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ORIGINAL PAPER

Biochemical analysis of cellobiose catabolism in *Candida pseudointermedia* strains isolated from rotten wood

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Abstract

We isolated two *Candida pseudointermedia* strains from the Atlantic rain forest in Brazil, and analyzed cellobiose metabolization in their cells. After growth in cellobiose medium, both strains had high intracellular β-glucosidase activity [~200 U (g cells)$^{-1}$ for 200 mM cellobiose and ~100 U (g cells)$^{-1}$ for 2 mM *p*NPβG] and negligible periplasmic cellobiase activity. During batch fermentation, the strain with the best performance consumed all the available cellobiose in the first 18 h of the assay, producing 2.7 g L^{-1} of ethanol. Kinetics of its cellobiase activity demonstrated a high-affinity hydrolytic system inside cells, with K_m of 12.4 mM. Our data suggest that, unlike other fungal species that hydrolyze cellobiose extracellularly, both analyzed strains transport it to the cytoplasm, where it is then hydrolyzed by high-affinity intracellular β-glucosidases. We believe this study increases the fund of knowledge regarding yeasts from Brazilian microbiomes.

Keywords Yeast · Hydrolysis · β-Glucosidase · Cellobiase · Fermentation · Atlantic rain forest

Introduction

Yeasts have been used by humans since the Neolithic Revolution. This was long before their role as fermenting microorganisms was discovered in the nineteenth century with the work of Louis Pasteur. Over these millennia human activity has primarily selected *Saccharomyces* spp. as industrial yeasts; nevertheless, several wild species have come to be known in the last century (Eliodório et al. 2019). Despite the fact that many of them have already been taxonomically described, the number of biochemically characterized species is much smaller. This is the case of *Candida pseudointermedia* (family Metschnikowiaceae, order Saccharomycetales, class Saccharomycetes subphylum Saccharomycotina, phylum Ascomycota), first described by Nakase et al. (1976), but hitherto poorly characterized in terms of carbohydrate metabolism.

The lasting relationship of yeasts with humans is mainly due to the broad spectrum of carbohydrates that they metabolize (Lagunas 1993; Gancedo 1998; Marques et al. 2016). Cellobiose, one of these sugars, is a disaccharide formed by two glucose molecules joined by a β-1,4 glycosidic bond, with one glucose unit rotated 180° with respect to the other. This disaccharide is mostly found in partially hydrolyzed cellulosic material (Parisutham et al. 2017). Cellobiose hydrolysis depends on the activity of β-glucosidases (also called cellobiases), enzymes primarily produced by representatives of the fungal kingdom (Srivastava et al. 2018; Alves et al. 2019). Both hydrolysis and fermentation of this sugar are interesting to the biofuel industry.

In yeasts, cellobiose metabolism also depends on β-glucosidases that can be secreted by cells in the medium in which they are found (Barbosa et al. 2010; Baffi et al.

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2011; Molnárová et al. 2014), or, they may be located either in the periplasm (Guo et al. 2015) or in the cytoplasm (Santos et al. 2011; Reis et al. 2014; Parisutham et al. 2017). With the exception of the species *Sporidiobolus pararoseus* (Baffi et al. 2011), *Naganishia albida* (*Cryptococcus albidus*) and *Aureobasidium pullulans* (Molnárová et al. 2014), which, respectively, belong to the orders Sporidiobolales (class Microbotryomycetes, subphylum Pucciniomycotina, phylum Basidiomycota), Filobasidiales (class Tremellomycetes, subphylum Agaricomycotina, phylum Basidiomycota) and Dothideales (class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota), all the other yeasts analyzed by those authors belong to the order Saccharomycetales (class Saccharomycetes, subphylum Saccharomycotina, phylum Ascomycota), being distributed in five different families: Metschnikowiaceae, Debaryomycetaceae, Phaffomycetaceae, Pichiaceae and Saccharomycodaceae. While the ability to extracellularly hydrolyze cellobiose is observed in the first three orders, the presence of β-glucosidases in the periplasm or in the cytoplasm of cells seems to be restricted to the order Saccharomycetales. If cellobiose hydrolysis occurs either extracellularly or in the periplasm, yeast cells only need to uptake the glucose molecules generated by this breakdown, then metabolize these hexoses through fermentative or respiratory metabolism. On the other hand, for cellobiose hydrolysis to occur in the cytoplasm, the yeast cell must have membrane permeases capable of transporting the molecule into the cell, where it can then be hydrolyzed by intracellular β-glucosidases, releasing glucose molecules that can be directly fermented or respired (Parisutham et al. 2017; Ríos-Fránquez et al. 2017; Eliodório et al. 2019).

Although several studies have already observed *C. pseudointermedia* on different substrates, including fish-paste (Nakase et al. 1976), lake (Rosa et al. 1995), coffee (Mausoud et al. 2004), human body (Putigani et al. 2011), coconut water (Maciel et al. 2016), rice phylloplane (Limtong and Kaewwichian 2015), tap water (Babič et al. 2016) and spoiled ready-to-eat meals (Feliciano et al. 2019), these studies have focused on ecological, taxonomic and phylogenetic aspects; there are no detailed metabolic characterizations of this yeast. Through simple physiological tests, used for taxonomic description of this species, it was discovered that this yeast assimilates cellobiose, among other sugars (Nakase et al. 1976). Nevertheless, the mechanism of this metabolism by yeast cells has not been detailed, nor has the role of their permeases and hydrolytic enzymes been analyzed. Therefore, with the purpose of broadening the base of knowledge regarding still poorly-characterized yeast species, we analyzed the cellobiose catabolism in two *C. pseudointermedia* strains isolated from rotting wood samples collected in the Atlantic rain forest of Southern Brazil. To the best of our knowledge, this is the first work to detail the metabolism of a disaccharide in *C. pseudointermedia*.

