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Economical alternatives for the production of fungal β-1,3-glucanase using easily obtainable industrial substrates

Alternativas econômicas para produção de β-1,3-glucanase fúngica utilizando substratos de fácil obtenção industrial

Alternativas económicas para la producción de β-1,3-glucanasa fúngica utilizando sustratos de fácil obtención industrial

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Abstract
The β-1,3-glucanases synthesized by filamentous fungi have wide applicability in the food, chemical, and pharmaceutical industries. However, its obtainment can be costly, especially due to substrates used to induce its synthesis. Therefore, the objective of this work was to produce β-1,3-glucanase by *T. harzianum* Rifai using free and immobilized cells in synthetic and plant sponges, using different inducing substrates that could provide better cost-effectiveness for the industrial production of the enzyme. The Petri dish zymogram technique proved to be efficient for screening substrates inducing β -1,3-glucanases against species of filamentous fungi. It was possible to perform the immobilization of *T. harzianum* in a synthetic sponge allowing the realization of repetitive batches for enzymatic production. All tested substrates resulted in the synthesis of β-1,3-glucanase, including succinoglycan, proposed innovatively in this study. Fungal biomass resulted in the best inducing substrate under conditions of free and immobilized cells, with a production of β-1,3-glucanases of 0.73 U and 0.80 U of β -1,3-glucanases. The substrates corn starch and cassava showed promise in the production of β-1,3-glucanase and maintained production until the fourth batch was evaluated, with values of 0.51 U and 0.46 U of β-1.3-glucanases, respectively. The results obtained in this study showed that the zymogram is a practical method for screening substrates induced by the fungus *T. harzianum*. Corn starch and cassava are accessible and low-cost sources for β-1,3-glucanase synthesis in repetitive batches, including the use of immobilized and free cells.
Keywords: *Trichoderma harzianum*; Starch; Immobilization; Zymogram; Succinoglycan.

Resumo
As β-1,3-glucanases sintetizada por fungos filamentosos possuem amplas aplicabilidades nas indústrias alimentícia, química e farmacêutica. Entretanto, sua obtenção pode ser onerosa, especialmente devido substratos utilizados para induzir sua síntese. Desta forma, o objetivo deste trabalho foi produzir β-1,3-glucanase por *T. harzianum* Rifai

utilizando células livres e imobilizadas em esponjas sintética e vegetal, empregando diferentes substratos indutores que pudessem proporcionar melhor custo-benefício para a produção industrial da enzima. A técnica de zimograma em placa de Petri se mostrou eficiente para triagem de substratos indutores de β-1,3-glucanases frente espécies de fungos filamentosos. Foi possível realizar a imobilização do *T. harzianum* em esponja sintética permitindo a realização de lotes repetitivos para produção enzimática. Todos os substratos testados resultaram em síntese de β-1,3-glucanase, incluindo a succinoglucana, proposto de forma inovadora neste estudo. A biomassa fúngica resultou no melhor substrato indutor em condições de células livres e imobilizadas, com uma produção de β-1,3-glucanases de 0,73 U e 0,80 U de β-1,3-glucanases. Os substratos amido de milho e mandioca mostraram promissores na produção de β-1,3-glucanase e mantiveram a produção até o quarto lote avaliado, com valores de 0,51 U e 0,46 U de β-1,3-glucanases, respectivamente. Os resultados obtidos neste estudo mostraram que o zimograma é um método prático para triagem de substratos indutores pelo fungo *T. harzianum*. O amido de milho e mandioca são fontes acessíveis e de baixo custo para síntese de β-1,3-glucanase em lotes repetitivos, inclusive com uso de células imobilizadas e livres.
Palavras-chave: *Trichoderma harzianum;* Amido; Imobilização; Zimograma; Succinoglucana.

