Original Article

ISOLATION, TAXONOMIC IDENTIFICATION AND INVESTIGATION OF THE BIOTECHNOLOGICAL POTENTIAL OF WILD-TYPE *Meyerozyma guilliermondii* ASSOCIATED WITH AMAZONIAN TERMITES ABLE TO FERMENT D-XYLOSE

ISOLAMENTO, IDENTIFICAÇÃO TAXONÔMICA E INVESTIGAÇÃO DO POTENCIAL BIOTECNOLÓGICO DE LINHAGEM SELVAGEM DE Meyerozyma guilliermondii FERMENTADORA DE D-XILOSE ASSOCIADA A CUPINS DA AMAZÔNIA

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ABSTRACT: A wild-type yeast that could ferment D-xylose was isolated from the abdominal content of *Nasutitermes sp.* collected in the Central Amazon rainforest using sugarcane bagasse hemicellulosic hydrolyzate (SBHH) as selective medium. The yeast was identified as *Meyerozyma guilliermondii*. Its ability to ferment D-xylose was assessed using liquid medium containing Durham tubes. A fermentometer assay showed a low ethanol yield using D-xylose as the carbon source. Cell viability after heat shock and ethanol shock was 39.8% and 56.0%, respectively. Cultivation in SBHH (pH = 5.0) showed its capability to perform saccharification of this substrate, increasing total reducing sugar concentration to 42.6%. The log phase was observed between 36 and 108 hours of cultivation with a highest specific growth rate (μ_{MAX}) of 0.10 h^{-1}. After 120 hours, 79.5% of total reducing sugar was consumed giving a biomass yield of 0.52 g/g. The final pH of SBHH (7.6) showed that *M. guilliermondii* was able to neutralize the acids of this substrate. These results agree with some predictions in the early eighties, which stated that investigations about microbial content of termite guts would provide new tools for bioconversion of lignocellulosic biomass to fuels and other added-value chemicals. This work is the first report for this species associated with termites in the Amazonian habitat.

KEYWORDS: *Meyerozyma guilliermondii*. Hemicellulosic hydrolyzate. *Nasutitermes sp.*

INTRODUCTION

Increasing greenhouse gas emissions creates the urgent demand for new alternative fuels that release less carbon than fossil fuels. In this context, biofuels are an interesting option for both economic and environmental sustainability (COCKERILL; MARTIN, 2008). Agriculture, besides its benefits for mankind, also produces hundreds of tons of waste products being, in Brazil, mostly in the form of lignocellulosic remains from sugarcane bagasse (BRASIL, 2011).

When compared to other agricultural residues, sugarcane bagasse has some advantages because, after hydrolysis, it produces a large amount of reducing sugar (Hernandez-Salas et al., 2009). Its composition is variable according to the environmental conditions, and is composed of cellulose (~50%), lignin (~15%), and hemicelluloses (~35%). Cellulose hydrolysis releases hexoses that are available for alcoholic fermentation by yeasts and bacteria. Hemicellulose is a heteropolymer of pentoses and hexoses (SAHA, 2003). According to many authors, D-xylose is the most abundant sugar in hemicelluloses ranging between 56 and 82% (CHENG et al., 2007; CANILHA et al., 2010).

There are a large number of yeasts able to ferment hexoses, but just a few species can produce ethanol from D-xylose with economic viability comparable to hexose fermentation (Agbogbo et al., 2006). In this context, studies aiming at the isolation and identification of new species or wild-type strains able to ferment D-xylose remain an important issue.

This work aimed to isolate and identify new wild-type species/strains associated with *Nasutitermes sp*. able to perform D-xylose fermentation using sugarcane bagasse hemicellulosic hydrolyzate (SBHH) as a carbon source. Moreover, the species/strains' thermotolerance, ethanol tolerance, sugar intake, and cell growth in SBHH were assessed to investigate its biotechnological potential.

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MATERIAL AND METHODS

Under authorization of the "Insituto Chico Mendes de Conservação da Biodiversidade" (ICMBio, protocol number 22161-3), the termites (*Nasutitermes sp*.) were collected from the Central Amazon Rain Forest (3º06'05.20" S, 59º58'23.14"W). The abdomen was disrupted and inoculated in isolation medium (IM) composed of SBHH and yeast nitrogen base without amino acids (YNB_{wa}, 6.7 $g.L^{-1}$, pH 5.5). The total reducing sugar (TRS) concentration in SBHH was 10 $g.L^{-1}$. After 24 hours, 100 µL of IM was spread in Petri dishes containing IM plus agar (15 $g.L^{-1}$). The yeasts colonies were recognized at optical microscopy and isolated in Petri dishes containing Sabouraud Agar (peptone 10 $g.L^{-1}$, dextrose 40 $g.L^{-1}$, agar 15 $g.L^{-1}$, pH 5.5). The SBHH was prepared as previously described (Matos et al., 2012).