Materials and methods

Yeast strain isolation and identification

The yeast strains used in this work were isolated from rotting wood samples collected from the Chapecó National Forest, 27° 5′ S and 52° 46′ W (strain FLONA-CE-3.4), and in the woods surrounding the Chapecó campus of the Federal University of the Southern Border, 27° 7′ S and 52° 42′ W (strain UFFS-CE-3.6), following the methodology described by Bazoti et al. (2017). Samples were stored in sterile plastic bags and were transported in isothermal boxes under refrigeration. One gram of each sample was inoculated in flaks with 20 mL minimal YNB media (yeast nitrogen base 6.7 g L^{-1}, pH 5.0) containing 10 g L^{-1} of cellobiose and 0.2 g L^{-1} of chloramphenicol. Flasks were incubated at 25 °C on a shaker at 145 rpm until growth was detected by turbidity. Subsequently, one loopful of each tube was streaked on plates containing the same media described above with addition of 20 g L^{-1} agar. Plates were incubated at 25 °C, and the various yeast morphotypes were purified by repeated streaking on YMA plates (D-glucose 10 g L^{-1}, peptone 5 g L^{-1}, yeast extract 3 g L^{-1}, malt extract 3 g L^{-1}, agar 20 g L^{-1}) and preserved at − 80 °C for later identification. The yeasts were characterized using standard methods (Kurtzman et al. 2011). Species identification was performed using analysis of the D1/D2 variable domains of the large subunit of rRNA (White et al. 1990; O'Donnell 1993; Kurtzman and Robnett 1998; Lachance et al. 1999). The amplified DNA was concentrated, cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies, California, USA) using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited, and aligned using the program MEGA6 (Tamura et al. 2013). The sequences obtained were deposited in Genbank (MT007799 and MT007800 for FLONA-CE-3.4 and UFFS-CE-3.6, respectively) and compared with those included in that database using the Basic Local Alignment Search Tool (BLAST, at https://www.ncbi.nlm.nih.gov). Furthermore, a phylogenetic tree was assembled with sequences from type material of the order Saccharomycetales, obtained through this BLAST. To this end, the sequences were first aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE, https://www.ebi.ac.uk/Tools/msa/muscle/), and then the alignment file were Gblock-curated on the Phylogeny.fr platform (Castresana 2000; Dereeper et al. 2008, 2010; https://www.phylogeny.fr). After that, the cured alignment file was used to generate the phylogenetic tree using Akaike Information Criterion (AIC) in Smart Model Selection (Lefort et al. 2017) implemented in the PhyML environment (Guindon

et al. 2010—https://www.atgc-montpellier.fr/phyml-sms), with a bootstrap analysis of 1000 replicates. For the management of the phylogenetic tree, we used the online tool Interactive Tree of Life (iTOL, https://itol.embl.de).

Media and culture conditions

Two culture media were used in this study: rich YP medium (10 g L^{-1} yeast extract and 20 g L^{-1} Bacto peptone), and minimal YNB medium, containing 6.7 g L^{-1} yeast nitrogen base without amino acids. Both media were supplemented with 20 g L^{-1} of the indicated carbon source (glucose or cellobiose), and the pH of the media was adjusted to pH 5.0 using HCl. Cells were grown aerobically at 25 °C with shaking (145 rpm) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium. Cellular growth was followed by turbidity measurements at 570 nm, and culture samples were harvested regularly, centrifuged (3500*g*, 3 min), and their supernatants were used for determination of sugars and ethanol.

For batch fermentations, yeasts were pre-grown on YP with 20 g L^{-1} of glucose until the exponential phase (~ 1 mg of dry yeast mL^{-1}), centrifuged (3500*g*, 3 min) and washed twice with cold water, and inoculated at high cell density (10 mg of dry yeast mL^{-1}) into minimal YNB medium containing 20 g L^{-1} of glucose or cellobiose. Samples were collected regularly and processed as described above. The data were expressed as averages and standard error from two completely independent experiments.

Sugars and ethanol determination

Samples of the supernatants were filtered through 0.45-µm filters before transfer to chromatographic vials. The concentrations of glucose, cellobiose and ethanol were determined using high performance liquid chromatography (HPLC; LCMS-2020, Shimadzu), with a refractive index detector (RID-10, Shimadzu) and a column for organic acids (Aminex HPX-87H, Bio-Rad). The mobile phase used 5 mM sulfuric acid at 50 °C with a flow rate of 0.6 mL min^{-1}. Calibration curves were established for all samples using seven concentrations ranging from 0.25 to 20 g L^{-1} for carbohydrates and 0.125 to 10 g L^{-1} for ethanol.

