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Efficient production of second generation ethanol and xylitol by yeasts from Amazonian beetles (Coleoptera) and their galleries

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Beetles of the Passalidae family live and feed on decaying wood and their guts are richly colonized by yeasts. The goal of this research was to prospect xylolytic yeasts with potential for the production of second-generation bioethanol. Therefore, 83 specimens of beetles belonging to the Passalidae and the Scarabaeidae families were collected in the Amazonian rainforest in Itacoatiara - AM, Brazil. Morphological differences of the beetles were identified and 25 chosen specimens were dissected. Yeasts from galleries inhabited by beetles and from insect guts were isolated. Isolates were previously selected through tolerance tests for temperature, ethanol and xylose assimilation capacity. Those isolates were then submitted to a panel of conditions related to ethanol production. The ethanol production reached 24.70 g.L^{-1} and the xylitol production reached 21.66 g.L^{-1}. One of the isolates with a promising profile was identified as *Spathaspora roraimanensis* and six as *Spathaspora passalidarum*. Three isolates showed to be more promising and, curiously, all came from the gut of the species *Popilius marginatus* (Percheron, 1835). In plate testing, however, the isolates obtained from galleries showed a greater capacity to assimilate xylose. As reported in this field of study, no isolate tolerated all conditions tested. Wild isolates with this profile may be used for testing larger-scale ethanol production, genetic engineering, or evolutionary techniques.

Key words: Beetles, bioethanol, *Popilius marginatus*, xylose.

INTRODUCTION

Fuels from renewable resources are becoming progressively important in times of increasing environmental concern. In the specific case of bioethanol production, researchers have sought to use plant biomass as the raw material (Brat et al., 2009).

Usually, production of bioethanol is a biological process in which sugars such as glucose, fructose and sucrose are converted into cellular energy by microbial

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fermentation and thus produce ethanol and carbon dioxide as metabolic residues (Tao et al., 2011). When production occurs from easily accessible sugars, it is referred to as first generation (1G) production, whereas, if lignocellulosic materials such as agricultural residues, forest materials and dedicated crops are used, they are called second-generation (2G) production (Joelsson et al., 2016).

Saccharomyces cerevisiae is the preferred microorganism for ethanol production, due to its capacity to grow into simple sugars such as disaccharides. However, wild strains of *S. cerevisiae* are unable to ferment D-xylose, which is one of the major polysaccharide constituents of lignocellulosic biomass. Also, D-xylose is the second most abundant sugar in the cell walls of plants and lignocellulosic biomass is of interest as a substrate for production of ethanol, xylitol and other microbial products (Carvalho et al., 2002; Hahn-Hägerdal et al., 2006; Doran-Peterson et al., 2008; Junyapate et al., 2014).

Although hydrolysis of plant biomass, which breaks hemicellulose and exposes cellulose to an enzymatic attack, is important to the production of 2G ethanol and it produces sugar mixtures rich in glucose and xylose, fermentation inhibitors are generated by the chemical pretreatment. Acetic acid, furfural and hydroxymethylfurfural (HMF) are considered key components among many inhibitors that are formed during pretreatment. According to Slininger et al. (2016), in order to advance the 2G ethanol process of production, research and procedures are required to allow evolution of yeast strains. It is necessary to work on the capability of surviving and functioning efficiently using both hexose and pentose sugars in the presence of such inhibitory compounds. Thus, an industrial strain, with high metabolic potential for xylose fermentation and good inhibitor tolerance would be potentially useful for industrial bioethanol production (Li et al., 2015).

Different approaches in genetic engineering have been used to allow fermentation of D-xylose by wild strains of *S. cerevisiae*, but growth and productivity rates are significantly lower in this sugar compared to glucose, and therefore the process is not industrially competitive (Hahn-Hägerdal et al., 2007; Brat et al., 2009). Therefore, there is strong pressure to improve the economic viability of 2G ethanol production, thus motivating researchers to explore alternatives beyond conventional *Saccharomyces* species (Radecka et al., 2015). Recently Slininger et al. (2016) used a wild strain of *Pichia stipitis* (NRRL Y-7124) with promising capacity for pentose fermentation and reported good results using evolutionary techniques to obtain more robust variants.

Alternatively, D-xylose can be converted into the polyol xylitol ($C_5H_{12}O_5$), which is an important chemical product and with higher financial value than ethanol. Xylitol is extensively used in food and pharmaceutical industries as sweetener (Guo et al., 2013; Li et al., 2013; Sena et al., 2016; Zhang et al., 2016).

Xylose-fermenting yeasts are commonly found into the digestive tract and/or feeding tubes of many xylophagous insects, suggesting an association with wood digestion (Suh et al., 2003, 2006). In fact, the gut of beetles and other insects is considered a hotspot of yeast diversity (Suh and Blackwell, 2004; Boekhout, 2005; Rivera et al., 2009; Cadete et al., 2009, 2012; Urbina et al., 2013; Gouliamova et al., 2015; Cadete et al., 2015, 2016).

In this context, it was hypothesized that the almost unexplored biodiversity of the Amazonian rainforest near Itacoatiara – AM, Brazil, could provide us yeast isolates from beetle guts and galleries in tree trunks with potential for 2G ethanol production.

MATERIALS AND METHODS

Collection of beetles and deposition in arthropod collection

Adult beetles (Passalidae) were collected in natural habitats due to their association with decomposing wood. Beetles were collected at two sites in the Amazon forest: Campus II of the Federal University of Amazonas (UFAM), kilometer 260 of Rodovia AM-010 (S03°05.654' W058°27.464'), and in the Sol Nascente community (S03°01.045' W058°28.830'), both in the municipality of Itacoatiara - AM.

