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MICROBIOLOGY

Comparative analysis of β-glucosidase activity in non-conventional yeasts

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Abstract: The objective of this study was to evaluate the β-glucosidase activity in the non-conventional yeasts under cellulose, glucose and sucrose substrates. The participation of the enzyme β-glucosidase and its contribution to the enzymatic degradation of tannins is known. Within the classification of tannins are ellagitannins, molecules of gallic acid and ellagic acid, which are considered as nutraceutical compounds due to the properties that they present and that they can be used in the design of food and new drugs, synthesis of materials with antimicrobial capacity. The extracellular β-glucosidase activity was mainly presented in the *Candida* and *Pichia* strains, being the glucose and sucrose media the most capable for inducing the activity that showed maximum values with *P. pastoris* in glucose (0.1682±0.00 µmol/min mg protein), and *C. utilis* in cellulose (0.1129±0.1349 µmol/min mg of protein), and sucrose (0.0657±0.0214 µmol/min mg protein). Additionally, *I. terricola* and *P. kluyvery* stood out in a qualitative cellulose degradation approach measured by Congo red method (9.60±0.04 mm and 9.20±0.05 mm respectively). These indicate that *P. pastoris* and *C. utilis* have potential as β-glucosidase producers, especially when growing under complex carbon sources for biomass conversion, new biofuels production and polyphenol degradation with more manageable bioreactor process.

Key words: β-glucosidase, non-conventional yeasts, biomass conversion, Tannins, Cellulolytic activity.

INTRODUCTION

Production of nutraceutical compounds with antimicrobial, antiviral and anticancer activity from tannins degradation has been widely explored where several strains of filamentous fungi, mainly of the genus *Aspergillus sp.*, have been used to transform different vegetal sources rich in these singular variety of polyphenols by the action of enzymes that present hydrolytic activity such as tannase, ellagitannase, and β-glucosidase (Aguilar-Zárate et al. 2013, Belmares et al. 2004, Huang et al. 2008, Shi et al. 2005). Nonetheless, the use of filamentous fungi presents an increment in the complexity of large-scale production due to the fermentation time and the formation of hyphae that modifies the rheology of the medium leading to limitations in the mass transference and a more complicated extraction and purification of the products (Gibbs et al. 2000). Non-conventional yeasts can contribute to resolving these problems due to their ability to form high cell densities cultures and to express a high number of extracellular proteins in a more manageable bioreactor process than filamentous fungi besides their capability to metabolize non-traditional substrates and lead to varied metabolic pathways make them potential candidates for bioprocesses improvement (Wagner & Alper 2016). In the attempt to improve nutraceutical

compounds production, we have developed a strategy using non-conventional yeasts to degrade tannins evaluating the activity of the hydrolytic enzymes tannase, ellagitannase, and β-glucosidase giving a particular emphasis on the analysis of β-glucosidase activity for its polyphenol degradation capacity. The β-glucosidase is a cellulase that in synergy with exoglucanases (EC 3.2.1.91) and endoglucanases (EC 3.2.1.4) breaks down the cellulose polymer to release small sugars fragments in a process known as saccharification, this process begins with the action of the endoglucanase that cut off randomly the cellulose polymer and release small fragments that then will be hydrolyzed to form cellobiose by exoglucanases and finally β-glucosidases converts cellobiose to glucose through hydrolysis of the β-1,4 linkages (Mohanram et al. 2013). In the application concerning to bioethanol production, the glucose released goes to fermentation proceed by extraction and purification stages. Finally, this study aims to evaluate the β-glucosidase activity in the non-conventional yeasts *Debaryomyces hansenii* PYC 2968, *Debaryomyces hansenii* PYC ISA 1510, *Candida utilis, Candida parapsilosis, Pichia kluyvery, Issatchenkia terricola* and *Pichia pastoris* under different polymeric (cellulose) and nonpolymeric (glucose and sucrose) substrates to their potential application in biotechnological processes.

MATERIALS AND METHODS

Non-conventional Yeastes and media

D. hansenii PYC 2968 and *D. hansenii* PYC ISA 1510 were obtained from the Higher Institute of Agronomy (Lisbon, Portugal) whereas *C. utilis*, *C. parapsilosis*, *P. kluyvery*, *I. terricola*, and *P. pastoris* were provided from the Instituto Tecnológico de Morelia, Biochemistry Laboratory (Morelia, Mexico). All strains were maintained in a modified Yeast Extract Peptone Dextrose medium (YEPD: In the evaluation of β-glucosidase activity, glucose, sucrose and carboxymethylcellulose (20 g/L) were used as inducing substrates) containing $MgSO_4$ (0.5 g/ L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), Na_2HPO_4 (3 g/L), $CaCl_2$ (0.02 g/L), casein peptone (10 g/L), yeast extract (10 g/L), glucose (20 g/L) and agar (20g/L) sterilized for 15 min at 121 °C and incubated for 24 h at 32 °C after inoculation.