β-Glucosidase assays

Periplasmic and intracellular β-Glucosidase activity was determined in cells grown up to the beginning of the exponential growth phase (OD_{570nm} ~ 3.5) in rich YP medium containing 20 g L^{-1} of glucose or cellobiose as carbon sources. Cells were initially centrifuged, washed twice with water at 4 °C, and resuspended to reach 20 g L^{-1}. Periplasmic cellobiose hydrolysis by β-glucosidase was determined in vivo with whole cells previously incubated with 50 mM sodium fluoride to block glycolysis (Silveira et al. 1996; Batista et al. 2004), using 100 mM cellobiose in 50 mM succinate-Tris buffer, pH 5.0. Intracellular β-glucosidase activity was determined in situ with permeabilized yeast cells (Stambuk 1999) using 200 mM cellobiose or 2 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβG) in 100 mM MOPS-NaOH buffer, pH 6.8. For the kinetic analysis, cellobiose concentration ranged from 12.5 to 400 mM. After incubation, the tubes were quickly boiled for 10 min and then cooled in an ice bath. The tubes were centrifuged for 5 min at 3500 rpm and the supernatants were used to quantify the glucose released from cellobiose hydrolysis or the *p*-nitrophenol released from *p*NPβG hydrolysis. Glucose was measured by the glucose oxidase and peroxidase method using a commercial kit (BioDiagnostica-Laborclin), and *p*-nitrophenol was measured on a spectrophotometer at 400 nm. Negative controls using previously boiled cells were used. Cellobiase activity was expressed as U (g dry yeast cells)$^{-1}$, where one unit corresponds to 1 µmol of glucose or *p*-nitrophenol produced per min at 30 °C. The presented data were averages and standard error from two independent experiments. The kinetic parameters K_m and V_{max} were calculated according to Lineaweaver and Burk (1934).

Additionally, β-glucosidase activity was also analyzed in the media, to verify if there was any secreted cellobiase by yeast cells. To this end, strains FLONA-CE-3.4 and UFFS-CE-3.6 were cultured in rich YP medium containing 20 g L^{-1} of cellobiose, and media was harvested by centrifugation at the beginning of the exponential growth phase (OD_{570nm} ~ 3.5) or after 48 h of culture. The harvested medium was mixed (1:3) with cellobiose (300 mM cellobiose in 150 mM succinate-Tris buffer, pH 5.0), and then incubated for 30 min at 30 °C or 50 °C. Extracellular β-glucosidase activity was assessed by glucose release as described above. For negative controls, the harvested medium was previously boiled.

Transport assays

β-Glucoside transport activity was determined using a colorimetric transport assay using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβG), as previously described for the determination of α-glucoside transport activity in yeasts (Hollatz and Stambuk 2001; Alves et al. 2007, 2008). Cells collected at the exponential phase of growth (OD_{570nm} ~ 3.5) in rich YP medium containing 20 g L^{-1} of glucose or cellobiose as carbon source were initially centrifuged, washed twice with water at 4 °C, and resuspended to reach 20 g L^{-1}. The cell suspension was incubated with 10 mM *p*NPβG in 100 mM succinate-Tris buffer, pH 5.0. After 5 min incubation at room temperature, the reaction was interrupted with 1.0 mL of 2 M sodium bicarbonate, and the *p*-nitrophenol

released was measured using a spectrophotometer at 400 nm. All assays were performed in triplicate, and controls with previously boiled yeast cells were used. Transport activity was expressed as U (g dry yeast cells)$^{-1}$, where one unit corresponds to 1 µmol of *p*NPβG transported per min. The data were expressed as averages and standard error from two completely independent experiments.

Results and discussion

The two *Candida pseudointermedia* yeast strains (strains FLONA-CE-3.4 and UFFS-CE-3.6) were isolated from rotting wood samples collected from the Atlantic rain forests of southern Brazil. The sequencing of the D1/D2 variable domains (see Materials and Methods) of both strains revealed an identity of 99.80% between them. When compared to a type strain of *C. pseudointermedia* (NCBI accession number MK394147.1), the identity was 99.80% for FLONA-CE-3.4 and 100.00% for UFFS-CE-3.6. Considering the proximity between the species *C. pseudointermedia* and *C. intermedia*, we also compared the D1/D2 variable domains of these strains with a type strain of *C. intermedia* (NCBI accession number NG_055404.1) and we found 99.39% and 99.60% of identity for FLONA-CE-3.4 and UFFS-CE-3.6, respectively. It is worth mentioning that the D1/D2 variable domains of *C. pseudointermedia* and *C. intermedia* type strains also share 99.60% of identity with each other. Thus, that led us to classify both FLONA-CE-3.4 and UFFS-CE-3.6 as members of the species *Candida pseudointermedia*.

Initially, we cultivated both strains in rich YP media and minimal YNB media containing cellobiose or glucose as carbon source. The two strains showed distinct profiles of cell growth and sugar consumption in the two types of medium employed (Fig. 1). Strain UFFS-CE-3.6 presented higher cell growth and sugar (glucose or cellobiose) consumption rate in rich YP medium (Fig. 1a, b), when compared with strain FLONA-CE-3.4; however, surprisingly, this last strain showed better performance in minimal YNB medium (Fig. 1c, d), with a 70% higher cellobiose consumption rate by the cells when cultivated in this medium (Table 1).

