabolism away from xylitol. RHH had a high osmotic pressure (1 539 m Osm kg^{-1}) implying a low solubility for oxygen. Very restricted aeration conditions favor the accumulation of NADH, which can inhibit the activity of NADPH-dependent xylose reductase, thus modifying the preference dependence cofactor NADPH to NADH. This modification results in the formation of NAD^+ by reducing the xylose, recovering xylitol dehydrogenase cofactor, thus diverting the fermentation of xylose to ethanol (Winkelhausen and Kuzmanova, 1998; Schimer-Michel et al., 2008).

Arabinose was metabolized in a later phase, when both glucose and xylose were exhausted, a similar metabolic profile observed

Table 1
Comparison of rice hulls broad composition used in this work with other residues and other compositions of the same reported in the literature.

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Brewers spent grain	17.0	28.0	23.0	Mussatto and Roberto (2006)
Soybean hull	38.4	10.2	2.8	Mielenz et al. (2009)
Rice hull	35	12	15	Saha et al. (2005)
Rice hull	34	13	29	This work

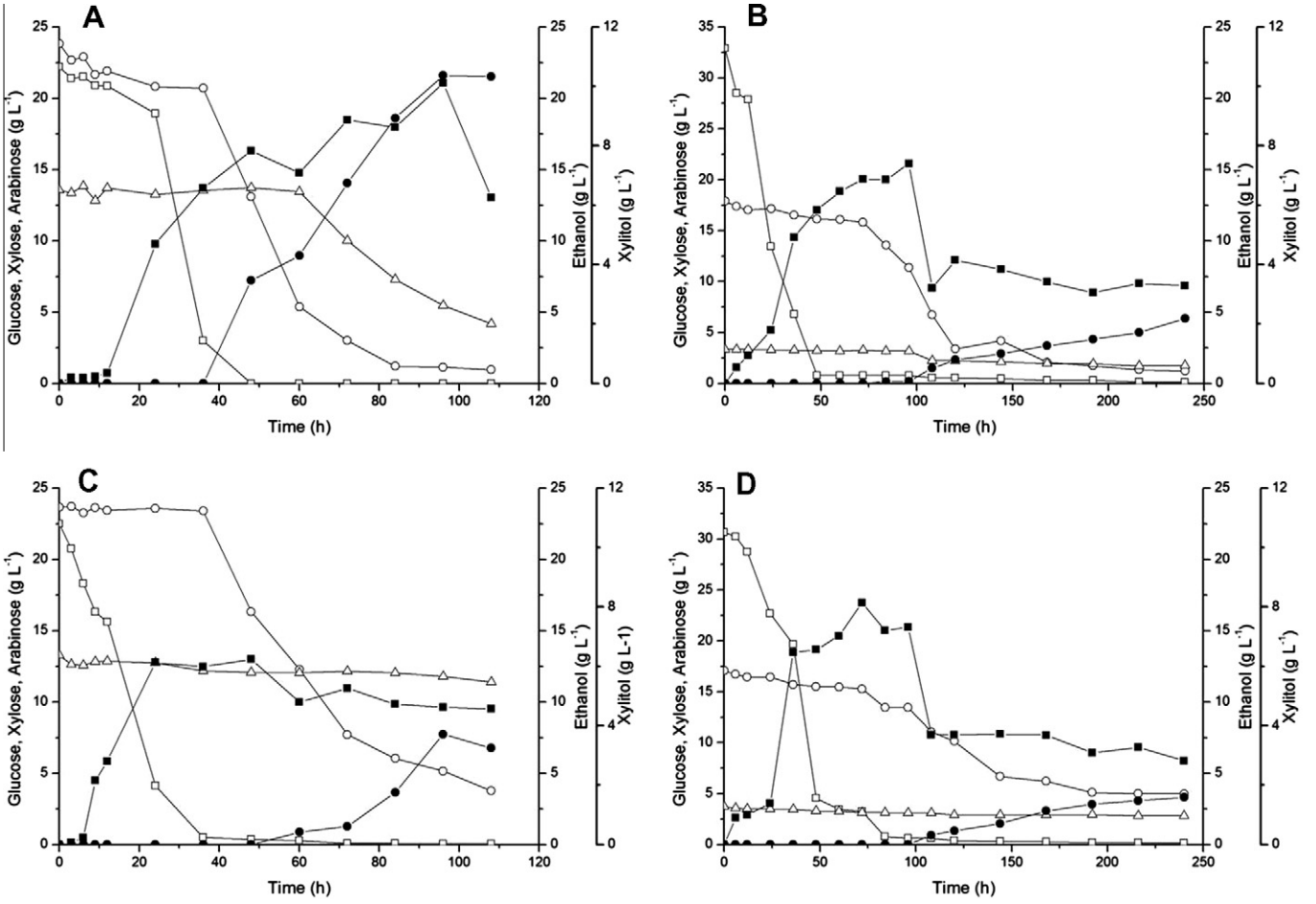

Fig. 1. Shake flask kinetics of substrate consumption, ethanol and xylitol production: (A) *C. shehatae* HM 52.2 cultivated in synthetic medium, and (B) in rice hull hydrolysate (RHH); (C) Co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in synthetic medium, and (D) in rice hull hydrolysate (RHH). Glucose (□); xylose (○); arabinose (△); ethanol (■); and xylitol (●). Results represent the mean of triplicates.

Table 2
Kinetic parameters obtained for pure cultures of *C. shehatae* HM 52.2, and for the co-cultivations of *C. shehatae* HM 52.2 and *S. cerevisiae* ICV 254D in synthetic medium and rice hull hydrolysate (RHH).

Yeast	Conditions		$G_{20}X_{20}A_{10}$		$G_{30}X_{15}A_5$		RHH	
			$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})	$Y_{P/S}$ (g g^{-1})	$Y_{X/X}$ (g g^{-1})
C. shehatae	Orbital shaker		0.40	0.45	–	–	0.40	0.13
S. cerevisiae + *C. shehatae*			0.42	0.20	–	–	0.51	0.13
S. cerevisiae + *C. shehatae*	Bioreactor	Anaerobic	–	–	0.50	0	0.48	0
		Oxygen limitation	–	–	0.51	0.24	0.44	0.11

$Y_{P/S}$, ethanol coefficient yield (g ethanol per g total sugar consumed); $Y_{X/X}$, xylitol coefficient yield (g xylitol per g xylose consumed).

