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Isolation and characterization of yeasts capable of efficient utilization of hemicellulosic hydrolyzate as the carbon source

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ABSTRACT. Few yeasts have shown the potential to efficiently utilize hemicellulosic hydrolyzate as the carbon source. In this study, microorganisms isolated from the Manaus region in Amazonas, Brazil, were characterized based on their utilization of the pentoses, xylose, and arabinose. The yeasts that showed a potential to assimilate these sugars were selected for the better utilization of lignocellulosic biomass. Two hundred and thirty seven colonies of unicellular microorganisms grown on hemicellulosic hydrolyzate, xylose, arabinose, and yeast nitrogen base selective medium were analyzed. Of these, 231 colonies were subjected to sugar assimilation tests. One hundred and twenty five of these were shown to utilize hydrolyzed hemicellulose, xylose, or arabinose as the carbon source for growth. The colonies that showed the best growth (N = 57) were selected, and their internal transcribed spacer-5.8S rDNA was sequenced. The sequenced strains formed four distinct groups in the phylogenetic tree, and showed a high percentage

of similarity with *Meyerozyma caribbica*, *Meyerozyma guilliermondii*, *Trichosporon mycotoxinivorans*, *Trichosporon loubieri*, *Pichia kudriavzevii*, *Candida lignohabitans*, and *Candida ethanolica*. The discovery of these xylose-fermenting yeasts could attract widespread interest, as these can be used in the cost-effective production of liquid fuel from lignocellulosic materials.

Key words: Yeasts; Pentoses; Hemicellulosic hydrolyzate; Internal transcribed spacer-5.8S rDNA

INTRODUCTION

Yeasts that can ferment both hexose and pentose are important for the large-scale production of ethanol from hemicellulose. Agro-industrial wastes resulting from the processing of raw materials are rich in these carbohydrate polymers, which can be exploited for bioconversion by microorganisms. The plant biomass must be hydrolyzed to ensure the bioavailability of these carbohydrates and a viable microbial metabolic process. This process promotes the disarrangement of polymers, allowing microbial enzymes to access polysaccharide chains (facilitating the releasing of oligomers and monomers) (Betancur and Pereira, 2009, 2010). Ethanol has been the major target of research for the development of new and efficient methodologies for the fermentation of hexose and pentose; however, the microorganisms used for the fermentation of ethanol, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, do not metabolize pentoses. One of the major obstacles preventing the large-scale production of ethanol from hemicellulose is the exorbitant cost of production. All sugars present in lignocellulosic material, including D-glucose, D-mannose, D-galactose, D-xylose, and L-arabinose, must be fermented for cost-effective ethanol production. Therefore, a critical feature of any industrial producer of ethanol from lignocellulose is its ability to effectively ferment pentose sugars (Watanabe et al., 2012).

A majority of research on the utilization of the hemicellulosic fraction of biomass has focused on the identification of microorganisms with specific characteristics required for this application. The microorganisms used in these fermentations must efficiently utilize pentose sugars as the major substrate for conversion to useful products, in addition to being tolerant to inhibitors generated by the pretreatment (furfural, hydroxymethylfurfural, and acetic acid) and the industrial reactor environment (Hahn-Hägerdal et al., 2006). However, *S. cerevisiae* lacks the ability to ferment pentose sugars. In addition, very few natural xylose-fermenting yeasts possess this ability; for example, *Pichia stipitis* naturally ferments xylose to ethanol (Lin et al., 2012), whereas *Meyerozyma* (*Candida*) *guilliermondii* naturally ferments xylose to xylitol (Mussatto et al., 2006; Coda et al., 2013). These yeast strains must be attuned to grow in environments containing microbial growth inhibitors (resulting from hydrolytic processes). In this context, the isolation of new wild yeast strains is a viable alternative to improve the performance of the target microorganisms. Environments that naturally contain hydrolyzed lignocellulosic material, such as the gut of wood-feeding insects or the gastrointestinal tract of other animals that feed on plant biomass (such as ruminants), are potential sources of microorganisms that are already adapted to these sugars. Therefore, this study attempted to isolate wild yeasts growing in lignocellulosic environments, which were naturally adapted for

pentose metabolism, which could then be used in order to achieve a better biotechnological utilization of the hemicellulosic biomass fraction.