Cellulolytic activity and halo essays by Congo Red

The cellulolytic activity was measured through a modification of the method proposed by Teather & Wood (1982). Yeasts were grown in the modified YEPD medium using carboxymethylcellulose CMC (10 g/L) as a carbon source for 48 h at 32 °C and Congo Red (1 % w/w) was added for 15 min as an indicator of cellulose degradation due to its ability to form a complex with this polymer indicated as a red coloration that disappears and leaves a degradation halo when cellulose is hydrolyzed (Pointing 1999, Zhang et al. 2006), then the plates were washed out applying HCl solution (0.1M) for 15 min and the size of the halos was measured. For the halo experiments, it was carried out in the same way as described, but the Petri dish was inoculated at an initial concentration of 1 X 10^7 cells / mL of each strain evaluated.

Growth kinetics

Growth kinetics were performed in the modified YEPD medium without agar and modifying the carbon source with glucose, cellulose, and sucrose (20 g/L) for 32 h at 32 °C and 180 rpm in a SI-300R orbital incubator. Samples were collected every 4 hours for cell concentration, potential for hydrogen (pH), reducing sugars, proteins, and β-glucosidase activity quantification. Logistic Model was applied to adjust growth curves and

kinetical parameters were obtained (González-Hernández et al. 2015).

Analytical techniques

Cell concentration was quantified via direct count in Neubauer chamber using 100 µL of the fermentation medium and methylene blue (1% v/v) for staining viable cells according to the technique taken and modified from González-Hernández et al. (2015) and pH variations were measured with a potentiometer (Hanna Instruments).

Reducing sugars were quantified through the method proposed by Miller (1959) using 1 mL of the centrifuged fermentation medium, the pellet was discharged and 10 µL of the supernatant were mixed with 3,5-dinitrosalicylic acid (DNS) for 5 min at 100 °C in a water bath (Felisa), the reaction was stopped by cooling it down into an ice bath for a posterior measure in a spectrophotometer Perkin Elmer Lambda 35 at 540 nm.

Extracellular proteins were analyzed by the Bradford technique (Bradford 1976) using 50 µL of the supernatant mixed with Bradford reagent and quantified in a spectrophotometer Perkin Elmer Lambda 35 at 595 nm.

β -glucosidase activity

The enzymatic assays were performed according to a modification of the protocol proposed by González-Pombo et al. (2011) that quantifies the release of *p*-nitrophenyl due to p-nitrophenyl-β-D-glucopyranoside hydrolysys by β-glucosidase, using an aliquot of 1 mL centrifuged and afterward separated from the pellet, then 100 µL of the supernatant were mixed with 900 µL of p-nitrophenyl-β-D-glucopyranoside in phosphate/citrate buffer (5 mM) and this reaction mixture was incubated for 1 h at 40 °C in a water bath (Felisa), the reaction was stopped with sodium carbonate solution (20% w/v), kept 30 min in incubation and measured in a Perkin Elmer Lambda 35 spectrophotometer at 400 nm.

Statistical analysis

The experiments carried out are a completely randomized design, in which the effect of different substrates (20 g / L) on non-conventional yeasts is being evaluated. Statistical analysis was performed using one-way ANOVA employing GraphPad Prism v8.01 for Windows (San Diego, California USA). Differences between measurements were considered significant when *p* values were lower than 0.05 and a further Tukey´s LSD test was performed for significant treatments. For the fit of the logistic model for kinetic data, the results were obtained with the JMP 6.0® program.

RESULTS

Cellulolytic activity

The qualitative evaluation performed on Congo Red media presented white areas of degradation mainly by the strains *C. parapsilosis* and *P. kluyveri* followed by *D. hansenii* PYC ISA 1510, *I. terricola* and *C. utilis*, even though, these results are not conclusive since the area can not be measured (Supplementary Material - Figure S1), this made necessary the implementation of a semiquantitative approach by inoculating an initial cell concentration of $1x10^7$ cell/mL at the center of the plates (Figure S2). The results obtained indicated that the best cellulolytic activity was presented by *I. terrícola* with degradation halos of 9.60±0.04 mm, followed by *P. kluyveri*, *C. parapsilosis*, and *C. utilis*, with 9.20±0.05, 8.67±0.03, and 8.57±0.04 mm respectively (Suplementary Material - Table SI).