for other *Candida* species, as reported by Schimer-Michel et al. (2008).

The observed ethanol yields ($Y_{P/S}$) was 0.40 g g^{-1}, while xylitol yields was 0.45 g g^{-1}. Comparatively, Yadav et al., 2011, reported that co-culture with *S. stipitis* NCIM 3498 and *C. shehatae* NCIM 3501 in shake flasks with varying concentrations of xylose (1–6%), at 30 °C, 150 rpm for 48 h obtained yields of 0.40 g g^{-1} for the highest xylose concentration. Chandel et al. (2011), investigated the metabolism of *S. stipitis* on synthetic medium to simulate wild-sugarcane bagasse hydrolysate with a complex mixture of sugars and toxic compounds, including the furan derivatives and acetic acid. They reported ethanol yields 0.44 g g^{-1} for

this yeast, while *S. cerevisiae* VS3 (control) achieved yields of only 0.22 g g^{-1}.

C. shehatae was able to grow in the RHH, where the presence of furan derivatives (0.23 g L^{-1}) and acetic acid (1.3 g L^{-1}) could disrupt its metabolism (Fig. 1B). Most of glucose and xylose, and a smaller amount of arabinose were consumed, with ethanol and xylitol yields of 0.40 and 0.16 g g^{-1} obtained, respectively. Apparently, the presence of toxic compounds did not affect ethanol production, but was strongly negative for xylose conversion into xylitol. Similar results were reported by Mussatto et al. (2005), for *C. guilliermondii* grown on hydrolysed brewers spent grain without detoxification. Sampaio et al. (2007), tested the influence of toxic compounds on xylose-to-xylitol bioconversion by *D. hansenii* UFV-170, with a set of experiments performed on semi-synthetic medium. They reported that xylitol and arabitol productions were negatively affected by furfural, not dependable to its concentration. Wahlbom and Hahn-Hägerdal (2002), reported that during xylose fermentation, xylitol excretion decreased after addition of furfural, possibly because NADH was oxidized to NAD^+ during its reduction to furfuryl alcohol, suggesting that furfural present in lignocellulosic hydrolysates could be beneficial for xylose fermentation to ethanol. These authors then postulated that HMF, which requires NADPH for reduction, did not affect xylitol excretion.

