EVALUATING β-GLUCOSIDASE ACTIVITY OF YEASTS ISOLATED FROM THE GUT OF FALL ARMYWORM

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ABSTRACT

Cellobiose, a disaccharide derived from cellulose, can be obtained from agricultural and forestry residues. Since *Saccharomyces cerevisiae*, commonly used in first-generation ethanol production, cannot naturally hydrolyze cellobiose, alternative yeasts with β-glucosidase activity are needed. Therefore, it is necessary to search for yeasts in the environment, such as in the digestive tract of herbivores, as in the case of the fall armyworm (*Spodoptera frugiperda* larvae). Thirteen yeast strains were evaluated for extracellular and intracellular β-glucosidase activity and cellobiose consumption. Four strains (CHAP-156, CHAP-157, CHAP-158, and CHAP-159) exhibited significant extracellular β-glucosidase activity, with higher activities at 50°C than at 30°C. Most strains consumed over 50% of the initial 20 g·L^{-1} cellobiose, indicating intracellular enzymatic activity. Strains CHAP-165, CHAP-166, CHAP-167, and CHAP-200 showed the highest intracellular activities, respectively 154.07 U·mg^{-1}, 196.58 U·mg^{-1}, 164.09 U·mg^{-1}, and 159.34 U·mg^{-1}. These findings suggest that the evaluated yeast strains have potential applications in Simultaneous Saccharification and Fermentation (SSF) processes, enhancing the viability of second-generation ethanol production from lignocellulosic biomass.

Keywords: cellobiose hydrolysis. β-glucosidase. ethanol 2G. *Spodoptera frugiperda*.

1 INTRODUCTION

Cellobiose is a disaccharide derived from the hydrolysis of cellulose, composed of β-1,4 linkages between glucose molecules. It can be obtained from the reuse of cellulose present in agricultural residues, forest management, and fruit growing[1]. Since the species most used in first-generation (1G) ethanol production, *Saccharomyces cerevisiae*, is unable to hydrolyze cellobiose, it is necessary to seek wild yeasts that possess cellobiases to be either employed, act in conjunction, or provide genes, to make second-generation ethanol production more viable[2].

β-glucosidases can be found in the enzymatic complex of cellulases used in cellulose hydrolysis. In this context, they are intrinsically necessary for the utilization of lignocellulosic biomass residues as a source for second-generation (2G) ethanol production[3]. Therefore, it is necessary to search for yeasts in the environment, such as in the digestive tract of herbivores, as in the case of the fall armyworm (*Spodoptera frugiperda* larvae), due to their plant biomass-based diet, which leads to a selective pressure over the microorganisms in their microbiota[4]. In this context, this study aimed to evaluate 13 yeast strains isolated from the intestine of the *S. frugiperda* larvae for β-glucosidase activity and cellobiose consumption.

2 MATERIAL & METHODS

The thirteen strains evaluated were previously isolated by our research group[5]. Precultures were performed for 48 hours in solid YPD media (10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone, and 20 g·L^{-1} glucose) and incubated at 30 °C. For cultivation, 125 mL Erlenmeyer flasks containing 25 mL of YPC (10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone, and 20 g·L^{-1} cellobiose) with pH adjusted to 5.0 were used, kept in a shaker at 145 rpm and 30 °C. The cultivation time varied according to the analysis. All analyses were performed in triplicate.

First, extracellular β-glucosidase activity and cellobiose consumption were evaluated. After 48 hours of cultivation, 2 microtubes of 1 mL of the culture medium were collected for both analyses, centrifuged at 9000 rpm for 3 minutes, and the supernatant was collected for the analyses. For extracellular β-glucosidase activity, 10 µL of the supernatant was added to new microtubes containing 15 µL of 0.15 M Succinate-Tris buffer (pH 5) containing 10 g·L^{-1} cellobiose and incubated at 30 °C and 50 °C for 1 hour. After the incubation period, the amount of glucose released after hydrolysis was measured using a commercial Analyza kit. For cellobiose consumption, the residual amount of cellobiose in the culture medium was measured in a microplate using DNS (1% 3,5-dinitrosalicylic acid and 30% sodium-potassium tartrate in 0.4 M NaOH)[6].