Growth kinetics

It was observed through the short duration of the exponential phase that the metabolism

of the analyzed yeasts was accelerated when utilizing glucose as the carbon source making it a suitable substrate for yeast growth, on the other hand, the adaptation phase of *I. terricola* was lower compared to the rest of the yeasts reaching the stationary phase at around hour 5 and for most of the strains this phase was reached in the hour 10 with the exception of *D. hansenii* PYC ISA 1510 (6 h) as shown in Figure 1a.

Sucrose evaluation presented a similar behavior to glucose kinetics showing a prolonged exponential growth phase that started at around 2 h and reached stationaty phase at around 8 h for *C. utilis* and *D. hansenii* PYC 2968 and at 12 h for the rest of the yeasts. *See* Figure 1b.

Finally, cellulose evaluation presented a lower growth in comparison with the other carbon sources, as seen in Figure 1c, prolonged exponential growth phases are observed for most of the strains that reached the stationary phase between 10 h and 14 h showing a low capacity to metabolize this carbon source, duplication times and the specific growth rates data is displayed in Table I.

The doubling times (tD) show us that for all the strains evaluated, the medium with cellulose presents the highest doubling times, therefore the metabolic capacity of the strains is diminished in this medium.

The specific growth rates (μ) in the non-conventional yeasts evaluated decreased when changing the growth medium from glucose to sucrose and cellulose, with the exception of the *C. utilis* strain, which presented a higher growth rate in the medium with sucrose. The strains that best adapted to growth on cellulose were from the genus *Candida*, particularly *C. utilis*, followed by *C. parapsilosis*.

pH

We described the pH decrement for glucose and sucrose media have a similar behavior (Figure 2a and 2b) possibly due to the release of protons and organic acids caused by the fermentation process (Serrano et al. 1986, González-Hernández et al. 2015). The strain *C. utilis* exhibited constant acidification of the media when evaluated on glucose and sucrose, but when the fermentation was performed using cellulose, an alkalinization tendency was observed (Figure 2c). It is observed that in the medium with YPD the yeasts tend to decrease the pH in the exponential phase due to the activity of the ATPase of the plasmatic membrane, which participates in the regulation of internal pH by pumping protons out of cells, subsequent to this shows an increase in pH at the beginning of the stationary phase due to a process of homeostasis (Serrano et al. 1986).

The alkalinization tendency of the extracellular pH is an important condition that influences or even determines many aspects of cell biology, such as growth and differentiation. Lamb & Mitchell (2003) proposed that a

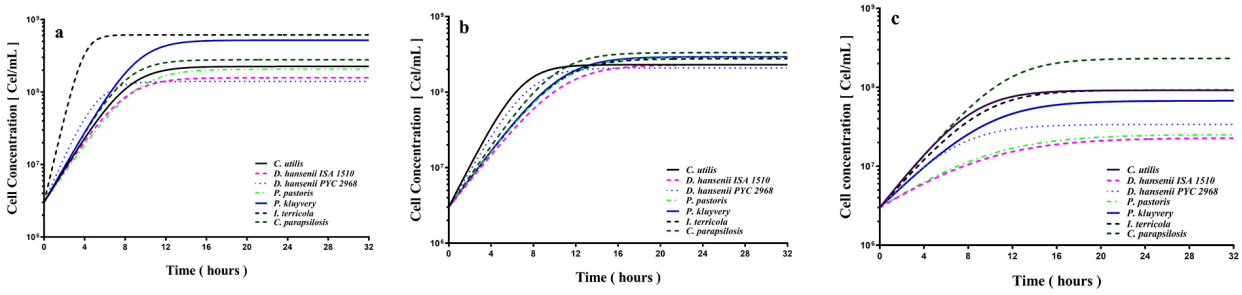

Figure 1. Growth kinetics in non-conventional yeasts during induction with (a) glucose, (b) sucrose, and (c) cellulose. The results are representative data from n = 2.

secondary effect of external alkalinization may be due to nutrient and ion limitation (Bensen et al. 2004) arising from disruption of the plasma membrane proton gradient (PMP). However, we did not test for a PMP disruption resulting from the pH gradient. Based on our DNS substrate results, it is suggested that it may be nutrient limitation (this behavior is more visual in the presence of sucrose and cellulose as substrate), which would not allow efficient plasma membrane ATPase activity. Alkalization mainly occurred to *C. utilis* and *P. kluyveri* yeasts, where the final pH values were increased by 0.9 and 0.8 units each compared to the initial value. Additionally, pH values between 6 and 7.5 have been reported as optimal for β-glucosidase production, and as a variable that has major influence over the enzymatic activity (Granados & Valderrama 2003).