MATERIAL AND METHODS

Isolation of microorganisms

Seven larvae of the insect order Coleoptera (one belonging to the family Cerambicidae and six to Scarabaeidae) were collected from decaying tree trunks in Manaus, Amazonas region in Brazil. The digestive tracts of the larvae were cut longitudinally to expose the content; these were then incubated in culture medium containing hemicellulosic hydrolyzate, xylose, and arabinose as the carbon source (1%), and 0.67% yeast nitrogen base (YNB; Difco-Becton Dickinson and Company). The pH of this medium was adjusted to 5.5. Feces and salivary samples from the oral cavity of cattle were collected and inoculated into the hemicellulosic hydrolyzate liquid medium; this was then incubated for 3 h, 10 days, and 45 days at 30°C. Subsequently, 100 µL aliquots of the liquid incubation inoculum were plated onto solid medium at 28°C for 24 h. The isolated colonies were separated based on macroscopic morphological distinctiveness, colored with blue lactophenol, and observed under a 400X optical microscope. The colonies selected from the sugar assimilation tests, as well as those adapted to the 5% xylose medium, as described below, were preserved in 20% glycerol and stored in a freezer at -80°C.

Sugar assimilation test

The assimilation test was performed using a modified version of the replica plate technique described by Barnet et al. (1990). A culture plate matrix with the original source of inoculants was prepared for this purpose. A Petri plate (140 x 15 mm) was filled with a 5-mm layer of solid medium (1% carbon source and 0.67% YNB). A grid was laid over this medium containing 96 inoculants. Xylose and arabinose (pentoses), glucose, mannose, and galactose (hexoses), lactose and sucrose (disaccharide), and hydrolyzed hemicellulose (with 1% total reducing sugar) were used as the carbon sources. Growth was assessed 48 h after incubation at 28°C.

Genotypic characterization

DNA was extracted from cultures grown on yeast extract, peptone, dextrose, and YPD medium (Difico), as described by Harju et al. (2004). Two oligonucleotide fungal primers were used for the amplification [internal transcribed spacer (ITS): ITS1, 5'-CGT AAC AAG GTT TCC GTA GG-3' and ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3']. The amplified region comprised a section of the rDNA 18S region, the noncoding ITS-1 and ITS-2 regions, the complete 5.8S region, and a section of the 26S region. The primers were synthesized by Life Technologies (Invitrogen). The PCR was performed according to the methodology described by Henry et al. (2000). Amplicons were sequenced with ITS1 primers on a Perkin-Elmer/ABI 373 DNA sequencer supplied by Life Technologies (Applied Biosystems) at the Human Genome Research Center (http://genoma.ib.usp.br/), using protocols supplied by the manufacturer. The sequences were compared against those available in the National Center

for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/blast. A phylogenetic tree was constructed using a hierarchical method for multiple alignments (neighbor-joining), based on the sequences and strain patterns obtained. Phylogeny was tested with 1000 bootstrap replications using the Molecular Evolutionary Genetics Analysis (MEGA) 5.0 software (Tamura et al., 2011), in order to identify the relationships among strains.

RESULTS

Two hundred thirty seven yeast strains were isolated from all biological sources; 127 of these were from insects, 74 from cattle, and 36 were isolated from cattle dung. Of the yeast isolates, 125 produced biomass using the three carbon sources of interest (xylose, arabinose, and hemicellulosic hydrolyzate). The profiles of the tested assimilating yeasts are summarized in Table 1.

Table 1. Yeasts classified based on the carbon source assimilation profile.

Carbon source			Number of yeast strains isolated
Xylose	Arabinose	Hydrolyzed hemicellulose	
+	+	+	125
+	+	-	1
+	-	+	51
+	-	-	0
-	+	+	6
-	+	-	0
-	-	+	47
-	-	-	0

"+" = Able to grow; "-" = Unable to grow.

A comparison of the sequences in GenBank verified similarities to seven different species (forming four groups). The distribution of the yeasts selected, classified according to the isolation source, is summarized in Table 2.

Table 2. Species from the National Center for Biotechnology Information GenBank database showing a high degree of sequence similarity with the colonies that were sequenced and classified based the source of isolation.

Species	Source of isolation and identified yeasts						
	Insect gut (Cerambycidae sp)	Insect gut (Scarabaeidae sp)	Insect gut (*Calosoma* sp)	Cattle saliva	Cattle dung	Cattle dung (rectum)	Subtotal (by species)
Candida ethanolica	0	0	0	0	1	0	1
Candida lignohabitans	9	2	0	0	0	0	11
Meyerozyma caribbica	11	17	0	1	1	0	30
Meyerozyma guilliermondii	2	2	2	2	1	0	9
Pichia kudriavzevii	0	0	0	1	1	0	2
Trichosporon loubieri	0	0	0	3	0	1	4
Trichosporon mycotoxinivorans	1	1	2	0	0	0	4
Total	23	22	4	7	4	1	61

Species deposited in GenBank with a high degree of similarity with the strains isolated and characterized in this study.