The beetles were collected individually with aid of sterile tweezers, deposited in sterile Petri plates and brought to the Mycology Laboratory of the Institute of Exact Sciences and Technology (ICET) at the Federal University of Amazonas (UFAM). The insects were transported alive with bark fragments collected from where they were found. A sampling of insects would be destroyed by the dissection process, so control specimens were collected from the same gallery and at the same time as the beetles that would be dissected (Suh and Blackwell, 2004). The entomologist Dr. Claudio Ruy Vasconcelos da Fonseca identified the specimens of beetles, and control specimens were deposited into the collection of the National Institute of Amazonian Research (INPA). A summary of the methods used to carry out the selection procedures was presented in Figure 1.

Isolation of yeasts from the intestinal tract of beetles

The beetles were kept in sterile Petri plates for three days, without feeding, before euthanasia (in 0.56% KCl solution) and dissection. According to Suh and Blackwell (2004), keeping specimens without food assists in eliminating some contaminating organisms that may eventually be isolated from their gut. The insects were submitted to a surface disinfection by submersion in 70% ethanol (5 min), bleach (5 min) and sterile water (10 min) prior to removal of their guts. Sterile water (100 µl) was seeded in acidified yeast malt YM extract agar (composition/L: 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g dextrose, 15 g agar, 1000 mL distilled water, supplemented with 0.1 g chloramphenicol; pH adjusted to 3.5 with hydrochloric acid), as a negative control (Suh and Blackwell, 2004).

Guts were removed aseptically with the aid of a stereomicroscope in a biological safety cabinet. After dissection, the large gut of the insects was cut into three parts with a sterile scalpel and crushed with a clamp into sterile microtubes containing 100 µL of saline solution, and this was spread on acidified YM agar (modified from Ravella et al., 2011). The cultures were incubated at 30°C for five days. After that, individual colonies with yeast morphology were taken from and purified by at least two successive

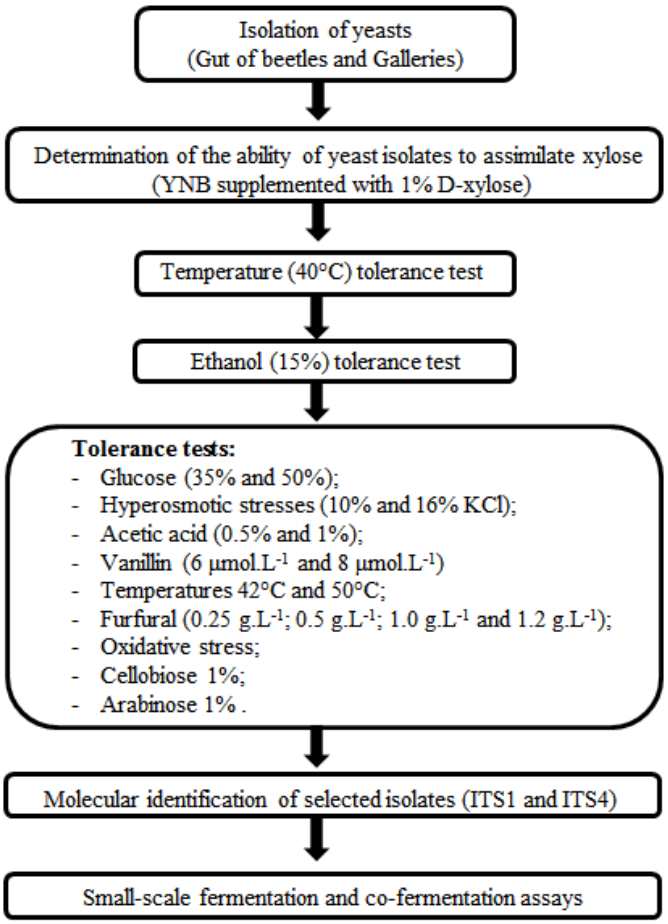

Figure 1. Flowchart showing the approach for the isolation of yeasts from beetles and their galleries and screening for ethanol and xylitol producers.

replications in YM agar. From each culture, the square root of the total colonies was calculated. That number was the number of colonies isolated for later identification.

Determination of the ability of yeast isolates to assimilate xylose

To determine the ability to utilize xylose, the isolates were seeded in yeast nitrogen-based medium (YNB) supplemented with 1% D-xylose per puncture and incubated at 30°C for 72 h. As a positive control, the same strains were inoculated in YNB supplemented with 1% glucose (Tanahashi et al., 2010). After growth, the growth rate was evaluated by measuring the diameter of the colonies considering a horizontal axis and its perpendicular axis, after the arithmetic mean was obtained of the measurements for each isolate, according to Golinski et al. (2008).

Tolerance tests

Tolerance tests evaluated the growth performance of yeast isolates under some typical stress conditions in fermentations for production of 2G ethanol. The conditions tested respectively were: Glucose (35% and 50%), hyperosmotic stresses (10 and 16% KCl), acetic acid (0.5 and 1%), temperatures of 42 and 50°C, vanillin (6 and 8 µmol.L^{-1}), furfural (0.25, 0.5, 1.0 and 1.2 g.L^{-1}) and oxidative stress. Also concentrations of cellobiose 1% and arabinose 1% (Ali and Khan, 2014; Li et al., 2015) were tested. For this, the strains were previously cultured overnight in YEPD agar medium (composition/L: 10 g yeast extract, 20 g peptone, 20 g dextrose, and 20 g agar, 1000 mL distilled water) and washed twice with sterile water. The optical density was adjusted (OD_{600}=1.0), dilution of the suspension (10^{-1}, 10^{-2} and 10^{-3}) was carried out, and for oxidative stress and temperature (42 and 50°C), the density was adjusted to OD_{600} = 2.0. 4 µL of each diluted suspension in each solid medium was applied. Except for the acetic acid test, where the plates remained incubated for four weeks at 30°C, and against furfural, with ten day incubation, the remaining tests were performed with 48-hour incubation at 30°C.