D-xylose assimilation was verified using the replica-plating method in Petri dishes containing D-xylose (50 mM) and YNB_{wa} (6.7 $g.L^{-1}$). Ability to ferment D-xylose was assessed by culturing the isolates in liquid medium composed of D-xylose (40 $g.L^{-1}$) and YNB_{wa} (6.7 $g.L^{-1}$), pH 7.0, containing a Durham tube to identify gas production, as described by Barnett et al. (1990). D-xylose fermentation yield was estimated using a fermentometer assay. Theoretical ethanol production was measured by stoichiometric calculation, as described by Dijck et al. (2000).

Taxonomic identification was assessed by molecular and biochemical methods. For molecular identification, genomic DNA was amplified by PCR using an ITS1 primer (5' TCCGTAGGTGAACCTGCGG 3'). The PCR products were sequenced, and the obtained DNA sequence was submitted to non-redundant nucleotide GenBank database using BLAST (http://www.ncbi.nlm.nih.gov), as described by Zhang et al. (2000). The biochemical profile was verified using the ID32C kit for biochemical tests developed by Biomerieux®. The results were plotted using the online application ApiWeb (Biomerieux ®).

A D-xylose-fermenting strain was selected for cultivation in liquid medium using SBHH (TRS = 42.6 $g.L^{-1}$) as the carbon source, supplemented with yeast extract (1.5 $g.L^{-1}$), urea (1.25 $g.L^{-1}$) and KH_2PO_4 (1.1 $g.L^{-1}$) during 120 hours (120 rpm, pH 5.0). The initial inoculum was 0.125 $g.L^{-1}$ (wet weight). Saccharification and sugar intake were monitored using the DNS method (Zhao et al., 2010). Cell growth was measured using optical density at 600 nm (OD_{600}) as described by Heer and Sauer (2008).

Specific growth rate (μ_{MAX}, h^{-1}) was calculated using linear regression by natural logarithm of optical density / initial optical density ($ln_{OD/ODi}$) versus time during the log phase, obtaining the equation [$ln_{OD/ODi} = \mu_{MAX}.t + a$] as described by Duarte et al. (2008). Yield biomass (Y_X, $g.g^{-1}$) was calculated by evaluating final biomass produced (wet weight) per gram of consumed sugar (DUARTE et al., 2008).

Thermotolerance was assessed using lethal heat-shock. The yeast was cultured in Erlenmeyer flasks of 125 mL containing 50 mL of YPD medium composed of yeast extract (10 $g.L^{-1}$), peptone (20 $g.L^{-1}$) and dextrose (20 $g.L^{-1}$) (30 ºC, 120 rpm, pH 5.5). After 72 h, the flasks were incubated in a water-bath at 52 ºC for 9 minutes and cell viability was measured. Ethanol tolerance was verified through ethanol shock. The yeast was cultivated in YPD (30 ºC, 120 rpm). After 72 hours, ethanol was added aseptically at final concentrations of 10% and 20%. The flasks were maintained at 30 ºC and 120 rpm for 9 minutes, and cell viability was measured. In both assays, cell viability was quantified using methylene blue staining, as described by Vianna et al. (2008).

Ability to grow in the presence of ethanol was assessed by culturing 1.0 mL of cell suspension (OD_{600} = 1.0) in YPD containing ethanol 10% (v/v). The control group was composed of Erlenmeyer flasks containing YPD without ethanol. This assay was performed in duplicate.

RESULTS

A total of 54 yeast colonies were isolated, with 12 colonies able to assimilate D-xylose and one able to ferment it. This D-xylose-fermenting strain was named LC27. The other isolates were stored in sterilized distilled water for future research. The fermentometer assay lasted a total of 120 hours. The maximum theoretical ethanol yield was estimated to be 3.8 $g.L^{-1}$, obtained after 108 hours.