Resumen
Las β-1,3-glucanasas sintetizadas por hongos filamentosos tienen amplia aplicabilidad en la industria alimentaria, química y farmacéutica. Sin embargo, su obtención puede resultar costosa, especialmente debido a los sustratos utilizados para inducir su síntesis. Así, el objetivo de este trabajo fue producir β-1,3-glucanasa por *T. harzianum* Rifai a partir de células libres e inmovilizadas en esponjas sintéticas y vegetales, utilizando diferentes sustratos inductores que pudieran brindar un mejor costo-beneficio para la producción industrial de la enzima. La técnica del zimograma de la placa de Petri demostró ser eficiente para la detección de sustratos inductores de β-1,3-glucanasas contra especies de hongos filamentosos. Fue posible inmovilizar *T. harzianum* en esponja sintética, permitiendo la realización de lotes repetitivos para la producción enzimática. Todos los sustratos probados dieron como resultado la síntesis de β-1,3-glucanasa, propuesto de forma novedosa en este estudio. La biomasa fúngica resultó ser el mejor sustrato inductor en condiciones de células libres e inmovilizadas, con una producción de β-1,3-glucanasas de 0,73 U y 0,80 U de β-1,3-glucanasas. Los sustratos de almidón de maíz y yuca se mostraron promisorios en la producción de β-1,3-glucanasa y mantuvieron la producción hasta el cuarto lote evaluado, con valores de 0.51 U y 0.46 U de β-1,3-glucanasas, respectivamente. Los resultados obtenidos en este estudio demostraron que el zimograma es un método práctico para el cribado de sustratos inductores del hongo *T. harzianum*. El almidón de maíz y mandioca son fuentes accesibles y de bajo costo para la síntesis de β-1,3-glucanasa en lotes repetitivos, incluido el uso de células inmovilizadas y libres.
Palabras clave: *Trichoderma harzianum*; Almidón; Inmovilización; Zimograma; Succinoglucano.

1. Introduction

The β-1,3-glucanases are enzymes that hydrolyze the glycosidic bonds between oligosaccharide and/or polysaccharide-forming monosaccharides (Usoltseva et al., 2020). Widely distributed in nature, β-1,3-glucanases can act in several functions, including the degradation of polysaccharides that will be used as an energy source for fungi and bacteria. The β-1,3-glucanases can be of the exo and endo type, synergistic action being common, in which at least two enzymes with different modes of action are used to degrade β-glucans (Pitson et al., 1993).

Several microorganisms can synthesize β-1,3-glucanases for the extracellular medium, especially yeasts such as Saccharomyces cerevisiae (Lopes et al., 2015), Pichiamem branifaciens (Masih & Paul, 2002), and *Aureobasidium pullulans* (Bauermeister et al., 2015), and filamentous fungi of *Penicillium*, *Aspergillus* and *Trichoderma* genders (Musoni et al., 2015). Some *Trichoderma species*, including *T. harzianum* (El-Katatny et al., 2000), are efficient biocontrol agents for phytopathogenic fungi of economic importance, such as *Botrytis*, *Fusarium*, *Pythium*, and *Rhizoctonia* (Howell, 2003; Sharma et al., 2009; Menezes et al., 2010), as they are capable of degrading the fungal cell wall, especially through the action of β-glucanases (Gerhardson, 2002).

The microorganism immobilization technique by passive adhesion to surfaces, such as natural and synthetic sponges, has great potential for industrial application and can be applied in the production of β-glucanases (Haapala et al., 1994). This process has several advantages over the conventional method with free cells, such as the ability to use the microorganisms in repetitive batches, easy recovery of cells from the fermentation medium, and a reduction in contamination risk. The ideal

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immobilization matrix must be strong, resistant, and porous. Natural and synthetic sponges have been used successfully to immobilize fungi (Hideno et al., 2007; Pazzetto et al., 2011; Santos & Cruz, 2016).

In addition to their use in agriculture, fungal β-1,3-glucanases have several applications such as in the production process of wines and beers for intensifying sensory characteristics, improving the digestibility of animal feed, in bioactive oligosaccharides production, including prebiotics and immunomodulators, becoming an important tool for the food, chemical and pharmaceutical industry (Bauermeister et al., 2010; González-Pombo et al., 2011).

The production of β-1,3-glucanases can be affected by some fermentation parameters, such as agitation, pH, temperature, incubation time, fermentation process, as well as culture medium components, such as the type and carbon source concentration (Vázquez-Garcidueñas, Leal-Morales, & Herrera-Estrella, 1998). Therefore, it is essential to choose the substrate used to induce its synthesis. Laminarin, isolated from *Laminaria digitata* alga, is one of the main substrates used for the synthesis and determination of β-1,3-glucanases, however, it has a high economic value which makes its industrial application difficult (Stubbs et al., 1999; Barsanti et al., 2001). Also, branched-chain fungal exopolysaccharides have stood out as inducers of β-glucanases, among them curdlan and botryosphere. In the case of botriosphere production, a lot of fungal biomass is generated and used as an industrial by-product (Giese et al., 2011), even so, when commercially available, these have high values due to their low productivity or commercial scarcity.

