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RESEARCH ARTICLE

Cultivation of *Trichosporon mycotoxinivorans* in sugarcane bagasse hemicellulosic hydrolyzate

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Abstract

Background: The yeast strain IB09 was isolated from the gut of *Calosoma* sp. (Carabidae, Coleoptera, Insecta) that were collected in the central Amazon rainforest. First, tolerance of the strain to ethanol and heat was tested. Then, IB09 was cultivated in a medium using sugarcane bagasse hemicellulosic hydrolyzate as a carbon source, and cell growth (OD_{600}), specific growth rate (μ_{MAX}, h^{-1}), biomass yield (Y_B, $g.g^{-1}$) and relative sugar consumption (RSC, %) were evaluated. Taxonomic identification was determined by sequencing the ITS1 region of IB09 and comparing it to sequences obtained from the GenBank database (NCBI). **Results:** IB09 showed both ethanol tolerance and thermotolerance. Relative sugar consumption indicated that IB09 was able to perform saccharification of sugarcane bagasse hemicellulosic hydrolyzate, increasing the total reducing sugar concentration by approximately 50%. The μ_{MAX} value obtained was 0,20, indicating that cell growth was slow under the assessed conditions. Biomass yield was 0,701 g per g of consumed sugar, which is relatively high when compared with other findings in the literature. After 120 hrs of cultivation, 80,1% of total reducing sugar had been consumed. Sequencing of the ITS1 region identified IB09 as *Trichosporon mycotoxinivorans*. **Conclusion:** This is the first report to document this species in the central Amazon rainforest at this host. *Trichosporon mycotoxinivorans* has great biotechnological potential for use in the saccharification of sugarcane bagasse hemicellulosic hydrolyzate and for biomass production with this substrate as carbon source.

Keywords: Amazon strain, biomass production, biotechnological potential, saccharification

INTRODUCTION

The increasing worldwide demand for oil and environmental sustainability has created the necessity for alternative fuels. Biofuels are attractive alternatives that have economic and environmental viability (Cockerill and Martin, 2008). The Brazilian biofuels program uses sugarcane juice as the main substrate for alcoholic fermentation. In Brazil, sugarcane production has increased by approximately 3.7% in the last thirty years (Goldemberg, 2008), and there has been an increase in waste production. In 2009, approximately 173,5 million tons of sugarcane bagasse were produced (IBGE, 2009). Currently, an increase in ethanol production has been achieved without increasing deforestation. In this

context, biomass constitutes an abundant carbon source for fermentation, thereby increasing the biofuels production yield per cultivated area (Hahn-Hägerdal et al. 2007).

Sugarcane bagasse is the most important and abundant agricultural waste in Brazil. It can be burned to generate thermoelectric energy (Goldemberg, 2008), or used for alkaline, enzymatic or acid hydrolysis, releasing the sugars available for alcoholic fermentation (Saha, 2003). When compared to other substrates, sugarcane bagasse hemicellulosic hydrolyzate (SBHH) presents the highest total reducing sugar (TRS) yield (Hernandez-Salas et al. 2009), high availability and low cost (Huang et al. 2011).

In addition to releasing fermentable sugars, pretreatment of sugarcane bagasse with diluted acid leads to the generation of unwanted microbial growth inhibitors, and some detoxification methods must be employed. There are many techniques that can be used for detoxification, including activated charcoal adsorption, ionic-exchange resins, evaporation and pH adjustment by adding hydroxides. However, each of these procedures has its limitations and may reduce TRS yield. So, detoxification of SBHH using simple techniques results in a high concentration of microbial growth inhibitors that can act as high selectivity media for the selection of microorganisms with high tolerance. Another alternative is biological detoxification, or biodetoxification, which uses microorganisms for inhibitors consumption. Biodetoxification offers an attractive alternative because it can be performed simultaneously with fermentation (Hou-Rui et al. 2009).

Yeasts that are able to use SBHH as their sole carbon source and that can assimilate D-xylose have high biotechnological potential. They can be used for many purposes, such as biodetoxification, alcoholic fermentation, protein supplement and biodiesel production (Huang et al. 2011).

Here, a D-xylose assimilating yeast strain (named IB09) was isolated, using SBHH as a selective medium, from the gut of a xylophage beetle *Calosoma sp.* (Carabidae Coleoptera, Insecta), collected in the central Amazon rainforest. The aim of this work was to determine the biotechnological potential of IB09 by evaluating its thermotolerance, ethanol tolerance, cell growth, TRS capacity consumption and biomass yield when cultivated in SBHH. Furthermore, it was identified using molecular biology tools.