Previous studies reported that some sugars may not be completely consumed if the cells are grown in minimal media (Hahn-Hagerdal et al. 2005; Alves et al. 2007), and the ethanol yield in media containing ammonia as nitrogen

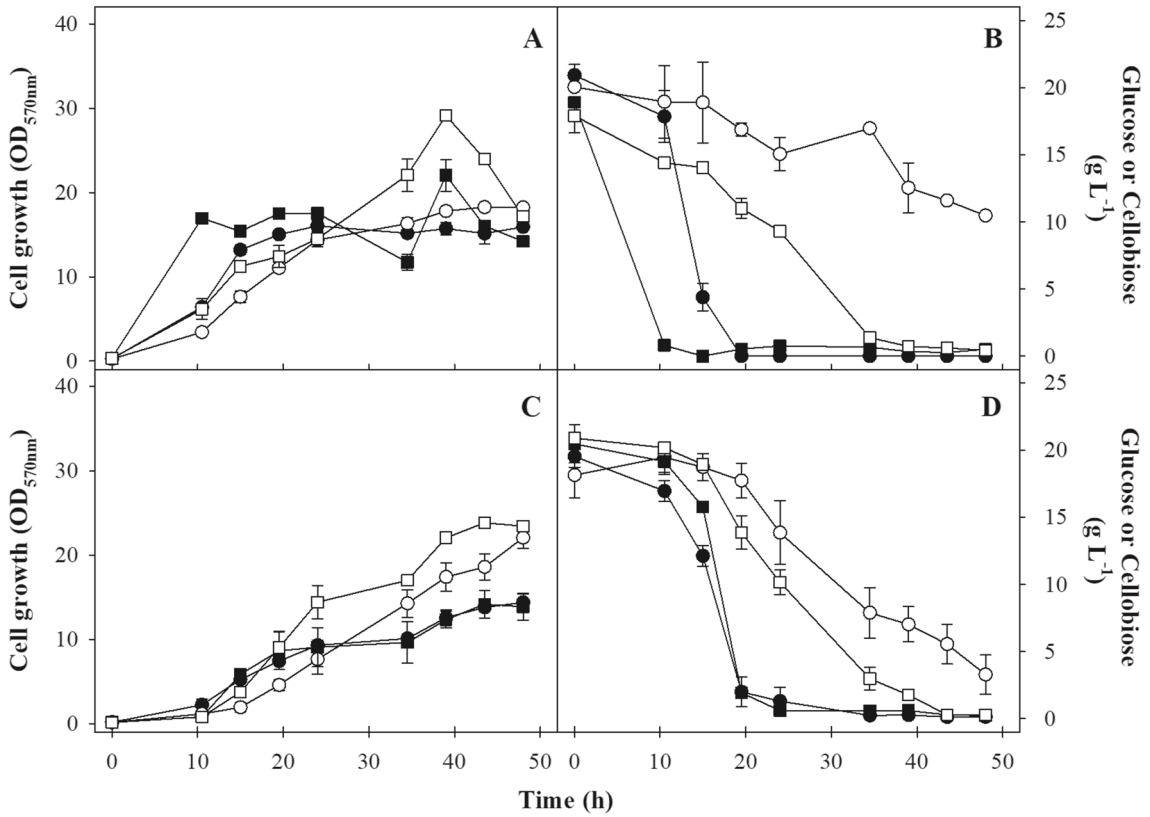


Fig. 1 Cellular growth (**a**, **c**) and sugar consumption (**b**, **d**) in rich YP medium (**a**, **b**) or minimal YNB medium (**c**, **d**) containing 20 g L^{-1} of cellobiose (open symbols) or glucose (closed symbols) by strains FLONA-CE-3.4 (circles) and UFFS-CE-3.6 (squares). Data represent averages and standard error from two independent experiments

Table 1 Ethanol yields and sugar consumption rate during cellular growth or batch fermentation[a]

Type of media and carbon source[b]	Ethanol yields (g g^{-1})		Sugar consumption rate (g L^{-1} h^{-1})	
	FLONA-CE-3.4	UFFS-CE-3.6	FLONA-CE-3.4	UFFS-CE-3.6
Cellular growth				
YP + 2% glucose	0.38 ± 0.05	0.37 ± 0.01	1.07 ± 0.06	1.72 ± 0.04
YP + 2% cellobiose	0.00	0.07 ± 0.00	0.20 ± 0.00	0.48 ± 0.05
YNB + 2% glucose	0.17 ± 0.02	0.19 ± 0.04	0.69 ± 0.15	0.83 ± 0.09
YNB + 2% cellobiose	0.00	0.00	0.34 ± 0.04	0.47 ± 0.00
Batch fermentation				
YNB + 2% glucose	0.38 ± 0.08	0.31 ± 0.02	8.75 ± 1.16	11.10 ± 1.28
YNB + 2% cellobiose	0.00	0.11 ± 0.05	0.46 ± 0.03	1.29 ± 0.07

[a]Data are expressed as mean ± standard error

[b]Percentages of sugar concentration refer to weight per volume

source (as in the minimal media used in the present work) is roughly half of the yield observed in media whose source of nitrogen comes from yeast extract and peptone (Hande et al. 2013). However, in the present study, this was true only for cellobiose consumption by strain UFFS-CE-3.6 that produced 1.1 g L^{-1} of ethanol from cellobiose in rich media, but not in YNB media (Table 1). During growth on glucose, both strains consumed this sugar in the first 10–20 h of incubation in rich YP media; however, they needed ~ 24 h to totally consume glucose from minimal YNB medium (Fig. 1). Consequently, the ethanol yield ($Y_{e/s}$) from glucose in rich media was around 0.38 g g^{-1}, while in YNB media the ethanol yield did not exceed 0.19 g g^{-1} (Table 1). The lower cellobiose consumption by FLONA-CE-3.4 in rich YP media may be explained by the presence of other sugars in yeast extract such as trehalose (Iwahara et al. 1993) that may interfere with cellobiose consumption in the rich YP media.