Another substrate for the β-1,3-glucanases synthesis was proposed in an innovative way in this research, as succinoglucyn being used for this function are not known in the literature. Succinoglycan are β-glucans synthesized by bacteria, such as *Agrobacterium* and *Sinorhizobium*, and constituted by monomers of galactose and glucose present in the proportion of 1-7, which are connected by β-glycosidic bonds (Ruiz et al., 2015). Succinoglycan have properties such as thickening, stabilizing, emulsifying and texturizing agents, which allows them to be used in different industrial sectors (Bakhtiyari et al., 2015).

Starch, which is already used in several enzymatic syntheses, becomes an alternative for the industrial production of β-1,3-glucanase as an inductor and carbon source (Marcello et al., 2010; Rao et al., 2016). Considering Brazil the third-largest corn producer in the world, followed by China and the United States, and one of the main producers of cassava root, reaching 18.96 million tons in 2020 (Da Silva & Castañeda-Ayarza, 2021), the use of corn starch and cassava starch can make β-1,3-glucanases a product that is easy to obtain and has a reduced cost when compared to the use of other inducing sources mentioned above.

Thus, considering the potential of β-1,3-glucanases in agriculture and their applications in the food, chemical, and pharmaceutical industry, the objective of this research was to synthesize these enzymes by free and immobilized cells of *T. harzianum* Rifai, using different inducing substrates, mainly starch, easily obtainable, and succinoglycan, as an innovative proposal in this research. Therefore, it is expected with this study to provide better cost-effectiveness in the industrial production of β-1,3-glucanases.

2. Methodology

2.1 Materials

Acurdluna was acquired from Takeda Chemical Industries Ltd. (Osaka, Japan) and succinoglycan (Rheozan®) was provided by the Rhodia Solvay Group (São Paulo, Brazil). The fungus biomass of *Botryosphaeria rhodina* MAMB 05 was provided by the Department of Chemistry of State University of Londrina (UEL) (Giese et al., 2005). The substrates cassava starch (Polvilho Doce, Alimentos Zaeli Ltd., Brazil) and corn starch (Maizena, Unilever Company), both food grade, were

purchased in the local market. Standard laminarin (*Laminaria digitata*) was purchased from Sigma Aldrich (St. Louis, MO, The USA). All other chemicals used in the study were of analytical grade.

2.2 Microorganism and Maintenance Conditions

The filamentous fungus *Trichoderma harzianun* Rifai and yeast *Aureobasidium pullulans* 1WA1 were kindly granted by Dr. Aneli Barbosa de Melo Dekker, Senior Professor of the Department of Chemistry, from State University of Londrina (PR, Brazil). The microorganism maintenance was carried out using the technique of replicating in Petri dishes every 30 days. Potato dextrose agar (BDA) was used for yeast and VXA solid medium for filamentous fungus (1% xylose (p/V); 2% agar (p/V); 2% Vogel solution (V/V) (1956)).

For reactivation and preparation of the inoculum of *T. harzianum* Rifai, it was cultivated in a VXA plate for 7 days. Sequentially, 15 discs of approximately 8 mm were removed and added to 250 ml Schott flasks containing 200 ml of saline (0.85% (w/V)), vigorously homogenized to release the conidia in the solution.

2.3 Screening of Inducing Substrates by Detection of Enzymatic Activity in Solid Media-Zymogram

The substrates were evaluated in relation to production of β-glucanases in the presence of *T. harzianum* Rifai fungus and the *A. pullulan* 1WA1 yeast, through the observation of hydrolysis halo in a Petri dish. The zymogram method was performed according to the methodology described by Bauermeister et al. (2015), with modifications, comprising the following steps: punctual inoculation of the microorganism tested with a bacteriological needle in a previously prepared Petri dish, containing 0.5% of specific substrate, solubilized in minimal Vogel medium (2% of 1: 50) containing agar (2%). The plates were incubated at 28 ºC for 48 to 120 h. After incubation, the presence of a hydrolysis halo around the microbial growth indicated enzymatic activity. The specific substrates studied were: curdlan, succinoglycan, corn starch, cassava starch, fungal biomass and lactose.