The 5.8S-ITS regions in the isolates and other strains identified from the GenBank database were compared by rRNA gene sequence comparison. High sequence similarities were observed among these regions, confirming the identification of these isolates to a species level (by ITS1 and ITS4 sequence analysis), as shown in Figure 1. In summary, all reference strains and isolates were correctly identified by ITS sequencing, yielding an identification rate of 100%.

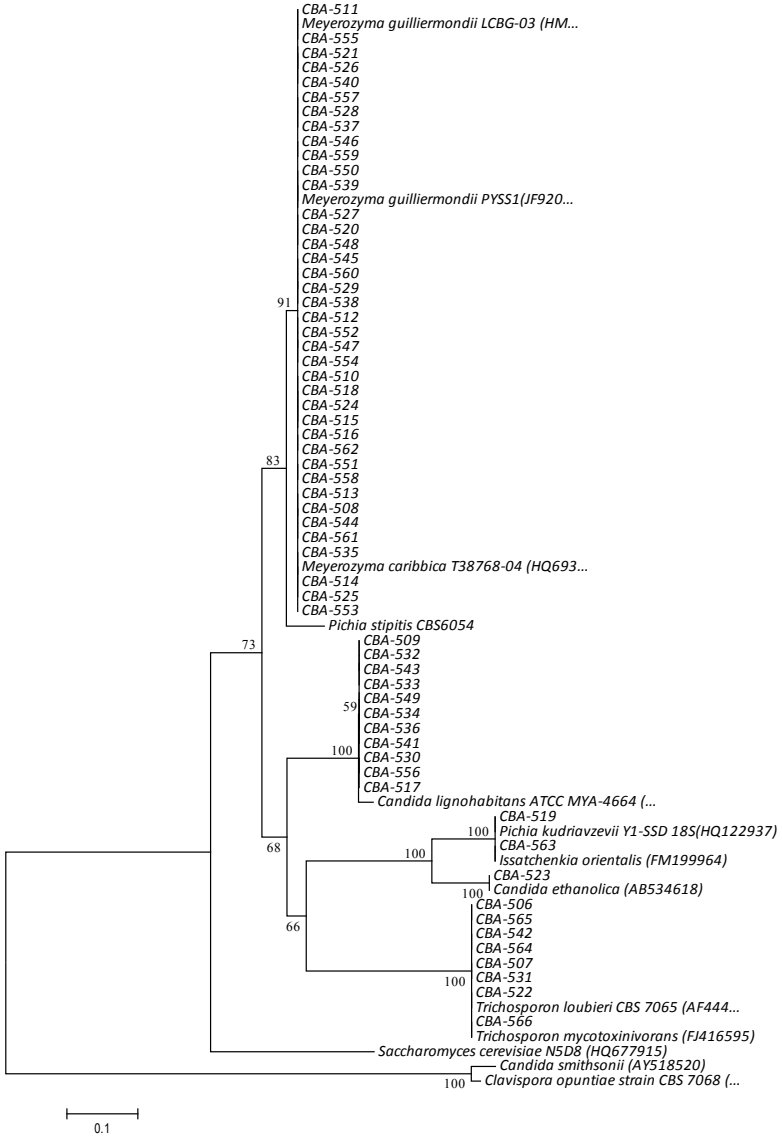

Figure 1. Phylogenetic tree constructed using the 5.8S rDNA internal transcribed spacer (ITS) region sequence data, depicting the relationship between the isolated yeast strains and the strain patterns. Numbers on the branches indicate bootstrap values. The scale bar represents a genetic distance of 0.01 (Bar = 1 nucleotide change per 100 nucleotides).