The two analyzed strains showed high intracellular β-glucosidase activity against cellobiose, and negligible periplasmic activity (Fig. 2). No cellobiase activity was detected in the medium, neither in the beginning of exponential growth nor at the end of cultivation, after 48 h (data not shown). Taking together, these results, therefore, suggest that the analyzed strains harbor only intracellular isoforms of β-glucosidase. Although cellobiose-grown cells showed high intracellular cellobiose hydrolysis rates, when the cells were pre-cultured in media with glucose, the expression of cellobiase was strongly inhibited in both strains. Indeed, glucose promotes the catabolic repression of a series of enzymes involved in the metabolism of alternative sugars (Santangelo 2006; Alves et al. 2014; Cadete and Rosa 2018).

Similar β-glucosidase activity was shown by Santos et al. (2011) in two strains of the cellobiose-fermenting yeast *Scheffersomyces* (*Candida*) *queiroziae*, although in that case, yeasts had higher ethanol yields than did FLONA-CE-3.4 and UFFS-CE-3.6, suggesting that cellobiose transport through the plasma membrane of this strains might limit the fermentation of this sugar. Indeed, Casa-Villegas et al.

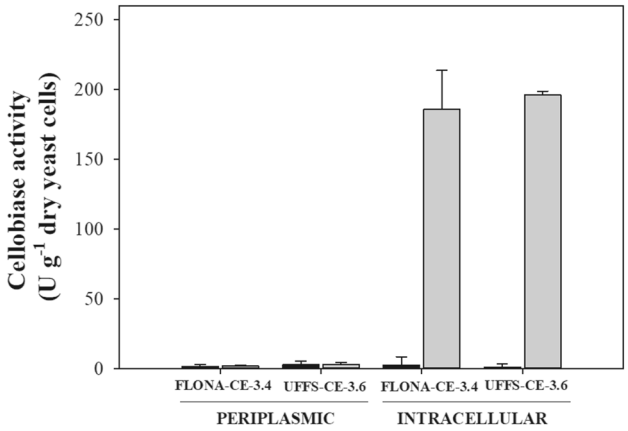


Fig. 2 Periplasmic and intracellular cellobiase activity against 200 mM cellobiose as substrate, determined in cells collected during growth in media with glucose (black bars) or cellobiose (gray bars) as carbon sources. Data represent averages and standard error from two independent experiments

(2018) recently showed that a genetically-modified *Saccharomyces cerevisiae* strain heterologously expressing intracellular β-glucosidases had its growth in cellobiose limited by low sugar intake. In our study, this was also corroborated by the fact that cellobiose consumption was slower than glucose consumption in both FLONA-CE-3.4 and UFFS-CE-3.6 strains (Table 1). When sugars are taken up slowly by yeast cells, their metabolism is essentially respiratory (Duval et al. 2010). Moreover, when the *p*NPβG (a cellobiose synthetic analog) transport and hydrolysis activities were analyzed in cells cultured in media with cellobiose (Fig. 3), we found that the transport system may indeed be the main bottleneck for cellobiose fermentation, because transport activity (Fig. 3a) reached no more than 12.5% of *p*NPβG hydrolysis rate (Fig. 3b). Regarding these analyses with *p*NPβG, it is also worth noting that not only the transport activity, but also hydrolytic activity was negligible when both strains were pre-grown in glucose. This corroborates cellobiase activity

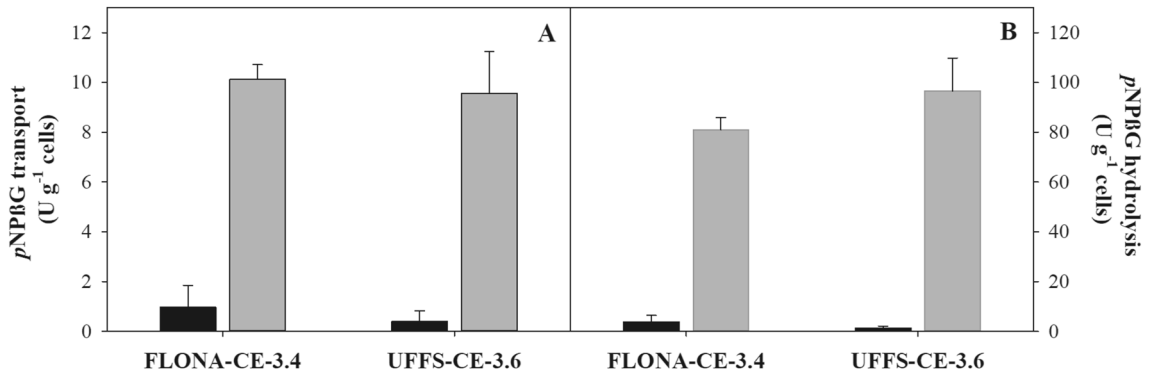


Fig. 3 Analysis of pNPβG transport (**a**) and intracellular hydrolysis (**b**) activity by cells previously grown in glucose (black) or cellobiose (gray) as carbon sources. Data represent averages and standard error from two independent experiments

data (Fig. 2), suggesting that cellobiose metabolization is impaired in the presence of this hexose.