Reducing sugars

The analysis of reducing sugars presents different behaviors in each of the carbon sources evaluated showing a decrement for all the strains in the media supplied with glucose (Figure 3a), on the other hand, for sucrose and cellulose media it is not possible to calculate the concentration of reducing sugars by this technique due to the characteristics of the carbon source as observed in Figures 3b and 3c, respectively. However, for fermentations with sucrose as carbon source, a gradual increase in the concentration and subsequent decay is exhibited attributed to the decomposition of sucrose to its reducing sugars components, fructose and glucose, being visible in *D. hansenii PYC ISA 1510*, *P. kluyvery* and *C. utilis*, where the increase of reducing sugars is observed around the hour 8 for *D. hansenii PYC ISA 1510* and around the hour 12 for *P. kluyvery* and *C. utilis*.

β-glucosidase activity

As presented in Figure 4a for the evaluation of the enzymatic activity with glucose, *P. pastoris* exhibited higher enzymatic activity than the remaining strains that started at around 12 h (0.1044±0.00 µmol/min mg of protein) and reached a maximum value at 28 h (0.1682±0.00 µmol/min mg of protein), followed by *C. parapsilosis* with a maximum at 28 h (0.0768±0.0103 µmol/min mg of protein), *C. utilis* (0.0734±0.00), *D. hansenii PYC ISA 1510* (0.0548±0.0047), *I. terrícola* (0.054±0.0000), *D. hansenii PYC 2968* (0.052±0.0072), and *P.*

Table I. Duplication time (tD) and specific growth rate (µ) in non-conventional induced with Glucose, Sucrose, and Cellulose. The data are presented as mean ± standard deviation, n=2.

Yeasts	Glucose		Sucrose		Cellulose		R^2 Logistic Model		
	µ (h^{-1})	t_D (h)	µ (h^{-1})	t_D (h)	µ (h^{-1})	t_D (h)	G	S	C
***D. hansenii* PYC 2968**	0.76 ± 0.03^{C}	0.91±0.04	0.56 ± 0.02^{B}	1.23±0.04	0.37 ± 0.06^{A}	1.91±0.33	0.85	0.98	0.97
C. utilis	0.55 ± 0.11^{A}	1.28±0.25	0.64 ± 0.02^{B}	1.08±0.04	0.42 ± 0.04^{C}	1.67±0.16	0.85	0.98	0.96
C. parapsilosis	0.56 ± 0.00^{A}	1.24±0.01	0.50 ± 0.13^{B}	1.44±0.39	0.40 ± 0.05^{C}	1.77±0.24	0.92	0.92	0.96
P. kluyvery	0.57 ± 0.00^{BC}	1.22±0.01	0.44 ± 0.01^{AC}	1.59±0.04	0.32 ± 0.02^{AB}	2.16±0.11	0.96	0.98	0.72
I. terricola	1.38 ± 0.10^{BC}	0.50±0.04	0.52 ± 0.22^{A}	1.45±0.60	0.37 ± 0.12^{A}	1.98±0.63	0.77	0.91	0.74
P. pastoris	0.45 ± 0.02^{A}	1.24±0.08	0.43 ± 0.01^{B}	1.62±0.06	0.24 ± 0.09^{C}	3.08±1.14	0.83	0.98	0.91
***D. hansenii* ISA 1510**	0.58 ± 0.27^{A}	1.35±0.63	0.41 ± 0.00^{B}	1.69±0.01	0.21 ± 0.02^{C}	3.28±0.29	0.78	0.99	0.92

Different letters mean statistical difference in Tukey test for α = 0.05, n = 2.
R^2 adjusted to the Logistic Model for Glucose (G), Sucrose (S), and Cellulose (C).

kluyvery (0.051±0.000 µmol/min mg of protein) in descending order at 28 h.