To determine the resistance to oxidative stress, the cells were mixed with 20 mL of YEPD agar (cooled to approximately 50°C) and immediately plated. Thereafter, sterile filter paper (0.5 mm diameter) was placed in the center of each plate with 6 µL of hydrogen peroxide 30% (H_2O_2), followed by incubation of two days at 30°C. The diameters of the growth inhibition zones (in mm) were recorded (Li et al., 2015).

Molecular identification of selected isolates

The molecular method for yeast identification was based on the amplification and sequence analysis of rDNA internal transcribed spacer (ITS) (Caggia et al., 2001). Prior to extraction, the yeasts were grown in a YEPD medium and incubated for 24 h at 30°C. For the extraction of the genomic DNA with a pipette tip, an isolated colony was resuspended in 1 mL of sterile water in microtube that was centrifuged for 1 min at 10,000 to 12,000 rpm. Then the supernatant was removed. After that, 100 µL of InstaGene™ Matrix (under continuous stirring) was then added to the pellet and incubated at 56°C for 30 min. The suspension was homogenized in a vortex for 10 s and the tubes incubated in boiling water (100°C) for 8 min, with further vortexing for 10 s and centrifuging at 10,000 to 12,000 rpm for 3 min. 2.5 µL of the supernatant result per 50 µL of the PCR reaction was used. The DNA sample was stored at -20°C, according to da Silva et al. (2012).

The primers used to amplify the rDNA ITS region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification reaction was performed in 50 µL (final volume) containing 1 µL of each primer, 25 µL of TopTaq® Master Mix Kit (Qiagen), 21 µL of miliQ water and 2 µL of genomic DNA (sample) in a thermocycler. Samples were sent to Macrogen (Rockville, USA) for sequencing. The PCR product sequences were compared to the ITS regions deposited in GenBank (http://www.ncbi.nlm.nih.gov) and the similarity compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (modified Tao et al., 2011).

Small-scale fermentation and co-fermentation assays

The fermentation tests were performed in 125 mL Erlenmeyer flasks in culture medium containing yeast extract (5 g.L^{-1}); peptone (5 g.L^{-1}); NH_4Cl (2 g.L^{-1}); KH_2PO_4 (1 g.L^{-1}); $MgSO_47H_2O$ (0.3 g.L^{-1}) and glucose and/or xylose under stirring at 120 rpm for 70 h. The strains were previously cultured in YP medium with 2% xylose, under stirring at 120 rpm, at 28°C. From the pre-inoculum, dilutions were performed so that the initial optical density $(OD)_{600nm}$ of all strains was equal to 1. Each isolate was inoculated in fermentation liquid medium (4% xylose), resulting in a final volume of 50 mL. The Erlenmeyer flasks were incubated in a horizontal shaker at 28°C for 70 h and at 120 rpm. At intervals of 0, 3, 6, 9, 12, 20, 30, 40, 50, 60 and 70 h, aliquots of 200 µL were withdrawn to evaluate OD_{600nm},

Table 1. Species of beetles used for the isolation of yeasts.

Family	Genus	Species	Specimens
Passalidae	Passalus	*Passalus latifrons* (Percheron, 1841)	11
Passalidae	Passalus	*Passalus interruptus* (Lin, 1758)	4
Passalidae	Veturius	*Veturius platyrhinus* (Westwood, 1845)	1
Passalidae	Popilius	*Popilius marginatus* (Percheron, 1835)	5
Passalidae	Passalus	*Passalus punctiger* (Lep & Serv. 1825)	1
Passalidae	Passalus	*Passalus convexus* (Dalman, 1817)	1
Passalidae	Veturius	*Veturius transversus* (Dalman, 1837)	1
Scarabaeidae	Cetoniinae sp	Unidentified species	1

the cell viability by serial dilution and an aliquot of 800 µL was centrifuged at 10,000 rpm for three minutes. The supernatant was filtered through a 0.2 µm membrane and subsequently frozen for analysis of xylose, xylitol, glycerol and ethanol levels through high performance liquid chromatography (HPLC). For the co-fermentation assays, glucose (2%) was added.

RESULTS

In this research, 83 specimens of beetles were collected from two different sites in the Amazon forest. Due to the easily observable morphological differences, 24 specimens of beetles were dissected and identified as belonging to the Passalidae family and one to the Scarabaeidae family (Table 1). From the intestinal contents of these insects and swabs rotated inside the log galleries they inhabited, 380 and 412 isolates suggestive of yeasts were obtained, totaling 792 isolates. The obtained isolates were then subjected to screening tests to verify their potential for production of 2G ethanol.

Screening of yeast strains for bioethanol production

The 792 isolates obtained were initially submitted to a screening to verify xylose assimilation capacity. Only twelve isolates, all from the intestinal contents of the insects, did not present growth in the medium containing xylose as the only carbon source. Considering the size of the colonies (see Materials and methods), the isolates obtained from galleries had higher xylolytic capacity when compared to the isolates obtained from the guts of the beetles (t test; $p<0.001$) (Figure 2).

The 780 strains that assimilated xylose were submitted to a temperature tolerance test of 42°C for 48 h. In this condition, 73 isolates showed growth. These 73 isolates were then tested against 15% ethanol. Sixteen strains showed growth after 48 h of incubation at 30°C.

The 16 selected strains were subsequently submitted to tolerance tests to evaluate the growth performance under typical stress conditions in fermentations. Growth on the plate containing 1% acetic acid was not observed. The G7-1.4 isolate showed growth on the plate containing 0.5% acetic acid after 22 days of incubation. There was growing in the plates with supplementation of furfural 0.25g.L^{-1}. The isolates were categorized according to the growth observed in the plates as: Absence of growth (-), slight growth (+), moderate growth (++) and intense growth (+++) (Table 2).