Some new yeast species have been described as cellobiose-fermenting yeasts, despite the fact that, in most studies, investigators have only performed assays in Durham tubes, and/or the fermentation was considered weak or slow in relation to that observed with glucose (Santos et al. 2011; Wang et al. 2015, 2016; Peter et al. 2017; Cadete and Rosa 2018). Nevertheless, Lopes et al. (2018) recently showed that yeast strains from the species *Candida jaroonii*, *Scheffersomyces queiroziae* as well as from a new species of *Yamadozyma* fermented 50 g L^{-1} cellobiose in rich YP medium with efficiencies ranging from 82.0 to 94.4% of the maximum theoretical ethanol yield (0.538 g of ethanol per g of cellobiose consumed). Therefore, in addition to the growth experiments in full aerobic condition with a low initial cell concentrations inoculum, we also carried out batch fermentations with a high cell concentration (~ 10 g L^{-1}) in minimal YNB media containing cellobiose or glucose as carbon source. Both strains consumed all available glucose in the first two hours of the experiment; however, it took 18 h for UFFS-CE-3.6 to deplete all the cellobiose from the medium, and 24 h of incubation was not sufficient to allow total cellobiose consumption by the strain FLONA-CE-3.4 (Fig. 4a). Even so, strain UFFS-CE-3.6 fermented cellobiose with an ethanol yield ($Y_{e/s}$) of 0.11 g g^{-1}, representing 57% more than that obtained during growth in this carbon source and one-third of the ethanol yield achieved from glucose (see Fig. 4b and Table 1). One might say that this delayed cellobiose consumption observed in batch fermentations can be explained by the repressive effect exerted by glucose, because the strains were pre-grown on media with glucose; nevertheless, when the strains were pre-grown in cellobiose, the fermentation of this β-glucoside was not improved at all (data not shown).

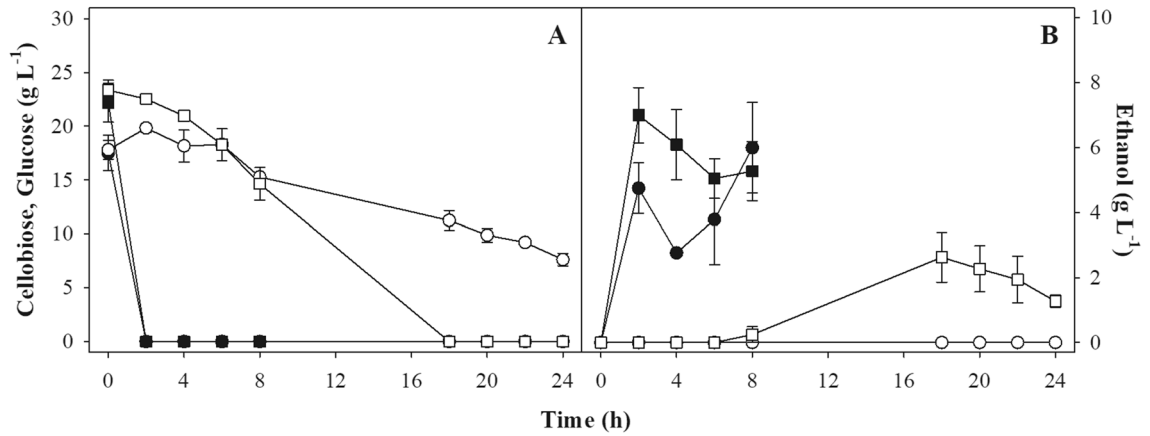


Fig. 4 Sugar consumption (**a**) and ethanol production (**b**) during batch fermentations in minimal YNB medium containing cellobiose (open symbols) or glucose (closed symbols) by strains FLONA-CE-3.4 (circles) and UFFS-CE-3.6 (squares). Data represent averages and standard error from two independent experiments