Using similar substrate as in this work (rice straw and hulls), Silva et al. (2012), used non-detoxified rice straw hydrolysate in shaker cultures of *S. stipitis* reporting ethanol yields of 0.37 g g^{-1}, while Saha et al. (2005) obtained ethanol yields of 0.43 g g^{-1} under micro-aerobiosis cultivation of a recombinant ethanogenic *Escherichia coli* (FBR 5) strain in RHH.

In order to understand the conversion kinetics of both xylose and glucose into ethanol by co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* in $G_{20}X_{20}A_{10}$ and RHH, shaker flask cultivations were set up and results are shown in Fig. 1C and D, respectively. In the co-culture, the same CCR profile observed for cultures of *C. shehatae* was in place, with xylose being consumed after the complete depletion of glucose. Interestingly, arabinose was not metabolized in the co-cultures. Several yeasts can utilize arabinose as a carbon and energy sources, but most of them are unable to ferment it into ethanol (Wisselink et al., 2007). Roberto et al. (1994), investigated the metabolism of *C. guilliermondii* in three different synthetic media containing xylose, glucose, and arabinose made up to simulate the compositions of sugarcane bagasse and rice straw hydrolysates and reported that arabinose was poorly metabolized in all simulations. Mussatto et al. (2006), suggested that many microorganisms, including ethanogenic yeasts, are able to regenerate co-factors necessary for the conversion of arabinose to xylulose, therefore producing xylitol from this pentose. In this work, values for $Y_{P/S}$ (ethanol) are similar in both cultivations. However, $Y_{X/X}$ (xylitol) was higher in the *C. shehatae* cultivation than in the co-culture of yeasts, suggesting that arabinose was also partially converted into xylitol (Table 2). This remarkable behavior concern-