DISCUSSION

Most yeasts selected were grouped under the *M. guilliermondii* and *Meyerozyma caribbica* species, whose sequences are deposited in GenBank. Thirty individuals were isolated in this study. The percentage of identity among the sequences deposited varied between 99 and 100%. Genus *Meyerozyma* is a reclassification of the genus *Pichia*; therefore, until recently, these microorganisms were classified as *Pichia guilliermondii* and *Pichia caribbica*. These results agree with those of other studies where *M. caribbica* and other species associated with the *M. guilliermondii* phylogenetic group were isolated from the gut of members of the order Coleoptera (Rao et al., 2007; Hammons et al., 2009; Rivera et al., 2009). Some studies have also reported the isolation of *M. caribbica* from other sources, such as the digestive tract of *Aedes aegypti* (Gusmão et al., 2007) and fermented beverages (Papalexandratou and Vuyst, 2011; Hidalgo et al., 2012). *M. guilliermondii* has also been isolated as an endophytic yeast (Zhao et al., 2010) from bovine milk (Zaragoza et al., 2011) and the genital tract of female camels (Shokri et al., 2010). These species have been cited in literature as producers of xylitol (Rao et al., 2007). *M. caribbica* has also been used in the saccharification of hydrolyzed hemicellulose, increasing the yield of fermentable sugars (Saucedo-Luna et al., 2011). A phylogenetic group was formed by six of the yeasts isolated in this study, two sequences identified in another study, and species deposited in GenBank (*Trichosporon loubieri* - NR_073253 and *T. mycotoxinivorans* FJ416595). The sequences related to yeast CBA-522 (isolated from feces) and yeasts CBA-566, CBA-564, and CBA-565 (isolated from cattle saliva) showed 99% identity with *T. loubieri*. The proximity of the yeast strains isolated in this study to *T. mycotoxinivorans* varied between 99% (CBA-506, CBA-531) and 100% (CBA-507) identity. These *Trichosporon* species have been previously described as pathogenic yeasts (Girmenia et al., 2005; Hickey et al., 2009; Sharman et al., 2010; Gabriel et al., 2011). These species share a close similarity in the phylogenetic tree based on the D1/D2 regions of the 26S ribosomal DNA, and the (approximately) 600-bp 18S ribosomal DNA. *T. mycotoxinivorans* was distinguished from *T. loubieri* based on its ability to assimilate inulin, and inability to grow at 40°C. *T. mycotoxinivorans* can be used to suppress the effect of mycotoxins, such as ochratoxin A and zearalenone, which cause damage to livestock, such as pigs and chickens. The addition of this yeast to broiler diets resulted in a decrease of toxins in the serum of these animal (Molnar et al., 2004; Politis et al., 2005; Hanif et al., 2011). *T. mycotoxinivorans* has shown great biotechnological potential in the saccharification of sugarcane bagasse hemicellulosic hydrolysate, and biomass production using this substrate as the sole carbon source (Matos et al., 2012). Eleven of the isolated species formed a group with *Candida lignohabitans*. This yeast has been isolated from the gastrointestinal tract of insects of the order Coleoptera, and formed a group together with *Candida shehatae* and *Sugiyamaella* sp (Houseknecht et al., 2011), isolated from hollow portions of cotton stems (Maganti et al., 2012) and forest plants (Kurtzman, 2007). These authors have described the isolation of the species, while not commenting on its physiological characteristics or biotechnological applications. Only one sequence (CBA-523) showed a 100% identity to *Candida ethanolica*. Two sequences (CBA-519, CBA-563) were grouped with *P. kudriavzevii* (*Issatchenkia orientalis*). Thermotolerant *Pichia kudriavzevii* has previously been reported to produce cellulosic ethanol (hydrolyzed ligno-enriched glucose) under conditions of stress, resulting from high temperature (40°-43°C) or high concentrations of sulfuric acid and salt (calcium sulfate generated by the neutralization

of hydrolyzate). This type of yeast was isolated from various sources, such as cotton stalks and straw (containing lignocellulosic hydrolyzates), subjected to different treatments, including enzymatic hydrolysis with cellulases. This strain showed better ethanol production (up to 200%) under conditions of high temperature, compared to *S. cerevisiae* (Dhaliwal et al., 2011; Isono et al., 2011; Kaur et al., 2011; Oberoi et al., 2012). Fonseca (2009) have reported to use of a strain of *I. orientalis* to reduce the toxic components of hemicellulosic hydrolyzates.

The use of hemicellulosic hydrolyzate (bagasse from sugarcane) as a selective medium has led to the isolation of yeast strains capable of utilizing this culture medium as the carbon source for biomass production. This reflects a relative tolerance to inhibitors of microorganisms generated by hydrolysis of this type of plant biomass. Several isolates were able to assimilate both hexoses and pentoses (such as xylose and arabinose), sugars that are normally available in hydrolyzates derived from lignocellulosic material. This feature is important for the better utilization of sugars present in plant biomass. After being imported to the microbial cell, the sugars may be applied to several metabolic pathways, ranging from fermentation (to xylitol and ethanol) and biomass accumulation, to production of other molecules of interest. The identification of yeast isolates showed the isolation of strains with the biotechnological potential to exploit lignocellulosic subproducts from selected sources; this contributed to the construction of a database of microbial cells that could be exploited to add value to agro-industrial waste, using carbohydrates that could be metabolized into products of interest.

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