For the evaluation of intracellular and periplasmic enzymatic activity, cultivation was performed in YPC media until the optical density reached the beginning of the exponential phase. The cells were concentrated to 20 g·L^{-1}, and intracellular and periplasmic activity was evaluated as described by Barrilli et al (2020)[7]. For periplasmic activity, whole cells incubated with 0.15 M sodium fluoride dissolved in 0.15 M Succinate-Tris buffer (pH 5) were used and then incubated in 0.3 M cellobiose. For intracellular activity, permeabilized cells were incubated in 0.2 M cellobiose in 0.1 M MOPS-NaOH buffer (pH 6.8). For the

negative controls, cells were boiled for 5 min. Glucose was measured using the supernatants collected after the hydrolysis step with a commercial kit (Gold Analyza). β-glucosidase activity was expressed as U·mg (dry yeast cells)$^{-1}$, where one unit (U) corresponds to 1 nmol of glucose produced per minute.

The data obtained from the enzymatic activity determinations were subjected to a null hypothesis ANOVA test using GraphPad Prism® v8.0.2 (StatSoft Inc) software to verify if there were any differences between sample groups ($p<0.05$), followed by a multiple comparison using Tukey's Test ($p<0.05$). The analyses were graphically presented with the means of the triplicates for each strain, along with standard deviation bars, and the samples that showed differences were indicated with letters.

3 RESULTS & DISCUSSION

For the evaluation of β-glucosidase activity, after 48 hours of cultivation in cellobiose, the supernatants from the cultivation were subjected to reaction in a buffer containing 1% of this disaccharide at temperatures of 50 °C and 30 °C. Of the 13 strains evaluated, only four showed statistically significant extracellular β-glucosidase activity: CHAP-156, CHAP-157, CHAP-158, and CHAP-159 (Figure 1A). Regarding the effect of temperature, the assays incubated at 50 °C showed the highest enzyme activity. The activities observed were 17.70 U·mL^{-1}, 29.35 U·mL^{-1}, 37.98 U·mL^{-1}, and 61.92 U·mL^{-1} for the strains CHAP-156, CHAP-157, CHAP-158, and CHAP-159, respectively, being significantly higher than those observed at 30 °C ($p<0.05$). Analyzing a strain of *Pseudozyma brasiliensis*, Kaupert Neto et al. (2016)[8] found extracellular activities below 10 U/mL. Similarly, Gao et al. (2022)[9], in tests with *Meyerozyma guilliermondii* NM218 and *Hanseniaspora uvarum* BF345, in media similar to wine fermentation musts, also obtained activities lower than those we observed, ranging from 4.8 to 5.4 U·mL^{-1}.

