PRODUCTION OF CELLULOLYTIC ENZYMES BY THE FUNGUS *Trichoderma asperellum* URM 6997/160821, VARYING THE CARBON SOURCE

Ádrian Guterres[1*], Pedro H. Trevisan[1], Isabela de L. Valente[2], Dinalva Schein[2] & Márcio Antônio Mazutti[3]

[1]*Chemical Engineering studente, Federal University of Santa Maria, Santa Maria-RS, Brazil.*
[2]*PhD student in Chemical Engineering, Department of Chemical Engineering, Chemical Engineering Graduate Program, Federal University of Santa Maria, Santa Maria-RS, Brazil.*
[3]*Professor Doctor, Department of Chemical Engineering, Chemical Engineering Graduate Program, Federal University of Santa Maria, Santa Maria-RS, Brazil.*
** Corresponding author's email address: Adrian.guterres@acad.ufsm.br*

ABSTRACT

Cellulolytic enzymes play a fundamental role in cellulose degradation, enabling its conversion into products of industrial interest. The fungus *Trichoderma asperellum* is widely used in the production of these enzymes due to its efficiency and versatility in degrading cellulose substrates. This study aims to evaluate the enzymatic activity of the fungus *Trichoderma asperellum* on different carbon sources such as glucose and sucrose. It is noticeable in the glucose-rich source that there was a peak of activity at 24 hours of fermentation followed by a sharp decline, which differs from sucrose, where there is a sustained low activity until the end of fermentation (96 hours). For glucose, activity decreases due to nutrient depletion within the first 24 hours, while for sucrose activity remains until the end, although nutrients are also depleted over time. While the results were significant, it is necessary to maintain fungal feeding to keep enzymatic activity high and constant.

Keywords: *Thichoderma asperellum*. Sucrose. Glucose. FPase analysis.

1 INTRODUCTION

Lignocellulosic materials are resources used for biofuel production, facing the challenge of biomass recalcitrance. Thus, cellulolytic enzymes are promising in this conversion process, along with other procedures for saccharification and fermentation of fermentable sugars to obtain second-generation ethanol[1]. Enzymatic methods for acting on cellulolytic materials have an advantage compared to chemical and physical methods due to lower energy consumption and pollution levels, as well as increased safety[2]. As a result, filamentous fungi such as Trichoderma species are options for synthesizing cellulases and xylanases[3].

Cellulases are enzymes belonging to the group of glycosyl hydrolases[4], composed of a mixture of enzymes such as endoglucanase, cellobiohydrolase, β-glucosidase that work synergistically in the degradation of cellulose biomass[5], hydrolyzing it into sugars and other oligomeric units. Just as cellulase acts in the enzymatic saccharification of cellulolytic biomass, it can also be used in the paper industry, either in the enzymatic deinking of office waste, or in pulping, as well as constituents in detergents, fabric softeners, denim bleaching, and fabric polishing[6].

Different species of the fungus Trichoderma, such as *T. reesei*[3], *T. harzianum*[7], and *T. asperellum*[8], are representative in the production of hydrolytic enzymes - cellulase. Therefore, this study aims to explore the cellulase activity produced by *Trichoderma asperellum* and evaluate different carbon sources for this microorganism, observing under which condition there is higher enzymatic activity.

2 MATERIAL & METHODS

The fungus *Trichoderma asperellum* URM 6997/160821 was cultivated in Potato Dextrose Agar (BDA) medium for 7 days at 25ºC with a photoperiod. A 9 mm disk of pure culture was inoculated into each 50 mL of fermentative medium consisting of KH_2PO_4 (2g/L), $(NH_4)_2SO_4$ (5g/L), $MgSO_4.7H_2O$ (0.3g/L), peptone (1g/L), and an oligoelement solution (1mL/L) containing $Na_2EDTA.2H_2O$ (15g/L), $ZnSO_4.7H_2O$ (4.5g/L), $MnCl_2.4H_2O$ (1g/L), $CoCl_2.6H_2O$ (0.3g/L), $CuSO_4.5H_2O$ (0.3g/L), $CaCl_2.2H_2O$ (4.5g/L), $FeSO_4.7H_2O$ (0.3g/L), and KI (0.1g/L), varying the carbon source, with 15g/L of glucose and 15g/L of sucrose, both adjusted to pH 4.5, and incubated at 180 rpm at 28°C for 96 hours. For the evaluation of enzymatic activity, samples were taken at 24h, 48h, 72h, and 96h, filtered, and quantified for FPase activity, according to Gelain et al., (2021) - with modifications.

The FPase activity analysis was performed according to Inforsato and Porto, (2016), and Gelain et al., (2021), consisting of the preparation, at room temperature, of the distribution of Whatman No. 1 filter paper strips (1cm x 6cm) spirally in test tubes, followed by the addition of 2mL of acetate buffer (50 mM, pH 5.0) and 1mL of filtered sample for subsequent incubation in a water bath at 50°C for 60min. A 1mL aliquot was then taken, 3mL of 3,5-dinitrosalicylic acid (DNS) reagent and 1mL of water were added, followed by incubation in a water bath at 100°C for 5 min, and subsequent ice bath until reaching room temperature. Quantification was performed by absorbance at 540nm in a spectrophotometer, and the calculation of enzymatic activity was performed using equation 1 (D: diluition; C: concentration determined from the DNS method; Vt: total volume of the reaction; T: reaction time; Ve: volume of enzyme solution).

$$x = \frac{D \times C \times V_t}{T \times V_e} \quad (1)$$

The quantification of reducing sugars proceeds with the determination of monosaccharides using the DNS (3,5-dinitrosalicylic acid) method according to Miller (1959) and Maldonade et al., (2013), with 500µL of sample, 500µL of DNS, heated at 95ºC for 5 min, cooled in an ice bath for 5 min, followed by the addition of 8ml of sodium potassium tartrate solution and reading in the spectrophotometer at 540nm. Disaccharides are quantified using the method described by Dubois et al., (1956), proceeding with 1mL of sample, 50µL of 80% phenol, and 5ml of concentrated sulfuric acid, kept at rest for 10 min, shaken for 20 min in a water bath at 25ºC, followed by reading in the spectrophotometer at 490nm.