MATERIALS AND METHODS

The IB09 strain was isolated from specimens of *Calosoma sp.*, that were collected in tree hollows in the central Amazon rainforest (03º05'56.25"S 59º58'12.84" W). This genus is commonly carnivorous, but some species may present xylophages habits during larval or adult phase (Lima, 1945). The organisms were washed in 70% ethanol for one minute for surface decontamination. After digestive tract dissection and fragmentation, intestinal contents were suspended in SBHH (TRS = 10 g.L^{-1}), and incubated for 24 hrs at 35ºC. One hundred microliters of this suspension was then plated in isolation medium, composed of SBHH (TRS = 10 g.L^{-1}) and yeast nitrogen base (YNB; 6.7 g.L^{-1}). The yeast was selected according to its ability to assimilate D-xylose.

The yeast strain was maintained in sterilized distilled water (room temperature) and reactivated in Petri dishes containing Sabouraud Agar (peptone 10 g.L^{-1}, glucose 40 g.L^{-1}) at 30ºC for 72 hrs. The pre-inoculum was prepared by cultivating a loop of IB09 in Erlenmeyer flasks (250 mL) containing 100 mL of YUKX medium, which consisted of yeast extract (1.5 g.L^{-1}), urea (1.25 g.L^{-1}), KH_2PO_4 (1.1 g.L^{-1}) and D-xylose (50 g.L^{-1}). The flasks were incubated at 35ºC with shaking at 120 rpm for 72 hrs.

Thermotolerance was assessed using lethal heat shock, according to Vianna et al. (2008). One milliliter of pre-inoculum was cultivated in Erlenmeyer flasks (125 mL) containing 50 mL of YUKX, at 35ºC with shaking at 120 rpm for 72 hrs. The flasks were then incubated in a 52ºC water-bath for 9 min, and the cell viability was measured.

Ethanol tolerance was assessed using ethanol shock, as described by Ogawa et al. (2000), with some modifications. A loop of IB09 was cultivated in Erlenmeyer flasks (125 mL) containing 40 mL of YPD medium (yeast extract 10 g.L^{-1}, peptone 20 g.L^{-1} and dextrose 20 g.L^{-1}) at 35ºC with shaking at 120 rpm for 72 hrs (*log* phase). After this period, 10 mL of absolute ethanol was aseptically added (final concentration of 20%). The flasks were maintained in stationary cultivation at 35ºC for 7 days. Cell

viability was measured and an aliquot of 100 µL was collected, cultivated in Petri dishes containing YUKX supplemented with agar (15 $g.L^{-1}$) and maintained at 30ºC for 72 hrs. At each 24 hrs time point the plates were analyzed to determine the number of colony forming units ($CFU.mL^{-1}$).

In all assays, cell viability was verified by staining with methylene blue and counting under an optical microscope as described by Vianna et al. (2008).

The SBHH was prepared with 400 mL of diluted sulfuric acid (3%, v/v) and 100 g of dry sugarcane bagasse, at a liquid to solid ratio of 4:1. This mixture was maintained at room temperature for 24 hrs, and autoclaved at 1.1 kgf/cm^2 for 40 min. The liquid phase was separated and solid waste was discarded. The pH (0.8 initial) was elevated to 5.5 upon addition of $Ca(OH)_2$ 1.0 M. The resulting SBHH was used as a carbon source in growth medium (GM), supplemented with yeast extract (1.5 $g.L^{-1}$), urea (1.25 $g.L^{-1}$) and KH_2PO_4 (1.1 $g.L^{-1}$).

The preinoculum was centrifuged for 40 min (4ºC, 3.500 x *g*), and the supernatant was discarded. The sediment was inoculated in Erlenmeyer flasks (125 mL) containing 50 mL of GM. The initial inoculum was approximately 0.125 $g.L^{-1}$. The flasks were maintained at 35ºC with shaking at 120 rpm for 120 hrs.

At four hrs time points, an aliquot of 100 µL was collected and diluted 10 times with sterilized distilled water. Cell growth was measured by determining the optical density at 600 nm (OD_{600}) (Heer and Sauer, 2008). The TRS concentration was assessed using the 3.5-dinitrosalicylic acid method (DNS), as described by Zhao et al. (2008).

Specific growth rate (μ_{MAX}, h^{-1}) was calculated by linear regression of ln (OD/ODi) *versus* time at *log* phase, obtained by the equation: ln (OD/ODi) = μ_{MAX}.t + a. Biomass yield (Y_B, g/g) was calculated verifying the total biomass produced per gram of sugar consumed. Relative sugar consumption (RSC, %) was calculated by assessing the percentage of consumed TRS relative to the initial concentration. All techniques have been performed as described by Duarte et al. (2008).