2.4 Microbial Immobilization Procedure

For microbial immobilization, natural and synthetic matrices were used, respectively, *Luffa cylindrica* bushing and synthetic polyurethane sponge for aquarium filters 25 ppi (pores per linear inch) (Elite HUSH, Hagen). *L. cylindrical* sponges were treated as described by Pazzetto et al. (2011). The matrices were prepared in disks form, each approximately 25 mm in diameter and thickness. After preparing the matrices, they were individually placed in 250 ml Erlenmeyer flasks containing 100 ml of VX medium (1% xylose (w/V); 2% Vogel solution (1:50)) and sterilized in an autoclave at 121°C for 20 min. Sequentially, 1 ml of *T. harzianum* Rifai inoculum was added, which were kept in an incubator with shaking for 72 h at 28 °C and 180 rpm, for immobilization by adsorption. After this procedure, the matrices with immobilized cells were transferred to the production medium to obtain β-1,3-glucanases.

2.5 Production and Recovery of β-1,3-glucanases

The production of β-1,3-glucanases was studied through the cultivation of *T. harzianum* Rifai using free and immobilized cells in synthetic sponges through repetitive batch processes. The following substrates were used as a carbon source (0.15%), solubilized in 2% minimum Vogel medium (1:50): curdlan, succininoglucan, corn starch, fungal biomass, and lactose. As well as succinoglycan (0.15%) plus glucose (0.1%). All enzymatic synthesis processes were performed in 250 ml Erlenmeyer flasks containing 100 ml of specific substrate plus minimal Vogel medium (2% of 1:50).

Inoculation was done with 1 mL of the inoculum solution as described above. The condition for the synthesis of β-1,3-glucanases, after preparation of medium and inclusion of the microorganism, consisted of incubation in a rotary shaker at 28 °C at 180 rpm for five days, initial pH of 5.5 with adjustments every 24 hours. After production, extracellular content was recovered by centrifugation, 7000 g for 10 min at 4 ºC (Bauermeister et al., 2015).

Tests were also carried out for the synthesis of β-1,3-glucanases with different concentrations of corn starch and cassava starch (0.15%; 0.5%; 1.0%; 3.0% and 5.0%), using only free cells.

In processes with repetitive batch at the end of each cycle was taken fungal recovery as follows: for free cells after the first inoculum (1º batch), the cells were recovered from the reaction medium by centrifuging 25 mL per 5 min at 2,940 xg and 25 °C, the sediment was washed with sterile saline solution and transferred to a new reaction medium. To recover the immobilized sponge discs, they were seized with tweezers, washed with saline solution, and transferred to a new reaction medium, the entire process is carried out in a sterile manner. In this way, successive operational cycles of β-1,3-glucanases production were carried out. All experiments were performed in triplicate.

2.6 Determination of Enzymatic Activity

The β-1,3-glucanase activity was determined by quantifying the reducing sugars released from the hydrolysis of the laminarin substrate of L. *digitata* (Sigma-Aldrich) according to the methodology of Bauermeister et al. (2015). Enzyme activity was determined in a final volume of 0.5 mL, 0.4% laminarin substrate, sodium acetate buffer, pH 5.0. Each reaction mixture was incubated at 37 ºC for 60 min and stopped by adding 50µl of 1.0 mol/L NaOH. The reducing sugars were determined according to the cuproarsenate method described by Somogyi and Nelson (Nelson, 1944). The unit of β-1,3-glucanases activity was defined as the number of µmoL of reducing sugars released per minute per mL of enzyme extract under the test conditions.

2.7 Statistical Analysis

The results of the synthesis of β-1,3-glucanases were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test, considering a significance level of 5% ($p \leq 0.05$).