The ITS1 sequence, deposited in GenBank with accession number JN974905, corresponds to *Pichia guilliermondii*, with a maximum identity of 100%. The biochemical profile of LC27 (Table 1) has 95.9% similarity with *Candida guilliermondii*. According to Lopes et al. (2009), this species is the asexual phase (anamorphous) of *P. guilliermondii*. Currently, this species has been repositioned in a new genus named *Meyerozyma* (Kurtzman and Suzuki, 2010).

Table 1. Biochemical profile of LC27 corresponding to *Candida guilliermondii* (according to ApiWeb / Biomerieux).

Substrates	Results	Substrates	Results
D-Galactose	+	D-Xylose	+
Cycloheximide	+	D-Ribose	–
D-Sucrose	+	Glycerol	+
N-Acetyl Glucosamine	+	L-Rhamnose	–
Lactic Acid	–	Palatinose	+
L-Arabinose	+	Erythritol	–
D-Cellobiose	+	D-Melibiose	+
D-Raffinose	+	Sodium glucuronate	–
D-Maltose	+	D-Melezitose	+
D-Threalose	+	Potassium Gluconate	–
2-Keto Gluconate	+	Levulinic Acid	–
α-Methyl D-Glucopyranoside	+	D-Glucose	+
D-Mannitol	+	L-Sorbose	+
D-Lactose	–	Glucosamine	+
Inositol	–	Esculin Iron Citrate	+
D-Sorbitol	+	Negative control	–

Legend: (+) Assimilate / (–) No assimilate

Cell viability after lethal heat-shock and ethanol shock was 39.8% and 56.0%, respectively. Despite its ethanol tolerance, *M. guilliermondii* LC27 was unable to grow in YPD containing 10% ethanol. Control flasks reached the log phase after 8 hours of cultivation. The stationary phase was observed after 32 hours, as shown in Figure 1.

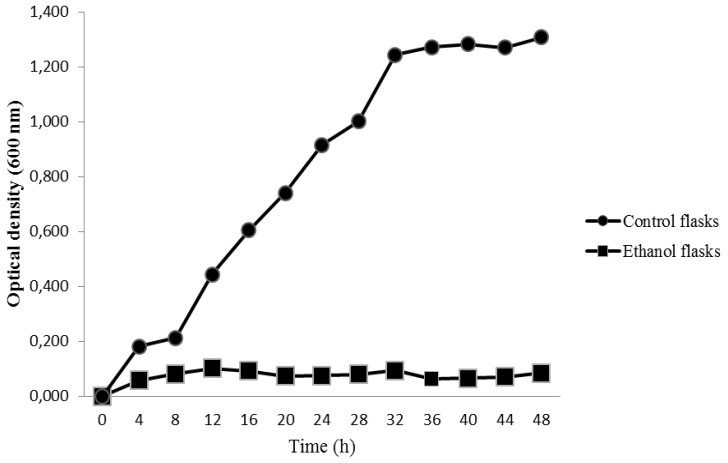

Figure 1. Growth curve of LC27 when cultivated in YPD containing ethanol (squares) and without ethanol (circles). Note the low cell growth in the presence of ethanol.

Cultivation of *M. guilliermondii* LC27 in SBHH had a lag phase of 36 hours. After this time, exponential growth (log phase) was observed until 84 hours, after which a slow cell growth occurred. The stationary phase was observed after 108 hours. The growth curve is presented in Figure 2.

Because of the heterogeneous log phase, the μ_{MAX} was calculated for each different time-point of this stage. The maximum value (0.1028 h^{-1}) was obtained between36 and 60 hours, and the lowest rate of cell growth (0.0102 h^{-1}) was observed between 60 and 108 hours of cultivation. If μ_{MAX} is considered during the entire log phase, the value would be 0.0369 h^{-1}. Linear regressions are presented in Figure 3.

TRS concentration increased 42.6% during the lag phase, obtaining the highest saccharification of SBHH after 4 hours of cultivation. Sugar intake was calculated based on the highest TRS concentration (61,0 $g.L^{-1}$) resulting in 79.5%

consumption. Saccharification and subsequent sugar intake are presented in Figure 4.

Final biomass produced was 25.26 $g.L^{-1}$ with Y_X=0.52 g of biomass produced per gram of consumed sugar. The final pH in SBHH was of 7.6 and final cell viability was of 90.3%.