Taxonomic identification was determined using molecular biology tools. The DNA extraction was performed according to Harju et al. (2004). The DNA was amplified by PCR (White et al. 1990) using the primer ITS1 5'-TCC GTA GGT GAA CCT GCG-3'. The amplified DNA was sequenced, and the generated sequence was edited using BIOEDIT software (Hall, 1999). The edited sequence was submitted to the non redundant nucleotide database at GenBank, and the BLAST program was used for yeast identification (Zhang et al. 2000).

RESULTS AND DISCUSSION

The IB09 strain showed a cellular survival rate of 86.3% in the thermotolerance test, indicating that the yeast was tolerant to lethal heat shock. Vianna et al. (2008) have described two types of heat-shock; non-lethal heat-shock, which subjects yeast to temperature of 52ºC with previous acclimatization at 40ºC for 60 min; and lethal heat shock, which is described in this work. IB09 cell viability was similar to that of the wild-type *Saccharomyces cerevisiae* UFMG-A905 and UFMG-A1007 strains, which have been described as thermotolerant when subjected to non-lethal heat shock. This is an important characteristic of yeasts with biotechnological potential because temperature is a limiting factor for microbial metabolism and it can reduce the yield of the bioprocess. Furthermore, the use of thermotolerant strains can reduce the waste water, that is required for cooling and permit the use of heat to eliminate contaminant microorganisms (Tomás-Pejó et al. 2009).

The IB09 strain was able to grow in YUKX medium (added of Agar, 15 $g.L^{-1}$) after ethanol-shock. Number of CFU was found to be 2.250 $CFU.mL^{-1}$ on average. Upon measurement of cell viability, was found a 53.7% of survival rate, which was similar to that observed by Vianna et al. (2008) for wild-type strains of *S. cerevisiae*. Thus, the IB09 strain can be considered an ethanol-tolerant yeast.

The growth curve of IB09 in GM is shown in Figure 1. Cultivation in GM showed a *lag* phase with 24 hrs. Exponential growth (*log* phase) was observed between 24 and 96 hrs. Stationary phase was observed after 108 hrs of incubation. After 120 hrs, the cell viability of IB09 was 65.5%.

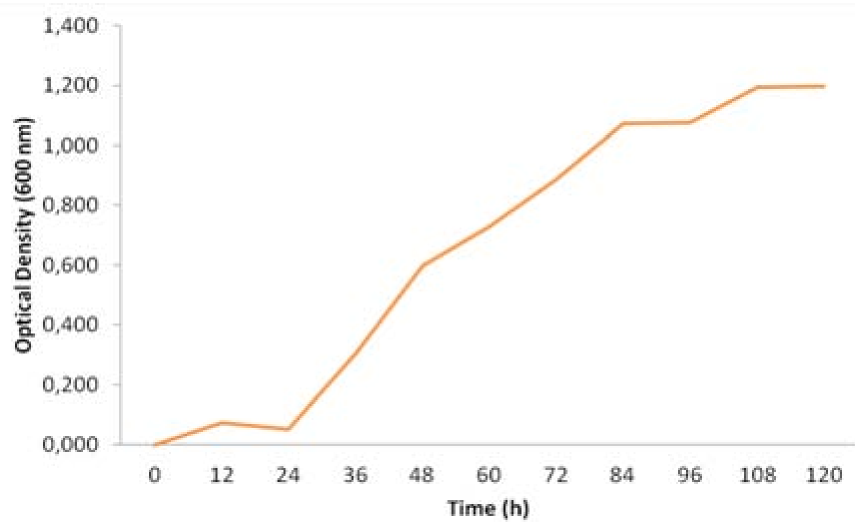

Fig. 1 Growth curve of the IB09 strain cultured in SBHH.

According to White et al. (2008), the acid pretreatment used in this work would result in the release of monosaccharides that would be available for fermentation. Despite this expectation, the TRS measurement indicated that saccharification occurred during the *lag* phase, as shown in Figure 2. The initial TRS concentration (42.5 g. L^{-1}) was increased to 63.4 g.L^{-1} (49.3%) and was probably due to oligosaccharides degradation. This saccharification rate was greater than that obtained by Hernandez-Salas et al. (2009) using commercial enzymatic kits, which obtained only approximately 20% of increasing in the TRS concentration. This result indicates that IB09 is able to perform saccharification. According to Olofsson et al. (2008), yeasts with this ability can perform simultaneous saccharification and fermentation (SSF) processes.

The RSC was calculated based on the highest reducing sugar concentration (63.4 g. L^{-1}), obtained after saccharification. The final concentration of TRS indicated that 80.16% of sugar was consumed. Converti et al. (1999) and Zhao et al. (2008) obtained similar RSC values (78.6% and 85%, respectively) when xylose-fermenting *Pachysolen tannophilus* was cultivated in SBHH.