Figure 4b exhibits the corresponding enzymatic activities to medium with sucrose, where the highest enzymatic activity was presented by *C. utilis* and *P. pastoris*, both of them showed a similar behavior, having a considerable increase at 20 h, reaching its maximum value at 28 h, with 0.0657±0.0214 and 0.0635±0.0027 µmol/min mg of protein, respectively followed by *I. terricola* (0.0558±0.0659 µmol/min mg of protein), *D. hansenii PYC2968* (0.0516±0.0102 µmol/min mg of protein), *P. kluyvery* (*0*.0468±0.0150 µmol/min mg of protein), *D. hansenii PYC ISA 1510* (*0*.0365±0.0162 µmol/min mg of protein), and *C. parapsilosis* (0.0162±0.0036 µmol/min mg of protein).

The evaluated non conventional yeasts metabolized cellulose as shown in Figure 4c, however, *C. utilis* is the only one that exhibited outstanding enzymatic activity starting at 20 h (0.0145±0.01 µmol/min mg of protein) and reaching the maximum activity at 28 h (0.1129±0.1349 µmol/min mg of protein) followed by *C. parapsilosis* that presented activity at 20 h (0.0211±0.0038 µmol/min mg of protein) and had a maximum level at 24 h (0.0412±0.0269 µmol/min mg of protein), subsequently, *I. terrícola*, *P. kluyvery*, *P. pastoris*, *D. hansenii* PYC ISA 1510, and *D. hansenii* PYC 2968 reached the maximum enzymatic activity at 20 h (0.0340±0.00 µmol/min mg of protein), 20 h (0.0189±0.00 µmol/min mg of protein), 28 h (0.0077±0.0042 µmol/min mg of protein), 28 h (0.0071±0.0029 µmol/min mg of protein), and 28 h (0.0038±0.0009 µmol/min mg of protein) respectively. The results indicate that yeasts of the genus *Candida* and *Pichia* are producers of β-glucosidase, which is correlated to previous research, including intracellular and extracellular activity (Polachek et al. 1987, Rosi et al. 1994, Mateo & Maicas 2016).

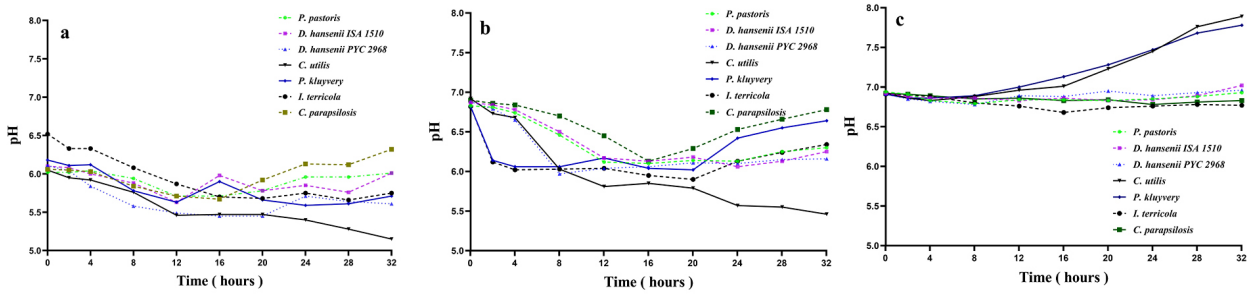

Figure 2. pH comparison for the growth in non-conventional yeasts during induction with (a) glucose, (b) sucrose, and (c) cellulose. The results are representative data from n = 2.

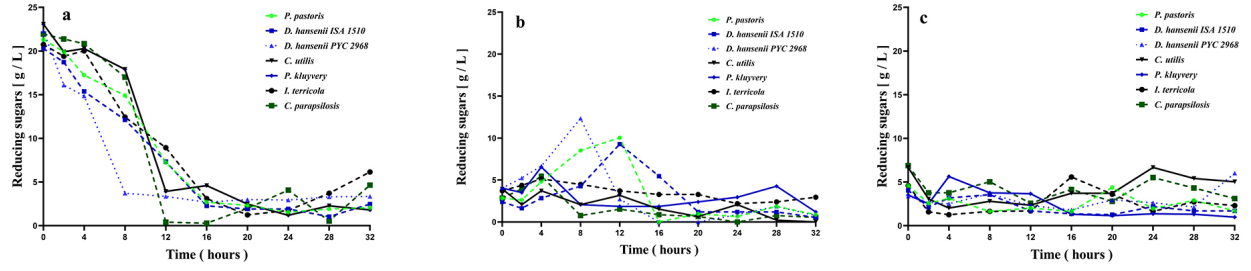

Figure 3. Reducing sugars uptake for the growth in non-conventional yeasts during induction with (a) glucose, (b) sucrose, and (c) cellulose. The results are representative data from n = 2.