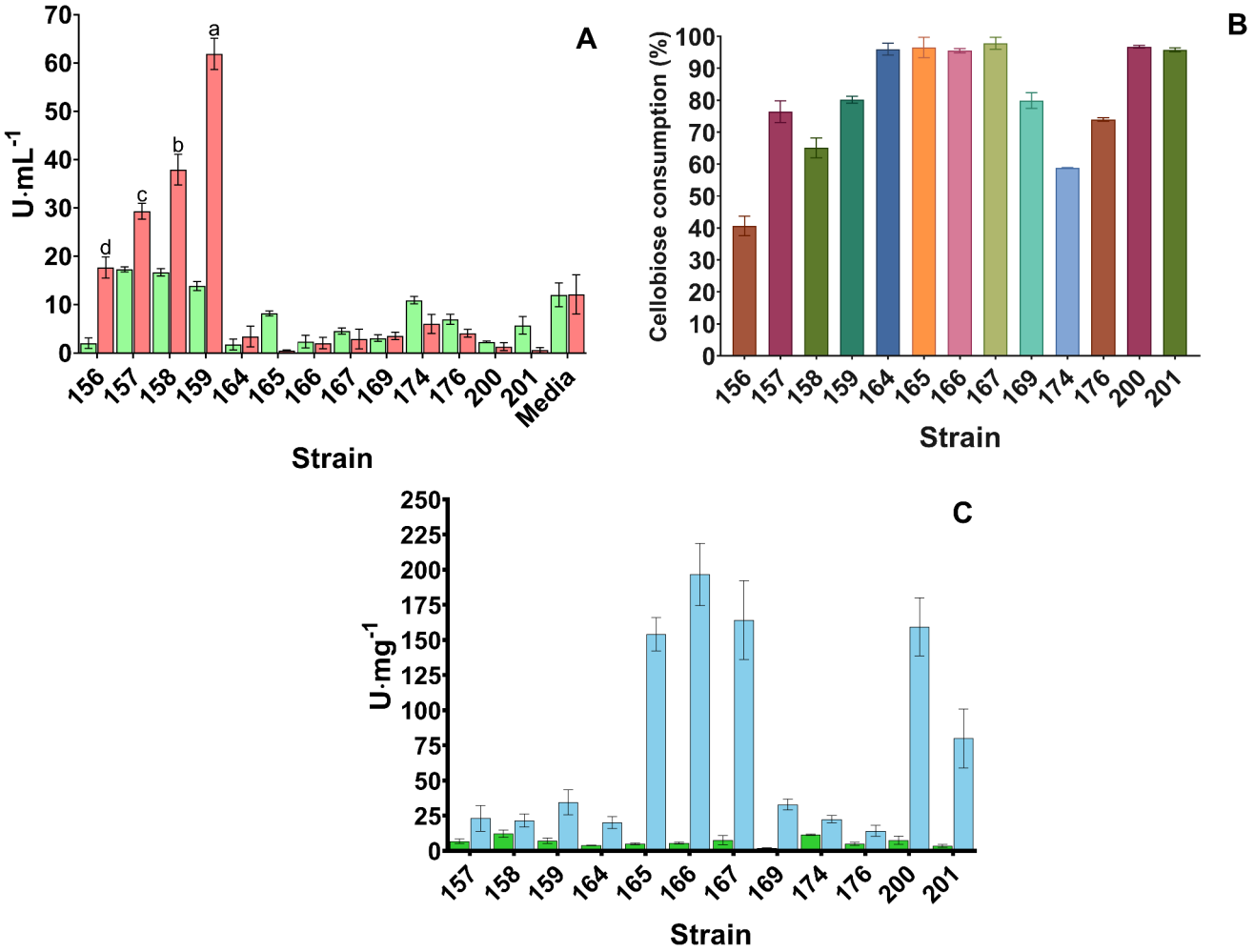

Figure 1 Cellobiose metabolism by fall armyworm-isolated yeasts. (A) Extracellular activity in 30 °C (Mint Green) and 50 °C (Salmon). (B) Percentage of cellobiose consumed. (C) Periplasmic (Lime Green) and Intracellular (Sky Blue) activity. Different lowercase letters next to the bars represent significant differences.

Regarding the consumption of cellobiose in the medium in which the cells were cultivated, it was observed that most strains consumed more than half of the initial 20 g·L^{-1} (Figure 1B), indicating that even though most did not show extracellular activity, they have enzymes that act in the periplasm or inside the cell. Indeed, a significant portion of yeasts tends to have a cellobiose transporter in the cell membrane and a cytoplasmic hydrolase to ensure cellobiose metabolism[7].

To verify the presence of enzymes in the periplasm and inside the cells, only the strains that consumed more than 50% of cellobiose were evaluated. Thus, as shown in Figure 1C, yeasts that did not show extracellular activity mainly have intracellular activity, with the most significant activities present in strains CHAP-165, CHAP-166, CHAP-167, and CHAP-200, respectively 154.07 $U \cdot mg^{-1}$, 196.58 $U \cdot mg^{-1}$, 164.09 $U \cdot mg^{-1}$, and 159.34 $U \cdot mg^{-1}$. In contrast, strains that showed extracellular activity exhibited low intracellular activity. Similar results were found by Barrilli et al. (2020)[7], where wild yeasts of the species *Candida pseudointermedia* also showed activities close to 200 $U \cdot mg^{-1}$; however, no extracellular activity was detected in their analyses.

4 CONCLUSION

Among the thirteen strains evaluated, only four have extracellular β-glucosidase activity, and twelve have predominantly intracellular activity. These yeasts have the potential to be applied in Simultaneous Saccharification and Fermentation (SSF) processes, where, together with cellulase enzymes, they can act both in the hydrolysis of plant biomass and in ethanol production.

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