The degree of resistance to oxidative stress was demonstrated by the diameter of the inhibition halo (mm). The experiment was performed in triplicate and expressed as the mean halo diameter. Four isolates (P22-1.2, P22-1.3, P22-2.19 and P22-2.20) were not inhibited by hydrogen peroxide (H_2O_2) in the tested condition (Table 2). Under the conditions used here, of the 16 isolates pre-selected and tested against this panel, three, P16-1.1; P21-1.7 and P21-2.1, showed superior tolerance (Table 2).

Molecular identification of the selected isolates

Considering the results obtained in tests for xylose assimilation capacity, temperature tolerance for 42ºC and tolerance for 15% of ethanol, 16 isolates were submitted to molecular identification. The P8-2.12 isolate showed high homology (98%) to *Spathaspora roraimanensis* XMD23.2 (JN099269.1); the P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 isolates showed high homology (99%) with *Spathaspora passalidarum* ATCC MYA-4345 (NR_111397.1). It was possible to obtain genera identification through the sequencing of isolates P19-1.1, P21-1.7, P21-2.1, P22-3.18 (*Candida* sp.) and P22-1.2, P22-1.3, P22-2.19, P22-2.20 (*Schwanniomyces* sp.). The G7-1.4 isolate has a good sequence, but low similarity (96%) with *Candida jeffriesii*, being able to be a new species (Table 3).

Small-scale fermentation tests

The 16 isolates selected were submitted to small-scale fermentations initially in media containing 4% xylose as the only carbon source. Isolates P22-1.2, P22-1.3 and P22-2.19 did not produce ethanol. After 30 h of

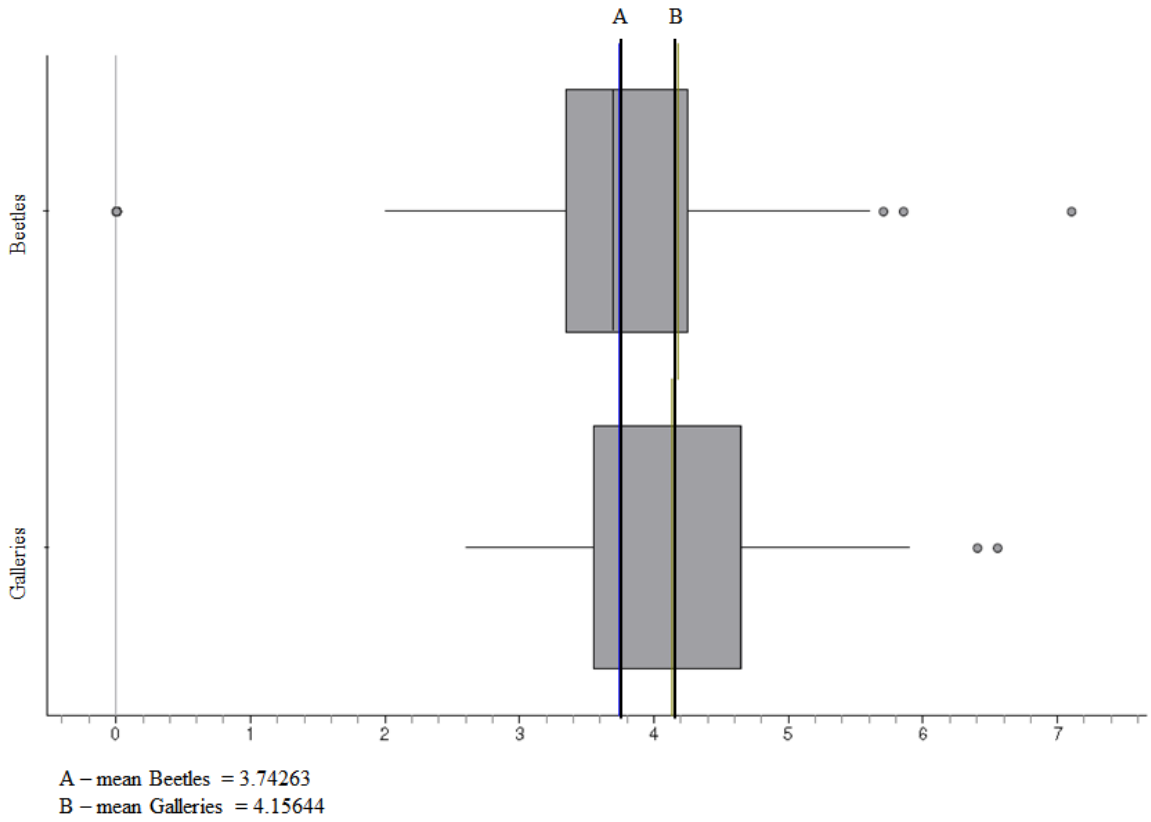

Figure 2. Difference between the dimension of the colonies (considering the mean of two perpendicular measures from the largest diameter) of yeasts isolated from the intestinal contents of beetles compared to yeasts isolated from inhabited galleries (** *t* test: p <0.001), when cultivated in nitrogen-based agar with 1% of D-xylose as the sole carbon source.

fermentation, isolates P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 showed a peak of ethanol production with approximately 85% fermentation efficiency (Figure 3), demonstrating considerable capacity to convert xylose into ethanol. The production of xylitol exceeded the yields of ethanol in the yeasts P8-2.12 (12.25 $g.L^{-1}$), P19-1.1 (21.66 $g.L^{-1}$), P21-1.7 (19.20 $g.L^{-1}$), P21-2.1 (19.14 $g.L^{-1}$) P22-2.20 (17.57 $g.L^{-1}$), P22-3.18 (22.10 $g.L^{-1}$) and G7-1.4 (20.01 $g.L^{-1}$) (Table 4). Ethanol or xylitol were the main products of xylose metabolism.