3. Results and Discussion

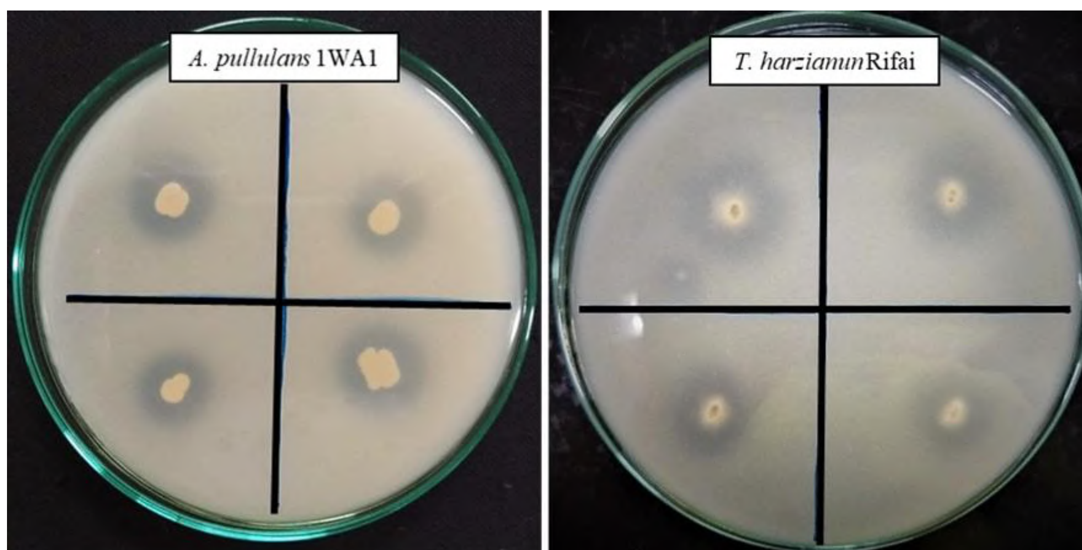
3.1 Screening of Inducing Substrates by Detection of Enzymatic Activity in Solid Media-Zymogram

The zymogram method is a qualitative, low-cost, and considerably fast technique, which allows the evaluation of substrates for the production of glycolytic complexes, as well as the possible microorganisms producing these complexes (Syed et al., 2013; Bauermeister et al., 2015). Therefore, it allows for a screening of substrates that stimulate the enzymatic synthesis of microorganisms already recognized as glucanase producers.

The activity of the complex of β-glucanases is considered positive when there is the formation of a halo transparent around the microbial growth.

The yeast A. *pullulan* 1WA1 was used as a positive control for screening new substrates with enzymatic induction potential, as it is recognized for its potential to produce β-glucanase (Vero et al., 2009; Zhang et al., 2010; Di Francesco et al., 2015). The yeast showed positivity for the Petri dish zymogram technique for all substrates analyzed in this research (curdlan, succinoglycan, corn starch, fungal biomass, and lactose) and the Figure 1 exemplifies the action of yeast on the succinoglycan substrate.

Figure 1. Petri dish containing 0.5% succinoglycan as the only carbon source. A clear halo around the growth of A. *pullulans* 1WA1 (standard) is observed, as well as the presence of a halo around the filamentous fungus *T. harzianum* Rifai (test).



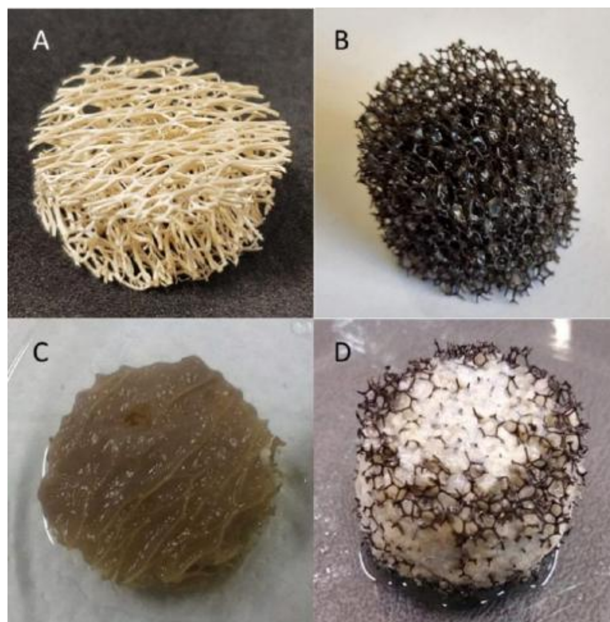
Source: Authors.

When the technique was used against the filamentous fungus *T. harzianum* Rifai, all the tested substrates showed positive for the production of β-glucanases. Figure 1 exemplifies the action of the fungus when the substrate succinoglycan was used. It was also observed that the diameters of the halos were larger than the diameters of microbial growth, indicating exocellular secretion of β-glucanases, which diffused through the agar medium and performed the substrate hydrolysis, as previously described by Bauermeister et al. (2015).