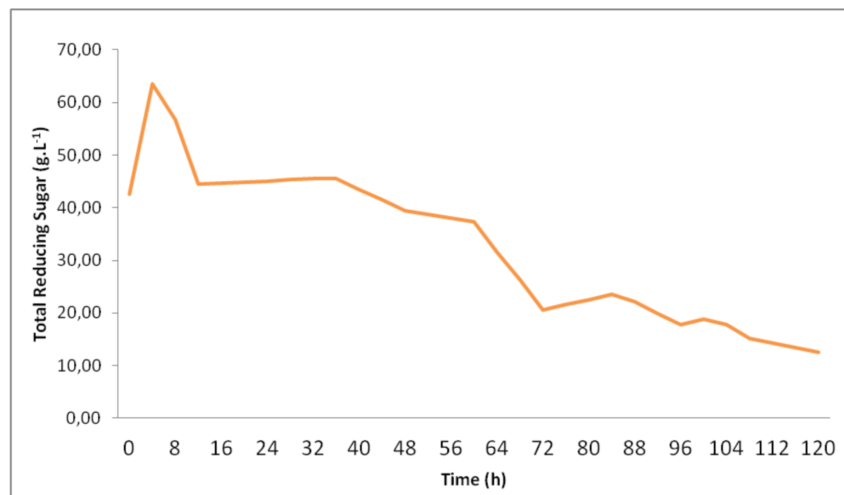

Fig. 2 Saccharification and subsequent sugar consumption by IB09 in SBHH.

The Y_B obtained was 0.701 $g.g^{-1}$, which means that each gram of consumed sugar was converted to 0.701 grams of biomass. This result indicates that there is a great potential for biomass production. The μ_{MAX} value of 0.20 h^{-1}, was relatively low when compared with the results that have been obtained in other studies and indicates that cell growth is slow under assessed conditions. This fact is likely associated with the low biomass of the inocula (Duarte et al. 2008; Yuan et al. 2011).

The pH of GM was elevated from 5.5 to 7.56, indicating that IB09 neutralized the acids that were diluted in the medium. It indicates that IB09 may be able to perform biodetoxification. Similar results have been obtained by Pessoa Jr. et al. (1996) in studies in which *Candida tropicalis* was cultivated in SBHH whereupon the pH was neutralized because acetic acid was fully consumed.

The results of the alignment of the sequenced region with the GenBank database showed that IB09 is a member of the *Trichosporon mycotoxinivorans* species, with 100% of identity (*e-value* = 0 and *query coverage* = 100%). This species was described for the first time by Molnar et al. (2004), isolated from the Australian termite species *Mastotermes darwinienses.* According to the study, *T. mycotoxinivorans* has the ability to neutralize many mycotoxins, and can use many carbon sources, such as D-galactose, D-xylose, L-arabinose and some organic acids. This explains the ability of IB09 to grow in SBHH and to perform its neutralizing functions. Even ethanol could be used as a carbon source, which may explain the ethanol tolerance observed in this work. After one week of growth on Petri dishes containing Sabouraud agar, *T. mycotoxinivorans* IB09 showed a filamentous appearance, which was in agreement with the phenotype described by Molnar et al. (2004).

Abe et al. (2010) have described the presence of *T. mycotoxinivorans* in fresh shavings of palm tissue that were damaged by beetle larvae (Coleoptera: Dryophthoridae) in Japan. It occurrence was also described in grape marc in southern Peloponnese, Greece, by Ntougias et al (2010), which reports it as closely related to an unidentified member of *Trichosporon* clade, isolated from the xylophagous beetle *Phrenapetes bennetti* (Tenebrionidae), collected in Panama by Suh et al. (2005). Hickey et al. (2009) have found *T. mycotoxinivorans* to be a novel respiratory pathogen in patients with cystic fibrosis. Together, these findings suggest a cosmopolitan habitat and opportunist pathogenicity, which has also been described by Chagas-Neto et al (2008) as common features of this genus.

This paper is the first to document the presence of *Trichosporon mycotoxinivorans* in Carabidae gut in the central Amazon rainforest.

CONCLUDING REMARKS

This work is the first to report the presence of *Trichosporon mycotoxinivorans* in Amazon habitats. The organism shows biotechnological potential, as it is able to perform saccharification in SBHH with yields greater than commercial enzymatic kits.

The successful cultivation in SBHH indicated that *T. mycotoxinivorans* IB09 could use SBHH as a carbon source for biomass production, with a yield of approximately 70% and it could also perform pH neutralization.

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