The results of the fermentative parameters ($Y_{P/S}$ = ethanol or xylitol yield; Q_P = ethanol or xylitol productivity; η% = etanol or xylitol fermentation efficiency; Y% = xylose consumption) relative to the comparison between fermentation in media containing only 4% xylose and co-fermentation in media with 4% xylose and 2% glucose are shown in Table 4. These results were calculated according to the fermentation time (maximum ethanol or xylitol production time). During the co-fermentation process, it was observed that glucose depletion occurred rapidly, that yeasts simultaneously consumed xylose and that, at the end of 70 h of fermentation, and xylose had not been totally consumed.

DISCUSSION

Ascomycetic yeasts that both ferment and assimilate xylose have been associated with insects that feed on decaying wood (Young et al., 2010; Ravella et al., 2011; Tao et al., 2011). Here the xylose assimilation capacity of 792 yeasts isolated from the gut of beetles and their galleries were tested. Only 12 did not show growth in the medium containing xylose as the sole carbon source. Interestingly, a significantly higher xylolytic capacity was observed in the isolates from galleries compared to those obtained from the intestinal contents of the beetles (Figure 2). This is preliminary data, however, to our knowledge there is no mention of this in the literature, which may be of interest in future native yeast screenings for bioethanol production.

Considering stress conditions involved in the fermentation processes for the production of 2G ethanol (Costa et al., 2014; Li et al., 2015), the sixteen isolates that showed tolerance to ethanol and were selected had different degrees of tolerance (Table 2), but none of those tolerated all the conditions imposed. This information is corroborated by Li et al. (2015), where none of the five strains of *S. cerevisiae* tolerated all

Table 2. Cultivation tests of sixteen isolates selected on a panel of conditions frequently observed in the production of second-generation ethanol.

Strains	Glucose 35%	Glucose 50%	Cellobiose 1%	KCl 16%	KCl 10%	Acetic acid 1%	Acetic acid 0.5%	42°C (OD_{600} 1.0)	50°C (OD_{600} 1.0)	42°C (OD_{600} 2.0)	50°C (OD_{600} 2.0)	Arabinose 1%	Vanillin 6 µmol L^{-1}	Vanillin 8 µmol L^{-1}	Furfural 1.2 gL^{-1}	Furfural 1.0 gL^{-1}	Furfural 0.5g L^{-1}	Furfural 0.25 gL^{-1}	H_2O_2 (mm)
P8-2.12	-	-	+++	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	37.34
P16-1.1	++	+++	+++	-	++	-	-	-	-	+	-	+	+++	++	-	-	-	++	17
P19-1.1	++	+++	+	-	++	-	-	-	-	-	-	+	+++	++	-	-	-	++	21.67
P21-1.7	++	+++	+	-	+	-	-	-	-	+	-	+	+++	+++	-	-	-	++	24.34
P21-2.1	++	+++	+++	-	+	-	-	-	-	++	-	+	+++	+++	-	-	-	++	21
P22-1.2	+++	+++	+	-	+	-	-	-	-	-	-	-	+++	++	-	-	-	-	0
P22-1.3	+++	+++	++	-	+	-	-	-	-	-	-	+	+++	++	-	-	-	-	0
P22-2.19	+++	++	+	-	+	-	-	-	-	+	-	-	+++	++	-	-	-	-	0
P22-2.20	+++	+++	++	-	++	-	-	-	-	-	-	+	+++	++	-	-	-	-	0
P22-3.18	++	+++	+++	-	++	-	-	-	-	-	-	+	+++	+++	-	-	-	+++	23.67
G7-1.4	++	++	+++	-	+	-	+	-	-	-	-	-	++	+	-	-	-	+	22.67
G13-2.1	+	++	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	28.67
G13-3.8	-	+	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	28.67
G14-1.8	-	+	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	27
G14-2.2	-	-	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	29.67
G18-3.7	-	-	+++	-	-	-	-	-	-	-	-	+	++	-	-	-	-	++	29.67

- = absence of growth; + = slight growth; ++ = moderate growth; +++ = intense growth. OD = optical density, mm = millimeters (for detailed information see materials and methods).

Table 3. Molecular identity of yeasts isolated by rDNA sequencing.

Isolate code	Organisms	Origin	Identification (%)	Genbank identification number/access number	rDNA[a]
P8-2.12	*Spathaspora roraimanensis*	Beetle gut	98	JN099269.1	ITS1/ITS4
P16-1.1	*Spathaspora passalidarum*	Beetle gut	99	NR_111397.1	ITS1/ITS4
P19-1.1	*Candida* sp.	Beetle gut	84	JQ901890.1; JQ647915.1	ITS1/ITS4
P21-1.7	*Candida* sp.	Beetle gut	90	JQ647915.1; JQ901890.1; FN4241041	ITS1/ITS4
P21-2.1	*Candida* sp.	Beetle gut	93	JQ647915.1; JQ901890.1; JX448364.1	ITS1/ITS4
P22-1.2	*Schwanniomyces* sp.	Beetle gut	99	JQ425347.1; HQ115736.1; AJ586527.1; EF198011.1; JQ425390.1	ITS1/ITS4
P22-1.3	*Schwanniomyces* sp.	Beetle gut	99	HQ115736.1; JQ425347.1; EF198011.1; AJ586527.1; JQ425390.1	ITS1/ITS4
P22-2.19	*Schwanniomyces* sp.	Beetle gut	99	HQ115736.1; JQ425347.1; EF198011.1; JQ425390.1	ITS1/ITS4
P22-2.20	*Schwanniomyces* sp.	Beetle gut	99	JQ425390.1; EF198011.1; AJ586527.1; JQ425347.1; JQ425390.1; LN875174.1	ITS1/ITS4
P22-3.18	*Candida* sp.	Beetle gut	99	FN424204.1; NR_137087.1; JF916546.1	ITS1/ITS4
G7-1.4[b]	*Candida jeffriesii*	Gallery	96	NR_111398.1	ITS1/ITS4
G13-2.1	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4

Table 3. Contd.