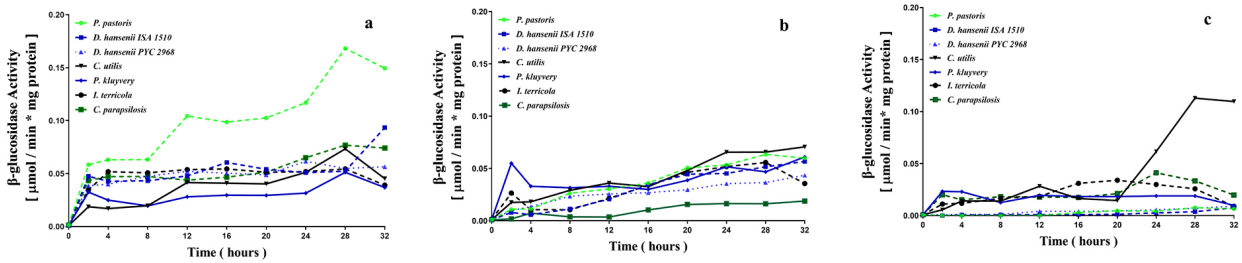

Figure 4. β-glucosidase activity in non-conventional yeasts during induction with (a) glucose, (b) sucrose, and (c) cellulose. The results are representative data from n = 2.

DISCUSSION

It is important to mention that *C. parapsilosis* and *C. utilis* exhibited better adaptation to the media due to its high biomass production and growth rate, but these results can provide only a general panorama on yeasts enzymatic activity.

These results compared with the ones obtained by Lu et al. (2005) who reported degradation halos of 6.4 cm for mesophilic bacteria collected from flower stems showed significantly lower activity for this research reaching nearly 15% of the halo size shown in bacteria. On the other hand, degradation halos of 0.2, 0.3 and 0.35 mm were reported by Gaitan & Pérez (2007) for cellulose-degrading bacteria isolated from a *Dendranthema grandiflora* culture, that presents a guideline to consider non-conventional yeasts as potential cellulolytic microorganism with a more hydrolytic activity than several bacterial strains (Figures S1 and S2).

The specific growth rate for glucose evaluation is maintained at similar values and within the expected range, when changing the carbon source, it can be observed a decrement in the growth rate. On the other hand, duplication time gives us information about the yeast metabolism employed as an indicator of the effect, either positive or negative, of a substrate on microorganisms growth and we can observe that for glucose *I. terricola* had the shortest duplication time followed by *D. hansenii* PYC 2968 and *P. kluyvery*, for sucrose, *C. utilis* had better adaptation to the medium followed by *D. hansenii* PYC 2968 and *C. parapsilosis*, and finally for the media with cellulose the three best-adapted yeasts were *C. parapsilosis*, *C. utilis* and *D. hansenii* PYC 2968, which were the yeasts that metabolize the three carbon sources with the lowest duplication times meaning that they can be utilized in degradation processes with complex carbon sources having a positive effect.

C. utilis is the microorganism best adapted to the different carbon sources according to the duplication times that were higher than most of the strains, also enzymatic activity stood out for its ability to metabolize complex molecules such as cellulose, sucrose and simple substrates such as glucose. In the same way, *C. parapsilosis* displayed a positive effect on cellulose degradation, similar to the enzymatic activity in the medium with glucose, this strain also presented outstanding results in Congo essays, *I. terricola,* on the other hand, exhibited viable enzymatic activity with sucrose and cellulose and *P. pastoris* is a microorganism whose enzymatic activity is benefited in simpler substrates.

Some investigations for β-glucosidases activity in non-conventional yeasts are presented in Table II and Figure 5, only the studies conducted by Sim & Hang (1996), Yanai & Sato (1999) and Turan & Zheng (2005) report enzymatic activity in units comparable to the ones expressed in this research, in comparison to these studies, *P.*

Table II. Comparison of the β-glucosidase activity reported in non-conventional yeasts.