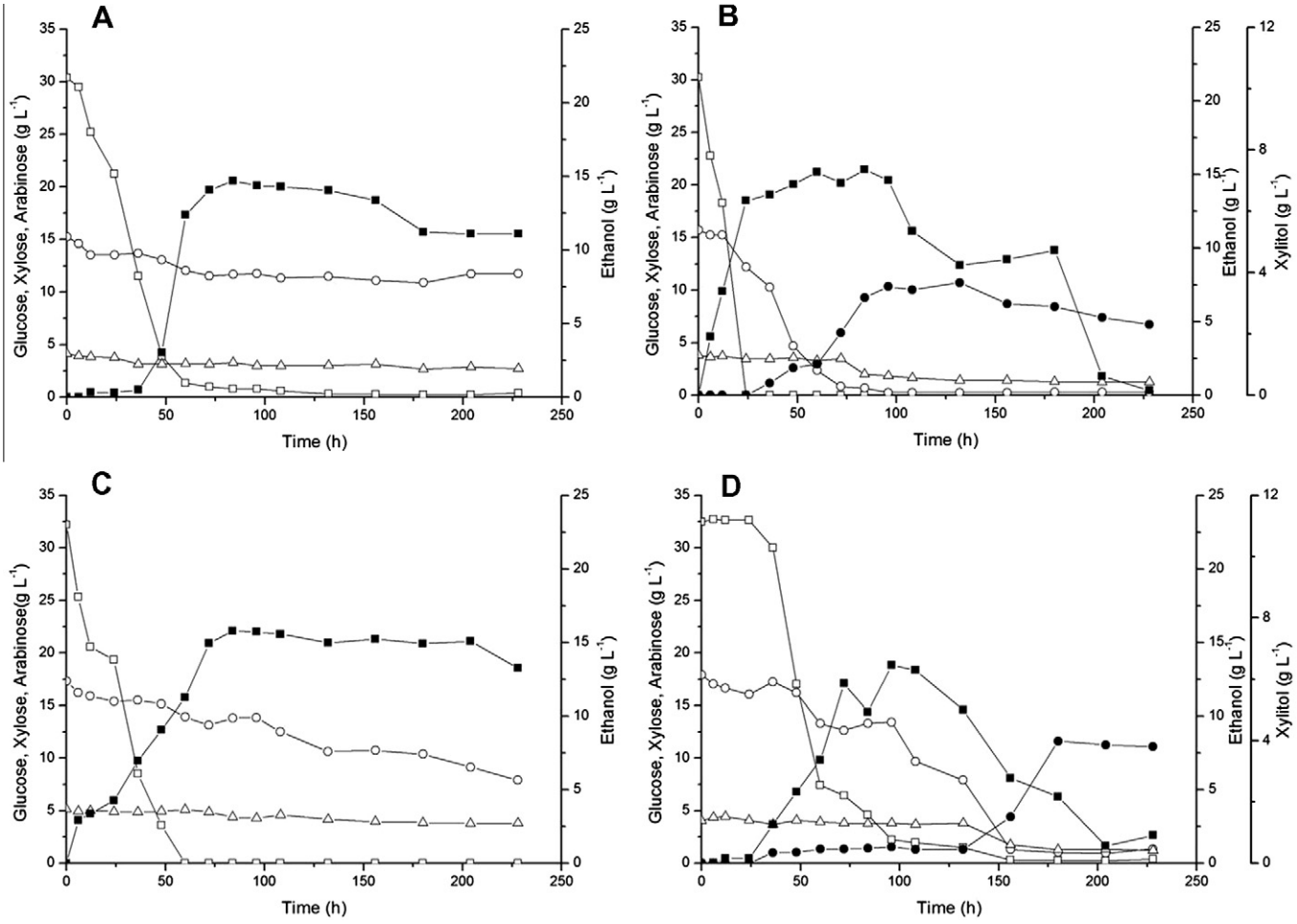

Fig. 2. Bioreactor kinetics of substrate consumption, ethanol and xylitol production of co-cultures of *S. cerevisiae* ICV 254D and *C. shehatae* HM 52.2 cultivated in (A) synthetic medium under anaerobiosis, and (B) under oxygen limitation; (C) in rice hull hydrolysate (RHH) under anaerobiosis, and (D) under oxygen limitation. Glucose (□); xylose (○); arabinose (△); ethanol (■); and xylitol (●). Results represent the mean of duplicates.

ing the metabolism of *C. shehatae* towards arabinose was similarly observed for other yeasts, suggesting the existence of CCR related to this sugar. Mussatto et al. (2006) reported that in cultures of *C. guilliermondii* FTI 20037 a concentration of xylose two times higher, or glucose ten times higher, than that of arabinose were enough to completely repress the uptake of the later, by inhibiting the action of enzymes involved on its metabolism. The kinetics shown in Fig. 1A and C for *C. shehatae* strongly support the same behavior for this yeast. Surprisingly, higher yields of ethanol were obtained in RHH (0.51 g g^{-1}) with the co-culture than in the synthetic medium (0.42 g g^{-1}) and for the isolated cultures of *C. shehatae* or *S. cerevisiae*, seem above. Similar behavior was observed for *S. stipitis* grown in either synthetic medium with only glucose or xylose, and in rice straw hydrolysate (RSH with $G_{17}X_{32}$), with higher ethanol yields in the RSH (Chen et al., 2012). Chandel et al. (2011), studied the co-cultures of *S. stipitis* NCIM 3498 and thermotolerant *S. cerevisiae*-VS3 in both sugarcane bagasse hydrolysate and synthetic medium, with ethanol yields of 0.48 and 0.49 g g^{-1}, respectively. Fig. 1C and D also show that small amounts of xylitol were produced in both media, with yields of 0.20 and 0.13 g g^{-1} in synthetic medium and RHH, respectively. The low production of xylitol most certainly is reflecting that culture conditions, especially oxygen concentration throughout cultivation, were not optimized to xylose conversion into xylitol, which requires tight controls, not possible to attain in shaker flask.