G13-3.8	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G14-1.8	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G14-2.2	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4
G18-3.7	*Spathaspora passalidarum*	Gallery	99	NR_111397.1	ITS1/ITS4

[a]Region of the rDNA gene used for identification. [b]Good sequence; may also represent a new species.

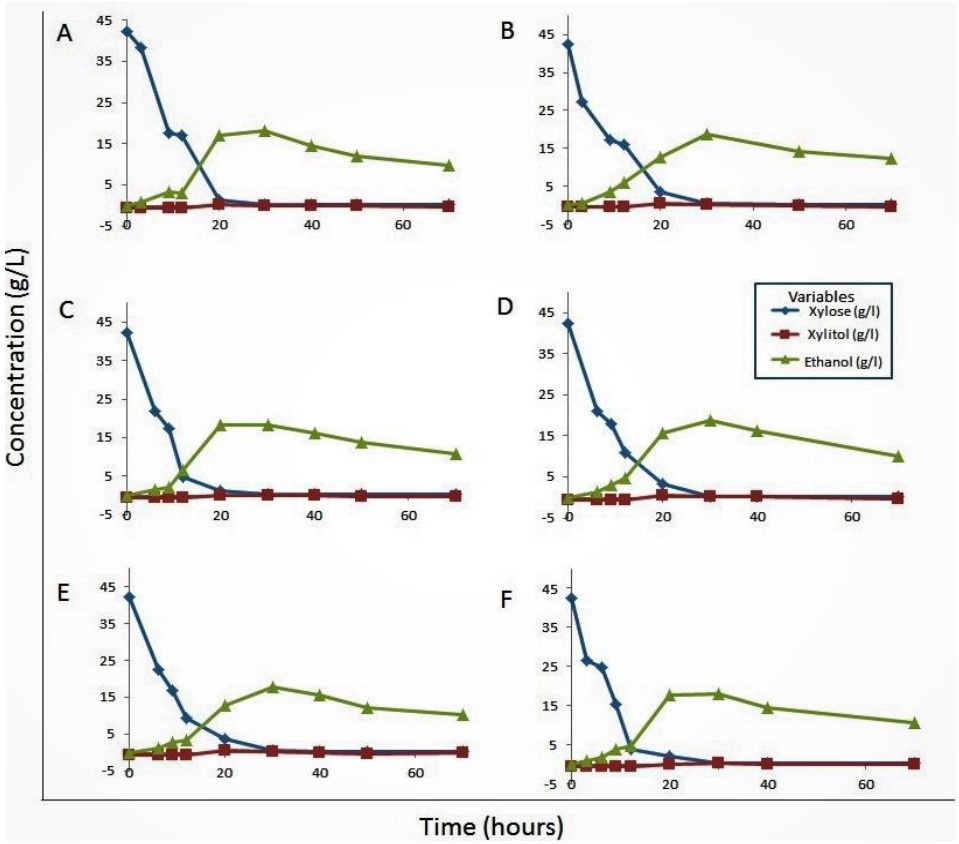

Figure 3. Fermentation kinetics in media containing 4% of xylose as a carbon source at 30°C and 120 rpm, where (A) P16-1.1 ethanol production peak - 18.04 $g.L^{-1}$, (B) G13-2.1 ethanol production peak - 18.85$g.L^{-1}$, (C) G13-3.8 ethanol production peak - 18.40$g.L^{-1}$, (D) G14-1.8 ethanol production peak - 18.68$g.L^{-1}$, (E) G14 -2.2 ethanol production peak - 17.85$g.L^{-1}$ and (F) G18-3.7 ethanol production peak - 17.92 $g.L^{-1}$.

Table 4. Fermentation parameters - comparison between fermentation in media with 4% of xylose and co-fermentation in medium with xylose 4% and glucose 2%.