Microorganism	β-glucosidase activity			References
C. molischiana	0.0048[a]			Gunata et al. (1990).
C. wickerhamii	0.0002[a]			
D. hansenii	0.121[b]			Yanai & Sato (1999).
Aurebasidium pallulans	2279[c]			
C. guillermondii	230[c]			McMahom et al. (1999).
C. parapsilosis	1313[c]			
H. anomala	587[c]			
K. apiculata	621[c]			
P. guillermondi	105[c]			
C. molischiana	0.0031[a]			Fernández-González et al. (2003).
D. hansenii	0.0000483[a]			
H. uvarum	0.0000217[a]			
M. pulcherrima	0.000205[a]			
K. thermotolerans	0.0000217[a]			
P. kluyveri	0.0000233[a]			
P. pastoris	0.11[b]			Turan & Zheng (2005).
I. terrícola	0.024[b]			González-Pombo et al. (2011).
C. utilis	0.12[b]			Sim & Hang (1996).
Microorganism	β-glucosidase activity			References
	Glucose[b]	Sucrose[b]	Cellulose [b]	
D. hansenii PYC 2968	0.0523±0.0072	0.0516±0.0102	0.0038±0.0009	This study
C. utilis	0.0734±0.0000	0.0657±0.0214	0.1129±0.1349	This study
C. parapsilosis	0.0768±0.0103	0.0162±0.0036	0.0334±0.0230	This study
P. kluyvery	0.0512±0.0000	0.0468±0.0150	0.0189±0.0000	This study
I. terricola	0.0544±0.0000	0.0558±0.0659	0.0258±0.0000	This study
P. pastoris	0.1682±0.0000	0.0635±0.0027	0.0077±0.0042	This study
D. hansenii ISA 1510	0.0548±0.0047	0.0365±0.0162	0.0071±0.0029	This study

[a] Data expressed in mmol of ρ-nitrophenol / mL min.
[b] Data expressed in mmol of ρ-nitrophenol / min mg of protein.
[c] Data expressed in mmol of ρ-nitrophenol / mg of cells for 30 min (dry weight).

pastoris evaluated on glucose media and *C. utilis* evaluated on cellulose are similar to what has been reported, nonetheless, other results may not be comparable in magnitude, however, others non conventional yeasts can be found in literature for β-glucosidase activity such as the ones of the genus *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Brettanomyces, and Kluyveromyces* (Strauss et al. 2001, Rodriguez et al. 2004, Cordero-Otero et al. 2003, Arévalo-Villena et al. 2006). Non-conventional yeasts studied in this work have also been explored as an alternative for microorganisms capable of producing the enzyme tannase which together with β-glucosidase are capable of degrading ellagitannins, which has multiple applications in the food and pharmaceutical industry. The activity on of both enzymes is very important due to their ability to produce ellagic acid (Márquez-López et al. 2020a, b). It is also important to mention the enzymatic bioprospecting that is being carried out on these non-conventional yeasts native to spontaneous fermentation in the production of artisanal mezcal in the Etucúaro, Michoacán, as well as others that were isolated from sawmills in Ciudad Hidalgo, Michoacan.

Our results obtained show a large standard deviation in the case of *C. utilis* grown on cellulose, as well as *I. terricola* grown on sucrose. However, the results obtained show that yeasts of the genus *Candida* and *Pichia* are producers of β-glucosidase, which have been reported with intracellular and extracellular activity (Rosi et al. 1994).

In addition, it can be seen that the enzymatic activity is closely correlated with growth, presenting the maximum during the stationary phase (hour 28 of growth kinetics). Glucose and sucrose were better inducers of β-glucosidase activity than cellulose which (Figure 5), regardless of its complex structure and large number of β-1-4 bonds, did not induce the presence of the enzyme of interest in non-conventional yeasts studied. However, it has been shown that other less complex sources, constituents of cellulose such as cellobiose, can act more effectively as an inducer (Villena et al. 2007). Cellulose has a complex structure, which