3.3. Kinetics of bioreactor cultivations of co-cultures of S. cerevisiae and C. shehatae under anaerobiosis and oxygen limitation conditions

The efficiency of a bioprocess is affected by medium composition and operational conditions used. The oxygen supply is one of the most important environmental factors in xylose fermentation by yeasts, affecting both the rates and the yields of xylitol and ethanol accumulation (Du Preez, 1994). In this research, two oxygen conditions (anaerobiosis and oxygen limitation) were analyzed in bioreactor co-cultures of *S. cerevisiae* and *C. shehatae* growing in $G_{30}X_{15}A_5$ and in RHH as shown in Fig. 2. Kinetic parameters are presented in Table 2 in comparison with the other cultivations. Under oxygen limitation, all sugars were metabolized in a CCR-positive profile, including arabinose, which was not used by cells in shaker co-cultures. Carbon catabolite repression can limit the industrial application of co-cultures with xylose-fermenting yeasts, because ethanol produced from glucose may inhibit xylose fermentation (Chen, 2011). In RHH, ethanol yields were 0.44 and 0.48 g g^{-1} under oxygen limitation and anaerobiosis, respectively, while in synthetic medium these values were up to 0.51 and 0.50 g g^{-1}. These results compare well with other reports on the literature. For instance, Fu and Peiris 2008, reported overall ethanol yields of 0.33 g g^{-1} for the total amount of sugars (0.49 g g^{-1} in the glucose fermentation stage, and 0.17 g g^{-1} in xylose fermentation stage) by a co-culture of *P. tannophilus* and *Z. mobilis*, using a synthetic medium with 60 g L^{-1} glucose and 40 g L^{-1} xylose as carbon sources, and different conditions of aeration in the glucose and xylose fermentation stages. Gutiérrez-Rivera et al., 2011, reported ethanol yields of 0.46 g g^{-1}, under oxygen limitation, and 0.20 g g^{-1} under anaerobic conditions for bioreactor co-cultures of *S. cerevisiae* and *S. stipitis* in synthetic medium containing glucose and xylose.

Concerning xylitol, yields of 0.24 g g^{-1} in synthetic medium and 0.11 g g^{-1} in RHH under oxygen limitation were obtained, while xylitol was not detected under anaerobiosis (Table 2). Xylose is reduced to xylitol using – preferentially or exclusively – NADPH, which is then oxidized to xylulose in a strictly NAD^+-dependent manner. The two steps use different redox factors, leading to the accumulation of NADH that cannot be recycled under anaerobiosis, thus inducing the accumulation of xylitol (Hou et al., 2009). Peng et al. (2012), investigated the metabolism of recombinant *S. cerevisiae* BSPX021 expressing xylose reductase–xylitol dehydrogenase (XR–XDH), in oxygen-limited shake flask cultivation with glucose and xylose. The authors reported xylitol yields of 0.27 g g^{-1}, similar to values obtained in this work in synthetic medium. Wikelhausen et al. (2004), growing *C. boidini* in synthetic medium free of inhibitory compounds, and with five times higher xylose concentrations than used in this work, reported xylitol yields of 0.16 g g^{-1}. Schimer-Michel et al. (2008) using soybean hull hydrolysate, with *C. guilliermondii* under oxygen limitation, reported xylitol yields of 0.22 g g^{-1}, with the formation of glycerol as a by-product (4.5 g L^{-1}). In this work, only 1.15 g L^{-1} of glycerol was detected. Van Maris et al. (2007), demonstrated that under anaerobic conditions, reoxidation of excess NADH could be accomplished via the production of compounds that are more reduced than xylose, such as xylitol and/or glycerol. Glycerol production is a well-known redox sink during hexose fermentation, especially under anaerobic conditions. Since there was low glycerol formation, it might be suggested that NADH was preferentially shuttled into xylitol formation in the co-culture used in this work.

4. Conclusion

It was demonstrated the possibility of using RHH as a substrate for ethanol production by co-cultures of *S. cerevisiae* and *C. shehatae*, which proved to be an efficient converter of hexoses and pentoses to ethanol. The co-culture was effective to simultaneously convert glucose and xylose, maximizing substrate utilization rates, increasing ethanol yields and production rates. Bioconversion of hexoses and pentoses can be influenced by the rate of oxygenation and furanic inhibitors in the medium. Further studies are granted in order to optimize cultures of *C. shehatae* in co-cultures with other ethanogenic microorganisms, under different oxygen conditions, especially on lignocellulosic hydrolysates.

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