Strains	Xylose concentration (%)	Glucose concentration (%)	Ethanol (g.L-1)	Xylitol (g.L-1)	$Y^1_{P/S}$ (g/g)	$Y^2_{P/S}$ (g/g)	Q^1_P (g/l.h)	Q^2_P (g/l.h)	η^1 (%)	η^2 (%)	Y (%)	Peak of production (hours)
P8-2.12	4	0	2.09	12.25	-	0.39	-	0.64	-	43.19	76.49	20
	4	2	6.51	10.59	-	0.23	-	0.15	-	24.90	99.62	70
P16-1.1	4	0	18.04	ND	0.43	-	0.60	-	82.25		99.19	30
	4	2	22.87	ND	0.47	-	0.76	-	82.25		99.35	30
P19-1.1	4	0	1.52	21.66	-	0.59	-	0.56	-	64.47	88.80	40
	4	2	5.02	16.76	-	0.13	-	0.87	-	14.58	75.51	20
P21-1.7	4	0	2.04	19.20	-	0.53	-	0.49	-	57.80	88.12	40
	4	2	7.28	11.80	-	0.26	-	0.18	-	28.72	95.71	70
P21-2.1	4	0	5.22	19.14	-	0.59	-	0.66	-	64.46	78.78	30
	4	2	6.03	17.99	-	0.43	-	0.37	-	46.40	88.81	50
P22-2.19	4	0	ND	13.60	-	0.44	-	0.20	-	47.96	76.15	70
	4	2	ND	ND	-	-	-	-	-	1.07	63.62	70
P22-2.20	4	0	ND	17.57	-	0.59	-	0.36	-	64.27	72.71	50
	4	2	ND	ND	-	-	-	-	-	8.04	18.06	70
P22-3.18	4	0	ND	22.10	-	0.54	-	0.32	-	58.65	99.57	70
	4	2	7.15	13.39	-	0.32	-	0.20	-	35.42	87.51	70
G7-1.4	4	0	1.94	20.01	-	0.58	-	0.69	-	62.89	84.28	30
	4	2	3.31	17.40	-	0.37	-	0.26	-	40.20	99.25	70
G13-2.1	4	0	18.85	ND	0.45	-	0.63	-	85.94	-	99.18	30
	4	2	23.81	ND	0.49	-	0.79	-	93.84	-	99.03	30
G13-3.8	4	0	18.40	ND	0.44	-	0.61	-	83.91	-	99.15	30
	4	2	23.69	ND	0.49	-	0.79	-	93.30	-	99.10	30
G14-1.8	4	0	18.68	ND	0.44	-	0.62	-	85.03	-	99.32	30
	4	2	24.70	ND	0.51	-	0.82	-	97.46	-	98.93	30
G14-2.2	4	0	17.85	ND	0.42	-	0.59	-	81.63	-	98.87	30
	4	2	23.95	ND	0.49	-	0.80	-	94.57	-	98.86	30
G18-3.7	4	0	17.92	ND	0.43	-	0.59	-	81.74	-	99.11	30
	4	2	24.26	ND	0.50	-	0.81	-	95.44	-	99.20	30

$Y^1_{P/S}$ = ethanol yield, $Y^2_{P/S}$ (g/g) = xylitol yield, Q^1_P = ethanol productivity, Q^2_P = xylitol productivity, η^1 % = ethanol fermentation efficiency, η^2 % = xylitol fermentation efficiency, Y% = xylose consumption, ND = not detected.

stress conditions imposed, and that seems to be a common condition for wild strains (Slininger et al., 2015).

The production of toxic compounds during pretreatment of lignocellulosic biomass negatively affects yeast growth and fermentation capacity, but removal of these compounds greatly increases the cost of ethanol production. Dubey et al. (2016) reaffirm the importance of these toxic compounds and, according to these authors, strains with greater tolerance to furfural and 5-hydroxymethyl furfural (5-HMF) would make the production of lignocellulosic ethanol economically viable. In the cited research, these authors described a wild strain with higher fermentation performance in the presence of 0.5 $g.L^{-1}$ of furfural and 7.6 $g.L^{-1}$ of 5-HMF compared to an industrial strain and a laboratory strain, demonstrating interest in searching for wild isolates with the appropriate profile.

Kumari and Pramanik (2012) selected mutant yeasts that showed good tolerance to high temperatures and ethanol. They reported that both growth and ethanol production processes in xylose fermenting yeasts were strongly inhibited at an initial vanillin concentration of 1.0 $g.L^{-1}$. Considering vanillin, furfural and acetic acid, the acetic acid showed the least toxic effect in all strains evaluated. This differed from our results, in which growth was not observed on the plate containing 1% acetic acid, except in the G7-1.4 isolate with 0.5% acetic acid. Eleven of the isolates showed growth against 0.2 $g.L^{-1}$furfural and all the isolates were to some degree inhibited by furfural at concentrations of 1.2, 1.0 and 0.5$g.L^{-1}$. Regarding supplementation with vanillin, only the isolates P8-2.12, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 did not show growth on the plates with 8 µmol L^{-1} vanillin (Table 2).

Molecular identification was done by the amplification of the regions ITS1 and ITS4 which are recommended universal primers for fungi identifications (Trost et al., 2004). The G7-1.4 isolate was potentially a yeast species not previously described from the *Candida* genus. *Candida* species have not been extensively reported as fermentative yeasts for industrial utilization such as the production of bioethanol nor in the production of other useful organic compounds except as causal agents of human diseases (Ebabhi et al., 2013). The yeasts that were identified as belonging to the *Candida* genus (Table 3) also did not produce ethanol, but they were capable of producing xylitol.

Morais et al. (2013) reported for the first time the conversion of D-xylose to ethanol by the yeasts *Schwanniomyces polymorphus* and *Wickerhamomyces pijperi*. In this study, isolates that were identified as *Schwanniomyces* sp. did not produced ethanol, but produced xylitol instead.

The isolated species found here differed from other studies, such as in Rivera et al. (2009). These authors isolated 403 yeasts from beetles (Coleoptera: Dendroctonus). The yeasts were isolated from the gut, ovaries, eggs and feces of insects collected from pines at 34 sites in Mexico, Guatemala and the USA. The yeasts were related to three genera: *Candida* species (*C. ernobii*, *C. piceae*, *C. membranifaciens*, *C. lessepsii*, *C. arabinofermentans* and *C. oregonensis*), *Pichia* spp. (*P. americana*, *P. guilliermondii*, *P. scolyti*, *P. mexicana*, *P. glucozyma* and *P. canadensis*) and *Kurashia* spp. (*K. capsulata* and *K. cf. molischiana*). Studies of the association between beetles and galleries conducted in China to investigate the fungal community of Chinese white pine beetles (*Dendroctonus armandi* Tsai and Li, Scolytidae) revealed that yeasts of the genus *Candida* predominated both in the insect and in its galleries (Hu et al., 2015).

In our research, isolates with high homology with the strain *S. passalidarum* obtained higher yields of ethanol in media containing 4% of xylose. Previous research (Cadete et al., 2009; Hou, 2012) has shown that all species of the *Spathaspora* clade isolated from decomposing wood trunks or insects associated with this substrate have converted xylose into ethanol more efficiently than the species of reference *Pichia stipitis*.