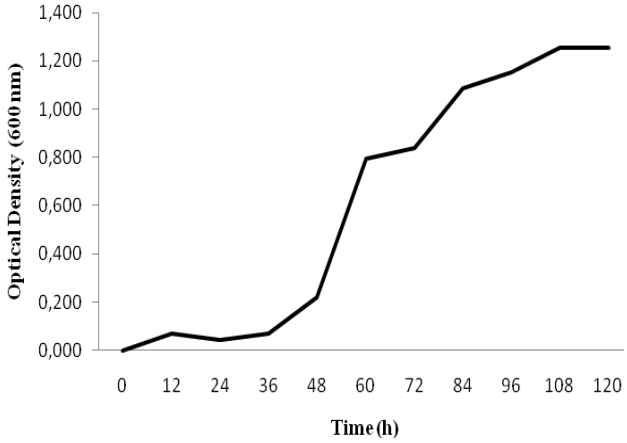

Figure 2. Growth curve of LC27 using sugarcane bagasse hemicellulosic hydrolyzate (SBHH) as carbon source.

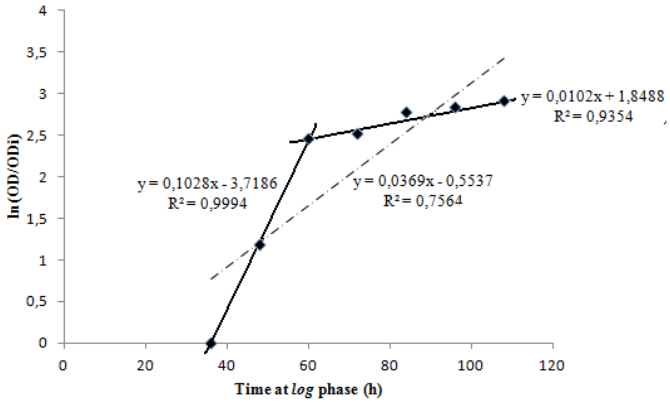

Figure 3. Specific Growth Rate (μ_{MAX}, h^{-1}) calculated for each different moment of the log phase. The maximum value was obtained between 36 and 60 hours (0,1028 h^{-1}). The slowest growth rate was observed between 60 and 108 hours (0.0102 h^{-1}). Dashed line means the average value (0.0369 h^{-1}) during the entire log phase.

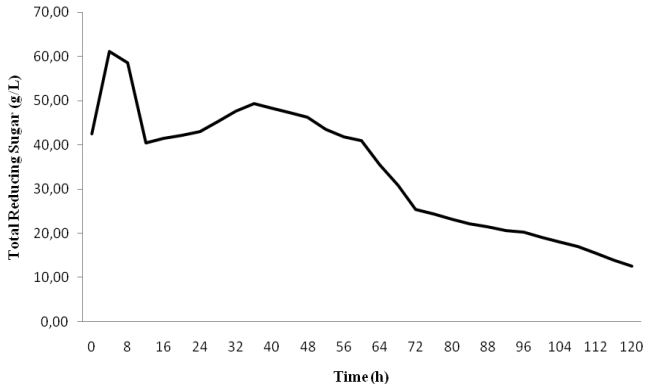

Figure 4. Saccharification and subsequent sugar intake by *Meyerozyma guilliermondii* LC27 in sugarcane bagasse hemicellulosic hydrolyzate.

DISCUSSION

The D-xylose-fermenting strain isolated from termites is a member of the *Pichia guilliermondii* species (anamorphous *Candida guilliermondii*). According to Kurtzman and Suzuki (2010), the genus *Pichia* is a polyphyletic group. They suggest that *P. guilliermondii* and *P. caribica* must be repositioned in a new genus named *Meyerozyma* based on phylogenetic analysis. Because this proposal has been largely accepted by mycologists, LC27 is referred to as *Meyerozyma guilliermondii*. There are no reports of this species being associated with termites in Amazonian habitats.

The estimated amount of ethanol produced corresponds to 18.6% of the maximum theoretical yield, meaning a low ability for ethanol production under the assessed conditions. Despite its capability to ferment D-xylose, optimal conditions are yet to be established.

The cell viability value obtained after lethal heat-shock implies a low tolerance of *M. guilliermondii* LC27 to high-temperature conditions. This characteristic can reduce its employability in ethanol industries. However, *M. guilliermondii* LC27 shows the same thermotolerance of some *Saccharomyces cerevisiae* strains employed in wine production, as demonstrated by Vianna et al. (2008). Furthermore, this isolate has shown ethanol tolerance, meaning that the use of *M. guilliermondii* LC27 cannot be discarded in industrial processes.