3.2 Immobilization of Filamentous Fungus in Synthetic and Natural Sponge

It was possible to immobilize *T. harzianum* Rifai in both synthetic and natural sponges (Figure 2). However, after exposing the natural sponge (L. *cylindrica*) to the fungus from the second repetitive batch of β-1,3-glucanases production, started a process of degradation of the natural support, preventing its use. The strain of *T. harzianum* Rifai studied was isolated from *Aspidosperma* sp. (Peroba) in decomposition and selected among 85 microorganisms as the best xylanase producer in submerged cultivation in sugarcane bagasse -of sugar. Resende et al. (2002), using the same strain, demonstrated the production of xylanase and cellulase in sugarcane bagasse fermentation under solid-state conditions, with the average yield of xylanase being higher than that of cellulose. This feature is shared by other *Trichoderma* species (De Souza, et al., 2019). Thus, considering that *L. cylindrica* bushing is chemically composed of cellulose (65.2%), hemicellulose (17.5%), and lignin (15.2%) (Siqueira et al., 2010), it is possible to suggest that the biodegradability of the plant support occurred through the action of the xylanase produced by the fungus. Consequently, the synthetic support was the only one selected for the subsequent tests of enzymatic synthesis in repetitive batches with immobilized cells.

Figure 2. Three-day immobilization of *T. harzianum* Rifai fungus in natural and synthetic sponge. (A) natural sponge before immobilization; (B) synthetic sponge before immobilization; (C) natural sponge after immobilization and (D) synthetic sponge after fungal immobilization.



Source: Authors.

3.3 Synthesis of β-1,3-glucanase and determination of enzymatic activity

Different carbohydrates were evaluated for their ability to produce β-1,3-glucanases by *T. harzianum* Rifai when cultivated under the same conditions over 5 days in repetitive batches using free cells. The different substrates used and the results obtained are shown in Table 1.

Table 1. β-1,3-glucanases production (U mL/min) by *T. harzianum* Rifai in repetitive batches using free cells in a reaction medium containing 0.15% of different inducing carbon sources in Vogel's minimal medium (28ºC, 180 rpm, 5 days, pH 5.5).

Inducing sources	Repetitive batches (β-1,3-glucanases U mL/min)			
	1ª	2ª	3ª	4ª
Curdlan	0.670 ± 0.174^{Aab}	0.036 ± 0.010^{Bd}	0.002 ± 0.000^{Bd}	0.005 ± 0.000^{Bb}
Succinoglycan	0.105 ± 0.013^{Ad}	0.073 ± 0.001^{Bd}	0.052 ± 0.007^{Cc}	0.004 ± 0.001^{Db}
Sucinoglycan and glucose	0.113 ± 0.027^{Ad}	0.111 ± 0.010^{Bc}	0.052 ± 0.131^{Cc}	0.005 ± 0.002^{Db}
Corn starch	0.382 ± 0.027^{Abc}	0.298 ± 0.001^{Ba}	$0,288 \pm 0.027^{Bb}$	0.166 ± 0.045^{Ca}
Fungal biomass	0.732 ± 0.023^{Aa}	0.313 ± 0.024^{Bb}	$0,300 \pm 0.070^{Ca}$	0.004 ± 0.001^{Db}
Lactose	0.272 ± 0.039^{Ac}	0.260 ± 0.080^{Ab}	$0,258 \pm 0.067^{Ab}$	0.020 ± 0.006^{Bb}

*Values represent mean ± standard deviation. U ml/min. Equal letters, uppercase in the row or lowercase in the column, do not present a statistically significant difference. Tukey Test 5%. P-value <0.0001. Source: Authors.

The inducing substrate that showed the best production in the first production batch of β-1,3-glucanases was fungal biomass, followed by curdlan and corn starch. However, fungal biomass had a sharp drop in production in the second batch, being negative in the fourth batch. The good performance of fungal biomass as an inducing substrate was already expected

since it is known in the literature that Trichoderma species synthesize a greater amount of extracellular β-glucanases when cultivated in a medium containing fungal cell wall (Ramada et al., 2016).