Cadete et al. (2016) classified some species of *Spathaspora* as ethanol producers and xylitol producers, according to the main product of xylose metabolism. In their research, ethanol was the main product for *S. passalidarum*. Among the xylitol producers, the *S. roraimanensis* species had higher production, with this being the physiological characteristic associated with the biochemical activity of xylose reductase (XR). The ethanol producers such as *S. passalidarum* revealed XR activities with both NADH and NADPH as cofactors. Xylitol producers had strictly NADPH-dependent XR activity. Considering this, in our research, the isolates P16-1.1, G13-2.1, G13-3.8, G14-1.8, G14-2.2 and G18-3.7 can be considered ethanol producers and the isolates P8-2.12, P19-1.1, P21-1.7, P21-2.1, P22-2.20, P22-3.18 and G7-1.4 can be considered xylitol producers.

According to Hou (2012), under aerobic conditions, glucose and xylose consumption occur simultaneously, which does not occur under anaerobic conditions, where xylose consumption begins after glucose depletion. It suggests that *S. passalidarum* may use different xylose transport systems under anaerobic and aerobic conditions.

Cadete et al. (2016), under severe oxygen limitation conditions, obtained ethanol production above 20 $g.L^{-1}$ for *S. passalidarum*. Compared with this study, the isolates P16-1.1 (18.04 $g.L^{-1}$), G13-2.1 (18.85 $g.L^{-1}$), G13-3.8 (18.40 $g.L^{-1}$), G14-1.8 (18.68 $g.L^{-1}$), G14-2.2 (17.85 $g.L^{-1}$) and G18-3.7 (17.92 $g.L^{-1}$) were efficient in ethanol production (Figure 3) and better results was obtained when co-fermentation was carried out (up to 24.7 $g.L^{-1}$). The peak of production was 30 h for both fermentation and co-fermentation. The fermentation efficiency (η%) for these isolates varied between 81 and 85.94%. During co-fermentation, a variation of 89-95.44% was obtained

(Table 4), which seems promising. Considering the results of Cadete et al. (2012), who, with *S. passalidarum* in a medium with D-xylose (50 $g.L^{-1}$) as the exclusive carbon source, obtained the maximum ethanol production in 24 h at a temperature of 30°C, with ethanol production ranging from 15 to 18 $g.L^{-1}$ (Q_P = 0.6 to 0.75 $g.L.h^{-1}$) and fermentation efficiency of approximately 70%. Khoja et al. (2015) found that the optimum yield was achieved in 34°C for *Zymomonas mobilis* with the bioethanol yield being 8.0% (v/v) with a fermentation efficiency of 88.96%. These authors observed that *S. cerevisiae* was suitable for low temperature process while *Z. mobilis* could be used in regions having an elevated-temperature process. In another study, Chibuzor et al. (2016) examined the production of bioethanol from cassava peels and found that the combination of *Rhizopus nigricans*, *Spirogyra africana* and *S. cereviceae* could be suitable for ethanol production, where they obtained the highest ethanol yield of 14.46 g/cm^3 and a concentration of 38% (v/v).

Strains with this profile, as recently reported by Slininger et al. (2015), may help techniques to force the evolution of these wild strains. These authors describe a significant improvement of the strain *Scheffersomyce stipitis* NRRL Y-7124, both for the quantitative ethanol production (55-60 $g.L^{-1}$ in the modified strain against 40-45 $g.L^{-1}$ in the parent strain) and for tolerance to toxic agents.

Regarding the xylitol producers, isolate P8-2.12 - *S. roraimanensis* was not efficient when compared to that reported by Cadete et al. (2016) for the same species. Our isolate produced 12.25 $g.L^{-1}$ of xylitol. The *S. roraimanensis* studied by these authors produced 27.4 $g.L^{-1}$. However, isolates P19-1.1, P21-1.7, P21-2.1, P22-2.19, P22-2.20, P22-3.18 and G7-1.4 showed good xylitol production with 21.66, 19.20, 19.14, 13.60, 17.57, 22.10 and 20.01 $g.L^{-1}$ respectively, but during the co-fermentation test good xylitol production was not obtained, in some cases not having any xylitol production (Table 4). Ping et al. (2013) determined the production of xylitol by *Candida tropicalis* using non-detoxified corn hemicellulose hydrolyzate, obtaining a maximum concentration of 38.8 $g.L^{-1}$ of xylitol. Among the species studied by Junyapate et al. (2014), *C. tropicalis* produced the highest concentration of xylitol (7.2 $g.L^{-1}$). Cadete et al. (2015) evaluated the production of xylitol from 50 $g.L^{-1}$ of xylose from five strains of the species *Cyberlindnera xylosilytica sp*. The maximum production of xylitol was reached in 72 h, which was practically the same among the isolates, with a mean production of 33.02 $g.L^{-1}$. It is considerably higher than the profile of the isolates of the study.

Three isolates (P16-1.1, P21-1.7 and P21-2.1) tolerant to several stress conditions were obtained which are normally encountered in the process of obtaining second-generation ethanol. Good comparative ethanol production from xylose was observed, with apparent potential for larger scale trials or tools for altering these wild strains.

These three isolates, with better performance, were curiously all isolated from the gut of the beetle *Popilius marginatus* (Percheron, 1835). Isolate P16-1.1, identified as *S. passalidarum* had, in particular, considerable potential for subsequent research. This study adds some information and possibly relevant isolates in the search for economically viable production of 2G ethanol, thus reaffirming the interest in the sampling of natural environments to obtain wild strains for bioethanol production. Furthermore, the results suggest that yeast isolated from insect galleries have a higher xylolytic capacity than yeasts isolated from the gut of beetles.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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