3 RESULTS & DISCUSSION

The FPase activity values (Figure 1) with carbon sources glucose (SG) and sucrose (SS) at 24, 48, 72, and 96 hours were 0.657, 0.046, 0.047, and 0.046 IU/mL, respectively, throughout the stage. The SG medium showed a significant increase at 24h followed by a sharp decline, stabilizing at 0.046 IU/mL. Comparing it to the amount of sugar consumed by the microorganism, the highest cellulase activity corresponded to the amount of glucose consumed by the fungus, and as the carbon source decreased, there was a proportional decline in cellulase activity.

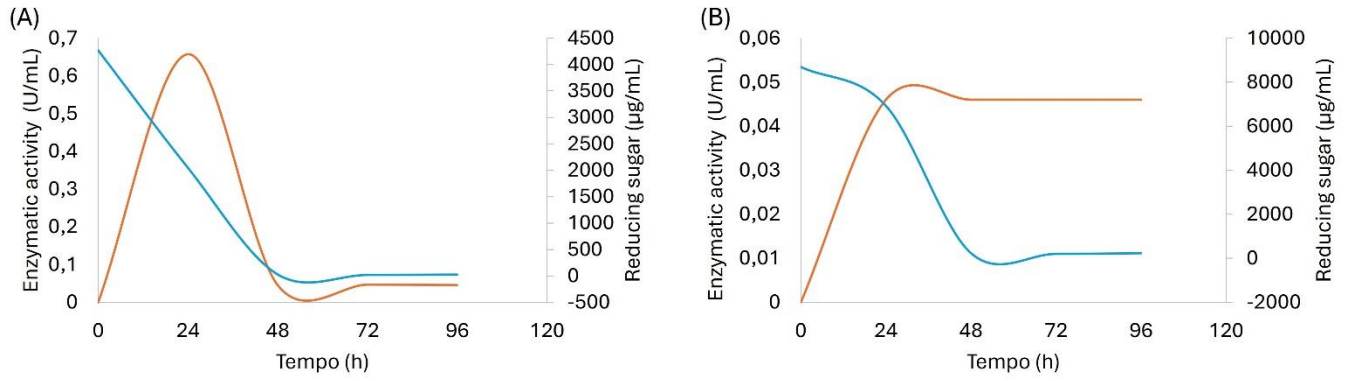

Figure 1 Graphical representation of the relationship between the metabolism of (A) glucose and (B) sucrose as carbon sources by Trichoderma and FPase enzyme production.

According to Szijártó et al. (2003), in cultivation with *T. reesei* Ru-C30 using glucose as the carbon source, the results were similar, as the initial glucose phase lasted 26h, with enzyme activity of 0.3 FPU/ml remaining constant, followed by glucose depletion. Similarly, Asad et al. (2015) demonstrated that the maximum activity of 147 U/mL of endoglucanase occurred within 24h when cultivated with *T. asperellum*. Alternatives for increasing extracellular enzyme production, as described by Gelain et al. (2021), would involve fermentations in continuous cultures using glucose as an easily assimilated source, as demonstrated in their work with *T. harzianum*, in addition to process optimization, as they achieved 1048 U/mol.h of β-glucosidase using the substrate p-nitrophenyl-β-d-glucopyranoside. Furthermore, Zhang et al. (2017) worked with a recombinant strain of RutC-30, which showed an increase in FPase activity from 4.66 to 12.15 compared to the non-recombinant strain within 48h.

On the other hand, SS maintained a constant enzyme concentration during fermentation, where even with the decrease in the carbon source, its activity remained constant throughout the period. Comparing with literature data, Ike and Tokuyasu (2018), in cultivation with *T. reesei* M2-1 using carbon sources like sucrose+cellobiose, there were no changes in sucrose, only in cellobiose, during cultivation. However, when using glucose+fructose+cellobiose, there was a preferential decrease in glucose and cellobiose, with fructose accumulating in the initial phase, nonetheless with cellulase production. Another test was conducted by applying invertase enzyme before inoculation, and strain M2-1 produced larger quantities of cellulase in 48h. Consequently, in accordance with the previous study, it is possible that in this study, *T. asperellum* produced the invertase enzyme capable of hydrolyzing sucrose into glucose and fructose. And, since it prefers glucose, it may have used it as the initial step for cellulase production. And at the end, it was maintained steadily by fructose.

In parallel to SS, Sanjeev et al. (2014) suggests exposing the strain *T. asperellum* RCK2011 to UV radiation and conducting tests on different carbon sources, in addition to solid-state fermentation, to increase FPase from 0.613 to 1.02 IU/g in the modified strain (SR1-7) compared to the original strain. Despite solid-state fermentation being an industrial challenge, it is an alternative as it mimics the natural habitat of filamentous fungi and, thus, better adapted, allowing for higher enzyme production capacities[9].

4 CONCLUSION

Glucose and sucrose are similar, metabolizable sugars that undergo central glycolysis metabolism, with the differentiation of invertase in the presence of the disaccharide. Another characteristic persists in continuous fermentation since maintaining the carbon source for longer periods results in higher enzymatic activity. It is necessary to optimize the process and investigate viable resources for cellulase production.

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