Succinoglycan is an acidic heteropolysaccharide synthesized by bacteria and consisting of β(1,3), β(1,4), and β(1,6) glucan bonds (Ruiz et al., 2015). Considering that its use in the synthesis of β-1,3-glucanases is unknown in the literature, the present research used succinoglycan as a hypothesis of an inducing carbon source, as it has a complex structure with a possible capacity to stimulate the synthesis of β- 1,3-glucanases. However, succinoglycan was the substrate that showed the lowest induction in the fungal synthesis of β-1,3-glucanases. It is given its complex structure and numerous ramifications, another proposal of this research was the addition of glucose together with succinoglycan in one of the formulations, to stimulate greater initial fungal multiplication, seeking to boost a subsequent production of β-1,3-glucanase. However, there was no significant difference between treatments with or without glucose.

Corn starch managed to maintain the production of β-1,3-glucanases until the fourth batch was evaluated, showing itself to be an interesting substrate since it is currently the cheapest and most accessible in the market among those evaluated in this research. Other authors have already reported on the induction of β-1,3-glucanases using starch as the main carbon source (Marcello et al., 2010). Rao, Raju and Ravisankar (2016) when evaluating the enzymatic production of *Trichoderma* spp. isolated from the rhizosphere of tobacco, obtained a β-1,3-glucanases production of 0.112 U when subjected to 0.2% starch as the only carbon source. Lactose also showed good results, maintaining production until the third repetitive batch. Curdlan, on the other hand, showed a significant decrease from the second batch.

The enzyme production, in repetitive batches, using *T. harzianum* Rifai cells immobilized in a synthetic sponge is shown in Table 2. In the first batch, about free cells, it was possible to observe an increase in the production of β-1,3-glucanases for the inducing media fungal biomass, lactose, and succinoglycan with and without glucose, which reached an enzymatic concentration around 8.74%, 47.80%, 78.10%, and 46.90% higher, respectively.

Table 2. Production of β-1,3-glucanases (U mL/min) by *T. harzianum* Rifai in repetitive batches, using cells immobilized in synthetic sponge containing 0.15% of different inducing carbon sources in Vogel's minimal medium (28 ºC, 180 rpm, 5 days, pH 5.5).

Inducing sources	**Repetitive batches (β-1,3-glucanases U mL/min)**			
	1ª	**2ª**	**3ª**	**4ª**
Curdlan	0.563 ± 0.132^{Ab}	0.007 ± 0.002^{Bd}	0.002 ± 0.000^{Bd}	0.002 ± 0.000^{Bd}
Succinoglycan	0.187 ± 0.017^{Ac}	0.129 ± 0.005^{Bc}	0.004 ± 0.001^{Cd}	0.004 ± 0.001^{Cd}
Sucinoglycan and glucose	0.166 ± 0.002^{Ac}	0.124 ± 0.011^{Bc}	0.006 ± 0.001^{Cd}	0.005 ± 0.001^{Cd}
Corn starch	0.302 ± 0.009^{Ac}	0.204 ± 0.009^{Bbc}	0.103 ± 0.009^{Cb}	0.039 ± 0.002^{Cc}
Fungal biomass	0.796 ± 0.146^{Aa}	0.267 ± 0.033^{Ba}	0.059 ± 0.013^{Cb}	0.039 ± 0.014^{Cc}
Lactose	0.402 ± 0.010^{Ac}	0.233 ± 0.075^{Bab}	0.185 ± 0.007^{Ba}	0.091 ± 0.005^{Ca}

*Values represent mean ± standard deviation. U ml/min. Equal letters, uppercase in the row or lowercase in the column, do not present a statistically significant difference. Tukey Test 5%. P-value <0.0001. Source: Authors.

In repetitive batches, the result of β-1,3-glucanases production using the microbial immobilization process was very similar to those obtained by free cells. Fungal biomass was also the best-inducing substrate in the first batch, but it was only relevant until the second batch. For substrates such as corn starch, fungal biomass, and lactose, immobilization was interesting, as it achieved production results in at least three repetitive batches, while the other inducing sources, only the first batch