The extensive lag phase and slow cell growth during log phase obtained in this work may be closely related to the low mass of inoculated cells, because there is a positive relationship between inoculum size and cell growth/biomass production (GORRET et al., 2004).

When saccharification is performed by microorganisms, it is referred to as biosaccharification. The increase of TRS obtained in this work was higher than that obtained by Hernandez-Salas et al. (2009) using commercial enzymatic kits for saccharification of SBHH. Commonly, biosaccharification of SBHH is obtained by releasing xylanases into fermented broth (KUMAR et al., 2012).

The pattern observed of sugar intake by *M. guilliermondii* LC27 is similar to that obtained by Converti et al. (1999) in *Pachysolen tannophilus* when cultivated in SBHH medium, with 78.6% of sugar consumed. Zhao et al. (2010) and Zou et al. (2010) achieved 85% of sugar intake when cultivating *P. tannophilus* and *Pichia guilliermondii* for alcoholic fermentation of D-xylose in synthetic medium.

Biomass yield obtained in this work was higher than the values obtained by Nigam (2000) using *Candida langeronii* for single-cell protein production in SBHH.

The increase of pH (from 5.0 to 7.6) and high final cell viability allows us to conclude that *M. guilliermondii* LC27 was able to perform neutralization of SBHH and presents a high tolerance against microbial growth inhibitors. According to Parawira and Tekere (2011), the anamorphous *Candida guilliermondii* is able to perform biodetoxification of SBHH and is able to neutralize inhibitor compounds of this substrate.

These results agree with Breznak (1982), who in the early eighties predicted that investigations about microbial content of termites' guts would provide new tools for bioconversion of lignocelluloses to fuels and other added-value chemicals.

This paper is the first to report *Meyerozyma guilliermondii* associated with *Nasutitermes sp.* in the Central Amazon rainforest.

CONCLUSIONS

The LC27 strain is a D-xylose-fermenting wild-type *Meyerozyma guilliermondii*, with a biochemical profile corresponding to the anamorphous *Candida guilliermondii*. It is associated with *Nasutitermes sp*., ratifying some early predictions about the biotechnological potential of the microbial community associated with termites.

M. guilliermondii LC27 presents the ability to neutralize sugar cane bagasse hemicellulosic hydrolysate and has a high potential for biomass production using this substrate as a carbon source, with higher biomass yields than some industrial strains.

The ethanol tolerance, thermotolerance and ability to perform saccharification allow an estimation for biotechnological applications.

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Biológica of the Universidade Federal do Amazonas (UFAM).

RESUMO: Uma levedura selvagem fermentadora de D-xilose foi isolada do conteúdo abdominal de *Nasutitermes sp.*, coletado na Amazônia Central usando Hidrolisado Hemicelulósico de Bagaço de Cana-de-açúcar (HHCA) como meio seletivo. A levedura foi identificada como *Meyerozyma guilliermondii*. Sua capacidade de fermentar D-xilose foi avaliada usando meio líquido contendo tubos de Durham. O isolado demonstrou moderada tolerância ao calor e ao etanol, com viabilidade celular de 39,8% e 56,0%, respectivamente, após submetida a estes fatores limitantes. O ensaio em fermentômetro demonstrou baixo rendimento de etanol usando D-xilose como fonte de carbono. O cultivo em HHCA (pH = 5,0) demonstrou sua capacidade de executar sacarificação e neutralização deste substrato, com aumento da concentração de açúcar redutor total em 42,6% e elevação do pH para 7,6. A fase *log* foi observada entre 36 e 108 horas de cultivo, com máxima taxa de crescimento específico (μ_{MAX}) de 0,10 h^{-1}. Depois de 120 horas, 79,5% do açúcar redutor total foi consumido, com rendimento de biomassa de 0,52 g/g. Estes resultados endossam as predições de alguns autores, os quais propuseram, no início dos anos 80, que a investigação da microbiota intestinal de cupins proveria novas ferramentas para utilização de biomassa lignocelulósica e seus derivados. Este trabalho é o primeiro a reportar a ocorrência de *Meyerozyma guilliermondii* associada a cupins da Amazônia Central.

PALAVRAS-CHAVE: *Meyerozyma guilliermondii*. Hidrolisado Hemicelulósico. *Nasutitermes sp.*

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