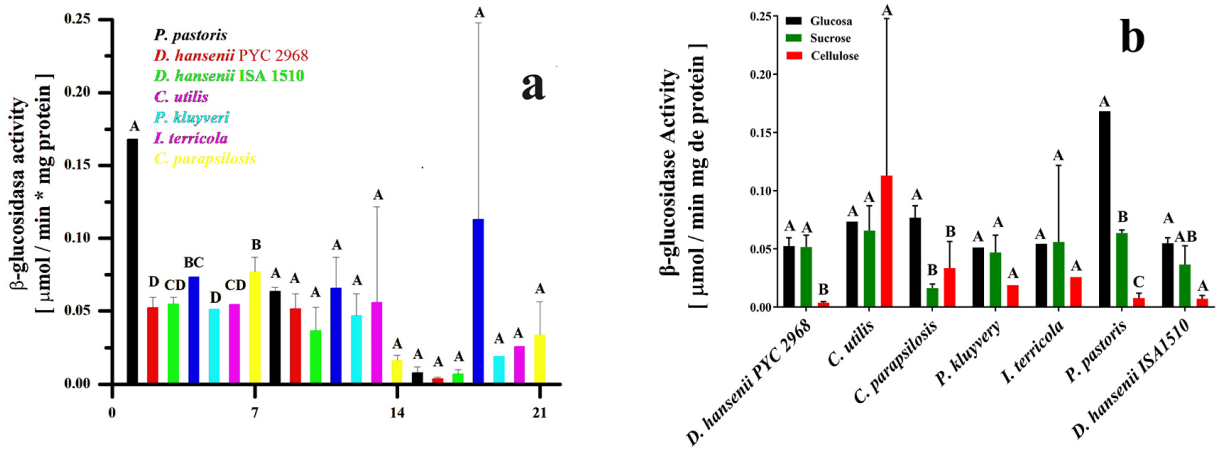

Figure 5. β-glucosidase activity in non-conventional yeasts at 28 h of growth. 5a) Describe the comparison of all yeasts with each substrate is shown with glucose (column 1-7), cellulose (column 8-14) and sucrose (column 15-21). 5b) Comparison of each yeast independently for each substrate: 1) *P. pastoris* , 2) *D. hansenii* PYC 2968, 3) *D. hansenii* ISA 1510, 4) *C. utilis*, 5) *P. kluyvery*, 6) *I. terrícola*, 7) *C. parapsilosis*. Different letters mean statistical difference in Tukey test for α = 0.05, n = 2.

can represent difficulties for hydrolysis due to effects such as the contact area between the enzyme and the molecule, the crystallinity of the cellulose, and the dimensions of the fiber pores. (Buschle-Diller et al. 1994).

In Figure 5, a multiple comparison procedure was applied to determine which means of the enzyme activity assays are significantly different. As a first part, the β-glucosidase activity of all the strains is compared with the glucose substrate (columns 1-7). when sucrose was used as a substrate (columns 8-14); finally, with cellulose as a substrate (columns 15-21) and as a second analysis, each yeast is compared independently for each substrate (Figure 5b).

The one-factor analysis of variance procedure for the β-glucosidase enzymatic activity with the evaluated substrates gives us the result that with glucose there are statistically significant differences between the means, where *P. pastoris* had the best β-glucosidase activity (Figure 5a). Likewise, it is described that with the presence of sucrose and cellulose used as substrates, when comparing the enzymatic activity between the studied strains, there are no statistically significant differences (Figure 5a).

Figure 5b describes the statistical analysis evaluating the differences of each strain against the different substrates used. The statistical analysis gives us the result that the strains *D. hansenii* PYC 2968, *D. hansenii* ISA 1510, *C. parapsilosis* and *P. pastoris* show statistically significant differences of the enzymatic activity when comparing the result obtained with glucose against cellulose as substrates. In Figures 5a and 5b it is described which strains show statistically significant differences at the 95.0% confidence level. The method used to discriminate between the means was Tukey's Honestly Significant Difference (HSD) procedure.

CONCLUSION

The extracellular β-glucosidase activity was mainly presented in the *Candida* and *Pichia* strains, being the glucose and sucrose media the most capable for inducing the activity that showed maximum values with *P. pastoris* in glucose (0.1682±0.00 µmol/min mg protein), and *C. utilis* in cellulose (0.1129±0.1349 µmol/min mg of protein), and sucrose (0.0657±0.0214µmol/min mg protein). Additionally, *I. terricola* and *P. kluyvery* stood out in a qualitative cellulose degradation approach measured by Congo red method (9.60±0.04 mm and 9.20±0.05 mm respectively).

On the other hand, the minimum values were obtained for *P. kluyvery* in the medium with glucose (0.0512±0.00 µmol/min mg of protein) followed by *C. parapsilosis* in the medium with sucrose (0.0162±0.0036 µmol/min mg of protein) and *D. hansenii PYC 2968* in the medium with cellulose (0.0038±0.0009 µmol/min mg of protein). These data indicate that *P. pastoris* and *C. utilis* have the potential as β-glucosidase producers for cellulose degradation, especially when growing under complex carbon sources favoring their introduction in renewable and more efficient processes.

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SUPPLEMENTARY MATERIAL

Figures S1, S2.
Table SI.

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