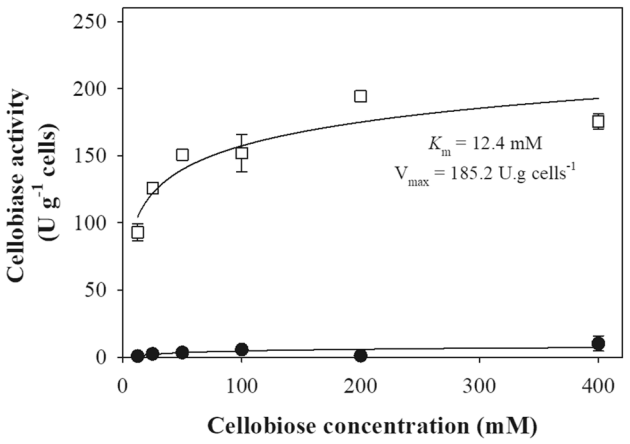


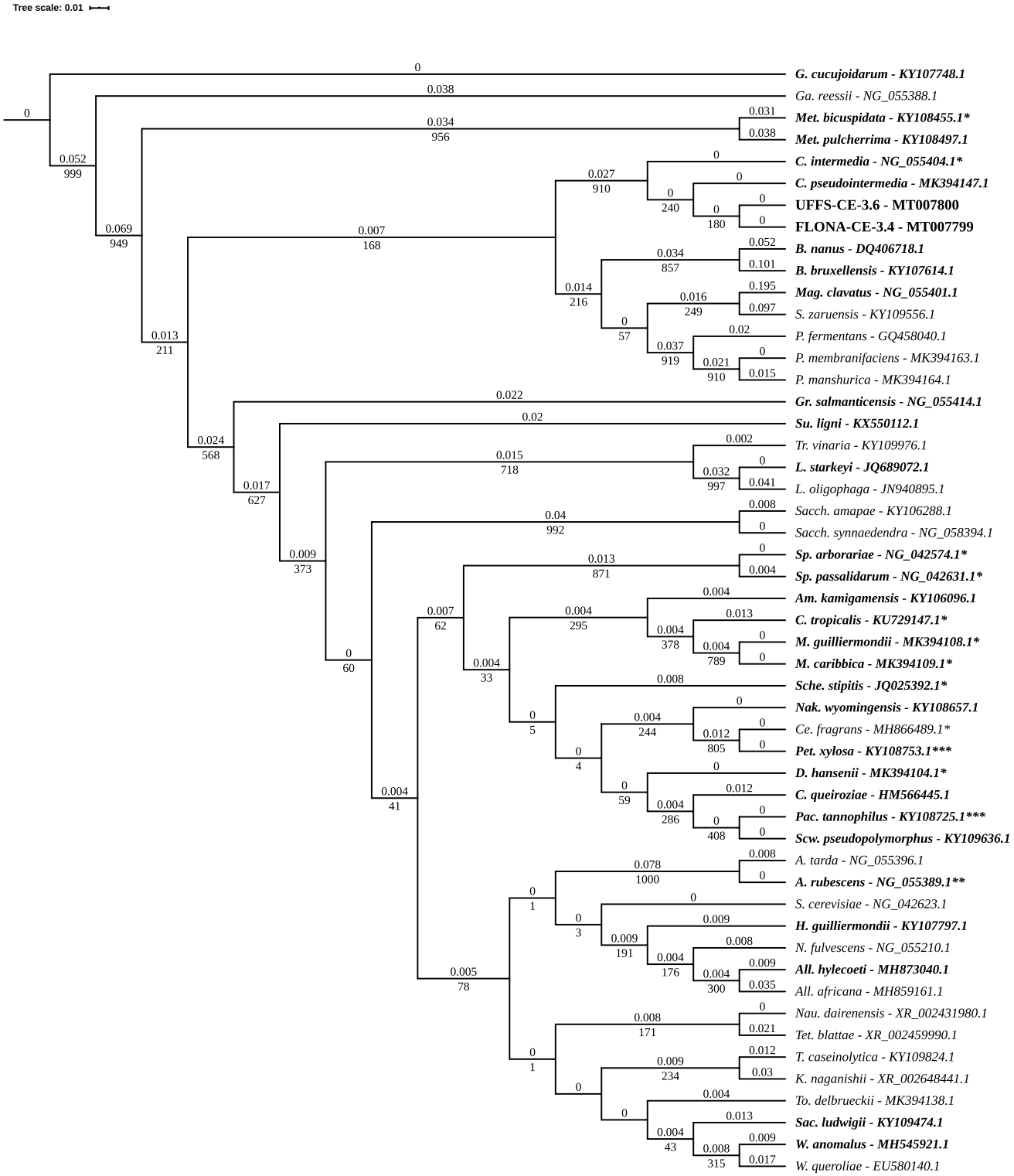
Fig. 5 Kinetics of cellobiase activity by strain UFFS-CE-3.6 precultured in media with glucose (closed circles) or cellobiose (open square). Data represent averages and standard error from two independent experiments

Because strain UFFS-CE-3.6 showed the best performance during fully aerobic growth and batch fermentation (Table 1) and showed slightly higher β-glucosidase activities than strain FLONA-CE-3.4 (Figs. 2, 3), the kinetics of its cellobiase activity were analyzed against 12.5–400 mM cellobiose (Fig. 5). We found that β-glucosidases in this yeast have high affinity ($K_m = 12.4$ mM) for cellobiose. This is approximately half of the Michaelis–Menten constant observed for the β-glucosidase BGLII from the thermotolerant yeast *Pichia etchellsii* (Wallecha and Mishra 2003) and one-sixth of the observed K_m for the *C. peltate* cellobiase (Saha and Bothast 1996). Therefore, this represents an affinity for cellobiose two and six times higher, respectively, than the affinities observed by those authors. In an extracellular β-glucosidase from the wine-associated yeast *Debaromyces pseudopolymorphus*, Villena et al. (2006) measured a K_m value (11.9 mM) similar to what we obtained for *C. pseudointermedia* UFFS-CE-3.6, although we found a V_{max} 2.6 times higher than the one found for that winery yeast. More recently, a thermostable β-glucosidase from *Aspergillus fumigatus* was shown to have a very high affinity for cellobiose ($K_m = 1.75$ mM), but a low V_{max} (Liu et al. 2012), with only 28.2% of the V_{max} we found for UFFS-CE-3.6.

Our results thus depict the metabolism of cellobiose in yet another non-domesticated yeast species. The assimilation of this disaccharide seems to be a constant in yeasts associated with processes of degradation of lignocellulosic material in nature (Riley et al. 2016). However, the data available in the literature do not seem to indicate close phylogenetic relationship among species capable of assimilating cellobiose, i.e., this characteristic is not restricted to closely related taxa, not even to a single phylum within the kingdom Fungi (Molnárová et al. 2014). Indeed, when comparing the D1/D2 variable domains of 28S rRNA gene of cellobiose-assimilating species with those of yeasts unable to metabolize this sugar, we see that positive-phenotype yeasts do not necessarily cluster together, and that the cellobiose-assimilation capacity is randomly spread among the members of the order Saccharomycetales (Fig. 6). On the other hand, recently Shen et al. (2018) showed that this trait is inferred as being from the budding yeast common ancestor (BYCA) and that those budding yeasts which are not able to metabolize this disaccharide probably have lost such trait.