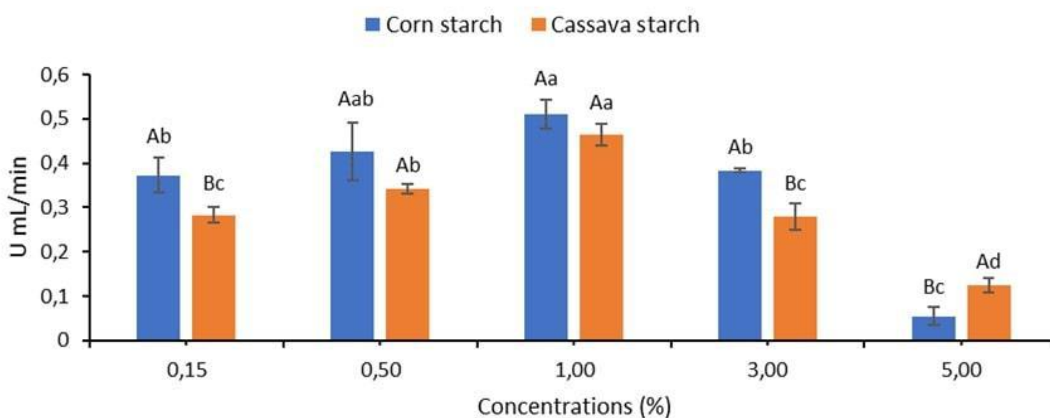
showed results. Considering that, during enzyme production, cell transfer is more practical with the microorganism immobilized in a sponge, not requiring centrifugation for cell rescue, this method requires prior preparation of the support with fungal immobilized, adding steps to the process. Therefore, an economic feasibility study is necessary for the use of the immobilization proposed in this research.

The decrease in enzyme synthesis during repetitive batches in the present research may be related to the viability, cell death, and interaction of the microorganism with the reaction medium. Yu et al. (2018) emphasize the importance of the balance between the interaction of the microorganism with the support, especially with the hydrophobic strength and pore size of the support. Therefore, a relatively loosely diffused fungal growth on the substrate is important so as not to block pores and allow the passage of nutrients.

Considering the good enzymatic production obtained using corn starch, and always aiming at the possibility of using a more cost-effective substrate, tests were carried out with free cells, comparing cassava starch and corn starch as a carbon source. A previous screening was performed with cassava starch, using the zymogram method, which was positive.

Results of β-1,3-glucanases production by free cells of *T. harzianum* Rifai using different starch sources (corn and cassava) and different concentrations, 0.15%; 0.5%; 1.0%; 3.0% and 5.0% are shown in Figure 3.

Figure 3. Synthesis of β-1,3-glucanases (free cells) by *T. harzianum* Rifai in corn starch and cassava starch at different concentrations (0.15% 1% 2% 3%) plus minimal Vogel medium (28°C/ 180 rpm/ 5 days/ pH 5.5). Equal letters do not present a statistically significant difference, with capital letters representing analysis between the two types of starch and lowercase letters representing analyzes between concentrations of the same substrate.



Source: Authors.

Corn starch showed the best result in this test and, for the two substrates studied, there was an increase in enzyme production against the increase in the concentration of up to 1.0% of the substrate, obtaining 0.51 U and 0.46 U for starch from corn and cassava starch, respectively. From 3% onwards, there was a decrease in the production of β-1,3-glucanases for the two substrates.

It is important to emphasize that even with lower production, the concentrations of 0.15% and 0.5% presented excellent benefits, compared to the production with 1% of the substrate, since with the amount of 1% it is possible to carry out six batches of concentration of 0.15% and two batches with 0.5% of the substrate, which would result in an enzyme production much higher than that obtained with a single production with 1% of the substrate. As an example, for the use of 0.15% corn starch, the increase would be about 338% higher and for cassava starch, 265% higher.

No significant difference was observed between corn and cassava starch when using concentrations of 0.5% and 1%. Similarities in production between substrates should be considered a relevant and positive factor, considering the possibility of replacing the raw material by industry in face of price variation, due to the seasonality of agricultural crops. This becomes even more relevant when it is observed that corn starch and cassava starch have similar properties and compete as a commodity in the international market (Vilpoux, 2011).

4. Conclusion

The zymogram technique proved to be efficient for screening inducing substrates. Fungal biomass resulted in good production of β -1,3-glucanases, however, its acquisition is restricted. Starch sources showed promise for enzyme production by *T. harzianum*, being easy to acquire industrially and at a low cost. Succinoglycan can be used as an inducing substrate in the production of fungal β -1,3-glucanases. The use of free and immobilized cells allowed the microorganism to be reused, allowing its use in repetitive batches.

The present study emphasizes the importance of future tests of economic viability, as well as, made possible suggestions for future research that include the characterization and bioactive action of the oligosaccharides obtained by enzymatic hydrolysis.

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