Conclusion

The present study showed that, to ferment cellobiose, the analyzed *C. pseudointermedia* strains rely on permeases responsible for transport of this disaccharide through the plasma membrane and on high-affinity intracellular β-glucosidases, whose expression is highly induced in cellobiose-grown cells and strongly inhibited by glucose. Our data also suggest that cellobiose transport activity might be a limiting step for efficient fermentation of cellobiose in these yeasts. To the best of our knowledge, this is the first study to analyze disaccharide metabolization in *C. pseudointermedia* strains. We believe it contributes to the fund of knowledge of Brazilian microbiota.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Met. bicuspidata - KY108455.1*
Met. pulcherrima - KY108497.1
C. intermedia - NG_055404.1*
C. pseudointermedia - MK394147.1
UFFS-CE-3.6 - MT007800
FLONA-CE-3.4 - MT007799
B. nanus - DQ406718.1
B. bruxellensis - KY107614.1
Mag. clavatus - NG_055401.1
S. zaruensis - KY109556.1
P. fermentans - GQ458040.1
P. membranifaciens - MK394163.1
P. manshurica - MK394164.1
Gr. salmanticensis - NG_055414.1
Su. ligni - KX550112.1
Tr. vinaria - KY109976.1
L. starkeyi - JQ689072.1
L. oligophaga - JN940895.1
Sacch. amapae - KY106288.1
Sacch. synnaedendra - NG_058394.1
Sp. arborariae - NG_042574.1*
Sp. passalidarum - NG_042631.1*
Am. kamigamensis - KY106096.1
C. tropicalis - KU729147.1*
M. guilliermondii - MK394108.1*
M. caribbica - MK394109.1*
Sche. stipitis - JQ025392.1*
Nak. wyomingensis - KY108657.1
Ce. fragrans - MH866489.1*
Pet. xylosa - KY108753.1***
D. hansenii - MK394104.1*
C. queiroziae - HM566445.1
Pac. tannophilus - KY108725.1***
Scw. pseudopolymorphus - KY109636.1
A. tarda - NG_055396.1
A. rubescens - NG_055389.1**
S. cerevisiae - NG_042623.1
H. guilliermondii - KY107797.1
N. fulvescens - NG_055210.1
All. hylecoeti - MH873040.1
All. africana - MH859161.1
Nau. dairenensis - XR_002431980.1
Tet. blattae - XR_002459990.1
T. caseinolytica - KY109824.1
K. naganishii - XR_002648441.1
To. delbrueckii - MK394138.1
Sac. ludwigii - KY109474.1
W. anomalus - MH545921.1
W. queroliae - EU580140.1

◂**Fig. 6** Phylogenetic tree assembled from the D1/D2 domains of 49 related yeast species, belonging to the order Saccharomycetales. Besides the two strains of the present study (UFFS-CE-3.6 and FLONA-CE-3.4) and the *Candida pseudointermedia* type strain, the following species were evaluated: *Geotrichum cucujoidarum, Galactomyces reessii, Metschnikowia bicuspidate, Metschnikowia pulcherrima, Candida intermedia, Brettanomyces nanus, Brettanomyces bruxellensis, Magnusiomyces clavatus, Saturnispora zaruensis, Pichia fermentans, Pichia membranifaciens, Pichia manshurica, Groenewaldozyma salmanticensis, Sugiyamaella ligni, Trigonopsis vinaria, Lipomyces starkeyi, Lipomyces oligophage, Saccharomycopsis amapae, Saccharomycopsis synnaedendra, Spathaspora arborariae, Spathaspora passalidarum, Ambrosiozyma kamigamensis, Candida tropicalis, Meyerozyma guilliermondii, Meyerozyma caribbica, Scheffersomyces stipites, Nakazawaea wyomingensis, Cephaloascus fragrans, Peterozyma xylosa, Debaromyces hansenii, Candida queiroziae, Pachysolen tannophilus, Schwanniomyces pseudopolymorphus, Ascoidea tarda, Ascoidea rubescens, Saccharomyces cerevisiae, Hanseniaspora guilliermondii, Nadsonia fulvescens, Alloascoidea hylecoeti, Alloascoidea africana, Naumovozyma dairenensis, Tetrapisispora blattae, Tortispora caseinolytica, Kazachstania naganishii, Torulaspora delbrueckii, Saccharomycodes ludwigii, Wickerhamomyces anomalus* and *Wickerhamomyces queroliae*. Accession numbers of the sequences used are shown to the right of species name. The numbers above each branch represent the branch lengths; the numbers below represent the bootstraps values. Bootstrap values were based on 1000 replications. In the assembled tree, yeasts capable of assimilating cellobiose are in bold. This feature was verified in the Westerdijk Fungal Biodiversity Institute (https://www.wi.knaw.nl/), with the exception of the following species, which have been checked in the literature: *Geotrichum cucujoidarum* (Suh and Blackwell 2006); *Galactomyces reessii* (Hoog et al. 1998); *Metschnikowia bicuspidata* (Miller and Phaff 1998); *Magnusiomyces clavatus* (Buchta et al. 2019); *Pichia manshurica* (Amoikon et al. 2018); *Spathaspora arborariae* (Cadete et al. 2009); *Cephaloascus fragrans* (Kurtzman and Hoog 2011); *Debaromyces hansenii* (Prillinger et al. 1999); *Candida queiroziae* (Santos et al. 2011); *Ascoidea tarda*, *Ascoidea rubescens*, *Alloascoidea hylecoeti* and *Alloascoidea africana* (Kurtzman and Robnett, 2013); and, *Wickerhamomyces queroliae* (Rosa et al. 2009). Yeasts labeled with *, ** and ***belong to CUG-Ser1, CUG-Ser2 and CUG-Ala clades, respectively (Shen et al. 2018)

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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